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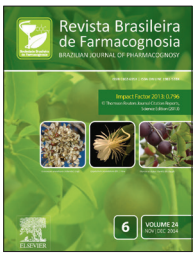

Original article

Chemical diversity and antileishmanial activity of crude extracts of *Laurencia* complex (Ceramiales, Rhodophyta) from Brazil

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ABSTRACT

Chemical profiles of extracts of four species from *Laurencia* complex (Ceramiales, Rhodophyta) from different populations collected along Southeast Brazilian coast were assessed by High Performance Liquid Chromatography coupled with a Diode Array Detector in order to observe geographic chemical variability. Aiming to evaluate the impact of chemical diversity on potential pharmaceutical uses, the extracts were tested against the promastigote form of *Leishmania amazonensis*. The most active extracts were submitted to anti-amastigote and cytotoxicity assays. Principal Component Analysis of the chromatograms resulted in four major groups of chemical profiles according to the presence of leishmanicidal chamigranes (-)-elatol and obtusol. The existence of chemotypes, displaying variable pharmacological action, is proposed for the differences observed in *L. dendroidea* samples. Although all extracts were found active against promastigote form of *L. amazonensis*, their efficacy was remarkably different and not related to the variation of (-)-elatol and obtusol, which indicates the presence of additional compounds with antileishmanial activity. Moreover, the active extracts also displayed anti-amastigote activity and none of them were considered cytotoxic. The results highlight that the knowledge of chemical geographic variability can be valuable in the search of new antileishmanial compounds from marine sources.

Introduction

Macroalgae are an important source of a wide range of compounds, endowed with relevant biological activity and potential applications in many different areas (Cardozo et al., 2007; Machado et al., 2010; Blunt et al., 2011). It is believed that these chemical compounds serve as defense mechanisms (Paul et al., 2011).

Red algae of *Laurencia* complex (Ceramiales, Rhodophyta), specifically genus *Laurencia*, represent one of the most studied marine organisms from environment (Suzuki et al., 2009; Wang et al., 2013). More than 700 halogenated compounds have already been reported for *Laurencia* genus (Kamada and Vairappan, 2012; Wang et al., 2013). Previous studies have focused on the discovery of chemical compounds with

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pharmaceutical or ecological potential in *Laurencia* complex (Blunt et al., 2011). In spite of the reports regarding antibacterial, antiviral and cytotoxic activities (Machado et al., 2010), there are only four studies on the antileishmanial action of *Laurencia* crude extracts (Freile-Pelegrin et al., 2008; Bianco et al., 2013) and their sesquiterpenes (Santos et al., 2010; Machado et al., 2011). Only few reports have addressed inter or intraspecific variation in secondary metabolite composition and none of them have considered the impact of chemical variability on bioactivity (Yuan et al., 2009; Stengel et al., 2011; Sudatti et al., 2011; Oliveira et al., 2013).

Currently, *Laurencia* complex is comprised by six genera: *Laurencia* J.V. Lamouroux, *Osmundea, Chondrophycus* (J.Tokida & Y.Saito) Garbary & J.T.Harper, *Laurenciella* V.Cassano, Gil-Rodríguez, Sentíes, Díaz-Larrea, M.C.Oliveira & M.T.Fujii, *Palisada* K.W. Nam and *Yuzurua* (Nam) Martin-Lescanne (Fujii et al., 2011; Cassano et al., 2012a). The genus *Laurencia* is represented by 419 species (and infraspecific) names in the database at present (Guiry and Guiry, 2014), of which 130 have been flagged as being currently taxonomically accepted. In Brazil, five of the six genera of the complex can be found: *Laurencia* itself, *Laurenciella, Osmundea, Palisada*, and *Yuzurua*, which are widely distributed along the coast in intertidal and shallow subtidal zones, except for the last, whose occurrence is restricted to the northeast.

The aim of this study was to evaluate the geographical variability of metabolite production by *Laurencia* complex, and the impact of chemical diversity on bioactivity. Chemical profiles and Principal Component Analysis were employed in the study. The samples were submitted to antileishmanial assay against promastigote and intracellular amastigote forms of *Leishmania amazonensis*, the etiological agent of leishmaniasis, which is a public health problem in many countries (Croft and Coombs, 2003; Chappuis et al., 2007). The most active samples were tested against amastigote forms and their cytotoxicity was evaluated in macrophages in order to estimate antiparasitic selectivity.

Materials and methods

General experimental procedures

NMR spectra was measured using a Bruker Advance spectrometer operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR in $CDCl_3$. Column chromatography was performed with silica gel 60 Merck (70-230 Mesh) and thin layer chromatography was carried out with silica gel GF254 plates using a 2% solution of Ce $(SO_4)_2$ in H_2SO_4 followed by heating to visualize spots.

