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Evidence for the involvement of descending pain-inhibitory mechanisms in the attenuation of cancer pain by carvacrol aided through a docking study

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ABSTRACT

Aims: The present study evaluated the carvacrol (CARV) effect on hyperalgesia and nociception induced by sarcoma 180 (S180) in mice.
Main methods: Carvacrol treatment (12.5–50 mg/kg s.c.) once daily for 15 days was started 24 h after injection of the sarcoma cells in the hind paw (s.c.). Mice were evaluated for mechanical sensitivity (von Frey), spontaneous and palpation-induced nociception, limb use and tumor growth on alternate days. CARV effects on the central nervous system were evaluated through immunofluorescence for Fos protein. Molecular docking studies also were performed to evaluate intermolecular interactions of the carvacrol and muscimol, as ligands of interleukin-10 and $GABA_A$ receptors.
Key findings: CARV was able to significantly reduce mechanical hyperalgesia and spontaneous and palpation-induced nociception, improve use paw, decrease the number of positively marked neurons in lumbar spinal cord and activate periaqueductal gray, nucleus raphe magnus and *locus coeruleus*. CARV also caused significant decreased tumor growth. Docking studies showed favorable interaction overlay of the CARV with IL-10 and $GABA_A$.
Significance: Together, these results demonstrated that CARV may be an interesting option for the development of new analgesic drugs for the management of cancer pain.

Introduction

Pain is one of the most common and distressing symptoms experienced by over half of all cancer patients (Schmidt et al., 2010) and its complex pathologic process is the main challenge for an effective treatment (Sarantopoulos, 2007). This symptom can interfere with daily activities of patients with neoplasms, reducing the quality of life and promoting important psychosocial disorders, in addition to an increase in the cost of treatment (Ling et al., 2012).

Throughout history, natural products and plant food supplements have contributed unequivocally to the pain and inflammation control (Di Lorenzo et al., 2013), the example of morphine isolated from *Papaver somniferum* and more recently, ziconotide, a peptide from snails which is used for the treatment of chronic pain. Hence, herbal medicines used in pain therapy can contribute to restoring the quality of life to a patient and may enhance conventional management of different types of pain, such as rheumatologic diseases, back pain, cancer, diabetic peripheral neuropathy and migraine (Zareba, 2009).

Carvacrol (CARV), a phenolic monoterpene found in Origanum oil (Lamiaceae family), has considerable analgesic and anti-inflammatory effects; it also modulates central neurotransmitter pathways, such as dopaminergic, serotoninergic and GABAergic systems, and the release of inflammatory mediators (Guimarães et al., 2010, 2012, 2013; Melo et al., 2010, 2011; Cavalcante Melo et al., 2012; Lima et al., 2013; Zotti et al., 2013). Although several studies describe its analgesic effects, there are no reports on the central nervous system (CNS) areas activated by CARV, as well as its potential use in cancer pain.

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Due to the emerging need for new therapeutic options for the treatment of cancer pain and the vast therapeutic potential of CARV, this study aimed to evaluate its effect on the nociception induced by Sarcoma 180 in mice and neuronal regions involved. We also performed a docking study between carvacrol and $GABA_A$, and IL-10 receptors, and a comparative docking between the carvacrol and the muscimol, a potent, selective agonist against the $GABA_A$ receptor.

Materials and methods

Chemicals

Carvacrol (5-isopropyl-2-methylphenol, CARV, 98% purity), cremophor, sodium chloride, trypan blue, glycerol, DABCO, glycine and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Morphine and lactated Ringer's solution were purchased from Cristália (São Paulo, São Paulo, Brazil). Rabbit anti-Fos k-25 was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and the donkey anti-rabbit Alexa Fluor 488 was purchased from Life Technologies (Carlsbad, California, USA).

Animals

Male *Swiss* mice used (28–32 g; 2–3 months of age) were randomly housed in appropriate cages at 21 ± 2 °C on a 12 h light/dark cycle with free access to food (Purina®, Brazil) and water. Experimental protocols were approved by the Animal Care and Use Committee (CEPA/UFS 43/09) at the Federal University of Sergipe, and all handling procedures were in accordance with the International Association for the Study of Pain (IASP) guidelines for the use of animals in pain research (Zimmermann, 1983).

Tumor cell and implantation

Sarcoma 180 (S180) tumor cells, which had been maintained in the peritoneal cavity of Swiss mice, were obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará. A suspension of 10^6 viable S180 cells per 25 μl of lactated Ringer's solution was implanted subcutaneously into the plantar region of mice. Animals of the sham group received only 25 μl of lactated Ringer's solution. This methodology was adapted from Kamioka et al. (1999) and Lee et al.(2009).

Treatment and behavioral studies

Twenty-four hours after administration of S180, animals (n = 10/group) were treated daily with vehicle (saline + cremophor 0.4% v/v), CARV (12.5, 25 or 50 mg/kg) or morphine (15 mg/kg) by subcutaneous route, until the fifteenth day, and submitted to behavioral evaluation on alternate days. The investigator responsible for the behavioral evaluation was blind to the experimental situation of each animal.

Mechanical hyperalgesia

The animals were screened for the sensitivity towards mechanical stimulation generated by a gradual increase in pressure of a hand-held force transducer (electronic anesthesiometer, model: EFF-301, Insight®, Brazil) adapted with a polypropylene tip. This stimulus evokes a hind paw flexion reflex that corresponds to the paw withdrawal followed by clear flinching movements.

