Role of secondary metabolites as defense chemicals against ice-ice disease bacteria in biofouler at carrageenophyte farms

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Abstract Carrageenophyte farming is an expanding economical activity in North Borneo Island, Malaysia. During routine monitoring of "ice-ice" disease and epiphyte outbreak at commercial farms, it was apparent that culture lines were heavily (60–80%) infested with biofoulers, particularly *Acanthophora* spp. and *Laurencia majuscula*. However, only *L. majuscula* showed dominance and flourished even during "ice-ice" disease outbreak. Presence of chemical defense against seaweed pathogens was investigated in two populations of *L. majuscula* collected from three major carrageenophyte farms in two districts; (A) Lohok Butun, Selakan Island, and Bum-Bum Island, in Semporna district, and (B) Telutuh, Carrington Reef, and Balambangan Island, in Kudat district. The first population contained elatol (**1**), and iso-obtusol (**2**), and, second population contained (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**), as their antibacterial metabolites. All four metabolites showed highly selective inhibition against "ice-ice" disease bacteria compared to human pathogens at 30 µg disk^{-1}. In addition, seasonal variation of these compounds at two representative farms (Selakan Island [P-1] and Balambangan Island [P-2]) revealed a 120–170% increase in concentration during "ice-ice" disease outbreak. Microscopy of fresh specimens showed the presence of *corps en cerise*, which is the synthesis and storage site of halogenated metabolites at superficial cortical cells, branch tips, and trichoblasts. This suggests the importance of these metabolites as defense chemicals against "ice-ice" disease bacteria in *L. majuscula* that grows on seaweed culture lines.

Keywords Carrageenophyte · Biofouler · Ice-ice disease · *Laurencia majuscula* · Chamigrenes · Defense chemicals

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Introduction

Commercial cultivation of carrageenophyte is an important economical activity among the coastal communities in North Borneo Island, Sabah, Malaysia. Total annual production is close to 10,000 t DW, and Malaysia is the third largest producer of carrageenophyte after the Philippines and Indonesia. Commercial scale seaweed farming is labor intensive and often plagued with seasonal outbreak of "ice-ice" disease and epiphyte infection (Vairappan 2006; Vairappan and Chong 2006; Vairappan et al. 2008). Since early detection of disease symptoms could assist in reducing crop loss, schedule field monitoring is carried out at intensively cultivated carrageenophyte farms in Semporna and Kudat districts in Sabah. During routine field surveys, it became apparent that the culture lines were heavily infested with biofoulers, particularly *Acanthophora* spp. and *Laurencia majuscula* (Harvey) Lucas. During peak infestation season, close to 80% of the exposed lines were occupied by these biofoulers.

Upon detailed analysis of field data obtained for the period of two years, it was apparent that *L. majuscula* was one of the dominant biofoulers, and it is the only species that flourished when the cultivated crops were infected with

"ice-ice" disease. Since, red algae of the genus *Laurencia* are known to synthesize and store a wide array of halogenated secondary metabolites in their *corps en cerise* at the surface cortex cells (Masuda et al. 1997a; Vairappan et al. 2004; Suzuki et al. 2005), detailed investigation by bioassay-guided isolation and identification of defense chemicals against bacteria isolated from "ice-ice" disease infected seaweed was carried out. Here, we report the isolation, identification and antibacterial activity of the secondary metabolites from two populations of *L. majuscula* found growing in two major seaweed cultivation districts in Sabah. The isolated metabolites were also tested against six strains of human pathogenic bacteria to show their selectivity as seaweed defense chemicals. In addition, specimens were taken every 3 months from Selakan Island (Population-1) and Balambangan Island (Population-2) to determine seasonal changes in halogenated metabolite production and their correlation to disease outbreak in *Kappaphycus alvarezii*.

