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ARTICLE

Anti-inflammatory activity of novel thiosemicarbazone compounds indole-based as COX inhibitors

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Abstract

Background In this article, a series of 20 new thiosemicarbazone derivatives containing indole were synthesized and evaluated for their anti-inflammatory potential.

Methods The compounds were obtained through a synthetic route of only two steps, with yields that varied between 33.6 and 90.4%, and characterized by spectroscopic and spectrometric techniques.

Results An initial screening through the lymphoproliferation assay revealed that compounds LT76, LT81, and LT87 were able to inhibit lymphocyte proliferation, with CC_{50} of 0.56 ± 0.036, 0.9 ± 0.01 and 0.5 ± 0.07 μM, respectively, better results than indomethacin ($CC_{50} > 12$ μM). In addition, these compounds were able to suppress the in-vitro production of TNF-α and NO, in addition to stimulating the production of IL-4. Reinforcing in-vitro assays, the compounds were able to inhibit COX-2 similar to Celecoxib showing greater selectivity for this isoform (LT81 SI: 23.06 versus Celecoxib SI: 11.88). Animal studies showed that compounds LT76 (64.8% inhibition after 6 h), LT81 (89% inhibition after 6 h) and LT87 (100% inhibition after 4 h) were able to suppress edema in mice after inoculation carrageenan with greater potency than indomethacin, and immunohistochemistry revealed that the groups treated with LT76, LT81 and LT87 reduced the expression of COX-2, similar or better results when compared to indomethacin. Complementarily, in-silico studies have shown that these compounds have a good pharmacokinetic profile, for respecting the parameters of Lipinski and Veber, showing their good bioavailability.

Conclusions These results demonstrate the potency of thiosemicarbazone derivatives containing indole and confirm their importance as scaffolds of molecules with notorious anti-inflammatory activity.

Keywords Thiosemicarbazone · Indole · Immunosuppressive drugs · Immunomodulatory agents · COX-2 · Immunohistochemistry

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Introduction

The response to tissue damage caused or not by an infectious agent triggers a series of essential vascular and cellular changes to promote the restoration of the injured structure [1]. The repair mechanism is mainly performed by chemical mediators such as prostaglandins, nitric oxide (NO), tumor necrosis factor alpha (TNF-α) and IL-1β, IL-6, IL-10, in addition to the activation of T cells and other cells inflammatory [2, 3].

These substances can modulate the inflammatory process determining its beginning, intensity, and evolution [4]. An exacerbated inflammatory response can lead to a physiological imbalance of the tissue with undesirable effects that cause pain and even destruction of the affected site [5, 6].

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Non-steroidal anti-inflammatory drugs (NSAIDs) are used as one of the therapeutic alternatives to control and to decrease the inflammatory process [7]. These drugs act by inhibiting the cyclooxygenase enzymes (COX) that are present in the body basically in two isoforms, COX-1, constitutively expressed in most tissues, and COX-2, induced in inflammatory processes [8].

In general, NSAIDs vary in the isoforms of COX. When they suppress the expression of COX-1, they end up preventing the production of prostaglandins, such as PGI_1 and PGE_2, responsible for the cytoprotection of organ mucosa (stomach and kidney, for example), resulting in gastric lesions, nephrotoxicity, and increased bleeding. This limits its use for longer treatments [9, 10] and justifies the need for more specific drugs without side effects.

The concept of privileged structures in the design of new drug prototypes is a widely used approach in medicinal chemistry [11]. Some studies point to the insertion of the thiosemicarbazone portion and the indole nucleus in several promising structures for anti-inflammatory activity [12, 13]. Thus, we aim to join these fragments to build new molecules of anti-inflammatory interest. For this, attachment of chemical groups in the *N*-4 position of thiosemicarbazone-indole fragment was planned according to previous work knowledge of structure–activity relationship [13, 14].

Thus this work aimed to investigate the anti-inflammatory potential of new indole-thiosemicarbazone derivatives. After synthesis and characterization of the compounds, the lymphoproliferation assay was employed as a screening method. The selected compounds were then tested against inflammatory cytokines and COX in vitro. To confirm the activity, the animal model of carrageenan-induced paw edema and immunohistochemistry were used. Finally, we describe in silico methods to confirm the pharmaceutical potential of the studied compounds.

Materials and methods

Chemistry

All reagents used were obtained commercially (Sigma Aldrich, Fluka and Merck). The development of the reactions was monitored using thin-layer chromatography (Merck, silica gel F_{254} on aluminum foil). The melting points were determined from capillary tubes with the Fisatom device (model 431D 60W, Brazil). The IR spectrum was generated with the Spectrum 400 equipment (Perkin Elmer). Mass spectrometry was performed using a MALDI-TOF Autoflex III device (Bruker Daltonics, USA). NMR spectra were obtained using Varian UnityPlus spectrometer 400 MHz (400 MHz for ^{1}H and 100 MHz for ^{13}C). DMSO-d_6, purchased from Sigma-Aldrich, was used as a solvent in the NMR experiments. Chemical shifts were recorded in δ units and coupling constants (*J*) were recorded in Hertz (Hz). The multiplicities were displayed as s (singlet), d (doublet), t (triplet), m (multiplet), dd (double doublet).

General procedures for obtaining thiosemicarbazide compounds

Equimolar amounts of hydrazine hydrate (1 mmol) were added slowly in solution with equimolar amounts of substituted isothiocyanate (0.5 mmol) and 20 of mL of dichloromethane. The reaction was kept in reflux for 2 h at room temperature. The product was filtered, rinsed with dichloromethane and dried in vacuum desiccator [15].

General procedures for obtaining thiosemicarbazone compounds

Thiosemicarbazides previously obtained (1 mmol) reacted with substituted 3-indole-carboxaldehyde (1 mmol) in absolute ethanol (10 mL), using a catalytic amount of glacial acetic acid (15 drops). The reaction was processed for 2 h at room temperature, followed by thin-layer chromatography. The product was precipitated from the reaction mixture, washed with absolute ethanol and dried [16].

(*E*)-2-((5-cyano-1*H*-indol-3-yl)methylene)-*N*-phenylhydrazinecarbothioamide (LQIT/LT70) Compound LT70 was obtained as white powder (78.8%). M.p. (°C): 230–231. Rf (4:6 *n*-hexane/AcOEt): 0.52. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.16 (s, 1H, NH indole), 11.62 (s, 1H, NH hydrazine), 9.84 (s, 1H, NH phenyl), 8.78 (s, 1H, HC=N), 8.42 (s, 1H, CH indole), 8.10 (s, 1H, CH indole), 7.62 (t, $J=8.4$ Hz, 2H, CH, phenyl), 7.55 (d, $J=8.4$ Hz, 2H, CH indole), 7.38 (t, $J=8$ Hz, 2H, CH phenyl), 7.21 (t, $J=7.6$ Hz, 1H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.0 (C=S), 140.2 (C indole), 139.4 (C=N), 138.8 (C indole), 133.4 (C phenyl), 128.0 (C phenyl), 127.6 (C phenyl), 125.7 (C phenyl), 125.6 (C indole), 125.1 (C indole), 123.5 (C indole), 120.5 (C indole), 113.1 (CN), 111.8 (C indole), 102.7 (C indole). IR (KBr): $\tilde{\nu}=3358$ (NH), 2220 (CN), 1553 (C=N), 1533 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{13}N_5S$: 320.092; found: 320.109.

(*E*)-2-((4-nitro-1*H*-indol-3-yl)methylene)-*N*-phenylhydrazinecarbothioamide (LQIT/LT71) Compound LT71 was obtained as red powder (90.4%). M.p. (°C): 245–246. Rf (4:6 *n*-hexane/AcOEt): 0.65. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.56 (s, 1H, NH indole), 11.76 (s, 1H, NH hydrazine), 9.57 (s, 1H, NH phenyl), 8.55 (s, 1H, HC=N), 8.48 (d, $J=2.8$ Hz, 1H, CH indole), 7.87 (d, $J=8.0$ Hz, 1H, CH indole), 7.83 (d, $J=8.0$ Hz, 1H, CH indole), 7.72 (d, $J=8.0$ Hz, 2H, CH phenyl), 7.38 (d, $J=8.0$ Hz, 2H, CH phenyl), 7.31 (d, $J=6$ Hz,

1H, CH indole), 7.18 (t, $J=7.6$ Hz, 1H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.3 (C=S), 142.4 (C indole), 139.8 (C=N), 139.1 (C indole), 139.0 (C indole), 133.5 (C phenyl), 128.1 (C phenyl), 124.8 (C phenyl), 124.3 (C phenyl), 121.5 (C indole), 118.1 (C indole), 117.1 (C indole), 115.7 (C indole), 109.5 (C indole). IR (KBr): $\tilde{\nu}$ 3348 (N–H), 3329 (N–H), 3152 (N–H), 1550 (C=N), 1502 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{16}H_{13}N_5O_2S$: 340.082; found: 340.017.

(*E*)-2-((1*H*-benzo[g]indol-3-yl)methylene)-*N*-phenylhydrazinecarbothioamide (LQIT/LT72) Compound LT72 was obtained as yellow powder (35.4%). M.p. (°C): 223–224. Rf (1:1 *n*-hexane/AcOEt): 0.59. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.58 (s, 1H, NH indole), 11.66 (s, 1H, NH hydrazine), 9.71 (s, 1H, NH phenyl), 8.51 (s, 1H, HC=N), 8.41 (d, $J=8.0$ Hz, 1H, CH indole), 8.34 (d, $J=8.8$ Hz, 1H, CH indole), 8.02 (d, $J=2.8$ Hz, 1H, CH indole), 7.97 (d, $J=7.6$ Hz, 1H, CH indole), 7.69 (d, $J=8.0$ Hz, 2H, CH phenyl), 7.61 (d, $J=9.2$ Hz, 1H, CH indole), 7.57 (d, $J=8.0$ Hz, 1H, CH indole), 7.46 (t, $J=7.6$ Hz, 1H, CH indole), 7.38 (d, $J=8.0$ Hz, 2H, CH phenyl), 7.20 (t, $J=7.6$ Hz, 1H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.0 (C=S), 141.5 (C=N), 139.7 (C phenyl), 132.2 (C indole), 130.7 (C indole), 129.2 (C indole), 128.5 (C phenyl), 126.1 (C phenyl), 125.5 (C phenyl), 124.7 (C indole), 121.8 (C indole), 121.6 (C indole), 121.2 (C indole), 120.7 (C indole), 113.0 (C indole). IR (KBr): $\tilde{\nu}$ 3307 (N–H), 3158 (N–H), 3000 (N–H), 1550 (C=N), 1505 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{20}H_{16}N_4S$: 345.112; found: 345.121.