Spontaneous and palpation-induced nociception

Mice were placed in boxes scattered and allowed to acclimate for 10 min. Flinching behaviors were counted during a 10-min observation period. Afterwards, non-noxious palpation of the tumor-bearing paw was performed during 2 min and the number of flinching behaviors was quantified for 2 min (Sabino et al., 2003).

Movement-evoked pain

In the same boxes scattered, the limb use was assessed as previously described by Luger et al. (2001), through the observation of the mouse while walking in a continuous motion. Limping and/or guarding behavior of the right (sarcoma-treated) hind limb was rated on the following scale: 0 = complete lack of use, 1 = partial non-use, 2 = limping and guarding, 3 = limping, and 4 = normal walking.

Measurement of paw volume

The effect of CARV on tumor growth caused by the plantar incubation of S180 was evaluated through right paw volume, which was measured by the dislocation of the water column of a plethysmometer (Insight®, Brazil) before (time zero) and on every other day up to 15 days.

Measurement of forelimb grip strength

In order to check for possible changes in neuromuscular function, we measured the tension force of limbs using the commercial grip strength meter (Insight®, Brazil), before the treatment (s.c.) of tumor-free animals with vehicle or CARV (12.5, 25 or 50 mg/kg) and 30, 60 and 120 min after treatment (Van Riezen and Boersma, 1969).

Immunofluorescence

Ninety minutes after the injection of CARV (50 mg/kg; s.c.), morphine (15 mg/kg; s.c.) or vehicle, the animals (n = 6, per group) were perfused and the brains and lumbar spinal cords (L4–L6) were collected and cryoprotected for immunofluorescence processing to Fos protein.

Frozen serial transverse sections (20 μm) of all brains and lumbar spinal cord lamina I were collected on gelatinized glass slides. The tissue sections were stored at −80 °C until use. The sections were washed with phosphate buffer (0.01 M) saline isotonic (PBS) 5 times for 5 min. and incubated with 0.01 M glycine in PBS for 10 min. Non-specific protein binding was blocked by the incubation of the sections for 30 min. in a solution containing 2% BSA. Then, the sections were incubated overnight with rabbit anti-Fos as primary antibodies (1:2000). Afterwards, the sections were incubated for 2 h with donkey anti-rabbit Alexa Fluor 594 as secondary antibodies (1:2000). The cover slip was mounted with glycerol solution (79% glycerol + 10% PBS + 1% DABCO). As an immunofluorescence control for non-specific labeling, sections were incubated without primary antibody. After each stage, slides were washed with PBS 5 times for 5 min. Pictures from Fos positive brain and spinal cord areas were acquired for each animal with an Axioskop 2 plus, Carl Zeiss, Germany. The brain regions were classified according to Paxinus and Watsu Atlas, 1997. Neurons were counted by the free software ImageJ (National Institutes of Health) using a plug-in (written by authors) that uses the same level of label intensity to select and count the Fos-positive cells.

Docking studies

The docking study investigated the intermolecular interactions of the carvacrol and muscimol, as ligands (Fig. 7) and 3 macromolecules available in the Protein Data Bank: crystal structure of human interleukin-10 (PDB ID 2ILK) and $GABA_A$ receptors (PDB IDs 1KJT and 3D32). The simulations were performed on the Auto-Dock 4.2 software (Morris et al., 2009). Receptor and ligand preparation was carried out using VEGA ZZ 3.0.1 (Pedretti et al., 2004) and Molegro Molecular Viewer 2.5.

Initially, the structures were saved in pqbqt format to be used for docking calculations. PyRx 0.8 software (Wolf, 2009) was used to aid the steps of job submission and analysis of the results. The grid maps were calculated with AutoGrid. The three-dimensional grid box with 60 Å grid size (x, y, z) with a spacing of 0.300 Å, was created. Each ligand was docked into this grid with the Lamarckian algorithm as implemented in AutoDock. The genetic-based algorithm ran 12 simulations per

