

Antinociceptive Effect of the Monoterpene *R*-(+)-Limonene in Mice

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In the present study were studied the antinociceptives properties of monoterpene *R*-(+)-limonene (LM) in chemical and thermal models of nociception in mice. The *R*-(+)-limonene was administered, intraperitoneally (i.p.), at doses of 25 and 50 mg/kg. The results showed significant inhibition produced on chemical nociception induced by intraperitoneal acetic acid and in the second phase of subplantar formalin test, but did not manifest a significant effect in hot-plate test. The *R*-(+)-limonene-induced antinociception in second phase of formalin test was insensitive to naloxone (1 mg/kg, s.c.). It was also demonstrated that *R*-(+)-limonene (25, 50 mg/kg) neither significantly enhanced the pentobarbital-sleeping time nor impaired the motor performance in rota-rod test, indicating that the observed antinociception is unlikely to be due to sedation or motor abnormality. In conclusion it may be suggested that the *R*-(+)-limonene presented antinociceptive activity and that, probably, this action can be related with peripheral analgesia, but, not with the stimulation of opioids receptors.

Key words *R*-(+)-limonene; antinociceptive; naloxone

Although a considerable number of analgesic drugs are available for the treatment of pain, search for development of new compounds as therapeutic alternatives have been increasingly realized, since the available analgesic drugs exert a wide range of side effects.^{1–4)}

Plant derived essential oils exhibit a variety of biological properties, such as anxiolytic,⁵⁾ anticonvulsant,⁶⁾ antinociceptive and anti-inflammatory.⁷⁾ Those effects are attributed to the monoterpenes, which are the major chemical components of these essential oils.

D-Limonene [*R*-(+)-isomer] is a monoterpene prevalent in essential oils of various plants, such as *Lippia alba* (MILL.) N. E. BROWN (Verbanaceae),⁸⁾ *Artemisia dracunculul* L. (Asteraceae),⁹⁾ and in other aromatic plants species.³⁾ Some studies with essential oils containing *R*-(+)-limonene as one of prevalent compounds or with pure limonene have demonstrated its anti-inflammatory activities. The pure *R*-(+)-limonene when administered orally was able to inhibit the LPS-induced inflammation including cell migration and inhibiting the production of nitric oxide, gamma-interferon and IL-4.¹⁰⁾ *R*-(+)-Limonene inhalation significantly prevented bronchial obstruction and was able to diminish peribronchiolar and perivascular inflammatory infiltrates in rats.¹¹⁾ Such results were obtained from pulmonary function tests and pathological data, respectively.

Recently, the essential oil of *Dracocephalum kotschy* (Labiateae) presented antinociceptive effect,³⁾ which was partly attributed to the presence of *R*-(+)-limonene (and α -terpineol), but the exact mechanism of action remains to be elucidated. Although literature data presents the antinociceptive effects of the essential oils containing *R*-(+)-limonene as one of prevalent compounds,³⁾ there are scarce reports with reference to those effects. Therefore, the main objective of the present paper were to study the analgesic effect of the pure *R*-(+)-limonene, in order to attempt to elucidate its mechanisms of action.

MATERIALS AND METHODS

Drugs and Chemicals The following drugs were used: *R*-(+)-limonene (Dierberger, Brazil) morphine hydrochloride (Cristalia, RJ, Brazil), diazepam (Novaquímica, Brazil) pentobarbital sodium, indomethacin, naloxone, acetic acid and formaldehyde (Sigma Chemical Co. MO, U.S.A.). Morphine hydrochloride, indomethacin, pentobarbital sodium, naloxone, diazepam were dissolved in physiological saline (0.9% NaCl). The other solutions (0.6% acetic acid and 1% formalin) were also prepared in 0.9% saline. The vehicles used alone had no effects *per se* on the nociceptive responses in mice.

Experimental Animals and Treatment Male albino Swiss mice, weighing between 20 and 30 g, maintained on a 12/12 h light–dark cycle were used in this study. The experiments were carried at an ambient temperature of 24 ± 2 °C with access to food and water *ad libitum*. The *R*-(+)-limonene was suspended in 2% Tween 80 in distilled water and administered intraperitoneally to mice at doses of 25 and 50 mg/kg. Control animals were given vehicle only (10 ml/kg) by the same route. Each experimental group consisted of 8 animals. Animals were treated in accordance to the current law and the NIH Guide for Care and Use of Laboratory Animals.

