CHEMOTAXONOMICAL EVALUATION OF THE RED ALGA *LAURENCIA NANGII* MASUDA (RHODOMELACEAE, CERAMIALES) IN THE COASTAL WATERS OF SABAH

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DECLARATION

I hereby declare that this thesis contains my original research work. Sources of findings reviewed herein have been duly acknowledged.

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TITLE	:	**CHEMOTAXONOMICAL EVALUATION OF THE RED ALGA *LAURENCIA NANGII* MASUDA (RHODOMELACEAE, CERAMIALES) IN THE COASTAL WATERS OF SABAH**
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ABSTRACT

CHEMOTAXONOMICAL EVALUATION OF THE RED ALGA *Laurencia nangii* Masuda (RHODOMELACEAE, CERAMIALES) IN THE COASTAL WATERS OF SABAH

Species discrimination in the red alga genus *Laurencia* is difficult due to a high degree of morphological variation within individual species. Chemical studies on a worldwide basis revealed that most species of *Laurencia* can be characterized by a specific set of halogenated secondary metabolites. Some species, however, produce unrelated sets of metabolites depending on geographical distribution. The morphologically similar but chemical distinct populations can be referred to as sibling species or chemical races. This study was carried out to identify the halogenated secondary metabolites that can be utilized as taxonomic markers for *Laurencia nangii* Masuda. Samples of *L. nangii* from three localities: Pulau Sulug, Kota Kinabalu; Pantai Bak-Bak, Kudat; and Dogoton, Pulau Banggi, Kudat were investigated. Voucher herbarium specimens of each population (BORH 37572–37577, 37587–37588) were deposited in the Borneensis Collection in the Institute for Tropical Biology and Conservation. Plants are purplish-green in colour and attain height of 3 – 6.5 cm, with several upright axes arise from discoid holdfast and stolon-like branches. Microscopy study showed that superficial cortical cells contain two to three *corps en cerise*, while trichoblast cells contain one or two *corps en cerise*. Tetrasporangia are formed on all four orders of branches and measure 100 – 120 μm in diameter. Ovoid cystocarps are formed on first- to fourth-order branches, measure 550 – 800 μm in height by 500 – 750 μm in diameter. Male trichoblasts are formed in the cup-shaped apical depression of first- to fourth-order branches. Samples were extracted and the crude extracts from these three populations showed different chemical profiles on TLC and RP-HPLC. Crude extracts were fractionated by column chromatography and isolation of secondary metabolites was carried out via RP-HPLC. The structures of the isolated compounds were deduced from ^{1}H-NMR, ^{13}C-NMR, and 2D NMR (COSY, HSQC, HMBC and NOESY) spectra. Three C_{15}-acetogenins (**C1** – **C3**) were isolated from Pulau Sulug's sample. **C1** present in 2.4% of crude extract, **C2**, 2.0% and **C3**, 1.0%. Five C_{15}-acetogenins (**C4** – **C8**) were isolated from Pantai Bak-Bak samples. **C4** present in 1.8%, **C5**, 1.3%, **C6**, 2.6%, **C7**, 1.1% and **C8**, 1.9%. And, one C_{15}-acetogenin, **C9** (15.0%) was isolated from Dogoton's sample. Thus it is conclusive that *L. nangii* is characterized by C_{15}-acetogenins and they could serve as chemotaxonomic markers for the species. Since these compounds are derived from similar metabolic pathway, sibling species or chemical races may not be present in this species.