Material and methods

Density of biofoulers was investigated at Selakan Island (Population 1) and Balambangan Island (Population 2) in January, April, July, and October 2007. Specimens of biofoulers were collected from farms that are cultivating more than 100 lines (each line 100 m) of *K. alvarezii*. A total of five samples (10 m in length each) were collected from five randomly chosen lines, cleaned off epiphytes and sediments, and voucher specimens (BOR37533–BOR37543; BOR37552–37562) are deposited at the Borneensis Herbarium of the Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah. Samples of *Laurencia majuscula* for chemical analysis were collected in April 2007, from six seaweed cultivation farms on the coastal waters of Sabah, Malaysia; Lohok Butun, Semporna (06°04.933′N, 117°59.833′E), Selakan Island, Semporna (04°35.007′N, 118°41.978′E), Bum-Bum Island, Semporna (04°29.491′N, 118°41.855′E), Telutuh, Kudat (07°04.017′N, 117°04.836′E), Carrington Reef, Kudat (07°07.538′N, 117°01.775′E), Balambangan Island, Kudat (07°05.433′N, 117°02.355′E). The voucher specimens (BOR37544–BOR37551; BOR37563–37570) are also deposited at the Borneensis Herbarium.

Extraction and isolation

Partially dried samples (80 g) from the respective populations were soaked in 1 L of methanol for 7 days. The extracts were concentrated in vacuo, partitioned between diethyl ether (Et$_2$O) and double distilled water (DDW). Et$_2$O fraction was further washed with two changes of DDW, dried over anhydrous Na$_2$SO$_4$ and evaporated to yield dark green crude extract. Chemical profiling of the crude extract was done by spotting crude extract on SiO$_2$ gel F$_{254}$ thin layer chromatography plates and developed in toluene (100%) and hexane:EtOAc (3:1) solvent systems, visualized by UV light (254 nm) and molybdophosphoric acid. HPLC profiles of crude extracts were obtained in gradient separation mode at 40ºC using Shimadzu VP class HPLC with dual single pump high pressure gradient system under these conditions; UV-detection at 220 nm, 10× 250 mm 5 μ phenyl hexyl (Phenomenex) column, 2 mL min^{-1} and at 30–70% acetonitrile (MeCN) in 45 min.

Further separation of active metabolites involved fractionation by Si gel column chromatography (CC) with a step gradient of hexane and ethyl acetate in the ratio of 9.5:0.5, 9.0:1.0, 8.0:2.0, 7.0:3.0, 6.0:4.0, and 5.0:5.0. Obtained fractions were spotted on SiO$_2$ gel F$_{254}$ TLC plates, visualized by molybdophosphoric spray and heated. Fractions with secondary halogenated metabolites were further purified using reverse phase HPLC under these conditions: isocratic mode, UV-detection at 220 nm, 10×250 mm 5 μ phenyl hexyl (Phenomenex) column, 70% acetonitrile at 2 mL min^{-1}.

Spectroscopic procedures

Spectroscopy data were measured using ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz), JEOL ECA 600 MHz NMR; CDCl$_3$; TMS as internal standard. Melting point was measured on a micro-melting point apparatus (Fisher Scientific) and was uncorrected. Optical rotations were measured on a JASCO DIP-140 polarimeter, and LR/HREIMS on a JEOL JMS-A500 spectrometer. Compounds were identified by comparison of the spectral data with those of the authentic samples.

Antibacterial bioassay

Antibacterial bioassays were performed *via* paper disk diffusion method as described by Vairappan et al. (2001) against six strains of bacteria isolated from "ice-ice" disease infected seaweed (*Alteromonas* sp. 1 (SP0103UMS), *Alteromonas* sp. 2 (SP0203UMS), *Proteus mirabilis* (0303UMS), *Proteus* sp. (SP0403UMS), *Cytophaga–Flavobacterium* (SP0503UMS), and *Vibrio* sp. (SP0603UMS)), and six strains of human pathogenic bacteria (*Staphylococcus aureus* (HP0105UMS), *Staphylococcus* sp. (HP0205UMS), *Streptococcus* sp. (HP0305UMS), *Citrobacter freundii* (HP0405UMS), *Escherichia coli* (SP0505UMS) and *Klebsiella pneumoniae* (HP0605UMS)Bacteria from diseased seaweed were isolated and identified using modified media and techniques described by Largo et al. (1995), and Vairappan and Chong (2006). Meanwhile, the human pathogenic bacteria were obtained from Department of Pathology, Queen Elizabeth Hospital, Kota Kinabalu, Sabah. One loopful of bacteria was pre-cultured in nutrient broth for 24 h and turbidity of

culture was adjusted to optical density of McFarland 0.5. Then, 0.1 mL of pre-cultured bacterial suspension was seeded on Nutrient Agar plates. Paper disks (Whatman 6 mm) impregnated with $30\,\mu g$ disk^{-1} pure compounds were placed on the seeded agar plates, and the diameters of inhibition zone were measured after 24 h incubation at 28°C.

