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Analysis of the antinociceptive effect of the flavonoid myricitrin. Evidence for a role of the L-arginine-nitric oxide and protein kinase C pathways.

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Abbreviations: L-NOARG, N^{ω}-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; BK, bradykinin; EPI, epinephrine; PMA, phorbol 12-myristate 13-acetate; PGE_2, prostaglandin E_2; PCKα protein kinase C-alpha; PCKε, protein kinase C-epsilon; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate; PI 3-kinases, phophoinositide 3-kinases; TRPV1, transient receptor potential vanilloid type 1; PBS, phosphate buffer saline; i.pl., intraplantar.; i.p., intraperitoneal; p.o., oral; s.c., subcutaneous.

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Abstract

The present study investigated the antinociceptive effects of the flavonoid myricitrin in chemical behavioral models of pain in mice and rats. Myricitrin given by intraperitoneal (i.p.) or oral (p.o.) route produced dose-related antinociception when assessed on acetic acid-induced visceral pain in mice. In addition, the i.p. administration of myricitrin exhibited significant inhibition of the neurogenic pain induced by intraplantar (i.pl.) injection of capsaicin. Likewise, myricitrin given by i.p. route reduced the nociception produced by i.pl. injection of glutamate and phorbol myristate acetate (PMA). Western blot analysis revealed that myricitrin treatment fully prevented the PCKα and PCKε activation by PMA in mice hindpaw. Myricitrin given i.p. also inhibited the mechanical hyperalgesia induced by bradykinin, without affecting similar responses caused by epinephrine and prostaglandin E_2 (PGE_2). The antinociception caused by myricitrin in the acetic acid test was significantly attenuated by i.p. treatment of mice with the nitric oxide precursor, L-arginine. In contrast, myricitrin antinociception was not affected by naloxone (opioid receptor antagonist) or neonatal pre-treatment of mice with capsaicin and myricitrin antinociceptive effects are not related with muscle-relaxant or sedative action. Together, these results indicate that myricitrin produces pronounced antinociception against chemical and mechanical models of pain in rodents. The mechanisms involved in their actions are not completely understood but seem to involve an interaction with nitric oxide-L-arginine and protein kinase C pathways.

Introduction

Flavonoids are a gamma-benzopyrone family that occur naturally and are widely spread in higher plants (Ramelet, 2000). They are plant secondary metabolites and are chemically defined by their common structure, which is composed of diphenylpropanes (C6-C3-C6) and consists of two aromatic rings linked through three carbons that usually form an oxygenated heterocycle (Harborne, 1989). In mammals, flavonoids occur only through dietary intake. The average daily human intake in the United Kingdom and United States has been present to be 20 mg to 1 g. These compounds are present in fruits, vegetables, grains, nuts, tea and wine. However, little is reported about quantify flavonoid in food and thus, only a few studies have attempted to assess the relationship between consumption of foods rich in flavonoids and the prevention of certain diseases (Birt et al., 2001).

A variety of biological effects have been ascribed to flavonoids (Birt et al., 2001; Havsteen, 2002; Calixto et al., 2003; 2004). Much attention has been given to their antioxidant (Edenharder and Grünhage, 2003) and anti-inflammatory properties, *in vitro* and *in vivo* (Calixto et al., 2003; 2004). In addition, some studies report antitumoral (Clifford et al., 1996) and hepatoprotective action (Ferrándiz et al., 1994). Flavonoids inhibit cytokine release from RAW264.7 cells (Xagorari et al., 2002) and may modulate the increasing number of cellular processes involving redox reaction, including the regulation of tyrosine phosphatase activity (Gamet-Payrastre et al., 1999). In contrast, little is known about the effects of flavonoids on the modulation of pain transmission.

Myricitrin (Fig 1) is a flavonoid that belongs to the flavonol sub-group. This flavonoid is found in fruits of genus *Pouteria* (Ma et al., 2004) and *Manilkara zapota* (Ma et al., 2003); leaves of *Eugenia uniflora* (Schmeda-Hirschmann et al., 1987) and latex of *Croton draco* (Tsacheva et al., 2004). In addition, myricitrin has been reported as having an antioxidant action (Edenharder and Grünhage, 2003). It comprises an anti-mutagenic effect, which is attributed to a free radical scavenger action (Edenharder and Grünhage, 2003). However, the ability of myricitrin to inhibit tumor promotion is also due to the activation of immune responses against tumors (Yasukawa et al., 1990). The intake of myricitrin from food is associated with the level of HDL cholesterol and negatively related to the level of triglycerides (Kimira et al., 1998).

Previous reports demonstrate that myricitrin is able to inhibit nitric oxide (NO) production. In addition, it reduces the over expression of nitric oxide synthase (iNOS) and NF-κB activation induced by lipopolyssacharide on RAW264.7 cells (Chen et al., 2000).

Taking into account the biological activities of myricitrin, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of this flavonoid up to this date. Here, we have therefore attempted to examine the possible antinociceptive action of myricitrin in chemical and mechanical models of nociception in mice and rats. Attempts have been made in order to further investigate some of the possible mechanisms, which underlie the antinociceptive action of myricitrin.

Materials and methods

Animals. Experiments were conducted using Wistar rats (200 – 300 g) or Swiss mice (25 – 35 g) of both sex, housed at 22 ± 2°C under a 12-h light/12-k dark cycle (lights on at 6:00) and with access to food and water *ad libitum*. Animals (Male and female rat or mice were homogeneously distributed among the groups) were acclimatized to the laboratory for at least 1 h before testing and were used only once throughout the experiments. The experiments were performed after protocol approval by the Institutional Ethics Committee and were carried out in accordance with current guidelines for the care of laboratory animals and the ethical guidelines for investigations of experimental pain in conscious animals as specified (Zimmermann, 1983). The number of animals and intensity of the noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments.

Abdominal constriction induced by acetic acid. The abdominal constrictions were induced according to procedures described previously (De Campos et al., 1997) and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an i.p. injection of acetic acid (0.6 %, 0.45 ml/mouse) at the time of the test. Mice were pre-treated with myricitrin by i.p. (0.01 - 10 mg/kg) or oral (p.o.) (1 - 100 mg/kg) routes, 30 or 60 min before the irritant injection. Control animals received a similar volume of vehicle (10 ml/kg). After the challenge, the mice were individually placed into glass cylinders of 20 cm diameter, and the abdominal constrictions were counted cumulatively over a period of 20 min.

Algogen-induced overt nociception in mice.

Glutamate-induced nociception. In an attempt to provide more direct evidence concerning the possible interaction of myricitrin with the glutamatergic system, we separately investigated whether myricitrin would be able to antagonize glutamate-induced licking in the mouse paw. The procedure used was similar to describe previously (Beirith et al., 2002). A volume of 20 µl of glutamate (10 µmol/paw prepared in saline) was injected intraplantarly (i.pl.) in the ventral surface to the right hindpaw. The mice were observed individually for 15 min following glutamate injection. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. Mice were treated with myricitrin (1 - 100 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.) 30 min before glutamate injection.

Phorbol myristate acetate (PMA)-induced nociception. The procedure used was similar to that described previously (Siebel et al., 2004). A volume of 20 µl of PMA (50 pmol/paw prepared in saline) was injected, i.pl., in the ventral surface of the right hindpaw. After the challenge, the mice were individually placed into glass cylinders of 20 cm diameter, serving as observation chambers. The mice were observed individually from 15 to 45 min after PMA injection and the amount of time spent licking the injected paw timed with a chronometer was considered indicative of nociception. The mice were treated with myricitrin (0.01 - 10 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.) 30 min before PMA injection.

