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STUDY OF VOLATILE COMPOUNDS EXTRACTION USING SPME-GCMS IN LOCAL SEAWEEDS OF *Kappaphycus alvarezii* , *Caulerpa lentillifera* AND *Sagassum polycystum.*

VIJAYA RATINAM A/L RAMAH

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SCHOOL OF FOOD SCIENCE AND NUTRITION
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2009

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries, and references, which have been duly acknowledged.

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ABSTRACT

A pioneer study with a new extraction method was applied to the volatile compounds of three local seaweeds from Borneo, *Caulerpa lentillifera, Kappaphycus alvarezii* and *Sagassum polycystum* using HS-SPME method. Dynamic headspace was optimized according to an experimental design, with appropriate temperature of 50^0C and 60^0C and extraction time as 20min, 30min, 40min and 50min. Two different fibres namely PDMS and PDMS-DVB were utilized in the extraction. The optimization result varies according to seaweeds with the temperature of 60^0C and extraction time of 50 minutes for both fibres for *K. alvarezii* and PDMS fibre extraction for *S. polycystum.* While for PDMS-DVB extraction for the same seaweed spesies, a 50^0C temperature with 20 minute extraction time provided optimum peak areas. *Caulerpa lentillifera* shows peaks with highest abundance when extracted in 50^0C with 20 minute for PDMS fibre and 50^0C with 40 minute extraction for PDMS-DVB fibre. GC-MS analysis was performed on this extract to identify the volatile components. Significantly, PDMS fibre extracted more compounds than PDMS-DVB. Thus PDMS is recommended as a better fibre comparatively to PDMS-DVB in volatile compounds extraction using SPME for these particular seaweeds. Qualitative study portray a total of 223 volatile constituents successfully extracted and identified with 82, 91 and 50 volatiles from each species of *K. alvarezii* , *C. lentillifera* and *S. polycystum* respectively. The volatile compounds are comprised of nine chemical compounds of hydrocarbons, aldehydes, ketones, esters, alcohols, halogenated compounds, acid compounds, aromatic compounds and some miscellaneous compounds. Comparison on the major volatile constituents among the seaweeds shows a similar percentage of volatile compounds in all seaweeds among the nine groups. Within them, hydrocarbon compounds were the most characteristic of all three algae, and more particularly, all three seaweeds mostly share the common constituents in their structures.

ABSTRAK

Kajian pelopor ini meliputi kaedah pengekstrakan yang diaplikasikan pada tiga jenis rumpai laut yang terdapat di Borneo, *Caulerpa lentillifera, Kappaphycus alvarezii* dan *Sagassum polycystum* dengan menggunakan kaedah mikro pengekstrakan phasa pepejal. Ruang udara dinamik dioptimumkan mengikut kaedah eksperimen dengan nilai yang sesuai bagi faktor suhu sebagai 50^0C dan 60^0C dan masa pengekstrakan selama 20min, 30min, 40min and 50min. Dua jenis fiber yang berlainan, PDMS dan PDMS-DVB digunakan dalam pengekstrakan. Keputusan ujian optimasi berbeza mengikut jenis spesis rumpai laut. Di mana, suhu 60^0C dengan masa pengekstrakan sebanyak 50min diperlukan oleh kedua-dua fiber bagi *Kappaphycus alvarezii* dan dengan masa dan suhu yang sama bagi fiber PDMS bagi *Sagassum polycystum.* Manakala fiber PDMS-DVB bagi spesis yang sama menunjukkan 50^0C dengan 20 minit masa pengekstrakan sebagai keadaan optimum. *Caulerpa lentillifera* pula menunjukkan puncak-puncak dengan taburan tertinggi apabila diekstrak selama 20 minit pada 50^0C dengan menggunakan fiber PDMS dan 50^0C dengan 40 minit ekstrak bagi fiber PDMS-DVB. Analisis GC-MS digunakan ke atas hasil-hasil ekstrak ini bagi tujuan pengecaman bahan-bahan meruap. Secara signifikasi, fiber PDMS-DVB mengekstrak lebih banyak bahan meruap daripada fiber PDMS-DVB. Maka fiber PDMS disyorkan digunakan bagi pengekstrakan bahan meruap dari rumpai laut tempatan berbanding fiber PDMS-DVB apabila menggunakan kaedah mikro pengekstrakan phasa pepejal atau SPME. Sejumlah 223 bahan meruap berjaya diekstrak dan dicam yang terdiri daripada sembilan kelas kimia yang termasuk hidrokarbon, aldehyde, ketone, alkohol, kumpulan halogen, acid, kumpulan beraroma dan beberapa kompoun yang berlainan. Perbandingan pada jumlah kompoun meruap mengikut kumpulan kimia menunjukkan ketiga-tiga spesis rumpai laut ini mengandungi jumlah kelas kimia yang hampir sama mengikut kumpulan berfungsi masing-masing di antara sembilan kumpulan. Didapati sebanyak 82, 91 dan 50 bahan meruap diperolehi daripada species rumpai laut *K. alvarezii , C. lentillifera* dan *S. polycystum* masing-masing. Di antaranya, kompoun hidrokarbon yang menguasai kebanyakkan bahan meruap di kesemua rumpai laut. Lagipun, ketiga-tiga rumpai laut ini mempunyai bahan-bahan meruap yang sama dalam strukturnya.

