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Antinociceptive activity of carvacrol (5-isopropyl-2-methylphenol) in mice

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Abstract

Objectives Carvacrol (5-isopropyl-2-methylphenol) is a monoterpenic phenol which is present in the essential oil of oregano and thyme. We have investigated the behavioural effects of carvacrol in animal models of pain, such as acetic acid-induced abdominal constriction, formalin and hot-plate tests in mice. The spontaneous motor activity of animals treated with carvacrol was investigated using open-field and rotarod tests.

Methods Carvacrol was administered orally, at single doses of 50 and 100 mg/kg while indometacin (5 mg/kg), morphine (7.5 mg/kg) and diazepam (2 mg/kg) were used as standard drugs. Naloxone (1 mg/kg) and L-arginine (150 mg/kg) were used to elucidate the possible antinociceptive mechanism of carvacrol on acetic acid-induced abdominal constriction and formalin tests.

Key findings The results showed that carvacrol produced significant inhibitions on nociception in the acetic acid-induced abdominal constriction, formalin and hot-plate tests. In the open-field and rotarod tests carvacrol did not significantly impair the motor performance. The effect of the highest dose of carvacrol in mice in the acetic acid-induced abdominal constriction and formalin tests were not reversed by naloxone or L-arginine.

Conclusions Based on these results, it has been suggested that carvacrol presents antinociceptive activity that may not act through the opioid system nor through inhibition of the nitric oxide pathway.

Introduction

Contemporary analgesics, such as opiates and nonsteroidal anti-inflammatory drugs (NSAIDS), have been widely used for treatment of chronic pain. These medications are usually associated with many side effects, including propensity to lead to tolerance (opiates). As a result, the continuing search for other alternatives is necessary.[1,2]

Since ancient times, natural products have been used as therapeutic agents.[3] Medicinal plants are known to be a significant source of new chemical substances with potential therapeutic effects.[4] Essential oils are natural products derived from herbs that have a wide use in medicine as antiseptics, antimicrobials and flavouring in the food industry and in perfumes.[5,6]

Carvacrol (5-isopropyl-2-methylphenol) is a monoterpenic phenol found in the essential oil of the family Labiatae including Origanum, Satureja, Thymbra, Thymus, and Corydothymus species. It is the major component of the essential oil fraction of oregano and thyme and has been used on a large scale in the food and cosmetic industries.[6–9] It has been reported that carvacrol has antibacterial, antifungal, antihelmintic, analgesic, antioxidant, antimutagenic, antigenotoxic, antispasmodic, anti-inflammatory, angiogenic, and hepatoprotective activity.[10]

Recently, our group made a central nervous system pharmacological screening for carvacrol, which showed antidepressant and anxiolytic effects in mice, when administered orally.[11,12]

Previous studies have demonstrated antinociceptive properties of carvacrol in mice when administered intraperitoneally.[13] This work was undertaken to evaluate the

antinociceptive effect of carvacrol through the oral route by using behavioural models of pain, such as acetic acid-induced abdominal constriction, formalin, and hot-plate thermal tests, to analyse the involvement of peripheral mechanisms (nitric oxide/cyclic guanosine monophosphate pathway) and central mechanisms (opioid system). We analysed the effects of carvacrol on locomotor activity in animal models using open-field and rotarod tests.

Materials and Methods

Animals

Male Swiss mice (25–30 g) were used in each experiment. The animals were provided by the Animal House of the Federal University of Ceará (Fortaleza, Ceará, Brazil) and maintained at a controlled temperature (23 ± 1°C) with a 12-h dark/light cycle and free access to water and food. Animals were treated in accordance with the current law and the National Institutes of Health Guide for Care and Use of Laboratory Animals. The protocol of the experiments were approved in the Ethical Committee on Animal Research number 95/10 at Federal University of Ceara, Brazil.

