

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

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ABSTRACT

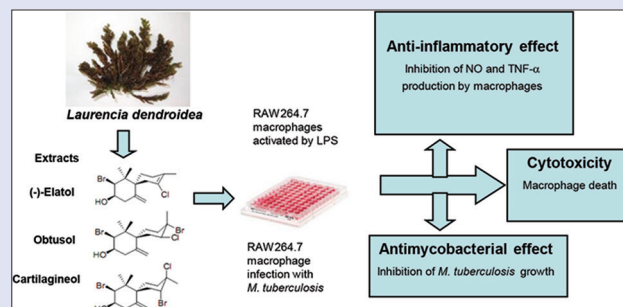
Background: Red algae of the genus *Laurencia* J. V. Lamouroux are a rich source of secondary metabolites with important pharmacological activities such as anti-tumoral, anti-inflammatory, anti-fungal, anti-viral, anti-leishmanial, anti-helminthic, anti-malarial, anti-trypanosomal, anti-microbial as 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* H₃₇Rv 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 [IC₅₀] 8.7 \pm 1.4 μ g/mL) and NO production by activated macrophages (IC₅₀ 5.3 \pm 1.3 μ g/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).



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DOI : 10.4103/0973-1296.172972

Access this article online

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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.

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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 dichloromethane: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 CH₂Cl₂, AcOEt, and *n*-BuOH. All fractions were analyzed by thin-layer chromatography (TLC). The CH₂Cl₂ 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: CH₂Cl₂ 6:4; 43 mg; 0.00125%, w/w, of *Alga* dry weight) and 2 (*n*-hexane: CH₂Cl₂ 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 CH₂Cl₂ (0–100% in steps of 10%, 50 mL each) in *n*-hexane.

The LDM extract (4.54 g) was partitioned with H₂O and CH₂Cl₂. The resulting CH₂Cl₂ 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 CH₂Cl₂ (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 (CH₂Cl₂-Hex 7:3); [α] D: -66.2 (*c* 0.13, CHCl₃); 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 (CH₂Cl₂-Hex 7:3); [α] D: +9.61 (*c* 0.05, CHCl₃); 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 (CH₂Cl₂-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 μ g/mL, corresponding to the following molar concentrations: Compound 1 (300, 60, and 12 μ M) and compounds 2 and 3 (241, 48, and 10 μ M), 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 μ g/mL) in the presence of 5% CO₂ at 37°C. These cells were seed in 96-well plates (1 × 10⁵ cells/well) in the presence or absence of various concentrations of the algal samples (100, 20, and 4 μ g/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 × 10⁵ 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)-2,5-diphenyl-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)-2,5-diphenyl-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 × 10⁶ 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 H₂O, 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* H₃₇Rv (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 × 10⁶ CFU/well) in the presence of each sample at three concentrations. The sealed plate was incubated at 37°C and 5% CO₂ for 7 days for *M. bovis* BCG or 5 days for *M. tuberculosis* H₃₇Rv. 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 (IC₅₀) 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

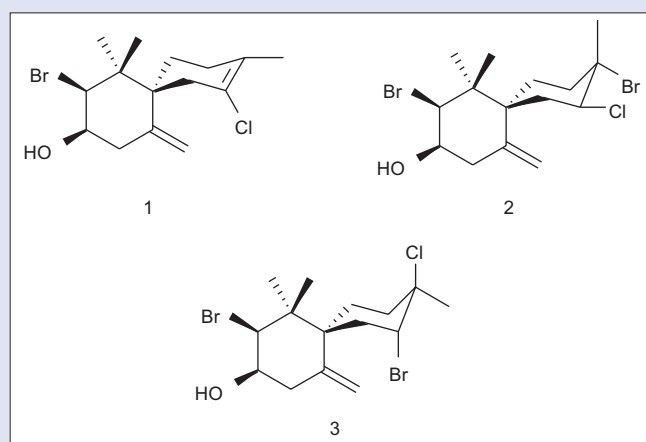


Figure 1: Chemical structures of (-)-elatol (1), obtusol (2), and cartilageol (3)

