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Bioassay-guided Evaluation of Antioxidant and Antinociceptive Activities of Carvacrol

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Abstract: We examined the antioxidant properties *in vitro* and the antinociceptive effect of carvacrol (CARV) in several models of pain in mice. CARV presented a strong antioxidant potential according to the TRAP/TAR evaluation; it also presented scavenger activity against nitric oxide and prevented lipid peroxidation *in vitro*. In mice, when evaluated against acetic acid-induced abdominal writhing, CARV (25, 50 and 100 mg/kg, i.p.) reduced ($p < 0.001$) the number of writhing compared to the control group, without opioid participation. In the formalin test, CARV also significantly inhibited both the early (neurogenic pain) and the late (inflammatory pain) phases of formalin-induced licking, with inhibition percentage values of 56.8% (100 mg/kg) for the neurogenic phase and 41.2% (25 mg/kg), 73.8% (50 mg/kg) and 99.7% (100 mg/kg) for the inflammatory phase. CARV also produced a significant inhibition of the pain caused by capsaicin (63.1, 67.1 and 95.8%, $p < 0.001$) and glutamate (46.4, 61.4 and 97.9%, $p < 0.01$). When assessed in a thermal model of pain, CARV (100 mg/kg, i.p.) caused a significant increase ($p < 0.05$) in the latency response on the hot-plate test. Such results were unlikely to be provoked by motor abnormality. Together, these results indicate that the properties of CARV should be more thoroughly examined in order to achieve newer tools for management and/or treatment of painful conditions, including those related to pro-oxidant states.

Although a considerable number of analgesic drugs are available for the treatment of pain, the search for new compounds as therapeutic alternatives continues since the available analgesic drugs exert a wide range of side-effects [1]. Because of their relatively low cost and easy availability in several countries, natural active products could be used as synthesis models of more selective and powerful drugs [2,3].

Essential oils are concentrated volatile aromatic compounds produced by aromatic plants, such as *Cymbopogon winterianus*, *C. citrates, Lavandula multifida* and *Thymus pubescens* that have been found to exhibit a variety of biological properties, such as analgesic [1], spasmolytic and anticonvulsant [4] activities. Monoterpenes are the main chemical constituents of the essential oils of these plants, and are found as mixtures of odoriferous components that can be obtained by steam distillation or solvent extraction from a large variety of aromatic plants. They are found in edible as well as in medicinal plants with a therapeutic property [4,5]. Additionally, monoterpenes and their derivative compounds exhibit several types of pharmacological properties, such as anxiolytic [6], antinociceptive [5,7], sedative [8,9], antidepressant [10] and anticonvulsant activities [11,12].

Carvacrol (CARV) is the predominant monoterpenic phenol in many essential oils of the family Labiatae including *Origanum*, *Satureja*, *Thymbra*, *Thymus* and *Corydothymus* species which have been used through the ages as a source of flavour in food [13]. Previous studies have demonstrated that CARV promoted inhibition of cyclooxygenase [14]. Carvacrol is also responsible for the biological activities of oregano, which include antimicrobial, antitumour, antimutagenic, antigenotoxic, analgesic, antispasmodic, antiinflammatory, angiogenic, antiparasitic, antiplatelet, inhibition of acetylcholinesterase, antielastase, insecticidal, antihepatotoxic and hepatoprotective activities [15]. There are no data on the possible antinociceptive activity of CARV.

Free radicals and related reactive species are in the realm of several pathological and physiological processes, including inflammation and pain [16]. Many natural products exert significant redox activities, which are related to their therapeutic properties or even a possible toxic effect. The evaluation of the redox properties of such compounds is crucial for both understanding the potential mechanisms of their biological actions and for determining possible toxic or harmful side-effects. Many of the biological properties associated to CARV include processes mediated by free radicals, such as mutagenicity and inflammation. Since CARV-containing natural products have been used by to alleviate pain and inflammation-related processes [15], and that most biological properties of compounds produced by plants have been related to their redox properties [17], the purpose of the present study was to evaluate both antinociceptive and antioxidant activities of CARV in various experimental models.

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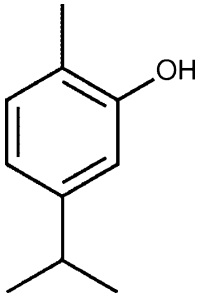

Fig. 1. Chemical structure of carvacrol (CARV).

Material and Methods

Chemicals. Acetic acid, cremophor, capsaicin, glutamate, formalin, AAPH (2,2′-azobis[2-methylpropionamidine]dihydrochloride), trichloroacetic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid), glycine, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), TBARS (thiobarbituric acid reactive species), phosphate buffer, TBA (thiobarbituric acid), sodium nitroprusside, Griess reagent and carvacrol (CARV, 98% purity) (fig. 1) were purchased from Sigma (USA). Morphine, naloxone and acetylsalicylic acid were purchased from União Química (Brazil).

