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Biologically Active Polybrominated Indoles in the Red Alga *Laurencia similis* from the Coastal Waters of Sabah (Rhodomelaceae, Ceramiales)

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ABSTRACT The red alga *Laurencia similis* Nam *et* Saito collected from six locations in coastal waters of Sabah, contained two polybrominated indoles in high quantities, 2,3,5,6-tetrabromoindole (**1**) and 1-methyl-2,3,5,6-tetrabromoindole (**2**). Structures of these compounds were determined based on their spectroscopic data and other physical characteristics. Total percentage of these compounds in samples analyzed were in this order; Tg Aru (58%) > Sepanggar Island (47%) > Kudat (41%) > Mantanani Island (32%) > Lankayan Island (28%) > Sipadan Island (26%). Isolated compounds showed a wide spectrum of activities against human pathogenic yeast, environmental bacteria and fungus. Compound **1**, 2, 3, 5, 6-tetrabromoindole, in particular showed significant activity against the tested microbes. Potencies of the brominated metabolites were also compared against 13 types of commercially available antibiotic sensitivity test bio-discs.

ABSTRAK Alga merah *Laurencia similis* Nam et Saito diambil dari 6 lokasi di persisiran pantai Sabah, mengandungi dua sebatian polibromoindol dalam kuantiti yang tinggi, 2,3,5,6-tetrabromoindole (**1**) dan 1-metil-2,3,5,6-tetrabromoindol (**2**). Struktur sebatian-sebatian ini telah ditentukan berdasarkan data spektroskopi dan cirri-ciri fizikalnya. Jumlah peratusan sebatian dalam sampel yang dianalisa adalah dalam turutan berikut: Tg. Aru (58%) > Pulau Sepanggar (47%) > Kudat (41%) > Pulau Mantanani (32%) > Pulau Lankayan (28%) > Pulau Sipadan (26%). Sebatian yang dipencilkan telah menunjukkan aktiviti antimikrob terhadap bakteria patogen manusia, serta bakteria dan kulat persekitaran. Sebatian **1**, 2,3,5,6-tetrabromoindole, khususnya telah menunjukkan aktiviti antimikrob yang amat berkesan. Potensi sebatian-sebatian bromoindol ini juga telah dibandingkan dengan 13 jenis antibiotik komersil.

(Bromo-indoles, *Laurencia similis*, biological activities, antimicrobial activities)

INTRODUCTION

Members of the red alga *Laurencia* are known as prolific producers of structurally interesting halogenated secondary metabolites. Numerous halogenated metabolites have been reported, most abundant being sesquiterpenes followed by non-terpenoid C_{15} acetogenins, diterpenes and triterpenes [1, 2, 3, 4, 5, 6, and 7]. Consistency in chemical synthesis of these halogenated metabolites and their chemotaxonomical value were described by Howard *et al.* [8] and Masuda *et al.* [9]. Chemical compositions of halogenated metabolites are also known to vary in variety and quantity depending on their species specification, geographical distribution and chemical races [10, 11, and 12].

Chemical constituents of several *Laurencia* species from South East Asian waters were reported by Masuda [9, 13] and Suzuki [5], however, only few reports are available on the chemical constituents of Malaysian *Laurencia* [5, 7, 13, 14 and 15]. Therefore, as part of our continuous effort to document chemical diversity in species of the Malaysian red algal genus

Laurencia, we have recently reported several studies on the potency of halogenated metabolites [14, 15]. Here, we report the chemical composition in samples of *Laurencia similis* Nam *et* Saito collected from six locations in the coastal waters of Sabah and their antimicrobial potentials against environmental and medically important microorganisms.

MATERIALS AND METHODS

Sampling Locations

Specimens of *Laurencia similis* were collected from six locations; Tanjung Aru (5°55'08"N, 116°03'10"E), Sepanggar Island (6°03'38"N, 116°04'11"E), Mantanani Island (6°43'15"N, 116°21'10"E), Lankayan Island (6°31'21"N, 117°54'05"E), Banggi Island (6°53'08"N, 116°48'11"E) and Sipadan Island (4°10'21"N, 118°48'19"E). Latitude and longitude in sampling locations were recorded using GPS 12XL (GARMIN Olathe, KS, USA).

