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Potent antibacterial activity of halogenated metabolites from Malaysian red algae, *Laurencia majuscula* (*Rhodomelaceae*, *Ceramiales*)

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Abstract

Red algae genus *Laurencia* (*Rhodomelaceae*, *Ceramiales*) are known to produce a wide range of chemically interesting secondary halogenated metabolites. This investigation delves upon extraction, isolation, structural elucidation and antibacterial activity of inherently available secondary metabolites of *Laurencia majuscula* Harvey collected from two locations in waters of Sabah, Malaysia. Two major halogenated compounds, identified as elatol (**1**) and iso-obtusol (**2**) were isolated. Structures of these compounds were determined from their spectroscopic data such as IR, ^{1}H-NMR, ^{13}C-NMR and optical rotation. Antibacterial bioassay against human pathogenic bacteria was conducted using disc diffusion (Kirby–Bauer) method. Elatol (**1**) inhibited six species of bacteria, with significant antibacterial activities against *Staphylococcus epidermis*, *Klebsiella pneumonia* and *Salmonella* sp. while iso-obtusol (**2**) exhibited antibacterial activity against four bacterial species with significant activity against *K. pneumonia* and *Salmonella* sp. Elatol (**1**) showed equal and better antibacterial activity compared with tested commercial antibiotics while iso-obtusol (**2**) only equaled the potency of commercial antibiotics against *K. pneumonia* and *Salmonella* sp. Further tests conducted using dilution method showed both compounds as having bacteriostatic mode of action against the tested bacteria.

Keywords: *Laurencia*; Halogenated metabolites; Pathogenic bacteria; Bactericidal; Bacteriostatic

1. Introduction

Red algae genus *Laurencia* is an extremely rich source of secondary halogenated metabolites with diverse structural features, particularly of three major classes, sesquiterpenes, diterpenes and acetylenes [1,2]. Most of these metabolites are characterized by the presence of halogen atoms in their chemical formula. Chemistry of halogenated compounds from *Laurencia* is a very interesting area of research and it never fails to offer the possibility of discovering new compounds with novel structure and properties [3–6]. Malaysian waters are rich with many species of genus *Laurencia*, but very few are documented and even fewer findings are published pertaining to their chemistry [7,8]. Substantial number of *Laurencia*'s halogenated metabolites are known to exhibit biological activity, particularly antibacterial activity [9–11]. Hence, it is reasonable to suggest some of these compounds as seaweed's defense chemicals in their endeavor to protect themselves against pathogens and survive in the marine ecosystem. In a previous study, it was shown that metabolites isolated from Malaysian *Laurencia majuscula* could inhibit its surface bacteria, suggesting the presence of an inherently available antibacterial defense mechanism [7]. Hence, the present study undertakes the isolation and structural elucidation of major halogenated secondary metabolites from two samples of *L. majuscula* collected from different locations in the coastal waters of Sabah, Malaysia. Isolated and characterized compounds were tested for their antibacterial activities against eight species of human pathogenic bacteria. Potencies of these compounds were determined by comparing their antibacterial activities against commercially available antibiotics. Finally, isolated compounds were subjected to antibacterial testing *via* dilution method to determine their possible mode of action, bactericidal or bacteriostatic activity.

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2. Materials and methods

2.1. Collection

Samples of *L. majuscula* were collected from Pulau Tikus (Sandakan, Sabah (6°04′06″N, 117°57′48″E); May 2002) and Pulau Bankawan (Sandakan, Sabah (6°04′56″N, 117°59′50″E); May 2002). The voucher specimens are deposited in the Herbarium (CSV) of the Borneo Marine Research Institute, University Malaysia Sabah.

2.2. Extraction and separation of L. majuscula

Partially dried sample (60 g) from Pulau Tikus was soaked in methanol for 1 week. The MeOH solution was concentrated in vacuo and partitioned between Et_2O and H_2O. Et_2O solution was washed with water, dried over anhydrous Na_2SO_4 and evaporated to leave dark green oil (1.24 g). Extract was fractioned by Si gel column chromatography with a step gradient (hexane and EtOAc; gradient ratios: 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). The fraction eluted with hexane–EtOAc (3:1) was repeatedly submitted to preparative Thick Layer Chromatography with hexane–EtOAc (3:1) to give elatol (**1**) (182 mg) and iso-obtusol (**2**) (48 mg).

