Pharmaceutical Biology

http://informahealthcare.com/phb
ISSN 1388-0209 print/ISSN 1744-5116 online
Editor-in-Chief: John M. Pezzuto
Pharm Biol, 2014; 52(6): 762–766
 DOI: 10.3109/13880209.2013.870582

informa
healthcare

ORIGINAL ARTICLE

Antinociceptive activity of *Syzygium cumini* leaves ethanol extract on orofacial nociception protocols in rodents

Jullyana S. S. Quintans[1], Renan G. Brito[1], Pedro Gregório V. Aquino[2], Paulo H. B. França[2], Pollyana S. Siqueira-Lima[3], Antônio E. G. Santana[2], Eurica A. N. Ribeiro[4], Marcos J. Salvador[5], João X. Araújo-Júnior[3,4], and Lucindo J. Quintans-Júnior[1]

[1]Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brazil, [2]Instituto de Química e Biotecnologia, Universidade Federal de Alagoas, Maceió, Alagoas, Brazil, [3]Programa de Pós-Graduação em Biotecnologia, Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, Brazil, [4]Escola de Enfermagem e Farmácia, Universidade Federal de Alagoas, Maceió, Alagoas, Brazil, [5]Departamento de Biologia Vegetal, Curso de Farmácia, BTPB, Instituto de Biologia, UNICAMP, Campinas, SP, Brazil

Abstract

Context: *Syzygium cumini* (L.) Skeels (Myrtaceae) is a tree with dark purple fruits, popularly known as ''jambolão'' or ''jambolan''. In folk medicine, this plant is used for the treatment of diabetes and inflammatory conditions.
Objective: We investigated the antinociceptive effect of ethanol extract (EE) from *S. cumini* leaves on orofacial nociception.
Material and methods: The antinociceptive effects of the EE obtained from the leaves of *S. cumini* were evaluated in mice using formalin- and glutamate-induced orofacial nociception.
Results: ESI-MS/MS analyses demonstrated that major constituents in the analyzed samples coincided with the mass of the phenolic acids and flavonoids. In pharmacological approach, pre-treatment with EE (100, 200, or 400 mg/kg, p.o.) significantly reduced ($p < 0.05$ or $p < 0.01$) the percentage of paw licks time during phase 2 (43.2, 47.1, and 57.4%, respectively) of a formalin pain test when compared to control group animals. This effect was prevented by pretreatment with glibenclamide and N^G-nitro-L-arginine (L-NOARG). The extract, all doses, also caused a marked inhibition ($p < 0.01$ or $p < 0.001$) of glutamate-induced orofacial nociception (38.8, 51.7, and 54.7%) when compared with the control group. No effect was observed with the rota-rod model.
Conclusions: We can suggest that the antinociceptive effect of the EE is mediated by peripheral mechanisms, possibly involving K_{ATP} channels and the nitric oxide pathways. These effects appear to be related to the presence of flavonoids compounds, such as quercetin.

Keywords

Formalin, glutamate, myrtaceae, orofacial pain, pain, quercetin

History

Received 29 June 2013
Revised 10 September 2013
Accepted 15 November 2013
Published online 21 February 2014

Introduction

Syzygium cumini (L.) Skeels (Myrtaceae) is a tree with dark purple fruits originating in Indo-Malaysia, China, and the Antilles, and is cultivated in various countries, including Brazil, where is popularly known as ''jambolão'' or ''jambolan''. It is reported that the bark is employed in folk medicine for the treatment of diabetes and inflammatory conditions (Schoenfelder et al., 2010). The juice of this plant's tender leaves, mango, and myrobalan are mixed and administered in milk and honey to treat dysentery with bloody discharge, whereas the juice of its tender leaves alone or in combination with carminatives, such as cardamom or cinnamon, is given in goat's milk to treat diarrhea in children (Ayyanar & Subash-Babu, 2012).