Sample collection and extract preparation

The algae were collected in intertidal and infralitoral regions at distinct areas of the Southeastern Brazilian coastline from September, 2007 to April, 2008. The species were identified by the authors, L.M. Gestinari and M.T. Fujii, based on morphological features. Voucher specimens were deposited at the Herbarium of the Rio de Janeiro Federal University, Brazil (RFA) and the voucher numbers of the collections are shown in Box 1. During the collection of algae, intrapopulation morphological differences were observed between samples from Manguinhos (LdM1, LdM2 and LdM3) and from Ubú (LdU1 and LdU2) beaches. The main morphological features of all collected samples are listed in Box 2.

The collected samples were washed in seawater to eliminate associated organisms, air-dried at ambient temperature, grounded and extracted at room temperature, four times

Box 1
Collection places.

Sample	Specie	Collection place	Collection date	Voucher number
LaFl	*Laurencia aldingensis*	Flexeira, State of Espírito Santo	March 7th, 2008	35921
LdAR	*Laurencia dendroidea*	Biscaia inlet, Angra dos Reis, State of Rio de Janeiro	September 26th, 2007	35918
LdM1	*Laurencia dendroidea*	Manguinhos, State of Espírito Santo	March 8th, 2008	35947
LdM2	*Laurencia dendroidea*	Manguinhos, State of Espírito Santo	March 8th, 2008	35887
LdM3	*Laurencia dendroidea*	Manguinhos, State of Espírito Santo	March 8th, 2008	35951
LdMa	*Laurencia dendroidea*	Macaé, State of Rio de Janeiro	May13th, 2008	35946
LdU1	*Laurencia dendroidea*	Ubú, State of Espírito Santo	March 7th, 2008	35949
LdU2	*Laurencia dendroidea*	Ubú, State of Espírito Santo	March 7th, 2008	35950
PfFl	*Palisada flagellifera*	Flexeira, State of Espírito Santo	March 7th, 2008	35937
PpFl	*Palisada perforata*	Flexeira, State of Espírito Santo	March 7th, 2008	35922
PpM	*Palisada perforata*	Manguinhos, State of Espírito Santo	March 8th, 2008	35917
PpU	*Palisada perforata*	Ubú, State of Espírito Santo	March 7th, 2008	35945

Box 2
Morphological description of collected samples.

Sample	Remarks
LaFl	Erect thallus forming dense red-wine tufts with live red tips, up to 10 cm high, attached to the substrate by a discoid holdfast and by descending branches.
LdAR	Erect thallus forming dense violet-greenish tufts, usually greenish in main axes and primary lateral branches, and violet in the remaining parts of the thallus, up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
LdM1	Erect thallus forming dense violet-greenish tufts, usually greenish in main axes and primary lateral branches, and violet in the remaining parts of the thallus, up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
LdM2	Erect thallus forming dense violet-greenish tufts, usually greenish in main axes and primary lateral branches, and violet in the remaining parts of the thallus, up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
LdM3	Erect thallus forming dense tufts, brown-purple in colour, with iridescent orange tips. Terete and cartilaginous thallus, up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
LdMa	Erect thallus forming dense violet-greenish tuft, with iridescent purple tips when alive. Terete and cartilaginous thallus, up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
LdU1	Erect thallus forming dense red to brown-purple tufts, up to 20 cm high, attached to the substrate by a discoid holdfast.
LdU2	Erect thallus forming dense tufts, brown-purple in colour. Terete and cartilaginous thallus up to 20 cm high; attached to the substrate by a discoid holdfast and by descending branches.
PfFl	Erect thallus, terete, cartilaginous forming dense dark to brown-purple tufts, up to 15 cm high, attached to the substrate by a small discoid holdfast.
PpFl	Erect thallus gregarious, cartilaginous forming loose or dense mats, olive-brown in colour, up to 10 cm high, densely branched, attached to the substrate by a single discoid holdfast or from an aggregation of discoid holdfasts.
PpM	Erect thallus solitary, cartilaginous, purple-brown in colour, up to 10 cm high, densely branched, attached to the substrate by a single discoid holdfast or from an aggregation of discoid holdfasts.
PpU	Erect thallus gregarious, cartilaginous forming loose or dense mats, olive-brown in colour, up to 10 cm high, densely branched, attached to the substrate by a single discoid holdfast or from an aggregation of discoid holdfasts.

