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Novel Phenylpropanoid Derivative from *Euploca procumbens* (Mill.) Diane & Hilger with Potential Anti-Inflammatory Activity

George L. D. dos Santos,[a] *Francisca S. V. Lins,*[a] *Yuri M. do Nascimento,*[a] *Thalisson A. de Souza,*[a] *Luiza C. F. Opretzka,*[b] *Cristiane F. Villarreal,*[b] *Ana C. F. de Albuquerque,*[c] *Lucas S. Abreu,*[c] *Fernando M. dos Santos Junior,*[c] *José I. M. de Melo,*[d] *Ivana M. Fechine,*[e] *Raimundo Braz-Filho,*[f] *Josean F. Tavares*[*,a] *and Marcelo S. da Silva*[a]

[a]*Instituto de Pesquisa em Fármacos e Medicamentos, Universidade Federal da Paraíba, 58050-585 João Pessoa-PB, Brazil*

[b]*Faculdade de Farmácia, Universidade Federal da Bahia, 40170-115 Salvador-BA, Brazil*

[c]*Departamento de Química Orgânica, Universidade Federal Fluminense, 24020-141 Niterói-RJ, Brazil*

[d]*Departamento de Biologia, Universidade Estadual da Paraíba, 58109-753 Campina Grande-PB, Brazil*

[e]*Departamento de Farmácia, Universidade Estadual da Paraíba, 58109-753 Campina Grande-PB Brazil*

[f]*Instituto de Química, Universidade Federal Rural do Rio de Janeiro, 23890-000 Seropédica-RJ, Brazil*

A new phenylpropanoid derivative, named euploic acid (**1**), was isolated from *Euploca procumbens* (Mill.) Diane & Hilger, Boraginaceae, along with lithospermic acid B (**2**), lithospermic acid (**3**), 9''-methyl lithospermate (**4**), and luteolin-7-*O*-glucoside (**5**). Compound structures were determined by mass spectrometry (MS), nuclear magnetic resonance (NMR) analysis and comparison with published data. Absolute configuration of **1** was established via electronic circular dichroism (ECD). Anti-inflammatory potential of euploic acid (**1**) was assessed by measuring its inhibition of cytokine and nitric oxide production in stimulated J774 macrophages. Compound **1** significantly reduced pro-inflammatory mediator production and release by stimulated macrophages at non-cytotoxic concentrations, comparable to the efficacy of dexamethasone. Our findings demonstrate the potential of euploic acid (**1**) as an effective anti-inflammatory agent. This study contributes to the structural elucidation of a new phenylpropanoid derivative and highlights its promising anti-inflammatory activity in the treatment of inflammatory disorders.

Keywords: Boraginaceae, phenolic acid, anti-inflammatory, structure elucidation

Introduction

The Boraginaceae family encompasses a significant group of plants that have garnered medicinal interest due to their diverse secondary metabolites. Various compounds, such as terpenoids, naphthoquinones, flavonoids, phenylpropanoids, alkaloids, and lignans, have been isolated from different species within this family.[1-3] These compounds have exhibited a wide range of biological activities, including antitumor, antiulcer, antimicrobial, antifungal, larvicidal, anti-inflammatory, analgesic, antileishmanial, and antidepressant properties.[4-6] Among the Boraginaceae genera, *Euploca* comprises approximately 100 species and is predominantly found in South America and Mexico.[7]

Euploca procumbens (syn. *Heliotropium procumbens*) is widely distributed throughout various phytogeographic domains in Brazil, including the Amazon, Cerrado, Caatinga, and Atlantic Forest regions.[8] In local communities, this species is referred to as "*borragem cinzenta*" in Bahia and "*erva azul*" in Pernambuco. Ethnomedicinal studies have reported the presence of steroids and pyrrolizidine alkaloids, namely heliotrine, indicine, indicinine, and supinine, in *E. procumbens*.[9] However, there is still a lack of scientific studies focused on the isolation and

*e-mail: josean@ltf.ufpb.br
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characterization of chemical constituents from this species, particularly regarding their different classes and biological activities.