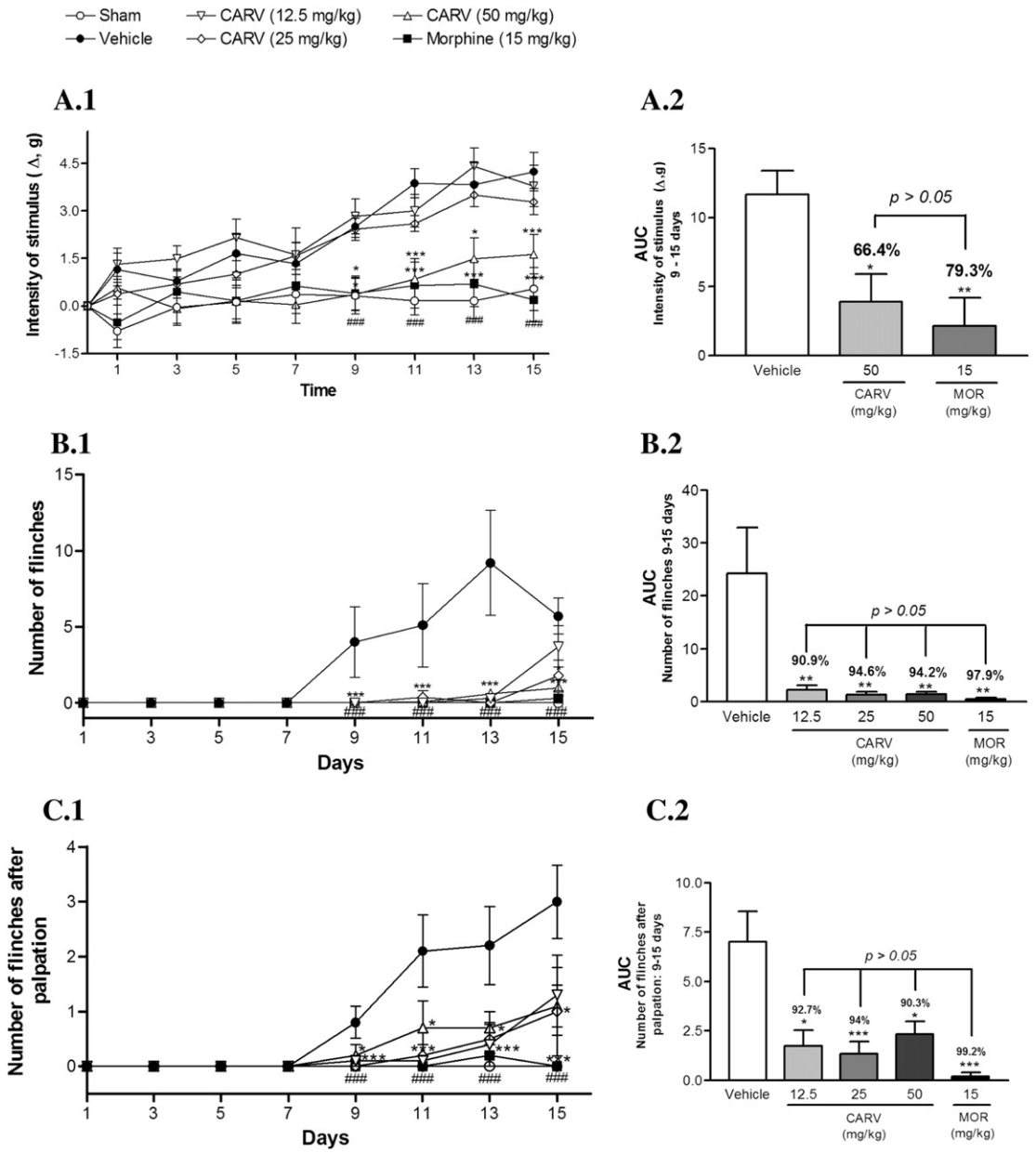

Fig. 1. Effect of carvacrol (CARV, 12.5 to 50 mg/kg, s.c.) on the mechanical hyperalgesia (A), spontaneous (B) and palpation-induced nociception (C) in mice with S180. *p < 0.05, **p < 0.01 and ***p < 0.001 vs. the control group, ###p < 0.001 vs. sham (ANOVA followed by Tukey's test).

substrate with 2,500,000 energy evaluations and a maximum number of generations of 54,000. The crossover rate was increased to 0.8, the rate of gene mutation was 0.02 and, the number of individuals in each population was 200. All other parameters were left at the AutoDock default settings. The results for each calculation were analyzed to obtain the affinity energy (kcal/mol) values for each ligand conformation in its respective complex and the probable structure inaccuracies were ignored in the calculations. In order, the number of hydrogen bonds and non-covalent interactions between each ligand conformation and the residues of the macromolecules were observed using the Molegro Virtual Docker 6.0 (Motohashi et al., 2013) and the ligand maps were generated.

Statistical analysis

The data obtained were evaluated by means of one- and two-way analyses of variance (ANOVA) followed by Tukey's test. Kruskal–Wallis followed by Dunn's test was applied to limb use. In all cases, differences were considered significant if $p < 0.05$, using the Graph Pad Prism (v 4.00) software (San Diego, CA, USA). The percent of inhibition was determined using the following formula: Inhibition% (PI) = 100.(control − experiment) / control, with the data obtained by the area under the curve (AUC).

Results

In this study, S180 cells promoted a significant reduction ($p < 0.001$) in the sensitivity threshold to mechanical stimulation on the control group (vehicle) when compared to tumor-free animals (sham group) which was maintained until the fifteenth day. After this period, paws of the animals started showing lesions, and then established the end point. Treatment of animals with CARV (50 mg/kg; s.c.) was able to significantly reduce hyperalgesia, with an average inhibition percentage of 66.4%. The treatment with morphine (15 mg/kg; s.c.) also promoted relief of hyperalgesic response with an average inhibition of 79.3% (Fig. 1.A).

