Chemico-Biological Interactions 227 (2015) 69–76

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Encapsulation of carvacrol, a monoterpene present in the essential oil of oregano, with β-cyclodextrin, improves the pharmacological response on cancer pain experimental protocols

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ARTICLE INFO

Article history:
Received 11 September 2014
Received in revised form 19 November 2014
Accepted 14 December 2014
Available online 31 December 2014

Keywords:
Cancer pain
Hyperalgesia
Nociception
Monoterpene
Carvacrol
β-Cyclodextrin

ABSTRACT

Cancer pain is a major public health problem worldwide due to the strong impact on the quality of life of patients and side effects of the existing therapeutic options. Monoterpenes, as carvacrol (CARV), have been extensively studied about their therapeutic properties, especially their importance in the control of painful conditions and inflammation, which can be improved through the use of inclusion complexes of β-cyclodextrin (β-CD). We evaluated the effect of encapsulation of CARV in β-CD (CARV/β-CD) on the nociception induced by tumor cells (Sarcoma 180) in rodents. Inclusion complexes were prepared in two different procedures and characterized through thermal analysis and scanning electron microscopy. CARV/β-CD complex was administered (50 mg/kg, p.o.) in mice with tumor on the hind paw and was able to reduce the hyperalgesia (von Frey) during 24 h, unlike the free CARV (100 mg/kg, p.o.), which promoted effects until 9 h. Administration on alternate days of complex of CARV/β-CD (12.5–50 mg/kg, p.o.) reduced hyperalgesia, as well as spontaneous and palpation-induced nociception. However, pure CARV (50 mg/kg) did not cause significant changes in nociceptive responses. Together, these results produced evidence that the encapsulation of carvacrol in β-cyclodextrin can be useful for the development of new options for pain management.

1. Introduction

Pain is a frequent symptom in malignant neoplasms. Decades after the publication of the World Health Organization's (WHO) analgesic ladder, cancer pain is still a major cause of suffering for patients with cancer and affects millions of people worldwide. Due to the increasing incidence of cancer and the increased life expectancy of patients, cancer-related pain is a major public health problem worldwide [1].

At present, opioids represent one of the few therapeutic options capable of controlling moderate and severe cancer pain. However, apart from analgesia, these drugs cause many consistent side effects such as sedation, euphoria, respiratory depression, constipation, addiction and pruritus [2]. These factors can lead to poor adherence of pharmacotherapy and decreased quality of life. For this reason, there is a growing interest of research centers and the pharmaceutical industry in the discovery of new pharmacological options for the treatment or management of cancer pain.

In this context, we highlight the monoterpenes, which can be found in the essential oil of aromatic herbs. They are shown to be interesting candidates for the development of new analgesic drugs, through clinical studies in cancer patients, as reviewed by Guimarães et al. [3], and some pre-clinical reviews by Da Silveira

Abbreviations: β-CD, β-cyclodextrin; CARV/β-CD, carvacrol-β–cyclodextrin complex; CARV, carvacrol; CDs, cyclodextrins; DSC, differential scanning calorimetry; PM, physical mixture; S180, Sarcoma 180; SC, slurry complex; TG/DTG, thermogravimetry/derivative thermogravimetry.

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http://dx.doi.org/10.1016/j.cbi.2014.12.020
0009-2797/

e Sá et al. [4], De Sousa [5] and Guimarães et al. [6], including studies for chronic pain [7].

Carvacrol (CARV) is a phenolic monoterpene found in essential oils of the family Lamiaceae, including the genera *Origanum* and *Thymus* [8]. Several studies have demonstrated that CARV presents pharmacological effects of interest, such as analgesic, anti-inflammatory, antitumor and antioxidant [9–12]. Recently, CARV was shown to be a pharmacologically relevant agent on cancer pain control, activating brain areas involved in descending inhibitory control of pain [13], besides being able to decrease the release of inflammatory mediators [14–16]. However, employment of these compounds by the pharmaceutical industry can present disadvantages from the technological point of view, in the preparation and storage process due to their instability, easy sublimation and the low water solubility, which can all limit their application.