Seasonal variation of bioactive metabolites

Seasonal changes in the production of the identified metabolites were investigated in detailed at two main commercial farms; (1) Selakan Island (Population 1), and (2) Balambangan Island (Population 2). *Laurencia majuscula* was collected every 3 months (January, April, July, and October) and the percentages of the bioactive secondary metabolites were quantified using HPLC. Analytical technique used in extraction, isolation, and quantification by HPLC are as described above.

Microscopy analysis

For the morphological study living specimens were examined. Sections were hand cut using a razor blade and immediately mounted in seawater on microscope slides. Other morphological observations were made on the basis of specimens preserved in 10% formalin/seawater. Sections of fixed materials were stained with 0.5% (*w/v*) cotton blue in a lactic acid/phenol/glycerol/water (1:1:1:1) solution and mounted in 50% glycerol/seawater on microscope slides. Presence of *corps en cerise* was confirmed under compound microscope under 40× magnification.

Results

A total of ten biofoulers growing on the culture lines were collected and identified and their relative composition and seasonality are shown in Table 1. In terms of abundance, their relative composition is as follows; Rhodophytes (60% (P-1) and 67% (P-2))>Phaeophyta (30% (P-1) and 33% (P-2)) >Chlorophyta (10% (P-1) and 11% (P-2)), at culture farms in Selakan Island (P-1) and Balambangan Island (P-2). Relative coverage of *L. majuscula* showed a dramatic increase between January and April, increasing from 12.2± 0.5 % to 78.5±2.9 % at Selakan Island, and from 14.4±1.2 % to 69.5±6.9 % at Balambangan Island, for Populations 1 and 2, respectively. *Laurencia majuscula* dominated and flourished in April and July at both the farms. During October and January, the dominant biofouler at Selakan Island and Balambangan Island were *Acanthophora spicifera* (Vahl) Børgesen and *Acanthophora* sp., respectively.

Collected *L. majuscula* specimens were subjected to extensive bioassay-guided separation technique using "ice-ice" disease derived bacteria as test organisms to yield four halogenated secondary metabolites with potent antibacterial activity. The first population of *L. majuscula* from three farms in Semporna district, produced elatol (**1**) and iso-obtusol (**2**); while the second population from three farms in Kudat district produced (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7 (14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**), as their antibacterial metabolites. Relative concentrations of the respective metabolites in each of the farms are given in Table 2. Identities of the chemical structures were elucidated based on detailed spectroscopic

Table 1 Seasonal variation in relative composition of biofoulers (%) on culture lines at Selakan Island (P-1) and Balambangan Island (P-2)

	Locations							
	Selakan Island (P-1)				Balambangan Island (P-2)			
	Months							
	J	A	J	O	J	A	J	O
Acanthophora spicifera (Vahl) Børgesen	**22.1±2.4**	5.5±0.4	15.4±2.6	**18.4±2.5**	17.3±2.1	4.8±0.3	14.4±2.1	15.8±2.1
Acanthophora sp.	14.3±3.2	6.2±1.1	12.3±1.1	16.6±1.2	**20.6±3.1**	3.2±0.1	16.5±2.3	**20.2±1.8**
Caulerpa racemosa Weber van Bosse	8.0±0.5	2.0±0.6	7.2±1.2	6.3±0.8	12.9±1.2	1.3±0.1	13.6±1.9	15.3±3.2
Hypnea valentiae (Turner) Montagne	14.5±2.5	3.1±1.2	18.5±3.2	12.7±1.9	12.8±2.2	1.4±0.1	8.3±1.5	10.9±0.9
Dictyota dichotoma (Hudson) Lamouroux	6.5± 0.4	0	2.4±1.1	8.3±0.4	8.6±1.4	5.8±0.2	6.5±0.5	7.5±0.6
Laurencia majuscula Harvey	12.2±0.5	**78.5±2.9**	**24.3±4.1**	12.8±1.1	14.4±1.2	**69.5±6.9**	**23.5±3.8**	12.6±1.2
Laurencia snackeyi Masuda van Bosse	8.7±0.4	1.4±0.5	6.7±0.4	9.4±0.7	6.4±0.4	3.2±0.5	3.9±0.2	5.8±0.3
Laurencia sp.	6.5±0.8	0	4.9±0.2	5.8±0.5	0	0	0	0
Turbinaria conoides (J.Agardh) Kützing	4.9±0.6	3.3±0.4	5.7±1.1	6.3±0.6	4.3±0.4	5.6±0.4	6.4±0.3	7.3±0.8
Sargassum duplicatum Bory	2.3±0.5	0	2.6±0.6	3.4±0.8	2.7±0.2	5.4±0.1	6.9±0.2	4.6±0.3