Total Radical-Trapping Antioxidant Parameter (TRAP). An adapted method of TRAP assay was used to determine the capacity of CARV to trap a flow of water-soluble peroxyl radical produced at constant rate, through thermal decomposition of AAPH [18]. Briefly, the reaction mixture containing 4 ml of the free radical source (AAPH 10 mM) in glycine buffer (0.1 M) pH 8.6, 10 ml of the test samples at different concentrations) and 10 ml luminol (4 mM) as external probe to monitor radical production were incubated at 25°C. Trolox (water-soluble vitamin E analogue) was used as reference peroxyl radical scavenger molecule. The chemiluminescence produced was directly proportional to the radical generation and measured in out-of-coincidence mode (Wallac 1409 DSA Liquid Scintillation Counter, Wallac Oy, Turku, Finland) as counts per minute (CPM). The TRAP of CARV was evaluated for instantaneous inhibition of chemiluminescence reading values as area under curve (AUC). The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after CARV addition (I) and expressed as percent of inhibition. AUC and radical basal production were acquired by software (GraphPad Software Inc. San Diego, CA, USA) analysis of data.

TBARS Assay. TBARS (thiobarbituric acid reactive species) assay was employed to quantify lipid peroxidation [19], and an adapted TBARS method was used to measure the antioxidant capacity of CARV using egg yolk homogenate as lipid-rich substrate [18]. Briefly, egg yolk was homogenized (1% w/v) in 20 mM phosphate buffer (pH 7.4), 1 ml of homogenate was sonicated and then homogenized with 0.1 ml of CARV at different concentrations. Lipid peroxidation was induced by addition of 0.1 ml of AAPH solution (0.12 M). Control was only CARV vehicle (DMSO 10%). Reactions were carried out for 30 min. at 37°C. After cooling, samples (0.5 ml) were centrifuged with 0.5 ml of trichloroacetic acid (15%) at $1200 \times g$ for 10 min. An aliquot of 0.5 ml from supernatant was mixed with 0.5 ml TBA (0.67%) and heated at 95°C for 30 min. After cooling, sample absorbance was measured using a spectrophotometer at 532 nm. The results were expressed as percentage of TBARS formed by AAPH alone (induced control).

Scavenging activity of nitric oxide (NO). Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Once generated, NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction [20]. The reaction mixture (1 ml) containing 10 mM sodium nitroprusside (SNP) in phosphate buffer and CARV at different concentrations were incubated at 37°C for 1 hr. A 0.5 ml aliquot was taken and homogenized with 0.5 ml Griess reagent. The absorbance of chromophore was measured at 540 nm. Percent inhibition of nitric oxide generated was measured by comparing the absorbance values of negative controls (only 10 mM sodium nitroprusside and vehicle) and assay preparations. Results were expressed as percentage of nitrite formed by SNP alone.

Animals. Adult (3-month-old) male albino Swiss mice (20–30 g) were randomly housed in appropriate cages at 21 ± 2°C with a 12-hr light: dark cycle (light from 06:00 a.m. to 06:00 p.m.), with free access to food (Purina®, Brazil) and tap water. All experimental observations were conducted between 12:00 a.m. and 05:00 p.m. All procedures were carried out in accordance with the Animal Care and Use Committee at the Federal University of Sergipe (CEPA/UFS 43/08).

Acetic acid-induced writhing. This study was performed according to Koster *et al.* (1959) [21] and Broadbear *et al.* (1994) [22]. Mice (n = 8, per group) were injected intraperitoneally (i.p.) with 0.85% acetic acid at a dose of 10 ml/kg. CARV (25, 50 and 100 mg/kg, i.p.), morphine (MOR, 3 mg/kg) and vehicle (saline + two drops of cremophor) were administered i.p. to different groups of the mice 0.5 hr before the acetic acid injection. An additional group was pre-treated with 1.5 mg/kg of naloxone (NAL), a non-selective opioid antagonist, 0.5 hr before the i.p. administration of CARV (100 mg/kg) or morphine (MOR, 3 mg/kg). Subsequently, the writhing was counted for 15 min. after a latency period of 5 min.

Formalin-induced pain. The procedure described by Hunskaar and Hole (1987) [23] was used with slight modifications. Pain was induced by injecting 20 µl of 1% formalin in distilled water in the subplantar of the right hind paw. Mice (n = 8 per group) were given CARV (25, 50 and 100 mg/kg, i.p.), acetylsalicylic acid (ASA, 200 mg/kg), morphine (MOR, 3 mg/kg) or vehicle (saline + two drops of cremophor) 0.5 hr prior to formalin injection. These mice were individually placed in a transparent acrylic glass cage (25 cm × 15 cm × 15 cm) observation chamber. The amount of time spent licking the injected paw was indicative of pain. This time was counted after injection of formalin from 0–5 min. (first phase) and 15–30 min. (second phase), which represented neurogenic and inflammatory pain responses, respectively.

Glutamate-induced nociception. A volume of 20 µl of glutamate solution (20 µmol), made up in sterile saline solution, was injected intraplantarly (i.pl.) under the ventral surface of the right hind paw. Mice (n = 8, per group) were given CARV (25, 50 and 100 mg/kg, i.p.), morphine (MOR, 3 mg/kg) and vehicle (saline + two drops of cremophor) 0.5 hr prior to injecting glutamate. After i.pl. injections of glutamate, these mice were individually placed in the same observation chamber of the formalin test. The mice were observed from 0 to 15 min. following glutamate injection in order to evaluate the duration of nociceptive behaviour, as described previously [24].

