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Vitexin Inhibits Inflammatory Pain in Mice by Targeting TRPV1, Oxidative Stress, and Cytokines

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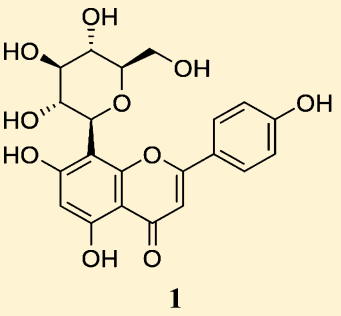
ABSTRACT: The flavonoid vitexin (**1**) is a flavone C-glycoside (apigenin-8-*C*-*β*-D-glucopyranoside) present in several medicinal and other plants. Plant extracts containing **1** are reported to possess antinociceptive, anti-inflammatory, and antioxidant activities. However, the only evidence that **1** exhibits antinociceptive activity was demonstrated in the acetic acid-induced writhing model. Therefore, the analgesic effects and mechanisms of **1** were evaluated. In the present investigation, intraperitoneal treatment with **1** dose-dependently inhibited acetic acid-induced writhing. Furthermore, treatment with **1** also inhibited pain-like behavior induced by phenyl-*p*-benzoquinone, complete Freund's adjuvant (CFA), capsaicin (an agonist of transient receptor potential vanilloid 1, TRPV1), and both phases of the formalin test. It was also observed that inhibition of carrageenan-, capsaicin-, and chronic CFA-induced mechanical and thermal hyperalgesia occurred. Regarding the antinociceptive mechanisms of **1**, it prevented the decrease of reduced glutathione levels, ferric-reducing ability potential, and free-radical scavenger ability, inhibited the production of hyperalgesic cytokines such as TNF-*α*, IL-1*β*, IL-6, and IL-33, and up-regulated the levels of the anti-hyperalgesic cytokine IL-10. These results demonstrate that **1** exhibits an analgesic effect in a variety of inflammatory pain models by targeting TRPV1 and oxidative stress and by modulating cytokine production.



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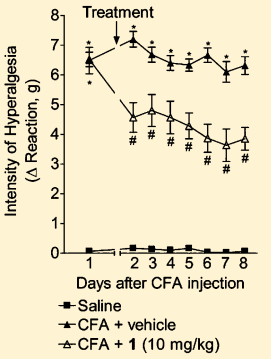

The flavonoid vitexin (**1**) is a flavone C-glycoside (apigenin-8-*C*-*β*-D-glucopyranoside)[1] found in medicinal and other plants such as *Crataegus* spp. (hawthorn), *Passiflora* spp. (passion flower), *Pennisetum glaucum* R. Br. (pearl millet), *Phyllostachys nigra* (Lodd. ex Lindl.) Munro (bamboo leaves), and *Vitex agnus-castus* L. (chasteberry or chaste tree).[2−4] There is evidence that **1** possesses anticancer, antioxidant, and anti-inflammatory effects. For instance, treatment with **1** suppresses tumor growth and induces apoptosis in a mouse model of human choriocarcinoma[5] and oral cancer OC2 cells in vitro.[6] Importantly, the antioxidant properties of **1** include scavenging cationic and anionic free radicals and ferric reducing ability.[7] Furthermore, **1** protects against myocardial ischemia/reperfusion by inhibiting the activation of the transcription factor NFκB and the consequent production of TNF-*α* and IL-1*β*.[8]

Recently, the analgesic effects of some plant extracts have been attributed to the presence of **1**.[9,10] Compound **1** inhibits the acetic acid-induced writhing response.[9,10] On the other hand, **1** did not inhibit acetic acid- and formalin-induced overt pain-like behavior at doses equivalent to its concentrations in the extract of *Pereskia bleo* DC. (Cactaceae)[11] and did not increase the thermal latency of naive mice in the hot plate test,[12] suggesting that it does not possess supraspinal analgesic effects.[13] Therefore, further evidence on the analgesic effect of **1** is necessary, and whether or not it inhibits inflammatory pain in other models and its analgesic mechanisms of action remain to be determined.

During inflammation, the sensitization of primary nociceptive neurons (nociceptors) occurs, producing an increase in pain sensation (hyperalgesia). This sensitization is caused by the direct action of inflammatory mediators such as prostaglandins (e.g., PGI_2, PGE_2) and sympathetic amines (e.g., epinephrine, dopamine) 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.[14−16] In the carrageenan model of paw inflammation, this cascade is initiated by the release of TNF-*α* and the chemokine CXCL1. These trigger the production of IL-1*β*, which, in turn, induces prostaglandin production.[14] Furthermore, TNF-*α* induces IL-6 production that contributes to prostaglandin production.[15] CXCL1 is also responsible for the stimulation of the sympathetic component of inflammatory pain.[13] Recently, it has been demonstrated that IL-33 acting on ST2 receptors contributes to carrageenan-induced inflammatory

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hyperalgesia through the production of TNF-α, CXCL1, and IL-1β.[16] On the other hand, there are anti-hyperalgesic cytokines, such as IL-10, which inhibit the action and production of pro-hyperalgesic cytokines including TNF-α and IL-1β.[15] Therefore, modulation of cytokine production is an important analgesic approach.

