IMMUNOMODULATING PROPERTIES OF SABAH SEAWEEDS *KAPPAPHYCUS STRIATUS* AND *EUCHEUMA DENTICULATUM*

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FACULTY OF SCIENCE AND NATURAL RESOURCES
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DECLARATION

I hereby declare that no part of this report has been previously submitted for a degree in this or any other university. All work were based on my own research except as cited in references and other part of the report where indicated.

13 October 2016

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ABSTRACT

Seaweeds which are high in vitamins, minerals, proteins, polysaccharides and dietary fibers have been consumed traditionally in many East Asia countries as functional food and as sources of medicinal drugs for health promotion and disease prevention. Despite of the vast benefits, documentation on the immunological properties of Sabah seaweeds is scarce. Immunomodulators are capable to augment the immune system in order to provide protection against infections and improve our health. Thus, this study investigated the water and ethanolic extracts of *Kappaphycus striatus* and *Eucheuma denticulatum* that are widely cultured in Sabah and of economic importance. *Ex vivo* experiments were conducted using Balb/c mice spleens for NK cells activities, splenocytes proliferation and serum was used for Th-1 cytokine IFN-γ and Th-2 IL-4. Macrophage proliferation assay, nitric oxide production and wound healing assay were evaluated *in vitro* using RAW 264.7 and 3T3 cells. The *K. striatus* water extracts and ethanolic extracts of both seaweed samples could significantly increase the proliferation of RAW264.7 cells in a concentration-dependent manner (50 – 200 μg/mL). Both water extracts at 12.5 – 200 μg/mL were able to significantly increase considerable amounts of nitrite, while the production of nitrite was significantly inhibited when LPS-induced RAW 264.7 were incubated with the water extracts. This indicates that the water extracts of *K. striatus* and *E. denticulatum* possess anti-inflammatory activity. *E. denticulatum* ethanolic extracts at 100 μg/mL stimulated the migration of 3T3 fibroblasts in scratch assay indicated they exhibited wound healing properties. In addition, Balb/c mice administered with 50 mg/kg of *E. denticulatum* ethanolic extracts showed significant proliferation in all *ex vivo* tests suggesting that it is the best concentration to significantly immunomodulate the immune system. Gene expression profile were further investigated in mice treated with *E. denticulatum* ethanolic extracts at 50 mg/kg and 100 μg/mL to further understand the molecular mechanism involved in the immunomodulation. Gene expression results showed that the ethanolic extracts were involved in T-cell signaling pathway via the *CD8B1* and *CD3G* cell surface receptors. This lead to subsequent multiple downstream signaling pathways including calcium-calcineurin-NFAT, MAPK and NF-κB signaling pathways which could further activate downstream targets *P38*, *NFAT*, *AP-1*, *NF-κB* to induce cell proliferation, differentiation and production of cytokines. Increased expressions were shown in *CD8B1* and *CD3G* surface receptor genes, at a fold-change of 1.60 (p=0.039) and 1.49 (p=0.026) respectively whereas slight increased expressions which were not significant were shown in *NFAT1*, at 1.21 (p=1.53) and *IFN-γ*, at 1.54 (p=0.14) indicating that the extracts serve as a strong modulator in activating the surface receptors which could lead to the proliferation and activity of lymphocytes (T cells and NK cells), macrophage activation which mediate calcium-calcineurin-NFAT signaling pathway. In addition, the molecular mechanisms of cell-adhesion activities were also investigated. Wound healing activity involves the signal transduction and interaction of ECM to cell adhesion receptor integrins, cytoplasmic kinases, growth factors and cytokines. *SPP1*, an ECM protein with a fold-change of 2.11 (p=2.11) which interacts and bind to *ITGB3* cell surface integrins, with a fold-change of 1.44 (p=0.004) followed by stimulating cell-to-cell and cell-to-ECM adhesion, were found to be significantly expressed in

this study. Activation of *SPP1* protein and *ITGB3* cell surface receptor could initiate the integrin-mediated signaling pathway followed by activation of downstream pathways such as cytoskeletal organization, PI3K-Akt signalling pathway and MAPK signalling pathway. Besides, *SPP1* and *ITGB3* genes play an important role in cell proliferation, migration, differentiation, growth regulation which could accelerate fibroblast migration thus enhancing wound healing activity. Gene expression studies showed that Sabah seaweeds especially *E. denticulatum* ethanolic extracts has immunomodulating activities by acting on cell surface receptors which could then mediate T-cell receptor signaling and integrin-mediated signaling pathway. This study stipulated that all water and ethanolic seaweed extracts especially 50 mg/kg *E. denticulatum* ethanolic extracts served as a strong immunomodulator in enhancing host defence mechanisms and immune system.