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LIST OF SYMBOLS, UNITS AND ABBREVIATIONS

%	percentage
Min	minute
µl	microliter
µm	micrometer
cm	centimeter
^{0}C	Degree Celcius
mm	milimeter
g	gram
nm	nanometer
HS-SPME	Head Space Solid Phase Micro Extraction
GC	Gas Chromotography
MS	Mass Spectrometer
MUFA	Mono Unsaturated Fatty Acids
PUFA	Poly Unsaturated Fatty Acids
PDMS	Polydimethylsiloxane
HPLC	High Performance Liquid Chromatography
DVB	Divinylbenzene
SDE	Solvent Distillation Extraction
SFE	Supercritical Fluid Extraction
Spp.	Species
ANOVA	Analysis of Variance
LSD	Least Significant Difference

CHAPTER 1

INTRODUCTION

1.1 Background of study

Malaysia has an extensive coastline with numerous islands form clusters along the coastlines of Peninsular Malaysia, and East Malaysia comprising of Sabah and Sarawak. Rocky shores and sandy bays alternate with mudflats and mangroves, while coral reefs fringe most islands. All these provide niches for the variety of seaweed species found in Malaysian waters (Ahmad, 1995; Phang, 2006). The last two decades have seen an increase in seaweeds as a potential economic resource in the Asia-Pacific region, including Malaysia. Presently 375 taxa of seaweed are recorded in Malaysia (Phang, 2006). But until now there has been very limited research and investigation from the organic biochemistry aspect of these seaweeds. So far many researches have been carried out in terms of its nutrient content, antioxidant properties, cultivation and processing, physiochemical properties and many more. Yet there is dearth of studies on the volatile constituents of these locally available seaweeds (Gardon & Sonia, 2000). Many scientifically interesting as well as commercially important species have been identified. Ecological information is scarce. Biomass assessments of natural seaweed areas, productivity determination and phenological studies of important species are also less, comparatively to other regions of world (Ahmad, 1995; Phang, 2006). So this present study is designed to contribute to the data on major group of volatile compounds of local seaweeds.

Caulerpa lentillifera is a species of seaweed which is commonly known as green macro alga under the family of *Caulerpaceae*. This species is also consumed under the names of sea grape or green caviar or umi-budo in other regions of the world. While *Eucheuma cottonii* or *Kappaphycus alvarezii* is a type of red algae from the class of Rhodophyceae (Ahmad, 1995; Phang, 2006). This seaweed accounts for the largest production worldwide. Today, the most important commercial seaweed for carrageenan production is the cultivated species *E.cottonii* (Chapman & Chapman, 1980).

