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Anti-allodynic property of flavonoid myricitrin in models of persistent inflammatory and neuropathic pain in mice

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ABSTRACT

The aim of the present study was to investigate the effects of myricitrin, a flavonoid with anti-inflammatory and antinociceptive action, upon persistent neuropathic and inflammatory pain. The neuropathic pain was caused by a partial ligation (2/3) of the sciatic nerve and the inflammatory pain was induced by an intraplantar (i.pl.) injection of 20 μL of complete Freund's adjuvant (CFA) in adult Swiss mice (25–35 g). Seven days after sciatic nerve constriction and 24 h after CFA i.pl. injection, mouse pain threshold was evaluated through tactile allodynia, using Von Frey Hair (VFH) filaments. Further analyses performed in CFA-injected mice were paw edema measurement, leukocytes infiltration, morphological changes and myeloperoxidase (MPO) enzyme activity. The intraperitoneal (i.p.) treatment with myricitrin (30 mg/kg) significantly decreased the paw withdrawal response in persistent neuropathic and inflammatory pain and decreased mouse paw edema. CFA injection increased 4-fold MPO activity and 27-fold the number of neutrophils in the mouse paw after 24 h. Myricitrin strongly reduced MPO activity, returning to basal levels; however, it did not reduce neutrophils migration. In addition, myricitrin treatment decreased morphological alterations to the epidermis and dermis papilar of mouse paw. Together these results indicate that myricitrin produces pronounced anti-allodynic and anti-edematogenic effects in two models of chronic pain in mice. Considering that few drugs are currently available for the treatment of chronic pain, the present results indicate that myricitrin might be potentially interesting in the development of new clinically relevant drugs for the management of this disorder.

1. Introduction

The pathological pain (clinical pain) differs from nociceptive pain (physiological pain) on the period of occurrence, threshold for stimulation and plastic alterations on the tissue. Pathological pain is generally associated with inflammation of peripheral tissue that arises from the initial damage (inflammatory pain) or from lesions to the nervous system (neuropathic pain) [1–3].

The neuropathic pain is usually difficult to treat because the etiology is heterogeneous and the underlying pathophysiology

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is complex. In addition, currently available drugs which provide relief of neuropathic and inflammatory pain are effective only in a fraction of such patients. In general, these drugs present low efficacy and numerous side effects [1,4]. Because no universally efficacious therapy for it exists, neuropathic pain research has been explored with different animal models where intentional damage is done to the sciatic nerve, branches of spinal nerves or in the spinal cord [2,3,5,6]. In animal models of neuropathic pain, the extent of hyperalgesia is related to the extent of the inflammatory response at the site of injury [7] and anti-inflammatory agents alleviate hyperalgesia in nerve-injured rats [7,8].

In this context, drugs that decrease the inflammatory condition could be successful applied in certain chronic pain states. Taking this into account, several works have described the powerful anti-inflammatory activity of flavonoids [9–12]. These compounds are broadly distributed in higher plants and known by their antioxidant; anti-inflammatory; immunomodulatory; anti-diabetic; anti-allergic; anti-cancer; hepatoprotective; neuroprotective; antinociceptive and anti-rheumatic properties [9–14].

We have previously demonstrated that myricitrin, a flavonoid that belongs to the flavonol sub-group, inhibits the nociceptive response in models of acute pain [14]. The effects of myricitrin have been attributed, mainly, to the inhibition of PI 3-kinase and PKC activities, NO production, nitric oxide synthase (iNOS) over expression and NF-κB activation [14–17]. More recently, myricitrin was found to cause a potent inhibition of calcium transport *in vitro*, additional to its *in vivo* effects (unpublished data).

Regarding these previous findings, we might suggest that myricitrin is a good candidate for the relief of both neuropathic and inflammatory pain. On the other hand, chronic pain differs substantially from acute pain in terms of its persistence and in relation to adaptive changes [1,2,18]. Taken together, these pieces of evidence provide the rationale for research into the effects of myricitrin on chronic pain. Therefore, the present study was designed to investigate the antinociceptive effects of myricitrin in models of neuropathic and inflammatory chronic pain. The neuropathic pain was induced by a partial constriction of the sciatic nerve and the inflammatory pain was induced by CFA injection. CFA consists of heat-killed mycobacteria suspended in a mineral oil vehicle, which produces a chronic inflammatory condition in rodents [19]. The mouse pain threshold was evaluated through tactile allodynia, using Von Frey Hair (VFH) filaments, and further analyses performed included paw edema measurement, leukocytes infiltration, morphological changes and MPO enzyme activity in the injured paw.