Cyclodextrins (CDs) are cyclic oligosaccharides that have recently been recognized as useful tools for optimizing the delivery of such problematic drugs and also for drugs having other undesirable properties such as objectionable taste and odor, and irritation potential. This excipient (FDA approved and described in pharmacopoeias of several countries) can be found in at least in 35 pharmaceutical products, as anticancer agent and anti-inflammatory drugs, predominantly as β-CD [17]. Besides, several recent studies have demonstrated that the complexation of monoterpenes with β-CD provides benefits in solubility and stability. It could also improve pharmacological response mainly to pain control [18–21].

Based on the foregoing, it is expected that the use of carvacrol–β-cyclodextrin complex (CARV/β-CD) can be an alternative for the treatment of pain associated with cancer. Hence, the aim of our study was to optimize the biopharmaceutical properties of CARV, through the use of inclusion complexes of β-cyclodextrin, in order to improve the physicochemical characteristics and pharmacological effect of CARV on the nociception induced by Sarcoma 180 (S180) in experimental animals.

2. Materials and methods

2.1. Chemicals

Carvacrol (5-isopropyl-2-methylphenol, CARV, 98% purity), β-cyclodextrin (β-CD), cremophor, sodium chloride, trypan blue were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). Morphine and lactated ringer's solution were purchased from Cristália (São Paulo, São Paulo, Brazil).

2.2. Preparation of inclusion complexes

Inclusion complexes were prepared in two different procedures. A physical mixture (PM) was prepared with the addition of CARV (150 mg, based on its molecular weight) to an agate mortar containing powdered β-CD under manual agitation. The CARV/β-CD molar ratio was maintained as described for inclusion complex preparation and the mechanical mixture was stored in airtight glass containers. Slurry complex (SC) was carried out by the addition of water to a beaker containing 1135 mg of β-CD (3:4, v/w, ml/mg). CARV (150 mg), which is equal to about a 1:1 M guest: host ratio, was added to the SC and stirred for 36 h by a magnetic stirring device operating at 400 rpm (Quimis Q 261A21, Brazil). Thereafter, the mixture was transferred to an agate mortar, and dried in a desiccators [22].

2.3. Thermal analysis

Differential scanning calorimetry (DSC) curves were obtained in a DSC-50 cell (Shimadzu Corporation, Kyoto, Japan) using aluminum crucibles with about 2 mg of samples, under dynamic nitrogen atmosphere (50 ml/min) and heating rate of 10 °C/min in the temperature range from 25 to 600 °C. The DSC cell was calibrated with indium (m.p. 156.6 °C; ΔHfus. = 28.54 J/g) and zinc (m.p. 419.6 °C). Thermogravimetry/derivative thermogravimetry (TG/DTG) curves were obtained with a thermobalance (TGA 60, Shimadzu Corporation, Kyoto, Japan) in the temperature range 25–900 °C, using platinum crucibles with ∼3 mg of samples, under dynamic nitrogen atmosphere (50 ml/min) and heating rate of 10 °C/min. Thermogravimetric system was calibrated using a $CaC_2O_4{\cdot}H_2O$ standard substance in conformity to the ASTM pattern.

2.4. Scanning electron microscopy (SEM)

The dried products were mounted on copper tape and visualized with a JEOL (Model JSM-7410-F SEM, JEOL USA, Inc. Peabody, MA, USA), at an accelerated voltage of 1 kV. Images were registered at 1000× and 2000× of magnification in order to study their surface.

2.5. Animals

For the realization of the experimental protocols, male *Swiss* mice were used (2–3 months of age; 28–32 g), which 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 [23]. All experiments involving the behavioral analysis were carried out by the same visual observer and in a double-blind manner.

2.6. Tumor cell and implantation

Sarcoma 180 (S180) tumor cells, obtained from the Laboratory of Experimental Oncology at the Federal University of Ceará (Brazil), were maintained in the peritoneal cavity of *Swiss* mice. Ten days after tumor implantation in the peritoneal cavity of the maintenance animals, Sarcoma 180 ascites tumor cells were checked for cell viability using trypan blue and a suspension of 10^6 cells per 25 μL of lactated ringer's solution were implanted subcutaneously in the hind paw of mice. This methodology was adapted from Kamioka et al. [24] and Lee et al. [25].

