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Anti-inflammatory effect of Schinus terebinthifolius Raddi hydroalcoholic extract on neutrophil migration in zymosan-induced arthritis

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Abstract

Ethnopharmacological relevance: Schinus terebinthifolius is a species of plant from the Anacardiaceae family, which can be found in different regions of Brazil. Schinus is popularly known as aroeirinha, aroeira-vermelha, or Brazilian pepper. In folk medicine, S. terebinthifolius is used for several disorders, including inflammatory conditions, skin wounds, mucosal membrane ulcers, respiratory problems, gout, tumors, diarrhea and arthritis. According to chemical analyses, gallic acid, methyl gallate and pentagalloylglucose are the main components of hydroalcoholic extracts from S. terebinthifolius leaves. In the present study, we demonstrated the ability of a hydroalcoholic extract to inhibit cell migration in arthritis and investigated the mechanisms underlying this phenomenon.

Materials and methods: The anti-inflammatory effect of S. terebinthifolius hydroalcoholic leaf extract (ST-70) was investigated in a zymosan-induced experimental model of inflammation. Male Swiss mice received zymosan (100 µg/cavity) via intra-thoracic (i.t.) or intra-articular (i.a.) injection after oral pre-treatment with ST-70. The direct action of ST-70 on neutrophils was evaluated via chemotaxis.

Results: ST-70 exhibited a dose-dependent effect in the pleurisy model. The median effective dose (ED_{50}) was 100 mg/kg, which inhibited 70% of neutrophil accumulation when compared with the control group. ST-70 reduced joint diameter and neutrophil influx for synovial tissues at 6 h and 24 h in zymosan-induced arthritis. Additionally, ST-70 inhibited synovial Interleukin (IL)-6, IL-1β, keratinocyte-derived chemokine (CXCL1/KC) and Tumor Necrosis Factor (TNF)-α production at 6 h and CXCL1/KC and IL-1β production at 24 h. The direct activity of ST-70 on neutrophils was observed via the impairment of CXCL1/KC-induced chemotaxis in neutrophils. Oral administration of ST-70 did not induce gastric damage. Daily administration for twenty days did not kill any animals. In contrast, similar administrations of diclofenac induced gastric damage and killed all animals by the fifth day.

Conclusions: Our results demonstrated significant anti-inflammatory effects of ST-70, suggesting a putative use of this herb for the development of phytomedicines to treat inflammatory diseases, such as joint inflammation.

Keywords: Schinus terebinthifolius Raddi, Arthritis, Neutrophils

Chemical compounds studied in this article:

Gallic acid (PubChem CID: 370); Methyl gallate (PubChem CID: 7428); Pentagalloylglucose (PubChem CID: 65238)

1. Introduction

Schinus terebinthifolius Raddi (Anacardiaceae) is a native plant from South America. It has been used in folk medicine as teas, infusions or tinctures; as an anti-inflammatory, febrifuge, analgesic, and depurative agent; and to treat urogenital system illnesses (Medeiros et al., 2007). Through ethnopharmacological research, the gastroprotective properties of S. terebinthifolius are remarkably effective, especially in the treatment of gastritis and ulcers (Carlini et al., 2010). Previous reports have demonstrated that S. terebinthifolius extracts or fractions rich in polyphenols, display antioxidant, antibacterial, antifungal and anti-allergic activities in different experimental models (Cavalher-Machado et al., 2008; de Lima et al., 2006; Schmourlo et al., 2005; Velázquez et al., 2003). Despite its importance in popular medicine for the treatment of inflammatory disorders, few scientific studies have examined the biological activities and chemical composition of Schinus terebinthifolius extracts.

Inflammation is a complex physiological response that occurs in vascularized tissues in response to harmful stimuli, such as pathogens, damaged cells or irritants. The inflammatory process is coordinated by different chemical mediators that induce vasodilation, plasma leakage and leukocyte margination. However, when the inflammatory response becomes prolonged or chronic, the same process can become destructive and has been linked to a number of diseases. Chronic inflammation can result from a failure to eliminate harmful stimuli, an abnormal autoimmune response or the persistence of a chronic, low-intensity irritant that continually causes acute inflammation response (Medzhitov, 2010).

Rheumatoid arthritis (RA) is a chronic autoimmune inflammatory disease characterized by pathological changes, such as persistent synovitis, vascular proliferation, infiltration of inflammatory cells, and damage to cartilage and bone (Scott et al., 2010). A critical factor that contributes to joint damage is the excessive production of inflammatory mediators by resident or infiltrating inflammatory cells. Cytokines (TNF-α and IL-1β) and eicosanoids (leukotrienes and prostaglandins) are involved in the pathogenesis of arthritis and participate in pain, neutrophil accumulation and tissue damage (Brennan and McInnes, 2008; Guerrero et al., 2008). Recently, the importance of IL-17 has been studied in experimental arthritis, wherein the cytokine was detected during neutrophil accumulation and cartilage degradation and in hyperalgesic symptoms (Pinto et al., 2010). The recruitment of neutrophils contributes to the local production of cytokines and joint damage and appears to be important in the

pathogenesis of human arthritis (Wright et al., 2014). In the last decade, the involvement of other cells, such as macrophages, synoviocytes, lymphocytes and mast cells, have been described, indicating that a wider variety of cells are also important in the perpetuating the articular inflammatory process (McInnes and Schett, 2011).

Current clinical treatments for RA include steroidal and nonsteroidal anti-inflammatory drugs (SAIDs and NSAIDs, respectively), disease-modifying antirheumatic drugs (DMARDs) and biological agents (Kalden, 2002). However, the prolonged use of SAIDS and NSAIDS has been associated with serious adverse effects, including gastrointestinal disorders, immunodeficiency and humoral disturbances (Roth, 2012), which are factors that have been attributed to treatment dropout.

In recent decades, the screening of safer and more potent anti-inflammatory drugs for clinical use has increased. In this context, plants with anti-inflammatory activities have shown promising effects against inflammatory diseases, such as arthritis (Lama and Saikia, 2011). A few reports have shown that a polyphenol from green tea extract displayed a protective effect in a model of inflammatory arthritis, largely through its ability to inhibit the production of key inflammatory mediators, such as interleukin (IL)-1β and IL-6, by RA synovial fibroblasts (Ahmed et al., 2006).

Considering the popular uses of teas and tinctures for medicinal purposes, we evaluated the anti-inflammatory effect of hydroalcoholic extracts from S. terebinthifolius Raddi to assess its ability to inhibit cell migration and inflammatory mediators in experimental arthritis. Furthermore, we explored the mechanisms involved in this phenomenon.