Drugs and treatment

Carvacrol was obtained from Sigma-Aldrich (St Louis, MO, USA). According to Sigma, the degree of purity is > 97%. Carvacrol was emulsified with 0.2% Tween 80 (Sigma, USA.) and dissolved in distilled water. Animals were treated with oral doses of 50 or 100 mg/kg, one hour before the experiments. In the acetic acid-induced abdominal constriction test, animals were also treated with carvacrol at doses of 12.5, 25 and 200 mg/kg. Controls received 0.2% Tween 80 (Sigma, USA) dissolved in distilled water at the same volume as the treated groups (10 ml/kg). The following drugs were used: diazepam (2 mg/kg; União Química, São Paulo, SP, Brazil), formalin (1%) and acetic acid (0.6%; VETEC QUÍMICA FINA LTDA), indometacin (5 mg/kg; i.p.), morphine hydrochloride (7.5 mg/kg; i.p.), naloxone (1 mg/kg; i.p.; opioid receptor antagonist) and L-arginine (150 mg/kg; i.p.; nitric oxide (NO) precursor) from Sigma (USA).

To assess the possible involvement of NO in the acetic acid-induced abdominal constriction test and formalin test, the animals were pretreated with carvacrol (100 mg/kg, p.o.) 30 min before treatment with L-arginine or vehicle. Thirty minutes after the last treatment the groups were submitted to the experiment.

To investigate the involvement of the opioid system in the acetic acid-induced abdominal constriction and formalin tests animals were pretreated with naloxone (1 mg/kg; i.p.), then the groups were treated with carvacrol (100 mg/kg; p.o.) or vehicle 15 min later. The groups were submitted to the experiment one hour after the last treatment.

Experimental protocol

The animals were tested during a lit period, illuminated with normal light, observed in a closed room with constant temperature (23 ± 1°C). All tests were performed on different days with distinct groups of animals.

Acetic acid-induced abdominal constriction

Abdominal constriction, induced by intraperitoneal injection of acetic acid 0.6%, consisted of a contraction of the abdominal muscles together with hind limbs stretching.[14] The animals were pretreated either intraperitoneally with indometacin (5 mg/kg), used as positive control, or orally with carvacrol (12.5, 25, 50, 100 or 200 mg/kg) one hour before the injection of acetic acid. The control group received the same volume as the treated groups (10 ml/kg; 0.2% Tween 80). After the treatment, pairs of mice were placed in separate boxes, and the abdominal constrictions started to be counted 10 min after acetic acid injection, for 20 min. Antinociceptive activity was expressed as the reduction in the number of constrictions i.e. the difference between control animals (0.2% Tween 80 dissolved in saline solution) and animals pretreated with carvacrol or indometacin.

Formalin test in mice

Mice were injected with formalin (20 µl 1% formalin) intraplantarly under the ventral surface of the right hind paw. The amount of time spent licking the injected paw was timed with a chronometer and was considered as indicative of nociception. The initial nociceptive response peaked 5 min after formalin injection (early phase) and 20–25 min after formalin injection (late phase), representing the tonic and inflammatory pain responses, respectively.[15] The animals were orally pretreated with carvacrol (50 or 100 mg/kg), one hour before the formalin injection or with morphine (7.5 mg/kg), which was used as positive control, 30 min before the experiment. The control group received the same volume (10 ml/kg) as the treated groups.

Hot-plate test in mice

The hot plate (UGO BASILE, model-DS 37) was used to measure the latencies according to the method described previously.[16] In the experiments, the hot plate was maintained at 55 ± 1°C. Before beginning the experiments, the basal reaction time response of all animals was taken (mice with baseline latencies of more than 15 s were eliminated from the study). The animals were pretreated with either vehicle (10 ml/kg, p.o.), morphine (10 mg/kg, i.p.), or carvacrol (50 or 100 mg/kg, p.o.), and they were put on the heated surface of the plate 30, 60, 90, and 120 min later. The time needed for the initial response to the painful stimulus (licking the paws

or jumping) was taken as the defining response, and it was termed as reaction time. To minimize damage to the animals' paws, the cut-off time was 45 s.

