Anti-microbial and anti-biofilm compounds from Indonesian medicinal plants

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Anti-microbial and Anti-biofilm Compounds From Indonesian Medicinal Plants

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For my families

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Chapter 1

General introduction

In recent times there has been an increased urgency to find new anti-microbial compounds as a growing number of microorganisms develop resistance to many commonly used anti-microbial agents upon exposure to anti-microbials. Infections caused by antibiotic-resistant microorganisms complicate conventional treatment, resulting in prolonged illness and greater risk of death (Barza and Travers, 2002).

The problem of microbial resistance to antibiotics appeared as soon as the mass-production of penicillin was started in 1945 (Shaughnessy, 2007), and has now become a major concern in medicine throughout the world. Bacterial resistance not only can occur to a single class of antibiotic, but also to multiple classes of antibiotics. The susceptibility of microorganisms to antibiotics can be categorized in two types:

- innate resistance, which means that some microorganisms are inherently resistant to many antibiotics;
- Acquired resistance, which can arise by a number of diverse mechanisms such as mutation and horizontal gene transfer.

Horizontal gene transfer can occur by several different processes such as bacterial conjugation, transfer of genetic material between bacterial cells by direct cell-to-cell contact. Also DNA can be transferred from one bacterium to another via a viral vector, or directly taken up from its surrounding through the cell membrane (Tenover, 2006).

One of the most serious cases of multidrug resistance is the resurgence of tuberculosis caused by the bacterium *Mycobacterium tuberculosis*, which results in around 2 million deaths per year. Multi-drug resistant *Mycobacterium* strains and the spread of the HIV/AIDS pandemic have contributed to this mortality (Amin, 2006). Tuberculosis, combined with other mycobacterium-based infections, has killed over 30 million people in the last decade, highlighting the need for new anti-bacterial compounds.

According to an annual World Health Organization (WHO) report (WHO, 2014), half of all *Staphylococcus aureus* infections in the United States (US) are resistant to penicillin, methicillin,

tetracycline and erithromycine. This left vancomycin as the only effective agent available at the time. However, glycopeptide intermediate-resistant *S. aureus* (GISA) or vancomycin intermediate-resistant *S. aureus* (VISA) strains began to appear in the late 1990s. The first identified case was in Japan in 1996, and thereafter strains have been found in hospitals in England, France and the US. The first documented strain with complete resistance to vancomycin, termed vancomycin-resistant *S. aureus* (VRSA) appeared in the US in 2002.

Resistance to antibiotics of pathogenic fungi is even a more serious problem, due to the limited number of available anti-fungal drugs. Fungi can counteract the fungicidal or fungistatic effects of all anti-fungals classes by three major mechanisms, namely: (i) reducing the accumulation of the drug within the fungal cell, (ii) decreasing the affinity of the drug for its target, and (iii) modifications of metabolism to counterbalance the drug effect (Vandeputte *et al*., 2012).

Taken together the increase in (multi) drug resistance of pathogenic microbes and the limited amount of antibiotics available for certain classes of microorganisms requires the discovery of new methods and drugs to defend humans and animals against infection by the pathogenic organisms. Another important reason to discover new classes of antibiotics is the recent recognition that a "biofilm" is the most common living environment of microorganisms (including those causing diseases) and that in this environment microorganisms are much more resistant against antibiotics. Biofilms were actually already discovered in 1650s by Anthonie van Leeuwenhoek when he examined the "animalcules" in the dental plaque on his own teeth, however the theory describing the biofilm process was not developed until Costerton and colleagues published their study in 1978. Costerton's theory states that the majority of bacteria grow in matrix-enclosed biofilms that adhere to biological or non biological surfaces in aqueous environments, and that these sessile bacterial cells differ greatly from their planktonic counterparts. Biofilms have been intensively studied in recent years due to increased awareness of the impact of biofilms on human health and industrial productivity (Costerton *et al.,* 1978; Donlan and Costerton 2002; Hall-Stoodley *et al.,* 2004).

Biofilms are defined as a community of cells that are attached to a surface, grow on this surface and can form structured layers. Within these layers cells develop properties as free living (planktonic) cells. One of these traits is the development of an increased antibiotic resistance and the expression of pathogenicity properties. Bacterial biofilm formation is likely to involve a cascade of gene expression events associating with a crossover of many sensing systems directed against environmental changes (Yamanaka *et al*., 2009). *Streptococcus mutans* biofilm proved to exhibit a different expression of some proteins involved in cell signalling, peptidoglycan biosynthesis, motility, and adhesion in comparison to its planktonic cultures (Svensäter *et al*., 2001). In the study by

Kreikemeyer *et al*. (2011) concerning biofilm structure, the determinants of *Streptococcus* pathogenicity are related to the discovery of long filamentous structures similar to the pilus observed on bacteria surfaces. These structures exhibit adhesive properties and may play a key role in adhering to host cells and tissues, as well as in biofilm formation by pathogens of the *Streptococcus* species. Exopolysaccharide (EPS) is one of the main constituents of the biofilm extracellular matrix (Flemming et al., 2007), and recent investigations have revealed that each biofilm-forming bacterium produces distinctive EPS components *e.g*. alginate and/or Psl found in *Pseudomonas aeruginosa* (Ryder *et al*., 2007), and polysaccharide intercellular adhesin (PIA) of *Staphylococcus* (Gotz, 2002). This EPS provides a protection for the biofilm microorganisms from a variety of environmental threats, such as UV radiation, pH shifts, osmotic shock, desiccation, and toxic compounds, and creates a micro niche favorable for long term survival and functioning of the microbial communities (Baumgartner *et al*., 2008). Therefore, when microorganisms grow as a biofilm they are less susceptible against antibiotics, biocides and other chemical and physical challenges (Lewis, 2001).

It is estimated that at least 65% of all hospital infections involve biofilms, since biofilms are not only efficiently formed on human tissues, but also on medical equipment such as catheters and biomaterials such as implants (Donlan, 2002). Furthermore, it is shown that biofilms are involved in developing resistance of bacterial infections (Furukawa *et al*., 2006) as biofilms form a very suitable environment for the selection and emergence of multi-drug resistant strains.

Previously, antibiotic discovery and characterization has been studied mostly with free living bacteria. Since bacterial cells in a biofilm are physiologically so different, it is clear that specific compounds that are able to degrade biofilms still need to be discovered. The anti-biofilm compounds are important tools in the successful treatment of infections and to prevent the development of resistance.

Quorum sensing (QS)

Microorganisms can communicate with one another. One of the communication mechanisms, called quorum sensing, has been named so after discovery of the mechanism underlying cell-density-dependent behavior (Nobile and Mitchell, 2007; Atkinson and Williams, 2009). It has been shown that bacteria assess their population density by producing and detecting the concentration of small, diffusible cell-to-cell signal molecules (auto-inducer), such as an *N*-acylhomoserine lactone (AHL), that they export into their environment. At high population density, a surplus of signaling molecules accumulates in the environment causing diffusing back of these molecules into the cell (Bassler and

Losick, 2006; Atkinson and Williams, 2009). When the signal molecules reaches a threshold level inside the cell, they activate (or repress) a response regulator. This regulator controls expression of quorum-sensing dependent genes such as genes encoding virulence factors or genes encoding proteins involved in biofilm formation (Shih and Huang, 2002; Hentzer *et al*., 2003; Atkinson and Williams, 2009), antibiotic production (Liu *et al*., 2007; Duerkop *et al*., 2009), pigment production, bioluminescence (Fuqua *et al*., 2001), and surface motility (Rasmussen *et al*., 2000) as well as gene transfer systems (Rodelas *et al*., 1999; Schaefer *et al*., 2002).

In Gram-negative bacteria, one of the best studied quorum-sensing systems is the LuxR/I system which consists of a four-component circuit: an AHL (*N*-Acyl homoserine lactones)-signal molecule, a LuxI-type signal synthase, a LuxR-type signal receptor, and the target gene(s) The AHL-signal is synthesized at a low basal level by the AHL-synthase, the product of the *luxI* gene. The AHL-signaling molecules diffuses out of the cell, and when the cell density is high, AHL diffuses back into the cell where it binds to and activates the transcriptional activator LuxR, which then stimulates transcription of *luxI* (Hentzer and Givskov, 2003).

In Gram-positive bacteria, the most common type of signal molecules is oligopeptides that are being exported from the bacterium by transporters (Miller and Bassler, 2001). These peptides will interact with two-component systems ultimately regulating gene transcription. Interaction with the peptide ligand initiates a series of phosphorylation events that culminate in the phosphorylation of a cognate response regulator protein. This phosphorylation allows the response regulator to bind to the promoter region of the quorum sensing–controlled target gene(s) and to alter in this way their transcription (Reading and Sperandio, 2005).

The existence of fungal QS systems was revealed after the discovery that farnesol inhibit biofilm formation in the pathogenic polymorphic fungus *Candida albicans*. In addition to farnesol, the aromatic alcohol tyrosol was also found to be a *C. albicans* QS-molecule (QSM) controlling growth, morphogenesis and biofilm formation. In *Saccharomyces cerevisiae*, two other aromatic alcohols, phenylethanol and tryptophol, were found to be QSMs regulating morphogenesis during nitrogen starvation conditions. Additionally, population density-dependent behaviors that resemble QS have been described in several other fungal species (Albuquerque and Casadevall, 2012).

There is evidence that interspecies communication via QS can occur. This is known as QS cross talk. Cross talk plays a significant role in microbiology as in nature micrrorganisms almost always exist in

mixed species communities. Among the many virulence factors and phenotypes that are controlled by quorum-sensing, there is a clear focus on biofilm formation (Kjelleberg and Molin, 2002).

Biofilm: a novel target

For decades, microbes are mostly considered as free-floating single-celled (planktonic) organisms living an independent lifestyle, rapidly multiplying and described based on their growth characteristic in nutritionally rich culture media (Donlan, 2002; Schachter, 2003). But in fact in nature, most of microbes live together in large aggregates, are attached to a surface, are growing on this surface and often form structured layers. This way of occurance of microbial populations is known as a biofilm. A biofilm community can comprises a single kind of microorganism, but in nature biofilms almost always consist of mixtures of many species of bacteria, as well as fungi, algae, yeasts, protozoa, and other microorganisms, along with non-living debris and corrosion products (Liu *et al*., 2000; Sutherland 2001). Biofilms are found in almost every habitat where the combinations of moisture, nutrient, and surface are available, such as in pipe-lines of industrial or drinkable water, on rocks, at the grime in the toilet bowls and showers or in moss or lichen on tree trunk. Growth as biofilms is likely to provide a better opportunity to adapt to changing conditions collectively than as single cells. Advantages of microbial life in a biofilm comprise protection from host defense and antibiotics, increased availability of nutrients by sequestration to nutrient-rich areas, and cooperative benefits of life in a community in general (Leid, 2009).

Biofilms also have an important impact on human health. The National Institutes of Health (NIH) estimated that biofilms are involved in at least more than 65% of nosocomial infections and up to 75% of microbial infections that occur in the human body (Richards *et al*., 2009). In human tissue for instance, biofilms are produced on the surface of teeth as dental plaque, lungs in cystic fibrosis patients, and as fungal infection on the woman reproductive system. Biofilms formed on medical instruments such as catheters, artificial heart valves, contact lenses, and artificial joints, putting patients at risk for local and systemic infection complications, including local-site infections, catheter-related bloodstream infections, and endocarditis (Donlan, 2002; O'grady *et al*., 2002). The establishment of microbial biofilms is often considered a pathogenicity trait during chronic infections, and biofilm formation has been recognized as an important virulence mechanism of many bacterial pathogens (Donlan and Costerton, 2002).

Biofilm communities can develop within hours. There are five stages of biofilm development, as depicted schematically in figure 1. The first step of biofilm formation is attachment of free-floating

cells, referred as ‘pioneer cells’ to a surface associated with proteins, glycoproteins, humic-like substances (HULIS), and other dissolved or colloidal organic matters (the so called "conditioning layer"). This conditioning layer is consider to play a vital role in initial microbial attachment as they neutralizes excessive surface charge and surface free energy which may prevent a microbial cell from approaching near enough to initiate attachment (Schneider and Leis, 2002; Palmer *et al.,* 2007; Renner and Weibel, 2011).

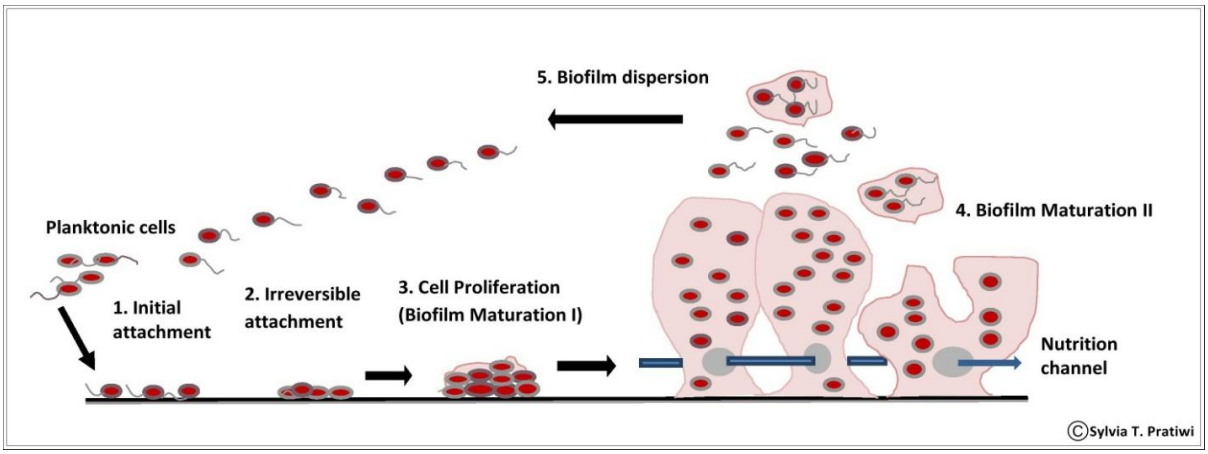

Fig. 1: Five stages of biofilm development: 1. Initial attachment of planktonic cells to the surface, 2. Irreversible (permanent) attachment, 3. Cells proliferation and formation of EPS (maturation I), 4. Formation of biofilm structure (maturation II), 5. Biofilm spreading (dispersion).

During initial attachment, the ‘pioneer cells’ adhere to the surface initially through weak, reversible Van der Waals forces. The bacteria may then form a transient association with the surface, and facilitate the arrival of other cells by providing more diverse adhesion sites to yield a basal layer and forms a stable association as a member of a microcolony (Watnick and Kolter, 1999). Afterwards, the attachment of the cells becomes irreversible as the microcolonies start joining together above the substratum to form a continuous layer of extracellular polymeric substances (EPS) that run into a complex, three-dimensional mushroom-like structure stick out from the surface, separated by “voids” through which the medium (e.g., water with nutrients) may flow relatively uninhibited. This EPS holds the biofilm together and serves as a barrier to defend the microbial colonies from anti-microbials. The last stage in the development of biofilms is the detachment of small or large clumps of cells, or by a type of "seeding dispersal" from the biofilm that releases individual cells**.** These cells move on to further colonize sites, and repeat the development cycle. Dispersal of cells from the biofilm colony is an essential stage of the biofilm lifecycle because it enables biofilms to spread and colonize new surfaces (Hall-Stoodley *et al.,* 2004; Richards *et al.*, 2009).

The EPS consists of polysaccharides, proteins, glycoproteins, glycolipids and contribute to biofilm integrity. Extracellular DNA (eDNA) has also been found in the EPS and is considered as a remnant of lysed cells (Cheng *et al*., 2011) All EPS biopolymers are highly hydrated. About 97% of the biofilm consists of water and forms a matrix that keeps the biofilm cells together and prevents desiccation (Zhang *et al*, 1998; Shuterland, 2001). It is clear from a number of studies that mutants unable to synthesize the EPS are unable to form biofilms although they may still attach to surfaces and form micro-colonies to a limited extent (Flemming and Wingender, 2010; Xiao *et al*., 2012).

Biofilm communities of bacteria are different from planktonic cells, most importantly being their increased resistance to anti-microbial agents. The dense EPS matrix serves as an initial barrier to prevent penetration of anti-microbial agents at the full depth of biofilm. As a consequence, cells inside the biofilm are about 10 to 1.000 times more resistant to anti-microbial agents than planktonic cells. Cells in biofilms are also extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living host (Stewart *et al.,* 2001). Hentzer, *et al.* in their experiments (2003), showed that classical antibiotics used to treat *P. aeruginosa* infections, e.g., tobramycin and piperacillin are required at concentrations 100-to-1000-fold higher in order to kill biofilm bacteria than in order to kill their planktonic counterparts.

Generally, the most rapid cell growth in a biofilm occurs at the biofilm surface where the oxygen and nutrients are plentiful. Cells that are embedded deep inside the biofilm might grow in a slow or starved state due to nutrients and oxygen limitations and accumulation of toxic metabolic product (Wimpenny and Kinniment, 1995). Slower growing or non-growing cells are not very susceptible to many anti-microbial agents. Penicillin which target cell wall synthesis kills only growing bacteria (Tuomanen *et al*., 1986; Stewart and Costerton, 2001). Also, anti-microbials hardly affect all bacteria within the biofilm due to heterogeneity within the biofilm, because unlike the growth of bacteria in liquid cultures which produce homogeneous populations of genetically identical cells, growth in biofilms generates a large amount of genetic diversity (Stewart, 1998; Costerton *et al*., 1999). Heterogeneity within the biofilm caused by cellular diversity formed by gradients of nutrients, waste products and signaling factors which can develop microniches within the biofilm (Mehrotra, 2007).

Biofilms also accommodate heterogeneous populations coming from both epigenetic and genetic phenomena. Epigenetic heterogeneity is the production of persister cells as well as in stationary-phase planktonic culture. Persister cells are cells that neither grow nor die in the presence of bactericidal agents (dormant), and tolerant to a lethal stress such as anti-microbial treatment, heat shock, cold shock, changes in pH and some chemicals that are lethal to growing cells (Hengge-Aronis,

1996; Lewis, 2007). The presence of persister cells explains the resistance of biofilms-based infection to anti-microbial therapy (Nobile and Mitchell, 2007).

The role of QS in biofilm

The precise role for QS in biofilm formation and maturataion is not always clear. Multiple reports have discussed the involvement of QS in biofilm formation, and conflicting conclusions have been drawn regarding the importance of QS in microbial biofilm formation. QS regulatory networks are very intricate and may include several genes whose products affect biofilm development at different stages (Solano *et al.*, 2014). The effect of AHL QS on *P. aeruginosa* biofilms appears to depend on different growth conditions, such as static or under flow, different growth phases observed, different supply of nutrients, or strain differences (Heilmann and Götz, 2010). It was shown that QS affected *P. aeruginosa* biofilms only in the presence of glucose and not citrate (De Kievit *et al*., 2001). Schaber *et al*., (2007) found that biofilms formed by *P. aeruginosa* in acute burn wound infections in mice to be unaffected by mutation of the *lasI* and *rhlI* QS genes, despite the fact that the mutant strains were significantly less virulent than their wild-type counterpart.

In *Listeria monocytogenes*, the effect of the agr (accessory gene regulation) system is to induce the cells to bind to a surface (Rieu *et al.*, 2008). A mutation in the *agrD* gene reduced its ability to attach to a PVC surface, thus makes it difficult to form biofilm. Similarly in *Enterococcus faecalis*, the *fsr* peptide base QS system has also been shown to positively influence biofilm formation (Hancock and Perego, 2004; Nakayama *et al*., 2006).In *Aeromonas hydrophila*, the deletion of the AHL synthase gene *ahyI* responsible for the synthesis of both C4 and C6 HSL results in the reduction of biofilm differentiation and the failure of the bacteria to form microcolonies (Lynch *et* al., 2002).

In several cases, such as in *P. aeruginosa* and a common co-habitat of Cystic Fibrosis (CF) patient lung infections, *Burkholderia cenocepacia*, QS does not appear to affect the initial attachment of cells to a surface, but rather the maturation of biofilm structure. Mutations in the QS systems of these bacteria result in the formation of flat, undifferentiated biofilms (Huber *et al.,* 2001). In *Serratia liquefaciens* and *S. marcescens*, QS system regulates the maturation stages of biofilm formation. A mutation in the AHL synthase gene *swrI* results in biofilm development which is apparently halted after 24 hours, forming a flat and thinner biofilm, with more homogenous cells which are poorly attached and less cellular aggregation. This is in contrast to the wild type, which forms aggregate cluster biofilms (Labbate *et al*., 2004; Rice *et al*., 2005).

Staphylococcus uses the *agr* system to regulate biofilm formation. The agr system downregulates the expression of genes encoding colonization factors, such as the microbial surface components recognizing adhesive matrix molecules (MCSRAMMS) and other adhesin that promote attachment and biofilm accumulation, or to induce the dispersal cells from a biofilm by initiate secretion of a variery of toxins (α-toxins, δ-toxins, and toxic shock syndrome toxins-1), and ezymes (such as proteases, lipases, hyaluronidase, and nuclease) that are involved in tissue destruction and/or biofilm detachment probably requires for dissemination of the infection and colonization of new sites (Heilmann and Götz, 2010).

However, not all microorganisms positively regulate biofilm formation using QS. High cells densities of *Vibrio cholerae* are known to inhibit biofilm formation (Karatan and Watnick, 2009), whilst studies have shown that *V. cholera* "locked" in a low-cell density mode, produce more pronounced biofilms (Teschler *et al*., 2015).

Model organism: *P. aeruginosa*

P. aeruginosa (figure 2), is a Gram-negative bacteria rod-shaped, about 1-5 µm in length and about 0.5-1.0 µm in breadth (Prescott *et al*., 2009). This bacteria is an obligate aerobe, however *P. aeruginosa* is considered by many as a facultative anaerobe, as it is well adapted to proliferate in conditions of partial or total oxygen depletion (Kelli *et al*., 2007; Prescott, *et al*., 2005).

P. aeruginosa produces two types of water-soluble pigments: pyocyanin, a blue-green, water- and chloroform-soluble, non-fluorescent pigment, and pyoverdine, a yellow-green, water-soluble, fluorescent pigment which are released in the medium resulting yellow-green iridescent colonies on plate agar. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is a characteristic of suppurative infections caused by *P. aeruginosa*. Pyoverdin is most often produced by *P. aeruginosa* and other Pseudomonads commonly isolated from humans. In contrast, *P. aeruginosa* is the only *Pseudomonas* spp. known to produce pyocyanin, a pigment that diffuses into the surrounding medium. Definitive clinical identification of *P. aeruginosa* often includes identifying the production of pyocyanin and pyoverdine, as well as its ability to grow at 42°C (Forbes *et al*., 2007; Todar, 2011; Tamang, 2014).

P. aeruginosa has an outer membrane which contains Protein F (OprF) OprF functions as a porin that enable certain molecules and ions to come into the cells. OprF also act as a structural protein to maintain the bacterial cell shape (Nestorovich *et al*., 2006; Fito-Boncompte, 2011). *P. aeruginosa* is

motile by single polar flagellum. The flagellum is very important during the early stages of infection, as it is required for the attachment and invasion of the tissues of the hosts. This bacterium also has *N*-methyl-phenyl-alanine (NMePhe) or type IV pili which contribute greatly to its ability to adhere to mucosal surfaces and epithelial cells (Mattick, 2002; Klaussen *et al*., 2003).

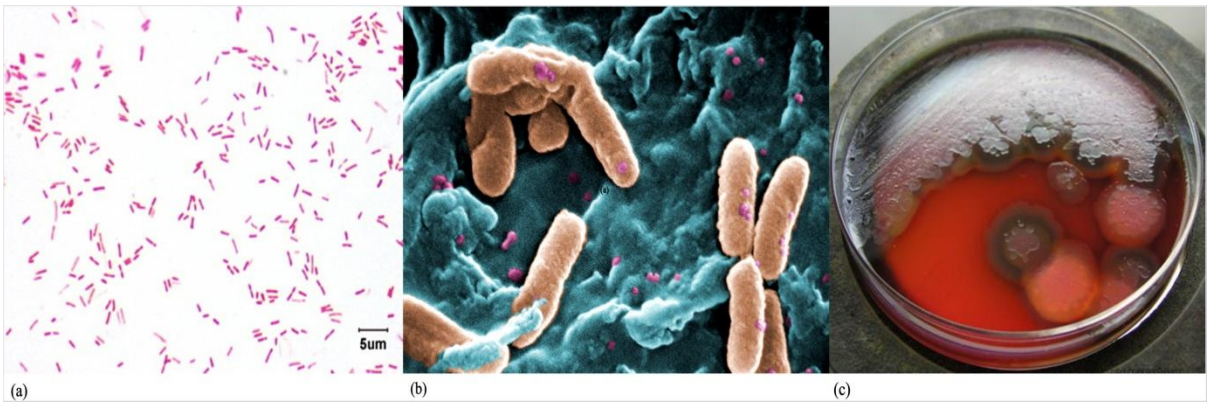

Fig. 2: Multiple views of *Pseudomonas aeruginosa*. (a) Gram stain of *P. aeruginosa*, (b) scanning electron micrograph (SEM) of *P. aeruginosa,* (c), colonies of *S. aureus* on blood agar plates showing hemolysis (Kaiser, 2009; Carr, 2006).

Under oxidative-stress conditions, *P. aeruginosa* synthesizes Fe- or Mn-containing superoxide dismutase (SOD) enzymes, which catalyze the very reactive O- to H_2O_2 and O_2. *P. aeruginosa also* produces several catalases that detoxify H_2O_2, leaving oxygen and water (Hassett *et al.,* 1993; Elkins *et al.,* 1999).

***P. aeruginosa* virulence factors and disease association**

P. aeruginosa is an opportunistic human pathogen that is commonly found in the environment e.g. soil, water and other moist locations. It is the most common Gram-negative bacterium found in nosocomial infections. For most healthy people, this bacterium seldom poses a problem. *P. aeruginosa* causes urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, bone and joint infections, gastrointestinal infections, and a variety of systemic infections, particularly in patients with severe burns and in cancer or AIDS patients who are immunosuppressed. In the expanding AIDS population, *P. aeruginosa* bacteremia is associated with 50% of deaths. CF patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population (Todar, 2011).

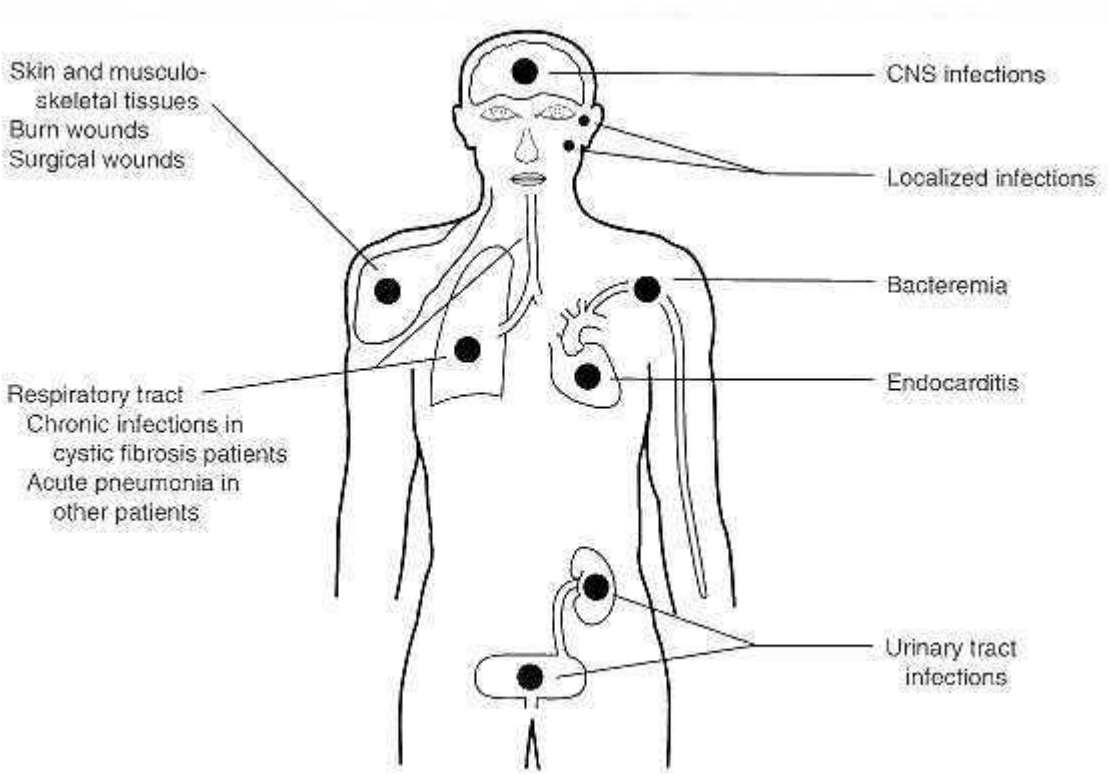

Fig. 3: Various sites of *P. aeruginosa* infection (Iglewski, 1996).

The capability of *P. aeruginosa* to produce infections is due to several extracellular and cell-associated virulence factors, (e.g, adhesins, invasins, toxins, antiphagocitic surface properties, defense against serum bactericidal reactions, and defense against immune responses) which play major roles in the toxicity and aggressivity of the bacteria. The bacterial capsule or alginate slime layer that forms matrix of the *Pseudomonas* biofilm effectively protects the bacteria from the opsonization by antibodies, complement deposition, and phagocyte engulfment (Todar, 2011).

Elastase and alkaline protease, are two extracellular proteases which have been associated with virulence that during the invasive stage. Elastase cleaves collagen, IgGs, IgAs, elements of the complement system. It also lyses fibronectin to expose receptors for bacterial attachment on the mucosa of the lung. Elastase activity also disrupts the respiratory epithelium and interferes with ciliary function. Alkaline protease interferes with fibrin formation and will lyse fibrin. It destroys the ground substance of the cornea and other supporting structures composed of fibrin (Todar, 2011).

P. aeruginosa secretes a number of toxins into the extracellular environment, but one set of toxins is injected directly into host cells. This occurs through a macromolecular syringe consisting of a type III secretion system (TTSS) that inject at least four toxins into host cells. Injection of these toxins leads

to disruption of Ras- and Rho-mediated signal transduction pathways, collapse of the actin cytoskeleton, and cell death (Frank, 1997; Engel and Balachandran, 2009).

Exotoxin A is the most potent toxic factor which is responsible for local tissue damage, bacterial invasion and immunosuppression. Exotoxin A catalyzes ADP-ribosylation and inactivation of elongation factor 2, an essential component of the protein synthesis machinery which leads to inhibition of protein biosynthesis and cell death (Todar, 2011).
About 60% of people with cystic fibrosis have a chronic respiratory infection caused by *P. aeruginosa* biofilm-growing mucoid (alginate-producing) strains that settle into the thick mucus trapped in the respiratory passages. *P. aeruginosa* mucoid biofilms allow bacterial cells to escape from the innate and adaptive defense mechanisms of the lungs, and intense antibiotic therapy (Høiby *et al*., 2010).

***P. aeruginosa* QS and biofilm formation**

There are two QS systems in *P. aeruginosa*, which have been extensively studied. The *las* system consists of the LasR transcriptional regulator and the LasI synthase protein. LasI is essential for the production of the AHL signal molecule *N*-(3-oxododecanoyl)-L-homoserine lactone (3O-C_{12}-HSL). LasR requires 3O-C_{12}-HSL in order to become an active transcription factor. A second QS system in *P. aeruginosa* consists of the RhlI and RhlR proteins. The RhlI synthase produces the AHL *N*-butyryl-L-homoserine lactone (C_4-HSL), and RhlR is the transcriptional regulator. Only when RhlR is complexed with C_4-HSL it is activated. Both 3O-C_{12}-HSL and C_4-HSL have been shown to freely diffuse out of bacterial cells; however, 3O-C_{12}-HSL diffusion is significantly slower than that of C_4-HSL. Recently, a third LuxR homologue termed QscR was identified, which has been shown to regulate the transcription of both *lasI* and *rhlI*. Although QscR exhibits significant homology to LasR and RhlR, it is currently unknown whether an AHL or similar molecule is needed to stimulate QscR function. Data indicate that *qscR* is important in regulating the production of several virulence factors but whether this regulation occurs through controlled expression of both the *las* and the *rhl* systems is currently unknown (Smith and Iglewski, 2003; Bjarnsholt, 2010).

At least three exo-polysaccharides, Psl, Pel, and alginate contribute to biofilm formation in *P. aeruginosa* that responsible to protect bacteria from environmental stressed and is required for bacterial cells to adhere to a substratum and maintaining biofilm structure (Matsukawa and Greenberg, 2004; Ryder *et al.,* 2007). The *Psl* (*p*olysaccharide *s*ynthesis *l*ocus) containing the *pslA*-*O* genes, is responsible for mannose and galactose rich EPS, whereas *pel* (referring to *pel*licle, a biofilm formed at the air-medium interface), containing the genes *pelA*-*G*, is responsible for synthesis of a glucose-rich component of the matrix (Khan *et al*., 2010).

The dispersal mechanism for bacterial cells to escape the biofim is induced by unfavorable conditions and important to spawn new communities of bacterial cells at new locations. It involves a reduction mechanism of bacterial adhesiveness, and a breakdown of the biofilm matrix (Harmsen *et al.,* 2010; Gjermansen *et al.,* 2005). Davies and Marques (2009) found that the compound *cis*-2-decenoic acid (CDA), an unsaturated fatty acid produced by *P. aeruginosa* is capable of inducing the dispersal of established biofilms and of inhibiting biofilm development. The authors suggested that this molecule is produced continuously by *P. aeruginosa* during growth in biofilms and that small microcolonies do not disperse because CAD is removed through diffusive and advective transport; However dispersal from larger microcolonies occurs because the rate of production of this molecule exceeds the rate of diffusion.

Model organism: *S. aureus*

S. aureus, means the "golden cluster seed" is a Gram-positive coccus, non-motile, non-spore forming bacteria, facultative anaerobic, with the diameter of 0.5-0.15 µm, that usually occur single, in pairs, tetrads, short chains (three or four cells) or irregular grape-like clusters. Typical colonies are yellow to golden-yellow in color, smooth, entire, slightly raised, often with hemolysis when grown on blood agar plates (figure 4). *S. aureus* is able to produce enzyme catalase (catalase positive) and able to convert hydrogen peroxidase (H_2O_2) to water and oxygen. The catalase test is useful to distinguish Staphylococci from Enterococci and Streptococci. The ability of *S. aureus* to clot blood plasma by the presence of enzyme coagulase is the main criteria to identify this bacterial, separating the virulent pathogen of *S. aureus* from the less virulent coagulase-negative Staphylococci species (Prescott *et al.,* 2005).

Resistance of *Staphylococcus* to many commonly used antibiotics started as soon as the first antibiotic, penicillin, was introduced in 1943. By 1950, 40% of hospital *S. aureus* isolates were penicillin-resistant (Chambers, 2001). The resistance is mediated by penicillinase, an enzyme that breaks-down the β-lactam ring of the penicillin molecule. β-Lactamase-resistant penicillins such as methicillin, oxacillin, cloxacillin, and fluconacillin that are able to resist degradation by staphylococcal penicillinase were developed to treat penicillin-resistant *S. aureus*. Methicillin was the first antibiotic in this class to be used in 1959, but only two years later, the first case of methicillin-resistant *S. aureus* (MRSA) was found and became endemic in 1990 (Givney *et al*., 1997).

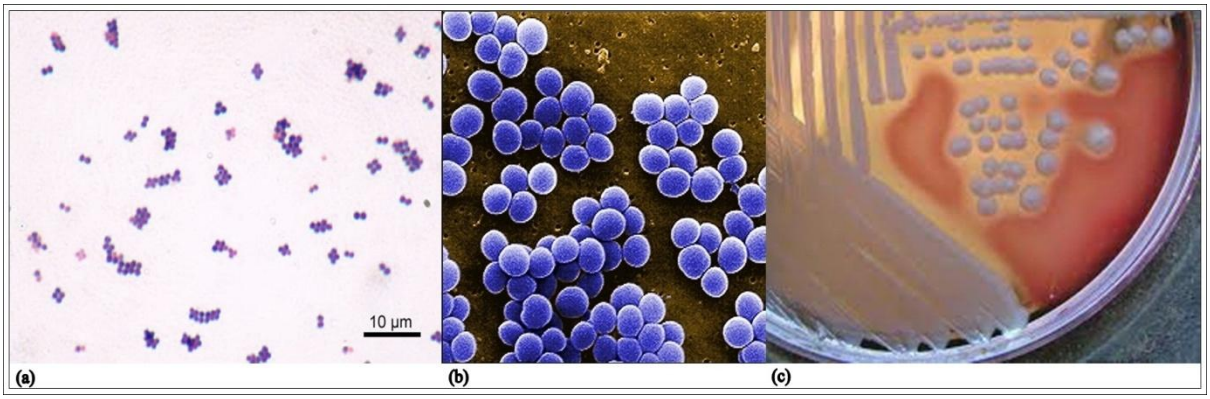

Fig. 4: Multiple views of *Staphylococcus aureus*. (a) Gram stain of *S. aureus*, (b). scanning electron micrograph (SEM) of *S. aureus*, (c) colonies of *S. aureus* on blood agar plates showing hemolysis (Tambe, 2005[a]; Carr, 2007; Buxton, 2013).

First line treatments for infections caused by MRSA are currently glycopeptide antibiotics (vancomycin and teicoplanin). Most of *S. aureus* isolates are susceptible to vancomycin. *Staphylococcus* bacteria are classified as vancomycin intermediate resistant *S. aureus* (VISA) if the MIC for vancomycin is between 4-8 µg/mL, and classified as vancomycin resistant *S. aureus* (VRSA) if the vancomycin MIC is ≥16µg/ml (Walters *et al*., 2015). The first case of vancomycin-intermediate *S. aureus* (VISA) was reported in Japan in 1996 (Hiramatsu *et al*., 1997), and the first case of *S. aureus* resistant to glycopeptide antibiotics was only reported in 2002 (Chang *et al.,* 2003). Surveillance data shows VISA and VRSA infections are uncommon, and only seen in people with significant underlying health conditions. However, the prevalence has been gradually rising and reports of cases have spread throughout the United States. Due to the high rates of vancomycin clinical treatment failure and associated morbidity and mortality rates of 60%–70%, these VISA and VRSA strains deserve serious attention (Walters *et al.,* 2015).

Virulence factors and disease association

S. aureus is one of the most common causes of nosocomial infection. *S. aureus* infections are often acute and pyogenic and, if untretated, may spread to surroundings tissue or, via bacteremia, to metastasic sites (involving organs) such as skin which is the cause Staphylococcal scalded skin syndrome (SSSS) in infants. Infection from Staphylococci can also cause life-threatening diseases such as pneumonia, meningitis, endocarditis, bacteremia, septicemia, cervicitis, and toxic shock syndrome (TSS) (Prescott *et al*., 2005).

The diverse range of *S. aureus* diseases has been attributed to its ability to produce an array of virulence factors. *S. aureus* expresses many potential virulence factors, such as surface proteins that promote colonization of host tissues; invasins that promote bacterial spread in tissues (i.e.

hyaluronidase); surface factors that inhibit phagocytic engulfment (capsule, Protein A); biochemical properties that enhance their survival in phagocytes (i.e. catalase production); immunological disguises (Protein A, coagulase); membrane-damaging toxins that lyse eucaryotic cell membranes (hemolysins, leukotoxin, leukocidin); exotoxins that damage host tissues or otherwise provoke symptoms of disease: Staphylococcus enterotoxins (SEs), TSST, exfoliative toxins (ET); and inherent and acquired resistance to anti-microbial agents (Todar, 2011; Cheung *et al.*, 2004).

Bacterial surface protein adhesins, known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) mediates *S. aureus* adherence to extracellular matrix (ECM) components to initiate colonization to the host colonize and resist physical removal (Foster and Höök, 1998). Certain MSCRAMMs acting as invasins, an adhesin (a surface protein in the cell wall of bacteria) molecule, can facilitate bacterial entry into the cell by phagocytosis, and place the bacteria in a vacuole. By entering the cytoplasm of the host cell, it has a ready supply of nutrients and is protected from complement, antibodies, and certain other body defenses. Once the bacterium escapes from the vacuole and is able to replicate in the cytoplasm of the host cell (Prescott *et al.,* 2005). This interaction is critical for infection and virulence and also permits internalization of pathogens into non-phagocytic host cells (Cambronne *et al.,* 2005; Niemann *et al*., 2004).

S. aureus is a clinically important capsule-forming bacterium, i.e., they produce uronic acid-containing, extracellular polysaccharides, which enhance microbial virulence by rendering the bacterium resistant to phagocytosis. The capsular polysaccharide (CPs) production appears to be an important virulence determinant, and occurs as different chemical structures depending on the serotype of the organism (Cook, *et al.,* 2009). This CPs are found in 90% of the clinical strains. Eleven serotypes have been described, type 5 and 8 are the most common among human isolates (80%) (Poutrel *et al.,* 1988).

Some pathogens can hide their unique antigenic surface components from the immunological system. *S. aureus* produces cell-bound coagulase and clumping factor that cause fibrin to clot and to deposit on the cell surface. It is possible that this disguises the bacteria immunologically so that they are not readily identified as antigens and targets for an immunological response (Todar, 2011).

According to the presence of enzyme coagulase, the virulence-pathogen *S. aureus* can be separated from the less virulent coagulase-negative Staphylococci species. There are three coagulase-positive Staphylococci species: *S. aureus* in humans and mammals, and *S. intermedius* and *S. hyicus* in vertebrates (Murray *et al.*, 1995). Coagulase negative staphylococci (CoNS) such as *S. epidermidis* do

not produce coagulase, are nonpigmented, and are generally less invasive but have increasingly been associated as opportunistic pathogens with serious nosocomial infections (Prescott *et al.,* 2005).

Two different forms of coagulase can be produced, free and bound. While free coagulase is an extracellular enzyme that is secreted extracellularly, bound coagulase is part of the larger family of MSCRAMMs. Bound coagulase, also known as clumping factor, reacts with fibrinogen to cause organisms to aggregate. Another factor, extracellular staphylocoagulase, reacts with prothrombin to form staphylothrombin, which can convert fibrinogen to fibrin. This results in clotting of the blood. Approximately 97% of human *S. aureus* isolates possess both of free and bound forms of coagulase. There are two different tests that can be performed to detect the presence of coagulase: a tube test to detect the presence of free coagulase, and a slide test to detect bound coagulase. Isolates that do not produce clumping factor must be tested for the ability to produce extracellular coagulase (free coagulase). Another way of *S. aureus* to hide its unique antigens from opsonizing antibodies or complement is by producing Protein A which is located on cell wall. Protein A is an IgG-binding protein which binds to the Fc end of an antibody, thus coating the bacteria with antibodies that helps inhibit phagocytic engulfment and acts as an immunological disguise (Todar, 2011).

Depending on the strain, *S. aureus* is capable of secreting several toxins that can be categorized into three groups: Pyrogenic toxin superantigens (PTSAgs), Exfoliative toxins (ET), and other toxins. Toxic shock syndrome (TSS) is associated with strains that produce and secrete exotoxin toxic shock syndrome toxin 1 (TSST-1), a member of PTSAgs family that has the ability to stimulate T-cells. Although first described in children, it was most frequently associated with women using tampons during menstruation. The staphylococcal enterotoxins which cause food poisoning also belong to PTSAgs family. These enterotoxins are divided into 6 types of heat-stable toxins, i.e. A, B, C1, C2, D, E, responsible for the gastrointestinal upset typical of food poisoning (Prescott *et al.,* 2005). Exfoliative toxins are implicated in the disease staphylococcal scalded-skin syndrome (SSSS), also known as Ritter disease, which occurs most commonly in infants and young children. The protease activity of exfoliative toxins causes peeling of the skin observed with SSSS (Bukowski *et al*., 2010).

Other Staphylococcal toxins that act on cell membranes include alpha hemolysin-toxin, beta hemolysin-toxin, delta hemolysin-toxin, and gamma hemolysin-toxin which are destroy erythrocytes, and several bicomponent toxins such as toxin Panton-Valentine Leukocidin (PVL), a pore forming toxin in the membranes of the infected cells, causing the cell's contents to leak out and cause cell death.

S. aureus is normal inhabitants of the upper respiratory tract, skin, intestine, and vagina, and known as the most important human staphylococcal pathogen. This bacterium, altogether with pneumococci and streptococci, is member of a group of invasive Gram-positive bacteria known as the pyogenic (or pus-producing) cocci, causing various supurative, or pus-forming diseases (e.g., boils, carbuncles, folliculitis, impetigo contagiosa, scalded-skin syndrome) in humans. *S. aureus* also cause medical device-related infections such as intravascular line sepsis and prosthetic joint infections (Prescott *et al.,* 2005).

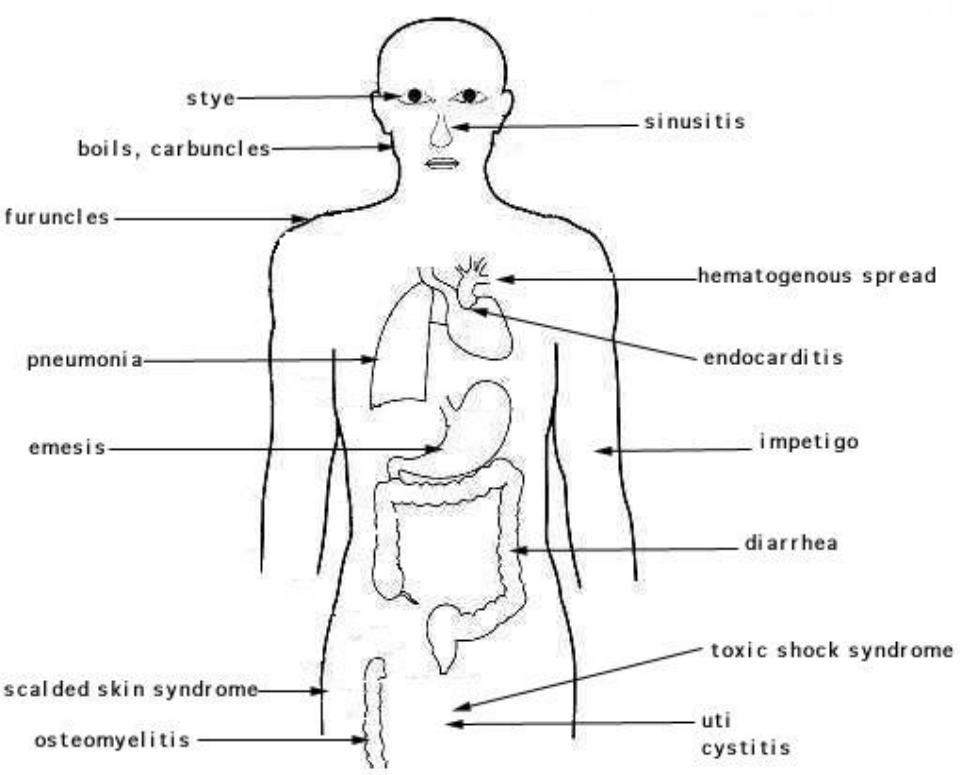

Fig. 5: Various sites of *S. aureus* infection (Todar, 2011).

***S. aureus* QS and biofilm**

The *S. aureus* quorum-sensing system is encoded by the accessory gene regulator (*agr*) locus, which consists of two divergent transcripts, RNAII and RNAIII, driven by the P2 and P3 promoters (Yarwood *et al*., 2004). RNAII contains four open reading frames designated *agrA* to *agrD,* that encode factors required to synthesize **a**uto-**i**nducing **p**eptide (AIP). The current working model suggests that the autoinducer peptide is generated from *agrD* and then processed, post-translationally modified, and secreted by a membrane-bound protein bearing an ABC-type transporter system known as AgrB. The sensing apparatus consists of the transmembrane receptor-histidine kinase AgrC. If sufficient

quantities of the appropriate AIP ligand have accumulated, AgrC phosphorylates AgrA. which then binds to the RNAIII transcript that encodes a small RNA that functions to modulate gene expression of *S. aureus* gene (Figure 6) (Chegelski *et al*., 2008).

A second quorum-sensing cascade, **R**NAIII **a**ctivating **p**rotein (RAP) and the **t**arget of **R**NAIII **a**ctivating **p**rotein (TRAP), is thought to serve as a precursor to the Agr system (Balaban and Novick, 1995). The RAP is secreted and accumulates outside the cell, and at a threshold concentration, RAP triggers the phosphorylation of the cytoplasmic protein TRAP, which induces expression of the RNAII transcript (Gov *et al*., 2004). Interestingly, a linear heptapeptide called RIP is known to block the activity of RAP, enabling small-molecule control over *S. aureus* pathogenicity (Balaban *et al.,* 1998).

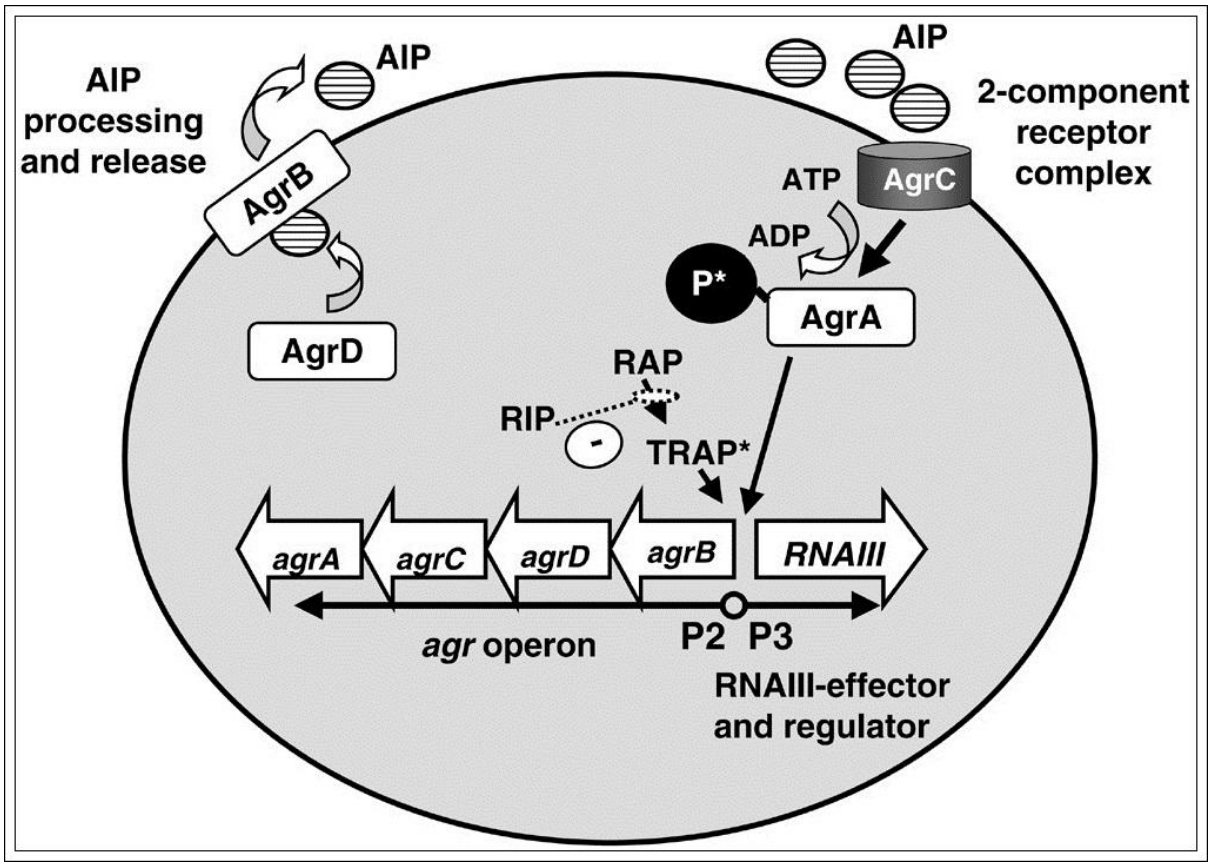

Fig. 6. Schematic diagram of quorum-sensing systems of *S. aureus*. The gene locus for the *agr* system contains two divergent transcripts, RNAII and RNAIII, driven by the P2 and P3 promoters. The RNAII transcript encodes the *agrBDCA* operon, which encodes the signal, processing and detection components for quorum-sensing in *S. aureus*. The RNAIII transcript is a regulatory RNA that up-regulates and down-regulates all genes related to quorum-sensing. Agr, accessory gene regulator; AIP, autoinducer peptide; P, promoter site; P*, phosphorylated protein; RAP, RNAIII-activating protein; RIP, RNAIII-inhibiting peptide; TRAP, target of RNAIII-activating protein (Asad and Opal, 2008).

The capability of *S. aureus* to form biofilms is one of the defense mechanisms that makes this bacterium difficult to eradicate with standard antibiotic treatment and makes it inherently resistant to host immune responses. The biofilm formation then consequently impedes the treatment of many chronic *S. aureus* biofilm related infections, including endocarditis, osteomyelitis and indwelling medical device infections (Costerton *et al*., 1999).

As well as *P. aeruginosa* biofilm, the development of a biofilm in *S. aureus* involves an initial attachment, maturation and detachment phase. Initial attachment can occur on tissues or after covering of an abiotic surface by host matrix proteins in the human body (specific, protein-protein interaction) or directly to an abiotic surface (nonspecific interaction) by surface hydrophobicity. MSCRAMMMs expressed by *S. aureus* have the capacity to bind to human matrix proteins such as fibrinogen or fibronectin (Patti *et al.,* 1994; Otto, 2008).

The major functional component responsible for intercellular adhesion essential for formation of multilayered *S. aureus* biofilm is the **p**olysaccharide **i**ntercellular **a**dhesin (PIA) which is also called **p**oly-***N***-**a**cetyl**g**lucosamine (PNAG). Together with other polymers such as **t**eichoic **a**cids (TA) and proteins, PIA forms the major part of slime, the extracellular matrix of biofilm-forming staphylococci (Otto, 2008). In *S. aureus* isolates from animals suffering from mastitis, a cell wall-bound surface protein named biofilm-associated protein, Bap, is involved in adherence to a polystyrene surface, intercellular adhesion, and biofilm formation (Cucarella *et al.,* 2001).

Biofilm detachment which may occur by the detachment of single cells or larger cell clusters is crucial for the dissemination of bacteria to other colonization sites. Some factors such as mechanical forces (e.g. blood vessel flow), cessation of the production biofilm building material such as exo-polysaccharide, and detachment factors such as enzymes that destroys the matrix, or surfactants-like molecules (e.g. δ-toxin or quorum-sensing-controlled surfactant peptides known as **P**henol-**s**oluble-**m**odulins (PSMs)) contributing to the detachment of biofilm (Boles and Horswill, 2008; Otto, 2008).

