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Kaurenoic Acid from *Sphagneticola trilobata* Inhibits Inflammatory Pain: Effect on Cytokine Production and Activation of the NO−Cyclic GMP−Protein Kinase G−ATP-Sensitive Potassium Channel Signaling Pathway

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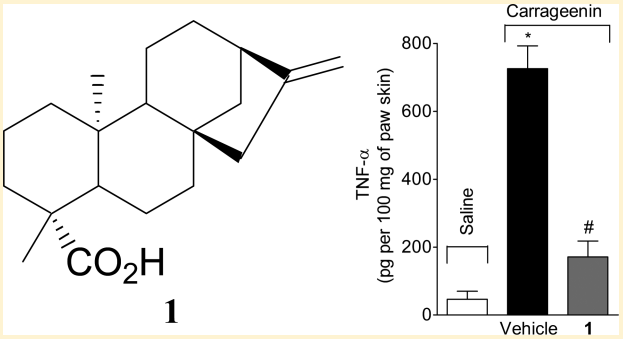
ABSTRACT: Kaurenoic acid [*ent*-kaur-16-en-19-oic acid (**1**)] is a diterpene present in several plants including *Sphagneticola trilobata*. The only documented evidence for its antinociceptive effect is that it inhibits the writhing response induced by acetic acid in mice. Therefore, the analgesic effect of **1** in different models of pain and its mechanisms in mice were investigated further. Intraperitoneal and oral treatment with **1** dose-dependently inhibited inflammatory nociception induced by acetic acid. Oral treatment with **1** also inhibited overt nociception-like behavior induced by phenyl-*p*-benzoquinone, complete Freund's adjuvant (CFA), and both phases of the formalin test. Compound **1** also inhibited acute carrageenin- and PGE_2-induced and chronic CFA-induced inflammatory mechanical hyperalgesia. Mechanistically, **1** inhibited the production of the hyperalgesic cytokines TNF-α and IL-1β. Furthermore, the analgesic effect of **1** was inhibited by L-NAME, ODQ, KT5823, and glybenclamide treatment, demonstrating that such activity also depends on activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway, respectively. These results demonstrate that **1** exhibits an analgesic effect in a consistent manner and that its mechanisms involve the inhibition of cytokine production and activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway.



Kaurenoic acid [*ent*-kaur-16-en-19-oic acid (**1**)] is a diterpene obtained from a number of plants and is a major compound in *Sphagneticola trilobata* (L.) Pruski (syn. *Wedelia paludosa*, *Acmella brasiliensis*; Asteraceae),[1] which is known popularly in Brazil as "arnica-do-mato", "pseudo-arnica", "picão-da-praia", and "vedélia". There is evidence that **1** exhibits its biological effects by inhibiting the inflammatory process such as in the carrageenin-induced paw edema[2,3] and TPA-induced ear edema models.[4] Furthermore, in a model of asthma in guinea pigs, **1** inhibited ovalbumin challenge-induced airway resistance in immunized animals as well as the production of histamine and activity of phospholipase A_2.[5] In vitro experiments also demonstrated that **1** inhibits LPS-induced production of nitric oxide[2,3] and prostaglandin E_2 (PGE_2) as well as the expression of cyclooxygenase-2 and inducible NO synthase (iNOS) in RAW 264.7 macrophages.[3] This inhibition of cyclooxygenase-2 and iNOS expression is probably related to the inhibition of NFκB activation.[3] On the other hand, there is also evidence that **1** does not inhibit LPS-induced NFκB activation, nitrite production, or mRNA expression of pro-inflammatory cytokines such as TNF-α and IL-1β, and cyclooxygenase-2.[6] Thus, the possible effects of **1** on these inflammatory pathways is still controversial and has been addressed only in vitro.

Additional relevant biological effects of **1** are the vasorelaxant effect via NO/cyclic guanosine monophosphate (cGMP)/protein kinase G (PKG)/ATP-sensitive potassium and calcium channels, depending on the experimental model,[7] and antimicrobial effects against *Bacillus cereus* and *Mycobacterium tuberculosis*.[8,9]

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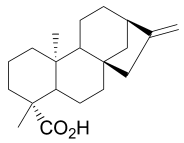
Sphagneticola trilobata is used popularly to treat rheumatic inflammatory diseases and fever.[1,11] Of note, rheumatic diseases such as rheumatoid arthritis are accompanied by pain.[12] As a consequence, it has been demonstrated that **1** inhibits the acetic acid-induced writhing response in mice.[11,13] However, whether or not **1** inhibits inflammatory pain in other models and also its analgesic mechanisms of action remain to be determined.

In terms of inflammatory pain, the sensitization of primary nociceptive neurons (nociceptors) occurs during inflammation, producing an increase in pain sensation (hyperalgesia). This sensitization is caused by the direct action of inflammatory mediators such as prostaglandins (e.g., PGE_2, PGI_2) and sympathetic amines (e.g., dopamine, epinephrine) on their receptors present in the membrane of nociceptors. It is also accepted that the release of these direct-acting hyperalgesic mediators is preceded by the release of a cascade of cytokines.[14,15] In the carrageenin model of paw inflammation, this cascade is initiated with the release of TNF-α and the chemokine CXCL1. These trigger the production of IL-1β, which, in turn, induces prostaglandin production. CXCL1 is also responsible for the stimulation of the sympathetic component of inflammatory pain.[16] Additionally, increasing attention has been drawn to the activation via the NO−cyclic GMP−PKG−ATP-sensitive potassium channel (nitric oxide/cyclic guanosine monophosphate/protein kinase G/ATP-sensitive potassium channel), as an important analgesic signaling pathway, since morphine and other drugs activate this signaling pathway.[17]

In view of the information presented above, it was investigated as to whether the antinociceptive effect of **1** depends on counteracting cytokine production/release and activation of the NO−cyclic GMP−PKG−ATP-sensitive potassium channel analgesic signaling pathway, in inflammatory pain.



