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The oral administration of trans-caryophyllene attenuates acute and chronic pain in mice

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ABSTRACT

Trans-caryophyllene is a sesquiterpene present in many medicinal plants' essential oils, such as *Ocimum gratissimum* and *Cannabis sativa*. In this study, we evaluated the antinociceptive activity of trans-caryophyllene in murine models of acute and chronic pain and the involvement of trans-caryophyllene in the opioid and endocannabinoid systems. Acute pain was determined using the hot plate test (thermal nociception) and the formalin test (inflammatory pain). The chronic constriction injury (CCI) of the sciatic nerve induced hypernociception was measured by the hot plate and von Frey tests. To elucidate the mechanism of action, mice were pre-treated with naloxone or AM630 30 min before the trans-caryophyllene treatment. Afterwards, thermal nociception was evaluated. The levels of IL-1β were measured in CCI-mice by ELISA. Trans-caryophyllene administration significantly minimized the pain in both the acute and chronic pain models. The antinociceptive effect observed during the hot plate test was reversed by naloxone and AM630, indicating the participation of both the opioid and endocannabinoid system. Trans-caryophyllene treatment also decreased the IL-1β levels. These results demonstrate that trans-caryophyllene reduced both acute and chronic pain in mice, which may be mediated through the opioid and endocannabinoid systems.

Introduction

Trans-caryophyllene is a bicyclical sesquiterpene and is one of the major active principles from *Cannabis sativa* (Malingré et al., 1975), *Ocimum gratissimum* (Silva et al., 1999) and *Cordia verbenaceae* (Carvalho Junior et al., 2004). It is widely used in seasoning mixtures, in various food products and in soaps and detergents (Sabulal et al., 2006).

Studies have demonstrated that trans-caryophyllene possess biological activities. For example, evidence from several experimental models show that trans-caryophyllene itself or trans-caryophyllene-containing plants possess antioxidant (Alma et al., 2003), antibacterial (Michielin et al., 2009), gastroprotective (Tambe et al., 1996), anxiolytic (Galdino et al., 2012), anti-inflammatory (Medeiros et al., 2007) and anesthetic (Ghelardini et al., 2001) activities. Trans-caryophyllene also exhibits neuroprotective effects (Chang et al., 2007) and augments the number of *natural killer* cells (Standen et al., 2006).

In addition to its biological functions, trans-caryophyllene has been shown to act as an agonist at the type 2 endocannabinoid receptors (Gertsch et al., 2008). Because the endocannabinoids play an important role in pain modulation, trans-caryophyllene may hold promise as a new pain treatment. Therefore, we evaluated the antinociceptive potential of this naturally occurring compound in acute and chronic pain models. In addition, we examined the possible mechanism of action of trans-caryophyllene by using opioid and cannabinoid antagonists and by measuring the IL-1β levels.

Materials and methods

Animals

Male C57BL/6J mice (3 months old and weighing 25–30 g) from the Centro de Desenvolvimento de Modelos Experimentais para Medicina e Biologia (CEDEME – UNIFESP) vivarium were used. Animals were housed in rooms with controlled temperature (22 ± 1 °C) and a 12 h light/dark cycle (lights on at 7:00 a.m.). Water and food were available *ad libitum*. A minimal number of experimental animals were utilized, and animals were used only once. The C57BL/6J strain was selected because of their higher sensitivity to behavioral tests and lower inter-animal variability compared with other inbred strains (Mogil et al., 2006; Balter and Dykstra, 2013). All

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studies were conducted ethically in accordance with the Research Ethical Committee of UNIFESP (protocol #0675/09). The number of animals ($n = 5$) and the intensity of painful stimuli were as small as needed to consistently demonstrate the analgesic effect of the drug (Zimmermann, 1983).

Drugs

Trans-caryophyllene was purchased from Sigma (St. Louis, USA) and diluted in corn oil. Formaldehyde and morphine were obtained from Merck (Darmstadt, Germany) and diluted in saline. The opioid antagonist naloxone (Sigma) was also diluted in saline. The cannabinoid antagonist AM630 and agonist JWH-015 (Sigma) were diluted in a solution containing saline and dimethyl sulfoxide (90/10%). Drugs were freshly prepared on the day of each experiment and administered intraperitoneally (i.p.) or orally (p.o.) in a volume of 0.1 ml/10 g body weight.

