ANTIMICROBIAL AND ANTI-QUORUM SENSING ACTIVTIES OF JAVANESE TURMERIC (Curcuma xanthorrhiza Roxb.) ETHANOLIC EXTRACT AGAINST Pseudomonas aeruginosa

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By

AHMAD FIQRI MUSTAQIM BIN OTHMAN

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Chair : Assoc. Prof. Yaya Rukayadi, PhD
Faculty : Food Science and Technology

Bacteria such as Pseudomonas aeruginosa uses quorum sensing (QS) mechanism to regulate the production of virulence factors, swarming motility and biofilm formation. As the synthetic quorum quenching compounds such as halogenated furanones reported to be toxic for human, using medicinal plant as an alternative as an anti-quorum sensing agent have been gaining attention. The objective of this study was to evaluate the antimicrobial and the antiquorum sensing activities of the C. xanthorrhiza Roxb. extract on the P. aeruginosa ATCC35554. The rhizome of the C. xanthorriza Roxb. was extracted using ethanol as the solvent. The crude extract were tested for antibacterial activity against P. aeruginosa in terms of well diffusion, minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) using the Clinical and Laboratory Standard Institute (CLSI) methods. Additional analysis on the antimicrobial activity of the extract was done on the bacterial growth using the Log10 colony forming unit assay. The quenching of QS mediated swarming was done by measuring the mean diameter of the swarming colonies treated with the C. xanthorrhiza Roxb. extract. The pyocyanin inhibition was evaluated colorimetrically by extraction with chloroform and 0.2 M hydrochloric acid (HCl). As for the alkaline protease and the LasB protease, the supernatant of the culture treated with the extract were exposed to the skim milk agar and the casein buffered broth. Biofilm formation prevention was done using the 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino) carbonyl]-2H-tetra-zolium-hydroxide (XTT) reduction assay on a pre-sterilized 96-wells microtiter plate. The results showed that the extract can inhibit and kill the growth of the P. aeruginosa with MIC and MBC values of 200 and 700 mg/mL, respectively. This indicates that the inhibition and killing of this bacterium need a relatively

high concentration. Up to 200 mg/mL of the extract was used in the antibacterial assay as in the quorum quenching assays, 50 mg/mL of the extract did not exhibited significant inhibition. Interestingly, the extract at 200 mg/mL showed 72.12% reduction of swarming motility, 84% inhibition of the pyocyanin production, 50.14% and 40% decrement of alkaline protease and LasB protease secretion, respectively and 78.35% decrease in the biofilm formation. Since production of the QS virulence factors, swarming and biofilm has been known to be regulated by the multiple QS circuits, the quorum quenching activities by the ethanolic extract of the C. xanthorriza Roxb. suggest that it can interfere on these systems. In conclusion, the C. xanthorriza Roxb. extract shows a high potential for an alternative natural quorum quenching agent.

Keywords: antimicrobial, Quorum quenching, antibiofilm, Curcuma xanthorriza Roxb., swarming motility, virulence factors

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

ANTIMIKROBIAL DAN ANTI-PENGESANAN KUORUM AKTIVITI KUNYIT JAVA (Curcuma xanthorrhiza Roxb.) EKSTRAK ETANOL TERHADAP Pseudomonas aeruginosa