Open-field test

The open-field area was made of acrylic (transparent walls and black floor, 30 × 30 × 15 cm) and divided into nine squares of equal area. This apparatus was used to evaluate the exploratory activity of the animals.[17] The observed parameters were as follows: number of squares crossed (with the four paws) and number of groomings and rearings. The animals were pretreated with vehicle (10 ml/kg, p.o.), diazepam (2 mg/kg, i.p.), or carvacrol (50 or 100 mg/kg, p.o.).

Rotarod test

For the rotarod test, animals were placed with the four paws on a 2.5 cm diameter bar, 25 cm above the floor, with the bar turning at 5, 15, or 40 rev/min.[18] The animals were pretreated with vehicle (10 ml/kg, p.o.), diazepam (2 mg/kg, i.p.), or carvacrol (50 or 100 mg/kg, p.o.). The time of permanence on the bar was measured (2 min for each animal), and different groups were used on all the rotating speeds.

Statistical analysis

Data were analysed by 4.0 Graphpad Prism software (San Diego, CA, USA). Results are shown as mean ± standard error of the mean (SEM). For the statistical analysis one-way analysis of variance followed by Student-Newman-Keuls multiple comparison test were used. *P*-values < 0.05 were considered to be significant.

Results

Acetic acid-induced abdominal constriction in mice

Carvacrol administered orally at doses of 50, 100 and 200 mg/kg significantly decreased the number of constrictions as compared with control (control 31.50 ± 2.976 (8); carvacrol-50 19 ± 2.821 (8); carvacrol-100 18.50 ± 2.318 (7); carvacrol-200 8.714 ± 2.112). Indometacin (10 mg/kg), as expected, decreased the number of constrictions compared with the control group (indometacin 12.08 ± 1.323 (10); Figure 1). Animals treated with carvacrol at 12.5 and 25 mg/kg did not significantly alter the number constrictions.

The results in Figure 2 show that the pretreatment with naloxone (1 mg/kg; i.p.) and L-arginine (150 mg/kg) was not able to reverse the antinociception promoted by carvacrol, with a dose of 100 mg/kg, as compared with the control group (control 27.73 ± 2.566 (10); carvacrol-100 15.32 ± 2.474 (10); carvacrol + L-arginine 8.500 ± 2.847 (8); carvacrol + naloxone 14.13 ± 1.894 (8)).

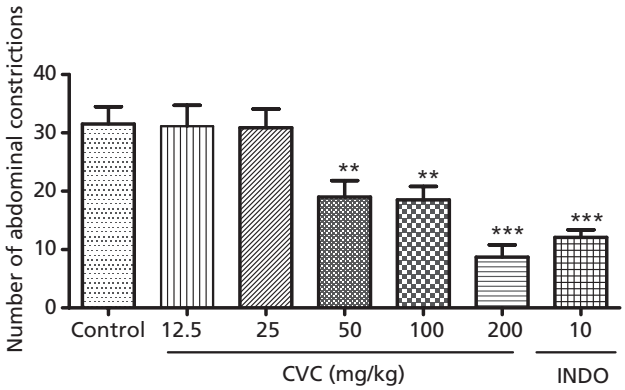

Figure 1 Acetic acid-induced abdominal constriction test of groups of mice which received either vehicle, carvacrol or indometacin. The figure shows number of abdominal constrictions. Carvacrol: (CVC) 12.5; 25, 50, 100 or 200 mg/kg; indometacin (INDO) 5 mg/kg. $^{**}P < 0.01$; $^{***}P < 0.001$ analysis of variance and Student-Newman-Keuls as the *post hoc* test.