In this study, we report the isolation and characterization of five phenylpropanoids from *E. procumbens*, including a novel compound named euploic acid, along with four known compounds: lithospermic acid B, lithospermic acid, 9''-methyl lithospermate, and luteolin-7-*O*-glucoside.[10] Furthermore, we investigated the potential anti-inflammatory activity of euploic acid by evaluating its effects on the production of nitric oxide (NO) and cytokines by stimulated macrophages *in vitro*.

Experimental

General experimental procedures

Maceration and liquid-liquid extraction were carried out using ethanol, methanol, hexane, chloroform, ethyl acetate and *n*-butanol analytical grade (Qhemis, Hexis, Brazil). The extracts and fractions were concentrated by a rotary evaporator (Buchi, St. Gallen, Switzerland). Sephadex® LH-20 (Sigma-Aldrich, Munich, Germany) was employed to purify the samples. Analytical high performance liquid chromatography (HPLC) was performed on a UFLC (Shimadzu, Kyoto, Japan) with LC-20AD binary solvent pump module, an SIL-10A auto-injector, a DGU-20 A_3 degassing system and an SPD-M10A diode array detector (DAD), and a CMB-20A interface was also used. A Kromasil, (Nouryon, Mains, Germany) C18 analytical column (250 mm × 4.6 mm × 5 µm) was used at 0.6 mL min^{-1} flow and 20 µL injection volume. Whereas an C18 preparative column (ACE, Aberdeen, UK), (250 mm × 21.2 mm × 5 µm), had 8 mL min^{-1} flow rate and 100 µL injection volume. The mobile phase was composed by ultrapure water, Milli-Q® (Merck, Rahway, USA) acidified with 0.1% (v/v) formic acid (A) and methanol (Biograde, Anápolis, Brazil) (B), a gradient elution mode with an analysis set time in minutes and detection at 254 and 280 nm was used.

Nuclear magnetic resonance (NMR) spectra were recorded on spectrometers at 500 and 400 MHz for ^1H, 100 and 125 MHz for ^{13}C (Bruker, Billerica, USA). The equipment were optimized for one-dimensional (BB, attached proton test (APT) and distortionless enhancement by polarization transfer (DEPT)-135) and two-dimensional techniques (heteronuclear single quantum coherence spectroscopy (HSQC), heteronuclear multiple bond correlation (HMBC) and correlated spectroscopy (COSY)) recorded on CD_3OD and dimethyl sulfoxide (DMSO-d_6, Sigma-Aldrich, Munich, Germany). Chemical shifts (δ) were expressed in parts *per* million (ppm). High-resolution mass spectrometer (HRMS) experiments were performed in micrOTOF-II (Bruker, Billerica, USA) coupled with electrospray ionization (ESI). ESI conditions were as follows: capillary voltage 4.0 kV in the negative mode; end plate offset 500 V; 49.3 psi fogging pressure; dry gas (N_2) with 8 L min^{-1} flow rate at 200 °C.

Plant material

Aerial parts and roots of *E. procumbens* were collected in March 2019 from the vicinity of Campina Grande, Paraíba (latitude 07º13'50"S, longitude 35º52'52"W). The plant material was identified by Prof Dr José Iranildo Miranda de Melo from the Department of Biological Sciences at the State University of Paraíba, and a voucher specimen (ACAM-1931) was deposited at the Herbarium Manuel de Arruda Câmara. The collection and handling of the plant material comply with the guidelines of the Convention on Biological Diversity, and the accesses information has been registered in the Sistema Nacional de Gestão do Patrimônio Genético (SisGen) under A9A4116 code.

Extraction and isolation

The roots of *E. procumbens* were dried in an air circulation oven at 40 ºC for 96 h and then ground into a powder using a knife mill grinder, yielding 220 g of powder. The powder was extracted with 96% ethanol (3 L) for 72 h. The ethanol extract was concentrated using a rotary evaporator, resulting in 10 g of extract. From this, 9 g were partitioned successively with hexane (2 × 200 mL), $CHCl_3$ (5 × 200 mL), EtOAc (4 × 300 mL), and *n*-butanol (4 × 250 mL), and each phase was concentrated using a rotary evaporator. The EtOAc and *n*-butanol phases were separated on a Sephadex LH-20 chromatographic column and eluted with 100% MeOH, yielding fractions 7 (Fr7) and 8 (Fr9), respectively.

