Pharmacology, Biochemistry and Behavior 93 (2009) 10–16

Contents lists available at ScienceDirect

Pharmacology, Biochemistry and Behavior

journal homepage: www.elsevier.com/locate/pharmbiochembeh

Mechanisms involved in the antinociception caused by ethanolic extract obtained from the leaves of *Melissa officinalis* (lemon balm) in mice

Giselle Guginski [a], Ana Paula Luiz [b], Morgana Duarte Silva [b], Murilo Massaro [b], Daniel Fernandes Martins [b], Juliana Chaves [a], Robson Willain Mattos [c], Damaris Silveira [c], Vânia M.M. Ferreira [c], João Batista Calixto [a], Adair R.S. Santos [a,b,*]

[a] Departamento de Farmacologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis, 88049-000, SC, Brazil
[b] Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, Florianópolis 88040-900, SC, Brazil
[c] Faculdade de Ciências da Saúde, Curso de Ciências Farmacêuticas, Universidade de Brasília, Campus Universitário Darcy Ribeiro (Asa Norte), Brasília 70910-900, DF, Brazil

ARTICLE INFO

Article history:
Received 11 April 2008
Received in revised form 23 March 2009
Accepted 31 March 2009
Available online 7 April 2009

Keywords:
Melissa officinalis
Rosmarinic acid
Nociception
Cholinergic system
Nitric oxide

ABSTRACT

The present study examined the antinociceptive effect of the ethanolic extract from *Melissa officinalis* L. and of the rosmarinic acid in chemical behavioral models of nociception and investigates some of the mechanisms underlying this effect. The extract (3–1000 mg/kg), given orally (p.o.) 1 h prior to testing, produced dose-dependent inhibition of acetic acid-induced visceral pain, with ID50 value of 241.9 mg/kg. In the formalin test, the extract (30–1000 mg/kg, p.o.) also caused significant inhibition of both, the early (neurogenic pain) and the late (inflammatory pain), phases of formalin-induced licking. The extract (10–1000 mg/kg, p.o.) also caused significant and dose-dependent inhibition of glutamate-induced pain, with ID50 value of 198.5 mg/kg. Furthermore, the rosmarinic acid (0.3–3 mg/kg), given p.o. 1 h prior, produced dose-related inhibition of glutamate-induced pain, with ID50 value of 2.64 mg/kg. The antinociception caused by the extract (100 mg/kg, p.o.) in the glutamate test was significantly attenuated by intraperitoneal (i.p.) treatment of mice with atropine (1 mg/kg), mecamylamine (2 mg/kg) or L-arginine (40 mg/kg). In contrast, the extract (100 mg/kg, p.o.) antinociception was not affected by i.p. treatment with naloxone (1 mg/kg) or D-arginine (40 mg/kg). It was also not associated with non-specific effects, such as muscle relaxation or sedation. Collectively, the present results suggest that the extract produced dose-related antinociception in several models of chemical pain through mechanisms that involved cholinergic systems (i.e. through muscarinic and nicotinic acetylcholine receptors) and the L-arginine-nitric oxide pathway. In addition, the rosmarinic acid contained in this plant appears to contribute for the antinociceptive property of the extract. Moreover, the antinociceptive action demonstrated in the present study supports, at least partly, the ethnomedical uses of this plant.

1. Introduction

Records concerning lemon balm use date back over 2000 years. Medicinal use throughout this early epoch includes a recommendation by Paracelsus (1493–1541) that the balm would be indicated for "all complaints supposed to proceed from a disordered state of the nervous system" (Kennedy et al., 2003; Allaverdiyev et al., 2004).

Melissa officinalis belongs to the Laminaceae family, is a perennial herb, up to 1 m high, growing in the Mediterranean region, western Asia, southwestern Siberia, and northern Africa. Parts mostly used are dried leaves; which often present flowering tops (Carnat et al., 1998; Herodez et al., 2003; Dastmalchi et al., 2008). Infusions prepared with the aerial part of *M. officinalis* are used in folk medicine for the treatment of fevers and colds, indigestion associated with nervous tension, hyperthyroidism, depression, mild insomnia, epilepsy, headaches, and toothaches among others (Carnat et al., 1998; Herodez et al., 2003, Salah and Jäger, 2005; Dastmalchi et al., 2008).

The scientific reported uses are: antioxidant (Carnat et al., 1998; Ribeiro et al., 2001), sedative (Kennedy et al., 2003; Müller and Klement, 2006), anti-inflammatory, hepatoprotective, digestive (Simmen et al., 2006; Schemann et al., 2006), anti-bacterial, antifungal, antiviral, antihistaminic (Carnat et al., 1998; Sandraei et al., 2003; Allaverdiyev et al., 2004), antikinetic, antilipidaemic (Bolkent et al., 2005), anxiolytic (Santos-Neto et al., 2006) and effective in controlling light to mild Alzheimer's cases (Akondzadeh et al., 2003; Ferreira et al., 2006).

Phytochemical studies carried out with *M. officinalis* have demonstrated the occurrence of many classes of constituents, including polyphenolic compounds (rosmarinic acid, caffeic acid and protocatechuic acid), essencial oils (citral), monotherpenoid aldehides, sesquiterpenes, flavonoids (luteolin) and tannins (Carnat et al., 1998; Heitz et al., 2000; Kennedy et al., 2003; Ziaková et al., 2003; Gazola et al., 2004;

* Corresponding author. Departamento de Ciências Fisiológicas, Universidade Federal de Santa Catarina, Campus Universitário, Trindade, 88040-900, Florianópolis, SC, Brazil. Tel.: +55 48 3721 9352; fax: +55 48 3721 9672.
E-mail address: arssantos@ccb.ufsc.br (A.R.S. Santos).

0091-3057/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pbb.2009.03.014

Bolkent et al., 2005; Salah and Jäger, 2005; Dastmalchi et al., 2008). In addition, the rosmarinic acid is the major compound of the *M. officinalis* ethanolic extract, in concentrations between 2 and 5% (Carnat et al., 1998). This substance has antiviral and antioxidant activities. Iuvone and col. (2006) demonstrated in a recent study that rosmarinic acid protects cells against neurotoxicity promoted by β-amiloid peptide, by *in vitro* assays.

