



**STUDY ON NATURAL PIGMENT OF CAROTENOID
AND CHLOROPHYLL CONTENT IN FIVE SPECIES
OF MALAYSIAN SEAWEED**

BY

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ABSTRACT

Seaweed, also known as macroalgae, are plant-like organisms that can be found in marine habitat. Various seaweed species have been gaining the interest of researchers due to the presence of functional bioactive compounds that can be extracted from their biomass. The study was conducted because of major colorants that have been used in the industries are derived from synthetic colorants. It is due to the comparative stability, cost effective and ease of use. Safety of the synthetic colorant usage is still being debated in order to identify the harmful effect towards the consumption. In the present study, three varieties of red macroalgae (*Eucheuma denticulatum*, *Glacilaria tikvahiae* and *Kappaphycus striatum*), a green macroalgae (*Caulerpa lentillifera*) and one brown macroalgae (*Padina pavonica*) were selected to determine their chlorophyll and carotenoid content by using UV-Vis spectrophotometer and HPLC, to identify their antioxidant properties by using DPPH scavenging method and antimicrobial activity by using Disc-diffusion method as well as to assess the stability of their natural pigment via CIELab. Characterisation study of the functional groups and volatile compounds in the selected seaweed were performed by using FTIR and GC-TOF/MS instrument. The findings of this research represents the preliminary stage in this study.

Overall results for GC-TOF/MS analysis lead to the identification of 37 compounds *K. striatum*, 32 compounds from in both *E. denticulatum* and *P. pavonica* as well as 27 compounds in *C. lentillifera* and *G. tikvahiae*. In terms of carotenoid content, *P. pavonica* contained the highest amount of carotenoid ($100.9 \pm 14.7 \mu\text{g/g DW}$) while *G. tikvahiae* recorded the lowest value ($25.1 \pm 9.4 \mu\text{g/g DW}$). Meanwhile, individual carotenoid content showed *K. striatum* had the highest lutein content ($38.6 \mu\text{g/g DW}$) while *P. pavonica* showed the lowest lutein content ($7.2 \mu\text{g/g DW}$). As for zeaxanthin, *C. lentillifera* ($21.3 \mu\text{g/g DW}$) showed the highest amount while *E. denticulatum* ($3.6 \mu\text{g/g DW}$) showed the lowest result. *C. lentillifera* also contained the most beta-carotene ($10.7 \mu\text{g/g DW}$) whereas *E. denticulatum* had the least amount at ($2.4 \mu\text{g/g DW}$). Last but not least, violaxanthin was only found in the green seaweed *C. lentillifera* ($8.9 \mu\text{g/g DW}$). The total chlorophyll content for *C. lentillifera* and *P. pavonica* were comparable at ($7.3 \mu\text{g/g DW}$) and ($7.5 \mu\text{g/g DW}$) respectively. In contrast, *E. denticulatum* recorded the lowest amount of total chlorophyll content at ($2.9 \mu\text{g/g DW}$). The antioxidant test showed that *P. pavonica* presented the strongest DPPH activity with percentage of inhibition (i%) of ($61.0\% \pm 0.9$) while *K. striatum* showed the weakest DPPH activity ($35.6\% \pm 1.7$). In the antimicrobial test, the strong antimicrobial action was shown by all the seaweed samples toward *E. coli* and *P. aeruginosa*. Moderate activity was observed in all seaweed extracts against MRSA and *S. epidermidis*. *S. pyogenes* and *B. subtilis* were least affected by all seaweed samples. Besides that, the overall results of the antifungal test demonstrated moderate antifungal action by all seaweed samples towards *M. gypseum* and *Fusarium sp.*

In the stability study, it was evident that the difference in salinity for every seaweed sample concentration was consistent from week 1 up to week 4. In terms of pH, seaweed colour change were more obvious in acidic condition compared to neutral and alkaline condition. Higher temperature also led to rapid colour changes in seaweed pigments. Finally, it was found that the colour changes in seaweed increased with longer exposure to UVA and UVB. As a conclusion, all the seaweed selected in this study possess bioactive compounds that have the potential to be utilized in various fields.