Acetic Acid-Induced Abdominal Constrictions The nociceptive response was evaluated after the intraperitoneal (i.p.) injection of acetic acid¹²⁾ which is a modification of the model originally described.¹³⁾ The *R*-(+)-limonene or vehicle were injected intraperitoneally to groups of the mice 30 min prior of the i.p. injection of acetic acid (0.6%, 10 ml/kg). The number of abdominal constriction associated with total stretching of the hind limbs was counted over a period of 20 min, commencing 10 min after injection of acetic acid. An indomethacin (INDO) (5 mg/kg; i.p.) pretreated group was included in the study as a positive control.^{13,14)}

Formalin-Induced Nociception Groups of mice treated

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as described before were injected with 20 μ l of 1% formalin (in 0.9% saline, subplantar) and the duration of paw licking was determined 0–5 min (first phase) and 20–25 min (second phase) after formalin injection.¹⁵ Morphine (7.5 mg/kg, i.p.) was used as a reference drug. To investigate the participation of the opioid system in the antinociceptive effect of *R*-(+)-limonene, animals were pre-treated with naloxone (non-selective opioid antagonist, 1 mg/kg, s.c.) 15 min before the administration of *R*-(+)-limonene (50 mg/kg, i.p.), morphine (7.5 mg/kg, i.p.) or saline (0.9% NaCl solution, 10 ml/kg, i.p.).

Hot-Plate Model The hot-plate test was used to measure the response latencies according to the method described previously.¹⁶ Animals were placed into a glass cylinder of 24 cm diameter on the heated surface, and the time between placement and shaking or licking of the paws or jumping was recorded as the index of response latency. Animals were pre-selected on the hot-plate at $51 \pm 0.5^\circ\text{C}$ and treated with vehicle, *R*-(+)-limonene or morphine (7.5 mg/kg, i.p.). The reaction time (s) for each mouse was determined on the hot-plate before and after drug administration at intervals of 30 min for a total period of 90 min. A cut-off period of 45 s was set to avoid possible injury.

Pentobarbitone-Induced Sleeping Time Groups of animals were treated with *R*-(+)-limonene, and vehicle (10 ml/kg) or diazepam (2.0 mg/kg, i.p.) 30 min before the injection of sodium pentobarbitone (40 mg/kg, i.p.). The loss and regain of righting reflex was considered as the duration of sleep time in minutes.¹⁷

Rota-Rod Test To verify a possible effect on motor coordination, *R*-(+)-limonene was examined on rota-rod apparatus.¹⁸ The apparatus consisted of a horizontal bar with a diameter of 5 cm, subdivided into four compartments (IN-SIGHT, RT-2002, Brazil). The mice were placed on the bar rotating at a speed of 4 rpm and mice that were able to remain on the rod longer than 60 s were selected 24 h before test. The animals were treated with *R*-(+)-limonene, and vehicle (10 ml/kg) or diazepam (2.0 mg/kg, i.p.). After 30 min following the treatments, each animal was tested on the rota-rod and the time of permanence (s) on the bar during a 1 min period was registered before (0 h), 1 and 2 h after drug administration.

Statistical Analysis Data are expressed as mean \pm S.E.M. ANOVA followed by Student Newman Keul's test was used to analyse the significance of differences between groups. Values were considered statistically significant at $p < 0.05$.

RESULTS

Effect on Nociception *R*-(+)-Limonene presented significant antinociception relative to the control group in two tests models of nociception induced by chemical agents. In the acetic acid-induced writhing test, performed in the present study, *R*-(+)-limonene in the doses of 25 and 50 mg/kg, i.p., significantly reduced the number of writhes (5.28 ± 1.92 and 0.00 ± 0.00 writhes/20 min), respectively, in relation to the control group (29.14 ± 2.36 writhes/20 min) (Fig. 1). The indomethacin (5 mg/kg, i.p.), a nonsteroidal anti-inflammatory drug, also promoted a significant reduction in the number of writhes (7.71 ± 2.17 writhes/20 min).

