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Characterization of volatile composition of *Laurencia dendroidea* by gas chromatography-mass spectrometry

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Abstract: In this study we report the characterization of the volatile compounds of *Laurencia dendroidea.* Solvent extracts (dichloromethane and methanol), hydrodistillation extracts and headspace solid-phase microextraction samples were obtained and analyzed by GC-MS. Forty-six volatile components were identified in *L. dendroidea*, among them hydrocarbons, alcohols, phenols, aldehydes, ketones, acids, esters and terpenes.

Article

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Introduction

Red algae, especially many species of the genus *Laurencia* J.V. Lamouroux (Ceramiales, Rhodomelaceae), have proven to be a prolific source of secondary metabolites and have been the subject of intensive research in the field of marine natural products (Faulkner, 2000; Guaratini et al., 2005; Cardozo et al., 2006; Cardozo et al., 2007). Many of *Laurencia*'s metabolites have been found to possess a variety of biological activities such as antibacterial (Vairappan, 2003; Kladi et al., 2008; Vairappan et al., 2008), cytotoxic (Kladi et al., 2006; Kladi et al., 2007; Cen-Pacheco et al., 2011; Lhullier et al., 2010), antioxidant (Li et al., 2009), antimalarial (Topcu et al., 2003), antihelmintic (Davyt et al., 2001), analgesic (Chatter et al., 2009), antitrypanossomal (Veiga-Santos et al., 2010) and antileishmanial (Machado et al., 2011) activities.

Volatile compounds play an important role in chemical communication for algae in marine ecosystems (Fink, 2007) and many of them also have other biological importance. These compounds can be obtained by several extraction processes like hydrodistillation (HD), hydrodestillation (HD) focused microwave-assisted hydrodistillation (FMA-HD), solvent extraction (SE), supercritical fluid extraction (SFE), headspace (HS) and headspace solid-phase microextraction (HS-SPME), and characterized by Gas Chromatography-Mass Spectrometry (GC-MS) (Gressler et al., 2009).

Laurencia species are found all over the world in the tropical and subtropical seas. This genus comprises more or less 150 species and around thirty of them occur in Brazil (Machado et al., 2010). Therefore, the purpose of the present investigation was to identify the volatile compounds of *L. dendroidea* obtained by different extraction procedures (hydrodistillation, solvent extraction and by headspace solid-phase microextraction) using the GC-MS technique.

Materials and Methods

Seaweed samples

The red macroalgae *Laurencia dendroidea* (Mont) M. Howe was collected at Parati beach in Anchieta, Espírito Santo State, Brazil, in October 2006, and identified by Dr. Mutue Toyota Fujii. A voucher specimen was deposited in the São Paulo State Herbarium "Maria Eneyda P. K. Fidalgo" as SP 399.947.

Hydrodistillation (HD)

The hydrodistillation was done as previously described (Gressler et al., 2011). In summary, around 400 g of fresh material was extracted in a Clevenger-type apparatus for 4 h. The essential oil was then diluted 100-

fold with ethyl acetate prior to analysis by GC-MS.

Solvent extraction (SE)

The solvent extracts were obtained as follow: freeze-dried algae (5 g) were subjected to extraction with 50 mL of dichloromethane in an ultrasonic bath for 30 min. After that, the solvent was filtered through Whatman 41 filter paper. The remaining alga was re-extracted two more times and the combined solvent fractions concentrated by rotary evaporation. The algal residue was then extracted with 50 mL of methanol, also in an ultrasonic bath, for 30 min. The solvent was filtered and the methanol extraction repeated 2 more times.

The residues of the dichloromethane extract (DE) and the methanol extract (ME) were resuspended in 1 mL of ethyl acetate and analyzed separately by GC-MS.

Headspace Solid-phase microextraction (HS-SPME)

Because of the high water content present in algae and the desire to avoid drop formation in the extraction flasks, the extraction of the volatile compounds by HS-SPME were performed with lyophilized algal material.

