Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Nitric Oxide Production Inhibition and Anti-Mycobacterial Activity of Extracts and Halogenated Sesquiterpenes from the Brazilian Red *Alga Laurencia Dendroidea* J. Agardh

Thatiana Lopes Biá Ventura[1,2], Fernanda Lacerda da Silva Machado[3,4], Marlon Heggdorne de Araujo[1,2], Lísia Mônica de Souza Gestinari[4], Carlos Roland Kaiser[3], Francisco de Assis Esteves[4], Elena B. Lasunskaia[1], Angélica Ribeiro Soares[3,4], Michelle Frazão Muzitano[2]

[1]Laboratory of Biology of Recognition, Center of Bioscience and Biotechnology, Darcy Ribeiro University of Northern Fluminense, Campos dos Goytacazes-RJ, [2]Laboratory of Bioactive Products, School of Pharmacy , Federal University of Rio de Janeiro, Macaé/ IMMT-RJ, [3]Department of Organic Chemistry, Institute of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, [4]Group of Natural Products from Aquatic Organisms, Center for Ecology and Socio-Environmental Development, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

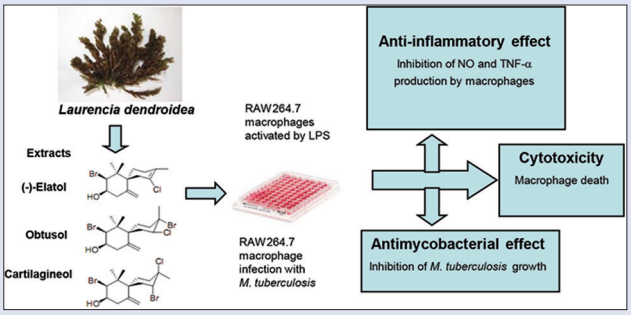
ABSTRACT

Background: Red algae of the genus *Laurencia* J. V. Lamouroux are a rich source of secondary metabolites with important pharmacological activities such as anti-tumoral, anti-inflammatory, anti-fungal, anti-viral, anti-leishmanial, anti-helminthic, anti-malarial, anti-trypanosomal, anti-microbial as well as anti-bacterial against *Mycobacterium tuberculosis*. **Objective:** In the present study, we evaluated the inhibition of nitric oxide (NO) and tumor necrosis factor-α production and the anti-mycobacterial activity of crude extracts from the red *Alga Laurencia dendroidea* (from the South-Eastern coast of Brazil). Halogenated sesquiterpenes elatol (1), obtusol (2) and cartilagineol (3), previously isolated from this *Alga* by our group, were also studied. **Materials and Methods:** The lipopolysaccharide-activated macrophage cells (RAW 264.7) were used as inflammation model. Cytotoxic effect was determined using a commercial lactate dehydrogenase (LDH) kit and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The growing *Mycobacterium* inhibition was verified against *Mycobacterium bovis Bacillus* Calmette–Guérin and *M. tuberculosis* $H_{37}Rv$ strains. **Results:** The crude extract from *Alga* collected at Angra dos Reis, RJ, Brazil, was the most active inhibitor of both mycobacterial growth (half maximal inhibitory concentration [IC_{50}] 8.7 ± 1.4 μg/mL) and NO production by activated macrophages (IC_{50} 5.3 ± 1.3 μg/mL). The assays with isolated compounds revealed the anti-mycobacterial activity of obtusol (2), whereas (-)-elatol (1) inhibited the release of inflammatory mediators, especially NO. To our knowledge, this is the first report describing an anti-mycobacterial effect of *L. dendroidea* extract and demonstrating the association of this activity with obtusol (2). **Conclusion:** The described effects of active compounds from *L. dendroidea* are promising for the control of inflammation in infectious diseases and specifically, against mycobacterial infections associated with exacerbated inflammation.

Key words: Anti-inflammatory, anti-mycobacterial, halogenated sesquiterpenes, *Laurencia dendroidea, Rhodomelaceae*

SUMMARY

- Inflammation is strongly involved in the pathogenesis of most infectious diseases, including TB. The treatment of TB is based on the use of anti mycobacterial drugs, however the most severe forms of TB, require additional anti inflammatory therapy to prevent excessive inflammation. A combination of these properties in one compound could provide additional therapeutic benefits. In this work, we studied L. dendroidea extracts and purified compounds and demonstrated that the LDA extract and (-)-elatol (1) were potent in inhibiting NO production by macrophages through the specific inhibition of iNOS expression. The LDA and LDM extracts and obtusol (2) were active against virulent strain of M. tuberculosis. This is the first report demonstrating that the anti-inflammatory activities of L. dendroidea were associated with the presence of (-)-elatol (1), whereas anti-mycobacterial activities of L. dendroidea extracts were associated with obtusol (2).



Correspondence:
Prof. Michelle Frazão Muzitano,
Laboratory of Bioactive Products, School of Pharmacy , Federal University of Rio de Janeiro, Macaé/ IMMT-RJ, Brazil.
E-mail: mfmuzitano@gmail.com
DOI : 10.4103/0973-1296.172972

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Red algae of the genus *Laurencia* J. V. Lamouroux are found in the tropical and subtropical regions throughout the world and are an extremely rich source of secondary metabolites with diverse structural features, mainly represented by sesquiterpenes, diterpenes, and C15-acetogenins, which are mainly halogenated.[1,2] The distribution of these compounds is known to vary in different species and in specimens from different regions.[3] Notably, the importance of these compounds as a chemical defense against herbivores,[4] fouling organisms, and pathogens[5] has been demonstrated.

Cite this article as: Biá Ventura TL, da Silva Machado FL, de Araujo MH, de Souza Gestinari LM, Kaiser CR, Esteves Fd, *et al*. Nitric oxide production inhibition and anti-mycobacterial activity of extracts and halogenated sesquiterpenes from the Brazilian red *alga laurencia dendroidea* J. Agardh. Phcog Mag 2015;11:611-8.