Morphological Studies

Approximately ten specimens from each sampling location were collected and fixed in 4 % formalin in seawater while others were prepared into herbariums. Some specimens were also transported alive to the laboratory at Borneo Marine Research Institute for examination of their structures and retractile organelles called *crops en cerise*. These organelles are known to be the production site for halogenated secondary metabolites as reported by Young *et al.* [16].

Chemical Analysis

Partially dried seaweed samples (100 g) were soaked in one L methanol for the duration of one week, separately. Resulting methanol solution was concentrated in *vacuo* and partitioned between diethyl ether (Et_2O) and water (distilled). Et_2O solution was washed with two changes of distilled water, dried over anhydrous Na_2SO_4 and evaporated to leave dark green oil. Presence of secondary metabolites was checked by spotting the crude extracts on SiO_2 gel F_{254} thin layer chromatography (Merck, Germany), developed in hexane: ethyl acetate (3:1) solvent system and visualized using molybdophosphoric acid spray. 200 mg of crude extract was then 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 (9.5:0.5) was repeatedly subjected to preparative Thick Layer Chromatography (Merck, Germany) with toluene solvent system to yield and 2,3,5,6-tetrabromoindole (**1**) and 1-methyl-2,3,5,6-tetrabromoindole (**2**) (Figure 2).

Spectroscopy data were obtained using ^{1}H-NMR (600 MHz) and ^{13}C-NMR (100 MHz), JEOL ECA 600 MHz; $CDCl_3$, TMS as internal standard. Melting point of both the compounds were recorded in °C and corrected (Fisher Scientific); column chromatography was prepared using silica gel (Merck, Kieselgel 60, 70 – 230 mesh); analytical thin layer chromatography and preparative thick layer chromatography were of silica gel $60F_{254}$ (Merck, Germany). Yields are based on weights of the crude extracts.

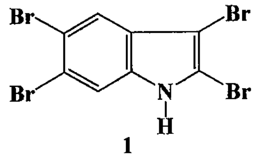
Antibacterial Bioassay

Isolated metabolites were subjected to antimicrobial bioassay using six strains of marine environmental bacteria, fourteen strains of human pathogen bacteria, seven strains of clinical yeasts and eleven strains of environmental fungi. Details of the test organisms are given in Table 1. One loopful of each bacteria and yeast was precultured in 10 ml of peptone water (3% NaCl) overnight. Culture turbidity was adjusted to an optical density equivalent to Mcfarland 0.5 [17, 18]. Antifungal bioassay was performed by preparing 4×10^5 spore suspensions in sterile distilled water, for each tested fungal species. 0.1 ml of the precultured suspension was used to seed Hilton Mueller agar plate (3% NaCl) for antibacterial and antiyeast test while potato dextrose agar (PDA) plates were used for antifungal bioassay. Paper discs (Whatman, 6 mm) impregnated with 30 □gdisc^{-1} of the respective pure compounds were placed on the seeded agar plates and diameters of inhibitory zones were measured upon incubation at 28°C (bacterial and yeast) and 25°C (fungi) for 24 ~ 36 hours. Potency of tested compounds were evaluated by comparing them against a range of commercially available antibiotics; Novobiocin (NB30) (BBL, France), Loracarbef (LOR30) (BBL, France), Minocycline (MI30) (BBL, France), Oxytetracycline (T30) (BBL, France), Ceftriaxone (CTX30) (Biomeneux, France), Cefuroxime (CRO30) (Biomeneux, France), Cefotaxime (CXM30) (Biomeneux, France), Vancomycine (VA30) (Biomeneux, France), Nalidixic Acid (NA30) (Biomeneux, France), Tetracycline (TE30) (Biomeneux, France), Cefazoline (CZ30) (Biomeneux, France), Kanamycine (K30) (Biomeneux, France) and Netilmicine (NET30) (Biomeneux, France).