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RESULTS AND DISCUSSION

Kaurenoic Acid (1) Inhibits the Writhing Response Induced by Acetic Acid and Phenyl-*p*-benzoquinone (PBQ). In the first series of experiments, the antinociceptive effect of **1** was evaluated in acetic acid and PBQ-induced pain-like behavior. Mice were treated ip (Figure 1A) or po (Figure 1B) with **1** (3−30 mg/kg; DMSO 2% diluted in saline) 30 min before ip injection of acetic acid 0.8%. In another group, mice were treated with **1** (10 mg/kg, po) 30 min before ip injection of phenyl-*p*-benzoquinone (1890 μg/kg, Figure 1C) or vehicle (saline or DMSO 2% diluted in saline, respectively). All doses of **1** inhibited the acetic acid-induced writhing response without differences between doses when administrated ip (Figure 1A). On the other hand, po treatment with **1** produced a dose-dependent inhibition of acetic acid-induced writhing with significant differences between the doses of 10 and 30 mg/kg, as compared to 3 mg/kg of **1**, and there was no difference between the 10 and 30 mg/kg doses of **1** (Figure 1B). This difference might be related to the pharmacokinetics of **1** since ip absorption is expected to be faster than po absorption, and there were no differences in the effect of **1** at doses of 10 and 30 mg/kg, irrespective of the route of administration. Therefore, it is unlikely that **1** is susceptible to the gastrointestinal environment. Furthermore, the po route is considered better than the ip route because it is a convenient mode of administration with great acceptance by patients and does not need a special environment such as a hospital to be administered. Therefore, the po route and a dose of 10 mg/kg of **1** was chosen for the next experiments. Nevertheless, the present data do demonstrate that **1** is active via other routes of administration such as ip, suggesting that should the po route be compromised, there would be alternative routes of administration. Previous data have indicated that **1** inhibits the acetic acid-induced writhing response.[11,13] The po treatment with **1** also inhibited PBQ-induced writhing. Acetic acid and PBQ models of writhing response share some mechanisms, while other mechanisms are model specific. For instance, both models involve the participation of prostaglandins.[18] In fact, in the present study, treatment with indomethacin (a cyclooxygenase inhibitor; 5 mg/kg, ip, 40 min) inhibited both the acetic acid (Figure 1A)- and PBQ (Figure 1C)-induced writhing responses.

Kaurenoic Acid (1) Inhibits Paw Flinch and Time Spent Licking the Paw Induced by Formalin and Complete Freund's Adjuvant (CFA). Mice were treated with **1** (10 mg/kg, po, 30 min) and received 25 μL of formalin 1.5% intraplantarly (ipl). Compound **1** inhibited both phases of the formalin test, regarding paw flinching (Figure 2A) and the time spent licking the paw (Figure 2B). Previous evidence demonstrates that the first phase of the formalin test depends on the direct effect of formalin in TRPA1 receptors present in primary nociceptive neurons[19] together with the involvement of histamine and serotonin release/action on nociceptors.[20] Considering that **1** inhibits histamine release in a model of asthma,[5] it is possible that inhibition of histamine release explains, at least in part, the inhibition of the first phase of the formalin test by treatment with **1**. The second phase of the formalin test depends on indirect mechanisms such as prostaglandin and cytokine production.[21] Therefore, **1** could inhibit the action of direct and indirect acting inflammatory mediators. The CFA-induced inflammation is also dependent on cytokine production.[8] In Figure 2C and D, it was detected that **1** (10 mg/kg, po, 30 min) inhibited both paw flinching and licking behaviors induced by CFA (10 μL/paw), respectively.

Treatment with Kaurenoic Acid (1) Inhibits Carrageenin- and CFA-Induced Mechanical Hyperalgesia. Next, the antinociceptive effect of **1** was tested in the carrageenin (100 μg/paw)- and CFA (10 μL/paw)-induced mechanical hyperalgesia models. The po treatment with **1** dose-dependently (1−10 mg/kg) inhibited carrageenin-induced mechanical hyperalgesia (Figure 3A). No significant effects were observed at a dose of 1 mg/kg. On the other hand, 3 mg/kg of **1** inhibited carrageenin-induced hyperalgesia significantly at 1 and 3 h after stimulus, and a 10 mg/kg dose inhibited carrageenin-induced mechanical hyperalgesia 1−5 h after stimulus, with significant differences compared to the dose of 1 mg/kg of **1** (Figure 3A). Therefore, a 10 mg/kg dose of **1** was selected for mechanical hyperalgesia-related experiments. Indomethacin treatment inhibited significantly carrageenin-induced mechanical hyperalgesia (Figure 3A). To further address the possible therapeutic usefulness of the compound, mice were treated daily with **1** (10 mg/kg, po) starting 1 h after CFA ipl injection for 7 days (Figure 3B). There was significant

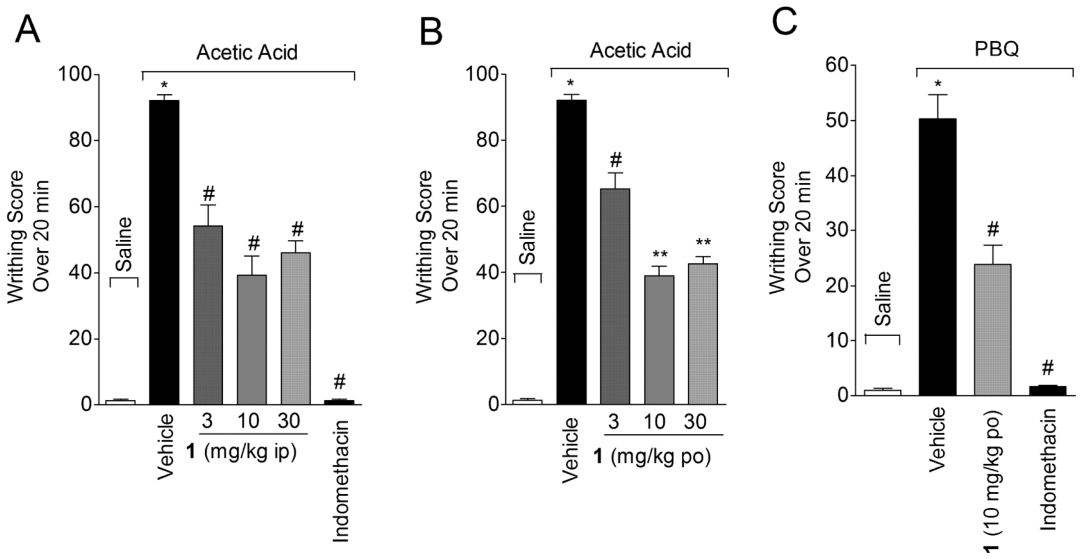

Figure 1. Kaurenoic acid (**1**) inhibits the writhing response induced by acetic acid and phenyl-*p*-benzoquinone (PBQ). Mice were treated ip (A) or po (B) with **1** (3−30 mg/kg) or vehicle (DMSO 2% diluted in saline) 30 min before an acetic acid (A) injection. In another group, mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle 30 min before a phenyl-*p*-benzoquinone (PBQ, panel C) injection. Control groups were treated with indomethacin (5 mg/kg, ip) 40 min before acetic acid or PBQ injection. The cumulative number of writhings (writhing score) was evaluated for 20 min ($n = 6$). [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group, and $^{**}p < 0.05$ compared to the vehicle group and the dose of 3 mg/kg of **1** (one-way ANOVA followed by Tukey's test).]