ABSTRAK

CIRI-CIRI IMMUNOMUDULASI RUMPAI LAUT SABAH KAPPAPHYCUS STRIATUS DAN EUCHEUMA DENTICULATUM

Rumpai laut mengandungi vitamin-vitamin, protein, polisakarida dan serat dietari telah digunakan secara tradisional dalam banyak negara-negara Asia Timur sebagai makanan fungsian dan sebagai sumber perubatan untuk promosi kesihatan dan pencegahan penyakit. Dokumentasi tentang imunologi rumpai laut di Sabah sukar didapati walaupun terdapat banyak laporan tentang faedah-faedah rumpai laut. Imunomodulator mempunyai kebolehan untuk mempertahankan sistem imun bagi memberi perlindungan terhadap jangkitan dan membaiki kesihatan kita. Maka, kajian ini menyiasat ekstark air dan etanol Kappaphycus striatus dan Eucheuma denticulatum yang banyak dikulturkan di Sabah dan mempunyai kepentingan ekonomi. Eksperimen-eksperimen ex vivo telah dijalankan dengan menggunakan limpa mencit Balb/c untuk aktiviti sel-sel NK, proliferasi sel-sel limpa dan serum mencit untuk sitokin Th1 IFN-γ dan sitokin Th2 IL-4. Proliferasi makrofaj, produksi nitrik oksida dan eksperimen penyembuhan luka dinilaikan in vitro dengan menggunakan sel-sel RAW 264.7 dan 3T3. Ekstrak air K. Sriatus dan ekstrak etanol daripada kedua-dua rumpai laut boleh menambahkan proliferasi RAW 264.7 dengan signifikan daripada 50 – 200 µg/mL. Kedua-dua ekstrak air pada 12.5 – 200 µg/mL dapat menambahkan nitrit dengan signifikan manakala pengeluaran nitrit direncat degan signifikan apabila RAW 264.7 yang dicetuskan dengan LPS dieramkan dengan ekstrak air. Ini menunjukkan ekstrak air K. striatus dan E. denticulatum memiliki aktiviti anti-radang. 100 µg/mL ekstrak etanol E. denticulatum merangsang penghijrahan fibroblas 3T3 dalam asai penyembuhan luka menunjukkan mereka mempunyai ciri-ciri penyembuhan luka. Sebagai tambahan, mencit Balb/c yang diberi dengan 50 mg/kg ekstrak etanol E. denticulatum menunjukkan proliferasi signifikan dalam semua kajian ex vivo dan ini mencadangkan bahawa kepekatan ini adalah kepekatan yang terbaik untuk imunomodulasikan sistem imun. Profil ekspresi gen seterusnya disiasat dalam mencit Balb/c yang diberi dengan 50 mg/kg ekstrak etanol E. denticulatum dan fibroblas 3T3 yang dieramkan dengan 100 µg/mL ekstrak etanol E. denticulatum untuk memahami mekanisma molekul yang terlibat dalam imunomodulasi. Keputusan ekspresi gen menunjukkan bahawa ekstrak etanol terlibat dalam laluan pengisyaratan sel-T melalui permukaan sel-sel reseptor CD8B1 dan CD3G. Ini menjurus kepada beberapa laluan pengisyaratan seperti laluan-laluan kalsium-calcineurin-NFAT, MAPK dan NF-κB. Laluan-laluan tersebut akan mengaktifkan gen-gen seperti P38, NFAT, AP-1 dan NF-κB yang boleh mendorong proliferasi sel, pembezaan dan pengeluaran sitokin. Peningkatan ekspresi telah ditunjukkan pada permukaan gen-gen reseptor iaitu gen CD8B1 dan CD3G yang menunjukkan perbezaan kali ganda iaitu 1.60 (p=0.039) dan 1.49 (p=0.026) manakala ekspresi yang tidak signifikan ditunjukkan di dalam NFAT1 dan IFN-γ, iaitu 1.21 (p=1.53) dan 1.54 (p=0.14) menunjukkan bahawa ekstrak rumpai laut ini berfungsi sebagai satu pemodulat kuat dalam mengaktifkan permukaan gen-gen reseptor yang boleh menjurus proliferasi dan aktiviti limfosit (sel-sel T dan sel-sel NK). Selain itu, ekstrak ini dapat mengaktifan makrofaj yang menjadi pengantara dalam laluan kalsium-calcineurin-NFAT. Sebagai tambahan, mekanisma molekul lekatan sel juga *disiasat. Kegiatan penyembuhan luka melibatkan transduksi isyarat dan interaksi ECM kepada reseptor lekatan seperti sel integrin, kinase sitoplasma, faktor pertumbuhan dan sitokin. Kajian ini menunjukkan perningkatan ekspresi gen secara signifikan apabila protein ECM, SPP1 yang menunjukkan perbezaan kali ganda iaitu 2.11 (p=2.11) berinteraksi dengan permukaan sel ITGB3, iaitu 1.44 (p=0.004) melalui perangsangan sel-kepada-sel dan perlekatan sel-kepada-ECM. Pengaktifan protein SPP1 dan reseptor permukaan sel ITGB3 boleh merangsangkan laluan pengisyaratan pengantara integrin diikuti oleh pengaktifan organisasi kerangka sel, laluan PI3K-Akt dan laluan MAPK. Selain itu, gen-gen SPP1 dan ITGB3 memainkan peranan yang penting dalam proliferasi, penghijrahan, pembezaan dan pertumbuhan sel-sel yang boleh mempercepatkan penghijrahan fibroblas maka mempertingkat kegiatan penyembuhan luka. Kajian-kajian ekspresi gen menunjukkan bahawa rumpai laut Sabah terutamanya ekstrak etanol E. denticulatum mengandungi akitiviti imumomodulasi dengan mengaktifkan permukaan sel reseptor yang boleh mengantara dan menyebabkan regulasi laluan pengisyaratan sel-T dan laluan pengisyaratan pengantara integrin. Kajian ini menunjukkan semua ekstrak air dan etanol terutama 50 mg/kg ekstrak etanol E. denticulatum berfungsi sebagai pemodulat kuat untuk meningkatkan mekanisma pertahanan badan dan sistem imun.*