Organic extracts and some purified metabolites from different species of *Laurencia*, including *Laurencia okamurae*, *Laurencia glandulifera*, and *Laurencia rigida*, showed important pharmacological activities such as anti-tumoral,[6] anti-fungal,[7] anti-viral,[8] anti-leishmanial,[9] anti-helminthic,[10] anti-malarial,[11] anti-trypanosomal,[12] as well as bactericidal effects against Gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and Gram-negative (*Moraxella catarrhalis*) bacteria,[13] and against *Mycobacterium tuberculosis*.[14] In addition, extracts from *L. okamurae* and *L. glandulifera* were reported to present anti-inflammatory activity.[15,16]

The pharmacological effects of *Laurencia dendroidea*, widely distributed along Brazilian coast, started to be investigated only recently, and strong anti-leishmanial and anti-viral effects have been demonstrated.[9,17] Further investigations demonstrated that the observed microbicidal effects are associated with sesquiterpenes, such as elatol and obtusol, which are abundantly expressed in this *Alga*.[9,17] These halogenated sesquiterpenes, elatol (1) and obtusol (2), belong with cartilagineol (3), were isolated, identified, and complete nuclear magnetic resonance (NMR) data for compounds (1–3) were presented by our group.[9]

Previous reports that have demonstrated anti-mycobacterial activity of the elatol isolated from *L. rigida*[14] and identification of a terpene as a principal component responsible for anti-inflammatory activity of *L. glandulifera*,[15] prompted us to test the extracts from *L. dendroidea* and specifically, the isolated halogenated sesquiterpenes: (-)-elatol (1), obtusol (2), cartilagineol (3), for their potential to inhibit nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production and for their anti-mycobacterial activity. In this study, we investigated the immunomodulatory and anti-mycobacterial activities of two crude lipophilic extracts of *L. dendroidea*, collected in different regions of the Brazilian coast, as well as those of the purified halogenated sesquiterpenes, (-)-elatol (1), obtusol (2), and cartilagineol (3), isolated from this *Alga*. In addition, the cytotoxicity of these compounds was evaluated in macrophage culture.

MATERIALS AND METHODS

Plant material

The red *Alga L. dendroidea* J. Agardh (*Rhodomelaceae*, *Ceramiales*) was collected at infralitoral and intertidal zones at two distinct areas on the South-Eastern coast of Brazil: Biscaia inlet, Angra dos Reis, Rio de Janeiro (23°01′53.36″S and 044°14′09.55″W) in September 2007 and at Manguinhos Beach, Espírito Santo (20°12′05.17″S and 040°11′37.70″W) in March 2008. The collections were identified by the botanist Lisia Monica de S. Gestinari. Voucher specimens (Biscaia Inlet: RFA 36068, Manguinhos Beach: RFA 35887) were deposited in the Herbarium of the Rio de Janeiro Federal University, Brazil (RFA).

Extraction and isolation

The air-dried specimens of *L. dendroidea* from Angra dos Reis (LDA, 34.3 g) and Manguinhos (LDM, 60.4 g) were extracted with a mixture of dicloromethane:methanol (1:1) at room temperature. Crude extracts were concentrated to give green residues of 3.99 g for LDA and 4.80 g for LDM. The LDA extract (1.08 g) was re-suspended in water and partitioned with CH_2Cl_2, AcOEt, and *n*-BuOH. All fractions were analyzed by thin-layer chromatography (TLC). The CH_2Cl_2 and AcOEt fractions (448 mg) were submitted to column chromatography on a Si-gel (1 cm × 30 cm) and were eluted with an increasing gradient of AcOEt (0–100%) in *n*-hexane (in steps of 10%, 40 mL each), and separated into 15 fractions on the basis of TLC analysis (A–O). The sesquiterpenes 1 (*n*-hexane: CH_2Cl_2 6:4; 43 mg; 0.00125%, w/w, of *Alga* dry weight) and 2 (*n*-hexane: CH_2Cl_2 1:1; 26 mg; 0.000758%, w/w, of *Alga* dry weight) were isolated from fraction E (*n*-hexane: AcOEt 9:1), following Si-gel column chromatography (1 cm × 35 cm) and elution with an increasing gradient of CH_2Cl_2 (0–100% in steps of 10%, 50 mL each) in *n*-hexane.

The LDM extract (4.54 g) was partitioned with H_2O and CH_2Cl_2. The resulting CH_2Cl_2 fraction (1.76 g) was submitted to column chromatography on a Si-gel 60 Merck (2 cm × 30 cm), eluted with an increasing gradient of AcOEt (0–100%, in steps of 10%, 120 mL) in *n*-hexane and separated into nine fractions on the basis of TLC analysis (A–I). The sesquiterpenes 2 (390 mg) and 3 (14 mg; 0.000232%, w/w, of *Alga* dry weight) were isolated from fraction D (*n*-hexane: AcOEt 8:2), following column Si-gel chromatography (1.5 cm × 30 cm) and eluting with an increasing gradient of CH_2Cl_2 (0–100%, in steps of 10%, 60 mL) in *n*-hexane.

(-)-elatol (1): Colorless oil; purity 99% (high-performance liquid chromatography [HPLC]); retention factor (Rf) =0.52 (CH_2Cl_2-Hex 7:3); [α] D: -66.2 (*c* 0.13, $CHCl_3$); infrared (IR) (mineral oil): 3458, 2970, 2947, 1718, 1676, 898, 817, 736/cm; NMR data: As previously described;[9] EI-MS: *m*/*z* (rel. int. %) =319 (2), 317 (1), 299 (3), 297 (3), 253 (8), 237 (40), 236 (18), 235 (100), 217 (7), 209 (15), 207 (29), 200 (9), 199 (36).

Obtusol (2): White solid; purity 96% (HPLC); Rf = 0.43 (CH_2Cl_2-Hex 7:3); [α] D: +9.61 (*c* 0.05, $CHCl_3$); IR (KBr): 3465, 2969, 1640, 1441, 907, 813, 792/cm; NMR data: As previously described;[9] EI-MS: *m*/*z* (rel. int. %) =319 (25), 318 (17), 317 (100), 316 (13), 315 (76), 299 (17), 297 (18), 235 (23), 217 (12), 200 (18), 199 (47).

Cartilagineol (3): White solid; purity 98% (HPLC); Rf = 0.45 (CH_2Cl_2-Hex 7:3); [α] D: -83.5 (*c* 0.14, CHCl3); IR (KBr): 3410, 2968, 1637, 1448, 969, 902, 736, 712/cm; NMR data: As previously described;[9] EI-MS: *m*/*z* (rel. int. %) =334 (3), 332 (2), 320 (4), 319 (24), 318 (16), 317 (100), 316 (13), 315 (75), 300 (4), 299 (25), 279 (8), 253 (7), 235 (13), 217 (31), 201 (10), 200 (22), 199 (49), 197 (21).