Capsaicin-induced nociception. The procedure used was similar to that described previously (De Campos et al., 1997). After an adaptation period, 20 μl of capsaicin (1.6 μg/paw) was injected, i.pl., in the ventral surface of the right hindpaw. The mice were observed individually for 5 min following capsaicin injection. The amount of time spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. The mice were treated with myricitrin (1, 10 and 100 mg/kg, i.p.) 30 min before capsaicin injection. Control animals received vehicle (10 ml/kg).

Bradykinin-, epinephrine- or prostaglandin E_2-induced hyperalgesia. The procedures used were similar to those described previously (De Campos et al., 1997). The rats were pre-treated, i.p., with myricitrin (30 mg/kg) or vehicle (10 ml/kg) 30 min before injection of 100 μl of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw), prostaglandin E_2 (10 nmol/paw) or only saline solution alone (control group) in the ventral surface of the right hindpaw. The nociception threshold (of squeak response or paw withdrawal) was assessed by applying increasing pressure to the dorsal site of inflamed or control paws, using a Basile analgesy meter (Ugo Basile, Milan, Italy) according to the method of Randall-Selitto (Randall and Selitto, 1957). The weight on the analgesy meter ranged from 0 to 759 g, and the threshold was expressed as load (g) tolerated. When bradykinin was used, animals were pretreated with the angiotensin-converting enzyme inhibitor captopril (5 mg/kg, s.c.) 60 min before experiments to prevent its degradation (De Campos et al., 1997).

Measurement of motor performance, locomotor activity and corporal temperature. In order to evaluate the possible non-specific muscle-relaxant or sedative effects of myricitrin, mice were submitted to the rota-rod task (Vaz et al., 1996) and open-field test (Rodrigues et al., 2002). Rota-rod apparatus consisted of a bar with a diameter of 2.5 cm, subdivided into four compartments by disks 25 cm in diameter. The bar rotated at a constant speed of 17 revolutions per min. The animals were selected 24 h previously by eliminating those mice which did not remain on the bar for two consecutive periods of 120 s. Animals were treated with myricitrin (30 or 100 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.) 30 min before being tested. The results are expressed as the time (s) for which animals remained on the rota-rod. The cut-off time used was 120 s.

The ambulatory behavior was assessed in an open-field test as described previously (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring 40 $\times$ 60 $\times$ 50 cm. The floor of the arena was divided into 12 equal squares, and the number of squares crossed with all paws crossing) was counted in a 6-min session. Mice were treated with myricitrin (30 and 100 mg/kg, i.p.), or vehicle (10 ml/kg, i.p.) 30 min beforehand.

In addition, some compounds cause antinociception by decreasing basal corporal temperature (hypothermia). To exclude this possibility, we assessed the skin temperature of mice 30 min after they received vehicle (saline) or myricitrin (30 and 100 mg/kg, i.p.). A thermosensor (Mallory, CE-Brazil) was placed on the skin in the sacral region and the procedure was carried out in accordance with the manufacturer's instructions.

Analysis of the possible mechanism of action of myricitrin.

Involvement of the opioid system. To investigate the possible participation of the opioid system in the antinociceptive effect of myricitrin, mice were pre-treated with naloxone (a non-selective opioid receptor antagonist, 5 mg/kg, i.p.), and after 20 min the animals received an injection of myricitrin (1 mg/kg, i.p.), morphine (5 mg/kg, s.c.) or vehicle (10 ml/kg, i.p). Other groups were pre-treated with vehicle and after 20 min received myricitrin, morphine or vehicle, 30 min before acetic acid injection.

Involvement of the L-arginine-nitric oxide pathway. To investigate the role played by the nitric oxide-L-arginine pathway in the antinociception caused by myricitrin, mice were pre-treated with L-arginine (40 mg/kg, i.p., a nitric oxide precursor) or D-arginine (40 mg/kg, i.p., an inactive isomer of L-arginine) and after 20 min they received myricitrin (1 mg/kg, i.p.), N^{ω}-nitro-L-arginine (L-NOARG, 25 mg/kg, i.p., a inhibitor of nitric oxide synthesis) or vehicle (10 ml/kg, i.p.). The nociceptive responses to acetic acid were recorded 30 min after the administration of myricitrin, L-NOARG, or vehicle. Other groups were pre-treated with vehicle (10 ml/kg, i.p.) and after 20 min received myricitrin, L-NOARG, or vehicle, 30 min before acetic acid injection (Abacioglu et al., 2001).

Involvement of capsaicin-sensitive fibers. To explore the role of capsaicin-sensitive fibers in the antinociceptive effect of myricitrin, newborn mice were anaesthetized with ether and treated subcutaneously with 50 mg/kg of capsaicin on the second day of life with the purpose of inducing the irreversible degeneration of

unmyelinated afferent neurons, especially C fibers (Holzer, 1991). Control animals received, by the same route, the same volume of vehicle used to dissolve capsaicin (10% ethanol, 10% Tween-80 and 80% saline). The antinociceptive effect caused by myricitrin (1 and 10 mg/kg, i.p.), against the nociceptive response induced by acetic acid, was analyzed at 6 to 7 weeks after the neonatal capsaicin or vehicle treatment. The efficiency of the treatment of mice with capsaicin was confirmed by means of a topical application of 50 µl capsaicin (0.01%) into right eye, and the number of wiping motions occurring during the subsequent 1 min was counted as described previously (Ikeda et al., 2001).

Effect of myricitrin on the PKCα and PKCε activation by PMA

Preparation of tissue for western blot studies. The mice received myricitrin (1 mg/kg; i.p.) or vehicle (10 ml/kg; i.p.), 30 min before PMA (50 pmol/paw) injection. The injected paw was isolated 15 min after PMA or PBS injection (Ferreira et al., 2005). The skin and connective tissues of the plantar portion of the hind paws were removed and disrupted using a glass Potter homogenizer in an ice-cold buffer containing protease and phosphatase inhibitors (100 mM Tris–HCl–pH 7.4; 2 mM EDTA; 2 µg aprotinin, 0.1 mM phenylmethanesulfhonyl fluoride, 200 mM NaF and 2 mM of sodium orthovanadate). The homogenate was centrifuged at 1.000 × g for 10 min at 4 °C; the pellet was discarded and the supernatant was further centrifuged at 35.000 × g for 30 min at 4 °C. The supernatant was collected as a cytoplasm-rich fraction. The resulting pellet was re-suspended and considered as a membrane-rich fraction. The protein concentration was determined using a protein

assay kit (Bio-Rad, Hercules, CA). The samples were aliquoted and stored at −80^{o} C until their western blot analysis.

Western blot analysis. In order to confirm the activation of PKCα and PKCε after PMA injection into the mouse paw, western blot analysis was carried out as previously described (Ferreira et al., 2005). Equivalent amounts of protein (10 and 50 µg for membrane- and cytoplasm-rich fractions, respectively) were mixed in buffer (Tris 200 mM, glycerol 10%, SDS 2%, β-mercaptoethanol 2.75 mM and bromophenol blue 0.04%) and boiled for 5 min. Proteins were resolved in 10% sodium dodecyl sulfate-polyacrilamide gel by electrophoresis (SDS-PAGE) and transferred on to polynilidene difluoride membranes, according to the manufacturer's instructions (Millipore). The membranes were saturated by incubation overnight with 10% non-fat dry milk solution and then incubated with anti-PKCα and anti-PKCε (Santa Cruz Biotechnology, USA). After washing, the membranes were incubated with adjusted peroxidase-coupled secondary antibodies. The immunocomplexes were visualized using the ECL chemiluminescence detection system (Amersham Biosciences, UK).