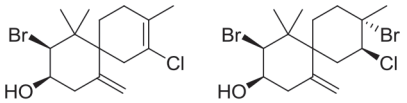
repeatedly, using a 1:1 mixture of dichloromethane and methanol with the assistance of ultrasound.

Chromatographic fingerprinting

The high-performance liquid chromatographic (HPLC) analysis was carried out on a VP-ODS Shim-pack column (5 µM, 250 × 4.6 mm) using a Shimadzu HPLC chromatograph equipped with a Diode Array Detector (DAD) and a coupled system consisting of a vacuum degasser, pump LC-20AT, and detectors SPD-M20A and CBM-20A. A binary gradient elution at flow rate 1.0 ml/min^{-1} was employed using water as solvent A and acetonitrile as solvent B, as follows: 20%-50% B at 0-10 min, 50%-70% B at 10-20 min, 70%-100% B at 20-40 min, 100% isocratic B for 5 min. The column was conditioned before next injection (by 20 min). The UV-Vis spectra were registered. Crude extracts were diluted in acetonitrile to a final concentration of 10 mg/ml and filtered in a 0.45 µM PTFE syringe filter (Millipore) prior injection. An aliquot of 20 µl of filtrate solution was injected for HPLC analysis.

Isolation and identification of sesquiterpenes (-)-elatol and obtusol

The crude extract of LdAR was submitted to a column chromatography on silica gel, eluting with an increasing gradient of *n*-hexanes and EtOAc (1:0-0:1) and fifteen fractions were separated on the basis of TLC analysis (fractions A-O). Column chromatography on silica gel of fraction E, eluted with an increasing gradient of *n*-hexanes and CH_2Cl_2 (1:0-0:1), resulted in the isolation of sesquiterpenes (-)-elatol (43 mg, **1**) and obtusol (26 mg, **2**). Their structures were unambiguously elucidated by interpretation of NMR spectra followed and compared to reported data (Machado et al., 2011).



1 **2**

(-)-Elatol (**1**): colorless oil; $[\alpha]_D$ -66.2 (c 0.13, $CHCl_3$); IR (mineral oil) 3458; 2970; 2947; 1718; 1676; 898; 817; 736 cm^{-1}; NMR ^{1}H (300 MHz, $CDCl_3$): 1.07 (s); 1.08 (s); 1.62 (ddd, 13.4; 12.5; 7.6); 1.81 (ddd, 14.8; 12.5; 2.1); 1.82 (ddd, 13.4; 4.1; 2.1); 1.96 (ddd, 14.8; 7.6; 4.1); 2.37 (brd, 16.1); 2.51 (dd, 14.4; 3.0); 2.59 (dl, 16.1); 2.63 (dd, 14.4; 3.0); 4.14 (ql, 3.0); 4.60 (d, 3.0); 4.79 (brs); 5.12 (brs). ^{13}C (75 MHz, $CDCl_3$): 19.4 (CH_3); 20.7 (CH_3); 24.2 (CH_3); 25.6 (CH_2); 29.3 (CH_2); 38.0 (CH_2); 38.6 (CH_2); 43.1 (C_0); 49.1 (C_0); 72.2 (CH); 70.8 (CH); 115.9 (CH_2); 128.0 (C_0); 124.1 (C_0); 140.8 (C_0).

Obtusol (**2**): white amorphous solid; $[\alpha]_D$ +9.61 (c 0.05, $CHCl_3$); IR (KBr) 3465; 2969; 1640; 1441; 907; 813; 792 cm^{-1}; NMR ^{1}H (300 MHz, $CDCl_3$): 1.08 (s); 1.56 (brs); 1.74 (m); 1.83 (s); 1.94 (dd, 14.0; 11.7); 2.30 (m); 2.36 (brs); 2.49 (dd, 14.1; 3.1); 2.62 (dd, 14.1; 3.1); 4.10 (sl); 4.47 (d, 3.0); 4.70 (dd, 11.7; 2.9); 5.05 (s); 5.39 (s). ^{13}C

(75 MHz, $CDCl_3$): 20.6 (CH_3); 23.9 (CH_3); 24.2 (CH3); 25.6 (CH_2); 37.1 (CH_2); 38.5 (CH_2); 40.5 (CH_2); 44.2 (C0); 50.3 (C_0); 67.6 (CH); 68.1(CH); 70.1 (CH); 71.9 (CH); 117.8 (CH_2); 141.2 (C_0).

The isolated compounds were used as external standards in the chromatographic analysis. The retention time recorded for (-)-elatol (**1**) was 32.74, and 30.78 min for obtusol (**2**). Additionally, the presence of both compounds in the unfractionated extracts was assessed by ^1H and ^{13}C NMR spectra.