2. Materials and Methods

2.1. Reagents

Zymosan serotype A, dexamethasone, potassium diclofenac, phosphate buffered saline (PBS), buffer perborate, o-phenylenediamine dihydrochloride (OPD), Bradford reagent, bovine serum albumin (BSA), ethylene diamine tetraacetic acid disodium salt (EDTA), RPMI 1640 medium and fMLP (N-formyl-methionyl-leucyl-phenylalanine) were all obtained from Sigma Chemical Co. (St. Louis, MO, USA). DMSO (for biological tests), ethyl ether, ethyl acetate, n-hexane, dichloromethane, methanol and acetone for chromatography were purchased from Vetec Química Fina, Ltda. (Xerém, RJ, Brazil). LTB_4 immuno-assay kit was obtained from Cayman Chemicals (Ann Arbor,

Michigan, USA). Purified anti-murine TNF-α, CXCL1/KC, IL-6 and IL-1β mAbs; biotinylated anti-TNF-α, CXCL-1/KC, IL-6 and IL-1β mAbs; and recombinant TNF-α, CXCL-1/KC, IL-6 and IL-1β were all obtained from R&D Systems (Minneapolis, MN, USA).

2.2. Preparation and analysis of ST-70 extract

Leaves were collected from 10 individual of S. terebinthifolius plants in the Atlantic Forest Campus FIOCRUZ, Jacarepaguá, Rio de Janeiro, RJ, Brazil, and a voucher specimen was deposited into the Rio de Janeiro Botanical Garden Herbarium under number RB-451742.

The collected material were dried at 40°C in an oven with air circulation, reduced to small fragments and extracted with 70% ethanol in a dynamic maceration for 24 h. Then, the extract was filtered, concentrated under reduced pressure and lyophilized, resulting in a hydroethanolic extract (ST-70) with a yield of 11.00%. These conditions were based on previous studies of extraction times.

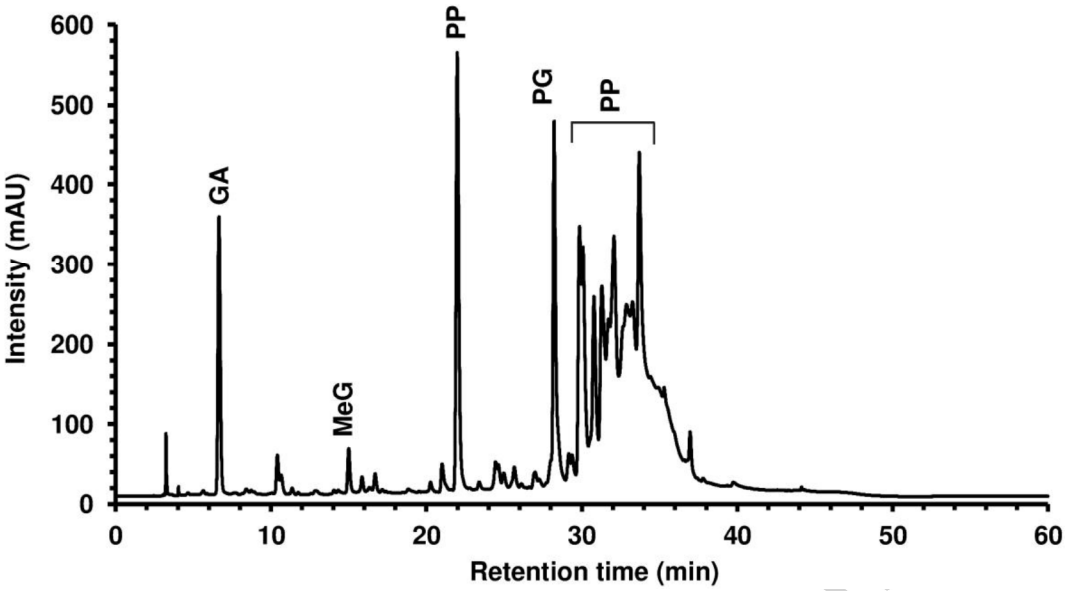
The ST-70 extract was analyzed using techniques such as adsorption column chromatography, thin layer chromatography, partition chromatography (countercurrent chromatography), gas chromatography coupled to mass spectrometry, high performance liquid chromatography (HPLC) and crystallization by traditional methodologies.

Several different methodologies were employed to isolate compounds from S. terebinthifolius. Spectrometric and spectroscopic analyses led to the identification of luteolin, quercetin, kaempferol, agathisflavone, gallic acid (GA), methyl gallate (MG), 1,2,3,4,6–pentagalloylglucose (PG), epicatechin, α-amyrin, β-amyrin, and lupeol.

The main secondary metabolite constituents in the extract, namely GA and MG, were assigned by HPLC analyses to the respective standard substances based on the similarities of their UV spectra on a diode array detector (DAD) at 220–400 nm and their retention times (6.6 min for GA and 15.0 min for MG). The HPLC chromatograms of ST-70 and the standards are shown in Fig. 1. The samples and the standard compounds were prepared in methanol through conventional dilution, and 20 μL was injected for analysis. A Supelcosil LC-18 column (250 × 4.6 mm, 5 μm) coupled to a Supelcosil LC-18 (4.0 mm × 20 mm, 5 μm) guard-column (Supelco, Bellefonte) was used. The following gradient program of the mobile phase, which consisted of aqueous trifluoroacetic acid at pH 2.5 (A) and methanol/acetonitrile 1:3 v/v (B), was performed at 40°C and 1.0 ml/min: 0-20 min, 3-20% B; 20-30 min, 20-30% B; 30-35 min, 30-50% B; 35-40 min, 50-96% B; 40-45 min, 96-3% B; and 45-60 min, 3% B. Detection was set at 270 nm. Separately, the extract was spiked with the standards to confirm peak

assignments. The GA and MG contents of ST-70 were determined based on calibration curves of the standards, which were tested in triplicate to confirm linearity and validity via ANOVA. The range (18.1 to 226 μg/ml for GA and 2.0 - 200 μg/ml for MG), linearity [$y_{GA} = 14.24 \times 10^3 \times$ (μg GA/ml) at r = 0.9998, $y_{MG} = 12.52 \times 10^3 \times$ (μg MG/ml) at r = 0.9997], precision (relative standard deviation for GA = 2.02 and for MG = 2.07) and limits of quantification (1 and 0.2 μg/ml for GA and MG, respectively) were determined following the International Conference of Harmonization guidelines (ICH, 2005) and literature recommendations (Ribani M et al., 2004). The extract was composed of 13.7 ± 0.3 mg/g of GA and 2.3 ± 0.1 mg/g of MG. A total of 172 ± 2.1 mg/g of eight main polyphenolic components presented UV spectra similar to that of GA, including 1,2,3,4,6–pentagalloylglucose (PG at 37.4 ± 0.9 mg/g), were estimated by an external calibration versus an MG curve.

