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Antinociceptive action of limonexic acid obtained from *Raulinoa echinata*

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Abstract

The antinociceptive effect of the limonexic acid isolate of *Raulinoa echinata* Cowan in four models of pain in mice is described. When evaluated against acetic acid-induced abdominal constrictions, limonexic acid (10, 30 and 60 mg kg^{-1}, i.p.) produced dose-related inhibition of the number of constrictions, with a mean ID50 value of 43 (2.3–79) μmol kg^{-1}, and was more potent than some standard drugs. In the formalin test, limonexic acid inhibited both the first and second phases of formalin-induced pain. Furthermore, the effect was more pronounced in the second phase, with a mean ID50 value of 13.66 (9.35–19.61) μmol kg^{-1}, and had a pharmacological profile that was similar to standard drugs such as acetaminophen and acetyl salicylic acid. Limonexic acid also produced dose-related inhibition of glutamate- and capsaicin-induced pain, with mean ID50 values of 11.67 (8.51–16.0) μmol kg^{-1} and 47.17 (36.51–60.93) μmol kg^{-1}, respectively. The mechanism of action is not completely understood, but seems to involve direct interaction with the GABAergic and nitroxidergic pathways.

Introduction

The Rutaceae family is characterized by a great diversity of uncommon metabolites, mainly alkaloids, coumarins and limonoids. *Raulinoa* is a monospecific genus, and the species *Raulinoa echinata* Cowan is a spiny shrub endemic to the Vale do Itajaí region of Santa Catarina, Brazil. It grows exclusively on the banks of the Itajaí River and is known as 'Cutia-de-espinho' or 'sarandi'.

From *R. echinata*, abundant quantities of limonexic acid and limonin were previously isolated and identified, together with three degraded limonoids (Biavatti et al 2005). Several biological activities were attributed to limonin. For example, it is a chemopreventive agent and an insect antifeedant. Cytotoxic (Roy & Saraf 2006) and antioxidant potential was also detected (Breksa & Manners 2006). Limonin is one of the major components of the Chinese medicinal species *Evodia rutaecarpa* (Iwata et al 2005). Limonin at 30 or 100 mg kg^{-1}, isolated from the dried fruits of *E. rutaecarpa* var. *bodinieri*, significantly decreased the frequency of licking and biting behaviour during a unit of time in the late phase without affecting that of the early phase in the formalin test. Limonin inhibited the acetic acid-induced increase in vascular permeability and the decrease in paw oedema induced by carrageenin, and also showed inhibitory effects on bradykinin-induced paw oedema and arachidonic acid-induced ear swelling, suggesting that limonin possesses an antinociceptive effect accompanied by an anti-inflammatory action (Matsuda et al 1998).

Limonexic acid has also been isolated from other traditional Chinese species, namely *Dictamnus angustifolius* (Wu et al 1999) and *Tetradium trichotomum* (Quader et al 1990), and is one of the main compounds of *Citrus nippokoreana* fruits (Connolly & Hill 2001). Although limonexic acid has been identified since 1948 (Kondo et al 1985), there is no report of significant bioactivity of this limonoid. Our previous work verified that limonexic acid significantly reduced the survival time of leaf-cutter ants (*Atta sexdens rubropilosa*) (11 days) compared with the control group (22 days) (Biavatti et al 2005). Limonexic acid also displayed weak inhibitory activity when assayed in-vitro against trypomastigote forms of *Trypanosoma cruzi* (Biavatti et al 2001). This paper evaluates for the first time the antinociceptive activity of limonexic acid (Figure 1) in several models of nociception in mice.

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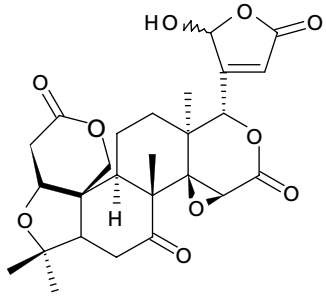

Figure 1 Structure of limonexic acid.

Materials and Methods

Plant material

Roots and stems of *R. echinata* were collected in Ressacada, Ibirama, SC, Brazil, and identified by A. Reis (Federal University of Santa Catarina) and J. R. Pirani (University of São Paulo). Voucher specimens (A. Reis & M. Biavatti 2.570 (26/07/98)) were deposited at the Herbário Barbosa Rodrigues, Itajaí, Santa Catarina, Brazil.