Principal Component Analysis

The two-dimensional chromatogram matrices from the HPLC-DAD system had the first 8 min of retention time removed prior the PCA analysis, because it was characterized by the strong overlapping signal from polar compounds and very low peak resolution. The software COW tool (http://www2.biocentrum.dtu.dk/mycology/analysis/cow/) was used to perform the baseline alignment of each chromatogram and also to correct the time shift of peaks among them using the Correlation Warping Algorithm (COW), as described by Nielsen et al. (1998). The LdAR sample was used as the index chromatogram. The data from 200 nm was extracted from each two-dimensional chromatogram to compose a matrix. This matrix was mean centered before running the PCA routine, which was done using R language and environment (www.R-project.org) using the package "ChemometricsWithR" (Wehrens, 2011).

Antileishmanial assay

Leishmania amazonensis (WHOM/BR/75/Josefa), transfected with the green fluorescent protein (GFP) gene (Rossi-Bergmann et al., 1999) was used. The parasites were periodically isolated from cutaneous lesions of experimentally infected mice and maintained in culture as the insect stage promastigote forms at 26°C in M199 Medium (Sigma) supplemented with 10% fetal calf serum (HIFCS, Cultilab) and 40 µg/ml of gentamycin (Schering-Plough, Rio de Janeiro, Brazil) for no more than four passages. Promastigotes were periodically cultured in 1000 µg/ml of geneticin (G418, Sigma) for bright green fluorescence selection.

For antipromastigote activity, fluorescent promastigotes were plated in triplicate at 105 parasites/well with varying concentrations of test compounds (0, 0.1, 1, 10, and 100 µg/ml) in a final volume of 200 µl of medium M199 containing 5% HIFCS and 1% hybri-max dimethyl sulfoxide (DMSO, Sigma). After 72 h at 27°C, the fluorescence intensity of the cultures was measured using a plate-reader fluorometer (Fluoroskan) set at 435 nm excitation/538 nm emissions. All cultures were performed in triplicate, and the results were expressed as percent inhibition in relation to controls cultured in medium alone.

For antiamastigote activity, mouse peritoneal macrophages were harvested from the peritoneal cavities of BALB/c mice in ice-cold DMEM medium (Sigma). The cells were plated at 2×10^6/ml (0.4 ml/well) in Lab-Tek 8-chamber slides (Nunc, Naperville, Ill.) and incubated at 37°C and 4% CO_2 for 1 h. Non-adherent cells were removed by washing with pre warmed phosphate-buffered saline (PBS). Adherent macrophages were infected with *L. Amazonensis* promastigotes (stationary growth phase) at a 5:1 parasite/macrophage ratio and incubated for 1 h at 35°C, 5% CO_2. The cell monolayers were washed three times with pre warmed PBS to remove free parasites, and 0.4 ml of the test compounds complete medium at different concentrations was added in duplicate for 72 h more. The cultures were then fixed with absolute methanol and stained with Giemsa. The numbers of intracellular amastigotes were determined by counting at least 100 macrophages per sample, and the results were expressed as the 50% effective dose (ED_{50}) determined by logarithm regression analysis. The reference drug amphotericin B (Sigma-Aldrich) was used at concentrations varying from 0.01 to 10 µg/ml.

After 72 h of treatment, the NO production by the infected macrophages was measured by nitrite level assessment in the culture supernatants. Briefly, 100 ml of fresh Griess reagent [1% sulfanilamide *p*-aminobenzenesulfonamide, 5% H_3PO_4, 0.1% *n*-(1-naphthyl) ethylenediaminedihydrochloride; Sigma] was added to equal volumes of culture supernatants. After 10 min of incubation at room temperature, the optical density at 570 nm was measured. Macrophages stimulated with IFN-γ (10 mg/ml) were used as positive control. The nitrite concentration was determined by using $NaNO_2$ diluted in DMEM as the standard and DMEM plus Griess reagent alone as the blank.

For cytotoxicity against mammalian cells, single-cell suspensions of cervical lymph nodes of BALB/c mice were freshly prepared in DMEM supplemented with 40 µg/ml of gentamicin sulphate (Schering-Plough), 25 mM HEPES (Sigma, USA), sodium bicarbonate (4.7 µg/ml) and β-mercaptoethanol (5 mm/l) and cultured for 72 h at 37°C at varying concentrations of the test compounds. The release of the cytoplasmic enzyme lactate dehydrogenase (LDH) into the culture medium was measured using an assay kit (Doles Reagentes, Brazil). Maximum and minimum release values were cells cultured with 2% Triton X-100 or 1% DMSO, respectively. The IC_{50} values were calculated by linear regression analysis. For cytotoxicity macrophages, the supernatants of the infected macrophages treated as described above for antiamastigote activity was assayed for (LDH) as described for lymph node cells.