Samples were evaluated for their biological activities at concentrations of 100, 20, and 4 μg/mL, corresponding to the following molar concentrations: Compound 1 (300, 60, and 12 μM) and compounds 2 and 3 (241, 48, and 10 μM), to allow a direct comparison with other known agents.

Determination of nitric oxide and tumor necrosis factor-α production by the RAW 264.7 macrophages

The RAW 264.7 cells obtained from the American Type Culture Collection (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and gentamicin (50 μg/mL) in the presence of 5% CO_2 at 37°C. These cells were seed in 96-well plates (1×10^5 cells/well) in the presence or absence of various concentrations of the algal samples (100, 20, and 4 μg/mL) and/or lipopolysaccharide (LPS-*Escherichia coli* 055:B5; Sigma-Aldrich). In the experiments, a NO inhibitor, N^G-methyl-l-arginine acetate salt (L-NMMA, Sigma-Aldrich, 98% purity), was used as a positive control. After a 24 h incubation period, culture supernatants were collected for NO and TNF-α assays. The concentration of nitrite, a stable NO metabolite, was determined using the Griess method[18] and TNF-α was measured by a L929 fibroblast bioassay, based on the sensitivity of L929 cells to the cytotoxic effect of TNF-α. For this, the L929 cells were seeded in 96-well plates (2×10^5 cells/well). After 24 h incubation, the resulting cell monolayers were treated with the macrophage culture

supernatants in the presence of actinomycin D (2 μg/mL). After 24 h of additional incubation, the viability of the L929 cells was assayed by the 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT) method.[19] The cytokine concentration was determined using a recombinant mouse cytokine to obtain a standard curve to correlate cellular viability and TNF-α concentration.

3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide and LDH assays for cytotoxicity

The cytotoxic effects of algal compounds on macrophages were examined by LDH (cytoplasmic enzyme lactate dehydrogenase) and MTT assays. The release of LDH from RAW 264.7 cells treated with the algal samples (as described above) was quantified in the culture supernatant and determined colorimetrically using a commercial LDH kit (Doles, GO, Brazil), as described previously.[20] Cell lysates obtained via treatment with 1% Triton X-100 were used as a positive control. The rate of LDH release was calculated using the formula: (Supernatant value − blank value)/(lysate value − blank value) × 100%.

The ability of the cells to metabolize MTT to formazan by mitochondrial dehydrogenase activity was used as an indicator of cell viability.[19] After incubation for 2 h with MTT solution (5 mg/mL in phosphate-buffered saline [PBS]), the optical density was measured at 570 nm employing a microplate reader. Cytotoxicity was calculated by subtracting the ratio of the mean absorbance value for treated cells from the mean absorbance value for nontreated cells.

Scavenging of nitric oxide from sodium nitroprusside

Sodium nitroprusside (SNP), a NO donor, spontaneously liberates NO in aqueous solution at physiological pH, which rapidly interacts with oxygen to produce nitrite. To determine whether extracts and isolated halogenated sesquiterpenes directly interact with NO, SNP (5 mM; Sigma) was incubated at room temperature for 2.5 h in the presence of the samples (100, 20, and 4 μg/mL). After this period, nitrite accumulation was determined using the Griess method detailed above.

Western blot analysis

The RAW 264.7 cells were seeded in 24-well plates (1×10^6 cells/well) in the presence or absence of LPS (1 μg/mL) and samples at the concentration of 4 and 20 μg/mL. After 24 h incubation, cells were rinsed with PBS and lysed with lysis buffer (Tris/HCl 1 M, pH 6.8, 25% distilled H_2O, 10% sodium dodecyl sulfate (SDS), 20% glycerol, and 5% β-mercaptoethanol). The thawed cell lysates were mixed with 2% bromophenol blue. Samples of cell lysates (60 μg protein/lane) were separated by SDS/polyacrylamide gel electrophoresis on 10% gels and transferred onto a polyvinylidene difluoride membrane (Hybond), which was blocked overnight at 4°C with 5% nonfat milk in PBS/Tween buffer (10 mM Tris/HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween 20). The blots were then incubated for 1 h at room temperature with rabbit polyclonal anti-inducible NO synthase (iNOS) antibody and for 1 h with peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc.). Immunoreactivity was detected using a solution containing enzyme substrate and 3,3′-diaminobenzidine reagent. In all electrophoresis experiments, a protein standard ladder (Full Range Rainbow-GE Healthcare) was used to estimate the molecular size of the proteins. Western blots were digitized using a high-resolution image size scanner and densitometrically evaluated by gel-analysis software, ImageJ version 1.45. The Bradford method was used to measure the protein concentration in the cell extracts, which were then stored in aliquots at −80°C.

Anti-mycobacterial activity

Samples were evaluated for their anti-mycobacterial activity using an MTT assay to measure mycobacterial growth in a liquid medium.[21] A suspension of the *Mycobacterium bovis Bacillus* Calmette-Guérin (BCG) strain Moreau and *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) was grown in Middlebrook 7H9 medium supplemented with 0.05% tween 80 and albumin-dextrose-catalase. During the logarithmic growth phase, the bacterial suspension was plated in a 96-well plate (1×10^6 CFU/well) in the presence of each sample at three concentrations. The sealed plate was incubated at 37°C and 5% CO_2 for 7 days for *M. bovis* BCG or 5 days for *M. tuberculosis* $H_{37}Rv$. After this period, the bacteria were incubated for 3 h with MTT solution and overnight with lysis buffer (20%, w/v, SDS/50% dimethylformamide in distilled water, pH 4.7). The optical density was measured at 570 nm. A bacterial suspension treated with the standard anti-mycobacterial drug rifampicin (Sigma-Aldrich, 95% purity), at concentrations of 0.0011, 0.0033, 0.0100, and 0.0300 μg/mL was used as a positive control; as a negative control, an untreated bacterial suspension was used. Growth inhibition was expressed as percentage inhibition using the formula (test sample × 100)/untreated control.