2. Materials and methods

2.1. Animals

Adult female Swiss mice (25–35 g) were kept in a temperature-controlled room (23 ± 2 °C) on a 12 h light–dark cycle. Food and water were freely available. The experiments reported were carried out in accordance with the current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified [20]. All experiments were approved by the institutional Ethics Committee for animal use. The numbers of animals and intensities of noxious stimuli used were the minimum necessary to demonstrate consistent effects of the drug treatments.

2.2. Mechanical allodynia induced by partial sciatic nerve injury

Mice were anesthetized with 7% chloral hydrate (6 mL/kg, i.p.). Then, a partial ligation of the sciatic nerve was performed by tying the distal third of the sciatic nerve, according to the procedure described in mice [5] and rats [6]. In sham-operated mice, the nerve was exposed using the same surgical procedure, but without ligation. Mice with ligated nerves did not present paw drooping or autotomy.

The mechanical allodynia was measured as described before [21], as the withdrawal response frequency to 10 applications of 0.6 g Von Frey Hair Filaments (VFH; Stoelting, Chicago, USA). To this end, mice were further habituated in individual clear Plexiglas boxes (9 cm × 7 cm × 11 cm) on an elevated wire mesh platform to allow access to the ventral surface of the hind paws. The frequency of withdrawal was determined before nerve injury (baseline), in order to obtain data purely derived from nerve injury-induced allodynia. The operated mice received myricitrin (30 mg/kg, i.p.) or vehicle 7 days after surgery. The withdrawal response frequency was recorded immediately before (0) and after (0.5, 2, 4, 6 and 24 h) treatment.

2.3. CFA-induced inflammation and mechanical allodynia

Mice were lightly anaesthetized with ether and received 20 μL of complete Freund's adjuvant (CFA; 1 mg/mL of heat killed *Mycobacterium tuberculosis* in 85% paraffin oil and 15% mannide monoleate) subcutaneously in the plantar surface of the right hind paw (ipsilateral paw).

Twenty-four hours after CFA injection the mice were treated with myricitrin (30 mg/kg, i.p.) or vehicle. Effects were evaluated against paw edema and mechanical allodynia. Paw edema was measured by use of a plethysmometer (Ugo Basile) at several time-points (0, 0.5, 2, 4 and 8 h) and was expressed (μL) as the difference between paw volume before (baseline) and subsequent to CFA injection, the difference indicating the degree of inflammation.

The mechanical allodynia was measured as described in Section 2.2. The frequency of withdrawal was determined before CFA injection (baseline), in order to obtain data purely derived from the treatments. The mechanical allodynia was examined immediately before (0) and after (0.5, 2, 4 and 8 h) myricitrin treatment.

2.4. Myeloperoxidase assay

The neutrophil infiltration and activation was evaluated indirectly by measuring the myeloperoxidase (MPO) activity, as previously described [22]. The experiments were carried out 24 h after CFA i.pl. injection. The animals were subdivided into

four groups ($n = 5$ per group) as follows: (1) vehicle (saline) i.p. plus PBS i.pl.; (2) vehicle (saline) i.p. plus CFA i.pl.; (3) and (4) myricitrin (30 mg/kg) i.p. plus CFA i.pl. Groups 1–3 were pretreated with vehicle or myricitrin i.p., 30 min before PBS or CFA i.pl. injection, while group 4 received myricitrin i.p. 22 h after CFA i.pl. injection.

Twenty-four hours after CFA injection the animals were killed and the subcutaneous tissue of the injected footpad was removed and placed in an Eppendorf tube containing 0.75 mL of 80 mM sodium phosphate buffer (pH 5.4) and 0.5% hexadecyltrimethyl ammonium (HTAB). Enzyme assay was then carried out as described [22]. The reaction product was determined colorimetrically using an ultra microplate reader (absorbance 652 nm), with a molar absorption coefficient of 3.9×10^4 for 3,3′,5,5′-tetramethylbenzidine (TMB) salt.