inhibition of mechanical hyperalgesia at all time points evaluated except in the first day after CFA injection for both **1** and the control drug indomethacin (Figure 3B).

In addition to inflammatory hyperalgesia, **1** also inhibits carrageenin-induced paw edema,[2,3] supporting a wider potential therapeutic applicability of **1** in inflammatory conditions.

Kaurenoic Acid (1) Inhibited TNF-α and IL-1β Production Induced by Carrageenin. Carrageenin-induced inflammatory hyperalgesia is mediated by a cascade of cytokines.[16] In turn, the hyperalgesic role of cytokines is mediated by the production of the final sensitizing mediators such as PGE_2 and sympathetic amines.[16,22,23] Mice were treated with **1** (10 mg/kg, po, 30 min) before carrageenin stimulus (100 μg/paw). Three hours after injection of carrageenin, mice were anesthetized terminally and the cutaneous plantar tissue was collected for cytokine (TNF-α and IL-1β) measurement (Figure 4). Compound **1** inhibited carrageenin-induced production of TNF-α (Figure 4A) and IL-1β (Figure 4B) in the paw tissue. This inhibition of cytokine production by **1** might account for the inhibition of cyclooxygenase-2 expression[3] since both cytokines induce it.[14,16] Furthermore, the present data demonstrating the inhibition of cytokine production by **1** shed light on a controversy of whether it inhibits NFκB activation and mRNA expression of TNF-α and IL-1β as determined in vitro,[3,6] indicating that these mechanisms might be of importance in vivo, and the inhibition of cytokine production is probably related to the inhibition of NFκB activity, as previously described.[3] To the best of our knowledge, this is the first study to demonstrate the inhibition of cytokine production by **1**. Besides being important cytokines in carrageenin hyperalgesia, TNF-α and IL-1β are considered major targets to control several inflammatory diseases.[24]

Kaurenoic Acid (1) Reduced PGE_2-Induced Mechanical Hyperalgesia. Mice were treated with **1** (10 mg/kg, po) or vehicle 30 min before ipl injection of PGE_2 (100 ng/paw) (Figure 5). There was a significant inhibition of PGE_2-induced mechanical hyperalgesia at 3 h by treatment with **1**. Since PGE_2 acts directly on nociceptive neurons to produce mechanical hyperalgesia, it is likely that the analgesic effect of **1** might be through activation of neuronal events.[14,16]

Kaurenoic Acid (1) Inhibits Inflammatory Pain by Activating the NO−Cyclic GMP−PKG−ATP-Sensitive Potassium Channel Signaling Pathway. Mice were treated with L-NAME (L-nitro-arginine methyl ester, NOS inhibitor, 10−90 mg/kg, ip) 60 min before **1** (10 mg/kg, po) treatment (Figure 6A). After an additional 30 min, mice received an ipl injection of carrageenin (100 μg/paw), and mechanical hyperalgesia was evaluated after 5 h (peak of hyperalgesia). Corroborating the data presented above, **1** inhibited carrageenin-induced mechanical hyperalgesia. In turn, L-NAME dose-dependently inhibited the analgesic effect of **1**. A dose of 10 mg/kg of L-NAME did not affect antinociception by **1**, but doses of 30 and 90 mg/kg of L-NAME significantly inhibited the effect of **1**. The 90 mg/kg dose was also statistically different from the 10 mg/kg dose of L-NAME. There was no difference between the doses of 30 and 90 mg/kg of **1** (Figure 6A). Similar results were obtained at 1 and 3 h after carrageenin injection (data not shown), but only those for the fifth hour are presented for clarity. Therefore, in addition to inhibition of cytokine production, the antinociceptive effect of **1** depends on the induction of NO production. Although the isoform of NOS responsible for **1**-induced NO production was not determined, it has been shown that selective inhibitors of nNOS and eNOS, but not iNOS, reduce the vasorelaxing effects of **1**.[7] This information[7] also explains the apparent contradiction with previous data demonstrating that **1** inhibits LPS-induced NO production and iNOS expression.[3,6] Therefore, it is likely that **1** exhibits a dual effect on NO production since it can increase NO production by activating nNOS and eNOS[7] and reduce NO production by inhibiting the expression of iNOS.[3] Under the present experimental conditions, it is very likely that **1** activates nNOS, because a series of studies demonstrated that nNOS-derived NO is a prominent analgesic mechanism shared by morphine, diclofenac, and other clinically