Motor coordination (rotarod)

The motor coordination of the mice was evaluated on the rotarod apparatus at a constant speed of 12 rotations per minute (rpm). Twenty-four hours prior to drug testing, animals were tested for rotarod performance. Animals that remained on the rotating bar for the full 60 s for 3 consecutive trials were used for the subsequent experiments. The selected animals were randomly distributed into groups of 5 mice and orally received corn oil (control) or trans-caryophyllene (20, 40 and 80 mg/kg). Rotarod tests were performed prior to drug administration (basal) and at 30, 60 and 120 min after administration. The length of time, up to 60 s, that each animal remained on the bar was recorded (Marques et al., 2004).

Acute pain tests

Hot plate

Groups of 5 mice received corn oil (control, p.o.) or trans-caryophyllene (1, 5 or 10 mg/kg, p.o.). An additional group received morphine (5 mg/kg, i.p.) and served as a positive control. After trans-caryophyllene administration, animals were placed on a hot plate (Ugo Basile, Biological Research Apparatus Company, Comerio, Italy) heated to 50 °C. The reaction time was defined as the latency for the animal to lick its paw(s) or jump from the plate. The maximum exposure time was 60 s (Hargraves and Hentall, 2005). Animals were submitted to testing 1, 2, 3 and 4 h after drug administration to verify the duration of the antinociceptive effects.

Formalin test

Groups of 5 mice received corn oil (control, p.o.) or trans-caryophyllene (1, 5 or 10 mg/kg, p.o.). An additional group received morphine (5 mg/kg, i.p.) and served as a positive control. After the drug or control treatment, each animal received an intraplantar injection of 2% formalin (20 μl/animal) into the right paw. Total time spent licking the injected paw was recorded during 2 phases: 5–10 min after injection and 15–30 min after injection (Hunskaar and Hole, 1987).

Mechanism of action (acute pain)

Animals were pretreated with naloxone (1 mg/kg, i.p.) or AM630 (1 mg/kg, i.p.) 30 min before receiving 10 mg/kg of trans-caryophyllene or corn oil (control) to evaluate the participation of the opioid and endocannabinoid systems in the acute antinociceptive activity of trans-caryophyllene. Two additional groups treated with morphine (5 mg/kg, i.p.) and JWH-015 (CB_2 agonist, 1 mg/kg, i.p.) served as positive controls. Thirty minutes after the treatment with the specific agonists or 1 h after the treatment with trans-caryophyllene or corn oil animals were subjected to the hot plate test, as described above.

Chronic pain tests

Induction of neuropathic pain

To induce neuropathic pain, mice were anesthetized (with a combination of xylazine and ketamine, 10 mg/kg, i.p.), and a constriction chronic injury of the sciatic nerve (CCI) was performed according as described by Bennett and Xie (1988) and modified for mice (Martucci et al. 2008). Briefly, the nerve was exposed in the mid-region of the hind limb and close to its trifurcation, was constricted with 4 loose silk thread ligatures (8-0). Sham-operated animals (with the sciatic nerve exposed but no ligature) were used as controls.

Mechanical hypernociception (von Frey test)

Groups of 5 mice were treated with corn oil (control and sham-operated), pregabalin (20 mg/kg, positive control, p.o.) or trans-caryophyllene (1, 5 or 10 mg/kg, p.o.) for 14 days after surgery. The animals were evaluated for tactile stimulation by a von Frey electronic device before surgery (baseline values) and at the 7th and 14th days post-surgery. For this test, a linear crescent pressure was applied in the center of the hindpaw plantar surface until the animal exhibited a characteristic "flinch" withdrawal or licking response. The intensity of the hypernociceptive response was quantified as the change in pressure applied by subtracting the mean of the 3 values obtained before the surgery from the mean of the three values observed on days 7 and 14 post-surgery (Cunha et al. 2004).