Drugs. The following substances were used: acetic acid, morphine hydrochloride (Merck, Darmstadt, Germany), bradykinin, epinephrine, prostaglandin E_2, capsaicin, naloxone hydrochloride, phorbol 12-myristate 13-acetate, glutamic acid, L-arginine, D-arginine, L-NOARG and phosphate buffer saline (PBS) tablets (Sigma, St. Louis, USA). All other chemicals were of analytical grade and obtained

from standard commercial suppliers. Drugs were dissolved in 0.9% of NaCl solution, with the exception of myricitrin, which was dissolved in tween 80 plus saline and capsaicin, dissolved in ethanol plus tween 80 plus saline. The final concentration of tween and ethanol did not exceed 5% and did not cause any "per se" effect. The myricitrin was isolated from the plant of genus *Eugenia* the Department of Chemistry, Federal University of Santa Catarina, Brazil, was characterized by spectral analyzes, (RMN-^{1}H) and (RMN-^{13}C) and by comparison the spectrum literature data (Agrawal, 1989) and showed a degree of purity greater than 98%.

Statistical analysis. The results are presented as mean ± S.E.M., except the ID_{50} values (i.e., the dose of myricitrin reducing the nociceptive response by 50% relative to the control value), which are reported as geometric means accompanied by their respective 95% confidence limits. When possible, the ID_{50} value was determined using at least three dosages of myricitrin by linear regression from individual experiments, using linear regression software (GraphPad software, San Diego, CA). Maximal inhibition values were calculated at the most effective dose used. The statistical significance of differences between groups was performed by ANOVA followed by Newman-Keuls test. *P*-values less than 0.05 ($P< 0.05$) were considered as indicative of significance.

Results

Abdominal constriction induced by acetic acid. The results in Fig. 2A show that myricitrin, given i.p. 30 min prior to testing, produced dose-related inhibition of the

acetic acid-induced abdominal constrictions in mice, with a mean ID_{50} value (and their respective 95% confidence limits) of 0.33 (0.20-0.54) mg/kg and the inhibition observed was 84±5% for the dose of 10 mg/kg. Furthermore, given by p.o. route 60 min beforehand, myricitrin (100 mg/kg) caused a partial, but significant inhibition (39±4%) of the acetic acid-induced pain (Fig. 2B). Hence, myricitrin was less efficacious and potent in preventing the nociception caused by acetic acid when it was given orally in comparison with given intraperitoneally. Thus, the administration of myricitrin by the i.p. route (time point 30 min beforehand) was chosen for all further studies with independent groups of animals.

Glutamate-induced nociception. The results presented in Fig. 3A show that myricitrin caused a dose-dependent and significant inhibition of the glutamate-induced nociception, with a mean ID_{50} value of 16.8 (10.3-27.5) mg/kg and the inhibition observed was 81±10% for the dose of 100 mg/kg.

Capsaicin-induced nociception. The i.p. administration of myricitrin produced partial, but significant, inhibition of the capsaicin-induced neurogenic nociception (Fig. 3B). The inhibition observed was 13±8, 42±8 and 37±6% for the doses of 1, 10 and 100 mg/kg, respectively (Fig. 3B).

PMA-induced overt nociception. The i.p. administration of myricitrin also produced a marked and dose-dependent inhibition of PMA-induced licking (Fig. 3C). The mean ID_{50} value from this result was 0.56 (0.33–0.95) mg/kg and the inhibition was 100% when administrated at 10 mg/kg.

Bradykinin-, epinephrine- or prostaglandin E_2-induced hyperalgesia. The results of Fig. 4 show that intraplantar administration of bradykinin (3 nmol/paw), epinephrine (450 nmol/paw) and PGE_2 (10 nmol/paw) significantly increased ($p<0.01$) the sensibility to mechanical stimuli (hyperalgesia) in rats, when assessed in the Randall-Selitto model. In addition, bradykinin, epinephrine and PGE_2 caused a decrease of 40±7, 58±2 and 75±3% on pressure supported in grams when compared with control group value. Furthermore, the treatment of rats with myricitrin (30 mg/kg, i.p) completely reversed the hyperalgesic effect caused by bradykinin ($p<0.01$), but did not reduce epinephrine- or PGE_2-induced hyperalgesia (Fig. 4).

Measurement of motor performance, locomotor activity and basal temperature. The myricitrin treatment (30 and 100 mg/kg, i.p.) did not alter response of mice in both tests, motor performance on the rota-rod task and locomotor activity in the open-field test, when compared with animals that received saline (control group) (Table 1). In addition, the basal temperature of mice was not altered by myricitrin (30 and 100 mg/kg) treatment; the temperatures were 29.4±0.6, 29.0±0.5 and 29.6±0.7 °C for animals that received saline and myricitrin (30 and 100 mg/kg), respectively (Table 1).

Involvement of the opioid system. The results presented in Fig. 5 show that the pre-treatment of mice with naloxone (5 mg/kg, i.p.), given 20 min beforehand, completely reversed the antinociception caused by morphine (5 mg/kg, s.c.)

against acetic acid-induced pain, without affecting the antinociception caused by myricitrin (1 mg/kg, i.p.).

Involvement of the L-arginine-nitric oxide pathway. The results presented in Fig. 6 show that mice pre-treatment with nitric oxide precursor L-arginine (40 mg/kg, i.p.), given 20 min prior to testing, but not with D-arginine (40 mg/kg, i.p.) significantly prevented ($p < 0.05$) the antinociception caused by L-NOARG (25 mg/kg, i.p.) and by myricitrin (1 mg/kg, i.p.) when analyzed against acetic acid-induced abdominal constrictions.

Involvement of capsaicin-sensitive fibers. Finally, the neonatal capsaicin (50 mg/kg, s.c.) treatment of mice produced partial, but significant, inhibition (27±7%) of the acetic acid-induced nociception (Fig. 7). In contrast, the same treatment of mice with capsaicin did not significantly change the antinociceptive effect of myricitrin (1 and 10 mg/kg, i.p.) when compared with the neonatal vehicle treatment group (Fig. 7). Furthermore, successful capsaicin treatment of newborn mice was confirmed by a significant reduction ($P<0.001$) in the response to topical application of capsaicin to the cornea in capsaicin-treated mice. The mean number of wiping motions was 4.6±1.6 and 18.5±1.7 in capsaicin and vehicle-treated mice, respectively.

Western blot analysis of protein kinase C. The possible participation of the PKC pathway on the antinociceptive effect of myricitrin was confirmed through western blot analysis. Injection of PMA (50 pmol/site) into the mouse paw activated PKCα

and, into a less extent, PKCε isoforms, as indicated by their translocation from cytosol- to membrane-rich homogenates achieved in administered tissues (Fig. 8 A-D). In addition, myricitrin (1 mg/kg) pre-treatment significantly prevented the activation of both PKCα and PKCε isoforms caused by PMA injection (Fig. 8 A-D).

Discussion

The present study demonstrates, for the first time, that systemic (i.p. or p.o.) administration of the flavonoid myricitrin, at doses that did not produce any important motor dysfunction, alterations in basal temperature or any other obvious side effects, induced a dose-dependent inhibition of acetic acid-induced visceral nociceptive response in mice. The most relevant additional findings of the present work were that, (i) i.p. administration of myricitrin caused significant inhibition of glutamate and capsaicin-induced nociception; (ii) caused dose-dependent inhibition of the nociceptive response caused by intraplantar injection of PMA; (iii) myricitrin antinociceptive effect in PMA-induced nociception is closely related with inhibition of both PKCα and PKCε; (iv) myricitrin comprised an anti-hyperalgesic effect upon the intraplantar injection of bradykinin, but not with epinephrine or PGE_2, in rats (v) its antinociception against the acetic acid test was significantly reversed by i.p. treatment of mice with L-arginine, but not by naloxone; (vi) the neonatal treatment of mice with capsaicin completely failed to affect myricitrin-induced antinociception in mice.