2.5. Histopathological analysis and stereology

In the histopathological analysis animals were subdivided into four groups ($n = 5$ per group). The schedule of treatment was the same as that for the MPO assay described above. Twenty-four hours after CFA or PBS i.pl. injection, the mice were killed and the injected paw was cut longitudinally into equal halves. The lateral half was fixed in 4% paraformaldehyde and the medial half was fixed in Zenker solution ($HgCl_2$ plus $K_2Cr_2O_7$) for 24 h. The tissues were rinsed, dehydrated and embedded in paraffin. Tissue blocks were then sectioned at 5 μm thickness using a rotary microtome. The distal half was stained with hematoxylin–eosin and observed by light microscopy using a 40× objective to examine morphological alterations. The medial half was stained by May–Grünwald–Giemsa and a representative area of inflammatory cellular response (reticular dermal and hypodermal layers) was selected for quantitative cell analysis, using a light microscope (100× objective) coupled to a camera. The number of neutrophils, eosinophils (polymorphonuclears), lymphocytes, macrophages, and mast cells was quantified in 20 fields using the cycloid test system, as described [23,24]. The results are expressed as the mean ± S.E.M. of the total number of cells in an area of 1 mm^2.

2.6. Drugs

The following substances were used: hexadecyltrimethyl ammonium bromide (HTAB), complete Freund's adjuvant (CFA), 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, St. Louis, USA); chloral hydrate, dimethylformamide and stain reagents May–Grünwald–Giemsa and hematoxylin–eosin were purchased from Vetec (Rio de Janeiro, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Drugs were dissolved in 0.9% NaCl solution, with the exception of TMB, which was dissolved in dimethylformamide. Myricitrin was dissolved in Tween 80 plus saline. The final concentration of Tween did not exceed 10% and did not cause any effect ''per se''. The myricitrin dose (30 mg/kg) was chosen based on Ref. [14]. Myricitrin was isolated from the plant of genus *Eugenia* in the Department of Chemistry, Federal University of Santa Catarina, Brazil. Analysis of the ^1H NMR and ^{13}C NMR spectra showed analytical and spectroscopic data in full agreement with its assigned structure [25]. The chemical purity of myricitrin (more than 98%) was determined by GC/HPLC.

2.7. Statistical analysis

The results are presented as mean ± S.E.M. The statistical significance of differences between groups was determined by ANOVA followed by Student–Newman–Keuls multiple comparison test. *P*-values less than 0.05 ($P < 0.05$) were considered as indicative of significance.

3. Results

To evaluate the effects of myricitrin upon neuropathic pain we performed a partial ligation of the sciatic nerve in mice. This injury produced a marked development of allodynia on the ipsilateral side 7 days after the surgical procedure (Fig. 1). The acute treatment with myricitrin (30 mg/kg, i.p.) significantly decreased the paw withdrawal response 30 min after its administration (60 ± 8%), and this effect was maintained for 4 h after myricitrin treatment (Fig. 1).

Next, we investigated the effects of myricitrin on an inflammatory pain model, through the immunologic reaction induced by i.pl. injection of CFA. The i.pl. injection of CFA produced a profound mechanical allodynia and paw volume enhancement, which were maintained throughout the test. The animals that received myricitrin (30 mg/kg) exhibited a reduction in the mechanical allodynia induced

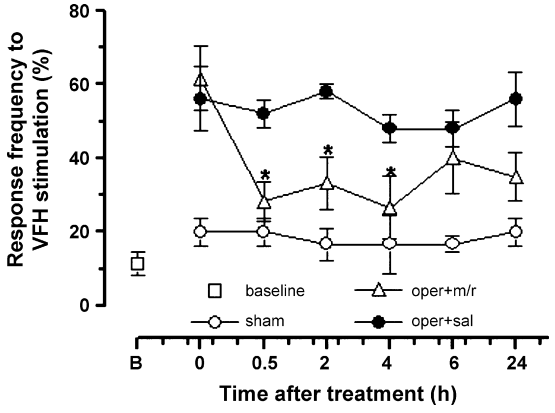

Fig. 1 – Effect of myricitrin on sciatic nerve injury-induced mechanical allodynia in response to 10 applications of 0.6 g VFH. The assessment was carried out in mice sham-operated (○), operated and treated with saline (●), or operated and treated with myricitrin 30 mg/kg, i.p. (△) 7 days after surgery. The baseline (□) was recorded before nerve injury. The results represent the mean ± S.E.M. of eight animals. The symbols denote a significant difference at $^*P < 0.05$ between operated mice plus vehicle and operated mice plus myricitrin, by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test.