(*E*)-2-((1*H*-pyrrolo[2,3-b]pyridin-3-yl)methylene)-*N*-phenylhydrazinecarbothioamide (LQIT/LT73) Compound LT73 was obtained as white powder (48.1%). M.p. (°C): 289–290. Rf (4:6 *n*-hexane/AcOEt): 0.52. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.18 (s, 1H, NH indole), 11.63 (s, 1H, NH hydrazine), 9.67 (s, 1H, NH phenyl), 8.67 (d, $J=8.0$ Hz, 1H, CH indole), 8.39 (s, 1H, HC=N), 8.31 (d, $J=6.0$ Hz, 1H, CH indole), 8.04 (d, $J=4.5$ Hz, 1H, CH indole), 7.62 (d, $J=8.0$ Hz, 2H, CH phenyl), 7.37 (t, $J=8.0$ Hz, 2H, CH phenyl), 7.21–7.17 (m, 1H, CH indole, 1H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.8 (C=S), 149.3 (C indole), 144.0 (C indole), 140.8 (C=N), 139.3 (C phenyl), 131.5 (C indole), 130.5 (C indole), 128.0 (C phenyl), 125.5 (C phenyl), 125.0 (C phenyl), 116.9 (C indole), 116.3 (C indole), 109.9 (C indole). IR (KBr): $\tilde{\nu}$ 3328 (N–H), 3134 (N–H), 3019 (N–H), 1553 (C=N), 1529 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{15}H_{13}N_5S$: 296.092; found: 296.129.

(*E*)-2-((7-methyl-1*H*-indol-3-yl)methylene)-*N*-phenylhydrazinecarbothioamide (LQIT/LT74) Compound LT74 was obtained as yellow powder (36.4%). M.p. (°C): 195–196. Rf (1:1 *n*-hexane/AcOEt): 0.47. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.69 (s, 1H, NH indole), 11.62 (s, 1H, NH hydrazine), 9.62 (s, 1H, NH phenyl), 8.45 (s, 1H, HC=N), 8.05 (s, 1H, CH indole), 7.94 (s, 1H, CH indole), 7.68 (d, $J=7.6$ Hz, 2H, CH phenyl), 7.38 (t, $J=8.0$ Hz, 2H, CH phenyl), 7.20 (d, $J=7.2$ Hz, 1H, CH phenyl), 7.08 (d, $J=7.6$ Hz, 1H, CH indole), 7.02 (s, 1H, CH indole), 2.50 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.3 (C=S), 141.2 (C indole), 139.2 (C=N), 136.5 (C phenyl), 130.9 (C indole), 128.1 (C phenyl), 124.9 (C phenyl), 124.8 (C indole), 123.8 (C indole), 123.2 (C indole), 121.0 (C indole), 120.9 (C phenyl), 119.2 (C indole), 111.3 (C indole), 16.6 (CH_3). IR (KBr): $\tilde{\nu}$ 3328 (N–H), 3134 (N–H), 3019 (N–H), 1553 (C=N), 1529 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{16}N_4S$: 309.112; found: 309.139.

(*E*)-*N*-(4-chlorophenyl)-2-((5-cyano-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT75) Compound LT75 was obtained as white powder (59.5%). M.p. (°C): 230–232. Rf (4:6 *n*-hexane/AcOEt): 0.48. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.06 (s, 1H, NH indole), 11.74 (s, 1H, NH hydrazine), 9.88 (s, 1H, NH phenyl), 8.77 (s, 1H, CH indole), 8.41 (s, 1H, HC=N), 8.10 (s, 1H, CH indole), 7.66 (d, $J=8.8$ Hz, 2H, CH phenyl), 7.61 (d, $J=8.8$ Hz, 1H, CH indole), 7.55 (d, $J=8.4$ Hz, 1H, CH indole), 7.43 (d, $J=8.8$ Hz, 2H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.0 (C=S), 140.6 (C indole), 138.8 (C indole), 138.4 (C=N), 133.5 (C phenyl), 129.1 (C phenyl), 127.9 (C phenyl), 127.6 (C indole), 127.3 (C indole), 125.6 (C phenyl), 123.5 (C indole), 120.5 (C indole), 113.1 (CN), 111.7 (C indole), 102.7 (C indole). IR (KBr): $\tilde{\nu}=3335$ (N–H) 3153 (N–H), 2953 (N–H), 2221 (CN), 1552 (C=N), 1498 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{12}ClN_5S$: 354.053; found: 354.056.

(*E*)-*N*-(4-chlorophenyl)-2-((4-nitro-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT76) Compound LT76 was obtained as red powder (74.2%). M.p. (°C): 235–236. Rf (4:6 *n*-hexane/AcOEt): 0.58. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.54 (s, 1H, NH indole), 11.86 (s, 1H, NH hydrazine), 9.63 (s, 1H, NH phenyl), 8.56 (s, 1H, HC=N), 8.50 (s, 1H, CH indole), 7.88 (d, $J=8$ Hz, 1H, CH indole), 7.84 (d, $J=7.6$ Hz, 1H, CH indole), 7.76 (d, $J=8.4$ Hz, 2H, CH phenyl), 7.43 (d, $J=9.2$ Hz, 2H, CH phenyl), 7.38 (t, $J=8.0$ Hz, 1H, CH indole). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.3 (C=S), 142.3 (C indole), 140.2 (C=N), 139.1 (C indole), 138.0 (C indole), 133.5 (C phenyl), 128.7 (C phenyl), 127.9 (C phenyl), 125.9 (C phenyl), 121.5 (C indole), 118.3 (C indole), 117.3 (C indole), 115.8 (C indole), 109.5 (C indole). IR (KBr): $\tilde{\nu}$ 3268 (N–H), 3116 (N–H), 2953 (N–H), 1541 (C=N), 1509 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{16}H_{12}ClN_5O_2S$: 374.043; found: 374.055.

(*E*)-2-((1*H*-pyrrolo[2,3-b]pyridin-3-yl)methylene)-*N*-(4-chlorophenyl)hydrazinecarbothioamide (LQIT/LT77) Compound LT77 was obtained as white powder (56.8%). M.p. (°C): 240–241. Rf (4:6 *n*-hexane/AcOEt): 0.44. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.17 (s, 1H, NH indole), 11.71 (s, 1H, NH hydrazine), 9.71 (s, 1H, NH phenyl), 8.68 (d, J = 8.0 Hz, 1H, CH indole), 8.38 (s, 1H, HC=N), 8.31 (d, J = 4.8 Hz, 1H, CH indole), 8.04 (s, 1H, CH indole), 7.66 (d, J = 8.8 Hz, 2H, CH phenyl), 7.42 (d, J = 8.8 Hz, 2H, CH phenyl), 7.20–7.17 (m, 1H, CH indole). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.8 (C=S), 149.3 (C indole), 144.0 (C indole), 141.1 (C=N), 138.3 (C phenyl), 131.6 (C indole), 130.6 (C indole), 129.0 (C phenyl), 127.8 (C phenyl), 127.2 (C phenyl), 116.9 (C indole), 116.3 (C indole), 109.8 (C indole). IR (KBr): $\tilde{\nu}$ 3143 (N–H), 3078 (N–H), 2923 (N–H), 1551 (C=N), 1493 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{15}H_{12}ClN_5S$: 330.053; found: 330.086.

(*E*)-2-((1*H*-benzo[g]indol-3-yl)methylene)-*N*-(4-chlorophenyl)hydrazinecarbothioamide (LQIT/LT78) Compound LT78 was obtained as white powder (33.6%). M.p. (°C): 220–222. Rf (1:1 *n*-hexane/AcOEt): 0.66. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.59 (s, 1H, NH indole), 11.72 (s, 1H, NH hydrazine), 9.77 (s, 1H, NH phenyl), 8.51 (s, 1H, HC=N), 8.40 (d, J = 8.4 Hz, 1H, CH indole), 8.35 (d, J = 8.8 Hz, 1H, CH indole), 8.02 (s, 1H, CH indole), 7.97 (d, J = 7.6 Hz, 1H, CH indole), 7.72 (d, J = 8.4 Hz, 2H, CH phenyl), 7.61 (d, J = 8.8 Hz, 1H, CH indole), 7.57 (d, J = 7.6 Hz, 1H, CH indole), 7.47 (d, J = 7.2 Hz, 1H, CH indole), 7.44 (d, J = 9.2 Hz, 2H, CH phenyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 175.0 (C=S), 141.8 (C=N), 138.8 (C phenyl), 132.2 (C indole), 130.7 (C indole), 129.3 (C phenyl), 128.8 (C indole), 128.4 (C phenyl), 127.2 (C indole), 126.1 (C phenyl), 124.7 (C indole), 122.2 (C indole), 121.9 (C indole), 121.6 (C indole), 121.2 (C indole), 120.7 (C indole), 113.0 (C indole). IR (KBr): $\tilde{\nu}$ 3404 (N–H), 3384 (N–H), 3289 (N–H), 1544 (C=N), 1493 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{20}H_{15}ClN_4S$: 379.073; found: 379.060.

(*E*)-*N*-(4-chlorophenyl)-2-((7-methyl-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT79) Compound LT79 was obtained as white powder (51.6%). M.p. (°C): 205–206. Rf (1:1 *n*-hexane/AcOEt): 0.63. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.66 (s, 1H, NH indole), 11.64 (s, 1H, NH hydrazine), 9.64 (s, 1H, NH phenyl), 8.41 (s, 1H, HC=N), 8.03 (d, J = 8.0 Hz, 1H, CH indole), 7.90 (s, 1H, CH indole), 7.67 (d, J = 8.4 Hz, 2H, CH phenyl), 7.39 (d, J = 8.8 Hz, 2H, CH phenyl), 7.02 (d, J = 8.0 Hz, 1H, CH indole), 6.97 (d, J = 7.2 Hz, 1H, CH indole), 2.46 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 174.3 (C=S), 141.5 (C indole), 138.3 (C=N), 136.5 (C phenyl), 130.9 (C indole), 128.7 (C phenyl), 127.9 (C phenyl), 126.6 (C phenyl), 123.8 (C indole), 123.2 (C indole), 121.0 (C indole), 120.8 (C indole), 119.3 (C indole), 111.2 (C indole), 16.6 (CH_3). IR (KBr): $\tilde{\nu}$ 3390 (N–H), 3288 (N–H), 3134 (N–H), 1548 (C=N), 1502 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{15}ClN_4S$: 343.073; found: 343.079.

(*E*)-2-((5-cyano-1*H*-indol-3-yl)methylene)-*N*-(naphthalen-1-yl)hydrazinecarbothioamide (LQIT/LT80) Compound LT80 was obtained as white powder (61.7%). M.p. (°C): 215–216. Rf (1:1 *n*-hexane/AcOEt): 0.63. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.10 (s, 1H, NH indole) 11.75 (s, 1H, NH hydrazine), 10.09 (s, 1H, NH naphthyl), 8.89 (s, 1H, CH indole), 8.49 (s, 1H, HC=N), 8.12 (s, 1H, CH indole), 8.01–7.89 (m, 3H, CH naphthyl), 7.68–7.52 (m, 2H, CH indole, 4H, CH naphthyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.6 (C=S), 140.5 (C indole), 138.8 (C=N), 135.8 (C indole), 133.7 (C naphthyl), 133.6 (C naphthyl), 130.6 (C naphthyl), 128.0 (C naphthyl), 127.7 (C naphthyl), 126.6 (C naphthyl), 126.3 (C naphthyl), 126.1 (C naphthyl), 125.9 (C indole), 125.7 (C naphthyl), 125.3 (C indole), 123.5 (C indole), 123.1 (C naphthyl), 120.6 (C indole), 113.0 (CN), 111.9 (C indole), 102.7 (C indole). IR (KBr): $\tilde{\nu}$ 3395 (NH), 2224 (CN), 1617 (C=N), 1557 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{21}H_{15}N_5S$: 370.108; found: 370.131.