Table 2 Concentrations of the respective metabolites in *L. majuscula* from each farm for the month of April 2007

	Compounds (%/DW)			
	1	2	3	4
Lohok Butun	25.3±1.8	10.3±1.1	–	–
Selakan Island	32.8±4.2	14.6±2.2	–	–
Bum-Bum Island	22.7±3.1	9.8±1.3	–	–
Terutuk	–	–	20.7±3.5	10.3±1.8
Caringhton Reef	–	–	14.1±1.3	8.7±0.7
Balambangan Island	–	–	18.4±2.1	12.9±1.1

data, and compared with data available from authentic compounds. Details of their spectroscopic properties are as below:

Elatol (**1**) and iso-obtusol (**2**)—as reported by Vairappan et al. (2001). ^{1}H and ^{13}C-NMR spectra were identical to that of the authentic samples (Sims et al. 1974; González et al. 1979).

Compound **3**, after repeated separation using reverse phase Shimadzu HPLC, (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene was isolated as an oil, [α]^D −7.0 (c, 0.5) [Coll and Wright 1989 ((+2.7 (*c*, 0.01)]; Suzuki and Kurosawa 1978 (−4.0 (*c*, 1.90))]. ^{1}H and ^{13}C-NMR spectra were identical to that of the authentic samples (Coll and Wright 1989).

Compound **4**, after repeated separation using reverse phase Shimadzu HPLC, (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene was isolated as an oil, [α]^D −4.2 (c, 0.5) [Coll and Wright 1989 (−40.0 (*c*, 0.01))]. ^{1}H and ^{13}C-NMR spectra were identical to that of authentic samples (Coll and Wright 1989) (Fig. 1).

The results of antibacterial screening assays for the four halogenated chamigrenes are summarized in Table 3. All four compounds showed potent inhibition against the bacteria isolated from "ice-ice" disease seaweed as compared to human pathogenic bacteria at 30 µg disk^{-1}. Strong

inhibition was observed against *Cytophaga–Flavobacterium*, *Vibrio* sp. and *Alteromonas* sp. 1, and moderate to weak inhibition against *Alteromonas* sp. 2, *P. mirabilis* and *Proteus* sp. Comparatively, these compounds only showed relatively weak inhibition against four of the human pathogenic bacteria. Compounds **1** and **2** exhibited inhibition against *Staphylococcus aureus* and *Staphylococcus* sp., while compounds **3** and **4** inhibited *E. coli* and *K. pneumoniae*. Positive controls in antibacterial bioassay were carried out using cefuroxime (CRO30) and vancomycin (VA30), and both showed strong inhibition against all the tested bacteria.

Seasonal variations in relative composition of all four bioactive chamigrenes were evaluated every three months; and these data is shown in Table 4. Highest concentrations of these compounds were in April and the lowest concentrations were in January. The difference between their maximum and minimum values was 125%, 143%, 170%, and 122% for compounds **1**, **2**, **3**, and **4**, respectively.