Table 1. Antimicrobial bioassay using six strains of marine environmental bacteria, fourteen strains of human pathogen bacteria, seven strains of clinical yeasts and eleven strains of environmental fungi

Test / Compounds Tested / Organisms	1	2	CTX 30	CRO 30	CXM 30	CZ 30	LOR 30	T 30	M I30	NA 30	NET 30	NB 30	TE 30	K 30	VA 30
Pathogenic Yeast															
Candida albicans ATCC	7	-	-	-	-	-	-	-	13	-	-	-	-	-	-
Candida albicans G561	7	-	-	-	-	-	-	-	20	-	-	-	-	-	-
Candida albicans G588	8	8	-	-	-	-	-	-	-	-	-	-	-	-	-
Candida albicans G670	-	-	-	-	-	-	-	-	19	-	-	-	-	-	-
Candida albicans U1515	7	-	-	-	-	-	-	-	18	-	-	-	-	-	-
Candida albicans U1580	8	-	-	-	-	-	-	-	14	-	-	-	-	-	-
Crytococcus neoformans	10	-	-	24	-	26	18	28	44	-	18	38	-	17	22
Pathogenic Bacteria															
Bacillus cereus	11	-	8	14	-	8	12	14	16	-	20	22	14	16	12
Enterococcus faecalis	7	-	-	-	-	16	-	-	-	-	20	-	-	14	18
Escherichia coli	-	-	10	10	-	12	12	-	18	10	18	20	20	12	-
Proteus mirabilis	-	-	14	16	10	14	10	-	-	-	20	-	-	16	-
Pseudomonas aeurelis	-	-	-	-	-	-	-	-	-	-	20	-	-	-	-
Salmonella enteritidis	-	-	-	30	-	-	26	20	20	20	18	-	22	18	-
Salmonella sp.	-	-	10	20	10	14	16	14	-	-	18	-	26	10	-
Salmonella typii	-	-	22	32	20	20	24	10	10	24	26	-	26	16	-
Staphylococcus aureus	-	-	26	24	-	26	30	30	28	-	22	-	26	16	-
Vibrio cholerae	-	-	16	20	10	10	14	28	36	30	16	22	22	16	-
Vibrio parahaemolyticus	-	-	20	20	12	-	10	14	22	16	28	16	14	16	-
Listeria monocytogenes	-	-	-	-	-	26	24	20	40	-	26	32	24	20	32
Environmental Bacteria															
Clostridium cellobioparum	10	10	24	30	28	30	34	32	36	-	32	20	26	20	24
Clostridium sordelli	10	-	12	16	-	24	18	20	18	18	20	26	20	16	14
Clostridium novyi	10	-	10	14	-	-	20	26	24	22	22	26	22	16	20
Proteus vulgaris	15	-	40	40	60	50	60	20	20	34	20	60	20	-	-
Vibrio alginolyticus	11	-	10	12	-	18	16	26	22	20	22	26	22	14	18
Vibrio parahaemolyticus	11	-	10	16	-	14	20	30	32	-	24	24	30	20	20
Terrestrial Fungi															
Aspergillus niger	9	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus orizae	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus terreus	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mucor sp.	11	-	-	-	-	-	-	-	11	-	-	-	-	-	-
Penicillium sp.	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Phytothora sp.	18	-	-	-	-	-	-	-	25	-	-	-	-	-	-

Inhibition zone diameter; ++++: 25 – 30 mm, +++:19 -24 mm, ++:12 - 18 mm, 7-12 mm, - : no inhibition. Compound concentration: 30□gdisc^{-1} (NCCLS level).



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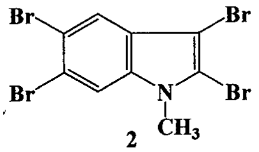

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Figure 2. Thick Layer Chromatography with toluene solvent system to yield and 2,3,5,6-tetrabromoindole (**1**) and 1-methyl-2,3,5,6-tetrabromoindole (**2**)