(*E*)-*N*-(naphthalen-1-yl)-2-((4-nitro-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT81) Compound LT81 was obtained as red powder (70.4%). M.p. (°C): 248–250. Rf (4:6 *n*-hexane/AcOEt): 0.63. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.55 (s, 1H, NH indole), 11.89 (s, 1H, NH hydrazine), 9.90 (s, 1H, NH naphthyl), 8.66 (s, 1H, HC=N), 8.59 (s, 1H, CH indole), 7.99–7.84 (m, 1H, CH indole, 4H, CH naphthyl), 7.58–7.53 (m, 1H, CH indole, 3H, CH naphthyl), 7.35 (t, J = 8.0 Hz, 1H, CH indole). ^{13}C NMR (100 MHz, DMSO-d_6): δ 177.2 (C=S), 142.4 (C indole), 140.2 (C=N), 139.6 (C indole), 135.9 (C indole), 134.1 (C naphthyl), 133.2 (CN), 130.9 (C naphthyl), 128.4 (C naphthyl), 127.2 (C naphthyl), 126.8 (C naphthyl), 126.5 (C naphthyl), 126.4 (C naphthyl), 125.8 (C naphthyl), 123.7 (C naphthyl), 121.6 (C indole), 119.1 (C indole), 118.1 (C indole), 116.7 (C indole), 110.3 (C indole). IR (KBr): $\tilde{\nu}$ 3342 (N–H), 3091 (N–H), 1531 (C=N), 1518 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{20}H_{15}N_5O_2S$: 390.098; found: 390.149.

(*E*)-2-((1*H*-pyrrolo[2,3-b]pyridin-3-yl)methylene)-*N*-(naphthalen-1-yl)hydrazinecarbothioamide (LQIT/LT82) Compound LT82 was obtained as yellow powder (51%). M.p. (°C): 269–270. Rf (4:6 *n*-hexane/AcOEt): 0.54. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.18 (s, 1H, NH indole), 11.72 (s, 1H, NH hydrazine), 9.91 (s, 1H, NH naphthyl), 8.82 (d, J = 8.0 Hz, 1H, CH indole), 8.44 (s, 1H, HC=N), 8.28 (d, J = 6 Hz, 1H, CH indole), 8.05 (s, 1H, CH indole),

7.99–7.89 (m, 3H, CH naphthyl), 7.60–7.53 (m, 4H, CH naphthyl), 7.10 (t, $J=8.0$ Hz, 1H, CH indole). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.7 (C=S), 149.3 (C indole), 144.0 (C indole), 140.9 (C=N), 135.9 (C naphthyl), 133.7 (C naphthyl), 131.5 (C indole), 130.9 (C indole), 130.7 (C naphthyl), 128.0 (C naphthyl), 126.7 (C naphthyl), 126.4 (C naphthyl), 126.0 (C naphthyl), 125.9 (C naphthyl), 125.4 (C naphthyl), 123.2 (C naphthyl), 116.8 (C indole), 116.3 (C indole), 110.0 (C indole). IR (KBr): $\tilde{\nu}$ 3313 (NH), 3140 (NH), 1582 (C=N), 1557 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{19}H_{15}N_5S$: 346.108; found: 346.135.

(*E*)-2-((1*H*-benzo[g]indol-3-yl)methylene)-*N*-(naphthalen-1-yl)hydrazinecarbothioamide (LQIT/LT83) Compound LT83 was obtained as yellow powder (85.3%). M.p. (°C): 285–286. Rf (1:1 *n*-hexane/AcOEt): 0.61. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.59 (s, 1H, NH indole), 11.75 (s, 1H, NH hydrazine), 9.96 (s, 1H, NH naphthyl), 8.58 (s, 1H, HC=N), 8.46 (d, 1H, $J=12$ Hz, indole), 8.41 (d, 1H, $J=10.8$ Hz, CH indole), 8.03 (d, $J=4$ Hz, 1H, CH indole), 8.01–7.89 (m, 4H, CH naphthyl), 7.69 (d, $J=9.2$ Hz, 1H, CH naphthyl), 7.61–7.52 (m, 3H, CH indole, 2H, CH naphthyl), 7.46 (d, $J=10.8$ Hz, 1H, CH indole). ^{13}C NMR (100 MHz, DMSO-d_6): 176.4 (C=S), 141.2 (C=N), 135.7 (C indole), 133.7 (C naphthyl), 131.8 (C naphthyl), 130.4 (C naphthyl), 130.2 (C indole), 128.9 (C indole), 128.3 (C naphthyl), 128.0 (C naphthyl), 126.5 (C naphthyl), 126.1 (C naphthyl), 126.0 (C indole), 125.9 (C indole), 125.6 (C naphthyl), 125.4 (C indole), 124.2 (C indole), 123.0 (C naphthyl), 121.7 (C indole), 121.6 (C indole), 121.0 (C indole), 120.7 (C indole), 120.2 (C indole), 112.7 (C indole). IR (KBr): $\tilde{\nu}$ 3440 (NH), 3321 (NH), 1688 (C=N), 1544 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{24}H_{18}N_4S$: 395.128; found: 395.132.

(*E*)-2-((7-methyl-1*H*-indol-3-yl)methylene)-*N*-(naphthalen-1-yl)hydrazinecarbothioamide (LQIT/LT84) Compound LT84 was obtained as yellow powder (52.6%). M.p. (°C): 255–256. Rf (4:6 *n*-hexane/AcOEt): 0.53. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.68 (s, 2H, NH indole; NH hydrazine), 9.84 (s, 1H, NH naphthyl), 8.48 (s, 1H, HC=N), 8.15 (t, $J=6.4$ Hz, 1H, CH indole), 7.99–7.93 (m, 3H, CH naphthyl), 7.87 (d, $J=10.8$ Hz, 1H, CH naphthyl), 7.67 (d, $J=10.8$ Hz, 1H, CH naphthyl), 7.58 (s, 1H, CH indole), 7.55 (d, $J=4.0$ Hz, 1H, CH indole), 7.53 (d, $J=4.0$ Hz, 1H, CH indole), 6.98 (d, 2H, $J=6.0$ Hz, CH naphthyl), 2.48 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.1 (C=S), 141.3 (C=N), 136.5 (C indole), 135.6 (C naphthyl), 133.6 (C naphthyl), 131.0 (C indole), 130.3 (C naphthyl), 128.0 (C naphthyl), 126.4 (C naphthyl), 126.0 (C naphthyl), 125.9 (C naphthyl), 125.8 (C naphthyl), 125.4 (C indole), 123.8 (C indole), 123.1 (C indole), 122.9 (C naphthyl), 120.9 (C indole), 120.7 (C naphthyl), 119.5 (C indole), 111.4 (C indole), 16.6 (CH_3). IR (KBr): $\tilde{\nu}$ 3413 (NH), 3326 (NH), 1557 (C=N), 1494 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{21}H_{18}N_4S$: 359.128; found: 359.141.

(*E*)-*N*-allyl-2-((5-cyano-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT85) Compound LT85 was obtained as yellow powder (73.8%). M.p. (°C): 202–203. Rf (6:4 *n*-hexane/AcOEt): 0.5. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.10 (s, 1H, NH indole), 11.29 (s, 1H, NH hydrazine), 8.67 (s, 1H, HC=N), 8.31 (s, 1H, CH indole), 8.29 (s, 1H, NH alkyl), 8.01 (s, 1H, CH indole), 7.58 (d, $J=10.8$ Hz, 1H, CH indole), 7.53 (d, $J=10.8$ Hz, 1H, CH indole), 6.01–5.92 (m, 1H, CH allyl), 5.23–5.10 (m, 2H, CH_2 allyl), 4.28 (t, $J=7.2$ Hz, 2H, CH_2 allyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.4 (C=S), 139.7 (C indole), 138.8 (C=N), 135.4 (C allyl), 133.1 (C indole), 127.3 (C indole), 125.6 (C indole), 123.5 (C indole), 120.5 (C indole), 115.1 (C allyl), 113.1 (CN), 111.9 (C indole), 102.7 (C indole), 45.7 (C allyl). IR (KBr): $\tilde{\nu}$ 3243 (NH), 3171 (NH), 3145 (NH), 2222 (CN), 1617 (C=N), 1539 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{14}H_{13}N_5S$: 284.092; found: 284.097.

(*E*)-*N*-allyl-2-((4-nitro-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT86) Compound LT86 was obtained as red powder (81.6%). M.p. (°C): 261–262. Rf (6:4 *n*-hexane/AcOEt): 0.65. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.50 (s, 1H, NH indole), 11.44 (s, 1H, NH hydrazine), 8.46 (s, 1H, HC=N), 8.37 (s, 1H, CH indole), 8.09 (t, $J=6$ Hz, 1H, NH alkyl), 7.86 (d, $J=8.0$ Hz, 1H, CH indole), 7.82 (d, $J=7.6$ Hz, 1H, CH indole), 7.34 (t, $J=8.0$ Hz, 1H, CH indole), 5.98–5.88 (m, 1H, CH allyl), 5.22–5.09 (m, 2H, CH_2 allyl), 4.24 (t, $J=5.6$ Hz, 2H, CH_2 allyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.4 (C=S), 142.1 (C indole), 139.0 (C=N), 134.9 (C allyl), 132.2 (C indole), 121.2 (C indole), 118.3 (C indole), 117.3 (C indole), 115.9 (C indole), 115.6 (C allyl), 109.8 (C indole), 45.7 (C allyl). IR (KBr): $\tilde{\nu}$ 3344 (NH), 3139 (NH), 1544 (C=N), 1524 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{13}H_{13}N_5O_2S$: 304.082; found: 304.099.

(*E*)-2-((1*H*-benzo[g]indol-3-yl)methylene)-*N*-allylhydrazinecarbothioamide (LQIT/LT87) Compound LT87 was obtained as white powder (31.1%). M.p. (°C): 200–202. Rf (1:1 *n*-hexane/AcOEt): 0.43. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.53 (s, 1H, NH indole), 11.33 (s, 1H, NH hydrazine), 8.40 (s, 1H, HC=N), 8.39 (d, $J=8.6$ Hz, 1H, CH indole), 8.31 (d, $J=8.7$ Hz, 1H, CH indole), 8.13 (t, $J=6$ Hz 1H, NH alkyl), 7.96 (d, $J=8.0$ Hz, 1H, CH indole), 7.93 (d, $J=2.9$ Hz, 1H, CH indole), 7.64–7.52 (m, 2H, CH indole), 7.48–7.43 (m, 1H, CH indole), 6.08–5.90 (m, 1H, CH allyl), 5.29–5.17 (m, 1H, CH allyl), 5.17–5.09 (m, 1H, CH allyl), 4.39–4.23 (m, 2H, CH_2 allyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.7 (C=S), 141.0 (C=N), 135.9 (C allyl), 132.2 (C indole),

130.6 (C indole), 129.0 (C indole), 128.8 (C indole), 126.1 (C indole), 124.7 (C indole), 122.1 (C indole), 121.9 (C indole), 121.5 (C indole), 121.2 (C indole), 120.5 (C indole), 115.7 (C allyl), 113.2 (C indole), 46.1 (C allyl). IR (KBr): $\tilde{\nu}$ 3403 (N–H), 3111 (N–H), 1610 (C=N), 1536 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{17}H_{16}N_4S$: 309.112; found: 309.184.