Muruganandan et al. (2001) demonstrated that *S. cumini* bark extract produces a potent anti-inflammatory effect against different phases of the inflammation process without any side effect on gastric mucosa. Recently, Ayyanar and Subash-Babu (2012) published a newsworthy review about phytochemical constituents and the traditional uses of *S. cumini*, which shows its hypoglycemic properties and its effectiveness in treating gastrointestinal disorders, fever, inflammation, and pain.

There are no studies exploring the analgesic profile of *S. cumini* and its possible role in orofacial nociception protocols. Thus, this study aims to investigate the antinociceptive effect of ethanol extract (EE) from *S. cumini* leaves on orofacial nociception induced by formalin and glutamate in mice.

Materials and methods

Plant material and preparation of plant extract

S. cumini was identified by Professor Flávia de Barros Prado Moura and a voucher sample (MUFAL 4080) is deposited in

Correspondence: L.J. Quintans-Júnior, Departamento de Fisiologia, Universidade Federal de Sergipe, 49.000-100 São Cristóvão, Sergipe, Brazil. Tel/Fax: +55 79 2105 6645. E-mail: lucindojr@gmail.com, lucindo@pq.cnpq.br

the Herbarium Prof. Honório Monteiro (Maceió-AL). The leaves of *S. cumini* were collected in July 2009 at the Federal University of Alagoas campus and subjected to drying at 40 °C for 72 h with subsequent grinding. The dried and ground material (700 g) was subjected to extraction with 2 L of a 70% hydroethanol solution at room temperature in three cycles of 72 h each. After each cycle, the solvent was removed under reduced pressure in a rotary device, furnishing 79.7 g of the EE.

Electrospray ionization mass spectrometry fingerprinting

The crude EE was diluted in a solution containing 50% (v/v) chromatographic grade methanol, 50% (v/v) deionized water, and 0.5% of ammonium hydroxide (Merck, Darmstadt, Germany). The fingerprinting ESI-MS analyses were performed according to Salvador et al. (2011); using UPLC-MS equipment, model ACQUITY TQD (Waters Corporation, Milford, MA). The general conditions were included a source temperature of 100 °C, capillary voltage of 3.0 kV, and cone voltage of 30 V. ESI-MS was performed by direct infusion using a syringe pump, with a flow rate of 10 μL/min/mL. Structural analysis of single ions in the mass spectra from the extract was performed by ESI-MS/MS. The ion with the *m*/*z* of interest was selected and submitted to 15–45 eV collisions with argon in the collision quadrupole. The collision gas pressure was optimized to produce extensive fragmentation of the ion under investigation. The compounds were identified by comparing their ESI-MS/MS fragmentation spectra to fragmentation spectra of authentic standard samples and literature data (Afify et al., 2011; Roesler et al., 2007; Salvador et al., 2011; Timbola et al., 2002; Ye et al., 2007).

Animals

Male Swiss mice (32–40 g), 2–3 months of age, were used throughout this study. The animals were obtained from the Animal Facilities of Federal University of Sergipe and they were randomly placed in appropriate cages, which maintained a temperature of 22 ± 2 °C on a 12 h light/dark cycle (lights on 6:00 am–6:00 pm) with free access to food (Labina®) and water. All experiments were carried out between 9:00 am and 4:00 pm in a quiet room and nociception tests were performed by the same visual observer in a double-blind study. Experimental protocols were approved by the Animal Care and Use Committee at the UFS (CEPA/UFS No. 49/09).

Formalin and glutamate tests

Orofacial nociception procedures were performed on mice by s.c. injection of 20 μL of 2% formalin or 40 μL of glutamate (25 mM) into the right upper lip (perinasal area) using a 27-gauge needle (Luccarini et al., 2006; Quintans-Júnior et al., 2010). Mice ($n = 6$, per group) were treated with the EE (100, 200, or 400 mg/kg, p.o.), morphine (MOR, 5 mg/kg, i.p.), and vehicle (Tween 80 0.2%, Sigma-Aldrich, St. Gallen, Switzerland) 1 h before algogen injections.

