VIBRIOSIS PREVENTION AND ANTI-ATHEROSCLEROSIS ACTIVITY OF SECONDARY METABOLITES FROM MARINE SPONGE, *Aaptos aaptos* AND *Callyspongia pseudoreticulata*

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Thesis Submitted in Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Institute of Marine Biotechnology Universiti Malaysia Terengganu

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DEDICATION

Special to my beloved husband ‘Muhammad Fajar’ and children ‘Eva Febrianty, Mohd.Afdhol Fadlurrahman, Muh.Abidzar Zulkarnain, and Afifah Nur Atifa
Supervisory committee
Laboratory staff
Many thanks for your support and inspiration

“….Allah will raise up to (suitable) ranks (and degrees), those of you who believe and have been granted Knowledge. And Allah is well-acquainted with all you do.”
(Al Mujadaalah: 11)

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Marine environment is a source of biological diversity and potential for discovery of novel drugs. Drugs produced by marine organisms have a broad spectrum of activity for treatment many important diseases without dangerous side effects. To date, the majority of marine natural products have been obtained from mostly sessile soft-bodied invertebrates, such as sponges and tunicates.

In search of potential antibacterial agents in preventing of vibriosis, six species of sponge were screened [*Aaptos aaptos* (I), *Aaptos suberitoides* (J), *Callyspongia* sp. (K), *Callyspongia pseudoreticulata* (L), *Haliclona* sp. (M), and *Clathria reinwardti* (N)] on *Vibrio harveyi* and *Vibrio* sp. by using paper disc diffusion method. Methanol extract of *Aaptos aaptos* and *Callyspongia pseudoreticulata* produced strong antibacterial activity and selected for further investigation. The methanol extracts of the two sponges were solvent partitioned into diethyl ether, butanol and aqueous extracts. These extracts were tested for antibacterial activity and butanol extracts of both sponges were found to be the most active with the inhibition zone of

23 ± 0.1 and 11± 0.1 mm, respectively. The antibacterial activity of these butanol extracts was lower than that of streptomycine (30 ± 0.1 and 28 ± 0.1 mm).

Bioactivity-guided isolation and purification led to the isolation of five known alkaloids aaptamine (CIII3236), 9-methoxyaaptamine (CIII22829), 4-*N*-methylaaptamine (CIVCC), 9-demethylaaptamine (1924K), and 9-demethyloxyaaptamine (1925C), one known amide (*p*-hydroxybenzamide (CVA) and one known sterol, 5α-cholestan-3β-ol (A10712) from *Aaptos aaptos* butanol extract (BUEI). Meanwhile, a new alkaloid (10a-methyl-8,9,10,10a-tetrahydrobenzo[*b*]pyrido[5,4,3,2-*lmn*]phenanthroline-1,7-dione (LH2636DA) was isolated from butanol extract of *Callyspongia pseudoreticulata* (BUEL). These compounds except CVA were tested for antibacterial assay and the result showed that they exhibited a strong antibacterial activity on *Vibrio harveyi* and *Vibrio* sp. with the inhibition zone of 12-25 and 17-25 mm, respectively.

The efficacy of the most active butanol extract of *Aaptos aaptos* in preventing vibriosis was further studied through *in vivo* toxicity and challenge test by using soaking method. The toxicity test of *Aaptos aaptos* butanol extract on tiger shrimp *Penaeous monodon* post larvae at the six different doses, 0, 62.5,125, 250, 500, and 1000 ppm was obtained that the dose of 62.5 (16.7 %) and 125 ppm (23.3 %) did not show any different mortality compared to the control (20 %) whilst, the concentrations of 250, 500 and 1000 ppm were found to be toxic on tiger shrimp post larvae with the mortality > 50 %. The histological observation on the three of doses was found to be no significant side effect on the haepatopancreas compared to normal tiger shrimp post larvae. Tiger shrimp post larvae challenged and treated with

Aaptos aaptos butanol extract at the safe dose of 32.25, 62.50 and 125 ppm demonstrated that the dose of 125 ppm gave the lowest mortality (43.33 %), followed by 62.50 ppm (66.67 %), 31.25 ppm (70%) and the positive control (80 %). This dose was also found to drastically decreased the *Vibrio harveyi* population in the rearing water with the lowest density namely 9.3 x 10^5 CFU/mL, followed by 62.5 (5.3 x 10^7 CFU/mL), 31.25 ppm (7.5 x 10^7) and the positive control (1.91 x 10^8 CFU/mL) 1 hour post treatment. The dose of 125 ppm also resulted in the lowest *V. harveyi* population in the tiger shrimp post larvae with the density of (1.77 x 10^3 CFU/mL), followed by 62.50 ppm (4.13 x 10^4 CFU/mL), 31.25 ppm (6.13 x 10^4 CFU/mL) and positive control (1.51 x 10^6 CFU/mL) at 6 hours post treatment. The histopathological examination on haepatopancreas of tiger shrimp post larvae challenged with *Vibrio harveyi* and treated with 125 ppm *Aaptos aaptos* butanol extract did not showed any changing on their cells and tissues such as cells necrosis, haemorrhagy, atropy, and lysis as occurred on the positive control and those two concentrations. The post larvae treated with this dose also displayed behavior and morphology which were backed to normal in the end of study. In addition, the water quality parameters observed during this experiment were suitable for the growth of the post larvae with the pH, temperature and dissolved oxygen ranging 7.19-8.08, 26.02-26.4 and 6.89-7.80, respectively for all concentrations.