(*E*)-2-((1*H*-pyrrolo[2,3-b]pyridin-3-yl)methylene)-*N*-allylhydrazinecarbothioamide (LQIT/LT88) Compound LT88 was obtained as white powder (41.6%). M.p. (°C): 237–239. Rf (8:2 *n*-hexane/AcOEt): 0.55. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.11 (s, 1H, NH indole), 11.28 (s, 1H, NH hydrazine), 8.62 (s, 1H, HC=N), 8.30 (s, 1H, NH alkyl), 8.29 (s, 1H, CH indole), 8.14 (s, 1H, CH indole), 7.95 (s, 1H, CH indole), 7.19–7.16 (m, 1H, CH indole), 6.00–5.90 (m, 1H, CH allyl), 5.19–5.08 (m, 2H, CH_2 allyl), 4.28 (s, 2H, CH allyl). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.4 (C=S), 149.2 (C indole), 144.0 (C indole), 140.3 (C=N), 135.5 (C allyl), 131.1 (C indole), 130.5 (C indole), 116.9 (C indole), 116.3 (C indole), 115.2 (C allyl), 110.0 (C indole), 45.7 (C allyl). IR (KBr): $\tilde{\nu}$ 3169 (NH), 3128 (NH), 3080 (NH), 1550 (C=N), 1529 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{12}H_{13}N_5S$: 260.092; found: 260.099.

(*E*)-*N*-allyl-2-((7-methyl-1*H*-indol-3-yl)methylene)hydrazinecarbothioamide (LQIT/LT89) Compound LT89 was obtained as white powder (49.3%). M.p. (°C): 265–266. Rf (4:6 *n*-hexane/AcOEt): 0.53. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.63 (s, 1H, NH indole), 11.28 (s, 1H, NH hydrazine), 8.35 (s, 1H, HC=N), 8.04 (s, 1H, NH alkyl), 8.02 (s, 1H, CH indole), 7.85 (s, 1H, CH indole), 7.09–7.00 (m, 2H, CH indole), 6.04–5.93 (m, 1H, CH allyl), 5.25–5.12 (m, 2H, CH allyl), 4.31 (t, $J=8$ Hz, 2H, CH_2 allyl), 2.50 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 176.6 (C=S), 141.3 (C indole), 137.2 (C=N), 135.7 (C allyl), 130.8 (C indole), 124.2 (C indole), 123.8 (C indole), 122.0 (C indole), 121.0 (C indole), 119.1 (C indole), 115.6 (C allyl), 111.5 (C indole), 46.1 (C allyl), 16.7 (CH_3). IR (KBr): $\tilde{\nu}$ 3317 (NH), 3133 (NH), 3129 (NH), 1606 (C=N), 1544 (C=S) cm^{-1}. HRMS *m/z* $[M+H]^+$ calcd for $C_{14}H_{16}N_4S$: 273.112; found: 273.129.

Biological section

In-vitro studies

J774 macrophages cytotoxicity J774 macrophages (1×10^4 cells/well) were distributed on a 96-well plate in DMEM medium. Each compound was solubilized in 1% DMSO. As a positive control, gentian violet was tested, whereas as a negative control, the wells received only DMEM and DMSO medium. The plate was then cultured for 72 h, at 37 °C and 5% CO_2. Then 20 µL of Alamar Blue was added to each well and the plate incubated for an additional 4 h. The plate was read on the plate reader at 570 and 600 nm [17].

Lymphoproliferation assay Splenocytes of BALB/c mice (5×10^6 cells/well, 200 µL) were cultured in DMEM medium and 96-well plates in triplicates, in the presence/absence of concanavalin A (2 µg/mL) and also in the presence/absence of the compounds analyzed. The compounds were solubilized in DMSO and diluted in DMEM medium and were tested at seven concentrations (12—0.05 µM), in triplicates. After 48-h incubation, at 37 °C and 5% CO_2, 1 µCi of [methyl-^{3}H] thymidine was added to the cultures, starting a new 18-h incubation period. Afterwards, the cells were collected for quantification of proliferation by thymidine uptake determination. The percent inhibition was determined by relating the thymidine incorporation of the treated cultures to the evaluated substances and to the incorporation of the only stimulated cultures. Dexamethasone and indomethacin were used as a positive control. Three independent experiments were carried out [18].

COX-1 and COX-2 inhibition assay The screen for COX-1 and COX-2 inhibitory activity was performed according to Ayoub, Flower and Seed [19]. Briefly, all positive compounds and controls (indomethacin and celecoxib) were solubilized in DMSO and assayed in triplicate at 25 µM concentration. COX-1 or COX-2 (Sigma-Aldrich) were added in 180 µL of the assay buffer containing 5 mM hematin (Sigma-Aldrich), 100 mM Tris–HCl buffer, pH 8.0. After addition of the test compound or positive control (10 µL), the reaction mixture was incubated for 5 min at room temperature. The reaction was started by adding 5 µL of arachidonic acid solution (Sigma-Aldrich) dissolved in methanol and *N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD). After incubating for 1 h, the reaction mixture had its absorbance measured at 610 nm. As the compounds inhibited more than 50% of COX-1 or 2 activity at a concentration of 25 µM, the EC_{50} (50% effective concentration) value was calculated by a five-point dose–response curve (0, 0.15, 3, 6.25, 12.5) in a 96-well plate by a Dose Response function fitting a nonlinear curve (Logistic) using ORIGIN 8.0 software (OriginLab Corporation). The following equation was adopted:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(LOGx0-x)p}}$$

When fitting this function, the *X* values are supposed to be the logarithm of dose, and *LOGx*0 is the center of the curve, that is, the concentration for half response. Then, we can compute the EC_{50} by $EC50 = 10^{LOGx0}$. The same calculation was performed with positive controls.

Immunological studies with mice Balb/c splenocytes Immunological assay performed with monocytes and lymphocytes cells from the spleen of mice female BALB/c (6–8 weeks old; 5 animals) were performed to confirm the results obtained from the J774 lineage. The animals were raised and maintained at the animal facilities of the Keizo Asami Immunopathology Laboratory—LIKA located in the Federal University of Pernambuco, Brazil. Mice were kept under standard laboratory conditions (20–22 °C and 12 h day and night cycle) with free access to a standard diet (Labina/Purina, Campinas, Brazil) and water. All experimental procedures were performed according to the Ethics Committee of Animal Use (CEUA) of the Federal University of Pernambuco (protocol number: 0048/2016). After the inoculation of 1 mg of anesthetic and euthanasia of the animals by displacement of vertebra, the spleen of each mouse was removed aseptically and placed in a Falcon tube containing RPMI 1640 with fetal calf serum (complete medium).

The spleens were vertically transferred onto a petri dish where they were soaked. The cell suspensions obtained from each spleen were transferred to Falcon tubes containing approximately 10 mL of incomplete medium. Spleen homogenates were overlaid onto the Ficoll-Paque™ PLUS layer, with the density adjusted to 1.076 g mL^{-1}, and centrifuged at $1000 \times g$ at room temperature for 25 min. The interface cell layer containing immune cells was recovered by Pasteur pipette, washed twice in phosphate-buffered saline (PBS 1X) and centrifuged twice at $500 \times g$ for 10 min. Cells were counted in a Neubauer chamber, and cell viability was determined by the trypan blue exclusion method. Cells were only used when viability was > 98%. Cells were cultured in plates (24 wells) with compounds LT76, LT81 and LT87 at 11 µM for 24 and 48 h.

Investigation of immunostimulation promoted by LT compounds Supernatants of splenocytes culture stimulated with compounds LT76, LT81 and LT87 were used to measure cytokines and nitric oxide release by these immune cells. Cultures were treated with 11 µM of the compound and, after 24 and 48 h, supernatants were collected to perform the assays. Cytokine assessment was carried out using mice Th1/Th2 cytokine kit II (BD Bioscience, San Jose, CA, USA) for simultaneous detection of seven cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ in culture supernatants samples. All data was generated on the FACSCalibur platform. The cytokine measurement was based on the principle of cytometric bead array (CBA) technology. Nitrite Oxide analysis was performed through the colorimetric Griess method [20]. NO concentration was estimated by the standard curve (3.12–100 µmol mL^{-1}). The reading was carried out in a microplate spectrophotometer (Thermo Scientific Multiskan FC, Waltham-USA) at 595 nm.

In-vivo studies

Animals Experiments were conducted with Swiss *Mus musculus* mice (30–35 g) obtained from the Ministry of Agriculture, Livestock and Supply (MAPA). Animals were maintained at the biotery of the Antibiotics Department/UFPE (Recife, Brazil), under controlled conditions (22 ± 3 °C for 12 h light/dark cycle, free access to food and water). All animals used in the determination of anti-inflammatory activity fasted for 4 h prior to the experimentation. The Animal Studies Committee of the Federal University of Pernambuco approved the experimental protocols (number 23076.017928/2010-25). The animals were treated according to the ethical principles of animal experimentation of COBEA (Brazilian College of Animal Experiments) and the norms of the National Institute of Health Guide for Care and Use of Laboratory Animals.

Acute oral toxicity A fixed-dose procedure was adopted to evaluate the acute oral toxicity of compounds LT76, LT81 and LT87 according to Organization for Economic Cooperation and Development (OECD) Guidelines for the Testing of Chemicals [21]. Groups of 3 mice were dosed in a stepwise procedure using the fixed dose of 2000 mg kg^{-1}. Clinical signs and conditions associated with pain, suffering and impending death are described in detail in a separate OECD Guidance Document [22]. The test was conducted in duplicate.

Carrageenan-induced paw edema test in mice Carrageenan-induced paw edema test in Swiss mice (*Mus musculus*) was used to evaluate the anti-inflammatory activity of LT76, LT81 and LT87. This experiment was performed on male mice as the previous report [23]. Eleven groups of mice (n = 8) were treated orally with DMSO 10% in PBS, LT76, LT81 and LT87 (10, 30 and 50 mg kg^{-1}) or indomethacin (10 mg kg^{-1}) 1 h before the carrageenan injection. Also, 1% (v/v) carrageenan (50 µL) was administered by subplantar injection to the right hind paws of mice. After carrageenan injection, the paw volume was measured at 1, 2, 3, 4, 5 and 6 h using the plethysmometer apparatus (7140 Ugo Basile, Italy). The increase of paw volume was measured and the inhibition of edema was calculated according to the previous method.

Evaluation of inflammation All samples were fixed in 10% paraformaldehyde and included in paraffin blocks, which were sliced and submitted to paraffin removal, rehydration and evaluated with an inverted microscope (Observer Z1, Zeiss Micro Imaging GmbH) equipped with a camera and a 4.7.4 image analysis program (AxionCam MRm Zeiss) at a magnification of 4009, according to the standard histological procedures performed at the Laboratory of Ultrastructure

of Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, Brazil. All tissue slices were stained with hematoxylin–eosin and evaluated. The inflammatory activity was evaluated based on the presence of inflammatory infiltrate, cytomegalic cells and intranuclear cytoplasmic inclusions.