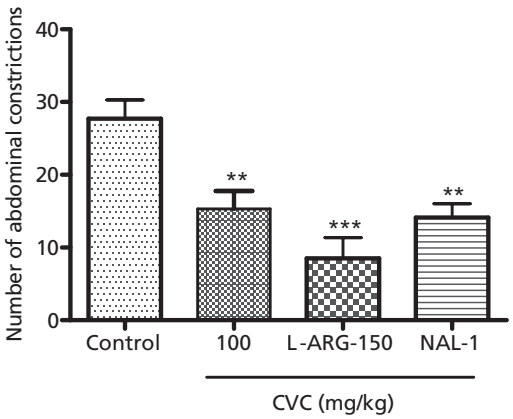

Figure 2 Acetic acid-induced abdominal constriction test of groups of mice which received vehicle, carvacrol, carvacrol + L-arginine or carvacrol + naloxone. The figure shows number of abdominal constrictions. Carvacrol (CVC) 100 mg/kg; L-arginine (L-ARG) 150 mg/kg; naloxone (NAL) 1 mg/kg. $^{**}P < 0.01$; $^{***}P < 0.001$ analysis of variance and Student-Newman-Keuls as the *post hoc* test.

Formalin test in mice

In the formalin test, the groups treated with carvacrol 100 mg/kg and morphine 7.5 mg/kg significantly decreased the licking time during the early phase (control 53.53 ± 2.661 (10); carvacrol-100 37.11 ± 3.307 (10); morphine 2.000 ± 0.8660 (8)) and late phase (control 21.88 ± 3.641 (10); carvacrol-100: 0.7207 ± 0.5415 (10); morphine 2.000 ± 0.8660 (8)) as compared with control. However, animals treated with carvacrol 50 mg/kg

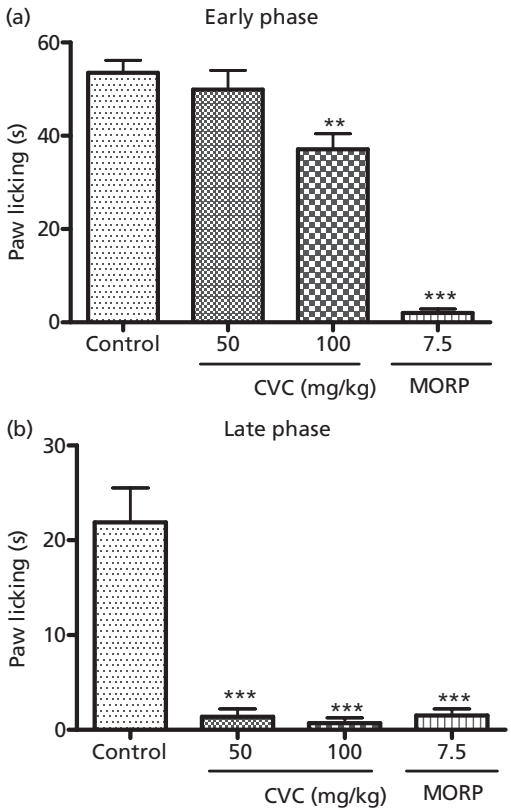

Figure 3 Formalin test of groups of mice which received vehicle, carvacrol or morphine. The figure shows paw licking time (s) at the early and late phases. Carvacrol: (CVC) 50 or 100 mg/kg; morphine (MORP) 7.5 mg/kg. $^{**}P < 0.01$; $^{***}P < 0.001$ analysis of variance and Student-Newman-Keuls as the *post hoc* test.