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ABSTRAK

Diskriminasi spesis alga merah genus Laurencia adalah sukar disebabkan oleh perbezaan morfologi yang wujud dalam sesuatu spesis. Kajian atas genus ini di pelosok dunia menunjukkan bahawa kebanyakan spesis dapat dicirikan dengan suatu set metabolit sekunder berhalogen yang tersendiri bagi sesuatu spesis tersebut. Namun, terdapat juga spesis yang menghasilkan set metabolit sekunder yang berlainan bergantung pada taburan geografi. Spesis sebegini yang mempunyai ciri morfologi yang sama tetapi kandungan sebatian kimia yang berlainan dikatakan mempunyai ras kimia (chemical race). *Kajian ini dilakukan untuk mengenalpasti metabolit sekunder berhalogen yang boleh dijadikan sebagai petanda taksonomi bagi* Laurencia nangii *Masuda. Persampelan dilakukan di tiga lokasi di persisiran pantai Sabah, iaitu: Pulau Sulug, Kota Kinabalu; Pantai Bak-Bak, Kudat; dan Dogoton, Pulau Banggi, Kudat. Specimen herbarium untuk setiap populasi (BORH 37572–37577, 37587–37588) didepositkan dalam Koleksi Borneensis di Institut Biologi Tropika dan Pemuliharaan. Talus berwarna hijau-keunguan dan mencapai ketinggian 3.0 – 6.5 cm; beberapa cabang tegak muncul dari pelekap berbentuk cakera dan dari cabang stolon. Kajian mikroskopi menunjukkan sel kortikal permukaan mengandungi dua hingga tiga 'corps en cerise', manakala sel trikoblas mengandungi satu atau dua 'corps en cerise'. Tetrasporangium yang berukuran 100–120 μm membentuk pada anak cabang. Sistokarpa berbentuk bujur dengan ketinggian 550 – 800 μm dan diameter 500 – 750 μm. Trikoblas jantan membentuk dalam liang pada hujung anak cabang. Sampel dari ketiga-tiga populasi diekstrak dan profil kimia (TLC dan HPLC) ekstrak kasar menunjukkan corak yang berlainan. Pembahagian ekstak kasar dilakukan melalui kromatogarfi kolum dan pemencilan metabolit sekunder dilakukan melalui RP-HPLC. Struktur kimia ditentukan melaui kaedah spektroskopi ^{1}H-RMN, ^{13}C-RMN, dan RMN 2D (COSY, HSQC, HMBC and NOESY). Tiga sebatian C_{15}-acetogenin (**C1-C3**) dipencilkan daripada sampel dari Pulau Sulug: **C1**, 2.4%, **C2**, 2.0%, **C3**, 1.0%; lima sebatian C_{15}-acetogenin (**C4-C8**) dipencilkan daripada sampel dari Pantai Bak-Bak: **C4**, 1.8%, **C5**, 1.3%, **C6**, 2.6%, **C7**, 1.1%, **C8**, 1.9%; dan satu sebatian C_{15}-acetogenin, **C9** (15%) dipencilkan daripada sampel dari Dogoton. Daripada kajian ini, adalah didapati bahawa* L. nangii *dicirikan dengan sebatian C_{15}-acetogenin yang berasal dari laluan metabolik yang sama, maka ras kimia tidak wujud dalam spesis ini.*

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LIST OF ABBREVIATIONS

CC	Column chromatography
$CDCl_3$	Deuterated chloroform
dH_2O	Distilled water
EtOAc	Ethyl acetate
Et_2O	Diethyl ether
FTIR	Fourier transform infrared
HSQC	Heteronuclear single quantum coherence
HMBC	Heteronuclear multiple bond correlation
HREIMS	High resolution electron ionization mass spectrometry
LREIMS	Low resolution electron ionization mass spectrometry
KBr	Potassium bromide
MeOH	Methanol
MeCN	Acetonitrile
Mp	Melting point
Na_2SO_4	Anhydrous sodium sulphate
NOESY	Nuclear Overhauser effect spectroscopy
RP-HPLC	Reverse phase High performance liquid chromatography
R_f	Mobility relative to front
T_R	Retention time
Si	Silica
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV-Vis	Ultra violet – visible
^1H-^1H COSY	Proton-proton correlation spectroscopy
^1H-NMR	Proton nuclear magnetic resonance
^{13}C-NMR	Carbon-13 nuclear magnetic resonance
2D	Two dimensions

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LIST OF SYMBOLS

v/v	volume to volume
w/v	weight to volume
%	percent
°C	degree Celsius
min	minute
g	gram
L	litre
mL	millilitre
μL	microlitre
cm	centimetre
mm	millimetre
μm	micrometre
nm	nanometre
MHz	mega hertz
Hz	hertz
δ	chemical shift
J	coupling constant
s	singlet
dd	doublet of doublet
ddd	doublet of doublet of doublet
dq	doublet of quadruplet
m	multiplets
t	triplet
$[\alpha]_D$	optical rotation
ν	wave numbers
m/z	mass to charge ratio

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CHAPTER 1

INTRODUCTION

1.1 Red Algae Genus *Laurencia*

Marine red algae genus *Laurencia* was first described by Lamouroux in the year 1813. It is a relatively large genus with approximately 135 species, which spans a wide geographical range, having been documented in tropical, sub-tropical and temperate waters (Masuda *et al.*, 1996). It is classified under the phylum Rhodophyta, class Rhodophyceae, subclass Florideophyceae, order Ceramiales, family Rhodomelaceae.