In Sabah, cultivation of *E.cottonii* for export had been carried out for almost three decades, where it is normally exported as sun-dried seaweed or processed into semi-refined carrageenan (Ahmad, 1995). Meanwhile, *Sagassum polycystum* also known as brown algae or seaweed is from the genus of *Sargassum* and from the class of Phaeophyta. They grow and reproduce on substrates such as dead coral and masive material on the reef flats and are found in the coastal waters all of the years (Balaji *et al.,* 2006). Even though it does not play an important role commercially, yet it exhibit major role in ecological balance.

Since antiquity, natural fragrances hold the attention of man and not surprisingly the first study in the field of natural products chemistry was carried out on aromatic substances isolated from terrestrial organisms. In contrast, volatiles of marine origin have been rarely studied although they could be used as a source of original flavouring agents in food and perfume industries (Hattab *et al.,* 2007). Even the general chemical substances in seaweeds are rarely being studied compared to those terrestrial plants. The volatile compounds which are part of chemical substances from seaweed are expected to be distinct among species due to its habitat distribution, genera it belong to, maturity and environment (Wong & Cheung, 2000). Volatile compounds such as propanal and hexanal can be identified through gas chromatography method.

For HS-SPME, it has gained increasing attention over traditional techniques like steam distillation and liquid solvent extraction. Moreover, commonly used methods for the extraction and preconcentration of analytes in solid samples are often too time consuming, involve multi-step procedures prone to loss of analytes, and use of toxic organic solvents (Antoniou *et al.,* 2005). Modern trends in analytical chemistry are developed towards the simplification of sample's preparation, as well as minimization of organic solvent used. This has turned in recognition that classical methods can be replaced with procedures that are faster, cheaper, and equal or better than classical methods (Moreno *et al.*, 2007).

Thus a HS-SPME extraction method is used to analyse the volatile constituents from the seaweeds. The volatile compounds of *C.lentillifer, K.alvarezii* and *S.polycystum* are expected to be extracted and identified by above mentioned way, before the components may classifieds according to their chemical groups.

1.2 Scope of study

The study involves the extraction of volatile compounds from three different species of seaweeds namely *Caulerpa lentillifera, Kappaphycus alvarezii* and *Sagassum polycystum* which are also known as green, red and brown seaweed respectively. The extraction technique involves the usage of Head Space Solid Phase Micro Extraction, which is a solvent free method, with two different types of fibres being used: a 100µm PDMS fibre and 65µm PDMS-DVB to trap the volatile constituents. This will lead to the analysis of constituents by gas chromatography which is fused with mass spectrometer as a detector. Eventually a qualitative study on the major volatile groups from the local seaweed species would be obtained together with the identification on the better fibre for extraction. Besides, a clear comparison and distinction in volatile compounds among the three different species of seaweed would be able to be made.

1.3 Objectives of study

There are three objectives in this study, which are:

i. To identify qualitatively by screening the major volatile compound groups in *Kappaphycus alvarezii* , *Caulerpa lentillifera* and *Sagassum polycystum* using HS-SPME-GCMS.

ii. To determine the most suitable fibre for HS-SPME extraction of volatile compounds from local seaweeds.

iii. To compare the volatile constituent of chemical groups among different species of local seaweeds.

1.4 Rationales of study

The rationales behind this study include the easy and abundance availability of these particular seaweed species in coastal areas of Sabah which leads to the selection of *Caulerpa lentillifera, Kappaphycus alvarezii* and *Sagassum polycystum* species. Besides, seaweed production and utilization in Malaysia is still as an infant industry which requires ample of exploration in terms of application by research (Ahmad, 1995). Whereby there is great chance of exploitation of these seaweeds in various industries. A check and balance can be created in the aspect of commercialization and ecological importance too. Importantly, there is very dearth of studies on volatile compounds in these seaweeds especially in the perspective of extraction, screening and qualitative analysis (Gardon & Sonia, 2000).