RESULTS AND DISCUSSION

Morphological Features

Plants were found growing on dead corals at reef flats and sandy subtidal slopes with a well developed discoid holdfast, upright axes and one or two stolon-like branches formed at the basal portion of axes (Figure 1A). They are also found attached to their substratum secondarily by small discoid holdfasts. Individual plant can attain a maximum height of 6 -13 cm with light red appearance. Main axis above the holdfast is 0.8 – 1.9 mm in diameter, width increases to the middle portion and then tapers gradually upwards reaching 0.5 – 0.9 mm (Figure 1B). Multiple branches are formed on primary and secondary axes in an alternate-spiral manner (Figure 1C & 1D). Each superficial cortical cell usually shows the presence of two *corps en cerise* (Figure 1E). Morphological features of this plant shows close resemblance to *Laurencia papilosa* except for several differences like; presence of *corps en cerise*, pale red as compared to dark brown and presence of secondary longitudinal pit-connection. *Crops en cerise* are known to be organelles responsible for the production of secondary halogenated metabolites and their presence confirms the fact that the plants studied here is not *L. papilosa*. The observed features agree with findings reported by Masuda *et al.* [9] on *L. similis* collected from coastal waters of Sabah, Malaysia. It is also impossible to distinguish both these species based on habitat but in field they could be differentiated based on their coloration. However, confirmative differentiation will have to be the presence of *corps en cerise* in the studied species as compared to *L. papilosa*.

Extraction/Isolation of Halogenated Metabolites

100 g of partially-dried sample was extracted in methanol for seven days, resulting methanol solution concentrated *in vacuo* to yield methanol extract. The concentrate was partitioned between diethyl ether and water, ether solution washed in two changes of distilled water, dried over anhydrous Na_2SO_4 for three hours, filtered and evaporated to yield an oily dark greenish extract. The methanol extract was fractioned by CC over silica gel with step gradient (hexane and EtOAc). The fraction eluted with hexane-EtOAc (9.5:0.5) was further subjected to preparative TLC with toluene to give 2, 3, 5, 6-tetrabromoindole (**1**) and 1-methyl-2, 3, 5, 6-tetrabromoindole (**2**) in various quantities.

2,3,5,6-tetrabromoindole (**1**), showed R_f value of 0.50 on silica gel thin-layer aluminium sheet (Merck, Kieselgel $60F_{254}$), mp 152 – 154°C , was assigned molecular formula $C_8H_3Br_4N$ (HR-EIMS). ^{1}H-NMR and ^{13}C-NMR data were similar to data reported by Carter *et al.* [19].
1-methyl-2,3,5,6-tetrabromoindole (**2**), showed R_f value of 0.65 on silica gel thin-layer aluminium sheet (Merck, Kieselgel $60F_{254}$), mp 170 – 172°C, was assigned molecular formula $C_9H_5Br_4N$ (HR-EIMS). ^{1}H-NMR and ^{13}C-NMR data were similar to data reported by Carter *et al.* [19].

Figure 3 shows distribution of these compounds in samples collected from 6 different locations in the coastal waters of Sabah. Content of active compounds are as follows; Tg Aru (58%) > Sepanggar Island (47%) > Banggi Island (41%)> Mantanani Island (32%)> Lankayan Island (28%)> Sipadan Island (26%). In all the samples examined, compound **1** was present in a higher percentage as compared to compound **2**; details are shown in Figure 3. Since, only two compounds were found consistently in samples of *L.similis* from Sabah waters, perhaps it is reasonable to assume that these two compounds are the chemotaxonomical markers for this seaweed in the coastal waters of Sabah. Besides, these findings also suggest the possibility that there might not be any chemical races in *L. similis*.

Both the isolated compounds were subjected to antimicrobial bioassays; antiyeast (7 pathogenic strains), antibacterial (6 environmental strains and 12 pathogenic strains), and antifungal (6 environmental strains). Details of the results are shown in Table 1. Compound 1 showed a wide spectrum of activity against the tested pathogenic yeast, environmental bacteria and fungus but not against the pathogenic bacteria. However, compound **2** showed only weak biologically activity against the tested organism.