Formalin test

The neurogenic phase (first phase) occurs within 0–5 min after the administration of the nociceptive substance and this phase is followed by a latency period of about 10 min. Then, an inflammatory phase (second phase) occurs within 15–40 min after the administration of the nociceptive substance.

Glutamate test

Mice were observed individually for 20 min following glutamate injection. Animals were observed individually in mirrored chambers (30 × 30 × 30 cm) to allow an unobstructed view of the orofacial region. The nociceptive behavior assessed during the two tests was the time during which the animals kept rubbing the orofacial region.

Possible antagonism of EE antinociceptive effect by pretreatment with naloxone, glibenclamide, or L-NOARG

To assess the possible participation of the opioid and nitric oxide (NO) pathways, mice were intraperitoneally (i.p.) pretreated ($n = 6$, per group) with 1.5 mg/kg of naloxone (NAL, a nonselective opioid antagonist), 20 mg/kg of glibenclamide (GLI, a K_{ATP} channel blocker), or 40 mg/kg of N^G-nitro-L-arginine (L-NOARG, a NO synthase inhibitor), 30 min before the administration of vehicle (control; p.o.), EE (400 mg/kg; p.o.), or morphine (5 mg/kg; i.p.). Subsequently, the formalin-induced orofacial nociception test was performed, as described above.

Motor performance assessment

The previously described test was conducted by Quintans-Junior et al. (2010). Mice were selected 24 h prior to the test by selecting only those that were able to remain successfully on the revolving bar (9 rpm) of the rota-rod apparatus (AVS®, Sao Paula, Brazil) for two consecutive periods of 120 s. The selected mice were divided into five groups ($n = 6$) and treated with the EE (100, 200, or 400 mg/kg, p.o.) and vehicle or diazepam (1.5 mg/kg, i.p.). Motor performance was evaluated at 60, 120, and 240 min following treatments, and the amount of time the mice remained (in seconds) on the revolving bar during a 180 s period was recorded.

Statistics analysis

Data obtained were expressed as mean ± standard error of the mean (SEM). Statistical evaluation of the data was performed using a one-way analysis of variance (ANOVA) followed by Tukey's test. p Values less than 0.05 were considered significant. The percent of inhibition by an antinociceptive agent was determined using the following formula (Reanmongkol et al., 1994): Inhibition $\% = 100 \times (\text{control} - \text{experiment})/\text{control}$.

Results

These analyses showed that major constituents in the analyzed samples coincided with the mass of the phenolic acids and flavonoids (Table 1 and Figure 1).

EE produced significant ($p < 0.05$ or $p < 0.01$) antinociception in the second phase of the formalin test (inflammatory phase) (Table 2). The previous treatment of mice with glibenclamide and L-NOARG significantly reversed the antinociception caused by EE; however, pretreatment with

Table 1. Compounds identified in ethanolic extract of *Syzigium cumini* leaves (EE) using negative ion mode ESI-MS/MS.

Compound	*Syzigium cumini* leaves ethanol extract	Deprotonated ions $[M-H]^-$, *m/z*	MS/MS ions *m/z*
Malic acid	+	133	25 eV: 133 → 115
Gallic acid	+	169	25 eV: 169 → 125, 79
Caffeic acid	+	179	25 eV: 179 → 135
Ferulic acid	+	195	25 eV: 193 → 178, 149, 134
Quercetin	+	301	25 eV: 301 → 179, 151, 121, 107
Myricitrin	+	463	25 eV: 463 → 435, 349, 319, 317
Myricetin 3-*O*-(4″-acetyl)-α-L-rhamnopyranoside	+	505	25 eV: 505 → 317

+, detected compound.