1.5 Benefits of study

The benefits which are expected to be gained by this study are a contribution on qualitative data on volatile constituents of local seaweeds. Besides, a clear distinction among three species of local seaweed's volatile content is able to be attained. In addition, a recommendation on an effective fibre for Head Space Micro Extraction of volatile compounds from local seaweeds could be delivered. Most importantly, a precedence benefit by further exploitation on the data provided in related local industries utilization is also able to be achieved in future.

CHAPTER 2

LITERATURE REVIEW

2.1 Seaweed

Sea weeds are macrophytic algae, a primitive type of plants lacking true roots, stems and leaves. Most seaweed belongs to one of three divisions: the chlorophyta (green algae), the phaeophyta (brown algae) and the Rhodophyta (red algae). There are about 900 species of green seaweed, 4000 red species and 1500 brown seaweeds found in nature (Gardon & Sonia, 2000). Some great variety of red seaweed is found in subtropical and tropical, waters while brown seaweeds are more common in cooler temperate waters. Some 221 species are of seaweeds are utilized commercially (Chapman & Chapman, 1980). Of these, about 145 species are used for food and 110 species for phycolloid production, such as agar (Ahmad, 1995).

In Malaysia, most seaweed production is in Sabah. Seaweed production in Sabah mostly takes place on farms in the Semporna region of Darvel Bay. Some seaweed farmings are also developing in the Banggi Island Region of Northwestern Sabah (Ahmad, 1995; Phang, 2006). From the aspect of food, seaweeds are an important part of the diet of many Asian countries (Phang, 2006). In Asia, seaweed has been used for centuries in the preparation of salads, soups and also as low-calorie foods. Although most Malaysians exhibit little interest in consuming seaweeds, it is consumed by small pockets of the population along the coastal of Peninsular Malaysia and East Malaysia Most people do not realize how important seaweeds are, both ecologically and commercially (Phang, 2006).

In reality, seaweeds are crucial primary producers in oceanic food webs. They are also valuable sources of foods, micronutrients and raw materials for the pharmaceutical industries (Phang, 2006). Seaweed has plenty of essential nutrients and several other bioactive substances. Like other plants, seaweeds contain various kinds of inorganic and organic substances which probably benefit human health. In food manufacturing, seaweeds have been developed as raw or semi-processed food products (Chapman & Chapman, 1980). That explains why seaweeds are considered as the food supplement for 21^{st} century as source for proteins, lipids, polysaccharides, mineral, vitamins and enzyme (Gardon & Sonia, 2000). Over the past several decades, seaweeds or their extracts have been studied as novel sources which have been shown to produce a variety of compounds and some of them have been reported to possess biological activity of potential medicine value (Flament & Ohloff, 1984).

2.2 The seaweed flora of Malaysia

The first checklist of the marine benthic algae in Malaysia was published in 1991 (Phang and Wee, 1991) together with a historical account of the study of marine algae in this region. In 1998 Phang updated the checklist of Malaysian marine algae including additions from Phang and a new species, *Sargassum stolonifolium*, described from Penang, west coast Peninsular Malaysia (Phang and Yoshida, 1997). Two hundred and sixty specific and infraspecific taxa (17 Cyanophyta, 92 Chlorophyta, 94 Rhodophyta and 57 Phaeophyta) were recorded (Phang, 2006). Rhodophyta dominated, as is expected of tropical seaweed flora. Many of the red algae are filamentous comprising mainly epiphyte species. These two checklists comprise many species that were reported in literature but were not verified due to absence of deposited material. A survey (1995–1999) was conducted by University of Malaya in collaboration with Hokkaido University, Japan, resulted in many additions to the checklist, and confirmation of others, especially of the Rhodophyta. Thirty-eight new records (Kawaguchi *et al.*, 2002; Yamagishi *et al.*, 2003), including one new species *Lomentaria gracillima* were added to the checklist. Further additions included taxa previously recorded by Zanardini (1872), 35 species of Rhodophyta, 13 species of Phaeophyta and 16 species of Chlorophyta recorded by Ahmad in 1995 and others (Phang, 2006). The tally of Malaysian marine algae now stands at 373 specific and intraspecific taxa (17 taxa of Cyanophyta, 102 Chlorophyta, 182 Rhodophyta and 72 Phaeophyta).