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 $\mu\text{g/mL}$), as shown in Figure 2a. The LDA extract (IC_{50} 5.3 \pm 1.3 $\mu\text{g/mL}$) was more active than LDM (IC_{50} 14.1 \pm 1.1 $\mu\text{g/mL}$). 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 \pm 2.96%, when tested at the noncytotoxic concentration of 20 $\mu\text{g/mL}$ (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 \pm 1.1 $\mu\text{g/mL}$, 49.5 \pm 3.2 μM), which inhibited NO production at a concentration of 20 $\mu\text{g/mL}$. This effect was not observed for obtusol (2) (IC_{50} 33.2 \pm 1.0 $\mu\text{g/mL}$, 80.2 \pm 2.5 μM) or cartilagineol (3) (IC_{50} > 100 $\mu\text{g/mL}$, >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 $\mu\text{g/mL}$ 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* extract 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 $\mu\text{g/mL}$ (53.01 \pm 2.49%) and 20 $\mu\text{g/mL}$ (35.05 \pm 1.92%) with an IC_{50} of 189.8 \pm 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

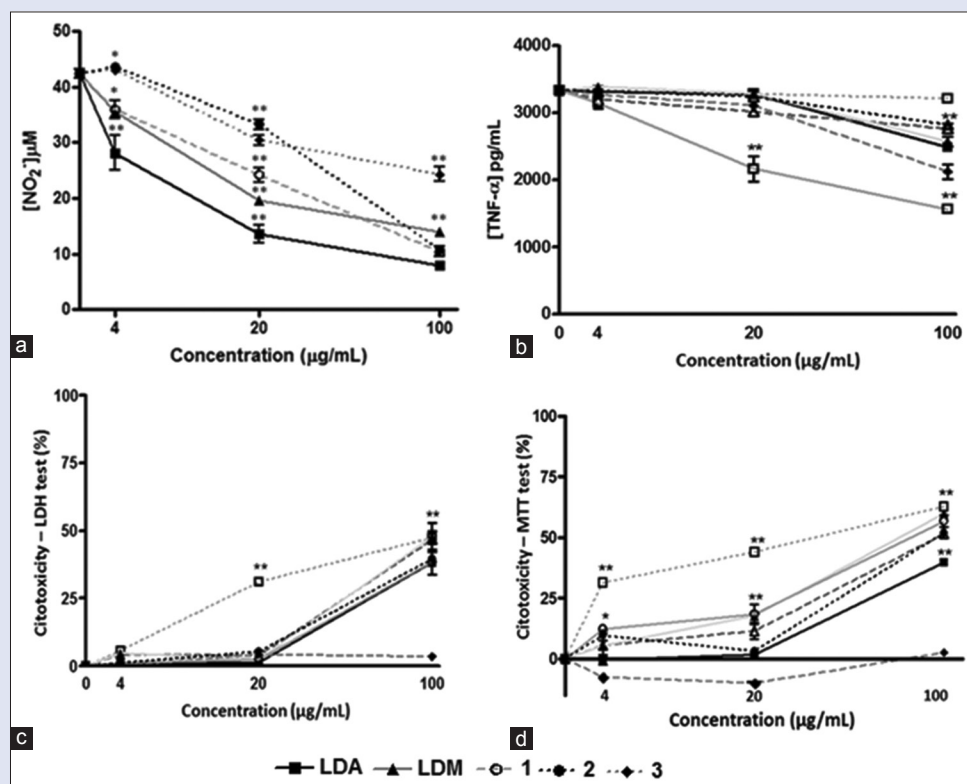


Figure 2: Potential anti-inflammatory (a and b) and cytotoxic activities (c and d) of *Laurencia dendroidea* extracts and their sesquiterpenes 1–3 at concentrations of 4, 20, and 100 $\mu\text{g/mL}$ in lipopolysaccharide-stimulated RAW 264.7 macrophages. (a) The inhibitory effect of algal compounds on nitric oxide production. Negative control: Macrophages stimulated with 1 $\mu\text{g/mL}$ lipopolysaccharide (42.4 \pm 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 \pm 2.96% at 20 $\mu\text{g/mL}$. (b) The inhibitory effect on tumor necrosis factor- α production was measured using an L929 fibroblast bioassay. Untreated macrophages were used as a negative control (5.95 \pm 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 \pm 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 $\mu\text{g}/\text{mL}$ and displayed a moderate cytotoxic effect (<30% of cell viability at 100 $\mu\text{g}/\text{mL}$) [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 $\mu\text{g}/\text{mL}$). 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 $\mu\text{g}/\text{mL}$. At the lowest concentration (4 $\mu\text{g}/\text{mL}$), 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.