Thermal hypernociception (hot plate test)

Thermal hypernociception was evaluated in treated mice for 14 days post-surgery using the hot plate test. Groups of 5 mice received corn oil (control and sham-operated), pregabalin (20 mg/kg, positive control) or trans-caryophyllene (1, 5 or 10 mg/kg, p.o.) for a period of 14 days after surgery. After 1 h of treatment, the animals were placed on a hot plate heated to 50 °C. The reaction time was defined as the latency for the animal to lick its paw(s) or jump from the plate, with a maximum exposure of 60 s. The test occurred before surgery (baseline values) and on the 7th and 14th postoperative days (Hargraves and Hentall, 2005).

Mechanism of action (chronic pain)

To verify the involvement of the opioid and endocannabinoid systems on the antihypernociceptive activity of trans-caryophyllene, animals were treated with corn oil (control surgery) or trans-caryophyllene (10 mg/kg) for 14 days. On the 14th day of treatment, the animals were pretreated with naloxone or AM630 at a dose of 1 mg/kg (i.p.) 30 min before receiving the trans-caryophyllene. The CB_2 agonist-treated group (JWH-015, 1 mg/kg, i.p.), was used as positive control. After 30 min of JWH-015 treatment and 1 h after trans-caryophyllene or corn oil treatment, animals were evaluated using the hot plate test as described above.

Biochemical assay

Enzyme-linked immunosorbent assay (ELISA) tissue sample preparation

After the behavioral tests evaluating neuropathic pain, the IL-1β levels were measured (Safieh-Garabedian et al. 1995). The animals were euthanized, and the sciatic nerve was collected and homogenized in phosphate buffered saline (PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin (BSA), 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM

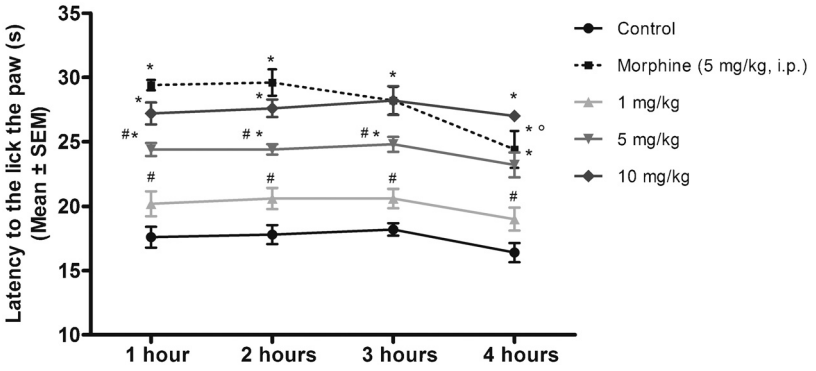

Fig. 1. The influence of trans-caryophyllene on the latency to withdraw from a thermal stimulus in mice. Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group at corresponding times, (#) from the morphine group at corresponding times and (°) the intragroup difference at 1 h (repeated-measures ANOVA followed by Tukey's *post hoc* test, $p < 0.05$, $n = 5$/group).

EDTA and 0.001% aprotinin (37.6 mg per 100 ml of PBS with EDTA). Then, the samples were centrifuged at 3000 rpm for 15 min at 4 °C. The IL-1β levels were measured using an ELISA kit (enzyme-linked immunosorbent assay), following the manufacturer's recommendations (R&D Systems™, Minneapolis, USA). The total amount of protein collected was measured by the bicinchoninic acid method using the Micro BCA™ Protein kit. The absorbance was measured using a microplate reader at 470 and 570 nm, and the results were expressed as pg/mg protein.

Statistical analysis

The data obtained in the formalin test, in the evaluation of opioid and endocannabinoid systems involvement and from the IL-1β measurements were analyzed by one-way ANOVA, followed by Tukey's *post hoc* test when needed. Rotarod motor coordination, hot plate tests and von Frey tests were evaluated by repeated measures ANOVA, followed by Newman–Keuls *post hoc* test. Data are presented as the mean ± standard error of the mean (SEM). The significance level was set at $p < 0.05$.

Results

Motor coordination (rotarod)

The mean time spent on the rotarod is presented in Table 1. The oral administration of 20, 40 or 80 mg/kg of trans-caryophyllene did not significantly affect the motor coordination of the mice at any of the time points examined.