PSMs is a novel class of small peptides with an amphipathic α-helical structure and strong surfactant-like properties, are produced by most staphylococci, especially pathogenic *S. aureus* and *S. epidermidis*. PSMs can induce the production of proinflammatory cytokines; recruit, activate and lyse neutrophils to help staphylococci evade immune damage; lyse erythrocytes, and are associated with the hemolysis of staphylococcal disease; facilitate the structuring and detachment of

staphylococcal biofilms and disseminate biofilm-associated infection; and kill competing microbes and act as weapons in interbacterial warfare. Therefore, PSMs are considered to be critical virulence-associated factors and to play important roles in the pathogenesis of staphylococci. PSMs can be subdivided in two classes: an alpha-type that is 20 amino acids in length and a beta-type that is approximately 40-45 amino acids in length. Under biofilm conditions, PSM expression is shifted to the beta-type of PSM peptides, which are encoded in an operon (Yao *et al.,* 2005). After attachment to a surface and actively expressing PSM beta-type peptides, some cell clusters discontinue expressing PSM beta-type for yet unknown reasons, possibly due to limited oxygen concentration. Cell clusters with active PSM beta expression detach, leaving gaps in the biofilm, which ultimately leads to the typical structure of a biofilm with mushroom-likes structure and fluid-filled channels that are believed to have a vital function in delivering nutrients to cells in deeper biofilm layers (Costerton *et al.,* 1995; Otto, 2008).

An anti-biofilm drug in staphylococci that would inactive a factor required for biofilm formation that appears to be involved in biofilm-associated infection such as polysaccharide intercellular adhesin (PIA), is worth considering. *Actinobacillus actinomycetemcommitans* produces PIAse, named dispersin B which is a potential anti-biofilm drug (Kaplan *et al*., 2003). Lysostaphin, a peptidoglycan-degrading enzyme, although not biofilm-specific, is being evaluated for therapeutic use against staphylococci biofilm (Wu *et al.,* 2003; Otto, 2008).

Model organism: *C. albicans*

C. albicans belongs to the Ascomycota class of fungi and the family *Saccharomycetaceae*. *C. albicans* is a diploid fungus that can exist in two morphological states (dimorphism): as unicellular yeast (blastopores and chlamydospores), as well as in filamentous forms: hyphae, or pseudohyphae, a chain of distinct cells formed by the failure of mother and daughter to separate after each cell division. This dimorphism is depending upon environmental factors such as nutrients, CO_2, neutral pH, high temperature (37^{o}C), and cell population density (Kohler and Fink, 1996; Brown and Gow, 1999; Romano, 2008). *C. albicans* switched to the chlamydospore morphology when cultured under certain nutrient-poor media (Staib and Morschhäuser, 2007). Among *Candida* species, *C. albicans* has the strongest tendency to form hyphae, which parallels its role as the most virulent *Candida* species. *C. albicans*mutants defective in hyphal formation have almost completely lost their virulence (Sonneborn *et al*., 1999) Strains of *C. albicans* which are 'locked' into either yeast or hyphal morphology are dramatically attenuated in their virulence (Lo *et al.,* 1997).

C. albicans is also well known for its capability to undergo a process called phenotypic switching, which is superficially resembles dimorphism, and is thought to be important for *Candida*'s virulence (Thompson *et al*., 2011). Switching usually is generated spontaneously and discriminated by colony morphology and in some cases by the phenotype of cells in the budding phase. For example *C. albicans* strains WO-1 produces two different colonies: one that grows as round cells in smooth white colonies and one that is rod-like and grows as flat gray colonies. The switching is reversible, and colony type can be inherited from one generation to another (Tao *et al*., 2014).

The capability of *C. albicans* to change its morphological pattern from the yeast (Y) form to the mold or mycelial (M) form in the external environment (YM shift) is important in pathogenesis of candidiasis. In the yeast state, *Candida* is a non-invasive, sugar-fermenting organism, while in mycelia state it is invasive and can produce rhizoids, a filament used to anchor the fungus down. Rhizoids can penetrate mucosa or intestinal walls, leaving microscopic holes and allowing toxins, undigested food particles, bacteria, and yeast to enter the bloodstream (Ryan and Ray, 2004).

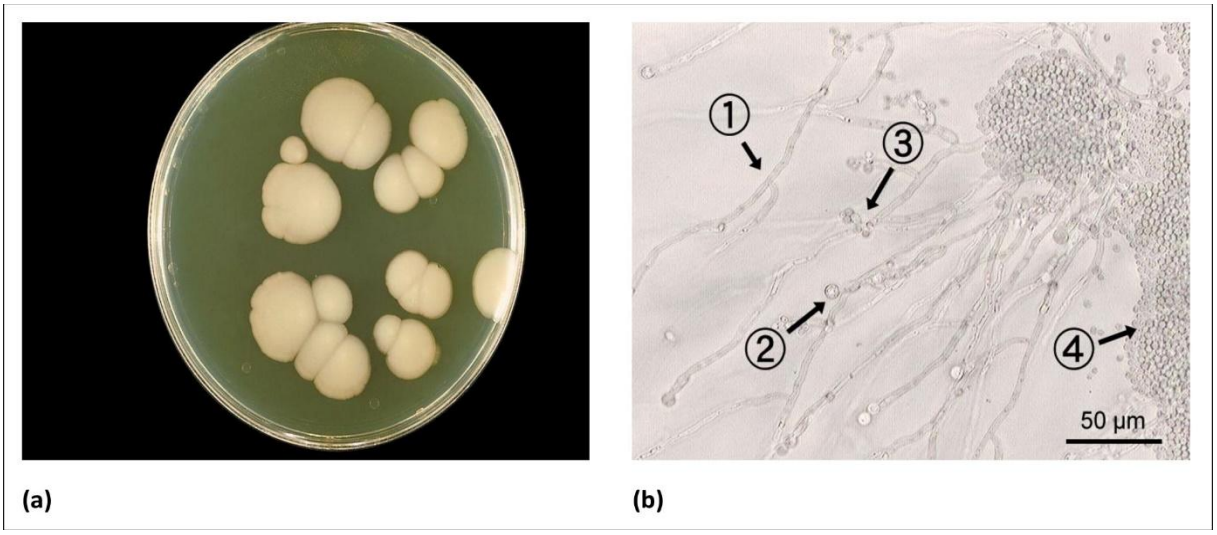

Fig. 7. Multiple views of *C. albicans*. (a) SABHI agar plate culture of the fungus *C. albicans* grown at 20°C, (b) Microscopic image (200-fold magnification) of *C. albicans* ATCC 10231, grown on cornmeal agar medium with 1% Tween80 : 1. Pseudohypha, 2. Chlamydospore, 3. Blastospore, 4. Yeast-like cells (Tambe, 2005[b]; Kaplan, 2009).

C. albicans virulence factors and disease association

Candida is the most common cause of fungal disease of mammals. Mycoses range from the common mild cutaneous or subcutaneous skin infections, to the potentially lethal acute or chronic infection of deep tissue. Of the *Candida* species afflicting humans, *C. albicans* is the most abundant and significant species, and the most commonly studied because it causes a variety of mycotic infections

in humans (Siqueira and Sen, 2004). This fungus has been claimed to be the fourth most common cause of hospital-acquired infectious disease in the United States (Miller *et al*., 2001).

One of the major reasons for *C. albicans* to be such an effective opportunist is that the organism has at its disposal an arsenal of virulence traits to cause a persistent infection in susceptible individuals. These traits include production of secreted aspartyl proteases and phospholipases, host recognition biomolecules (adhesins), phenotypic switching and the ability to undergo morphogenic shift from yeast-to-hyphae (Navarro-Garcia *et al.,* 2001; Si *et al*., 2013). The transition from unicellular yeast form to filamentous form (pseudohyphae or hyphae) is strongly required for *C. albicans* pathogenesis. Yeast forms are more suited for dissemination in tissues and to other hosts, whereas hyphal forms are required for tissue damage and invasion. Yeast cells, when phagocytosed by macrophages, produce hyphae and secrete hyphae-associated proteinases that kill macrophages. These factors prevent hyphal cells from being killed by neutrophils. In addition, hyphal cells have been shown to induce epithelial cell endocytosis. *C. albicans* cells induce epithelial cells to produce pseudopods that surround them, and pull them into the cell, helping *Candid*a cells to invade epithelial cells (Gow *et al*., 2012). The ability of *Candida* to form biofilms on catheters, endotracheal tubes, pace-makers, and other prosthetic devices has contributed to its predominant prevalence in nosocomial infections. Dental plaque is a well-known example of biofilm formation from *Candida* cells, and is responsible for oral candidiasis. Biofilm formation on such tissues is favored by a high concentration of glucose, serum, and other proteins (Calderone and Fonzi, 2001).

Normal amounts of *Candida* live in the mouth, stomach, and vagina, and do not cause infections. Candidiasis (*Candida* infections) can occur in the mouth, vagina, skin, stomach, and urinary tract when there is an overgrowth of *Candida*. Causes may include taking certain drugs (especially antibiotics, corticosteroids, and some birth control pills), pregnancy, being overweight, having a bacterial infection, or several different health conditions (for example, a weakened immune system, diabetes, and psoriasis). About 75% of women will get a vaginal yeast infection during their lifetime, and 90% of all people with HIV/AIDS develop *Candida* infections. Oral infections, called oral thrush, are most common in infants, elderly people, and those with a weakened immune system (Calderone and Fonzi, 2001).

***Candida* QS and biofilm**

QS has a contribution to morphogenic control in *C. albicans*. The yeast-to-hyphal shift *in vitro* is dependence on cell density, a phenomenon called the inoculum size effect. The inoculum effect is seen when yeast cell are diluted to concentrations less than 10^6 CFU in culture medium, under

conditions which affect the cells to germinate into the hyphal form (pH 7.5, 37^{o}C). Inoculation of the cells at higher concentration will maintain the cells in yeast morphology (Kruppa, 2008).

The basis for this cell-density-dependent control of morphogenesis is similar to that which is seen with bacterial cells regulating their activities via QS. The QS molecule in *C. albicans* is farnesol [(2E,6E)-3,7,11-trimathyldodeca-2,6,10-trien-1-ol], farnesoic acid [(2E,6E)-3,7,11-trimethyldodeca-2,6,10-trienoic acid], and tyrosol [4-(2-hydroxyethyl)phenol]. Farnesol was shown to prevent the germination of yeast cells into mycelia, a phenomenon that may be pertinent to *C. albicans* biofilm formation. Farnesoic acid, like farnesol, acts to block the yeast to filament transition, but it is far less active than farnesol and produced by only one known strain of *C. albicans* 10231, while tyrosol is a promoter of hyphal development, shortens the lag-time for cells to begin germinating when present under hyphal-inducing conditions (Oh *et al.,* 2001; Hornby *et al.,* 2001; Alem *et al.,* 2006).

The capacity of *C. albicans* to cause disease is closely associated with their ability to grown as biofilm communities, in contrast to planktonic growth. The biofilm of *C. albicans* formed in almost any medical devices including catheters, joint prostheses, cardiac valves, artificial bypass devices, pacemakers, contact lens, dentures, and intrauterine devices (IUDs). Candidiasis associated with indwelling devices can result in serious medical complications, expensive care and limiting the prolonged use of the devices, since removal of the device is nearly always necessary to cure the infections (Nett and Andes, 2006; Lal *et al.,* 2008).

Candida biofilm formation occurs in three developmental phases over a period of 24-72 hours. The initial phase (0-11 h) begins with the adherence of yeast cells to the substrate. The intermediate phase (12-30 h) involves the attached cells proliferations to form microcolonies and producing carbohydrate-rich extracellular matrix. The maturation phase (31-72 h) occurs when adherent cells are achieved with a biofilm consisting of interspersed yeast/hyphae cells encased in the thick extracellular matrix. Finally, newly formed yeasts on the surface of biofilm are released from the biofilm which allows further dissemination of the organism (Blankenship and Mitchell, 2006).

Biofilm-associated fungal cells are much more resistant to the traditional anti-fungal agents. Resistance of biofilm cells can be up to 1000-fold greater than that of planktonic cells and can also resist host immune factors (Hawser and Douglas, 1995). Several factors have been proposed for the increased anti-fungal resistance of *C. albicans* biofilms. These include altered growth／metabolic rate of cells within biofilm, presence of extracellular matrix with delayed penetration of the anti-

microbial agent, expression of resistance genes and presence of persister (drug tolerant) cells (Tobudic *et al.,* 2011). A biofilm consists of a heterogeneous population of cells with different growth rates; therefore, a subpopulation of cells could also confer anti-fungal resistance because of their slower growth rate (Kumamoto, 2002). Extracellular matrix can act as a physical barrier that prevents the access of anti-microbials to cells embedded in the biofilm community. This barrier is depends on both, amount and nature of the extracellular matrix, as well as the physicochemical properties of the drug (Tobudic *et al.,* 2011).

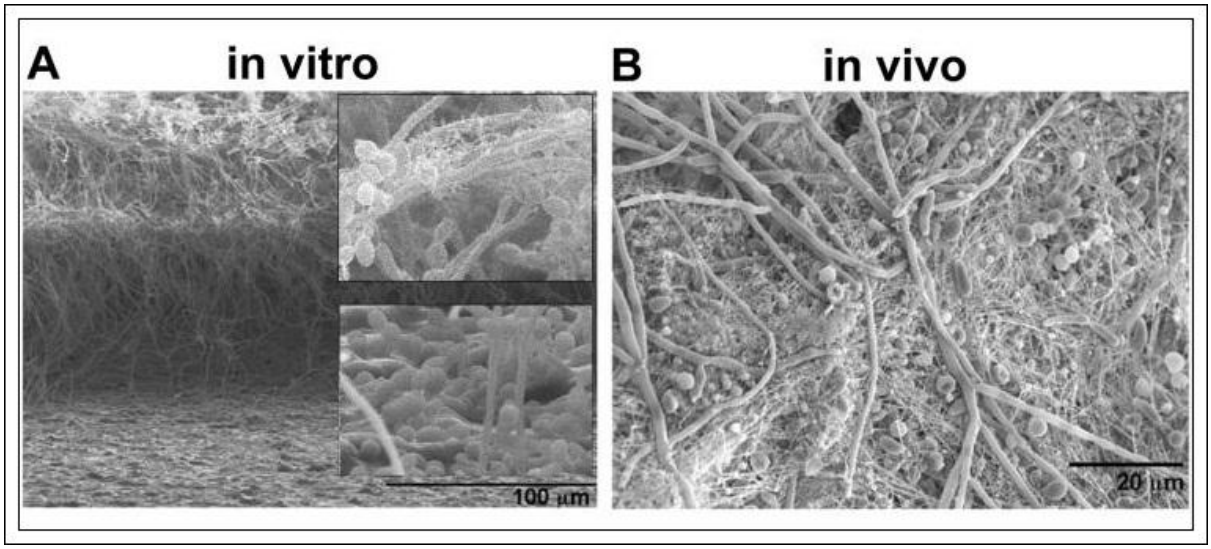

Fig. 8: *C. albicans* biofilm structure *in vitro* and *in vivo.* A) Scanning electron micrograph (SEM) of an *in vitro* biofilm. The biofilm sample was sliced to reveal three layers in a cross-sectional view. The basal layer includes primarily yeast cells, as evident in the lower enlarged inset. The central layer is mainly hyphae. The upper layer has yeast cells budding from the hyphae. The upper enlarged inset shows extracellular matrix material, which appears fibrous in this preparation. B) SEM of an *in vivo* biofilm from the rat catheter model. Yeast cells, hyphae, and some pseudohyphal cells are evident, along with extracellular matrix material (Finkel and Mitchell, 2011).

The involvement of two different types of membrane-localised efflux pumps, adenosine triphosphate-binding cassette transporters encoded by the ***C****andida* **d**rug **r**esistance (CDR) genes and major facilitators encoded by the **m**ulti-**d**rug **r**esistance (MDR) genes increased azole anti-fungal resistance in planktonically grown of *C. albicans*. The expression of MDR and CDR genes in biofilms is phase-specific, contributing to azole resistance only during the early phases of biofilm growth. Moreover, changes in the membrane sterol composition also contribute to azole resistance in the intermediate and mature phases of C*. albicans* biofilm (Mukherjee *et al*., 2003).

Drug discovery from natural sources

Natural products are products from natural origin, including an entire organisms (plants, animals, or microbes) that have not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g., drying); part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ); an extract of an organism or part of an organism, exudates, and pure compounds (e.g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc.) isolated from plants, animals, or microorganisms (Sarker *et al*., 2006).

Traditional medicinal use of natural products plays a major role since in ancient civilizations and in many parts of the world. The importance of natural products as a source of medicines is acknowledged by the World Health Organization (WHO). There are many example of drugs derived from natural product, such as compounds from plants: artemisinin from *Artemisia annua* (Weathers *et al.,* 2011), Taxol from *Taxus baccata* (Malik *et al*., 2011); compounds from microbes: menisporopsin B from *Menisporopsis theobromae* (Chinworrungsee *et al.,* 2006), hirsutellide A from *Hirsutella kobayasii* (Vongvanich *et al*., 2002); and compounds from animals (Trabectedin from *Ecteinascidia turbinata* (Monk *et al*., 2012), and Ziconotide from *Conus magus* (McGivern, 2007).

Natural products remain to play an important role in the discovery of new drugs in the coming years as a rich source of novel chemotypes. It is because of plants and other organisms produce many biologically active compounds for defense and other purposes that are often very complex and offer incomparable structural diversity. Synthetically prepared drugs can never compete with the natural variation therefore the screening and isolation from natural sources is the only feasible way to access them. Natural products have also been an invaluable source to synthesize novel drug candidates. There are three roles of natural products to the development of new drugs; by acting as new drugs to be used directly for the treatment of disease; by indicating new modes of pharmacological action which allow complete synthesis of novel analogs (e.g. synthesis analogs of penicillin from *Penicillium notatum*); and by providing natural blueprint used for the synthesized of more complex molecules (e.g. diosgenin from *Dioscorea floribunda* for the commercial synthesis of cortisone, pregnenolone, progesterone, and other steroid products) (Sarker *et al.,* 1999; Li and Vederas, 2009). The structural analysis of natural compounds and the ability to synthesize them allowed chemist to modify them in order to suppress or enhance certain characteristics such as solubility, efficiency or stability in the human body (Shelley, 2009; Ji *et al.,* 2009).

Plants as anti-microbial therapies

The search for pharmaceutically active compounds to combat new and existing diseases is ongoing, and higher plants have often been targeted as part of this search because they contain an abundance of potentially active secondary metabolites. It is estimated that there are 250.000 to 500.000 species of plants on Earth (Boris, 1996). About 155.000 seed plants occur in the tropics, with about 120.000 in the tropical moist forest alone (only 7-8% of the land surface on earth). This is due to the high humidity, elevated temperature, species density and a continuous growing season. The result is an enormous diversity of chemical structures, including secondary metabolites involved in many biological processes and interactions, e.g., as attractants of pollinators, signal products, defensive substances against predators and parasites, or in resistance against pests and diseases. Since only 5-15% of the higher plants have been systemically investigated for the presence of the bioactive compounds, nature's biodiversity remains largely unexplored (Cragg *et al*., 1997; McGee, 2006).

Plants have always played a central role in the prevention and treatment of disease since the ancient time. Many modern medicines are derived from traditional medicinal plants. The illnesses treated by medicinal plants cover an incredibly broad range, from minor ailments such as cuts and skin infections, to more severe diseases including typhoid fever, diabetes, and cancer-like conditions (Clardy and Walsh, 2004). The goals of using plants as sources of therapeutic agents are: to isolate bioactive compounds for direct use as drug, e.g., atropine, scopolamine, digoxin, digitoxin, morphine, reserpine, taxol, vinblastine, vincristine; to produce bioactive compounds from novel or known structures, using them as lead compounds for (semi)synthesis of novel patentable entities with better activity and/or lower toxicity, e.g., metformin, nabilone, oxycodone, taxotere, taniposide, terapamil, podophyllotoxin; to use natural product as pharmacological tools, e.g., lysergic acid diethylamide, mescaline, strychnine, yohimbine; and to use the whole plant or part of it as a herbal remedy, e.g., cranberry (*Vaccinium macrocarpon*), *Echinacea*, feverfew (*Tanacetum parthenium*), garlic (*Allium sativum*), Ginkgo (*Ginkgo biloba*), St. John's wort (*Hypericum perforatum*), and saw palmetto (*Serenoa repens*). There are four standard approaches available for selecting plants for medicinal purposes: random selection followed by chemical screening, random selection followed by anti-microbial assay, follow-up of anti-microbial assay reports, and follow-up of ethnomedical or traditional uses of plants against infectious diseases (Fabricant and Farnsworth, 2001).

Following the Amazon rain forests, Indonesia has the second biggest biodiversity in the world

expressed by a high number of indigenous medicinal plants. As an archipelagic state with thousands of islands, Indonesia is endowed with a rich and unique biodiversity. The area of Indonesian tropical forests covers about 143 million hectares and is inhabited by about 80% of the world's medicinal plants. It is estimated that the Indonesian tropical forests inhabit 28,000 plant species. There are various reports concerning the inventory of higher plant in Indonesia. The Indonesian Country Study on Biodiversity (ICSBD 1993) puts the number of flowering plants species in Indonesia between 25,000 and 30,000. Some 40 million Indonesians depend directly on the country's biodiversity, and the Indonesian community makes use of around 6,000 plant species. Data of the number of medicinal plants also vary. Zuhud *et al.* (2001) identified 1,845 species with medicinal potential in the forests of Indonesia. These numbers are potentially to be updated due to the continuing inventory and investigation of yet unidentified species (Damayanti *et al.,* 2011).

A large number of anti-microbial agents derived from traditional medicinal plants are available for treating various diseases caused by microorganisms. There are vast amount of published scientific information from around the globe describing the anti-microbial activities of plant extracts against a range of bacteria, or fungi. The anti-bacterial activity also could be due to various chemical components. The useful major groups of anti-microbial phytochemicals can be divided into several categories that include alkaloids, flavones (flavonoids, flavonols, quinones), essential oils, lectins, polypeptides, phenolics, polyphenols, tannins and terpenoids (Samy and Gopalakrishnakone, 2008).

The use of medicinal plants in Indonesia has always been a part of culture that has been passed down from generation to generation. Throughout the centuries, Indonesia's indigenous people developed traditional medicines from plants identified by their forefathers for curing illness and keeping their health (Bermawie, 2004). By trial-and-error the country's early inhabitants learns how to distinguish useful plants with beneficial effects from those that were either toxic or non-active. They picked, kept and used medicinal plants to satisfy their basic needs and even experimented on combinations of plants or processing methods to gain optimal results. The abundantly available medicinal plants in Indonesia might be the great potential source for the isolation of novel, naturally-occuring anti-microbial active compounds with high activity against microbial biofilms.

Main hypotheses and aims of this thesis

Hypothesis: Some traditionally used Indonesian medicinal plants have anti-infective properties which may affect biofilm formation of human pathogenic microorganisms such as *P. aeruginosa*, *S. aureus* and *C. albicans*.

Specifically this research will investigate of the presence of anti-microbial and anti-biofilm activity of Indonesian medicinal plants against *P. aeruginosa*, *S. aureus* and *C. albicans* planktonic growth and biofilm. The construction of extract libraries of plant material will provide the source for screening and elucidation of anti-microbial (anti-biofilm) compounds. The capability of these plants in inhibiting QS systems also will be determined. Purification and characterization together with determination of the mode of action of the active compound(s) from these plants species is important in order to obtain compound(s) that may be used for drug candidate against biofilm infection and will be powerful tools in the successful treatment of microbial infections.

Aim and Thesis Outline

Microbial biofilms are responsible for several chronic diseases that are difficult to treat. Biofilm protects the microbial from killing by antibiotics. Even sensitive microbials that do not have a known genetic basis for resistance can have profoundly reduced susceptibility when they form a biofilm. In biofilms, poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, and formation of persister cells are hypothesized to constitute a multi-layered defense. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms (Stewart and Costerton, 2001; Stewart, 2002). This thesis aims to discover new anti-microbials from medicinal plants with a high activity against microbial biofilms and to analyze if such compounds will exhibit a synergistic effect with other known antibiotics.

Chapter 1 reviews the discovery of drugs from natural sources, the use of plants as anti-microbial therapies, the quorum sensing mechanism in microbial related to biofilm formation, biofilm involved infections, antibiotic resistance of biofilm cells and emergence of multi-resistant microbial pathogens and strategies to discover anti-biofilm therapeutics.

Chapter 2 describes the screening for anti-microbial compounds on microbial biofilms. Compounds are isolated from Indonesian medicinal plants by ethanol extraction to discove new candidate antibiotics to be used against microbial biofilms.

Chapter 3 and 4 are focused on the discovery of novel essential oils as anti-microbials from Indonesian medicinal plants. The effect of the plant essential oils on bacterial cells and microbial biofilms is studies using viability staining, fluorescence microscopy analysis and colony forming unit (CFU) determination along with toxicity assay of the isolated compound(s).

Chapter 5 focuses on the screening of the Indonesian medicinal plants ethanol extract and essential oils for their capability to inhibit the quorum sensing mechanisms.

The purification and structural identification of the most potential compounds to obtain a novel anti-biofilm compound along with the analysis of a synergistic effect of novel anti-biofilm activities isolated from medicinal plants in combination with known antibiotic used to treat biofilms is described in **Chapter 6**.

In **Chapter 7** a general discussion is given about the results of the research described in this thesis.

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Chapter 2

Anti-microbial effects of Indonesian Medicinal Plants Extracts on Planktonic and Biofilm Growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

The increasing rates of antibiotic-resistant microbial infections requiring continuous development of new anti-microbial agents. Moreover, microbial biofilms are exhibits elevated resistance to both most anti-microbial drugs and the host defense systems, which often results in persistent and difficult-to-treat infections. The discovery of anti-infective agents which are active towards planktonic and biofilm microbial are consequently required to deal with these biofilm-mediated infections. The aim of this study is to evaluate the activity of Indonesian medicinal plants extracts on planktonic growth and biofilm of *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I. Fifty four (54) ethanol extracts were obtained from a variety of known Indonesian medicinal plants. The growth inhibitory concentration (PMIC), effects on biofilm formation and biofilm breakdown, and biofilm architecture in the absence and presence of the extracts by confocal laser-scanning microscopy along with LIVE/DEAD staining were performed. The extracts showed an inhibitory effect on planktonic growth of these bacteria and also on their biofilm formation. At a concentration as low as 0.12 mg/mL, biofilm formation of *P. aeruginosa* PAO1 and *S. aureus* Cowan I is inhibited by 5 plant ethanol extracts : *Kaempferia rotunda* L., *Caesalpinia sappan* L., *Cinnamomum burmannii* Nees ex Bl., *C. sintoc* and *Nymphaea nouchali* Burm.f. Limited bacteriostatic activity was evident. The results obtained clearly indicate the extracts obtained are interesting sources of putative anti-biofilm agents. This research can contribute to the development of new strategies to prevent and treat biofilm infections.

Keywords : medicinal plants, anti-biofilm, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* Cowan I.

INTRODUCTION

In the past, it was thought that microorganisms are free-floating single-celled (planktonic) organisms. They rapidly multiply and are living an individualistic lifestyle in nutrient rich media. However, in nature most microorganisms mostly live together in large numbers, attached to a surface, forming structured layers. This feature is known as a biofilm. A biofilm community can be formed by a single kind of microorganism, but in nature biofilms can also consist of mixtures of many species of bacteria, as well as fungi, algae, yeasts, and protozoa (Hall-Stoodley *et al*., 2004; Jones, 2007).

Biofilms of infectious microorganisms play an important role in human health (Jefferson, 2004) and because of their resistance to detergents and anti-microbial agents they are difficult to treat. The National Institute of Health (NIH) estimates that biofilms are involved in at least more than 65% of nosocomial infections and up to 75% of microbial infections occurring in the human body (Richards and Melander, 2009). Biofilms of infectious microorganisms are also formed on medical instruments and implants such as catheters, artificial heart valves, contact lenses, and artificial joints, putting patients at risk for local and systemic infectious (Donlan and Costerton, 2002; O'grady *et al*., 2002). In addition, the prevalence of microbial resistance to many commonly used antibiotics tends to increase. These findings enlarge the need for new anti-microbial compounds.

Since ancient times, man has used plants for healing, although incapable to find a rational explanation for their curing effects. According to the World Health Organization, the use of traditional medicine (TM) continues to play an important role in health care. In many parts of the world, it is the preferred form of health care. About 80% of people in developing countries, especially in rural areas, use TM as the primary source of medicine (Kim, 2005). There are approximately 500.000 plant species occurring worldwide, and less than 1% has been screened for biologically active compounds (Palombo, 2006). The Indonesian Country Study on Biodiversity (ICSBD, 1993) places the number of species of flowering plants in Indonesia between 25.000 and 30.000. Of the total flora of Indonesia, 10% is expected to have pharmaceutical potential. There is a large variety of plants that are used as medicine (Sunesi and Wiryono, 2007).

Previously, antibiotic discovery and characterization has been performed mostly with planktonic bacteria. Therefore, it can be predicted that compounds that are suitable to inhibit biofilm formation still need to be discovered. Up to now, only a few compounds, isolated from natural products with activity against microbial biofilm formation have been reported (Hentzer *et al*., 2003). Eugenol isolated from clove showed inhibition of *Candida albicans* biofilm formation (Shufford *et al*., 2005;

He *et al*., 2007). *Aeromonas hydrophylla* biofilm formation is inhibited by vanillin (Ponnusamy *et al*., 2009). Usnic acid, a secondary lichen metabolite, is also capable to inhibit *Pseudomonas aeruginosa* biofilm formation (Francolini *et al*., 2004). In this study, we screened extracts of Indonesian medicinal plants with respect to their capacity to inhibit biofilm formation and or to breakdown the biofilms of two known human opportunistic pathogens, the Gram negative strain *P. aeruginosa* PAO1 and the Gram positive strain *Staphylococcus aureus* Cowan I. *P. aeruginosa* and *S. aureus* are bacteria that cause nosocomial infections worldwide and can form biofilms which play an important role in various acute infections.

The plants investigated in this paper were those predicted and known to have anti-microbial properties based on the studies and local uses of the plants (Nawawi *et al*., 1999; Sangat and Larashati, 2002; Batugal *et al*., 2004; Elfahmi *et al*., 2014). However, very few studies have investigated Indonesian medicinal plants for their anti-biofilm activities. This study focused particularly on the idea that Indonesian medicinal plants might be the source of new anti-biofilm compounds. Therefore, specific focus was given to the screening programs for anti-microbial and anti-biofilm activity, which may yield candidate compounds for developing new anti-biofilm compounds that may provide a powerful tool in the discovery of a successful treatment for biofilm infections.

Our result demonstrated the effectiveness of *Kaempferia rotunda* L., *Caesalpinia sappan* L., *Cinnamomum burmannii* Nees ex Bl., *C. sintoc* L., and *Nymphaea nouchali* Burm.f extract towards *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm. This property can be applied clinically to treat infectious biofilm along with conventional antibiotics, or applied industrially as to remove biofilm from water pipes.

MATERIALS AND METHODS

Plant material and extraction

Indonesian medicinal plants were collected from Yogyakarta, Indonesia and its surroundings on the basis of ethnopharmacological information during January – May 2009. The plant materials were identified, authenticated and preserved at Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia for further reference.

Plants samples were washed, cut into small pieces and oven dried at 40°C for 48-72 hours (Salie *et al*., 1996; Zakaria *et al*., 2007; Farooq *et al*., 2014). The drying process prevents degradation through metabolic process, and prevents microbial development. The drying temperature may vary from 35°C to 70°C depending on the part of the plant and sensitivity of the active principles. For the leaves, a temperature range of 20°C – 40°C is recommended. Drying plant material in oven with low drying temperatures between 30°C and 50°C is faster than exposure plant materials to fresh air (shaded from direct sunlight), and still capable to protect sensitive active ingredients (Cechinel-Filfo, 2002). The dried plant materials were ground into a fine powder. The pulverized materials were extracted by maceration using Petroleum Ether (PE) in a ratio of 1 g (plant material) : 10 mL PE to remove the lipids. The plant material of which lipids have previously been removed were again extracted with 70% ethanol (EtOH) using a ratio of 1 g (plant material) : 10 mL (EtOH) to obtain crude ethanol extract. Furthermore, extracts were dried and concentrated under reduced pressure using a rotary evaporator. Stock solutions (100 mg/mL) of crude ethanol extract in dimethyl sulfoxide (DMSO) were prepared, filter-sterilized (0.2 µm) and stored at 4°C.

Determination of planktonic growth inhibitory concentration (PMIC)

Preparation of the inoculums used for inoculums standard and the growth phase of the microbial tested at the time of testing are important factors that needs to be carefully controlled. *P. aeruginosa* PAO1 and *S. aureus* Cowan I was grown on LB agar plates at 28°C and 37°C, respectively. A single colony was inoculated in 5 mL LB broth. After overnight growth, the OD_{600} was set to a 0.5 McFarland standards, i.e. optical density 600 nm 0.01 equal to 10^7 CFU/mL. Cells were incubated for 2 hours and the final OD_{600} was diluted to 10^5 CFU/mL.

Inhibiting concentration of extracts was determined by the microtiter broth method in sterile flat-bottom 96-well polystyrene plates using Mueller-Hinton broth medium (Difco). Experiments were perfomed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007), with concentration ranging from 0.06 to 1 mg/mL. Controls were: media control, and infected untreated control (100% growth). DMSO used as vehicle control, and streptomycin concentration of 1024 µg/mL was used as positive control. All tests were performed in triplicate. Culture plates were incubated overnight at 37 °C for *S. aureus* Cowan I and 28 °C for *P. aeruginosa* PAO1. Optical density readings were obtained using microplate reader (Bio-Rad 680 XR) at 595 nm.

Growth reduction was calculated as % of inhibition by using the formula mentioned below. The % of inhibition of replicate tests was used to determine the final Planktonic Minimum Inhibitory

Concentration (PMIC) values. The concentration at which the extract depleted the growth of bacterial by at least 50% was labeled as the PMIC50.

$$\% \text{ inhibition} = \left(1 - \left(\frac{ODt24 - ODt0}{(ODgc24 - ODgc0)}\right)\right) \times 100\%$$

ODt24 = optical density (595 nm) of the test well at 24 h post-inoculation; ODt0: optical density (595 nm) of the test well at 0 hour post-inoculation; ODgc24: optical density (595 nm) of the growth control well at 24 hours post-inoculation; ODgc0: optical density (595 nm) of the growth control well at 0 hour post-inoculation (Quave *et al*., 2008). The OD time zero was used to account for the effect of plant extract color on the OD595 nm reading.

Effect on biofilm formation and biofilm breakdown

To test for the inhibition activity of plant extract on biofilm formation, a PVC (polyvinyl chloride) flexible U bottom 96 wells plates were used (Falcon 3911, Becton Dickinson, Franklin Lakes, NY). To determine biofilm formation inhibition and biofilm breakdown activity, extracts at sub inhibitory concentration (concentration under $PMIC_{50}$) ranging from 0.03-0.5 mg/mL were used to ensure a concentration that is not affecting the microbial growth. Negative controls (cells + media: TSB for *S. aureus* Cowan I and M63 supplemented with 20% casamino acid, 20% glucose and 1mM MgSO4 for *P. aeruginosa* PAO1), positive control (cells + media + streptomycin), vehicle controls (cells + media +DMSO), and media controls were included. For the positive controls concentrations of 1024 µg/mL streptomycin were used, prepared by serial dilution techniques. Blanks undergo the same treatment as samples, but without incubation. All tests were performed in triplicate.

Plates were incubated for 24 h at 28^{o}C for *P. aeruginosa* and 48h at 37 ^{o}C for *S. aureus*. After 24-48 h incubation, the content of the well was aspired, rinsed 3 times with distilled water, and dried at room temperature for 10 min. Then, 125 µL of 1% crystal violet stain was added to the wells for staining for 15 min. The excess stain was rinsed off with tap water and 200µL methanol was added to the wells, and transferred to a flat-bottom 96-well plates. Optical density readings were obtained by a plate reader at 595 nm. Biofilm formation inhibition was calculated as % of inhibition by using the formula mentioned below. The % of inhibition of replicate tests was used to determine the final

minimum biofilm inhibitory concentration (MBIC) values. The concentration at which the extract depleted the bacterial biofilm by at least 50% was labeled as the $MBIC_{50}$.

$$\% \text{ inhibition} = \left(1 - \left(\frac{\bar{X}\ ODt - \bar{X}\ ODmc}{(\bar{X}\ ODvc)}\right)\right) \times 100\%$$

ODt= optical density (595 nm) of the test well; ODmc: optical density (595 nm) of the media control well; ODvc: optical density (595 nm) of the vehicle control well (Quave *et al*., 2008; Sandasi *et al*., 2009).

The efficacy of plant extract on established biofilm (biofilm breakdown) was also studied, as described by Nostro *et al.* (2007) with some modifications. Biofilms were grown on 96-well plates for 24-48 h. At post-inoculation time, planktonic cells and media were removed, and fresh media was added together with the test extract. Plates were placed back into the incubator for another 24-48 h. The staining methods have been described above. Percentage of inhibition was calculated, as described before, to determine the final minimum biofilm eradication concentration (MBEC) values. The concentration at which the plant extract capables to breakdown the bacterial established biofilm by at least 50% was labeled as the $MBEC_{50}$.

Biofilm architecture

Confocal laser-scanning microscopy (CLSM) was used to study the structure of the *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilms (Jin *et al*., 2005). Bacterial biofilms were grown under static conditions on glass slides in sterile tubes. To examine effect of extract on inhibiting biofilm formation, fifteen mL of LB media in a sterile tube with or without plant ethanol extract was inoculated with the different bacteria to an OD_{600} of 0.1 from overnight grown LB cultures. Glass slides were submerged in this suspension and tubes were incubated for 24h or 48h at 28 °C or 37 °C. For analysis the effect of extract in breaking down the biofilm, bacterial biofilm were grown under static conditions on glass slides in sterile tubes for 24h or 48h at 28 °C or 37 °C. Following the incubation period, the suspensions of bacteria were removed and glass slides were rinsed with 0.15 M phosphate-buffered saline (PBS, pH 7.0) to remove unattached cells. Fifteen mL of LB media with or without plant ethanol extract were poured into the tubes, and the tubes then incubated for another 24h or 48h at 28 °C or 37 °C.

Prior to CLSM analysis, glass slides were rinsed with 0.15 M phosphate-buffered saline (PBS, pH 7.0) to remove unattached cells. After a washing with PBS, the bacterial biofilm on the cover-glass slide was incubated for 15 min with 1.5 µL of 3.34 mM SYTO9 in anhydrous DMSO to stain the living organisms, and with 1.5 µL of 20mM Propidium Iodide (PI) in anhydrous DMSO to stain the dead organisms. SYTO9 penetrates intact bacterial membranes (life) and stains the cells green; while PI penetrates only cells with damaged membranes (dead) and stains the cells red. The life organisms, freshly cultured and subsequently harvested, were used for staining control. Cells killed by heating in 100 ^{o}C were used for PI staining control. Stained biofilms were observed with a Carl Zeiss LSM 5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany). A 40× oil immersion objective was used with 488 nm Ar laser excitation and 500–640 nm band pass emission setting. The images were subsequently analysed using the freely available image processing software imageJ version 1.46 (Rasband, National Institutes of Health (NIH), Bethesda, Maryland, USA: http://rsb.info.nih.gov/ij/) including the LSM reader plugin to open LSM5 formatted image stack created by the microscope software. The images' scale bar used to calibrate the ImageJ area measurement algorithm. The observations were made in triplicates and representative images are presented here (Dusane *et al*., 2012).

The image obtained has 2 channels (red and green) and converted into a composite image with: Image>Color>Make composite. By default, it will assign red to channel #1, green to #2. Brightness and contrast levels were then adjusted to give the best differentiation between the live (green) and dead (red) areas. The scale bar was determined with: Analyze > Tools > Scale bar. Estimated 3D surface plot was obtained using: Plugins > 3D > Interactive 3D Surface Plot. Data containing arrays of the type (x, y, z) where x and y are the coordinates of the pixel positioning and the luminance of an image is interpreted as height for the plot (z): http://rsbweb.nih.gov/ij/plugins/surface-plot-3d.html.

Statistical analysis

For statistic analysis, all data were initially analyzed by a normal distribution using the one-sample Kolmogorov-Smirnov test. Following the confirmation of normal distribution, differences for individual parameters between treated group and control were tested by one way ANOVA, followed by Dunnett's test. A *P* value of 0.05 or less was considered to be statistically significant.

RESULT AND DISCUSSION

Preparation of ethanol extract from 54 Indonesian plants

During this study, fifty four plants (Table 1) were collected from Yogyakarta, Indonesia and its surroundings. The ethanol extracts of these plants were obtained as described in Material and Methods. Briefly, plant materials of which lipids were previously been removed were macerated in an ethanol solution (70%). Crude ethanol extracts were obtained after filtration and the evaporation of this solution (Zhang *et al*., 2004; Kosar *et al*., 2007).

Table 1: Indonesian medicinal plants tested for anti-biofilm activity

Voucher Number	Plant name	Part used
STP001	*Curcuma xanthorrhiza* Roxb.	Rhizome
STP002	*C. heyneana* Val. & v.Zijp	Rhizome
STP003	*C. aeruginosa* Roxb.	Rhizome
STP006	*Zingiber officinale* Roxb.	Rhizome
STP007	*Zingiber officinale Roscoe* (*var. rubrum Theilade*)	Rhizome
STP004	*C. domestica* L.	Rhizome
STP011	*Kaempferia galanga* L.	Rhizome
STP013	*Boesenbergia pandurata* (Roxb.) Schlecht.	Rhizome
STP005	*C. mangga* Val. & v.Zijp	Rhizome
STP012	*Kaempferia rotunda* L.	Rhizome
STP014	*Languas galanga* (L.) Stuntz.	Rhizome
STP008	*Z. aromaticum* Val.	Rhizome
STP009	*Z. zerumbet* (L.)J.E. Smith	Rhizome
STP015	*Elettaria cardamomum* (L.) Maton	Fruit
STP018	*Cosmos caudatus* H.B.K.	Leaves
STP016	*Sonchus arvensis* L.	Leaves
STP019	*Pluchea indica* (L.) Less.	Leaves
STP017	*Elephantophus scaber* L.	Leaves
STP020	*Blumea balsamifera* (L.) DC.	Leaves
STP021	*Psidium guajava* L.	Leaves
STP022	*Syzygium aromaticum* (Linn.) Merr.	Flower
STP024	*Apium graveolens* L.	Leaves
STP026	*Foeniculum vulgare* Mill.	Fruit
STP027	*Piper betle* L.	Leaves
STP029	*P. retrofractum* Vahl.	Fruit
STP040	*Terminalia catappa* L.	Leaves
STP041	*Azadirachta indica* A.Juss	Leaves
STP039	*Averrhoa bilimbi* L.	Leaves

STP031	*Citrus aurantifolia* Swingle	Leaves
STP032	*Tamarindus indica L.*	Leaves
STP034	*Caesalpinia sappan* L.	Bark
STP042	*Sesbania grandiflora* L.PERS var. Rubra	Leaves
STP033	*Andropogon citratus* (DC.) Stapf.	Leaves
STP044	*Clerodendron serratum* (L.) Spreng.	Leaves
STP043	*Sauropus androgynus* (L.) Merr.	Leaves
STP045	*Andrographis paniculata* (Burm.f) Nees	Leaves
STP035	*Myristica fragrans* Houtt.	Seeds
STP036	*Guazuma ulmifolia* Lmk.	Leaves
STP046	*Ocimum basilicum* L.	Leaves
STP047	*Orthosiphon stamineus* Benth.	Leaves
STP048	*Coleus scutellaroides* (L.) Benth.	Leaves
STP038	*Anredera scandens* (L.) Moq.	Leaves
STP037	*Phaleria macrocarpa* (Scheff.) Boerl.	Leaves
STP050	*Litsea cubeba*(Lours.) Pers.	Bark
STP051	*Cinnamomum burmannii* Nees ex Bl.	Bark
STP052	*C. sintoc* Bl.	Bark
STP049	*Tinospora tuberculata* Beumee.	Leaves
STP054	*Paederia foetida* L.	Leaves
STP055	*Melastoma polyanthum* Bl.	Leaves
STP056	*Stelechocarpus burahol* (Blume) Hook F. & Thomson	Leaves
STP058	*Stachytarpheta mutabilis* (Jacq.) Vahl.	Leaves
STP059	*Alyxia stellate* Roem & Schult.	Bark
STP060	*Parameria laevigata* (A.Juss.) Moldenke	Bark
STP061	*Nymphaea nouchali* Burm.f.	Flower

Effects of ethanol extracts on planktonic growth, biofilm formation and biofilm breakdown of *P. aeruginosa* PAO1 and *S. aureus* Cowan I

Plant extracts assayed in this research were selected based on their anti-bacterial activity that was reported in the literature. The maximum concentration of 1 mg/mL of plant ethanol extracts for testing was chosen based on the previous study by Rios and Recio (2005) who reported that extracts should be avoided exhibiting minimum inhibitory concentration (MIC) values higher than 1 mg/mL or isolated compounds exhibiting MIC values higher than 0.1 mg/mL. The inoculums concentration used in this study is 10^5 CFU/mL. According to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2007) and protocols for broth microdilution minimum inhibitory concentration (MIC) testing (Rankin, 2005), a $5x10^5$ CFU/mL inoculum provides an acceptable challenge dose for assessing the biological activity of anti-microbial agents, and is large enough to provide statistically satisfactory data. If the inoculum is too small, significant bacteria resistance may not be detected. As

demonstrated by Barry *et al.* (1983), the growth phase may not have significant effect on MIC determination assay, however, it does critical in the MBC assay because if a stationary phase of inoculums is used, the number of surviving cells tends to increase after 24 hours incubation and makes the MBC result artificially high.

The percentage of planktonic growth inhibition was measured from the reduction in absorbance level of the treatments wells which is indirectly measure the bacterial cells biomass, compare to negative control well (without the presence of the test compounds). The negative control was assumed to have 0% activity compare to the compounds tested. The $PMIC_{50}$ concentration was calculated using probit analysis. Based on the probit analysis data, extract concentration around $PMIC_{50}$ and below (sub-PMIC) were used for anti-biofilm assay, to ensure a concentration that is not affecting the microbial growth.

As shown in Table 2, most of the crude extracts used in this study have limited anti-bacterial activity against planktonic growth of *P. aeruginosa* PAO1 and *S. aureus* Cowan I. As much as 20 out of 54 plant extracts tested failed to give inhibition on planktonic and biofilm growth of bacteria tested. The lowest concentration of plant ethanol extract to inhibit growth of the bacteria tested shown by *C. xanthorrhiza* and *M. fragrans* which give 50% growth inhibition of *P. aeruginosa* PAO1 at concentration of 0.25 mg/mL, whereas *C. xanthorrhiza* and *M. fragrans* ethanol extract showed a $PMIC_{50}$ against the growth of *S. aureus* Cowan I at concentration of 0.12 mg/mL. In addition to testing of the plant extracts for inhibition of planktonic growth we also have investigated their effect on biofilm formation and biofilm breakdown. Crystal violet staining has been widely adopted by microbiologist to investigate biofilm formation and attachment of microorganisms to diverse surfaces. This staining method is inexpensive, relatively quick, and adaptable for use in high-throughput screening with microtiter plates (Niu and Gilbert, 2004).

Using the crystal violet method, we have found that the inhibition of biofilm formation and biofilm breakdown by plant ethanol extract was dose dependent (Figure 1 and 2) in both *P. aeruginosa* PAO1 and *S. aureus* Cowan I. Plant ethanol extract concentration of 0.12 mg/mL is the lowest concentration which shows 50% inhibition on *P. aeruginosa* biofilm formation (Table 2). Only five of the 54 extracts tested inhibit ≥ 50% of *P. aeruginosa* PAO1 biofilm formation at that concentration (Figure 1). Ethanol extract of *N. nouchali* at concentration of 0.12 mg/mL inhibit *P. aeruginosa* biofilm formation as much as 54.7±0.3 % (**P<0.01). As much as 51.1±0.6 % and 53.4±0.5 % (**P<0.01) inhibition of *P. aeruginosa* biofilm formation were obtained by ethanol extract of *C. sappan* and *C. burmannii* respectively, and 51.0±0.5 % and 53.0±0.3 % by *K. rotunda* and *C. sintoc*

respectively (**P<0.01). In addition, 4 of 54 extracts show 50% inhibition on *P. aeruginosa* biofilm formation at an extract concentration of 0.25 mg/mL and 6 extracts at an extract concentration of 0.5 mg/mL (Table 2).

The lowest concentration which shows 50% of biofilm breakdown of *P. aeruginosa* (Table 2) is 0.5 mg/mL and only three of extracts tested shows that activity. *N. nouchali* extract at a concentration of 0.5 mg/mL shows as much as 52.8±0.3 % (**P<0.01) degradation of the *P. aeruginosa* PAO1 preformed biofilm, and ethanol extracts of *C. sappan* and *K. rotunda* shows 52.2±0.6 % and 50.6±0.5 % degradation respectively (**P<0.01) (Figure 3A).

The lowest concentration of ethanol extracts which causes 50% inhibition on *S. aureus* Cowan I biofilm formation was also 0.12 mg/mL. As much as 51.29±0.61% inhibition of *S. aureus* biofilm formation was observed by incubation of *S. aureus* with *K. rotunda* ethanol extract at concentration of 0.12 mg/mL (**P<0.01). At the same concentration, *N. nouchali, C. sappan, C. burmannii* and *C. sintoc* cause 53.4±0.6 %, 52.5±0.32 %, 51.4±0.6 % and 50.6±0.5 % inhibitions of *S. aureus* biofilm formation (**P<0.01) (Figure 2). In addition, 4 of the 54 extracts show 50% inhibitions on *P. aeruginosa* biofilm formation at an extract concentration of 0.25 mg/mL and 10 extracts at an extract concentration of 0.5 mg/mL (Table 2).

Similar as for breakdown of preformed *P. aeruginosa* biofilm, only 5 of the 54 ethanol extracts caused 50% breakdown of preformed biofilm of *S. aureus* Cowan I at an extract concentration of 0.5 mg/mL. In the presence of the ethanol extract of *C. sappan* at a concentration of 0.5 mg/ml, preformed biofilm of *S. aureus* was decreased as much as 53.8±0.4 % (**P<0.01). At the same concentration, *C. burmannii, K. rotunda* and *N. nouchali* show the capability to degrade *S. aureus* biofilm as much as 50.0±0.3 %, 50.2±1.0 % and 52.9±0.3 % (**P<0.01), respectively (Figure 3B).

Qualitative analysis of *P. aeruginosa* and *S. aureus* biofilm

The activity of the extracts on the biofilm formation inhibition and biofilm breakdown was analysed by confocal laser scanning microscope (CLSM), along with LIVE/DEAD staining as described in Material and Methods. Examples of estimated 3D surface plot of the biofilm are shown in Figures 4-7. Qualitative analysis of biofilm structure by CLSM indicated an evident disruption of the biofilm structure resulting from exposure to plant extract. Viability staining using LIVE/DEAD staining showed that both life and dead cells were present in the analyzed biofilms. The control cells fluorescened green indicating that the cells were alive, embedded in a polysaccharide matrix that stimulates cell clustering.

Table 2: Effects of ethanol extracts on planktonic growth, biofilm formation and biofilm breakdown of *P. aeruginosa* PAO1 and *S. aureus* Cowan I.

Plant	Planktonic anti-bacterial activity ($PMIC_{50}$) in µg/mL*		Anti-biofilm formation activity ($MBIC_{50}$) in mg/mL*		Biofilm breakdown activity ($MBEC_{50}$) in mg/mL*	
	***P.aeruginosa* PAO1**	***S. aureus* Cowan I**	***P.aeruginosa* PAO1**	***S. aureus* Cowan I**	***P.aeruginosa* PAO1**	***S. aureus* Cowan I**
Curcuma xanthorrhiza Roxb.	0.25	0.12	-	-	-	-
C. heyneana Val. & v.Zijp	-	0.51	-	0.58	-	-
C. aeruginosa Roxb.	-	0.25	0.25	-	-	-
Zingiber officinale Roxb.	-	0.55	0.25	-	-	-
Z. officinale Roscoe var. rubrum Theilade	-	0.52	0.25	-	-	-
Kaempferia galanga L.	-	0.50	0.25	-	-	-
C. mangga Val.&v.Zijp	-	1.03	-	0.53	-	-
Kaempferia rotunda L.	-	0.50	0.11	0.11	0.50	0.50
Languas galanga (L.) Stuntz.	0.52	0.50	-	-	-	-
Z. zerumbet (L.)J.E. Smith	-	-	-	0.27	-	-
Elettaria cardamomum (L.) Maton	-	-	-	0.52	-	-
Cosmos caudatus H.B.K	-	-	-	0.62	-	-
Pluchea indica (L.) Less.	-	-	0.54	-	-	-
Elephantophus scaber L.	-	1.06	-	-	-	-
Blumea balsamifera (L.) DC.	-	-	0.53	-	-	-
Psidium guajava L.	1.08	1.02	-	-	-	-
Apium graveolens L.	-	-	-	0.52	-	-
Piper betle L.	1.05	0.50	0.51	0.25	-	-

P. retrofractum Vahl.	1.05	0.25	0.57	-	-	-
Terminalia catappa L.	-	1.14	-	-	-	-
Azadirachta indica A.Juss	1.05	1.08	-	-	-	-
Tamarindus indica L.	-	-	-	0.51	-	-
Caesalpinia sappan L.	0.52	0.25	0.11	0.10	0.49	0.49
Sesbania grandiflora L. PERS Leaves	-	-	-	0.55	-	-
Clerodendron serratum (L.) Spreng *(*Leaves)	1.12	1.05	-	-	-	-
Sauropus androgynus (L.) Merr.	-	-	0.62	0.25	-	-
Myristica fragrans Houtt.	0.25	0.12	-	-	-	-
Ocimum basilicum L.	-	-	-	0.53	-	-
Orthosiphon stamineus Bth.	-	-	-	0.57	-	-
Litsea cubeba (Lours.) Pers.	-	1.05	-	-	-	-
Cinnamomum burmannii Nees ex Bl.	1.01	1.01	0.09	0.11	-	0.50
C. sintoc L.	1.03	0.54	0.10	0.12	-	-
Melastoma polyanthum Bl.	-	-	-	0.62	-	-
Nymphaea nouchali Burm.f.	0.54	0.52	0.10	0.10	0.48	0.48

*****A dash (−) represents that no $PMIC_{50}$, $MBIC_{50}$ or $MBEC_{50}$ was identified within the concentration range tested.