Flavonoids exert important pharmacological actions, such as anti-oxidant, anti-inflammatory, anti-allergic, and anti-ischemic properties, suggesting their

potential protective function against cardiovascular and coronary heart diseases and against certain forms of cancer (Gamet-Payrastre et al., 1999; Birt et al., 2001; Havsteen, 2002; Edenharder and Grünhage, 2003; Calixto et al., 2003; 2004). The flavonoid myricitrin produces important anti-oxidant and anti-mutagenic effects, which are attributed or not to its free radical scavenger action (Yasukawa et al., 1990; Edenharder and Grünhage, 2003). Although myricitrin is known to possess a strong antioxidant effect, the putative antinociceptive activities of myricitrin, as well as the mechanisms of its action, *in vivo*, have never been reported.

The results reported here indicate that i.p. administration of myricitrin produced consistent and dose-related antinociception when assessed in acetic acid-induced visceral nociception in mice. When compared with standard analgesic drugs (results obtained by our group), myricitrin was about 36.6- to 72.7-fold more potent than acetaminophen, aspirin and diclofenac in attenuating acetic acid-induced pain (Vaz et al., 1996; Santos et al., 1998). As expected, oral administration of myricitrin was less potent and efficacious than its intraperitoneal administration in preventing the nociception. In fact, it is generally recognized that intact flavonoid glycosides, like myricitrin, are poorly absorbed when given by oral route (Murota and Terao, 2003). Thus, the present result is in agreement with the data from literature, demonstrating that the bioavailability (antinociceptive activity) of myricitrin is notably decreased when given orally in comparison to when given intraperitoneally.

The acetic acid-induced writhing reaction in mice, described as a typical model for inflammatory pain, has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents (Collier et

al., 1968). This method presents a good sensitivity; however it shows poor specificity, leaving scope for the misinterpretation of results. This can be avoided by complementing the test with other models of nociception and by a performance motor test. For this reason, myricitrin was examined for its possible inhibitory action in the rota-rod and open-field tests. In both tests, we could observe that there was no statistically significant interference in performance motor patterns at higher doses than those produced marked suppression of the writhing response.

Another interesting finding of the present study, is the demonstration that myricitrin, given intraperitoneally, produced a dose-dependent inhibition of the nociceptive response caused by injection of glutamate into the mouse hindpaw. Glutamate nociception appears to involve peripheral, spinal and supraspinal sites of action and its greatly mediated by both NMDA and non-NMDA receptors, as well as by the release of nitric oxide and nitric oxide related substances (Beirith et al., 2002). Thus, these previous findings and data of the present results may indicate, at least in part, that the antinociceptive action of myricitrin could be associated with its ability to inhibit NO production or through interaction with the glutamatergic system.

Results of the present study also strongly suggest the involvement of protein kinase C, but not protein kinase A, in the antinociception caused by myricitrin. This notion derived from the data showing that i.p. administration of myricitrin dose-dependently inhibited the overt nociception by intraplantar PMA injection (a protein kinase C activator). Another piece of evidence that support this view was the results demonstrating that myricitrin suppressed the mechanical hyperalgesia induced by bradykinin, but not that induced by prostaglandin E_2 in rats. Some

studies propose that, in nociceptor, bradykinin binds to B_2 receptor causing a direct activation of protein kinase C and the indirect activation of the protein kinase A (Ferreira et al., 2004). Furthermore, the results of the current study show that myricitrin, at a dose that abolished bradykinin-induced mechanical hyperalgesia, was not able to inhibit epinephrine-induced mechanical hyperalgesia. It has been suggested that mechanical hyperalgesia produced by epinephrine depends on the activation of both PKC and PKA (Khasar et al., 1999). Thus, we can speculate that epinephrine produced a powerful hyperalgesic effect by acting in the β-adrenergic receptor via cAMP/PKA independent of PKC second-messenger pathways.

Of note, mice treated with myricitrin did not show PKC activation, as indicated by western blot analyzes. PKC activity requires an intracellular translocation from cytosol to cytoskeletal and membrane sites of action. Thus, translocation of PKC from a cytosolic to a membrane-associated location within the cell is a sensitive indicator of activation (Ferreira et al., 2005). In accordance with previous results (Ferreira et al., 2005), we found that i.pl. injection of PMA induced the translocation of a classical (PKCα) and, to a less extend, a novel (PKCε) protein kinase C isoforms from cytosolic to membrane. The present results show that myricitrin given systemically was able to prevent the activation of both PKC isoforms. Taken together these results strongly suggest that the antinociceptive effect of myricitrin in PMA-induced nociception is closely related with inhibition of PKC.

In fact, several experimental evidence now indicate that flavonoids, such as myricitrin, can inhibit PI 3-kinases and, consequently, they inhibit protein kinase C

isoenzymes activation (Gamet-Payrastre et al., 1999). However, flavonoids can also inhibit PKC directly (Agullo et al., 1997). The major structural characteristics in potent PI 3-kinases and PKC inhibition by flavonoids are the presence of 3',4' OH group on the B ring (Gamet-Payrastre et al.,1999) and myricitrin shares this condition.

Another interesting result of the present study was the demonstration that the L-arginine-nitric oxide pathway is likely to be involved in the antinociception caused by myricitrin. This conclusion derives from the fact that the pre-treatment of mice with the substrate of nitric oxide synthase (NOS), L-arginine, at a dose that produced no significant effect on acetic acid-induced pain, significantly reversed the antinociception caused by both myricitrin and L-NOARG (a known nitric oxide inhibitor). In marked contrast, the pre-treatment of animals with the inactive isomer of L-arginine, D-arginine, had no significant effect against both myricitrin- and L-NOARG-induced antinociception. In agreement with these findings, it has been reported that myricitrin inhibits nitric oxide (NO) production and reduces the over expression of inducible nitric oxide synthase (iNOS) in RAW264.7 cells (Chen et al., 2000).

Our data also demonstrate that the activation of the opioid naloxone-sensitive pathway is probably not involved in the antinociception produced by myricitrin because naloxone significantly reversed morphine, but not myricitrin, antinociception in the acetic acid test.

Previous studies have demonstrated the involvement of vanilloid receptor (TRPV1) in acetic acid-induced writhing (Ikeda et al., 2001). Hence, TRPV1 is stimulated by capsaicin, heat and pH alterations (Julius and Basbaum, 2001).

Capsaicin activates TRPV1, which in turn, induces membrane depolarization and increases cation influx, leading to noxious stimulus (Julius and Basbaum, 2001). Capsaicin administration in newborn mice (48 h old) causes persistent desensitization due to a non-selective loss of small sensory fibers, mostly C-fibers (Jancsó et al., 1977). Our results confirm these observations by demonstrating that neonatal treatment of mice with capsaicin significantly blocked the writhing responses induced by acetic acid. However, the capsaicin newborn treatment did not significantly modify myricitrin-induced antinociception. Furthermore, the i.p. administration of myricitrin produces partial, but significant, inhibition of capsaicin-induced neurogenic nociceptive behavior on mouse hindpaw. Capsaicin is known to evoke the release of neuropeptides, excitatory amino acids, nitric oxide and pro-inflammatory mediators from the periphera C fibres, and transmits nociceptive information to the spinal cord or causes spinal sensitization through the protein kinase C and A activation (Calixto et al., 2005). Thus, the ability of myricitrin to interact with PKC might explain, at least partially, its antinociceptive effect on acetic acid, glutamate, capsaicin and PMA-induced nociceptive responses. In addition, it's been demonstrated that inhibitors of PKC prevent the phosphorylation of TRPV1, reducing the sensitization of this capsaicin sensitive receptor (Prekumar and Abern, 2000, Ferreira et al., 2004, Calixto et al., 2005), thus, making it less responsive to agonist action.