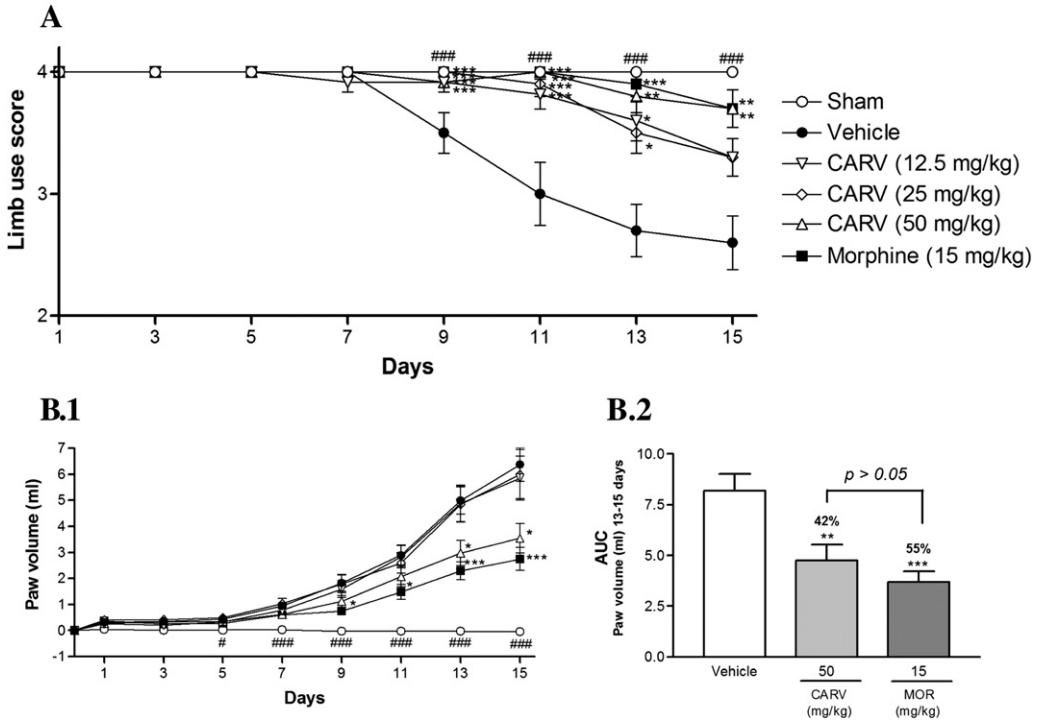

Fig. 2. Effect of carvacrol (CARV, 12.5 to 50 mg/kg, s.c.) on the paw use (A) in mice with S180 and on tumor growth (B). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ vs. the control group, ###$p < 0.001$ vs. sham (ANOVA followed by Tukey's test for paw volume and Kruskal–Wallis followed by Dunn's test for limb use).

Mice of the control group also exhibited an increased number of spontaneous flinches as compared to the sham group ($p < 0.001$), a reflection of ongoing cancer pain. CARV (12.5 and 25 mg/kg) promoted a significant reduction ($p < 0.001$) of this nociceptive response up to the 13th day, with average inhibition percentages of 90.9 and 94.6%, respectively. CARV (50 mg/kg) and positive control were able to reduce the spontaneous nociception until the fifteenth day, presenting an inhibition of 94.2 ($p < 0.001$ until the 13th day and $p < 0.05$ on the 15th day) and 97.7% ($p < 0.001$ all days), respectively (Fig. 1.B).

Mice with sarcoma also exhibited a greater number of palpation-evoked flinches as compared to shams ($p < 0.001$). A reduction of flinches induced by non-nocive palpation was observed (Fig. 1.C) in mice treated with CARV at doses of 12.5 ($p < 0.001$ up to the 13th day; PI = 92.7%), 25 ($p < 0.001$ until the 11th day and $p < 0.05$ on 13

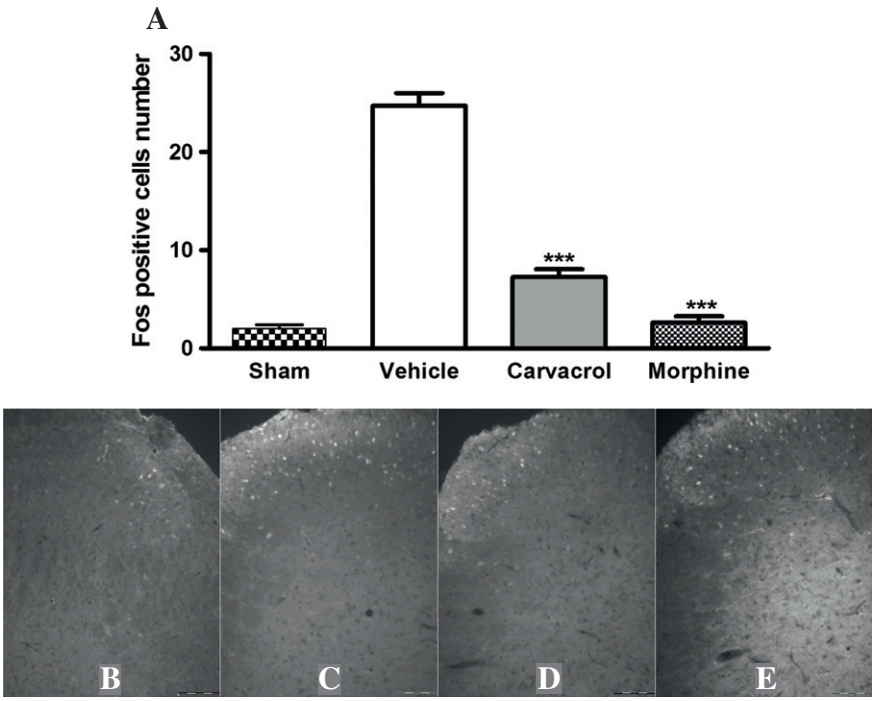

Fig. 3. Effect of carvacrol (CARV, 50 mg/kg, s.c.) on the activation of Fos positive neurons in lamina I of the lumbar cord. (A) The values were expressed as mean ± E.P.M. ($n = 6$/group). ***$p < 0.001$ vs. the control group (ANOVA one way followed by Tukey's test). Immunofluorescence for cFos protein in neurons (white labels) in lamina I of the lumbar cord in group sham (B), vehicle (C), carvacrol (D) and morphine (E) (magnification: 20×; scale bars 20 μm).