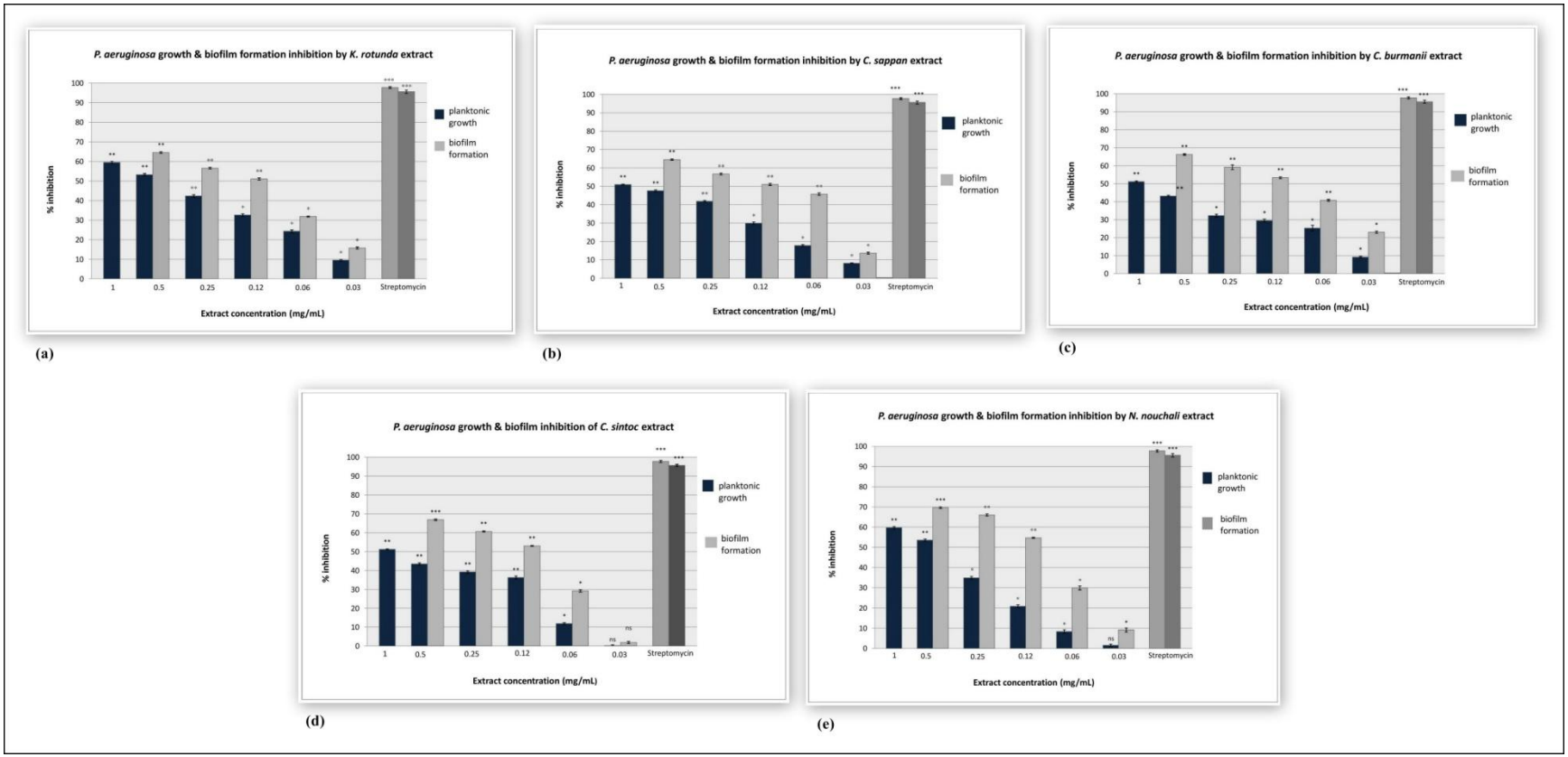

Fig. 1: Percentage of inhibition in planktonic growth and biofilm formation of *Pseudomonas aeruginosa* PAO1 by plant ethanol extracts at different concentrations. (a) *Kaempferia rotunda*, (b) *Caesalpinia sappan*, (c) *Nymphaea nouchali*, (d) *Cinnamomum burmannii*, (e) *Cinnamomum sintoc*. P: Planktonic growth, B: Biofilm formation. Streptomycin concentration used is 1024 µg/mL. The standard deviation in the percentages are indicated by bar. Asterisks indicate a significant difference between treatment and negative control.

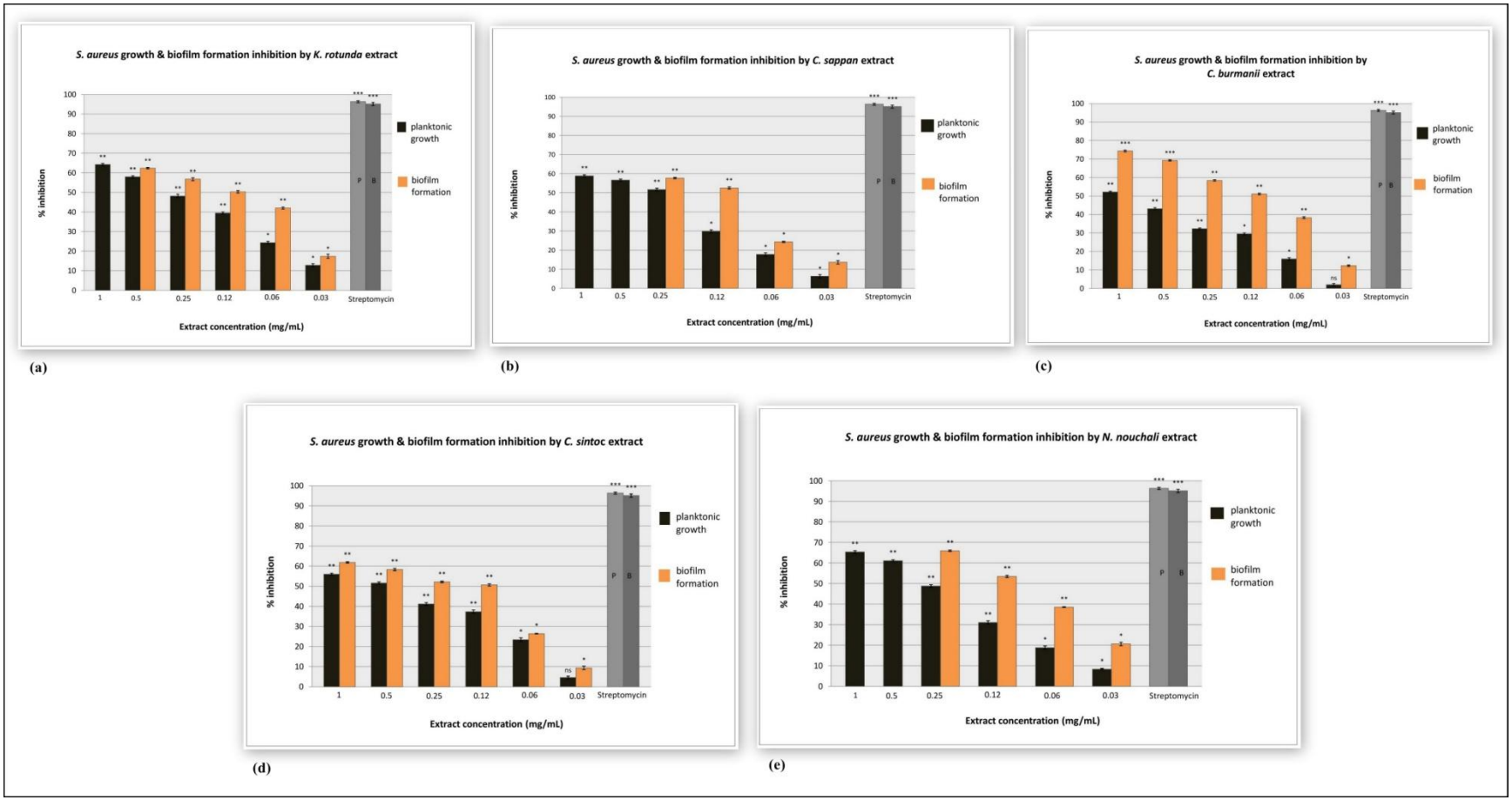

Fig. 2: Percentage of inhibition in planktonic growth and biofilm formation of *Staphylococcus aureus* Cowan I by plant ethanol extracts at different concentrations. (a) *Kaempferia rotunda*, (b) *Caesalpinia sappan*, (c) *Nymphaea nouchali*, (d) *C. burmannii*, (e) *Cinnamomum sintoc*. P: Planktonic growth, B: Biofilm formation. Streptomycin concentration used is 1024 µg/mL. The concentration of Streptomycin used is 1024 µg/mL. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

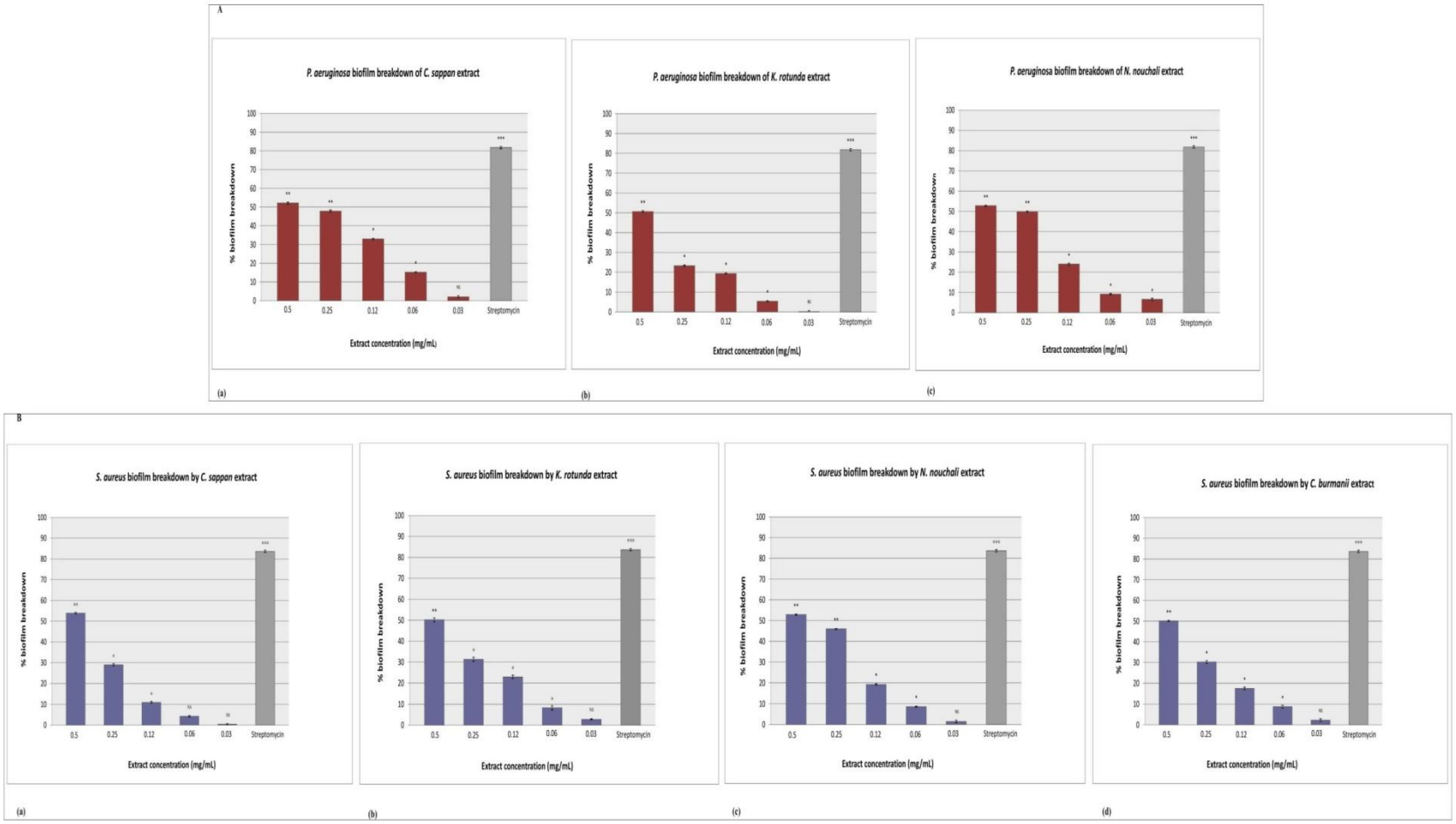

Fig. 3: Percentage degradation of biofilm of A) *Pseudomonas aeruginosa* PAO1 or B) *Staphylococcus aureus* Cowan I by plant ethanol extracts at different concentrations. The concentration of Streptomycin used is 1024 µg/mL. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Ethanol extracts from *K. rotunda, C. sappan , C. burmannii*, *C. sintoc*, and *N. nouchali* at concentration of 0.12 mg/mL significantly reduced *P. aeruginosa* PAO1 and *S. aureus* Cowan I initial biofilm formation compared to the negative control (biofilm cells without addition of plant extract) which is densely packed. The initial biofilm formation inhibition by plant ethanol extracts was found to be concentration dependent. The presence of 0.25 mg/mL extract resulted in loss of aggregates structures. The cells were found scattered individually along the substratum (Fig 4-5).

At concentration of 0.5 mg/mL, the ethanol extracts from *C. sappan*, *K. rotunda* and *N. nouchali* showed capability in breaking down preformed biofilm of both bacterial tested even more than at a concentration of 0.25 mg/mL (Fig 6-7). The biofilm exposed to the plant extracts were disrupted, leaving small aggregates which are remained attached to the substrate compare to the densely packed cells in untreated biofilm control (without the presence of extract) (Fig 6-7).

CLSM images showed that plant ethanol extracts tested significantly prevent the formation of biofilm at concentration of 0.12 mg/mL. Compared to the cells in the control that formed cells clusters and attached to the substratum, the amount of cells in the clusters, embedded in the EPS matrix was diminished with the presence of plant extract. It seems that bacterial growth was inhibited before the cells were able to promote attachment on the surface. However, the result showed that activity on biofilm breakdown was more difficult to achieve than inhibition in cell attachment. The concentration of plant extract needed to disrupt preformed biofilm was higher (0.5 mg/mL) than the concentration needed to inhibit the initial attachment. It is evident that cells in a biofilm are more resistant to anti-microbial agents compare to free floating cells (Stewart, 2002).

The cell attachment is the initial stage in biofilm formation following formation of film consists of nutrients, organic and inorganic molecules adsorbed on a surface (surface conditioning). The surface conditioning is important for the growth of cells and often creates a favorable environment for bacterial attachment, which in turn promotes cell adhesion to surfaces which subsequently leads to infections (Sandasi *et al.*, 2009). It can therefore be postulated that the presence of plant extracts in growth media produced an unfavorable condition that could inhibit cell attachment or reduce the surface adhesion (Sharon and Ofek, 2002; Klueh *et al*., 2000).

The reduced susceptibility of bacteria in a biofilm is thought to be due to a combination of several factors. The presence of extracellular polymer substances (EPS) containing mainly polysaccharides, proteins and nucleic acids and other compounds that surrounds biofilm cells contribute to the anti-microbial resistance properties of biofilms by impeding the mass transport of antibiotics through the biofilm (Donlan and Costerton, 2002). The anti-microbial agent is adsorbed onto the EPS and effectively diluted before it reaches the individual bacterial cells in the biofilm (Dibdin *et al.,* 1996). Killing by many anti-microbial agents is growth dependent by targeting macromolecular synthesis. Reduction in oxygen and nutrients availability in biofilm leads to cell growth limitation and bacterial macromolecular synthesis is arrested. This among others makes the bacterial cells in the biofilm less susceptible to anti-microbial agents (Stewart, 2002; Lewis, 2001). Our study suggests that the inhibition activity of the plant ethanol extract of bacterial biofilm formation and the disperse existing of biofilms appears to be coupled with biocidal/biostatic activity. These results are helpful for designing novel biofilm inhibitors and developing more effective therapeutic methods.

The activity of *K. rotunda, C. burmannii, C. sappan, C. sintoc* and *N. nouchali* ethanol extracts to inhibit *P. aeruginosa* PAO1 *and S. aureus* Cowan I initial biofilm formation and degradation of formed biofilm has not been reported previously. It has been reported that *K. rotunda* contains flavonoids, crotepoxide, chalcones, quercetin, flavonols, β-sitosterol, stigmasterol, benzoic acid, syringic acid, protocatechuic acid and some hydrocarbons such as camphor. The abundant presence of flavonoids in this plant has interpreted as the involvement in antioxidant mechanisms as a prime role (Mohanty *et al*., 2008). Resins, tannin and essential oils which contain cinnamaldehyde, cinnamyl acetate, eugenol and anethole are present in *C. burmannii* bark. Other chemical components of the essential oil include ethyl cinnamate, beta-caryophyllene, linalool, and methyl chavicol. Eugenol oil that can be used as an ingredient in cosmetics is also present in *C. sintoc* bark (Sangat and Larashati, 2002; Jantan *et al*., 2005). Especially cinnamaldehyde and eugenol are proved to be active against many pathogenic bacteria and fungi (Ooi *et al*., 2006; Shan *et al*., 2007; Gende *et al*., 2008). Nuryastuti *et al*., (2009) reported the potency of *C. burmannii* oil to combat both planktonic and biofilm cultures of clinical *Streptococcus epidermidis* strains, with MICs ranging from 0.5 to 1%, and 1 to 2%, respectively. A study based on cell permeability assay and electron microscopy observation on cinnamaldehyde revealed that anti-bacterial mechanism of cinnamaldehyde are possibly due on its interaction with cell membrane causes disruption on membrane permeability, and the leakage of intracellular constituents (Gill and Holley, 2004; Huang *et al*., 2014).

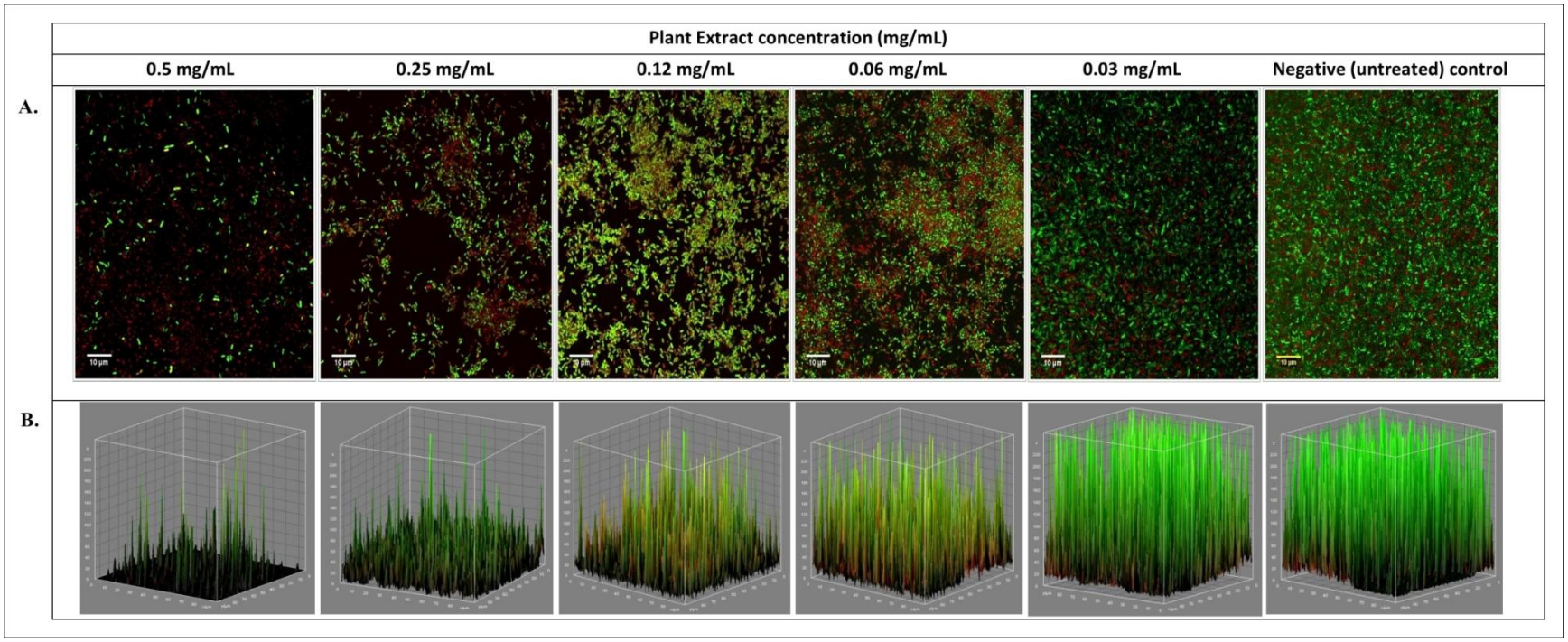

Fig. 4: Biofilm inhibition activity of *Cinnamomum burmannii* ethanol extract against *Pseudomonas aeruginosa* PAO1. A: projected upper view of the biofilm; B: estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensitycollected using ImageJ. Extract concentration from 0.5 mg/mL – 0.03 mg/mL. Negative control is *P. aeruginosa* PAO1 biofilm growth without the presence of extract.

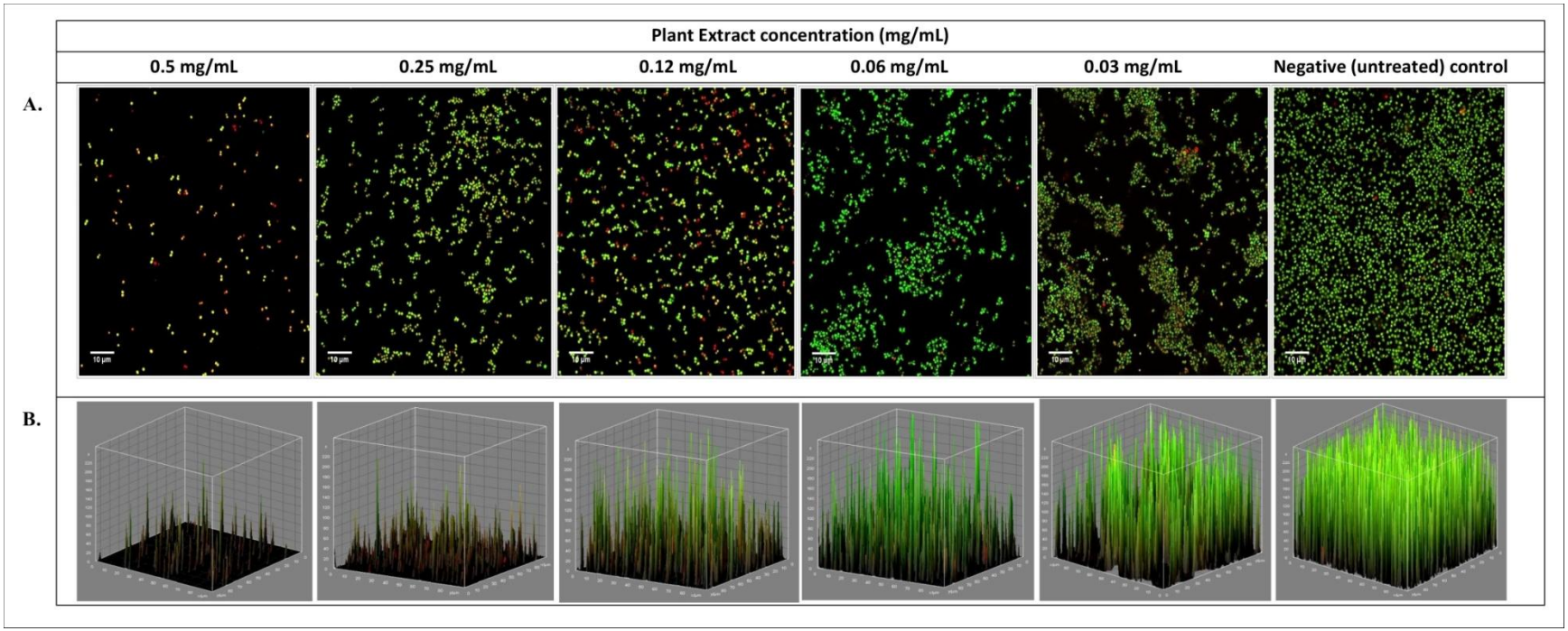

Fig. 5: Biofilm inhibition activity of *Nymphaea nouchali* ethanol extract against *Staphylococcus aureus* Cowan I. A: projected upper view of the biofilm; B: estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using ImageJ. Extract concentration from 0.5 mg/mL – 0.03 mg/mL. Negative control is *S. aureus* Cowan I biofilm growth without the presence of extract.

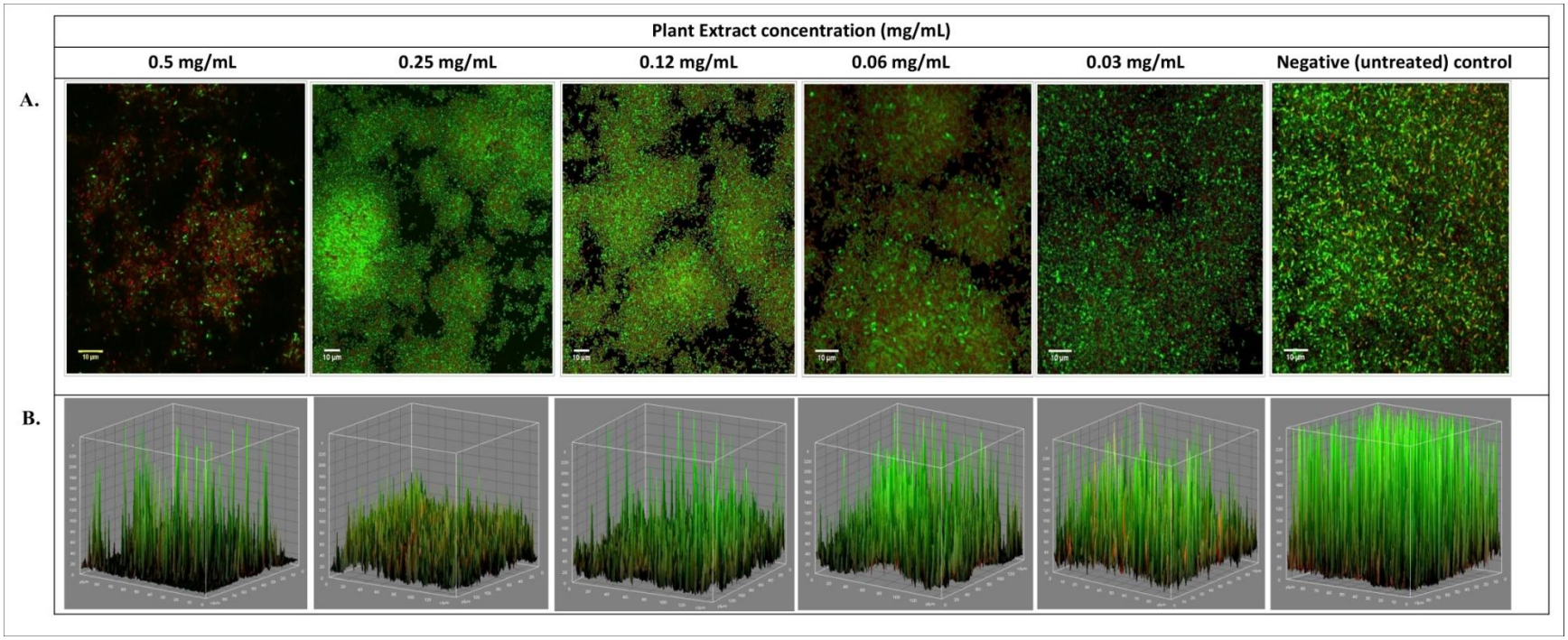

Fig. 6: Biofilm breakdown activity of *Kaempferia rotunda* ethanol extract against *Pseudomonas aeruginosa* PAO1. A: projected upper view of the biofilm; B: estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using ImageJ. Extract concentration from 0.5 mg/mL – 0.03 mg/mL. Negative control is *P. aeruginosa* PAO1 biofilm without the presence of extract.

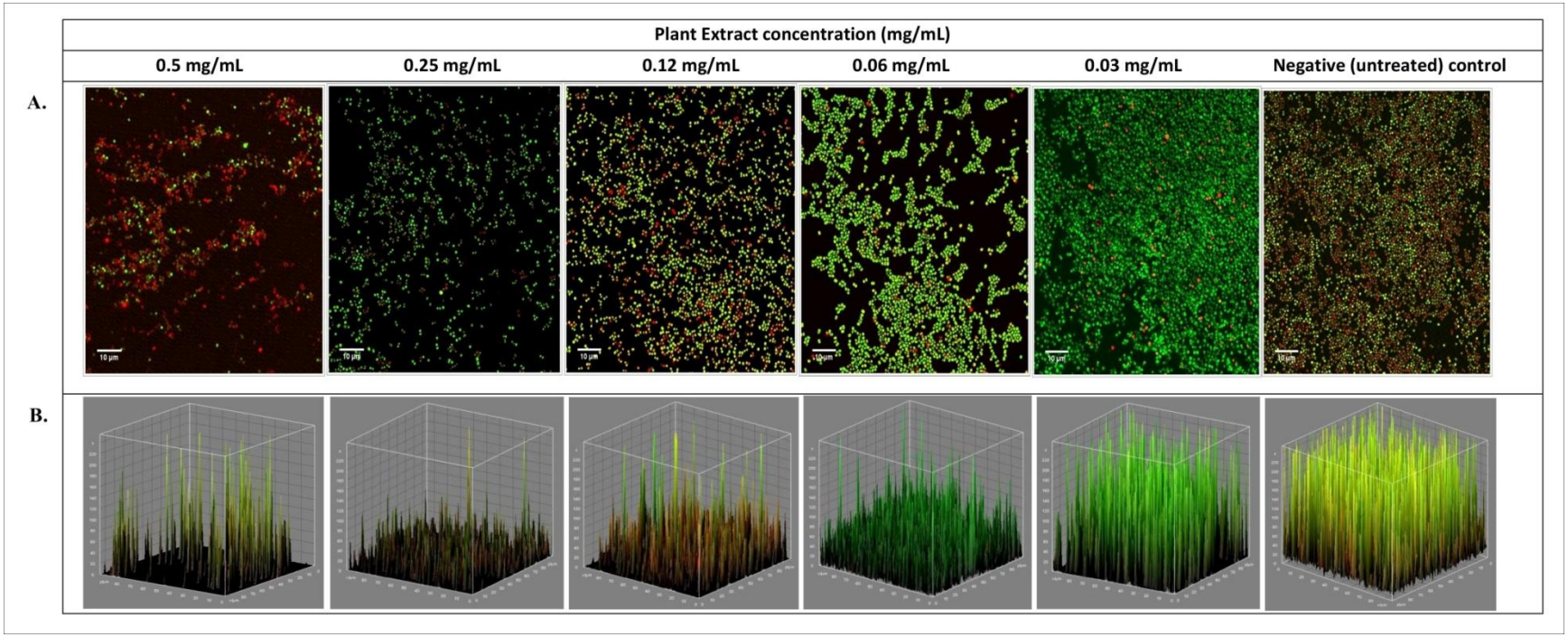

Fig. 7: Biofilm breakdown activity of *Caesalpinia sappan* ethanol extract against *Staphylococcus aureus* Cowan I. A: projected upper view of the biofilm; B: estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using ImageJ. Extract concentration from 0.5 mg/mL – 0.03 mg/mL. Negative control is *S. aureus* Cowan I biofilm without the presence of extract.

Phytochemical investigations on heartwood and other parts of *C. sappan* (sappan wood), have resulted in reports of various compounds including triterpenoids, lipids, amino acids, flavonoids, and phenolic compounds such as 4-*O*-methylsappanol, protosappanin A, protosappanin B, protosappanin E, brazilin, brazilein, caesalpin J, brazilide A, neosappanone A, caesalpin P, sappanchalcone, 3-deoxysappanone, 7,3',4'-trihydroxy-3-benzyl-2H-chromene (Nagai *et al*., 1986; Namikoshi and Saitoh, 1987). Brazilin is known to have anti-bacterial activity. It has also the potency to be developed into an antibiotic. A study from Xu and Lee (2004) suggested that the antibacterial activity of brazilin could be attributes to its capability on inhibit bacterial DNA and protein synthesis. However, the exact anti-bacterial mechanism of action of brazilin remains unknown at this time.

The chemical constituents of *N. nouchali* (red and blue water lily), synonym *N. stellata* Willd flowers, contain quercetin, luteolin, isoquercitrin, kaempferol, galuteolin, and alkaloids. The seeds are rich in starch, and also contain raffinose, proteins, fats, carbohydrates, calcium, phosphorus, iron, nuciferine, oxoushinsunine, N-norarmepavine. The rhizome contains starch, protein, asparagine, and vitamin C. It also contains catechol, d-gallocatechol, neochlorogenic acid, leucocyanidin, leucodelphinidin, and peroxidase. The roots contain tannic and asparagine. The leaves of this plant contain roemerine, nuciferine, nornuciferine, armepavine, pronuciferine, N-nornuciferine, DN-methylcoclaurine, anonaine, liriodenine, quercetin, isoquercitrin, nelumboside, citric acid, tartaric acid, malic acid, gluconic acid, oxalic acid, succinic acid, and tannin. Oxoushinsunine, found on the seed coat, suppress the development of throat cancer while the seeds and stalks have efficacy as anti-hypertension (ICSBD, 1993; Nagavani and Rao, 2010). Studies found that quercetin, a flavonoid compound, has anti-bacterial properties which, possibly due to the DNA gyrase inhibition that is required for DNA synthesis (ICSBD, 1993; Cushnie and Lamb, 2010).

Biofilm formation can be controlled by quorum sensing, a bacterial communication system which causes a rapidly and coordinately change of expression pattern in the bacterial population in response to population density. The fact that in sub-PMIC concentration, the *K. rotunda, C. sappan, C. burmannii, C. sintoc* and *N. nouchali* ethanol extracts are capable of disturbing biofilm formation and biofilm breakdown suggests that this disturbance may have been caused by the presence of compounds inhibiting quorum sensing. Similarly, Rasmussen *et al.* (2005) reported that carrot, garlic, habanero (chili), and water lily produce compounds that interfere with bacterial quorum sensing. Halogenated furanone compounds isolated from marine algae *Delisea pulchra* inhibit biofilm formation influence microbial biofilm formation by interfering with bacterial quorum sensing (Manefield *et al*., 1999). Other plant's compounds could attenuate biofilm development by inhibit bacteria's peptidoglycan synthesis (Ogunlana *et al*., 1987), disrupt the permeability barrier of

microbial membrane structures, causing the cell to leak out (Cox *et al*., 2000), modify bacterial membrane structure hydrophobicity (Turi *et al*., 1997; Das, 2014), disturbing the extracellular polymeric matrix in the biofilm to release biofilm from the surface of the solid substratum (Traba and Liang, 2011). Further studies need to be performed to confirm the actual mode of action of anti-biofilm activity from these extracts.

Assignment of the active compound to one of these groups is often the first step in determining the identity of the compound. Therefore, characterization of the active anti-biofilm compound(s) is needed to gain a deeper understanding of the active compounds that affect the biofilm formation of *P. aeruginosa* PAO1 and *S. aureus* Cowan I and to develop a possible anti-biofilm therapeutic.

CONCLUSION

The results obtained in this study have made the extract of *K. rotunda, C. sappan, C. burmannii*, *C. sintoc* and *N. nouchali* an interesting source for anti-biofilm agents in the development of new strategies to treat infections caused by *P. aeruginosa*, and *S. aureus* biofilm. The future scope of this work is to isolate the biologically active compounds responsible for anti-biofilm activity from *K. rotunda, C. sappan, C. burmannii, C. sintoc* and *N. nouchali* ethanol extracts to use in pharmaceutical applications.

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Chapter 3

Effect of Essential Oils of Indonesia Medicinal Plants on Planktonic Growth and Biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus In Vitro*

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ABSTRACT

Biofilms are communities of microorganisms that can be found in almost every habitat. They are attached to a surface and embedded in an extracellular matrix of biomolecules that substantially protect microorganisms and make them more resistant to anti-microbials and to the defense mechanism of the host than single cell planktonic microorganism, which makes them a progressive source of infections. The discovery of anti-biofilm agents is required to deal with these biofilm-mediated infections. This study aimed to explore the potency of essential oils from various selected Indonesian medicinal plants against planktonic growth and biofilm of, two opportunistic pathogens, *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I. Anti-biofilm activity was studied using microtiter broth dilution method. Confocal laser-scanning microscopy (CLSM) with Live/Dead staining was used to observe the ultrastructure of the *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilms. Major compounds in *Cinnamomum burmanniii* and *Massoia aromatica* oils were identified by gas chromatography-mass spectrometry (GC-MS). Essential oil from *C. burmannii* and *M. aromatica* showed a 50% growth inhibition of *P. aeruginosa* and *S. aureus* I at a concentration of 0.12 % v/v. The result also demonstrated an evident activity of those oils in preventing and breaking down biofilm formation of *P.aeruginosa* PAO1 and *S.aureus* Cowan I at sub-PMIC concentration (0.03 % v/v). The analysis by GC-MS showed cinnamic aldehyde (92.0 %) to be the major component of *C. burmannii* oil, whereas Massoialactone (92.1 %) was the main constituent of *M. aromatica* oil. The results obtained clearly indicate that *C. burmannii* and *M. aromatica* oil represents an interesting source for anti-biofilm agents in the development of new strategies to treat and prevent biofilm associated infections.

Keywords : Biofilms, *Cinnamomum burmannii* Nees ex Bl., *Massoia aromatica* Becc., *Pseudomonas aeruginosa* PAO1*, Staphylococcus aureus* Cowan I.

INTRODUCTION

Biofilms are communities of microorganisms that attached to a surface and are protected by an extracellular matrix of biomolecules (O'Toole *et al*., 2000). Because of the matrix-enclosed mode of growth among others, a biofilm has a high resistance against antibiotics which makes them hard to treat. Treatment of biofilm infections with antibiotics is often not sufficient. The demand for new and effective anti-microbial agents is increasing considerably (Harrison *et al*., 2005).

In recent years there has been a growing interest in discovery of GRAS (generally regarded as safe) compounds from natural substances to combat new and existing diseases. Higher plants are attractive species for this discovery because they contain an abundance of potentially active secondary metabolites (Clardy and Walsh, 2004). Extracts of these plants, like e.g. essential oils, are generally assumed to be more acceptable and less hazardous than synthetic compounds. Moreover, essential oils that are registered as food grade material, could even form a better alternative source for anti-fungal and anti-bacterial compounds. Therefore essential oils have been extensively studied for its anti-microbial and anti-fungal activities (Reichling *et al*., 2009; Bagamboula *et al*., 2004; Hadizadeh *et al*., 2009).

Essential oils consist of many chemical constituents and the makeup of the oil quite often varies between species. It is difficult to correlate the activity to single or classes of compounds, and it seems that the anti-fungal and anti-bacterial effects are often the result of many compounds acting synergistically. This means that the individual components by themselves are not always sufficiently effective (Hadizadeh *et al*., 2009). Because of a negligible chance for fungi and bacteria to of develop a resistance towards these oils, makes essential oil one of the most promising candidate groups of natural compounds for the development of safer and active anti-microbial agents (Nadoushani *et al*., 2010).

In this study we examined the anti-microbial and anti-biofilm activities of several essential oils, isolated from Indonesian medicinal plants towards *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I, as well as their chemical compositions. The purpose of this research is to generate data to identify those oils which potentially can be used for the development of anti-biofilm drugs.

MATERIALS AND METHODS

Plant Material

Indonesian medicinal plants were collected from Yogyakarta, Central Java and its surroundings, and the bark of *Massoia aromatica* Becc. was collected from Nabire district, West Papua, Indonesia, on the basis of ethnopharmacological information. The species were identified, authenticated, and the voucher specimens were preserved in Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia for further reference (Table 1).

Isolation of the essential oils

Plant materials were triturated and steam hydrodistilled for 6 hours to obtain volatile oils. The essential oils yielded were sealed and kept in a dark glass vial for further analysis. Stock solutions of 50% plant essential oil in 90% methanol (MeOH) was made to enhance the solubility of essential oil in suspension and used for the following dilution to obtain an essential oil concentration ranging from 1 to 0.01 % v/v.

Bacterial strains and culture conditions

P. aeruginosa PAO1 and *S. aureus* Cowan I were inoculated in Luria Bertani (LB) media and incubated in a shaking incubator at 28 ^{o}C for *P. aeruginosa* PAO1 and 37 ^{o}C for *S. aureus* Cowan I overnight. Cultures were then diluted 100 fold into LB media and allowed to grow to an OD_{600} of 0.1 (approximately $1 \cdot 10^8$ CFU/mL) for the different assays described below.

Anti-microbial susceptibility testing

Anti-microbial susceptibitily test for essential oils was performed according to Clinical and Laboratory Standards Institute (CSLI) guidelines (2007). A culture of *S. aureus* and *P. aeruginosa* (5 mL) in LB broth was grown overnight at 37 °C for *S. aureus* and at 28 °C for *P. aeruginosa*, and diluted to an OD_{600} of 0.01 (10^7 CFU mL^{-1}). The cultures were incubated for an additional 2 h and finally the OD_{600} was diluted to 10^5 CFU/mL. Inhibitory concentrations of essential oils were determined by microdilution method in sterile flat-blottom 96-well polystyrene using Mueller Hinton (MH) broth medium. Concentrations of essential oils used for the test are ranging from 1 to 0.06 % v/v. Negative controls (cells + media), positive controls (cells + media + antibiotic: streptomycin), vehicle controls (cells + media + MeOH), and media controls were included. Streptomycin sulphate was obtained from Duchefa Biochemie, Netherlands. All tests were performed in triplicate. Culture

plates were incubated overnight at 37 °C for *S. aureus* and 28 °C for *P. aeruginosa*, and optical density readings were taken using plate reader at 595 nm. The % of inhibition of replicate tests was used to determine the final MIC_{50} values. Since the oils obtained has a clear/transparent appearance, the formula from Pirbalouti *et al.* (2010) instead of formula from Quave *et al*., (2008) where the OD time zero was measured (Chapter 2, this thesis), was used to determine the planktonic minimum inhibitory concentration (PMIC) of the EOs:

Inhibition % = [(ODc –OD t) / ODc] ×100

Where ODc is the OD_{595} for the negative control (containing no essential oil) at 24 h post-inoculation and Odt is the OD_{595} for the anti-microbial compounds tested at 24 h post-inoculation. The concentration at which the essential oils depleted the growth of bacteria by at least 50% was labeled as the $PMIC_{50}$.

Effect on biofilm formation and established biofilm

Biofilm inhibition was studied using a sterile flat-bottom 96-well polystyrene plate. In order to determine the activity of biofilm inhibition and biofilm breakdown, essential oils at subinhibitory concentration (below $PMIC_{50}$ concentration) were used to ensure a non-toxic concentration (Quave *et al*., 2008). A 5 µL culture of 10^7 CFU/mL of *S. aureus* I or *P. aeruginosa* was dispensed into each well of 96-well polystyrene flat-bottomed microtitre plates containing medium in the presence of 100 µL subinhibitory concentrations (0.01 – 0.5 % v/v) of essential oils. For negative controls, as much as 100 µL TSB medium for *S. aureus* and M63 medium supplemented with 20% casamino acid, 20% glucose and 1mM MgSO4 for *P. aeruginosa* were used. A medium with streptomycin concentration of 1024 µg/mL used as positive control. All tests were performed in triplicate. As a measure of efficacy, OD_{595} of the negative control was subtracted from corresponding absorbance reading and compared to that of positive control and multiplied by 100. The concentration at which the essential oil depleted the biofilm biomass by at least 50% was labeled as the minimum biofilm inhibition concentration 50 ($MBIC_{50}$).

Plates were incubated overnight at 28^{o}C for *P. aeruginosa* and 48 hours at 37 ^{o}C for *S. aureus*. Following the incubation, the contents of the wells were removed, rinsed 3 times with distilled water, and air dried at room temperature for 10 min. Then, 125 µL of 1% crystal violet was added to the wells to stain for 15 min. The excess stain was rinsed off with tap water and 200 µL ethanol (EtOH) was added to the wells, and transfer to flat-bottom 96-well plates. Optical density was determined at 595 nm. The % inhibition of replicate tests was used to determine the final $MBIC_{50}$

values. Comparing the average of OD of the growth control wells with that of the sample wells, the inhibition percentages for each concentration of the oil was defined by the following formula:

[(ODgrowth control – Odsample) / ODgrowth control] x 100

The efficacy of plant essential oils on established biofilm was also studied, as described by Nostro *et al.* (2007) with some modifications. Biofilms were grown on 96-well plates for 24 for *P. aeruginosa* and 48 h for *S. aureus*. At post-inoculation, planktonic cells and media were removed and fresh media was added together with the test essential oils. Plates were placed back into the incubator for 24 h. The staining methods are described above. Percentage of minimum biofilm eradication (breakdown) concentration (MBEC) was calculated, as described before.

Qualitative analyses: Microscopic and image acquisition

Confocal laser-scanning microscopy (CLSM) (Jin *et al*., 2005) was used to observe the ultrastructure of the *P. aeruginosa* and *S. aureus* biofilms. Bacterial biofilms were grown under static condition on glass slides. Twenty millilitres media in a sterile tube with or without essential oil was inoculated to an OD_{600} of 0·1 from overnight cultures grown in LB. Glass slides were submerged and the cultures were incubated for 24 h at 28°C or for 48 h at 37 °C. Prior to CLSM analysis, glass slides were rinsed with 0.15 M phosphate-buffered saline (PBS, pH 7.0) to remove unadsorbed cells. After washing with PBS, the bacterial biofilm on the cover-glass was stained for 15 min with 1.5 µL of 3.34 mM SYTO9 in dimethylsulfoxide (DMSO) for the live organisms, and with 1.5 µL of 20mM Propidium Iodide (PI) in DMSO for the dead organisms. SYTO9 penetrates all bacterial membranes and stains the cells green, while PI penetrates only cells with damaged membranes and stains the cells red. The live organisms, freshly cultured and subsequently harvested, were used for control staining. The dead organisms, killed by heating in 100 ^{o}C were also used for control staining. Stained biofilms were observed with with a Carl Zeiss LSM 5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany), using high magnification: 40x - oil immersion microscopy. The images were processed for display using freely available image processing software imageJ version 1.46 (Rasband, National Institutes of Health (NIH), Bethesda, Maryland, USA: http://rsb.info.nih.gov/ij/) including the LSM reader plugin to open LSM5 formatted image stack created by the microscope software. The images' scale bar used to calibrate the ImageJ area measurement algorithm (Dusane *et al*., 2012).

The image obtained has 2 channels (red and green) and was converted into a composite image with: Image > Color > Make composite. By default, it will assign red to channel #1, green to #2. Brightness and contrast levels were then adjusted to give the best differentiation between the live (green) and dead (red) areas. The scale bar was determined with: Analyze > Tools > Scale bar. Estimated 3D surface plot was obtained using: Plugins > 3D > Interactive 3D Surface Plot, whereas estimated 3D view was obtained using: Plugins > 3D > 3D viewer. Data containing arrays of the type (x, y, z) where x and y are the coordinates of the pixel positioning and the luminance of an image is interpreted as height for the plot (z): http://rsbweb.nih.gov/ij/plugins/surface-plot-3d.html.

GC-MS analysis

Gas chromatography mass spectrometry (GC-MS) is a useful tool for quantitative and qualitative analysis of a wide range of relatively volatile compounds, and the technique has been widely applied in medical, biological, and food research. Essential oil extracts were analysed by GC-MS according to the method of Wu *et al*. (2008) on a GC-2010 gas chromatography (Shimadzu, Japan) equipped with a GC-MS-QP2010 Plus mass spectrometer (Shimadzu, Japan). An Rxi-5MS capillary column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Shimadzu, Japan) was used for separation. A split injector was used and diluted samples (1/100 in ethyl acetate, v/v) of 1.0 mL were injected by an autosampler in the split mode (1/153). The oven temperature was programmed from 60^{o}C to 290^{o}C at a rate of 10^{o}C ml^{-1}. Helium was used as the carrier gas. Identification of compounds was based on comparisons of their mass spectra with those recorded in the National Institute of Standards and Technology (NIST) database. Quantitative analysis of each essential oil component (expressed as area percentage) was carried out by peak area normalization measurement. For dead-time and Kovats Retention Index determination were conducted under isothermal condition (70^{o}C).

Kovats Retention Index Determination

The accurate determination method of dead-time is very crucial role in calculating other retention parameters such as, the adjusted retention time (t_r'), retention factor (k'), Retention Index (RI) and Separation Factor (α). Kovats retention index (shorter Kovats index, retention index; plural retention indices) is a concept firstly used in gas chromatography system by Ervin Kovats in year 1968 (Kovats, 1968) to quantify the retention of an analyte by comparing it with a pair of n-alkane adjacent chromatograms. This index is claimed independent of column packing, temperature, or any other chromatographic conditions. In an extensive study for determining chromatographic dead time, some researchers (Smith *et al*., 1985; Idroes & Oesman, 2011) concluded that iteration method

established by Guardino *et al*., (1976) not only the fastest but also the most accurate compared to the other methods. The iterative method in this research developed based on the flow chart that has been given by Guardino *et al.,* and the written source code by Furr (1989).

Statistical analysis

The data were initially analyzed by a normal distribution using the one-sample Kolmogorov-Smirnov test. The differences for individual parameters between treated group and control were tested using one way ANOVA, followed by Dunnett's test. The bars in all of the graphs indicate the standard deviation of the mean. A *P* value of 0.05 or less was considered to be statistically significant.

RESULT

Essential oil yields

As much as 29 Indonesian medicinal plants were collected and screened for inhibition of biofilm formation and biofilm breakdown. Plant essential oils assayed in this research were selected based on their ethnopharmacological application. Also their abundant availability in Indonesia was considered. The scientific, family, and local names of the samples as well as their common usage are presented in Table 1**.** The yield of the plant samples oils is presented in Table 1. Most plants had oil yield around 0.25-2.5 % v/w, and largest quantities of oil were obtained from the leaves part of *Litsea cubeba* (6 % v/w).

Screening and determination of MIC plant oils for *P. aeruginosa* and *S. aureus* planktonic growth

The values of growth inhibition of bacterial strains by essential oils are shown in Figure 1 and Table 2. The essential oils used in this study showed anti-bacterial activity against *P. aeruginosa* PAO1 and *S. aureus* Cowan I, at concentrations ranging from 0.12 to 1 % v/v.

Bacterial planktonic growth inhibition by essential oils was measured from the reduction in absorbance level of the wells which consist of essential oil tested compare to the negative control wells. The negative control was assumed to have 0% activity compare to the compounds tested. The $PMIC_{50}$ concentration was calculated using probit analysis. To ensure a concentration that is not affecting the microbial growth, oil concentration around $PMIC_{50}$ and below (sub-PMIC) were used for anti-biofilm assay.

Table 1: Indonesian medicinal plants tested for the anti-biofilm activity

No	Family	Binomial name	Local name	Voucher number	Indication
1	Zingiceaberae	*Zingiber zerumbet* (L.) Smith	Lempuyang gajah	STP009	Dysentery, skin disease, appetite enhancer, carminative, ulcus pepticum
		Z. aromaticum Val.	Lempuyang wangi	STP008	asthma, stimulate appetite, stimulate gastric mucous membranes, reduce pain, blood cleanser, enhancing appetite, birth control, cramps relief, arthritis, whooping cough, cholera, anemia, malaria, neurological diseases, abdominal pain
		Z. officinale Roxb.	Jahe	STP006	Cough, Heartburn, Flatulence, Itching, Injury, headache, cold. immunostimulant
		Z. officinale Roscoe (var. rubrum Theilade)	Jahe merah	STP007	Antihistamine, cold, antirheumatism, cough, aphrodisiac, asthma, immunostimulant
		Kaempferia rotunda L.	Temu putri (Temu putih)	STP012	scabies, dermatitis, flatulence, and other disorders of the digestive tract
		Languas galanga (L.) Stuntz.	Lengkuas	STP014	Skin disease: Pitriyasis versicolor, ringworm, scabies, sores, ulcers; rheumatism, headache, chest pain, improves digestion
		Elettaria cardamomum (L.) Maton	Kapulaga	STP015	cough, osteoporosis
		Curcuma domestica L.	Kunyit	STP004	Diabetes mellitus, Typhoid, appendix, Dysentery, stomach pains during menstruation, immunostimulant
		Kaempferia galanga L.	Kencur	STP011	Toothache, headache, cold, rheumatism, dyspepsia, stomachic, inflammation, cough, carminative, tonic and stimulant
		Zingiber purpureum Roxb	Bangle	STP065	Fever, headache, cough, stomach aches, colds, constipation, jaundice, worms, rheumatic, medicinal herbs, obesity; Decrease the abdomen after childbirth;
		C. heyneana Val. & v.Zijp	Temu giring	STP002	acne, scar, scab, chickenpox
2	Myristicaceae	*Myristica fragrans*	Pala	STP035	Dysentery, Maag, Diarrhea, vomiting, Nausea,

		Houtt.			rheumatism
3	Lauraceae	*Litsea cubeba* (Lours.) Pers.	Krangean	STP050	Antiinsect, improve digestion and to promote a restful sleep
		Cinnamomum burmannii Nees ex Bl.	Manis jangan	STP051	stomach ulcers, nausea, vomiting, flatulence
		C. sintoc Bl.	Sintok	STP052	Vermicida, antiacne, flatulence, fever
		Massoia aromatica Becc.	Masoyi	STP053	To treat vaginal discharge, abdominal cramps, pain in the bones, cathartic, fever, and as an herbal medicine postpartum
4	Myrtaceae	*Syzygium aromaticum* (Linn.) Merr.	Cengkeh	STP022	Toothache, carminative, stomachic, antiseptic anticarcinogenic, Cholera, dysentery, measles
		S. polyanthum (Wight) Walp.	Salam	STP023	Stomachache, gout, stroke, high cholesterol, diarrhea, rashes, diabetes
5	Piperaceae	*Piper cubeba* L.	Kemukus	STP028	For inflammation of the urinary tract's mucous membranes
		P. nigrum L.	Lada putih	STP030	Dysentriae, cholera, rheumatism, headache, menstruation pain, cold
		P. retrofractum Vahl.	Cabe jawa	STP029	Stomach cramps, vomiting, flatulence, colic, dysentery, diarrhea, headache, toothache, cough, fever
6	Apiaceae	*Coriandrum sativum* L.	Ketumbar	STP025	facilitating digestion, carminative, lactago, and appetite enhancer (stomachica)
		Foeniculum vulgare Mill.	Adas	STP026	Abdominal pain (heartburn), abdominal bloating, nausea, vomiting, diarrhea, jaundice (jaundice), coughing, asthma, menstrual pain, insomnia, colic
7	Sapindaceae	*Schleichera oleosa* (Lour.) Oken	Kesambi	ST062	Skin diseases
8	Rutaceae	*Citrus aurantifolia* Swingle	Jeruk nipis	STP031	Tonsils, Malaria, Ambeien, influenza, cough, constipation, dysentery, Nausea, Fatigue
9	Araceae	*Acorus calamus* L.	Dlingo	STP063	Sedatives, Drugs for stomach and spleen, cosmetic raw materials
10	Labiatae	*Ocimum basilicum* L	Kemangi	STP046	Acne, for disease in kidneys, bladder and urethra
11	Leguminosae	*Caesalpinia sappan* L.	Secang	STP034	Diarrhea, dysentery, tuberculosis, wounds, syphilis,

					malaria, tetanus, tumors, inflammation of the mucous membranes
12	Annonaceae	*Cananga odorata* (Lamk) Hook.f. & Thomson	Kenanga	STP057	for personal adornment and decoration at festivities and other celebrations

Indication is/are the usage of the plant for medical application(s) according to Indonesian National Health Department, INHD (1985).