Extraction and isolation of limonexic acid

The dried crushed stems were exhaustively extracted with methanol after solvent elimination in a rotary evaporator. The crude extract obtained (80 g) was partitioned with dichloromethane (yielding 9 g), then chromatographed over silica gel (70–230 mesh) using an increasing polarity gradient of hexane/acetone (9:1, 7:3, 5:5, 3:7 and 0:10) as eluent, yielding 17 sub-fractions (A–P). Sub-fractions D–G (4.1 g) were grouped and re-chromatographed under the same conditions as above (over silica gel and hexane/acetone gradient as eluent), yielding sub-fractions A–T. Sub-fraction T (1.2 g) was purified by preparative TLC using hexane/benzene (1:1), yielding 650 mg of limonexic acid, which presented spectral data consistent with the literature (Biavatti et al 2001).

Drugs and reagents

The following substances were used: acetic acid, formalin acetaminophen, acetyl salicylic acid and morphine hydrochloride (Merck, Darmstadt, Germany); N^{ω}-nitro-L-arginine, L-arginine hydrochloride, capsaicin, clonidine hydrochloride, L-glutamic acid hydrochloride, naloxone hydrochloride, yohimbine hydrochloride (Sigma Chemical Co., St Louis, USA); prazosin (Pfizer, New York, USA); baclofen, phaclofen and muscimol (RBI, Natick, MA, USA). The drugs were dissolved in saline, with the exception of capsaicin, which was dissolved in absolute ethanol. Limonexic acid was dissolved in Tween 80 and diluted just before use in 0.9% NaCl. The final concentration of Tween 80 and ethanol did not exceed 5% and did not have any effect in itself. All the test doses of drugs were chosen based on our pilot experiments and due to the limited amount it was not possible to test them orally.

Animals

The experiments were conducted using male Swiss mice (25–35 g), housed at 22±2°C under a 12-h light–dark cycle (lights on at 0600 hours) and with free access to food and water. The animals were acclimatized to the laboratory for at least 1 h before testing, and were used only once throughout the experiments. The experiments were carried out after approval of the protocol by the institutional ethics committee (113/2005-03 CEP-UNIVALI) and in accordance with the Current Guidelines for the Care of Laboratory Animals and the Ethical Guidelines for Investigations of Experimental Pain in Conscious Animals (Zimmermann 1983). The number of animals (six to eight per group) and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of the drug treatments. Afterwards, the animals were killed by exposure to CO_2.

Acetic acid-induced nociception

Abdominal constrictions were induced according to procedures described previously (Collier et al 1968), with minor modifications. The animals were pre-treated with limonexic acid (10–60 mg kg^{-1}, i.p.) 30 min before acetic acid injection (0.6%, 0.45 mL/mouse). The control animals received a similar volume of vehicle. After the challenge, pairs of mice were placed in separate boxes and the number of abdominal constrictions was cumulatively counted over a period of 20 min. Antinociceptive activity was expressed as the reduction in the number of constrictions in mice pre-treated with limonexic acid. Acetaminophen and acetyl salicylic acid were used as positive controls to compare the potency of the antinociceptive effect of limonexic acid in this model.

Formalin-induced nociception

The procedure used was similar to that described previously (Hunskaar et al 1985; Hunskaar & Hole 1987), with minor modifications. The animals received 20 μL (2.5%) of formalin (0.92% formaldehyde) made up in phosphate buffer solution, under the surface of the right hindpaw. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of pain. The initial nociceptive scores normally peaked 5 min after formalin injection (first phase) and 15–30 min after formalin injection (second phase), representing the neurogenic and inflammatory pain responses, respectively. The animals were treated with limonexic acid (10–60 mg kg^{-1}, i.p.) and/or vehicle (saline 0.9%/10 mL kg^{-1}, i.p.) 30 min before formalin injection. After intraplantar injection of formalin, the animals were immediately placed in a glass cylinder, 20 cm in diameter, and the time spent licking the injected paw was determined.

Capsaicin-induced nociception

The method used was similar to that described previously by Sakurada (1992). The animals were placed in individual transparent glass cylinders (20 cm in diameter), which served

as observation chambers. After treatment with limonexic acid (10–60 mg kg^{-1}) and/or vehicle (10 mL kg^{-1}, i.p.), 20 μL of capsaicin (1.6 μg/paw prepared in phosphate-buffered solution) was injected under the skin of the dorsal surface of the right hind paw. Pairs of mice were placed in different glass cylinders for 5 min following capsaicin injection. The amount of time spent licking the injected paw was timed using a chronometer and was considered as indicative of nociception.