For further purification, the analyzed fractions were subjected to preparative HPLC-DAD with a flow rate of 8 mL min^{-1} and an injection volume of 100 µL. Gradient elution was performed using solvent A (0.1% formic acid in H_2O) and solvent B (MeOH). Fr5 (EtOAc, 50 mg) was subjected to the following elution profile: 0.0-100 min (2-53% B), resulting in the isolation of compound **1** (t_R = 78 min, 6.4 mg) and compound **2** (t_R = 97 min, 8.3 mg). Fr5 (*n*-butanol, 70 mg) was subjected to the elution profile: 0.0-100 min (0-60% B), leading to the isolation of compound **3** (t_R = 83 min, 5.8 mg) and compound **4** (t = 91 min, 3.6 mg)

The aerial parts of *E. procumbens* were treated similarly. A total of 2.830 g of powder was obtained and extracted with 96% ethanol (5 L) for 72 h. Subsequently, 50 g of the ethanolic extract was partitioned with hexane (5 × 400 mL), $CHCl_3$ (5 × 300 mL), EtOAc (4 × 400 mL), and *n*-butanol (4 × 200 mL). The resulting soluble fractions were concentrated using a rotary evaporator. The EtOAc fraction was further fractionated into eleven fractions, and Fr5 (EtOAc, 67 mg) was subjected to analytical and preparative HPLC. A gradient elution (0.0 to 100 min, 2-60% B) resulted in the isolation of compound **5** (t_R = 94 min, 5 mg).

Cytotoxicity to mammalian cells

The Alamar Blue method as previously described was used to evaluate cell viability.[10] Murine macrophage-like J774 cells were plated in 96-well plates at a cell density of 2×10^5 cells well^{-1} in Dulbecco's modified Eagle medium (DMEM; Life Technologies, GIBCO-BRL, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO), and 50 µg mL^{-1} gentamicin (Novafarma, Anápolis, GO, Brazil) and incubated for 72 h at 37 °C and 5% CO_2. The cells were then stimulated with lipopolysaccharides (LPS) (500 ng mL^{-1}, Sigma Chemical Co., St. Louis, USA) and interferon-gamma (IFN-γ) (5 ng mL^{-1}, Sigma). Substance **1** was added to the medium at five concentrations ranging from 12.5 to 200 µM in triplicate and incubated for 72 h. Afterwards, 20 µL well^{-1} of Alamar Blue (Invitrogen, Carlsbad, USA) were added to the plates, followed by incubation for 6 h. Colorimetric readings were performed at 570 and 600 nm. Gentian violet (Synth, São Paulo, Brazil) at 10 µM was used as the positive control.

Assessment of cytokine and nitric oxide production by stimulated macrophages

J774 cells were seeded in 96-well tissue culture plates at 2×10^5 cells well^{-1} in DMEM medium supplemented with 10% of FBS and 50 µg mL^{-1} gentamicin for 2 h at 37 °C and 5% CO_2, as described previously, for cytokine and nitric oxide evaluations.[11] Cells were then stimulated with LPS (500 ng mL^{-1}) and IFN-γ (5 ng mL^{-1}) in the presence of **1** at different concentrations (12.5 to 200 µM), medium (control group), or dexamethasone (20 µM, gold-standard drug), and incubated at 37 ºC. Cell-free supernatants were collected 24 h after the incubation for IL-1β and nitrite quantification. IL-1β concentrations in supernatants from J774 cultures were determined by enzyme-linked immunosorbent assay (ELISA), using the DuoSet kit from R&D Systems (Minneapolis, MN, USA), according to the instructions of the manufacturer. The results were expressed in pg mL^{-1} IL-1β. Quantification of nitrite, as an indicator of nitric oxide (NO) production, was performed using the Griess method.[12]

Statistical analysis

Data are presented as mean ± standard deviation (SD), n = 3. Comparisons between groups were made using one-way analysis of variance (ANOVA) with Tukey's post-hoc test. Analyses were performed using Prism 8.4.3 computer Software[13] with statistical significance at $p < 0.05$.