Fig. 1



2.3. Animals

Male Swiss-44 and C57BL/6 mice (20-30 g) from CECAL-FIOCRUZ bioterium were maintained on a 12 h-light/dark cycle with controlled temperature and free access to food and fresh water. All experiments were conducted under license number CEUA LW-43/14 in accordance with the ethical guidelines of the International Association for the Study of Pain and the institutional guidelines for animal use.

2.4. Treatments

Mice that were fasted overnight received ST-70 (3.125 - 200 mg/kg) orally (p.o.) in a final volume of 200 µl 1 h prior to stimulation. As reference drugs, dexamethasone (10 mg/kg, 100 µl) was administered i.p., and potassium diclofenac (100 mg/kg, 200 µl) was administered p.o. 1 h prior to stimulation. Equivalent volumes of vehicle were administered to the control groups.

2.5. Zymosan-induced pleurisy

Swiss mice were orally pre-treated with diclofenac (100 mg/kg) or different doses of extract (3,125 - 200 mg/kg) 1 h before zymosan-induced pleurisy in order to determine the ST-70 effective dose to reduce inflammation by half (ED_{50}). The control group received an i.t. injection of an equal volume of vehicle. The animals were euthanized 4 h after stimulus by carbon dioxide (CO_2) inhalation, and their thoracic cavities were washed with 1 ml of PBS containing EDTA (10 mM) at pH 7.4. Total

leukocyte counts were performed on all washes from thoracic cavities using an automatic particle counter Z2 Counter (Coulter Z1, Beckman Coulter Inc., Brea, CA, USA). Differential cell counts were performed under light microscopy (1000x) using cytospin smears (Cytospin 3, Shandon Inc., Pittsburgh, PA, USA) stained according to the May-Grunwald-Giemsa method. The counts were reported as the numbers of cells ($\times 10^6$) per cavity. Then, pleural washes were centrifuged at 400 × g for 10 min. Supernatants were stored at -80ºC for further EIA and ELISA. The total protein contents of, the supernatants were quantified using the Bradford method according to the manufacturer's instructions.

2.6. Experimental arthritis

Joint inflammation was induced by intra-articular (i.a.) injection of zymosan (500 μg/cavity) that was diluted in sterile saline to a final volume of 25 μl, according to the technique of (Conte et al., 2008). Control animals (C57BL/6 mice) received i.a. injections of equal volumes of sterile saline. Knee-joint swelling was evaluated by measuring the transverse diameters of each knee joint using digital calipers (Digmatic Caliper, Mitutoyo Corporation, Kanagawa, Japan). Animals were pre-treated 1 h prior with ST-70 (100 mg/kg) or dexamethasone. Values of knee joint thickness were expressed in millimeters (mm) as the difference of the knee-joint diameter before and after the induction of articular inflammation (Δ). After 6 h or 24 h of joint inflammation induction, the mice were euthanized by carbon dioxide inhalation. Knee synovial cavities were washed with 300 μl of PBS containing EDTA (10 mM). Total leukocyte counts were performed in an automatic particle counter Z2 (Coulter Z2, Beckman Coulter Inc., Brea, CA, USA). Differential cell counts were performed under light microscopy (1000×) using cytospin smears (Cytospin 3, Shandon Inc., Pittsburgh, PA, USA) stained according to the May-Grunwald-Giemsa method. The counts were reported as numbers of cells per cavity ($\times 10^5$). The synovial washes were centrifuged at 400 × g for 10 min and stored at -80ºC for further analyses.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Levels of the CXCL1/KC and IL-6 were evaluated in Swiss mice pleural washes. CXCL1/KC, IL-6, TNF-α and IL-1β levels were quantified in C57BL/6 mice synovial washes by sandwich ELISA using matched antibody pairs from R&D Systems (Quantikine. Minneapolis, MN, USA) according to the manufacturer's instructions.

2.8. Determination of cytotoxicity

To investigate the treatment of GA on cellular viability, J77A-4 cells were plated onto black, flat-bottomed 96-well plates at a density of 2.5×10^6 cells/well. After 1 h of incubation in a controlled atmosphere (5% CO_2, 37°C), the cells received fresh medium with or without Tween 20 (3%), DMSO (0.5%) and GA (1, 10 or 100 nM) in a quadruplicate assay. After 21 h of further incubation, 20 µL of an Alamar Blue solution were added to each well. After an additional 3 h, the fluorescence was read on a SpectraMax M5/M5e microplate reader (Molecular Devices; k_{exc} 555 nm, k_{em} 585 nm).

2.9. Murine neutrophil purification

C57BL/6 mice were euthanized, and the femurs from both hind legs were dissected, removed and freed of soft tissue attachments. The distal tip of the femur extremity was excised, and each femur was washed with 2 ml of Ca^{2+}/Mg^{2+}-free HBSS–EDTA solution. Cell suspensions were then centrifuged at 400 × g for 15 min at 20°C and resuspended in 2 ml Ca^{2+}/Mg^{2+}-free HBSS–EDTA. The cells were purified via Percoll discontinuous gradient centrifugation (65% and 72% diluted in Ca^{2+}/Mg^{2+}-free HBSS–EDTA). Briefly, the cells were centrifuged at 1200 × g for 35 min at room temperature without braking, and polymorphonuclear cells were recovered from the 65%/72% interface. Then, neutrophils were counted in a Neubauer chamber, identified by cytospin centrifugation, and followed by Wright-Giemsa coloration according to the manufacturer's instructions.

2.10. Human neutrophil purification

Human neutrophils were isolated from citrate (3.8%)-treated peripheral venous blood of healthy volunteers using a four-step discontinuous Percoll gradient (Amersham Biosciences, San Francisco, CA, USA). Erythrocytes were removed by hypotonic lysis. Isolated neutrophils (98% purity), estimated to be >96% viable by Trypan blue exclusion, were resuspended in RPMI 1640 medium.