2.2.1 Cyanophyta

Of the marine blue-green algae, the most visible are the larger forms including the cushion shaped *Brachytrichia* which grow on rocks and dead corals, and the long filamentous *Lyngbya majuscula* and species of *Oscillatoria*. The latter two genera produce slimy films over mudflats and sandy-muddy areas (Ahmad, 1995; Gardon & Sonia 2002; Phang 2006).

2.2.2 Chlorophyta

The Chlorophyta consists of the second highest number of taxa in Malaysian waters. Thirteen species and seven varieties of *Caulerpa* have been recorded, mainly in coral reefs. Recent collections indicate that eight of these, namely *C. lentillifera, C. peltata, C.racemosa, C.scalpelliformis, C. serrulata, C. sertulariodes,C. taxifolia* and *C. verticillata* are still commonly found (Phang and Wee, 1991). The coral reefs are also dominated by species of *Halimeda* (*H. discoidea, H. opuntia, H. tuna*), the erect coralline algae which contribute towards reef building with the calcium carbonate retained in their cell walls (Kawaguchi *et al.*, 2002; Yamagishi *et al.*, 2003). Other green seaweeds inhabiting coral areas include *Anadyomene plicata, Boodlea montagnei, Cladophoropsis* species, *Dictyosphaeria cavernosa, Valonia aegagropila, Cladophora fascicularis, Bryopsis pennata, Codium* species, *Udotea javensis, Udotea flabellum, Tydemmania expeditionis, Bornetella* species and *Neomeris* species (Phang and Yoshida, 1997). Several species of *Enteromorpha* and *Ulva* are found in the nutrientrich shores and mudflats. *Enteromorpha intestinalis, E. chlathrata, Ulva lactuca* and *U. fasciata* are commonly seen covering small rocks, stones, driftwood and sandy patches along beaches which are frequently visited by people (Phang, 2006).

a. *Caulerpa lentillifera*

Caulerpa Lentillifera or which is also known as Green Caviar or Sea Grapes is a type of green algae from the class of Caulerpaceae, order of Caulerpales, family of Caulerpaceae, genus of *Caulerpa* and species of *Lentillifera* (Chapman & Chapman, 1980). They are common components of seaweed communities in tropical and subtropical waters. In South East Asian countries it is usually eaten raw as salad or cooked due to its soft and succulent texture (Ahmad, 1995).

Figure 2.1 *Caulerpa lentillifera*

2.2.3 Rhodopyhta

The red seaweeds comprise the highest number of taxa. The large foliose species of *Halymenia* dominate the subtidal bedrock areas, while the proliferous branching thalli of *Laurencia* and *Hypnea* species inhabit the bedrocks at the intertidal regions (Phang and Wee, 1991). These grow mainly in the cleaner deep waters. Five species of *Eucheuma* and two species of *Kappaphycus*, sources of carrageenan, have been collected from lower intertidal to upper sub-tidal areas in Sabah and around islands in Peninsular Malaysia. Except for the cultivated *Kappaphycus*, many of the *Eucheuma* species seem to have disappeared from Peninsular Malaysia. Twenty species of the agarophytic genus *Gracilaria* have been reported, many of which inhabit mangroves, sandy mudflats and rocky shores (Lim and Phang, 2004). Rhodophytes are commonly found in the coral reefs especially in the cleaner deep waters around the islands. In the mangroves small tufted thalli of *Bostrychia*, *Laurencia microcladia*, *Caloglossa adnata*, and *Catenella* grow commonly with the green filaments of *Chaetomorpha linum* and *Cladophora*. Common epiphytic taxa include *Champia parvula*, *Centroceras*, *Ceramium*, *Spyridia*, *Polysiphonia*, *Heterosiphonia*, *Herposiphonia* and *Tolypiocladia glomerulata* (Phang, 2006).