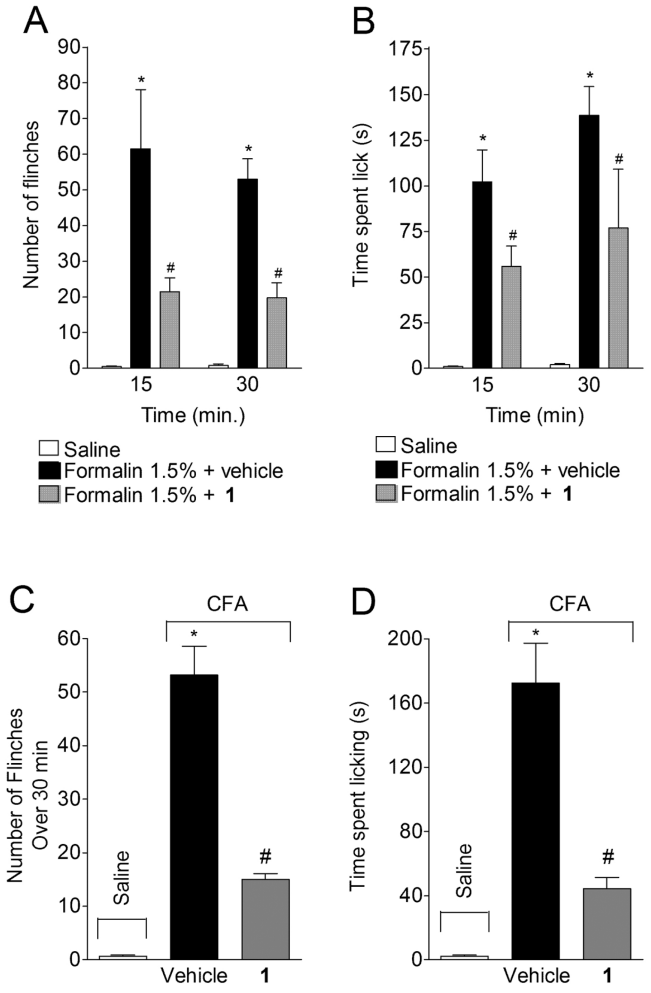

Figure 2. Kaurenoic acid (**1**) inhibits paw flinch and time spent licking the paw induced by formalin and complete Freund's adjuvant (CFA). Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the injection of formalin (25 μL of 1.5% formalin in saline, panels A and B) or CFA (10 μL/paw, panels C and D). The total number of flinches (A and C) and time spent licking the paw (B and D) was evaluated for 30 min and expressed between 0 and 15 and 15−30 min (A and B) or the cumulative number (C and D) (n = 6). [*p < 0.05 compared with the saline group, $^{\#}p$ < 0.05 compared to the vehicle group (one-way ANOVA followed by Tukey's test).]

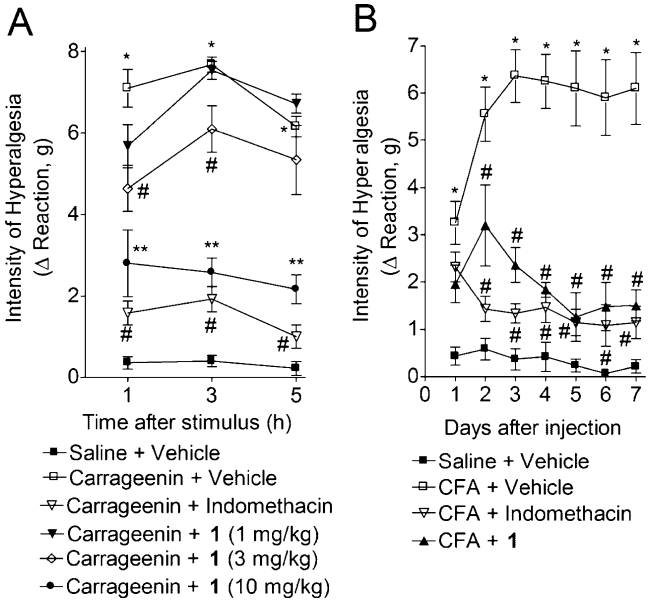

Figure 3. Treatment with kaurenoic acid (**1**) inhibits carrageenin- and CFA-induced mechanical hyperalgesia. Mice were treated with **1** (1−10 mg/kg, po, 30 min) or vehicle before the carrageenin (100 μg/paw) injection (A). In another group, mice were treated daily with **1** (10 mg/kg, po) starting 1 h after CFA (10 μL/paw) injection (B). Control groups were treated with indomethacin 40 min (5 mg/kg, ip) before carrageenin or every 24 h starting 1 h after CFA. The intensity of hyperalgesia was measured 1−5 h (A) or every 24 h (B) by the electronic pressure-meter test (n = 5). [*p < 0.05 compared with the saline group, $^{\#}p$ < 0.05 compared to the vehicle group, and **p < 0.05 compared to the dose of 1 mg/kg of **1** (one-way ANOVA followed by Tukey's test).]

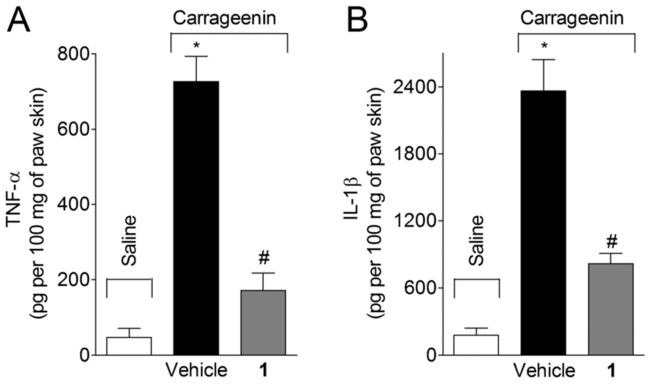

Figure 4. Kaurenoic acid (**1**) inhibited TNF-α and IL-1β production induced by carrageenin. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before the ipl injection of carrageenin. Three hours after carrageenin injection, mice were sacrificed and paw skin samples were collected for the determination of TNF-α and IL-1β production (n = 5). [*p < 0.05 compared with the saline group, and $^{\#}p$ < 0.05 compared to the vehicle group (one-way ANOVA followed by Tukey's test).]

available drugs.[17] NO also inhibits the hyperalgesia induced by PGE_2 and other direct-acting hyperalgesic mediators.[17] Thus, the present results on the role of NO in the analgesic effect of **1** explains the inhibition of PGE_2-induced hyperalgesia (Figure 5). NO activates the cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway to induce analgesia. Accordingly, the next experiments assessed whether **1** would also activate similar mechanisms to reduce inflammatory mechanical hyperalgesia. Mice were treated with ODQ (Figure 6B, soluble cGMP inhibitor; 0.3 mg/kg, ip, diluted in 2% DMSO in saline, 30 min), KT5823 (Figure 6C, PKG inhibitor; 0.5 μg/mice, ip, diluted in 2% DMSO in saline, 5 min), glybenclamide (Figure 6D, ATP-sensitive potassium channel blocker; 0.3 mg/kg, ip, diluted in 5% Tween 80 in saline, 45 min), or the respective vehicle before **1** (10 mg/kg, po). After an additional 30 min, mice received an ipl injection of carrageenin (100 μg/paw). Again, **1** inhibited the carrageenin-induced mechanical hyperalgesia significantly at 1, 3, and 5 h after the stimulus, and treatment with ODQ (Figure 6B), KT5823 (Figure 6C), or glybenclamide (Figure 6D) inhibited the analgesic effect of **1** significantly. Thus, the analgesic effect of **1** depends on the activation of the NO−cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway, which diminishes the nociceptive transmission, resulting in diminished inflammatory mechanical hyperalgesia.[17]