2.11. Chemotaxis assay

Neutrophil chemotaxis was assayed in a 48-well Boyden chamber (Neuroprobe, Inc. Cabin John, MD, USA) using a 5-µm PVP-free polycarbonate filter (Nuclepore, Pleasanton, CA, USA). Briefly, purified murine neutrophils were incubated with ST-70 (500 µg/ml) or dexamethasone (50 nM) for 1 h prior to the chemotaxis assay. Then, the cells were centrifuged at 400 × g for 10 min at room temperature and assayed in a chemotaxis chamber. The bottom wells of the chamber were filled with 29 µl of a chemoattractant stimulant, CXCL-1/KC (250 nM). Pre-treated neutrophils (1×10^5

cells/50 µl) were seeded in the upper compartment. The chamber was then incubated at 37°C in 5% CO_2 atmosphere for 1 h, and the filter was fixed and stained by Wright-Giemsa coloration according to the manufacturer's instructions. In another set of assays, purified human neutrophils were incubated with one of the main polyphenols of ST-70, GA (100 nM), or dexamethasone (50 nM) for 1 h prior to the chemotaxis assay. Then, the human cells were assayed in chemotaxis chambers, as described above, under fMLP (10 µM) stimulation. Neutrophils that had migrated through the membrane were counted under a light microscope (1000 × magnification) in fifteen random fields. The result is expressed as the mean number of migrated neutrophils per fifteen fields.

2.12. Ulcerogenic activity and survival

Swiss mice that were fasted for 24 h were orally administered either different doses of ST-70 (100 or 400 mg/kg) or potassium diclofenac (100 mg/kg) diluted in filtered water to a final volume of 200 µl. After 5 h, the mice were euthanized. The stomachs were removed, opened along the greater curvature and gently washed with PBS. Gastric lesions were analyzed under a stereoscopic microscope (Olympus, Japan) by a single-blind method. Additionally, Swiss mice received daily p.o. ST-70 (100 mg/kg), potassium diclofenac (100 mg/kg) diluted in filtered water to a final volume of 200 µl, or only filtered water (200 µl) for twenty days to assess survival rates.

2.13. Statistical analysis

All results are expressed as the means ± standard errors of the means (SEM) and were evaluated using one-way ANOVA followed by the Student-Newman-Keuls test. A log-rank (Mantel-Cox) test was used to compare the survival rates. The significance level was set at $P \leq 0.05$.

3. Results

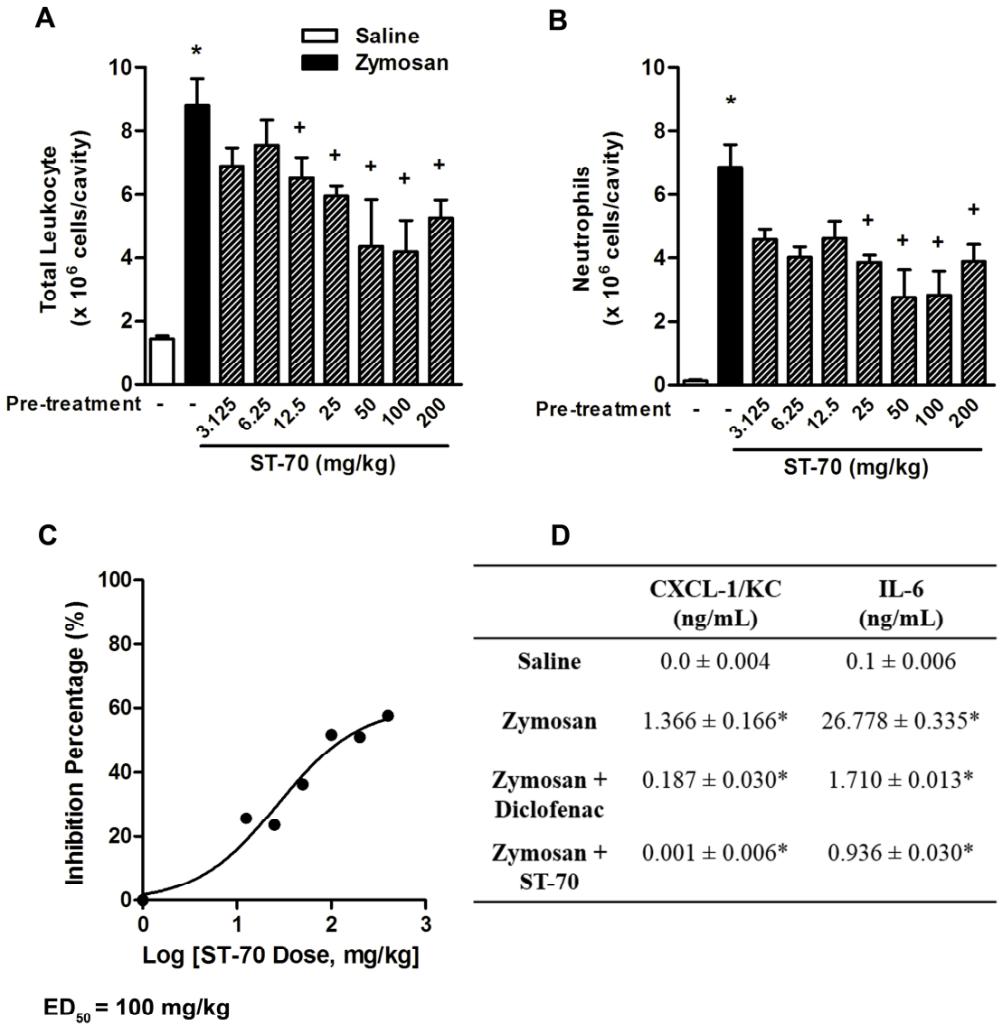
3.1. ST-70 inhibits leukocyte infiltration and inflammatory mediator production in pleurisy.

To determine the dose-response effect of ST70, it was applied to zymosan-induced pleurisy. As shown in Figs. 2A-B, the oral pre-treatment with different doses of ST-70 (3.125 - 200 mg/kg) significantly inhibited (from 25 mg/kg) the migration into pleural sites of total leukocytes, including neutrophils, 4 h after stimulation in a dose-dependent manner. According to the dose-response curve for leukocyte infiltration, the ED_{50} of ST-70 was 100 mg/kg (Fig. 2C). Potassium diclofenac, a nonsteroidal anti-

inflammatory drug (NSAID), was used as a reference inhibitor. As was observed with 100 mg/kg ST-70, the oral pre-treatment with 100 mg/kg diclofenac similarly inhibited total leukocyte and neutrophil accumulation (Fig. S1).

The migration and activation of neutrophils during inflammation results from several events, such as cytokine and chemokine action. In zymosan pleural washes, increased CXCL-1/KC and IL-6 levels were detected when compared with control groups. The oral pre-treatment with ST-70 (100 mg/kg) decreased the concentrations of CXCL-1/KC and IL-6 by 99.92% and 96.52%, respectively (Fig. 2D). A smaller decrease was observed after potassium diclofenac (100 mg/kg) pre-treatment (Fig. 2D).