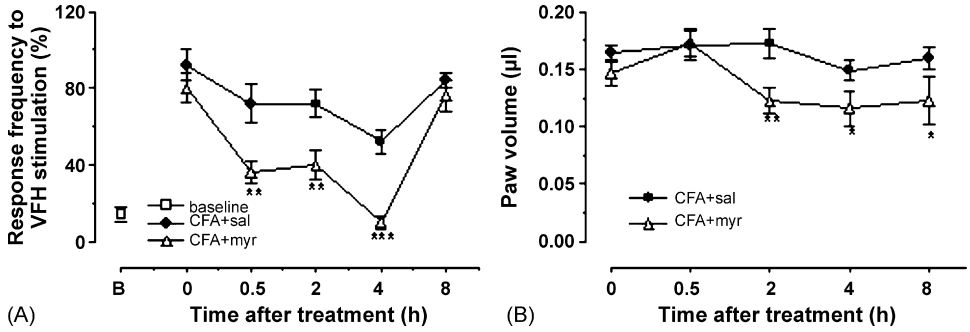

Fig. 2 – Effect of myricitrin on mechanical allodynia (A) in response to 10 applications of 0.6 g VFH and paw edema (B) induced by CFA in mice. The animals received saline (●) or myricitrin (△) 24 h after CFA injection. (A) The baseline (□) was recorded before CFA injection and in (B) baseline paw volume was discounted from total volume to give the absolute edema value. Data were obtained 24 h after CFA-injection (0) and (0.5, 2, 4, and 8 h) subsequent to myricitrin (30 mg/kg, i.p.) treatment. The results represent the mean ± S.E.M. of eight animals. The symbols denote a significant difference at $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ between saline treated and myricitrin treated mice, by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test.

by CFA. This began 30 min after myricitrin administration and was maintained for up 4 h. The most pronounced effect was observed at 30 min, where myricitrin inhibition reached 50 ± 8%, while at 4 h the animals' sensitivity was similar to baseline values (Fig. 2A). Furthermore, CFA injection caused an increase in paw volume 24 h after its administration. This effect was significantly reversed by myricitrin (30 mg/kg) beginning 2 h after treatment. The effect was most marked at 2 h with paw edema reduction of 25 ± 7% (Fig. 2B).

Regarding the anti-allodynic and anti-edematogenic effects of myricitrin on mechanical allodynia and paw edema induced by CFA, we analyzed MPO activity in mouse paw. MPO activity in injured tissue reflects neutrophils infiltration and degranulation since this enzyme is the most abundant in neutrophils [22]. The results in Fig. 3 show that MPO activity was enhanced four-fold at 24 h after CFA administration. Both treatment schedules, with myricitrin 30 min before and 22 h after CFA injection, reduced MPO activity to basal levels ($P > 0.05$ from baseline) (Fig. 3).

To confirm the effect of myricitrin upon neutrophil migration, we carried out histochemical staining and quantified the leucocytes present in subcutaneous mouse paw tissue. The CFA administration caused marked migration of neutrophils into the mouse paw. On the other hand, the mast cells, lymphocytes and macrophages were not altered by CFA 24 h i.pl. administration (Table 1). A massive number of inflammatory cells was found in the dermis and hypodermis. The neutrophils, mast cells, macrophages, and lymphocytes (plasmocytes) identified in the tissue can be seen in Fig. 4D. Another cellular alteration was the greater presence of fibroblasts rather than fibrocytes in the CFA-injected footpad, characterizing a pathological condition. The myricitrin treatment was unable to reduce neutrophil migration regardless of whether it was applied before or after CFA administration (Table 1).