Results and Discussion

Ethanol extracts (EE) from roots and aerial parts of *E. procumbens* were subjected to chromatographic processes resulting in the isolation of compounds **1-5** (Figure 1). Compound **1** was isolated as a yellow powder with a positive optical rotation of $[\alpha]^{25}_D$ +14 (*c* 0.1, MeOH). Its molecular formula was determined to be $C_{36}H_{28}O_{16}$ by high-resolution electrospray ionization mass spectrometry (HRESIMS) ion of *m/z* 715.1321 $[M - H]^-$ (calcd. for $[C_{36}H_{27}O_{16}]^-$, 715.1305, Δ = -2.3 ppm, where theoretical *m/z* value - experimental *m/z* value), indicating 19 indices of hydrogen deficiency (IHD) (Figure S17, Supplementary Information (SI) section).

The set of signals in ^{1}H NMR spectrum of compound **1** showed a characteristic arrange of aromatic protons typical from an ABX system at δ_H 6.84 (d, *J* 2.0 Hz, 1H), δ_H 6.87 (d, *J* 8.2 Hz, 1H), δ_H 6.91 (dd, *J* 8.2 and 2.0 Hz, 1H), signals for a trisubstituted aromatic system δ_H 6.83 (d, *J* 2.0 Hz, 1H) and δ_H 6.75 (m, 2H), two doublets δ_H 7.11 (d, *J* 8.5 Hz, 1H) and δ_H 6.85 (d, *J* 8.5 Hz, 1H), two *trans*-olefinic protons at δ_H 7.33 (d, *J* 15.9, 1H) and δ_H 5.98 (d, *J* 15.9, 1H), and two doublets corresponding to aliphatic protons at δ_H 6.10 (d, *J* 6.3 Hz, 1H) and δ_H 4.34 (d, *J* 6.3 Hz, 1H), suggesting a structure similar to lithospermic acid B (**2**).[14-16]

At the same spectrum, two singlets at δ_H 5.91 (s, 1H) and δ_H 5.82 (s, 1H) differs from the pattern observed for compound **2**, indicating substitutions at 1,3,4,6 position of one aromatic moiety. In the ^{13}C NMR spectrum, the presence of four carbonyl carbons at δ_C 167.1, 173.1, 173.5 and 172.7 together with the signals at δ_C 88.6 and δ_C 59.3 led to a compound derived from lithospermic acid.

In the HMBC spectrum, evident correlations were observed between the signal at δ_H 6.10 (H-7'') and the carbons at δ_C 59.3 (C-8''), δ_C 172.6 (C-9''), δ_C 133.9 (C-1''), δ_C 113.7 (C-2''), and δ_C 118.6 (C-6''), confirmed the dehydrobenzofuran unit. Additional correlations between δ_H 5.69 (H-8''') and δ_C 173.0 (C-9'''), δ_C 172.6 (C-9''), and