The cytotoxicity activity and the effects on increasing the transcriptional activity of peroxisome proliferator responsive element (PPRE) and scavenger receptor-type B1 (SR-B1 promoter) of these secondary metabolites were also determined. The cytotoxicity assay was performed by using MTS method on HepG2 cell line and the result exhibited that aqueous, diethyl ether and butanol extracts of (I) produced

cytotoxicity effect on HepG2 cell with the EC_{50} value of 2.97, 6.8 and 13.1 µg/mL, respectively. However, methanol extract of (I) and extracts of (L) aqueous, methanol, diethyl ether, and butanol did not have any cytotoxicity effects on HepG2 cell with the EC_{50} value of 48.7, 50.1, 77.6, > 100, and > 100 µg/mL, respectively. The non cytotoxicity effect on HepG2 cell was also produced by compounds CIII3236 and LH2636DA with the EC_{50} value which was not recovered up to 10 µg/mL. Interestingly, compounds (A10712), (CIII22829), (1924K), and (CIVCC) were found to be cytotoxic on HepG2 cells with EC_{50} values of 0.25, 2.03, 5.6, and 9.6 µg/mL, respectively.

Potential of the toxic and nontoxic extracts and compounds as an anti-atherosclerotic agent was carried out by treating all extracts and compounds onto HepG2 cells transfected with PPRE. Butanol and aqueous extract as well as diethyl ether extract were treated onto HepG2 cells transfected with PPRE in various concentrations ranging from 0.485 to 3.125 and 0.097 to 6.25 µg/ml whilst, the nontoxic diethyl ether, butanol and aqueous extract from *Callyspongia pseudoreticulata* were treated onto HepG2 cells transfected with PPRE in various concentrations ranging 1.563 to 50 µg/mL. Result showed all extracts either toxic or nontoxic extracts were able to increase the transcriptional activity of PPRE promoter. Diethyl ether, butanol and aqueous extract of *Aaptos aaptos* gave the PPRE transcriptional activity by 1.29, 7.3 and 1.06 fold than that of negative control at the effective concentration of 0.390, 0.390 and 1.563 µg/mL, respectively whilst, diethyl ether, butanol and aqueous extract of *Callyspongia pseudoreticulata* were able to increase the PPRE transcriptional activity by 2,11, 8.46 and 1.46 fold than that of negative control at the effective concentration of 25, 50 and 6.25 µg/mL, respectively. Compounds treated

onto HepG2 cells transfected with PPRE also exhibited a positive result in inducing the transcriptional activity of PPRE and SR-B1 promoter activity in depending of their effective concentrations. Interestingly, those six compounds (CIII3236, CIII22829, A10712, CIVCC, 1924K, and LH2636DA) significantly increased the activity of PPRE about 1.82 up to 4.48-fold than to that of the negative control in depending of their effective concentrations. The best compound to increase the transcriptional of PPRE promoter was compound A10712. This compound was able to regulate the PPRE transcriptional activity by 4.48 fold when compared to negative control and was higher compared to positive control (TZD). All six compounds also demonstrated the significant ability in increasing of SR-B1 promoter activity between 1.09 to 13.62-fold than to that of the negative control. Compound CIVCC was the best transcriptional activator for SR-B1 promoter by 13.62 fold than to that of negative control and was higher compared to the positive control (LRH-1).

The result of this study shows that secondary metabolite from *Aaptos aaptos* was able to inhibit the growth of *Vibrio harveyi* population in rearing water and tiger shrimp *Penaeous monodon* post larvae body through quorum sensing which will prevent vibriosis. Aaptaminoid isolated from *Aaptos aaptos* butanol extract are the compounds which were responsible in vibriosis prevention. The result also exhibits that compounds isolated from *Aaptos aaptos* and *Callyspongia pseudoreticulata* butanol extract posses a potential activity in increasing the transcriptional regulation of SR-B1 promoter which will subsequently increase the efficiency of the Reverse Cholesterol Transport (RCT) and finally lower the risk of atherosclerosis in the body.