In the histological study, the CFA-injected footpad showed some morphological alterations in the epidermis and connective tissues. In these samples we observed a hyperplasia of epidermal cells, scattering of collagen fibers in papilar and reticular dermis, a marked presence of inflammatory cells, granulomas, angiogenesis and the presence of active fibroblasts. Both treatments, myricitrin 30 min before or 22 h after CFA, decreased morphological changes in epidermis and dermis papilar, without affecting alterations in the dermis

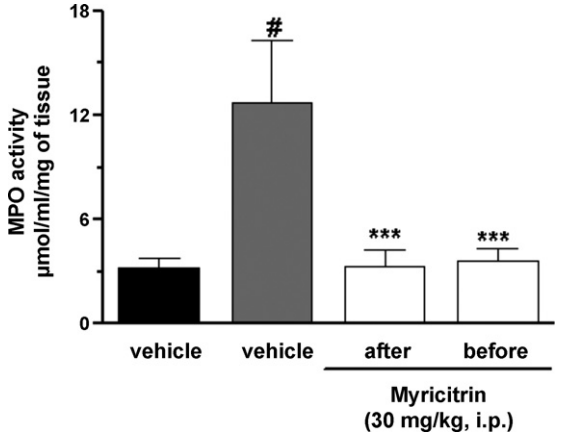

Fig. 3 – Effect of myricitrin on MPO activity in CFA-injected mouse paw. The animals were subdivided into four groups: (1) saline i.p. 30 min before PBS i.pl.; (2) saline i.p. 30 min before CFA i.pl.; (3) myricitrin i.p. 30 min before CFA i.pl.; (4) myricitrin i.p. 22 h after CFA i.pl. Twenty-four hours after CFA-injection the subcutaneous tissue from paws was removed and homogenized in buffer to determine MPO activity. The results are expressed as the concentration of TMB oxidized with a molar absorption coefficient of 3.9×10^4 for TMB salt. Each bar represents the mean ± S.E.M. of five animals. The statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test and the symbols denote a significant difference among groups: $^{\#}P < 0.001$ when compared to PBS i.pl. group; $^{***}P < 0.001$ when compared to vehicle i.p. plus CFA i.pl. group.

Table 1 – Leucocytes infiltration in the CFA-injected footpad

Treatment	Neutrophils (mm^2)	Mast cells (mm^2)	Macrophages and lymphocytes (mm^2)
Saline i.p. plus PBS i.pl.	5.2 ± 3.1	16.9 ± 4.5	15.9 ± 7.0
Saline i.p. plus CFA i.pl.	$141.6 \pm 13.9^*$	22.8 ± 7.9	15.2 ± 5.9
Myricitrin i.p. (before) plus CFA i.pl.	$119.8 \pm 8.8^*$	11.2 ± 3.6	12.0 ± 3.2
Myricitrin i.p. (after) plus CFA i.pl.	$133.0 \pm 4.3^*$	15.9 ± 10.7	15.7 ± 3.5

The number of cells was counted in 20 fields in a representative area of inflammation on the reticular dermal and hypodermal layers. The statistical analyses were performed by one-way analysis of variance (ANOVA), followed by Student–Newman–Keuls test and the symbol denotes a significant difference when compared to the group saline i.p. plus saline i.pl., $^*P < 0.001$.

reticular. This effect was best observed when myricitrin was administered 30 min before CFA (Fig. 4A–C).

4. Discussion

Pathological (chronic) pain is an unrelenting condition that often becomes debilitating. At present, few drugs are effective in treating this disorder and many of these are known for their side effects [1,4]. In this concern, the search for new compounds that could be applied in chronic pain therapy has been essential. In the current study, we demonstrated, for the first time, that systemic (i.p.) administration of the flavonoid myricitrin produced an inhibition of tactile allodynia induced by two chronic pain models: sciatic nerve partial constriction and chemically (CFA)-induced inflammation in mice. It should be noted that the anti-allodynic effect of myricitrin appeared 30 min after treatment (first measurement) and was maintained for up to 4 h. The additional findings of this study were that myricitrin treatment reduced CFA-induced paw edema, MPO activity, and morphological alterations in epidermis and dermis papilar, all without affecting leukocytes infiltration.

Previous studies have demonstrated that myricitrin interacts with certain proteins such as PI-3 kinase, PKCα and PKCε decreasing their activities [14,15,17]. In addition, this flavonoid has been reported to inhibit inducible nitric oxide synthase (iNOS) over expression, NO production, and NF-κB activation [16]. More recently, a study by our group found that myricitrin produces a potent inhibition of calcium transport (unpublished data). In accordance with this, further investigations showed that myricitrin exerts an antinociceptive action in models of acute pain. This antinociceptive action seems to involve PKC and L-arginine-nitric oxide pathways [14]; $G_{i/o}$ protein and K^+ channels activation, and Ca^{2+} movement inhibition (unpublished data).