Table 2: Effects of different essential oils on planktonic growth, biofilm formation and biofilm breakdown of *P. aeruginosa* PAO1 and *S. aureus* Cowan I. The $PMIC_{50}$, $MBIC_{50}$, and $MBEC_{50}$ results were determined based on probit analysis data. The $PMIC_{50}$ was tested in the oil's concentration range of 0.06–1 % v/v, whereas the $MBIC_{50}$ and $MBEC_{50}$ were tested in the oil's concentration range of 0.01–0.5 % v/v. A dash (–) represents that no $PMIC_{50}$ or $MBIC_{50}$/$MBEC_{50}$ was identified within the concentration range tested.

No	Plant (Binomial name)	Sample (plant part used)	Volume oil obtained (mL)	Sample fresh weight (g)	Oil yield (% v/w)[a]	Planktonic growth inhibition activity ($PMIC_{50}$) in % v/v		Biofilm formation inhibition activity ($MBIC_{50}$) in % v/v		Biofilm breakdown activity ($MBEC_{50}$) in % v/v	
						PA[b]	SA[b]	PA[b]	SA[b]	PA[b]	SA[b]
1	*Zingiber zerumbet* (L.) Smith	Rhizome	7	7000	0.10	0.51	0.49	0.49	0.25	-	-
2	*Z. aromaticum* Val.	Rhizome	7	3000	0.23	1.03	0.98	0.49	0.12	-	-
3	*Z. officinale* Roxb.	Rhizome	3	3000	0.10	1.01	0.96	0.25	0.25	-	-
4	*Z. officinale* Roscoe (var. rubrum Thelidae)	Rhizome	3	3000	0.10	1.01	0.96	0.25	0.25	-	-
5	*Kaempferia rotunda* L.	Rhizome	5	6000	0.08	0.53	0.25	0.12	0.12	0.55	0.51
6	*Languas galanga* (L.) Stuntz.	Rhizome	7	3000	0.23	1.08	0.53	0.25	0.12	-	-
7	*Elettaria cardamomum* (L.) Maton	Seed	60	5500	1.09	0.51	0.50	0.12	0.12	-	-

8	*Curcuma domestica* L.	Rhizome	6	5000	0.12	1.04	0.97	0.25	0.25	-	-
9	*Z. purpureum* Roxb	Rhizome	12	5000	0.24	0.52	0.25	0.12	0.25	-	-
10	*K.galanga* L.	Rhizome	9	3000	0.30	1.04	0.51	0.12	0.06	-	-
11	*C. heyneana* Val. & v.Zijp	Rhizome	5	5000	0.10	1.04	1.01	0.12	0.12	-	-
12	*Myristica fragrans* Houtt.	Seed	6	3000	0.20	1.06	0.25	0.12	0.12	-	-
13	*Litsea cubeba* (Lours.) Pers.	Bark	30	3000	1.00	0.53	0.51	0.25	0.12	-	0.51
		Leaves	60	1000	6.00	0.53	0.51	0.25	0.12	-	0.51
		Seeds	40	1000	4.00	0.53	0.25	0.12	0.06	0.54	0.51
14	*Cinnamomum burmannii* Nees ex Bl.	Bark	3	3000	0.10	0.12	0.12	0.03	0.03	0.12	0.10
15	*C. sintoc* Bl.	Bark	5.3	3000	0.18	0.53	0.50	0.12	0.12	-	-
16	*Syzygium aromaticum* (Linn.) Merr.	Leaves	6	3000	0.20	1.03	0.25	0.25	0.12	-	-
17	*Piper cubeba* L.	Fruit	10	1000	1.00	-	-	0.5	0.12	-	-
18	*Coriandrum sativum* L.	Seed	10	3000	0.33	-	0.25	0.12	0.12	-	0.5
19	*Schleichera oleosa* (Lour.) Oken	Seed	20	1000	2.00	-	-	0.5	0.25	-	-
20	*Citrus aurantifolia* Swingle	Leaves	20	3000	0.67	0.97	0.54	0.25	0.25	-	-
21	*Acorus calamus* L.	Stem	9	5000	0.18	-	-	0.55	1.12	-	-
22	*Foeniculum vulgare* Mill.	Seed	17	1000	1.70	-	0.25	0.55	-	-	-
23	*C. sappan* L.	Leaves	8	4000	0.20	-	-	-	-	-	-
24	*Canangium odoratum* Lamk.	Flower	6	1000	0.60	-	0.98	0.5	-	-	-
25	*P.retrofractum* Vahl.	Fruit	0.5	1000	0.05	1.03	1.00	0.52	0.51	-	-
26	*S.polyanthum* (Wight.) Walp.	Leaves	1	3000	0.03	1.05	1.00	0.52	0.50	-	-
27	*P. nigrum* L.	Seeds	15	1000	1.50	0.54	0.51	0.25	0.25	-	-
28	*Ocimum basilicum* L.	Leaves	5	3000	1.17	0.51	0.51	0.12	0.12	0.48	0.25
29	*Massoia aromatica* Becc	Bark	2.5	5000	0.05	0.12	0.12	0.03	0.04	0.12	0.12

[a] % oil yield (v/w) = (volume of the oil obtained)/(weight of ground plant part) x 100%

[b]PA = *P. aeruginosa* PAO1; SA = *S. aureus* Cowan I

Effect of plant essential oils on *C. albicans* biofilm formation and established biofilm

Against bacterial biofilms, both essential oils showed 50% inhibition on biofilm formation at concentration of 0.03 % v/v against *P. aeruginosa* PAO1, whereas higher concentration (0.06 % v/v) needed by *Massoia* oil to give 50% inhibition of *S. aureus* Cowan I biofilm formation (Figure 1). The biofilm formation of *P. aeruginosa* was partially reduced (50.2± 0.5 %) (***P*<0.01) in the presence of *C. burmannii* oil at concentration of 0.03 % v/v, whereas as much as 51.3±0.8 % (***P*<0.01) reduction in biofilm was shown by the *M. aromatica* oil at concentration of 0.03 % v/v. Similar result was shown against *S. aureus* biofilm formation. At concentration of 0.03 % v/v, *C. burmannii* oil reduced the formation of *S. aureus* biofilm as much as 51.7±0.9 % (***P*<0.01), whereas at the same concentration, massoia oil capable to give 45.3±0.4 % (***P*<0.01) reduction of *S. aureus* biofilm development.

Both *C. burmannii* and *M. aromatica* oil needs higher concentrations (0.12 % v/v) in order to be able to break the biofilm of the bacteria tested (Figure 2). $MBEC_{50}$ of both oils against *P. aeruginosa*, as well as against *S. aureus* was at concentration of 0.12 % v/v. As much as 49.5±0.6 % (***P*<0.01) of *P. aeruginosa* biofilm was degraded in the presence of *C. burmannii* oil (concentration of 0.12 % v/v), whereas 49.7±0.5 % (***P*<0.01) breakdown of *P. aeruginosa* biofilm was given by concentration of 0.12 % v/v massoia oil. The activity in breakdown established biofilm were shown by both oils against *S. aureus* established biofilm. At concentration of 0.12 % v/v, *C. burmannii* oil gave 52.5±0.8 % (***P*<0.01) breakdown, while at the same concentration, massoia oil capable to disrupt *S. aureus* settled biofilm as much as 50.1±0.5 % (***P*<0.01), accordingly.

Qualitative analyses

The inhibition of biofilm formation and biofilm breakdown of *C. burmannii* and *M. aromatica* essential oils was confirmed by confocal laser scanning microscope (CLSM) analysis, along with LIVE/DEAD staining for monitoring live/dead cells. From CLSM observations, we observed an inhibition of biofilm formation and a breakdown of an established biofilm for both bacteria when essential oils were applied.

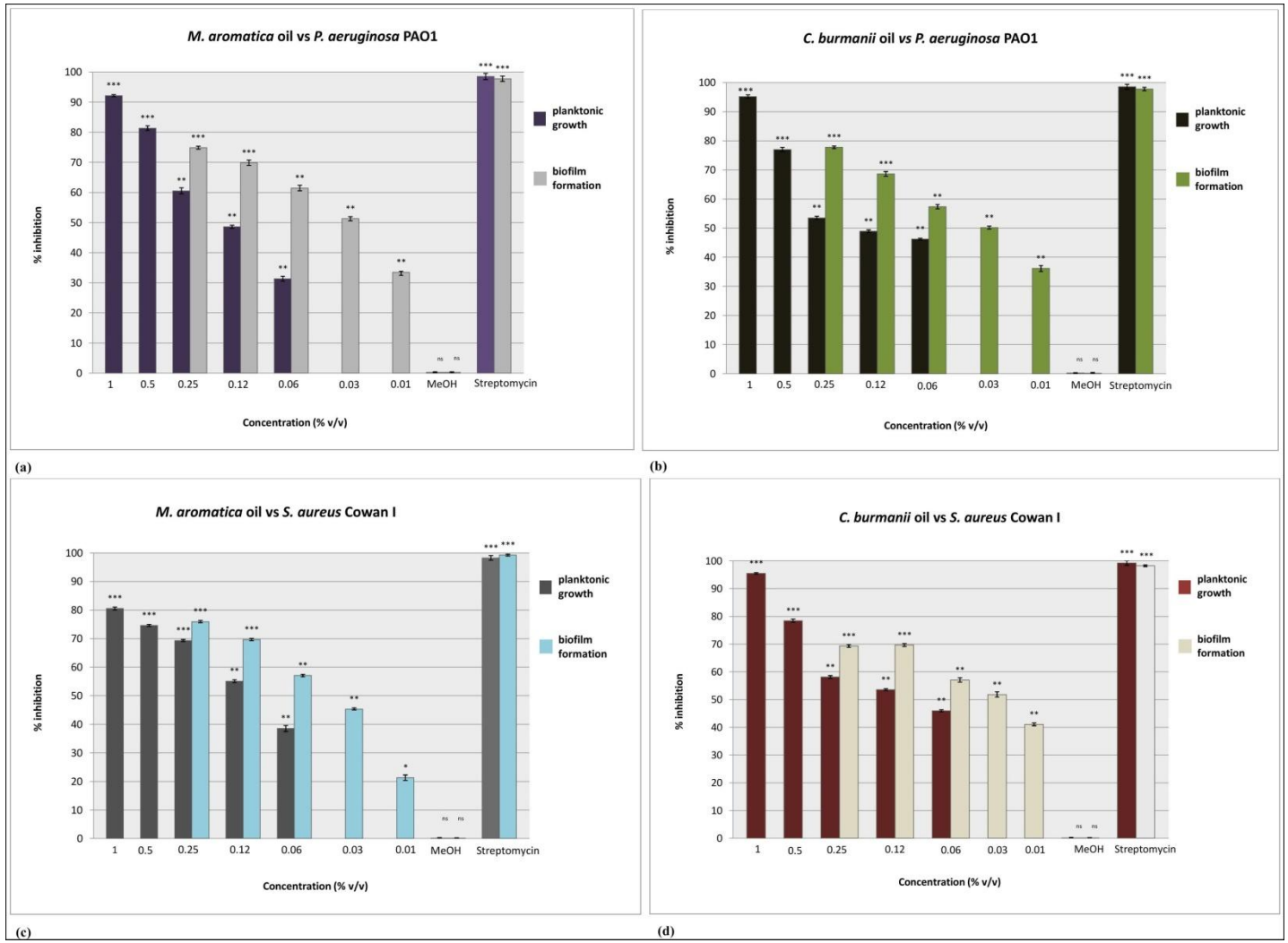

Fig. 1: **The percentage inhibition of planktonic growth and biofilm formation activity of plant essential oils against (a-b) *Pseudomonas aeruginosa* PAO1, and (c-d) *Staphylococcus aureus* Cowan I at different concentrations. The concentration of Streptomycin used is 1024 µg/mL. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.**

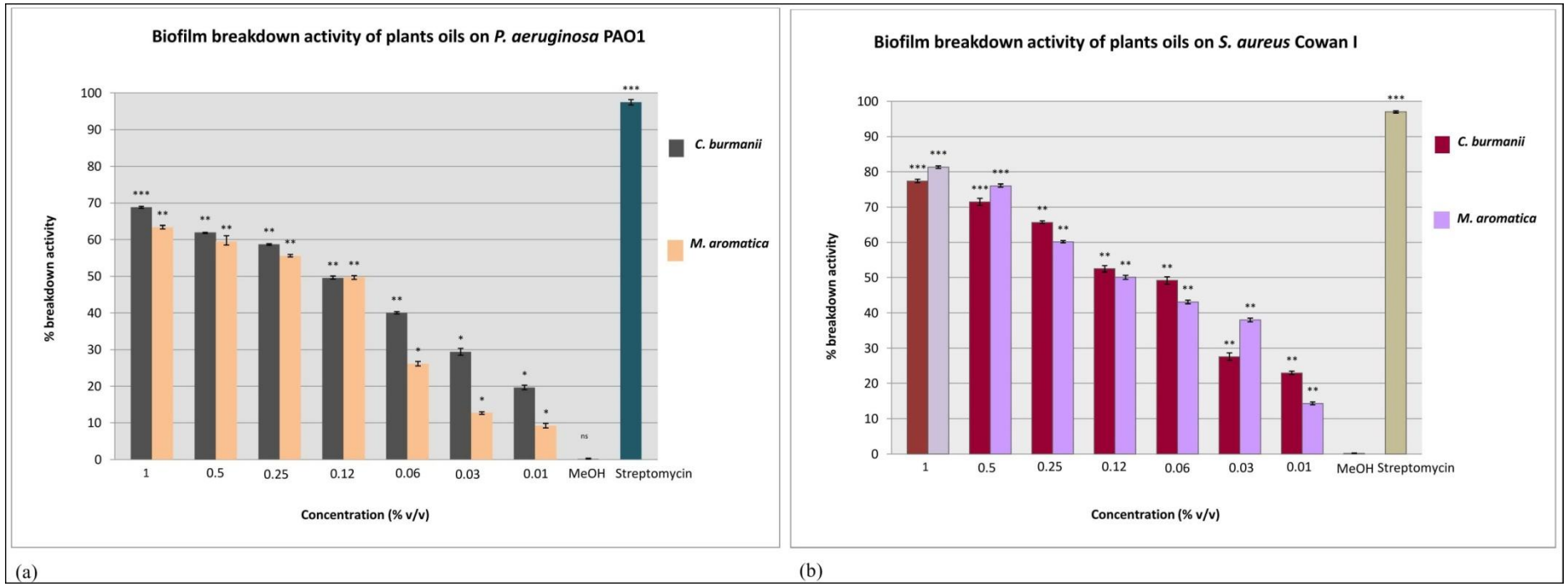

Fig. 2: The percentage activity in biofilm breakdown of plant essential oils against *Pseudomonas aeruginosa* PAO1 (a), and *Staphylococcus aureus* Cowan I (b) at different concentrations. The concentration of Streptomycin used is 1024 µg/mL. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

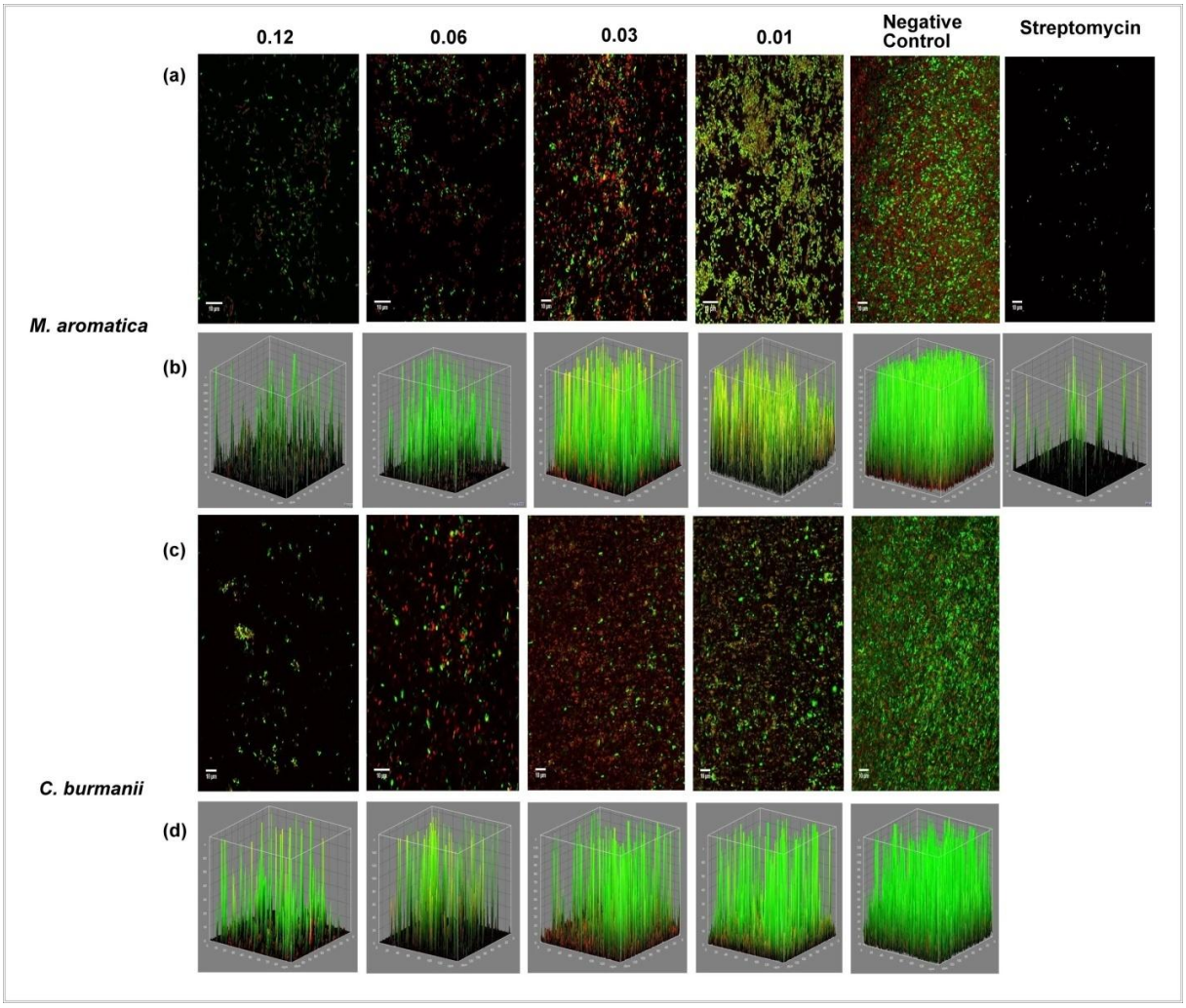

Fig. 3: Representative confocal scanning micrograph images of *Pseudomonas aeruginosa* PAO1 initial biofilm formation grown in the presence or absence of *Massoia aromatica* (a & b), and *Cinnamomum burmannii* (c & d) essential oil at concentration of 0.12 – 0.01 % v/v. Streptomycin (positive control) at concentration of 1024 µg mL^{-1}; (a & c): projected upper view of the biofilm, (b & d): estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using ImageJ. The cells in biofilms were stained with SYTO9 (green), and PI (red). Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

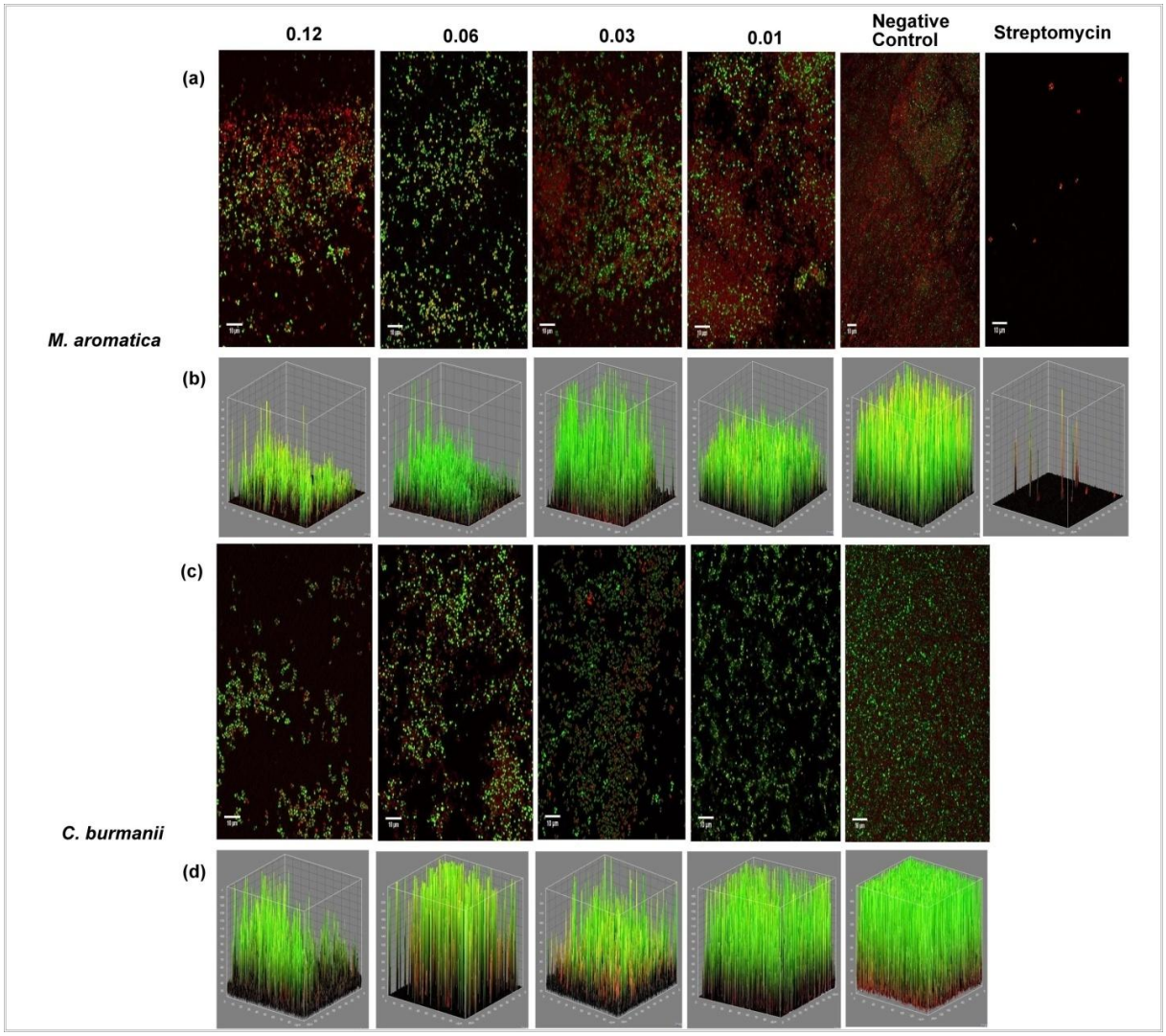

Fig. 4: Representative confocal scanning micrograph images of *Staphylococcus aureus* Cowan I initial biofilm formation grown in the presence or absence of *M. aromatica* (a & b), and *Cinnamomum burmannii* (c & d) essential oil at concentration of 0.12 – 0.01 % v/v. Streptomycin (positive control) at concentration of 1024 µg mL^{-1}; (a & c): projected upper view of the biofilm, (b & d): estimated three-dimensional surface plot of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning, and z is the intensity collected using ImageJ. Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

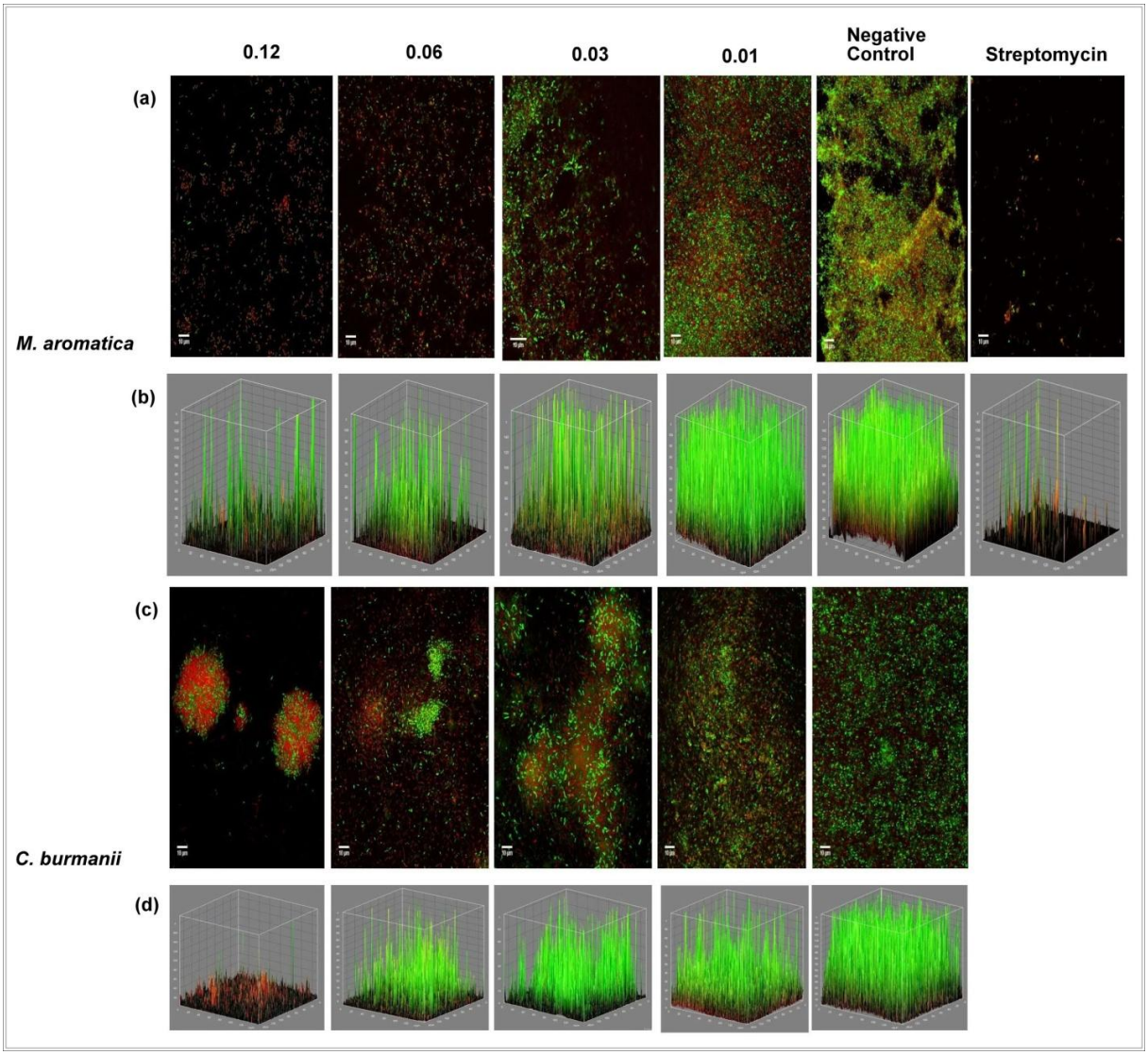

Fig. 5: Representative confocal scanning micrograph images of biofilm breakdown activity of *Massoia aromatica* (a & b), and *Cinnamomum burmannii* (c & d) essential oil at concentration of 0.12 – 0.01 % v/v against *P. aeruginosa* PAO1. Streptomycin (positive control) at concentration of 1024 µg mL^{-1} ; (a & c): projected upper view of the biofilm, (b & d): estimated three-dimensional view of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning, and z is the intensity collected using ImageJ. Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

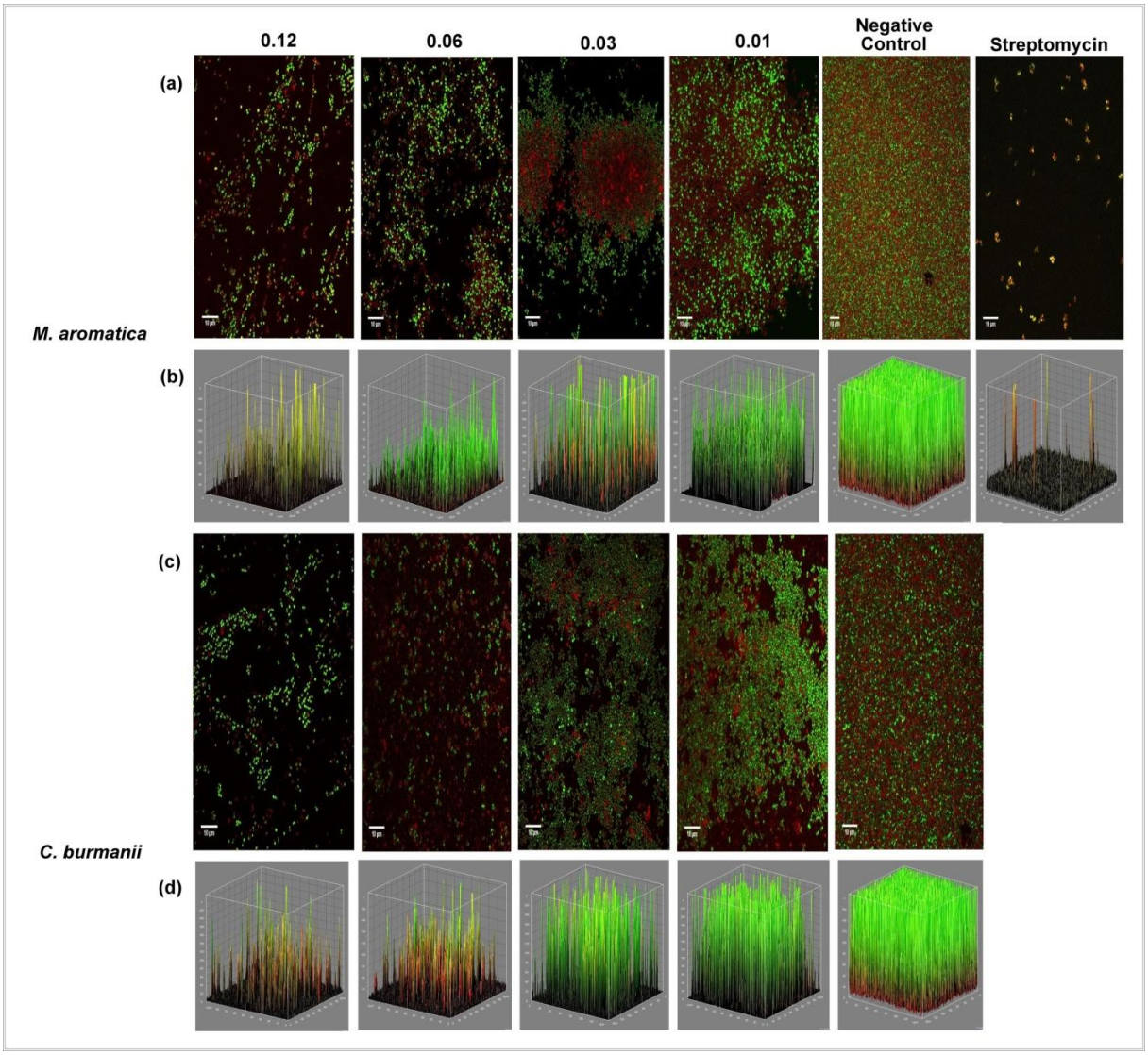

Fig. 6: Representative confocal scanning micrograph images of biofilm breakdown activity of *Massoia aromatica* (a & b), and *Cinnamomum burmannii* (c & d) essential oil at concentration of 0.12 – 0.01 % v/v against *S. aureus* Cowan I. Streptomycin (positive control) at concentration of 1024 µg mL^{-1} ; (a & c): projected upper view of the biofilm, (b & d): estimated three-dimensional view of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning, and z is the intensity collected using ImageJ. Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

At the highest concentration tested (0.12 % v/v), a clear inhibitory effect was observed for the essential oil tested against both *P. aeruginosa* and *S. aureus* biofilm compared to the untreated control (figure 3 and figure 4). The biofilm formed in the presence of 0.12 % v/v oil was less dense and consisted of scattered cell clusters compared to the normal biofilm without the presence of oil. At the lowest concentration tested (0.01 % v/v), both oils of *M. aromatica* and *C. burmannii* failed to inhibit the biofilm. From CLSM studies, in the presence of sub-PMIC concentration (0.01 % v/v) oils these biofilms showed dense microcolonies intersected by irregular channels. When we tested the oil at concentration higher than sub-PMIC (0.25 – 0.5 % v/v) no biofilm was formed and the few

planktonic cells were dead (data not shown). This is probably related to the anti-bacterial activity of the oils, which provide no growth of the bacteria tested in the presence of the oils, therefore the biofilm could not be established.

The similar characteristic was also observed when the oil activity was tested for disruption of preformed biofilms (Figure 5 & 6). At concentrations higher than subMIC (0.25 – 0.5 % v/v), the biofilms largely disappeared and a few dead cell clusters were evident (data not shown). At lower concentrations (0.12 % v/v), the oils have less capability in disrupting the preformed biofilm as shown by surviving cell clusters and dispersion on the glass slide surface. The oils were least effective at concentration of 0.01 % v/v against the adherent cells. In the presence of oil at concentration of 0.01 % v/v the microcolonies within the biofilm are more compact and formed layers which increase the thickness of the biofilm developed on the glass side surface.

GC-MS analysis

The principal components of the oils of *M. aromatica* and *C. burmannii* were determined using gas chromatography and identified by comparing the mass spectra of chemical compounds in essential oils with library mass spectra from NIST02 (www.nist.gov/index.html). In this study, the analysis by GC-MS showed cinnamic aldehyde (92.0 %) to be the major component of *C. burmannii* essential oil, whereas massoialactone (92.1 %) was the main constituent of *M. aromatica* essential oil. The chemical composition of the volatile oils from *C. burmannii* and *M. aromatica* obtained from GC-MS were described in Table 3; Figure 7 & 8.

The identification of the essential oils chemical constituents was assigned on the basis of comparison of their retention indices mass spectra with data published in the literature. Kovats retention Index of *C. burmannii* and *M. aromatica* oil by isothermal GC were shown at Table 4**.** The dead-time was estimated 1.82 minutes with a very good of coefficient correlation 0.9992. These calculations particularly involved approximately 25 iterations. N-alkanes homologous series was used not only as standards described by Kovats but also for estimating dead-time simultaneously. The obtaines Kovats Retention Index for *C. burmannii* oil and *M. aromatica* oil are 1123.46, 1168.63 respectively

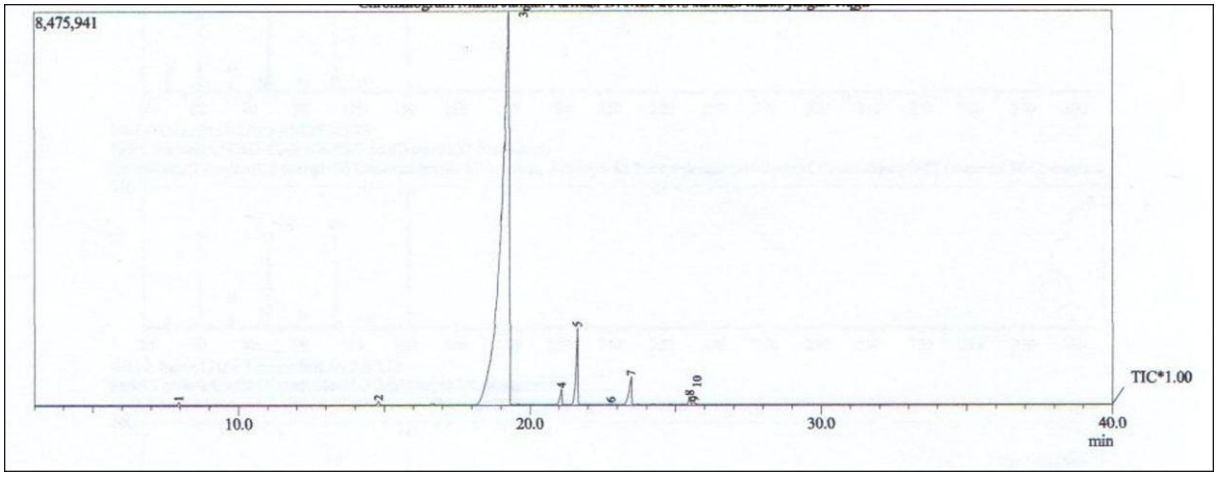

Fig. 7: GC-MS profile of *Cinnamomum burmannii* oil. The indicated compounds in the chromatograms were shown in Table 3.

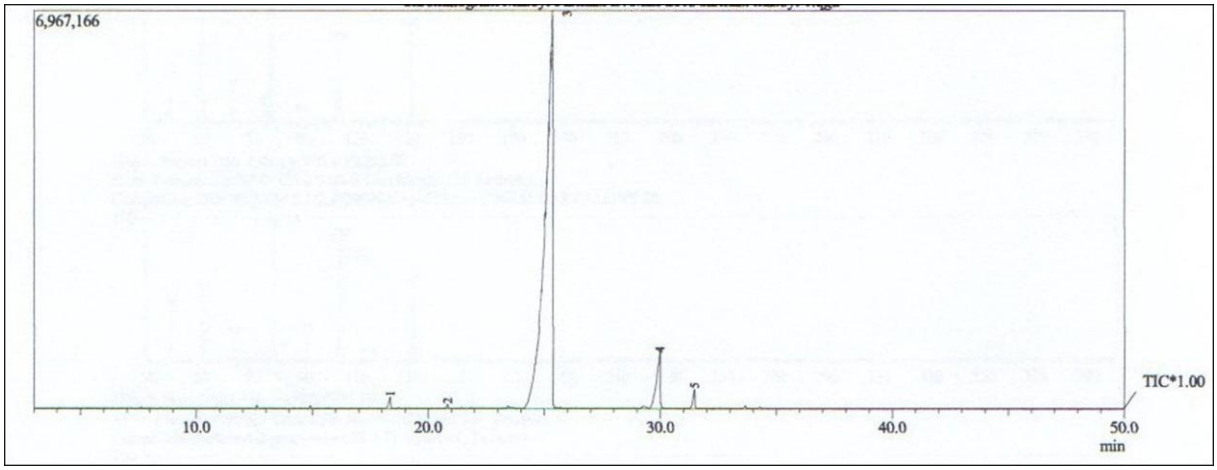

Fig. 8: GC-MS profile of *Massoia aromatica* oil. The indicated compounds in the chromatograms were shown in Table 3.

Table 3: Major chemical constituents of *C. burmannii* and *M. aromatica* essential oil as identified by GC-MS

Essential oil sample	Peak	Retention time (RT)	Kovats Retention Index (RI)	Area	Area (%)	Similarity index (SI)	Chemical Component
C. burmannii	3	19.296	1123.459	164665734	92.02	95	Cinnamaldehyde
	5	21.630	1142.506	7342238	4.10	93	Alpha-Copaene
	7	23.472	1156.016	3708184	2.07	91	3-Phenyl-2-prophenyl acetate
M. aromatica	3	25.346	1168.629	156590945	92.05	94	5-Hydroxy-2-Decenoic acid lactone (Massoialactone)
	4	29.961	1195.847	10522375	6.19	86	5-Hydroxy-2-Decenoic acid lactone (Massoialactone) *

*The compound obtained is predicted to be a derivate of 5-Hydroxy-2-Decenoic acid lactone (Massoialactone) with higher boiling point and have the similar fragmentation pattern (86%) to Massoialactone.

Table 4: Kovats retention Index of *C. burmannii* and *M. aromatica* oil by isothermal GC on an AGILENT J&W DB-1 GC column.

Estimation of Coloumn Dead-time (t_o)				
Estimated dead time : 1.81510 Estimated Coef Corr Semi-log plot : 0.99915 Calculated Slope Semi-log plot : 0.00658 Calculated Intercept Semi-log plot : -4.53096				
***n*-Alkane**	**Carbon Number**	**Retention Time (*min*)**	**Adjusted Retention Time (calculated/min)**	***k'***
Hexane	6	2.381	0.558	0.311
Heptane	7	2.850	1.077	0.570
Oktane	8	3.899	2.080	1.148
Nonane	9	6.098	4.018	2.359
Decane	10	9.281	7.758	4.113
Estimation of Kovats Index				
Analyte	**Retention Time (*min*)**	**Adjusted Retention Time (*min*)**	***k'***	**Retention Index**
C. burmannii	19.296	17.480	09.630	1123.459
M. aromatica	25.346	23.530	12.963	1168.629

DISCUSSION

The result obtained from this study showed that the anti-bacterial and anti-biofilm activity of plant essential oils varied for the studied microorganisms. It is very likely that different compounds within the essential oils are responsible for the inhibitory effect on biofilm formation and biofilm breakdown activity. Many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth. Some anti-microbial agents at sub-inhibitory concentrations could influence bacterial virulence factors, such as adherence, cell surface hydrophobicity, biofilm formation, sensitivity to oxidative stress and motility (Cowan, 1999; Nadoushani *et al*., 2010).

Essential oils, which are mixtures of numerous organic chemicals, are biosynthesized by plants and their anti-microbial properties, their constituents and their mechanism of action have been extensively studied (Bagamboula *et al*., 2004). Phenolic compounds such as eugenol and cinnamaldehyde are present in essential oils of many plants and are proved to be active against many pathogenic bacteria and fungi (Suresh *et al*., 1992; Pacheco *et al*., 1993; Ooi *et al*., 2006; Chami *et al*., 2004; Bennis *et al*. 2004).

From this study we found out that cinnamon and massoia oils possess the anti-bacterial and anti-biofilm activity against the studied microorganisms. It is very likely that different compounds within the essential oils are responsible for the inhibitory effect on biofilm formation and biofilm breakdown activity. Many plant essential oils, which are mixtures of numerous organic chemicals, contain compounds that inhibit microbial growth. Some anti-microbial agents at sub-inhibitory concentrations could influence bacterial virulence factors, such as adherence, cell surface hydrophobicity, biofilm formation, sensitivity to oxidative stress and motility (Cowan *et al*., 1999; Bennis *et al*., 2004).

C. burmannii and *M. aromatica* are a rare plant species that grow in Indonesia. *Massoia* plants grown in Indonesia, especially in Maluku and Papua. Wood and bark of *Massoia* contains oil which has a distinctive sweet coconut fragrance that has been traded for centuries. *M. aromatica* has a long history to be used as a traditional medicine (Rali *et al*., 2007). Canoes made from this tree resist insect and fungal predation that might be due to its anti-fungal properties (Suresh *et al*., 1992). Massoia lactone, the major compound in *Massoia* oil, is a rare essential oil component in nature that was first characterized by Abe in 1937 (Pacheco *et al*., 1993). Widely used as a

natural coconut flavouring, natural massoia lactone has been largely superseded by a synthetic alternative because the extraction process is expensive and the tree is killed during the process of removing the bark (Ooi *et al*., 2006).

C. burmannii also known as Indonesian cinnamon, is distributed in Southeast Asia and is cultivated in parts of Indonesia and Philippines. The dried bark of the plant is found in the market in the form of rolls and quills, which is used for cooking and flavoring. The dried inner bark of the plant is used as flavoring agent in foods, beverages, chewing gums, etc. The distilled bark oil and the oleoresin of the bark of the plant are used in soap and perfume manufacturing. The powdered bark is also used in traditional medicine (Nadoushani *et al*., 2010; Suresh *et al*., 1992; Chami *et al*., 2004).

Up to now, there is no information in the literature regarding the influence of sub-PMIC essential oils of *M. aromatica* Becc. on inhibition and breakdown of *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm. However, a study has been conducted on activity of cinnamaldehyde, the main compound of *C. burmannii* Nees ex Bl. essential oil, against *S. aureus* biofilm (Jia *et al*., 2011).

Jia *et al*. (2011) showed that cinnamaldehyde was able to kill *S. aureus* and detach existing biofilms. Also it was found that the expression of *sarA*, encoding a staphylococcal accessory regulator which is a central regulatory element that controls the production of virulence factors and is essential for biofilm development of *S. aureus,* was decreased upon exposure to sub-PMICs of cinnamaldehyde.

In the quantification measurement of (static) biofilms, crystal violet (CV) staining has been widely used as a indirect methods with 96-well microtiter plates (Burt, 2004; Mastelic *et al*., 2005). The major disadvantage is that CV, a basic dye which binds to negatively charged surface molecules and polysaccharides in the extracellular matrix, stains both living and dead cells. Therefore it is difficult to evaluate killing of biofilm cells (Burton *et al*., 2007).

In order to gain a clear view about the effect of essential oils on *P. aeruginosa* and *S. aureus* biofilm, the Live/Dead staining was performed and viewed using CLSM. Examining the permeability of a compound through biofilms using CLSM and Live/Dead staining capable to show the dead organisms which are differentiated from the live ones with the respective labeling of SYTO9 and propidium iodide (PI) (Djordjevic *et al*., 2012).

We observed inhibiting activity on biofilm formation when we inoculate the biofilm together with the essential oils using concentration lower than concentration for 50% inhibition of planktonic growth (0.01 - 0.12 % v/v). We found that the essential oil activity against the biofilm formation was concentration dependent. In the presence of the oil, biofilms of both bacterial tested was poorly developed and most of the cells were eventually killed (Table 4).

The concentration of plant essential oils needed to degrade preformed biofilms was higher than the one needed to inhibit the initial attachment. The reduced susceptibility of bacteria in biofilms is thought to be due to a combination of several factors such as the presence of extracellular polymer substances (EPS) surrounding the biofilm cells. The anti-microbial agents is absorbed onto the EPS and effectively diluted in its concentration before it reaches the individual cells in the biofilm (Peeters *et al*., 2008; Takenaka *et al*., 2001).

The cinnamaldehyde and eugenol in the *C. burnamii* essential oil has been proven to have anti-bacterial and anti-fungal activity (Ooi *et al*., 2006). These two compounds and massoia lactone as the main compound of *M. aromatica* essential oil might have an influence in inhibiting biofilm formation of *P. aeruginosa* PAO1 and *S. aureus* Cowan I.

CONCLUSION

According to the findings of this study, the essential oils of *M. aromatica* and *C. burmannii* have anti-biofilm formation and biofilm breakdown activity against *P. aeruginosa* PAO1 and *S. aureus* Cowan I. The present study suggests that the essential oils of these plants are a potential source of natural anti-biofilm agents. After this screening process, further work on phytochemical characterization will be performed to identify and isolate active constituents responsible for the anti-biofilm activity.

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Chapter 4

Efficacy of Indonesia Medicinal and Spices Plants Essential Oils Against Planktonic Growth and Biofilm of *Candida albicans*

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ABSTRACT

The emerging resistance to anti-fungal agents encourage the exploration of new and effective natural product showing anti-fungal activity to significantly impact the treatment and the management of biofilm associated fungal infections. Essential oils which are mixture of multiple heterogenous compounds can exhibit anticandida activity because many essential oils are capable to disrupt fungal cell membranes with their lipophilic compounds. The aim of this study is to find a possible source of new anti-fungal for the treatment of *C. albicans* biofilms from Indonesian medicinal and spices plant essential oils. Essential oils from collected plants were obtained by water-steam distillation process. The susceptibility of the oils against *C. albicans* planktonic cells were determined using microdilution method. Microtiter plates method was used to test for the anti-biofilm activity. Effect of essential oil on *C. albicans* biofilm architecture was assessed using Confocal Laser Scanning Microscopy (CLSM) along with LIVE/DEAD staining. ANOVA and Dunnett's test using SPSS19 is used for statistical analysis. Differences were considered significant with P-values of 0.05 or less. Our study revealed that the essential oil from *Cinnamum burmannii, Massoia aromatica, Ocimum basilicum* and oil from the seeds part of *Litsea cubeba* showed an evident anti-fungal activity against planktonic growth of *C. albicans,* inhibited the formation of *C. albicans* biofilm and demonstrated the efficacy in disrupting *C. albicans* established biofilm at a sub-PMIC concentration. We conclude that the oils from *C. burmannii, M. aromatica, O. basilicum* and the seeds of *L. cubeba* are an interesting sources of innovative plant derived anti-fungal agents, thus can be used in the development of new strategies to treat biofilms of medical relevance.

Keywords: Essential oil, *Candida albicans* biofilm, CLSM, *Cinnamomum burmannii, Massoia aromatica, Ocimum basilicum, Litsea cubeba*

INTRODUCTION

Candida is a common commensal fungus of humans and has been shown to be the third most commonly isolated bloodstream pathogen from US hospitals, now surpassing gram-negative rods in frequency (Ramage *et al.,* 2001). They can convert to an opportunistic pathogen causing superficial infections through the invasion of oral and vaginal epithelial surfaces. *Candida* also causing lethal systemic infections in susceptible host including neonates, individual who are immunocompromised or otherwise compromised by severe underlying disease, surgery or hospitalization, through dissemination in the bloodstream and invasion of internal organs (Shapiro *et al.,* 2011; Bandara *et al.,* 2009).

Candida albicans is known as the most common causal agent of invasive candidiasis, and a major fungal pathogen responsible for the majority of nosocomial infections (Ramage *et al*., 2001). The capacity of *C. albicans* to cause disease is closely associated with its ability to grow as biofilm communities by adhere both to cellular and inanimate surfaces of implanted devices such as catheters, prostetic cardiac valves, contact lenses, artificial joints and intraurine devices (IUDs) (Kojic and Darouiche, 2004; Lal *et al*., 2008).

The majority of microorganisms grow in biofilms instead of planktonic (floating) state. They adhere to a wide variety of surfaces in all nutrient-sufficient aquatic ecosystems except deep ground water and abyssal oceans (Donlan and Costerton, 2002; Costerton *et al.,* 1995). *Candida albicans* biofilm development occurs in three phases. The early (adherence) phase consists of budding-yeast cells which are adhered to the substratum. The attached cells proliferate to form microcolonies and begin to deposit extracellular matrix. Dimorphic switching occurred with a transition of the yeast cells forms to pseudo-filamentous and true hyphal forms, and microcolonies became interlinked by the hyphal extensions, forming a confluent monolayer. The complexity of the biofilm increased with the time and after 24 h, *C. albicans* biofilm consisted of a mixture of yeast cells, pseudohyphae and true hyphae (Jabra-Rizk, 2004).

Microorganisms involved in biofilms are generally more resistant to anti-microbials than single cell planktonic microorganism and also against the defense mechanism of the host. This fact makes them a progressive source of infections (Donlan and Costerton, 2002). Although the mechanisms of the drug resistance of *Candida* biofilms are still poorly understood, the action of anti-fungal may be limited by their penetration and chemical reaction into biofilm matrix (Agarwal, 2007). Besides this,

biofilm heterogeneity is thought to be a contributing factor for the drug resistance phenomenon. Biofilm communities develop differential gradients in nutrients and metabolic waste, so that groups of cells in the same biofilm react variably in response to environmental cues, leading to a variety of phenotypes (Jin *et al.,* 2005).

The emerging resistance to anti-fungal agents encourages the exploration of new and effective natural product showing anti-fungal activity to significantly impact the treatment and the management of biofilm associated fungal infections. Secondary plant metabolites such as essential oils which are mixture of multiple heterogenous compounds can exhibit anticandida activity because many essential oils are capable to disrupt fungal cell membranes with their lipophilic compounds (Pires *et al*., 2011).

The present study aimed to find a possible source of new anti-fungal for the treatment of *C. albicans* biofilms. We compared the anti-fungal activity of several Indonesian medicinal and spices plant essential oils against planktonic cells of *C. albicans*. We also investigated anti-biofilm properties of the essential oils that demonstrated anticandida activity against planktonic cells. The *Candida* biofilm architecture in the presence or absence of the essential oil also observed using confocal laser scanning microscope (CLSM) to gain better information of the essential oil effect on *Candida* biofilm.

MATERIALS AND METHODS

Fungal isolate and culture conditions

Candida albicans ATCC 10231 was cultured in Sabouraud Dextrose Broth (SDB) and incubated for 24 h at 30˚C with agitation (120 rpm). Following incubation, cells were sedimented by centrifugation (5000 x *g* for 15 min at 4˚C), washed twice with 5 mL of sterile PBS (phosphate buffered saline) buffer pH 7.2, and finally suspended to 10^7 CFU/mL by adjusting the optical density of the suspension to 0.38 at 520 nm. This cell concentration was selected because previous workers have demonstrated that optimal biofilm formation occurs at this particular density (Kuhn *et al*., 2002; Kruppa, 2009). Densities that are too high or too low result in poorly formed biofilms (Pierce *et al*., 2008; Chandra *et al*., 2008).

Medicinal plants and essential oil extraction

A list of the plants studied, including the botanical name, voucher specimen and data related to traditional use are listed in Table 1, Chapter 3 this thesis. The plant samples were collected from Yogyakarta, Central Java and its surroundings, and the bark of *Massoia aromatica* Becc. was collected from Nabire district, West Papua, Indonesia, on the basis of ethnopharmacological

information. The species were identified, authenticated, and the voucher specimens were preserved in Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia for further reference. From the collected plants, a total of 29 essential oils were obtained by water-steam distillation process. Oil samples obtained were dried over anhydrous sodium sulfate (Na_2SO_4), filtered using a Whatman filter paper no. 40, and stored in sealed dark glass vial at 4°C for further use.