Acute pain tests

Hot plate

Dose-dependent antinociceptive effects were observed after trans-caryophyllene treatment in the hot plate test (Fig. 1). Mice receiving 5 or 10 mg/kg of trans-caryophyllene exhibited a significant increase in the latency to lick the paw(s) or to jump from the plate when compared with the control group, up to 4 h after treatment ($p < 0.01$). Furthermore, the 5 mg/kg-treated group showed reduced latencies compared with the morphine group during the first three hours of testing, suggesting an intermediate effect.

Table 1
Effect of oral trans-caryophyllene administration on motor coordination of mice in the rotarod test.

Treatment	Permanence time on Rota-rod (s) (Mean ± standard error from mean) Observation intervals (min)			
	Baseline	30′	60′	120′
Control	60 ± 0	57 ± 3	56 ± 4	55 ± 5
20 mg/kg	60 ± 0	59 ± 1	56 ± 4	60 ± 0
40 mg/kg	60 ± 0	57 ± 2	59 ± 1	60 ± 0
80 mg/kg	60 ± 0	59 ± 1	60 ± 0	60 ± 0

Formalin test

The highest dose (10 mg/kg) of trans-caryophyllene significantly reduced time spent licking the formalin-injected paw in both phases of the test ($p < 0.01$, Fig. 2). The 5 mg/kg dose was effective only against the inflammatory pain evaluated in the second phase ($p < 0.05$; Fig. 2). The smallest dose used (1 mg/kg) was not effective in any phase of the test. The administration of trans-caryophyllene produced a dose-dependent effect as in the other tests.

Mechanisms of action (acute pain)

Morphine (opioid agonist) and JWH-015 (cannabinoid agonist) significantly decreased the latencies in the hot plate test. Naloxone (opioid antagonist) and AM630 (cannabinoid antagonist) completely reversed the antinociceptive effect induced by their respective agonists. Both antagonists also significantly attenuated the antinociceptive effects of trans-caryophyllene (10 mg/kg) ($p < 0.05$), indicating the involvement of the opioid and endocannabinoid systems in the observed effects (Fig. 3).

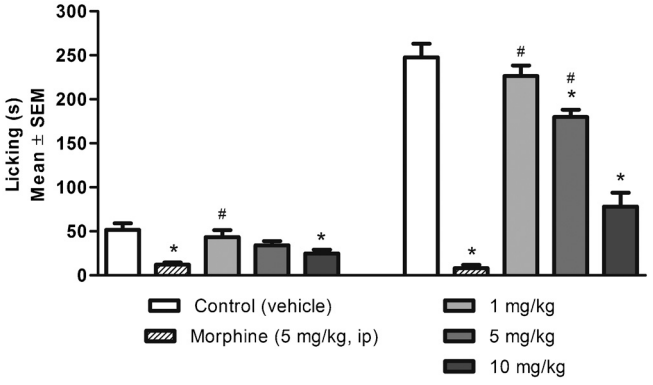

Fig. 2. Duration (in seconds) of paw licking after intraplantar formalin injection of trans-caryophyllene pre-treated mice. Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group and (#) from the morphine group (ANOVA followed by Tukey's *post hoc* test, $p < 0.05$, $n = 5$/group).

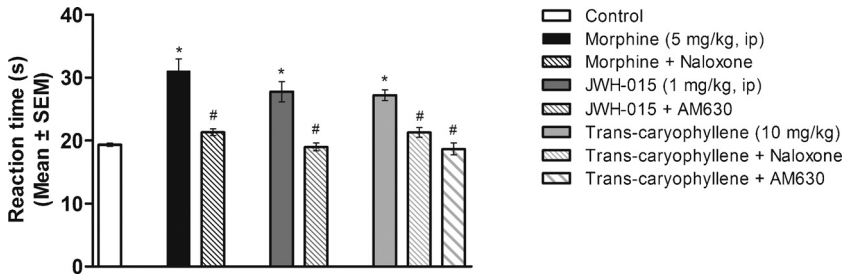

Fig. 3. The effects of different antagonists on the antinociceptive activity of trans-caryophyllene in thermal hypernociception. Animals were pre-treated with naloxone (1 mg/kg, i.p.) or AM630 (1 mg/kg, i.p.) 30 min prior to trans-caryophyllene administration (10 mg/kg, p.o.). Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group and (#) from their respective agonists (ANOVA followed by Tukey's *post hoc* test, $p < 0.01$, $n = 5$/group).