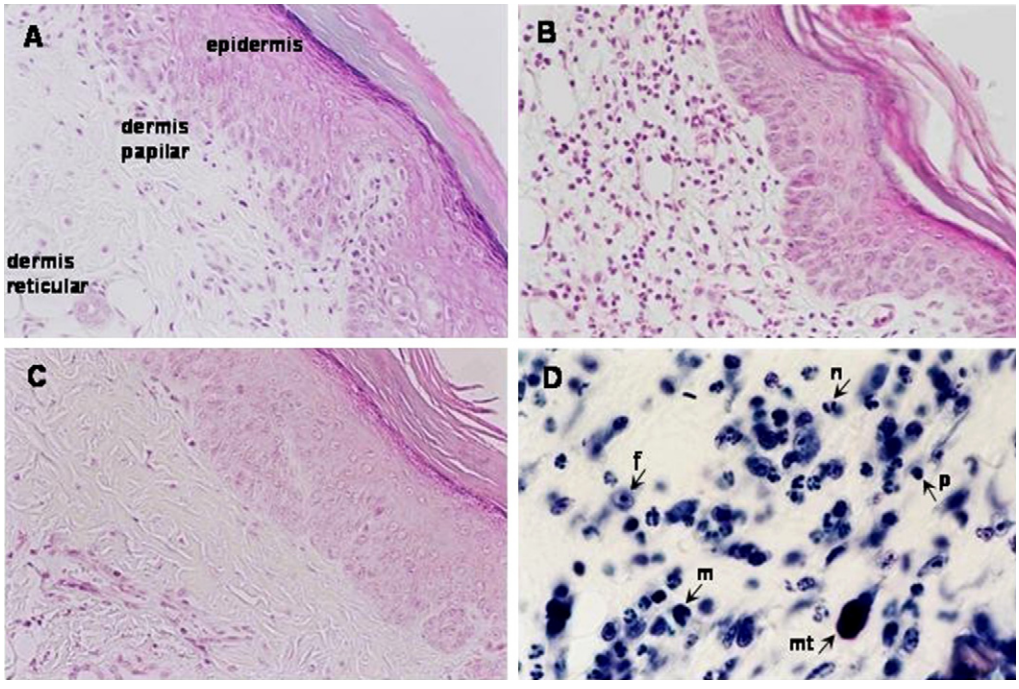

Fig. 4 – Effect of myricitrin on subcutaneous morphological changes and leucocytes infiltration in CFA-injected mouse paw. The mice were killed and their footpads were removed exactly 24 h after CFA injection, in all groups. The photomicrographs represent: (A)–(C) footpad sections stained by hematoxylin–eosin (40× objective); (A) saline i.p. 30 min before PBS i.pl.; (B) saline i.p. 30 min before CFA i.pl.; (C) myricitrin i.p. 30 min before CFA i.pl.; (D) footpad section, stained by May–Grünwald–Giemsa (100× objective), from a representative animal that received saline i.p. and CFA i.pl. Cell migration in an inflammatory condition is shown in (D): mast cells (mt); plasmocytes (p); neutrophils (n); macrophages (m); active fibroblast (f).

Taking into account these previous findings, the purpose of the present study was to investigate the effects of myricitrin on two chronic pain models. It is now well recognized that persistent pain resulting from peripheral injection of CFA or sciatic nerve partial constriction, leads to the release of multiple inflammatory and nociceptive mediators, resulting in increased long-lasting discharge of primary sensory fibers that modifies neuronal, neuro-glial and neuro-immune cell phenotype and function in the central nervous system. These alterations can occur at translational or post-translational levels and affect receptors, ion channels, soluble mediators and other molecules involved in cell signaling [1,3,26]. In this context, valuable effects of myricitrin in counteracting nerve injury- and CFA-induced inflammatory nociception are probably associated with its ability to interfere in cell signaling, particularly that related to PKC, NO, Ca^{2+} and K^+ pathways.