Capsaicin-induced nociception. The method used was similar to that described previously [25]. About 20 µl of capsaicin (1.6 µg/paw) was diluted in DMSO 25% in distilled water (v/v) and injected under the skin of the dorsal surface on the right hind paw. An additional group pre-treated with vehicle of carvacrol (saline + two drops of cremophor) received a similar volume of capsaicin vehicle (Water/DMSO 25%). The mice (n = 8 per group) were pre-treated with CARV (25, 50 and 100 mg/kg, i.p.), morphine (MOR, 3 mg/kg) and vehicle (saline + two drops of cremophor) 30 min. before injection of the nociceptive agent. After this process, pairs of mice were placed individually in different acrylic glass cages for 5 min. following capsaicin injection. The amount of time spent licking the injected paw was

timed with a chronometer and was considered indicative of nociception.

Hot-plate test. The hot-plate test described by Jacob *et al.* (1974) [26] and by Jacob and Ramabadran (1978) [27] was used. The animals were placed on a hot-plate apparatus (Insight®, Brazil) that was adjusted to 55 ± 0.5°C. The reaction time was noted by observing either the licking of the hind paws at basal, 0.5, 1.0, 1.5 and 2.0 hr after i.p. administration of 25, 50 and 100 mg/kg of CARV or the vehicle (saline + two drops of cremophor) to different groups of eight mice each. Morphine (MOR), 3 mg/kg (i.p.), was used as reference drug.

Evaluation of the motor activity. To investigate if the treatments influenced the motor activity of the animals and consequently impaired the assessment of the nociceptive behaviour in the experimental models, the motor activity of the animals was evaluated in a rota-rod apparatus, according to Dunham and Miya (1957) [28] with some modifications. Initially, the mice able to remain on the Rota-rod apparatus (AVS®, Brazil) longer than 180 sec. (7 rpm) were selected 24 hr before the test. Then, the selected animals were divided into five groups (n = 8) and treated i.p. with vehicle (control), CARV (25, 50 and 100 ml/kg, i.p.) and diazepam (DZP, 1.5 mg/kg). Each animal was tested on the rota-rod and the time(s) they remained on the bar for up to 180 sec. was recorded 0.5, 1 and 2 hr after administration.

Statistical analysis. The obtained data were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. In all cases, differences were considered significant if $p < 0.05$. The percent of inhibition by an antinociceptive agent was determined using the following formula [29]:

$$\text{Inhibition}\% = 100.(\text{control-experiment})/\text{control}$$

Results

Antioxidant properties.

The general antioxidant potential of CARV was first evaluated by the TRAP/TAR assays, which are widely adopted to assess the non-enzymatic antioxidant activity of a given compound. TRAP and TAR measurements show a strong antioxidant capacity of CARV at different concentrations (fig. 2). All doses were observed to effectively inhibit the free radical-induced luminescence generated by AAPH, with $p < 0.001$ for all concentrations.

To confirm such ability, we tested the effect of CARV on the lipoperoxidation (oxidative damage to lipids) induced by AAPH *in vitro*. CARV showed a pronounced ability to prevent AAPH-induced lipid peroxidation (fig. 3), with the highest doses decreasing AAPH-induced lipid damage to basal levels.

To determine the ability of CARV to act as a reactive nitrogen species (RNS) scavenger, we evaluated the NO-scavenging activity by incubating CARV with sodium nitroprusside (SNP), a chemical inducer of NO production. CARV at 100 μg/ml and 1 mg/ml showed a significant NO-scavenging activity with $p < 0.01$ (fig. 4).

Acetic acid-induced writhing.

CARV significantly reduced writhing and stretching induced by 0.85% acetic acid at a dose of 10 ml/kg. The significant protective effects were observed as 61.5%, 69.2% and 73.4% ($p < 0.001$) at 25, 50 and 100 mg/kg of CARV, respectively,

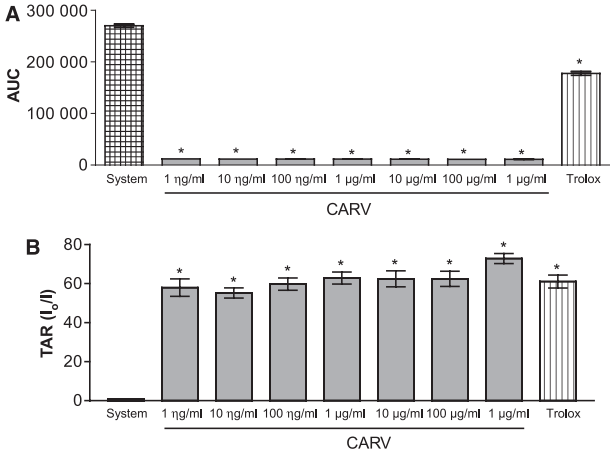

Fig. 2. *In vitro* antioxidant activity of carvacrol (CARV). (A) Total Radical-Trapping Antioxidant Parameter (TRAP) of CARV at different concentrations. Bars represent arbitrary units of the area under curve (AUC) generated by the readings of AAPH-induced chemiluminescence along the assay in the presence of different concentrations of CARV. The system was the area generated during reading of luminescence by AAPH alone, and Trolox was the standard antioxidant. (B) The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in absence of samples and the light intensity right after CARV addition and expressed as percent of inhibition (I_0/I) using the same raw data. Values represent mean ± S.E.M., n = 5, experiments in triplicate, $*p < 0.001$ *versus* system (ANOVA followed by Tukey's test).