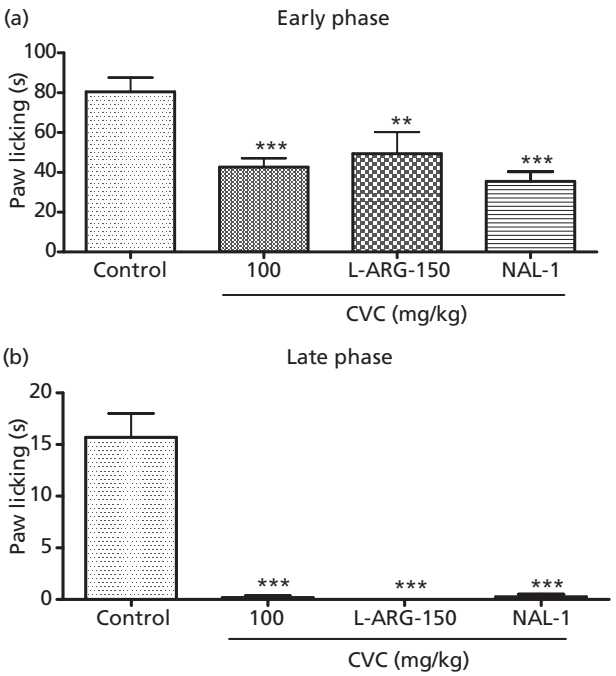

Figure 4 Formalin test of groups of mice which received vehicle, carvacrol, carvacrol + L-arginine or carvacrol + naloxone. The figure shows paw licking time (s) at the early and late phases. Carvacrol (CVC) 100 mg/kg: L-arginine (L-ARG) 150 mg/kg; naloxone (NAL) 1 mg/kg. $^{*}P < 0.01$; $^{***}P < 0.001$ analysis of variance and Student-Newman-Keuls as the *post hoc* test.

significantly decreased the licking time only during the late phase (carvacrol-50 1.381 ± 0.8284 (10)) as compared with control (Figure 3).

Similarly to those results observed in acetic acid-induced abdominal constriction test, Figure 4 shows that pretreatment with naloxone (1 mg/kg; i.p.) or L-arginine (150 mg/kg) was not able to reverse the antinociception promoted by carvacrol with a dose of 100 mg/kg on both early (control 80.47 ± 7.169 (10); carvacrol-100 42.61 ± 4.418 (10); carvacrol + L-arginine 49.39 ± 10.78 (8); carvacrol + naloxone 35.49 ± 4.914 (8)) and late phase (control 15.70 ± 2.317 (10); carvacrol-100 0.1931 ± 0.1931 (10); carvacrol + L-arginine 0.2575 ± 0.2575 (8); carvacrol + naloxone 35.49 ± 4.914 (8)) when compared with the control group.

Hot-plate test in mice

In the hot-plate test (Table 1), carvacrol increased the reaction time (latency time) at 60 min at doses of 50 and 100 mg/kg. In addition, morphine (7.5 mg/kg) caused, as expected, a significant increase in the reaction time in the hot-plate test at all temperatures measured.

Open-field test

In the open-field test (Figure 5), at dose of 100 mg/kg, carvacrol did not significantly alter the number of crossings and rearings compared with respective controls, however, it significantly decreased the number of groomings as compared with control. Animals treated with diazepam (2 mg/kg) decreased the number of crossings (control 44.71 ± 3.998 (7); carvacrol-100: 37 ± 5.487 (8); diazepam 27.14 ± 1.280 (7)), groomings (control 2.250 ± 0.25 (7); carvacrol-100 1.125 ± 0.125 (8); diazepam 0.9 ± 0.2769 (7)) and rearings (control 4.375 ± 0.6797 (7); carvacrol-100: 4.875 ± 0.8952 (8); diazepam 2.125 ± 0.5154 (7)) as compared with the control group.

Rotarod test

Carvacrol at doses of 100 mg/kg did not alter the time of permanence on the bar at 5, 15, or 40 rev/min, as compared with the control group. Diazepam (2 mg/kg) decreased these parameters (at 5 rev/min: control 119.2 ± 0.3329 (8); carvacrol-100 119.3 ± 0.5261 (8); diazepam 97 ± 5.041 (8);

Table 1 Hot-plate test basal reaction time (s) after oral administration of vehicle, carvacrol or morphine to mice