Taking into account the biological activities of *M. officinalis*, it is surprising that no pharmacological study has been carried out on the possible antinociceptive effects of the extract up to now. Here, we have therefore examined the possible antinociceptive action of the extract in chemical models of nociception in mice. Attempts have been made to further investigate some of the possible mechanisms that underlie the antinociceptive action of the extract. In addition, we also analysed the possible antinociceptive effect of the rosmarinic acid present in this plant.

2. Material and methods

2.1. Animals

Experiments were conducted using Swiss mice (25–35 g) of both sexes, housed at 22 ± 2 °C under a 12 h light/dark cycle (lights on at 6:00 a.m.) and with access to food and water *ad libitum*. Animals (male and female mice 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 treatment.

2.2. Preparation of ethanolic extract of M. officinalis and HPLC analysis

The dried leaves of *M. officinalis* were kindly supplied by Centroflora Group (Botucatu, Brazil), that also provided a certificate of identity and quality. The powdered plant material (1.9 kg) was extracted by maceration at room temperature (24 ± 3 °C) using ethanol as solvent. After solvent elimination under vacuum and temperature lower than 40 °C, the ethanolic extract was obtained (13% yield) and stored at − 18 °C until use.

HPLC analysis of ethanolic extract was performed on a Shimadzu LC-MS 2010A apparatus with a Diode Array detector (SPD M20A, Shimadzu), coupled with an auto injector (SIL-20A, Shimadzu), both using the software LC MS Solutions 3.0 (Shimadzu). A Zorbax ODS column (5 µm, 4.6/250 mm: Agilent) coupled with a guard-column (5 µm, 4.6/12.5 mm: Agilent) was used. The mobile phase consisted of a gradient solvent system of aqueous formic acid 0.1% (A) and acetonitrile with 0.1% formic acid (B). The elution profile was, 0 to 25 min: 10 to 40% B (linear gradient); 25 to 30 min: 40 to 30% B (linear gradient); 31 to 35 min: 10% B. The Flow rate was 1 mL/min UV detection was set at 330 nm.

A sample of the extract (55.6 mg) was extracted using ODS C18 extraction cartridges (Accubond, Agilent) as follows: (1) the cartridge was preconditioned by rinsing with 3 ml of each of the following in sequence, methanol, water; (2) the sample was applied to the cartridge; (3) the analytes were eluted by rinsing the cartridge with 3 ml of ethanol: water (1:1). Then the solvent was evaporated to dryness at 37 °C under a gentle stream of nitrogen. The residue was dissolved in 4 ml of the mobile phase, filtered on a 0.45 µm PTFE membrane (Millex) and submitted to HPLC analysis, by injection of 5 µL.

The identity of the peaks relative to rosmarinic acid was established by comparison of retention time and UV spectra. A quantitative analysis was performed by the external standard method, plotting calibration curves at concentrations of 270, 360, 450, 600, 800 and 1000 µg/ml in mobile phase. Each determination was carried out in duplicate. Concentration is shown as percentage of rosmarinic acid in extract from *M. officinalis*.

2.3. Reagents

The following reagents were employed: acetic acid, tween 80, morphine hydrochloride from Merck, A.G. (Darmstadt, Germany); Nω-nitro-L-arginine (L-NOARG), naloxone hydrochloride, glutamic acid, L-arginine, D-arginine, atropine sulfate and pilocarpine hydrochloride (Sigma Chemical CO, St. Louis, USA), nicotine hydrochloride and mecamylamine hydrochloride (Tocris Cookson Inc., Ellisville, USA), rosmarinic acid (Sigma Chemical CO, St. Louis, USA) and saline (NaCl 0.9%) (LabSynth, São Paulo, Brazil). All other chemicals were of analytical grade and obtained from standard commercial suppliers. All reagents were dissolved in 0.9% NaCl solution, except the extract which was dissolved in tween 80 plus saline. The final concentration of tween 80 did not exceed 5% and did not cause any effect *per se*.

The solvents employed for HPLC analysis were HPLC grade (Mallinckrodt). HPLC grade water (18 mΩ) was prepared using a Milli-Q system (Millipore).

2.4. Assessment of the antinociceptive effect of the extract and rosmarinic acid

2.4.1. Abdominal constriction response caused by intraperitoneal injection of acetic acid

The abdominal constrictions were induced according to procedures previously described (Collier et al., 1968) and resulted in contraction of the abdominal muscle concomitant 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 pretreated with the extract by p.o. (3–1000 mg/kg) route, 60 min before 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. Antinociceptive activity was expressed as the reduction in the number of abdominal constrictions, i.e., the difference between control animals (mice pre-treated with vehicle) and animals pre-treated with extract.

2.4.2. Formalin-induced nociception

The procedure used was essentially the same as that previously described (Santos and Calixto, 1997). Animals received 20 µl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline, injected intraplantarly (i.pl.) in the ventral surface of the right hindpaw. Animals were observed from 0–5 min (neurogenic phase) and 15–30 min (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative of nociception. Animals received the extract (30–1000 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.) 60 min before formalin injection.

2.4.3. Glutamate-induced nociception

In an attempt to provide more direct evidence concerning the interaction of the extract with the glutamatergic system, we separately investigated whether or not the extract was able to antagonize glutamate-induced licking of the mouse paw. The procedure used was similar to that previously described (Beirith et al., 2002). A volume of 20 µl of glutamate (20 µmol/paw prepared in phosphate buffered saline) was intraplantarly injected in the ventral surface of the right hindpaw. Animals were observed individually for 15 min following glutamate injection. The amount of time they spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. Animals were treated with the extract (10–1000 mg/kg, p.o.) or rosmarinic acid (0.3–3 mg/kg, p.o.) 60 min before glutamate injection. Control animals received a similar volume of vehicle (10 ml/kg) by p.o. route.