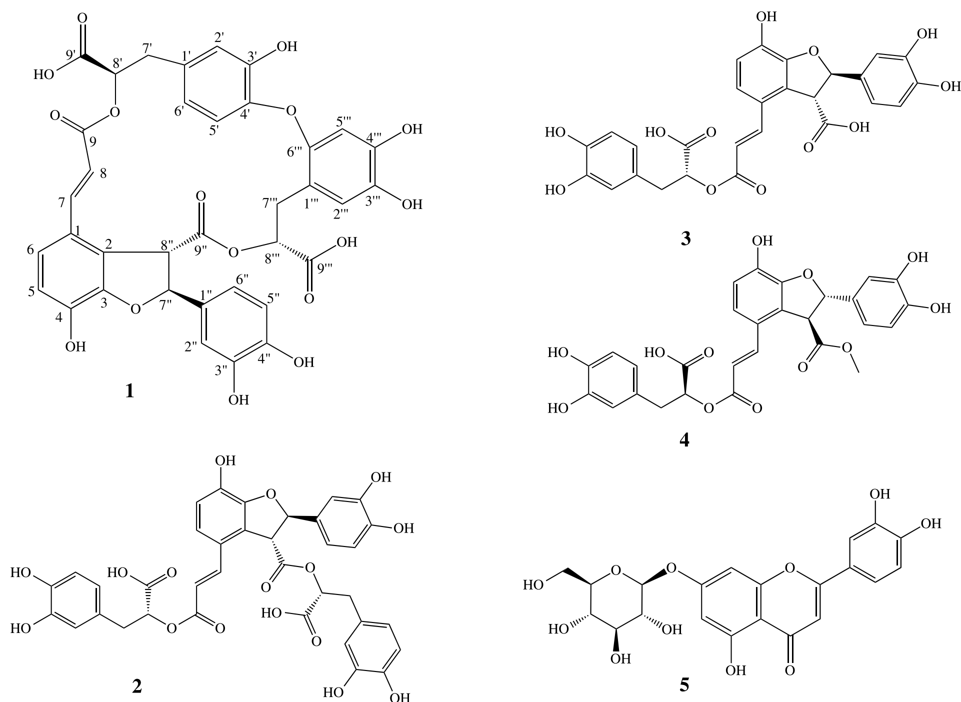

Figure 1. Compounds isolated from *Euploca procumbens.*

δ_C 33.8 (C-7’’’), indicated C-8’’’–O–C-9” linkage. The mutual correlations of the signal at δ_H 5.91 (H-2’’’) with δ_C 117.1 (C-1’’’), δ_C 33.8 (C-7’’’), and δ_C 150.2 were assigned to C-6’’’, confirming the 1,3,4,6 oxygenation pattern of the aromatic ring. The ether bonding by C-6’’’ explains the chemical shift of C-7’’’ at δ_C 33.8 due to a gamma protection effect. In the same spectrum, correlations of the signal at δ_H 5.98 (H-8) with δ_C 167.0 were assigned to C-9 and of δ_H 5.06 (H-8’) with δ_C 167.0 (C-9) and δ_C 173.4 were assigned to C-9’ and with δ_C 37.6 (C-7’), confirming another C-8´–O–C-9 bond. The signal at δ_H 3.08 (H-7’) correlated with δ_C 126.1, confirming C-6’.

Through the HMQC spectrum, the correlation of C-6’ established the chemical shift of the proton at δ_H 6.91. This signal in turn correlated with the carbon at δ_C 149.5 corresponding to C-4’. C-4’ (δ_C 149.5) and C-6’’’ (δ_C 150.3) exhibited higher chemical shift values compared to the other oxygenated sp2 carbons of the related units, such as C-3’ (δ_C 146.2), C3’’’ (δ_C 141.1), and C-4’’’ (δ_C 146.2). This finding, combined with the established molecular mass through high-resolution analysis, supported the presence of a C-4–O–C-6’’’ bonding, which involved the coupling of two C6-C3 units. Experimental and calculated ECD spectra were consistent with the same configuration found in lithospermic acid[14] (Figure S18, SI section). Notably, this coupling through two C6-C3 units in lithospermic acid derivatives has not been reported in the literature. Therefore, compound **1** was identified as a new natural product named euploic acid. The other correlations are presented in Table 1 and Figure 2.

Comparing ^{1}H and ^{13}C NMR data and values reported in the literature, the structures of known compounds were identified as: lithospermic acid B (**2**) and lithospermic acid (**3**)[14] 9”-methyl lithospermate (**4**),[17] and luteolin-7-*O*-glucoside (**5**).[18]

The anti-inflammatory activity of compound **1** was assessed using stimulated J774 macrophages as an *in vitro* model. Macrophages are immune cells that are implicated in inflammation and immune regulation processes and contribute to tissue homeostasis.[19,20] Upon activation by inflammatory stimuli, macrophages produce a myriad of pro-inflammatory mediators, such as chemokines, cytokines and nitric oxide (NO).[19]

First, the non-cytotoxic concentration range was determined by testing the cell viability of macrophages treated with compound **1**. To define this non-cytotoxic concentration range to perform the subsequent experiments is an important step, as the reduction of cell viability