Determination of planktonic minimum inhibitory concentration (PMIC) of plant oils

Minimum Inhibitory concentration (MIC) of plant essential oils against *C. albicans* planktonic cells (PMIC) were determined using microdilution method measured by optical density. The PMIC assays were performed in triplicate in microtiter plates and used an inoculum of 5 x 10^7 CFU/mL in SDB. The final volume including SDB, cells and test compound in each well was 200 µL. The assay of plant essential oils involved in five serial, twofold dilutions using SDB, starting at 1% v/v and ending at 0.06% v/v in methanol. As positive control, fluconazole with concentration of 1 mg/mL in Dimethyl sulfoxide (DMSO) was used. Negative control wells received only SDB and *C. albicans* inoculum. Inhibition was monitored and calculated based on OD_{595} of treated vs control after 48 hours of incubation at 37°C (Faria *et al*., 2011).

Effect of plant essential oils on *C. albicans* biofilm formation inhibition and breakdown

Biofilms were formed on polystyrene flat bottom 96-well microtiter plates (Sarstedt Inc., Newton, NC, USA). Briefly, 100 µL of a standardized cell suspension (10^7 CFU/mL) on RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine (Sigma) was transferred into each well of a microtiter plate, and the plate was incubated for 90 minutes at 37 ˚C of adhesion phase. RPMI 1640 medium was used because this medium capable to induce hyphal formation in *C. albicans*. For media control, wells should be unseeded, and in negative control, biofilms were not exposed to antifungal agent. Following the adhesion phase, the cell suspensions were aspirated and each wells was washed twice with 150 µL of PBS to remove loosely adhered cells. A total of 100 µl RPMI media containing concentration of plant oils ranging from 0.5 % v/v to 0.01 % v/v were added to the washed wells. Fluconazole concentration of 1 mg/mL was used as a positive control in this study, and methanol (MeOH) was used for vehicle control. The plates were then incubated at 37˚C for 8 hours for early phase biofilm, 24 hours for intermediate phase biofilm and 48 hours for mature phase biofilm. Quantification of biofilm formed was done using XTT reduction assay using microtiter plate reader (Bio-Rad 680 XR) at 495 nm. Testing was performed in triplicate. Plant oils found to reduce at least 50% biofilm formation were considered as biofilm preventive (Ramage *et al*., 2002).

To determine the effects of essential oils on *C. albicans* pre-formed biofilms, *C. albicans* biofilms were grown for 8, 24, and 48 h at 37˚C on the wells of microtiter plates using the protocol described by Ramage *et al.* (2002). Briefly, 100 µL of a standardized cell suspension (10^7 CFU/mL) on RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine (Sigma) was transferred into each well of a microtiter plate, and the plate was incubated for 8, 24 and 48 hours at 37˚C. Following biofilm formation, the medium was aspirated and nonadherent cells were removed by washing the biofilms three times in 150 µL sterile PBS per well. After each wash, residual PBS was removed by blotting the microtiter plates in an inverted position with paper towels. Serial double dilutions of plant essential oils (in RPMI 1640 medium) with concentrations ranged from 0.5 % v/v to 0.01 % v/v were then added to the washed wells and the plates were incubated at 37˚C for another 8, 24, and 48 h. The capability of plant essential oils to breakdown *C. albicans* established biofilms were estimated using the XTT reduction assay as describes below.

XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma) solution (0.5 g $liter^{-1}$ PBS) was prepared, filter-sterilized using a 22-µm pore size filter, aliquot into 10 mL working volumes, and stored at -70˚C. A stock solution of 10 mM menadione (Sigma) in 100% acetone was prepared, filter sterilized, aliquout into smaller volume (about 50 µL) and stored at -70˚C. Prior to each assay, tubes containing 10 mL XTT solution were thawed and 1 µL of the stock solution of menadione was ad

The biofilm were first washed three times with 200 µl PBS, and then 100 µL of the XTT-menadione solution were added to each of the prewashed wells. The microtiter plate was then covered with aluminium foil and incubated in the dark for 2-3 h at 37˚C. Following incubation, 75-80 µL of the resulting colored supernatant from each wells was transferred to a new microtiter plate and the color change in the solution was measured with a microtiter plate reader at 495 nm. The absorbance values for the media controls were substracted from the values for the test wells to calculate the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC). $MBIC_{50}$ and $MBEC_{50}$ is the antifungal concentrations at which a 50% decrease in absorbance is detected in comparison with the control biofilms formed by the fungal isolate in the absence of antifungal drug (Pierce *et al*., 2008).

***C. albicans* biofilm architecture analyses**

A Carl Zeiss LSM 5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany) was used to observe the architecture of the *C. albicans* biofilms in the presence or absence of essential

oils tested. To obtained information about the effect of oil on *Candida* biofilm formation, *Candida* biofilms were formed by dispensing standardized cell suspension (3 ml of a suspension containing 1.0 x 10^7 cells/mL in LB medium) onto 25 mm diameter pre-sterilized plastic coverslips (Thermanox; Nulge Nunc International) placed in the wells of pre-sterilized flat bottomed six-well plates (Iwaki). The plate was incubated at 37˚C for 90 minutes (adherent phase). Following incubation period, the supernatant was removed and a total of 3 mL RPMI medium containing different concentrations of plant oils were added to the washed wells. The plate then incubated for 8 h (early phase), 24 h (intermediate phase), and 48 h (mature phase) at 37˚C. To obtained information about the efficacy of oil on breaking down the established biofilm, *C. albicans* biofilm were formed for 8, 24, and 48 h on 25 mm diameter pre-sterilized plastic-coverslips (Thermanox; Nulge Nunc International) placed in the bottom of 6 wells microtiter plate. Following incubation period, the supernatant was removed and a total of 3 mL RPMI medium containing different concentrations of plant oils were added to the washed wells. The plate then incubated for 8, 24, and 48 h at 37˚C.

The coverslips then were washed twice with PBS and stained using the LIVE/DEAD fluorescent stain (10 µL of 3.34 µM SYTO9 and 10 µL of 20 µM Propidium Iodide (PI) both in DMSO) (Molecular Probes, USA) before examined under CLSM. Serial sections in the xy plane were obtained at 1 µm intervals along the z axis. A 40× oil immersion objective were used with 488 nm Ar laser excitation and 500–640 nm band pass emission setting. The image were subsequently analysed using the freely available image processing software ImageJ version 1.46 (Rasband, National Institutes of Health (NIH), Bethesda, Maryland, USA : http://rsb.info.nih.gov/ij/) including the LSM reader plugin to open LSM5 formatted image stack created by the microscope software. The images' scale bar used to calibrate the ImageJ area measurement algorithm. The observations were made in triplicates and representative images are presented here (Ramage *et al*., 2001; Dusane *et al.,* 2012; Jin *et al*., 2005).

Statistical methods

The data were initially analyzed by a normal distribution using the one-sample Kolmogorov-Smirnov test. Following the confirmation of normal distribution, statistical significance of the data were determined using one way ANOVA, followed by Dunnett's test. Differences were considered significant with *P* values of 0.05 or less.

RESULT

Essential oil yields

Oil yields of the plant samples is presented in Table 1. Most plants had oil yield around 0.25-2.5 % v/w, and largest quantity of oil was obtained from leaves part of *Litsea cubeba* (6 % v/w).

Screening and determination of MIC plant oils for *Candida* planktonic growth

The screening for anticandida activity and determination of PMIC of plant oils obtained by microdilution method are shown in Table 1 and Figure 1. Majority of oils tested were found to have anticandida activity and only three of the oils (*Piper cubeba*, *Schleichera oleosa* and *Cananga odoratum*) were found failed to inhibit *C. albicans* growth at the highest concentration tested which was 1 % v/v. From probit analysis, we calculated the $PMIC_{50}$. The oil from *C. burmannii, M. aromatica, O. basilicum* and the seeds of *L. cubeba* at lowest concentration tested (0.06 % v/v) were effective at inhibiting partial (50%) growth ($PMIC_{50}$) of *C. albicans*. *C. burmanii* oil has $PMIC_{50}$ at oil concentration of 0.04 % v/v. *M. aromatica* oil and *L. cubeba* (seeds part), gave $PMIC_{50}$ at concentration of 0.05 % v/v, whereas *O. basilicum* oil showed $PMIC_{50}$ at concentration of 0.06 % v/v.

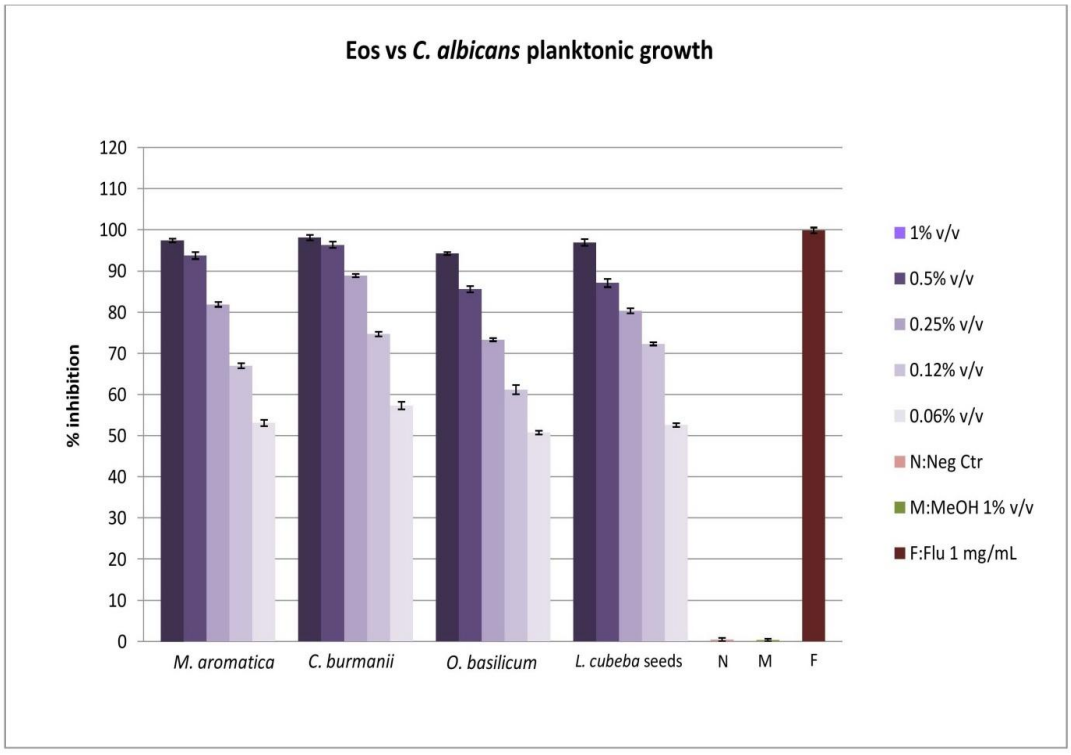

Fig. 1: Comparison of anticandida activity of various essential oils: Ma = *M. aromatica*; Cb = *C. burmannii*; Ob = *O. basilicum*; Lc – s = *L. cubeba* seeds; N = Negative control; M = Methanol 1% v/v; F = Fluconazole 1 mg/mL (positive control). The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Table 1: Effects of different essential oils on planktonic growth, biofilm formation, and biofilm breakdown of *Candida albicans* ATCC 10231. The $PMIC_{50}$, $MBIC_{50}$, and $MBEC_{50}$ results were determined based on probit analysis data. A dash (–) represents that no $PMIC_{50}$ or $MBIC_{50}$ and $MBEC_{50}$ was identified within the concentration range tested.

No	Plant Binomial Name)	Plant part used)	Vol. oil obtained (mL)	Sample fresh weight (g)	Yield (%v/w)[a]	$PMIC_{50}$ (% v/v)	$MBIC_{50}$ oil (% v/v)			$MBEC_{50}$ oil (% v/v)		
							8h	24h	48h	8h	24h	48h
1	*Zingiber zerumbet* (L.) Smith	Rhizome	7	7000	0.10	1.04	0.51	0.51	-	-	-	-
2	*Z. aromaticum* Val.	Rhizome	7	3000	0.23	0.96	-	-	-	-	-	-
3	*Z. officinale* Roxb.	Rhizome	3	3000	0.10	0.24	0.12	0.25	0.25	-	-	-
4	*Z. officinale* Roscoe (var. rubrum Thelidae)	Rhizome	3	3000	0.10	0.22	0.06	0.25	0.25	-	-	-
5	*Kaempferia rotunda* L.	Rhizome	5	6000	0.08	0.25	0.25	0.25	0.5	-	-	-
6	*Languas galanga* (L.) Stuntz.	Rhizome	7	3000	0.23	0.18	0.50	0.50	0.52	-	-	-
7	*Elettaria cardamomum* (L.) Maton	Seed	60	5500	1.09	0.48	0.25	-	-	-	-	-
8	*Curcuma domestica* L.	Rhizome	6	5000	0.12	1.04	-	-	-	-	-	-
9	*Zingiber purpureum* Roxb	Rhizome	12	5000	0.24	0.27	0.12	0.12	0.5	0.5	-	-
10	*Kaempferia galanga* L.	Rhizome	9	3000	0.30	0.97	0.25	0.5	0.5			
11	*C. heyneana* Val. & v.Zijp	Rhizome	5	5000	0.10	0.52	0.52	-	-	-	-	-
12	*Myristica fragrans* Houtt.	Seed	6	3000	0.20	0.52	0.25	-	-	-	-	-
13	*Litsea cubeba* (Lours.) Pers.	Bark	30	3000	1.00	0.25	0.06	0.25	0.5	0.25	0.5	-
		Leaves	60	1000	6.00	0.23	0.12	0.50	-	0.50	-	-
		Seeds	40	1000	4.00	0.05	<0.01	0.03	0.06	0.03	0.03	0.12
14	*Cinnamomum burmannii* Nees ex Bl.	Bark	3	3000	0.10	0.04	0.01	<0.01	0.03	0.03	0.03	0.06
15	*C. sintoc* Bl.	Bark	5.3	3000	0.18	0.14	0.06	0.25	0.5	0.5	-	-

16	*Syzygium aromaticum* (Linn.) Merr.	Leaves	6	3000	0.20	0.25	0.12	0.12	0.52	0.51	-	-
17	*Piper cubeba* L.	Fruit	10	1000	1.00	-	-	-	-	-	-	-
18	*Coriandrum sativum* L.	Seed	10	3000	0.33	0.25	0.12	0.25	0.54	0.51	-	-
19	*Schleichera oleosa* (Lour.) Oken	Seed	20	1000	2.00	-	-	-	-	-	-	-
20	*Citrus aurantifolia* Swingle	Leaves	20	3000	0.67	0.12	0.25	0.25	0.51	0.51	-	-
21	*Acorus calamus* L.	Stem	9	5000	0.18	0.58	0.25	0.25	0.55	-	-	-
22	*Foeniculum vulgare* Mill.	Seed	17	1000	1.70	0.54	0.25	0.55	-	-	-	-
23	*C. sappan* L.	Leaves	8	4000	0.20	1.03	0.54	-	-	-	-	-
24	*Canangium odoratum* Lamk.	Flower	6	1000	0.60	-	-	-	-	-	-	-
25	*Piper retrofractum* Vahl.	Fruit	0.5	1000	0.05	0.54	-	-	-	-	-	-
26	*Syzygium polyanthum* (Wight.) Walp.	Leaves	1	3000	0.03	0.51	0.25	0.25	0.52	-	-	-
27	*Piper nigrum* L.	Seeds	15	1000	1.5	0.97	-	-	-	-	-	-
28	*Ocimum basilicum* L.	Leaves	5	3000	0.16	0.06	<0.01	<0.01	0.04	0.01	0.02	0.02
29	*Massoia aromatica* Becc	Bark	2.5	5000	0.05	0.05	<0.01	<0.01	0.01	0.01	0.02	0.07

[a] % oil yield (v/w) = (volume of the oil obtained)/(weight of ground plant part) x 100%

Effect of plant essential oils on *C. albicans* biofilm formation and established biofilm

C. albicans biofilm formation proceeds through three distinct developmental phases: early or adherence period (0 - 11 h), intermediate (12 – 30 h), and mature (38 – 72 h) (Jabra-Rizk, *et al*., 2004). Plant essential oils at concentrations of 0.5% - 0.01% v/v were tested against *C. albicans* adherent cells populations at different stages of biofilm development. XTT assay was performed to determine the metabolic activity of *C. albicans* biofilms in the presence or absence of the oils tested.

The result of this experiment is shown in Table 2, demonstrated that partial (50%) inhibition of *C. albicans* early, intermediate and mature biofilms occurred in the presence of oils tested except for *Z. aromaticum, E. cardamomum, C. domestica, C. heyneana, M. fragrans, P. retrofractum* and *P. nigrum* oil which were failed to prevent *C. albicans* biofilm formation at the highest concentration tested (0.5 % v/v).

Only 4 from 29 essential oils tested showed capability in both inhibiting and breaking down *C. albicans* biofilms, i,e. *C. burmannii* oil, *M. aromatica* oil, *O. basilicum* oil and the oil from seeds part of *L. cubeba*. The oils from the bark of *C. burmannii* and *M. aromatica* exhibited the $MBIC_{50}$ at concentration below 0.01 % v/v in order to inhibit early and intermediate phase of *C. albicans* biofilm, however higher concentration of these oils (0.03% v/v) needed to give 50% inhibition of the *C. albicans* mature biofilm (Figure 2). At concentration 0.01 % v/v, the metabolic activity of intermediate phase (24 hours) *C. albicans* biofilm in the presence of *C. burmannii* was 41.2 ± 0.5 % (P<0.001), while *M. aromatica* oil addition to the media for *C. albicans* biofilm formation capable to reduce metabolic activity of *C. albicans* intermediate biofilm up to 35.6 ± 0.5 % (P<0.001).

Compare to *C. burmani* and *M. aromatica* oils, the oil obtained from *O. basilicum* and the seeds of *L. cubeba* showed less potent activity in inhibit intermediate and mature phase of *C. albicans* biofilm. Higher concentration (0.03 and 0.06% v/v) was needed by these oils to inhibit intermediate and mature phase of *C. albicans* biofilm (Figure 2). The metabolic activity of a 24 hours *C. albicans* biofilm in the presence of *O. basilicum* oil at concentration of 0.03 % v/v was 43.1±0.4 % (P<0.001), whereas in the presence of *L. cubeba* oil concentration of 0.03 % v/v, the metabolic activity of *C. albicans* intermediate biofilm was 47.9±0.4 % (P<0.001) accordingly. Higher concentration (0.03 and 0.06% v/v) were needed by *C. burmani* and *M. aromatica* oils (0.03 % v/v), and by *O. basilicum* and *L. cubeba* oils (0.06 % v/v) to inhibit mature phase (48 hours) of *C. albicans* biofilm (Figure 2 and 3).

In order to eradicate the established biofilm, higher concentrations of essential oils were needed. As shown in figure 2, the oils from the bark of *C. burmannii* and *M. aromatic*a showed capability in partial disrupting (50%) the early and intermediate phase of established *Candida* biofilm at concentration of 0.03, meanwhile against mature phase of *C. albicans* established biofilm, those oils were shown activity in partially reducing biofilm at higher concentration (0.06%). In the presence of *C. burmannii* oil at a concentration of 0.06 % v/v, the metabolic activity of mature phase preformed biofilm of *C. albicans* was 48.4±0.7 % (P<0.01), while in the presence of *Massoia* oil at the same concentration, metabolic activity of 48 hours *C. albicans* biofilm was 43.7±0.5 % (P<0.01), respectively (Figure 2).

Higher concentration (0.12 % v/v) was also needed by the oil from *O. basilicum* and *L. cubeba* to be able to breakdown *C. albicans* mature biofilm. In the presence of concentration of 0.12 % v/v *L. cubeba* oil, mature phase preformed biofilm of *C. albicans* metaboilic activity was 35.6±0.4% (P<0.001), while metabolic activity of mature phase *C. albicans* biofilm in the presence of *O. basilicum* oil at concentration 0f 0.12 % v/v was 34.4±0.7 % (P<0.001), respectively (Figure 3).

Biofilm architecture analyses

The antibiofilm activity of plant essential oils tested in microtiter plate using XTT method was confirmed by CLSM analysis of *C. albicans* biofilm. The biofilms were grown on the surface of coverslips and stained with SYTO9 and PI for the monitoring of live/dead cells. We observed a preventive activity on biofilm formation, when we inoculated *C. albicans* with different concentration of *C. burmannii, M. aromatica, O. basilicum* and *L. cubeba* oil tested. Growth suppression of *C. albicans* biofilm by essential oils was clearly seen by CLSM with the Live/Dead stain, compared with the dense biofilm growth of the control (Figure 4).

The architecture of *C. albicans* biofilm in the presence of tested oils in biofilm formation inhibition and biofilm breakdown assays were showed in Figure 5-8**.** The estimated three-dimensional view of the biofilm refers to the total area in the x-y-z dimension, where x and y are the coordinates of the pixel positioning and z is the intensity collected using ImageJ. Live cells are labeled in green (SYT09), and dead cells are labeled in red (propidium iodide).

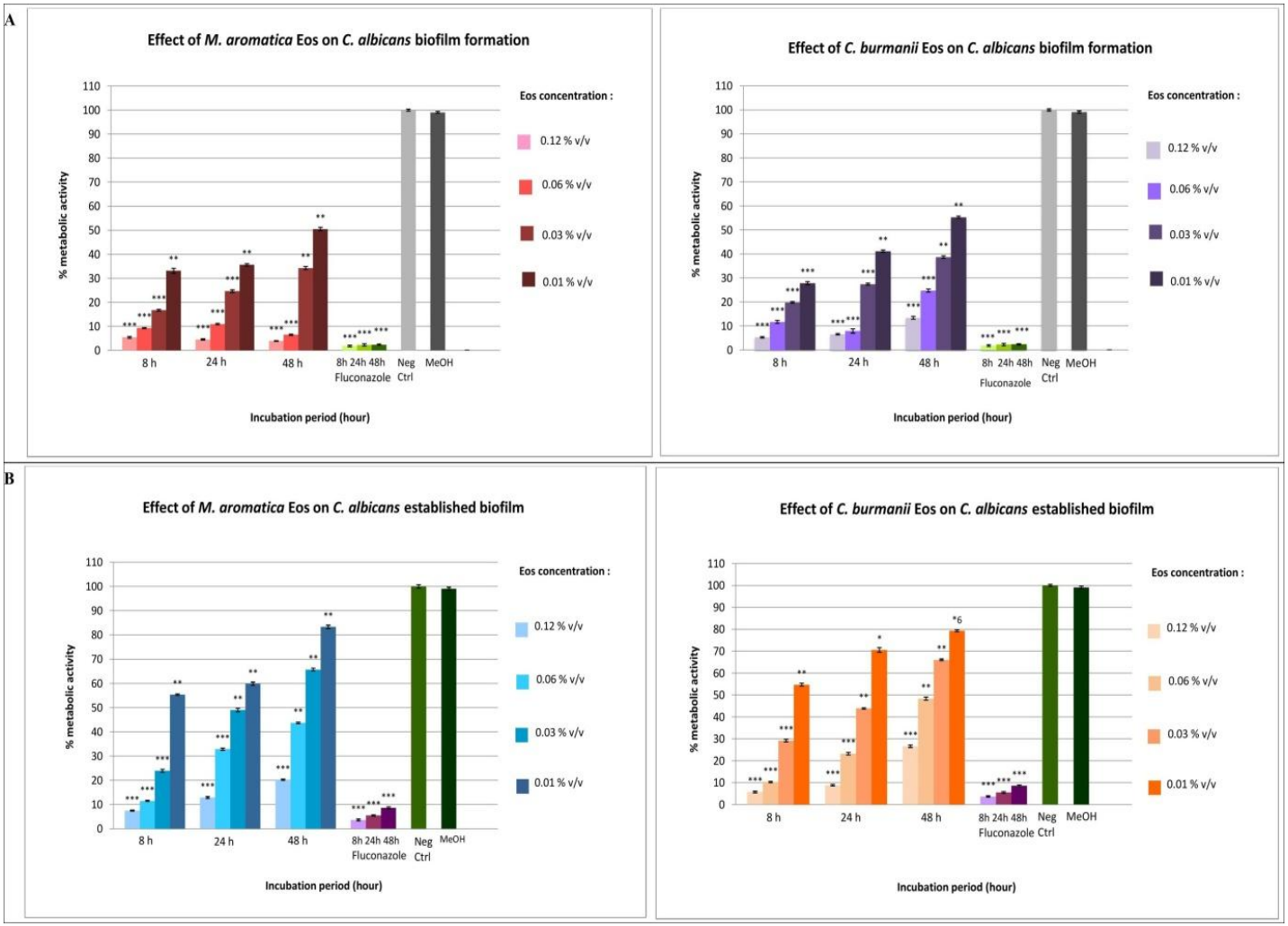

Fig. 2: The percentage activity of *Massoia aromatica* oil and *Cinnamomum burmannii* oil: (A) in inhibit *Candida albicans* biofilm formation, and (B) in breaking down established *C. albicans* biofilm. The concentration of Fluconazole used is 1 mg/mL. The bars indicated the standard deviations of the menas. Asterisks indicate a significant difference between treatment and negative control.

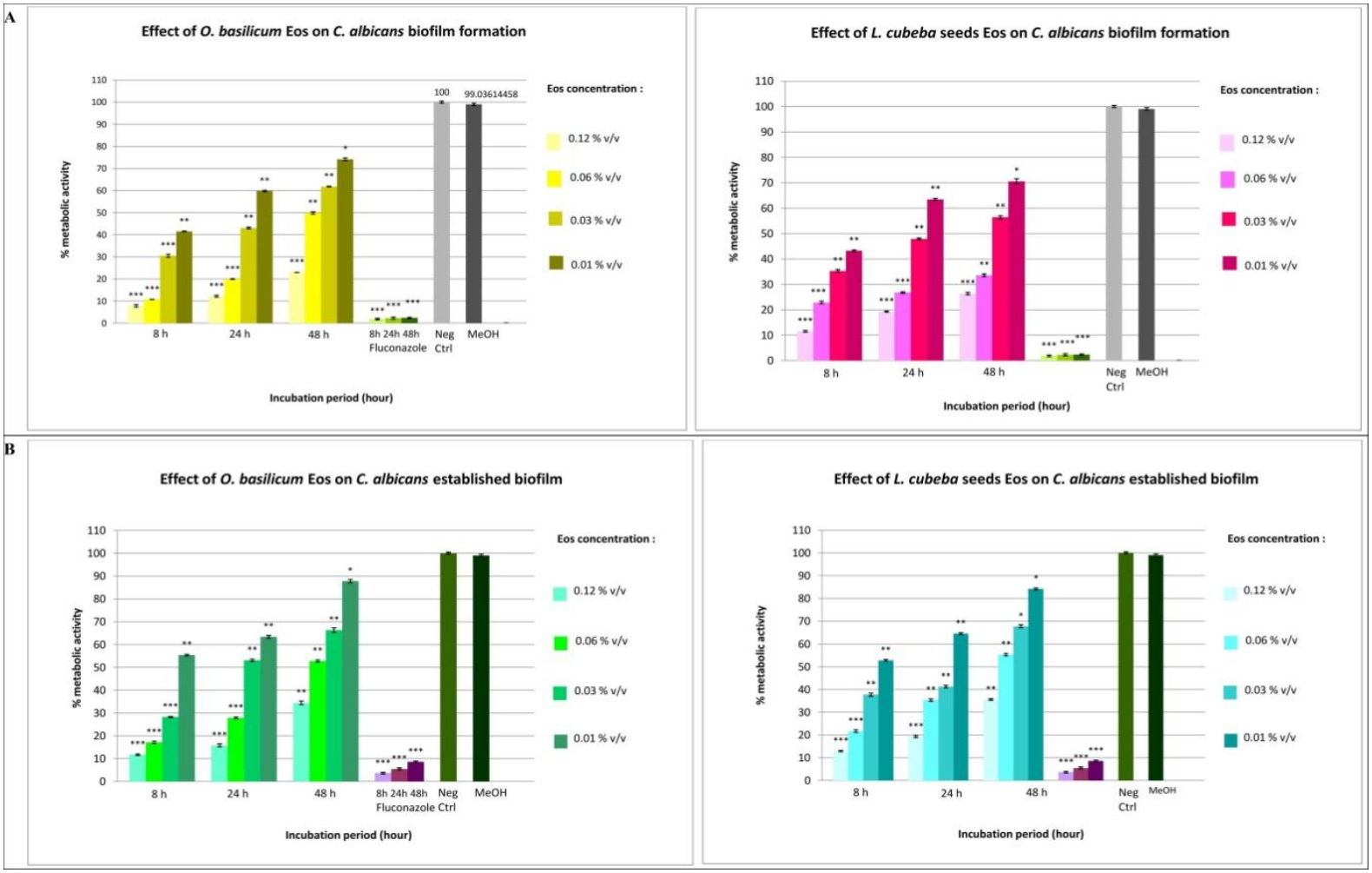

Fig. 3: The percentage activity of *Ocimum basilicum* oil and *Litsea cubeba* oil: (A) in inhibit *Candida albicans* biofilm formation, and (B) in breaking down established *C. albicans* biofilm. The concentration of Fluconazole used is 1 mg/mL. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Propidium iodide (PI) is a non-permeant fluorescent nucleic acid stain that enters dead/nonviable cells by binding to double-stranded nucleic acids through intercalation between base pairs with no preference to purine or pyrimidine base pairs. Figure 4 shows intense green fluorescence resulting from membrane permeant Syto9 binding to DNA of *C. albicans* intact cells while PI stains the cells red. Thus, areas of red fluorescence represent dead cells, and green fluorescent indicates healthy cells. The yellow color (formed as a result of overlapping of red and green images which were representative of SYTO9 and PI colocalization due to the presence of dead cells or eDNA, a DNA which is present in all environments as a source of organic nutrients and provide structural support for biofilm matrix.

The result of this experiment, as assessed by colorimetric assay and microscopy, demonstrated that negative control (biofilm formed in the absence of essential oil) exhibited typical biofilm architecture. At early phase of biofilm development, *C. albicans* biofilm was found to be mainly composed of yeast cells. Elongated pseudohyphae and hyphae were found at intermediate phase of *C. albicans* biofilm, and at mature phase biofilms composed mainly of intertwining mycelial structures, and a basal layer of blastospores (Figure 4).

Pretreatment of *C. albicans* biofilm with various essential oil concentrations showed that there was a dose-dependent effect on biofilm formation. CLSM study shows that the number of viable cells (green) decreased when the concentration of extract exposed increased. Figure 5-8 showed that in the presence of essential oils tested (concentration of 0.03% v/v), a significant decrease of *C. albicans* biofilm biomass compare to negative control was evident. Preincubation of biofilm with the concentration of essential oil equal to $PMIC_{90}$ prevented successful germination of the adherent yeast cell, resulting in nonexistent biofilm (data not shown). When the concentration was decreased, pseudohyphae and mycelia were observed. The antibiofilm activity thus may be due to the antifungal activity of the essential oils.

An interesting antibiofilm effect was determined against 2 h and 24 h old biofilm by using *C. burmannii*, *M. aromatica, O. basilicum and L. cubeba* (seeds part) essential oils, which are found to be the most potential source to inhibit *C. albicans* biofilm. Our results clearly demonstrate that essential oil of *C. burmannii*, *M. aromatica, O. basilicum and L. cubeba* (seeds part) not only able to kill *C. albicans* cells efficiently ($PMIC_{50} \leq 0.06\%$ v/v), but also inhibit biofilm formation at sub-PMIC concentration. At the lowest concentration tested (0.01% v/v), essential oil of *C. burmannii, M. aromatica* and *O. basilicum* were still capable to inhibit 50% the formation of early phase *C. albicans*

biofilm. However, as shown in Figure 5 and 7, the same concentration of oils was found failed to exhibit 50% inhibition of *C. albicans* mature (48 h) biofilm formation as the treated biofilms displayed minor defects in the overall biofilm architecture compare to the control biofilm.

As can be seen in figure 6 and 8, CLSM images revealed that exposure of *C. burmannii*, *M. aromatica, O. basilicum and L. cubeba* (seeds part) essential oil could eradicate the established *C. albicans* biofilm, however higher concentration needed to breakdown the established biofilm compare to the concentration needed to disturb the biofilm formation. The concentration of oils tested required to remove 50% mature phase of *C. albicans* established biofilm mass were two-four times higher than those needed for breaking down intermediate phase established biofilm.

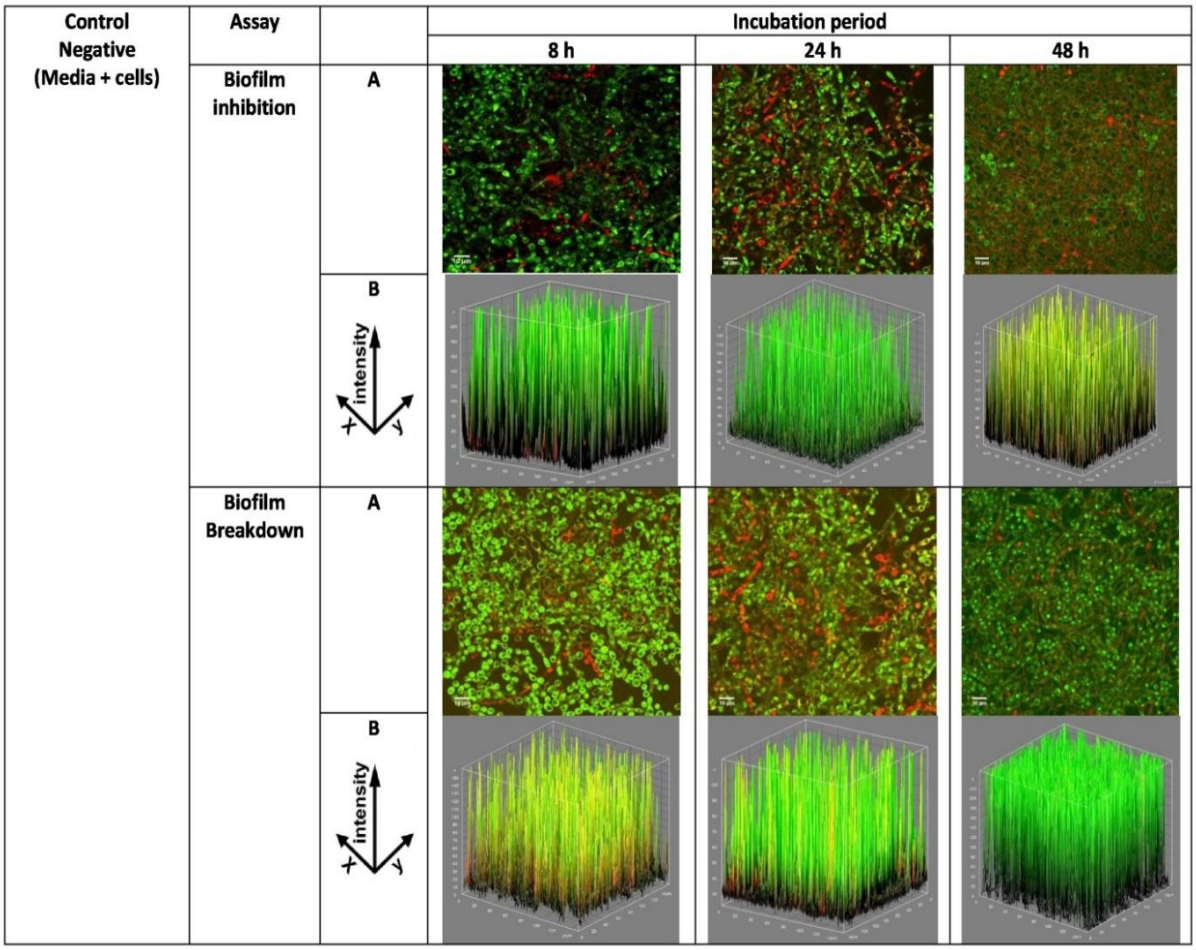

Fig. 4: Representative confocal scanning micrograph images of negative control *Candida albicans* biofilm obtained from biofilm formation inhibition assay and biofilm breakdown assay. A: projected upper view of the biofilm, B : estimated three-dimensional view of the biofilm.

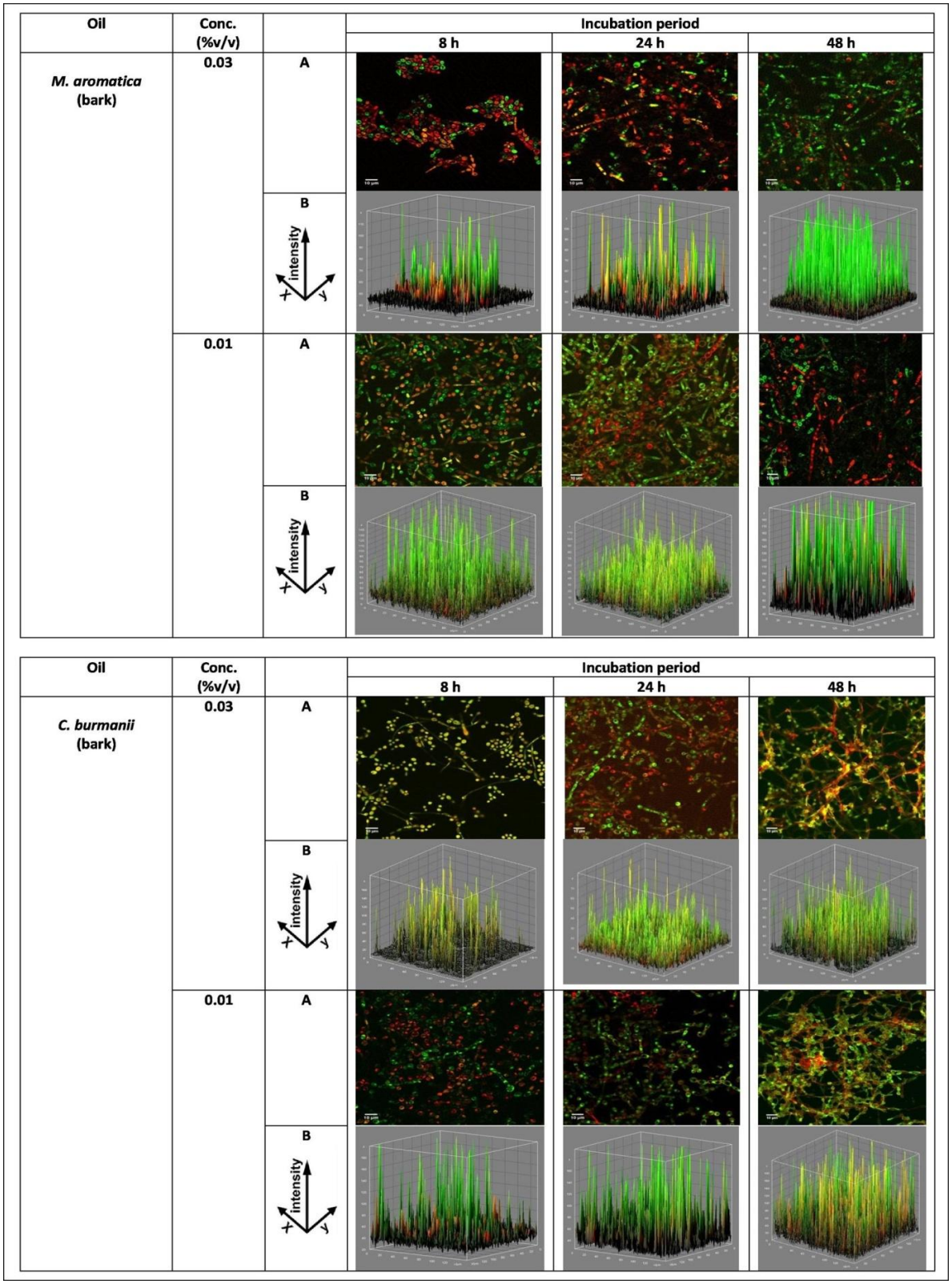

Fig. 5: Representative confocal scanning micrograph images of biofilm inhibition activity of *Massoia aromatica* and *Cinnamomum burmannii* essential oil at concentration of 0.03 and 0.01 % v/v against *Candida albicans*. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm.

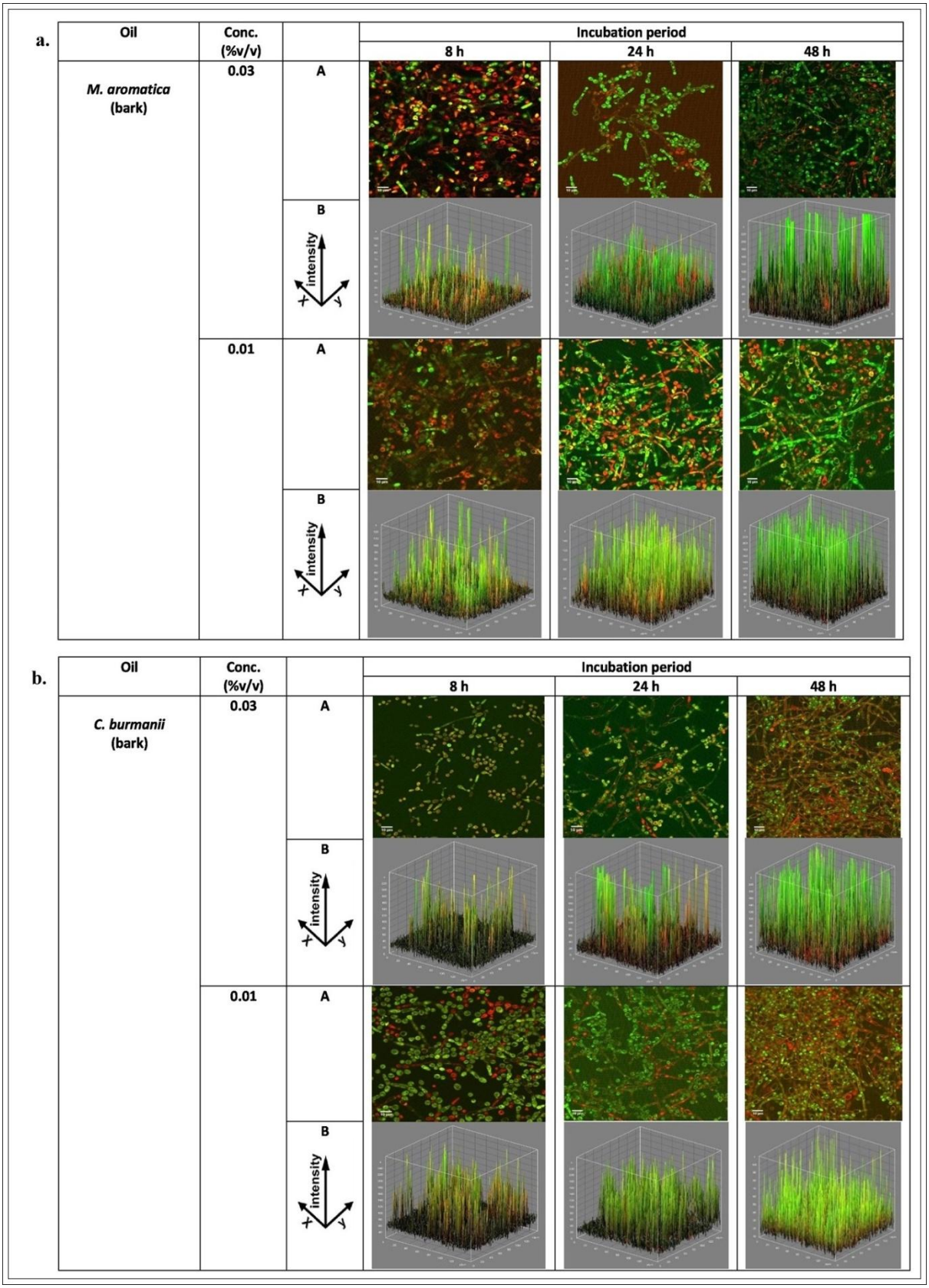

Fig. 6: Representative confocal scanning micrograph images of biofilm breakdown activity of *Massoia aromatica* and *Cinnamomum burmannii* essential oil at concentration of 0.03 and 0.01 % v/v against *Candida albicans* established biofilm. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm.

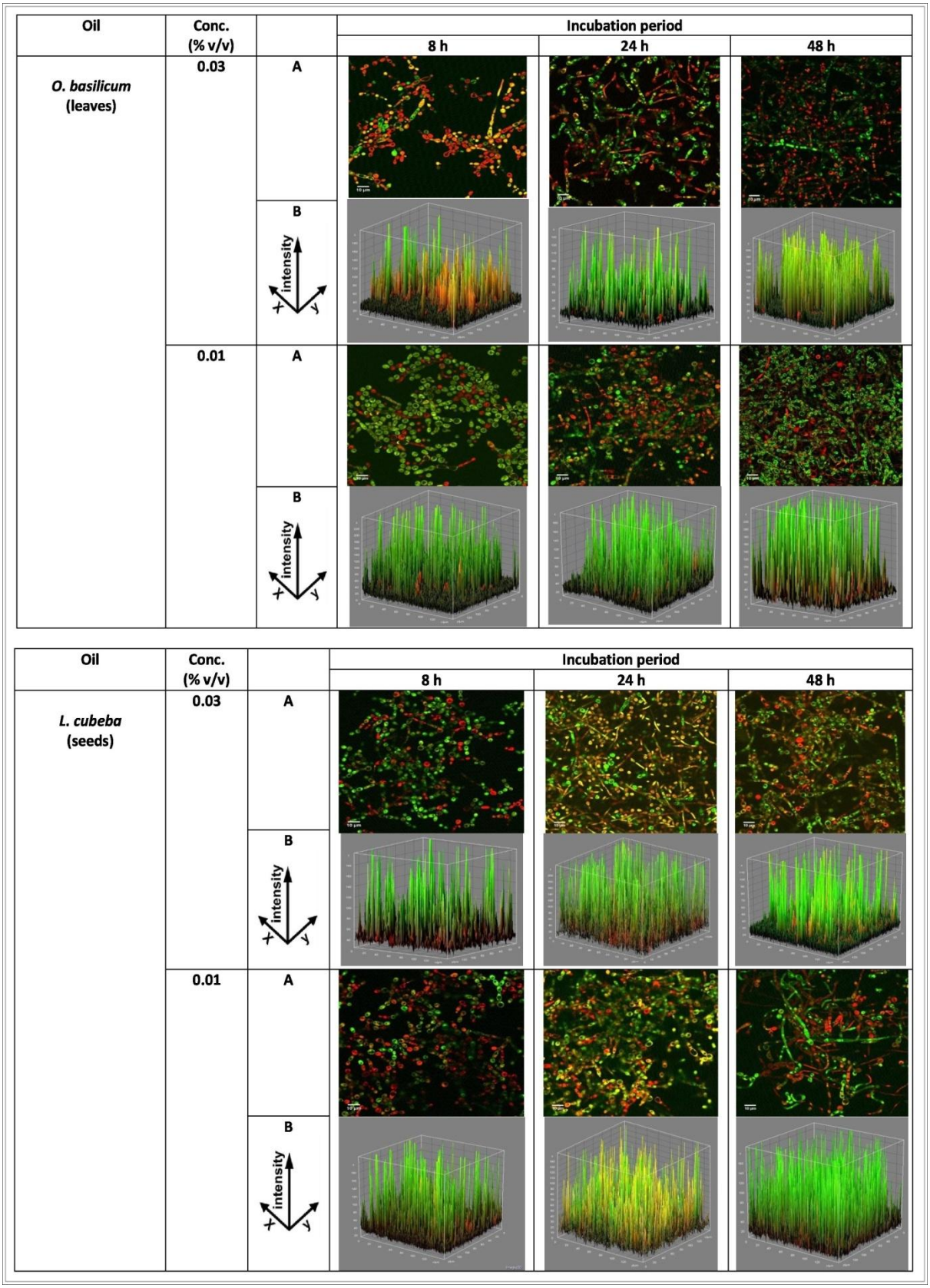

Fig 7. Representative confocal scanning micrograph images of biofilm inhibition activity of *Ocimum basilicum* and *Litsea cubeba* (seeds) essential oil at concentration of 0.03 and 0.01 % v/v against *Candida albicans* established biofilm. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm.

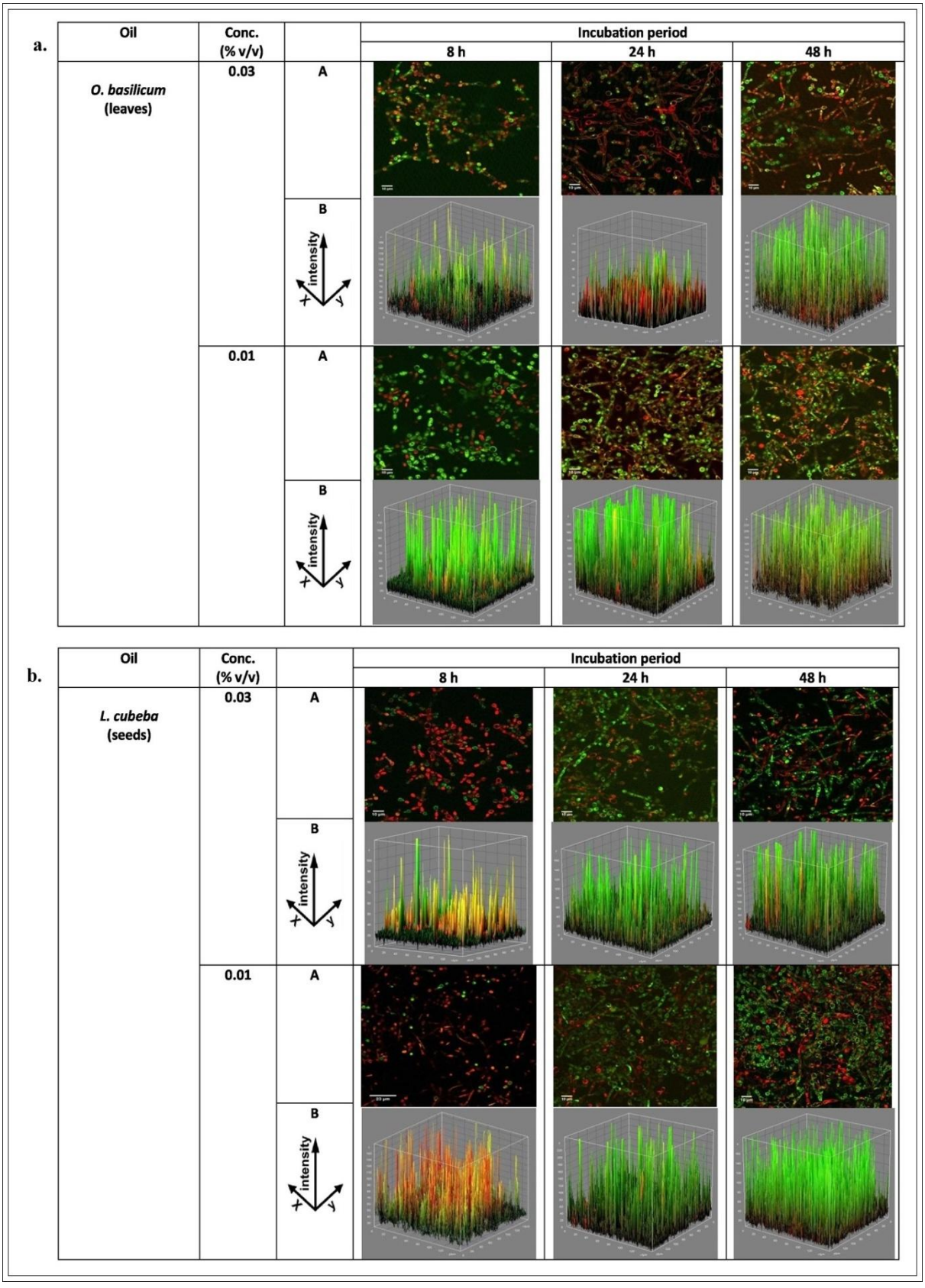

Fig. 8: Representative confocal scanning micrograph images of biofilm breakdown activity of *Ocimum basilicum* and *Litsea cubeba* (seeds) essential oil at concentration of 0.03 and 0.01 % v/v against *Candida albicans* established biofilm. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm.

GC-MS analysis

The chemical compositions of *C. burmannii* and *M. aromatica* bark, *O. basilicum* leaves and *L. cubeba* seeds essential oils determined using GC-MS are shown in Table 2. A total of three major compounds were identified in *C. burmannii* oil, 2 major compounds were identified in *M. aromatica* oil, 6 major compounds were identified from *L. cubeba* seed oil and five major compounds were identified from *O. basilicum* oil. Cinnamaldehyde (92.0%) was found to be main compound of *C. burmannii* oil and massoia lactone (92.1 %) was found to be the major consituent of *M. aromatica* oil. Among six major identified compounds found in oil of *L. cubeba* seed, citronella (41.3%) was found to be the most abundant compound followed by limonene (23.5%), whereas 2,6-Octadienal, 3,7-dimethyl-, (E)-(CAS) Geranial (36.3 %) and Z-citral (29.2 %) were found to be major compounds in *O. basilicum* oil.

DISCUSSION

C. albicans is a major human fungal pathogen, since its capable of invading deep tissue and organs, superficial sites such as skin, nails and mucosa. Like many other microbial organisms, *C. albicans* is capable to adhere to and colonize surfaces of medical devices, resulting in the development of a biofilm. The increasing incidence of *C. albicans* biofilm infections raises the urgent need for new antibiofilm agent, as the number of therapeutic options for *Candida* biofilm-related disease is very small. The discovery of antiinfective agents, which are not only against planktonic microorganisms but also against microbial biofilms, represents an important goal. Plant materials are important sources of starting materials for bioactive compounds in drug discovery, and many of them exhibit potent activity against fungal pathogen (Tsang *et al*., 2012).

In this study we evaluated the effect of essential oils on various stages of *in vitro C. albicans* biofilm development. Our result indicated that essential oil of *C. burmannii, M. aromatica, O. basilicum* and *L. cubeba* (seeds part) have significant effect on the adhesion of *C. albicans* to polystyrene surfaces. Established *C. albicans* biofilms formed in polystyrene surface were effectively disturbed by the exposure of essential oils tested, athough higher concentration of oils needed to breakdown the already established biofilm. The reduced antibiofilm activity towards the established biofilm proved that cells within the biofilm are more resistant to antimicrobial agents compare to planktonic cells (Sandasi *et al*., 2009).