Statistical analysis

The test was performed in triplicate, and values were expressed as mean ± standard error of the mean. Statistical analyses were performed by one-way ANOVA, followed by Tukey's *post-hoc* test. The results were considered statistically significant for $P < 0.05$. The half maximal inhibitory concentration (IC_{50}) values were calculated by nonlinear regression.

RESULTS AND DISCUSSION

L. dendroidea extracts obtained from samples collected at two sites of the South-Eastern Brazilian coast: Angra dos Reis (LDA) and Manguinhos (LDM) were evaluated for their anti-inflammatory and anti-mycobacterial activities. Halogenated sesquiterpenes (1–3), previously isolated from this *Alga* by our group,[9] were also studied [Figure 1].

To study the immunomodulatory properties of the *Alga*, focusing on pro-inflammatory mediators, we verified whether *L. dendroidea* extract and halogenated sesquiterpenes could inhibit the production of NO and TNF-α induced in RAW 264.7 macrophages by bacterial LPS.

NO is a chemical mediator with microbicide activity that is produced by activated phagocytes during inflammation. However, tissue

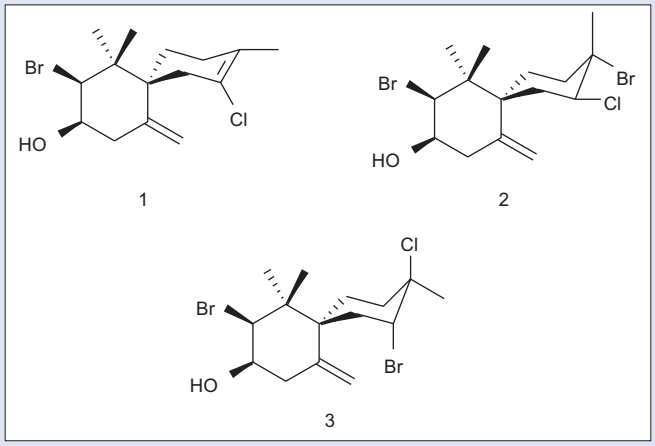


Figure 1: Chemical structures of (-)-elatol (1), obtusol (2), and cartilagineol (3)

concentrations of NO required for microbicide action are toxic to the host cells and must be tightly regulated.[22] Another important pro-inflammatory mediator is TNF-α, which is involved in systemic inflammation; TNF-α is produced by activated macrophages and can cause degranulation of neutrophils, followed by the release of proteolytic enzymes and tissue injury.[23]

In patients with various infectious diseases, including tuberculosis (TB), increased expression of iNOS in alveolar macrophages,[24] together with excessive production of TNF-α, responsible for fever, wasting, and necrosis of pulmonary tissue,[25] was observed. The production of NO and TNF-α could be induced in macrophages by a variety of bacterial components through the mechanism mediated by toll-like receptors.[26] In this study, production of these mediators was induced by LPS, the TLR4 agonist.

NO production by LPS-activated RAW 264.7 cells was inhibited by *L. dendroidea* extracts, reaching more than 50% inhibition within the range of the tested concentrations (4, 20, and 100 μg/mL), as shown in Figure 2a. The LDA extract (IC_{50} 5.3 ± 1.3 μg/mL) was more active than LDM (IC_{50} 14.1 ± 1.1 μg/mL). Even at the lowest concentration tested, the LDA extract was able to inhibit NO production by 50%, whereas a selective iNOS synthase inhibitor, L-NMMA, suppressed NO production by 59.22 ± 2.96%, when tested at the noncytotoxic concentration of 20 μg/mL (80.5 μM).

All the tested halogenated sesquiterpenes showed a moderate-to-high inhibitory capacity. The most active compound was (-)-elatol (1) (IC_{50} 16.5 ± 1.1 μg/mL, 49.5 ± 3.2 μM), which inhibited NO production at a concentration of 20 μg/mL. This effect was not observed for obtusol (2) (IC_{50} 33.2 ± 1.0 μg/mL, 80.2 ± 2.5 μM) or cartilagineol (3) (IC_{50} > 100 μg/mL, >241.2 μM) [Figure 2a]. Interestingly, none of the sesquiterpenes was more active than the LDA extract. Since both (-)-elatol (1) and obtusol (2) were isolated as major components from the crude LDA extract, a synergistic effect of these compounds or the presence of an additional powerful bioactive compound in this extract can be postulated. Conversely, the less active compounds obtusol (2) and cartilagineol (3) were mainly isolated from the crude LDM extract, which might explain the lower activity of LDM in comparison to LDA. The IC_{50} values of isolated compounds were shown as μg/mL and μM to allow a direct comparison between crude extracts and isolated compounds.

The production of TNF-α by macrophages was also evaluated and *L. dendroidea* extracts exhibited a poor capacity to inhibit TNF-α production by LPS-activated RAW 264.7 cells [Figure 2b]. However, (-)-elatol (1) reduced TNF-α production at 100 μg/mL (53.01 ± 2.49%) and 20 μg/mL (35.05 ± 1.92%) with an IC_{50} of 189.8 ± 3.6 μM.

To exclude the possibility that the inhibitory effects of the studied samples on macrophages were due to their cytotoxicity, we monitored macrophage viability via two experiments: Measuring lactate