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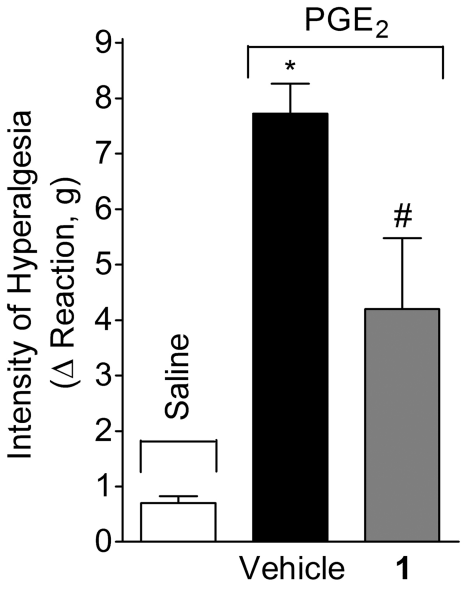

Figure 5. Kaurenoic acid (**1**) reduced PGE_2-induced mechanical hyperalgesia. Mice were treated with **1** (10 mg/kg, po, 30 min) or vehicle before PGE_2 (100 ng/paw) injection. The intensity of mechanical hyperalgesia was measured 3 h after stimulus injection by the electronic pressure-meter test (n = 5). [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test).]

Kaurenoic Acid (1) Showed No Muscle-Relaxing or Sedative Effects. Per oral treatment with 10 mg/kg of **1** did not alter the motor response of the test animals 1.5, 3.5, and 5.5 h after treatment (n = 6). These time points were based on the 30 min of pretreament plus 1, 3, and 5 h until mechanical hyperalgesia measurement in the carrageenin model. The vehicle control responses and **1**-treated animals in the rota-rod test were 120 s vs 120 s (1.5 h), 120 s vs 120 s (3.5 h), and 120 s vs 120 s (5.5 h), respectively (data not shown). These results support the notion that **1** diminishes the nociceptive threshold induced by inflammation and not that the mice are incapable of responding because of muscle-relaxing or sedative effects.[25] Previous data have shown that **1** reduces methamphetamine-induced locomotor enhancement, suggesting sedative effects.[13] However, this effect was achieved with 300 mg/kg of **1**, a 30-fold higher dose compared to the present experimental conditions used.

Kaurenoic Acid (1) Did Not Exhibit Any Effect in the Hot Plate Test in Naive Mice. Mice were treated with **1** (10 mg/kg, po route) or morphine hydrochloride (8 mg/kg, ip route), and the thermal nociception was evaluated before and 1.5, 3.5, and 5.5 h after treatment (data not shown), in the same manner as for the rota-rod test. Morphine hydrochloride treatment increased the thermal nociceptive threshold as expected because of its central analgesic effects. On the other hand, **1** did not alter the thermal threshold of mice (data not shown). This result further supports a peripheral neuronal effect of **1** upon inflammatory pain, since the hot plate test is considered to be modulated by supraspinal mechanisms.[26]

Kaurenoic Acid (1) Did Not Alter the Plasma Levels of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT), and Myeloperoxidase Activity in the Stomach. For Figures 7 and 8, the same protocol was used. Mice received po treatment daily with vehicle (2% DMSO in saline or tris[2-amino-2-hydroxymethylpropan-1,3-diol]/HCl buffer, pH 8.0, with the data for the vehicles pooled and no difference evident between the vehicles in Figures 7 and 8), **1** (10 mg/kg), or indomethacin (2.5 mg/kg) for 7 days. Vehicle and **1** exhibited similar plasma levels of AST (Figure 7A) and ALT (Figure 7B), indicating that **1** does not induce liver damage. On the other hand, treatment with indomethacin induced a significant increase of AST and ALT plasma levels in a similar manner to previous reports.[27,28]

There is evidence that po treatment with indomethacin or other cyclooxygenase inhibitors (e.g., acetylsalicylic acid) induces gastric mucosa tolerance, described as showing no detectable gastric mucosal lesions concomitant with an increase in polymorphonuclear cell infiltrate as determined by myeloperoxidase (MPO) activity.[30] In agreement with that, the positive control indomethacin induced a significant increase of MPO activity in the stomach tissue compared to group treated with vehicle (Figure 8). There was no statistical difference between groups treated with vehicle or **1** (Figure 8). Therefore, **1** seems to be a safe test compound regarding liver damage and stomach inflammation as assessed over a seven-day period of treatment.

In conclusion, the present study has evaluated further the antinociceptive activity of kaurenoic acid (**1**) upon inflammatory pain and has provided new evidence for the effects of this compound in a variety of inflammatory pain models and for its mechanism of action. The antinociceptive mechanisms of action of **1** may depend on (a) inhibition of pro-hyperalgesic cytokine production, TNF-α and IL-1β; (b) inhibition of cyclooxygenase-2 and consequently PGE_2 production;[2,3] (c) reduction of hyperalgesia produced by a directly acting mediator such as PGE_2; and (d) activation of the NO−cyclic GMP−PKG−ATP-sensitive potassium channel signaling pathway. As a consequence, there is reduced sensitization of nociceptors due to reduced cytokine (present data) and PGE_2[2,3] production and reduced nociceptive transmission related to activation of the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway. Therefore, the presence in high concentrations of kaurenoic acid (**1**) in different plants supports its possible commercial utilization. Considering that it exhibits promising antinociceptive activity, this compound merits further preclinical and possible clinical investigation.