Fig. 2



D

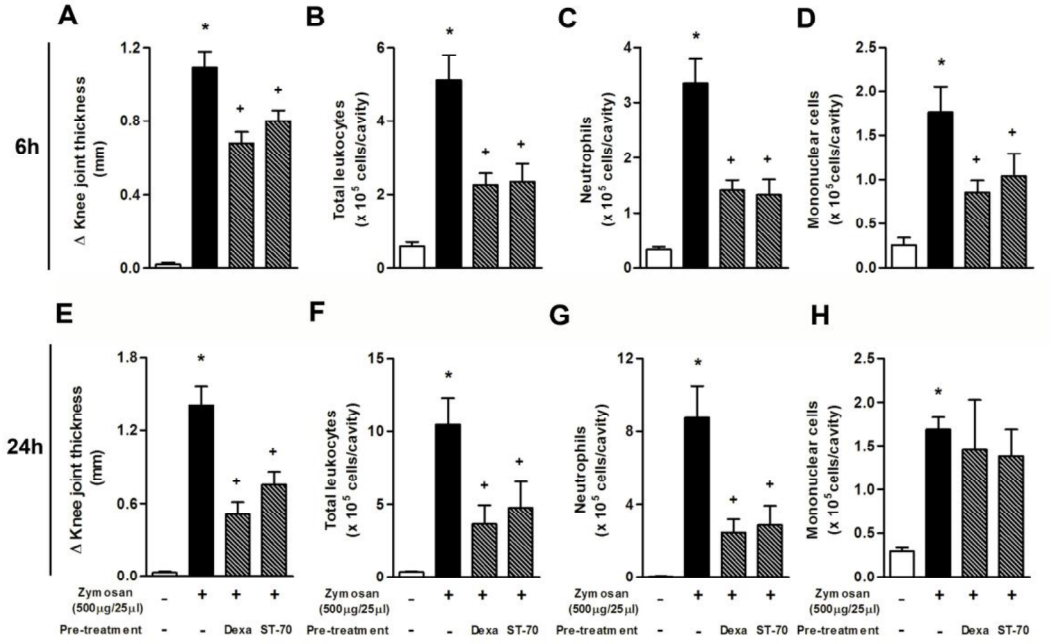
	CXCL-1/KC (ng/mL)	IL-6 (ng/mL)
Saline	0.0 ± 0.004	0.1 ± 0.006
Zymosan	1.366 ± 0.166*	26.778 ± 0.335*
Zymosan + Diclofenac	0.187 ± 0.030*	1.710 ± 0.013*
Zymosan + ST-70	0.001 ± 0.006*	0.936 ± 0.030*

3.2. ST-70 inhibits neutrophils migration on zymosan-induced arthritis

We previously reported that the i.a. injection of zymosan (500 µg/cavity) induced an articular inflammatory response after 6 h, which was characterized by a significant increase in edema formation and massive neutrophil influx peaking at 24 h (Conte et al., 2008; Penido et al., 2006). In the present study, the analyses were performed at 6 h and 24 h after stimulation because marked inflammatory reactions were already observed at these time points (Figs. 3 and 4). As shown in Figs. 3A and 3E, the oral pre-treatment with ST-70 (100 mg/kg) 1 h prior to zymosan injection markedly reduced knee-joint thickness at 6 h and 24 h after stimulation. Notably, the oral treatment with ST-70 induced a similar effect as that achieved via the i.p. administration of dexamethasone (10 mg/kg) (Figs. 3A and 3E). The treatments with ST-70 or dexamethasone also inhibited total leukocyte, mononuclear cell and neutrophil infiltration when compared with non-treated mice 6 h after i.a. zymosan injection (Fig.

3B-D). However, 24 h after stimulation, the ST-70 pre-treatment inhibited total leukocyte and neutrophil migration but failed to reduce mononuclear cell influx into the synovial cavity (Figs. 3F-H). Interestingly, a single oral treatment with ST-70 (100 mg/kg) was able to inhibit edema formation and leukocyte migration at all times analyzed after i.a. zymosan injection. It is noteworthy that ST-70 also inhibited murine neutrophil chemotaxis induced by CXCL-1/KC in vitro, demonstrating a direct effect of ST-70 on neutrophils (Fig. S2).