Glutamate-induced nociception

The procedure used in this test was similar to that described previously by Beirith et al (2002). The animals were observed individually for 15 min following injection of glutamate (30 μmol/paw). The amount of time spent licking the injected paw was timed with a chronometer and was considered indicative of nociception. The animals were treated with limonexic acid (10–60 mg kg^{-1}) 30 min before the glutamate injection. The control group received a similar volume of vehicle (10 mL kg^{-1}, i.p.) used to dilute limonexic acid.

Mechanism of action of limonexic acid

To determine some of the mechanisms by which limonexic acid causes antinociception in the acetic acid-induced visceral nociception, the animals were pre-treated with different drugs. The doses of the drugs used were selected based on data found in the literature, and on previous results from our laboratory. To assess the possible participation of α-adrenergic receptors in the antinociception caused by limonexic acid, the animals were pre-treated with yohimbine (α_2 antagonist, 0.15 mg kg^{-1}, i.p.) or with prazosin (α_1 antagonist, 0.15 mg kg, i.p.) 15 min before administration of limonexic acid (60 mg kg^{-1}, i.p.), phenylephrine (α_1 agonist, 10 mg kg^{-1}, i.p.) or clonidine (α_2 agonist, 0.15 mg kg^{-1}, i.p.). The pain response caused by injection of acetic acid (0.6%) was analysed 30 min after the administration of limonexic acid or agonists. Other groups of animals received only limonexic acid (60 mg kg^{-1}, i.p.), phenylephrine, clonidine or the vehicle (saline, 10 mL kg^{-1}) 30 min before acetic acid injection.

In separate experiments, we investigated the possible participation of the nitric oxide L- arginine pathway on the antinociceptive effect caused by limonexic acid. For this, the animals were pre-treated with L-arginine (600 mg kg^{-1}, i.p.) and after 15 min they received limonexic acid (60 mg kg^{-1}, i.p.) or N^G-nitro-L-arginine (a nitric oxide inhibitor, L-NOARG, 75 mg kg^{-1}, i.p.). The pain caused by acetic acid injection was analysed 30 min after treatment of the animals with limonexic acid or with L-NOARG. The other groups received limonexic acid or the vehicle (saline, 10 mL kg^{-1}) 30 min before acetic acid injection.

In other experiments, we investigated the possible participation of the opioid system on the analgesic effect caused by limonexic acid. The animals were pre-treated with limonexic acid (60 mg kg^{-1}, i.p.) and morphine (5 mg kg^{-1}, s.c.) 30 min before acetic acid injection. In a separate group of mice, we analysed the effect of naloxone (5 mg kg^{-1}, i.p.) injected 15 min before against the analgesic effect caused by both morphine and limonexic acid. The control animals received a similar volume of 0.9% NaCl (10 mL kg^{-1}, i.p.). Finally, in the same model, the possible participation of the GABAergic system in the limonexic acid-induced analgesic effect was investigated. For this purpose, the animals were pre-treated with bicuculin (1.0 mg kg^{-1}, i.p.), a $GABA_A$ antagonist, or phaclofen (2.0 mg kg^{-1}, i.p.), a $GABA_B$ antagonist, and after 15 min they received limonexic acid (60 mg kg^{-1}, i.p.), or muscimol (2.0 mg kg^{-1}, i.p.) or baclofen (2.0 mg kg^{-1} i.p.), which are $GABA_A$ and $GABA_B$ receptor agonists, respectively. The pain caused by acetic acid injection was analysed 30 min after treatment of animals with limonexic acid, muscimol or baclofen. The other groups received limonexic acid or the vehicle (saline, 10 mL kg^{-1}), 30 min before the acetic acid injection.

Statistical analysis

The results are represented as the mean ± s.e.m., except for the ID50 values (dose of extracts that reduced responses by 50% relative to the control values), which are presented as the geometric means with respective 95% confidence limits. The ID50 values were determined using linear regression GraphPad software (GraphPad Software, San Diego, CA, USA). The statistical significance between groups was calculated by analysis of variance followed by the Student–Newman–Keuls test. P values of less than 0.05 were considered as indicative of significance.

Results

Limonexic acid was isolated in crystalline form from the stems of *R. echinata*, with melting point and spectral data in accordance with previously published data (Biavatti et al 2001).