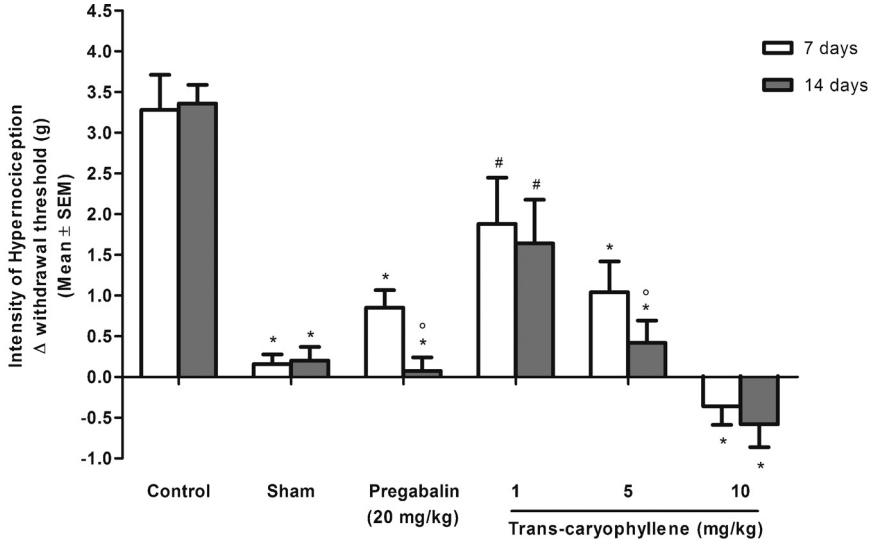

Fig. 4. Effects of varying doses of trans-caryophyllene on mechanical hypernociception in mice subjected to the von Frey test. Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from control group at the respective times, (#) from sham-operated animals at the respective times and (°) the intragroup difference between 7 and 14 days of treatment (repeated measures ANOVA followed by the Newman–Keuls test, $p < 0.05$, $n = 5$/group).

Chronic pain tests

Mechanical hypernociception (von Frey test)

Pregabalin treatment (positive control) or 5 and 10 mg/kg trans-caryophyllene treatments significantly inhibited the mechanical hypernociception induced by the chronic constriction injury as evaluated by the von Frey test compared with the control group ($p < 0.05$) at the 7th and 14th days post-surgery (Fig. 4).

Thermal hypernociception (hot plate test)

The results presented in Fig. 5 shows that the trans-caryophyllene at doses of 5 and 10 mg/kg statistically increased the permanence of the animals on the heated plate at the 7th and 14th days post-surgery when compared with the control group ($p < 0.05$). The 1 mg/kg dose did not promote antihypernociception.

Mechanism of action (chronic pain)

Fig. 6 shows that administration of JWH-015 (1 mg/kg, i.p.) and trans-caryophyllene (10 mg/kg) promoted antihypernociception in mice subjected to CCI when compared with the animals receiving vehicle. The mice treated with trans-caryophyllene spent more time on the hot plate compared with the positive control group (JWH-015, $p = 0.0004$). The administration of the cannabinoid antagonist AM630 (1 mg/kg, i.p.) completely inhibited the antihypernociceptive effect observed by the agonist (JWH-015), as expected. The antagonists, naloxone (opioid) or AM630 (cannabinoid), also inhibited the antihypernociceptive effect of

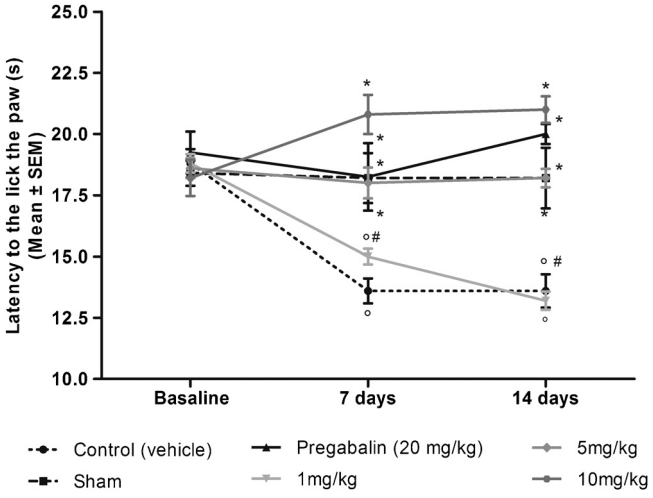

Fig. 5. The influence of trans-caryophyllene on thermal nociception after a chronic constriction injury (CCI). Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group at the corresponding times, (#) from the sham group at the corresponding times and (°) the intragroup difference at basal evaluation (repeated-measures ANOVA followed by Tukey's *post hoc*, $p < 0.05$, $n = 5$/group).