Species discrimination in *Laurencia* is complicated by high degree of morphological variations within individual species, which at times, lead to reclassification or reduction of some species to heterotypic synonyms of other species (Masuda *et al.*, 1996). Members of this genus can be found in small sizes (a few mm long) to large size ones (up to 50 cm long), each of which has been characterized by a combination of many morphological features (Masuda and Suzuki, 1997). Based upon morphological and chemical studies (Masuda *et al.*, 1996), species of *Laurencia* can be generally categorized into 2 main groups: i) Species that do not posses *corps en cerise* and hence contain no halogenated secondary metabolite. Examples of species in this group include: *L. cartilaginea* Yamada, *L. capituliformis* Yamada, *L. concreta* A.B. Cribb, *L. intermedia* Yamada, *L. palisada* Yamada, *L. papillosa* (C. Agardh) Greville, and *L. undulata* Yamada, and, ii) Species that posses *corps en cerise* and produce halogenated secondary metabolites. These include: *L. aldingensis* Saito & Womersley, *L. brongniartii* J. Agardh. *L. distichophylla* J. Agardh, *L. elata* (C. Agardh) J.D. Hooker & Harvey, *L. intricata* J.V. Lamouroux, *L. japonensis* T. Abe & Masuda, *L. majuscula* (Harvey) Lucas, *L. mariannensis* Yamada, *L. microcladia Kützing, L. nana* (C. Agardh) Greville, *L. nangii* Masuda, *L. nidifica* J. Agardh, *L. nipponica* Yamada, *L. obtusa* (Hudson) J.V. Lamouroux, *L. okamurae* Yamada, *L. omaezakiana* Masuda, *L. pacifica* Kylin, *L. palisada* Yamada, *L. pannosa* Zanardini, *L. pinnata* Yamada, *L.*

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pinnatifida (Hudson) J.V. Lamouroux, *L. pygmaea* Weber-van Bosse, *L. saitoi* Perestenko, *L. similis* Nam & Saito, *L. snackeyi* (Weber-van Bosse) Masuda, *L. snyderae* Dawson, *L. venusta* Yamada, *and L. viridis* Gil-Rodríguez & Haroun (Suzuki and Vairappan, 2005).

Corps en cerise are intracellular refractive globular inclusions, which distribute exclusively in superficial cortical cells and trichoblast cells, where halogenated secondary metabolites are synthesized and/or stored (Young *et al.*, 1980). The presence or absence and their number have a valuable taxonomic meaning at specific level within the genus *Laurencia* (Furnari *et al.*, 2001).

1.2 Chemotaxomomy Study of *Laurencia*

The genus *Laurencia* has attracted intense interest among organic chemists due to its ability to produce diverse types of halogenated secondary metabolites. The first chemical investigation on the red algae *Laurencia* was done in 1953 when Obata and Fukushi studied the chemistry of *Laurencia nipponica* Yamada (as *Laurencia glandulifera* Kützning) from Oshoro Bay, Hokkaido, Japan and reported the odoriferous components of the essential oil to be sesquiterpenoids. In 1963, Irie and coworkers started their continuous studies on the chemical constituents of *Laurencia* that led to the isolation of Laurencin, the first brominated acetogenin, from the same alga collected at the same location in 1965. They isolated laurinterol and debromolaurinterol from *L. okamurae* Yamada (as *L. intermedia* Yamada) the following year. Since then, various groups around the world have carried out research on this aspect of the algae. To date, more than 500 diverse and unique halogenated secondary metabolites, in particular, sesquiterpenoids, diterpenoids, triterpenoids and C_{15} acetogenins, have been discovered from more than 60 *Laurencia* species worldwide (Suzuki and Vairappan, 2005).

Various studies (Erickson, 1983; Suzuki and Vairappan 2005) led to the observation that, while there is a certain degree of overlap, most species in this genus can be characterized by at least one specific compound not found in the others or a particular set of compounds, render them as useful taxonomic characters for species discrimination. In the year 1975, Fenical and Norris

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differentiated three different entities passing under the name of *Laurencia pacifica* Kylin from the Gulf of California based on comparative thin layer chromatography of lipid components. Progressively, in the year 1980, Howard *et al.* investigated a few species of *Laurencia* under culture and found that the production of secondary metabolites were unaffected by varying photoperiod and temperature conditions and the chemical compounds produced were both quantitatively and qualitatively identical to those of the natural populations.