Data were analyzed using Student's t-test when comparing two groups or one-way ANOVA for more than two groups followed by Tukey's multiple comparisons post-hoc test, using the GraphPad Program; p-values less than 0.05 were considered statistically significant.

Results

Chemical profiles of twelve crude extracts from four different species of *Laurencia* complex collected in different sites of the Brazilian coast were obtained by HPLC/DAD (Fig. 1) and compared using Principal Component Analysis (PCA).

During collection, two populations were distinguished at Ubú beach (LdU) and three at Manguinhos beach (LdM). While LdU1 displayed dense red to brown-purple tufts, LdU2 exhibited brown-purple patterns (Box 2). Differently, the samples LdM1, LdM2 and LdM3 were greenish or brown-purple with orange tips. Additionally, it remarkable differences were observed on

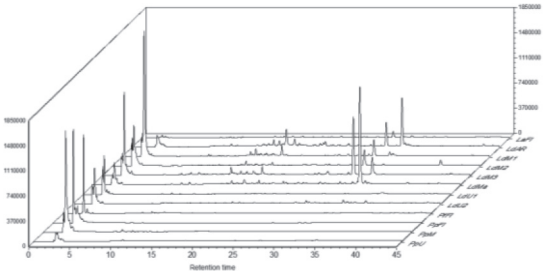


Figure 1 – HPLC fingerprint chromatograms (λ=200 nm) of *Laurencia* complex.

the chemical profiles (Fig. 1). Chemical profiles of LdAR, LdM1, LdM2, LdM3 and LdMa displayed greater chemical diversity than LaFl, LdU1, LdU2, PfFl, PpFl, PpM and PpU.

In this study, the first eight minutes of the chemical fingerprint were removed for PCA, since they comprise a set of overlapping peaks of more polar compounds that do not have useful discriminant information. The two first components of PCA accounted for 96.1% (PC1 = 53.3% and PC2 = 42.8%) of the total chromatographic variance. The resulting PCA score plot discriminated the samples (Fig. 2A) according to the strong influence of two main substances, with a strong weight in CP1 and CP2, as depicted by the plot of PCA loadings against the retention time (Fig. 2B and 2C). Basically, PC1 discriminated the samples according to the presence of a compound with retention time of 32.74 min while PC2 according to a compound eluting in 30.78 min. The identification of these compounds was accomplished by comparison of their retention times with purified chamigranes (-)-elatol (**1**) and obtusol (**2**), respectively.

The PCA score plot grouped the sample of *L. dendroidea* from Macaé (LdMa) alone at the lower left plot quadrant, characterized mostly by the presence of (-)-elatol (**1**) confirmed after the analysis of ^{1}H NMR spectra. Conversely, the samples of *L. dendroidea* from Manguinhos (LdM1, LdM2 and LdM3) were characterized by an increased signal of obtusol (**2**) and no signal of (-)-elatol and were grouped at the upper right quadrant. Similarly, NMR data of these extracts also revealed the presence of obtusol. Containing both (-)-elatol and obtusol and placed alone at the upper left quadrant was the *L. dendroidea* from Angra dos Reis (LdAr). Finally, at the lower right quadrant were the samples characterized mostly by flat chromatograms, with the absence of (-)-elatol and obtusol, which corresponds to the species *P. flagellifera* from Flexeira (PfFl1) and P. perforata from Flexeira, Manguinhos and Ubú (PpFl, PpM and PpU), along with *L. dendroidea* from Ubú (LdU1 and LdU2) and *L. aldingensis* from Flexeira (LaFl). Fig. 3 shows the comparison of the ^{1}H NMR spectra of purified (-)-elatol and obtusol with the crude extracts LdMa, LdM1, LdM2, LdM3 and the LdAR characterized by the presence of these compounds. Additionally, Table1 shows the relative area of the pure compounds obtained by HPLC/DAD analysis.