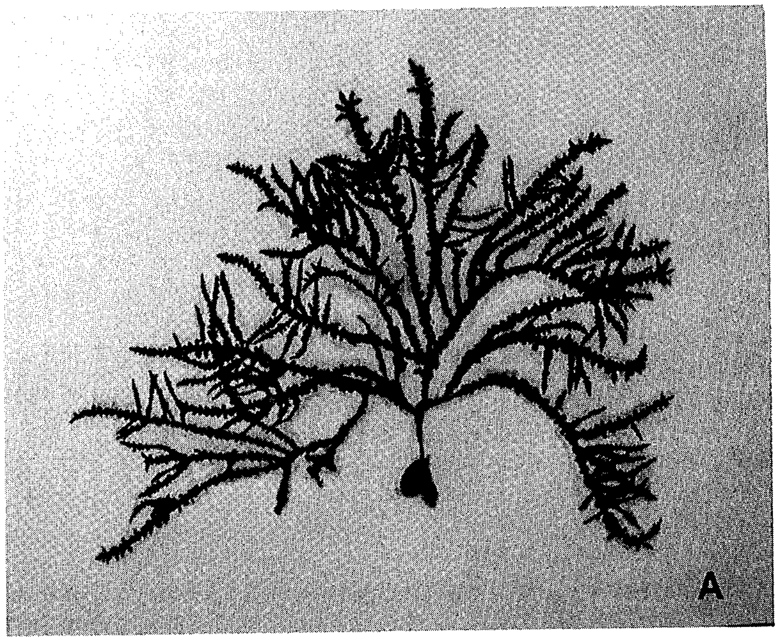

Figure 1A. Plants Herbarium tetrasporangial specimen (scale bar = 1cm).

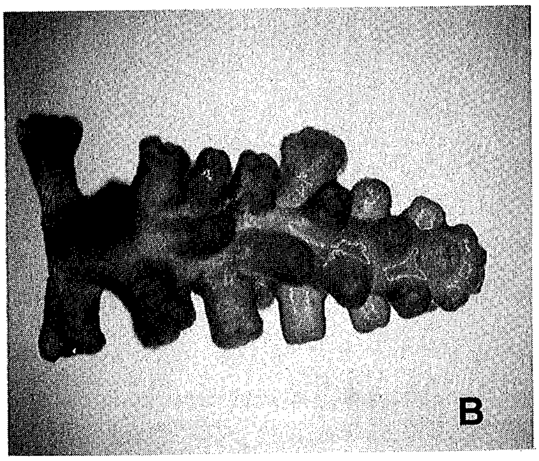

Figure 1B. Main axis of the plant above holdfast showing gradual tapering of its width towards the tip (scale bar = 1 mm).

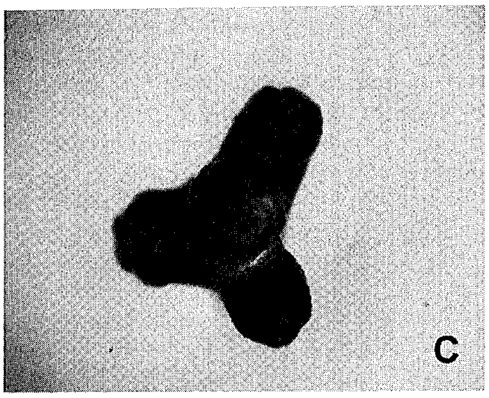

Figure 1C. Main axes produce numerous first-order branches in an alternate-distichous manner at an angle of 120° (scale bar = 0.5 mm).

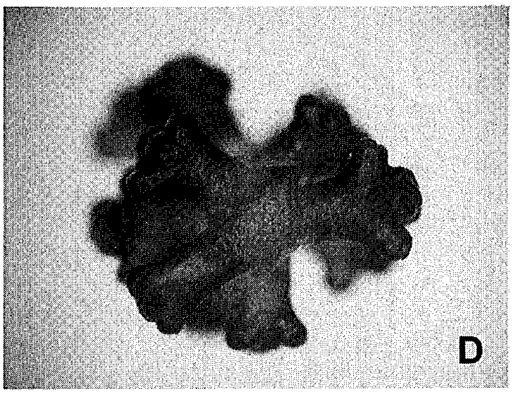

Figure 1D. Multiple branches are formed on primary and secondary axes in an alternate-spiral manner

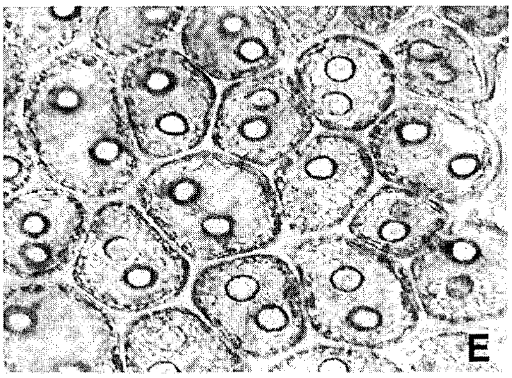

Figure 1E. Surface view near the apex of an ultimate branch; note each superficial cortical cell contains two *corps en cerise*.