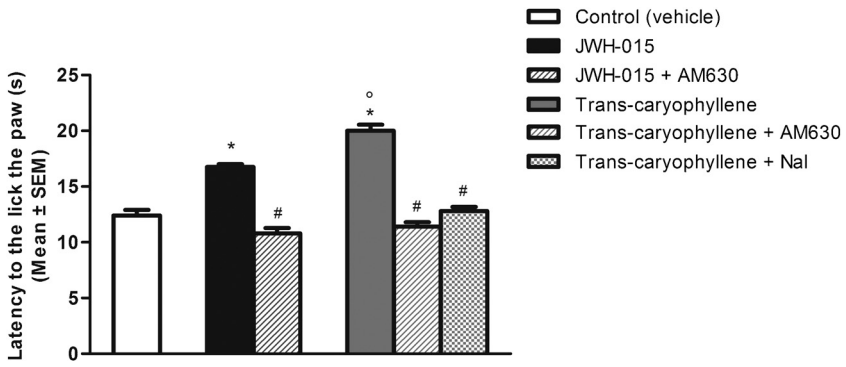

Fig. 6. The effects of opioid and cannabinoid antagonists on the thermal hypernociceptive response of trans-caryophyllene. Animals were pre-treated with naloxone or AM630 for 30 min before the oral administration of trans-caryophyllene (10 mg/kg). Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group, (#) from the respective agonists, (◦) from the JWH-015 group (ANOVA followed by Tukey's *post hoc* test, $p < 0.05$, $n = 5$/group).

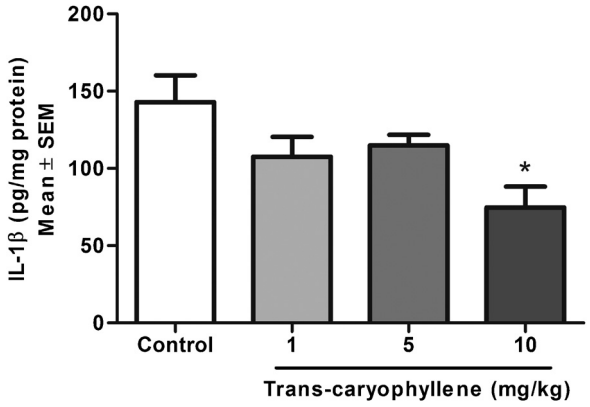

Fig. 7. IL-1β levels in the sciatic nerve after a chronic constriction injury in the presence or absence of trans-caryophyllene. Data are expressed as the mean ± SEM. Statistical significance is indicated as (*) from the control group (ANOVA followed by Tukey *post hoc* test, $p < 0.05$, $n = 5$/group).

trans-caryophyllene (10 mg/kg), indicating the involvement of the opioid and endocannabinoid systems ($p < 0.05$).

Effect on IL-1β production

The IL-1β levels of the sham-operated animals were undetectable. Conversely, the control group showed increased levels of this cytokine. Only the 10 mg/kg dose of trans-caryophyllene was effective at reducing the IL-1β levels in the mice subjected to a CCI of the sciatic nerve when compared with the control group (Fig. 7, $p < 0.05$).

Discussion

We investigated the antinociceptive effects of the oral administration of trans-caryophyllene using classic models of acute and chronic nociception described in the literature. In a dose-dependent manner, trans-caryophyllene increased the latency to lick or to jump from the hot plate and inhibited pain in both phases of the formalin test when compared with the control group. Trans-caryophyllene was also effective at reducing the neuropathic pain induced by a sciatic nerve injury and at decreasing the levels of IL-1β. In addition, we demonstrated that both opioid and cannabinoid antagonists were able to attenuate these antinociceptive effects.