Table 2: Major chemical constituents of plant essential oils as identified by GC-MS.

Plant Essential Oil	Peak	Retention time (RT)	Kovats Retention Index (RI)	Area	Area (%)	Similarity index (SI)	Chemical Component
C. burmanii	3	19.296	1123.459	164665734	92.02	95	Cinnamaldehyde
	5	21.630	1142.506	7342238	4.10	93	Alpha-Copaene
	7	23.472	1156.016	3708184	2.07	91	3-Phenyl-2-prophenyl acetate
M. aromatica	3	25.346	1168.629	156590945	92.05	94	5-Hydroxy-2-Decenoic acid lactone (Massoialactone)
	4	29.961	1195.847	10522375	6.19	86	5-Hydroxy-2-Decenoic acid lactone (Massoialactone) *
L. cubeba	6	9.976	1007.68695	12572648	23.54	93	Limonene
	7	10.025	1008.59674	1007044	1.89	88	1,8-cineole
	12	14.284	1072.10950	22032578	41.26	93	citronella
	17	16.439	1096.33838	2860892	5.36	94	citronellol
	22	20.098	1130.27734	994918	1.86	90	Alpha-terpinyl-acetate
O. basilicum	1	8.346	973.82391	2600710	5.33	95	6-Methyl-5-hepten-2-one
	4	12.183	1044.06531	1876093	3.84	95	Alpha-terpinolene
	9	17.088	1102.93787	14268004	29.22	94	Z-citral
	11	18.066	1112.37109	17991094	36.85	95	2,6-Octadienal, 3,7-dimethyl-, (E)- (CAS) Geranial
	13	22.558	1149.46313	377404	0.77	91	Trans-alpha-bergamotene

*The compound obtained is predicted to be a 5-Hydroxy-2-Decenoic acid lactone (Massoialactone) derivate with higher boiling point and have the same fragmentation as Massoialactone.

CLSM was employed as a qualitative analytical tool to refeal the architecture of *C. albicans* biofilm in the presence or absence of essential oils tested. CLSM images confirmed the inhibition of biofilm formation and breakdown of biofilm biomass in the presence of essential oils. Microbial cells within the biofilm are protected by extracellular polymeric substances (EPS) composed mainly by proteins, glycoconjugates, and polysaccharides. EPS is secreted by microorganisms to their environment, and provide a barrier to the diffusion of antimicrobials and thus, limit the access of drugs to the cells deep in the biofilm (Baillie and Douglas, 2000). Since many antimicrobial agents fail to penetrate extracellular polymeric substances (EPS) biofilm which protect the microbial cells within, the use of compounds which capable to degrade the EPS biofilm become a worthwhile alternative to consider (Walker *et al*., 2007). Deprivation of microbial adherence and biofilm formation without disturbing bacterial growth is a characteristic of antipathogenic therapies which become an alternative to combat microbial resistance to known antimicrobial agents (Escaich, 2008).

C. burmannii, M. aromatica, O. basilicum and *L. cubeba* are ones among Indonesian plants which are used in food not only to enhance flavor and fragrance qualities and appetizing effects but also for their preservation. We determined the compositions of *C. burmannii, M. aromatica, O. basilicum* and *L. cubeba* seeds essential oils using GCMS. It has been shown that *C. burmannii* oil contains cynnamaldehyde as major compounds, whereas massoialactone was found to be the major compound of *M. aromatica* oil. Citronella and limonene is reportedly detected as main constituent of *L. cubeba* seed oil geranial and z-citral were found to be major compounds in *O. basilicum* oil.

According to Khan and Ahmad (2012), cynnamaldehyde possesses antifungal and antibiofilm activity against *C. albicans*. Study taken by Kim and Park (2012) and Mesa-Arango et al. (2009) showed that geranial and citral have antifungal activity against *Candida* and *Aspergillus*. Previous studies also reported that limonene and citronelal possess antifungal activity (Agarwal *et al*., 2008; Chee e*t al*., 2009; Li *et al*., 2013).

The possible mechanism of an active compound(s) to attenuate the development of a biofilm, or to breakdown a mature biofilm, may be due to the the compound(s) capability to disturb microorganism's quorum sensing (Manefield *et al*., 1999), disrupt the permeability or to modify the hydrophobicity of microbial membrane structures (Cox *et al*., 2000), or to break the extracellular polymeric matrix in the biofilm to release biofilm from the surface of the solid substratum (Traba and Liang, 2011).

Although Khan and Ahmad (2012) already reported that cynnamaldehyde, the major components of *C. burmannii* essential oil posesses antibiofilm activity against *C. albicans* biofilm, in our knowledge the essential oil of *M. aromatica, O. basilicum* and *L. cubeba* (seeds part) have not previously been tested as *C. albicans* antibiofilm agents. The efficacy of these essential oils should be considered as an important factor in evaluating them as an interesting source of innovative plant derived antifungal agents that can be used in the development of new strategies to treat biofilms of medical relevance. Further studies would be required to isolate, identify and characterize the active compounds responsible for the observed antibiofilm activity.

CONCLUSION

In conclusion, the finding from this study conclusively demonstrated the *in vitro* antifungal and antibiofilm activity of *C. burmannii, M. aromatica, O. basilicum* and *L. cubeba* (seeds part) essential oil against *C. albicans.* This study highlights the significance of essential oils in producing novel antibiofilm compound. Further purification of the active compounds may be suggested on the basis of the present study.

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Chapter 5

Screening of Indonesian Medicinal Plants for Quorum Sensing Inhibitory Compounds

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ABSTRACT

Targeting the quorum sensing system and interrupting bacterial communication, is an example of an anti-pathogenic effect and may provide a solution to increasing antibiotic resistance. The use of plants as medicine in Indonesia has always been a tradition. Previous anti-infective studies on Indonesian medicinal plants have focused mainly on anti-microbial drug discovery perspectives. However, no systemic effort has been made to explore the anti-quorum sensing activity of these plants. Our objective is to investigate several Indonesian medicinal plants' ethanol extracts and essential oils on anti-quorum sensing activity using a quorum sensing violacein biosensor, *Chromobacterium violaceum* CV026. Also the effect of extracts and essential oils on the motility of *Pseudomonas aeruginosa* PAO1 is of interest, since bacterial motility has been shown to be associated with its virulence. Of the 54 plant ethanol extracts and 30 essential oils screened, *Nymphaea nouchali* ethanol extract, *Syzygium aromaticum* essential oil and *Massoia aromatica* essential oil demonstrated violacein production inhibition in the reporter strain. A significant reduction in quorum sensing related motility of *P. aeruginosa* PAO1 was also observed. This screening system forms an interesting potential for the selection of active principles from plant ethanol extracts and essential oils.

Keywords : Quorum sensing, *Chromobacterium violaceum* CV026, motility, *Pseudomonas aeruginosa* PAO1, *Nymphaea nouchali*, *Syzygium aromaticum*, *Massoia aromatica*.

INTRODUCTION

Quorum sensing is a process of cell to cell communication in bacteria mediated by a small diffusible molecule called autoinducers (oligopeptides in Gram positive and *N*-acyl homoserine lactone (AHL) in Gram negative bacteria). These autoinducers diffuse freely from the bacterial cell and accumulate in the surrounding environment. When a threshold concentration (quorum) has been reached they diffuse back into the cell and regulate transcription of specific genes as in a response to their changing environmental conditions (Waters and Bassler, 2005). Fungi, like bacteria, also use quorum sensing to affect population-level behaviors such as biofilm formation and pathogenesis (Hogan, 2006; Nickerson *et al.,* 2006; Albuquerque and Casadevall, 2012). In the human commensal and pathogenic fungus *Candida albicans*, two quorum sensing molecules have been described: farnesol and tyrosol (Chen *et al*., 2004; Hornby *et al*., 2001). In *Candida albicans*, accumulated farnesol and tyrosol affect both dimorphism and biofilm formation. As well as in *C. albicans*, another QS molecules in fungi, phenylalanine and tryptophan, were found in *Saccharomyces cerevisiae* (Albuquerque and Casadevall, 2012).

Examples of cellular processes modulated by quorum sensing are biofilm formation (McClean *et al*., 1997), bioluminescence (Nealson and Hasstings, 2006) and motility (Eberl *et al*., 1999). These traits have also shown to be involved in the pathogenicity of bacteria (Choo *et al*., 2006). It has been suggested that targeting the quorum sensing system by interruption of bacterial communication, instead of killing bacteria, is an example of an antipathogenic effect and may give a solution to antibiotics resistance (Hentzer and Givskov, 2003). Therefore, anti-quorum sensing (anti-QS) compounds can be of great interest in the treatment of bacterial infections (Fuqua *et al*., 2001; Rice *et al*., 2005).

Anti-QS compounds like halogenated furanones produced by the marine alga *Delisea pulchra* (Manefield *et al*., 1999 have limited or no therapeutic application due to their toxicity and high reactivity on the host. Other anti-QS molecules have been found from garlic extract and *Penicillium* species. During screening of 100 extracts from 50 Penicillium species, Rasmussen *et al.* (2005) found patulin and penicillic acid as biologically active QS inhibitor (QSI) compounds. However, patulin is found to be toxic and today it is belongs to a list of mycotoxins that level in food is regulated. The US Food and Drug Administration (FDA) limits patulin to 50 µg/L (Puel *et al*., 2010). As well as patulin, penicillin acid also belongs to mycotoxin group. It found in many fruits and vegetables, and toxic effects may result from the accidental ingestion of the material. Animal experiments indicate that ingestion of less than 40 gram

may be fatal or may produce serious damage to the health of the individual (Anonym, 2010). By bioassay-guided fractionation of garlic extracts, Jakobsen *et al*. (2012) determined the primary QS inhibitor present in garlic to be ajoene, and the toxicity investigation revealed that ajoene has a very low cytotoxicity effects on human epithelium cells. Therefore, the discovery of non-toxic, broad spectrum quorum sensing inhibitors is still needed for successful exploitation in inhibiting bacterial communication (Choo *et al.,* 2006).

Plants have long been a source of medicines and continues to contribute significantly to the development of today's pharmaceuticals for therapeutics and source of new bioactive compounds (Cragg *et al*., 1997). Indonesia harbors a very high flora of diverse species used in traditional ways as medicine (Damayanti *et al*., 2001). Previous anti-infective studies on Indonesian medicinal plants have focused mainly on antimicrobial drug discovery perspectives. However, no systemic effort has been made to explore its anti-QS activity. Furthermore, shifting the focus from antibacterial activity to anti-QS properties may disclose new quorum quenching compounds (Adonizio *et al*., 2006). For this reason, research in determining anti-QS activity of a compound is generating potential for development of a new therapeutic.

Motility of *P. aeruginosa* in aqueous and dry environments has been shown to be associated with its virulence. In the presence of a quorum sensing inhibitory compound, the motility and therefore the virulence will be limited (Drake and Montie, 1988; Häse, 2001; Rasmussen *et al*., 2011; Majik *et al*., 2013). Six different forms of bacterial movement have been described including swimming, swarming, twitching, gliding, sliding and darting (Henrichsen, 1972). These various forms of surface motility enable bacteria to increase their efficiency of nutrient uptake, avoid toxic substances, move to preferred hosts and provide access to optimal colonization sites within, and spread themselves into the environment (Rashid and Kornberg, 2000). Bacterial motility plays a different role in biofilms. They can promote adhesion of the cell to the surface for biofilm maturation process and/or in be involved in the dispersal process (Marchal *et al*., 2010). However, motility is not critical to biofilm formation, which was shown for biofilms produced by *P. aeruginosa* PAO1 mutant strain which is lack flagella and type IV pili (Chow *et al*., 2011).

In this study, we provide the screening result of some commonly used Indonesian medicinal plants for anti-QS activity using the quorum sensing violacein biosensor, *Chromobacterium violaceum* 31532 wild

type (WT) strain, *C. violaceum* CV026 mutant strain, and *P. aeruginosa* PAO1. The wild type strain *C. violaceum* ATCC 31532 produces a purple pigment, violacein, when AHL molecules reach a threshold level, whereas the mutant strain CV026 lacks the ability to produce violacein unless exogenous AHLs are detected. We also investigated the correlation of the quorum sensing inhibition in reducing quorum sensing related motility of *P. aeruginosa* PAO1. Swimming, swarming and twitching motility have been studied in this research. We have found anti-QS activity in 10 out of 54 plant ethanol extracts and 12 out of 29 plant essential oils, with 1 extract and 2 essential oils demonstrating a high anti-quorum sensing activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The bacterial strains used in this study were *Chromobacterium violaceum* ATCC 31532 wild type (WT) strain*, C. volaceum* CV026 mutant strain and *P. aeruginosa* PAO1 strain. The C6-homoserine lactone (HHL) (Sigma Aldrich, Germany) was dissolved in dimethylsulfoxide (DMSO) and used as autoinducer for violacein pigment production in *C. violaceum* CV026. The bacterial strains were grown on Luria-Bertani (LB) Agar and incubated for 24 hours at 30^{o}C. Following the incubation on agar plate, the colonies were transferred to LB broth and incubated for another 24 hours at 30^{o}C with shaking (250 rpm). Cell density was adjusted to 10^{8} CFU/mL by altering the optical density of the suspension to 0.1 at 600 nm.

Indonesian medicinal plants

Fifty four commonly used medicinal plants were collected from Yogyakarta, and its surroundings, and the bark of *Massoia aromatica* Becc. was collected from Nabire district, West Papua, Indonesia, on the basis of ethnopharmacological information. Crude ethanol extracts were prepared by washing and cutting the plant samples into small pieces followed by oven drying (40^{o}C) for 48-72 hours. The dried plant materials were grind into a fine powder. The pulverized materials were soaked in Petroleum Ether (PE) in a ratio of 1 g (plant material) : 10 ml PE to remove the lipids. Secondly, plant materials were also extracted with 70% ethanol (EtOH) using a ratio of 1 g (plant material) : 10 mL (EtOH) to obtain crude ethanol extract. Extracts were dried and concentrated under reduced pressure using a rotary evaporator (Ika RV 10 Basic, Ika HB 10, Heidolph). Stock solutions (100 mg/mL) of crude ethanol extracts in the excipient DMSO were prepared, filter-sterilized (0.2 µm) and stored at 4^{o}C. A total of 29 essential oils were obtained from water-steam distillation process. The oils obtained were dried over anhydrous sodium sulphate (Na_2SO_4), filtered using a Whatman filter paper no. 40, and stored in sealed dark glass

vial at 4°C. Stock solutions of 50% (v/v) essential oils were prepared in methanol (MeOH), for the following dilution to obtain essential oil concentration ranging from 1 to 0.01 % v/v.

Screening for anti-quorum sensing assay using disc diffusion method

Standard disc diffusion assay was used to detect anti-quorum sensing activity of the plant ethanol extracts and essential oils. 100 µL of *C. violaceum* WT strain adjusted to $OD_{600\ nm}$ = 0.1 (approximately 1 x 10^8 CFU/mL) was spread on LB agar plates. A cork-borer (6 mm in diameter) was used to make wells on the agar plates. Each plant ethanol extract (1 mg/mL) in DMSO and essential oil 1% v/v in MeOH was loaded in the wells. DMSO concentrations of 1 % v/v and methanol concentration of 1 % v/v were used as the background control. Streptomycin at a concentration of 100 µg/mL was used as positive (growth inhibition) control. Bacterial growth inhibition by the plant extracts and essential oils was measured the radius (r1) in mm from the obtained halo, while plant extracts and essential oils showing both growth and pigment inhibition was measured as radius (r2) in mm. The pigment inhibition was determined by substracting bacterial growth inhibition radius (r1) from the total radius (r2) thereby, quorum sensing inhibition is r2-r1 in mm, as illustrated in Figure 1 (Zahin *et al*., 2010).

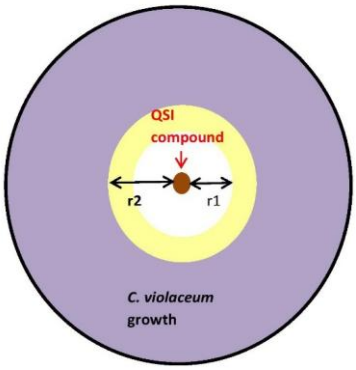

Fig. 1: Illustration of quorum sensing inhibition diameter measurement on *C. violaceum* growth on a petridish.

Planktonic Minimal Inhibitory Concentration (PMIC)

Planktonic minimal inhibitory concentrations (PMIC) of plant ethanol extracts and essential oils which showed anti-QS activity against *C. violaceum* WT strain were determined by the broth microdilution test. Briefly, LB broth containing two-fold increments of plant extracts and essential oils was added to a 96-wells plate micro dilution tray. The bacterial suspension equal to a 0.5 McFarland standard was further

diluted and added to the tray to achieve a final inoculum of 5×10^5 CFU/mL per well. Inoculated 96-well plates were incubated for 18–24 h at 30 °C. The $PMIC_{50}$ concentration, i.e. the extract or oil concentration showing 50% growth inhibition on the bacterial tested, was calculated using probit analysis.

Quantification of violacein production

Properly labeled tubes containing: (i) LB broth and HHL; (ii)LB broth, HHL and methanol; (iii) LB broth, HHL and different dilutions of essential oils; were used to measure the amount of violacein production. 100 µL of a bacterial suspension (10^8 CFU/mL) was added to each tube. Tubes were incubated for 24 hours at 30°C (Table 1).

Table 1: Composition of the tubes for determining cell growth inhibition and antiquorum sensing activity.

Content \ Tube	A	B	C	Final Concentration
LB	895 µL	895 µL	895 µL	
CV026	50 µL	50 µL	50 µL	1 x 10^8 CFU/mL
HHL	5 µL	5 µL	5 µl	15 µmol/mL
Ethanol extracts/Essential oils	-	-	50 µl	½ PMIC
DMSO/Methanol	50 µL	50 µL	-	≤ 1% (v/v)
Total Volume	1000 µL	1000 µL	1000 µL	

After incubation, tubes were centrifuged at 13.000 rpm for 10 minutes to precipitate the insoluble violacein. The culture supernatant was discarded and 1 mL of DMSO or MeOH was added to the pellet. The solution was vortexed vigorously for 30 seconds to completely solubilize violacein and centrifuged at 13.000 rpm for 10 minutes to remove the residue of cells. 200 µL of the violacein containing supernatant was added to a 96-well flat bottomed microplate, four wells per each sample and the absorbance was measured at 595 nm with a microplate reader (Perkin Elmer's Enspire® 2300 multimode plate reader) (Choo *et al*., 2006).

***Pseudomonas aeruginosa* PAO1 motility test**

The plant ethanol extract and essential oils which inhibited violacein production were further tested to explore their effects on quorum sensing related swarming, swimming and twitching motility of *P. aeruginosa* PAO1. Swimming, swarming and twitching motility assays were performed by the method of Rashid and Kornberg (2000). Briefly, LB agar (0.3%) plates (for swimming motility), and LB agar (0.5%) plates (for swarming motility) containing sub-inhibitory concentrations of ethanol extract or essential oils were prepared and allowed to dry for 3-4 h at 30^{o}C. Plates were point inoculated with freshly grown culture cells using a blunt ended sterile toothpick. For twitching motility, LB agar (1%) plates were used bacterial cells were inoculated by using a sharp end toothpick and stabbing through the agar to the bottom of the petridish. After 24 hours of incubation in upright position at 30^{o}C, the extent of motility was determined by measuring the diameter of the bacterial colony.

Statistical analysis

All data were initially analyzed by a normal distribution using the one-sample Kolmogorov-Smirnov test. Following the confirmation of normal distribution, differences for individual parameters between treated groups and control were tested by one way ANOVA, followed by Dunnett's test. Differences were considered significant with P-values of 0.05 or less.

RESULT

Samples preparations

A list of the plant extracts studied, including the botanical name, voucher specimen and data related to traditional use is listed in Table 1 Chapter 2, whereas the list of essential oils is indicated in Table 1 Chapter 3. Fifty four plants ethanol extracts and 29 essential oils were obtained as described in Material and Methods of Chapter 2 and 3, this thesis. The yields of plant oils are presented in Table 2 Chapter 3, this thesis.

Anti-quorum sensing activity

The inhibition in quorum sensing activity of the evaluated plant ethanol extract and essential oils are shown in figure 2 and 3. The disappearance of the violet colored (violacein) pigment in *C. violaceum* is an indication of anti-QS activity by the added plant ethanol extracts and essential oils. From out of 54 plant ethanolis extracts screened for anti-quorum sensing activity, 10 plants (*Cinnamomum burmannii*,

Zingiber officinale, *Terminalia catappa*, *Sesbania grandiflora*, *C. sintoc*, *Piper bettle*, *Caesalpinia sappan*, *Kaempferia rotunda*, *Nymphaea nouchali* and *Syzygium aromaticum*) showed anti-QS activity, 12 out of 29 plants essential oil extracts (*Massoia aromatica, Ocimum basilicum, Z. purpureum, Litsea cubeba* (leaves and bark part), *C. sintoc*, *S. polyanthum, S. aromaticum, Myristica fragrans, Z. officinale var rubrum, Coriandrum sativum, K.rotunda,* and *K.galanga*) proved to be effective. Strong anti-QS activity was observed in essential oils from *M. aromatica* (Table 2).

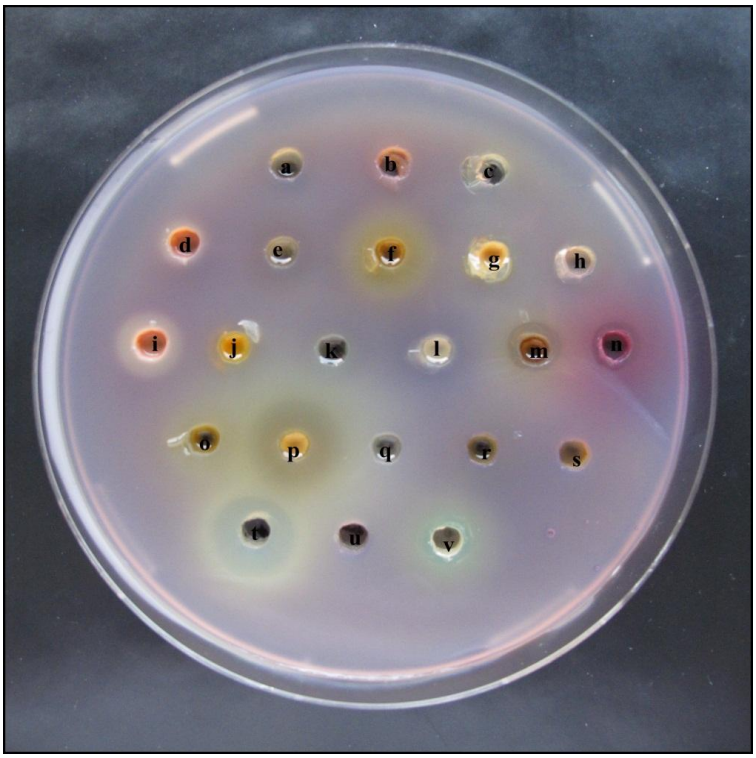

Fig. 2: Inhibition in violacein production and cell growth in *Chromobacterium violaceum* ATCC 31532 (wild type strain) by ethanol extracts. Anti-QS activity and growth inhibition was demonstrated for (a) *Cinnamomum burmannii* , (e) *Zingiber officinale*, (f), *Syzigium aromaticum,* (i) *Sesbania grandiflora*,(l) *Cinnamomum sintoc*, (m) *Piper bettle*, (n) *Caesalpinia sappan*, (o) *Kaempferia rotunda*, (p) *Nymphaea nauchalli*, and (v) *Terminalia catappa* ethanol extracts. As a positive control (t), Streptomycin 50 µg/mL was used as antibiotic control, and (u) DMSO was used as a solvent control.

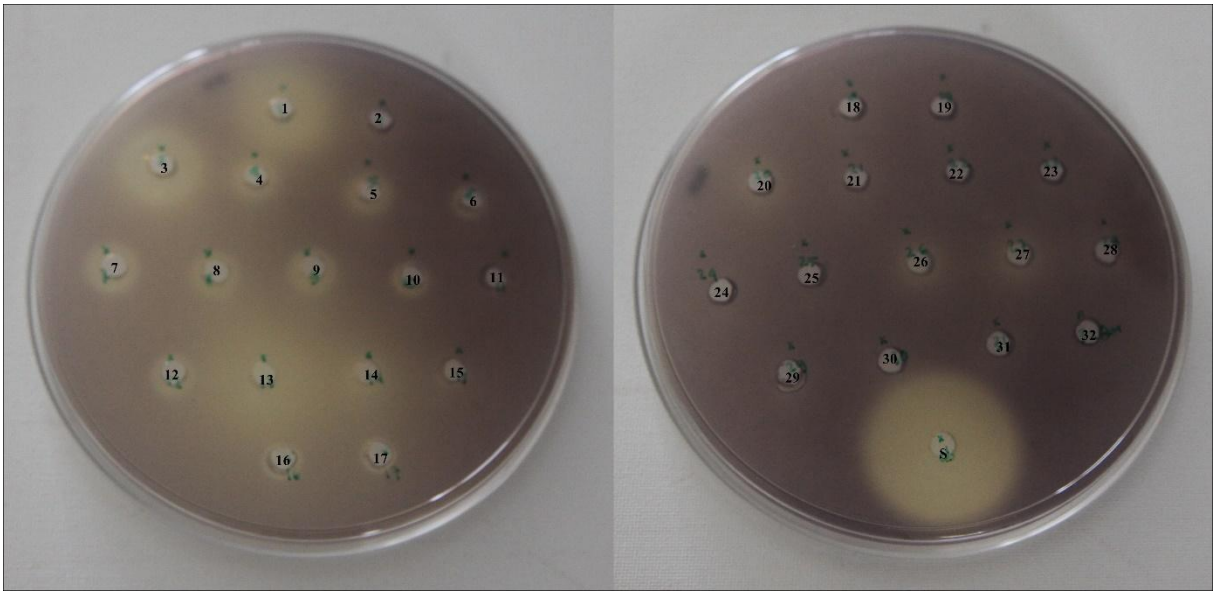

Fig. 3: Inhibition in violacein production and cell growth in *Chromobacterium violaceum* ATCC 31532 (wild type strain) by essential oils : (1) *Massoia aromatica*, (2) *Ocimum basilicum*, (3) *Cinnamomum burmannii*, (4) *Zingiber purpureum*, (5) *Litsea cubeba* (leaves), (6) *L. cubeba* (seeds), (7) *L. cubeba* (bark), (8) *Cinnamomum sintoc*, (9) *Elettaria cardamomum*, (10) *Citrus aurantifolia*, (12) *Z. officinale var rubrum*, (13) *Syzigium aromaticum*, (14) *S. polyanthum*, (15) *Coriandrum sativum*, (16) *Piper nigrum*, (17) *Caesalpinia sappan*, (20) *Myristica fragrans*, (21) *Kaempferia rotunda,* (27) *K. galanga*. As a positive control (S), Streptomycin 100 µg/mL was used.

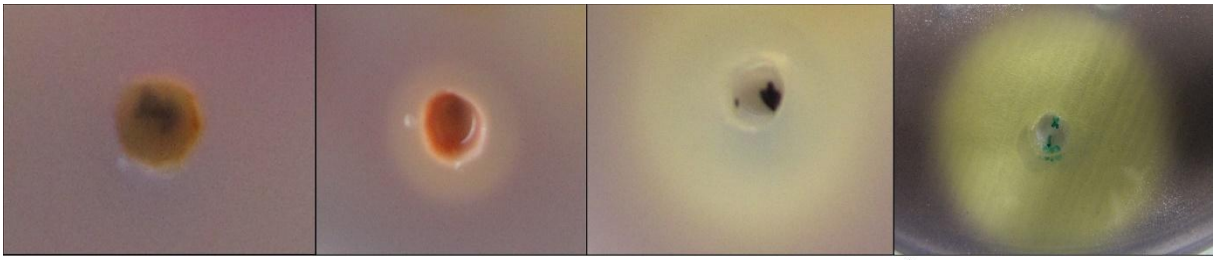

Fig. 4: Anti-QS activity versus antibacterial activity. (a) no anti-QS nor antibacterial activity, (b) anti-QS activity, (c) anti-QS and antibacterial activity, (d) antibacterial activity.

Table 2: Antiquorum sensing activity of Indonesian medicinal plants ethanol extracts by agar well diffusion method (Values are mean ±SD of 3 experiments).

No	Plants	Sample form	Part used	Zone of inhibition (mm)		
				Total radius (r1)*	Growth Inhibition (r2)*	QS Inhibition (r1-r2)
1	*C. burmannii*	Extract	Bark	13.0±0.0	11.5±0.0	1.5±0.0
2	*Z. officinale*	Extract	Rhizome	13.0±0.0	9.5±0.0	3.5±0.0
3	*S. aromaticum*	Extract	Flower	21.5±0.0	9.5±0.0	12.0±0.0
4	*Sesbania grandiflora*	Extract	Leaves	13.0±0.0	8.0±0.0	5.0±0.0
5	*C. sintoc*	Extract	Bark	13.5±0.0	9.0±0.0	4.5±0.0
6	*P. betle*	Extract	Leaves	12.0±0.0	9.5±0.0	2.5±0.0
7	*C. sappan*	Extract	Bark	12.5±0.0	8.0±0.0	5.5±0.0
8	*K. rotunda*	Extract	Rhizome	11.5±0.0	8.0±0.0	3.5±0.0
9	*Nymphaea nouchali*	Extract	Flower	24.5±0.0	11.5±0.0	13.0±0.0
10	*Terminalia catappa*	Extract	Leaves	16.0±0.0	9.2±0.0	6.8±0.0
11	*Massoia aromatica*	Oil	Bark	24.2±0.3	-	24.2±0.3
12	*Syzygium aromaticum*	Oil	Flower	23.7±0.6	8.3±0.56	15.3±0.6
13	*Ocimum basilicum*	Oil	Leaves	9.7±0.6	-	9.7±0.6
14	*Zingiber purpureum*	Oil	Rhizome	12.2±0.3	-	12.2±0.3
15	*Z. officinale var rubrum*	Oil	Rhizome	10.0±0.00	8.0±0.0	2.0±0.0
16	*Litsea cubeba*	Oil	Leaves	12.2±0.3	-	12.2±0.3
			Bark	12.0±0.0	9.3±0.6	2.7±0.6
			Seeds	8.0±0.0	8.0±0.0	-
17	*S. polyanthum*	Oil	Leaves	12.0±0.0	-	12.0±0.0
18	*Myristica fragrans*	Oil	Seeds	10.2±0.3	-	10.2±0.3
19	*Kaempferia rotunda*	Oil	Rhizome	10.0±0.0	-	10.0±0.0
20	*K. galanga*	Oil	Rhizome	10.0±0.0	-	10.0±0.0
21	*Coriadrum sativum*	Oil	Seeds	9.5±0.5	-	9.5±0.5
22	*Cinnamomum burmannii*	Oil	Bark	18.0±0.0	18.0±0.0	-
23	*C. sintoc*	Oil	Bark	8.2±0.3	-	8.2±0.3
24	*Elettaria cardamomum*	Oil	Bark	12.0±0.0	12.0±0.0	-
25	*Piper nigrum*	Oil	Fruit	9.2±0.3	9.2±0.3	-
26	*Caesalpinia sappan*	Oil	Bark	9.2±0.2	9.2±0.3	-
27	*Citrus aurantifolia*	Oil	Leaves	9.3±0.6	9.3±0.6	-
28	Control (Streptomycin 50 µg/mL)			24.8±0.0	24.8±0.0	-
29	Control (Streptomycin 100 µg/mL)			36.2±0.3	36.2±0.3	-

r1= radius of growth + pigment inhibition (mm); r2= radius of growth inhibition (mm); (r1-r2)= radius of pigment inhibition (mm).

*including 6mm wells diameter on agar plate

PMIC determination

The antibacterial activity of plant ethanol extracts and essential oils against *C. violaceum* CV026 was determined using the microbroth dilution method. The results obtained from this assay are presented in Table 3**.**

S. aromaticum and *N. nouchali* ethanol extracts showed the capability to inhibit 50% of *C. violaceum* CV026 growth ($PMIC_{50}$) at a concentration of 0.12 mg/mL and 0.25 mg/mL. Higher concentrations to inhibit CV026 growth were needed for the other ethanol extracts. A total of four essential oils (*M. aromatica*, *O. basilicum*, *K. rotunda*, and *C. sativum*) had a $PMIC_{50}$ at a concentration of 0.06% (v/v). Essential oils from *C. burmannii* showed a $PMIC_{50}$ at 0.03% (v/v).

Violacein quantification

Quantification of violacein production by *C. violaceum* CV026 was used to measure the inhibition quantity in quorum sensing activity. *N. nouchali* ethanol extract, *S. aromaticum* essential oil and *M. aromatica* essential oil showed the highest quorum sensing inhibition activity by using the agar well diffusion method. Figure 5 shows the result of reduction in violacein production by *N. nouchali* ethanol extracts, *S. aromaticum* essential oil and *M. aromatica* essential oil.

Nymphaea nouchali ethanol extracts, *S. aromaticum* essential oil and *M. aromatica* essential oil showed a concentration dependent anti-quorum sensing activity derived from the amount of violacein produced. At elevated concentrations, both the ethanol extract and essential oils tested demonstrated antibacterial activity besides the anti-QS activity (Figure 5).

At the lowest concentration tested (0.06% v/v) of *M. aromatica* oil, the amount of violacein pigment produced by *C. violaceum* was 38.9±0.6% (***P*<0.01), whereas the percentage of cell growth on that concentration was 72.0±0.5% (**P*<0.05). In the presence of concentration of 0.06% v/v of *S. aromaticum* oil, violacein production of *C. violaceum* was 43.7±0.4% (***P*<0.01), and the percentage of total cell growth was around 64.4±0.6% (***P*<0.05), respectively. This result indicates that the anti-quorum sensing activity of *M. aromatica* and *S. aromaticum* oil is independent of cell growth (Figure 5).

The violacein production of *C. violaceum* CV026 impregnated with HHL was respectively decreased by the ethanol extract of *N. nouchali* at a concentration of 0.25 mg/mL, with total amount of violacein

produced as much as 49.2±0.9% (**P<0.01) . However, at the same concentration the percentage of cell growth was found to be 51.2±0.8% (**P<0.01) indicates that for *N. nouchali* extract, the violacein production inhibition is basically dependent on the cell growth of bacterial reporter strain (Figure 5).

The ethanol extracts and essential oils which showed anti-QS activity by inhibition of violacein production were further tested to explore their effect on quorum sensing related swimming, swarming and twitching motility for *P. aeruginosa* PAO1.

Table 3: Antiquorum sensing activity of Indonesian medicinal plants essential oils by microdillution method.

No	Plants	Materials	$PMIC_{50}$ [a]
1	*N. nouchali*	Extract	0.25 mg/mL
2	*Syzygium aromaticum*	Extract	0.12 mg/mL
3	*Massoia aromatica*	Oil	0.06% (v/v)
4	*Syzygium aromaticum*	Oil	>1.00% (v/v)
5	*Ocimum basilicum*	Oil	0.06% (v/v)
6	*Zingiber purpureum*	Oil	0.12% (v/v)
7	*Z. officinale var rubrum*	Oil	0.25% (v/v)
8	*Litsea cubeba* (bark)	Oil	0.25% (v/v)
	L. cubeba (leaves)	Oil	0.25% v/v
	L. cubeba (seeds)	Oil	0.12% (v/v)
9	*S. polyanthum*	Oil	0.25% (v/v)
10	*Myristica fragrans*	Oil	0.12% (v/v)
11	*Kaempferia rotunda*	Oil	0.06% (v/v)
12	*K. galanga*	Oil	0.12% (v/v)
13	*Coriadrum sativum*	Oil	0.06% (v/v)
14	*Cinnamomum burmannii*	Oil	0.03% (v/v)
15	*C. sintoc*	Oil	0.12% (v/v)
16	*Elettaria cardamomum*	Oil	0.50% (v/v)
17	*Piper nigrum*	Oil	0.25% (v/v)
18	*Caesalpinia sappan*	Oil	0.12% (v/v)
19	*Citrus aurantifolia*	Oil	0.50% (v/v)

[a] $PMIC_{50}$: plant ethanol extract concentration (mg/mL) or essential oil concentration (% v/v) at which 50% respectively of *C. violaceum* CV026 planktonic growth is inhibited.

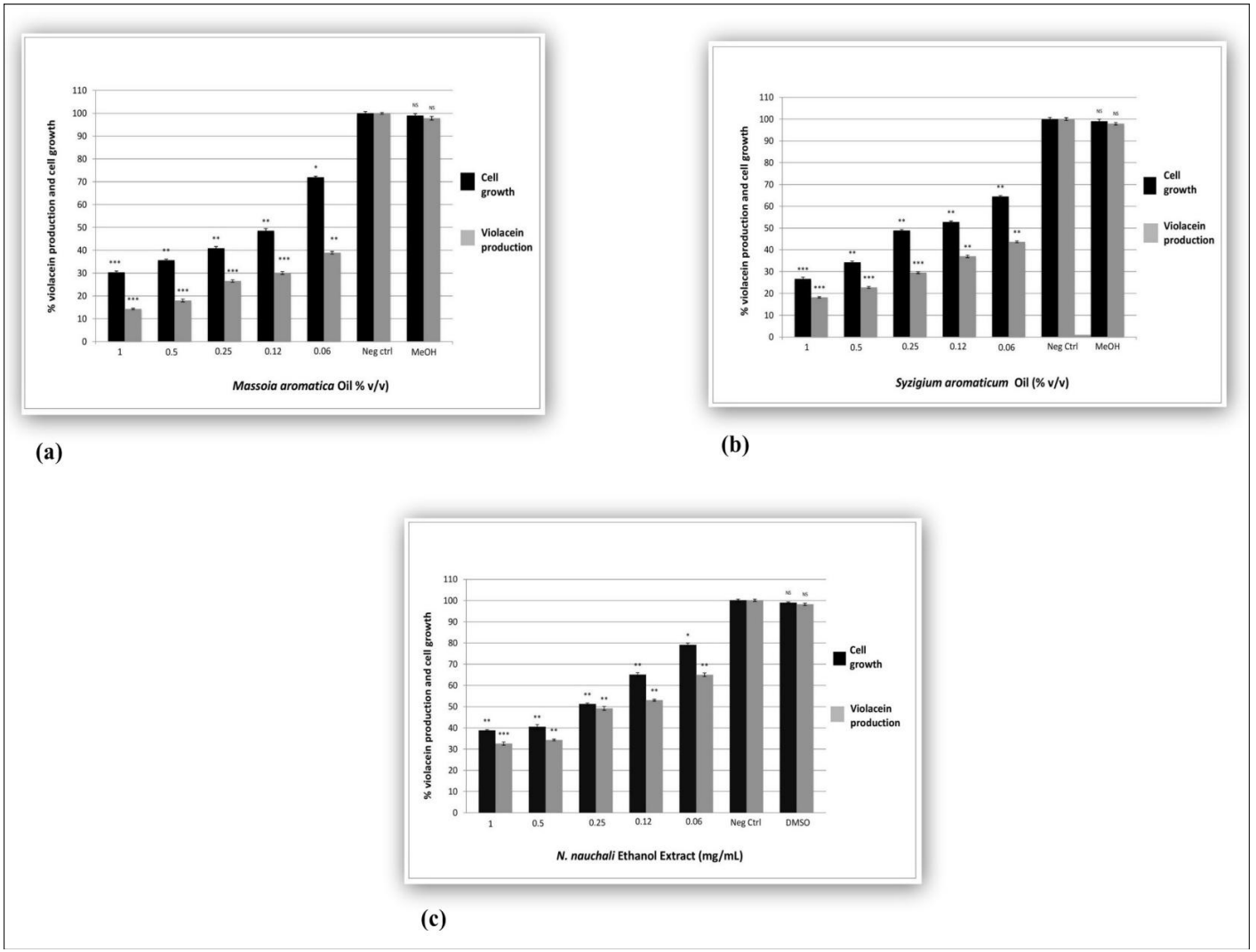

Fig. 5: Inhibition of *Chromobacterium violaceum* CV026 growth and its violacein production by : (a) *Massoia aromatica* essential oil , (b) *Syzigium aromaticum* essential oil, and (c) *Nymphaea nouchali* ethanol extract. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Inhibition of bacterial motility

We investigated if the extracts and essential oils which inhibited quorum sensing had any effect on quorum sensing related motility in the human opportunistic pathogen *P. aeruginosa* PAO1. Our result, shown in figure 6, indicated that ethanol extracts of *N. nouchali* at a concentration of 1 and 0.5 mg/mL significantly reduced the swimming motility of *P. aeruginosa* PAO1 by 74.7±0.9 % (***P<0.001) and 54.4±0.6 % (**P<0.01), respectively. The swarming motility of *P. aeruginosa* PAO1 was also reduced as much as 67.8±0.3 % (***P<0.001) and 53.4±0.3 % (**P<0.01) when concentrations of 1 mg/mL 0.5 mg/mL were applied. The same concentrations of the extract also showed a decrease in twitching motility namely, 75.8±0.0 % (***P<0.001) and 62.1±0.3 % (**P<0.001) respectively.

These results are in accordance with our previous study (chapter 2 this thesis) which showed that ethanol extracts from *N. nouchali* have inhibitory effects on the biofilm of *P. aeruginosa* PAO1 at the concentrations of 0.06 mg/mL. According to O'Toole and Kolter (2002), twitching motility of the type IV pili has been shown to be important for the adherence adhesion to eukaryotic cell surfaces and is necessary for biofilm development. At the microscopic level, the edge of the colonies in twitching motility is highly irregular and this is thought to be a consequence of the surface movement associated with type IV pili (Figure 7).

Both concentrations of essential oils from *M. aromatica* (1% (v/v) and 0.5% (v/v)) that were tested significantly decreased the swarming motility of *P. aeruginosa* PAO1 by 29.57 ± 0.57 % (***$P\leq0.001$), and 51.4 ± 0.0 % (**P<0.01) respectively. As much as 100±0.0 % and 50.9 ± 0.6 % inhibition in twitching motility for *P. aeruginosa* PAO1 was demonstrated by these essential oils with concentrations ranging from 1 to 0.06 % (v/v) (Figure 8). The capability of essential oils from *M. aromatica* in reducing *P. aeruginosa* PAO1 twitching motility correlated siginificantly with a decrease in *P. aeruginosa* PAO1 biofilm formation when these cells were incubated with the same essential oil concentration of 0.06 % (v/v) and higher (data chapter 3 this thesis). However, at concentrations lower than 0.5 % (v/v) essential oils from *M. aromatica* failed to reduce more than 50% of the swimming motility of *P. aeruginosa* PAO1.

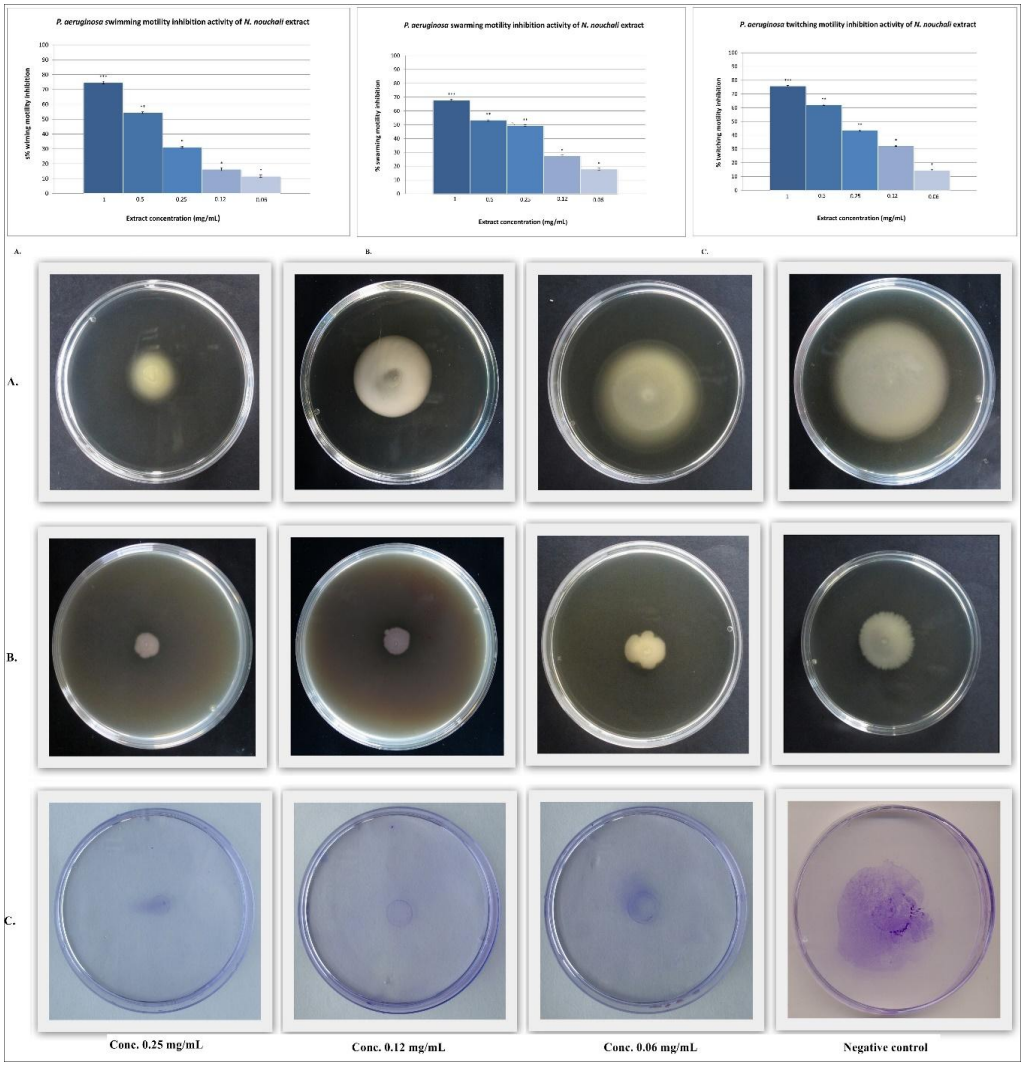

Fig. 6: Effect of *Nymphaea nouchali* ethanol extracts on swimming, swarming and twitching motility of *Pseudomonas aeruginosa* PAO1. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

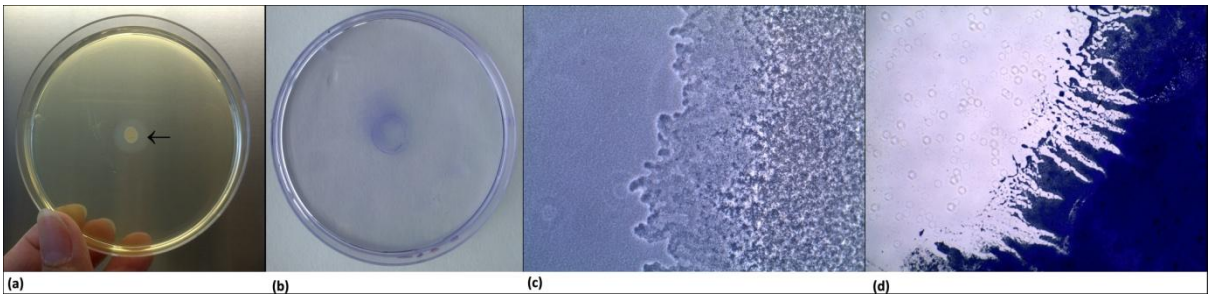

Fig. 7: *Pseudomonas aeruginosa* PAO1 twitching motility. (a) Arrow shows twitching motility of *P. aeruginosa* PAO1 on 1% LB agar, (b) Twitching motility of *P. aeruginosa* PAO1 on 1% LB agar stained with crystal violet, (c) Direct visual inspection of the colony edges by microscopy and (d) colony edges stained with crystal violet. The edge is highly irregular. Micrographs were taken at 100x (b) and 400x (c) magnification using a Leica Light microscope.

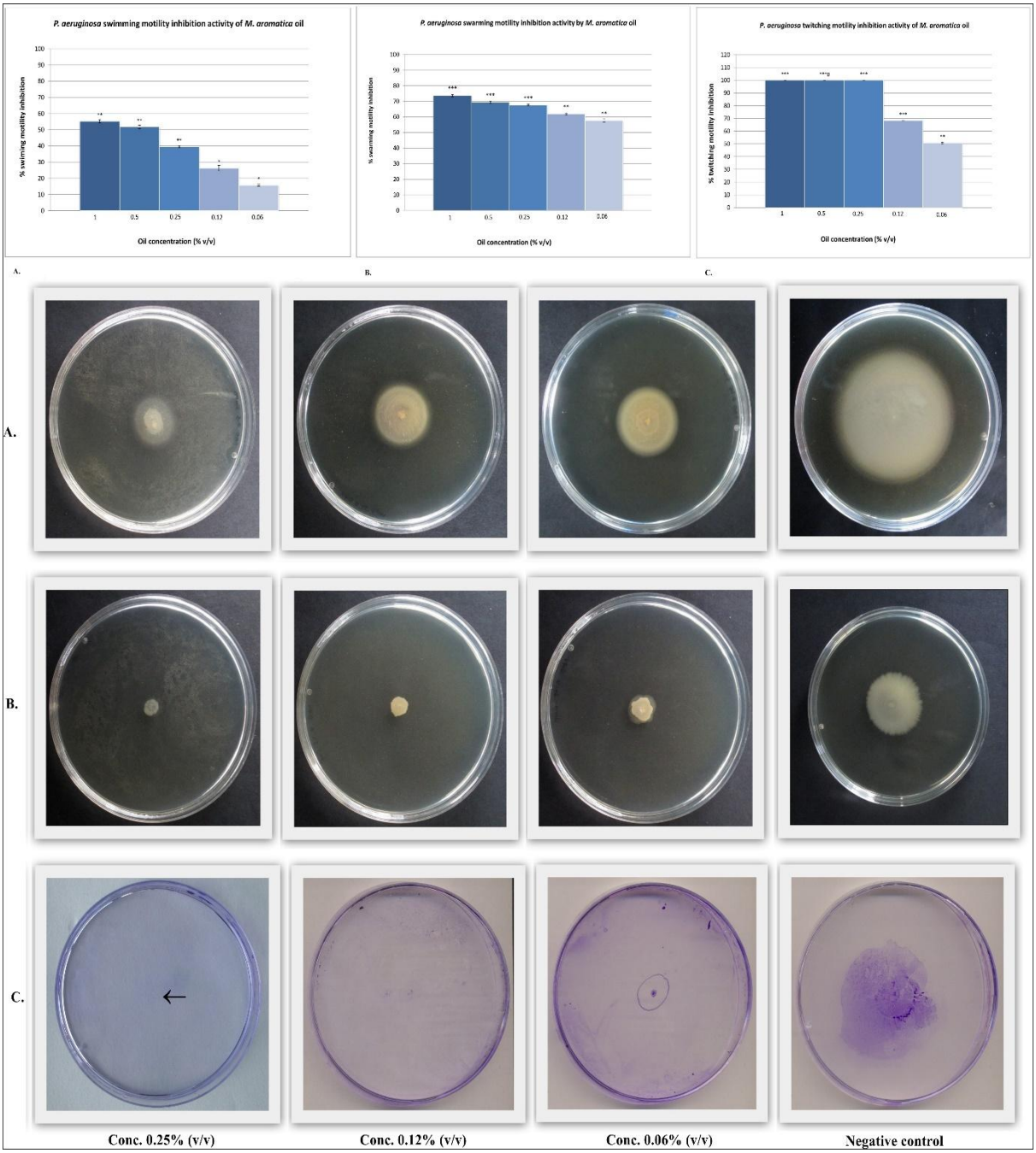

Fig. 8: Effect of *Massoia aromatica* essential oils on swimming, swarming and twitching motility of *Pseudomonas aeruginosa* PAO1. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

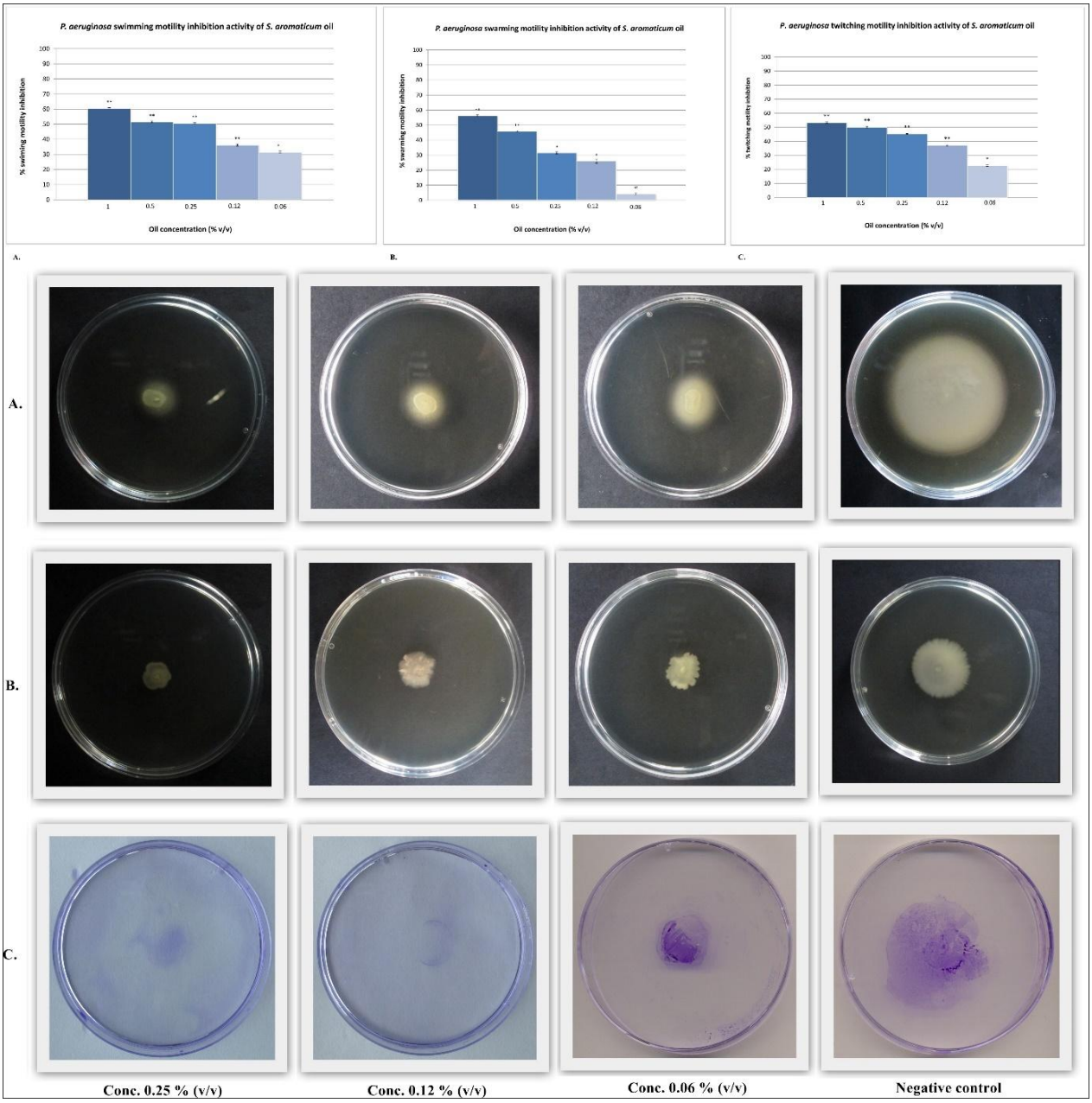

Fig. 9: Effect of *Syzigium aromaticum* essential oils on swimming, swarming and twitching motility of *Pseudomonas aeruginosa* PAO1. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

At concentrations ranging from 1 – 0.25 % (v/v), essential oils from *Syzygium aromaticum* significantly (***P*<0.01) decreased the swimming motility of *P. aeruginosa* PAO1 from 60.5±0.3 % to 50.5±0.6 %, respectively. However this essential oil failed to reduce the swarming motility of *P. aeruginosa* PAO1 at concentrations lower than 1 % (v/v) (Figure 9). As much as 53.2±0.8 to 50.0 ± 0.6 % (***P*<0.01) inhibition in twitching motility of *P. aeruginosa* PAO1 was shown by this essential oil with a concentration range from 1 to 0.5 % (v/v). The capability of the essential oil from *S.*

aromaticum in reducing *P. aeruginosa* PAO1 twitching motility correlated with a siginificant decrease in *P. aeruginosa* PAO1 biofilm formation when incubated with the same essential oil concentration of 0.5 and 1 % (v/v) (chapter 3, this thesis).