Acetic acid-induced nociception

The results in Figure 2 show that limonexic acid, administered intraperitoneally 30 min prior to testing, produced a dose-dependent inhibition of acetic acid-induced abdominal constrictions in mice, with 58 ± 80.0% inhibition at a dose of 60 mg kg^{-1}. In addition, the potency of limonexic acid was determined and gave a calculated ID50 value of 43.0 (2.3–79)

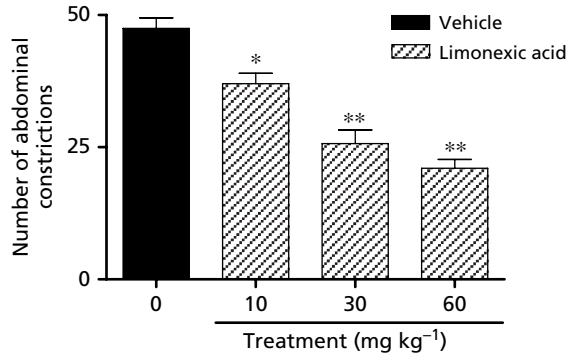

Figure 2 Effect of intraperitoneal injection of limonexic acid in the acetic acid-induced abdominal constriction test in mice. Each column represents the mean for six to eight animals and the error bars indicate the s.e.m. The closed column represents the control group (animals treated with the vehicle). *$P < 0.05$, **$P < 0.01$ significantly different compared with the control group.

Table 1 Antinociceptive effect of limonexic acid and some well known analgesic drugs in formalin and acid acetic-induced pain models

Treatment	Acetic acid		Formalin test	
	ID50 (μmol kg^{-1})	MI (%)	ID50 (μmol kg^{-1})	MI (%)
Limonexic acid	43.0 (2.3–7.9)	58.80	13.66 (9.35–19.96)[a]	78.69
Acetaminophen	25.8 (105.9–152.08)	79.18	34.52 (25.4–48.53)[a]	72.04
Acetyl salicylic acid	133.0 (72.2–244)	83.12	123.45 (76.22–209.56)[a]	85.34

95% confidence limits are given in parentheses. MI, maximal inhibition obtained with a dose of 60 mg kg^{-1}. [a]Second phase of formalin test.

μmol kg^{-1} (Table 1), indicating that it was about 3- to 4-times more active than acetylsalicylic acid and acetaminophen in the acetic acid-induced abdominal constriction test. This suggest that limonexic acid is a partial agonist in the acetic acid-induced abdominal constriction test, since the maximal response is lower than that of the other compounds tested.

Formalin-induced nociception

In the formalin test, the results showed that limonexic acid was effective in preventing the first and second phases of formalin-induced pain (neurogenic and inflammatory pain, respectively) (Figure 3). In the first phase, the maximal inhibitory value was 33.90% at a dose of 60 mg kg^{-1}; it was not possible to calculate the ID50. Limonexic acid was more effective against inflammatory pain, with 78.69% inhibition of pain, presenting a calculated ID50 value of 13.66 (9.35–19.61) μmol kg^{-1}. Limonexic acid was about 2- and 9-times more potent than acetaminophen and acetylsalicylic acid, respectively, in the second phase of formalin-induced pain (Table 1). In addition, this experimental model showed that limonexic acid reduced the paw oedema induced by formalin (results not shown), which suggests a possible anti-inflammatory effect of limonexic acid.

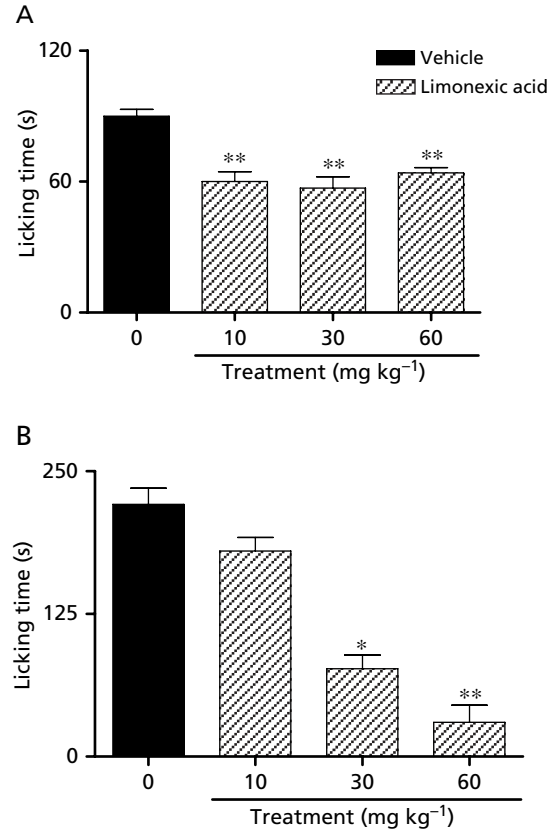

Figure 3 Effect of intraperitoneal injection of limonexic acid in the formalin test in mice. The total time (mean ± s.e.m.) spent licking the hindpaw was measured against the first phase (0–5 min; A) and the second phase (15–30 min; B) after intraplantar injection of formalin into the hindpaw. Each column represents the mean for six to eight animals and the error bars indicate the s.e.m. The closed column represents the control group (animals treated with the vehicle). *$P < 0.05$, **$P < 0.01$ significantly different compared with the control group.