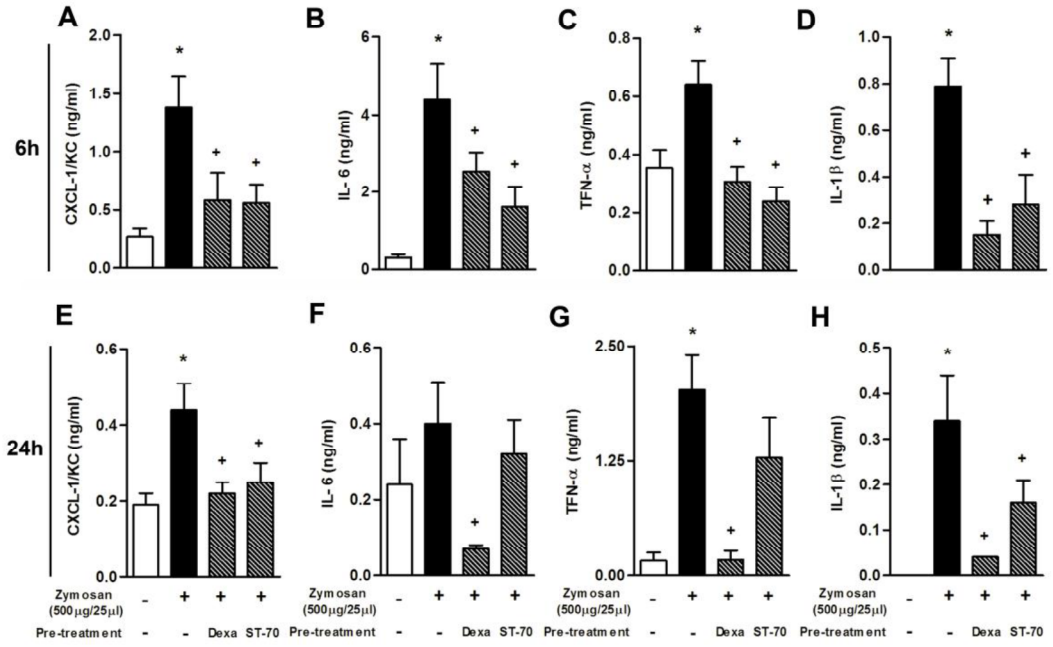
Fig. 3



3.3. ST-70 reduces pro-inflammatory mediators in articular zymosan-injected knee joints

To investigate whether ST-70 exerted its anti-inflammatory effect by modulating the production of inflammatory mediators, we analyzed its effect on the production of CXCL-1/KC, IL-6, TNF-α and IL-1β. As demonstrated in Figs. 4A-H, the articular cavity levels of CXCL-1/KC, TNF-α, IL-1β and IL-6 increased at 6 h and at 24 h after i.a. zymosan injection. ST-70 oral pre-treatment markedly reduced zymosan-induced CXCL-1/KC, IL-6, TNF-α, and IL-1β production 6 h after i.a. injection (Figs. 4A-D). ST-70 (100 mg/kg) only significantly inhibited CXCL-1/KC and IL-1β production 24 h after i.a. zymosan injection (Figs. 4E and 4H). Pre-treatment with dexamethasone reduced the production of CXCL-1/KC, IL-6, TNF-α and IL-1β at both time points investigated.

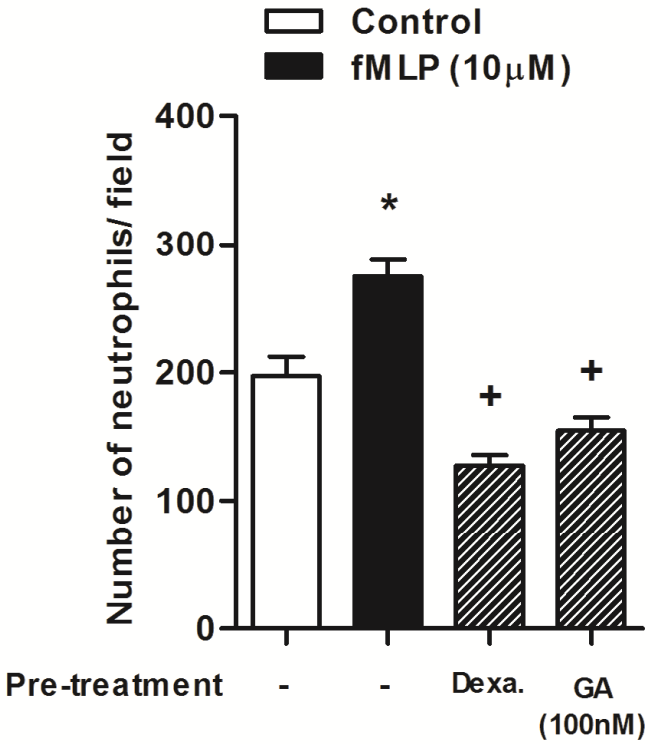
Fig. 4



3.5. Gallic acid from ST-70 exhibits anti-inflammatory activity

Phytochemical studies revealed that GA is one of the major polyphenol components of the ST-70 extract. To determine whether this substance is responsible for the observed effect on neutrophil migration, we evaluated the effect of GA on human neutrophil chemotaxis. First, the effect of GA (1, 10 and 100 nM) was assessed on cell viability using the resazurin assay; none of tested concentrations were cytotoxic (100 nM ≥95% viability). Pre-treatment with GA (100 nM) significantly reduced neutrophil chemotaxis (Fig. 5). This result suggests that GA is one of the bioactive component present in ST-70 extracts.

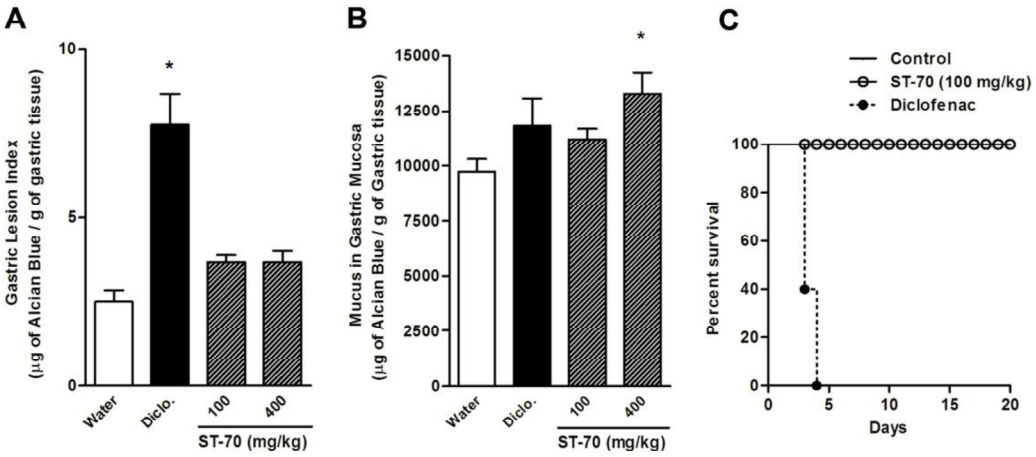
Fig. 5



3.6. ST-70 does not induce gastric injury or death in mice

As shown in Fig. 6, animals that received a single oral dose of sodium diclofenac (100 mg/kg) showed high indices of gastric lesions (Fig. 6A) but did not exhibit decreased mucus secretions (Fig. 6B). Furthermore, 100% of the mice died up to the fifth day after daily sodium diclofenac oral administration (Fig. 6C). However, ST-70 oral administration (100 or 400 mg/kg) induced few gastric lesions when compared with the control group (non-treated animals) and did not decrease the production of mucus secreted in the stomach (Figs. 6A-B). Moreover, the animals that received a 400 mg/kg dose displayed increased gastric mucus production. Additionally, the mice that were orally administered ST-70 daily for twenty days remained alive at the end of the experiment (Fig. 6C), showing no acute toxicity.

Fig. 6



4. Discussion

In the present work, we showed that the hydroalcoholic extract from Schinus terebinthifolius Raddi leaves (ST-70) exhibited important anti-inflammatory properties in pleurisy and arthritis, including a marked inhibition of neutrophil influx and reduced inflammatory mediators induced by zymosan. Previous results from our group demonstrated an anti-allergic effect of the ethyl acetate fraction from S. terebinthifolius leaves (ST fraction), in which HPLC analysis identified gallic acid, methyl gallate and 1,2,3,4,6–pentagalloylglucose as polyphenol components of ST fraction (Cavalher-Machado et al., 2008). In this work, the ST-fraction was shown to reduce eosinophil migration in allergic pleurisy. To date, the effect of S. terebinthifolius on experimental arthritis and neutrophil migration in acute inflammatory responses has not been studied.