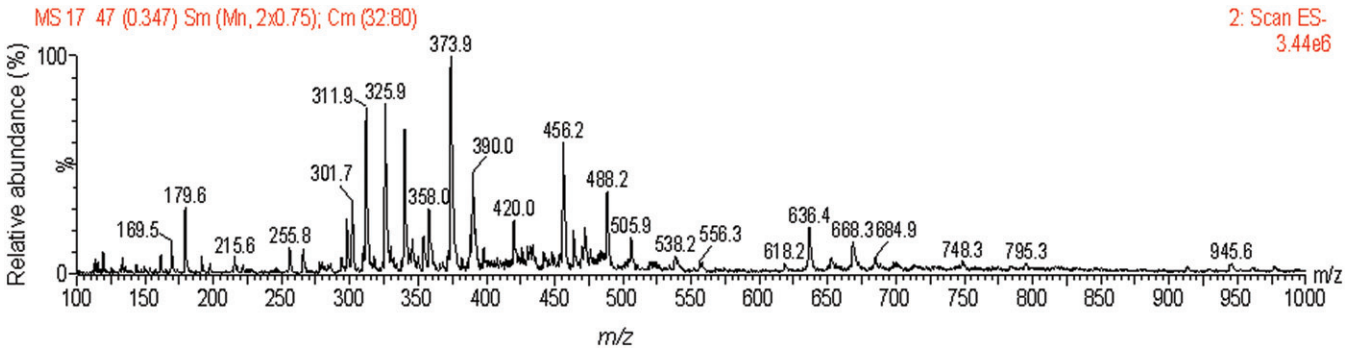

Figure 1. ESI-MS fingerprints of ethanol extract of *Syzigium cumini* leaves (EE).

naloxone did not change the antinociceptive action in formalin-induced orofacial nociceptive test (Table 2). Oral pretreatment with EE (all doses) inhibited the nociceptive response caused by the right upper lip injection of glutamate in mice (Table 2).

At the same dosages used in the nociceptive assays, the EE did not modify the performance of the mice in the rota-rod apparatus (Figure 2).

Discussion

The ESI-MS fingerprints technique with direct infusion (Roesler et al., 2007; Salvador et al., 2011) was used to characterize the presence of compounds in the EE. The extracts were analyzed by direct insertion in the negative ion mode. This method is sensitive and selective for the identification of polar organic compounds with acidic sites, such as the phenolic organic acids. Deprotonated forms of the compounds of interest were then selected and dissociated and their ESI-MS/MS were compared to those which were considered standard, along with literature data (Afify et al., 2011; De Brito et al., 2007; Hoffmann-Ribani et al., 2009; Reanmongkol et al., 1994; Roesler et al., 2007; Salvador et al., 2011; Timbola et al., 2002; Ye et al., 2007). The investigation by direct infusion electrospray ionization mass spectrometry (ESI-MS) analyses provided important information about the bioactive components present in these extracts.

The orofacial formalin model in rodents is the most widely used pattern to assess nociceptive processes in the orofacial region (Raboisson & Dallel, 2004). Perinasal injection (in the right upper lip) of formalin induces two distinct phases of pain sensitivity (Luccarini et al., 2006). In the first phase, the neurogenic pain is caused by the direct activation of type C nociceptive nerve endings, releasing neuropeptides, such as substance P, among others. The second phase is characterized by liberation of inflammatory mediators such as histamine, serotonin, bradykinin, prostaglandins, and excitatory amino acids, which can be inhibited by painkillers and anti-inflammatory drugs (Le Bars et al., 2001). The previous treatment of mice with glibenclamide and L-NOARG suggests that EE produces an antinociceptive response, at least by involving K_{ATP} channels and the NO pathway.

The nociceptive response caused by glutamate seems to involve peripheral, spinal, and supra-spinal sites. Its action is mediated by *N*-metil-D-aspartate (NMDA) and non-NMDA receptors (Quintans-Júnior et al., 2010). The glutamate *N*-methyl-D-aspartate receptor (NMDAR) is essential for the function of the nervous system, and, therefore, its de-regulation contributes to the pathophysiology of many neurological disorders. The midbrain periaqueductal gray (PAG) is densely innervated by glutamatergic projections from the forebrain, and there is robust morphine–NMDAR colocalization in the dendrites and somata of ventrolateral PAG neurons. The PAG is of physiological relevance to the nociceptive modulation network that operates both at the supraspinal level and through dorsal horn interneurons (Lipton, 2006; Marinelli et al., 2002; Narita et al., 2008; Rodríguez-Muñoz et al., 2012). The antinociception evoked from the PAG is opioid receptor mediated and can be attenuated by concurrent administration of an opioid antagonist such as naloxone (Brito et al., 2013). Thus, the inhibition of glutamate-induced orofacial nociceptive behavior by EE treatment can be associated with its interaction with the glutamatergic system.