Capsaicin-induced nociception

The capsaicin test in mice has been used to access the antinociceptive effect of the tachykinin neurokinin-1 receptor antagonist, glutamate receptor antagonist, nitric oxide synthase inhibitor and morphine (Sakurada 1992). Limonexic acid was tested intraperitoneally in mice. The results indicate that limonexic acid reduces the inhibition of capsaicin-induced pain in a dose-dependent manner, with a calculated ID50 value of 11.67 (8.51–16.0) μmol kg^{-1} (Figure 4). Limonexic acid was effective against neurogenic pain, as observed before in the first phase of the formalin test.

Glutamate-induced nociception

The results given in Figure 5 show that, at different doses, limonexic acid exhibited significant inhibition of the glutamate-induced nociceptive response in mice, with an ID50 value of 47.17 (36.51–60.93) μmol kg^{-1}.

Mechanism of action of limonexic acid

The results of the analysis of the possible mechanism of action of limonexic acid are given in Table 2. Pre-treatment of the mice with naloxone, 15 min before injection of morphine, largely reversed the antinociception caused by morphine when analysed in the acetic acid model, leaving the antinociceptive effect of limonexic acid unaffected. Pre-treatment of the animals with α-adrenergic antagonists, prazosin (0.2 mg kg^{-1}, i.p.) or yohimbine (0.2 mg kg^{-1}, i.p.), 30 min before morphine injection, caused marked inhibition of the analgesic effect induced by phenylephrine (10 mg kg^{-1}, i.p.) and clonidine (0.2 mg kg^{-1}, i.p.) but failed to reverse the

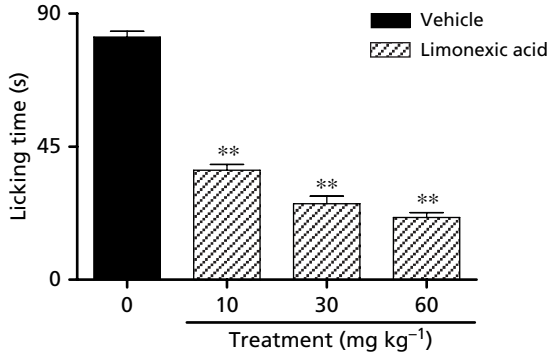

Figure 4 Effect of intraperitoneal injection of limonexic acid in the capsaicin test in mice. Each column represents the mean for six to eight animals and the error bars indicate the s.e.m. The closed column represents the control group (animals treated with the vehicle). $^{**}P < 0.01$ significantly different compared with the control group.

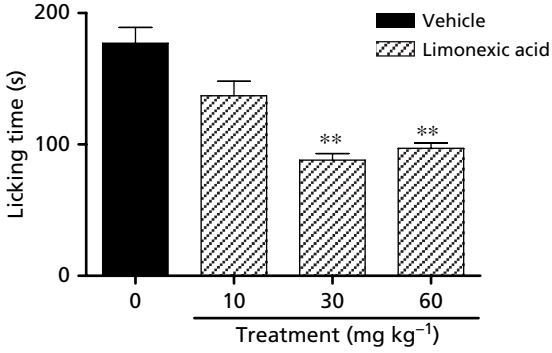

Figure 5 Effect of intraperitoneal injection of limonexic acid in the glutamate test in mice. Each column represents the mean for six to eight animals and the error bars represents the s.e.m. The closed column indicates the control group (animals treated with the vehicle). $^{**}P < 0.01$ significantly different compared with the control group.

analgesic effect of limonexic acid. Treatment of the mice with L-arginine (600 mg kg^{-1}, i.p.), a precursor of nitric oxide synthase, almost completely reversed the antinociceptive action caused by injection of L-NOARG (75 mg kg^{-1}, i.p., 30 min before) and limonexic acid. Finally, bicuculine, a $GABA_A$ antagonist, and phaclofen, a $GABA_B$ antagonist, reversed the analgesic response of muscimol, baclofen ($GABA_A$ and $GABA_B$ receptor agonists, respectively) and limonexic acid.