In formalin test, control group showed the mean licking

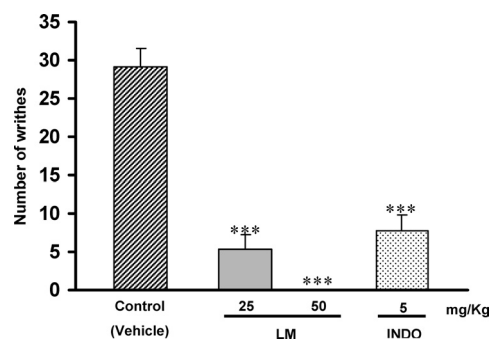


Fig. 1. Effects of Intraperitoneally Administered Limonene (LM) and Indomethacin (INDO) on Acetic Acid-Induced Writhings in Mice

The vehicle (control, 10 ml/kg), LM (25, 50 mg/kg) or INDO (5 mg/kg) were administered intraperitoneally, 30 min before the intraperitoneal administration of acetic acid (0.6%, 10 ml/kg) and the number of writhes were counted over a period of 20 min. Each column represents the mean \pm S.E.M. ($n=8$). Asterisks indicate significant difference from control. *** $p < 0.001$ (ANOVA followed by *t*-Student Newman Keul).

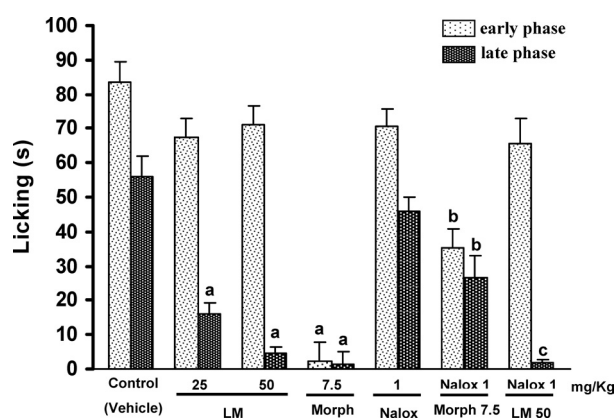


Fig. 2. Effects of Intraperitoneally Administered Limonene (LM) and Morphine (Morph) on Formalin-Induced Nociception in Mice

The total time spent (s) in licking the injected hind-paw was measured in the early phase (0–5 min) and the late phase (20–25 min). The vehicle (control, 10 ml/kg), LM (25, 50 mg/kg) or morphine (7.5 mg/kg) were administered intraperitoneally, 30 min before the test. Naloxone (Nalox 1 mg/kg s.c.) were administered 15 min before LM or morphine. Each column represents the mean \pm S.E.M. ($n=8$). ^a $p < 0.001$ vs. control; ^b $p < 0.01$ vs. Morph; ^c $p < 0.001$ vs. control (ANOVA followed by *t*-Student Newman Keul).

times (s) of 83.33 ± 6.21 in the first phase and 55.83 ± 6.17 in the second phase (Fig. 2). Treatment with *R*-(+)-limonene caused significant reduction only in the second phase (15.83 ± 3.42 , 4.50 ± 1.76 s), at the tested doses of 25 and 50 mg/kg, respectively. Morphine (7.5 mg/kg), an opioid agonist, was used as positive control group and significantly suppressed the formalin-response at both phases (first phase, 2.00 ± 0.86 and second phase, 1.50 ± 0.70 s).

The naloxone (1 mg/kg, s.c.), an opioid antagonist, significantly inhibited the antinociceptive effect of the morphine in the two phases of the test (naloxone+morphine: first phase, 35.25 ± 5.60 and second phase, 26.57 ± 6.31 s). However, naloxone was not capable to revert the antinociception *R*-(+)-limonene (50 mg/kg, i.p.) induced (naloxone+*R*-(+)-limonene: 1.75 ± 0.90 s) in second phase of the test, when compared to *R*-(+)-limonene alone. Naloxone failed to modify the formalin-induced nociceptive responses in a significant manner, when administered alone (Fig. 2).

In the hot-plate test, the *R*-(+)-limonene (25, 50 mg/kg, i.p.) failed to demonstrate any statistically significant influence on reaction latency. However, morphine (7.5 mg/kg,

i.p.) demonstrated significant antinociception at the time points of 30 and 60 min (Fig. 3).