In conclusion, the present results provide convincing evidence indicating that myricitrin, a flavonoid occurring naturally and widespread in higher plants, produces systemic antinociception when assessed in chemical models of nociception in mice, as well as producing anti-hyperalgesic effects in models of

painful mechanical hypersensitivity in rats, at doses that reveal no interference with locomotor activity. The precise mechanisms through which myricitrin exerts its action are currently under investigation, but inhibition of the L-arginine-nitric oxide and blockade of the PKC pathways seems largely to account for myricitrin antinociceptive effect. However, the opioid system, unmyelinated C-fibers sensitive to capsaicin and PKA pathway seem unlikely to participate in the antinociception caused by myricitrin.

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Footnotes

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Legends for Figures

Figure 1: Molecular structure of myricitrin.

Figure 2: Effect of myricitrin administered intraperitoneally **(A)** and orally **(B)** against acetic acid-induced writhing response in mice. Each column represents the mean of six to eight mice and the error bar indicates the S.E.M. Control values (C) indicate the mice injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman-Keuls test): *P<0.05; ** P<0.01 and *** P<0.001.

Figure 3: Effect of myricitrin given intraperitoneally (0.01-100 mg/kg, i.p.) against the glutamate (10 µmol/paw, **A**)-, capsaicin (5.2 nmol/paw, **B**)- (B) and PMA (50 nmol/paw, **C**)-induced licking in mice. Each column represents the mean of six to eight mice and the error bar indicates the S.E.M. Control values (gray bar) indicate the mice injected with vehicle i.p. and the asterisks denote the significance levels, when compared with control groups (one-way ANOVA followed by Newman-Keuls test): *P<0.05; ** P<0.01 and *** P<0.001.

Figure 4: Effects of intraperitoneal injection of the myricitrin (30 mg/kg) on mechanical hyperalgesia induced by bradykinin (BK, 3 nmol/paw); epinephrine (EPI, 450 nmol/paw) or prostaglandin E_2 (PGE_2, 10 nmol/paw) in rat paw. The closed columns indicate the control values (C, phosphate-buffered solution, injection paw), and the diagonally hatched column indicates the BK-, EPI-, or

PGE_2-injected paws, in the absence or the presence (open column) of the myricitrin. Each column represents the mean of six rats and the error bars indicate the S.E.M. The symbols denote significance levels ***P*<0.01 and ****P*<0.001 compared to saline group; #*P*<0.001 compared to vehicle i.p. plus BK i.pl. group (one-way ANOVA followed by Newman-Keuls test).

Figure 5: Effect of pre-treatment of mice with naloxone (5 mg/kg, i.p.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and morphine (5 mg/kg, s.c.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice and the error bars indicate the S.E.M. The symbols denote significance levels ****P*<0.001 compared to control group; #*P*<0.001 compared to morphine and myricitrin without naloxone group (one-way ANOVA followed by Newman-Keuls test).

Figure 6: Effect of pre-treatment of mice with L-arginine (50 mg/kg, i.p.) or D-arginine (50 mg/kg, i.p.) on the antinociceptive profiles of myricitrin (1 mg/kg, i.p.) and L-NOARG (25 mg/kg, i.p.) against the acetic acid-induced writhing in mice. Each column represents the mean of six to eight mice and the error bars indicate the S.E.M. The symbols denote significance levels ****P*<0.001 compared to control group; #*P*<0.05 compared to L-NOARG and myricitrin without L-arginine group (one-way ANOVA followed by Newman-Keuls test).

Figure 7: Effect of neonatal treatment with vehicle (clear bars) and capsaicin (50 mg/kg, dark bars) on the antinociceptive action of myricitrin (1 and 10 mg/kg)

against the acetic acid-induced writhing in mice. Each column represents the mean of eight to ten mice and the error bars indicate the S.E.M. The symbols denote significance levels *P<0.05 and ***P<0.001 compared to control group (vehicle neonatal plus saline i.p.); #P<0.001 compared to capsaicin neonatal plus saline i.p. group (one-way ANOVA followed by Newman-Keuls test).

Figure 8: Western blots showing the inhibitory effect of myricitrin (1 mg/kg, i.p.) in the translocation from cytossol (A and C) to membrane (B and D) of PKC-α (upper panel) and PKCε (lower panel) in response to i.pl. injection of PMA into mice paw. Mice paw tissues were obtained from basal (PBS) or PMA injected. Membrane and cytosolic levels of PKC-α and PKCε were determined using specific antibody. Results were normalized by arbitrarily setting the densitometry of the basal group and are expressed as mean ± SEM (n=3). #P<0.05, as compared with basal values and *P<0.05 compared to control groups (saline i.p. plus PMA i.pl.), one-way ANOVA followed by Student-Newman-Keuls' test.

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Table 1 – Effect of myricitrin in the motor performance (rota-rod test), locomotor activity (Open-field test) and corporal temperature of mice.

	Rota-rod (s)	**Open field (crossing number)**	**Temperature (^{o}C)**
Saline	97.8 ± 14.0	113.2 ± 4.5	29.4 ± 0.6
Myricitrin 30 mg/kg	97.6 ± 11.4	122.3 ± 29.5	29.0 ± 0.5
Myricitrin 100 mg/kg	120.0 ± 0	120.4 ± 7.2	28.6 ± 0.5

Data are expressed as mean ± S.E.M of 6 animals.