The opioid and endocannabinoid pathways participate actively in pain management (Giordano, 2005). Few studies directly examine the antinociceptive activity of trans-caryophyllene, but some evidence exists in the literature. Trans-caryophyllene is an agonist of the cannabinoid CB_2 receptors (Gertsch et al., 2008) and is present in many plants with antinociceptive properties, including *C. sativa* (3–37.5% in essential oil) (Mediavilla and Steinemann, 1997) and in *C. Verbenaceae* (Medeiros et al., 2007). Both the CB_1 (Costa et al., 2006) and CB_2 receptors (Jhaveri et al., 2008) of the endocannabinoid system are established mediators of potent antinociceptive effects. Compounds targeting this system are used to treat painful and inflammatory processes (Dyson et al., 2005; Beltramo, 2009). Substances that act as agonists at the CB_2 receptor are widely studied for their potent effects against acute and neuropathic pain and their scarce deleterious side effects compared with drugs acting on the CB_1 receptors (Guindon and Hohmann, 2008; Xu et al., 2010).

In conjunction with other recent reports, our results clearly demonstrate the antinociceptive effects of trans-caryophyllene. Recent studies suggest that its analgesic effect may be due to the interaction of the glutamatergic pathway with the opioid system and with L-arginine/nitric oxide (Celedônio, 2008; Katsuyama et al., 2013). Other possible mechanisms of action for trans-caryophyllene include pain modulation through the endocannabinoid pathways, the inhibition of the nuclear transcription factor *kappa*-B (NF-κB) pathway, and a reduction in cycloxygenase-2 expression or TNFα and PGE_2 release (Fernandes et al., 2007; Medeiros et al., 2007). The results from our opioid and cannabinoid antagonist studies demonstrate that both systems are involved in the acute and chronic antinociceptive activity of trans-caryophyllene. Naloxone and AM630 were able to reverse the antinociception induced by treatment with 10 mg/kg of trans-caryophyllene.

The CB_2 receptor is widely found in peripheral tissues, especially in immune cells, but is less widespread in the central nervous system compared with the CB_1 receptor (Atwood and Mackie, 2010). Studies indicate that CB_2 receptor activity can promote antinociception through either the direct or indirect activation of the final pain modulation pathway (Ibrahim et al., 2006; Hsieh et al., 2011). Thus, selective agonists of CB_2 receptors, such as trans-caryophyllene, have been suggested to induce the release of endogenous β-endorphin peptide precursors from keratinocytes, leading to the activation of μ-opioid receptors in primary afferent neurons and, consequently, analgesia (Ibrahim et al., 2005). Alternatively, the analgesic effects may be due to the direct activation of CB_2 receptors in peripheral neurons (Anand et al., 2008) or even in brain regions related to pain modulation, such as the thalamus, cerebellum and brainstem (Jhaveri et al., 2008; Yamamoto et al., 2008; Brownjohn and Ashton, 2012). There is support for both pathways in the literature. Therefore, the study of new cannabinoid compounds specific for CB_2 receptors holds promise.

Trans-caryophyllene treatment was effective at reducing the IL-1β levels in the injured sciatic nerve of mice. IL-1β is a cytokine mostly expressed in response to inflammatory or infectious agents and endotoxins (Lopez-Castejon and Brough, 2011). Studies have shown an inadequate production of IL-1β in a range of pathologies, including painful neuropathies such as peripheral nerve injuries (del Rey et al., 2011; Kiguchi et al., 2012). In contrast, other studies have shown that some drugs that reduce painful hypersensitivity in neuropathic pain animal models prevent inadequate IL-1β production (Silva et al., 2011; Amin et al., 2012). Because the induction of this cytokine is important for the generation and maintenance of neuropathic and inflammatory pain, the substances capable of reversing this increase should be investigated more thoroughly for the development of new therapeutics.

The present work demonstrates that trans-caryophyllene, an active principle found in many medicinal plants, showed marked oral antinociceptive properties in both acute and chronic models of nociception in mice. The mechanisms through which trans-caryophyllene exerts its antinociceptive actions still remain incompletely understood, but they seem in part to involve interaction with the opioid and endocannabinoid pathways, besides reduction of IL-1beta at the injury site.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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