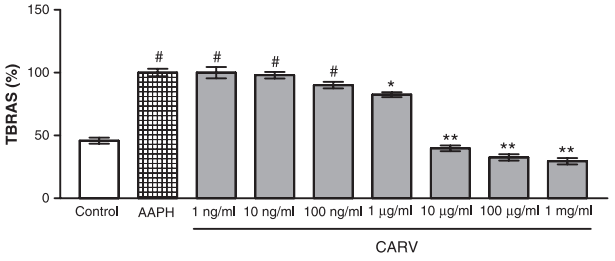

Fig. 3. TBARS *in vitro*. Lipid extracted from egg yolk was subjected to oxidative damage by incubation with AAPH and the ability of different concentrations of CARV to prevent TBARS formation was analysed. Control means basal lipid peroxidation with vehicle alone (DMSO 10%); AAPH alone group is considered 100% of oxidative damage. Values represent mean ± S.E.M., n = 5, experiments in triplicate. $^{\#}p < 0.001$ *versus* control, $*p < 0.05$ and $**p < 0.001$ *versus* (ANOVA followed by Tukey's test).

while MOR (3 mg/kg) had 87.7% ($p < 0.001$). Naloxone did not reverse the effect of CARV (100 mg/kg) but antagonized the antinociceptive effect of MOR to the acid-induced writhing response (fig. 5).

Formalin-induced pain.

CARV had analgesic effects on both the first (0–5 min.) and second phase (15–30 min.) of formalin-induced pain. These phases corresponded to neurogenic and inflammatory pains,

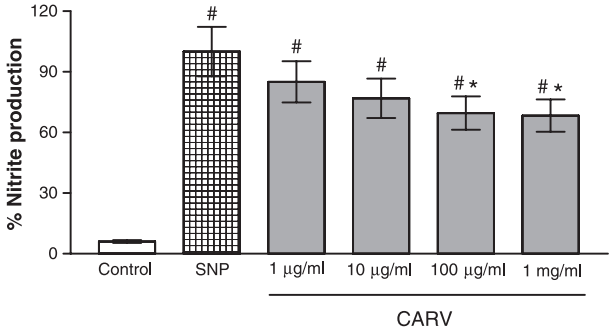

Fig. 4. NO-scavenging activity. Control means basal NO production of vehicle (DMSO 10%) in the absence of a NO generator source (without SNP); SNP group reflects nitrite production by sodium nitroprusside alone, considered 100% of NO production. The effect of different concentrations of CARV against SNP was determined by the Griess method. Values represent mean ± S.E.M., n = 5, experiments in triplicate. $^{\#}p < 0.001$ *versus* control, $*p < 0.01$ *versus* SNP (ANOVA followed by Tukey's test).

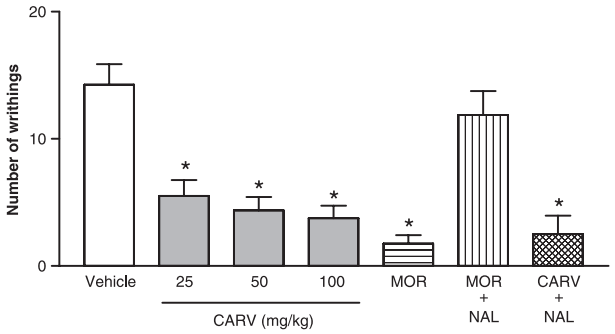

Fig. 5. Effects of CARV on the acetic acid-induced writhing test in the absence and presence of naloxone in mice. Vehicle (control), CARV (25, 50 and 100 mg/kg) or morphine (MOR) were administered i.p. 0.5 hr before acetic acid injection. Pre-treatment with naloxone (NAL, 1.5 mg/kg, i.p.) was performed 0.5 hr before treatment (i.p.) with CARV (100 mg/kg), or MOR (3 mg/kg). Each column represents mean ± S.E.M. (n = 8). $*p < 0.001$ *versus* control (ANOVA followed by Tukey's test).