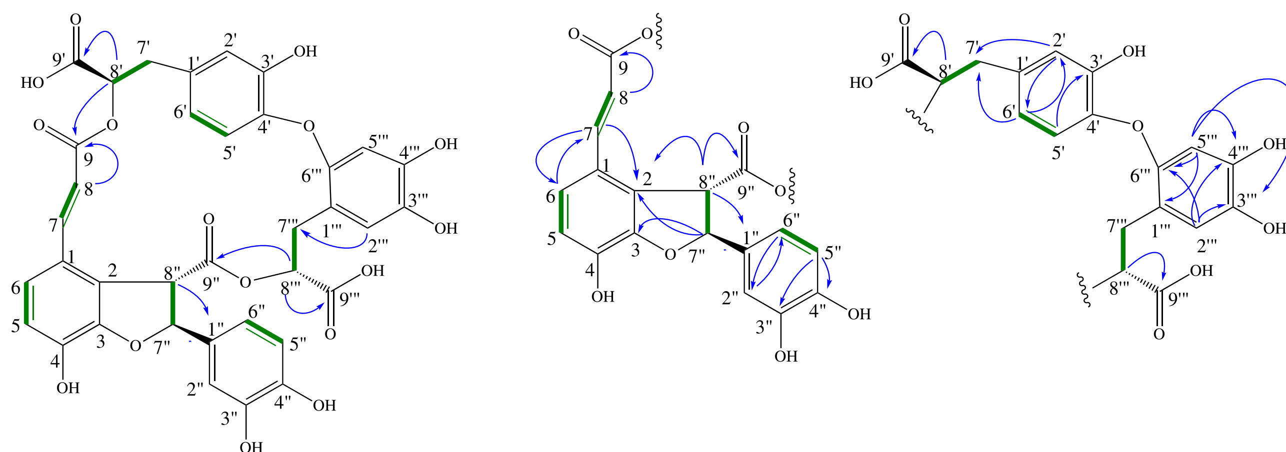

Figure 2. The key HMBC (arrow) and COSY (green line) correlations for compound **1**.

Table 1. NMR spectroscopic data for compound **1**

Position	δ_C[a,b] / ppm	δ_H[a,c] (*J* / Hz) / ppm	Position	δ_C[a,b] / ppm	δ_H[a,c] (*J* / Hz) / ppm
1	123.9	–	9”	172.7	–
2	126.9	–	1’’’	117.3	–
3	149.0	–	2’’’	119.1	5.91 (s)
4	144.9	–	3’’’	141.1	–
5	118.6	6.85 (d, 8.5)	4’’’	146.2	–
6	122.1	7.11 (d, 8.5)	5’’’	105.7	5.82 (s)
7	142.0	7.33 (d, 15.9)	6’’’	150.3	–
8	116.1	5.98 (d, 15.9)	7’’’	33.9	3.61 (dd, 13.9, 2.9)
9	167.1	–			2.48 (dd, 13.9, 12.3)
1’	131.4	–	8’’’	72.9	5.69 (dd, 12.3, 2.9)
2’	123.1	6.84 (d, 2.0)	9’’’	173.1	–
3’	146.2	–			
4’	149.5	–			
5’	117.7	6.87 (d, 8.2)			
6’	126.2	6.91 (dd, 8.2, 2.0)			
7’	37.7	3.08 (dd, 14.6, 1.8)			
		3.00 (dd, 14.6, 11.1)			
8’	74.6	5.06 (dd, 11.1, 1.8)			
9’	172.5	–			
1”	134.0	–			
2”	113.7	6.83 (d, 2.0)			
3”	146.0	–			
4”	146.9	–			
5”	116.5	6.75 (m)[d]			
6”	118.6	6.75 (m)[d]			
7”	88.7	6.10 (d, 6.3)			
8”	59.4	4.34 (d,6.3)			

[a]Measured in CD_3OD, [b]125 MHz, [c]500 MHz; [d]multiplicity not defined, signal overlap.

would consequently cause a decrease in inflammatory mediator production, which could be wrongly interpreted as a possible anti-inflammatory activity. Macrophages stimulated with LPS (500 ng mL^{-1}) and IFN-γ (5 ng mL^{-1}) were treated with the vehicle (DMSO 2% in culture medium; CTRL), different concentrations of compound **1**,

or gentian violet (used as positive control). Stimulation with LPS (500 ng mL^{-1}) and IFN-γ (5 ng mL^{-1}) did not affect cell viability, as the unstimulated cells and stimulated cells showed similar cell viability (data not shown). The data showed that compound **1** did not exert cytotoxic effects in stimulated J774 macrophages at concentrations up to 200 µM (Figure 3). Thus, the following assays were carried out using the concentration range tested.

Next, the effect of compound **1** on the production/release of pro-inflammatory mediators was evaluated.