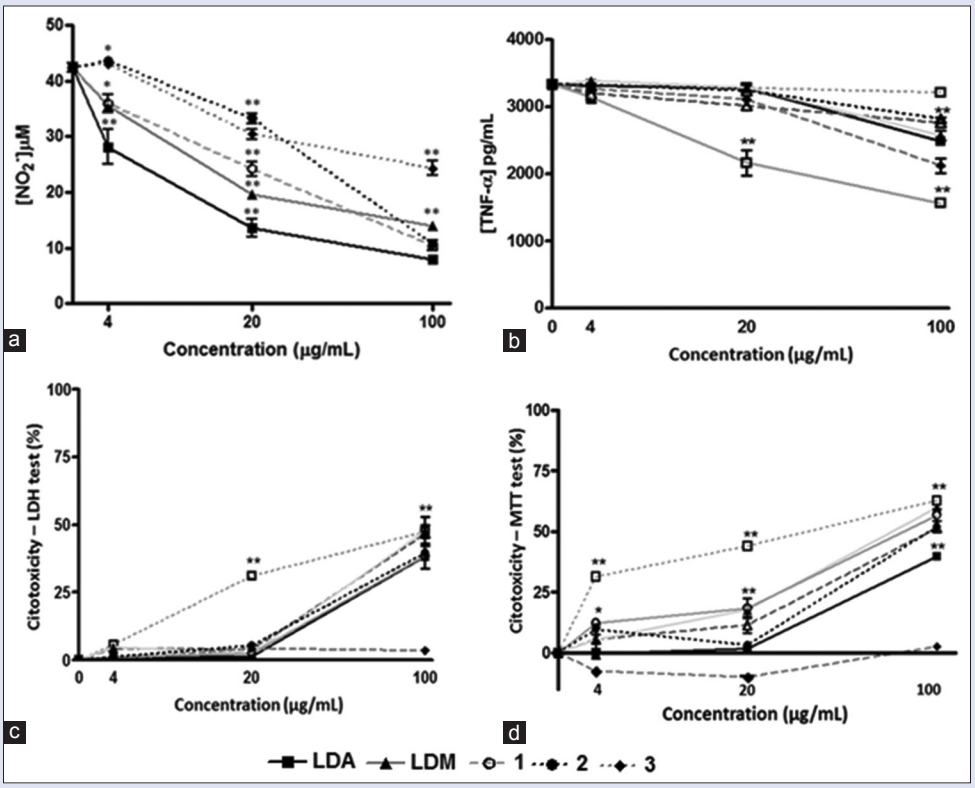


Figure 2: Potential anti-inflammatory (a and b) and cytotoxic activities (c and d) of *Laurencia dendroidea* extracts and their sesquiterpenes 1–3 at concentrations of 4, 20, and 100 μg/mL in lipopolysaccharide-stimulated RAW 264.7 macrophages. (a) The inhibitory effect of algal compounds on nitric oxide production. Negative control: Macrophages stimulated with 1 μg/mL lipopolysaccharide (42.4 ± 0.96 μM). Treatment with N^G-methyl-l-arginine acetate salt was used as a positive control of nitric oxide inhibition, reducing nitric oxide production by 59.22 ± 2.96% at 20 μg/mL. (b) The inhibitory effect on tumor necrosis factor-alpha production was measured using an L929 fibroblast bioassay. Untreated macrophages were used as a negative control (5.95 ± 3.16 pg/mL). (c) Specific release of lactate dehydrogenase. Lipopolysaccharide-stimulated macrophages were used as a negative control (O.D. 0.238, cytotoxicity – 0%) and 1% Triton X-100 detergent-treated macrophages were used as positive controls (O.D. 1.098, cytotoxicity – 100%). (d) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Lipopolysaccharide-stimulated macrophages were used as a negative control (O.D. 1.170, cytotoxicity – 0%) and 1% Triton X-100 detergent-treated macrophages as a positive control (O.D. 0.078, cytotoxicity – 100%). Arithmetic means ± standard deviation ($n = 3$). $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***) compared to the cells treated with lipopolysaccharide only

dehydrogenase release from dying cells and evaluating the ability of macrophages to metabolize MTT to formazan. Crude extracts of LDA and LDM were not toxic for macrophages at concentrations of 4 and 20 μg/mL and displayed a moderate cytotoxic effect (<30% of cell viability at 100 μg/mL) [Figure 2c and d].

Among the purified compounds, cartilagineol (3) did not show significant toxicity, whereas (-)-elatol (1) and obtusol (2) displayed weak cytotoxicity [Figure 2c and d]. However, it is unlikely that cytotoxicity mediated the inhibitory effects of (-)-elatol (1) and obtusol (2) on the production of pro-inflammatory mediators, since these compounds were also active at noncytotoxic concentrations.

To study the mechanism of NO inhibition, we evaluated the ability of the extracts and purified compounds to scavenge NO radicals derived from a NO donor, SNP, and to suppress the induction of iNOS, a key enzyme in NO production by activated macrophages. As seen in Figure 3a, the algal samples scavenged only low amounts of SNP-derived NO (about 25–30% of the NO scavenging activity). Therefore, these data demonstrated that the scavenging of the generated NO could not explain the strong inhibitory activity of the tested compounds on NO production by macrophages.

To examine the level of expression of iNOS by the treated macrophages, we carried out a Western blot analysis using specific anti-iNOS anti-bodies [Figure 3b]. For these experiments, we used concentrations of the samples that were not toxic to cultured cells (4 and 20 μg/mL). According to densitometry analysis of the bands, iNOS protein expression was significantly reduced by crude LDA extract and (-)-elatol (1) by 91.6% and 70.7%, respectively, at a concentration of 20 μg/mL. At the lowest concentration (4 μg/mL), these samples retained their ability to inhibit iNOS by 34.8% and 18.4%, respectively. These results agree with the results of NO inhibition shown in Figure 2a.