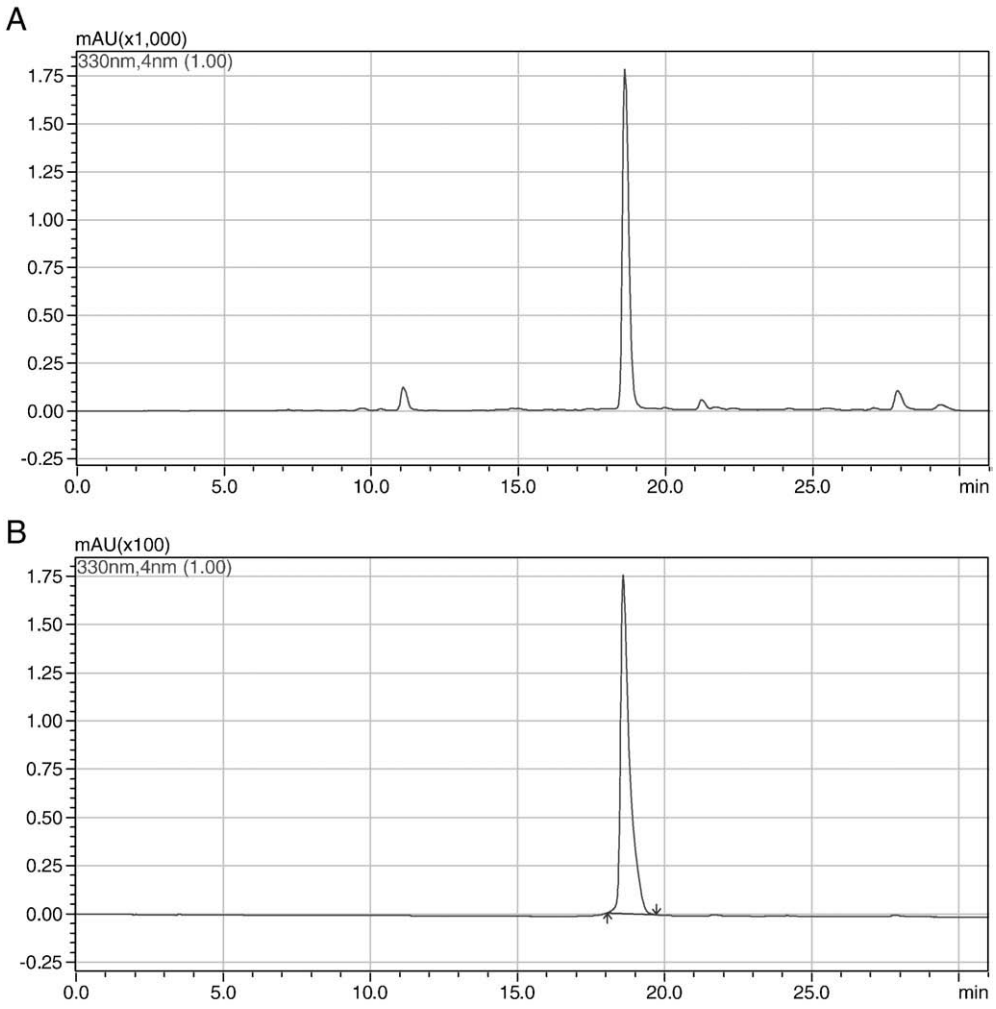

Fig. 1. A) Chromatogram obtained for ethanolic extract of *Melissa officinalis*. Retention time of rosmarinic acid was 18.59 min, λ = 330 nm. B) Chromatogram obtained for standard solution of rosmarinic acid (270 μg/mL). Retention time was 18.59 min, λ = 330 nm.

2.5. Measurement of locomotor activity

In order to evaluate a possible non-specific muscle-relaxant or sedative effect of the extract, mice were submitted to the open-field test. The ambulatory behavior was assessed in an open-field test as previously described (Rodrigues et al., 2002). The apparatus consisted of a wooden box measuring of 40 × 60 × 50 cm. The floor of the arena was divided into 12 equal squares, and the numbers of squares crossed with all paws were counted in a 6-min session. Mice were treated with extract (30–1000 mg/kg, p.o.) or vehicle (10 ml/kg, p.o.) 60 min beforehand.

2.6. Analysis of possible mechanism of action of the extract

To evaluate some mechanisms by which the extract causes antinociception in the glutamate-induced nociception, animals were treated with some classical drugs. The doses of the used drugs were selected based on previous studies (Santos et al., 1999, 2005; Abacioglu et al., 2001) and also based on previous results from our laboratory.

2.6.1. Involvement of the opioid system

To assess the possible participation of the opioid system in the antinociceptive effect of the extract, mice were pre-treated with naloxone (1 mg/kg, i.p.), and after 20 min the animals received the extract (100 mg/kg, orally), morphine (2.5 mg/kg, subcutaneously) or vehicle (10 ml/kg, orally). The nociceptive responses to glutamate were recorded 60, 30 or 60 min after the administration of the extract, morphine, or vehicle, respectively. Another group of animals was pre-treated with vehicle and after 20 min, received the extract, morphine or vehicle, 60, 30 or 60 min before glutamate administration, respectively.

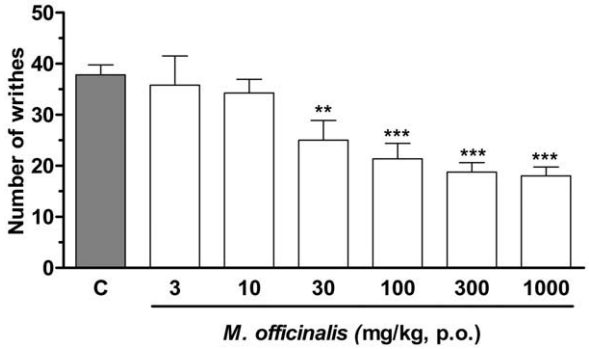

Fig. 2. Effect of the extract administered orally against acetic acid-induced writhing response in mice. Each column represents the mean of 6–8 animals and the error bars indicate the SEM. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test), $^{**}p<0.01$ and $^{***}p<0.001$.

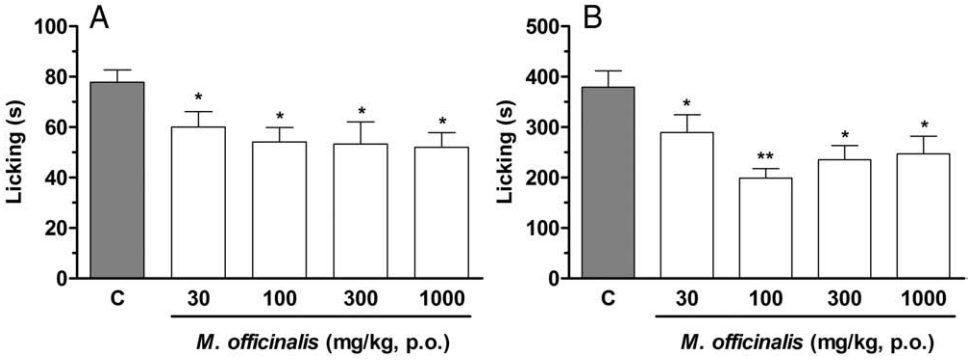

Fig. 3. Effect of EE of *M. officinalis* administered orally against formalin-induced licking (first phase, panel A, and second phase, panel B) in mice. Each column represents the mean of 6–8 animals and the error bars indicate the SEM. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls test), *$p<0.05$ and **$p<0.01$.