a. *Kappaphycus alvarezii*

Kappaphycus alvarezii or which is also known as *Eucheuma cottoni* is a type of red algae from the class of Rhodophyceae, Order of Gigartinalase, Family of Solieracea, Genus of *Eucheuma* and species of *Cottonii. Eucheuma Cottonii* is cultivated in many tropical countries (Phang, 2006). Commercial seaweed such as *E.Cottonii* can be grown in subtidal or intertidal coasts with certain preferred surroundings such as stable salinity (around 35%), clear waters (5m visibility), moderate current or flow of water (20-40/min), stable temperature (27^{0}C -30^{0}C), plenty of sunshine, free or protected from waves, and free from predators. It is a popular species for aquaculture, being farmed at places with strong wave action such as on the reef edge (Chapman & Chapman, 1980; Phang, 2006). Its main product of commercial importance is carrageenan. In Sabah, cultivation of *E.Cottonii* had been carried out for the past three decades where it is normally exported as sun-dried seaweed (Phang, 2006).

Figure 2.2 *Kappaphycus alvarezii*

2.2.4 Pheaophyta

The large thalli of the brown seaweeds make them most conspicuous, especially in the coral reefs. Generally the high algal biomass (Phang and Maheswary, 1989) on reefs is attributed to the brown algae. While *Sargassum* and *Dictyota* dominate in terms of species number, *Padina* are the most frequently found species. They inhabit a variety of substratum including mangroves, sandy areas, mudflats, coral reefs and rocky shores. *Turbinaria* and the encrusting *Lobophora variegate* often accompany the *Padina* on the intertidal coral reefs (Phang and Wee, 1991). The new species *Sargassum stolonifolium* which Phang and Yoshida described from Penang Island, west coast Peninsular Malaysia, is the first in the genus to exhibit the phenomena of new plantlets derived from the first leaves (Phang and Yoshida, 1997).

a. Sagassum polycystum

Sagassum polycystum is a brown seaweed species from Phaeophyta group which grows in the sea depth of 0.5 to 10 meters down with the presence of waves and sea water current. This macro alga adheres to the substrate at sea depth mainly on coral reefs. It also serves as a habitat for many marine lives (Chapman & Chapman, 1980; Phang, 2006). Sulphated polysaccharide (e.g. fucoidan) is structurally unique that is found only in the cell walls of several types of brown seaweed including in *Sagassum polycystum* species (Balaji *et al.,* 2006). Brown algae substantially differ from algae of other divisions and terrestrial plants in their composition. A perusal of literature study has shown that the brown algae contain essential minerals, vitamins, free amino acids, mannitol, glucitols, sulphated polysaccharides and phlorotannins. These compounds in brown algae are found to have wide spectrum of biological properties (Gardon & Sonia, 2000; Balaji *et al.,* 2006).