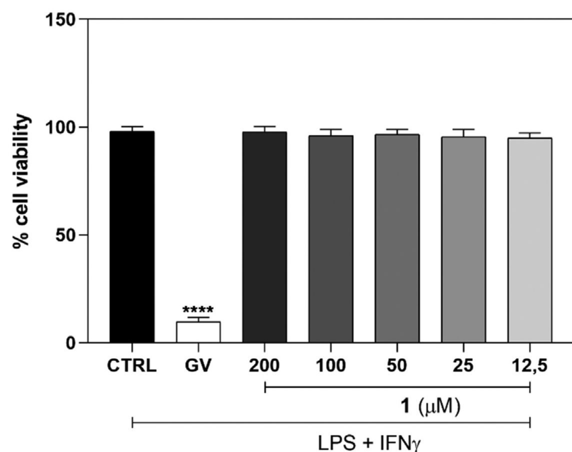

Figure 3. Effect of compound **1** on cell viability of stimulated J774 macrophages. Different concentrations of compound **1** (12.5-200 µM) or gentian violet (GV; 10 µM, positive control) were added to the macrophage culture and incubated for 72 h. Control group (CTRL) represents untreated cells stimulated with LPS (500 ng mL^{-1}) and IFN-γ (5 ng mL^{-1}).

Untreated macrophages stimulated with LPS and IFN-γ (CTRL) showed increased levels of NO and IL-1β compared to unstimulated cells, indicated as the basal group (Figure 4a, $p < 0.01$; Figure 4b, $p < 0.001$, respectively). Treatment with compound **1** (50 to 200 µM) reduced nitric oxide production in a dose-dependent manner compared to untreated stimulated cells ($p < 0.001$; Figure 4a). Moreover, the inhibitory effect of compound **1** at 200 µM on NO production was similar to that of dexamethasone (20 µM), the gold-standard drug (Figure 4a; $p < 0.001$). NO is a molecule with a ubiquitous role, but during inflammatory events, it will have mainly a pro-inflammatory role. NO acts as an oxidant agent or a signaling mediator, and it amplifies the inflammatory response by activating cascades that lead to the production of more inflammatory mediators.[21]

Similarly, cytokines are pivotal mediators in the inflammatory response. Therefore, the potential anti-inflammatory activity of compound **1** was further evaluated by measuring the levels of the pro-inflammatory cytokine IL-1β produced by stimulated macrophages. The inhibitory effect of compound **1** on the release of IL-1β can also be observed. Stimulated macrophages treated with compound **1** in concentrations from 12.5 to 200 µM showed a dose-dependent reduction in the production/release of IL-1β compared to stimulated untreated cells ($p < 0.0001$, Figure 4b). This effect was comparable to that of dexamethasone (20 µM; $p < 0.001$). IL-1β is