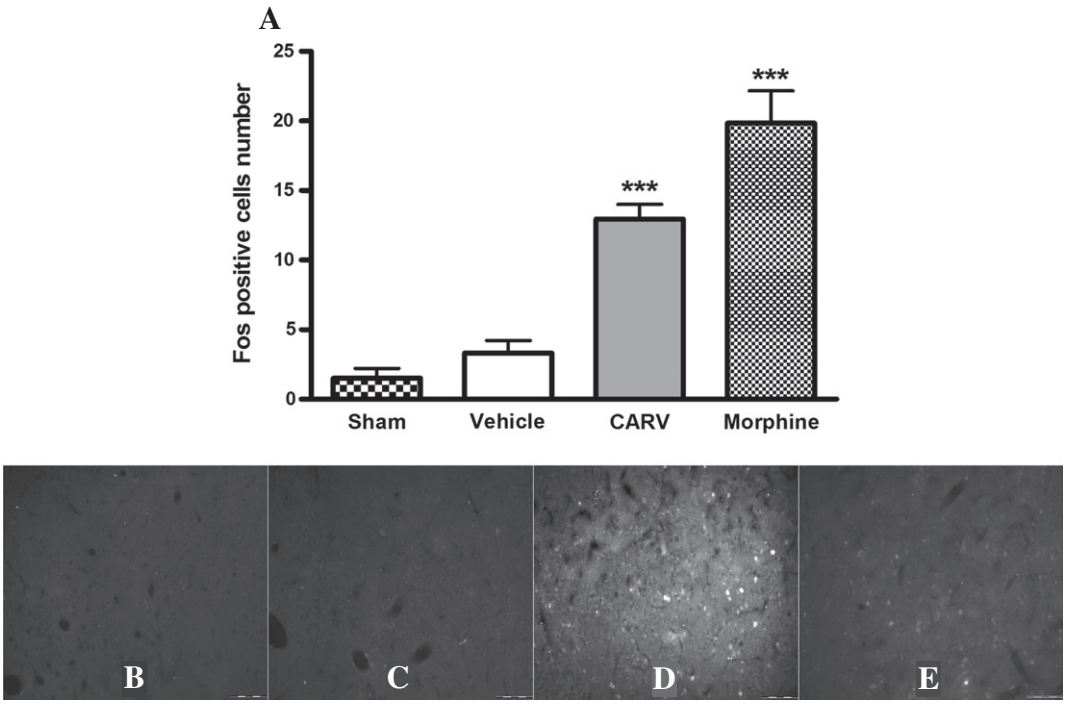

Fig. 4. Effect of carvacrol (CARV, 50 mg/kg, s.c.) on the activation of Fos positive in the periaqueductal gray (PAG). (A) The values were expressed as mean ± E.P.M. (n = 6/group). ***p < 0.001 vs. the control group (ANOVA one way followed by Tukey's test). Immunofluorescence for cFos protein in neurons (white labels) of PAG in group sham (B), vehicle (C), carvacrol (D) and morphine (E) (magnification: 20×; scale bars 20 μm).

and 15 days PI = 94%) and 50 mg/kg ($p < 0.05$; PI = 90.3%), and morphine ($p < 0.001$; PI = 99.2%).

These mice also exhibited pain-induced impairment of their limbs as evidenced by a reduction in limb use during normal ambulation ($p < 0.001$ versus sham; Fig. 2.A). Ttreatment with CARV promoted an improvement in the limb use at doses of 12–25 mg/kg ($p < 0.05$ up to the 11th day) and 50 mg/kg ($p < 0.01$ and 0.05 until the 11th and 15th days, respectively), as observed by morphine ($p < 0.01$).

The treatment effects of CARV and morphine on tumor development were assessed through the paw volume. CARV and morphine were able to reduce significantly paw volume with average inhibition percentages of 42.0 and 55.0%, respectively (Fig. 2.B).