In spite of the chemical differences, all the tested samples displayed antileishmanial action (Table 2). Comparing the

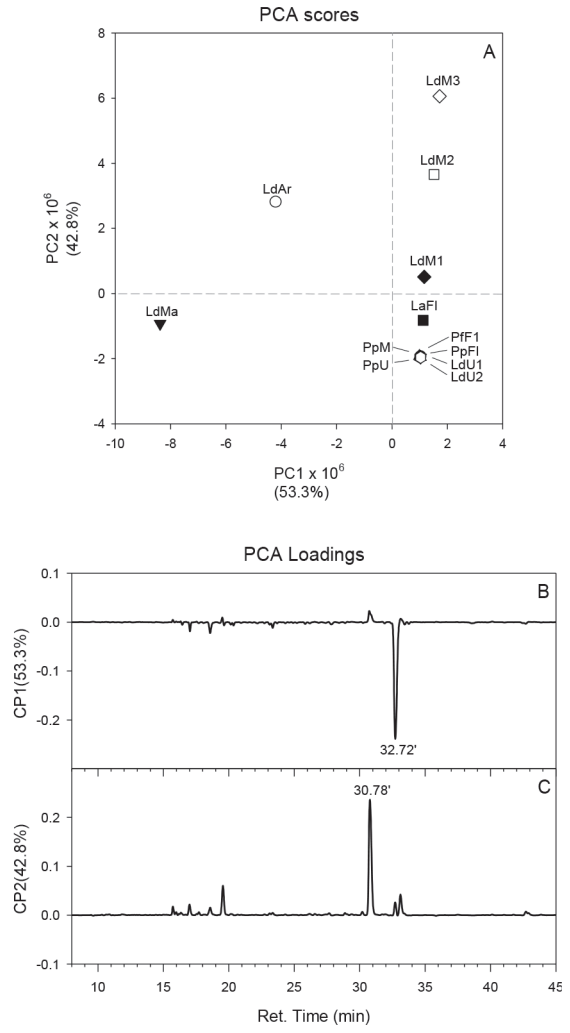


Figure 2 – Principal Component Analysis of HPLC fingerprints at 200 nm obtained from twelve populations of *Laurencia* complex. Components 1 and 2 account for 96.1% of the total chromatographic variation among the populations. Factor scores (**A**) and loads plotted against retention time (**B** and **C**) are given.

results against promastigote form with the groups showed on PCA, important differences can be observed on bioactivity even within a cluster. *L. dendroidea* from Angra dos Reis (LdAR) was the most active (IC_{50} 17.9 ± 1.3 µg/ml). Although eight samples were identified as *L. dendroidea* their bioactivity was remarkably different with IC_{50} values ranging from 17.9 ± 1.3 µg/ml (LdAR) to 97.2 ± 2.0 µg/ml (LdU1). It is important to mention that isolated compounds (-)-elatol and obtusol are known to possess antileishmanial action with IC_{50} 9.7 ± 1.2 µg/ml and 6.2 ± 0.5 µg/ml against promastigote forms (Machado et al., 2011). Despite the lack of chamigranes, *P. flagellifera* from Fleixeira (PfFl) was, along with LdU2, the third most active crude extract. Nevertheless, in the anti-amastigote assay, more relevant in terms of chemotherapy, LaFl was the most active sample (IC_{50} 12.5 ± 1.4 µg/ml) followed by LdU2 (IC_{50} 16.8 ± 2.8