Table 2. Effect of EE or morphine (MOR) on formalin-induced (in the presence and absence of naloxone (NAL), glibenclamide (GLI), or N^G-nitro-L-arginine (L-NOARG)) or glutamate-induced orofacial nociception tests.

		Formalin test				Glutamate test	
Treatment	Dose (mg/kg)	0–5 (min)[a]	Inhibition (%)	15–40 (min)[a]	Inhibition (%)	Pain[a]	Inhibition (%)
Vehicle	–	65.2 ± 7.3	–	129.3 ± 7.9	–	64.2 ± 5.5	–
EE	100	61.5 ± 8.9	6.1	73.5 ± 8.5[b]	43.2	39.3 ± 7.7[c]	38.8
EE	200	58.3 ± 7.5	10.6	68.4 ± 9.1[b]	47.1	31.0 ± 11.4[d]	51.7
EE	400	61.0 ± 9.2	6.4	55.1 ± 7.3[c]	57.4	29.1 ± 7.3[d]	54.7
EE + NAL	400 + 1.5	57.7 ± 8.8	11.5	69.5 ± 11.4[c]	46.2	–	–
EE + GLI	400 + 20	55.1 ± 11.7	11.8	104.5 ± 10.2	19.2	–	–
EE + L-NOARG	400 + 40	64.5 ± 5.9	1.1	111.4 ± 14.3	13.8	–	–
MOR	5	11.4 ± 3.2[d]	82.5	31.2 ± 7.8[d]	75.9	12.8 ± 5.9[d]	80.1
MOR + NAL	5 + 1.5	53.5 ± 12.4	17.9	97.4 ± 15.7	24.6	–	–

$n = 6$, per group.
[a]Values represent mean ± SEM.
[b]$p < 0.05$ (one-way ANOVA and Tukey's test), significantly different from the control group.
[c]$p < 0.01$ (one-way ANOVA and Tukey's test), significantly different from the control group.
[d]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from the control group.