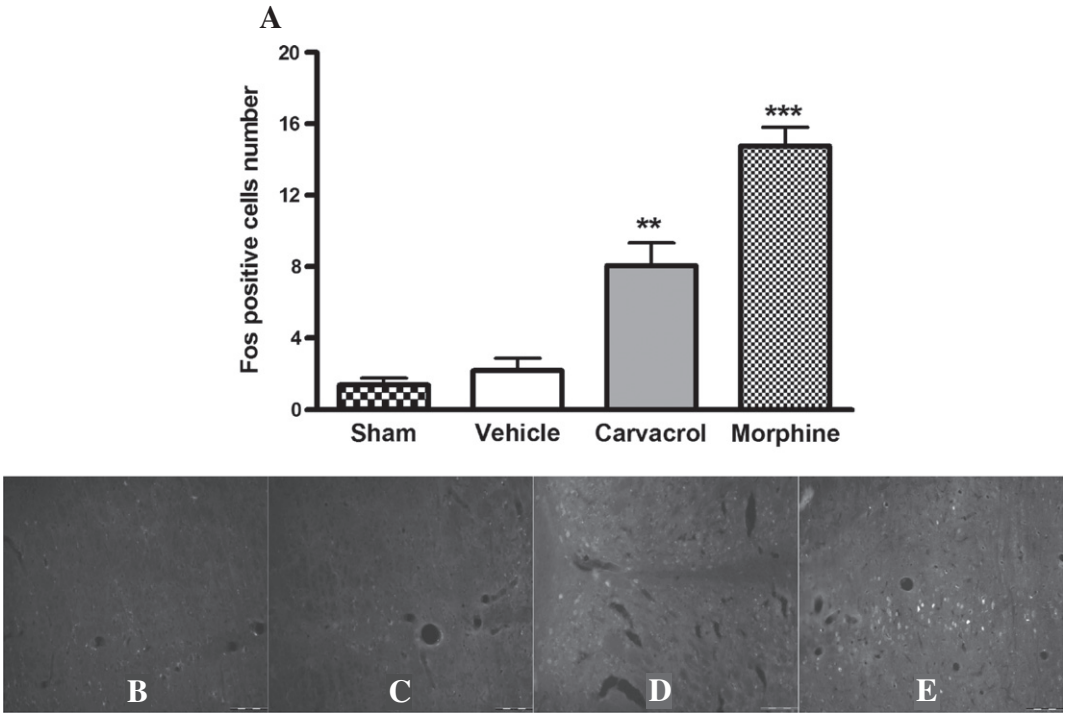

Fig. 5. Effect of carvacrol (CARV, 50 mg/kg, s.c.) on the activation of Fos positive in the nucleus raphe magnus (NRM). (A) The values were expressed as mean ± E.P.M. (n = 6/group). **p < 0.01 and***p < 0.001 vs. the control group (ANOVA one way followed by Tukey's test). Immunofluorescence for cFos protein in neurons (white labels) of NRM in the sham group (B), vehicle (C), carvacrol (D) and morphine (E) (magnification: 20×; scale bars 20 μm).

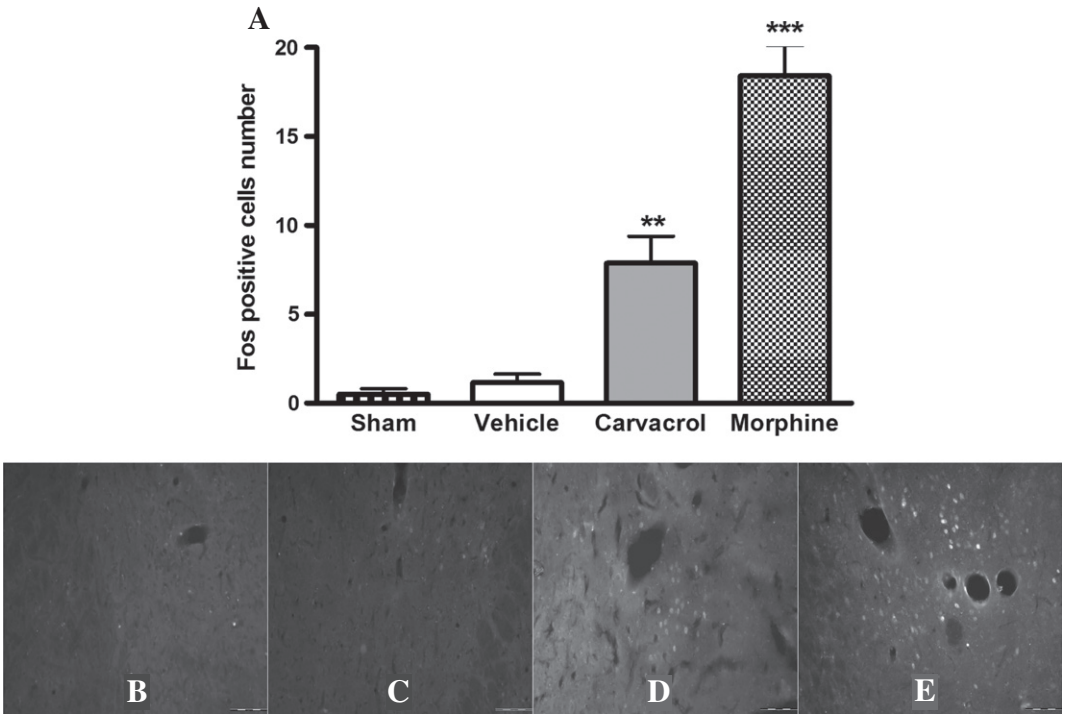

Fig. 6. Effect of carvacrol (CARV, 50 mg/kg, s.c.) on the activation of Fos positive in the *locus coeruleus*. (A) The values were expressed as mean ± E.P.M. (n = 6/group). **p < 0.01 and***p < 0.001 vs. the control group (ANOVA one way followed by Tukey's test). Immunofluorescence for cFos protein in neurons (white labels) of *locus coeruleus* in the sham group (B), vehicle (C), carvacrol (D) and morphine (E) (magnification: 20×; scale bars 20 μm).