خلاصة البحث

الأعشاب البحرية، والمعروفة أيضاً باسم الطحالب الكبيرة، هي كائنات شبيهة بالنباتات يمكن العثور عليها في المواطن البحرية. وقد اكتسبت أنواع مختلفة من الأعشاب البحرية اهتمام الباحثين بسبب وجود مركبات فعالة بيولوجياً وظيفياً يمكن استخلاصها من كتلتها الحيوية. أجريت الدراسة بسبب مواد ملونة كثيرة تم استخدامها في الصناعات مشتقة من ألوان اصطناعية. وبالمقارنة فهي مستقرة وفعالة وقليلة التكلفة وسهلة الاستخدام. لا يزال يجري مناقشة سلامة استخدام الملونات الاصطناعية من أجل تحديد التأثير الضار تجاه المستهلك. في هذه الدراسة، هناك ثلاثة أنواع من الطحالب الحمراء الحمراء (Euचेuma Glacilaria tikvahiae ، Kappaphycus striatum و denticulatum)، والطحالب الخضراء (Caulerpa lentillifera) وتم اختيار أحد الطحالب البنية البنية (Padina pavonica) لتحديد محتواها من الكلوروفيل والكاروتينات باستخدام مطياف UV-Vis و HPLC، لتحديد خصائصها المضادة للأكسدة عن طريق استخدام طريقة النبض DPPH والنشاط المضاد للميكروبات باستخدام طريقة diffusion-Disc بالإضافة إلى تقييم استقرار صبغتها الطبيعية عبر CIELab. أجريت دراسة توصيف المجموعات الوظيفية والمركبات الطيارة في الأعشاب البحرية المختارة باستخدام مطياف الأشعة تحت الحمراء FTIR GC-MS/TOF.

تمثل نتائج هذا البحث المرحلة الأولية في هذه الدراسة. وقد حددت النتائج الإجمالية ل GC-MS/TOF 37 مركباً من *K. striatum*، 32 مركباً من كل من *E. denticulatum* و *P. Pavonica*، فضلاً عن 27 مركباً في *C. Lentillifera* و *G. Tikvahiae*. من حيث المحتوى كاروتينات، *P. pavonica* تحتوي على أعلى كمية من الكاروتينات (100.89 ± 14.71 ug/g DW) في حين سجلت *G. Tikvahiae* أدنى قيمة (25.13 ± 9.42 ug/g DW). وفي الوقت نفسه، أظهر المحتوى الكاروتيني الفردي *K. striatum* أعلى محتوى ليوتين (38.6 ug/g DW) في حين أظهرت *P. pavonica* أدنى محتوى الليوتين (7.21 ug/g DW). أما بالنسبة لأكسازانثين، فقد أظهر *C. lentillifera* (21.30 ug/g DW) أعلى كمية بينما أظهرت *E. denticulatum* (3.61 ug/g DW) أدنى نتيجة. *C. lentillifera* يحتوي أيضاً على معظم بيتا كاروتين (10.70 ug/g DW) في حين أن *E. denticulatum* كان أقل (2.44 ug/g DW). وأخيراً وليس آخراً، تم العثور على فيولاكسانثين فقط في الأعشاب البحرية الخضراء *C. lentillifera* (8.93 ug/g DW).

كان محتوى الكلوروفيل الكلي ل *C. lentillifera* و *P. pavonica* قابلة للمقارنة في (7.29 ug/g DW) و (7.52 ug/g DW) على التوالي. في المقابل، سجلت *E. denticulatum* أقل كمية من محتوى الكلوروفيل الكلي (2.97 ug/g DW). وأظهر الاختبار المضاد للأكسدة أن *P. pavonica* قدمت أقوى نشاط DPPH مع نسبة تثبيط (%I) من (61.0 ± 0.9 %) في حين أظهرت *K. Striatum* أضعف نشاط DPPH (35.6 ± 1.7 %). أما في الاختبار المضاد للميكروبات، فقد أظهرت عمل جميع عينات الأعشاب البحرية عمل مضاد قوي تجاه *E. Coli* و *P. pavonica*. لوحظ نشاط معتدل في جميع مستخلصات الأعشاب البحرية ضد MRSA و *S. epidermidis*، *S. pyogenes* و *B. subtilis* كانت أقل تأثيراً من جميع عينات الأعشاب البحرية. إلى جانب ذلك، أظهرت النتائج الإجمالية للفطريات اختبار معتدلة عمل مضاد للفطريات من قبل جميع عينات الأعشاب البحرية نحو *M. Gypseum* و *Fusarium sp*.