There is a link between cytokines and oxidative stress through many facets. For instance, TNF-α and IL-1β activate NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase), resulting in the production of superoxide anion. In turn, superoxide anion activates NFκB and the consequent production of cytokines.[1,17,18] Furthermore, TNF-α and IL-1β induce the activation and expression of cyclooxygenase-2 to produce prostanoids that sensitize the nociceptor, causing hyperalgesia.[15] Activation of NADPH oxidase by TNF-α and IL-1β is a crucial step to induce the activation and expression of cyclooxygenase-2.[19] Therefore, this intimate relationship of cytokines and oxidative stress serves to explain the importance of oxidative stress in hyperalgesia.[17,19,20]

Additionally, transient receptor potential vanilloid 1 (TRPV1) is an important ligand-gated ion channel involved in pain modulation.[21] In support of this, the flavonoid eriodictyol reduces nociceptive behavior by acting as an antagonist of the TRPV1 channel.[22] Also, nociceptors are, in part, characterized by the expression of TRPV1.[23]

In view of the information presented above, the aim of this study was to investigate the analgesic effects of **1** in overt pain-like behavior and mechanical and thermal hyperalgesia models, in addition to assessing cytokine production/release and oxidative stress caused by this compound.

RESULTS AND DISCUSSION

Vitexin (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 assessed in acetic acid- and PBQ-induced overt pain-like behavior (Figure 1). Considering that **1** at a 10 mg/kg dose inhibited 91% of an acetic acid-induced writhing response,[9] it was chosen as the highest dose to be tested together with two lower doses. Mice were treated ip with **1** (0.3−10 mg/kg; diluted in saline) 30 min before ip stimulus with 0.8% acetic acid (Figure 1A). Treatment with **1** induced a dose-dependent inhibition of an acetic acid-induced writhing response with a significant difference observed between the doses of 1 and 3 mg/kg of **1** when compared to the acetic acid group (Figure 1A). The 10 mg/kg dose was able to inhibit significantly the acetic acid-induced writhing response when compared to both the acetic acid group and the 0.3 mg/kg dose of **1** (Figure 1A). Thus, the 10 mg/kg dose was chosen for the experiments following on overt pain-like behavior. Previous data that administration of **1** by the ip route inhibited the acetic acid-induced abdominal writing response corroborate the present finding.[9] On the other hand, it was reported that **1** is not the molecule responsible for the antinociceptive activity of the *Pereskia bleo* ethyl acetate extract in the acetic acid model.[11] However, considering that 0.8% (in the present study) versus 2.0% of acetic acid was used,[11] it is likely that the different doses of stimulus explain the divergent data. In a subsequent experiment, mice were treated ip with **1** (10 mg/kg) 30 min before ip stimulus with PBQ (1890 μg/kg) (Figure 1B). The PBQ-induced writhing response was inhibited by **1**. The acetic acid and PBQ models show similarities in their nociceptive mechanisms including the participation of prostanoids, IL-33, and spinal mytogen-activated protein kinases[24−26] as well as

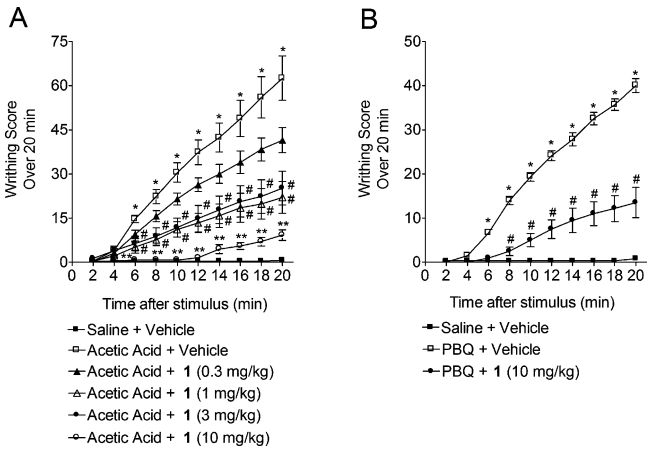

Figure 1. Vitexin (**1**) inhibits the writhing response induced by acetic acid and phenyl-*p*-benzoquinone (PBQ). Mice were treated with **1** (0.3−10 mg/kg) or vehicle (saline) 30 min before acetic acid (0.8%) (A) or PBQ (1890 μg/kg) (B) intraperitoneal stimulus. The vehicle of acetic acid was saline and the vehicle of PBQ was 2% DMSO in saline. The cumulative number of abdominal contortions (writhing score) was evaluated over 20 min and is presented at 2 min intervals. Results are provided as means ± SEM of six mice per group per experiment and are representative of two separate experiments [*p < 0.05 compared to the saline group; #p < 0.05 compared to the vehicle group and with a dose of 0.3 mg/kg of **1** (A) or compared only to the vehicle group (B); **p < 0.05 compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

model-specific mechanisms such as the participation of endothelin-1.[25] Therefore, it is reasonable that **1** inhibits mechanisms that are shared by both models of overt pain-like behavior, such as cytokine production.

Vitexin (1) Inhibits Paw Flinch and Time Spent Licking the Paw Induced by Formalin, Capsaicin, and Complete Freund's Adjuvant (CFA). Mice received **1** (Figure 1B) before the intraplantar (ipl) injection of 25 μL of formalin 1.5%. Compound **1** inhibited both phases of the formalin test regarding paw flinching (Figure 2A) and time spent licking the paw (Figure 2B). Formalin injected into the mice paw causes an overt pain-like behavior (0−30 min) characterized by a first phase (0−5 min), which depends on neurotransmitters such as serotonin, molecules from resident cells such as histamine, and activation of TRPA1 (transient receptor potential ankyrin 1) receptors expressed by neurons, and a second phase (10−30 min) that depends on inflammatory mediators produced in response to formalin stimulus including cytokines.[15,27] Similar to the acetic acid model, previous work did not show significant activity of **1** in the formalin test,[11] and the likely explanation is that a lower intensity of stimulus and higher dose of treatment were used in the present study than in the earlier investigation.[11] The analgesic effect of **1** was also evaluated in a capsaicin (TRPV1 agonist)-induced pain-like behavior model (Figure 2C and D). Mice received **1** (Figure 1B) before an ipl capsaicin (1.6 μg/paw) injection.[28] The paw flinches (Figure 2C) and the time spent licking (Figure 2D) induced by capsaicin were significantly inhibited by **1**. Capsaicin activates TRPV1 channels to induce nociception,[23] and together with the evidence that some flavonoids such as eriodictyol and baicalin act as an antagonist of the TRPV1 receptor[22] and reduce the expression and function of TRPV1,[29] respectively, it may be suggested that targeting TRPV1 channel activity is a contributing factor to the antinociceptive mechanism of **1**. Furthermore, TRPV1-induced