2.7. Time-effect curve

A time-effect curve was designed to evaluate the effect of the complexation in the pharmacological response of CARV on cancer pain. On the tenth day after the implantation of S180 in the hind paw of mice, they were treated with vehicle (saline), CARV/β-CD (50 mg/kg) or CARV (50 and 100 mg/kg) orally (p.o.). The sensibility to mechanical stimulation was assessed 1, 3, 6, 9, 12, 24 and 27 h after treatment.

2.8. Behavioral studies

Indicative parameters of cancer pain as mechanical hyperalgesia, movement-evoked pain, spontaneous and palpation-induced nociception were evaluated after the treatment with vehicle or CARV/β-CD (12.5, 25 and 50 mg/kg) from the 1st to 15th days following S180 administration.

2.9. Mechanical hyperalgesia

Mice were evaluated as to the sensitivity towards mechanical stimulation generated by gradual increase in pressure of a hand-held force transducer on the plantar surface of the hind paw (electronic anesthesiometer, model: EFF-301, Insight®, Ribeirão Preto, São Paulo, Brazil) adapted with a polypropylene tip. In this test, there is the automatic recording of the intensity of the pressure able to evoke a hind paw flexion reflex that corresponds to the paw withdrawal followed by clear flinching movements, an indication of hyperalgesia. The intensity of stimulus was obtained by averaging five measurements performed with minimal intervals of 3 min. The results are expressed by the Δ withdrawal threshold (in grams) calculated by subtracting the zero-time (basal) mean measurements from the time interval mean measurements [26].

2.10. Spontaneous and palpation-induced nociception

Animals were placed in scattered boxes and allowed to acclimate during 10 min. Spontaneous pain was represented by flinching behaviors carried by the animals with S180 counted during a 10-min period. After that, animals was submitted to non-noxious palpation of the paw with tumor during 2 min and the number of flinching behaviors was quantified for 2 min [27].

2.11. Movement-evoked pain

We also evaluated the limb use through the observation of the mouse while walking in the same boxes scattered [28]. Limping and/or guarding behavior of the right (sarcoma-implanted) hind limb was rated on the following scale: 0 = complete lack of use, 1 = partial non-use, 2 = limping and guarding, 3 = limping, 4 = normal walking.

2.12. Measurement of forelimb grip strength

The grip strength meter was carried before the treatment (p.o.) of tumor-free animals with vehicle or CARV/β-CD (12.5, 25 or 50 mg/kg) and 30, 60 and 120 min after treatment, using the commercial grip strength meter (EFF 305, Insight®, Ribeirão Preto, São Paulo, Brazil), that digitally displays the maximum force applied (in grams) by mice. The mean of three consecutive trials was taken as an index of limb grip strength. To assess the limb grip strength measurement, the mice were held gently by the base of their tail over the top of the grid so that paws were able to grip the grid platform/T-bar [29].

2.13. Statistical analysis

The data obtained were evaluated using the Graph Pad Prism (v 4.00) software (San Diego, CA, USA) by one- and two-way analysis of variance (ANOVA) followed by Tukey's test. Kruskal–Wallis followed by Dunn's test was applied forelimb use. In all cases, differences were considered significant if $p < 0.05$.

3. Results

3.1. Thermal analysis

In the characterization of the complex, the DSC curve of CARV (Fig. 1) showed two endothermic events up to 120 °C corresponding to its volatilization. The TG/DTG curves (Fig. 2) corroborate with this result, showing one step of weight loss of 100% up to 168 °C. DSC curve of β-CD showed four events of 27–600 °C. The first event is related to the water releasing of 27–120 °C (Δ_{m1} = 11.8%). With the non-significant mass loss in the TG/DTG, the second event of 210–230 °C was related the phase transition of β-CD. The other events are endothermically and exothermically assigned to fusion with thermal decomposition. TG/DTG curves showed thermal decomposition of β-CD started above 270 °C and occurring with elemental carbon formation because of the sample carbonization. Between 360 and 900 °C, the elemental carbon was slowly released.