Discussion

Limonexic acid was isolated from the stems of *R. echinata*, and differs from limonin only by the oxidation of the furane ring, being considered a direct limonin biogenetic precursor. Several limonoids and their glucosides are found in the seeds of *Citrus* fruits, being responsible for their characteristic bitterness. Their occurrence in the plant kingdom is confined to plant families of the Rutales order, and they occur more abundantly in Meliaceae and Rutaceae.

Table 2 Effect of various drugs on the antinociception caused by limonexic acid assessed in the acetic acid-induced abdominal constriction test

Treatment	Dose (mg kg^{-1})	Number of constrictions
Control	0	54 ± 3.2
Naloxone	5	52 ± 4.21
Morphine	5	6.8 ± 2.1***
Limonexic acid	60	24.65 ± 3.42**
Morphine + naloxone	5/5	48 ± 3.42
Limonexic acid + naloxone	60/5	23.62 ± 2.92^{NS}
Control	0	58 ± 3.42
L-Arginine	600	55 ± 2.48
L-NOARG	75	12 ± 2.35***
Limonexic acid	60	22.8 ± 2.44***
L-NOARG + L-arginine	75/600	49.24 ± 4.6†
Limonexic acid + L-arginine	60/600	53.14 ± 0.55†
Control	0	42.50 ± 2.29
Prazosin	0.15	32.16 ± 4.19
Phenylephrine	10	4.0 ± 0.89***
Phenylephrine + prazosin	10/0.15	48.40 ± 2.53†
Yohimbine	0.15	38.6 ± 3.24
Clonidine	0.1	2.5 ± 1.39***
Clonidine + yohimbine	0.1/0.15	48.6 ± 2.6†
Limonexic acid	60	19.45 ± 4.2
Limonexic acid + yohimbine	60/0.15	21.5 ± 2.33^{NS}
Limonexic acid + prazosin	60/0.15	17.54 ± 3.36^{NS}
Control	0	38.90 ± 4.25
Bicuculine	1.0	37.45 ± 1.17
Phaclofen	2.0	41.24 ± 0.85
Baclofen	2.0	21.14 ± 2.41**
Muscimol	2.0	16.24 ± 3.12**
Limonexic acid	60	18.37 ± 1.22**
Bicuculine + muscimol	1.0/2.0	36.68 ± 3.41†
Phaclofen + baclofen	2.0/2.0	41.26 ± 2.54†
Bicuculine + limonexic acid	2.0/60	32.26 ± 0.86†
Phaclofen + limonexic acid	2.0/60	39.18 ± 1.36†

NS, not significant. $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different compared with the control value. $^{\dagger}P < 0.05$ significantly different compared with morphine, phenylephrine, clonidine, muscimol or baclofen.

There are ever continuing efforts to discover new analgesic agents with increased efficacy and improved side-effect profiles, and compounds obtained from medicinal plants have been extensively studied (Calixto et al 2000; Soneera & Vijay 2005). Limonin has been previously investigated and found to have an antinociceptive effect that may be accompanied by an anti-inflammatory action (Matsuda et al 1998), and the antinociceptive activity of *E. rutaecarpa* (the fruits of *E. rutaecarpa* Benth (Rutaceae) have long been used for inflammatory disorders) is partially attributable to limonin. In the present work, we studied the antinociceptive effect of limonexic acid. Attempts have been made to further investigate some of the possible mechanisms underlying the antinociceptive action of this compound. For this purpose, limonexic acid was studied in the acetic acid-induced abdominal constriction test and in the formalin-, capsaicin- and glutamate-induced pain models. The use of different models