Basal reaction time (s)					
Group	0 min	30 min	60 min	90 min	120 min
Control	9.078 ± 1.722 (9)	9.578 ± 1.659 (9)	6.278 ± 1.315 (9)	7.289 ± 1.243 (9)	6.700 ± 1.097 (9)
Carvacrol 50 mg/kg	11.23 ± 1.276 (10)	17.21 ± 3.448 (10)	13.00 ± 2.258 (10)*	9.340 ± 1.274 (10)	9.980 ± 1.613 (10)
Carvacrol 100 mg/kg	8.170 ± 1.412 (10)	18.40 ± 3.727 (10)	12.06 ± 1.278 (9)*	7.600 ± 2.315 (10)	6.760 ± 0.7267 (10)
Morphine 7.5mg/kg	20.30 ± 1.169 (10)***	30.01 ± 4.220 (10)**	19.40 ± 2.267 (10)***	25.70 ± 3.358 (8)***	28.01 ± 2.545 (10)***

The results are given as mean ± SEM. The number in parentheses refer to number of animals tested. Significant differences compared with the control. *$P < 0.05$; ***$P < 0.001$: analysis of variance and Student-Newman-Keuls as the *post hoc* test.

at 15 rev/min: control 116.8 ± 0.9086 (8); carvacrol-100: 113.6 ± 2.519 (8); diazepam 93 ± 4.166 (8); at 40 rev/min: control 81.89 ± 4.120 (8); carvacrol-100: 72.3 ± 4.637 (8); diazepam 66 ± 1.390 (8)) as compared with the control group (Figure 6).

Discussion

The acetic acid-induced abdominal constriction method is one of the most well-described and utilized models used in studying antinociceptive activity.[1,19,20] The abdominal constriction response induced by acetic acid is a sensitive procedure to evaluate peripheral and central acting analgesics. It has previously been reported that reduction in the amount of constriction induced by acetic acid may be associated with several drugs by acting through different mechanisms, then being considered a nonselective antinociceptive test.[21] In general, acetic acid causes pain through the release of endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinins and substance P.[2,19] The method has been associated with the production and release of arachidonic acid metabolites via cyclooxygenase and prostaglandin biosynthesis, increasing levels of PGE_2, $PGF_{2\alpha}$ and PGI_2 in peritoneal fluids, as well as lipoxygenase products.[22]

Our results indicated that carvacrol at doses of 50, 100 and 200 mg/kg was able to reduce the number of constrictions in mice, showing for the first time its antinociceptive effect; doses lower than 50 mg/kg did not show antinociceptive activity. To assess a possible antinociceptive mechanism, we examined the effect of naloxone (a nonselective opioid receptor antagonist) in the acetic acid-induced abdominal constriction test. The dose of naloxone (1 mg/kg, i.p.) used in the experiments was high enough to block opiate receptors, as demonstrated previously in the pain-induced functional impairment model.[23] However, in this study, naloxone was not able to reverse antinociception caused by carvacrol (100 mg/kg), which suggested that the activation of opioid receptors might not have been involved in the antinociceptive effect of carvacrol.

To investigate the possible peripheral mechanisms in the antinociceptive action of carvacrol, the involvement of the NO pathway was analysed. It is well known that NO and cyclic guanosine monophosphate (cGMP) are involved in antinociception.[24] NO is an endogenous activator of guanylyl cyclase and causes intracellular cGMP accumulation.[25] NO is found to be involved in all three levels of the pain pathway, at the peripheral, the dorsal horn and the cerebral cortex.[26] However, researchers have reported that NO plays both nociceptive and antinociceptive roles in the L-arginine/NO/cGMP pathway in peripheral tissues.[27–30] Our group has shown that L-arginine increased nociceptive-related behaviour in the second phase of the formalin test and reversed the antinociceptive effect of drugs acting under the NO-pathway.[31] This fact suggested that NO had a pronociceptive effect in this test. In our research, antinociception of carvacrol in the acetic acid constriction test was not reversed when used in combination with L-arginine, a precursor of NO synthesis.