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%	-	Percent
°C	-	Degree celcius
µg	-	Microgram
µL	-	Microliter
µm	-	Micrometer
µM	-	Micromolar
bp	-	Base pair
CRAC channels	-	Calcium release activated channels
DMSO	-	Dimethyl Sulfoxide
dNTP	-	Deoxynucleoside triphosphate
EDTA	-	Ethylenediamine tetraacetic acid
ITAM	-	Immunenoreceptor tyrosine-based activation motif
LPS	-	Lipopolysaccharide
M	-	Molarity
mg	-	Milligram
min	-	Minutes
mL	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
NK	-	Natural killer
nm	-	Nanometer
PCR	-	Polymerase Chain Reaction
pmol	-	pico molar
RNA	-	Ribonucleic Acid
rpm	-	Revolution per minute
RQ	-	Relative quantity
sec	-	Second
v/v	-	Volume per volume
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Marine macroalgae or seaweeds are classified into red (Rhodophyta), brown (Ochrophyta) and green (Chlorophyta) macroalgae based on their pigmentation (Chan, Ho, and Phang, 2006). Seaweeds are low in calories, rich in vitamins, minerals, proteins, polysaccharides and dietary fibers (Dhargalkar and Pereira, 2005; Gupta & Abu-Ghannam, 2011b; Holdt and Kraan, 2011). Seaweeds are also known for their diverse claims health promotion and disease prevention. Red, brown and green seaweeds have been reported to exhibit antibacterial, antitumor, antioxidant, immunomodulatory, anti-inflammatory activities and anticoagulant properties (Zhou, Sun, Xin, Zhang, Li, and Xu, 2004; Cox, Abu-Ghannam and Gupta, 2010; Na, Kim, Kim, Park, Lee, Kim, Synytsya, and Park, 2010; Vairappan, Kamada, Lee and Jeon, 2013).

The demand of healthy food is progressively gaining attention over the years. Application of synthetic preservatives and additives such as sodium nitrite, sodium benzoate, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) to preserve the quality of food have been reported to be toxic and exert carcinogenic effect (Yamazaki, Yamaguchi, Yamauchi, and Kakiuchi, 1994; Massey, 1997; Sarhan, Shati, and Elsaid, 2014). This has opened up the necessity to search for natural food preservatives (Shan, Cai, Brooks, and Corke, 2007). The demand of seaweeds to be used as food, fodder, fertilizer and sources of medicinal drugs have also increased and thus lead to the study of seaweeds as potential for functional food development (Sánchez-Machado, López-Cervantes, López-Hernández, and Paseiro-Losada, 2004; Hong, Hien, and Son, 2007). In this regards, cultivated tropical seaweeds were chosen for their diverse claims for health promotion and disease prevention.