DISCUSSION

The aim of this study was to determine the anti-QS potential of ethanol extracts and essential oils from Indonesian medicinal plants with a potential to a possible use in controlling detrimental infections. Quorum sensing in *P. aeruginosa* has been well studied. This organism has two QS systems, LasR/I and RhlR/I systems. The lasI produces diffusible extracellular signal, N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) which binds with LasR to activate a number of virulence genes and biofilm maturation, and to regulate the expression of LasI. In the Rhl system, the RhlI synthase produces N-butyryl-L-homoserine lactone (C4-HSL), which interacts with RhlR protein and activated C4-HSL-RhlR complex, further stimulates the expression of RhlI, virulence genes and biofilm associated genes and biofilm associated genes (Whiteley *et al*., 1999; De Kievit, 2009; Desai and Gala, 2014).

P. aeruginosa produces a third signaling molecule, 2-heptyl-3-hydroxy-4(1H)-quinoline, called Pseudomonas quinolone signal (PQS). It is produced by the *pqs* (*pqsABCDE*) operon and pqsH gene, wherein the *pqsABCDE* operon products synthesize 2-heptyl-4-hydroxyquinoline (HHQ), and the PqsH converts HHQ into PQS. PQS diffuses in and out of the cell, accumulates in the environment, and when a threshold concentration reached, it binds to the regulator protein, PqsR, which modulates genes encoding virulence factors and the synthesis of PQS itself, resulting in autoinduction (Whiteley *et al*., 1999; Desai *et al*., 2014).

There are several reports in literature that propose different mechanisms of quorum sensing inhibition by natural products, either by inhibition of the signal molecule biosynthesis (Vattem *et al*., 2007), preventing the binding of the AHL molecules to its receptors (Hentzer and Givskov, 2003), or enzymatic inactivation and biodegradation of the quorum sensing signaling molecules (Defoirdt *et al*., 2004).

Plants are the sources of varied bioactive metabolites which useful for the development of successful and effective drugs. Various plants have demonstrated the ability to interfere with microbial QS systems that further and control its virulence. Vanilla (*Vanilla planifolia*), garlic (*Allium sativum*), weeping bottlebrush (*Callistemon viminalis*), Zaragoza mangrove (*Conocarpus erectus*), graceful sandmat (*Chamaesyce hypericifolia*), black olive (*Bucida buceras*), Florida clover ash

(*Tetrazygia bicolor*), and southern live oak (*Quercus virginiana*), showed quorum sensing inhibitory properties against *C. violaceum* and *Agrobacterium tumefaciens* (Choo *et al*., 2006; Adonizio *et al*., 2006). Study from Zahin *et al*., (2010), Chong *et al*., (2011) and Priya *et al*., (2013) also revealed quorum sensing property of *Mangifera indica*, *Punica granatum*, *Myristica cinnamomea* and *Phyllanthus amarus*, which showed efficacy in regulating violacein production of *C. violaceum* and inhibiting motility of *P. aeruginosa*. The result in our study have revealed that the ethanol extract of *N. nouchali*, *M. aromatica* and the essential oils from *S. aromaticum* significantly inhibit the quorum sensing mechanism of *P. aeruginosa* PAO1 as indicated by a reduction in violacein production of the reporter strain *C. violaceum.* They are also able to inhibit the motility of *P. aeruginosa* PAO1 which is an important trait for its pathogenicity.

To interfere with signal reception, plants produce molecules that structurally mimic the AHLs, and the competitive binding is effective to block activation of AHL-mediated QS (Koh et al., 2013). Halogenated furanones isolated from *D. pulchra* were found to inhibit QS regulated behaviors by competitively bind to the LuxR type proteins, thus, promote their rate of proteolytic degradation without killing the bacteria (Manefield *et al*., 1999). Chong *et al*., (2011) extracted malabaricone C from nutmeg *Myristica cinnamomea*, whose structure is not similar to AHL but possesses anti-QS activity by inhibits both lasR and rhlR systems in *P. aeruginosa*, CviR system in *C. violaceum*. However, that compound does not inhibit AHL production in *P. aeruginosa*. ,

S. aromaticum essential oil also known as clove oil has a long history of use in traditional medicine, and is a proven antimicrobial (Indonesian National Health Department, INHD, 1985). The oil contains majority of eugenol, which comprises around 72-90% of the oil. Other important consituents of the oil include tannins, flavonoids, and several sesquiterpenes (Joseph and Sujatha, 2011). Eugenol found to be toxic in relatively small quantities, as low as 5 mL (Jirovetz *et al*., 2006). Krishnan *et al*., (2012) discovered that hexane and methanol extract of *S. aromaticum* bud inhibited QS-controlled virulence production in *P. aeruginosa*, including reducing the bioluminescence of *P. aeruginosa* PAO1 *lecA::lux* (by hexane extract), swarming (maximum inhibition by methanol extract), pyocyanin (maximum inhibition by hexane extract). Inhibition of *P. aeruginosa lecA::lux* fusion suggests that *S. aromatica* extracts might interfere with *lux*, *rhl* and *las* QS-regulated system. Flavonoids contains in *S. aromaticum* is thought to be the compound responsible for the anti-QS mechanism. Flavonoids such as catechin, naringenin and taxifolin were found to have capability to reduce the expression of QS genes: lasI, lasR, rhlI and rhlR, and attenuate virulence factors such as pyocyanin, elastase and biofilm formation in *P. aeruginosa* (Vandeputte *et al*., 2010; Sarabhai *et al.,* 2013).

Beside used as spices, massoia oil from *M. aromatica* bark traditionally used in Indonesia to cure diarrhea, sore throat and stomach ache. Balinese people use this oil in the massage therapy (Hartnoll *et al*., 1993). From GC-MS analysis we found out that massoia oil contains massoia lactone (77.06%) (Chapter 4, this thesis). Amaya *et al*., (2012) discovered anti-QS activity of six sesquiterpenes lactones isolated from *Centratherum punctatum*. At low concentration, those lactones showed capability to prevent biofilm formation, elastase activity controlled by AHL, and production of AHLs. Similar to Amaya *et al*., Cartagena *et al*., (2007) studies acetogenin and squamocin, a plant sesquiterpene lactones isolated from Asteraceae and Hepaticae, which found to be able to inhibit *P. aeruginosa* QS-regulated biofilm formation.

Similar to study from Krishnan *et al*., (2012), the result from this study showed that *S. aromaticum* oil possess antibacterial as well as anti-quorum sensing activity. The fact that at elevated concentrations, *N. nouchali* extract, *M. aromatica* and *S. aromaticum* essential oils also demonstrated antibacterial activity along with anti-QS activity raised a question on whether the antibacterial and anti-QS effect in the ethanol extract and essential oils are from the same or distinct chemical compounds.

Bacterial motility plays a pivotal role in microbial surface colonization and the spreading of bacteria across the surface. These motilities contribute to the formation of structured surface-associated communities of bacteria called biofilms. The gram-negative bacterium, *P. aeruginosa,* can undergo the flagellum-mediated swimming motility and the surface-associated swarming and twitching motilities (Chow *et al*., 2011].

Swimming and swarming motility are dependent on flagella, whereas twitching has been shown to require type IV pili (Harshey, 2003). Swimming motility of bacteria formed on the plates with a low concentration of agar (0.3%) is not a social event unlike swarming, swimming motility. This motility represents individual cell movement and bacteria are fully immersed within the semisolid medium. The cells move separately in an unorganized manner resulting in an unorganized pattern, and this does not involve differentiation into polar hyperflagellated cells (Rashid and Kornberg, 2000; Inoue *et al*., 2008). Flagellum-mediated swimming motility is not required for *P. aeruginosa* biofilm structure development (Wu *et al*., 2011) however, this type of motility increases initial attachment to surfaces during initial biofilm development (O'Toole and Kolter, 2002).

Swarming is a cell density dependent way of movement and is considered as a model of bacterial social behavior. This motility is assessed on more solidified media (0.5% agar). This multicellular migration, generally dependent on flagella, takes place when the fluid layer on a surface is relatively

thin and involves a complex process of cell differentiation (elongated and hyperflagellated) leading to co-ordinated movement (Soto *et al*., 2002). In addition to flagella, swarmer cells require an increased production of certain extracellular components known as wetting agents that reduce surface frictions and enable the smooth migration of a group of cells on viscous surfaces (Rashid and Kornberg, 2000; Inoue *et al*., 2008).

Type IV pili extend and retract from the poles of the cell to mediate a form of surface translocation termed twitching motility, which is critical for host infection. The cells move in short, intermittent jerk of up to several micrometers and movement only appears when cells are within several micrometers of each other. The morphological pattern of twitching is less organized than in swarming motility (Rashid and Kornberg, 2000).

According to Shrout *et al.,* (2006), swarming motility promotes cell movement on the surface and results in the formation of a flat, uniform biofilm. Limitations in swarming motility resulted in biofilms containing cell aggregates, whereas twitching motility has been shown to be important in later steps in the formation of a structured biofilm. Klausen *et al.*, (2003) demonstrated that the bacterial migration which forms biofilm cap is mediated by type-IV pili. Cap formation is a process during biofilm maturation, by which a motile subpopulation in older biofilms moves across the surface and accumulates on an immobile subpopulation of cells. This immobile subpopulation is an aggregate of cells and has been termed “the stalk”.

The capability of ethanol extract from *N. nouchali* to inhibit swarming and twitching motility more severe than swimming motility of *P. aeruginosa* suggest that these extracts probably contain compound(s) that are capable to reduce the amount of quorum sensing signal in *P. aeruginosa* PAO1. Subsequently they also showed a reduction in violacein production by *C. violaceum* CV026 impregnated with HHL (Figure 3).

It is also still not clear why essential oils from *M. aromatica* at a low concentration (0.06% v/v) show a larger inhibition on swarming than swimming motility of *P. aeruginosa*. A possible explanation can be that essential oils from *M. aromatica* contain compounds capable to reduce the amount of quorum sensing signal in *P. aeruginosa*, as again demonstrated by the reduction in violacein production, Such low concentrations showed less activity in inhibition of single cell swimming motility, followed by no planktonic growth inhibition at a concentration of 0.06 % (v/v) of *M. aromatica* oil (chapter 3 this thesis).

The inhibition of quorum sensing signals could block cell-to-cell communication but fail to interfere with surface colonization by swimming motility. The blocking of cell-to-cell communication reduced

the production of several virulence factors and cytotoxic compounds such as elastase, rhamnolipids, cyanide and pycocyanine, which in turn strongly reduced the swarming motility (which also requires rhamnolipids (RLs) and 3-(3-hydoxylalkanoyloxy) alkanoic acids (HAAs) (Reimmann *et al*., 2002; Tremblay and Déziel ,2010).

The capability of *S. aromaticum* in reducing the swimming motility to a larger extend than reducing the swarming and twitching motility of *P. aeruginosa* PAO1 could be due to the antibacterial compounds present in the essential oil. These antibacterial compounds might disturb the single cell movement as in swimming instead of groups of cells movement in swarming motility.

In order to have a better insight whether the antibacterial and anti-QS effect from essential oils from *M. aromatica* are from the same or distinct chemical compounds, the bioautography test using *C. violaceum* WT strain should be performed. Further work will focus on chemical identification of the anti-quorum sensing compounds using chromatographic methods and mass spectrometry.

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Chapter 6

Efficacy of Massoia lactone as an Anti-biofilm Agent

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ABSTRACT

Massoia lactone ($C_{10}H_{16}O_2$), also known as *(R)*-5,6-dihydro-6-penthyl-2H-Pyrane-2-one was isolated from *Massoia aromatica* bark essential oil. Preparative Thin Layer Chromatography (TLC), followed by Gas Chromatography-Mass Spectrometry (GC-MS) and 1D- ^{1}H-Nuclear Magnetic Resonance (NMR) analysis were used to isolate and elucidate this compound. The effect of massoia lactone on microbial biofilm was evaluated. The result showed that this compound exhibit anti-biofilm activity against *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I ($MBIC_{50}$ at concentration of 25, 50 and 100 µg/mL). The anti-biofilm activity of massoia lactone also found to be three times greater when in combinations with anti-fungal/anti-bacterial. These result indicated that massoia lactone displayed potent activity against microbial biofilms *in vitro* and therefore has potential therapeutic implication for biofilm-associated microbial infections.

Keywords : massoia lactone, TLC, GC-MS, NMR, biofilm

INTRODUCTION

Massoia aromatica (synonym : *Cryptocarya massoy* (Oken) Kosterm) is an evergreen tree belongs to the family of Lauraceae. It is a medium-sized tree, growing up to 15 m tall, with optimal growth in rainforests between 400 m and 1000 m altitude. This plant is a rare plant species that grow in Indonesia, distributed mostly in Maluku and West Papua (Gunawan, 1999).

Local people common used massoia woods as building materials, and the bark is used as a mixture of red dye in batik making. In the islands of Seram, Maluku Indonesia, fishermen are commonly utilize masoyi leaves to fill pillows, and used to warm their heads when they go to sea (Guenther, 1972). Javanese and Balinese women are commonly use massoia bark powder to prepare a warming ointment, called bobory, which is used to lubricate the body, especially on days cold and rainy. The result makes the body feels warm, and it is believed to help alleviate complaints to various diseases. Massoia tree bark has a pleasant, sweet coconut-like aroma and is steam distilled to yield massoia bark oil that is used as the fragrance materials in incense manufacture (Hapsari and Simanjuntak, 2010).

The pleasant smell of massoia oil is believed to be capable of stimulating the emergence of sexual desire. In some areas in Indonesia, this plant is used as a medicinal plant to treat asthma, for a carminative (a herb or preparation that either prevents formation of gas in the gastrointestinal tract or facilitates the expulsion of said gas, thereby combatting flatulence), diaphoretic (herbs that induce prespiration, sweating and in return it will help to reduce fever, cool the body and speed the elimination of toxins from the system) (Heyne, 1987).

Previous studies mentioned that massoia oil was traditionally used to treat diarrhea, abdominal cramps, fever, tuberculosis, muscle pain, headache and chronic constipation, vaginal discharge, fever, anti-inflammatory agent, and as herbal postpartum. It is also believed that massoia oil has antiinsect and antifungal activity, since canoe or boat made of wood masoyi known to be able to resist insects and fungi (Lily, 1980; Gunawan, 1999; Bustanussalam *et al*, 2012).

Up to now, not much scientific research on the efficacy of massoia oil for health treatment that has been done. Thus, the mechanism underlying the efficacy of oil masoyi in modern medicine has not been widely known. From several studies it is known that massoia wood could act as a counter irritant (moisten capillaries) and aromaticum (fragrance materials, used as a mixture of fragrant incense). A study by Widowati and Pudjiastuti (1999) noted that infuse (water decoction) of massoia bark has properties of analgesics (pain relievers). A research by Sa'roni and Adjirni (1999) revealed that massoia bark also has activity as an anti-inflammatory.

Our previous study showed that beside the capability of inhibition of planktonic growth and biofilm formation, *M. aromatica* essential oil also showed activity in breaking the biofilm of *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus* Cowan I and *Candida albicans* ATCC 10231. The oil also showed activity in inhibit quorum sensing (QS) using QS reporter strain *Chromobacterium violaceum* CV026 and *P. aeruginosa* PAO1. In the experiments reported here, the active compound found in *M. aromatica* oil was isolated and screened for antibiofilm activity against *P. aeruginosa* PAO1, *S. aureus* Cowan I and *C. albicans* ATCC 10231.

MATERIALS AND METHODS

Plant Material

As much as five kilograms of *M. aromatica* bark were obtained from Nabire district, West Papua, Indonesia. A voucer specimen was identified and deposited at Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Indonesia. The essential oil was obtained by steam-hydrodistillation process. The oil sample was dried over anhydrous sodium sulphate (Na_2SO_4), stored in sealed dark glass vial, and kept at 4°C.

Planar chromatographic separation and analysis

Chromatography was performed on 10 × 20 cm silica gel 60 F_{254} aluminium sheet Thin Layer Chromatography (TLC) plates (Merck, Germany). Before use, the plates were preconditioned by heating at 120°C for 3 h. Without this procedure adsorbent layers became partly detached when soaked. Pure samples of eugenol, cynamaldehyde, and 1,8-cineole used as standards were obtained from Sigma Aldrich, dissolved in toluene to give solutions containing 1 µL/30 µL, and 3 µL was applied to the 10 x 20 cm TLC silica gel F_{254} type plates, using Minicaps capillary pipettes (West Germany). For the solution of the essential oils 100 µL was dissolved in 1 mL ethanol, and 5 µL of it was applied to the 10 x 20 cm TLC silica gel F_{254} type plates with Minicaps capillary pipettes. The position of the starting line was 1.0 cm from the bottom and 1.0 cm from the left side. The standards of the main components of the essential oils were applied to the TLC plates next to the spots of the oils. After the sample application, the TLC plates were developed with the previously optimized mobile phase. For the separation of essential oils, toluene:ethyl acetate, 93:7, as a mobile phase is recommended (Wagner and Bladt, 1996). The development mode was ascendant chromatography in a saturated twin-trough chamber (Camag, Switzerland). All TLC separations were performed at room temperature (20°C). For the bioautographic assay, solvents applied in TLC developing systems were also tested.

After chromatographic separation, the absorbent layers were dried in an oven at 90°C for 5 min to remove the solvent completely. An alcoholic vanillin–sulphuric acid reagent was used to visualize the separated compounds. The developed layers were dipped into this reagent and heated for 5 min at 100°C. Detection of the separated compounds was performed according to R_f (retardation factor) value: the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front; and colour of the standards. The TLC plate for bioautography was processed in parallel without final development with reagent (Wagner and Bladt, 1996; Horváth *et. al*., 2010).

Bioautography analysis

Agar diffusion or contact bioautography was used for the bioautography analysis. *P. aeruginosa* PAO1, *S. aureus* Cowan I and *C. violaceum* ATCC 31532 (wild type strain) were grown overnight in 100 mL Luria Bertani (LB) broth, at 37°C for *S. aureus* and 28°C for *P. aeruginosa* and *C. violaceum* in a shaker incubator for 24 h to an optical density (600 nm) of 1.0 or greater. The bacterial suspension was diluted with fresh LB to OD_{600} = 0.1, which corresponds to approximately 1×10^8 colony-forming units (CFU)/mL. *C. albicans* ATCC 10231 was cultured in Sabouraud Dextrose Broth (SDB) and incubated for 24 h at 30^{o}C with agitation (250 rpm). Following incubation, cells were finally resuspended to a concentration of 10^7 CFU/mL by adjusting the optical density of the suspension to 0.38 at 520 nm.

A 100 µL sample of the microbial culture was spreaded onto 15 mL of LB Agar for bacteria and Sabouraud Dextrose Agar (SDA) for yeast in petri dishes. After the agar solidified, the chromatogram plate developed was placed face down onto the inoculated agar layer and left for 24 hour at 4^{o}C to enable diffusion. Following the incubation, the chromatogram plate was removed and the agar layer was incubated at 37°C for 24 hours. The inhibition zone was observed on the agar surface in the places where the spots of compounds are diffuses into the agar. The presence of anti-bacterial activity within a sample was indicated by a clear halo on the agar surface were the separated compounds are present. The Rf value of the inhibition zone was measured by the distance of the substance over the distance moved by the solvent (Horváth *et. al*., 2010).

Isolation of *M. aromatica* Oil Active Compound

A Preparative TLC separation of *M. aromatica* oil was performed on 10 × 20 cm silica gel 60 F_{254} aluminium sheet TLC plates (Merck, Germany) as described above by spotting the essential oil over the whole width of the starting line (streaking the plate). UV_{254} instead of stain reagent was used for

the visualization. The area (band) which is showed the same Rf value as the Rf of the inhibition zone was marked, and carefully rubbed out off using a spatula. The loosened silica then transferred onto a glass funnel where glass wool is present and ethylacetate as mobile phase is poured over them as a solvent. The eluted fraction that has been collected, dried using SpeedVac, and dissolved in ethanol was further analysed by TLC and visualized under UV_{254}. GC-MS analysis also performed to obtain information about the chemical composition of the eluted fraction.

Gas Chromatography-Mass Spectometry (GC-MS) analysis

The isolated fraction of *M. aromatica* essential oil was analysed by GC-MS according to the method of Wu *et al.* (2008) on a GC-2010 gas chromatography (Shimadzu, Japan) equipped with a GC-MS-QP2010 Plus mass spectrometer (Shimadzu, Japan). An Agilent J&W DB-1 column (30 m length, 0.25 mm diameter, 0.25 µm film thickness, Agilent) was used for separation and also a s split injector. Diluted samples (1/100 in ethyl acetate, v/v) of 1.0 mL were injected by an autosampler in the split mode (1/153). The oven temperature was programmed from 70C to 230C at a rate of $5^oC\ ml^{-1}$. Helium was used as the carrier gas. Identification of compounds was based on comparisons of their mass spectra with those recorded in the MS library database NIST (National Institute of Standards and Technology) 12, NIST62 and WILEY229. Quantitative analysis of each essential oil component (expressed as area percentage) was carried out by peak area normalization measurement. For dead-time and Kovats Retention Index determination were conducted under isothermal condition (70^oC).

NMR analysis

In order to obtain information about the structure of the isolated active compound, ^{1}H-NMR (Nuclear Magnetic Resonance) spectroscopy analysis was performed. NMR spectra was recorded using Bruker DRX 500 (^{1}H: 500 MHz, ^{13}C: 125.77 MHz). The isolated fraction (20 mg/1.0mL) was dissolved in $CDCl_3$ into a 5mm NMR tube, and the solvent signal was used for the spectra calibration (^{1}H at 7.26 ppm).

Effect of essential oil isolated compound on bacterial biofilm formation and fungal preformed biofilms

Biofilms of *C. albicans* were formed on polystyrene flat bottom 96-well microtiter plates (Sarstedt Inc., Newton, NC, USA). Briefly, 100 µL of a standardized cell suspension (10^7 CFU/mL) grown on RPMI 1640 medium without sodium bicarbonate supplemented with L-glutamine (Sigma) was transferred into each well of a microtiter plate, and the plate was incubated for 24 h at 37^oC to yield a intermediate biofilm phase. RPMI 1640 medium was used because in this medium *C. albicans* is

capable to induce hyphal formation in *C. albicans*. Following incubation, the cell suspension was aspirated and each wells was washed twice with 150 µL of PBS to remove loosely adhered cells. A total of 100 µL concentrated compound ranging from 100 to 6.25 µg/mL in methanol (MeOH) were added to the washed wells. For vehicle control we used ethanol at concentration 1% v/v, and for the negative control the biofilm was not exposed to anti-fungal agent. The plates were then incubated at 37^{o}C for 24 h. Quantification of the biofilm formed was done using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay describes below with a microtiter plates reader (Bio-Rad 680 XR) at 550 nm. Testing was performed in triplicate. Fluconazole concentration ranging from 1,024 – 128 µg/mL was used as a positive control in this study. When a compound was found to reduce at least 50% of biofilm formation (Minimum biofilm inhibition concentration, $MBIC_{50}$), it was considered as biofilm preventive (Ramage *et al.,* 2002).

To test for the inhibition of active compounds on *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm formation inhibition, a PVC (polyvinyl chloride) flexible U bottom 96 wells plates was used (Falcon 3911, Becton Dickinson, Franklin Lakes, NY). Compound concentrations ranging from 100-6.25 µg/mL in MeOH were used. Negative control (cells + media : TSB for *S. aureus* Cowan I and M63 supplemented with 20% casamino acid, 20% glucose and 1mM MgSO4 for *P. aeruginosa* PAO1), positive control (cells + media + streptomycin), vehicle controls (cells + media +methanol concentration 1% v/v), and media controls were included. For the positive controls of the streptomycin concentration of 1000-100 µg/mL were used. Blanks undergo the same treatment as test samples, but without incubation. All tests were performed in triplicate. The MTT assay was used to estimate and validate the effects of plant essential oils on the biofilms formation. Inhibition of biofilm formation was calculated as % of inhibition on the basis of color absorbance by using the formula mentioned below to determine the final $MBIC_{50}$ values:

[(ODgrowth control – Odsample) / ODgrowth control] x 100

ODt= optical density (595 nm) of the test well; ODmc24: optical density (595 nm) of the media control well; ODvc: optical density (595 nm) of the vehicle control well (Sandasi *et al*., 2009). The concentration at which the extract depleted the bacterial biofilm by at least 50% was labeled as the $MBIC_{50}$.

Essential oils isolated compound in combination with anti-microbial against microbial biofilm

The anti-microbial activity of isolated plant essential oils isolated in combination with fluconazole or streptomycin was assessed by the chequerboard method in triplicate (Karpanen *et al.,* 2008).

Microtiter plates containing *C. albicans* biofilms were washed three times with 200 µL sterile PBS per well to remove any unbound cells. The isolated compounds used at concentration that inhibit 50% planktonic cells and fluconazole at various concentration were prepared in RPMI 1640 medium. Next, 50 µL of the compound was added to the wells of the microtiter plates in diminishing concentrations, and 50 µL of fluconazole was added to the columns. The isolated compound alone at various concentration, fluconazole alone and saline were used as controls. The microtiter plates were incubated at 37^{o}C for 24 h (intermediate phase *Candida* biofilm).

The compound isolated from essential oil at concentration that inhibited 50% of planktonic cells and streptomycin were prepared in LB medium. Next, 50 µL of the essential oil's isolated compound solution was added to the rows of 96 well microtiter plates in diminishing concentrations, and 50 µL of streptomycin was added to the columns in diminishing concentrations. The compounds isolated from essential oil alone at various concentrations, streptomycin alone and saline were used as controls. The microtiter plates were incubated at 37^{o}C for 24 h (*P. aeruginosa* biofilm) and 48 h (*S. aureus* biofilm). Following the incubation time, the effects of plant essential oil's isolated compound on the fungal and bacterial biofilms were estimated using the MTT assay as describes below. The test compound found to reduce at least 50% of the biofilm formation was considered as biofilm preventive (Ramage *et al.,* 2002).

The analysis of the combination of massoia lactone and streptomycin or fluconazole was obtained by calculating the Fractional Inhibitory Concentration Index (FICI) as follows: FICI = (MIC of the combination of massoia lactone and streptomycin or fluconazole /MICa alone) + (MIC of the combination of massoia lactone and streptomycin or fluconazole /MICb alone), where MICa is the Minimal Inhibitory Concentration of massoia lactone from *M. aromatica* oil, and MICb is the Minimal Inhibitory Concentration of streptomycin or fluconazole. The FICI was interpreted as follows: (1) a synergistic effect when FICI ≤ 0.5; (2) an additive or indifferent effect when FICI >0.5 and <1 and (3) an antagonistic effect when FICI >1 (konate *et al*., 2012).

MTT assay

The activity of the active compound in inhibit fungal and bacterial biofilm was quantified by determining biofilm cell viability using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. MTT (Sigma) solution (5 mg/mL in PBS) was prepared, filter-sterilized using a 0.22-µm pore size filter. The preformed biofilms were first washed three times with 200 µL PBS, and then 100 µL of PBS buffer and 20 µL of the MTT solution were added to each of the prewashed wells. The microtiter plate was then incubated for 2 h at 37^{o}C. Following incubation, the PBS-MTT solutions

were removed from the wells and replaced with 100 µL of DMSO in each well to dissolve the water soluble purple formazan crystal. The color intensity of the soluble formazan was determined using microplate reader (Bio-Rad 680 XR) at 550 nm. Percentage of cell survival is expressed as the formula below:

$$\textit{Percentage cell survive} = 100\% \text{ x } \frac{\textbf{absorbance of treated cells}}{\textbf{absorbance of control cells}}$$

Qualitative analysis on *C. albicans* biofilm

A Carl Zeiss LSM 5 Exciter Laser Scanning Confocal Microscope (Leica Microsystems, Germany) was used to observe the architecture of the *C. albicans* biofilms in the presence of massoia lactone. Candida biofilms were formed by dispensing standardized cell suspension (500 µL of a suspension containing 1.0 x 10^7 cells/mL in LB medium) onto 13 mm diameter pre-sterilized plastic coverslips (Thermanox; Nulge Nunc International) placed in the wells of pre-sterilized flat bottomed twelve-well plates (Iwaki). The plate was incubated at 37^0C for 90 minutes (adhesion phase). Following incubation period, the supernatant was removed and a total of 500 µL RPMI medium containing different concentrations of massoia lactone (100 – 6.25 µg/mL) were added to the washed wells. The plate then incubated for 8 h (adherence phase), 24 h (intermediate phase) and 48 h (maturation phase) at 37 ^{0}C.

The coverslips then were washed twice with PBS and stained using the LIVE/DEAD fluorescent stains (10 µL of 3.34 µM SYTO9 and 10 µL of 20 µM Propidium Iodide (PI) both in DMSO) (Molecular Probes, USA) before examined under CLSM. Serial sections in the xy plane were obtained at 1 µm intervals along the z axis. A 20× and 40x oil immersion objective were used with 488 nm Ar laser excitation and 500–640 nm band pass emission setting. The image were subsequently analysed using the freely available image processing software imageJ version 1.46 (Rasband, National Institutes of Health (NIH), Bethesda, Maryland, USA : http://rsb.info.nih.gov/ij/) including the LSM reader plugin to open LSM5 formatted image stack created by the microscope software. The images' scale bar used to calibrate the ImageJ area measurement algorithm. The observations were made in triplicates and representative images are presented here (Ramage *et al.,* 2002; Jin *et al.,* 2005; Dusane *et al.,* 2012).

Measurement of Hemolytic Activity

The hemolytic activity was investigated by incubating 100 µL of serially diluted massoia lactone (100-6.25 µg/mL) in phosphate-buffered saline (PBS) with 100µL of a suspension of 1% red blood cells in 96-wells V-bottom plate (Greiner). Fresh human red blood cells (RBCs, 30 mL) were suspended in 10 mL of PBS and washed 3 times by centrifugation (5 minutes at 1500 rpm) until the supernatant was clear and resuspended in PBS. Erythrocyte suspensions (5×10^6 cells/mL) were incubated with different concentrations of massoia lactone (100 – 6.25 µg/mL) at 37°C for 1 hour and then centrifuged at 2000 rpm for 10 minutes to precipitate intact erythrocytes and debris. The supernatant in each well was transferred to a new plate and were assayed for absorbance at 450 nm to determine the percentage of hemoglobin released from the lysed erythrocytes. 100% hemolysis data was obtained by adding 10 mL of TRITON-X solution (5 % v/v in DMSO), a strong surfactant, to the above-prepared RBC suspension. The hemolytic activity of the massoia lactone was expressed as the percentage of absorbance compared with that observed after 100% lysis induced by Triton X-100. The supernatant of an untreated erythrocyte suspension in PBS was used as a spectrophotometric blank.

Statistical methods

Statistical analysis was conducted with one way ANOVA followed by Dunnett's tests. Differences were considered significant with P-values of 0.05 or less.

RESULT AND DISCUSSION

TLC profile and Bioautography assay of *M. aromatica* oil

The essential oil obtained had a yield of 0.5 % (w/w).The TLC profile of the *M. aromatica* essential oil separation using Toluene:ethylacetate 93:7, and anysaldehyde sulphuric acid as stain solution showed three spots (Table 1). Using GC-MS analysis, the majority of the oil constituents were identified using the data sources available (Table 2). Among the identified compounds, Massoia lactone (77.06 %) shown to be the main component in *M. aromatica* oil.

Table 1: TLC profiling of *M. aromatica* Essential Oil

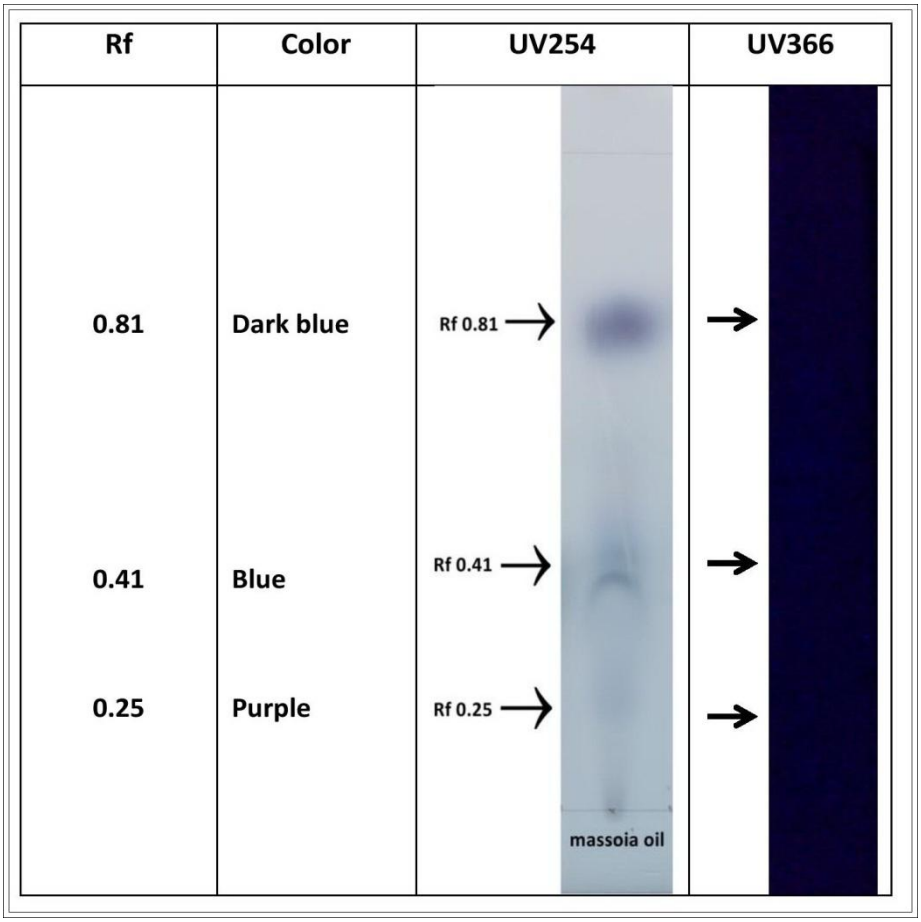

Table 2: Chemical composition of the essential oil from the bark of *M. aromatica*

No	Compound	Retention Time (min)	Peak Area (%)
1	L-Linalool $$	10.491	1.95
2	Alpha Copaene	15.243	2.94
3	Beta Elemene $$	15.467	1.24
4	Alloaromadendrene $$	16.411	3.17
5	5-Hydroxy-2-Decenoic Acid Lactone $$ 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-, (R)-(CAS) (-)-Massoia lactone	17.242	77.06
6	Cyclopentane, 1,1'-ethylidinebis-$$ Ethane, 1,1-dicyclopenthyl-$$ 1,1-Dicyclopenthylethane	19.591	8.54
7	Benzyl Benzoate	20.443	5.11

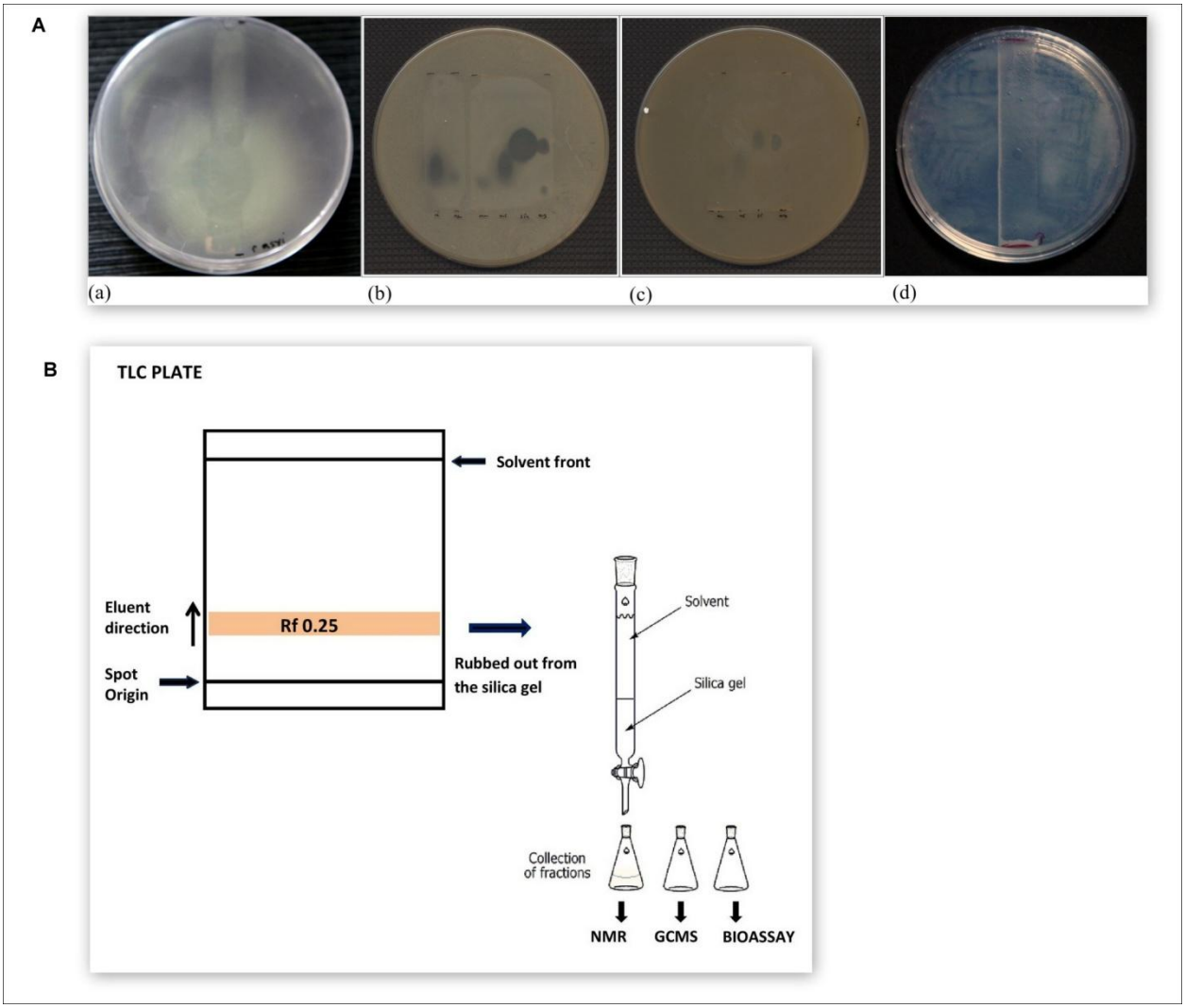

Fig. 1: Isolation of *M. aromatica* oil's active compound. (A) Bioautography analysis of (a) *M. aromatic*a oil on *C. violaceum* WT plate, (b) *M. aromatica* oil, isolated *M. aromatica* oil active compound (Mlact), cynamaldehyde and eugenol on *C .albicans* 10231 plate, on *P. aeruginosa* PAO1 plate, and (d) on *S. aureus* Cowan I plate. (B) Scheme of isolation procedure for *M. aromatica* active compound using preparative TLC.

Figure 1 shows a bioautography profile applied for *M. aromatica* essential oil using *P. aeruginosa* PAO1. The method resembles a disk assay, and combined with TLC separation provide a valuable screening method that quickly detection of components of an essential oil with anti-microbial activity. In this experiment we used contact bioautography, where the microbials diffuse from a TLC plate to an inoculated agar plate. The bioautography against *P. aeruginosa* showed that the component at Rf value 0.25 appear to be the main one responsible for the biological properties of the oil. This compound was then subjected to the TLC preparative analysis to isolate the active.

Isolation of *M. aromatica* oil active compound, GC-MS and NMR analysis

A preparative thin layer chromatography method was used to isolate the active compound of the *M. aromatica* essential oil. The result gave 3 isolated compound (24, 22, and 32 mg/mL). TLC profiling of the isolated compound showed one single dark blue spots at Rf value of 0.25.

In order to obtain information about the chemical compounds contain in the *M. aromatica* essential oil, GC-MS analysis was performed. Table 2 and Figure 2 show the chemical composition of *M. aromatica* essential oil and the GC-MS spectra. Massoia lactone was shown to be the major compound (77%).

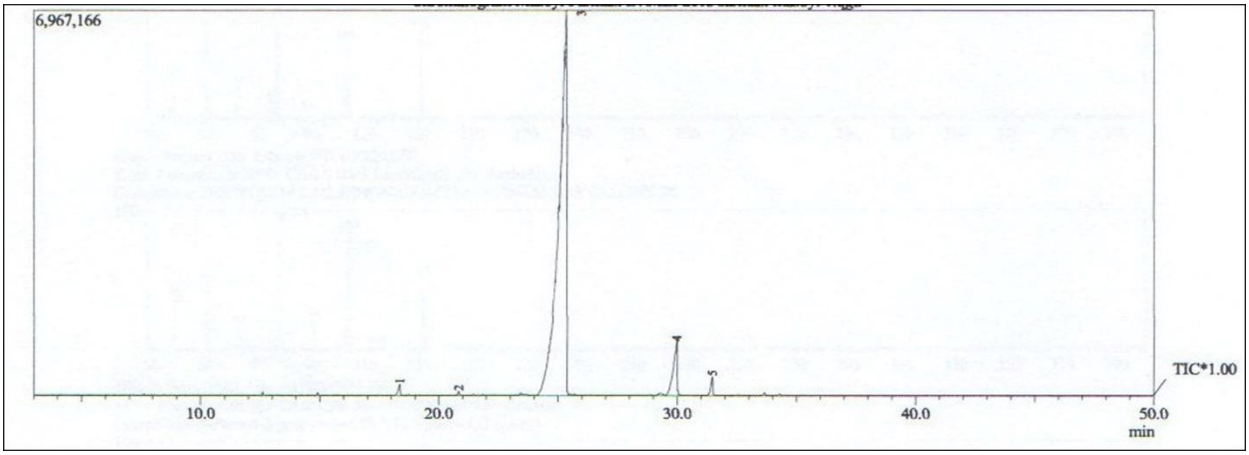

Fig. 2: GC-MS profile of *M. aromatica* essential oil. The indicated compounds in the chromatograms are shown in Table 3.

GC-MS analysis was also performed to check the chemical composition of the isolate compound. Table 3 and figure 3 showed that the isolated compound from *M. aromatica* oil obtained by preparative chromatography method followed by GC-MS analysis is 5-Hydroxy-2-Decenoic Acid Lactone $$ 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-, (R)-(CAS) (-)-Massoia lactone $C_{10}H_{16}O_2$ (94%). In our previous study, we determined the amount of massoia lactone in massoia oil using TLC-densitometry at 211 nm maximum wavelength. The content of massoia lactone in 1% v/v massoia oil was 0.55 % w/v respectively (Hertiani *et al*., manuscript in preparation).

1D ^{1}H-NMR elucidation was performed to ensure the structure of the massoia lactone obtained. H-NMR profile obtained (Figure 4) convinced the proposed structure recommended by the GC-MS i.e. $C_{10}H_{16}O_2$ which was supported further by comparison to the data reported by Siminiatt (2007) and Harbindu and Kumar (2011) reccorded on $CDCl_3$ as well (Table 4). The present of an aliphatic chain was confirmed by resonance peaks at the upfield region integrated as 11H which support a propyl

substituent. A shift to more down field area was observed at δ 4.40-4.46 (1H, m) which represent an H-containing chiral carbon next to the lactone moiety (H-6). A cis-vicinal H-H coupling of an alkene was observed between resonances at δ 6.87-6.92 and δ 6.03-6.06 having a coupling constant of 9.75 Hz. These finding support the present of an α,β unsaturated lactone. Further splitting of the resonance peak is resulted from the present of an adjacent methylene (H-5). Considering that the only the R isomer is available in nature, the isolate is elucidated as Figure 4.

We also assessed the anti-biofilm activity of massoia lactone in combination with antibiotic and anti-fungal drug. The result showed that the median MBIC of individual fluconazole for *C. albicans* biofilm was 512 μg/mL, and the median MBIC of individual streptomycin for *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm was 500 μg/mL. The MBIC of fluconazole in combination with massoia lactone decreased four fold for *C. albicans* biofilm. The MBIC of streptomycin, in combination with massoia lactone decreased from 500 μg/mL to 125 μg/mL (Table 5, Figure 7). Therefore, the combination of streptomycin/massoia lactone yielded synergism activity against the biofilm of *P. aeruginosa* and *S. aureus* (FICI 0.5), whereas the combination of fluconazole/massoia lactone also gave a synergistic effect where the effect of the combination is greater than the sum of the individual effects, with FICI value of 0.375. The use of anti-microbial combinations may improve the management of microbial biofilm-associated infection, disrupt the biofilm and prevent the emergence of microbial resistance. Although the result showed that there is a synergistic activity of massioa lactone with streptomycin and fluconazole, the exact mechanism are still unknown and has to be elucidated.

Table 3: Major chemical constituents of *M. aromatica* essential oil as identified by GC-MS

Peak	Retention time (Rt)	Kovats Retention Index (RI)	Area	Area (%)	Similarity index (SI)	Chemical Component
3	25.346	1168.629	156590945	92.05	94	5-Hydroxy-2-Decenoic acid lactone (Massoia lactone)
4	29.961	1195.847	10522375	6.19	86	5-Hydroxy-2-Decenoic acid lactone (Massoia lactone) *

*The compound obtained was predicted to be a 5-Hydroxy-2-Decenoic acid lactone (Massoia lactone) derivate with higher boiling point and have the same fragmentation as Massoia lactone.

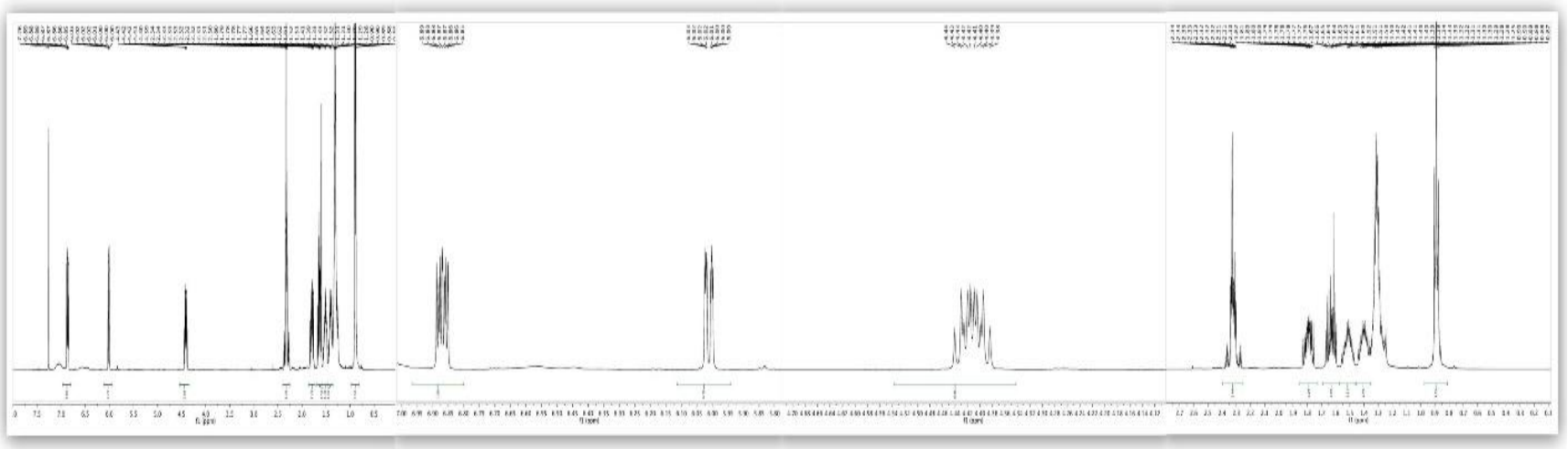

Fig. 3: ^{1}H-NMR profile of massoia lactone

Table 4: NMR data of isolate in comparison to (-)-Massoia lactone ($C_{10}H_{16}O_2$)

Atom No.	**Isolate** [a]		(-)-Massoia lactone ($C_{10}H_{16}O_2$)[b]		(-)-Massoia lactone ($C_{10}H_{16}O_2$)[c]	
	^{1}H		^{1}H		^{1}H	
	δ	Integration, multiplicity, *J* in Hz	δ	Integration, multiplicity, *J* in Hz	δ	Integration, multiplicity, *J* in Hz
H-4	6.87-6.92	1H, ddd, *J* = 9.75, 5.4, 4.4	6.78-6.83	1H, m	6.87-6.90	1H, m
H-3	6.03-6.06	1H, ddd, *J* = 9.75, 1.25, 1.4	5.93-5.97	1H, m	6.04	1H, d, *J* = 10
H-6	4.40-4.46	1H, m	4.32-440	1H, m	4.41-4.45	1H, m
H-5a	2.33-2.36	1H, dd, *J* = 1.40, 4.4	2.23-2.29	2H, m	2.32-2.38	2H, m
H-5b	2.34-2.37	1H, dd, *J* = 1.25, 5.4				
H-7	1.63-1.86	3H, m			1.64-1.82	3H, m
H-8	1.63-1.86	5H, m				
H-9	1.50-1.57					
H-10	1.39-1.47					
	1.31-1.36		1.24-1.93	8H, m	1.26-1.34	5H, m
H-11	0.92	1H, t, 7.1 Hz	0.82	3H, brs	0.90	3H, t, *J* =6.9 Hz

[a]Data were recorded in $CDCl_3$, at 500 MHz, multiplicities and coupling constant are given in Hz; [b] $CDCl_3$, at 400 MHz Siminionatto *et al.*, (2007); $CDCl_3$, at 200 MHz Harbindu and Kumar (2011)

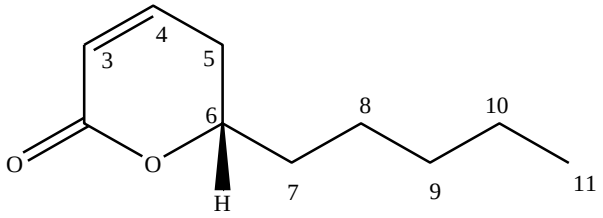

Fig. 4: Massoia lactone chemical structure

Effect of massoia lactone on microbial biofilms

Biofilm infections are a major medical problem since they are difficult to eradicate and the source of many chronic infections. The discovery of anti-infective agents, which are active not only against cells but also against microbial biofilms, represents an important goal (Schillaci *et al.,* 2008). Essential oils are mixtures of numerous organic chemicals, and some individual components are known to exhibit anti-microbial properties.

The MTT assay was used to measure the efficacy of massoia lactone against microbial biofilm. In this assay, microorganisms with active electron transport system metabolically reduce the yellow tetrazolium salt (MTT) to water soluble purple formazan product. The color intensity of the soluble formazan was determined using a microplate reader at A_{550} (Mossmann, 1983). The colorimetric assay using MTT is quicker, more sensitive compare to the traditional anti-bacterial assay that use colony counting of bacteria on nutrient agar plates, and less expensive compare to XTT (Stevens and Olsen, 1993; Roehm *et al*., 1991). The disadvantage of MTT is that the formazan forms a crystalline presipitate needs to be solubilized first prior to absorbance measurements.

In our study, we found the anti-biofilm potential of the massoia lactone. As the main compound isolated from *M. aromatica* bark essential oil, massoia lactone showed anti-biofilm activity against all the test organisms. A reduced level of biofilm formation was clearly observed against control (biofilm without the presence of the test compound). At concentration of 100 µg/mL, showed capability in inhibit the formation of *C. albicans* intermediate biofilm as much as 82.25 ± 0.54% (***P<0.001), whereas at lower concentration (25 µg/mL) massoia lactone capable to inhibit *C. albicans* intermediate biofilm formation as much as 49.98 ± 0.32% (**P<0.01). The median biofilm inhibition concentration ($MBIC_{50}$) for massoia lactone was at concentration of 50 µg/mL for *P. aeruginosa* biofilm (50.14 ± 0.08) (**P<0.01), and 100 µg/mL for *S. aureus* biofilm (67.28 ± 0.28) (**P<0.01) (Figure 5).

Efficacy massoia lactone in inhibit microbial biofilm tested in microtiter plate using MTT method was confirmed by CLSM analysis. Intermediate phase (24h) of *C. albicans* biofilms were grown on the surface of coverslips and stained with SYTO9 and PI for the monitoring of live/dead cells. We observed a preventive activity on biofilm formation, when we inoculated *C. albicans* with different concentration of massoia lactone. Growth suppression of *C. albicans* biofilm by massoia oil was clearly seen by CLSM with the Live/Dead stain, compared with the dense biofilm growth of the control (Figure 6).