On the 15th day, animals with sarcoma showed a significant increase ($p < 0.001$) in the number of positively marked neurons in the lamina 1 of the lumbar spinal cord (L4–L6), when compared to the sham group. This neuronal labeling was reduced ($p < 0.001$) by the treatment with CARV (50 mg/kg) and morphine (Fig. 3), with an inhibition percentage of 70.8%.

Furthermore, animals treated with CARV (50 mg/kg) presented a significant increase in positively marked neurons in periaqueductal gray (PAG; $p < 0.001$; Fig. 4), nucleus raphe magnus (NRM) and *locus coeruleus* (LC) ($p < 0.01$; Figs. 5 and 6), similar to morphine ($p < 0.001$). The activation percentages in the PAG, NMR and LC, were, respectively, 74.4, 93.6 and 85.8%.

Acute treatment of healthy animals with CARV at the doses tested was not able to change the grip strength of animals, ruling out the hypothesis of a myorelaxing activity of this compound (data not shown).

Table 1 shows the binding energies obtained in the formation of the ligand–macromolecule complexes and Fig. 8 reports the ligand maps calculated with the best conformations. When CARV is complexed with the IL-10, we find a negative energy value of −3.30 kcal/mol, indicating that the binding with the target is possible and favorable, also indicating that the CARV is a better ligand than is the muscimol for $GABA_A$ receptor, as it can be seen in Fig. 8.

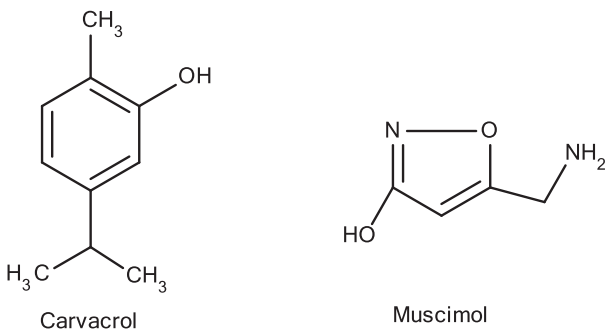

Fig. 7. Structures of the ligands used in docking studies.

Discussion

In this study, the S180 administration in the plantar region of mice was able to promote behavioral changes indicative of nociception through different methods of assessment such as mechanical hyperalgesia, spontaneous and palpation-induced nociception and movement-evoked nociception, which were attenuated by carvacrol.

Hyperalgesia indicates increased pain sensitivity and may include both a decrease in the threshold and an increase in the suprathreshold response of C and A-δ fibers (Sandkühler, 2009). Spontaneous nociception has been associated with the sensitization of C-fiber nociceptors in several nociception models (Uhelski et al., 2013). The palpation evoked nociception in animals with cancer mimics the allodynia, defined as pain in response to a non-nociceptive stimulus induced by low-threshold fibers, as A–β fibers (Sandkühler, 2009). Both palpation-evoked pain and ambulatory behavior reflect what occurs from weight-bearing touching of affected limbs in advanced cancer patients (Sabino et al., 2003).

This sensitization of such fibers can be generated by immune-system cells such as polymorphonuclear cells (PMN) found in the peripheral regions of the S180 (Sato et al., 2005), which secrete various factors that sensitize or directly excite, as prostaglandins, tumor necrosis factor-α (TNF-α), endothelins, interleukin-1 (IL-1) and -6 (IL-6), among others (Mantyh et al., 2002). In this context, the effect of carvacrol on the mediator and cytokine release may have contributed to the control of pain (Wagner et al., 1986; Hotta et al., 2010; Guimarães et al., 2012; Jalali et al., 2013; Lima et al., 2013).

It was demonstrated that S180 promotes an increase in the number of positively marked neurons in the lamina 1 of the lumbar spinal cord, which was reduced by CARV. The expression of c-Fos in the superficial dorsal horn of the spinal cord has been used as a marker of activation of primary afferent terminals, and sensitization of terminals in

Table 1
Binding energies of the complexes.

	Crystal structure of human interleukin-10 (kcal/mol) (2ILK)	Crystal structure of the GABA(A) (kcal/mol) (1KJT)	Crystal structure of the GABA(A) (3D32) (kcal/mol)
Muscimol	–	−3.66	−3.80
Carvacrol	−3.30	−4.24	−4.89

inflammatory and sarcoma-induced bone cancer pain states (Mantyh et al., 2002).

Furthermore, CARV was able to increase a number of positively marked neurons in regions involved in descending inhibitory pain pathway such as PAG, NRM and LC. This pain control circuit can be modulated by different transmitters and specific receptor types and is an important site of action to pain-relieving drugs including opiates, cannabinoids, NSAIDs, and serotonin/norepinephrine reuptake blockers that mimic, in part, the actions of opiates (Ossipov et al., 2010).

We can see the binding energies of the ligand–receptor complexes through docking studies, in which negative energy values represent favorable interactions with the receptors. Our results showed that the interaction overlay of the CARV, how much each ligand atom contributes to the overall binding interaction with the targets $GABA_A$ is greater than muscimol, besides the favorable interaction with IL-10.