The HS-SPME parameters, including algae sample weight, extraction temperature, equilibrium time and extraction time, were optimized. For this optimization, three of the parameters were kept invariant and the other parameter changed, as described by Guo et al. (2006). The final optimal conditions resulted in the following procedure: lyophilized algae (50 mg) were placed in a 10 mL glass flask and the flask hermetically sealed. In parallel, a second flask with the same amount of sample was sealed with a thermometer and used as temperature controller for the headspace. Both flasks were placed on a magnetic stirrer with heater. The temperature of the headspace (60 °C) was equilibrated for 15 min and then the SPME fiber exposed to the headspace. The HS-SPME extraction was performed during 15 min and, immediately afterward, the fiber was inserted into the injector of the GC-MS for analysis. The fiber was left inside the injector during 10 min to assure that all compounds were desorbed. Four different types of adsorbents were used in order to extract different groups of analytes, these being: polydimethylsiloxane (PDMS), 100 µm; polydimethylsiloxane-divinylbenzene (PDMS-DVB), 65 µm; carboxen-polydimethylsiloxane (CAR-PDMS), 75 µm; and polyacrylate (PA), 85 µm.

Gas chromatography/mass spectrometry (GC-MS) analysis

The samples of volatile compounds extracted by the different procedures were analyzed in a gas chromatograph coupled to a mass spectrometer (GCMS-QP2010 Plus, Shimadzu, Japan). The chromatographic separation was achieved with an Rtx-5MS capillary column (30 m x 0.25 mm x 0.1 µm) and helium was used as the carrier gas (with a flow rate of 1 $mL.min^{-1}$). The oven temperature was increased at a rate of 3 $°C.min^{-1}$ from 60 to 260 °C and held at the latter temperature for 40 min. Injection and transfer line temperatures were 220 and 240 °C. The detection was performed in the full scan mode, with a mass range of 50-650 *m/z*. Electron impact ionization was employed with a collision energy of 70 eV and the mass spectrometer ion source was maintained at 240 °C.

Qualitative and quantitative analysis

The volatile compounds were identified by matching mass spectra with spectra of reference compounds present in the National Institute of Standards and Technology (NIST 08) mass spectral library and by comparison with the literature Kovats Index (KI). When there was no spectrum for comparison, no identification was proposed. The relative amounts of individual components were expressed as percent peak areas relative to the total peak area.

Antimicrobial and antioxidant tests

The antimicrobial and antioxidant activities were done as previously described (Gressler et al., 2010; 2011).

For the antimicrobial test, the microorganisms used were: *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212), *Streptococcus pneumoniae* (ATCC 49619), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella typhi* (ATCC 19430), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), and one yeast strain, *Candida albicans*, (ATCC 10231). The Minimal Inhibitory Concentration (MIC) was determined according to NCCLS M100-S15 and M38-A (NCCLS, 1998 and 2005).

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) antioxidant test was done according to Yen & Chan (1995) with some modifications. The chemiluminescence antioxidant test was performed according to Krol et al. (1994).

Results and Discussion

Optimization of HS-SPME extraction

In order to identify the largest possible number of volatile compounds of the algae by HS-SPME, four different fibers, *i.e.*, PDMS, PDMS-DVB, CAR-PDMS and PA, were chosen. The PDMS fiber is more suitable for the analysis of low molecular weight polar and non-

polar compounds, PDMS-DVB for alcohols and amines, CAR-PDMS for hydrocarbons and PA for phenols and carboxylic acids.

To optimize the HS-SPME extraction conditions, the total peak area was used as a parameter. First, the amount of lyophilized algae was investigated. For this, the intermediate values of extraction temperature (60 °C), equilibrium time (15 min) and extraction time (15 min) were selected. Analysis with 50, 100 and 200 mg of lyophilized algae, showed a gradual increase of the total peak area with increasing amount of algae. However, when 50 mg was used, the chromatograms obtained were cleaner, with less peak overlap and without loss of a number of peaks. This result was observed for all types of fibers tested, with the exception of the PA fiber and *L. intricata*, where 200 mg of algae was used.

Having selected the amount of algae, different equilibrium and extraction times were evaluated (5, 15 and 25 min each) at the extraction temperature of 60 °C. The total peak area increased with the increase in the temperature used. Regarding the equilibrium time, no relevant differences were observed in the composition and relative amount of the extracted volatile compounds. On the other hand, the extraction time intervals showed a tendency toward fiber saturation, leading to broadening and overlapping of some of the peaks and a rise in the base line of the chromatograms. Thus, in order to reduce the extraction time and to obtain more informative chromatograms, equilibrium and extraction times of 15 min were chosen.