Extraction of sample (40 g) from Pulau Bankawan was carried out in the same manner as explained above and yielded 0.82 g. This sample contained iso-obtusol (**2**) as its major metabolite; elatol (**1**) (36 mg) and iso-obtusol (**2**) (86 mg).

Spectroscopy data were obtained using ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz); $CDCl_3$, TMS as int. standard (coupling constant, *J* in Hz). Optical rotations: $CHCl_3$; CC: silica gel (Merck, Kieselgel 60, 70–230 mesh); preparative Thick Layer Chromatography: silica gel $60F_{254}$ (Merck). Yields are based on the weights of the extracts.

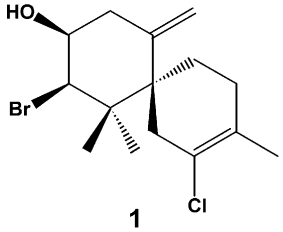
2.3. Antibacterial bioassay

Antibacterial bioassays were carried out using eight strains of human pathological bacteria obtained from specimens of clinical patients, i.e. sputum, stool, blood, wound and urine. These bacteria are β-hemolytic *Streptococcus* sp. (BP981), *Citrobacter freundii* (BP982), *Escherichia coli* (BP983), *Klebsiella pneumoniae* (BP984), *Pseudomonas* sp. (BP985), *Salmonella* sp. (BP986), *Staphylococcus aureus* (BP987) and *Staphylococcus epidermis* (BP988). One loopful of each organism was precultured in 20 ml of peptone water (3% NaCl) overnight. The turbidity of the culture was adjusted to an optical density (OD) of McFarland 0.5 [11,12]. Then 0.1 ml of the precultured bacterial suspension was used to seed Nutrient Agar plates (3% NaCl). Paper discs (Whatman, 6 mm) impregnated with 30 μg disc^{-1} of the respective pure compounds were placed on the seeded agar plates and diameters of inhibitory zones were measured after the plates were incubated at 28 °C for 24 h.

Similar antibacterial bioassays were also conducted for comparative analysis using six types of Oxoid antimicrobial susceptibility discs; Augmentin (AMC), Latamoxef (MOX), Ceflaclor (CEC), Ceftriaxone (CRO), Kanamycin (K) and Netilmicin (NET). Concentration used were according to NCCL levels; 30 μg per disc.

2.4. Minimum inhibition concentration

Minimum inhibition concentrations (MIC) of elatol (**1**) and iso-obtusol (**2**) against the tested pathological bacterial strains were determined using dilution method. Elatol (**1**) and iso-obtusol (**2**) were diluted in 500 μl dimethyl sulfoxide then mixed with bacterial strains cultured in 9.5 ml of nutrient broths, respectively. The initial concentration of elatol (**1**) and iso-obtusol (**2**) was 4 mg ml^{-1}, concentrations of 2 and 1 mg ml^{-1} were obtained by serial dilutions. Bacterial growth were monitored and quantified as Colony Forming Unit based on McFarland optical density method [11]. A second test was performed by transferring 100 ml of each bioassay culture (bacterial culture with elatol (**1**) and iso-obtusol (**2**), respectively) into a new test tube containing only nutrient broth. Observations were made every hour for 24 h, to determine possible bacterial growth in the respective culture broths. Optical density of treated cells reflects their viability and provides sufficient information pertaining to the mode of action of the tested metabolites. Bacterial growth in culture tubes will suggest the tested compounds as bacteriostatic, while lack of growth will indicate that the compounds were bactericidal.



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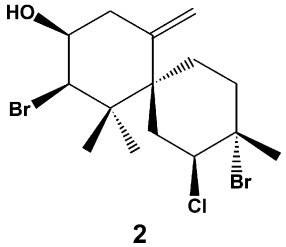

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3. Results and discussion

3.1. Halogenated secondary metabolites

Laurencia specimens were extracted in methanol separately and each gave crude greenish paste like extract. Crude extract was then fractioned via column chromatography with a step gradient (hexane:EtOAc). Fractions eluted with hexane:EtOAc (9:1) were further subjected to preparative TLC in hexane:EtOAc (3:1) solvent system to give compound **1**. In addition, fractions eluted with hexane:EtOAc (70:30) were also subjected to preparative TLC and gave compound **2**. Both these compounds were subjected to IR, ^{1}H-NMR, ^{13}C-NMR, optical rotation and melting point measurements. Based on these spectroscopic data, structures were determined as elatol (**1**) [13] and iso-obtusol (**2**) [14]. Spectroscopic data of elatol (**1**) and iso-obtusol (**2**) are as shown below.