The formalin model of nociception is a well-described method that discriminates pain in its central and/or peripheral components. It has been reported that formalin-induced persistent pain in mice paws produced a distinct biphasic nociception.[28,32] The early phase (0–5 min after formalin injection), characterized by intense neurogenic pain, starts immediately after the injection and is probably a direct result of stimulation of nociceptors in the paw and reflects centrally mediated pain. The late phase of moderate pain (20–40 min) seems to be caused by a release of serotonin, histamine, bradykinin, and prostaglandins and at least to some degree, the sensitization of central nociceptive neurons.[22,33,34]

Central analgesics, such as narcotics, inhibit both phases, while peripherally-acting drugs, such as steroids (hydrocortisone, dexamethasone) and NSAIDs suppress mainly the late phase.[35] In this test, carvacrol at 100 mg/kg significantly reduced the duration of the paw licking (s) in both first and second phase of the formalin test. However, at 50 mg/kg carvacrol was only able to reduce paw licking (s) in the second phase. For the evaluation of the possible antinociceptive mechanism on formalin test, animals were pretreated with naloxone (1 mg/kg) or L-arginine (150 mg/kg). Results showed that, similar to previous results shown in the acetic acid abdominal constriction test, naloxone and L-arginine were not able to reverse the antinociceptive effect of carvacrol (100 mg/kg).

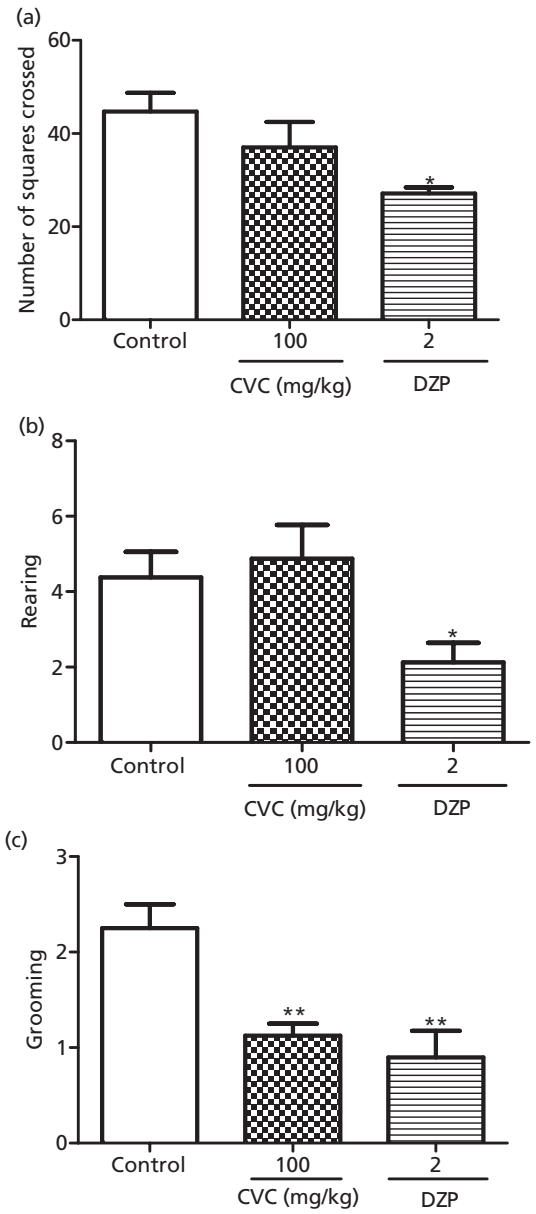

Figure 5 Open-field test of groups of mice which received vehicle, carvacrol or diazepam. (a) Number of squares crossed. (b) Rearing. (c) Grooming. Carvacrol (CVC) 100 mg/kg; diazepam (DZP) 2 mg/kg. The results are presented as mean ± SEM. Significant difference compared with control (*$P < 0.05$; **$P < 0.01$). Analysis of variance and Student-Newman-Keuls as the *post hoc* test.