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured in $CHCl_3$ using a Perkin-Elmer 241 polarimeter. Nuclear magnetic resonance (NMR) spectra were run on a Bruker DPX 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). Samples were dissolved in $CDCl_3$, and the spectra were calibrated with the solvent signals at δ 7.26 (^{1}H) and 77.0 (^{13}C). Mass spectrometric analysis was performed at low resolution on a Micromass Quattro-LC instrument (Manchester, UK) provided with an ESI ion source and a triple quadrupole mass analyzer. Solutions were dissolved in MeOH−H_2O, 8:2 (v/v), and infused into the ESI source at a flow rate of 5 μL/min, using a Harvard apparatus model 1746 (Holliston, MA, USA) syringe pump. Vacuum-liquid chromatography (VLC) was carried out using silica gel 60H (Merck, art. 7736) in glass columns with 5−10 cm i.d.[29] High-performance liquid chromatography (HPLC) analysis was accomplished using a Shimadzu CBM-20A liquid chromatography controller, operating with LC solution software, equipped with a Shimadzu UV-DAD detector SPD-M20A and a Shimadzu ODS column (4.6 × 250 mm, 5 μm, 100 Å).

For the in vivo experiments, mice received per oral (po, 1, 3, 10, and 30 mg/kg) or intraperitoneal (ip, 3, 10, and 30 mg/kg) treatment with kaurenoic acid (**1**) or vehicle (2% DMSO in saline) 30 min before

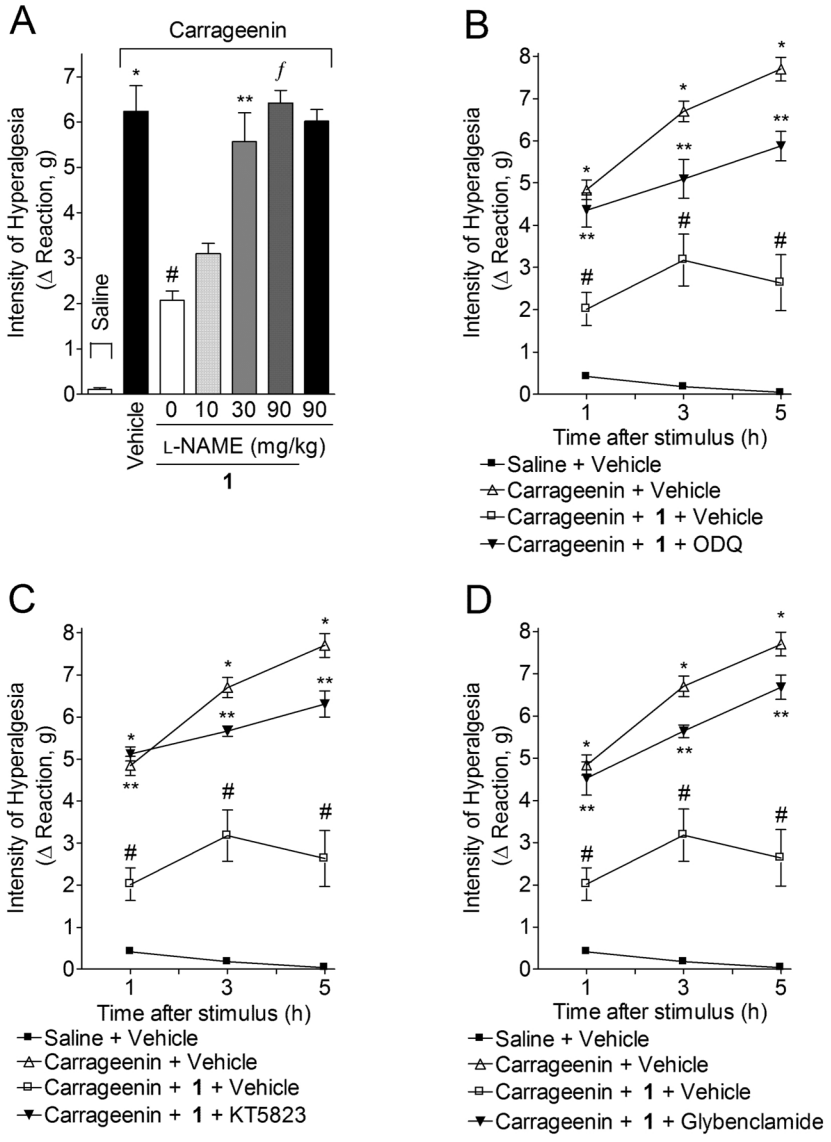

Figure 6. Kaurenoic acid (**1**) inhibits inflammatory pain by activating the NO−cyclic GMP−protein kinase G−ATP-sensitive potassium channel signaling pathway. Mice were treated with L-NAME (NOS inhibitor, 10−90 mg/kg, ip) 60 min before treatment with **1** (10 mg/kg, po) (A). After an additional 30 min, mice received an ipl injection of carrageenin (100 μg/paw), and mechanical hyperalgesia was evaluated after 5 h (peak of hyperalgesia). Mice were treated with ODQ (panel B, soluble cGMP inhibitor; 0.3 mg/kg, ip, diluted in 2% DMSO in saline), KT5823 (panel C, PKG inhibitor; 0.5 μg/mice, ip, diluted in 2% DMSO in saline, 5 min), glybenclamide (panel D, ATP-sensitive potassium channel blocker; 0.3 mg/kg, ip, diluted in 5% Tween 80 in saline, 45 min), or the respective vehicle before **1** (10 mg/kg, po). After an additional 30 min mice received an ipl injection of carrageenin (100 μg/paw). Mechanical hyperalgesia was measured 1, 3, and 5 h after stimulus injection ($n = 5$). [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle(s) group, $^{**}p < 0.05$ compared to the groups treated with **1** + vehicle(s) or **1** + L-NAME 0 mg/kg, and $^{f}p < 0.05$ compared to the dose of 10 mg/kg of L-NAME (A) (one-way ANOVA followed by Tukey's test).]

inflammatory stimulus. The doses of inflammatory stimuli were determined previously in our laboratory in pilot studies and were based on previous work.[16,18,25,30−32] Mechanical hyperalgesia was evaluated 1−5 h after carrageenin (100 μg/paw), 3 h after PGE_2 (100 ng/paw), or 1−7 days after CFA (10 μL/paw) stimulus. All inflammatory stimuli induced only ipsilateral (in the paw in which the stimulus was injected) mechanical hyperalgesia. IL-1β and TNF-α levels were evaluated 3 h after carrageenin (100 μg/paw) injection. The writhing response was evaluated for 20 min after po injection of acetic acid or phenyl-*p*-benzoquinone. The paw flinching and licking nociceptive responses were quantified for 30 min after formalin 1.5% (25 μL/paw) or CFA (10 μL/paw) injection. The plasma levels of AST and ALT, and MPO activity in stomach samples, were determined in mice treated po daily with vehicle, **1** (10 mg/kg), or indomethacin (2.5 mg/kg) for 7 days.