Microscopic evaluation of *L. majuscula* revealed the presence of one to two refractile inclusions or *corps en cerise* in both cortical and trichoblast cells as shown in Fig. 2a–d. The presence of these organelles confirms the ability of this seaweed to partition compounds into specialized storage structures that are distributed at their surface and ready to be used in times of need.
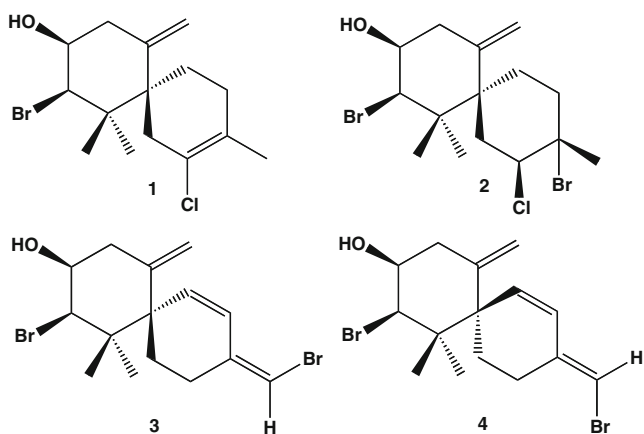
Discussion

Bacterial infection of cultivated carrageenophytes commonly called "ice-ice" disease was first reported by Doty (1973) and Uyenco et al. (1981) before the involvement of complex mixture of opportunistic bacteria and their dynamics was fully understood. Subsequent studies have examined and identified the abiotic factors that contribute to disease emergence, causative organisms and seasonality of disease outbreak (Largo et al. 1995; Vairappan and Chong 2006). However, the dynamics of biofoulers on the culture lines and their possible resistance towards the "ice-ice" disease bacteria has not been reported to date. The present study represents the first report on the dynamics of

Fig. 1 Chemical structures of elatol (**1**), iso-obtusol (**2**), (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**)

Table 3 Antibacterial activity of the halogenated compounds isolated from *Laurencia majuscula* of both populations against "ice-ice" disease pathogens and human pathogenic bacteria at 30 μg disk^{-1}

Tested bacteria	Tested metabolites (30 μg disk^{-1})					
	1	2	3	4	CRO	VA
Seaweed pathogens						
Alteromonas sp1.	++++	++++	++	++	+++	+++
Alteromonas sp2.	++	+	+	+	+++	+++
Proteus mirabilis	++	++	++	++	++	++
Proteus sp.	+	+	+	+	+++	+++
Cytophaga–Flavobacterium	++++	++++	++	++	+++	+++
Vibrio sp.	++++	++++	+	+	+++	+++
Human pathogens						
Staphylococcus aureus	++	++	–	–	+++	+++
Staphylococcus sp.	++	++	–	–	+++	+++
Streptococcus sp.	–	–	–	–	++	++
Citrobacter freundii	–	–	–	–	++	+++
Escherichia coli	–	–	+	+	+++	++++
Klebsiella pneumoniae	–	–	+	+	+++	++++

Inhibition zone diameter ++++ 25–30 mm, +++ 19–24 mm, ++ 12–18 mm, + 7–12 mm, – no inhibition

biofoulers in relation to disease outbreak and the presents of antibacterial metabolites in biofouler.

During our routine monitoring survey to access disease outbreaks in culture farms, the presence and dynamics of ten biofoulers on culture lines of both the investigated populations in Selakan Island (P-1) and Balambangan Island (P-2) was recorded. Moreover, only *L. majuscula* showed consistent trend of resistant towards the "ice-ice" disease outbreak and flourish during the peak of infection. The sudden increase in *L. majuscula* between January and April correlated well with "ice-ice" disease outbreak seasons in North Borneo Island as reported by Vairappan and Chong (2006). Since, red algae genus *Laurencia* are known to produce and store halogenated secondary metabolites, we collected these specimens and studied the inherently available antibacterial defense metabolites against bacteria isolated from diseased *K. alvarezii*, by bioassay-guided separation.

Bioassay-guided separation of both the *L. majuscula* populations against bacteria isolated from "ice-ice" disease seaweed revealed two sets of compounds based on collection location. Specimens of population 1 contained elatol (**1**) and iso-obtusol (**2**) as their antibacterial metabolites, while population 2 contained (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**).