2.6.2. Involvement of the cholinergic system

In order to investigate the participation of the cholinergic system in the antinociceptive effect of the extract, mice were pre-treated with mecamylamine (2 mg/kg, i.p.), atropine (1 mg/kg, i.p.) or vehicle (10 ml/kg, i.p.), and after 20 min the animals received the extract (100 mg/kg, p.o.), nicotine (1 mg/kg, i.p.), pilocarpine (3 mg/kg, i.p.) or vehicle (10 ml/kg, p.o.). The nociceptive responses to glutamate were recorded 60, 30, 30 or 60 min after the administration of the extract, nicotine, pilocarpine or vehicle, respectively. Another group of animals was pre-treated with vehicle and after 20 min, received the extract, nicotine, pilocarpine or vehicle, 60, 30, 30 or 60 min before glutamate administration, respectively.

2.6.3. Involvement of L-arginine-nitric oxide pathway

To investigate the role played by the L-arginine-nitric oxide pathway in the antinociception caused by the extract, mice were pre-treated with L-arginine (40 mg/kg, i.p.) or D-arginine (40 mg/kg i.p.) and after 20 min, they received the extract (100 mg/kg, p.o.), Nω-nitro-L-arginine (L-NOARG, 75 mg/kg, i.p.) or vehicle (10 ml/kg, p.o.). The nociceptive responses to glutamate were recorded 60, 30 or 60 min after the administration of the extract, L-NOARG, or vehicle, respectively. Another group of animals was pre-treated with vehicle (10 ml/kg, i.p.) and after 20 min received the extract, L-NOARG or vehicle, 60, 30 or 60 min before glutamate administration, respectively.

2.7. Statistical analysis

The results are presented as mean + SEM, except the ID50 values (i.e., the dose of extract or rosmarinic acid which reduces the nociceptive response by 50% relative to the control value), which are reported as the geometric means accompanied by their respective 95% confidence limits. The ID50 value was determined by linear regression from individual experiments using linear regression GraphPad software (GraphPad software, San Diego, CA, USA). The statistical significance of differences between groups was detected by ANOVA followed by Newman–Keuls' test. *P*-values less than 0.05 ($P<0.05$) were considered as indicative of significance.

3. Results

3.1. HPLC analysis of ethanolic extract from M. officinalis

The HPLC analysis revealed that rosmarinic acid is a major compound of the extract from *M. officinalis* (Fig. 1 A and B). The calibration curves were made by plotting the ratio of rosmarinic acid peak areas versus concentrations, and good linearity was obtained with the standard solutions. The regression equations for rosmarinic acid were $y = 15937x - 483967$ ($r = 0.9999$). The retention times in the system developed was 18.59 min and the concentration of rosmarinic acid in the extract was 4.37% (Fig. 1 A and B).

3.2. Abdominal constriction response caused by intraperitoneal injection of acetic acid

The results depicted in Fig. 2 show that the extract (3–1000 mg/kg), given by p.o. route 60 min beforehand, produced dose-related inhibition of acetic acid-induced abdominal constrictions in mice, with mean ID_{50} values (and their 95% confidence limits) of 241.92 (203.92–289.37) mg/kg and inhibitions of 52 ± 5% at a dose of 1000 mg/kg.

3.3. Formalin-induced nociception

The results in Fig. 3 show that the extract (30–1000 mg/kg), given by p.o. route 60 min beforehand, caused significant inhibition of both

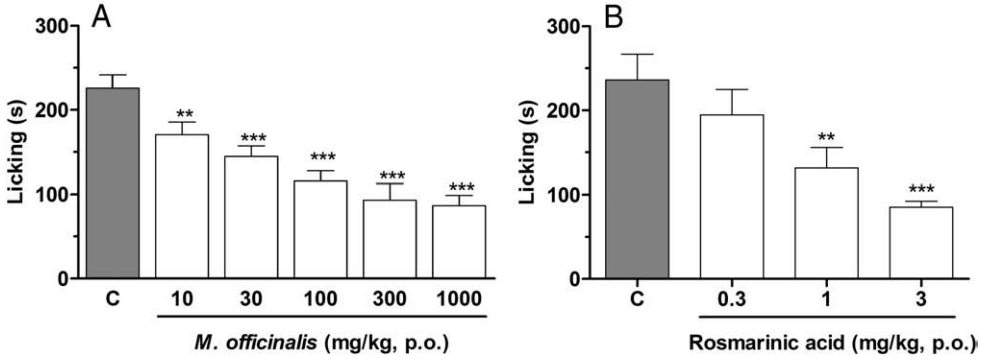

Fig. 4. Effect of the ethanolic extract (A) and rosmarinic acid (B) obtained from *M. officinalis* administered orally against glutamate-induced nociception in mice. the mean of 6–8 animals and the error bars indicate the SEM. Control values (C) indicate the animals injected with vehicle and the asterisks denote the significance levels when compared with control groups (one-way ANOVA followed by Newman–Keuls' test), **$p<0.01$ and ***$p<0.001$.

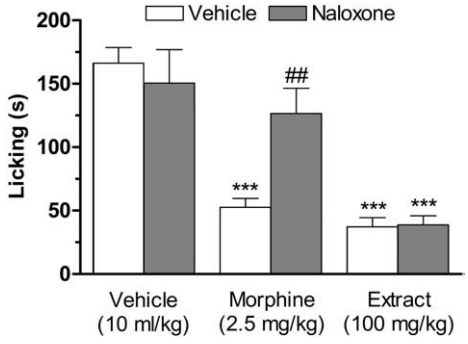

Fig. 5. Effect of pretreatment of mice with naloxone (1 mg/kg, i.p.) on the antinociceptive profiles of the extract (100 mg/kg, p.o.) and morphine (2. 5 mg/kg, s.c.) against the glutamate-induced nociception in mice. Each column represents the mean of 6–8 animals and the error bars indicate the SEM. The symbols denote significance levels: ***$p<0.001$ compared with control group (animals injected with the vehicle alone); and ##$p<0.01$ compared with the extract and morphine treatment (one-way ANOVA followed by Newman–Keuls' test).