Detection of cyclooxygenase 2 (COX-2) expression An Ultrathin section (5 µm thickness) of each group was cut and fixed to slides treated with 3-aminopropyl-triethoxy-silane [APES (Sigma, USA)]. Briefly, sections were deparaffinized with xylene and rehydrated in graded ethanol (70–100%). The sections were heated for 30 min in a sodium citrate buffer (0.01 M, pH 6.0) to increase epitope exposure. The slides were treated with 0.3% (v/v) H_2O_2 in water for 5 min to minimize endogenous peroxidase activity. The sections were washed with 0.01 M PBS (pH 7.2) and then blocked with 1% BSA, 0.2% Tween 20 in PBS for 1 h at room temperature. Then, the sections were incubated for 12 h at 4 °C with COX-2 antibody (ABCAM, CA, USA). The antigen–antibody reaction was visualized with avidin–biotin–peroxidase (Dako Universal LSAB_Kit, Peroxidase) using 3,3-diaminobenzidine as a chromogen. The slides were counterstained in hematoxylin. Positive staining resulted in a brown reaction product. Negative controls were treated as above, but with the omission of the first antibody [24].

Immunohistochemical images from different regions were assessed separately. Five pictures at the same magnification were quantitatively analyzed using a software program (GIMP 2.10.8 GNU Image Manipulation Program, UNIX platforms).

In-silico ADME

To further strengthen the results of our in-vitro studies, we also performed in-silico molecular studies. The ADME (absorption, distribution, metabolism and excretion) profiles of these compounds were investigated with SwissADME [25] and pkCSM [26] web platforms.

Statistical analysis

To test the normality of the hypothesis of the variables involved in the study, the Shapiro–Wilk test was applied and sample averages were assessed using non-parametric tests. The one-way ANOVA followed by post-test of Newman–Keuls or Bonferroni of multiple comparisons were used to determine the statistical significance of comparisons between groups in the studies. The results were considered statistically obtained when a value of $p < 0.05$ otherwise indicated in the respective caption graphic. All statistical analysis were performed using the GraphPad Prism program version 5.01 or up. A nonlinear regression curve was used to calculate the CC_{50} values of the cytotoxicity and lymphoproliferation assays.

Results

Chemistry

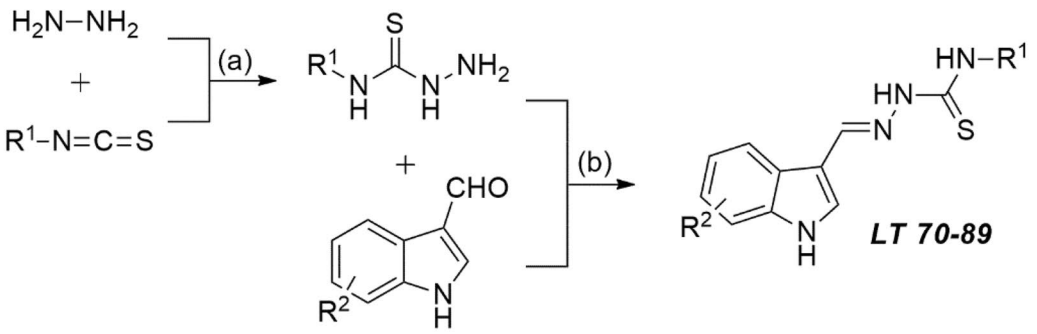
Synthesis and structural characterization of thiosemicarbazones

The compounds of interest in this study were designed based on the importance of the indole nucleus and thiosemicarbazone portion in molecules of biological interest. Thus, five different indole aldehydes were used and four different isothiocyanates were chosen to generate the twenty compounds based on previous studies by the group (unpublished data). The synthetic route of LT's compounds is represented in Scheme 1.

The synthesis route for obtaining the compounds has two phases. Initially, hydrazine and isothiocyanate reacted to originate thiosemicarbazides (intermediate compounds). Subsequently, the thiosemicarbazides were condensed with substituted indole-carboxaldehyde, leading to the formation of the thiosemicarbazones that were studied in this work. The compounds obtained showed yields that varied between 33.6 and 90.4%, validating the effectiveness of the synthetic route used.

For structural confirmation, spectroscopic and spectrometric techniques were used. The absorption spectroscopy in the infrared (IR) region showed characteristic bands of the NH bond referring to the indole nucleus, the hydrazine portion and NH^4 present in the structure of the compounds obtained (2923–3413 cm^{-1}). The presence of stretches in 1531–1688 and 1493–1557 cm^{-1}, referring to the groups C=N and C=S, respectively, are strong indications of the formation of the intended compounds.

In the ^1H NMR spectra, the main characteristic signals for the formation of compounds were evidenced. In the displacement range δ 8.35–8.78 ppm, it was possible to observe signs of the azomethine hydrogens (HC=N), which confirm the condensation with aldehydes. In addition, the presence of the shift related to NH indole at δ 11.63–12.59 ppm reaffirms the success of the reaction. Other important signals are NH hydrazine and NH^4, which were in the displacement range of δ 11.28–11.89 and δ 9.57–10.09 ppm, respectively. For compounds with the allyl group, the displacement of the NH^4 hydrogen varied from δ 8.04 to 8.30 ppm. Compounds containing the allyl group in N^4 show chemical shifts in a more shielded region of the ^1H NMR spectrum when compared to those substituted with aryl groups.



Cpd.	R^1	R^2
LT70	phenyl	5-cyano
LT71	phenyl	4-nitro
LT72	phenyl	benzo[g]
LT73	phenyl	pyrrolo[2,3-b]pyridin
LT74	phenyl	7-methyl
LT75	4-chlorophenyl	5-cyano
LT76	4-chlorophenyl	4-nitro
LT77	4-chlorophenyl	pyrrolo[2,3-b]pyridin
LT78	4-chlorophenyl	benzo[g]
LT79	4-chlorophenyl	7-methyl

Cpd.	R^1	R^2
LT80	1-naphthyl	5-cyano
LT81	1-naphthyl	4-nitro
LT82	1-naphthyl	pyrrolo[2,3-b]pyridin
LT83	1-naphthyl	benzo[g]
LT84	1-naphthyl	7-methyl
LT85	allyl	5-cyano
LT86	allyl	4-nitro
LT87	allyl	benzo[g]
LT88	allyl	pyrrolo[2,3-b]pyridin
LT89	allyl	7-methyl

Scheme 1 Synthesis of indole-thiosemicarbazones derivatives. Reagents and conditions: step **a** CH_2Cl_2, room temperature; step **b** ethanol, acetic acid (catalytic amount), room temperature

Signals referring to characteristic carbons were also elucidated in the ^{13}C NMR. The chemical shifts referring to C=N and C=S were observed in the δ 137.2–141.8 and 174.3–177.2 ppm ranges, respectively. The latter signal confirms the obtaining of compounds in the thione form. In addition, the HRMS spectrum confirmed the molecular mass of all the compounds.

The thiosemicarbazones of this study were obtained in a single diastereoisomer in view of the presence of a single signal referring to the azomethine hydrogen (HC = N–) in the ^1H NMR spectra. To confirm the stereochemistry more precisely, the 2D NMR NOESY experiment was carried out with the compound LT76.

The NOESY spectra (Supplementary material) showed a spatial correlation between hydrazine hydrogen at 11.86 ppm (=N–NH–) and azomethine hydrogen at 8.55 ppm (HC=N–), in addition to the correlation between indole hydrogen at 12.58 ppm (NH) and hydrogen at C2-indole (8.50 ppm). The absence of correlation between azomethine hydrogen and hydrogen at C2-indole, added to the previous results, confirms that the compounds were obtained as *E* isomers.

Anti-inflammatory activity in vitro

Cytotoxicity and lymphoproliferation assays

All twenty new synthesized compounds (LT70-89) were evaluated in-vitro for their cytotoxicity. The test carried out on J774 macrophages revealed that all compounds were less cytotoxic than gentian violet ($CC_{50} = 3.8 \pm 0.2$ µM). The results ranged from 7.0 ± 0.6 to > 75 µM, highlighting compounds LT73, LT75, LT77, LT80, LT82, LT83, LT85, LT86 and LT88, which showed CC_{50} greater than 75 µM, suggesting that these compounds have low toxicity in normal cells.

Even those derivatives that showed CC_{50} values below 75 µM, as in the case of LT76, LT81 and LT87, these were 6.4, 5.7 and 4.9 times, respectively, less cytotoxic than gentian violet. In addition, the positive controls, indomethacin and dexamethasone, were also non-toxic in these conditions. All values are described in Table 1.

Subsequently, we proceeded with the lymphoproliferation assay, which was used for the purpose of selecting promising candidates for anti-inflammatory activity. Results are shown in Table 1. The results indicated that

Table 1 Inhibitory activity of the new indole-thiosemicarbazone derivatives tested against splenocyte proliferation and cytotoxicity in macrophages J774

Compound	Cytotoxicity (CC_{50}) μM ± SEM[a]	Lymphoproliferation (IC_{50}) μM ± SEM[b]
LT70	64.7 ± 5.1	4.3 ± 0.5
LT71	37.9 ± 0.9	2.0 ± 0.060
LT72	34.9 ± 1.9	ND
LT73	> 75	4.6 ± 0.58
LT74	32.9 ± 2.5	1.9 ± 0.1
LT75	> 75	1.0 ± 0.36
LT76	24.5 ± 1.3	0.56 ± 0.036
LT77	> 75	9.3 ± 0.9
LT78	7.0 ± 0.6	5.3 ± 0.052
LT79	30.0 ± 2.6	3.5 ± 0.07
LT80	> 75	1.1 ± 0.042
LT81	21.8 ± 1.8	0.9 ± 0.01
LT82	> 75	10.3 ± 1.13
LT83	> 75	4.9 ± 0.58
LT84	38.7 ± 3.9	2.3 ± 0.21
LT85	> 75	8.9 ± 1.02
LT86	> 75	> 12
LT87	18.7 ± 5.0	0.5 ± 0.07
LT88	> 75	> 12
LT89	60 ± 4.3	> 12
Indomethacin	> 75	> 12
Dexamethasone	> 75	0.012 ± 0.005
Gentian Violet	3.8 ± 0.2	–

[a]Determined 72 h after drug exposure using AlamarBlue

[b]Determined 48 h after drug exposure using thymidine incorporation

the derivatives LT76, LT81 and LT87 were the most effective in reducing the proliferation of lymphocytes, since they had CC_{50} of 0.56 ± 0.036 μM, 0.9 ± 0.01 μM and 0.5 ± 0.07 μM, respectively. Derivatives LT86, LT88 and LT89, in turn, proved to be inactive since they had CC_{50} greater than the maximum concentration tested.

The derivatives were planned to obtain substitutions in thiosemicarbazone (position *N*-4) and in the indole nucleus. The evaluation between chemical structure and biological activity of the derivatives showed that the series presents great diversity of results due to the substitutions that were made. For example, it is possible to note the good activity of compounds LT71, LT76 and LT81 as opposed to LT86 (CC_{50} > 12 μM), even though all of them have the 4-nitro group added to the indole nucleus. The same can be seen when we fix the *N*-4 substitution in thiosemicarbazone by the allyl group. Derivatives LT85, LT86, LT88 and LT89 were little active or inactive, while LT87 (benzo[*g*]indole) was the most potent among all tested compounds.

Th2 cytokines and low Nitric oxide production promoted by compounds LT76, LT81 and LT87 in mice splenocytes cultures

Results obtained with macrophage J774 lineage treated in-vitro with LT76, LT81 and LT87 compounds showed the immunological regulation to Th2 response. Parameters like the decrease of IL-6 and TNF-α cytokines, associated with lower nitric oxide release and lymphoproliferation, reinforce this finding. Before the in-vivo assay, we isolated splenocytes from mice Balb/c and treated these cells, in-vitro, only with those three compounds for 24 h. Our results confirm the standard observed for the J744 lineage. The LT76, LT81 and LT87 compounds were not able to induce the production of cytokines related to the proliferation of lymphocytes, like IL-2 and IL-6 (Fig. 1a and b).