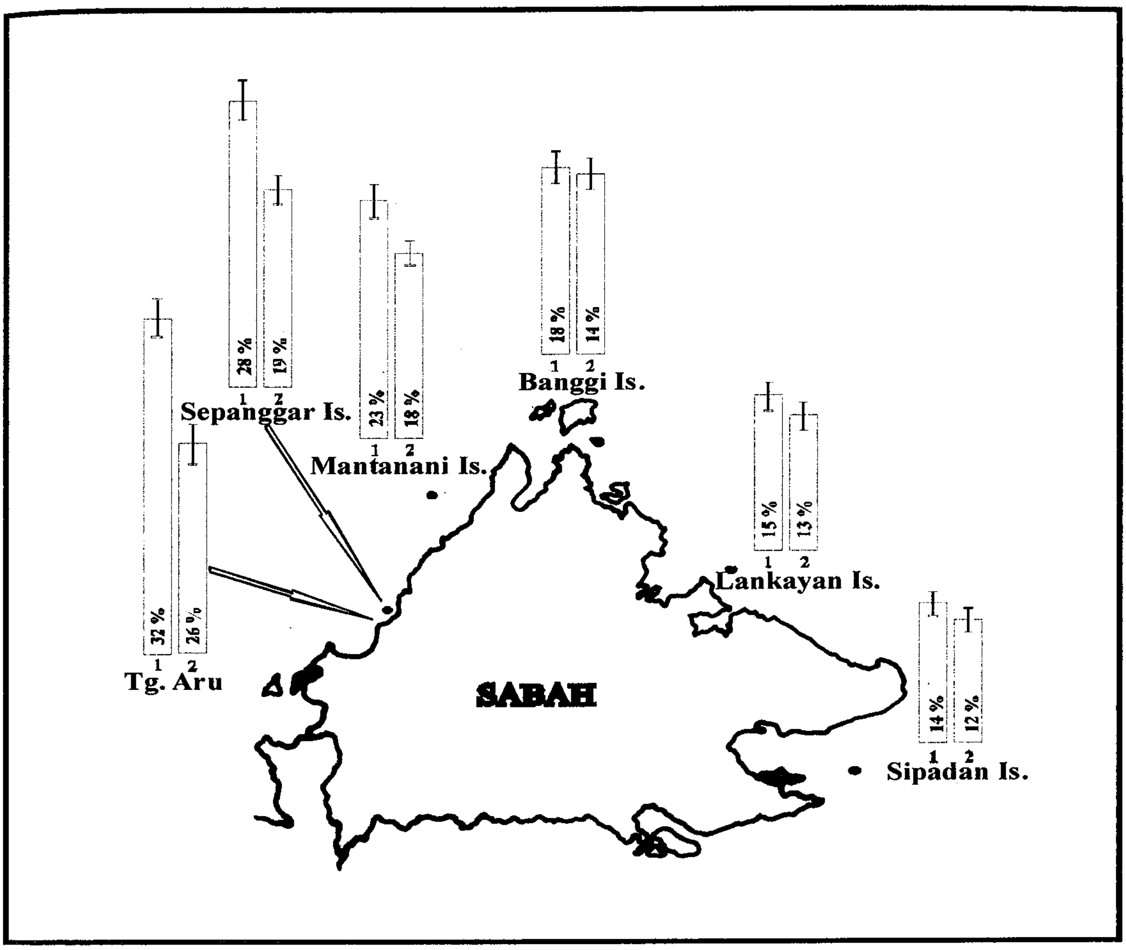

Figure 3. Distribution of bromoindoles (% of crude extract) in samples of *L. similis* collected from 6 locations in the coastal waters of Sabah.

CONCLUSION

Data presented in this study suggests that *L. similis* contains only two halogenated compounds, 2,3,5,6-tetrabromoindole (**1**) and 1-methyl-2,3,5,6-tetrabromoindole (**2**). Besides, data obtained from this study also showed that samples collected from various locations in the coastal waters of Sabah did not show any differences in compound diversity, hence, suggesting the absence of chemical race in *L.similis*. These compounds could be regarded as its chemotaxonomical marker. Isolated compounds also showed medium antimicrobial potency as compared to the commercially available antibiotics.

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