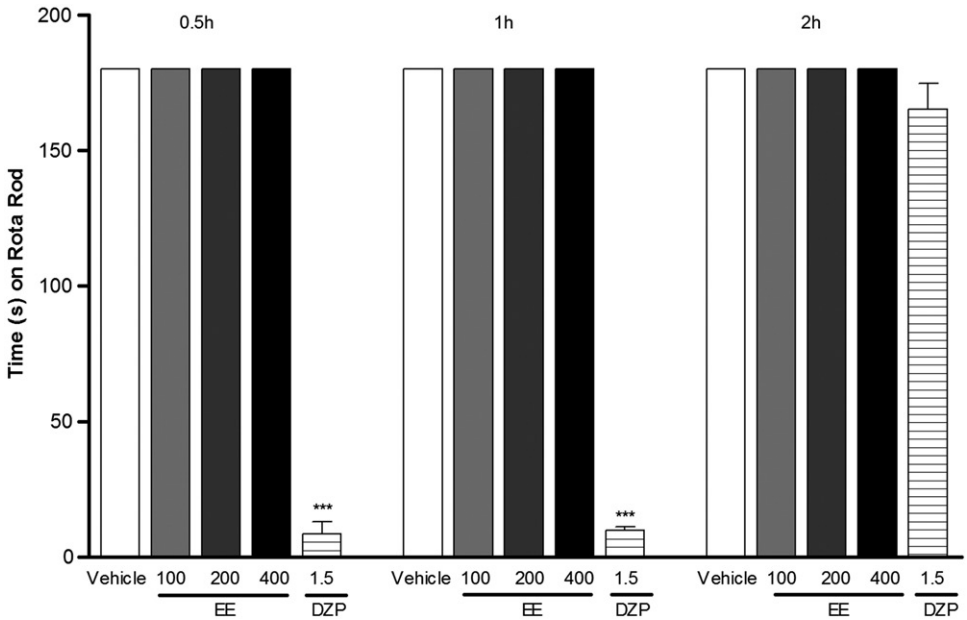

Figure 2. Time (s) on the rota-rod observed in mice after p.o. treatment with vehicle (control), ethanol extract of *Syzigium cumini* leaves (EE, 100, 200, or 400 mg/kg), or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 s after drug treatment. Statistical differences versus control group were calculated using ANOVA, followed by Tukey's test ($n = 8$, per group). *$p < 0.001$, ***$p < 0.001$.

Studies have suggested that the CNS depression and the non-specific muscle relaxation effects can reduce the response of motor coordination and might invalidate the results (Abbadie et al., 2003; Quintans-Júnior et al., 2010). This observation confirms that the action of EE on orofacial pain, observed in this study, is not entirely due to an inhibitory effect on the CNS.

According Ayyanar and Subash-Babu (2012) leaves from *S. cumini* are rich in acylated flavonol glycosides, quercetin, myricetin, myricitin, myricetin, triterpenoids, and tannin. These chemical compounds may act synergistically and contribute with analgesic profile of *S. cumini* leaves. In a study focusing on neuroinflammation, it was demonstrated that a honey flavonoid-rich extract was able to reduce the levels of a series of pro-inflammatory cytokines (Candiracci et al., 2012). A recent study demonstrated that quercetin was able to inhibit inducible NO synthase in the skeletal muscle of mice (Anhê et al., 2012). In a study with various models of chronic and acute inflammation, a glycosylated form of myricetin showed anti-inflammatory activity (Hiermann et al., 1998). With the ESI-MS fingerprinting, it was possible to identify the presence of three of these flavonoids (quercetin, myricitrin, and myricetin) that could explain, in part, the observed effects.

This study suggests that the antinociceptive potential of EE may be attributed, at least in part, to modulate inflammatory pain in the tests of orofacial nociception induced by formalin and glutamate. Considering the widespread use of *S. cumini* leaves in folk medicinal preparations (such as tea or juice), its analgesic profile may be of pharmacological and nutraceutical interest and its action in human should be confirmed by future clinical studies.

Acknowledgements

We would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq/Brazil) and the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the financial support. We would also like to thank Ashley Martin for reviewing the grammar on this manuscript.

Declaration of interest

The authors report no conflict of interest.

References

Abbadie C, Lindia JA, Cumiskey AM, et al. (2003). Impaired neuropathic pain responses in mice lacking the chemokine receptor CCR2. *Proc Natl Acad Sci USA* 100:7947–52.

Afify AE-MMR, Fayed SA, Shalaby EA, El-Shemy HA. (2011). *Syzygium cumini* (pomposia) active principles exhibit potent anticancer and antioxidant activities. *Afr J Pharm Pharmacol* 5:948–56.

Anhê GF, Okamoto MM, Kinote A, et al. (2012). Quercetin decreases inflammatory response and increases insulin action in skeletal muscle of ob/ob mice in L6 myotubes. *Eur J Pharmacol* 689:285–93.

Ayyanar M, Subash-Babu P. (2012). *Syzygium cumini* (L.) Skeels: A review of its phytochemical constituents and traditional uses. *Asian Pak J Trop Biomed* 2012:240–6.

Brito RG, Santos PL, Prado DS, et al. (2013). Citronellol reduces orofacial nociceptive behaviour in mice – Evidence of involvement of retrosplenial cortex and periaqueductal grey areas. *Basic Clin Pharmacol Toxicol* 112:215–21.