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk Sarjana Doctor Philosophy

RAWATAN VIBRIOSIS DAN AKTIVITI ANTI-ARTERIOSKLEROTIK DARIPADA SPAN MARIN, *Aaptos aaptos* DAN *Callyspongia pseudoreticulata*

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Dalam pencarian agen antibakteria yang berpotensi dalam rawatan vibriosis, telah diuji aktiviti antibakteria enam spesies span (*Aaptos aaptos* (I), *Aaptos suberitoides* (J), *Callyspongia* sp. (K), *Callyspongia pseudoreticulata* (L), *Haliclona* sp. (M), and *Clathria reinwardti* (N) terhadap *Vibrio harveyi* dan *Vibrio* sp. dengan kaedah pembauran cakera. Ekstrak metanol *Aaptos aaptos* (I) dan *Callyspongia pseudoreticulata* telah menunjukkan kesan antibakteria yang kuat sehingga dipilih untuk dikaji lebih lanjut. Ekstrak metanol dari kedua-dua span tersebut telah difraksi menjadi fraksi dietil ether, butanol dan air. Ekstrak butanol dari kedua-dua span tersebut didapati memberikan kesan antibakteria dengan zon perencatan 23 $\pm$ 0.1 dan 11 $\pm$ 0.1 mm, setiap satu.

Pengasingan dan penulenan ekstrak butanol *Aaptos aaptos* berpandukan biocerakin telah membawa kepada penemuan lima sebatian alkaloid (aaptamin (CIII3236), 9-metoksiaaptamin (CIII22829), 4-*N*-metilaaptamin (CIVCC), 9-demetilaaptamin (1924K), and 9-demetiloksiaaptamin (1925C), satu amida *p*-hidroksibenzamid (CVA) and satu sterol (5α-kolestan-3β-ol (A10712). Satu sebatian baru alkaloid

yang diasingkan daripada ekstrak butanol *Callyspongia pseudoreticulata* telah dicirikan sebagai 10a-metil-8,9,10,10a-tetrahidrobenzo[*b*]pirido[5,4,3,2-*lmn*]fenantrolin-1,7-dion (LH2636DA). Semua sebatian ini kecuali 1925C dan CVA menunjukkan aktiviti antibacteria yang kuat terhadap *Vibrio harveyi* dan *Vibrio* sp. dengan zon perencatan 12-25 dan 17-25 mm, setiap satu.

Kajian *in vivo* ketoksikan pada ekstrak butanol *Aaptos aaptos* ke atas pasca larva udang harimau menunjukkan bahawa ekstrak butanol ini selamat untuk udang ini pada 62.5 (16.7 %) dan 125 (23.3 %) ppm dengan kesan kematian udang yang tidak berbeda dengan kawalan negatif (20.0 %). Ujian histologi juga menunjukkan tiada kesan sampingan yang ketara ke atas haepatopankreas berbanding kawalan negatif. Kajian potensi ekstrak ini dalam rawatan vibriosis dengan kaedah perendaman ekstrak didapati bahawa ekstrak pada konsentrasi 125 ppm dapat menurunkan jangkitan vibriosis pada pasca larva udang harimau dengan rata-rata kematian lebih rendah (43.33 %) daripada kawalan positif (80 %). Ekstrak butanol 125 ppm juga didapati penurunan terhadap pertumbuhan bakteria *Vibrio harveyi* dalam air pemeliharaan dengan kepadatan lebih rendah iaitu 9.3×10^5 CFU/mL berbanding kawalan positif (1.91×10^8 CFU/mL) setelah satu jam rawatan. Penurunan populasi bakteria ini juga didapati di dalam pasca larva iaitu 1.77×10^3 CFU/mL lebih rendah berbanding kawalan positif (1.51×10^6 CFU/mL) setelah enam jam rawatan. Ujian histologi juga menunjukkan tiada kesan sampingan yang ketara ke atas haepatopankreas. Tingkah laku dan morfologi pasca larva udang harimau juga didapati kembali normal pada akhir kajian. Kualiti air pemeliharaan juga didapati sesuai untuk pertumbuhan udang harimau dengan pH antara7.19-8.08, suhu antara 26.02-26.4 dan oksigen terlarut antara 6.89-7.80 pada semua perlakuan.