It is noted too that some species produce unrelated sets of metabolites depending on geographical distribution, i.e. each chemical types is characterized by a specific end product of halogenated secondary metabolism. In the case of morphologically similar but chemical distinct populations, these can be referred to as sibling species or chemical races. The difference between these two is that, reproductive isolation is presence among differentiated populations for the formal, as exemplified by *L. pacifica* Kylin (Fenical and Norris, 1975); and the opposite for the latter. *L. nipponica* Yamada is the classical example of a species that has several chemical races, the first report in marine algae (Masuda *et al.*, 1997a). By virtue of their stability of specificity, halogenated secondary metabolites proved to be useful taxonomic feature at species level when the metabolites are comprehensively studied throughout the algal geographical range (Masuda *et al.*, 1997a; Suzuki and Vairappan, 2005).

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1.3 *Laurencia* species in Malaysian Waters

Documentations on the genus *Laurencia* in Malaysian waters are relatively limited (Ismail, 1995; Masuda *et al.*, 2001; Yamagishi *et al.*, 2003). Even fewer reports were published on its chemical compositions: *L. snackeyi* (Masuda *et al.*, 1997b), *L. similis* (Masuda *et al.*, 1999; Vairappan *et al.*, 2004), *L. nangii* (Masuda *et al.*, 2002a), *L. majuscula* (Vairappan *et al.*, 2001a, 2003), *L. pannosa* (Suzuki *et al.*, 2001), and *L. pygmeae* (Vairappan *et al.*, 2001a; Yamagishi *et al.*, 2003).

Laurencia nangii Masuda (Figure 1.1) is a relatively new species originally described from Vietnam (Masuda, 1997) and was reported from Malaysian water as recent as the year 2002 (Masuda *et al.*, 2002a). It is readily recognizable by its

fleshy and soft, green colour thalli. It is characterized by several upright terete, percurrent axes (2 – 6 cm in length) arise from a common discoid holdfast and from stolon-like branches, and produce first-order branches, which are arranged in an irregular spiral and bear progressively shorter branches of up to four order. Our survey (unpublished data) revealed that this species is widely distributed along the coastal waters of Sabah, commonly found growing epiphytically on various algae or on dead coral in the lower intertidal to upper subtidal zone.

Figure 1.1: *Laurencia nangii* Masuda

Masuda *et al.* (2002) reported the isolation of three C_{15} acetogenins: *cis*-pinnatifidenyne (**1**), obtusenyne (**2**) and (3*Z*)-laurenyne (**3**) from a sample collected from Pulau Tiga, Kuala Penyu; while a sample from Pulau Bai, Sandakan, contained (**1**) and (**2**) and diterpenoid, aplysiadiol (**4**) instead of (**3**). Figure 1.2 shows the chemical structures of (**1**) – (**4**). The authors suggested the presence of two chemical races in this species in its Malaysian populations: 1) a race characterized by the production of only acetogenins and distributed on the west coast of Sabah, and 2) another race characterized by the production of acetogenins along with aplysiadiol (diterpenoid) on the east coast of Sabah.