Effect on Pentobarbital Sleeping Time The *R*-(+)-limonene at 25 and 50 mg/kg (68.71 ± 8.42 , 76.00 ± 1.58 min, respectively) was not able to increase the sleeping time produced by pentobarbitone (40 mg/kg, i.p.) as compared to control (65.86 ± 7.15 min). Diazepam, a sedative drug, prolonged the sleeping time significantly (169.12 ± 9.31 min) (Table 1).

Effect in Rota-Rod Test *R*-(+)-Limonene (25, 50 mg/kg, i.p.) did not affect the motor coordination in mice, while, diazepam (2 mg/kg, i.p.) treatment significantly

($p < 0.001$) decreased the time of permanence on bar in this test when compared to controls (Table 2).

DISCUSSION

The writhing behavior, in mice, by the intraperitoneally injection of acetic acid in the chemical nociception, is used to evaluate, essentially, central and peripheral analgesic activity. The algnesia acetic acid-induced promote the endogenous substance release and many others that stimulate nervous terminations of pain.^{19,20} The acetic acid-induced nociceptive reponse can involve the direct stimulation of nociceptive afferent fibers from the reduction of pH and the synthesis of inflammatory mediators.²¹

In accordance with the percentage of inhibition of the number of the writhes obtained through *R*-(+)-limonene use, in the different tested doses, it was observed that the intensity of its analgesic effect was significantly similar to the indomethacin. The indomethacin and other nonsteroidal anti-inflammatory drugs (NSAIDs) can inhibit cyclooxygenase in peripheral tissues reducing the synthesis and/or the release of inflammatory mediators intervening thus, with the mechanisms of transduction of primary afferent nociceptors.²⁰

The analgesic mechanism of action of the *R*-(+)-limonene can, probably, involve inhibition of the synthesis and/or release of inflammatory mediators who promote pain in the nervous terminations, similarly to the indomethacin and the other NSAIDs suggesting a peripheral analgesic action. However, the test of abdominal constrictions is non-specific, since NSAIDs, opioid analgesics and even tricyclic antidepressants may inhibit the nociceptive response in the acetic acid model.^{21–23}

The formalin test is a model of an acute and tonic pain, being considered a more valid model for clinical pain of what the tests with mechanical or thermal stimulation.^{14,20,24,25} The formalin-induced nociceptive reponse possess two phases that can involve different mechanisms. The first phase (neurogenic pain) results of the direct chemical stimulation of myelinated and unmyelinated nociceptive afferent fibers, mainly C fibers. The second phase (inflammatory pain) results of the action of set free inflammatory mediators in the peripheral tissues and of functional changes in the neurons of spinal dorsal horn that, in the long run, promote to the facilitation of the synaptic transmission in the spinal level.^{14,21,23,24}

Experimental results have indicated that substance P and bradykinin participate of first phase, that can be suppressed, mainly, by analgesic drugs of central action (opioids anal-

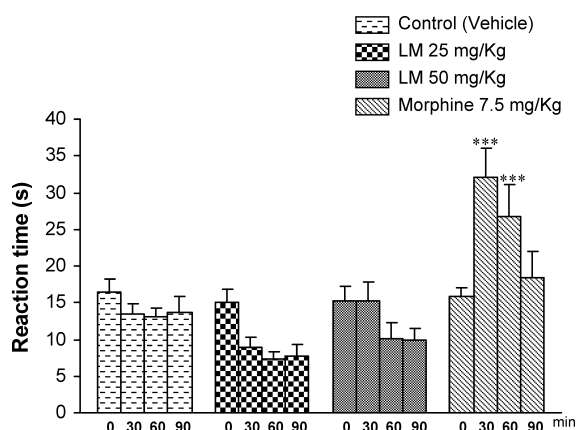


Fig. 3. Effect of Limonene (LM) in the Hot-Plate Test

The reaction time was measured in seconds (s) before (0 min) and 30, 60 and 90 min after drug treatment. Horizontal axis shows time intervals (min) and the bars represent reaction time (s) in each animal group treated with vehicle (control), LM 25 mg/kg, LM 50 mg/kg or morphine 7.5 mg/kg. Each column represents the mean \pm S.E.M. ($n=8$). Asterisks indicate significant difference from control. *** $p < 0.001$ (ANOVA followed by *t*-Student Newman Keul).