Oleh

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Bakteria seperti Pseudomonas aeruginosa menggunakan mekanisme pengesanan kuorum (QS) untuk mengawal pengeluaran faktor-faktor virulasi, “swarming” motiliti dan pembentukan “biofilm”. Oleh kerana kompoun penghalang quorum sintetik seperti “halogenated furanones” dilaporkan sebagai toksik untuk manusia, penggunaan herba-herba semula jadi sebagai alternatif ejen pengesan anti-kuorum semakin mendapat perhatian. Objektif kajian ini adalah untuk menilai aktiviti antimikrob dan anti-kuorum ekstrak C. xanthorrhiza Roxb. pada P. aeruginosa ATCC35554. Rizom C. xanthorriza Roxb. telah diekstrak menggunakan etanol sebagai pelarut. Etanol ekstrak telah duji untuk aktiviti antibakteria terhadap P. aeruginosa dari segi “well diffusion” , konsentrasi penghambatan minimum (MIC) dan kepekatan bakterisida minimum (MBC) berdasarkan kaedah Standard Klinikal dan Makmal Institut (CLSI). Analisis tambahan terhadap aktiviti antimikrob ekstrak juga dilakukan terhadap pertumbuhan bakteria menggunakan ujian unit pertumbuhan koloni pada Log10. Penghalangan pergerakan “swarming” melalui pengesanan kuorum telah dilakukan dengan mengukur diameter koloni “swarming” yang dirawat dengan C. xanthorrhiza Roxb. ekstrak. Perencatan pyocyanin dinilai secara kolimetrik selepas pengekstrakan dengan kloroform dan 0.2 M asid hidroklorik (HCl). Cecair supernatan telah diekstrak daripada kultur P. aeruginosa telah dirawat dengan ekstrak terhadap agar susu skim untuk ujian alkali “protease” dan “buffer” kasein untuk ujian “LasB protease”. Pencegahan pembentukan “biofilm” dilakukan dengan menggunakan perwarna 2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl) -5 -[(phenyl-amino) karboksil] -2H-tetra-zolium-hidroksida (XTT) pada piring microtiter 96 lubang. Hasilnya menunjukkan bahawa ekstrak boleh menghalang dan membunuh pertumbuhan P. aeruginosa dengan nilai 200 mg/mL (ujian MIC) dan 700 mg/mL (ujian MBC). Ini menunjukkan bahawa perencatan dan pembunuhan bakteria ini memerlukan kepekatan yang agak tinggi. Ekstrak C. xanthorrhiza Roxb. yang digunakan pada ujian antibakteria

telah dilakukan sehingga 200 mg/mL kerana pada ujian penghambatan pengesanan kuorum, konsentrasi ekstrak pada 50 mg/mL tidak menunjukkan pengurangan yang memberansangkan. Menariknya, ekstrak pada 200 mg/mL menunjukkan pengurangan “swarming” berkurang sebanyak 72.12%, 84% daripada produksi pyocyanin, 50.14% terhadap “protease” alkali dan 40% untuk ujian LasB “protease”. Keputusan penghambatan pengesanan daripada ekstrak juga telah menunjukkan 78.35% penurunan terhadap pembentukan biofilm. Oleh kerana produksi faktor virulasi pengesanan kuorum, “swarming” dan “biofilm” telah dikawal oleh pelbagai litar dalam sistem pengesanan kuorum, penghambatan terhadap faktor-faktor virulasi seperti di atas oleh ekstrak etanol C. xanthorriza Roxb. menandakan bahawa ia boleh mengganggu sistem pengesanan kuorum. Kesimpulannya, ekstrak C. xanthorriza Roxb. menunjukkan potensi yang tinggi sebagai ejen alternatif penghalang sistem pengesanan kuorum.

Kata-kata kunci: Penghalangan kuorum, anti-“biofilm”, Curcuma xanthorriza Roxb., antibakteria, faktor-faktor virulasi

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

UPM	Universiti Putra Malaysia
QS	Quorum sensing
AI	Autoinducer
OdDHL	N-(3-oxododecanoyl)-homoserine lactone
BHL	*N*-butanoyl-$_L$-homoserine lactone
^oC	Degree celcius
%	Percentage
mL	Milimeter
µL	Microliter
nm	Nanometer
g	Gram
mg	Miligram
Rpm	Rotation per minute
N	Normality
mM	MiliMolar
w/v	Weight per volume
XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carbo-xanilide
OD	Optical density

CHAPTER 1

INTRODUCTION

1.1 Background

Foodborne illnesses and food spoilage has been crucially affecting the global food safety and economy (Fukuda, 2015; Gustavsson *et al.,* 2011). There have been a lot of cases reported due to consumption of foodborne pathogens that has led to mild or serious diarrhea, vomiting, abdominal pain (Adam and Moss, 2000) and even deaths (WHO, 2014; Scallan *et al.,* 2011). Such symptoms affect more on the elderly, children and immune-compromised individuals (FDA, 2018). Spoilage of food products by these organisms have also increase the food wastage and food loss throughout the globe thus affecting the economy (Bhunia, 2008). While well developed countries such as the United States and United Kingdom are able to produce good statistical reports on the foodborne illnesses nor the economic loss due to food wastage (Teisl and Roe, 2010), less urbanized countries are facing difficulties in tackling this issue due to the lack of cases report (Soon *et al.,* 2011). This is also true for Malaysia, a country that holds multiple races with varieties of delicacies that some are produced in an unhygienic environments and lack of awareness regarding food safety protocols (Abdul-Mutalib *et al.,* 2015).