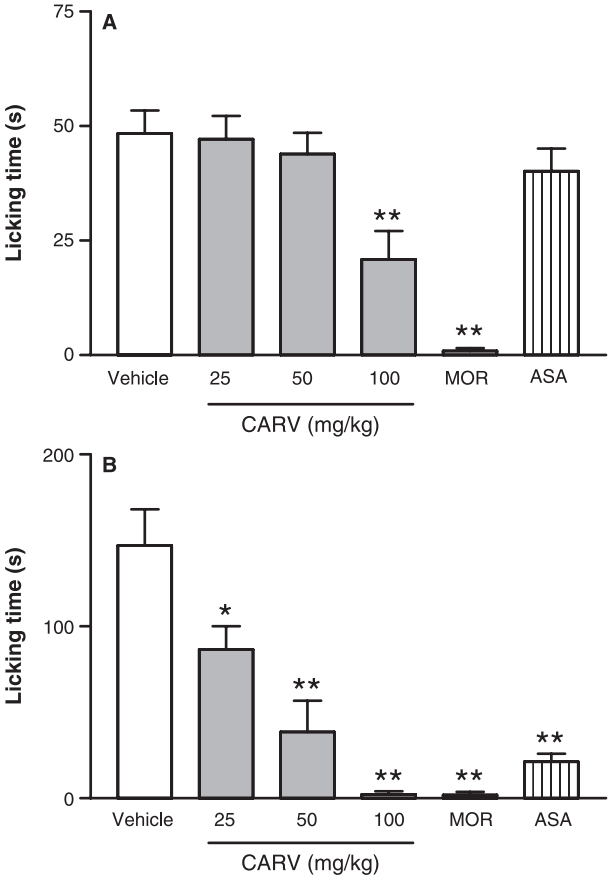

Fig. 6. Effects of CARV on the formalin-induced nociception in mice. Vehicle (control), CARV (25, 50 and 100 mg/kg), acetylsalicylic acid (ASA) or morphine (MOR) were administered i.p. 0.5 hr before formalin injection. (A) Represents the first phase and (B) represents second phase of formalin-induced nociception. Each column represents mean ± S.E.M. (n = 8). $*p < 0.01$ or $**p < 0.001$ *versus* control (ANOVA followed by Tukey's test).

respectively. Its neurogenic-induced pain was blocked only at 100 mg/kg (56.8%, $p < 0.001$) whereas all the doses of CARV significantly blocked the inflammatory pains (41.2, 73.8 and 99.7%) at 25, 50 and 100 mg/kg, respectively. ASA (200 mg/kg) was significantly active (44.8%, $p < 0.01$) only in the second phase (inflammatory pain). MOR inhibited both phases (first phase, 98.1%, $p < 0.001$) and (second phase, 99.2%, $p < 0.001$) (fig. 6).

Glutamate-induced nociception.

Fig. 7 shows that CARV 25, 50 and 100 mg/kg (i.p.) significantly reduced (46.4, 61.4 and 97.9%, $p < 0.001$) the licking time behaviour induced by administration of glutamate. The dose of 100 mg/kg produced a similar effect to morphine (97.4%; $p < 0.001$).

Capsaicin-induced nociception.

The results of this test (fig. 8) indicate that this compound reduces capsaicin-induced pain presenting a significant inhibition (63.1, 67.1 and 95.8%, $p < 0.001$) at 25, 50 and 100 mg/kg and even as MOR (97.4%, $p < 0.001$). The group that received only the diluent of capsaicin (DMSO 25%) did not present any significant behavioural alteration (data not shown).

Hot-plate test.

Table 1 shows the results of the hot-plate test. The reaction time parameter was only significantly increased where higher dose of CARV (100 mg/kg, i.p.) induced a nociceptive inhibition. Similar results were noted with MOR (3 mg/kg, i.p.) treatment.

Evaluation of the motor activity.

In the rota-rod test, CARV-treated mice did not show any significant motor performance alterations with the doses of 25, 50 or 100 mg/kg (fig. 9). As might be expected, the CNS depressant diazepam (3 mg/kg, i.p., standard drug), reduced the time of treated animals on the rota-rod after 30 min. (7.9 ± 3.0 sec.) and 60 min. (34.6 ± 21.1 sec.) compared with the control group.

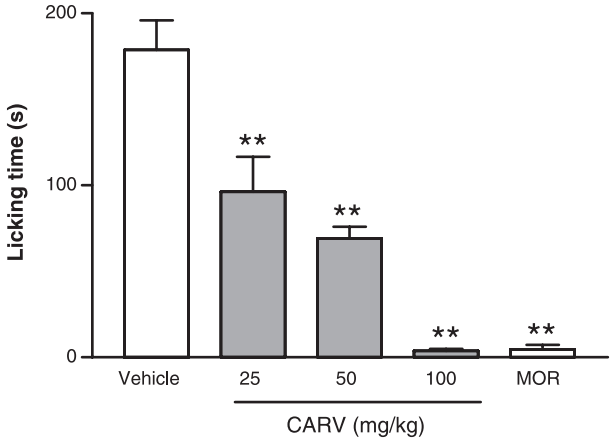

Fig. 7. Effects of CARV on the glutamate-induced nociception test in mice. Vehicle (control), CARV (25, 50 and 100 mg/kg) or morphine (MOR) were administered i.p. 0.5 hr before i.pl. glutamate injection. Each column represents mean ± S.E.M. (n = 8). $^{**}p < 0.001$ *versus* control (ANOVA followed by Tukey's test).