is significant in the detection of antinociceptive properties of a substance, considering that a variety of stimuli can be used to recognize different types of pain and reveal the actual nature of the antinociceptive test drug (Bergerot et al 2006). Limonexic acid exhibited antinociceptive activity in all four animal models studied. However, the absolute and relative potencies of this compound varied depending on the experimental model used. Limonexic acid exhibited greater antinociceptive activity in animal models of inflammatory pain (acetic acid-induced abdominal constriction and formalin tests) and was less effective in models of acute pain (glutamate and capsaicin tests). Past studies have postulated that acetic acid acts indirectly by inducing the release of endogenous mediators that stimulate the nociceptive neurons, which are sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids (Collier et al 1968). The acetic acid-induced abdominal constriction test in mice, 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. Our results showed inhibition of abdominal constrictions at all doses of limonexic acid used in this experiment, indicating an antinociceptive effect, although the abdominal constrictions induced by acetic acid represent a peripheral nociception model (Wei et al 1986). The acetic acid-induced abdominal constriction test is a widely used animal model of visceral pain, involving intraperitoneal injection of an irritant that induces abdominal constriction, twisting and turning of the trunk, arching of the back and extension of the hind limbs. This is considered by several authors as a non-specific model, since several compounds, such as opioids, analgesics (Gray et al 1998; Singh et al 2003a, b), tricyclic antidepressants (Gonzalez et al 2001; Carter & Sullivan 2002), cannabinoids (Calixto et al 2000) and antihistamines (Yeh 1985) inhibit the abdominal constrictions induced by acetic acid. Therefore, there is a need for further studies using other antinociceptive models. Nevertheless, the acetic acid-induced abdominal constriction test in mice has long been used for the study of the mechanism of action of several analgesic compounds (Santos et al 2005; Meotti et al 2006).

Limonexic acid was also evaluated in the formalin test, with excellent results in terms of effect. This test, which causes local tissue injury to the paw, has been used as a model for tonic or neurogenic pain and localized inflammatory pain (Neves et al 2007). Neurogenic pain is caused by the direct activation of the nociceptive nerve terminals, while inflammatory pain is mediated by a combination of peripheral input and spinal cord sensitization (Hunskaar & Hole 1987; Tjolsen et al 1992). It has been demonstrated that intraplantar injection of formalin in rodents produces significant increases in spinal levels of different mediators, such as excitatory amino acids, prostaglandin E_2, nitric oxide and tachykinin and kinins, among other peptides (Tjolsen et al 1992; Santos et al 2005). Furthermore, systemic spinal and supraspinal administration of tachykinin receptor antagonists, nitric oxide synthase inhibitors, NMDA receptor antagonists, opioids, α_2-adrenoceptor agonists and NSAIDs were found to be effective in antagonizing formalin-induced nociception (Tjolsen et al 1992; Malmberg & Yaksh 1992, 1994, 1995; Chaplan et al 1997; Santos & Calixto 1997; Santos et al 1998). In addition, all of the mediators mentioned above are also involved in the inflammatory process, and the use of compounds that reduce their inflammatory effects are designated as being anti-inflammatory (Calixto et al 2000; Cabrini et al 2001; Arya & Kumar 2005). More specifically, the major transmission pathway for inflammatory pain has been documented as comprising peripheral polymodal receptors around small vessels that signal to the central nervous system via sensory afferent C-fibres entering the dorsal horn (Kumazawa et al 1996). In this study, limonexic acid was effective, although both phases of the response were significantly inhibited and the effect was more predominant in phase 2 (involving inflammation). In addition, limonexic acid was effective in reducing formalin-induced paw oedema. The fact that this compound was effective in both the acetic acid-induced abdominal constriction and the formalin tests suggests a peripheral antinociceptive effect and possible anti-inflammatory activity.

Sakurada et al (1993) proposed the capsaicin-induced pain model for the study of compounds that act on pain of a neurogenic origin. Capsaicin is a neurotoxic amine extracted from red pepper which, when applied to the skin or injected into animals, produces irritation, a painful reaction and subsequent desensitization to chemically induced pain (Jancsón et al 1981). Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide and pro-inflammatory mediators in the peripheral sensory neurons, and transmits nociceptive information to the spinal cord (Sakurada et al 1996, 2003). Our results indicate a significant reduction in neurogenic nociception caused by the intraplantar injection of capsaicin, showing that limonexic acid caused significant effects when administered intraperitoneally at different doses. This is an interesting finding because the capsaicin-induced neurogenic paw licking response was similar to the first phase of the formalin test. Compounds with this action are good candidates for the treatment of neuropathic conditions, for which effective treatment is difficult (Akada et al 2006).

We also investigated the glutamatergic systems on limonexic acid-induced analgesia. Several glutamatergic receptors such as $NMDA_R$ and metabotropic glutamate receptors have been shown to be involved in the modulation of glutamate-induced nociception (Hizue et al 2005; Santos et al 2005; Yoon et al 2006). Beirith et al (2002) found that the glutamate-induced nociceptive response appears to involve peripheral, spinal and supraspinal sites of mediated action, which are modulated by their receptors (NMDA and non-NMDA) as well as by the release of nitric oxide or by some nitric oxide-related substance. Choi et al (2001) demonstrated that aspirin and acetaminophen reduced nociceptive behaviour induced by glutamate in a dose-dependent manner when administered intrathecally, which suggests that the antinociceptive effect of these drugs occurs not only through selective inhibition of prostaglandin synthetase. In fact, the intraplantar injection of glutamate induced direct stimulation of the nociceptive neurons, causing the liberation of various anti-inflammatory and neuropeptide mediators involved in the transmission of pain (Yashpal et al 2001). In this work, completing the study of the antinociceptive effect of limonexic acid, we verified that this compound was effective in reducing glutamate-induced pain.