FIGURE 1

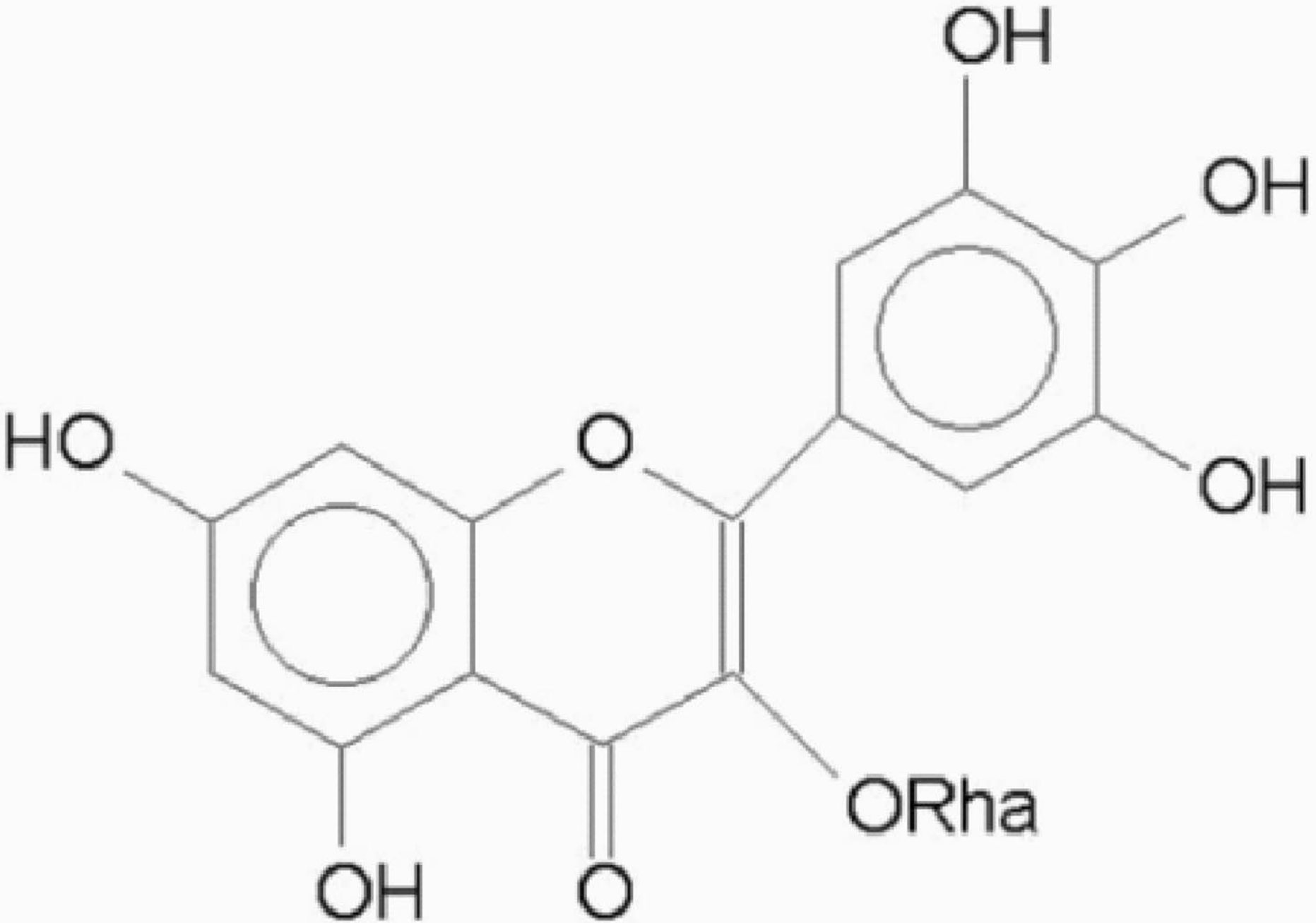

FIGURE 2

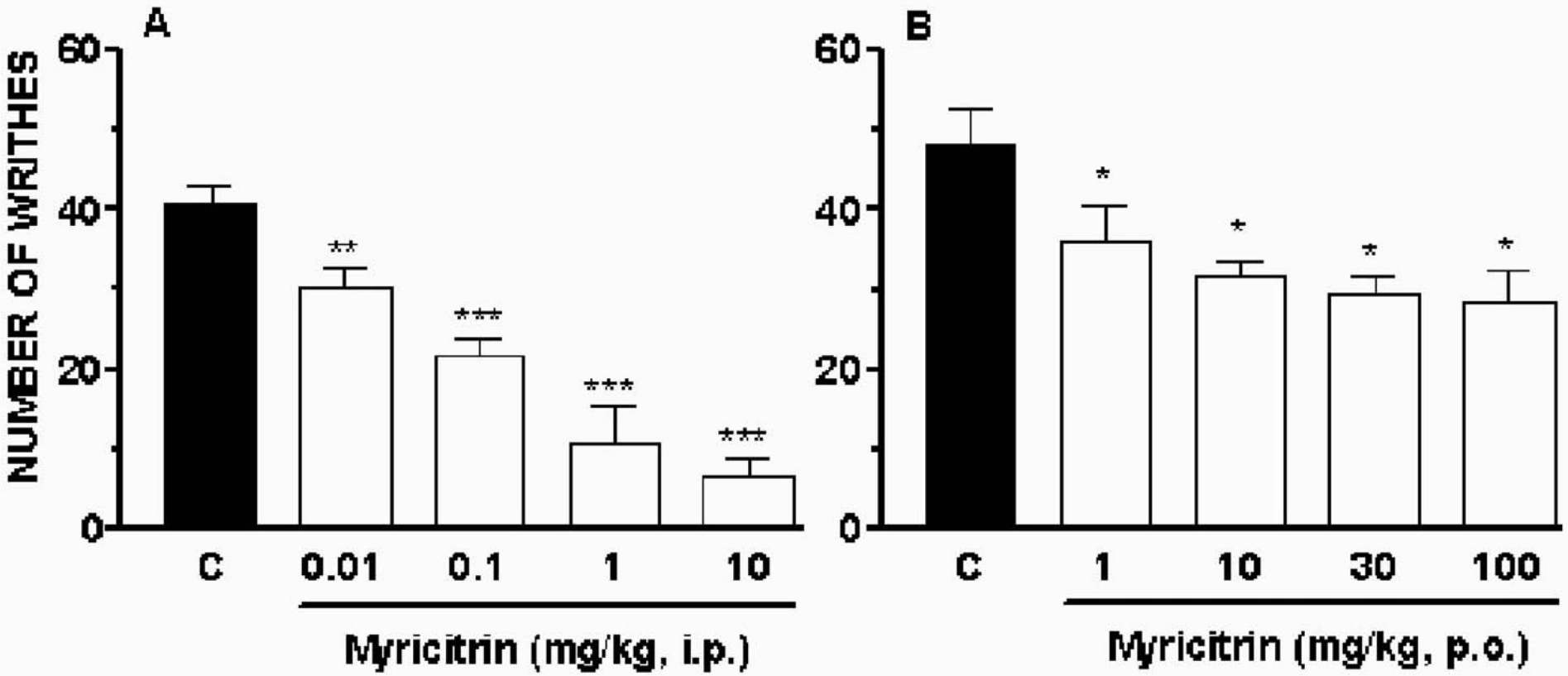

FIGURE 3

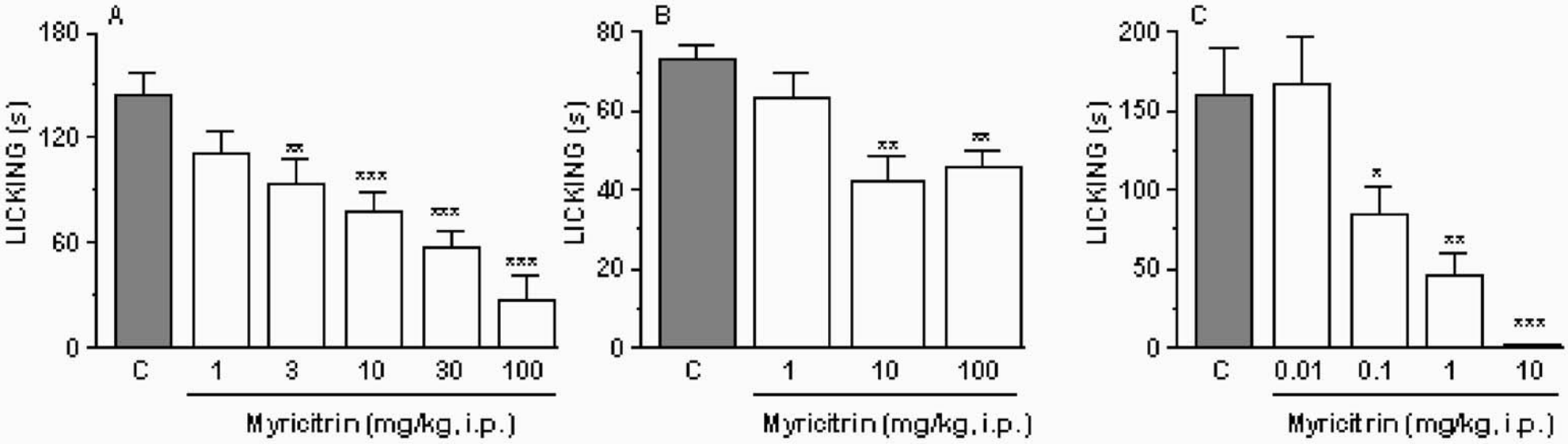