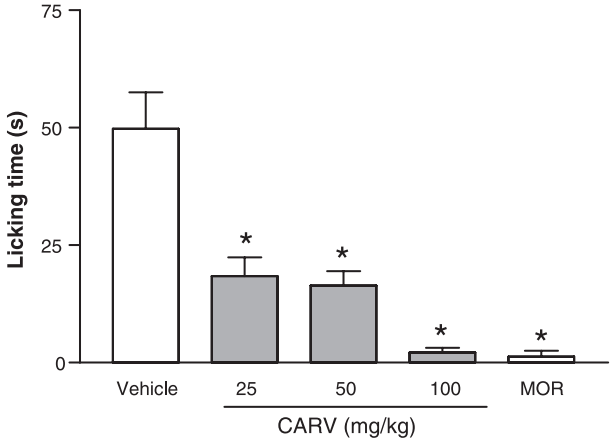

Fig. 8. Effects of CARV on the capsaicin-induced nociception test in mice. Vehicle (control), CARV (25, 50 and 100 mg/kg) or morphine (MOR) were administered i.p. 0.5 hr before i.pl. capsaicin injection. Each column represents mean ± S.E.M. (n = 8). $^{*}p < 0.001$ *versus* control (ANOVA followed by Tukey's test).

Discussion

The aim of this study was to evaluate the antioxidant and antinociceptive effects of carvacrol (CARV). The present data indicate that CARV has a pronounced antioxidant effect against free radicals generated *in vitro* as well as antinociceptive properties in distinct *in vivo* models of nociception.

The antioxidant activity of some essential oils is mainly attributed to one or more compounds with hydroxyl groups (OH) linked to aromatic ring or unsaturation. Some studies have shown that oxygenated monoterpenes constitute one of the main classes of antioxidant compounds largely due their functional groups (alcohols). As CARV has a weak acid character when it reacts with a free radical, it probably donates hydrogen atoms with an unpaired electron ($H^{\bullet}$), producing another radical that is stabilized by electron scattering generated at a molecule resonance structure [30] (fig. 10).

Carvacrol was able to scavenge peroxyl radicals produced at TRAP/TAR assay with an activity similar to Trolox, a hydrophilic α-tocopherol synthetic analogue widely used as standard antioxidant. Although concentrations of CARV as low as 1 ng/ml were effective in scavenging AAPH-derived peroxyl radicals produced *in vitro* at the TRAP/TAR assays, a significant lipoperoxidation-preventive effect was observed only with CARV at 1 μg/ml or higher concentrations. Lipoperoxidation involves a series of chain reactions in which a radical initiator extracts a hydrogen atom from a lipid, and oxygen molecules react with such lipid radicals; this process forms lipid peroxyl and peroxides, which in turn extracts hydrogen atoms from other lipids [31]. Thus, it is possible that CARV, a hydrophobic molecule, interacts more strongly with specific types of lipids, and in a lipid-rich system such as in the TBARS assay, lipids with lesser affinity to CARV and/or hydrophilic portions of amphipatic lipids are more susceptible to radical attack, allowing the initiation of lipoperoxidation chain reaction.

At the NO-scavenging assay, CARV reduced the production of nitrite, indicating a potential role as NO-scavenging agent. Nitric oxide plays an important role in various types of inflammatory processes and thus might be involved in a potential antinociceptive action by CARV [32]. Reaction

Table 1.

Antinociceptive effect of carvacrol (CARV) or morphine on the hot-plate test in mice.

Treatment	Dose (mg/kg)	Reaction time (licking of the hind paws) (s)[a]				
		Basal	0.5 hr	1 hr	1.5 hr	2 hr
Vehicle	–	8.9 ± 1.6	9.8 ± 1.7	8.8 ± 1.1	8.6 ± 1.1	10.4 ± 1.3
CARV	25	8.2 ± 2.4	8.8 ± 1.5	11.0 ± 1.7	11.9 ± 2.7	9.3 ± 1.5
CARV	50	8.2 ± 2.2	10.8 ± 1.5	8.3 ± 1.1	9.2 ± 1.8	8.0 ± 0.9
CARV	100	9.0 ± 2.3	15.5 ± 3.0[b]	14.5 ± 2.0[b]	16.4 ± 2.4[b]	10.8 ± 1.7
Morphine	3	7.6 ± 1.9	30.0 ± 0.0[c]	29.5 ± 0.4[c]	29.0 ± 0.9[c]	22.7 ± 4.9[c]

n = 8.

[a]Values represent mean ± S.E.M.

[b]$p < 0.05$ (one-way ANOVA and Tukey's test), significantly different from control.

[c]$p < 0.001$ (one-way ANOVA and Tukey's test), significantly different from control.