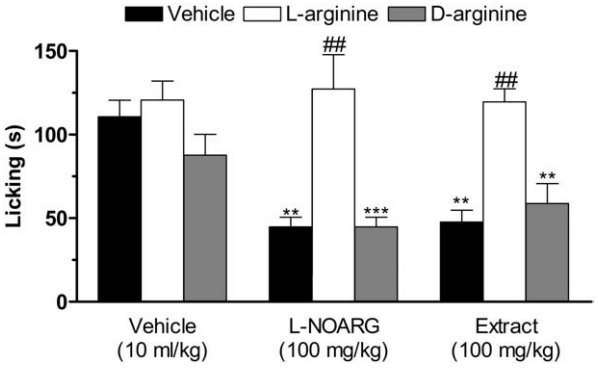

Fig. 7. Effect of pretreatment of mice with L-arginine (40 mg/kg, i.p.) or D-arginine (40 mg/kg, i.p.) on the antinociceptive profiles of the extract (100 mg/kg, p.o.) and L-NOARG (25 mg/kg, i.p.) against the glutamate-induced nociception in mice. Each column represents the Mean of 6–8 animals and the error bars indicate the SEM. The symbols denote significance levels: **$p<0.01$ and ***$p<0.001$ compared with control group (animals injected with the vehicle alone); ##$p<0.01$ e ###$p<0.001$ compared with the extract and L-NOARG treatment. (one-way ANOVA followed by Newman–Keuls' test).

neurogenic (0–5 min) and inflammatory (15–30 min) phases of the formalin-induced licking. The calculated inhibition values for these effects were 33 ± 7 and $48 \pm 5\%$, respectively, for the dose of 100 mg/kg.

3.4. Glutamate-induced nociception

The results presented in Fig. 4A show that the extract (10–1000 mg/kg, p.o.) caused a dose-related inhibition of the glutamate-induced nociception, with a mean ID_{50} value of 198.54 (146.37–261.21) mg/kg and inhibition of $62 \pm 5\%$ at a dose of 1000 mg/kg. Interestingly, when the rosmarinic acid (0.3–3 mg/kg), present in the *M. officinalis*, was administered orally to mice it produced dose-related inhibition of glutamate-induced pain, with a mean ID_{50} value of 2.64 (2.50–2.78) mg/kg and the peak of inhibition observed was $64 \pm 3\%$ (Fig. 4B). In addition, the rosmarinic acid was 75-fold more potent than extract when analysed in the glutamate test.

3.5. Evaluation of locomotor activity

The extract (30–300 mg/kg, p.o.) did not affect the locomotor activity in the open-field test when compared with animals that received vehicle (control group). The means ± SEM of crossed squares were 117.3 ± 19.9; 133.0 ± 5.5; 133.5 ± 3.4 and 139.8 ± 6.2 for the control, 30, 100 and 300 mg/kg group, respectively.

3.6. Analysis of possible mechanism of action of the extract

The results presented in Fig. 5 show that the pre-treatment of mice with naloxone (1 mg/kg, i.p.), given 20 min beforehand, completely reversed the antinociception caused by morphine (2.5 mg/kg, s.c.) when assessed against glutamate-induced pain. Under the same conditions, naloxone did not significantly modify the antinociception caused by the extract in the glutamate test (Fig. 5).

The systemic pre-treatment of animals with atropine (1 mg/kg, i.p.) or mecamylamine (2 mg/kg, i.p.), given 20 min beforehand, significantly reversed the antinociception caused by the extract (100 mg/kg, p.o.), pilocarpine (3 mg/kg, i.p.) and nicotine (1 mg/kg, i.p.), respectively, when analysed against glutamate-induced pain (Fig. 6 A and B).

The results depicted in Fig. 7 show that the previous treatment of mice with L-arginine (40 mg/kg, i.p.), given 20 min earlier, but not D-arginine (40 mg/kg, i.p.), completely reversed the antinociception caused by L-NOARG (100 mg/kg, i.p.) and by the extract (100 mg/kg, p.o.) against glutamate-induced pain (Fig. 7).

4. Discussion

The results presented here extend literature data and clearly demonstrate, for the first time, that the extract from *M. officinalis* administered by oral route elicited a significant and dose-dependent antinociception in a chemical model of inflammatory pain in mice, namely acetic-acid visceral nociception. The systemic (p.o.) administration of the extract also causes significant inhibition against both neurogenic and inflammatory pain responses induced by formalin. Furthermore, both extract and rosmarinic acid, present in the leaves of *M. officinalis*, also greatly inhibited the nociceptive response caused by

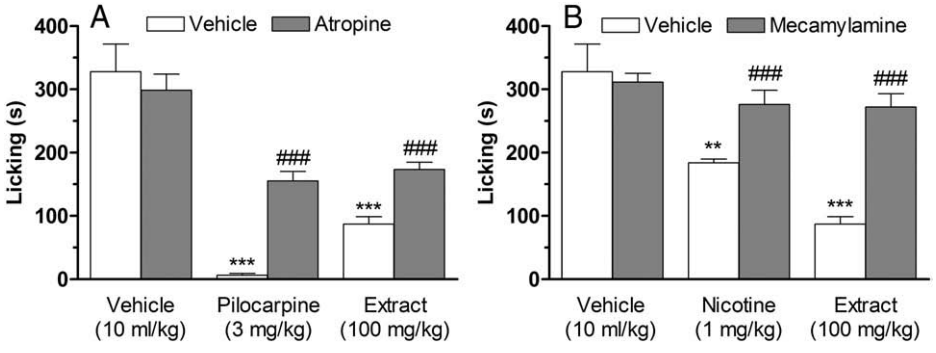

Fig. 6. Effect of pretreatment of mice with atropine (1 mg/kg, i.p., panel A) or mecamylamine (2 mg/kg, i.p., panel B) on the antinociceptive profiles of the extract (100 mg/kg, p.o.), pilocarpine (3 mg/kg, i.p.) and nicotine (1 mg/kg, i.p.) against the glutamate-induced nociception in mice. Each column represents the Mean of 6–8 animals and the error bars indicate the SEM. The symbols denote significance levels: **$p<0.01$ and ***$p<0.001$ compared with control group (animals injected with the vehicle alone); and ##$p<0.01$ compared with the extract, pilocarpine and nicotine treatment (one-way ANOVA followed by Newman–Keuls' test).

glutamate. Moreover, the antinociceptive action of the extract in the glutamate test was significantly reversed by i.p. treatment of animals with atropine, mecamylamine and L-arginine, but not by D-arginine and naloxone. In addition, the dose of the extract that caused significant antinociception did not produce any statistically significant motor dysfunction.