Furthermore, IL-17 (Fig. 1c) was produced in basal values (similar to control). Th2 cytokines, like IL-4 and IL-10, were produced in high values, but only LT76 induced the higher production of IL-4 cytokine (Fig. 1d). TNF-α was produced in lower values for all compounds tested (Fig. 1f). Reinforcing the inhibition of Th1 profile, it was noted the lower production of nitric oxide in cells treated with LT76, LT81 and LT87 compounds associated with no stimulation of IFN-γ cytokine (Fig. 2a and b).

Cyclooxygenase (1 and 2) in-vitro inhibition assay

Compounds LT76, LT81 and LT87 were tested for COX-1 and COX-2 inhibition at four concentrations (from 1.5 to 100 μM) to determine the 50% effective concentration (EC_{50}) and their selectivity index (SI). Celecoxib and indomethacin were used as reference drugs. As demonstrated in Table 2, compounds LT81 and LT87 displayed COX-2 inhibitory activity (0.049 and 0.14 μM, respectively) higher than celecoxib (0.26 μM). However, compound LT76 was less active than positive control and the other tested compounds. In relation to the selectivity index (SI), compounds LT76 and LT87 were not selective for COX-2, while LT-81 showed SI value two-fold higher than celecoxib.

Acute toxicity after single dose administration

During the experiment, neither death nor changes in the physiological and hematological parameters were observed (Supplementary Material). Based on these results, the in vivo anti-inflammatory assay was conducted at 10, 30, and 50 mg kg^{-1} doses for compounds LT76, LT81 and LT87.

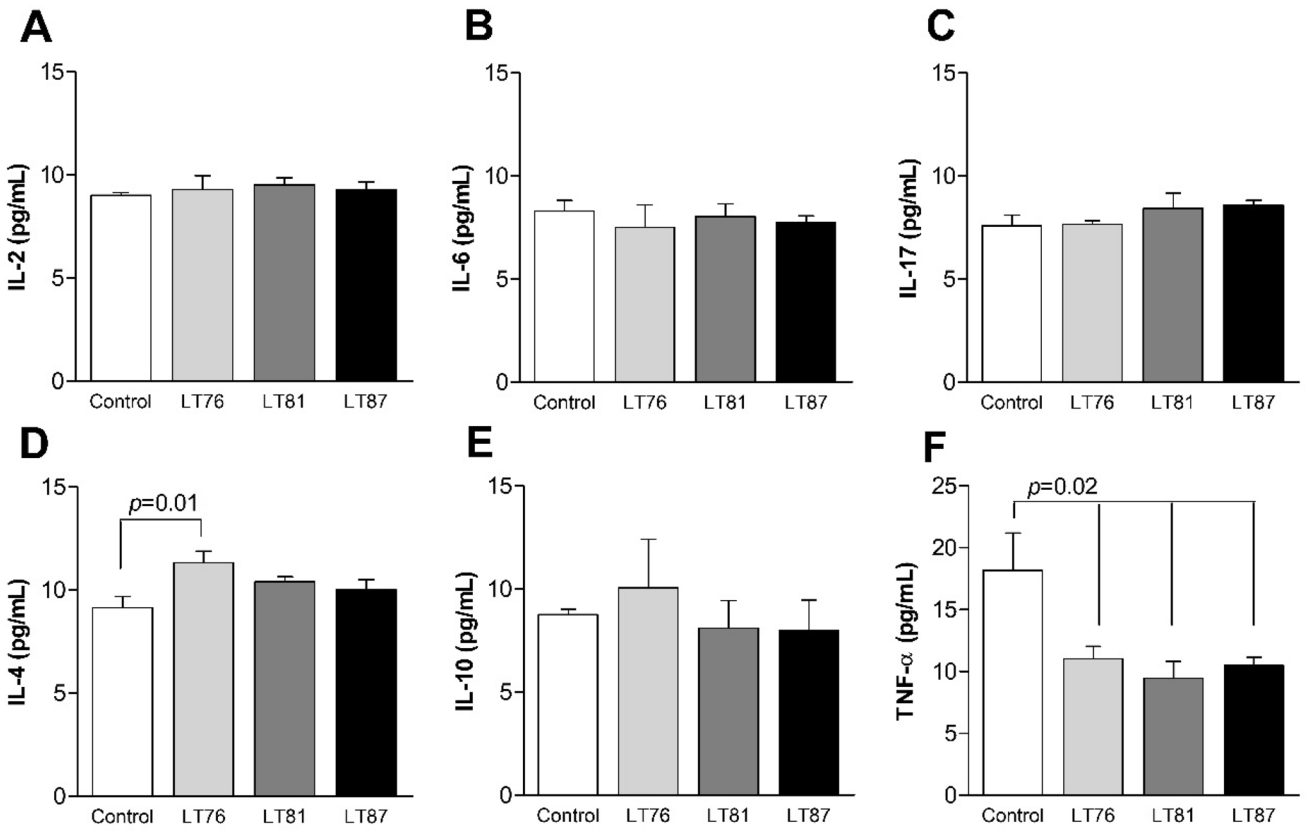


Fig. 1 Profile of cytokines production in splenocytes treated with LT76, LT81 and LT87 compounds in 24 h of incubation. Vertical white bars represent control samples (cells + cultures medium) and black vertical bars represent cells treated with different compounds. Two independent experiments were performed, each concentration in triplicate. one-way ANOVA: $*p < 0.001$. Calculated F Parameter (ANOVA): TNF (F 3, 12 = 20.46); IL4 (F 3, 12 = 11.00). Tabulated F Parameter (ANOVA): TNF (F 3, 12 = 3.4903); IL4 (F 3, 12 = 3.4903)

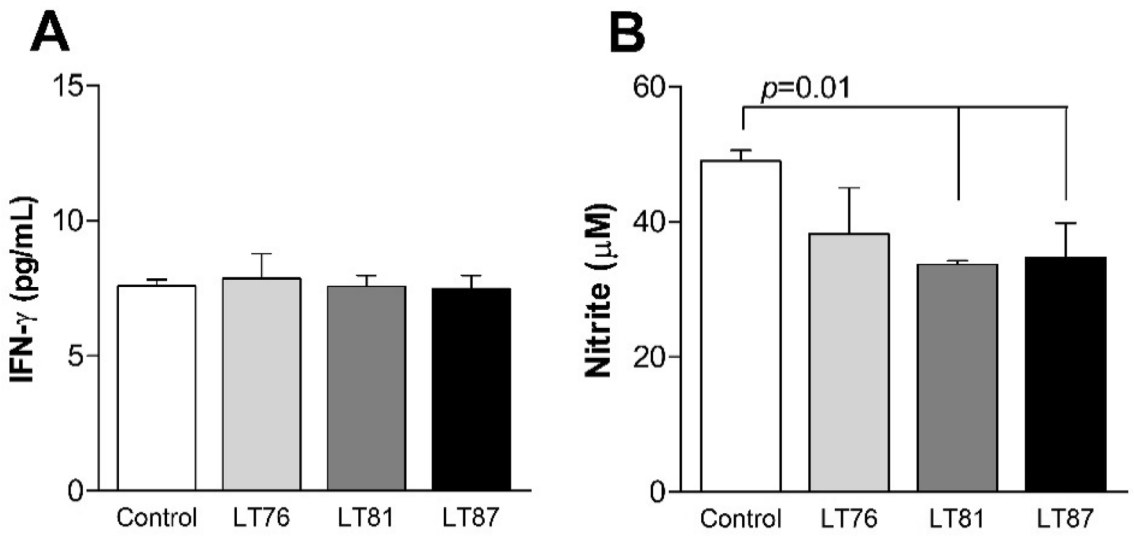


Fig. 2 Production of IFN-γ and nitric oxide (NO) by splenocytes treated with LT76, LT81 and LT87 compounds in 24 h of incubation. Vertical white bars represent control samples (cells + cultures medium) and black vertical bars represent cells treated with different compounds. Two independent experiments were performed in triplicate

Table 2 Inhibitory activities of the enzymatic activity for COX-1 and COX-2 and selectivity index (SI) of tested compounds

Compound	EC_{50} (μM)[a] COX-1	EC_{50} (μM) COX-2	SI (COX-1/COX-2)[b]
LT76	0.22 ± 0.12	0.29 ± 0.10	0.76
LT81	1.13 ± 0.38	0.049 ± 0.06	23.06
LT87	0.14 ± 0.12	0.14 ± 0.52	1
Celecoxib	3.09 ± 0.02	0.26 ± 0.02	11.88
Indomethacin	2.16 ± 0.04	0.84 ± 0.06	2.57

[a]EC_{50} value is the compound concentration required to produce 50% inhibition of COX-1 or COX-2 for means of three determinations

[b]SI: ratio (EC_{50} COX-1/EC_{50} COX-2)

Efficacy of compounds on carrageenan-induced paw edema

The experimental compounds of series LT were tested as anti-inflammatory agents administrated orally by gavage in the paw edema model and efficacy determined in comparison to untreated group. Indomethacin at a dose of 10 mg kg^{-1} was employed as a reference anti-inflammatory agent. The results can be seen in Table 3.

The LT76 compound showed a moderate result in this inflammation model, with maximum inhibitions for the doses 10 mg kg^{-1} ($t = 5$ h, 40.6%), 30 mg kg^{-1} ($t = 5$ h, 57.5%) and 50 mg kg^{-1} ($t = 6$ h, 64.8%). It is possible to suggest a dose-dependent effect of the studied compound and significant effectiveness after the fourth hour of the experiment (Table 3). Additionally, the LT81 compound showed anti-inflammatory activity at all times and doses used in the experiment (Table 3). It is noteworthy that the doses of 10 and 30 mg kg^{-1} promoted greater reductions in the paw volume of the treated animals, for which the maximum effect was seen at $t = 6$ h with an inhibition of 81.5% (10 mg kg^{-1}) and 89% (30 mg kg^{-1}). The 50 mg kg^{-1} dose promoted a greater reduction in the inflammatory process after 5 h (76.8%) and 6 h (79.7%) of carrageenan injection. It was possible to evidence the action of LT81 in both phases of the inflammatory process, reducing edema in a similar way to that presented by indomethacin.