Usage of antibiotics in various of antimicrobial agents such as detergents, preservatives (Gram *et al.,* 2002) are common in fighting and preventing contamination on food products and processing equipment (Wise and Soulsby, 2002). These antimicrobial agents often use the concept of selective toxicity whereby the difference between the microorganism metabolism and the structure and the corresponding features of human cells are exploited (Levinson, 2014), such as the cell wall biosynthesis, nucleic acid metabolism as well as protein synthesis (Sigma Aldrich, 2018). However, the frequent usage of these antimicrobial agents especially antibiotics have been reported to cause an increase of antibiotic resistance strain bacteria (Bhunia, 2008). Moreover, production of extracellular enzymes contributes to the host immune system evasion and protection of the bacterial colonies against antimicrobial agents (Hoge *et al.,* 2010; Hung *et al.,* 2005; Miyoshi *et al.,* 2002). As biofilm is composed of extracellular matrix of polysaccharides, it can help establishing suitable environments for wide spectrum of bacteria as well as providing enough nutrients for growth while protecting bacterial cells against host immune system via intracellular adhesions (Gotz, 2002; Mack *et al.,* 1994; Costerton *et al.,* 1987). This makes cleaning the equipment used during manufacturing of food products even challenging as the surviving bacteria could potentially act as a contamination source (Bai and Rai, 2011; Frank, 2001).

Therefore, numerous alternatives have been researched to reduce the dependencies on the usage of antibiotics in preventing microbial food contamination (Dong *et al.,* 2007). One of them includes quorum sensing (Dong *et al.,* 2007; Zhang and Dong, 2004; Hentzer and Givskov, 2003), a bacterial communication system that is used by various single-celled microorganisms such as bacterial and fungal pathogens in respond to the changing environments by regulating its virulence productions (Dong *et al.,* 2007; Fuqua and Winans, 1994). Example of the bacteria that uses quorum sensing in regulating its virulence include *Eschericia coli, Staphylococcus aureus, Pseudomonas aeruginosa* and many more (Waters and Bassler, 2005; Zhang and Dong, 2004; Hornby *et al.,* 2001). These foodborne pathogens have been reported to form biofilm on food equipment and food product surfaces, causing serious health issues and economic losses (Kumar and Anand, 1998). *Pseudomonas aeruginosa* uses quorum sensing to regulate production of virulence factors such as alkaline protease, pyocyanin, swarming motility as well as initiate and maintain biofilm formation (Venturi, 2006; Caballero *et al.,* 2001; Davies *et al.,* 1998).

Therefore, the development of quorum quenching is deeply invested in as it would serve as an alternative method in preventing bacterial growth without killing them (Bacha *et al.,* 2016; Lee and Zhang, 2014, Hong *et al.,* 2012). Numerous non-antibiotic agents such as quorum quenching have been reported to be developed and tested on their efficacy on preventing the quorum mediated virulence production (Chatterjee *et al.,* 2016). For example, synthetic antibiotics macrolide antibiotic azithromycin (AZM) is one of the quorum quenching agents that shows an improvement against quorum sensing inhibitors during the clinical trial (Imperi *et al.,* 2014; van Delden *et al.,* 2012; Høiby, 2011). However, according to Kohler *et al.* (2010), the usage of anti-virulence such as AZM treatments to pose a long term disadvantages, whereby discontinuation from the treatment would lead to more severe infections by *Pseudomonas aeruginosa* (Köhler *et al.,* 2010). Therefore, the usage of traditional medicinal plants as quorum sensing inhibitors have gained much interest as several of them have been reported to shown quorum quenching activities (Jakobsen *et al.,* 2012; Kandasamy *et al.,* 2012; Krishnan *et al.,* 2012).

Traditional medicinal plants have always been widely used by the locals as food as well as medicine due to their highly beneficial active compounds, even now (Choo *et al.,* 2006; Vining, 1990). For example, *Curcuma xanthorrhiza* Roxb. or commonly known as Javanese turmeric or temu lawak is commonly found in Indonesia, Malaysia as well as Thailand (Sylvester *et al.,* 2015). Extensive studies have reported that the beneficial bioactive compounds present in the Javanese turmeric such as terpenoids and curcuminoids among others that show high potential as anti-cancer, anti- bacterial, anti-diuretic, anti-fungal and more (Aznam and Atun, 2016; Rukayadi and Hwang, 2007; Rukayadi *et al.,* 2006; Siagian, 2006; Afifah, 2005; Choi *et al.,* 2004; Hwang *et al.,* 2000; Ozaki, 1990). Recent studies have also shown that these traditional medicinal plants possess anti quorum sensing mechanism (Packiavathy *et al.,* 2012; Wang *et al.,* 2012; Song. *et al.,* 2010). Therefore,

this study aims to explore the effect of the Javanese turmeric extract on inhibiting the quorum sensing mechanism by the *Pseudomonas aeruginosa*.