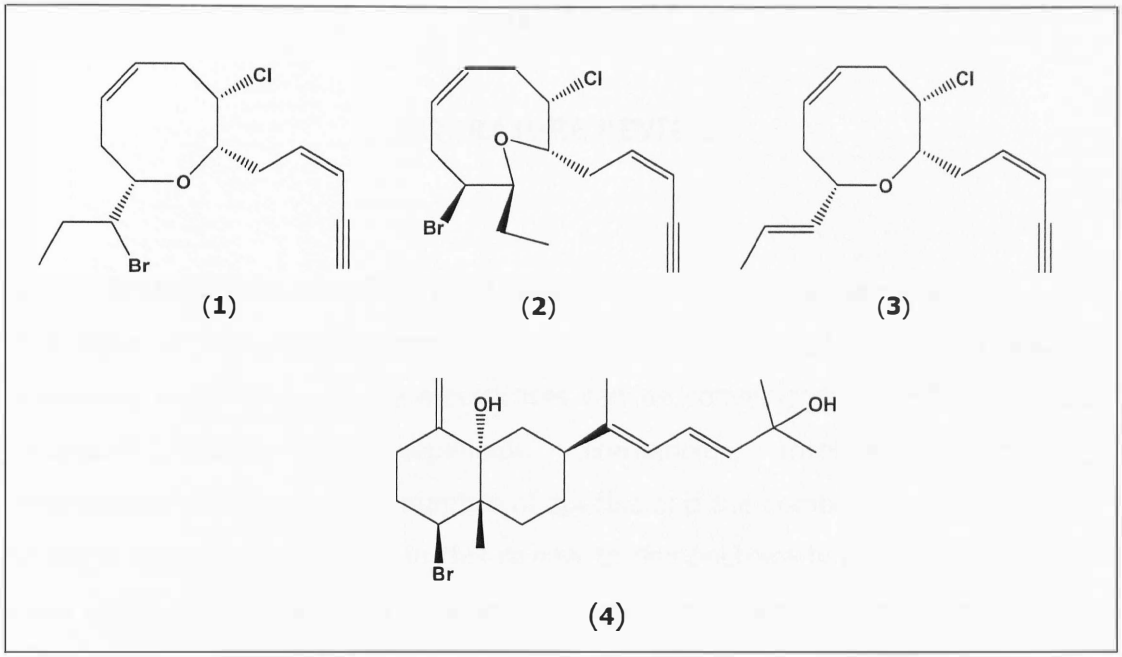

Figure 1.2: C_{15} acetogenins and diterpenoid isolated from *Laurencia nangii* Masuda.

To date, this is the only report published on the chemical composition of *L. nangii.* Hence, present research is undertaken to identify chemotaxonomical markers for *Laurencia nangii* Masuda as part of the effort to document the distribution and the chemical composition of red algae genus *Laurencia* in Malaysian waters.

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1.4 Objectives

The objectives of this study are:

1. To describe morphological features of *L. nangii.*
2. To quantify crude extract contents and chemical profiles of *L. nangii* collected from three localities in the coastal waters of Sabah.
3. To isolate and characterize halogenated secondary metabolites from these three populations.

CHAPTER 2

LITERATURE REVIEW

2.1 Distribution and Chemical Diversity of *Laurencia* Species

Red algae of the genus *Laurencia* produce a wide variety of structurally unusual secondary metabolites. These metabolites can be conveniently classified into four structural classes: sesquiterpenoids, diterpenoids, triterpenoids and C_{15} acetogenins. Due to the vast number of species and the compounds they produce, selected species are included in this review to demonstrate how these compounds have aided in species discrimination in this taxonomically troublesome genus. Information regarding the geographical distribution of the species mentioned in section 2.1.1 – 2.1.11 below was obtained from Guiry and Guiry, 2007.

2.1.1 *Laurencia brongniartii* Agardh

Laurencia brongniartii has been reported from various localities of tropical to subtropical regions in the world: Ireland, Canary Islands, Cuba, Ghana, Madagascar, South Africa, the Philippines, Sri Lanka, Japan, Taiwan, Indonesia, Australia and New Zealand.

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This species is characterized by polyhalogenated indoles. Sample collected in the Caribbean Sea was found to produce 1-methyl-2,3,5,6-tetrabromoindole (**5**), 2,3,5,6-tetrabromoindole (**6**), 1-methyl-2,3,5-tribromoindole (**7**), 1-methyl-2,3,6-tribromoindole (**8**). One the other hand, a Taiwanese population and a southern Japanese population were reported to contain four methylthiobromoindoles in common: (**9**), (**10**), (**11**) and (**12**). The Japanese population further contains two methylsulfinylbromoindoles: Itomanindole A (**13**) and itomanindole B (**14**), two simple bromoindoles: (**15**) and (**16**) and methylthiobromoindoles (**17**) and (**18**) (Masuda *et al.*, 1999; Suzuki and Vairappan, 2005). Figure 2.1 shows the chemical structures of (**5**) – (**18**).