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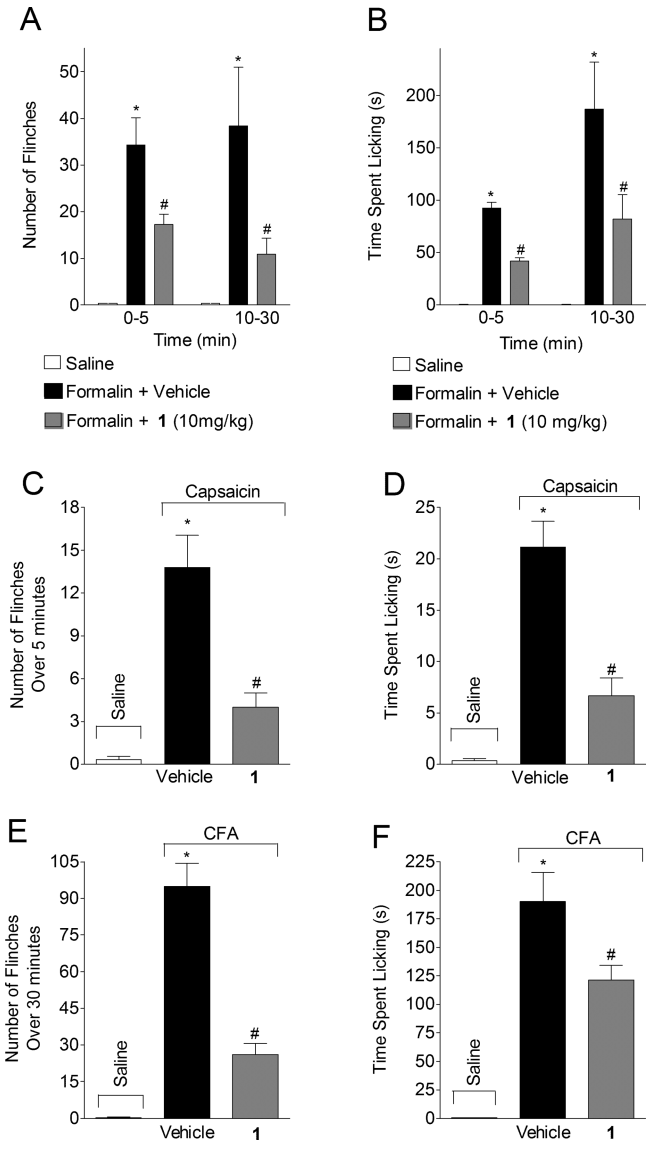

Figure 2. Vitexin (**1**) inhibits paw flinch and time spent licking the paw induced by formalin, capsaicin, and complete Freund's adjuvant (CFA). Mice were treated with **1** (10 mg/kg, ip, 30 min) or vehicle before the injection of formalin (25 μL of 1.5% formalin in saline, A and B), capsaicin (1.6 μg/paw, TRPV1 agonist, C and D), or CFA (10 μL/paw, E and F). The total number of flinches (A, C, and E) and time spent licking the paw (B, D, and F) were evaluated for 5 min during the capsaicin test and for 30 min during the formalin and CFA tests. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

calcium influx together with nociceptor activation by inflammatory mediators accounts for the activation of TRPA1 during inflammation;[30] so this could be a contributing mechanism to explain the inhibition of the first phase of formalin, which is TRPA1-dependent.[31] Therefore, the modulation of TRP channels may account for the analgesic effect of **1**. In complete Freund's adjuvant (10 μL/paw)-induced overt pain-like behavior, pretreatment with **1** inhibited both paw flinching (Figure 2E) and licking behavior (Figure 2F).

Vitexin (1) Inhibits Carrageenan-, Capsaicin-, and CFA-Induced Mechanical and Thermal Hyperalgesia. The antinociceptive effect of **1** was tested on carrageenan (300 μg/paw)-, capsaicin (dose as in Figure 2C)-, and CFA (10 μL/paw)-induced mechanical and thermal hyperalgesia models. Treatment with **1** (1−10 mg/kg, ip) inhibited carrageenan-induced mechanical (Figure 3A) and thermal (Figure 3B) hyperalgesia in a dose-dependent manner. Doses of 1 and 3 mg/kg of compound **1** inhibited carrageenan-induced mechanical hyperalgesia at 3 h after the stimulus, while a dose of 10 mg/kg inhibited carrageenan-induced mechanical hyperalgesia between 1 and 5 h after the stimulus, with significant differences compared to the 1 and 3 mg/kg dose levels at 5 h after the stimulus (Figure 3A). In the hot plate test, carrageenan-induced thermal hyperalgesia was abolished by the 3 and 10 mg/kg doses of compound **1** between 1 and 5 h after the stimulus, with significant differences when compared to the 1 mg/kg dose, at which no effect was observed (Figure 3B). Therefore, the 10 mg/kg dose of **1** was selected for subsequent experiments. Treatment with **1** inhibited the capsaicin (1.6 μg/paw)-induced mechanical hyperalgesia 1−5 h after the stimulus (Figure 3C) and thermal hyperalgesia 1−3 h after stimulus, without differences at 5 h after the stimulus (Figure 3D). In another experimental setting, mice were treated daily with **1** (10 mg/kg, ip) starting 24 h after CFA ipl injection for seven days for assessment of mechanical and thermal hyperalgesia (Figure 3E and F, respectively). There was significant inhibition of both CFA-induced mechanical (Figure 3E) and thermal (Figure 3F) hyperalgesia at all time points evaluated for treatment with **1** (2−8 days after stimulus). These results indicate the potential therapeutic use of vitexin in conditions associated with chronic inflammatory pain.