Our earlier studies of *L. majuscula* collected from degraded coral reef beds in the coast waters of Sabah revealed the presence of compounds **1** and **2** as their common metabolites (Vairappan et al. 2001; Vairappan 2003). Based on the chemotaxonomy perspective, these specimens resemble *L. majuscula* population from Woodmans Point, Perth, Western Australia, and the Canary Islands (Capon et al. 1988; Masuda et al. 1998). However, this is the first record of *L. majuscula* population that produces compounds **3** and **4** in Sabah coastal waters. Similar chemical types were reported from specimens collected at Tanegashima and Okino-shima Islands, Japan and Magnetic and Hinchinbrook islands, Australia (Suzuki and Kurosawa 1978; Suzuki et al. 1979; Coll and Wright 1989; Wright et al. 1990). Hence, our investigation confirms the presence of two chemical populations in *L. majuscula* in North Borneo. The first type contained elatol (**1**), and iso-obtusol (**2**) while the second type contained (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**), as their respective major metabolites. Variation in

Table 4 Seasonal variations in composition of compounds **1**, **2**, **3**, and **4** for both the population as represented in specimens from Selakan Island (P-1) and Balambangan Island (P-2)

	Locations			
	Selakan Island (P-1)		Balambangan Island (P-2)	
	Compound 1	Compound 2	Compound 3	Compound 4
January	14.6±1.7	6.0±0.6	6.8±0.5	5.8±0.2
April	32.8±4.2	14.6±2.3	18.4±2.1	12.9±1.1
July	28.5±3.8	12.1±2.1	13.7±1.5	9.1±0.2
October	18.3±2.9	6.7±0.7	9.4±0.4	8.8±0.2

Fig. 2 Herbarium and microscopic images of *L. majuscula*: **a** Herbarium specimen (10 mm=30 mm). **b** Surface view showing the presence of two or three *corps en cerise* in superficial cortical cells (10 mm=500 μm). **c** Longitudinal section showing only superficial cortical cells contain *corps en cerise* (10 mm=500 μm). **d** Presence of two *corps en cerise* in each trichoblast cell (10 mm=500 μm)
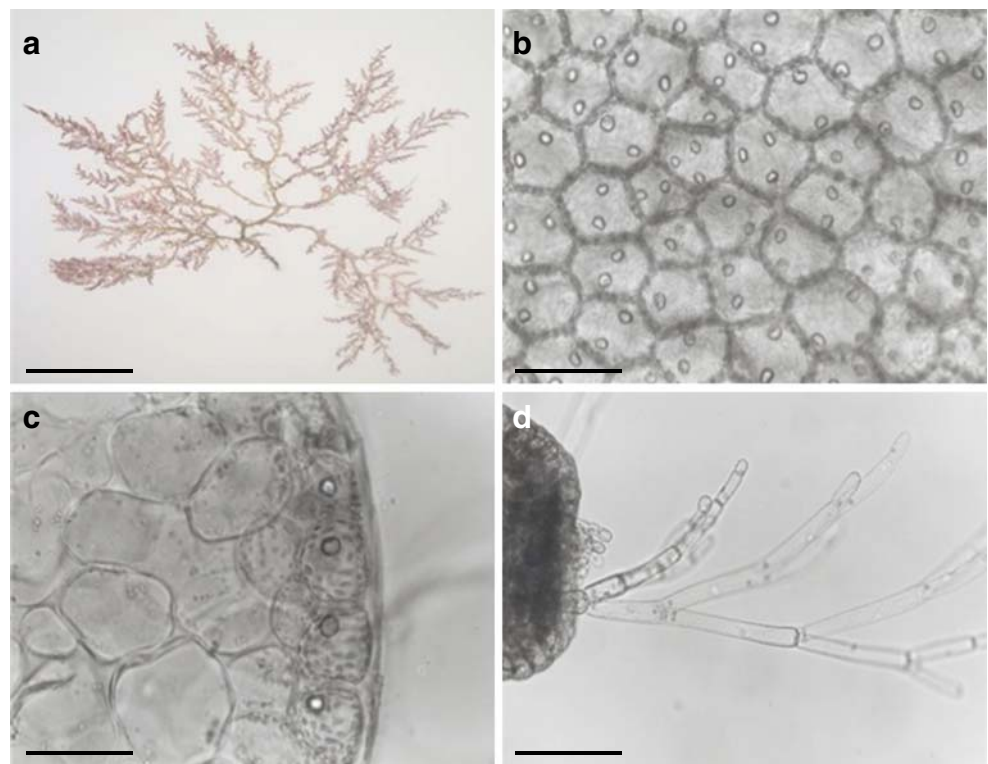
secondary metabolite production is not a new phenomenon, it has been observed in red algae genus *Laurencia*, and differences in seawater quality and environmental factors has been suggested as the factors causing the differences. However, in this investigation, since both the populations were found growing on carrageenophyte culture lines, it is not possible to suggest seawater or environmental condition to be the causal factor. Carrageenophytes are very sensitive towards changes in seawater and/or environmental parameter. Therefore, the ability of the two populations to produce different sets of metabolites could be regarded as their inherent ability. Based on the finding from this investigation, it is clear that there are two chemical types of *L. majuscula* in North Borneo waters. To date, *L. majuscula* populations are known to exist as four distinct chemical types and the other remaining types are the pacifenol producing population in Eastern Sicily, Italy (Caccamese et al. 1986) and a (2*R*,3*R*,5*S*)-5-acetoxy-2-bromo-3-chloro-chamigra-7(14),9-dien-8-one producing population found in Ryukyu Islands, Japan (Masuda et al. 1997b).