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 okamurai* 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* H₃₇Rv 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 (IC₅₀ 8.7 \pm 1.4 $\mu\text{g}/\text{mL}$). The extract showed similar activity in the H₃₇Rv culture at concentrations of 100 and 20 $\mu\text{g}/\text{mL}$, as observed previously (IC₅₀ 12.36 \pm 0.97 $\mu\text{g}/\text{mL}$). The LDA extract was more active than the LDM extract in both evaluated mycobacterial cultures, in *M. bovis* BCG (IC₅₀ 33.4 \pm 0.3 $\mu\text{g}/\text{mL}$) and in *M. tuberculosis* H₃₇Rv (IC₅₀ 44.76 \pm 0.14 $\mu\text{g}/\text{mL}$). 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 IC₅₀ of 31.4 \pm 0.8 $\mu\text{g}/\text{mL}$ (equivalent to 75.8 \pm 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

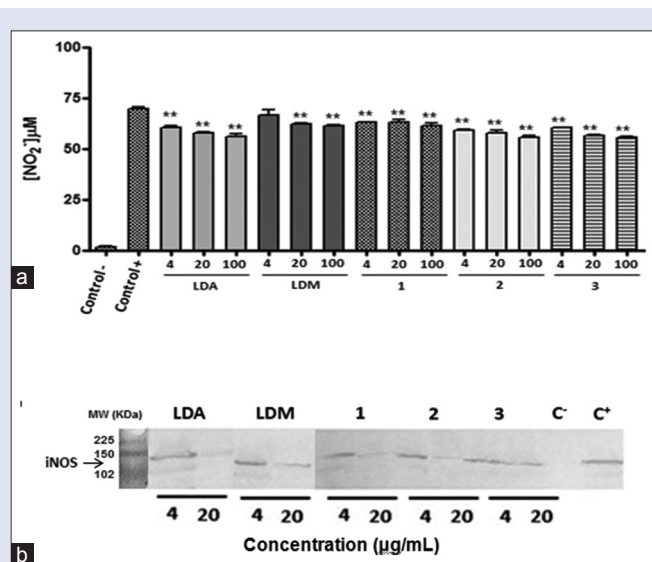


Figure 3: Effect of *Laurencia dendroidea* extracts and their sesquiterpenes 1–3 on the scavenging of nitric oxide and expression of inducible nitric oxide synthase in RAW 264.7 cells. (a) Scavenging of nitric oxide derived from sodium nitroprusside. Medium with sodium nitroprusside and samples (4, 20, and 100 $\mu\text{g}/\text{mL}$) was incubated for 2.5 h. The negative control of nitrite accumulation: culture medium without sodium nitroprusside (0.01 \pm 0.04 μM nitric oxide₂⁻). Positive control of nitrite accumulation: culture medium with 5 mM sodium nitroprusside (69.66 \pm 1.41 μM nitric oxide₂⁻). Data were expressed as the mean \pm 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 $\mu\text{g}/\text{mL}$, in the presence of each of the studied compounds, at concentration of 4 or 20 $\mu\text{g}/\text{mL}$ 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-)

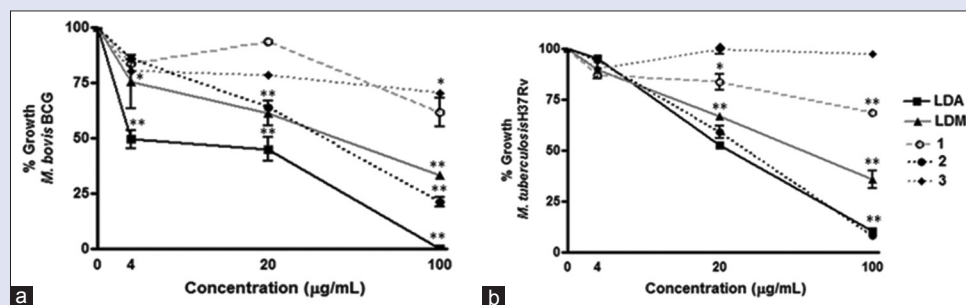


Figure 4: Effect of *Laurencia dendroidea* extracts and isolated sesquiterpenes on the growth of *Mycobacterium bovis* Bacillus Calmette-Guérin and *Mycobacterium tuberculosis* H₃₇Rv evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. Extracts (*Laurencia dendroidea* from Angra dos Reis and Manguihos) 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* H₃₇Rv 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 H₃₇Rv culture (IC₅₀ 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-mycobacterial 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.

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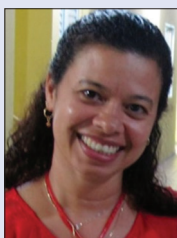
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