Natural products have been tested in a wide range of inflammatory models, including zymosan-induced pleurisy (Penido et al., 2006). In fact, during zymosan-induced inflammatory responses, it is possible to study the mechanisms of phagocytosis, cell migration and the production of inflammatory mediators (Underhill, 2003). Herein, we performed a dose-response analysis using zymosan-induced pleurisy to pharmacologically investigate the link between ST-70 levels and its effect on inflammatory processes. The ED_{50} of ST-70 in the reduction of leukocyte infiltration into the thoracic cavity was calculated to be 100 mg/kg.

Inflammatory signaling in response to zymosan requires nuclear factor (NF)-κB activation. NF-κB is a pivotal regulator in the expression of many pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, and chemokines, e.g., CXCL-1/KC (Underhill, 2003), detected at early time points in murine models of inflammation

(Ajuebor et al., 1999). Previous reports have shown that TNF-α, IL-1, IL-6, and CINC (a member of the α-CXC chemokine family in rats) were sequentially produced during the early stages of zymosan-induced pleurisy in rats (Utsunomiya et al., 1998). Our results observed the production of IL-6 and CXCL-1/KC 4 h after zymosan intra-thoracic injection in mice. Oral pre-treatments with ST-70 (100 mg/kg) inhibited these pro-inflammatory mediators by more than 70%. Thus, our results suggest that ST-70 reduces neutrophil migration by affecting cytokine and chemokine production.

RA is a disease characterized by neutrophil accumulation. Elevated numbers of neutrophils are found in the synovial fluid of patients with RA. These activated neutrophils secrete proteases that contribute to the destruction of cartilage and related joint structures (Wright et al., 2014). Zymosan-induced arthritis has been used as an experimental model to assess the anti-inflammatory effects of different compounds (Gegout et al., 1995). At early time points following zymosan i.a. stimulation, edema formation was accompanied by massive neutrophil infiltration into the synovial tissue and the fluids of inflamed joints (Conte et al., 2008). Previous reports demonstrated that zymosan i.a. injection in rodents is characterized by a marked increase in knee-joint thickness within 6 h and peaks within 24 h, remaining significantly above control values for 20 days (Penido et al., 2006). Accordingly, we observed that zymosan i.a. injections in mice resulted in a marked increase in knee-joint thickness within 6 h and 24 h, as characterized by protein extravasation into the joint space. This phenomenon was inhibited by ST-70 oral pre-treatment at a dose of 100 mg/kg. However, the mechanism of action of ST-70 in the inhibition of zymosan-induced knee-joint edema remains unknown.

Neutrophils are predominant in the synovial exudates of a variety of inflammatory arthropathies, including rheumatoid arthritis; the participation of these cells in the pathogenesis of tissue lesions has long been recognized (Harris, 1991). However, neutrophils do not seem to be responsible for the perpetuation of chronic synovitis. Neutrophils promote cartilage breakdown and bone resorption by producing important chemicals, such as reactive oxygen and nitrogen species, lysosomal enzymes and metalloproteases (Hampton et al., 1998). Therefore, strategies to limit neutrophil trafficking or activation have received attention as potential alternatives to treat arthritis. In the present study, neutrophil influx at 6 h and 24 h after zymosan injection was reduced by a single oral dose of ST-70, as observed in synovial washes. Thus, the inhibition of neutrophil migration into the synovial cavity reduced the production of inflammatory mediators and, consequently, tissue damage.

Currently, TNF-α is believed to be the primary mediator in the inflammatory cytokine cascade and in the pathogenesis of RA. TNF-α is primarily produced by activated macrophages in inflamed synovial membrane tissue and induces the production of other pro-inflammatory cytokines, such as IL-1β and IL-6, and chemokines, such as CXCL-1, leading to the destruction of articular cartilage (Brennan and McInnes, 2008; van den Berg et al., 1999).

IL-1β and TNF-α induce neutrophil infiltration when injected into the joints of normal animals. The combination of these cytokines exhibits synergistic activity (Henderson and Pettipher, 1989). A considerable number of studies have shown that blockades of IL-1β and TNF-α reduce inflammation and joint destruction in arthritic animal models (Maini and Taylor, 2000). In clinical studies, antagonism of IL-1β and TNF-α inhibition reduces joint destruction and the signs and symptoms of RA (Bendele et al., 2000). We observed that the i.a. injection of zymosan induced high levels of TNF-α and IL-1β production 6 h and 24 h after stimulation. The oral administration of ST-70 reduced the production of these cytokines at 6 h post stimulus; only IL-1β production was inhibited 24 h after i.a. zymosan injection.

IL-6 displays pleiotropic effects, including effects on the maturation and activation of macrophages, osteoclasts, chondrocytes and endothelial cells. The critical role of IL-6 in the pathogenesis of RA has been demonstrated in clinical trials wherein a specific anti-IL-6 antibody was able to suppress the progression of joint erosion in RA (Brennan and McInnes, 2008). The oral pre-treatment with ST-70 resulted in a significant decrease in IL-6 in the first 6 h after zymosan-induced joint inflammation. In our model, the reduction in IL-6 in the early stages of arthritis seems to be associated with a reduction in edema and cell migration.

The chemokine CXCL-1/KC binds the CXCR1 and CXCR2 receptors, which signal through G-proteins. CXCR2 and CXCR1 signaling plays key roles in neutrophil function, especially in chemotaxis, degranulation, phagocytosis and oxidative burst (Boppana et al., 2014). In our RA experimental model, we observed high levels of CXCL1/KC production and a reduction in the levels of this chemokine after oral pre-treatment with ST-70. Furthermore, neutrophils stimulated with CXCL-1/KC migrated in smaller quantities when pre-incubated with ST-70. Our data suggest that the inhibition in neutrophil tissue accumulation in the joint cavity may be related to impaired CXCL1/KC generation during zymosan-induced arthritis. Here, we report for the first time a direct inhibition of neutrophil migration in arthritis by a hydroalcoholic extract from S. terebinthifolius leaves.