It has been observed that carrageenan- and CFA-induced mechanical and thermal hyperalgesia were reduced by TRPV1 antagonists or in TRPV1-deficient mice,[32−34] and CFA-induced increase of discharges of wide dynamic range neurons in response to thermal noxious stimulus were inhibited by TRPV1 antagonists.[35] Therefore, TRPV1 mediates mechanical and thermal hyperalgesia induced by carrageenan and CFA, and considering the results of Figures 2C,D and 3C,D, it is likely that inhibition of TRPV1-mediated pain is a contributing mechanism of action of **1**.

Furthermore, the hyperalgesia induced by carrageenan and CFA depends on the production of cytokines and oxidative stress.[14−17,20,36,37] Capsaicin can also activate TRPV1 to induce the production of cytokines including IL-6 in conditions not related to pain[38] as well as induce the expression of TNF receptor I (p55) in a reactive oxygen species-dependent manner in dorsal root ganglia neurons, which might be a mechanism of nociceptor sensitization.[39] Moreover, the thermal hyperalgesia induced by the reactive oxygen species hydrogen peroxide was diminished in TRPV1-deficient mice.[40] Therefore, it was considered reasonable to investigate whether the analgesic effect of **1** might depend on the inhibition of oxidative stress and cytokine production.

Vitexin (1) Prevents the Decrease in Reduced Glutathione (GSH) Concentrations, Ferric Reducing Ability Potential (FRAP), and Ability to Scavenge 2,2′-Azinobis(3-ethylbenzothiazoline 6-sulfonic acid) (ABTS) Assays Induced by Carrageenan. GSH levels, and FRAP and ABTS assays in paw tissue were assessed to determinate the antioxidant activity of **1** (Figure 4). Mice were treated with **1** (10 mg/kg, ip, 30 min) before the ipl injection of carrageenan (300 μg/paw), and after an additional 3 h, samples of cutaneous plantar tissue were collected for GSH concentration, ferric-reducing ability potential (FRAP assay), and free-radical scavenging ability (ABTS) assay assessment. Carrageenan

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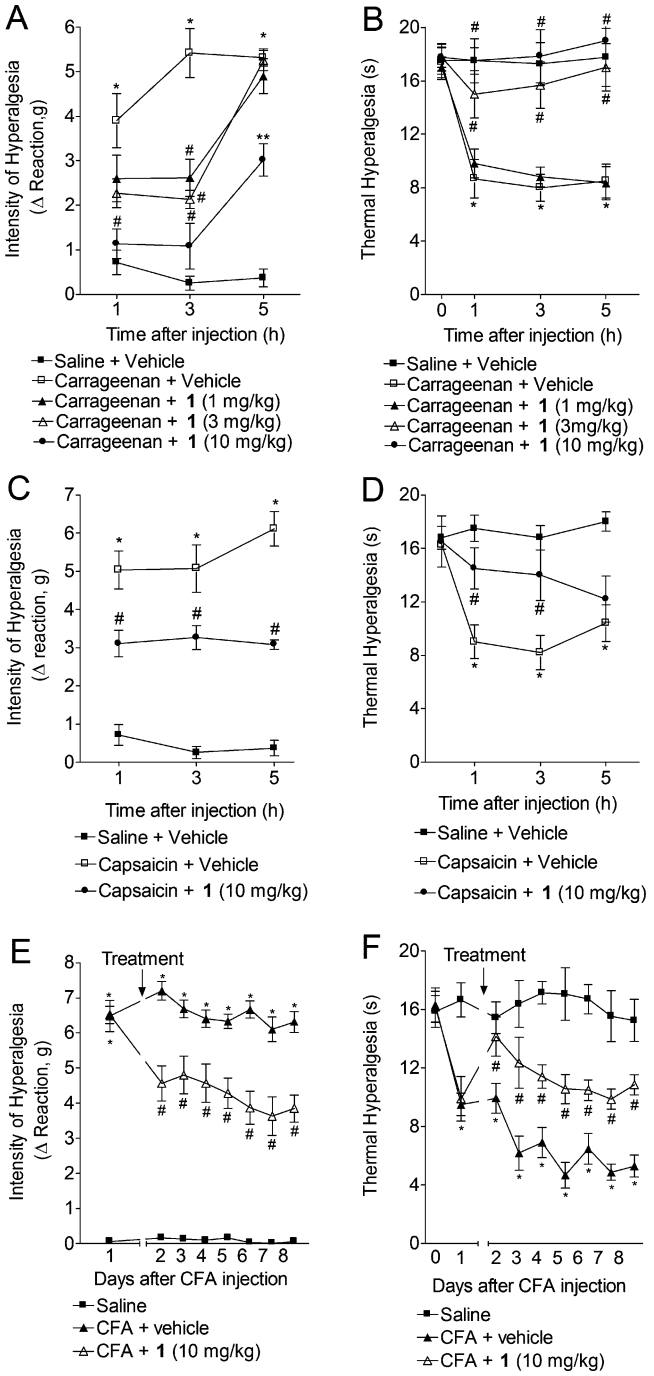