The use of a high sampling temperatures can induce degradation of volatiles. Therefore, the temperature range studied was restricted to 40-80 °C. The extraction and equilibrium times were kept constant and the extraction carried out at 40, 60 and 80 °C. This resulted in expressive differences between the chromatograms. In general, at 40 °C, some peaks disappeared and others decreased in intensity when compared with 60 and 80 °C. Comparing the chromatograms at extraction temperatures of 60 and 80 °C, the same number of peaks was observed. However, an increase in the peaks intensities, peak overlap and an increase of the chromatographic base line were seen when 80 °C was employed. Considering these factors, the extraction temperature that gave the best response was 60 °C. According to Guo et al. (2006), the diffusion coefficients of the analytes increase with an increase in temperature and the concentration of the volatile components in the headspace should be increased, favoring extraction at higher temperatures. On the other hand, the partition coefficients of the analytes between the microdrop and the headspace decrease with increasing temperature and, consequently, decrease the amount extracted.

Analysis of the volatile compounds

The species *L. dendroidea* showed 203 different volatile compounds by the extraction methods performed, some of them being particular to one type of extraction and others common to two or more samples. It was possible to identify only 46 metabolites, probably because of the absence of algae-derived compounds in the GC-MS libraries. Among the compounds identified, terpenes, alcohols, acids, esters, ketones and hydrocarbons were found.

As previous reported by Gressler et al. (2011), the sesquiterpenes 7-*epi*-silphiperfolan-6β-ol and silphiperfolan-7β-ol have the same retention time and the identifications were done by the comparison of the mass spectral overlap obtained with laboratory standards of both compounds and by the individual KI. Table 1 shows the composition of *L. dendroidea* in more details.

Antimicrobial and antioxidant activities

The dichloromethane and methanol extracts were tested to verify their antimicrobial and antioxidant activities. No significant antimicrobial inhibition was observed for the microorganism at the concentrations tested.

The results summarized in Figure 1 show that both extracts were effective towards the scavenging of DPPH radicals. The dichloromethane extract showed a better antioxidant activity (65.4±2.4% at 500 $\mu g.mL^{-1}$) comparing to the methanol extract (32.7±1.1% at 500 $\mu g.mL^{-1}$) by the DPPH method and by the chemiluminescence method (~100% at 500 $\mu g.mL^{-1}$ for DE and 25.9±1.2% at 500 $\mu g.mL^{-1}$ for ME). For the DE, the IC50 obtained were 340.9 $\mu g.mL^{-1}$ by the DPPH method and 48.5 $\mu g.mL^{-1}$ by the chemiluminescence method. The antioxidant profile of both extracts by the two methods is shown in Figure 1. The antimicrobial and antioxidant activities of the essential oil of *L. dendroidea* have already been published (Gressler et al., 2011).

The DPPH radical scavenging assay has often been performed for evaluation of the anti-radical activity of antioxidants since DPPH possesses an odd electron, responsible for the strong absorption peak at 517 nm (Ayhan-Kilcigil et al., 2004). However, formation of free radical intermediates from oxidized compounds, which could become important propagators of free radical chain reactions, cannot be detected by this assay (Okawa et al., 2001). The chemiluminescence method, the other experimental model used, is based on the antioxidant-dependent quenching of the chemiluminescence generated by luminol/peroxidase/H_2O_2 (Dodeigene et al., 2000). According to Hirayama et al. (1997), the chemiluminescence method is more sensitive because of the high light emission. Those methods are often used as complementary assays in order to provide comparative results.

Table 1. Compounds identified by GC-MS in the samples extracted by HS-SPME (PDMS, PDMS/DVB, CAR/PDMS and PA) and in the essential oil (EO) and dichloromethane (DE) and methanol (ME) extracts of *Laurencia dendroidea*.