Concerning the mechanism by which limonexic acid exerts its antinociceptive action, the antinociception elicited by limonexic acid seems to be independent of the activation of important endogenous analgesic systems, namely the opioidergic and noradrenergic systems. In fact, the antinociceptive action of limonexic acid, in contrast to that reported for morphine, was not reversed by naloxone, a non-selective opioid antagonist. Furthermore, the α_1- and α_2-adrenoceptors seem unlikely to be involved in the antinociceptive action of limonexic acid, given that selective antagonists of these receptors failed to alter the limonexic acid-induced antinociception in conditions where they produce significant inhibition of the antinociception provoked by the selective agonists. Our results also show that the antinociceptive profile of this compound appears, initially, to be related to the interaction of the L-arginine–nitric oxide pathway. Also relevant are our findings that limonexic acid was able to produce dose-dependent systemic inhibition of algesia induced by intraperitoneal injection of glutamate. These results also suggest that the antinociceptive effect of limonexic acid is related to interaction with glutamate receptors, but some nociceptive actions produced by glutamate are mediated by nitric oxide–cGMP pathway activation (Ferreira et al 1999; Beirith et al 2002). Nitric oxide is a modulator of the nociceptive processes and is able to produce hyperalgesia or antinociceptive effects depending on the experimental model and the dose or administration site tested (Moore et al 1991; Synder 1992; Haley et al 1998; Riedel & Neeck 2001). We have confirmed this hypothesis since L-NOARG markedly antagonized acetic acid-induced nociception, these effects being selectively reversed by intraperitoneal injection of the nitric oxide precursor, L-arginine. Also, in this study, the same treatment with L-arginine reversed the analgesic effect of limonexic acid, suggesting that L-arginine-derived or a nitric oxide-related pathway appears be involved in the antinociceptive of this compound. Another interesting finding of the present study was the involvement of the GABAergic pathway in the antinociceptive effect of limonexic acid. The modulation of ionotropic γ-aminobutyric acid (GABA) receptors (GABA-gated Cl(–) channels) by a group of natural products such as flavonoids was studied in electrophysiological experiments. Quercetin, apigenin, morine, chrysin and flavone inhibited ionic currents mediated by $\alpha(1)$ $\beta(1)$ $\gamma(2s)$ GABA(A) and GABA(C) receptors expressed in *Xenopus laevis* oocytes in the micromolar range (Goutman et al 2003). Baclofen, a GABA derivative, is a potent $GABA_B$ receptor agonist, which is clinically used for the treatment of spasticity, but also induces analgesia in certain animal models of pain (Aran & Hammond 1991; Shafizedeh et al 1997; Franek et al 2004). Our results confirm that baclofen markedly antagonizes acetic acid-induced nociception. In addition, the $GABA_A$ or $GABA_B$ antagonists bicuculine or phaclofen reversed the antinociceptive effect of limonexic acid, demonstrating that the antinociceptive effect of this compound also involves the GABAergic pathways. Considering the present data, we speculate that the antinociceptive action of limonexic acid is probably linked to the inhibition of both tachykinergic and glutamatergic pathways. This conclusion is derived in part from the fact that pre-treatment of animals with limonexic acid inhibited capsaicin and glutamate induced nociception, respectively. On the other hand, limonexic acid could be inhibiting the liberation of inflammatory and neuropeptide mediators involved in the pain process. This assertion is supported by the demonstration of the antinociceptive effect of this compound on both formalin and acetic acid-induced pain in the animal models of inflammatory pain. Further studies are necessary to elucidate the molecular events and pathway involved, as well as the structure–activity relationships.

Conclusion

Our results indicate that limonexic acid isolated from *R. echinata* presents an interesting profile of antinociceptive action in several models of pain. This compound was more potent than some clinically used drugs against acetic acid- and formalin-induced pain. Additional studies are necessary to determine the exact mechanism by which limonexic acid exerts its antinociceptive action, but our results suggest direct interactions with GABAergic and nitroxidergic pathways.

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