Despite the vast reports, documentation on immumodulating properties of Sabah seaweeds is scarce and majority of the researches were focused on its chemical contents, anti-cancer and anti-oxidant activities. Natural immunomodulators are host defense stimulators and are are being used as immunotherapy in some diseases such as cancer, immunodeficiency disorders, inflammation, and infectious diseases (Masihi, 2000; Peakman, Dayan and Thomas, 2001; Heo, Yoon, Kim, Ahn, Kang, Kang, Affan, Oh, Jung, and Jeon, 2010). Immunomodulators are capable to enhance host defence mechanisms which involve immune system cells such as lymphocyte subsets, macrophages and natural killer (NK) cells in order to provide protection against infections (Peakman, Dayan, & Thomas, 2001).

Marine bioresources are known to contain promising potential for discovering novel compounds with a diversity of bio-activities (Fujikura, Lindsay, Kitazato, Nishida, and Shirayama, 2010; Radulovich, Umanzor, Cabrera, and Mata, 2015). The rich marine biodiversity has attracted biomedical and medical scientists on the immunomodulating activities of the seaweeds. To date, researches on immunomodulating activities have been focused on red, brown, green seaweeds obtained from China, Japan, Korea and Spain. Polysaccharides from green seaweeds *Enteromorpha intestinalis* and *Enteromorpha prolifera* collected from China and Japan exerted immunomodulating activities as they induced cytokines production, nitric oxide production, T-cells splenocytes proliferation and also inhibit S-180 tumours (Jiao, Jiang, Zhang and Wu, 2010; Kim, Cho, Karnjanapratum, Shin, and You, 2011). Besides, sulphated polysaccharides obtained from *Ulva rigida* harvested from Spain could augment the immune system by inducing nitric oxide production, iNOS production from RAW 264.7 cells and increase chemokines expression (Leiro, Castro, Arranz, and Lamas, 2007). Also, polysaccharides obtained from brown and red seaweed *Hizikia fusiformis* and *Meristotheca papulosa* harvested in Japan could induce the proliferation of human lymphocytes indicated seaweeds exhibit immunomodulating activities and they might be useful in clinical applications for the treatment of tumours (Shan, Yoshida, Kuroda, and Yamashita, 1999).

Functional food is defined to have vast nutritional effects and thus improving the state of health or reducing the risk of illness and disease (Roberfroid, 2002). Seaweed showed potential as functional food ingredients as they possess medicinal characteristics, anticancer and anti-inflammatory and immunomodulatory activity (Hong *et al.*, 2007; Karnjanapratum, Tabarsa, Cho, and You, 2012; Vairappan *et al.*, 2013). Seaweeds such as Nori, Kombu, Wakame and Hijiki are widely used in food showed diverse health benefits (Okai, Higashi-Okai, Ishizaka, Ohtani, Matsui-Yuasa, and Yamashita, 1998; Ismail and Tan, 2002; Kuda, Hishi, and Maekawa, 2006; Negishi, Mori, Mori, and Yamori, 2013). Carrageenan, agar, and alginates from seaweeds were applied as functional food ingredients (Tseng, 2001).

To date, seaweeds also serve as a major food ingredient in food products especially in Japan, Korea and China (Ismail and Tan, 2002). However, seaweed as a food in Malaysia is not common and it is occasionally eaten as a salad dish (Norziah and Ching, 2000). Researches showed that seaweeds may be applied as functional food ingredient for lowering cholesterol and glycaemic index (Matanjun, Mohamed, Mustapha, and Muhammad, 2009). Red seaweeds *Eucheuma* spp. and *Kappaphycus* spp. and serve as an economically important source in east Malaysia as they are used for the extraction of iota and kappa carrageenan for the application in food industries (Phang *et al.*, 2010b; Cai *et al.*, 2013; Hurtado *et al.*, 2014). Hence, the study of immunomodulating activities of *Eucheuma denticulatum* and *Kappaphycus striatus* might promote adding seaweeds as functional food.

1.2 Problem Statement

Seaweeds widely consumed as food contain vast beneficial effects ranging from antibacterial, antitumor, antioxidant, anticoagulant and anti-inflammatory activities. Hence, do the *Eucheuma denticulatum* and *Kappaphycus striatus* red seaweeds contain immunomodulating properties and how do these seaweeds exert the immunomodulating activities.

1.3 Hypothesis of the Study

Eucheuma denticulatum and *Kappaphycus striatus* obtained from Sabah exhibit immunomodulating activities including splenocytes proliferation, natural killer activity, macrophage proliferation, nitric oxide production, cytokine production and wound healing activity.