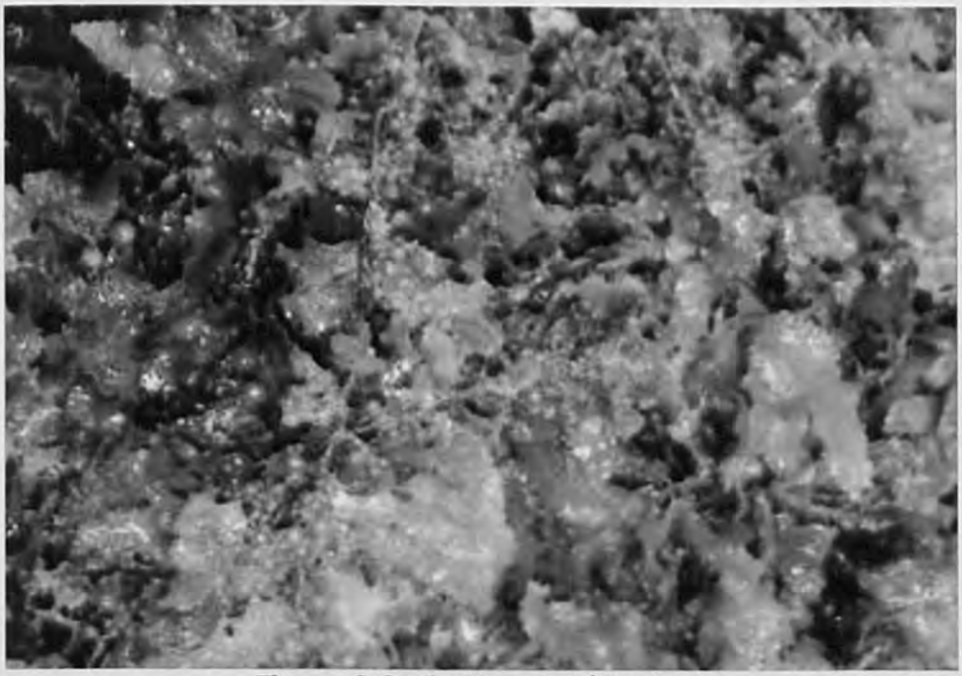

Figure 2.3 *Sagassum polycystum*

2.3 Economical important of seaweeds in Malaysia

Traditionally seaweeds in Malaysia have been documented as being utilised for food, animal feed, fertilizer and traditional medicine (Kawaguchi *et al.*, 2002; Yamagishi *et al.*, 2003; Phang, 2006). Several species of *Gracilaria* and *Gelidium* are used as salads and for agar extraction, while *Eucheuma* is cultivated for food as well as for the extraction of carrageenan in Sabah. *Corallina* (*Amphiroa*) is crushed and fed to children to expel worms while a soup made up of *Sargassum* and *Turbinaria* is a source of iodine and 'cools' the body system, popularly used by the Chinese. *Caulerpa, Ulva* and *Sargassum* are also collected for use as salads. *Halimeda opuntia, Acanthophora spicifera, Eucheuma spinosum, Gracilaria* sp., *Hypnea musciformis, Dictyopteris* sp., and *Sargassum* spp. are recognised for their antibiotic properties.

2.3.1 Seaweed as food

Although the use of seaweeds as food, especially by the fishing and coastal communities in Malaysia, has been documented (Phang & Maheswary, 1989), recent years have seen a decline in this usage. Seaweeds collected and eaten either as raw or blanched in salads include the Rhodophytes *Gracilaria changii, Gracilaria tenuispitata, Eucheuma* (*Kappaphycus*) species and the Chlorophytes *Caulerpa lentillifera* and *Caulerpa racemosa* (Phang and Wee, 1991). In the Chinese medicine shops one can buy dried *Sargassum* and *Turbinaria*, however their origin is unknown. Of course popularly eaten in Japanese and some Chinese restaurants are the temperate species of *Porphyra* (nori), *Undaria* (wakame), *Laminaria* (kombu) and *Nostoc* (fa' tsoi). Except for the *Eucheuma* species, the seaweeds are not cultivated (Phang, 2006).

2.3.2 Seaweed as a source of industrial product

Of the Malaysian seaweeds, *Eucheuma (Kappaphycus)* is presently cultivated in Sabah for the extraction of semi-refined carrageenan. Fishing families around Semporna, east coast Sabah, are involved in the mariculture of the *Eucheuma* using the monofilament techniques in the reefs fringing the islands near Semporna. The average cultivation period is 45 days and continues for eight months of the year. The monthly production from Semporna was around 60 to 100 tones dry wt per month (Phang, 1998). Biomass increases from the initial 0.5 kg per stocking bunch to 7 kg in 45 days. Two semi-refining factories were established in Sabah. *Gracilaria changii*, a good source of high quality agar and agarose (Phang, 2006) has also been experimentally cultivated in shrimp ponds, mangrove ponds and irrigation canals with daily growth rates of 3.6, 3.3 and 8.4% per day respectively (Phang and Yoshida, 1997).

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