3.2. Elatol (*1*)

Oil; $[\alpha]_D^{24}+75.3^\circ$ (MeOH; c 0.40); ^{1}H-NMR and ^{13}C-NMR spectrums were identical to that of the authentic sample and as reported by Vairappan et al. [7].

3.3. Iso-obtusol (*2*)

Mp 109-114 °C; $[\alpha]_D^{24}+24.6^\circ$ ($CHCl_3$; c 0.50); ^{1}H-NMR and ^{13}C-NMR spectrums were identical to that of authentic sample and as reported by Vairappan et al. [7].

L. majuscula collected from P. Tikus contained elatol (**1**) (15%) as its major metabolite and iso-obtusol (**2**) (4%) as minor. On the other hand, sample from P. Bankawan gave iso-obtusol (**2**) (11%) as its major compound and elatol (**1**) (4%) as its minor. Present finding indicates that Malaysian *L. majuscula* produces two distinct halogenated metabolites, elatol (**1**) and iso-obtusol (**2**) as its main metabolites. Hence, these metabolites can be used as chemical markers to facilitate the identification process of this species. Percentages of these compounds in crude extract are a good indication of the importance of these compounds to the seaweed and its possible role in antibacterial defense mechanism as suggested by Vairappan et al. [7]. However, the biological function of the many halogens contained in the metabolites produced by red algae remains a topic of speculation. In the marine ecosystem, methods to discourage predators, as well as defense against pathogenic microorganisms, could be biologically useful. But, due to the difficulty to conduct field experiments, whether these substances provide any advantage or not is still unclear [2]. On the other hand, percentage of isolated compounds also gives an indication of its abundance and such information can be used to calculate its availability in the wild.

3.4. Antibacterial activity

Antibacterial activities of elatol (**1**) and iso-obtusol (**2**) were determined by testing them against eight species of human pathogenic bacteria obtained from clinical patients. Results of paper disc diffusion assay of the isolated halogenated compounds as compared with commercially available antibiotics are shown in Table 1. Elatol (**1**) showed antibacterial activity against six of the tested bacteria. Significant antibacterial activities were seen against *S. epidermis*, *K. pneumonia* and *Salmonella* sp. Iso-obtusol (**2**) exhibited antibacterial activity against four species of bacteria, with significant inhibition against *K. pneumonia* and *Salmonella* sp. Comparative antibacterial studies showed that elatol (**1**) showed equivalent or better activity as compared with commercial antibiotic when tested against *K. pneumoniae*, *Salmonella* sp. and *S. epidermis*. On the other hand, iso-obtusol (**2**) was only equally potent as the commercial antibiotic against *K. pneumoniae* and *Salmonella* sp. As for the commercial antibiotics, all except NET showed potent inhibition against β-hemolytic *Streptococcus* β-hemolyticus, *C. freundii*, *E. coli*, *K. pneumoniae*, *Salmonella* sp., *S. aureus* and *S. epidermis*. NET showed intermediate inhibition against *K. pneumoniae*, *Pseudomonas* sp. and *S. epidermis*. In addition, *Pseudomonas* sp. was relatively resistant towards the antibiotics tested (Table 1).

3.5. Minimum inhibitory concentration

Investigation was further carried out to determine minimum concentration of elatol (**1**) and iso-obtusol (**2**) required to inhibit the growth of bacteria strains tested. Elatol (**1**) showed minimum inhibitory concentration in this following tendency; *K. pneumoniae* and *Salmonella* sp. (1 mg ml^{-1}) < *S. epidermis* (2 mg ml^{-1}) < *Pseudomonas* sp. (4 mg ml^{-1}) < β-hemolyticus *Staphylococcus* sp. and *S. epidermis* (10 mg ml^{-1}). Figs. 1 and 2 show results of time course experiments carried out to determine MIC value for elatol (**1**) against *K. pneumoniae* and *Salmonella* sp., respectively. Similar experiments were also performed using iso-obtusol (**2**) and their MIC values were; *K. pneumoniae* (1.5 mg ml^{-1}) < *Salmonella* sp. (1 mg ml^{-1}) < *Escheichia coli* (4 mg ml^{-1}) < *C. freundii*(8 mg ml^{-1}). Figs. 3 and 4 show results of time course experiments and MIC value obtained for iso-obtusol (**2**) against *K. pneumoniae* and *Salmonella* sp. Second set of tests to determine bactericidal or bacteriostatic activities were performed by culturing 1 ml culture broth of bioassay test tubes, that show no growth, in nutrient broth void of elatol (**1**) or iso-obtusol (**2**). Samples from test tubes that show no growth might contain bacteria that are dead or just inhibited and sub-culturing them in broth without chemotherapeutic agent will enable us to differentiate