Plant Material. The air-dried roots of *Sphagneticola trilobata* were collected in September 2011 at Horto de Plantas Medicinais (23°19′41″ S/51°12′14″ W) of Centro de Ciências Agrárias at Universidade Estadual de Londrina (UEL). A voucher specimen was deposited at the "Herbário da Universidade Estadual de Londrina (FUEL)" no. 49306, collected by one of the authors (N.S.A.). The voucher specimen was identified by Profa. Dra. Vieira, Departamento de Biologia Animal e Vegetal (Centro de Ciências Biológicas, UEL).

Extraction and Isolation. The air-dried roots were pulverized and then extracted exhaustively with dichloromethane (900 mL) at room temperature, to give 1.2 g of crude extract, which was suspended in 300 mL of methanol−H_2O (9:1) and filtered. The soluble fraction was partitioned using *n*-hexane (300 mL, four times), which resulted in a 0.7 g *n*-hexane-soluble fraction after solvent evaporation under reduced pressure. The *n*-hexane-soluble fraction was chromatographed over silica gel 60 (0.063−0.200 mm) using vacuum-liquid chromatography

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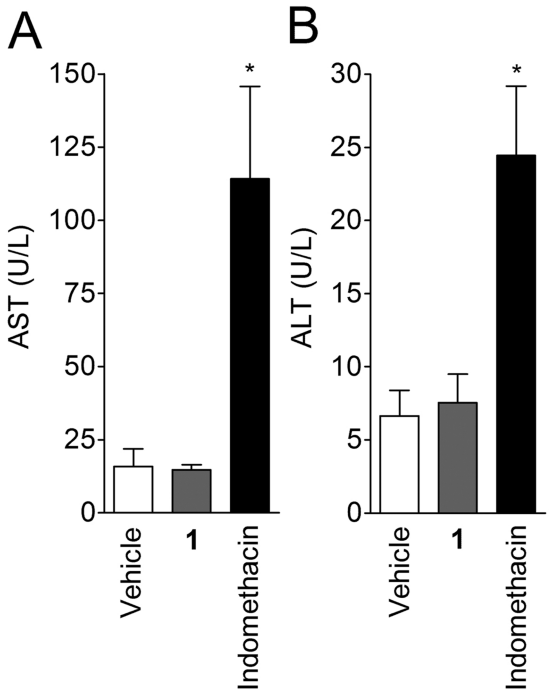

Figure 7. Effect of kaurenoic acid (**1**) on the plasma levels of aspartate aminotransferase (AST) and alanine aminotrasferase (ALT). Mice received po treatment daily with vehicle (DMSO 2% diluted in saline or Tris/HCl buffer, pH 8.0), **1** (10 mg/kg), or indomethacin (2.5 mg/kg). After seven days of treatment, mice were terminally anesthetized and blood was collected in heparinized microcentrifuge tubes. The blood was centrifuged and plasma collected for analysis of AST (A) and ALT (B) levels ($n = 8$). [$^*p < 0.05$ compared with the vehicle or **1** groups (one-way ANOVA followed by Tukey's test).]

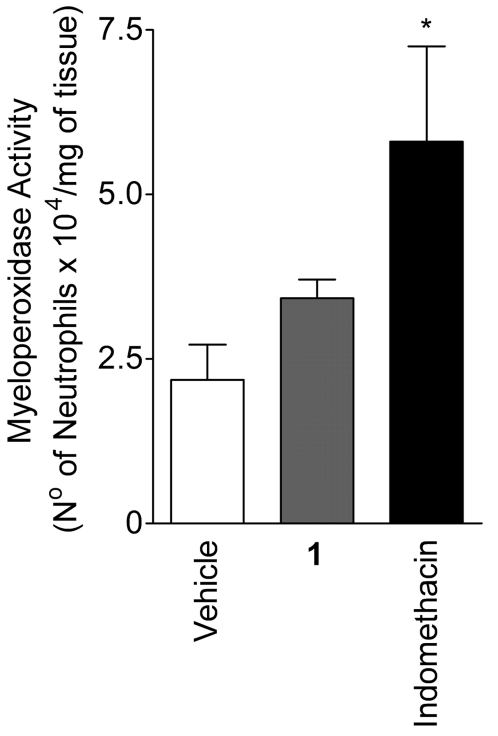

Figure 8. Effect of kaurenoic acid (**1**) on the myeloperoxidase (MPO) activity in the stomach. Mice received po treatment daily with vehicle (DMSO 2% diluted in saline or tris/HCl buffer, pH 8.0), **1** (10 mg/kg), or indomethacin (2.5 mg/kg). After seven days of treatment, mice were terminally anesthetized and samples of the stomach were collected for MPO activity measurement ($n = 8$). [$^*p < 0.05$ compared with the vehicle group (one-way ANOVA followed by Tukey's test).]

with *n*-hexane and increasing amounts of ethyl acetate as eluents (250 mL each fraction). The second fraction (0.41 g) was washed with cold methanol, to afford **1** (*ent*-kaur-16-en-19-oic acid; 800 mg, purity 96%, as determined by HPLC), exhibiting $[\alpha]^{20}{}_D$ −110, similar to a previous report,[12] EIMS *m*/*z* 325 $[M + Na]^+$ and compared by ^1H ($CDCl_3$, 400 MHz) and ^{13}C ($CDCl_3$, 100 MHz) spectroscopy with an authentic standard and literature data.[33,34]

Test Compounds. The compounds used in this study were carrageenan (FMC Corp, Philadelphia, PA, USA), L-NAME (Research Biochemicals, Natick, MA, USA), acetic acid and formaldehyde (Mallinckrodt Baker, S.A., Mexico City, Mexico), KT5823 (Calbiochem, San Diego, CA, USA), 1H-(1,2,4)-oxadiazolol-(4,3-a)-quinoxalin-1-one (ODQ) from Tocris Cookson (Baldwin, MO, USA), complete Freund's adjuvant, glibenclamide, phenyl-*p*-benzoquinone, and prostaglandin E_2 (PGE_2) from Sigma Chemical Company (St. Louis, MO, USA), and indomethacin from Prodome (Campinas, SP, Brazil).