Figure 3. Treatment with vitexin (**1**) inhibits carrageenan-, capsaicin-, and CFA-induced mechanical and thermal hyperalgesia. Mice were treated with **1** (1−10 mg/kg, ip, 30 min) or vehicle before a carrageenan (300 μg/paw) injection (A and B). In another groups, mice were treated with **1** (10 mg/kg, ip, 30 min) or vehicle before a capsaicin (1.6 μg/paw, TRPV1 agonist) injection (C and D) or daily with **1** (10 mg/kg, ip) starting at 24 h after CFA (10 μL/paw) injection (E and F). The intensity of mechanical and thermal hyperalgesia was measured at 1−5 h (A−D) or every 24 h (1−8 days) (E and F) by an electronic pressure-meter test and using a hot plate, respectively. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared to the saline group; $^{\#}p < 0.05$ compared to the carrageenan group; $^{**}p < 0.05$ compared to the carrageenan group and with the doses of 1 and 3 mg/kg of **1** groups (one-way ANOVA followed by Tukey's test)].

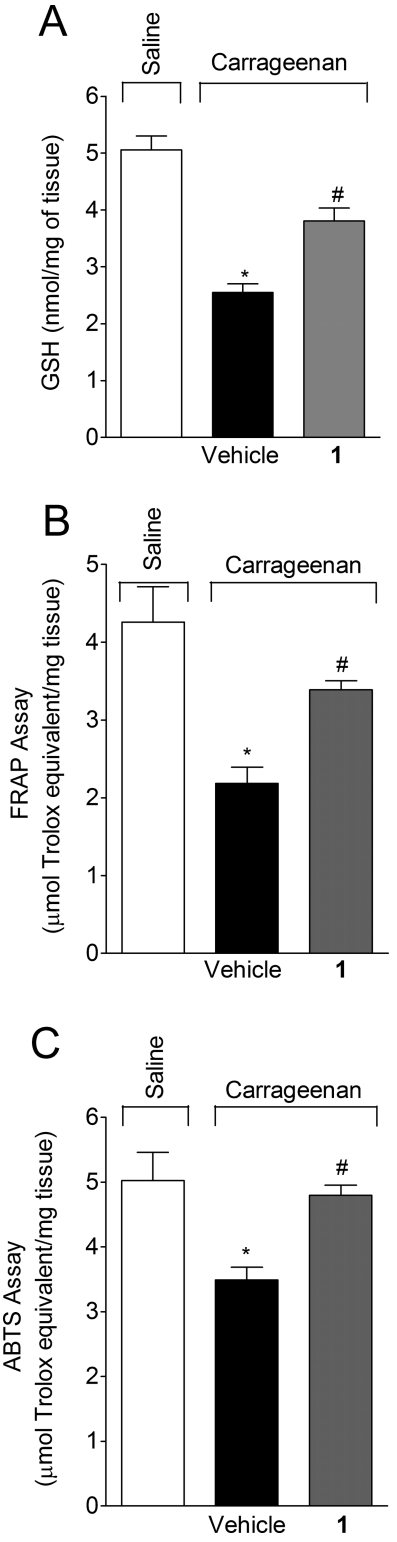

Figure 4. Vitexin (**1**) prevents the carrageenan-induced decrease of reduced glutathione (GSH) levels, ferric-reducing ability potential (FRAP assay), and free-radical scavenging ability (ABTS assay). Mice were treated with **1** (10 mg/kg, ip, 30 min) or vehicle before a carrageenan or saline injection. Samples of subcutaneous plantar tissue were collected 3 h after the stimulus and were processed for GSH (A), FRAP (B), and ABTS (C) assays. Samples of four (GSH) or two (FRAP and ABTS) mice were pooled for processing, and the measurements made in three pools per experiment are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, and $^{\#}p < 0.05$

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Figure 4. continued

compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

reduced the endogenous GSH skin levels (Figure 4A), ferric reducing ability potential (FRAP assay; Figure 4B), and free radical scavenging ability (ABTS assay; Figure 4C), and treatment with **1** prevented these reductions of antioxidant defenses in the inflammatory foci, demonstrating its in vivo antioxidant effect, which is an analgesic mechanism.[20,37]