Candiracci M, Piatti E, Dominguez-Barragán M, et al. (2012). Anti-inflammatory activity of a honey flavonoid extract on lipopolysaccharide-activated N13 microglial cells. *J Agric Food Chem* 60: 12304–11.

De Brito ES, Araújo MCP, Lin L-Z, Harnly J. (2007). Determination of the flavonoid components of cashew apple (*Anacardium occidentale*) by LC-DAD-ESI/MS. *Food Chem* 105:1112–18.

Hiermann A, Schramm HW, Laufer S. (1998). Anti-inflammatory activity of myricetin-3-*O*-β-D-glucuronide and related compounds. *Inflamm Res* 47:421–7.

Hoffmann-Ribani R, Huber LS, Rodriguez-Amaya DB. (2009). Flavonols in fresh and processed Brazilian fruits. *J Food Comp Anal* 22:263–8.

Le Bars D, Gozariu M, Cadden SW. (2001). Animal models of nociception. *Pharmacol Rev* 53:597–652.

Lipton SA. (2006). Paradigm shift in neuroprotection by NMDA receptor blockade: Memantine and beyond. *Nat Rev Drug Discov* 5:160–70.

Luccarini P, Childeric A, Gaydier AM, et al. (2006). The orofacial formalin test in the mouse: A behavioral model for studying physiology and modulation of trigeminal nociception. *J Pain* 7: 908–14.

Marinelli S, Vaughan CW, Schnell SA, et al. (2002). Rostral ventromedial medulla neurons that project to the spinal cord express multiple opioid receptor phenotypes. *J Neurosci* 22:10847–55.

Muruganandan S, Srinivasan K, Chandra S, et al. (2001). Anti-inflammatory activity of *Syzygium cumini* bark. *Fitoterapia* 72: 369–75.

Narita M, Hashimoto K, Amano T, et al. (2008). Post-synaptic action of morphine on glutamatergic neuronal transmission related to the descending antinociceptive pathway in the rat thalamus. *J Neurochem* 104:469–78.

Quintans-Júnior LJ, Melo MS, Sousa De DP, et al. (2010). Antinociceptive activity of citronellal in formalin-, capsaicin- and glutamate-induced orofacial nociception in rodents and its action on nerve excitability. *J Orofac Pain* 24:305–12.

Raboisson P, Dallel R. (2004). The orofacial formalin test. *Neurosci Biobehav Rev* 28:219–26.

Reanmongkol W, Matsumoto K, Watanabe H, et al. (1994). Antinociceptive and antipyretic effects of alkaloids extracted from the stem bark of *Hunteria zeylanica*. *Biol Pharm Bull* 17:1345–50.

Rodríguez-Muñoz M, Sánchez-Blázquez P, Vicente-Sánchez A, et al. (2012). The mu-opioid receptor and the NMDA receptor associate in PAG neurons: Implications in pain control. *Neuropsychopharmacology* 37:338–49.

Roesler R, Catharino R, Malta L, et al. (2007). Antioxidant activity of *Annona crassiflora*: Characterization of major components by electrospray ionization mass spectrometry. *Food Chem* 104:1048–54.

Salvador MJ, Andreazza NL, Lourenço CC, et al. (2011). Antioxidant capacity and phenolic content of four Myrtaceae plants of the south of Brazil. *Nat Prod Commun* 6:977–82.

Schoenfelder T, Warmlin CZ, Manfredini MS, et al. (2010). Hypoglycemic and hypolipidemic effect of leaves from *Syzygium cumini* (L.) Skeels, Myrtaceae in diabetic rats. *Braz J Pharmacol* 20: 222–7.

Timbola AK, Szpoganicz AB, Branco A, et al. (2002). A new flavonol from leaves of *Eugenia jambolana*. *Fitoterapia* 73:174–5.

Ye M, Han J, Chen H, et al. (2007). Analysis of phenolic compounds in rhubarbs using liquid chromatography coupled with electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom* 18:82–91.