Table 1. The Vehicle (Control, 10 ml/kg), LM (25, 50 mg/kg) or Diazepam (2 mg/kg) Were Administered Intraperitoneally, 30 min before the Intraperitoneal Administration of Pentobarbital (40 mg/kg) and the Sleeping Time Was Observed

Treatment	Dose (mg/kg, i.p.)	Sleeping time (min)
Vehicle (control)	—	65.86 ± 7.15 (8)
<i>R</i> -(+)-Limonene (LM)	25	68.71 ± 8.42 (8)
	50	76.00 ± 1.58 (8)
Diazepam	2	169.12 ± 9.31 *** (8)

The loss and regain of righting reflex was considered as the duration of sleep time in minutes. The results are expressed as mean \pm S.E.M., with the numbers of animals in parentheses ($n=8$). *** $p < 0.001$ as compared to controls (ANOVA followed by *t*-Student Newman Keul).

Table 2. The Vehicle (Control, 10 ml/kg), LM (25, 50 mg/kg) or Diazepam (2 mg/kg) Were Administered Intraperitoneally

Treatment	Dose (mg/kg, i.p.)	Time of permanence (s)/1 min		
		0 h	Period (observation) 1 h	2 h
Vehicle (control)	—	57.88 ± 0.63	58.25 ± 0.86	58.88 ± 0.47 (8)
<i>R</i> -(+)-Limonene (LM)	25	57.50 ± 0.73	57.63 ± 0.98	58.38 ± 0.49 (8)
	50	58.63 ± 0.59	59.13 ± 0.51	59.63 ± 0.26 (8)
Diazepam	2	59.13 ± 0.39	30.38 ± 1.77 ***	35.88 ± 1.34 *** (8)

After 30 min following the treatments, each animal was tested on the rota-rod and the time of permanence in seconds (s) on the bar during a 1 min period was registered before (0 h), 1 and 2 h after drug administration. The values represent mean \pm S.E.M., with the numbers of animals in parentheses ($n=8$). *** $p < 0.001$ as compared to controls (ANOVA followed by *t*-Student Newman Keul).

gesics as the morphine); whereas in second phase, some excitatory mediators such as histamine, serotonin, bradykinin, amino acids and prostaglandins can be involved in the inflammatory pain, that is very sensible the action of the majority of the NSAIDs, including acetylsalicylic acid, indomethacin and naproxen.^{23,24)}

In this model, *R*-(+)-limonene inhibited, significantly, the response of licking and biting of the injected paw only in second phase of the test; whereas the morphine, opioid agonist, significantly inhibited the nociception formalin-induced in the two phases of the test. However, naloxone, an opioid antagonist, reversed the antinociceptive effect of the morphine, but it showed no influence on the antinociceptive action of the *R*-(+)-limonene (50 mg/kg, i.p.). This can suggest nonparticipation of the opioid system in the modulation of pain for *R*-(+)-limonene.

The hot-plate test is known by being sensible only for drugs that act the supraspinal level.^{14,21)} The evaluating analgesic analogous to the morphine in mice, have demonstrated that the hot-plate at 55 °C produced false negatives results and suggested the use of lower temperatures.²⁶⁾ Therefore, we use the hot plate with low temperature (51 ± 0.5 °C). *R*-(+)-Limonene, in the studied doses, failed to demonstrate any significant influence in the latency of reaction to the hot plate, indicating lack of efficacy in suppressing supraspinal nociception. Although, in the intervals of time of 30, 60 and 90 min after the injection of *R*-(+)-limonene 25 mg/kg, the results have demonstrated a tendency to the hypersensitivity, those were not statistically significant. However, morphine, a well-known opioid agonist, produced a profound antinociceptive effect to the hot-plate test in the period of 30 and 60 min.

Previous studies suggest that the sedation of the central nervous system and the effect non-specific muscle-relaxation can reduce the response of motor coordination as, for example, the licking of the paw in the formalin test.^{18,21,25,27)} The antinociception caused by *R*-(+)-limonene seems to be unrelated to motor impairment or sedation since mice tested in rota-rod test and barbiturate-sleeping time test showed no significant effects on these behaviors.

In conclusion, the study demonstrates the antinociceptive activity of the *R*-(+)-limonene in the models of chemical nociception induced by acetic acid and formalin. The study also suggests that its antinociception action probably is unrelated to classical opioid receptor stimulation, but may involve predominantly inhibition of the synthesis or action of inflammatory mediators.

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