1.4 Objectives

a. To identify the potential of immunomodulatory activities of *Eucheuma denticulatum* and *Kappaphycus striatus* from Semporna, Sabah

b. To compare the immunomodulating properties between *Eucheuma denticulatum* and *Kappaphycus striatus*

c. To elucidate the molecular mechanism of *Eucheuma denticulatum* and *Kappaphycus striatus* in their immunomodulating activities using animal models

CHAPTER 2

LITERATURE REVIEW

2.1 General overview of seaweeds

Marine algae are simple chlorophyll-containing organisms, composed of single cells or multiple cells, and sometimes collaborating together as simple tissues. They vary greatly in size, from unicellular organisms of 3-10 μM to giant kelps up to 70 m length. Thus, marine algae can be classified into macroalgae and microalgae based on their size inhabiting terrestrial, freshwater, brackish water and marine habitats (El Gamal, 2010; Phang, 2010). Seaweeds are macroalgae which are included in the general category of plants but they differ from terrestrial plants as they do not have true roots, stems and leaves nor vascular tissues (Rinaudo, 2007). The frond is the main part of the seaweed and the holdfast is the region of the attachment for the seaweed (Fish and Fish, 1989). Phychologists define seaweed as marine algae that are multicellular and macrothallic (Rinaudo, 2007). Seaweeds are a group of photosynthetic organisms which are able to grow where as low as 0.1% photosynthetic light is available. Seaweeds are commonly dominant in the rocky surfaces of the shallow subtidal areas in the temperate and polar regions. Some seaweeds are also found available in the intertidal and deep, clear seas up to 250 meters (Chapman and Chapman, 1980; Dhargalkar and Pereira, 2005; Rinaudo, 2007; Chopin and Sawhney, 2009).

The most common feature employed to classify and identify macroalgae is based on the presence of the accessory photosynthetic pigments of the light harvesting complex (LHC). These colored compounds, thereby, form the basis in the classification into three groups, known as red (phylum Rhodophyta), brown (phylum Ochrophyta) and green (phylum Chlorophyta) macroalgae (Chopin and Sawhney, 2009; Guiry and Guiry, 2016). Rhodophytas contain phycobiliproteins that absorb light and gives them distinct red colour, pheophytas contain carotenoid fucoxanthin which gives them brown colour while cholorophyta consists mainly of chlorophylls and hence they are green in colour (Raghavendran, Sathivel, & Devaki, 2004; Wiencke and Kai, 2012). To date, the numbers of seaweeds reported were around 1500 species for green seaweeds, around 1800 species for brown seaweeds and 6500 species for red seaweed where green seaweeds are amongst the majority (Guiry, 2016).

Chlorophyta are green algae seaweeds which are found mainly in freshwater. They are also found in seawater, soil, tree barks and even snow. Their chloroplasts are surrounded by two membranes which contain chlorophylls *a* and *b* as their main light-capturing pigments. They contain α-, β-, γ-carotene and xanthophylls The main storage product of green seaweed is starch (α-1,4-glucan) (Fish and Fish, 1989; Menzel, 2004; Glimn-Lacy and Kaufman, 2006; Barsanti, Coltelli, Evangelista, Frassanito, Passarelli, Vesentini and Gualtieri, 2008).

Brown algae (phylum Ochrophyta, in class Phaeophyceae) are olive-green to brownish black colour. They contain chlorophylls *a* and *c*, β-carotene and xanthophyll pigments fucoxanthin and violaxanthin which give them the brown or olive-green colour characteristics. Their main storage product is laminarin with the absence of starch and the cell walls are made up of cellulose (Glimn-Lacy and Kaufman, 2006; Rinaudo, 2007; Barsanti *et al.*, 2008).

Rhodophytas, red seaweeds are mainly marine algae, found in intertidal and subtidal to depths up to 40 m or above (Seckbach and Chapman, 2010). They contain chlorophyll *a* but is masked by phycobilin pigments phycoerythrin and phycocyanin. The presence of pigment phycoerythrin with strong absorption peaks to absorb green and blue light enable red algae to dominate in deeper parts of photic zone. Phycoerythrin is the dominant light harvesting pigment which gives red algae pink to reddish-brown colour. However, not all rhodophytas are pink to reddish-brown colour with the regards of little or absence of red pigment phycoerythrin. Cyanidia algae (*Cyanidioschyzon, Cyanidium,* and *Galdieria*) appeared blue-green colour because they do not contain phycoerythrin (Seckbach and Chapman, 2010). Their main storage product of red algae is floridean starch (α-1,4-glucan) in the cytoplasm and the cell walls are made of cellulose, with the