أما في دراسة الاستقرار والثبات فقد كان واضحاً أن الفرق في الملوحة لكل تراكيز عينات الأعشاب البحرية قد ظل متسقاً من الأسبوع الأول وحتى الأسبوع الرابع. ومن حيث الرقم الهيدروجيني، كان تغير لون الأعشاب البحرية أكثر وضوحاً في حالة حامضية مقارنة مع الحالة المحايدة والقلوية. أدى ارتفاع درجة الحرارة أيضاً إلى تغيرات اللون السريع في أصباغ الأعشاب البحرية. وأخيراً، وجد أن تغيرات اللون في الأعشاب البحرية زادت مع التعرض لفترة أطول ل UVA و UVB. وختاماً، فإن جميع الأعشاب البحرية المختارة في هذه الدراسة تمتلك مركبات نشطة بيولوجياً ولديها القدرة على استخدامها في مختلف المجالات.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master in Science (Halal Industry Science)

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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ABBREVIATIONS

g	Gram
mg	Milligram
µg	Microgram
L	Litre
mL	Milliliter
µL	Microliter
mm	Millimeter
µm	Micrometer
nm	Nanometer
w/w	weight over weight
w/v	weight over volume
v/v	volume over volume
min	Minute
ATCC	American Type Culture Collection
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
DW	dry weight
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
MHA	Müeller-Hinton Agar
MHB	Müeller-Hinton Broth
NaCl	Sodium chloride
HPLC	High performance liquid chromatography
UV	Ultraviolet
Chl a	Chlorophyll a
Chl b	Chlorophyll b

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

This study explore the carotenoid and chlorophyll contents found in different marine seaweeds, which are red, green, and brown seaweed from sea water in Malaysia. Marine seaweeds comprise few thousands of species and they represent a considerable part of the littoral biomass. Based on their nutritional value and chemical composition, they are classified as red (Rhodophyta), brown (Phaeophyta), and green seaweeds (Chlorophyta) (Dawczynski *et al.*, 2007). Besides, the photosynthetic pigments, seaweed contains different groups of light harvesting and photoprotective pigments. Thus, classification and biodiversity studies are very important to determine the impact on their value as reserved food and other valuable products. (Vimala & Poonghuzhali, 2013). The bioactive compounds found in seaweed contribute to the antioxidant, antimicrobial and antifungal properties.

The most important reason to conduct study on bioactive properties of seaweed is the demand by the consumers for fresh and natural products in order to enhance the safety and quality. That is the reason for the synthetic additives and ingredient to be questioned by the industrial players (Cox *et al.*, 2010). Many seaweed species are used in the industry, principally for the extraction of phycocolloids (Jimenez-Escrig and Sanchez-Muniz, 2000) and as a source of pharmaceutical substances. In the food, pharmaceutical, cosmetic, and biomedicine industries, seaweed has been used as a valuable source of bioactive compounds. Many compounds being antiparasitic, antiviral or antibacterial are effective. (Pérez, *et al.* 2016). Maxilien *et. al.*, (1998) mentioned

that production of secondary metabolites of seaweed could defend itself against bacterial fouling by preventing the attachment and growth of bacterial colonizers.

Furthermore, many natural antioxidants have been found from various kinds of land plants, such as cereals, vegetables, fruits, herbs and algae, in which tocopherol, vitamin C, carotenoid and flavonoid are good sources of antioxidants (Larson, 1988). In general, for antioxidant activity, free radicals and peroxy radicals will be transformed into non-radicals by donating the electrons, hydrogen, chelating transition metals and dissolving generated peroxidation compounds by the secondary metabolites that may present in seaweed. Gupta & Abu-Ghannam, 2011).

Active Pharmaceutical Ingredient (API) include substances used in a finished pharmaceutical product, intended to furnish pharmacological activity or contribute direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease. Besides, it provides direct effect in restoring, correcting or modifying physiological functions in human beings (World Health Organization, 2011). Other than that, food carotenoid analysis has been carried out to different extents, determining only the provitamin A carotenoid, the principal provitamin A and non- provitamin A carotenoids and the complete carotenoid composition (Kimura & Rodriguez-Amaya, 2002).

Several studies have described methodologies to analyse these pigments. These investigative efforts are based on the evaluation of parameters such as solvents, number of extraction steps and required biomass, all of which are aspects that vary across different organisms. Moreover, an array of techniques is used in the analysis and quantification of these pigments, among which UV-visible light spectrophotometry and high performance liquid chromatography (HPLC) (Torres *et al.*, 2014).

Finally, this research aimed to explore the chlorophyll and carotenoid contents from five species of Malaysian seaweed as potential Active Pharmaceutical Ingredient

(API) and natural pigment. It is also important to study the stability of seaweed pigment as it derived from natural sources.