Results for compound LT87 showed that the 10 mg kg^{-1} dose a significant control in edema from $t = 3$ h (40.5% inhibition of edema) was achieved until the end of the experiment at $t = 6$ h (89.8% inhibition of edema). The 30 and 50 mg kg^{-1} doses demonstrated a dose-dependent effect, in addition to being effective in controlling edema at all times during the experiment. We emphasize that this compound showed maximum inhibition at $t = 4$ h (100% inhibition of edema) at doses of 30 and 50 mg kg^{-1}, compared to indomethacin at the same time (84.81%). Therefore, we can

Table 3 Anti-inflammatory effect of the indole-thiosemicarbazone derivatives in the carrageenan-induced paw edema model

	$t = 1$ h	$t = 2$ h	$t = 3$ h	$t = 4$ h	$t = 5$ h	$t = 6$ h
Negative control	37.6 ± 7.7	41.5 ± 11.5	52.2 ± 10.2	65.5 ± 6.1	66.5 ± 6.7	75.9 ± 8.9
Indomethacin 10 mg kg^{-1}	13.6 ± 4.3**	12.4 ± 3.9***	12.4 ± 3.9***	21.0 ± 4.8***	10.1 ± 3.1***	6.3 ± 2.5***
LT-76 10 mg kg^{-1}	31.1 ± 2.4	28.4 ± 8.5	36.1 ± 12.4	75.5 ± 2.7	39.5 ± 15.1*	64.8 ± 6.4
LT-76 30 mg kg^{-1}	33.4 ± 1.6	54.8 ± 4.3	38.2 ± 6.0	34.4 ± 5.1**	28.2 ± 4.6**	35.2 ± 5.8***
LT-76 50 mg kg^{-1}	21.1 ± 6.0	24.2 ± 6.4	31.9 ± 4.7	35.7 ± 4.6**	31.0 ± 6.1***	26.7 ± 5.9***
LT-81 10 mg kg^{-1}	15.5 ± 2.7*	12.3 ± 2.6***	23.5 ± 6.3***	24.8 ± 2.9***	12.6 ± 3.4***	14.0 ± 3.4***
LT-81 30 mg kg^{-1}	8.1 ± 3.0**	9.5 ± 3.2***	8.2 ± 3.0***	15.6 ± 3.7***	8.3 ± 2.6***	8.3 ± 2.6***
LT-81 50 mg kg^{-1}	18.8 ± 4.1***	17.6 ± 5.0**	25.5 ± 5.0**	27.5 ± 6.2***	15.4 ± 5.1***	15.4 ± 5.1***
LT-87 10 mg kg^{-1}	31.6 ± 4.8	29.5 ± 4.5	31.0 ± 2.9*	24.3 ± 5.4***	10.8 ± 4.4***	7.7 ± 3.7***
LT-87 30 mg kg^{-1}	6.2 ± 1.2***	6.2 ± 1.9***	7.5 ± 2.9***	2.5 ± 1.4***	0.0 ± 0.0***	0.0 ± 0.0***
LT-87 50 mg kg^{-1}	18.0 ± 2.3*	16.0 ± 4.8**	9.2 ± 3.8***	4.2 ± 2.5***	0.0 ± 0.0***	0.0 ± 0.0***

Data were expressed as the mean ± SE of 6 animals per group. Results were considered significant when $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$ (in comparison to the negative control group), determined by analysis of variance (one-way ANOVA), followed by Bonferroni post hoc test

Calculated F Parameter (ANOVA): LT76 (F 20, 125 = 2.778); LT81 (F 20, 147 = 2.017); LT87 (F 20, 146 = 4.122). Tabulated F Parameter (ANOVA): LT76 (F 20, 125 = 1.5705); LT81 (F 20, 147 = 1.5705); LT87 (F 20, 146 = 1.5706)

conclude that the compound was able to act in both phases of the carrageenan-induced inflammation.

Immunohistochemical analysis of LT76, LT81 and LT87 on the expression of cyclooxygenase 2 (COX-2) in carrageenan-induced paw swelling model

The immunohistochemical analysis of COX-2 expression was performed in the paw edema of mice, after the intraplantar injection of carrageenan. COX-2 markings in the positive control group (carrageenan) were evident in the epidermis and dermis, with greater expressiveness in the epidermis (Fig. 3a). The LT76 derivative showed results similar to those of indomethacin. On the other hand, the COX-2 expression of the LT81 and LT87 derivatives was significantly reduced when compared to the carrageenan and indomethacin group. The LT87 derivative being the best of the three in view of the inhibition of the response to COX-2 (Fig. 3f). The difference in the mean between the samples was statistically significant with $p < 0.05$ (0.0436). These results corroborate that the histological findings that demonstrate, after the administration of derivatives LT76, LT81 and LT87, reduction of edema and inflammatory infiltrate.

In-silico prediction of pharmacokinetic characteristics for indole-thiosemicarbazone compounds

A set of physico-chemical parameters known as the Lipinski rule or RO5 (rule of five) establishes that a molecule with good absorption or permeability when administered orally must have: molar mass ≤ 500 Da; log$P \leq 5$ (or M log$P \leq 4.15$); hydrogen bond acceptors (HBA) ≤ 10; hydrogen bond donors (HBD) ≤ 5. By meeting these standards or by violating only one of the points, it is possible to trace the profile of oral bioavailability. The data obtained for compounds LT76, LT81 and LT87 are shown in Table 4. Among the four parameters established by Lipinski, we can say that the compounds did not violate RO5 and could be considered orally bioavailable.

In addition, the solubility parameter (logS) can be used in pharmacokinetic predictions since the dissolution of an non-ionized substance in water (drug or prototype compound) reflects on absorption and bioavailability. The solubility of the compounds in water was estimated with logS, following these judgment parameters: logS = insoluble < −10 < poorly soluble < −6 < moderately soluble < −4 soluble < −2 < very soluble < 0 < highly soluble. The results revealed that compounds LT76 and LT81 were classified as poorly soluble, whereas LT87 was classified as moderately soluble. Structurally, compounds LT76 and LT81 are similar since both have the 4-nitro-indole group, differing only in *N*-4 at thiosemicarbazone (LT76: 4-chlorophenyl; LT81: 1-naphthyl). In contrast, LT87 has the allyl group at *N*-4.

In addition, all compounds are in accordance with the parameters number of rotatable bonds and polar surface area and, complementing the Lipinski rule, can be considered orally bioavailable.

The intestinal absorption of orally administered drugs is measured by the logarithm of the apparent permeability coefficient (log Papp, log cm s^{-1}). The compounds showed log Papp that varied between 0.921 and 1.344 cm s^{-1}, which implies the good intestinal absorption of these compounds. In addition, confirming these results, derivatives LT76, LT81 and LT87 showed 87.63, 93.46 and 90.65% of intestinal absorption, respectively.

For the pharmacokinetic analysis of the distribution, the parameter of the stationary volume of distribution log (VDss) was used, where higher values indicate a higher distribution of a compound in tissues (log VDss > 0.45) and low values indicate a distribution in plasma (low for log VDss < − 0.15). The results suggest that the compounds LT76 and LT81 have good plasma-tissue distribution. In contrast, LT87 has high distribution in tissue (VDss = 0.474 L kg^{-1}). These results may be associated with the pattern of substitution of compounds that caused an increase in the value of logS and a decrease in the value of logP. In addition, indole-thiosemicarbazone derivatives showed unbound fraction values ranging from 0 to 0.118.

The total clearance of compounds was studied and the results showed a total clearance of 0.037, 0.230 and 0.328 log ml/min/kg, for compounds LT76, LT81 and LT87, respectively. Indole-chalcone derivatives also showed low values of total clearance (hepatic and renal) corroborating the good elimination profile of compounds containing indole. Therefore, it was possible to conclude that the compounds in this study have good ADME properties and can be considered as probable lead compounds for anti-inflammatory activity.

Discussion

When obtaining promising compounds for biological activities, structural characterization is an important step. In this sense, characterization techniques are necessary. Thiosemicarbazones can be presented in the thione-thiol tautomeric form. However, the IR spectrum showed the absence of signs in the region between 2500 and 2600 cm^{-1} reinforced that the compounds were obtained in thione tautomeric form [27]. In relation to the NMR spectrum, the signals NH hydrazine and NH^4 presented results that are in agreement with the ones in the literature involving thiosemicarbazones [28, 29]. Compounds containing the allyl group in N^4 show chemical shifts in a more shielded region of the ^1H NMR spectrum when compared to those substituted with aryl groups [15, 30].

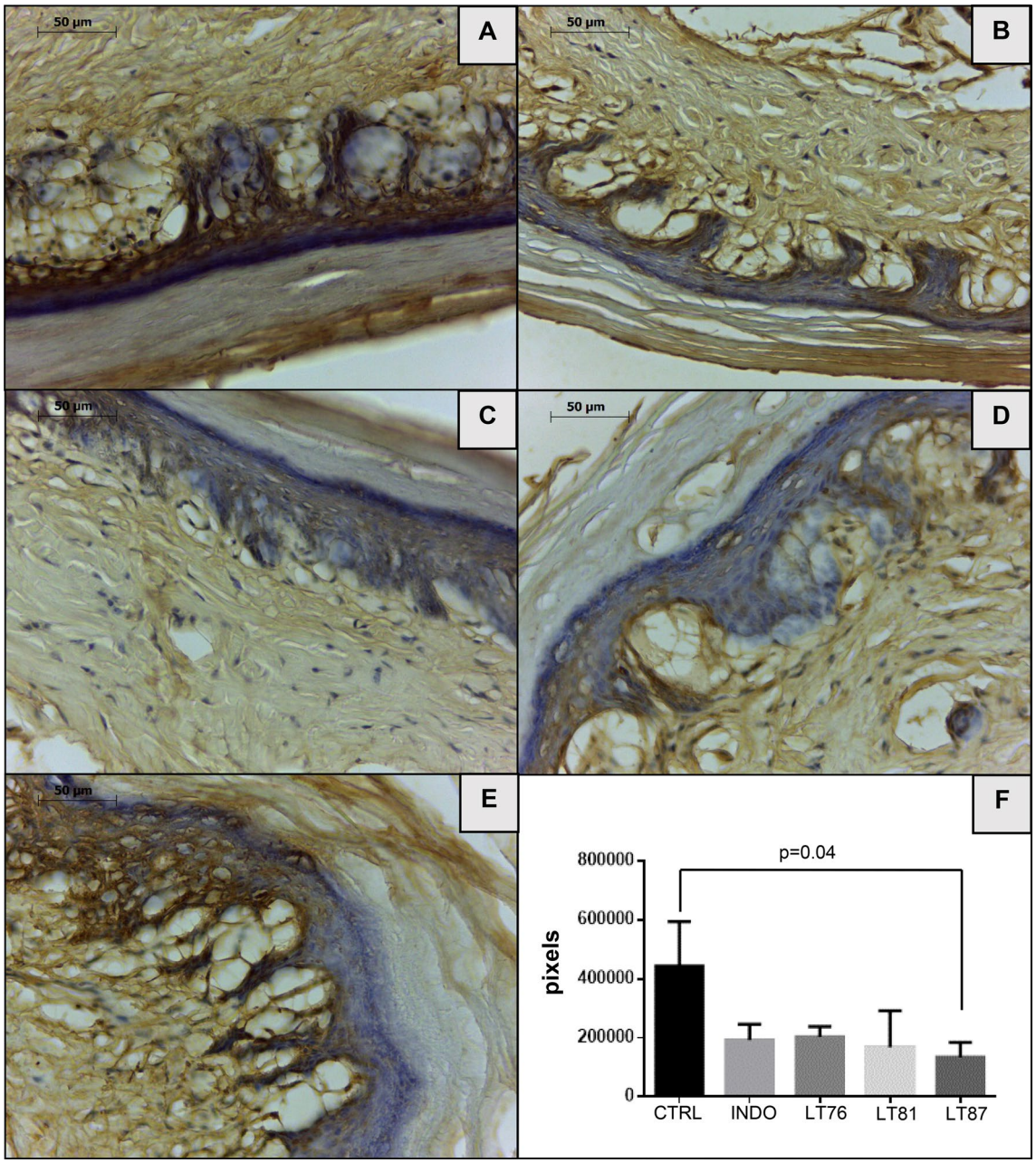


Fig. 3 Immunohistochemical localization of COX-2 in the carrageenan-induced paw swelling model. Positive staining in the epidermis and dermis was detected. **a** carrageenan, **b** indomethacin and derivatives LT76 (**c**), LT81 (**d**), LT87 (**e**). Data were expressed as mean ± SD; one-way ANOVA: $*p < 0.05$; Scale bar = 50 µm. Calculated F Parameter (ANOVA): LT87 (F 4, 7 = 4.376). Tabulated F Parameter (ANOVA): LT87 (F 4, 7 = 4.210)

The reaction of carbonyl compounds with primary amines leads to the formation of imines that can exist in two isomer forms *E* or *Z* [14]. Our results are in agreement with data in the literature involving thiosemicarbazone derivatives [15, 16, 30, 31].