Melo et al. (2010) demonstrated that the CARV modulate GABAergic transmission, corroborating with the results observed in this study once there is a significant contribution of GABAergic neurons to the spinal projections that originate lateral to the NRM about pathway pain (Reichling and Basbaum, 1990). This monoterpene is also able to stimulate IL-10 expression (Lima et al., 2013), an anti-inflammatory cytokine that contributed to control neuropathic pain (Milligan et al., 2012). These central and peripheral effects may contribute to decreased stimulation of nociceptive pathways, facilitating pain control.

Moreover, the highest dose of CARV decreased the tumor growth, corroborating with other studies that showed its anti-tumor effect

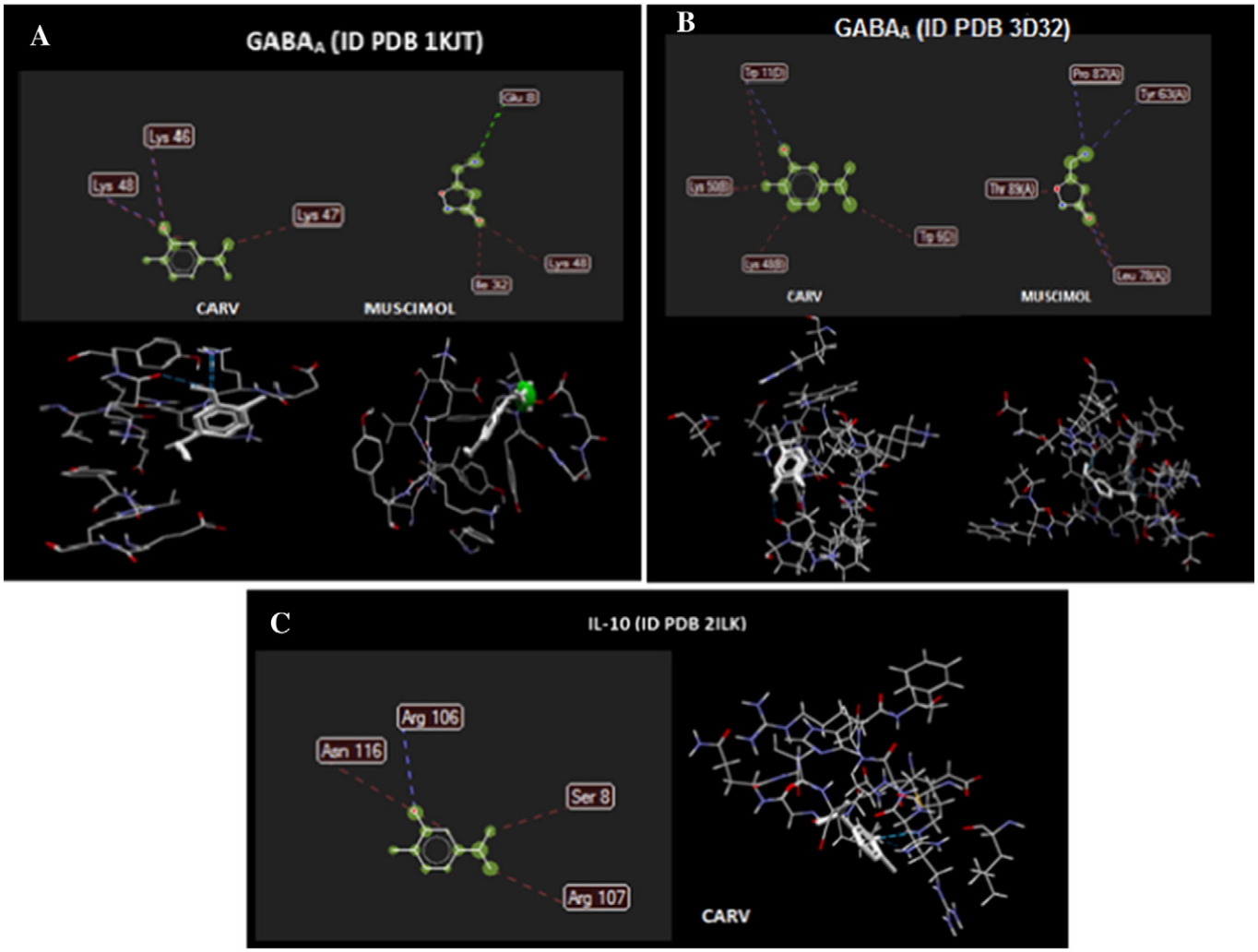

Fig. 8. Ligand maps. Blue lines—hydrogen bonds, red lines—steric interactions, green lines—eletrostatic interactions and green circles—interaction overlay.

(Arunasree, 2010). Besides, its potent anti-inflammatory action may have contributed with this finding, once several studies have demonstrated that anti-inflammatory drugs are capable of reducing tumor initiation and/or progression of several cancers (Valle et al., 2013; Alfonso et al., 2014; Grabosch et al., 2014).

Finally, we demonstrated that CARV did not promote alterations in the intensity of grip strength, discarding the hypothesis of myorelaxing effect, corroborating with previously data published (Guimarães et al., 2010, 2012).

Conclusions

The big diversity of the action mechanisms of carvacrol makes it a candidate of interest for cancer pain treatment. Among them, stand out the neuromodulatory properties of the brain nuclei that comprise the descending pathway pain controls, such as PAG, NRM and LC, possibly because of their effect on the GABA receptors, which characterizes an important mechanism of analgesics with central action and is also of great relevance for the treatment of cancer pain.

Conflict of interest statement

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

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