1.2 PROBLEM STATEMENT

Halal industries is one of the fastest growing business worldwide. It creates the market force that attracts Muslims or even non-Muslim with healthy, hygienic and contamination-free principles in food and pharmaceutical production., Halal is not only focuses on the halal material but it includes the safety purpose of the products to be consumed by consumer and no interference of harmful ingredient. In the food and pharmaceutical industries, the usage of food colorant and additives have been widely used as it contributes to the appearance and value added for certain products. For example, vitamin C product is expected to have orange in colour and for plant based products must be green in colour. Major colorants that have been used in the industries are derived from synthetic colorants. The major usage is due to the comparative stability, cost effective and ease of use. Safety of the synthetic colorant usage is still being debated in order to identify the harmful effect towards the consumption. Hence, natural colorant is an important area to be explored especially for the halal food colorant industries. (Bahrudin *et al*, 2011)

On top of that, the bioactive compounds found in seaweed provide a special value added for the products of the industries such as the antioxidant and antimicrobial properties of seaweed extract. Thus, both important contributions of the bioactive compound in seaweed can be utilized and added in the food and pharmaceutical products. All those natural sources will help to reduce the demand on the synthetic ingredient that has been used by the industries, which may cause harmful effect to the consumers.

1.3 RESEARCH AIM AND OBJECTIVES

This research aimed to explore the chlorophyll and carotenoid content from five species of Malaysian seaweed as potential Active Pharmaceutical Ingredient (API) and natural pigment. In order to achieve that, three objectives have been set as follow:

1. To identify the composition of chlorophyll and carotenoid content in five species of Malaysian seaweed by using UV-Vis and HPLC.
2. To determine the antioxidant properties and antimicrobial activities of extracted chlorophyll and carotenoid in Malaysian seaweed.
3. To assess the stability of natural pigment extracted from Malaysian seaweed.

1.4 RESEARCH QUESTIONS

1. What types of chlorophyll and carotenoid that can be found in various species of Malaysian seaweed?
2. How is the stability of the chlorophyll and carotenoid content in the Malaysian seaweed?
3. What is the potential of chlorophyll and carotenoid content in Malaysian seaweed to be used as Halal Active Pharmaceutical Ingredient (API) and natural pigment?

1.5 RESEARCH HYPOTHESIS

Chlorophyll and carotenoid extracted from various species of Malaysian seaweed are applicable to be used as Halal Pharmaceutical ingredient (API) and natural pigment.

1.6 SIGNIFICANCE OF THE STUDY

1. To provide alternative natural sources from marine plant that is valuable and renewable.
2. To prove that seaweed extract contains bioactive component that can be used as antioxidant and antimicrobial agents.

CHAPTER TWO

LITERATURE REVIEW

2.1 SEAWEED

2.1.1 Seaweed Ecology and Physiology

Since more than 70% of the world's surface is covered by oceans, the wide diversity of marine organisms offers a rich source of natural products. Marine environment contains a source of functional materials, including polyunsaturated fatty acids, polysaccharides, essential minerals and vitamins, antioxidants, enzymes and bioactive peptides. Therefore, one of the examples of popular marine organism is seaweed, which is a type of macroalgae. Among marine organism, marine algae is a rich source of structurally diverse bioactive compounds with various biological activities. (Rajasekar *et al.*, 2013)

Thus, the present of secondary metabolites in seaweed contributes to its unique characteristic, which is biological active that is not found in other organisms. These compounds are produced in response to situations of oxidation and extreme environmental conditions where the seaweed lives. Seaweed has been given huge concern because of its high nutritional value and short-term growth, which is only 45 days per cycle. Seaweed is mainly cultivated in Sabah due to its environmental suitability and geographical factors compared to peninsular Malaysia. In addition, Malaysia lies entirely in the equatorial region. Thus, Malaysia receives 4.21 to 5.56 kWh/m² of solar radiation a year on average. Furthermore, 3rd Economic Transformation Programme (3ETP) that has been launched in Malaysia focus mainly on transforming the system in seaweed to develop it one of the industries, which is also standardized, independent and competitive like other agriculture sectors. Thus, as stated by Khan *et al.* (2014), it also will provide a lot of job opportunities for the people in that

particular area, hence decrease the poverty of people especially in west coast of Sabah and increase household income of farmers. Seaweed industry also will attract investors to come and invest in Malaysia.