All four halogenated metabolites showed potent antibacterial activities against the seaweed pathogens as compared to the human pathogenic bacteria. Antibacterial activities of compound (**1**) and compound (**2**) against selected environmental bacteria had been reported earlier (Vairappan et al. 2001). Both compounds (**3**) and (**4**) showed similar level of activities against all the bacteria. As suggested by Vairappan et al. (2001), these halogenated compounds may have a role as defense substance's in *Laurencia* spp. against bacterial

infection since they are synthesized and stored in *corps en cerise* in superficial cortical cells (Young et al. 1980; Salgado et al. 2008), making it possible for them to inhibit the growth of penetrating marine bacteria. The presence of "ice-ice" disease bacteria on the surface of both *K. alvarezii* and *L. majuscula* during disease outbreak was detected *via* bacterial enumeration technique. Hence, *Falvobacterium–Vibrio* complex was present in a density of $42\pm2 \sim 64\pm 4$ CFU cm^{-2} on the surface of *L. majuscula*. Details of total bacterial count and bacterial species composition on *K. alvarezii* surface during disease outbreak were reported by Vairappan and Chong 2006.

The proposed defense role of halogenated metabolites in *Laurencia* was further substantiated with the detailed investigation of Sudatti et al (2008), which proved a transportation mechanism of halogenated metabolites from "corps en cerise" to the surface of *L. obtusa*. Based on these findings, it is conceivable that "ice-ice" disease bacteria and halogenated metabolites can come into contact on the surface of *L. majuscula*, where these compounds could function as a defense against pathogens.

The seasonality and variability of the isolated antibacterial metabolites examined from specimens collected from two representative farms of both populations seems to support the theory of them having a role in antibacterial defense during the "ice-ice" disease outbreak. All four compounds showed a drastic increase (more than double) in their production during the onset of infection on cultured *Kappaphycus*. Our earlier investigation of *L. majuscula*

collected from coral rubble in the coastal waters of North Borneo did not indicated the presence of seasonal variation in the production of halogenated compounds (Vairappan 2003). Based on this information, it is suggestive that *L. majuscula* increases the production of its halogenated metabolites during disease outbreak as a strategy to defend itself and flourish in the absence of other biofoulers on the culture lines.

In conclusion, out of ten biofouler species found growing on *Kappaphycus* culture lines, *L. majuscula* showed dominance and flourished during "ice-ice" disease outbreak. Bioassay-guided separation against "ice-ice" disease bacteria gave four bioactive chamigrenes, indicating the presence of two chemical types of *L. majuscula* in North Bornean waters. Populations on the eastern coast were characterized by elatol (**1**) and iso-obtusol (**2**), while populations on the western and northern coasts were characterized by (*Z*)-10,15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**3**) and (*E*)-10-15-dibromo-9-hydroxy-chamigra-1,3(15),7(14)-triene (**4**). All four halogenated metabolites showed various levels of selective activities against seaweed pathogens and could have a role as defense metabolites for these red algae.

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