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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 (Schachter, 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). ¹H-NMR analysis confirmed that the compound obtained is massoia lactone (C₁₀H₁₆O₂). 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 hinder the 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 substances, 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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