Other than the potential nutritional values found in seaweed, there are many reasons for seaweed to be highly valuable such as cheap and economical, easily cultivated and adaptation to extreme condition. (Ragaza *et al.*, 2013). Thus, seaweed production is considered as an important economic commodity and has the potential to be boosted to meet global demand (Neish, 2009). In order to have a continuous supply of the seaweed to sustain for the demand, farming activity is approached because it is easy to grow and maintain. However, a continuous farming activity that takes place at the same location throughout the year will cause a decrease in production. Thus, it is important to plan the farming schedule in order to come out with an optimum outcome of seaweed yield. Moreover, Hurtado and Agbayani (2002) showed that production of seaweed is higher by planting in long line system placed along the shoreline compared to vertical method in the water column (Wenno, *et al.*, 2016).

2.1.2 Types and Varieties of Seaweed

Seaweed is classified in three different categories, which are red (Rhodophyta), brown (Phaeophyta), and green seaweed (Chlorophyta). Besides, one of the popular Rhodophyta species, *Kappaphycus alvarezii*, *Kappaphycus striatum* and *Eucheuma denticulatum* belongs to a group of commercially important species known in the trade as *cottoni* and *spinosum* and known as Eucheumoid algae. Furthermore, there are the main sources of kappa and iota carrageenan, being responsible for about 88% of worldwide raw material. (Husin, 2014)

In general, the genera *Eucheuma* and *Kappaphycus* are the examples of important genera of carrageenophytes, which are abundant in tropical Asia and Western Pacific including Philippines. They belong to the family Solieriaceae of the Order Gigartinales. *Kappaphycus alvarezii*, *Eucheuma denticulatum*, *Kappaphycus striatum*, and *Kappaphycus cottonii* are the most common species of *Eucheuma* and *Kappaphycus*. Other than that, *Eucheuma* and *Kappaphycus* thalli are very cartilaginous, erect in habit and may be prostrate or consist of cylindrical to compressed branches. Male thalli appear to be uncommon while the fertile female thalli develop distinct cystocarps, which appear as mammillate structure (Glenn & Doty, 1990).

The first common species of this genus is *Eucheuma denticulatum* or *Eucheuma spinosum* that consists of tapering to acute tips of many terete branches thallus. Hence, branchlets arrange in whorls, forms distinct with nodes and internodes especially at the terminal portion of the branches whereby these are usually densely covered with 1 to 8 mm long spinose. Besides, cross section of a branch reveals a dense core of thick wall and very small rhizoidal cells at the center of medulla. Thus, it may derive from a dominant component of the algal community even though it is not as widely distributed as *K. alvarezii* and *K. cottonii*. To add with, this species thrives very well on coarse sandy-chorally to rocky substances, which means in areas that constantly exposed to moderate and strong water currents (Gerang & Ohno, 1997).

The second species is *Kappaphycus striatum*, also known as *Eucheuma striatum*. The thallus branches of this species are roughened by the presence of spinose processes or determinate branchlets, and erected or decumbent whereby the axis not procumbent Besides, these three species are presented in two types; the elkhorn type is varied in morphology, erected and irregularly branched the branch axil acute. The second form generally forms thick dense decumbent clump with typical dichotomous

branching, and the branches roughened by short processes. Hence, both forms are characterized by the presence of axial hyphae in the branches that are not more than 5 mm in diameter (Trono, 1992).

The only brown seaweed that has been used in this study is *Padina pavonica* species. It is widely found in the Indian River Lagoon. The thallus or body of the algae has fan-shaped clusters shape and brown to tan colour. Furthermore, each blade is calcified, more heavily above and lightly below, and curls inward near the edges. Both the upper and lower blade surfaces bear minute surface hairs arranged in a series of bands approximately 1.5 to 6 mm apart. Thus, the blades attach to the substratum via a holdfast, which is often matted. Other than that, the range of *P. pavonica* extends throughout the world in warm temperate to tropical locales. Besides, cluster is commonly attached to shell fragments and rocks from the lower intertidal zone down to 20 m, in seagrass beds and coral reefs, on tidal flats and attached to mangrove prop roots (https://www.sms.si.edu/irlspec/Padina_pavoni.htm).

2.2 SEAWEED AS HALAL SUBSTANCE

2.2.1 Role of Seaweed

Seaweed is one of the important agricultural crops in Malaysia. The government put high concern on seaweed industry and had allocated RM 58.87 million in 2011 and 2012 to boost this industry. There are a lot of strategies and incentives been taken under 3rd Economic Transformation Programme (3ETP) such as Algae Farming via Mini Estate System in Sabah, Seaweed Identification Grant, Seaweed Cultivation and Grant from National Key Economic Area (NKEA). These are the initiatives by the government to help the community to improve the technology and income with these valuable marine algae (Ali *et. al.*,(2014).