Pretreatment of *C. albicans* biofilm with various concentrations of massoia lactone showed that there was a dose-dependent effect of massoia lactone on *C. albicans* biofilm formation. CLSM study showed that in the presence of massoia lactone (concentration of 100 µg/mL), a significant decrease of *C. albicans* biofilm biomass compare to negative control was evident (figure 6). When the concentration was decreased, we observed the increase amount of blastopores, pseudohyphae and mycelia in *C. albicans* biofilm biomass. The anti-biofilm activity thus may be due to the anti-fungal activity of the massoia lactone.

The effect of massoia lactone to microbial biofilms has yet been scrutinized. Massoia lactone possess a characteristic α,β-unsaturated δ-lactone, which is also a common feature in a number of biologically active natural products such as (+)-geniothalamin, (-)-callystatin A, canolide A and the kava lactones, either different in the substituents at the C6 position or have a molecular structure architecture that maintains the α,β-unsaturated δ-lactone skeleton (Rali *et al*., 2007). The mechanism of action of lactone against microorganims is not fully understood but it is speculated to be involved in membrane disruption, resulting in alterations in membrane permeability and leakage of intracellular materials. This compound could be able to penetrate through the polysaccharide matrix of a biofilm and dissolve lipids in the biofilm matrix (Cowan, 1999; Trombetta *et al*., 2005). In the presence of lactone, *Yarrowia lipolytica* lossed its cultivability and membrane integrity and addition of lactone in the medium provoked a decrease in the concentration of ergosterol (Ngoc Ta *et al*., 2010). However, a study by Kishimoto *et al.* (2005) suggested that the anti-fungal activity of massoia lactone is possibly due to its capability in inhibit respiratory system of *C. albicans,* because it arrests oxygen consumption by *C. albicans*. Since the exact mechanism of anti-biofilm activity of massoia lactone remains unclear, study on a gene expression of *C. albicans* cells in response to massoia lactone will be required.

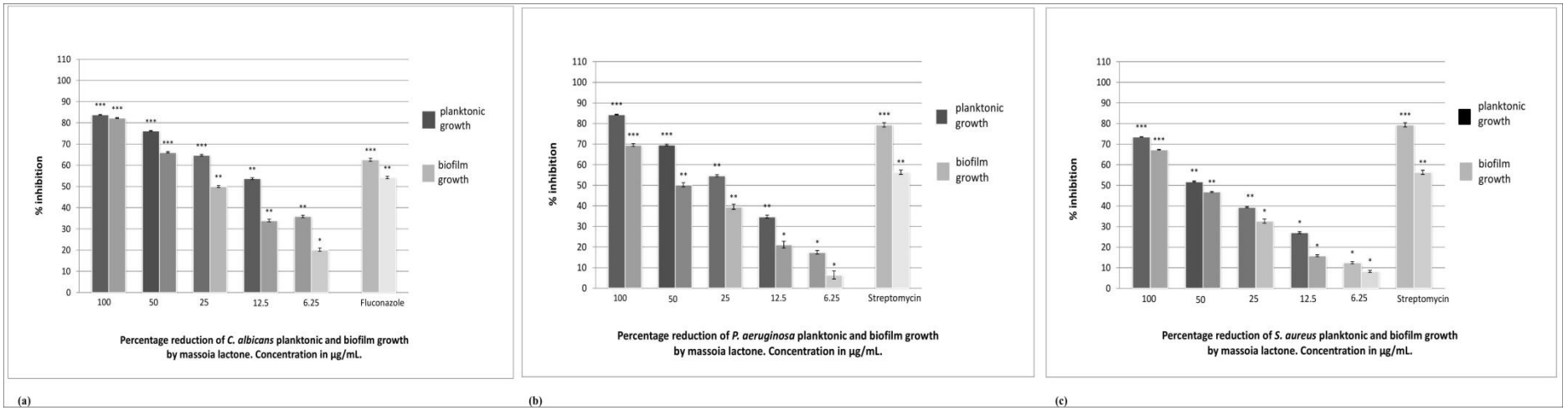

Fig. 5: Activity of massoia lactone against *C. albicans* ATCC 10231 (a), *Pseudomonas aeruginosa* PAO1 (b), and (c) *Staphylococcus aureus* Cowan I planktonic growth and biofilm formation. Concentration in µg/mL. Streptomycin concentration of 500 µg/mL and and Fluconazole concentration of 512 µg/mL were used as positive control. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

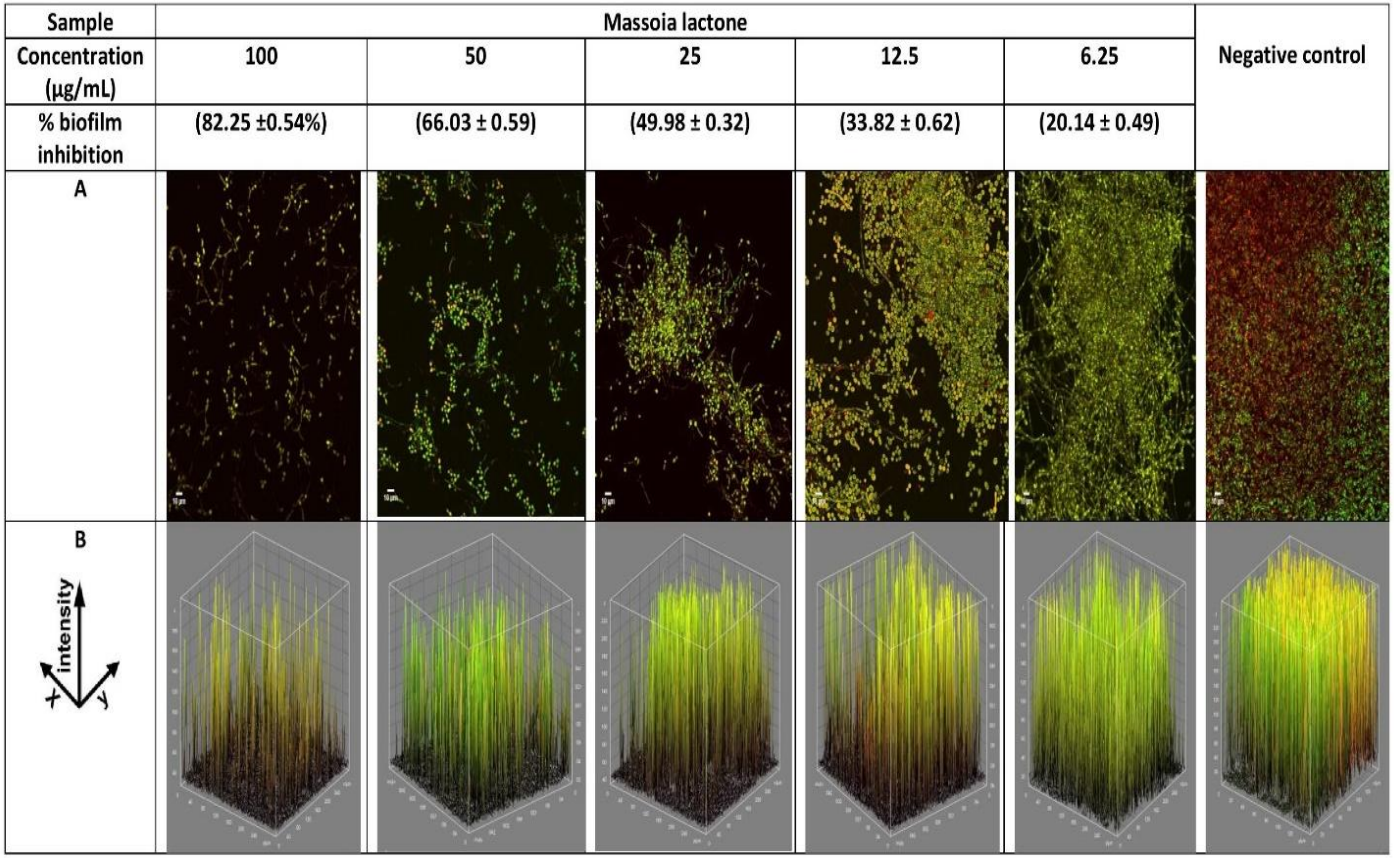

Fig. 6: Representative confocal scanning micrograph images of biofilm inhibition activity of massoia lactone at concentration of 100 – 6.25 µg/mL against intermediate phase of *Candida albicans* biofilm. A: projected upper view of the biofilm, B: estimated three-dimensional view of the biofilm. CLSM images were taken at 20x magnification.

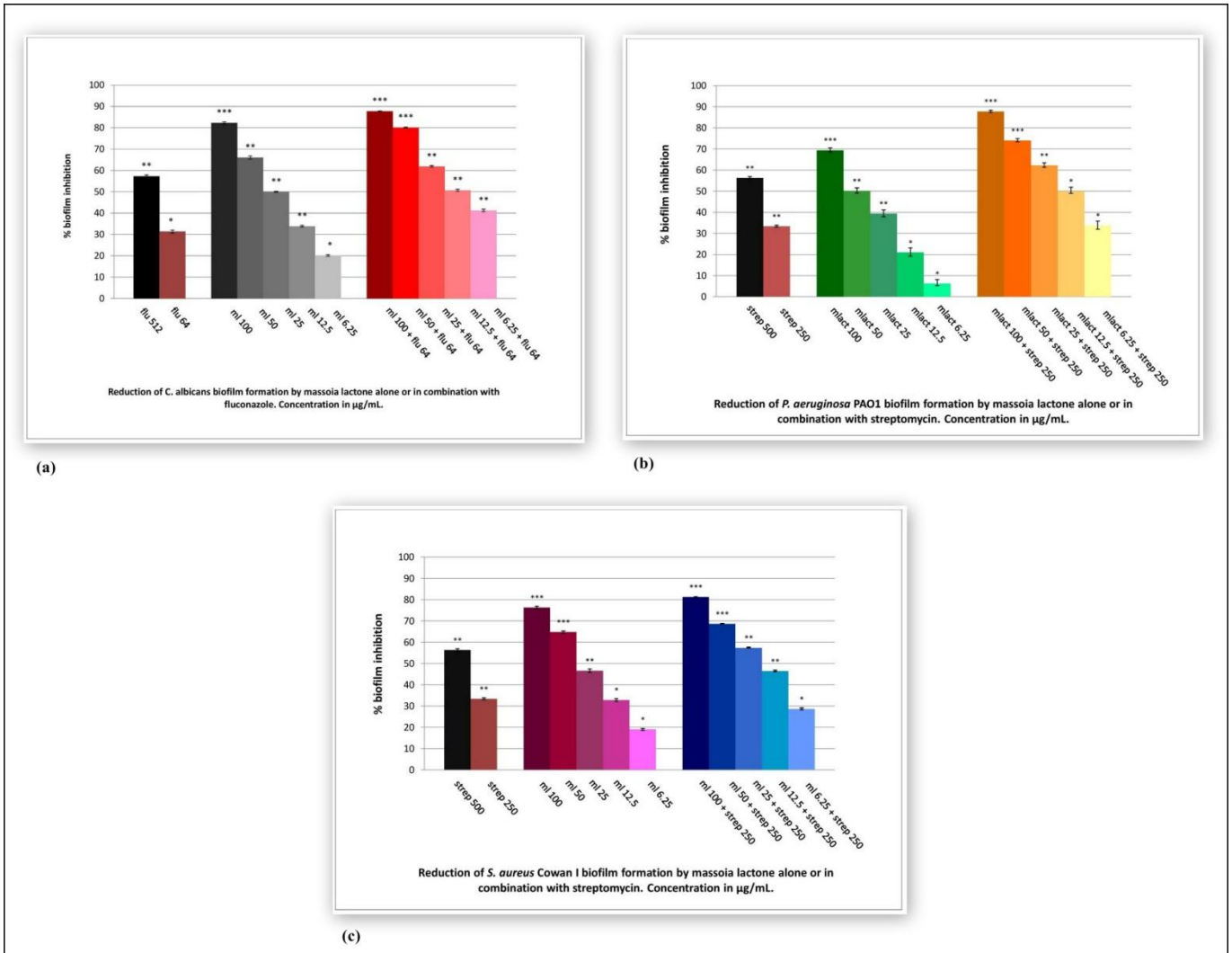

Fig. 7: Efficacy of massoia lactone (ml) alone and in combination with: (a) fluconazole (flu) against *C. albicans* 10231, (b) streptomycin (strep) against *Pseudomonas aeruginosa* PAO1, and (c) streptomycin (strep) against *Staphylococcus aureus* Cowan I. The bars indicated the standard deviations of the means. Asterisks indicate a significant difference between treatment and negative control.

Table 5: MBICs of Massoia lactone and antibiotics alone and in combination, and fractional inhibitory concentration index (FICI) values against *P. aeruginosa, S. aureus* and *C. albicans* biofilm formation

Microorganism	Compound Test (µg/ml)	$MBIC_{50}$-o (µg/mL)	$MBIC_{50}$-c (µg/mL)	FIC_{50}	$FICI_{50}$	Result (Interpretation)
P. aeruginosa PAO1	massoia lactone	50	12.5	0.25	0.5	synergy
	streptomycin	500	125	0.25		
S. aureus Cowan I	massoia lactone	100	25	0.25	0.5	synergy
	streptomycin	500	125	0.25		
C.albicans ATCC 10231	massoia lactone	50	12.5	0.25	0.375	synergy
	fluconazole	512	64	0.125		

Massoia lactone has been synthesized from various methods (Ramachandran *et al.,* 2000, Sabitha *et al*., 2007; Sato *et al.,* 2001), however no investigation about its anti-biofilm activity has ever been conducted. In future research, the effect on *P. aeruginosa* and *S. aureus* biofilm architecture in the presence of massoia lactone could be examined.

Hemolysis assay

In order to measure the cytotoxicity of the massoia lactone, hemolytic activity test was performed using human red blood cells. Result demonstrated that massoia lactone showed a dose dependent increase in destroying red blood cell and release hemoglobin (Figure 8). At to the highest concentration tested (100 µg/mL), exerted as much as 12.4 ± 0.9 % human red blood cells hemolysis compare to Triton X-100 which gave 100% hemolysis. This result indicates that at the range concentration tested, massoia lactone is less toxic to the human erythrocytes.

In nature massoia lactone is a rare essential oil component that was first characterized by Abe in 1937 (Abe, 1937). It is also present in *Hierochloe odorata* and *H. australis* that are commonly used in vodka production (Bernreuther *et al*., 1990), and has been isolated from mandibular glands formicine ants of the genus *Camponotus* as an alarm pheromone (Cavill *et al.,* 1968). The pleasant and sweet light coconut odor of massoia lactone make this compound very useful as a flavor for milk and butter, in the manufacturing of alcoholic drinks and as aromatizer in perfumes (Mineeva, 2012). IFRA (International Fragrance Association) prohibited the use of *Massoia aromatica* oil and massoia lactone because it was shown to be skin irritant and cause sensitization when it is applied in aromatheraphy. According to the study by Benoni and Hardebeck (1964), the oral toxicity (LD_{50}) for

massoia lactone is 1890 mg/kg, however the recommended safety dosage for massoia lactone to be use as a fragrance ingredient is not yet available (Anonim, 2009).

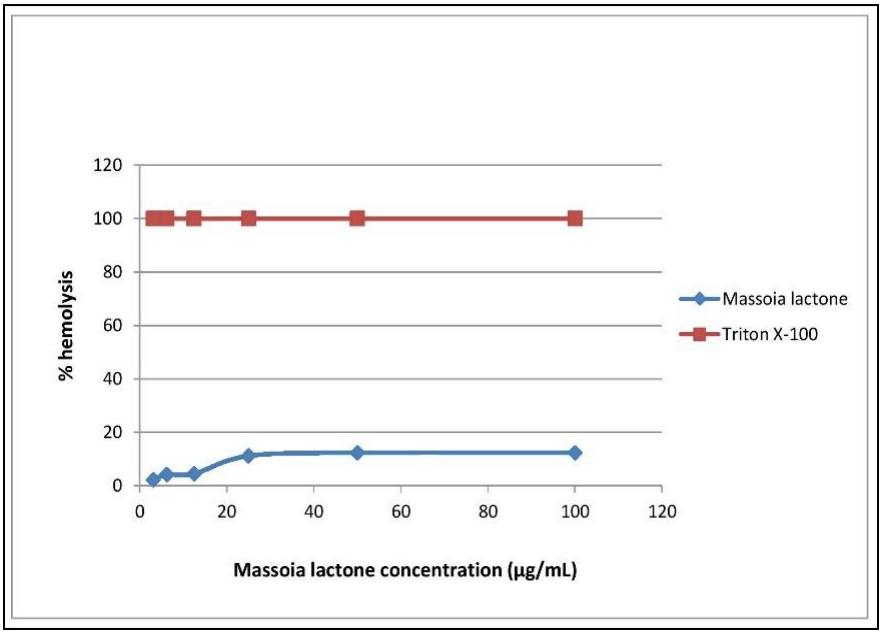

Fig. 8: Hemolytic activity of massoia lactone. Triton X-100 used as positive control.

In the future, massoia lactone could be used for therapeutical formulations to treat diseases caused by microorganisms. However, to be used as a drug for human and animal illness in pharmaceutical industry, safety tests in animal models have to determine the dose that causes no adverse effect.

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Chapter 7

General discussion

Instead of the occurrence of freely swimming single (planktonic) cells, microorganisms in nature have the tendency to interact with surfaces or each other, initially in a reversible association and eventually in an irreversible adhesion, resulting in the development of adherent microbial biofilm. The formation of a biofilm is a universal microbial strategy for survival under unfavorable conditions such as desiccation, nutrient starvation, and anti-microbial treatments. The multicellular structure of the biofilm makes it possible for the microbes to differentiate (e.g. going into dormancy or hibernation), enabling them to survive the harsh conditions (Bordi and de Bentzmann, 2011). The microbials within a biofilm are generally much more resistance to biocides and antibiotics, and less well recognized by phagocytic cells or antibodies compared to their planktonic counterparts, which makes them a source of many uncontrollable infections, and extremely difficult to eradicate (Schahter, 2003).

Pseudomonas aeruginosa, Staphylococcus aureus, and *Candida albicans* are opportunistic pathogens capable of causing biofilm infections on both natural body surfaces as well as medical devices such as contact lenses, central venous catheters and needleless connectors, endotracheal tubes, intrauterine devices (IUDs), mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, ear tubes, urinary catheters, and voice prostheses. They are major contributors to diseases that are characterised by an underlying microbial infection and chronic inflammation, e.g. periodontal disease, cystic fibrosis, chronic acne and osteomyelitis (Costerton *et al.*, 1999). Chronic biofilm infections cause inflammation, which is linked to carcinogenesis (Coussens and Werb, 2002). Biofilm related chronic mucocutaneous candidiosis (CMC) has been associated with a significant risk for oral cancer *in vivo* (Marttila *et al.,* 2013). Biofilms are also found in wounds and are suspected to delay healing. Electron microscopy of biopsies from chronic wounds found that 60% of the specimens contained biofilm structures in comparison with only 6% of biopsies from acute wounds (James *et al*., 2008). According to a recent public announcement from the National Institutes of Health (NIH), approximately 65% of microbial infections in humans are biofilm-related (Soto, 2014). This makes the discovery of anti-infective agents which are active against planktonic and biofilm microbial represents an important goal (Veeh *et al*., 2003; Soto, 2014; Mathé and Van Dijck, 2013; Tran *et al*., 2009).

Medicinal plants have been a source of wide variety of biologically active compounds for many centuries and used extensively as crude material or as pure compounds for applied for the treatment of inflammations, wounds, certain forms of cancer, infections due to bacteria, virus or fungi, and many more (Arif *et al*., 2009). It is estimated that there are 250.000 to 500.000 species of plants on earth and 1 to 10% of these plants are used by humans (Cowan, 1999). The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in the plant (Saranraj and Sivasakhti, 2014). In many cases, these substances serve as plant defense mechanism against microorganisms, insect, and herbivores. Some substances are responsible for plant odor, flavor and pigment (Cowan, 1999).

The plant's role is twofold in the development of new drugs. They may become the natural template for the development of a new medicine, and they may become a phytomedicine to be used to treat various disease conditions. The use of anti-microbials and other drugs derived from plants is widely accepted by modern (conventional) medicine, as traditional antibiotics become ineffective (Arif *et al*., 2009). Clinical microbiologists have two reasons to be interested in the topic of anti-microbial plant extracts. Firstly because there is a need to develop alternative anti-microbial drugs as the increasing resistance against many microbial infections due to the misuse of commonly used anti-microbial drugs. Secondly, because the scientists realize that the effective life span of any antibiotic is limited and new sources of antibiotics, including from plant sources, are required. In addition, the increasing interest in natural product as an alternative form of medical treatments contributes the developing of anti-microbial from plant extract into medicines (Cowan, 1999). Many commercially exploited drugs in modern medicine were initially used in crude form in traditional medicine indicating potentially useful biological activity. The primary benefits of using plant derived medicines are the perception that they are relatively safer, and have a long history of use in folk medicine for the cure and prevention of infections and diseases, offering profound therapeutic benefits and more affordable treatment (Sandasi *et al*., 2009; Ciocan and Bara, 2007). Application of phytochemical methods in medical treatments has led to the isolation of a wide range of natural compounds from the various plant species and include quinones, numerous flavonoids, tannins, comarins, terpenoids and essential oils, alkaloids, and other compounds of plant extracts (Samy and Gopalakrishnakone, 2010; Doughari 2012).

Indonesia is an archipelago country of more than 17000 islands straddling the equator in Southeast Asia. Indonesia considered to be rich in biodiversity, the second richest after Brazil, with at least 47 distinct natural ecosystems which are rich in plant and animal resources and large number of islands endemics, with the total known species about 1.46 million. Indonesia also plays a key role in the

herbal medicine industry in regards to its rich biological heritage, cultural background and population (Nitis, 1999). Indonesia has more than 38.000 plants species, about 9.600 are listed as medicinal plants and according to Indonesian National Agency of Drug and Food Control, NADFC (Badan Pengawas Obat dan Makanan Republik Indonesia, BPOM RI), only around 300 plant species have been studied scientifically for their medicinal properties and are officially registered and used commercially as traditional medicine. The larger remaining part still requires research and screening for their potential medicinal properties. The use of plants to treat common ailments to fertility aid (BPOM RI, 2008; Schonhardt, 2010; Neubauer, 2002) is common and important among Indonesian people from generations to generations, but much of the information regarding medicinal plants is empirical and lacking logical validation (Diba *et al*., 2013). Most of the Indonesian people have ever used traditional herbal medicines which are popularly known as jamu. Jamu is a word in Javanese tribe language, meaning the traditional medicine from plants. Today, jamu has been adopted into Bahasa Indonesia with the similar meaning (Limyati and Juniar, 1998; Elfahmi *et al.*, 2014). Many institutions in Indonesia, especially governmental institutions such as the Ministry of Health, and the NADFC (BPOM RI) as well as the universities are actively involved in plant research in the area of medicine, pharmacy, chemistry, biology, agriculture, forestry, marine, environment and engineering. Lots of study have been conducted on the biological activities of the most common plants used in Indonesian traditional medicine, e.g. *Piper betle, Caesalpinia sappan, Cinnamomum burmannii, C. sintoc, Syzigium aromaticum, Nymphaea nouchali, Kaempferia rotunda* and *Massoia aromatica*, as reported in the literatures (Nawawi *et al*., 1999; Sangat and Larashati, 2002; Batugal *et al*., 2004; Atmadja *et al*., 2009; Elfahmi *et al.,* 2014, Wijaya *et al*., 2014), however, very few studies have investigated Indonesian medicinal plants for their anti-quorum sensing and anti-biofilm activities, including in *P. aeruginosa, S. aureus* and *C. albicans*.

From our study, we found biofilm formation inhibition and biofilm breakdown activities from ethanol extracts of *K. rotunda, C. burmannii, C. sintoc, C. sappan*, and *N. nouchali* as well as essential oils of *C. burmannii, M. aromatica, O. basilicum* and *L. cubeba* against *P. aeruginosa* and *S. aureus* biofilm (Chapter 2 and 3, this thesis). Biofilm formation of *C. albicans* was suppressed by *C. burmannii* and *M. aromatica* essential oils. Both oils also showed capability in disturbing established *C. albicans* biofilm (Chapter 4, this thesis). Further research involving *C. burmannii* and *M. aromatica* oils revealed that both oils also have capacity to impede violacein pigment production related quorum sensing activity of *Chromobacterium violaceum*, a reporter strain in quorum sensing (Chapter 5, this thesis).

According to Shan *et al*., (2007), the extract of *C. burmannii* showed significant anti-bacterial activity against five common food-borne pathogenic bacteria such as *Bacillus cereus, Listeria monocytogenes, S. aureus, Escherichia coli*, and *Salmonella anatum.* Among the strains, the highest activity observed was against *S. aureus* and the least activity was observed against *E. coli*. Highly positive relationships were observed between anti-bacterial activities and phenolic content of the tested extracts against each bacterium. The study suggested that the anti-bacterial activity of the tested extracts were closely associated with their phenolic constituents. Nuryastuti *et al*., (2009) showed the potency of *C. burmannii* oil to combat both planktonic and biofilm cultures of clinical *Streptococcus epidermidis* strains, with MICs, ranging from 0.5 to 1% and 1 to 2%, respectively. CLSM images indicated that cinnamon oil is able to detach and kill existing biofilms.

Trans-cinnamaldehyde (TCA) has been identified as one of the bioactive compounds in *C. burmannii (Lv et al., 2010).* Jia *et al*., (2011) found that cinnamaldehyde, a major constituent of cinnamon essential oils, occurs naturally in the bark and leaves of cinnamon trees of the genus Cinnamomum could inhibit the biofilm formation of *S. aureus* ATCC 25923 in a dose-dependent manner, with MICs and MBCs were in the range of 0.06–0.5% (v／v).

Cinnamaldehyde is also known to possess anti-fungal properties. MIC of cinnamaldehyde against clinical isolate of *C. albicans* and *C. tropicalis* was 400 μg/mL and 500 μg/mL (Shreaz *et al.*, 2010). Study from Khan and Ahmad (2011) revealed that cinnamaldehyde alone or in combination with fluconazole and amphotericin B exhibit strong anti-biofilm activity against *C. albicans* biofilm, with the SMICs of 200–400 mg/L for *C. albicans* 04 and 100–360 mg/L for *C. albicans* SC5314. Further studies to mechanism of action suggested that cinnamaldehyde has an anti-fungal activity by targeting cell membrane integrity of yeast cells (Bennis *et al*., 2004), decreased ergosterol content (Mukherjee *et al*., 2003) and diminished level of ergosterol biosynthesis gene expression (Garcia-Sanchez *et al*., 2004). Inhibition of H (+) (-) ATPase leads to intracellular acidification and cell death (Shreaz *et al*., 2010). The defects in the cell wall are coupled with altered morphology and the inability of *Candida* cells to form hyphae and generate a biofilm. These defect in cell membrane integrity was notably translate into reduced pathogenicity of the *Candida* strain (Tan *et al*., 2014).

In chapter 6 of this thesis**,** we isolated the major compound of *M. aromatica* oil using preparative thin layer chromatography (TLC). ^{1}H-NMR analysis confirmed that the compound obtained is massoia lactone ($C_{10}H_{16}O_2$). Massoia lactone is a rare essential oil component and has only been found in a few other plants such as in cane sugar, tobacco and *Osmanthus fragrans*. Massoia lactone has an odour that is described as sweet, coconut-like and slightly fruity (Rali *et al.,* 2007). We discovered that massoia lactone exhibit anti-biofilm activity against *P. aeruginosa, S. aureus*, and *C. albicans.*

However, we found no activity of massoia lactone toward established biofilms of the microorganisms tested.

Various studies reported *in vitro* biological activity of massoia lactone related compounds against bacteria or fungi. Simionatto *et al.* (2007) reported that massoia lactone isolated from *Aeolanthus suaveolens* Mart. ex Spreng has anti-bacterial activity against *P. aeruginosa*. Anti-fungal activity of massoia lactone has been reported by Walter *et al.* (2000), and Kishimoto *et al.* (2005). It was suggested that the anti-fungal activity of massoia lactone is possibly due to its capability to inhibit the respiratory system of *C. albicans* because it arrested oxygen consumption by *C. albicans*. Efficacy of several lactone compounds against bacterial and fungal biofilm has also been reported. Plant sesquiterpene lactones are known as an inhibitory agent of *P. aeruginosa* biofilm formation (Cartagena *et al.,* 2007). A 3-oxo-C12 homoserine lactone is also reported to hinderithe filamentation of *C. albicans* by blocking the yeast-to-hyphal shift, an essential step for the adherence of *Candida* cells to a substrate to form a biofilm (Hall *et al*., 2011). Little is known about effects of massoia lactone on *Candida* biofilm formation or breakdown on the molecular level. A gene expression study of *C. albicans* biofilm cells in response to massoia lactone will be interesting to shed some further light on the molecular mechanism by which massoia lactone affects biofilm formation or breakdown.

Some selected natural products have already been found exhibit efficacy in influencing microbial biofilm formation. For example, halogenated furanone isolated from red algae *Delisea pulchra*, are thought to have evolved to interfere bacterial quorum sensing (Mannefield *et al.,* 1999). Other compounds disturb microbial biofilm by inhibit peptidoglycan synthesis (Ogunlana *et al*., 1987), damage microbial membrane structure (Cox *et al.,* 2000), change bacterial membrane surface hydrophobicity (Turi *et al*., 1997), and inhibit quorum sensing (Gao *et al*., 2003; Shayan and Saeidi, 2013).

It remains an important challenge to develop massoia lactone that has been isolated from this study into a new anti-biofilm drug. The process of drug discovery and development is a long-term, competitive, expensive and complicated (Mandal *et al*., 2009). The hit compound obtained from a screening process still has to be evaluated and undergo limited optimization to identify promising lead compound. The hit compound which shows biological activity has to be evaluated to determine its drug-like properties. Christopher A. Lipinski in 1997 formulated 5 rules, known as Lipinski's rule-of-five or Pfizer's rule-of-five, based on the observation that most orally administered drugs are relatively small and moderately lipophilic molecules. The rule requires that a hit molecule must have

no more than 5 hydrogen bond donors, not more than 10 hydrogen bond acceptors, a molecular mass less than 500 dalton, a partition coefficient (logP – a measure of hydrophobicity) less than 5 (Lipinski, 2004; Hefti, 2008). Massoia lactone is found to have 1 hydrogen bond donor, 2 hydrogen bond acceptors, a molecular mass of 168.24 dalton, and a partition coefficient of 2.419 (NCBI, 2014; ChemAxon, 2014), thus it fits Pfizer's rule-of-five requirements.

The lead compound has to undergo extensive optimization and trial programs before it can be considered as a successful drug. For this purpose, the lead compound is tested in cells (*in vitro*) and in animals (*in vivo*) to study its pharmacokinetics which include absorption, distribution, metabolism, excretion (ADME) and toxicology. The successful lead candidate, now called drug candidate, must be non-toxic, have a good bioavailability, can be distributed to the drug target in the body, can be metabolized efficiently and effectively as well as successfully excreted from the body. This part of the development process is referred to as the 'preclinical phase' and drug candidate is prepared for testing in humans. This phase is followed by the 'clinical phase' of development, in which the efficacy and safety of a drug candidate is scrutinized in patients (Saha, 2014). Although the road is well mapped out, it is certainly not easy or guaranteed to end in success (Hefti, 2008).

Therefore, further studies need to be conducted to examine the potency of massoia lactone as biofilm related drug, especially in immunosuppressed patient. The effect of massoia lactone to immune response has yet been investigated. However, other lactone subtances, such as homoserine lactone (Khajanci *et al.,* 2011), macrolides lactone (Nau and Tauber, 2008), and sesquiterpene lactone (Niphade *et al*., 2009), do have immunomodulatory properties. Due to its potential adverse side effects, massoia lactone also has to be tested for its selective toxicity towards normal cell. Cytotoxicity on host cells is a very important standard for assessing the selectivity of the observed pharmacological activities (Cos *et al*., 2006). Many drug development process are discontinued due to safety problems such as unfavorable toxic side effects, cardiac toxicity, etc (Mandal *et al.,* 2009). The toxicity of massoia lactone towards normal cells needs to be determined.

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Chapter 8

Summary

Microbial resistance to most commonly antibiotics is increasing and therefore requiring continuous development of new anti-microbial agents. Moreover, recent research has revealed that microbial biofilms causing elevated resistance to both most anti-microbial drugs and the host defense systems, which often results in persistent and difficult-to-treat infections. The discovery of anti-infective agents which are active against planktonic and biofilm microorganisms are therefore urgently required to deal with these biofilm-mediated infections.

Recently, scientists have focused on the anti-pathogenic potential of natural products. Plants are an interesting and important source for finding novel anti-biofilm compounds. They are a rich source of new molecules with pharmacological properties for the development of new drugs. Indonesia is one of the countries which has a very diverse flora and a rich tradition in the use of medicinal plants. Since several Indonesian medicinal plants contain anti-microbial compounds it was considered conceivable that they might also be a source of new anti-biofilm compounds. Therefore the research present in this thesis has been focused on the screening and identification of mixtures with anti-microbial and anti-biofilm activity, which may contain candidate compounds for developing new anti-biofilm drugs.

In **Chapter 1** a description is given about microbial biofilms which colonize polymer surfaces and forms a multilayered cell cluster. The biofilms are difficult to control due to their recurrence and high inherent resistance to anti-microbial agents and host immune responses. Quorum sensing, a cell-to-cell communication between microorganisms, is responsible for biofilm formation by microorganisms and plays an important key in microbial pathogenicity and antibiotic resistance. Infections due to the presence of microbial biofilms are major clinical concerns since they have the ability to adhere to and colonize surfaces of medical devices like e.g. implants. In many cases, this device has to be removed in order to cure the infections. These medical issues can be prevented if novel anti-microbials with high activity against microbial biofilms are discovered and identified. Medicinal plants are considered as an interesting source for novel anti-biofilm compounds. Plant-derived compounds have gained widespread interest in the search of drugs from natural sources. The compounds are widely accepted because of the perception that they are safe and have a long

history of use in folk medicine or treatment and prevention of the diseases and infections. In this chapter, we also discussed *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* as model microorganisms which are capable to form biofilm, and their pathogenicity.

Chapter 2 describes the screening of 54 ethanol extracts of different Indonesian medicinal plants for the presence of anti-microbial and anti-biofilm compounds against *P. aeruginosa* PAO1 and *Staphylococcus aureus* Cowan I. The extracts showed an inhibitory effect on planktonic grown bacteria but even more interestingly also on the formation of biofilm structures. At a concentration as low as 0.12 mg/mL, biofilm formation of *P. aeruginosa* PAO1 and *S. aureus* Cowan I is inhibited by 5 plant ethanol extracts : *Kaempferia rotunda* L., *Caesalpinia sappan* L., *Cinnamomum burmanii* Nees ex Bl., *C. sintoc* L., and *Nymphaea nouchali* Burm.f. The same extracts showed activity in degradation of the established biofilm of bacterial strains tested with higher concentration. Limited bacteriostatic activity was evident. This study demonstrated the effectiveness of *Kaempferia rotunda* L., *C. sappan* L., *C. burmanii* Nees ex Bl., *C. sintoc* L., and *N.a nouchali* Burm.f extract towards *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm. This property can be applied clinically to treat infectious biofilm along with conventional antibiotics, or applied industrially e.g. to remove biofilms from water pipes (Kim and Park, 2013).

In **Chapter 3 and 4** we report screening for anti-biofilm compounds in 29 essential oils from Indonesian medicinal plants against *P. aeruginosa* PAO1, *S. aureus* Cowan I, and towards *C. albicans* ATCC 10231 biofilms. *Candida* cells, like many other microorganisms, are able to adhere to and colonize surfaces of medical devices, resulting in development of a biofilm. The anti-biofilm activity of essential oils was confirmed by confocal laser scanning microscope analysis, along with LIVE/DEAD staining for the monitoring of live/dead cells. Essential oil from *C. burmanii* and *M. aromatica* showed a 50% inhibition of *P. aeruginosa* PAO1 and *S. aureus* Cowan I planktonic growth ($PMIC_{50}$) at concentration of 0.25 % v/v. Essential oil from *C. burmanii* and *M. aromatica* have been demonstrated to exhibit 50% ($MBIC_{50}$) of *P. aeruginosa* PAO1 and *S. aureus* Cowan I biofilm formation at concentration of 0.03 % v/v, whereas higher concentration (0.12 % v/v) was needed by both oils to disrupt 50% of *P. aeruginosa* PAO1 and *S. aureus* Cowan I established biofilms.

The essential oil of *C. burmanii, M. aromatica, O. basillicum* and *L. cubeba* (seeds part) showed an evident antifungal activity against planktonic growth of *C. albicans* and inhibited the formation of *C. albicans* biofilm at three different stages of development at a sub-PMIC concentration. The initial biofilm formation inhibition by plant essential oils was found to be concentration dependent. In the

presence of the essential oils, a significant decrease of biofilm biomass compared to negative control (biofilm cells without addition of plant essential oil) was evident. We also analyzed major components contained in Cinnamomum oil and Massoia oil by GC-MS. Cinnamic aldehyde (92.02 %) was found to be the major component of *C. burmanii* essential oil, and massoia lactone (92.05 %) is the main constituent of *M. aromatica* essential oil. The results obtained in this study indicate that the oil of *C. burmanii* and *M. aromatica* is an interesting source for anti-biofilm agents in the development of new strategies to treat infections caused by *P. aeruginosa*, *S. aureus* and *C. albicans* biofilm.

Chapter 5 reported the screening of the 54 ethanol extracts of Indonesian medicinal plants and 29 essential oils for their capability in inhibiting microbial quorum sensing mechanism using quorum sensing biosensor *Chromobacterium violaceum* ATCC 31532 (wild-type strain) and *C. violaceum* CV026 mutants strain. We also investigated the effect of the extracts and essential oils on the motility of pathogen *Pseudomonas aeruginosa* PAO1 strain, since bacterial motility has been shown to be associated with its virulence. Of the 54 plant extracts and 29 plants essential oils screened, *Nymphaea nouchali* ethanol extract, *Syzygium aromaticum* essential oil and *Massoia aromatica* essential oils demonstrated varying level of inhibition in violacein production of the reporter strains. A significant reduction in quorum sensing related motility of *P. aeruginosa* PAO1 has also been observed compare to the control. These plants extracts and essential oils may be selected for activity guided fractionation to identify and characterize the active principle.

Based on research described in Chapter 3, 4 and 5 which showed the activity of *M. aromatica* essential oil to inhibit biofilm formation of *P. aeruginosa, S. aureus and C. albicans*, and also violacein production of *C. violaceum* and *P. aeruginosa* motility related quorum sensing activity, the isolation of the active compound from this plant sample has been described in **Chapter 6**. Preparative thin layer chromatography along with GC-MS and ^{1}H-NMR elucidation were used to isolate and identify the active compound. Result from bioautography analysis revealed that massoia lactone (synonym: 5-Hydroxy-2-Decenoic Acid Lactone; 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-) is the active compound of massoia oil. The microtiter broth method was performed to detect the antifungal and antibacterial activity, as well as anti-biofilm activity of massoia lactone, alone and in combinations with antibacterial and antifungal agents. Massoia lactone found to exhibit anti-biofilm activity against *C. albicans* ATCC 10231 at concentration of 25 µg/mL. Biofilm formation of *P. aeruginosa* PAO1 and *S. aureus* Cowan I are also partially hindered by massoia lactone at concentration of 50 and 100 µg/mL. The anti-biofilm activity of massoia lactone also found to be

three to four times greater in combinations with antifungal/antibacterial drug. We performed initial toxicity study of massoia lactone by hemolysis assay using human red blood cells. At the highest concentration tested (100 µg/mL), massoia lactone showed 12.4±0.9 % human red blood cells hemolysis compare to Triton X-100 which gave 100% hemolysis. This result indicates that at the range concentration tested, massoia lactone is less toxic to the human erythrocytes. The result obtained in this thesis indicated that massoia lactone displayed potent activity against microbial biofilms *in vitro* and therefore has potential therapeutic implication for biofilm-associated microbial infections.

In **Chapter 7** a brief discussion is given about the results described in this thesis

CHAPTER 9

Nederlandse Samenvatting

Microbiële resistentie tegen de meeste gebruikte antibiotica neemt steeds meer toe en daarom is het noodzakelijk om nieuwe anti-microbiële medicijnen te ontwikkelen. Daarnaast heeft recent onderzoek laten zien dat biofilms van micro-organismen een verhoogde resistentie bezitten tegen bijna alle antibiotica en het menselijke afweersysteem, wat vaak resulteert in blijvende of moeilijk te bestrijden infecties. De identificatie van nieuwe anti-microbiële middelen, die zowel actief zijn tegen vrij bewegende (planktonische) micro-organismen als micro-organismen aanwezig in biofilms, is daarom erg urgent om infecties beter te kunnen behandelen.

Recent onderzoek wijst uit dat er natuurlijke producten de potentie hebben om pathogene micro-organismen te bestrijden. Daarbij is gebleken dat planten een belangrijke en interessant bron zijn van verbindingen die een anti-biofilm werking kunnen hebben en dus een rijke bron zijn voor nieuwe moleculen met farmacologische eigenschappen, waarmee nieuwe geneesmiddelen kunnen worden ontwikkeld. Indonesië is een van de landen met een enorme diverse flora en een rijke traditie in het gebruik van medicinale planten. Omdat verschillende Indonesische medicinale planten anti-microbiële verbindingen bevatten leek het waarschijnlijk dat ze ook een bron konden zijn voor anti-biofilm verbindingen. Daarom heeft het onderzoek beschreven in dit proefschrift zich gericht op het screenen en identificeren van mengsels met anti-microbiële en anti-biofim activiteit, welke mogelijk kandidaat verbindingen kunnen bevatten voor de ontwikkeling van nieuwe therapeutica.

In **Hoofdstuk 1** wordt een uitleg gegeven over biofilms, een cluster van meerdere lagen van cellen, die oppervlakten koloniseren. De bestrijding van een biofilm is moeilijk door het steeds maar weer vormen van nieuwe lagen van cellen en de hoge inherente resistentie tegen anti-microbiële verbindingen en immuun response van de gastheer. Quorum sensing , een cel tot cel communicatie systeem tussen micro-organismen, is onder andere verantwoordelijk voor de vorming van biofilms en speelt dan ook een belangrijke rol bij microbiële pathogeniciteit en antibiotica resistentie. Infecties veroorzaakt door de aanwezigheid van micro-organismen die biofilms vormen zijn van grote zorg in de medische wereld omdat ze in staat zijn oppervlakte van medische hulpmiddelen zoals bijv. implantaten te koloniseren en daardoor te infecteren.

In vele gevallen moeten die implantaten worden verwijderd om de infectie te genezen. Nieuwe antimicrobiële middelen die een hoge anti-biofim activiteit hebben kunnen hier een oplossing bieden. Zoals eerder genoemd, een interessante bron voor nieuwe anti-biofilm verbindingen zijn met name medicinale planten. Dit komt mede omdat extracten van planten al decennia lang een brede belangstelling hebben gekregen voor het zoeken naar medicijnen van natuurlijke oorsprong. Deze verbindingen worden breed geaccepteerd vanwege de perceptie dat ze veilig zijn en een lange geschiedenis hebben als volksmedicijn ter behandeling en preventie van ziektes en infecties. In dit hoofdstuk worden *Pseudomonas aeruginosa*, *Staphylococcus aureus* en *Candida albicans* besproken als model micro-organismen voor infecties via biofilm formatie.

De screening van 54 ethanol extracten van verschillende Indonesische medicinale planten op de aanwezigheid van antimicrobiële en antibiofim activiteit tegen *P. aeruginosa* PAO1 en *S. aureus* Cowan I is beschreven in **hoofdstuk 2**. De extracten vertoonden een remmend effect op planktonische groei maar, nog interessanter, op de vorming van biofilm-achtige structuren. Bij een minimale concentratie van 0.12 mg/mL, wordt biofilm vorming van *P. aeruginosa* PAO1 geremd evenals die van *S. aureus* Cowan I. De volgende 5 plant ethanol extracten vertonen deze remmende activiteit: *Kaempferia rotunda* L., *Caesalpinia sappan* L., *Cinnamomum burmanii* Nees ex Bl., *C. sintoc* L., en *Nymphaea nouchali* Burm.f. Dezelfde extracten lieten ook een verstoring van reeds gevormde biofilms door eerder genoemde bacteriën zien maar wel bij hogere concentraties. Ook beperkte bacteriostatische activiteit werd waargenomen. Deze eigenschappen kunnen mogelijk klinisch toegepast worden om infectieve biofilms te behandelen in combinatie met bestaande antibiotica. Daarnaast zou een toepassing ookindustrieel kunnen zijn zoals bijvoorbeeld het verwijderen van gevormde biofilms uit waterpijpleidingen (Kim en Park, 2013).

In **hoofdstuk 3 en 4** is de screening voor anti-biofilm verbindingen in 29 essentiële oliën van Indonesische medicinale planten tegen *P. aeruginosa* PAO1, *S. aureus* Cowan I, en *C. albicans* ATCC 10231 biofilms beschreven . *Candida* cellen, zoals vele andere micro-organismen, zijn in staat zich aan oppervlakten van medische hulpmiddelen te hechten, te koloniseren en biofilms te vormen. De anti-biofilm activiteit van essentiële oliën werd bevestigd door confocale laser scanning microscopie analyse, samen met een dood/levende kleuring om dode en levende cellen te monitoren. Essentiele olie van *C. burmanii* en *M. aromatica* lieten 50% remming bij een concentratie van 0.25% v/v zien van *P. aeruginosa* PAO1 en *S. aureus* Cowan I planktonische groei ($PMIC_{50}$). Tevens werd aangetoond dat essentiële olie van *C. burmanii* en *M. aromatica* 50% inhibitie laat zien op de ($MBIC_{50}$) biofilm-

vorming van *P. aeruginosa* PAO1 en *S. aureus* Cowan bij een concentratie 0.03 % v/v, terwijl een hogere concentratie (0.12 % v/v) nodig was van beide oliën om 50% van de reeds gevormde biofilms van *P. aeruginosa* PAO1 en *S. aureus* Cowan I af te breken.

De essentiele olie van *C. burmanii, M. aromatica, Ocimum basillicum* en *Litsea cubeba* (zaden) lieten een duidelijke anti-schimmel activiteit zien tegen de planktonische groei van *C. albicans.* Deze zelfde olie veroorzaakt ook remming van de vorming van *C. albicans* biofilm gedurende de drie verschillende stadia van biofilm ontwikkeling (sub-PMIC concentratie). Gevonden werd dat de remming van initiële biofilm vorming door deze essentiële oliën concentratie afhankelijk is. In de aanwezigheid van de geteste oliën , wordt een significante afname van biofilm biomassa gevonden vergeleken met de negatieve controle (biofilm cellen zonder toevoeging van essentiële oliën die duidelijk dicht op elkaar gelegen lagen van cellen lieten zien). Door GC-MS zijn ook de belangrijkste componenten die aanwezig zijn in cinnamomum oil en massoia olie geïdentificeerd. Cinnamic aldehyde (92.0 %) is de meest aanwezige component in *C. burmanii* essentiële olie, en Massoialactone (92.1 %) is de hoofdcomponent van *M. aromatica* essentiële olie. De resultaten verkregen in deze studie laten duidelijk zien dat de olie van *C. burmanii* en *M. aromatica* een interessante bron zijn voor mogelijke anti-biofilm verbindingen waarmee strategieën kunnen worden ontwikkeld om infecties veroorzaakt door *P. aeruginosa*, *S. aureus* end *C. albicans* biofilms te bestrijden.

In h**oofstuk 5** is de screening van de 54 ethanol extract en 29 essentiële oliën van Indonesische medicinale planten beschreven op mogelijke remming van het microbiële quorum sensing mechanisme met behulp van de quorum sensing biosensor *Chromobacterium violaceum* ATCC 31532 (wild type stam) en *C. violaceum* CV026 mutant stam (maakt geen signaal moleculen). Ook is het effect van de extracten en essentiële oliën op de beweeglijkheid van de pathogene *P. aeruginosa* PAO1 stam geanalyseerd, omdat is aangetoond dat beweeglijkheid geassocieerd is met de virulentie van deze stam en dat bewegelijkheid nodig is voor biofilm vorming. Van de 54 gescreende planten ethanol extracten en 29 planten essentiële oliën vertoonde *N. nouchali* ethanol extract, *Syzygium aromaticum* essentiële olie en *M. aromatica* essentiële olie een variërend remmend niveau van violacein-productie. Een significante reductie in quorum sensing, gerelateerd aan beweeglijkheid van *P. aeruginosa* PAO1 ten opzichte van de controle, werd ook gevonden. Deze planten extracten en essentiële oliën kunnen gebruikt worden voor fractionering, identificatie en karakterisering van de actieve verbinding(en).

Gebaseerd op het onderzoek beschreven in hoofdstuk 3, 4 en 5 waarin de activiteit van *M. aromatica* essentiële olie om zowel biofilm vorming van *P. aeruginosa, S. aureus* en *C. albicans*, te remmen als de violacein productie van *C. violaceum* en ook de beweeglijkheid- gerelateerde quorum sensing activiteit van *P. aeruginosa* te verminderen, is de actieve verbinding uit dit planten preparaat geïsoleerd en dit staat beschreven in **hoofdstuk 6**. Preparatie van dunne laag chromatografie tezamen met GC-MS en ^{1}H-NMR werden gebruikt om de actieve verbinding te isoleren en te identificeren. De resultaten van bioautografie analyse lieten zien dat massoia lactone (synonym: 5-Hydroxy-2-Decenoic Acid Lactone; 2H-Pyrane-2-one, 5,6-dihydro-6-penthyl-) de actieve component is in massoia oil. De microtiter broth methode werd uitgevoerd om zowel de anti-schimmel, anti-bacteriële activiteit en de anti-biofilm activiteit van massoia lactone te bepalen. Ook werden deze activiteiten in combinatie met standaard anti-bacteriële en anti-schimmel verbindingen (antibiotica) bepaald. Massoia lactone vertoont anti-biofilm activiteit tegen *C. albicans* ATCC 10231 bij een concentratie of 25 µg/mL. Biofilm vorming van *P. aeruginosa* PAO1 en *S. aureus* Cowan I wordt ook gedeeltelijk geremd door massoia lactone bij concentraties van 50 en 100 µg/mL. De anti-biofilm activiteit van massoia lactone is drie tot vier keer groter in combinatie met antibiotica. Ook werd een initiële toxiciteit studie met massoia lactone d.m.v. een hemolysis assay met humane rode bloedcellen uitgevoerd. Bij de hoogste concentratie (100 µg/mL), veroorzaakt massoia lactone bij 12.4±0.9 % menselijke bloedcellen, hemolyse t.o.v. Triton X-100 dat 100% hemolyse gaf. Dit resultaat laat zien dat in de range concentraties die getest zijn, massoia lactone minder toxisch is voor human erythrocyten. De resultaten die in dit proefschrift beschreven zijn laten zien dat massoia lactone een duidelijke activiteit heeft tegen microbiële biofilms *in vitro* en daarom een duidelijke potentie heeft als therapeuticum voor biofilm-geassocieerde microbiële infecties.

In **hoofdstuk 7** staat een korte discussie over de samengevatte resultaten behaald in dit proefschrift.

CURRICULUM VITAE

Sylvia Utami Tunjung Pratiwi was born on May 15, 1973 in Surabaya, Indonesia. She completed her undergraduate degree in Biology at Udayana University, Bali Indonesia in June 1996. A year later she moved to Yogyakarta, Indonesia and started to work as a permanent lecturer at Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University. She received a master degree in Biotechnology at Gadjah Mada University in October 2004. In 2008, she obtained a scholarship from The Directorate General of Higher Education (DIKTI)-Ministry of Education Indonesia, for her PhD study at Institute Biology Leiden, Leiden University under the supervision of Prof. C.A.M.J.J. van Den Hondel. Her research project is focused on the identification of bioactive compounds from Indonesian medicinal and spices plants affecting planktonic and biofilm growth of microorganisms, as presented in detail in this thesis. At this moment she is living in Yogyakarta Indonesia, and is continuing to work as a lecturer at Faculty of Pharmacy, Gadjah Mada University.

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List of abbreviations

AHL	*N*-acylhomoserine lactone
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	Analysis of variance
ATCC	American type culture collection
C4-HSL	N-butyryl-L-homoserine lactone
$CDCl_3$	Deuterated chloroform
CDR	*Candida* drug resistance
CF	Cystic Fibrosis
CFU	Colony forming unit
CLSI	Clinical and laboratory standard institute
CLSM	Confocal laser scanning microscope
CMC	chronic mucocutaneous candidiosis
CoNS	Coagulase negative staphylococci
CP	capsular polysaccharide
DMSO	Dimethyl sulfoxide
eDNA	Extracellular DNA
ECM	Extracellular matrix
EOs	Essential oils
EPS	Extracelullar polymeric substances, Exopolysaccharide
ETs	exfoliative toxins
EtOH	Ethanol
FDA	Food and Drug Administration
FICI	Fractional inhibitory concentration index
GC-MS	Gas chromatography-mass spectrometry
HHL	C6-Homoserine lactone
HULIS	Humic-like substances
ICSBD	Indonesian Country Study on Biodiversity
IUD	Intrauterine device
LB	Luria bertani
MCSRAMMS	Microbial surface components recognizing adhesive matrix molecules
MDR	Multi-drug resistance
Mm	Milimeter
MIC	Minimum inhibitory concentration
MBIC	Minimum biofilm inhibitory concentration
MBEC	Minimum biofilm eradication concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
µm	Micrometer
NIH	National Institutes of Health
NIST	National institute of standards and technology
nm	Nanometer
NMR	Nuclear magnetic resonance
OD	Optical density
PBS	Phosphate buffer saline
PE	Petroleum ether
PI	Propidium iodide
PIA	The polysaccharide intercellular adhesion
PMIC	Planktonic minimum inhibitory concentration

PNAG	Poly-*N*-acetylglucosamine
PSMs	Phenol-soluble-modulins
PTSAgs	Pyrogenic toxin superantigens
PVC	Polyvinyl chloride
QS	Quorum sensing
QSI	Quorum sensing inhibition
QSM	Quorum sensing molecule
RI	Retention index
RT	Retention time
SDB	Sabouraud dextrose broth
SEs	Staphylococcal enterotoxins
SI	Similarity index
SOD	superoxide dismutase
SSSS	Staphylococcal scalded skin syndrome
TLC	Thin layer chromatography
TM	Traditional medicine
TSS	Toxic shock syndrome
TTSS	Type III secretion system
v/v	Volume/volume
WT	Wild type
XTT	2-3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide