UNIVERSITI PUTRA MALAYSIA

MECHANISMS OF ANTI-MICROBIAL ACTION OF TUNICATE (Polycarpa papillata) EXTRACT AGAINST METHICILLIN RESISTANT Staphylococcus aureus AND METHICILLIN SUSCEPTIBLE Staphylococcus aureu

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master Science

MECHANISMS OF ANTI-MICROBIAL ACTION OF TUNICATE *(Polycarpa papillata)* EXTRACT AGAINST METHICILLIN RESISTANT *Staphylococcus aureus* AND METHICILLIN SUSCEPTIBLE *Staphylococcus aureu*

By

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August 2013

Chairman: Professor Mariana Nor Shamsudin, Phd

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The spread of multi drug-resistant (MDR) organism particularly Methicillin Resistance *Staphylococcus aureus* (MRSA) has become a public health concern worldwide. The failure to respond to antibiotic therapy has been a great challenge to clinicians in treatment of infection caused by this pathogen. This phenomenon stimulated the escalation to discovery of new antibacterial compounds from various natural resources worldwide including marine organism. A series of researches revealed that marine organism possess biologically active metabolites that signify roles such as antimicrobial, antioxidant, antifungal and antiviral. The objectives of the study are to elucidate the efficacy of tunicate extracts, as antimicrobial agent against MRSA and Methicillin Sensitive *Staphylococcus aureus* (MSSA) as well as to determine the mode of inhibition. Two different extraction methods were employed at the beginning of this study involving solvent extractions and supercritical fluid extractions (SFE). For solvent extractions, methanol and hexane were used whereas for SFE, the extractions were optimized for pressure, carbon dioxide (CO_2) flow rate and the used of co-solvent. Higher yield was obtained by solvent extraction than SFE extraction. As for SFE extraction, the optimum extraction conditions were obtained at 400 bar, 28 g/min CO_2 with 3 ml/min co-solvent (methanol) introduced intermittently giving high yield. The antimicrobial activity of all extracts against MRSA and non-MRSA isolates showed that SFE extracts have greater antimicrobial activity against both pathogenic bacterial strains tested when compared to solvent extract. Among all SFE isolates, SFE-F extract is the most potent followed by SFE-C, SFE-B and SFE-A. However SFE-D and SFE-E extract which extracted with methanol continuously only inhibit the growth of MSSA. The composition of the most and the least potent SFE extracts in comparison to solvent extracts, identified through gas chromatography mass spectrometry revealed that the chemical composition of both SFE-D and SFE-F tested were almost similar to hexane extract. However there were additional compounds present in SFE-

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D and SFE-F extract such as 2-decenal, 9-hexadecenoic acid, octadecanoic acid methyl ester and hexadecanoic acid ethyl ester. A number of these compounds were also present in methanol extract. Apart from that the presence of fatty acids, fatty acid esters and aldehydes which were higher in the SFE-F extract may contribute to the antimicrobial activity shown by the extract. Bioassay study of SFE-F and methanol extracts revealed that both *Polycarpa papillata* extracts showed positive effect in inhibiting the growth of all MRSA and MSSA isolates tested. The time kill assay showed bactericidal activity against both MRSA and MSSA at MIC and 2 times MIC. Cytotoxic effects of SFE-F and methanol extracts were shown to inhibit the growth of vero cells at IC_{50} 2.693 mg/ml and 6.509 mg/ml respectively. The mechanism of action of tunicate extracts was investigated through cellular based approach involving leakage assay. This assay showed evidence of membrane impairment through detection of cytoplasmic material measured at A_{260} upon treatment with tunicate extracts. Bacterial membrane permeabilizing ability was also utilized in this study whereby the penetration of fluorescence dye into the impaired membrane was investigated through fluorescence microscopy with the aid of SYTO 9 and propidium iodide fluorescence stains. Higher dye penetration rate observed in treated cells than the untreated cells. With this principle, effect of tunicate extracts on membrane synthesis targeting fatty acid synthesis II (FASII) pathway were investigated via molecular biotechnological approaches. The gene expression study of enoyl-acyl carrier protein reductase (*fabI*) gene was determined by real time polymerase chain reaction. The treatment on both MRSA and MSSA resulted in down-regulation of *fabI* expression thus induced a conditional lethal phenotype in comparison to untreated sample. In summary, the present study showed that *Polycarpa papillata* methanol and SFE-F extracts possessed significant *in vitro* antibacterial activity and the effectiveness of both extracts in affecting membrane synthesis are significantly noted. These findings indicate the promising applications of local marine organism as an alternative antimicrobial agent against MRSA.

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Sebagai memenuhi keperluan untuk ijazah Master Sains

MEKANISME TINDAKAN ANTI-MIKROB EKSTRAK TUNIKAT (*Polycarpa papillata)* TERHADAP METISILIN RINTANG *Staphylococcus aureus* DAN METISILIN SENSITIF *Staphylococcus aureus*

Oleh

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Penyebaran organisma rentan pelbagai ubat-ubatan (MDR) terutamanya metisillin rintang *Staphylococcus aureus* (MRSA) telah menarik perhatian orang ramai tentang kesihatan di dunia. Kegagalan untuk bertindak balas terhadap terapi antibiotik menjadi satu cabaran besar dalam rawatan klinikal bagi menangani jangkitan yang disebabkan oleh bakteria ini. Fenomena ini telah mendorong kepada peningkatan dalam pencarian sebatian antibakteria daripada pelbagai sumber semulajadi di seluruh dunia termasuklah organisma marin. Satu siri penyelidikan telah mendedahkan bahawa organisma marin mempunyai metabolit-metabolit yang aktif secara biologi yang berperanan sebagai antimikrob, antioksidan, antikulat dan antivirus. Oleh yang demikian, objektif kajian ini adalah untuk menjelaskan keberkesanan ekstrak tunikat sebagai agen antimikrob terhadap MRSA dan Methicillin sensitif *Staphylococcus aureus* (MSSA) serta menentukan mekanisme perencatan. Untuk tujuan itu, dua kaedah yang berbeza telah digunakan pada awal kajian ini yang melibatkan pengekstrakan pelarut dan pengekstrakkan cecair terlampau atau dikenal sebagai '*Supercritical Fluid Extraction'* (SFE). Bagi pengekstrakan pelarut, metanol dan heksana telah digunakan, manakala bagi SFE pengekstrakkan dilakukan dengan mengoptimumkan beberapa parameter seperti tekanan, kadar aliran karbon dioksida dan penggunaan pelarut-bersama. Kajian mendapati hasil yang lebih tinggi telah diperolehi melalui pengekstrakan pelarut berbanding pengekstrakan SFE. Bagi SFE, kaedah pengekstrakan yang optimum telah diperoleh pada tekanan 400 bar, 28 g/min CO_2 dengan 3 ml/min pelarut bersama (metanol) yang diberikan secara berkala (SFE-F) telah memberikan hasil yang tinggi. Aktiviti antimikrob kesemua ekstrak terhadap MRSA dan MSSA menunjukkan bahawa ekstrak SFE mempunyai aktiviti antimikrob yang lebih baik terhadap kedua-dua strain bakteria yang diuji berbandingkan ekstrak pelarut. Malah, ekstrak SFE-F telah terbukti berkesan merencat pertumbuhan bakteria yang diuji

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diikuti oleh SFE-C, SFE-B dan SFE-A. Walau bagaimanapun ekstrak SFE-D dan SFE-E yang diekstrak menggunakan metanol secara berterusan hanya menghalang pertumbuhan MSSA. Komposisi ekstrak SFE-D and SFE-F,yang dikenalpasti melalui gas kromatografi, spectrometri jisim mendedahkan bahawa komposisi kimia kedua-dua SFE-D dan SFE-F yang diuji adalah hampir sama dengan ekstrak heksana. Walau bagaimanapun terdapat sebatian tambahan yang hadir dalam SFE-D dan SFE-F seperti 2-desenal, 9-heksadesenoik asid, ester asid metil oktadekanoik dan asid heksadekanoik etil ester. Beberapa sebatian ini juga turut hadir dalam ekstrak metanol. Selain daripada itu kehadiran asid lemak, ester asid lemak dan aldehid yang lebih tinggi dalam ekstrak SFE-F dipercayai menyumbang kepada aktiviti antimikrobial yang ditunjukkan oleh ekstrak tersebut. Kajian bioassai SFE-F dan ekstrak metanol mendedahkan bahawa kedua-dua ekstrak *Polycarpa papillata* menunjukkan kesan yang positif dalam menghalang pertumbuhan semua MRSA dan MSSA yang diuji. Ujian '*Time-kill*' menunjukkan kedua-dua ekstrak tunikat yang diuji pada kepekatan MIC dan 2 kali MIC memberi kesan bakterisidal terhadap MRSA dan MSSA. Kajian sitotoksik kedua-dua ekstrak ini yang telah menunjukkan perencat pertumbuhan sel-sel vero di IC_{50} 2.693 mg/ml dan 6.509 mg/ml. Bagi, mengkaji mekanisme tindakan ekstrak tunikat, pendekatan berasaskan sel yang melibatkan ujian kebocoran (*leakage assay*) telah dijalankan. Data yang diperolehi menunjukkan membran bakteria telah terjejas akibat tindakan ekstrak tunikat berikutan pengesanan kandungan sitoplasma yang diukur pada A_{260}. Keupayaan ekstrak untuk menembusi membran bakteria juga telah kaji dalam kajian ini di mana penembusan pewarna pendafluor ke dalam membran yang terganggu telah dikaji melalui mikroskopi dengan bantuan SYTO 9 dan propidium iodida. Kadar penembusan propidium iodida yang lebih tinggi telah diperhatikan pada sel-sel yang dirawat berbanding sel-sel yang tidak dirawat. Berikutan itu, kesan ekstrak tunikat pada sintesis membran juga telah disiasat melalui pendekatan bioteknologi molekul dengan mensasarkan mekanisme sintesis asid lemak II (FASII). Kajian mengenai kesan ekstrak tunikat terhadap gen ungkapan-acyl pembawa protein enoyl reductase gen (*fabI*) telah dijalankan menggunakan tindak balas rantaian polimerase masa nyata (*real-time PCR*). Rawatan kedua-dua ekstrak yang dikaji terhadap MRSA dan MSSA mengakibatkan pengurangan ekspresi *fabI* yang mendorong fenotip yang mudah mati berbanding dengan sampel yang tidak dirawat. Secara ringkasnya, kajian ini menunjukkan bahawa *Polycarpa papillata* ekstrak, metanol dan SFE-F bertindak sebagai antibakteria dan keberkesanan kedua-dua ekstrak ini dalam mempengaruhi sintesis membran adalah amat ketara. Penemuan ini adalah merupakan salah satu aplikasi organisma marin tempatan ini sebagai ejen antimikrob terhadap MRSA.

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I certify that a Thesis Examination Committee has met on 30 August 2013 to conduct the final examination of Haslinda Ayu Binti Mohd Yaakob on her thesis entitled "Mechanisms of Anti-Microbial Action of Tunicate *(Polycarpa papillata)* Extract Against Methicillin Resistant *Staphylococcus aureus* and Methicillin Susceptible *Staphylococcus aureus*" in accordance with Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the candidate be awarded the Master of Science.

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master Science.
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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

HASLINDA AYU BINTI MOHD YAAKOB

Date: 30 August 2013

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LIST OF ABBREVIATIONS

CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
MBC	Minimal Bactericidal Concentration Test
MIC	Minimal Inhibitory Concentration Test
MRSA	Methicillin Resistant *Staphylococcus aureus*
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SFE	Supercritical Fluid Extraction

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Methicillin resistant *Staphylococcus aureus* (MRSA) is a concern to public health worldwide. It has been recognized as an important pathogen in the clinical setting causing clinical manifestation ranging from wound infection to severe endocarditis and septicemia. MRSA was predominantly a nosocomial pathogen causing hospital-acquired (HA) infections. In Malaysia, increment in MRSA incidence has been documented, a study conducted in three institutes, revealed that the rate of MRSA has increased from 25.7% to 28.7%, 27.9% and 33.0% in 1996, 1998, and 2000, respectively (2000). In 2007, the rates of MRSA in 13 of Malaysia's state hospitals ranged from 6.8% to 44.1% (Ministry of Health, 2007). The MRSA prevalence in European countries is also high and the increasing trend is worrisome. An increase of MRSA prevalence was noted from 1% in 1990 to 20% in 2007 and the occurrence were stable within the range of 20% to 26% in the years between 2001 and 2007 (Dulon *et al.,* 2011). The prevalence is expected to be increase due to the emergence of community-acquired MRSA (CA-MRSA) that increasingly isolated from infections without established risk factors for the acquisition of MRSA.