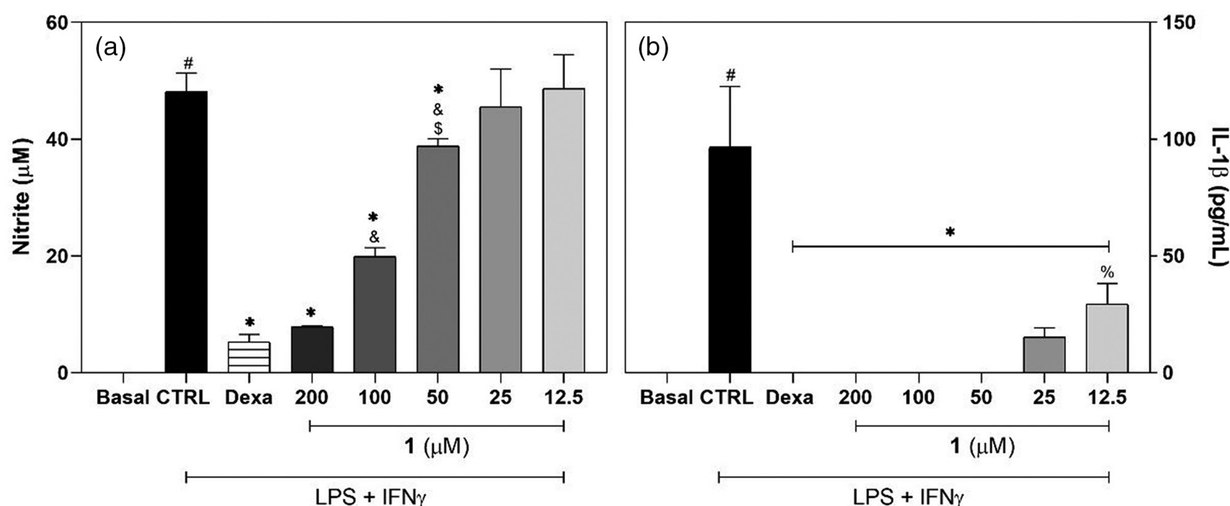

Figure 4. Inhibitory effect of **1** on the production of nitric oxide and IL-1β by stimulated J774 macrophages. (a) Nitrite levels determined by the Griess method. Different concentrations of compound **1** (12.5-200 µM) or dexamethasone (Dexa; 20 µM, reference drug) were added to J774 macrophages cultures in the presence of LPS (500 ng mL^{-1}) + IFN-γ (5 ng mL^{-1}). Nitrite quantifications were performed 24 h after treatments. The control group (CTRL) represents untreated cells stimulated with LPS + IFN-γ. The basal group shows data from untreated and unstimulated cells. #Different from the unstimulated (Basal) group ($p < 0.05$). &Different from 200 µM and Dexa groups ($p < 0.05$). $Different from the 100 µM group ($p < 0.05$). *Different from the control (CTRL) group ($p < 0.0001$). (b) IL-1β quantification data by ELISA. Different concentrations of 1 (12.5-200 µM) or dexamethasone (Dexa; 20 µM, reference drug) were added to J774 macrophages cultures stimulated with LPS + IFN-γ. The control group (CTRL) represents untreated cells stimulated with LPS + IFN-γ. The basal group shows data from untreated and unstimulated cells. IL-1β levels were determined 24 h after treatments. #Different from the basal group ($p < 0.001$). *Different from the control (CTRL) group ($p < 0.05$). %Different from 200, 100 and 50 µM and Dexa groups ($p < 0.05$). Data are expressed as mean ± SD of 3 replicates. One-way ANOVA followed by Tukey's test.

implicated as a key cytokine in inflammatory events. It serves primarily as a pro-inflammatory cytokine, plus it can induce its own production on a positive feedback loop that increases inflammatory signaling.[22] It is widely recognized that an enhanced secretion/induction of IL-1β has been associated with the pathogenesis of autoinflammatory diseases, metabolic syndrome, acute inflammation, chronic inflammation and malignancy.[23] Therefore, the aforementioned capacity to decrease the levels of pro-inflammatory mediators NO and IL-1β during the inflammatory response is an interesting feature for anti-inflammatory compounds.

In agreement with the present results, other compounds that are similar in structure to compound **1** were found to have anti-inflammatory activity. Lithospermic acid (**2**), structurally similar to compound **1**, exhibited anti-inflammatory activity by reducing monosodium urate crystal-induced paw edema in rats.[24] Additionally, a set of compounds chemically related to compound **1** obtained from *Salvia miltiorrhiza* roots, including lithospermic acid, also inhibited the production of pro-inflammatory cytokines TNF-α, IL-1β and IL-6.[25]

Conclusions

The current phytochemical investigation of *E. procumbens* led to the isolation of a new phenylpropanoid derivative (euploic acid) and four compounds previously cited in the literature (lithospermic acid B, lithospermic acid, 9''-methyl lithospermate and luteolin-7-*O*-glucoside). Data presented here demonstrate for the first time the biological activity of *Euploca procumbens* and its compounds. Among them, compound **1** (euploic acid) inhibited the production/release of pro-inflammatory mediators, which is an important attribute of anti-inflammatory compounds, with similar efficacy as that of dexamethasone, which is considered the gold-standard anti-inflammatory. Therefore, these results evidence the anti-inflammatory potential of euploic acid (**1**).

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

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Author Contributions

GLDS was responsible for conceptualization, investigation and writing original draft; FSVL, YMN and TAS for methodology and investigation (HPLC, MS, and NMR analysis); CFV and LCFO for methodology and investigation (biological activity analysis); JIMM for investigation (plant material); ACFA, FMSJ for formal analysis and investigation (ECD analyses); RBF for validation; IMF for supervision; JFT and LSA for project administration and writing review and editing; MSS for supervision, validation and funding acquisition. All authors have read the final manuscript and approve the submission.

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