Table 1
Comparative antibacterial activity of elatol (**1**), iso-obtusol (**2**) and six types of commercially available antibiotics against tested strains of human pathogenic bacteria

	Compounds tested							
	Elatol (**1**)	Iso-obtusol (**2**)	AMC	MOX	CEC	CRO	K	NET
Streptococcus -hemolyticus	+	−	+++	+++	+++	+++	+	−
C. freundii	−	+	+	++	+++	+++	+++	−
E. coli	−	++	+++	++	+++	++	−	−
K. pneumoniae	+++	+++	+	+++	++	+++	+++	+
Pseudomonas sp.	++	−	−	−	−	+	−	++
Salmonella sp.	++++	+++	+++	+++	+++	+++	+++	−
S. aureus	+	−	−	++	++	++	−	++
S. epidermis	+++	−	+++	+++	+++	++	+	−

Inhibition zone diameter; ++++: 25–30 mm, +++: 19–24 mm, ++: 12–18, +: 7–12 mm, −: no inhibition. Compound concentration: 30 μg per disc (NCCLS level).

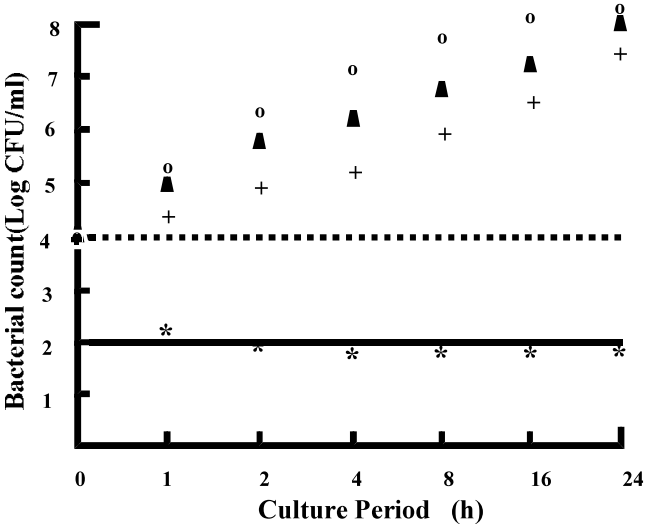

Fig. 1. Dynamics of bacterial growth (*K. pneumoniae*) as a function of time in various concentration of elatol (**1**). (○: control, ▲: 4 mg ml^{-1}, +: 2 mg ml^{-1}, *: 1 mg ml^{-1}, ----: inoculated bacterial concentration, —: lowest optical absorbance).

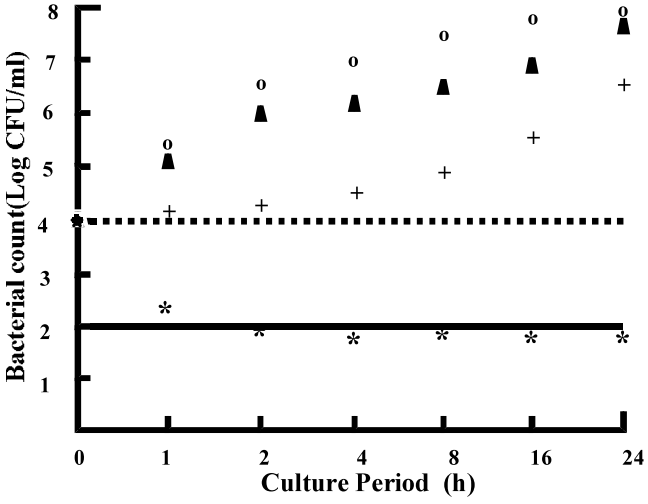

Fig. 2. Dynamics of bacterial growth (*Salmonella* sp.) as a function of time in various concentration of elatol (**1**). (○: control, ▲: 4 mg ml^{-1}, +: 2 mg ml^{-1}, *: 1 mg ml^{-1}, ----: inoculated bacterial concentration, —: lowest optical absorbance).

chemicals with bactericidal or bacteriostatic activity. All the sub-cultured test tubes showed bacterial growth, hence increase in bacterial optical density. Therefore, it can be concluded that both elatol (**1**) and iso-obtusol (**2**) have bacteriostatic activities.