Nº	RT (min)	$KI_{obtained}$	$KI_{literature}$	Compound	%							
					PDMS	PDMS/DVB	CAR/PDMS	PA	EO	DE	ME	Similarity
1	3.8	-	662[1]	3-penten-2-one, (*E*)-	-	-	-	-	-	0.21	-	96
2	5.0	-	735[1]	carbamic acid, dimethyl-, ethyl ester	-	-	-	-	0.88	-	-	96
3	5.9	925	925[2]	cumene	-	-	-	-	3.05	-	49.47	97
4	6.9	962	962[3]	benzaldehyde	-	-	-	-	-	-	2.28	97
5	10.6	1072	1069[4]	diethyl malonate	-	-	-	-	0.19	-	-	92
6	13.1	1133	1088[1]	4-acetyl-1-methylcyclohexene	0.17	0.36	0.46	0.20	0.21	-	-	97
7	15.3	1188	1188[5]	*o*-acetyltoluene	0.18	0.59	-	0.35	0.11	-	-	94
8	16.1	1207	1199[1]	1-cyclopentene-1-methanol, 2-methyl-5-(1-methylethyl)-	0.13	0.26	0.35	0.16	0.19	-	-	90
9	23.5	1380	1382[6]	α-gurjunene	3.46	3.51	1.90	2.11	-	-	-	85
10	23.6	1382	1368[1]	(5-methyl-2H-[1,2,4]triazol-3-yl)acetonitrile	0.33	0.44	0.45	0.15	1.87	-	-	91
11	23.9	1390	1394[1]	1H-indene, 2,3,3a,4-tetrahydro-3,3a,6-trimethyl-1-(1-methylethyl)-	0.80	0.84	0.88	0.27	-	-	-	90
12	24.0	1393	1398[1]	1H-cyclopropa[a]naphthalene, decahydro-1,1,3a-trimethyl-7-methylene-,[1aS-(1aα,3aα,7aβ,7bα.)]-	0.57	0.59	0.63	0.16	-	-	-	90
13	24.3	1399	1394[1]	butyl 3,4-dimethylphenyl ether	1.01	1.01	1.37	0.48	-	0.64	-	92
14	24.3	1400	1400[7]	tetradecane	-	-	-	-	0.23	-	-	91
15	26.0	1441	1440[8]	caryophyllene	0.35	0.32	0.39	0.22	-	-	-	88
16	27.5	1478	1470[9]	β-guaiene	0.27	0.21	0.37	0.18	-	-	-	87
17	28.9	1514	1520[10]	α-bisabolene	0.52	0.29	-	5.99	-	-	-	85
18	29.5	1529	-	7-*epi*-silphiperfolan-6β-ol and silphiperfolan-7β-ol mixture	6.36	6.42	9.96	0.53	61.67	5.69	-	-
19	29.6	1532	1526[9]	cadina-1(10),6,8-triene	0.33	0.54	-	-	-	-	-	85
20	30.7	1559	1557[11]	β-iraldeine	-	-	-	-	0.15	1.45	-	85
21	30.8	1562	1560[12]	globulol	-	-	-	-	0.16	1.16	-	87
22	30.9	1566	1550[12]	β-himachelene	5.04	5.03	4.71	4.56	-	-	-	86
23	31.1	1571	1570[13]	spathulenol	0.60	0.95	0.85	0.46	-	-	-	85
24	31.5	1580	1581[1]	2-butenal, 2-methyl-4-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	-	-	-	-	0.12	-	-	91
25	31.6	1584	1567[1]	3-butanone,1-(2,3,6-trimethylphenyl)-	-	-	-	-	0.17	-	-	90
26	32.0	1594	1582[1]	neoisolongifolene, 8-chloro-	0.32	0.50	0.59	0.41	-	0.31	-	93
27	32.2	1600	1603[14]	diethyl Phthalate	-	0.20	-	-	0.20	-	-	90
28	33.9	1645	1649[1]	1-hydroxy-6-(3-isopropenyl-cycloprop-1-enyl)-6-methyl-heptan-2-one	-	-	-	-	0.42	-	-	91

29	34.0	1648	1657[15]	cedre-3-ol-8	0.71	0.67	2.43	0.70	0.41			90
30	34.1	1650	1657[15]	cedren-13-ol, 8-	0.36	0.36	-	0.48	-	0.34	-	92
31	34.3	1656	1651[1]	widdrol	0.33	0.48	0.27	0.42	-	0.44	-	90
32	34.6	1662	1662[16]	α-cadinol	-	-	0.17	0.16	-	-	-	88
33	35.2	1681	1682[17]	1-heptadecene	0.79	0.87	0.27	0.39	-	0.55	-	93
34	35.9	1699	1681[1]	1,3,5-trimethyl-2-(2-nitroallyl)benzene	-	-	-	-	1.11	-	-	94
35	36.2	1708	1711[7]	heptadecane	6.15	7.06	-	0.13	-	3.54	-	96
36	36.6	1719	1717[18]	pentadecanal	0.94	0.97	-	-	-	-	-	89
37	36.9	1727	1726[19]	methyl tetradecanoate	-	-	-	-	0.51	-	-	90
38	39.5	1802	1816[1]	4-cyclohexylcarbonyl-1,3-dimethylbenzene	-	-	-	-	0.31	-	-	93
39	41.9	1874	1871[20]	isobutylphthalate	0.35	0.39	0.17	0.59	0.29	0.34	1.25	87
40	42.0	1877	1888[1]	1,2,3,4-tetrahydroisoquinoline-2-propionamide	-	-	-	-	0.20	-	-	90
41	43.6	1924	1918[21]	(E,E)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene	0.28	0.28	0.12	0.32	-	-	-	85
42	43.7	1928	1928[22]	hexadecanoic acid, methyl ester	-	-	-	-	1.23	-	0.95	86
43	45.0	1967	1965[20]	dibutyl phthalate	1.74	1.99	1.77	2.80	3.28	2.08	0.80	86
44	49.6	2118	2118[23]	phytol	0.27	0.19	-	0.34	-	0.36	-	96
45	61.4	2549	2546[24]	bis(2-ethylhexyl) phthalate	-	-	-	0.18	-	-	3.36	86
46	61.5	2555	2572[1]	benzeneethanamine, *N*-[(pentafluorophenyl)methylene]-.beta.,3,4-tris[(trimethylsilyl)oxy]-	-	-	-	-	-	-	2.36	90
Total number of analyzed peaks					94	93	83	108	58	53	12	-
Number of identified peaks					26	27	20	26	23	13	7	-
Total identified (%)					32.36	35.32	28.11	22.74	76.98	17.11	60.47	-