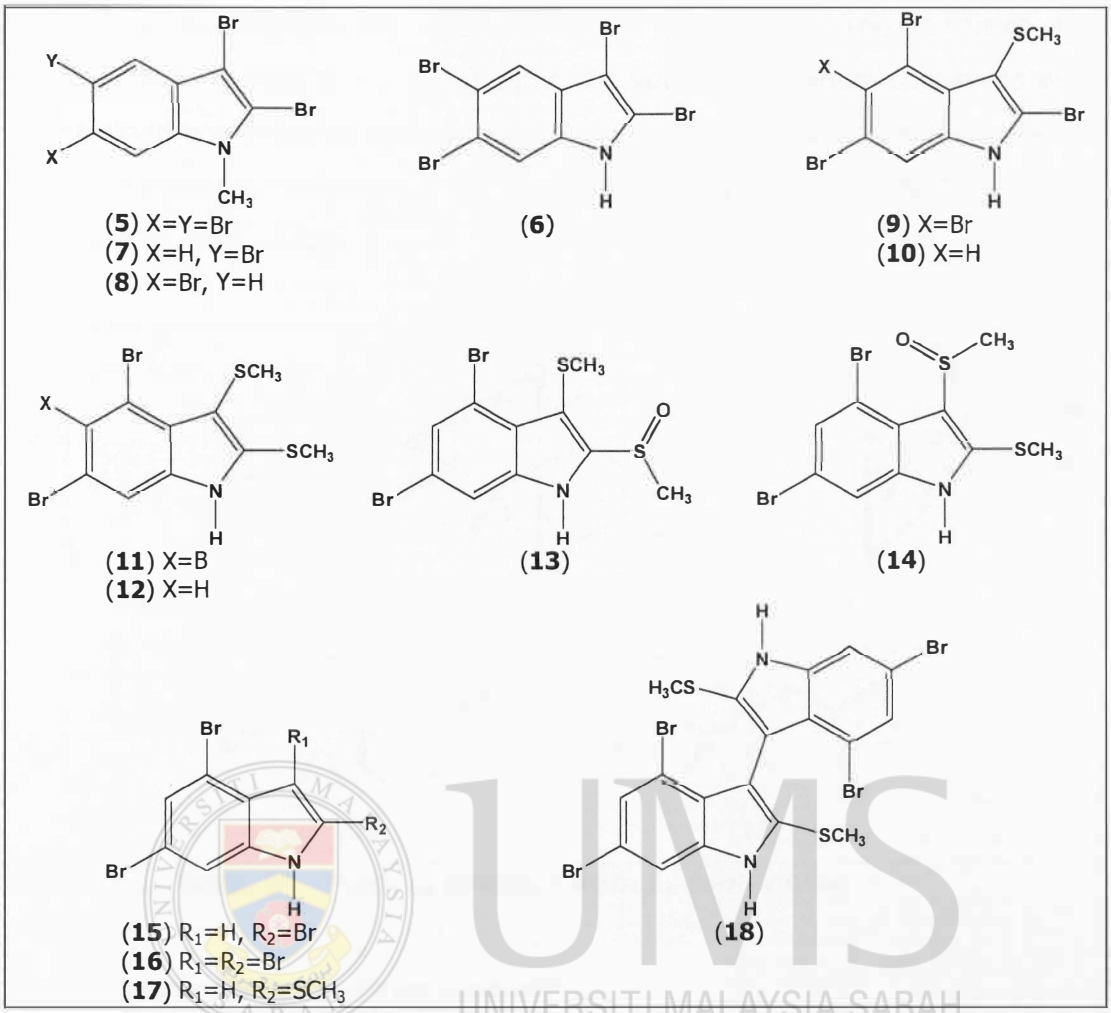

Figure 2.1: Polybromoindoles isolated from *L. brongniartii*.

2.1.2 *Laurencia composita* Yamada

Laurencia composita has been reported from Caribbean Islands, the Philippines and several localities along the Pacific coasts of Japan. This species is characterized by oxygenated chamigrane-type sesquiterpenoids. Masuda *et al.* (1996) reported pacifenol (**15**), prepacifenol epoxide (**16**) and johnstonol (**17**) from this species collected at Matsuura, Nagasaki Prefecture (Pref.), Awaji, Hyogo Pref. and Tateyama, Chiba Pref. Population collected from Tanegashima Island, Kagoshima Pref. also contains these three compounds along with another two chamigrenes: 2,10-dibromo-3-chloro-α-chamigrene (**18**) and 2,10-dibromo-3-chloro-9-hydroxy-α-chamigrene (**19**) (Masuda *et al.*, 2002b). Earlier on, Suzuki and Kurosawa (1985) confused this species as *L. okamurae* when they reported that the population from Iwaizaki, Mie Pref. contains two sets of sesquiterpenoids, chamigranes (pacifenol,

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