In essence, this investigation suggests the possibility that *L. majuscula* from Malaysian waters has elatol (**1**) and iso-obtusol (**2**) as its main halogenated metabolites. Further investigations are in progress and findings from these ongoing studies may establish the importance of secondary halogenated metabolites, particularly elatol (**1**) and iso-obtusol (**2**) as chemotaxonomical markers for *L. majuscula* found in Malaysian waters. Antibacterial activity of elatol (**1**) was significantly good against three bacterial strains, *K. pneumoniae*, *Salmonella* sp. and *S. epidermis*. Its antibacterial potency was as good as or better than the six commercially available antibiotics. On the other hand, iso-obtusol (**2**) showed significant inhibition against two bacterial strains, *K. pneumoniae* and *Salmonella* sp. Minimum inhibitory concentrations of both the compounds were not significantly low to be considered as a potential antibiotic. At the same time, results from second dilution test also indicated that antibacterial activities shown by elatol (**1**) and iso-obtusol (**2**) are actually bacteriostatic activities and not bactericidal.

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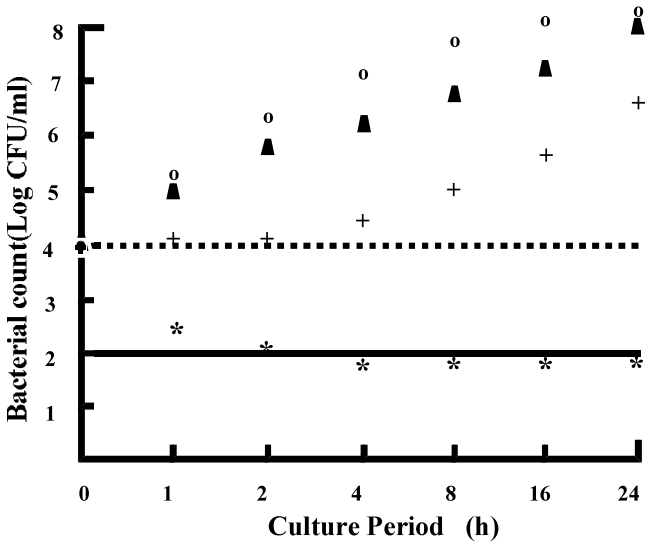

Fig. 3. Dynamics of bacterial growth (*K. pneumoniae*) as a function of time in various concentration of iso-obtusol (**2**). (○: control, ▲: 4 mg ml^{-1}, +: 2 mg ml^{-1}, *: 1 mg ml^{-1}, ----: inoculated bacterial concentration, —: lowest optical absorbance).

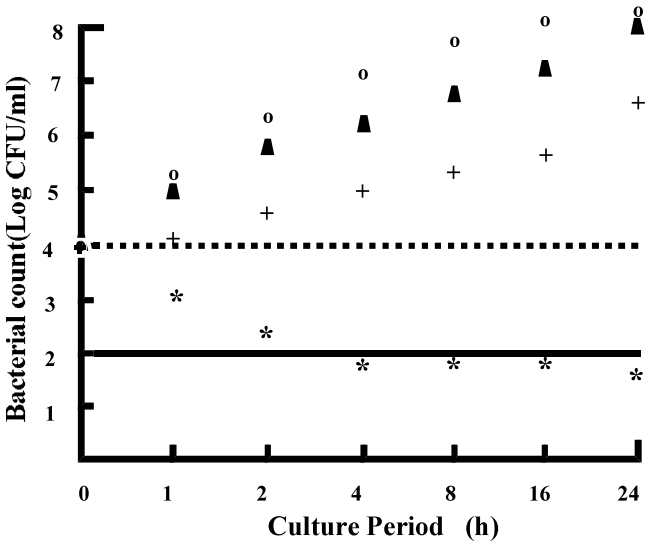

Fig. 4. Dynamics of bacterial growth (*Salmonella* sp.) as a function of time in various concentration of iso-obtusol (**2**). (○: control, ▲: 4 mg ml^{-1}, +: 2 mg ml^{-1}, *: 1 mg ml^{-1}, ----: inoculated bacterial concentration, —: lowest optical absorbance).

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