Some studies with different herbal formulations that contain *M. officinalis* demonstrate analgesic activity, especially against visceral pain (Savino et al., 2005; Vejdani et al., 2006; Capasso et al., 2007). This way, we analyzed the possible antinociceptive effect of *M. officinalis* in the visceral nociceptive response induced by acetic acid in mice.

The results reported here indicate that oral administration of the extract produced marked and dose-related antinociception when assessed in acetic acid-induced visceral nociception, at doses that did not produce any statistically significant motor dysfunction. To our knowledge this is the first report of this kind in the literature. The acetic acid-induced writhing reaction in mice, described as a typical model of visceral inflammatory pain, has been used as a screening tool for the assessment of analgesic or anti-inflammatory agents (Collier et al., 1968). Acetic acid derivative protons can directly activate non selective cation channels, located at primary afferent pathways (Julius and Basbaum, 2001). Moreover, acetic acid injection in mice peritoneal cavity promotes the release of many inflammatory mediators such as PG, BK, SP, TNF-α, IL-1β, IL-8 and others (Collier et al., 1968; Vinegar et al., 1979; Ribeiro et al., 2000; Ikeda et al., 2001). These substances will stimulate primary afferent neurons, enhancing aspartate and glutamate release at cerebrospinal fluid (Feng et al., 2003; Zhu et al., 2004).

Another interesting finding of the present study was the demonstration that the extract from *M. officinalis*, given by oral route was effective in inhibiting both phases (neurogenic and inflammatory) of formalin-induced nociception. The neurogenic phase is elicited by direct activation of nociceptive terminals; on the other hand the inflammatory phase is mediated by a combination of peripheral and central mechanisms (Hunskaar and Hole, 1987; Tjølsen et al., 1992).

Our results also show that p.o. administration of the extract produced a significant and dose-dependent inhibition of the nociceptive response caused by intraplantar injection of glutamate into mouse hindpaw. This nociceptive response caused by glutamate seems to involve peripheral, spinal and supra-spinal sites and its action is mediated by NMDA and non NMDA receptors, as well as by the nitric oxide release or some nitro derivate-regulate pathways (Beirith et al., 2002). Several studies have demonstrated that excitatory aminoacids receptors are involved in nociceptive primary afferent transmission, both in the development and maintenance of painful response (Aanonsen and Wilcox, 1987, 1990; Coggeshall and Carlton, 1997; Ferreira et al., 1999). Thus, the suppression of glutamate-induced nociception by the extract treatment can be associated with its interaction with the glutamatergic system or inhibition of nitric oxide production (Ferreira et al., 1999).

The result of the present study clearly confirms that the L-arginine-nitric oxide pathway is involved in the antinociception caused by the extract. This conclusion derives from the fact that the pretreatment of mice with the substrate of nitric-oxide synthase, L-arginine, at a dose that produced no significant effect on glutamate-induced pain, significantly reversed the antinociception caused by both the extract and L-NOARG (a known nitric oxide inhibitor). In marked contrast, the pretreatment of animals with the inactive isomer of L-arginine, D-arginine, had no significant effect against both extract- and L-NOARG-induced antinociception (Haley, 1998; Ferreira et al., 1999).

The endogenous opioid system is largely involved in the regulation of the experience of pain, and in the action of analgesic opiate drugs (Bodnar and Klein, 2005). The present study suggests that opioid naloxone-sensitive pathway is not involved in the extract-induced antinociception. This hypothesis is based on the fact that naloxone, a nonselective opioid receptor antagonist, completely inhibited the antinociceptive effect of morphine (a nonselective opioid agonist, positive control), however, the same treatment of animals with naloxone completely failed to affect the extract-induced antinociception in mice.

Several evidences demonstrate that the cholinergic system has broad therapeutic potential for efficacy against a number of clinically relevant pain states including inflammatory, neuropathic, visceral pain and pain due to arthritis (for review see Jones and Dunlop, 2007). In addition, acetylcholine mediates its effects through both nicotinic acetylcholine receptors (ligand-gated ion channels) and the G protein-coupled muscarinic receptors (for review see Jones and Dunlop, 2007). Furthermore, the results of the present study provide consistent evidence supporting the involvement of the cholinergic system in the antinociception caused by the extract, evident by the fact that, mecamylamine (a preferential $\alpha_2\beta_3$ selective nicotinic receptor antagonist) (Puttfarcken et al., 1999), at a dose similar to that known to prevent antinociception induced by the $\alpha_2\beta_3$ selective nicotinic receptor agonist (Dussor et al., 2004), consistently attenuated both nicotine (a nonselective nicotine receptor agonist)- and the extract-induced antinociception in the glutamate test. In addition, muscarinic acetylcholine receptors also appear to account for the antinociceptive action of the extract. This notion comes from the data showing that targeting the muscarinic acetylcholine receptor sensitive to atropine (a ligand nonselective for the muscarinic acetylcholine receptor), with the doses and treatment scheme in which this substance effectively antagonizes responses mediated by activation of muscarinic acetylcholine receptors (Demarco et al., 2003), largely prevents the antinociception caused by both pilocarpine (a non-selective muscarinic acetylcholine receptor) and the extract in the glutamate test. In accordance with these findings, it has been reported in a randomized, placebo-controlled, double-blind study, that commercial *Melissa* extract displaced [(3)H]-(N)-nicotine and [(3)H]-(N)-scopolamine from nicotinic and muscarinic receptors in the human cerebral cortex tissue (Kennedy et al., 2003). However, the extract utilized in that study did not exhibit cholinesterase inhibitory properties. Therefore, the cholinergic systems seem to play a critical role in the antinociception caused by the extract.