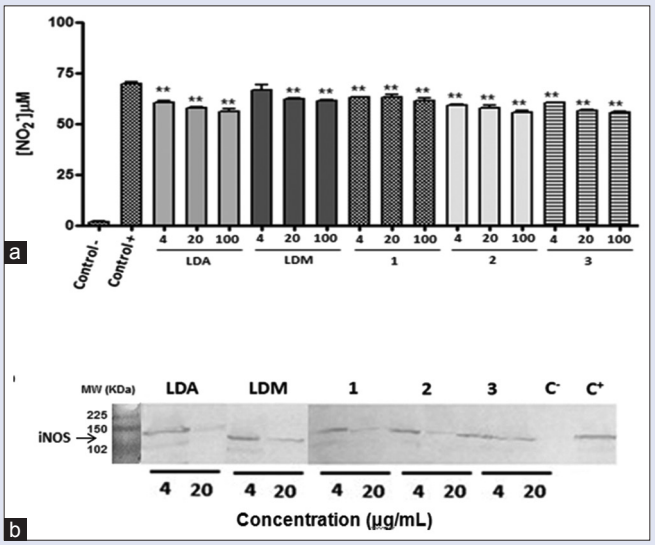


Figure 3: Effect of *Laurencia dendroidea* extracts and their sesquiterpenes 1–3 on the scavenging of nitric oxide and expression of inducible nitric oxide synthase in RAW 264.7 cells. (a) Scavenging of nitric oxide derived from sodium nitroprusside. Medium with sodium nitroprusside and samples (4, 20, and 100 μg/mL) was incubated for 2.5 h. The negative control of nitrite accumulation: culture medium without sodium nitroprusside (0.01 ± 0.04 μM nitric oxide$_2^-$). Positive control of nitrite accumulation: culture medium with 5 mM sodium nitroprusside (69.66 ± 1.41 μM nitric oxide$_2^-$). Data were expressed as the mean ± standard deviation of three independent experiments performed in triplicate. P<0.001 (**) in relation to the sodium nitroprusside-treated group. (b) Effects of the algal compounds on lipopolysaccharide-induced inducible nitric oxide synthase expression. The cells were incubated with lipopolysaccharide, 1 μg/mL, in the presence of each of the studied compounds, at concentration of 4 or 20 μg/mL for 24 h, lysed, and submitted to western blotting analysis employing an inducible nitric oxide synthase-specific anti-body. The positive control (C+) consisted of macrophages stimulated with lipopolysaccharide only. Untreated macrophages were used as the negative control (C–)

Our data demonstrating anti-inflammatory activities of *L. dendroidea* agree with those of previous reports, in which the anti-inflammatory potential of other *Laurencia* species was observed. *Laurencia okamurae* was reported to be a potent inhibitor of the production of pro-inflammatory mediators, such as prostaglandin E2, interleukin-6 (IL-6), NO, and TNF-α.[16] Extract of *L. undulata* was found to inhibit airway hyper-responsiveness and inflammation to ovalbumin antigen in a murine model of asthma, associated with a reduction in IL-4, IL-5, and TNF-α levels.[27] Neorogioltriol, a tricyclic brominated diterpenoid isolated from the organic extract of the red *Alga L. glandulifera*, showed anti-inflammatory effects *in vitro*, on LPS-treated RAW 264.7 macrophages, and *in vivo*, using the carrageenan-induced paw edema model.[15] To the best of our knowledge, anti-inflammatory activities of *L. dendroidea*, as well as its purified compounds (1-3), are reported here for the first time.

Inflammation is strongly involved in the pathogenesis of most infectious diseases, including TB. In general, the production of pro-inflammatory mediators by the infected macrophages, such as NO and TNF-α, is essential for protection from mycobacteria. The treatment of TB is based on the use of anti-mycobacterial drugs. However, the most severe forms of TB, such as military TB or tuberculous meningitis, require additional anti-inflammatory therapy to prevent excessive inflammation.[25] For instance, treatment with dexamethasone[28] and prednisolone[29] was demonstrated to reduce mortality and residual neurological deficit in patients surviving tuberculous meningitis.

Considering the anti-inflammatory potential of the tested samples, their anti-mycobacterial activity was additionally verified in a view of a combined anti-TB therapy. In this study, we tested the ability of *L. dendroidea* extracts and the halogenated sesquiterpenes (1–3) to inhibit the growth of *M. bovis* BCG in culture and subsequently to *M. tuberculosis* $H_{37}Rv$ in culture. As can be seen in Figure 4a, crude LDA extract showed a high anti-mycobacterial activity for *M. bovis* BCG in culture, inhibiting bacterial growth even at the lowest concentration tested (IC_{50} 8.7 ± 1.4 μg/mL). The extract showed similar activity in the $H_{37}Rv$ culture at concentrations of 100 and 20 μg/mL, as observed previously (IC_{50} 12.36 ± 0.97 μg/mL). The LDA extract was more active than the LDM extract in both evaluated mycobacterial cultures, in *M. bovis* BCG (IC_{50} 33.4 ± 0.3 μg/mL) and in *M. tuberculosis* $H_{37}Rv$ (IC_{50} 44.76 ± 0.14 μg/mL). These results showed that although the extracts were obtained from the same *Alga* species, samples collected in different regions can exhibit distinct levels of biological activity. This might be explained by a different distribution of compounds according to collection sites.[9]

The tested halogenated sesquiterpenes, elatol (1) and obtusol (2), displayed a moderate-to-high anti-mycobacterial activity in the growth of *M. bovis* BCG. Obtusol (2) was the most active compound with an IC_{50} of 31.4 ± 0.8 μg/mL (equivalent to 75.8 ± 1.8 μM) [Figure 4a]. Notably, structural differences between obtusol (2) and cartilagineol (3) only existed at the bromine and chlorine positions and at the absolute configuration of these halogenated carbon atoms. The enantiomeric form of a common sesquiterpene, (-)-elatol (1), found in many species of *Laurencia* and known for its potent anti-bacterial activity[30] was less active than compound 2 [Figure 4a]. The obtusol (2) was also the most active in

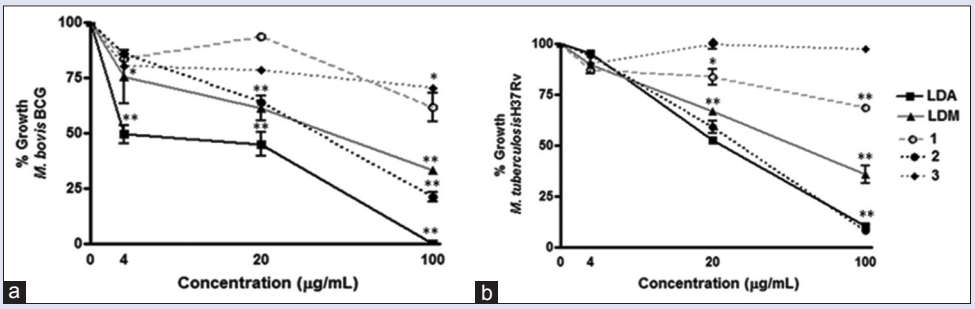


Figure 4: Effect of *Laurencia dendroidea* extracts and isolated sesquiterpenes on the growth of *Mycobacterium bovis Bacillus* Calmette-Guérin and *Mycobacterium tuberculosis* $H_{37}Rv$ evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Extracts (*Laurencia dendroidea* from Angra dos Reis and Manguinhos) and isolated sesquiterpenes (1–3) were studied at concentrations of 4, 20, and 100 µg/mL. (a) As a positive control, *Mycobacterium bovis Bacillus* Calmette-Guérin suspension treated with the anti-biotic rifampicin at concentrations of 0.0011, 0.0033, 0.0100, and 0.0300 µg/mL was used, demonstrating 67.42 ± 1.31%, 88.69 ± 4.09%, 91.40 ± 2.18%, and 97.13 ± 3.27% growth inhibition, respectively. (b) As a positive control, *Mycobacterium tuberculosis* $H_{37}Rv$ suspension treated with the anti-biotic rifampicin at concentrations of 0.0016, 0.008, 0.04, 0.2, and 1 µg/mL was used, demonstrating 29.27 ± 0.67%, 49.37 ± 0.52%, 81.75 ± 0.43%, 93.75 ± 1.09%, and 99.59 ± 1.28% growth inhibition, respectively. For both, negative control-untreated bacterial suspension. Arithmetic mean ± standard deviation ($n = 3$); $P < 0.05$ (*) and $P < 0.001$ (**) in relation to the nontreated group (0 µg/mL)