Aktiviti sitotoksik dan pengaruh ekstrak dan sebatian dalam meningkatkan aktiviti penggalak peroxisome proliferator responsive elemen (PPRE) dan scavenger receptor type B1 (SR-B1) juga telah dikaji. Uji aktiviti sitotoksik telah dikaji menggunakan kaedah MTS terhadap titisan sel hati hepatosellular karsinoma manusia (HepG2) dan hasil menunjukkan bahawa ekstrak air, dietil eter dan butanol (I) menghasilkan kesan sitotoksik terhadap sel HepG2 dengan nilai 50 % perencatan 2.97; 6.8 dan 13.1 µg/mL, setiap satu. Namun demikian, ekstrak metanol (I) dan ekstrak air, dietil eter dan butanol (L) tidak memberikan kesan sitotoksik terhadap sel HepG2 dengan nilai 50 % perencatan 48.7; 50.1; 77.6; > 100, dan > 100 µg/mL, setiap satu. Kesan tidak toksik terhadap sel HepG2 juga ditunjukkan oleh sebatian CIII3236 dan LH2636DA dengan nilai 50 % perencatan tidak dikesan sehingga 10 µg/mL. Sebatian A10712, CIII22829, 1924K, dan CIVCC telah menunjukkan kesan toksik terhadap sel HepG2 dengan nilai 50 % perencatan 0.25; 2.03; 5.6; dan 9.6 µg/mL, setiap satu.

Potensi ekstrak toksik dan tidak toksik dan juga sebatian diatas sebagai anti-arteriosklerotik telah diuji ke atas sel HepG2 yang dijangkitkan dengan pGL3-PPRE. Ekstrak butanol dan air dan juga dietil eter *Aaptos aaptos* diuji ke atas sel HepG2 yang dijangkitkan dengan pGL3-PPRE dalam pelbagai kepekatan pada julat 0.485 sehingga 3.125 dan 0.097 sehingga 6.25 µg/ml manakala, ekstrak dietil eter, butanol dan air yang tidak toksik diuji keatas sel HepG2 yang dijangkitkan dengan pGL3-PPRE dalam pelbagai kepekatan pada julat 1.563 sehingga 50 µg/ml. Kajian menunjukkan bahawa semua ekstrak baik yang toksik maupun yang tidak toksik dapat meningkatkan ungkapan transkripsi pGL3-PPRE. Ekstrak dietil eter, butanol dan air *Aaptos aaptos* telah meningkatkan ungkapan transkripsi pGL3-PPRE

sebanyak 1.29, 7.3 dan 1.06 kali ganda apabila dibandingkan dengan kawalan negative pada kepekatan efektif masing-masing iaitu 0.390, 0.390 dan 1.563 , 12.5, 50.0 μg/ml manakala, ekstrak dietil eter, butanol dan air *Callyspongia pseudoreticulata* telah meningkatkan ungkapan transkripsi pGL3-PPRE sebanyak 2.11, 8.46 dan 1.46 kali ganda apabila dibandingkan dengan kawalan negative pada kepekatan efektif masing-masing iaitu 25, 50 dan 6.25 μg/ml. Sebatian yang berhasil diasingkan juga diuji ke atas sel HepG2 yang dijangkitkan dengan pGL3-PPRE dan SR-B1 dan kajian menunjukkan hasil yang positif dalam meningkatkan aktiviti penggalak PPRE dan SR-B1 pada kepekatan tertentu. Keenam-enam sebatian yang diuji dapat meningkatkan aktiviti penggalak PPRE sekitar 1.82 sehingga 4.48 kali berbanding kawalan negatif. Sebatian yang paling berkesan untuk meningkatkan transkripsi promoter PPRE adalah sebatian A10712. Sebatian ini dapat meningkatkan ungkapan promoter PPRE sebanyak 4.48 kali ganda apabila dibandingkan dengan kawalan negatif dan adalah lebih tinggi berbanding kawalan positif (TZD). Hasil kajian juga menunjukkan bahawa keenam-enam sebatian tersebut juga dapat meningkatkan aktiviti penggalak SR-B1 sebanyak 1.09-13.62 kali kawalan negatif. Sebatian CIVCC adalah sebatian yang paling berkesan untuk meningkatkan transkripsi promoter SR-B1. Sebatian ini dapat meningkatkan ungkapan promoter SR-B1 sebanyak 13.62 kali ganda apabila dibandingkan dengan kawalan negatif dan adalah lebih tinggi berbanding kawalan positif (LRH-1).

Hasil kajian menunjukkan bahawa sebatian bioaktif daripada *Aaptos aaptos* mampu menghambat pertumbuhan daripada populasi *Vibrio harveyi* di dalam air pemeliharaan dan pasca larva udang harimau melalui mekanisme kuorum sensing dan seterusnya akan merawat vibriosis. Sebatian aaptaminoid yang diasingkan

daripada ekstrak butanol *Aaptos aaptos* adalah sebatian yang memberi kesan di dalam rawatan vibriosis. Hasil kajian juga menunjukkan bahawa sebatian yang diasingkan daripada butanol ekstrak *Aaptos aaptos* dan *Callyspongia pseudoreticulata* berpotensi di dalam meningkatkan ungkapan transkripsi promoter SR-B1 yang kemudiannya akan meningkatkan kecekapan pengangkutan berbalik kolesterol (RCT) dan seterusnya mengurangkan risiko arteriosklerosis di dalam badan.