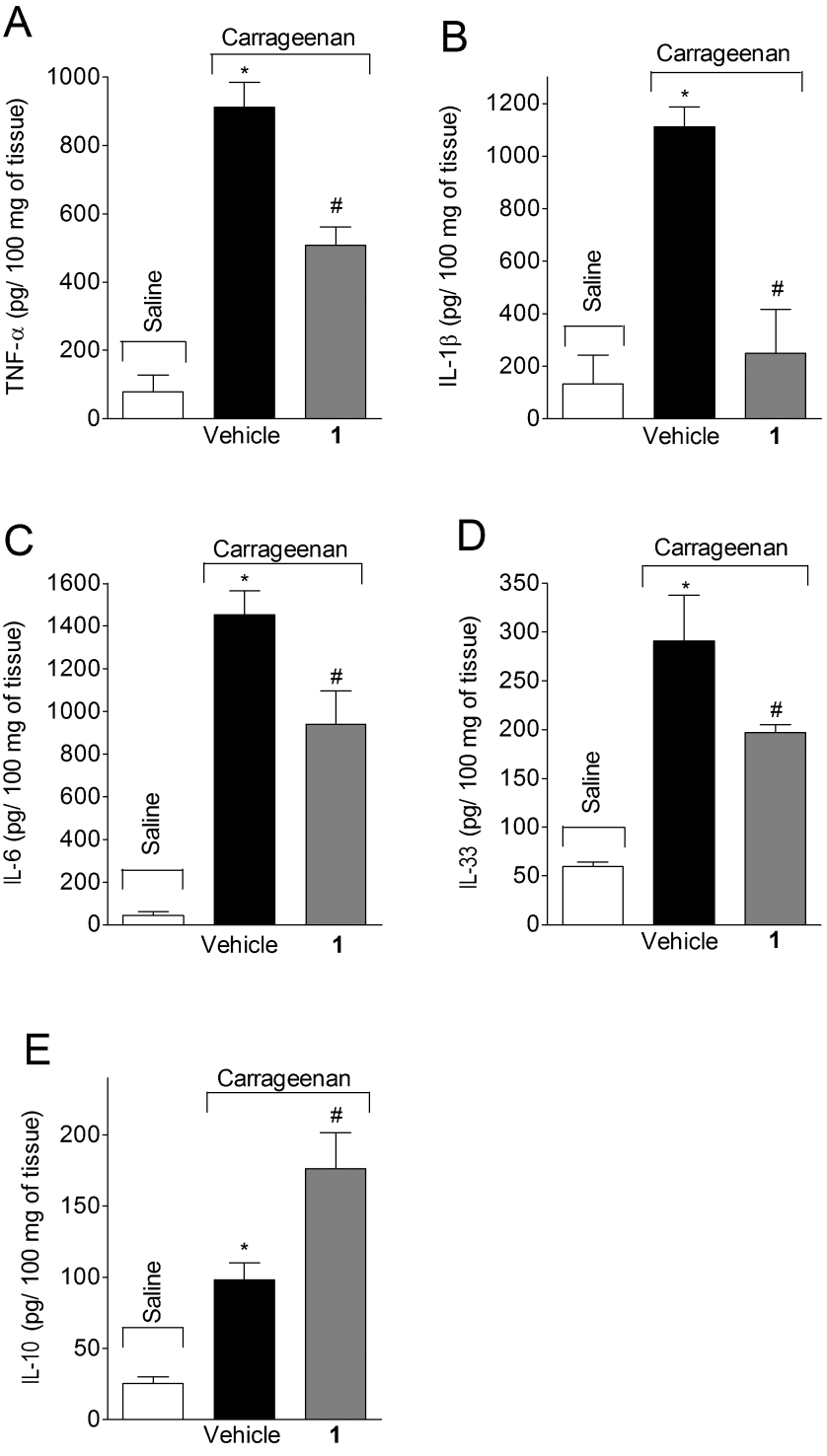

Figure 5. Vitexin (**1**) reduces TNF-α, IL-1β, IL-6, and IL-33 and enhances IL-10 production in carrageenan paw inflammation. Mice were treated with **1** (10 mg/kg, ip, 30 min) or vehicle before the ipl injection of carrageenan. Three hours after a carrageenan injection, mice were sacrificed and paw skin samples were collected for the determination of TNF-α (A), IL-1β (B), IL-6 (C), IL-33 (D), and IL-10 (E) levels. Results are presented as means ± SEM of six mice per group per experiment and are representative of two separate experiments [$^{*}p < 0.05$ compared with the saline group, and $^{\#}p < 0.05$ compared to the vehicle group (one-way ANOVA followed by Tukey's test)].

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Vitexin (1) Inhibits Pro-inflammatory Cytokine (TNF-α, IL-1β, IL-6, and IL-33) and Enhances Anti-inflammatory Cytokine (IL-10) Production Induced by Carrageenan. Inflammatory hyperalgesia induced by carrageenan is modulated by cytokines. There are pro-hyperalgesic cytokines, including TNF-α, IL-1β, IL-6, and IL-33, and anti-hyperalgesic cytokines such as IL-10.[14−16,25,41] In this context, the effect of **1** in cytokine production was evaluated. Mice received **1** (as in Figure 4) before the administration of a carrageenan stimulus, and after an additional 3 h, samples of cutaneous plantar tissue were collected for cytokine (TNF-α, IL-1β, IL-6, IL-33, and IL-10) level determination (Figure 5). Compound **1** inhibited carrageenan-induced production of TNF-α (Figure 5A), IL-1β (Figure 5B), IL-6 (Figure 5C), and IL-33 (Figure 5D). Moreover, treatment with **1** increased the production of the anti-hyperalgesic cytokine IL-10 (Figure 5E). There is evidence that **1** inhibits cytokine production in a model of ischemia/reperfusion-induced increase of myocardial TNF-α and IL-1β in an ex vivo setting.[8] To the best of our knowledge, the present study is the first to demonstrate that **1** inhibits the production of other pro-hyperalgesic cytokines such as IL-6 and IL-33 in addition to TNF-α and IL-1β and increases the production of anti-hyperalgesic IL-10 and also that this modulatory effect on cytokine production is one of its analgesic mechanisms.