Finally, a phytochemical study has demonstrated that the leaves from *M. officinalis* contained a great amount (2–5%) of rosmarinic acid (Carnat et al., 1998). In the present study, we demonstrated by HPLC analyses that the rosmarinic acid is in fact the major compound of the extract from *M. officinalis*. In addition, the extract analyzed in this study contained 4.37% of rosmarinic acid. Furthermore, it has been demonstrated that the rosmarinic acid exhibited several activities, including adstringent, antioxidant, anti-inflammatory, anti-mutagenic, anti-bacterial and antiviral activities (Petersen and Simmonds, 2003). Moreover, our results also show that oral administration of rosmarinic acid produced a significant and dose-dependent inhibition of the nociceptive response caused by intraplantar injection of glutamate into the mouse hindpaw. In addition, at the ID_{50} level, this compound had a 75-fold greater potency than the extract when analysed in the glutamate test. Furthermore, the rosmarinic acid seems, at least in part, to contribute to the explanation of the antinociceptive properties of the extract from *M. officinalis*.

5. Conclusion

The results of the present study demonstrate for the first time that the extract from *M. officinalis* produce dose-related antinociceptive action in chemical (acetic acid-induced visceral pain, formalin- or glutamate-induced nociception) models of nociception in mice. The extract effects were unrelated to a disability to respond to stimulus since it did not alter the locomotor activity of the animals. The precise mechanisms through which the extract exerts its action are currently

under investigation, but inhibition of the L-arginine-nitric oxide pathway and activation of cholinergic (e.g. nicotinic and muscarinic acetylcholine receptors) systems seems largely to account for the extract antinociceptive effect. However, the opioid system seems unlikely to participate in the antinociception caused by the extract. In addition, the rosmarinic acid contained in the leaves from *M. officinalis* contributes to the explanation of the antinociceptive properties reported for the extract. Furthermore, the antinociceptive action demonstrated in the present study supports, at least partly, the ethnomedical use of this plant.

Acknowledgements

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Programa de Apoio aos Núcleos de Excelência (PRONEX), Fundação de Apoio à Pesquisa Científica Tecnológica do Estado de Santa Catarina (FAPESC) and Financiadora de Estudos e Projetos [FINEP, Rede Instituto Brasileiro de Neurociência (IBN-Net)], Brazil. We thank Daniela Tagliari Longhi, Juliana Geremias Chichorro and Francisco José Cidral Filho for critical review of the manuscript.

References

Aanonsen LM, Wilcox GL. Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and *sigma* agonists. J Pharmacol Exp Ther 1987;1:9-19.

Aanonsen LM, Wilcox GL. Excitatory amino acid receptors and nociceptive neurotransmission in rat spinal cord. Pain 1990;41:309–21.

Abacioglu N, Ozmen R, Cakici I, Tunçtan B, Kanzik I. Role of L-arginine/nitric oxide pathway in the antinociceptive activities of morphine and mepyramine in mice. Arzneimittelforschung 2001;51:977–83.

Akondzadeh S, Noroozian M, Mohammadi M, Ohadinia S, Amshidi AH, Khani M. *Melissa officinalis* extract in the treatment of patients with mild to moderate Alzheimer's disease: a double blind, randomized, placebo controlled trial. J Neurol Neurosurg Psychiatry 2003;74:863–6.

Allaverdiyev A, Duran N, Ozguven M, Koltas S. Antiviral activity of the volatile oils of *Melissa officinalis* L. against Herpes simplex virus type-2. Phytomedicine 2004;11:657–61.

Beirith A, Santos ARS Calixto JB. Mechanisms underlying the nociception and paw oedema caused by injection of glutamate into the mouse paw. Brain Res 2002;924:219–28.

Bodnar RJ, Klein GE. Endogenous opiates and behaviour. Peptides 2005;26:2629–711.

Bolkent S, Yanardag R, Karabulut-Bulan O, Yesilyaprak B. Protective role of *Melissa officinalis* L. extract on the liver of hyperlipidemic rats: a morphological and biochemical study. J Ethno-Pharmacol 2005;99:391–8.

Capasso R, Savino F, Capasso F. Effects of the herbal formulation ColiMil on upper gastrointestinal transit in mice in vivo. Phytother Res 2007;21:999-1101.

Carnat AP, Carnat A, Fraisse D, Lamaison JL. The aromatic and polyphenolic composition of lemon balm (*Melissa officinalis* L. subsp. officinalis) tea. Pharm Acta Helv 1998;72:301–5.

Collier HOJ, Dinneen JC, Johnson CA, Schneider C. The abdominal constriction response and its suppression by analgesic drugs in the mouse. Br J Pharmacol Chemother 1968;32:295–310.

Coggeshall RE, Carlton SM. Receptor localization in the mammalian dorsal horn and primary afferent neurons. Brain Res Rev 1997;24:28–66.

Dastmalchi K, Dorman HJD, Oinonen PP, Darwis Y, Laakso I, Hiltunen R. Chemical composition and in vitro antioxidative activity of a lemon balm (*Melissa officinalis* L.) extract. LWT – Food Sci Technol 2008;41:391–400.

Demarco GJ, Baghdoyan HA, Lydic R. Differential cholinergic activation of G proteins in rat and mouse brainstem: relevance for sleep and nociception. J Comp Neurol 2003;457:175–84.

Dussor GO, Helesic G, Hargreaves KM, Flores CM. Cholinergic modulation of nociceptive responses in vivo and neuropeptide release in vitro at the level of the primary sensory neuron. Pain 2004;107:22–32.

Feng Y, Cui M, Willis W. Gabapentin markedly reduces acetic acid-induced visceral nociception. Anesthesiology 2003;98:729–33.

Ferreira J, Santos ARS, Calixto JB. The role of systemic, spinal and supraspinal L-arginine-nitric oxide-cGMP pathway in thermal hyperalgesia caused by intrathecal injection of glutamate in mice. Neuropharmacology 1999;38:835–42.

Ferreira A, Proença C, Serralheiro MLM, Araújo MEM. The in vitro screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnoharmacol 2006;108:31–7.

Gazola R, Machado D, Ruggiero C, Singi G, Alexandre MM. *Lippia alba*, *Melissa officinalis* and *Cymbopogon citratus*: effects of the aqueous extracts on the isolated hearts of rats. Pharmacol Res 2004;50:477–80.