According to previous studies, plant-derived compounds that can modulate the expression of pro-inflammatory signals, such as polyphenols, may have anti-arthritic potential (Khanna et al., 2007). In the present report, the phytochemical analyses of ST-70 revealed the presence of the major polyphenols, GA, methyl gallate and 1,2,3,4,6–pentagalloylglucose. The anti-inflammatory effects of GA have been demonstrated in a few pharmacological studies (Kang et al., 2009). Recently, the mRNA expression levels of pro-inflammatory cytokines (IL-1β and IL-6), chemokines (CCL-2/MCP-1 and CCL-7/MCP-3), cyclooxygenase-2, and matrix metalloproteinase-9 by fibroblast-like synoviocytes (FLS) from patients with RA were shown to be suppressed by GA treatment in a dose-dependent manner (Yoon et al., 2013). Therefore, we evaluated the effect of GA from ST-70 on human neutrophil migration to determine 1) whether GA was responsible for the inhibitory action of ST-70 in neutrophil migration and 2) whether this compound was active against human neutrophils. Indeed, the significant reduction of human neutrophil chemotaxis upon pre-incubation with non-cytotoxic GA concentrations and stimulation with fMLP suggests that GA was at least partly responsible for the anti-inflammatory effects of ST-70.

The present study is the first to report an anti-inflammatory role for the hydroalcoholic extracts of S. terebinthifolius leaves (ST-70). The long-term oral administration of ST-70 did not induce lethality and gastric damage, in contrast to mice that received potassium diclofenac. This suggests that such an extract could be used to treat inflammatory conditions like arthritis. Plant-derived compounds have been historically valued as a source of anti-inflammatory agents (Calixto et al., 2004) with well-documented therapeutic efficacy and fewer side effects. Hence, the search for new anti-inflammatory drugs from herbal derivatives is justified.

5. Conclusion

Taken together, our results demonstrated that ST-70 inhibited leukocyte (primarily neutrophils) migration, cytokine and chemokine production in inflammatory models such as zymosan-induced arthritis. For these reasons, ST-70 may have potential as a phytomedicine for the treatment of inflammatory conditions.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgment

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Legends

Fig. 1: HPLC-270 nm chromatogram of the hydroalcoholic extract from S. terebinthifolius leaves. GA, gallic acid. MeG, methyl gallate. PG, 1,2,3,4,6–pentagalloylglucose. PP, other gallic acid derivatives.

Fig. 2: Study of the dose-response effect of the hydroalcoholic extract from S. terebinthifolius (ST-70) on cell migration and the production of inflammatory mediators. Effect of pre-treatment with ST-70 (3.125 – 200 mg/kg, p.o., 1 h before stimulation) on (A) total leukocyte and (B) neutrophil recruitment were analyzed 4 h after zymosan i.t. injection (100 µg/cavity; black columns). Control animals received saline i.t. injection (100 µl/cavity; white columns). (C) Dose-response log-curve of ST-70 treatment based on neutrophil recruitment analysis; the ED_{50} was calculated as 100 mg/kg. (D) CXCL-1/KC and IL-6 production from thoracic cavity washes were measured by ELISA. Data are presented as the means ± SEM (n = 6). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (+).

Fig. 3: ST-70 effect on edema and leukocyte migration during zymosan-induced arthritis. Knee-joint diameter was measured with a digital caliper, and knee synovial cells were recovered 6 h or 24 h after zymosan stimulation. Pre-treatment with ST-70 (100 mg/kg, p.o., 1 h before stimulation) was analyzed in (A) knee-joint thickness, (B) total leukocyte, (C) neutrophil and (D) mononuclear cell migration 6 h after zymosan i.a. injection (500 µg/25 µl/cavity; black columns) and in (E) knee-joint thickness, (F) total leukocyte, (G) neutrophil and (H) mononuclear cells migration 24 h after zymosan i.a. injection (500 µg/25 µl/cavity; black columns). Control animals received sterile saline i.a. injection (25 µl/cavity; white columns). Dexamethasone (Dexa, 100 mg/kg) was applied i.p. 1 h before stimulation. Data are presented as the means ± SEM (n = 7). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (+).

Fig. 4: Effect of ST-70 on the production of inflammatory mediators 6 and 24 h after zymosan-induced arthritis. Pre-treatment with ST-70 (100 mg/kg, p.o., 1 h before stimulation) was analyzed in synovial cavity washes collected 6 h or 24 h after zymosan i.a. injection (500 µg/25 µl/cavity; black columns), and (A and E) CXCL-1/KC, (B and F) IL-6, (C and G) TNF-α and (D and H) IL-1β were measured by ELISA.

Dexamethasone (Dexa, 100 mg/kg) was applied i.p. 1 h before stimulation. Data are presented as the means ± SEM (n = 7). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (+).

Fig. 5: Effect of gallic acid (GA) on human neutrophil chemotaxis. Peripheral human neutrophils were isolated and subjected to Boyden chamber chemotaxis assays in response to fMLP stimulation (10 µM; black column). The cells were pre-incubated for 1 h with RPMI (control), dexamethasone (50 nM) or GA (100 nM) before stimulation. Data are presented as the means ± SEM (n = 4). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (+).

Fig. 6: Effect of ST-70 on mice gastric mucosa and survival. Animals received oral administration of ST-70 (100 or 400 mg/kg, 1 h before stimulation), potassium diclofenac (100 mg/kg) or water (200 µl). (A) Gastric lesion index and (B) mucus production in gastric mucosa were analyzed 5 h after oral administration. (C) Survival rates for animals that received daily water (solid line), ST-70 (100 mg/kg; ○) or potassium diclofenac (100 mg/kg; ●). The log-rank test revealed differences in the survival curves between ST-70 and potassium diclofenac oral administration. Data are presented as the means ± SEM (n = 10). Significant differences ($P < 0.05$) are indicated between animals that received diclofenac and non-treated animals (*).

Fig. S1: Comparison between the effects of potassium diclofenac and ST-70 on zymosan-induced pleurisy. Oral pre-treatment with potassium diclofenac (Diclo) or ST-70 (100 mg/kg, p.o., 1 h before stimulation) were analyzed on (A) total leukocyte and (B) neutrophil recruitment 4 h after zymosan i.t. injection (100 µg/cavity; black columns). Animals control and stimulated with zymosan received only water by gavage as pretreatment (200 µl/animal white and black columns, respectively). Non-stimulated animals received saline i.t. injection (100 µl/cavity; white columns). Data are presented as the means ± SEM (n = 6). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (±).

Fig. S2: Inhibitory effect of ST-70 on murine neutrophil chemotaxis. Bone marrow mouse neutrophils were isolated and subjected to a Boyden chamber chemotaxis assay in response to CXCL-1/KC stimulation (250 nM; black columns). The cells were pre-incubated with RPMI (control, white column), dexamethasone (Dexa, 50 nM) or ST-70 (500 µg/ml) for 1 h at 37 ºC/5% CO2. Control cells were stimulated with RPMI. Data are presented as the means ± SEM (n = 4). Significant differences ($P < 0.05$) are indicated between stimulated and non-stimulated groups (*) and between treated and non-treated groups (+).