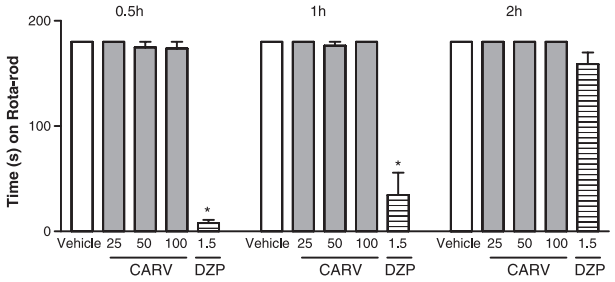

Fig. 9. Time (s) on the Rota-rod observed in mice after i.p. treatment with vehicle (control), CARV (25, 50 and 100 mg/kg) or diazepam (DZP, 1.5 mg/kg). The motor response was recorded for the following 180 sec. after drug treatment. Statistical differences *versus* control group were calculated using ANOVA, followed by Tukey's test (n = 8). *$p < 0.001$.

between some ROS (notably superoxide) and NO are generally fast and may result in 'cleaning' of NO in some cells, with consequent inhibition of some NO-triggered biological effects [16]. Nonetheless, combination of several ROS and NO invariably results in the formation of peroxynitrite, which is a strong oxidizing agent that reacts with several biomolecules at physiological pH, leading to severe cell damage [31]. Thus, CARV may preclude formation of peroxynitrite by its ability of separately scavenging both ROS and NO. Altogether, these results suggest that CARV is able to act as a general antioxidant and protects biomolecules such as membrane lipids against free radical-induced damage. Antioxidants have recently been suggested to be important regulators of inflammation since ROS and RNS have been increasingly implicated in triggering inflammatory processes [33,34]. Besides, the antioxidant action of CARV observed in the lipoperoxidation-induction assay suggests that this compound may act as a protective agent against oxidative damage to membrane polyunsaturated fatty acids (PUFAs) such as arachidonic acid, which is a very important component in the response to pain via the cyclooxygenase (COX) pathway [16].

Carvacrol showed both peripheral and central antinociceptive properties in different tests of nociception. In acetic acid-induced abdominal constriction, CARV significantly reduced the nociceptive behaviour in all doses. This is a standard, simple and sensitive test for measuring analgesia induced by both opioids and peripherally-acting analgesics [23,35]. This test, besides being the most appropriate antinociceptive model for opioids [36], is also commonly employed as a visceral inflammatory pain model [37]. In acetic acid-induced abdominal writhing, pain is elicited by the injection of an irritant such as acetic acid into the peritoneal cavity which produces episodes of characteristic stretching (writhing) movements, and inhibition of the number of episodes by analgesics is easily quantifiable [37]. Our results show that CARV produced an inhibition of the inflammatory pain in mice as determined by a significant reduction in acetic acid-induced abdominal writhing, not antagonized by naloxone. This suggests that the opioid system is not involved in the modulation of pain provoked by CARV. However, CARV may also participate in the inhibition of prostaglandin synthesis, as nociceptive mechanisms involve the processing or release of arachidonic acid metabolites via COX and prostaglandin biosynthesis [37]. Since it has been described that COX may be activated by NO [38,39], a possible link between the antioxidant and antinociceptive activities exerted by CARV might be its NO-scavenging ability.

The formalin test is a very useful method not only for assessing antinociceptive drugs but also helping in the elucidation of the action mechanism [40]. The neurogenic phase is probably a direct result of stimulation in the paw and reflects centrally mediated pain with release of substance P while the late phase is due to the release of histamine, serotonin, bradikynin and prostaglandins [37]. CARV (100 mg/kg) was able to block both phases of the formalin response but its effect was more prominent in the second phase. Intraplantar injection of formalin has been described to induce the production and release of NO [41], which in turn is suggested to be an essential component of the pro-inflammatory/nociceptive response by the stimulation of the production and release of cytokines, ROS and prostanoids [34,42]. Scavenging activity by CARV on NO and other reactive species may be an important mechanism of the antinociceptive action by this molecule.

It is well established that glutamate is involved in transmission of nociceptive signals from the peripheral nervous system to the dorsal horn of the spinal cord. Moreover, it has been reported that the glutamate injection evoked pronounced nociceptive responses, which are mediated by neuropeptides (substance P) released from C fibres. Additionally, activation of glutamate receptors (e.g. NMDA) can stimulate the production of a variety of intracellular second messengers such as NO, and pro-inflammatory cytokines such as TNF-α and IL-1β, which act synergistically in the

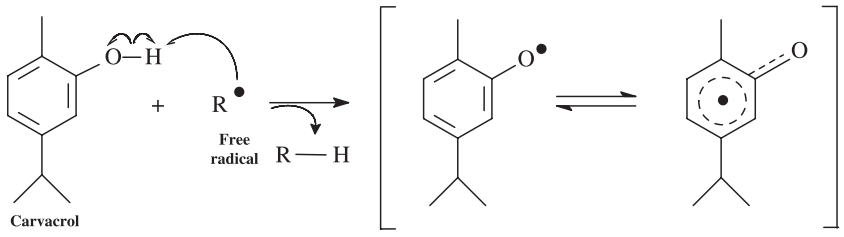

Fig. 10. Reaction of CARV and a free radical.

excitation of neurons [43]. ROS and RNS have also been related to act as pro-inflammatory signals *in vivo* by stimulating the activation of TNF-α, IL-1β and IL-6 genes through the activation of the redox-sensitive transcription factor NF-kB [44]. The antinociception by CARV could thus also be a consequence of its antioxidant ability, which may prevent the free radical-induced NF-kB activation and consequent pro-inflammatory cytokine production, a cycle that perpetuates inflammatory processes. To confirm such a hypothesis, studies to elucidate the role of CARV in the inhibition of inflammatory response in cell cultures are currently being addressed by our group.