Haley JE. Gases as neurotransmitters. Essays Biochem 1998;33:79–91.

Heitz A, Carnat A, Fraisse D, Carnat AP, Lamaison JP. Leotelin 3′-glucoronide, the major flavonoid from *Melissa officinalis* subdp. officinalis. Fitoterapia 2000;71:201–2.

Herodez SS, Hadolin M, Skerget M, Knez Z. Solvent extraction study of antioxidants from Balm (*Melissa officinalis* L.) leaves. Food Chem 2003;80:275–82.

Hunskaar S, Hole K. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. Pain 1987;30:103.

Ikeda Y, Ueno A, Naraba H, Oh-Ishi S. Involvement of vanilloid receptor VR1 and prostanoids in the acid-induced writhing responses of mice. Life Sci 2001;69:2911–9.

Iuvone T, De Filippis D, Esposito G, D'Amico A, Izzo A. The spice sage and its active ingrediente rosmarinic acid protect PC12 cell from amyloid-β peptide-induced neurotoxicity. J Pharmacol Exp Ther 2006;317:1143–9.

Jones PG, Dunlop J. Targeting the cholinergic system as a therapeutic strategy for the treatment of pain. Neuropharmacology 2007;53:197–206.

Julius D, Basbaum AI. Molecular mechanisms of nociception. Nature 2001;413:203–10.

Kennedy DO, Wake G, Savelev S, Tildesley NTJ, Perry EK, Wesnes KA, et al. Modulation of mood and cognitive performance following acute administration of single doses of *Melissa officinalis* (Lemon Balm) with human CNS nicotinic and muscarínico receptor-binding properties. Neuropsycopharmacology 2003;28:1871–81.

Müller SF, Klement S. A combination of valerian and lemon balm is effective in the treatment of restlessness and dysomnia in children. Phytomedicine 2006;13:383–7.

Petersen M, Simmonds MS. Rosmarinic acid. Phytochemistry 2003;62:121–5.

Puttfarcken PS, Manelli AM, Arneric SP, Donelly-Roberts DL. Evidence for nicotinic receptors potentially modulating nociceptive transmission at the level of the primary sensory neuron: studies with F11 cells. J Neurochem 1999;69:930–8.

Ribeiro RA, Vale MV, Thomazzi SM, Paschoalato ABP, Poole S, Ferreira SH, et al. Involvement of resident macrophages and mast cells in the writhing nociceptive response induced by zymosan and acetic acid in mice. Eur J Pharmacol 2000;387:111–8.

Ribeiro MA, Bernardo-Gil MG, Esquível MM. *Melissa officinalis*, L.: study of antioxidant activity in supercritical residues. J Supercrit Fluids 2001;21:51–60.

Rodrigues AL, da Silva GL, Mateussi AS, Fernandes ES, Miguel OG, Yunes RA, et al. Involvement of monoaminergic system in the antidepressant-like effect of the hydroalcoholic extract of *Siphocampylus verticillatus*. Life Sci 2002;70:1347–58.

Salah SM, Jäger AK. Screening of traditionally used Lebanese herbs for neurological activities. J Ethnopharmacol 2005;97:145–9.

Sandraei H, Ghannadi A, Malekshahi K. Relaxant effect of essential oil of *Melissa officinalis* and citral on rat ileum contrations. Fitoterapia 2003;74:445–52.

Santos ARS, Calixto JB. Rhutenium red and capzasepine antinociceptiva effect in formalin and capsaicin models of pain in mice. Neurosci Lett 1997;235:73–6.

Santos ARS, Miguel OG, Yunes OG, Calixto JB. Antinociceptive properties of the new alkaloid, cis-8, 10-di-Npropyllobelidiol hydrochloride dihydrate isolated from *Siphocampylus verticillatus*: evidence for the mechanism of action. J Pharmacol Exp Ther 1999;289:417–26.

Santos ARS, Gadotti VM, Oliveira G, Tibola D, Paszuk AF, Neto A, et al. Mechanisms involved in the antinociception caused by agmatine in mice. Neuropharmacology 2005;48:1021–34.

Santos-Neto LL, Toledo MAV, Medeiros-Souza P, Souza GA. The use of Herbal medicine in Alzheimer's disease — a sistematic review. Adv Acc Public 2006;23:441–5.

Savino F, Cresi F, Castagno E, Silvestro L, Oggero R. A randomized double-blind placebo controlled trial of a standardized extract of *Matricariae recutita*, *Foeniculum vulgare* and *Melissa officinalis* in the treatment of breastfed colicky infants. Phytother Res 2005;19:335–40.

Simmen U, Kelber O, Okpaneji SN, Jaeggi R, Buetler B, Weiser D. Binding of STW 5 (Iberogast) and its components to intestinal 5-HT, muscarinic M3 and opioid receptors. Phytomedicine 2006;13:51–5.

Schemann M, Michel K, Zeller F, Hohenester B, Rühl A. Region-specific effects of STW 5 (Iberogast) and its components in gastric fundus, corpus and antrum. Phytomedicine 2006;13:90–9.

Tjølsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an evaluation of the method. Pain 1992;51:5-17.

Vejdani R, Shalmani HR, Mir-Fattahi M, Sajed-Nia F, Abdollahi M, Zali MR, et al. The efficacy of an herbal medicine, Carmint, on the relief of abdominal pain and bloating in patients with irritable bowel syndrome: a pilot study. Dig Dis Sci 2006;51:1501–7.

Vinegar R., Truax J.F., Selph J.L., Johston P.R. Antagonism of pain and Hyperalgesia. Anti-inflammatory Drugs. In: Vane JR, Ferreira SH, editors. Handbook of Experimental Pharmacology. Vol. 50/II,. Springer: Verlag, Berlin. 208-22, 1979.

Zhu CZ, Wilson SG, Ikusa JP, Wismer CT, Gauvin DM, Lynch III JJ, et al. Assessing the role of metabotropic glutamate receptor 5 in multiple nociceptive modalities. Eur J Pharmacol 2004;506:107–18.

Ziaková A, Bradsteterová E, Blahová E. Matrix solid-phase dispersion for the liquid chromatographic determination of phenolic acids in *Melissa officinalis*. J Chromatogr A 2003;983:271–5.

Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 1983;16:109–10.