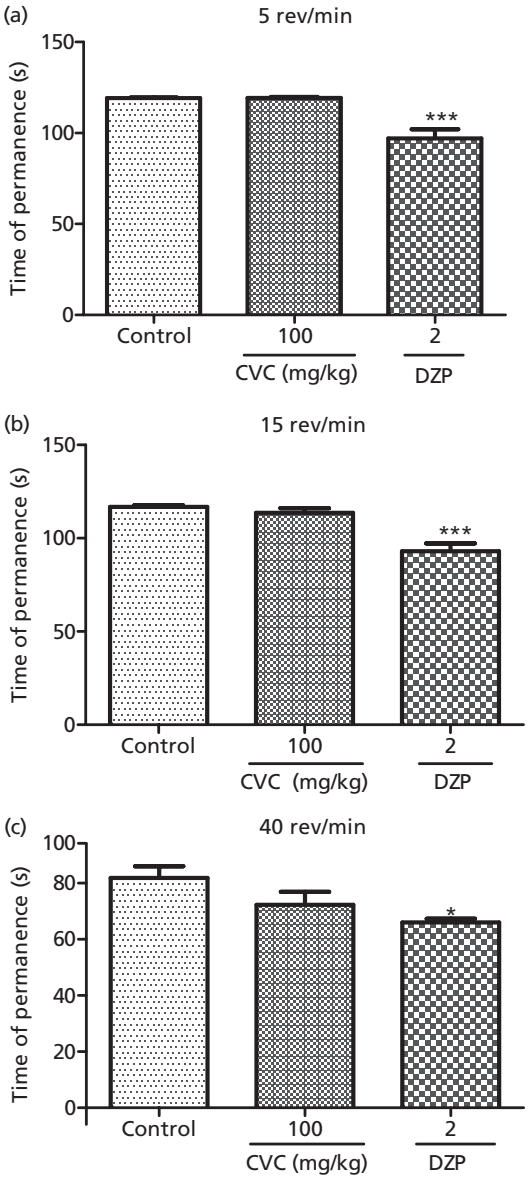

Figure 6 Rotarod test of groups of mice which received vehicle, carvacrol, or diazepam. The figure shows time of permanence (s). Carvacrol (CVC) 100 mg/kg; diazepam (DZP) 2 mg/kg. The results are presented as mean ± SEM. Significant difference compared with control (*$P < 0.05$; ***$P < 0.001$). Analysis of variance followed by Student-Newman-Keuls as the *post hoc* test.

The hot-plate test is a central antinociceptive test that produces, at constant temperature, two kinds of behavioural response, which are paw-licking and jumping. Both of these behaviours are considered to be supraspinally integrated responses.[36–38] The present data showed that carvacrol at both doses (50 and 100 mg/kg) increased the reaction time in the hot-plate test only at 60 min.

These results suggested a central involvement in the antinociceptive effect of carvacrol, however the opioid receptor probably was not related to this action as well as NO.

Data in the literature demonstrated that drugs such as muscle relaxants, sedatives and psychomimetics may show activity in the acetic acid abdominal constriction and hot-plate tests. Our group previously studied the effects of oral administration of 50 mg/kg carvacrol in the open-field and

rotarod tests.[11] Carvacrol at 100 mg/kg, similar to our previous findings, had no significant effect on the open-field and rotarod tests, suggesting that carvacrol did not present sedative and myorelaxant activity.

Conclusions

The main goal of this work was to demonstrate for the first time the antinociceptive activity of carvacrol in chemical and thermal-induced nociception models. The central effects of carvacrol were not clear once naloxone failed to revert the action of carvacrol in the acetic acid abdominal constriction and formalin tests, showing the lack of participation of the opioid system in antinociceptive effects of carvacrol. In addition, L-arginine failed in reverting the effect of carvacrol in both tests, probably signifying no NO involvement in this action. Therefore, further studies are required to clarify the mechanisms involved in the antinociception effect of carvacrol.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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