Beirith *et al.* [24] have found that the nociceptive response induced by glutamate appears to involve peripheral, spinal and supraspinal sites of action and is greatly mediated by both NMDA and non-NMDA receptors. CARV produced an inhibition of the nociception induced by glutamate. However, (−)-linalool, another monoterpene, is involved in a central antinociceptive effect associated with ionotropic glutamatergic receptors [45], so it might be possible that CARV-like effects could include such a non-opioid central mechanism. Besides, the antioxidant action of CARV could exert some effect in signal pathways controlled by some glutamatergic receptors; it has been observed, for instance, that free radicals may decrease NMDA-evoked signalling [46]. Thus, modulation of ROS and RNS concentrations by antioxidants may interfere with NMDA-signalling at varying degrees.

Sakurada *et al.* [47] proposed the capsaicin-induced pain model for the study of compounds that act on pain of neurogenic origin. Capsaicin is a neurotoxic compound extracted from red pepper which, when applied to the skin or injected into animals, produces irritation, a painful reaction, and subsequent desensitization to chemically-induced pain [24]. Studies have shown that capsaicin evokes the release of neuropeptides, excitatory amino acids (glutamate and aspartate), nitric oxide and pro-inflammatory mediators in the periphery, and transmits nociceptive information to the spinal cord [48]. Waning *et al.* [49] demonstrated that the capsaicin-sensitive transient receptor potential vaniloid 1 (TRPV1), which plays an important role in pain transduction, is one of the Ca^{+2} influx channels involved in cell migration. It was also shown that TRPV1 activation increases the expression of the TNF receptor 1 (TNFR1) by a ROS-dependent mechanism [50]. Our results indicate a significant reduction in neurogenic nociception caused by the intraplantar injection of capsaicin, showing that CARV caused significant effects in this test. This effect, similar to the effects observed in other tests, may be mediated at least in part by the antioxidant properties of CARV, which may inhibit the ROS-dependent pro-inflammatory TRPV1 signalling. The capsaicin-induced neurogenic paw licking response was similar to the first phase of the formalin test at CARV 100 mg/kg. Compounds with this action may be potential candidates for the treatment of neuropathic conditions in which effective treatment is difficult [51].

Additionally, a number of monoterpenes have also been described as agonists or antagonists of different members of the TRPV channel family [52]. The ability of camphor, a naturally occurring monoterpene produced by the Camphor Laurel (*Cinnamomum camphora*) to modulate sensations of warmth in humans, has been attributed to its ability to activate TRPV3 [53]. TRPV3 is expressed in keratinocytes, the dorsal root ganglia, brain and spinal cord [52]. It has been implicated in hyperalgesia, inflamed tissues and possibly skin sensitization [54]. According to Vogt-Eisele *et al.* [52], some monoterpenes aromatic (as carvacrol and thymol) have been shown more potent agonists to activate TRPV3 than camphor.

The analgesic action presented by CARV involves supraspinal as well as spinal components, as demonstrated by the utilization of the hot-plate test [37,55]. The results suggest that CARV (100 mg/kg, i.p.) has a central analgesic effect, as evidenced by the prolonged delay in response time when mice were subjected to a nociceptive stimulus during the hot-plate test. Previous studies suggested that the CNS depression and the non-specific muscle relaxation effect can reduce the response of motor coordination and might invalidate the behaviour tests results [9]. Our results revealed that all mice treated with CARV, at these doses, did not have any performance alteration in the Rota-rod test.

The antinociceptive effects of CARV reported here are very likely to be associated at some degree to its antioxidant activity. Different studies have demonstrated that inhibition of superoxide and superoxide-derived reactive species is an attractive strategy to control peripheral and central sensitization associated with several painful states [56], which occurred in the present study. The antioxidant agents have been reported to be effective against acute inflammatory pain, glutamate-induced hyperalgesia, neuropathic pain, trigeminal pain, fibromyalgia and temporomandibular joint dysfunction [16]. However, redox properties of isolated compounds may vary from *in vitro* to *in vivo* conditions. Natural products such as retinol and β-carotene have demonstrated to exhibit antioxidant activity *in vitro*, but at some concentrations retinol may act *in vivo* as a pro-oxidant agent [57]. Our results show that, in comparison to the standard antioxidant Trolox, CARV has a potent antioxidant potential, and probably exerts a protective action against free radicals *in vivo* as well. Additional experiments to determine the exact degree of the antioxidant effect of CARV against reactive species in cells (e.g. cultured cells challenged with H_2O_2) and involvement of ROS and RNS in inflammatory and nociceptive processes will be performed using animal and cell culture models, in which we will be able to establish the concentration range of antioxidant activity *in vivo*.

Thus, it can be concluded that CARV is effective as an analgesic compound in various pain models, probably mediated via inhibition of peripheral mediators (as prostaglandin synthesis) as well as central inhibitory mechanisms (non-opioid central receptors) which could be related with its strong antioxidant effect observed *in vitro*. Anyway, further studies currently in progress will enable us to understand the precise mechanisms of action of CARV on nociception and oxidation tests.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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