FIGURE 4

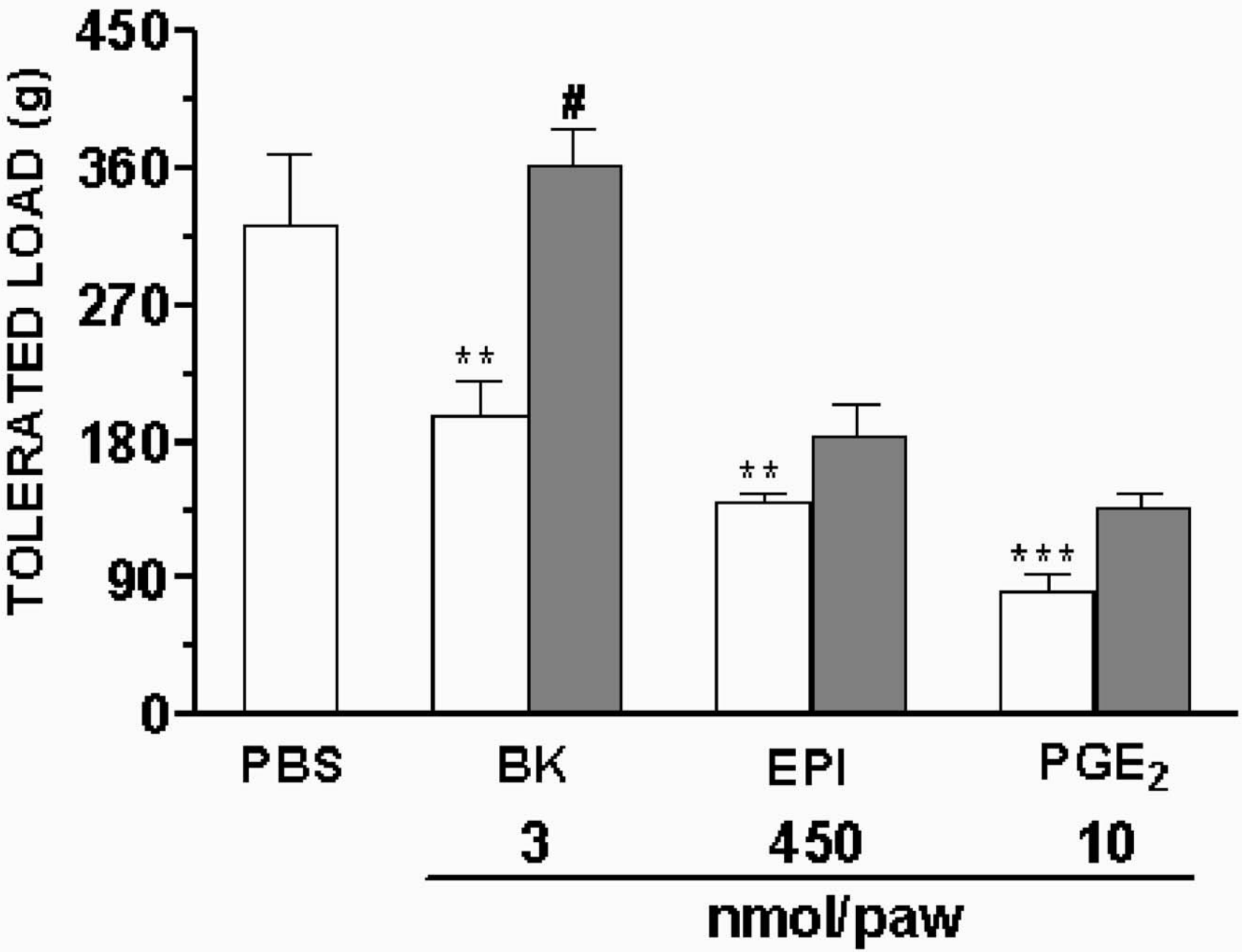

FIGURE 5

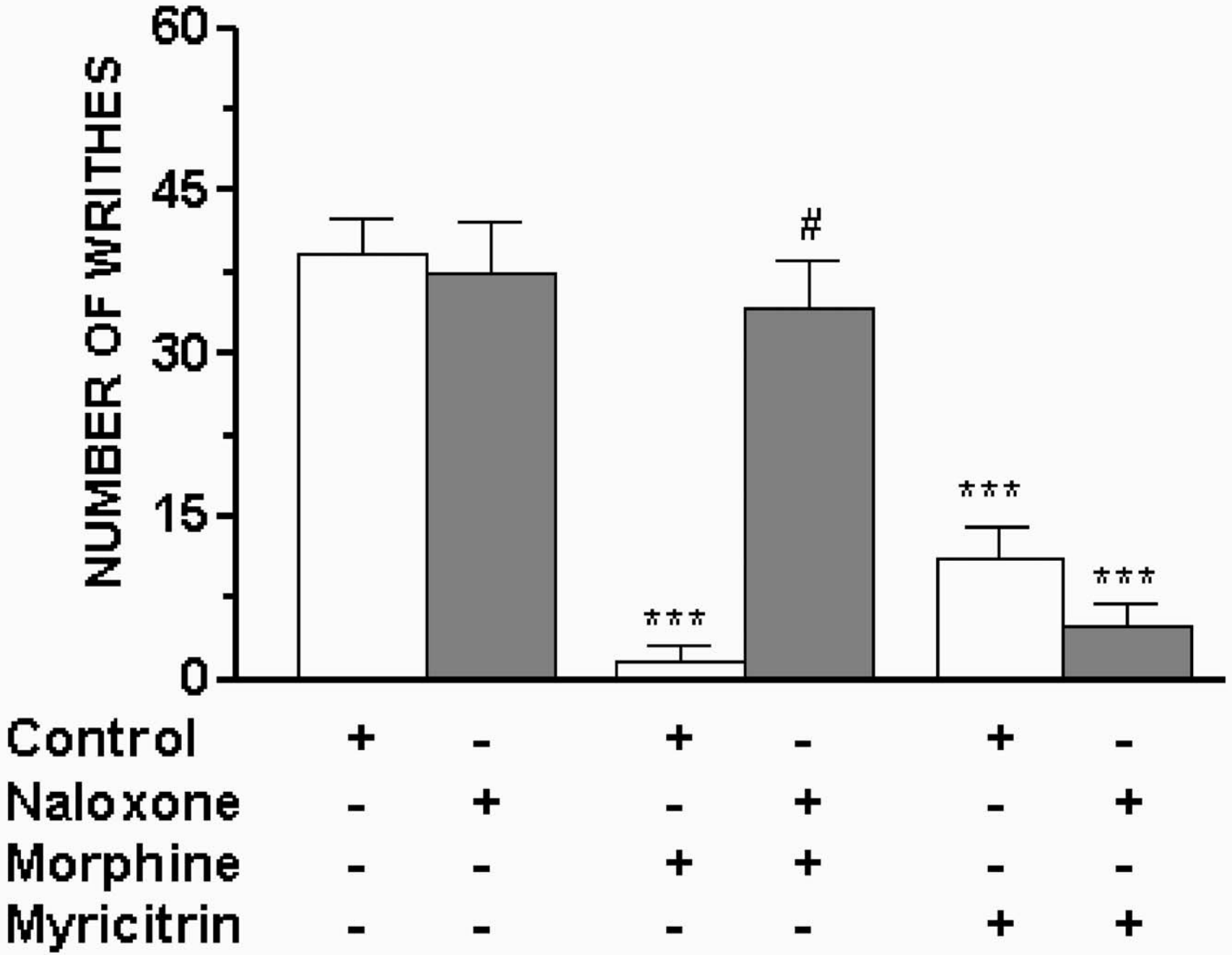

FIGURE 6