The cytotoxicity assay is an important parameter to assess the selectivity of test compounds in the screening phase [32]. Several studies show that thiosemicarbazones have little or no cytotoxicity in normal cells and relevant biological activity, which gives them high rates of selectivity [33, 34].

The inhibition of the lymphocyte proliferation induced by concanavalin A, expressed by CC_{50}, revealed increased anti-inflammatory activity through an immunosuppressive action [35]. The difference in results presented by the compounds studied here, in the lymphoproliferation assay, results from the steric and electronic diversity found

Table 4 In silico pharmacokinetic data for indole-thiosemicarbazone derivatives estimated at SwissADME or pkCSM webservices

	LT76	LT81	LT87
MW (g/mol)[a]	373.82	389.43	308.40
HBA[b]	3	3	1
HBD[c]	3	3	3
log*P*[d]	2.61	2.86	2.18
log*S*[e]	− 6.16	− 6.81	− 5.08
TPSA (Å^2)[f]	130.12	130.12	84.30
Rotatable bonds[g]	6	6	6
Caco-2 perm.[h]	0.921	1.160	1.344
Int. abs. (%)[i]	87.63	93.46	90.65
VDss (log L/kg)[j]	− 0.128	− 0.125	0.474
Fract. Unb.[k]	0	0	0.118
Total clearance (log mL/min/kg)[l]	0.037	0.230	0.328

[a]SwissADME Molecular Weight

[b]SwissADME Number H-bonds acceptors

[c]SwissADME Number H-bonds donors

[d]SwissADME Moriguchi log of octanol–water partition coefficient

[e]SwissADME Ali log of aqueous solubility

[f]SwissADME calculation of Topological Polar Surface Area (TPSA)

[g]SwissADME Number rotatable bonds

[h]pkCSM prediction of Caco-2 cell permeability as an estimation of absorption at the human intestinal mucosa

[i]pkCSM prediction of the proportion of compound absorption through the human small intestine

[j]pkCSM prediction of the log of steady state volume of distribution (VDss)

[k]pkCSM prediction of compound fraction unbound in plasma (not bound to serum proteins)

[l]pkCSM prediction of the log of total drug clearance

in their structures. Moraes et al. [14] evaluated a series of indole-*N*-acylhydrazone compounds, in which the structural diversity presented by substitutions in the indole group was responsible for the variation in responses in the lymphoproliferation assay. Thus, due to low cytotoxicity and high activity in inhibiting leukocyte migration, compounds LT76, LT81 and LT87 were selected for further studies.

Th1 and Th2 are two subsets of helper T lymphocytes, which are responsible for several functions, such as the production of cytokines. The balance of the Th1/Th2 response plays an important role in the physiological maintenance of humans. Therefore, an imbalance in these responses can contribute to the pathogenesis of inflammatory diseases [36, 37].

The tumor necrosis factor alpha (TNF-α) is a Th1 cytokine produced by macrophages that plays an important role in the host's immune response to infectious agents. However, the overproduction of TNF-α is related to the pathogenesis of inflammatory diseases, such as cancer and rheumatic diseases [38, 39].

Biological therapies for inflammatory diseases include TNF inhibitors as a form of treatment [40]. The study conducted by Cardoso et al. [41] showed that phthalimide-thiosemicarbazone derivatives thalidomide-based were able to inhibit the production of TNF-α at a concentration of 10 μg mL^{-1}. Thalidomide, an inhibitor of nuclear factor kappa B (NF-kB) activation, is involved in TNF-α expression[45]. Indole, a privileged structure found in our compounds, is considered a phthalimide bioisostere, which, associated with the thiosemicarbazone portion, can be considered important in the immunomodulatory activity.

Nitric oxide is produced from the oxidation of L-arginine to L-citrulline in a reaction catalyzed by inducible nitric oxide synthase (iNOS). Pro-inflammatory cytokines, such as TNF-α, can activate iNOS leading to an increase in the production of nitric oxide and the inflammatory process [42, 43]. The significant decrease in the production of nitric oxide by the compounds LT76, LT81 and LT87 indicates their promising anti-inflammatory activity.

Interleukin-4 (IL-4) is a Th2 cytokine of anti-inflammatory character that participates in the protection of injured neurons in the central nervous system and reduces asthma exacerbation [44, 45]. Our results showed a significant increase in the production of IL-4 for compound LT-76, which corroborates the anti-inflammatory action of the molecules of our study.

The synthesis of prostaglandins is regulated in the human body by the cascade of arachidonic acid. Cyclooxygenase (COX) is one of the key enzymes in this pathway and is present in two isoforms (COX-1 and COX-2). COX-1 is a constitutive enzyme and is related to the maintenance of homeostasis. COX-2 is an inducible enzyme under inflammatory conditions [46, 47].

Non-steroidal anti-inflammatory drugs are used as treatment (NSAID). However, non-selective NSAIDs, such as ibuprofen, promote gastrointestinal side effects, such as gastric ulcerations, that require caution in their use. Alternatively, selective COX-2 drugs, such as celecoxib, can be used since they significantly reduce gastric adverse effects [48, 49].

The search for selective compounds in COX inhibition shows a large number of studies involving structures containing the indole nucleus [12, 14, 50, 51], which highlights the importance of this privileged structure in the design of anti-inflammatory drugs. LT76, LT81 and LT87 have indole in their structure. However, LT81 was more selective (23 times COX-2/COX-1) than the other compounds. This does not rule out the anti-inflammatory activity of compounds LT76 and LT87, since the inflammatory process is extremely complex and various inflammatory mediators participate in the regulation of inflammation [52].

The acute oral toxicity test (AOTT) is widely used since it provides valuable preliminary results which are useful in selecting a dosage for subsequent in-vivo studies [53]. Oral administration of compounds LT76, LT81, and LT87 did not lead to significant toxic effects at the highest dose of 2000 mg kg^{-1}, as recommended by Organization for Economic Cooperation and Development [21].

The carrageenan-induced paw edema assay was used to investigate the anti-inflammatory potential of the selected compounds. The carrageenan-induced paw edema model is a biphasic process. The first phase (1 h) involves the release of serotonin and histamines, and the second phase (> 1 h) is mediated by prostaglandin, a cyclooxygenase product [23]. The use of carrageenan as a phlogistic agent is responsible for promoting an acute inflammatory process, triggering the action of several mediators present in the inflammatory response. These mediators are driven to the injury site by the increasing vascular permeability [54, 55].

The presence of kinins, nitric oxide, and prostanoid derivatives in the second phase of the inflammatory process induced by carrageenan [56, 57] indicates that the LT76 derivative may be acting in the suppression of these inflammatory mediators.

Compounds LT76 and LT81 are structurally similar since both have the 4-nitro-indole group. The importance of the indole nucleus in the design of anti-inflammatory molecules is already well reported [14, 58]. Furthermore, the presence of the nitro group ($–NO_2$) seems to increase the anti-inflammatory activity compared to other substituents [59, 60]. Roriz et al. [61] showed that (*E*)-2-(((4-nitrophenyl)imino) methyl)phenol was able to inhibit leukocyte recruitment, exudation, and edema on carrageenan-induced inflammatory models, emphasizing the importance of these groups for anti-inflammatory activity. However, the mechanism of action of anti-inflammatory drugs compounds containing the nitro group is still unknown.

It was noted that compounds LT81 and LT87 had greater anti-inflammatory activities for the dose of 30 mg kg^{-1}, with better results than the dose of 50 mg kg^{-1}. One of the major problems in the in-vivo screening of synthetic compounds is the low aqueous solubility. This factor can limit bioavailability or decrease the permeation rate of compounds, so that a dose-dependent effect is not observed. Such results were also noticed by Oliveira et al. [13] in the anti-inflammatory activity in-vivo of a 2-thiophene-thiosemicarbazone derivative. However, pharmaceutical technology strategies can be employed in order to guarantee the bioavailability and effectiveness of promising compounds [62].

COX-2 is one of the key enzymes that catalyzes the conversion of arachidonic acid to inflammatory mediators, such as prostaglandins and prostanoids, and your expression plays a crucial role in acute inflammation. Carrageenan-induced mouse paw edema is a model of acute inflammation that enables COX-2 expression to be measured [63, 64].

Hayashi et al. [65] showed the suppression of PGE_2 production in response to COX-2 inhibition in rats treated with {2-[(4-ethylpyridin-2-yl)carbonyl]-5-(trifluoromethyl)-1H-indol-3-yl}acetic acid after the injection of carrageenan. Kassab et al. [66], in their study with new indomethacin-based analogs, showed that compound 9-(4-chlorobenzoyl)-6-(methylsulfonyl)-1,2,3,9-tetrahydro-4H-carbazol-4-one was more capable of suppressing plasma PGE_2 production than Celecoxib, in addition to having lower ulcerogenic activity than indomethacin.

The decrease in PGE_2 is an important parameter to assess the anti-inflammatory potencies of the COX-2 inhibitors. Barbosa et al. [24] showed that the decrease in lung injury in a pleurisy model induced by carrageenan after treatment with a thiazolidine derivative containing indole and this effect is linked to the inhibition of the expression COX-2. The studies corroborate our results and show the importance of indole as a scaffold when planning new anti-inflammatory molecules.

One way of accelerating the drug discovery and development process is through estimation and knowledge of pharmacokinetic properties, mainly those involved in the absorption, distribution, metabolization, and excretion [67].

In the in silico assay, the presence of hydrophobic groups, such as 4-chlorophenyl and 1-naphthyl) in *N*-4 at thiosemicarbazone, is responsible for the decrease in aqueous solubility [30]. The number of rotatable bonds and polar surface area are considered important predictive parameters of bioavailability, where 10 or fewer rotatable bonds and polar surface area equal to or less than 140 $Å^2$ (or 12 or fewer H-bond donors and acceptors) are highly likely to have good oral bioavailability [68]. Caco-2 cells are an enterocyte cell-line from human colon adenocarcinoma which are characterized by the absorption of substances [69]. Substances that have log Papp values > 0.90 cm s^{-1} are considered to have good intestinal absorption [70].

Conclusions

In summary, a series of indole-thiosemicarbazone derivatives were obtained and evaluated for their anti-inflammatory potential. The results showed that the compounds were able to inhibit leukocyte migration, decrease the expression of pro-inflammatory cytokines and inhibit the enzymatic activity of COX-2. In addition, in-vivo tests showed that the compounds were able to reduce both carrageenan-induced edema in the paw and the expression of COX-2 in the treated tissues. Finally, in-silico simulations predicted the good pharmacokinetic profile of the derivatives obtained. These

results show the importance of the indole and thiosemicarbazone fragments in the design of anti-inflammatory drugs.

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Compliance with ethical standards

Conflict of interest The authors declare that there were no competing interests.

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