Cytokines are also closely related to oxidative stress. For instance, TNF-α and IL-1β activate NADPH oxidase, inducing the production of superoxide anion, starting the generation of reactive oxygen species.[17,18,37] In turn, superoxide anion can induce cytokine production by activation of NFκB.[1,37] This relationship adds to the explanations on the role of oxidative stress as a contributing factor to inflammatory hyperalgesia together with inflammatory cytokines.[37]

Vitexin (1) Does Not Produce Muscle-Relaxation or Sedative Effects. Intraperitoneal treatment with 10 mg/kg of **1** did not alter the motor response of the animals 1.5, 3.5, and 5.5 h after treatment (n = 6). These time points were based on the 30 min pretreatment plus 1, 3, and 5 h until mechanical and thermal hyperalgesia measurement in the carrageenan model (Figure 3A and B). The responses of the control group (nontreated naive animals) and animals treated by **1** in the rota-rod test were 180 s vs 180 s (1.5 h), 180 s vs 180 s (3.5 h), and 180 s vs 180 s (5.5 h), respectively. These results support the hypothesis that **1** reduces inflammatory pain and not that the mice are incapable of responding because of muscle-relaxing or sedative effects.[42]

Vitexin (1) Does Not Induce Liver Damage or Stomach Lesions. With the objective of evaluating whether **1** induces liver damage or stomach lesions, plasma levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and myeloperoxidase (MPO) activity in gastric mucosa, were measured, respectively. For both experimental procedures, the same experimental protocol was used. Mice were treated daily ip with vehicle [saline or tris(2-amino-2-hydroxymethylpropan-1,3-diol)/HCl buffer, pH 8.0, with the data for the vehicles pooled due to no difference between the vehicles], **1** (10 mg/kg, ip), or indomethacin (positive control, 2.5 mg/kg, ip, diluted in tris/HCl buffer) for 7 days.[42] Vehicle and animals treated with **1** exhibited similar plasma AST and ALT levels (data not shown), indicating that the compound does not induce liver damage. On the other hand, indomethacin (a nonselective cyclooxygenase inhibitor) treatment induced significant increases in AST and ALT plasma levels (data not shown), in a similar manner to previous reports.[33,42]

Although in the present study the analgesic effect of **1** was evaluated using ip treatment, it was felt important to determine whether the test compound induces gastric lesions, because these are related to inhibition of cyclooxygenase-1 and not to a direct irritant effect of the compound on the gastric mucosa when orally administrared.[43] Importantly, most anti-inflammatory drugs are used in a seven-day treatment schedule,[44] and, in this period, **1** did not induce alteration of myeloperoxidase activity in stomach samples compared to the vehicle-treated group, indicating that no gastric lesions occurred (data not shown). On the other hand, indomethacin induced a significant increase of stomach myeloperoxidase activity, indicating gastric lesions (data not shown).[42,44] Thus, compound **1** was found to be safe regarding liver damage and gastric mucosa injuries, as assessed over a seven-day period of treatment, using mice. In agreement, it was shown following the OECD guideline number 420 that **1** did not induce signs of acute toxicity over a period of 14 days in rats and mice at 2000 mg/kg dose.[45]

In conclusion, the present study has demonstrated the analgesic effect of vitexin (**1**) in a variety of inflammatory pain models as well as the possible mechanisms of action involved. The antinociceptive action of **1** may depend on (a) targeting TRPV1 channel activity as observed by inhibition of capsaicin (a TRPV1 agonist)-induced nociceptive responses; (b) antioxidant activity as observed by prevention of inflammation-induced depletion of GSH, ferric reducing ability potential (FRAP assay), and free radical scavenger ability (ABTS assay); and (c) modulation of cytokine production with concomitant inhibition of pro-hyperalgesic (TNF-α, IL-1β, IL-6, and IL-33) and stimulation of anti-hyperalgesic (IL-10) cytokine production. Therefore, the promising analgesic activity of vitexin (**1**) indicates that it merits further preclinical and possible clinical investigation.

EXPERIMENTAL SECTION

General Experimental Procedures. During the experiments, mice received intraperitoneal (ip, 1, 3, and 10 mg/kg) treatment with vitexin (**1**) or vehicle (saline) 30 min before or 24 h after inflammatory stimulus as indicated. The doses of inflammatory stimuli were determined previously in our laboratory in pilot studies and are based on previous work.[14−16,20,24,44] The writhing response was evaluated for 20 min after ip injection of acetic acid (0.8%) or phenyl-*p*-benzoquinone (1890 μg/kg). 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 and for 5 min after capsaicin injection (1.6 μg/paw). Mechanical and thermal hyperalgesia were evaluated 1−5 h after carrageenan (300 μg/paw) or capsaicin (1.6 μg/paw) treatment or 1−8 days after CFA (10 μL/paw) stimulus. All inflammatory stimuli induced only ipsilateral (in the paw in which the stimulus was injected) mechanical and thermal hyperalgesia. GSH, FRAP, ABTS, TNFα, IL-1β, IL-6, IL-33, and IL-10 levels were evaluated 3 h after carrageenan (300 μg/paw) injection. The plasma levels of AST, ALT, and MPO activity in stomach samples were determined in mice treated ip daily with vehicle, **1** (10 mg/kg), or indomethacin (2.5 mg/kg) for 7 days. Different individuals prepared the solutions to be injected, performed the injections, and performed the measurements.

Test Compounds. The compounds used in this study were saline (NaCl 0,9%; Fresenius Kabi Brasil Ltd.a. Aquiraz, CE, Brazil), dimethyl sulfoxide (DMSO), vitexin (**1**; purity ≥95% as determined by HPLC), capsaicin, complete Freund's adjuvant, and phenyl-*p*-benzoquinone (Sigma-Aldrich, St. Louis, MO, USA), carrageenan (Santa Cruz Biotechnology, Santa Cruz, CA, USA), acetic acid and formaldehyde (Mallinckrodt Baker, S.A., Mexico City, Mexico), and indomethacin (Prodome, Campinas, SP, Brazil).

Animals. Male Swiss mice (25−30 g), from the Universidade Estadual de Londrina, Londrina, Paraná, Brazil, were used in this study.