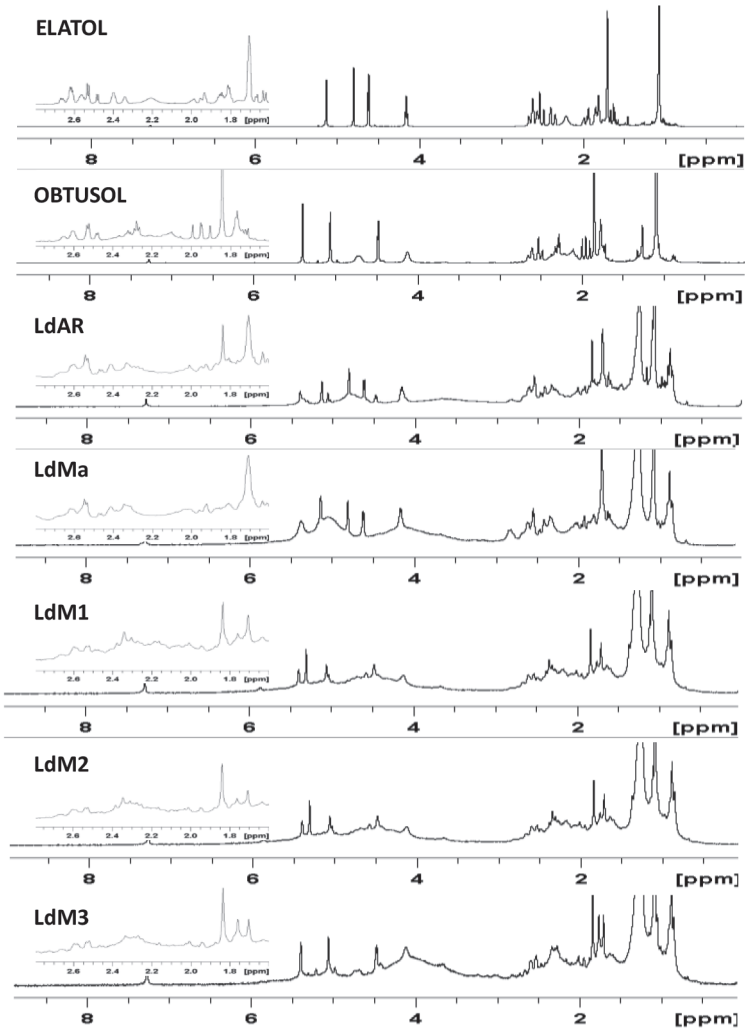


Figure 3 – Comparison of ^{1}H NMR spectra (200 MHz, $CDCl_3$) of (-)-elatol, obtusol and crude extracts LdAR, LdMa, LdM1, LdM2 and LdM3.

Table 1
Relative area of (-)-elatol and obtusol after HPLC/DAD analysis on samples containing chamigrene.

Sample	(-)-elatol (1)	obtusol (2)
LdAR	19.50%	8.93%
LdM1	ND	16.36%
LdM2	ND	71.82%
LdM3	ND	57.27%
LdMa	76.92%	ND

ND, not detected.

µg/ml) and LdM3 (IC_{50} 19.2 ± 4.6 µg/ml). Moreover, none of the tested samples displayed significant cytotoxic activity against macrophages with IC_{50} greater than 198 ± 6.1 µg/ml. Since none of the samples significantly activated the production of NO by infected macrophages, their antileishmanial activity is likely to be direct on the parasites.

Discussion

Chemical profiling has been proved to be useful in the study of chemical variability of natural products (Yuan et al., 2009; Nylund et al., 2011; Payo et al., 2011; Hou et al., 2012). These approaches are relevant because of the existence of inter- and intrapopulation chemical differences between marine organisms, described in several studies (Miller et al., 2001; Sacristan-Soriano et al., 2011). Basically, the sources of chemical variation can be either genetic or environmental.

The genetic influence on chemical differences was proposed by Oliveira et al. (2013) for elatol content on *L. dendroidea*. Additionally, Howard et al. (1980), using unialgal culture, observed that the chemical compounds produced by *Chondrophycus undulatus* (as *Laurencia undulata*), *L. gardneri, L. nippponica, L. intermedia, L. okamurai,* and *Osmundea spectabilis* (as *L. spectabilis*), were qualitatively and quantitatively identical to those produced in natural populations suggesting that the secondary metabolites of *Laurencia* complex populations reflect genetic differences. Crossing experiments were used to study

Table 2
Antileishmanial action of crude extracts.

CODE	Promastigotes IC_{50} (µg/ml)	Amastigotes IC_{50} (µg/ml)	Toxicity Macrophages IC_{50} (µg/ml)	Δ NO (µg/ml)
LaFl	24.5 ± 1.4	12.5 ± 1.4	259.8 ± 2.7	1.8 ± 0.2
LdAR	17.9 ± 1.3	22.4 ± 3.2	240.0 ± 5.3	0.5 ± 0.1
LdM1	48.1 ± 1.7	ND	ND	ND
LdM2	41.9 ± 1.6	ND	ND	ND
LdM3	34.2 ± 1.5	19.2 ± 4.6	187.0 ± 8.4	1.2 ± 0.4
LdMa	40.6 ± 1.6	ND	ND	ND
LdU1	97.2 ± 2.0	ND	ND	ND
LdU2	30.1 ± 1.5	16.8 ± 2.8	214.0 ± 7.3	1.6 ± 0.5
PfFl	30.7 ± 1.5	34.5 ± 3.4	198.0 ± 6.1	0.3 ± 0.2
PpFl	36.1 ± 1.6	29.7 ± 4.2	203.0 ± 5.0	0.4 ± 0.1
PpM	36.8 ± 1.6	29.5 ± 3.5	267.0 ± 6.5	0.6 ± 0.2
PpU	46.7 ± 1.7	ND	ND	ND
Amphotericin B	0.42 ± 0.14	0.74 ± 0.18	19.4 ± 0.55	0
IFN-γ(1.0 ng/ml)	-	-	-	12.5 ± 1.4

Arithmetic means ± SD (n = 3); ND, not determined; IFN-γ, Interferon-gamma.
Δ NO, Nitric oxide production by macrophages (drug stimulated – spontaneous).

the chemical diversity in *Laurencia nipponica* revealing that genetic variation is a determining factor for the synthesis of halogenated metabolites (Masuda et al., 1997).