RT: retention time;[1] theoretical KI;[2] Wang et al., 1994;[3] Spadone et al., 1990;[4] Boulanger & Crouzet, 2001;[5] Hamm et al., 2004;[6] Senatore et al., 1998;[7] von Kováts, 1958;[8] Quere & Latrasse, 1990;[9] Elias et al., 1997;[10] Larsen & Frisvad, 1995;[11] Tudor, 1997;[12] Larsen & Frisvad, 1994;[13] Zunino et al., 1997;[14] Ramarathnam et al., 1993;[15] Roscigno, 1998;[16] Tzakou et al., 2000;[17] Flamini et al., 2003a;[18] Flamini et al., 2003b;[19] Boulanger et al., 1999;[20] Weber, 1986;[21] Heinrich et al., 2002;[22] Ansorena et al., 2001;[23] Esteban et al., 1999;[24] Fukui et al., 1997. *GC column utilized in the literature is different with that used in this study.

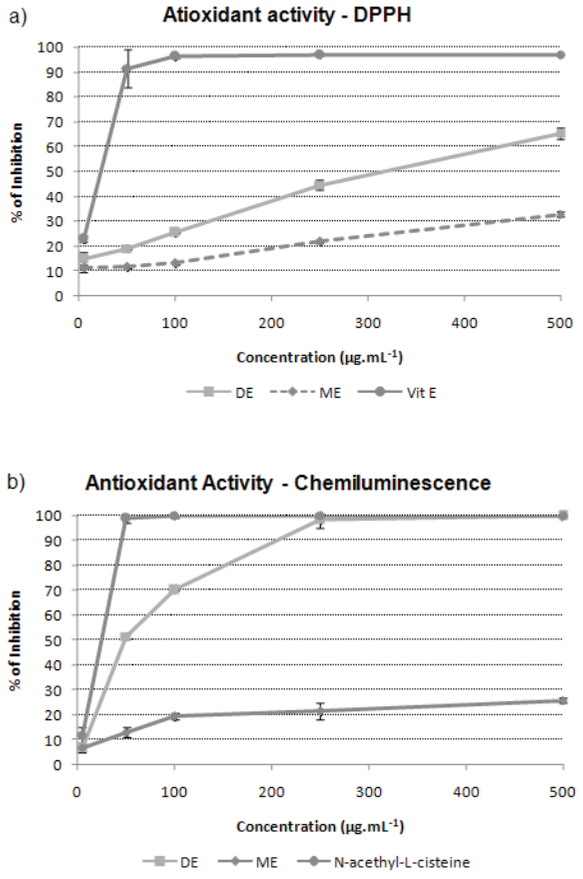

Figure 1. Comparative graphs of the antioxidant activity.

Conclusions

Volatile organic compounds obtained by different extraction procedures (hydrodistillation, solvent extraction and headspace solid-phase microextraction) provide complementary information of the organic compound profile, which is very important when an overview of the composition is required. In *L. dendroidea*, 46 volatile compounds were identified and the higher antioxidant activity of the dichloromethane extract indicates that the most active antioxidant components are in this fraction, although it is not possible to distinguish between the possibilities of the presence of just a few compounds with high potency or many constituents with low potencies. As indicated by this and previous reports, this species of algae could have many important applications and should be more thoroughly studied.

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