M. tuberculosis $H_{37}Rv$ culture (IC_{50} of 97.1 ± 1.4 µM), whereas elatol (1) was not able to inhibit mycobacterial growth [Figure 4b]. It is important to highlight that LDA and obtusol (2) showed anti-mycobacterial activity at nontoxic concentrations for macrophages [Figure 2b and c].

Although marine organisms are a promising source of anti-mycobacterial compounds,[31] only one study has reported the anti-mycobaterial activity of natural products produced by *Laurencia*, in which elatol was also tested.[14] Thus, the present study describes for the first time, anti-mycobacterial activity for *L. dendroidea*, obtusol (2) and cartilagineol (3).

CONCLUSION

Our results demonstrate that the *L. dendroidea* LDA extract and previously isolated (-)-elatol (1) were the most active immunomodulatory (anti-inflammatory) samples, which strongly inhibited NO and TNF-α production by activated macrophages. The observed inhibitory activity against NO production was mediated mainly by the specific inhibition of iNOS expression in activated cells. Additionally, LDA was the most active extract in the inhibition of mycobacterial growth, and this effect was mediated predominantly by obtusol (2). The data obtained in this study showed that *L. dendroidea* is a promising source of anti-inflammatory and anti-mycobacterial drugs. In the future, the most active compounds identified in this study will be tested for anti-inflammatory and anti-mycobacterial activity *in vivo*, employing models of animal infection with *M. tuberculosis*.

Acknowledgments

The authors thank FINEP (3175/06), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and the Fundação de Amparo à Pesquisa do Rio de Janeiro for financial support. F.L.S. Machado and A.R. Soares thank the CNPq for Ph.D. fellowships and productivity fellowships, respectively. T. Ventura also thanks the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior for a Ph.D. fellowship.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

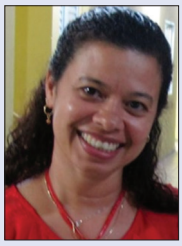
1. Blunt JW, Copp BR, Hu WP, Munro MH, Northcote PT, Prinsep MR. Marine natural products. Nat Prod Rep 2007;24:31-86.
2. Wang BG, Gloer JB, Ji NY, Zhao JC. Halogenated organic molecules of *Rhodomelaceae* origin: Chemistry and biology. Chem Rev 2013;113:3632-85.
3. Masuda M, Abe T, Sato S, Suzuki T, Suzuki M. Diversity of halogenated secondary metabolites in the red *Alga Laurencia nipponica* (*Rhodomelaceae, Ceramiales*). J Phycol 1997;33:196-208.
4. Pereira RC, Da Gama BA, Teixeira VL, Yoneshigue-Valentin Y. Ecological roles of natural products of the Brazilian red seaweed *Laurencia obtusa*. Braz J Biol 2003;63:665-72.
5. König GM, Wright AD. *Laurencia rigida*: Chemical investigations of its antifouling dichloromethane extract. J Nat Prod 1997;60:967-70.
6. Mohammed KA, Hossain CF, Zhang L, Bruick RK, Zhou YD, Nagle DG. Laurenditerpenol, a new diterpene from the tropical marine *Alga Laurencia intricata* that potently inhibits hif-1 mediated hypoxic signaling in breast tumor cells. J Nat Prod 2004;67:2002-7.
7. Morales JL, Cantillo-Ciau ZO, Sanchez-Molina I, Mena-Rejon GJ. Screening of anti-bacterial and anti-fungal activities of six marine macroalgae from coasts of Yucatán peninsula. Pharm Biol 2006;44:632-35.
8. Sakemi S, Higa T, Jefford CW, Bernardinelli G. Venustatriol. A new, anti-viral, triterpene tetracyclic ether from *Laurencia venusta*. Tetrahedron Lett 1986;27:4287-90.
9. da Silva Machado FL, Pacienza-Lima W, Rossi-Bergmann B, de Souza Gestinari LM, Fujii MT, Campos de Paula J, *et al.* Antileishmanial sesquiterpenes from the Brazilian red *Alga Laurencia dendroidea*. Planta Med 2011;77:733-5.
10. Davyt D, Fernandez R, Suescun L, Mombrú AW, Saldaña J, Domínguez L, *et al.* New sesquiterpene derivatives from the red *Alga Laurencia scoparia*. Isolation, structure determination, and anthelmintic activity. J Nat Prod 2001;64:1552-5.
11. Martínez JM, Hernández H, Acuña D, Esquivel M, Lizama RS, Payrol JÁ. Inhibitory activity of Laurencia algal extracts on *in vitro* growth of Plasmodium falciparum. Rev Cubana Med Trop 2005;57:192-5.
12. Veiga-Santos P, Pelizzaro-Rocha KJ, Santos AO, Ueda-Nakamura T, Dias Filho BP, Silva SO, *et al. In vitro* anti-trypanosomal activity of elatol isolated from red seaweed *Laurencia dendroidea*. Parasitology 2010;137:1661-70.
13. Vairappan CS, Kawamoto T, Miwa H, Suzuki M. Potent antibacterial activity of halogenated compounds against antibiotic-resistant bacteria. Planta Med 2004;70:1087-90.
14. König GM, Wright AD, Franzblau SG. Assessment of antimycobacterial activity of a series of mainly marine derived natural products. Planta Med 2000;66:337-42.
15. Chatter R, Ben Othman R, Rabhi S, Kladi M, Tarhouni S, Vagias C, *et al. In vivo* and *in vitro* anti-inflammatory activity of neorogioltriol, a new diterpene extracted from the red algae *Laurencia glandulifera*. Mar Drugs 2011;9:1293-306.
16. Yang EJ, Moon JY, Kim MJ, Kim DS, Kim CS, Lee WJ, *et al.* Inhibitory effect of Jeju endemic seaweeds on the production of pro-inflammatory mediators in mouse macrophage cell line RAW 264.7. J Zhejiang Univ Sci B 2010;11:315-22.