The outcome of genetic differences, proposed in previous studies, is the existence of chemical types, once chemical diversity is observed not only between species but also among populations from different sites, as observed for *Laurencia nipponica* (Masuda et al., 1997), *L. obtuse* (Caccamese et al., 1981), *L. pacifica* (Fenical, 1976) and recently for *L. nangii* (Kamada and Vairappan, 2012).

In the present study, four groups of *L. dendroidea*, were recognized by PCA analysis. Therefore, four chemical types were distinguished, determined by collection site. Previous reports, with samples from another regions, revealed that some Brazilian populations of *L. dendroidea* (as *L. scoparia*) also produce a different array of chamigrane and bisabolene sesquiterpenes (Davyt et al., 2001; 2006), corroborating the existence of a specific chemical profile for each collection site.

Environmental factors can modulate the levels of secondary metabolites in marine organisms. In this regard, Kuwano et al. (1998) reported that an increase in salinity led to higher levels of sesquiterpenes in *L. okamurae*. In another study, temperature and salinity were reported to influence the amount of sesquiterpenes and severe conditions decreasing the level of elatol in *L. dendroidea* (Sudatti et al., 2011).

Environmental conditions are also considered to be the cause of morphological plasticity in algae (Oliveira et al., 2013). It is known that *Laurencia dendroidea* display morphological differences that may potentially complicate the identification of species (Cassano et al., 2012b). In the present study, intrapopulation and interpopulation morphological differences were observed (Box 2). However, chromatographic profiles indicated that intrapopulation morphological differences do not represent important chemical variations. For instance, morphologically different samples LdM1, LdM2 and LdM3 displayed similar chemical profiles, and the same pattern is observed for LdU1 and LdU2. Conversely, despite the quantitative difference on obtusol content (Table 1), antileishmanial action of LdM1, LdM2 and LdM3 are very similar, while LdU1 (IC_{50} 97.2 ± 2.0 µg/ml) is about three times less active than LdU2 (IC_{50} 30.1 ± 1.5 µg/ml). These results suggest that microscale factors acting at Úbu beach (LdU1 and LdU2) result, not only on morphological differences, but also on antileishmanial action. Once chemical profiles are very similar, the pharmacological dissimilarities may be explained as quantitative variation on active metabolites produced.

Although three different collection sites were compared, all *Palisada* spp. samples displayed similar chromatograms, with the absence of sesquiterpenes (-)-elatol and obtusol, as reported previously (Fujii et al., 2011). Nevertheless, samples PfFl, PpFl, PpM and PpU displayed antileishmanial action comparable to *Laurencia* spp., samples containing chamigranes (LdAR, LdMa, LdM1, LdM2 and LdM3). This result indicates the existence of additional active compounds that may be eluting in the first eight minutes of the chromatograms, or the presence of additional compounds that were not detected by DAD. In both cases, it is clear that compounds other than (-)-elatol and obtusol are active against *Leishmania amazonensis*.

The antileishmanial action also differed among the samples that contained (-)-elatol (**1**) and obtusol (**2**). The most active extract against promastigote form was LdAR, which contained both sesquiterpenes. The presence of (-)-elatol and obtusol

on LdAR could explain the antileishmanial action observed. Samples with only one of these two compounds (Table 1) were less potent and displayed similar antileishmanial action, for instance IC_{50} for LdMa was 40.6 ± 1.6 while for LdM2 was 41.9 ± 1.6 µg/ml.

In conclusion, the present results showed a geographic variation among the metabolites produced by *Laurencia* complex from Brazilian coast, especially within *L. dendroidea* species, with small scale factors affecting the levels of leishmanicidal compounds and morphological features at Ubú beach. The antileishmanial action was not restricted to the samples containing chamigranes, suggesting the existence of other classes of active compounds. This fact reinforces that *Laurencia* complex can be a source of new antileishmanial compounds other than chamigrane-type sesquiterpenes.

Authors' contributions

FLSM (PhD student) performed the experiments, analyzed the data and wrote the paper. WPL and BRB carried out biological studies. LMG and MTF were responsible for sample identification. LMG also contributed in collecting plant material. HMD was responsible for the PCA, contributed with the collections, wrote the paper and did critical reading of the manuscript. CRK performed NMR analysis. ARS collected the plant material, supervised the entire work, analyzed the data, wrote the paper and did critical reading of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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