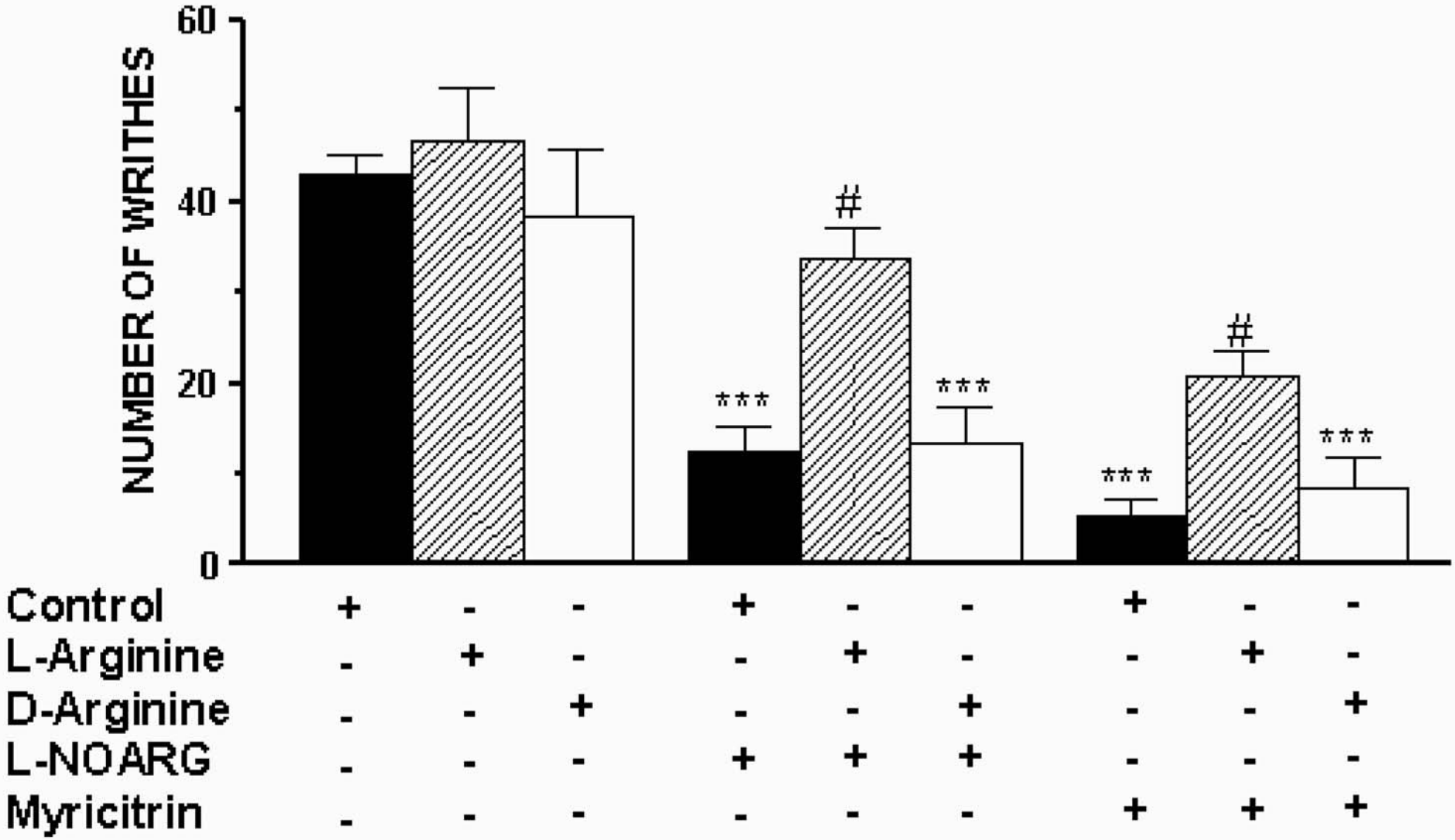

FIGURE 7

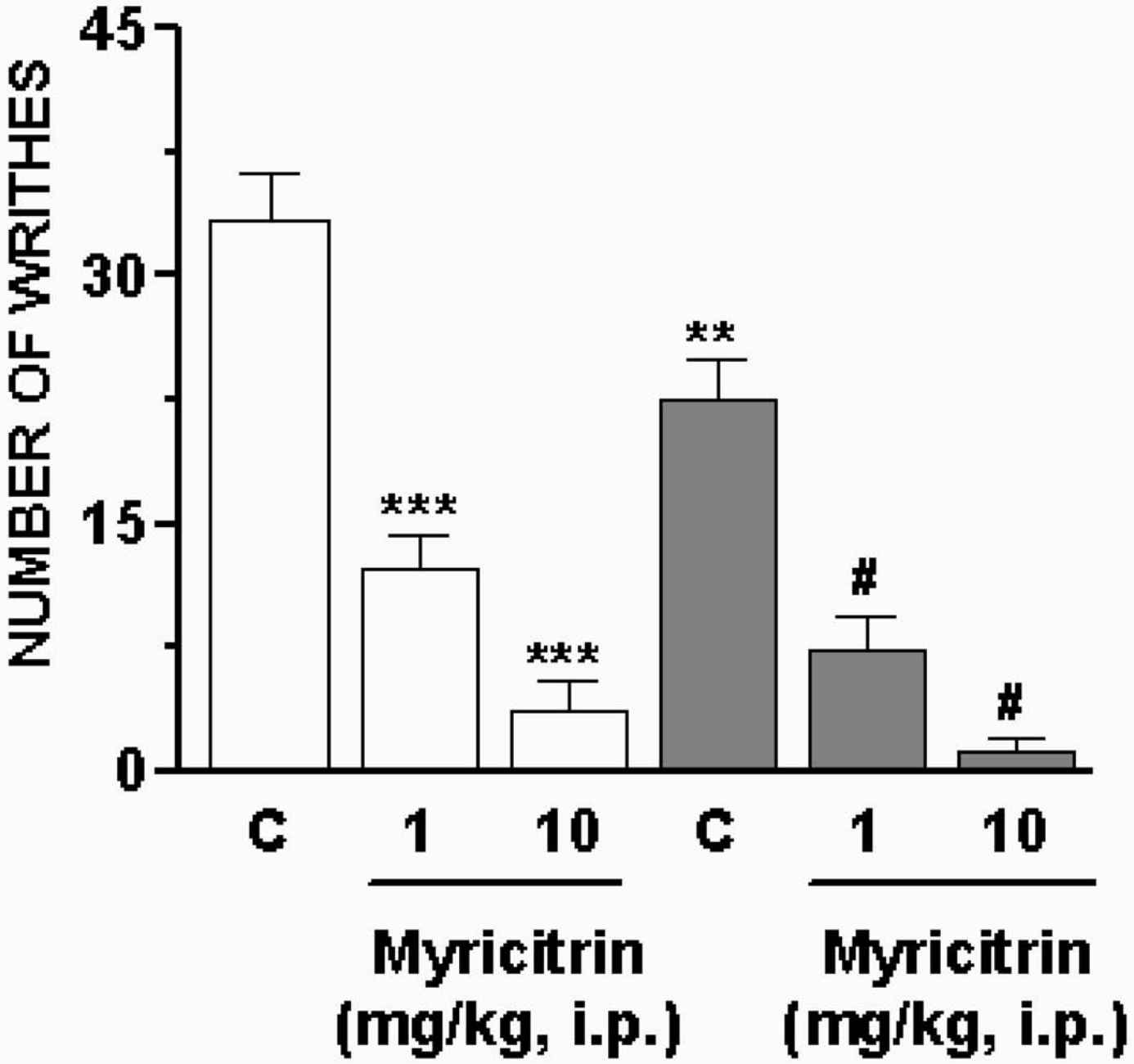

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FIGURE 8