17. Soares AR, Robaina MC, Mendes GS, Silva TS, Gestinari LM, Pamplona OS, *et al.* Antiviral activity of extracts from Brazilian seaweeds against herpes simplex virus. Rev Bras Farmacogn 2012;22:714-23.
18. Da-Silva SA, Costa SS, Rossi-Bergmann B. The anti-leishmanial effect of *Kalanchoe* is mediated by nitric oxide intermediates. Parasitology 1999;118:575-82.
19. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
20. Muzitano MF, Cruz EA, de Almeida AP, Da Silva SA, Kaiser CR, Guette C, *et al.* Quercitrin: An antileishmanial flavonoid glycoside from *Kalanchoe pinnata*. Planta Med 2006;72:81-3.
21. Gomez-Flores R, Gupta S, Tamez-Guerra R, Mehta RT. Determination of MICs for *Mycobacterium avium*-M. *intracellulare* complex in liquid medium by a colorimetric method. J Clin Microbiol 1995;33:1842-6.
22. Shen SC, Lee WR, Lin HY, Huang HC, Ko CH, Yang LL, *et al. In vitro* and *in vivo* inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E2 production. Eur J Pharmacol 2002;446:187-94.
23. Kundu JK, Surh YJ. Inflammation: Gearing the journey to cancer. Mutat Res 2008;659:15-30.
24. Guzik TJ, Korbut R, Adamek-Guzik T. Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol 2003;54:469-87.
25. Garlanda C, Di Liberto D, Vecchi A, La Manna MP, Buracchi C, Caccamo N, *et al.* Damping excessive inflammation and tissue damage in *Mycobacterium tuberculosis* infection by Toll IL-1 receptor 8/single Ig IL-1-related receptor, a negative regulator of IL-1/TLR signaling. J Immunol 2007;179:3119-25.
26. Sodhi A, Tarang S, Kesherwani V. Concanavalin A induced expression of Toll-like receptors in murine peritoneal macrophages *in vitro*. Int Immunopharmacol 2007;7:454-63.
27. Jung WK, Choi I, Oh S, Park SG, Seo SK, Lee SW, *et al.* Anti-asthmatic effect of marine red *Alga* (*Laurencia undulata*) polyphenolic extracts in a murine model of asthma. Food Chem Toxicol 2009;47:293-7.
28. Buonsenso D, Serranti D, Valentini P. Management of central nervous system tuberculosis in children: Light and shade. Eur Rev Med Pharmacol Sci 2010;14:845-53.
29. Prasad K, Singh MB. Corticosteroids for managing tuberculous meningitis. Cochrane Database Syst Rev. 2008;23:CD002244.
30. Cabrita MT, Vale C, Rauter AP. Halogenated compounds from marine algae. Mar Drugs 2010;8:2301-17.
31. Laport MS, Santos OC, Muricy G. Marine sponges: Potential sources of new antimicrobial drugs. Curr Pharm Biotechnol 2009;10:86-105.


Dr. Elena Lasunskaia


Michelle Muzitano


Thatiana Ventura


Lísia Gestinari


Angelica Soares


Carlos Kaiser


Francisco Esteves


Fernanda Machado


Marlon Heggdorne

ABOUT AUTHORS

Research program of **Dr. Elena Lasunskaia, PhD, MD,** addresses immunologic bases of how mycobacterial pathogens cause disease employing murine models of experimental infection. The studies in pathogenesis of tuberculosis are aimed on a deeper understanding of the host-pathogen interface to find new approaches to treatment.

Michelle Muzitano, PhD, is an Adjunct Professor in Federal University of Rio de Janeiro School of Pharmacy. Dr.Muzitano specializes in Pharmacognosy and Chemistry of Natural Products. Her research interests include Medicinal plants, New Drugs Research, HPLC-MS, NMR, Tuberculosis and Immunopharmacology.

Thatiana Ventura obtained MSc and PhD degrees from the University of Northern Fluminense. Her doctoral research has focused on evaluation of anti-inflammatory and anti-mycobacterial activities of natural products and synthetic compounds in vitro and *in vivo*.

Lísia Gestinari, PhD. Expert in taxonomy, ecophysiology and molecular biology of Marine Benthic Seaweeds, acting on the knowledge for the conservation of biodiversity in marine ecosystems.

Angelica Soares obtained her BSc in chemistry and Ph.D. in Organic Chemistry at the Federal Fluminense University. Her main research area concentrates on the marine natural products, marine chemical ecology, drug discovery; exploration, conservation and development of marine biodiversity.

Carlos Kaiser is graduated in Chemistry and received his Ph.D degree from the University of Campinas/Brazil. He is expert in Chemistry, focusing on Chemistry of the Natural Products, NMR, Organic Synthesis and Medicinal Chemistry. He is a full professor and NMR Sector Coordinator (LABRMN-IQ/UFRJ) at the Chemistry Institute of the Federal University of Rio de Janeiro, Brazil.

Francisco Esteves obtained his PhD at the Max-Plack Insitut für Limnologie - Universitat Kiel (Christian-Albrechts) in 1978. He was founder and currently is a general director of Center for Ecology and Socio-Environmental Development (NUPEM/UFRJ). Has experience in Ecology, with emphasis on Ecology of Aquatic Ecosystems.

Fernanda Machado obtained her Master and PhD degrees in Chemistry from the Federal University of Rio de Janeiro. Her research interests involve Chemistry of Natural Products, focusing on Laurencia, Rhodophyta and chamigrane.

Marlon Heggdorne is a PhD student in Pharmaceuthical Science program at the Federal University of Rio de Janeiro. He received his Master degree in Bioscience and Bioactive Products focusing on antimycobacterial activity of natural products obtained from plant species.