CA-MRSA which acquired within community is genetically distinct than HA-MRSA (Baba *et al.,* 2002; Ma *et al.,* 2002). It is treatable by the use of non β-lactam antibiotics, though it often gives greater health implication (DeLeo *et al.,* 2010). As for HA-MRSA, the treatment of infection is often problematic as it is usually resistant to multiple drugs including ß-lactam antibiotics, cephalosporins, macrolides and aminoglycosides (Kurosu *et al.,* 2013). This strain of MRSA which is sometimes referred as multiple drug resistant *Staphylococcus aureus* (MDRSA) frequently resulted in increment of morbidity and mortality rates as well as healthcare cost due to treatment failure and prolonged hospital stay (Cosgrove 2006). Resistance of this pathogen to a wide range of antibiotics has limited the treatment options to very few agents such as vancomycin and teicoplanin. Furthermore, the global health care setting is now threatened by another emerging pathogen, the vancomycin-intermediate *S. aureus* (VISA) and vancomycin resistant *S. aureus* (VRSA) reported in recent years has made therapy of MRSA infections even more challenging for clinicians. Emergence of virulent strain has raised the need to find new antibiotics against this organism. Thus tremendous research were conducted involving biodiversity screening program to seek for therapeutic drugs worldwide (Burt and Reinders 2003; Kim Jung-Eun *et al.,* 2008; Mandalari *et al.,* 2010).

Exploration of drug candidates has been extensively carried out from natural resources including marine organisms as the ocean is a unique source that provides diverse natural products. Studies have shown that marine environment provides novel leads against fungal, parasitic, bacterial, and viral diseases. Many marine natural products have successfully advanced to the final stages of clinical trials,

including dolastatin 10, ecteinascidin-743, kahalalide F, and aplidine (Donia and Hamann 2003). Increasing number of candidates mainly invertebrates such as sponges and tunicates have been widely studied. Tunicate or also known as sea squirt is a rich source of unique and biologically active metabolites (Abas *et al.,* 1996; Bowden and Atta ur 2000; Wang *et al.,* 2008). It has been shown to harbour metabolites that are active as antioxidant, anticancer, antifungal, antiviral and antimicrobial agents (Rinehart *et al.,* 1981; Abourriche *et al.,* 1999; Aknin *et al.,* 1999; Abourriche *et al.,* 2000; In *et al.,* 2001; Jang *et al.,* 2003). Bioactive compound from various species of tunicate such as halocidin, epidioxysterol, clavanin, clavaspirin, styelins and cynthichlorine were reported to be effective against gram positive and gram negative bacteria. Many other studies involving tunicates bioactive compound of different species from all over the world have been described in the literature (Kobayashi *et al.,* 2000; Rudi *et al.,* 2000). However in Malaysia, tunicate has yet to gain importance and popularity. There is so much still unknown about the tunicates present in our coastal region. Research is required on many levels as data are deficient on many aspects. This study is one of the attempts to improve the knowledge on this organism in which the potential of this organism as pharmaceutical agent particularly as an antibacterial agent will be explored. Therefore the present research aims at assessing the potential of local tunicate crude extracts as antimicrobial agent for treatment of MRSA in order to fill the high demand for alternative treatment.

1.2 Hypothesis

1. Tunicate extracts will inhibit the growth of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA).
2. The mechanism of action may involve the disruption of bacterial membrane.

1.3 Objectives

To elucidate the antimicrobial potential of *Polycarpa papillata* crude extracts in treatment of methicillin resistant *Staphylococcus aureus* (MRSA) and methicillin sensitive *Staphylococcus aureus* (MSSA) as well as to investigate the mode of inhibition at the molecular level.

1.3.1 Specific objectives:

1. To determine the most potent extract(s) of tunicate with antibacterial activity from different extraction techniques.
2. To identify the chemical constituents of the most potent extract(s) of tunicate
3. To examine the antimicrobial kinetics and cytotoxicity of tunicate extracts on MRSA and MSSA.
4. To elucidate the effect of tunicate extract treatments on bacterial membrane and determine the mechanism of action.

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