Animals. Male Swiss mice (25−30 g), from the Universidade Estadual de Londrina, Londrina, Parana, Brazil, were used in this study. Mice were housed in standard clear plastic cages with free access to food and water, with a light/dark cycle of 12:12 h, at 21 °C. All behavioral testing was performed between 9 a.m. and 5 p.m. in a temperature-controlled room. Animal care and handling procedures were approved by the Ethics Committee of the Universidade Estadual de Londrina (protocol number 1440.2011.09). Every effort was made to minimize the number of animals used and their suffering.

Nociception Tests. Electronic Pressure-Meter Test. Mechanical hyperalgesia was tested in mice, as previously reported.[35] In a quiet room, mice were placed in acrylic cages (12 × 10 × 17 cm) with wire grid floors, 15−30 min before the start of testing. The test consisted of evoking a hindpaw flexion reflex with a hand-held force transducer (electronic anesthesiometer; Insight, Ribeirao Preto, SP, Brazil) adapted with a 0.5 mm^2 polypropylene tip. The investigator was trained to apply the tip perpendicularly to the central area of the hindpaw with a gradual increase in pressure. The end point was characterized by the removal of the paw followed by clear flinching movements. After the paw withdrawal, the intensity of the pressure was recorded automatically. The value for the response was an average of three measurements. The animals were tested before and after treatment. The results are expressed by delta (Δ) withdrawal threshold (in g) calculated by subtracting the zero-time mean measurements from the mean measurements 1, 3, or 5 h after stimulus. Withdrawal threshold was 9.1 ± 0.4 g (mean ± SEM; $n = 30$) before injection of the hyperalgesic agents (e.g., PGE_2, CFA, or carrageenin).

Writhing Response Tests. The phenyl-*p*-benzoquinone and acetic acid-induced writhing models were performed as previously described.[18] PBQ (diluted in DMSO 2%/saline, 1890 μg/kg), acetic acid (0.8% v/v, diluted in saline, 10 mL/kg), or vehicle was injected into the peritoneal cavities of mice pretreated with kaurenoic acid (**1**) (3−30 mg/kg, ip route). Each mouse was placed in a large glass cylinder, and the intensity of nociceptive behavior was quantified by counting the total number of writhes occurring between 0 and 20 min after stimulus injection. The writhing response consisted of a contraction of the abdominal muscle together with a stretching of the hind limbs. The intensity of the writhing response was expressed as the cumulative writhing score over 20 min. Different individuals administered each test, prepared the solutions to be injected, and performed the injections.

Formalin Test. The number of paw flinches and time spent licking the paws were determined between 0 and 30 min after intraplantar injection of 25 μL of formalin 1.5%, as previously described.[25,36] The period was divided in intervals of 5 min and clearly demonstrated the presence of the first and second phases, which are characteristic of the method.[25,36] Results were obtained for both the first (0−15 min) and second (20−30 min) phases.

Hot Plate Test. Mice were placed in a 10 cm wide glass cylinder on a hot plate (IITC Life Science, Inc., Woodland Hills, CA, USA) maintained at 55 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 5−9 s. The latency was also evaluated 1.5, 3.5, and 5.5 h after test compound administration. The reaction time was scored when the animal jumped or licked its paws. A maximum latency (cutoff) was set at 30 s to avoid tissue damage.[23]

Measurement of Motor Performance. In order to discard possible nonspecific muscle relaxant or sedative effects of kaurenoic acid (**1**), mice motor performance was evaluated using the rota-rod test.[30] The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into six compartments by disks 25 cm in diameter (Ugo Basile, model 7600). The bar rotated at a constant speed of 22 rotations per min. The animals were selected 24 h previously by eliminating those mice that did not remain on the bar for two consecutive periods of 120 s. Animals were treated with vehicle (Tween 80 20% in saline) or **1** (10 mg/kg, po), and testing was performed 1.5, 3.5, and 5.5 h after treatement. The cutoff time used was 120 s.

Cytokine Measurement. Mice were treated with vehicle or kaurenoic acid (**1**) (10 mg/kg, po) 30 min before carrageenin (100 μg/paw) stimulus. Three hours after the injection of carrageenin, mice were terminally anesthetized, and the skin tissues were removed from the injected and control paws (saline and naive). The samples were homogenized in 500 μL of buffer containing protease inhibitors, and IL-1β and TNF-α levels were determined as described previously[31,32] by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as picograms (pg) of cytokine/paw. As a control, the concentrations of these cytokines were determined in animals injected with saline.

Hepatotoxicity. Plasma levels of aspartate aminotransferase and alanine aminotransferase were used as indicators of hepatotoxicity.[27,28] These assays were performed using a diagnostic kit from Labtest (Lagoa Santa, Minas Gerais, Brazil).

Myeloperoxidase Activity. The neutrophil migration to the stomach tissue was evaluated by the MPO kinetic-colorimetric assay.[25] Stomach samples were collected in 50 mM K_2PO_4 buffer (pH 6.0) containing 0.5% HTAB and were homogenized using a Polytron (PT3100). After the homogenates were centrifuged at 16100*g* for 2 min, the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (SpectraMax) with three readings within 1 min. The MPO activity of the samples was compared with a standard curve of neutrophils. In these experimental conditions, MPO activity was not detected from peritoneal macrophages (data not shown). Briefly, a 10 μL sample was mixed with 200 μL of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The results were presented as the MPO activity (number of neutrophils × 10^4/mg of tissue).

Statistical Analysis. Results are presented as means ± SEM of experiments made on 5 (Figures 3A, 4−6), 6 (Figures 1 and 2, 3B), or 8 (Figures 7 and 8) animals per group and are representative of two separate experiments. Differences between groups were evaluated by analysis of variance (one-way ANOVA) followed by the Tukey's *t* test. Statistical differences were considered to be significant at $p < 0.05$.

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Notes

The authors declare no competing financial interest.

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