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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 in accordance with the International Association for Study of Pain (IASP) guidelines and approved by the Ethics Committee of the Universidade Estadual de Londrina (process number 32813.2012.03). Every effort was made to minimize the number of animals used and their suffering.

Nociception Tests. Writhing Response Tests. The PBQ- and acetic acid-induced writhing models were performed as previously described.[25] PBQ (DMSO 2% diluted in 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. 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.

Formalin Test. The number of paw flinches and the time spent licking the paws were determined between 0 and 30 min after intraplantar (ipl) injection of 25 μL of formalin 1.5%, as previously described.[20,27] 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.[20,27] Results were obtained for both the first (0−5 min) and second (10−30 min) phases.

Complete Freund's Adjuvant Test. The number of paw flinches and time spent licking the stimulated paw were determined between 0 and 30 min after ipl injection of 10 μL of CFA. Results were expressed by the total number of flinches and licks performed in 30 min.[42]

Capsaicin Test. The procedure used was similar to that described previously.[28] After an adaptation period, capsaicin (1.6 μg/paw) was injected ipl. Animals were observed individually for 5 min following capsaicin injection. The amount of time spent licking and flinches in the injected paw were considered as indicative of nociception.

Electronic Pressure-Meter Test. Mechanical hyperalgesia was tested in mice, as previously reported.[46] 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 9.0 ± 0.4 g (mean ± SEM; n = 30) before injection of the hyperalgesic agents carrageenan (300 μg/paw), capsaicin (1.6 μg/paw), and CFA (10 μL/paw).

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 approximately 15 s. The latency was also evaluated 1, 3, and 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.[47] The inflammatory stimuli were carrageenan (300 μg/paw), capsaicin (1.6 μg/paw), and CFA (10 μL/paw).

Reduced Glutathione Assay. Mice were treated with vehicle or vitexin (**1**) (10 mg/kg, ip) 30 min before carrageenan (300 μg/paw) stimulus. Three hours after the stimulus injection, the levels of paw GSH (pool of four paw samples) were determined using a spectrophotometric method.[37] Samples were homogenized (IKA T10) in 4 mL of 0.02 M EDTA. Homogenates (2.5 mL) were treated with 2 mL of H_2O Milli Q plus 0.5 mL of trichloroacetic acid 50%. After 15 min, the homogenates were centrifuged at 1500g for 15 min, and 1 mL from the supernatant was added to 2 mL of a solution containing Tris 0.4 M (pH 8.9) plus 50 μL of DTNB. After 5 min, the measurements were performed in 412 nm against a blank control [UV−vis spectrophotometer (UV-1650, Shimadzu)]. The results were presented as nmol of GSH per miligram of tissue.

Ferric-Reducing Ability Potential and Free-Radical Scavenging Ability Assays. A paw skin sample was collected, immediately homogenized with 500 μL of 1.15% KCl, and centrifuged (10 min × 200g × 4 °C). The ability of the sample to resist oxidative damage was determined as ferric-reducing ability using the FRAP assay and as free-radical scavenging ability using the ABTS assay.[37] For the FRAP assay, 50 μL of supernatant was mixed with 150 μL of deionized water and 1.5 mL of freshly prepared FRAP reagent. The reaction mixture was incubated at 37 °C for 30 min, and the absorbance was measured at 595 nm. For the ABTS assay, the ABTS solution was diluted with phosphate buffer saline at pH 7.4 to an absorbance of 0.80 at 730 nm. Then, 1.0 mL of diluted ABTS solution was mixed with 20 μL of supernatant. After 6 min, the absorbance was measured at 730 nm. The results were equated against a Trolox standard curve (1.5−30 μmol/L, final concentrations). The results are expressed as Trolox equivalents per milligram of tissue weight in both assays.

Cytokine Measurement. Mice were treated with vehicle or **1** (10 mg/kg, ip) 30 min before carrageenan (300 μg/paw) stimulus. Three hours after the injection of carrageenan, mice were euthanized, and the skin tissues were removed from the injected and control paws (saline). The samples were homogenized in 500 μL of buffer containing protease inhibitors, and TNF-α, IL-1β, IL-6, IL-33, and IL-10 levels were determined as described previously[48,49] by an enzyme-linked immunosorbent assay (ELISA) using eBioscience kits. The results are expressed as picograms (pg) of cytokine/100 mg of tissue. As a control, the concentrations of these cytokines were determined in animals injected with saline.

Measurement of Motor Performance. In order to discard possible nonspecific muscle-relaxant or sedative effects of **1**, mice motor performance was evaluated using the rota-rod test.[44] The apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four 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 180 s. Animals were treated with vehicle (DMSO 2% in saline) or **1** (10 mg/kg, ip), and testing was performed 1.5, 3.5, and 5.5 h after treatment. The cutoff time used was 180 s.

Hepatotoxicity. Plasma levels of AST and ALT were used as indicators of hepatotoxicity.[42] 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.[45,50,51] 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 16100g for 2 min, the resulting supernatant was assayed spectrophotometrically for MPO activity determination at 450 nm (Spectra max), 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 measurements made on six mice in each group per experiment and are representative of two separate experiments. Two-way analysis of variance (ANOVA) was used to compare the groups and doses at all times (curves) when the hyperalgesic responses were measured at different times after the administration or enforcement of the stimuli. The factors analyzed were treatment, time, and time versus treatment

interaction. When there was a significant time versus treatment interaction, one-way ANOVA followed by Tukey's *t* test was performed on each occasion. On the other hand, when the hyperalgesic responses were measured once after the administration or enforcement of the stimuli, the differences between responses were evaluated by one-way ANOVA followed by Tukey's *t* test. Additionally, comparative statistical analysis between two groups were performed using the *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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