Another interesting finding of this work was the number of leucocytes in the CFA-injected paw. Previous studies had consistently reported that CFA i.pl. administration causes massive infiltration of neutrophils [19], however, this had not been quantified prior to the present investigation. Twenty-four hours after CFA i.pl. administration, the number of subcutaneous neutrophils increased around 27-fold, whereas the number of macrophages/lymphocytes and mast cells remained similar to the saline i.pl. group. Hence, it is very important to point out that an i.pl. treatment of 24 h with CFA causes intense migration of neutrophils alone, preserving the resident numbers of the other leucocytes. Interestingly, myricitrin, under both treatment schedules, did not modify leucocyte migration. This was an unexpected finding, since myricitrin treatment inhibited CFA-induced paw edema, tactile allodynia and MPO activity. The direct relationship between MPO activity and the presence of neutrophils has been well described, since this enzyme is the most abundant in neutrophils [22]. In this work we found that a decrease in MPO activity did not directly reflect a decrease in neutrophil numbers. A reasonable explanation would be because flavonoids, particularly those that contain a cathecol group (like myricitrin), are good substrates for the MPO enzyme at concentrations corresponding to circulating plasma flavonoids levels [27]. In agreement with this, when incubated *in vitro*, myricitrin was able to inhibit MPO activity from subcutaneous tissue of CFA-injured mice (data not shown). Hence, MPO-catalyzed flavonoid oxidation can prevent the oxidation of other targets, diminishing oxidative damage caused by inflammation. Thus, it can be postulated that anti-inflammatory drugs with oxidizable functional groups can inhibit MPO and this explains, in part, their anti-inflammatory effects [28]. The ability of myricitrin to inhibit MPO activity also would explain its effects against allodynia, edema and morphological changes caused by CFA.

Furthermore, myricitrin showed itself to be a powerful antioxidant agent, since it inhibited, at low concentrations, the lipid peroxidation in a condition where Fe^{2+} is releasing free radicals (data not shown). This antioxidant action can be attributed mainly to the scavenger ability of flavonoids, specially since flavonoids that contain a cathecol group have been described as scavengers and mimics of superoxide dismutase, representing an important role in the oxidative stress process [29,30].

Oxidative stress is defined as a disturbance in the pro-oxidant-antioxidant balance in favor of the pro-oxidant, thereby leading to potential damage [31–33]. The major pro-oxidant agents are the reactive oxygen species (ROS), which play a crucial role in the initiation and progression of pathological conditions, including the inflammatory process through induction of mediators such as interleukins [33,34]. In addition, ROS can be released in response to TNF-α and LPS [35] and can serve as intracellular signals for the activation and regulation of redox-sensitive transcription factors [36]. This is corroborated by the finding that antioxidant substances can act as inhibitors of cytokines at both transcriptional and post-transcriptional levels [33,36,37]. Regarding these mechanisms, the antioxidant action and MPO inhibition exerted by myricitrin demonstrate its potential beneficial effects in inflammatory and neuropathic pain conditions.

Although myricitrin did not reduce neutrophil migration it did succeed in decreasing paw edema and morphological alterations in CFA-induced local inflammation. It has been assumed that microvessel permeability can increase independently of leukocyte adhesion and the cell migration process, however, it is dependent on a mechanism involving the release of ROS [38]. These results support the idea that myricitrin, as an antioxidant agent, is a potential candidate for anti-inflammatory drugs research.

In summary, the current study provides convincing evidence that myricitrin, a flavonoid occurring naturally and widespread in higher plants, produces a systemic anti-allodynic effect in two models of persistent inflammatory (CFA-i.pl. injected) and neuropathic (sciatic nerve injured) pain, when evaluated using a mechanical stimulus (VFH) in the hindpaw. In addition, myricitrin decreased the CFA-induced increases in MPO activity, paw edema and, consequently, the subcutaneous morphological footpad alterations. The beneficial effects of myricitrin appear to occur through molecular mechanisms including inhibition of PKC and NO cell signaling, Ca^{2+} and K^+ transport. Furthermore, additional means by which myricitrin exerts its effects are largely related to its antioxidant activity. Together, the present results indicate that myricitrin might be of potential interest in the development of new clinically relevant drugs for the management of persistent neuropathic and inflammatory conditions.

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