1.2 Problem Statements

P. aeruginosa is an opportunistic and persistent gram negative bacteria that is commonly associated with foodborne illnesses and food spoilage such as in milk, vegetables and fruits (Motarjemi *et al.,* 2013; Hossain, 2013; Arslan *et al.,* 2011). *Pseudomonas* spp. can cause spoilage in dairy products (Cousin *et al.,* 2001) via the production of the stable extracellular enzymes (Herrera, 2001) including lecithinases (Ray, 2004; Herrera, 2001), protease and lipases (Arslan *et al.,* 2011; Ray, 2004). These enzymes are able to impairs the milk coagulation process causing it to elicit bitter or rancid flavors (Richter and Vedamuthu, 2001). *P. aeruginosa* can also cause food spoilage in blue milk which often is pathogenic towards human and animals as a secondary infection (Collins *et al.,* 1989). *P. aeruginosa* has also been reported to cause high fever, headache and diarrhoea as well as bacterecemia and septicemia in the host blood stream (Motarjemi *et al.,* 2013).

Quorum sensing (QS) is a bacteria cell-to-cell communication mechanism used to determine the bacterial physiology such as local population density as well as applying strategies to adapt to the changes in the environment (Ahmad *et al.,* 2015; Skandamis and Nychas, 2012; Szabó *et al.,* 2010; Choo *et al.,* 2006; Fuqua and Greenberg, 2002). Quorum sensing allows the bacteria to increase their resistance towards antibacterial agents and facilitate bacterial pathogenesis while protecting the growing quorum from the harsh environment (Wei and Ma, 2013). However, usage of synthetic chemical quorum quenchers that focuses on their bactericidal effect have been reported to increase the selection of resistant strains (Ugurlu *et al.,* 2016; Gyawali and Ibrahim, 2014; Lewis, 2008). Synthetic quorum quencher might also have other detrimental effect for example, halogenated furanone have been reported to be chemically reactive, unstable and toxic to human cells (Hentzer and Givskov, 2003).

Therefore, with the increasing emergence of multidrug resistant pathogens, rather than killing or inhibiting bacterial growth, alternative approaches that rely on inhibition of pathogenic traits such as quorum sensing using natural products have gain popularity in the research society (Ahmad *et al.,* 2015; Lew *et al.,* 2015). Javanese turmeric (*Curcuma xanthorrhiza* Roxb.) or locally known as “temu lawak” can be found in tropical countries such as Malaysia and Indonesia (Musfiroh *et al.,* 2013). *C. xanthorrhiza* has been traditionally used for food and medicinal purposes (Lim, 2016; Park *et al.,* 2008). Studies have reported that *C. xanthorrhiza* possesses several of biological activities such as anti-tumor (Itokawa *et al.,* 1985), anti-bacterial (Hentschel *et al.,* 1996; Rukayadi *et al.,* 2006; Hwang *et al.,* 2000) and anti-inflammatory (Lee *et al.,*

2002; Claeson *et al.,* 1996) among others. The Javanese turmeric have been reported to have bioactive compounds including curcuminoids, camphor, geranyl acetate, zerumbone, β-curcumene, zingiberene, ar-curcumene and xanthorrhizol (Jantan *et al.,* 2012). These bioactive compounds such as xanthorrhizol have been proven to elicit biological properties such as antifungal (Lew *et al.,* 2015; Rukayadi *et al.,* 2006), antimicrobial (Lew *et al.,* 2015), anticancer (Choi *et al.,* 2004) activities. Therefore, the aim of this study is to determine the QS inhibition activity of the Javanese turmeric (*C. xanthorrhiza* Roxb.) on the quorum sensing mechanisms of *P. aeruginosa in vitro*.

1.3 Objectives

The objectives of this study are:

1. To extract the rhizome of the *C. xanthorrhiza* Roxb. and determine the non-killing or inhibiting concentration of against growth of *P. aeruginosa*
2. To evaluate the inhibitory activity of the *C. xanthorrhiza* Roxb. on the *P. aeruginosa* quorum sensing regulated virulence factors and biofilm formation.

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