Thus, the DSC curve of the PM indicated endothermic peaks: the first in the range of 62–86 °C, the second in the range of 86–130 °C (which corresponds to the release of water molecules as well as the

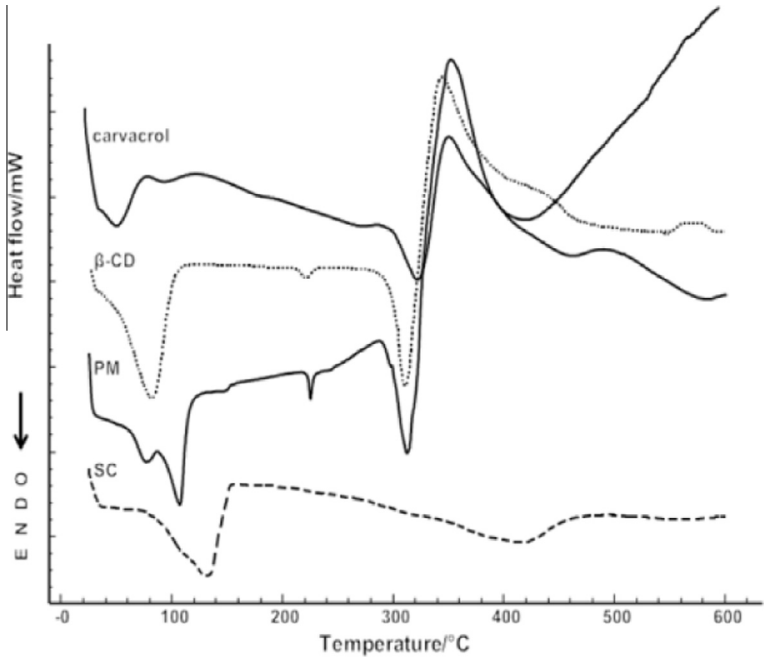

Fig. 1. DSC curves of carvacrol, β-cyclodextrin (β-CD), physical mixture (PM), and slurry complex (SC) in dynamic nitrogen atmosphere (100 ml min^{-1}) and heat rate of 10 °C min^{-1}.

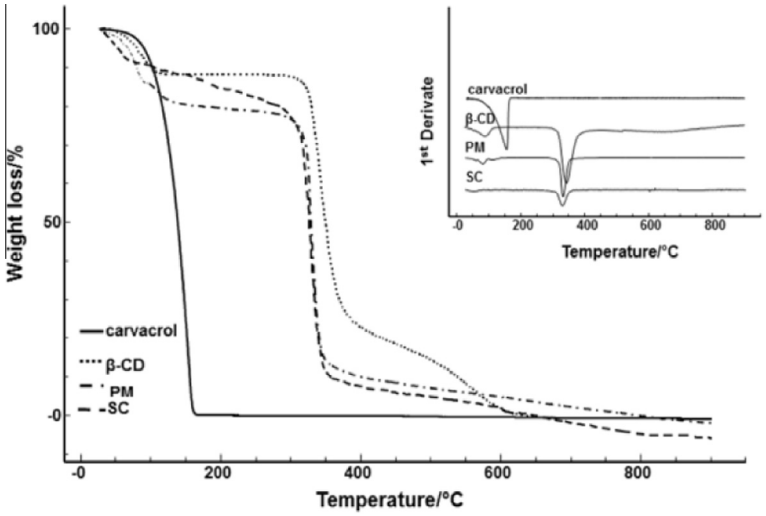

Fig. 2. TG curves of carvacrol, β-cyclodextrin (β-CD), physical mixture (PM), and slurry complex (SC) in dynamic nitrogen atmosphere (100 ml min^{-1}) and heat rate of 10 °C min^{-1}.

Table 1
Mass losses for carvacrol, β-CD, PM and SC (CARV/β-CD) complex.

	Weight loss/%			
	1st step	2ndstep	3rdstep	4th step
Carvacrol	100[a]	–	–	–
β-CD	11.8[b]	–	72.88[e]	10.76[f]
PM	18.6[c]	3.4[d]	65.7[e]	14.3[f]
SC	11.2[c]	8.7[d]	70.9[e]	15.2[f]

[a] Percentage of the carvacrol evaporates up to 168 °C.
[b] Percentage of water releasing up to 120 °C.
[c] Mass loss related to evaporation of the carvacrol and the water release up to 130 °C.
[d] Mass loss probably attributed to carvacrol release in the interval from 130 to 280 °C.
[e] Thermal decomposition in the interval from 280 to 365 °C.
[f] Elemental carbon formation due to sample carbonization in the interval from 365 to 900 °C.

release of CARV, probably adsorbed in the surface), and an event similar to the phase transition of β-CD of 220–230 °C. However, the DSC curve of the SC indicates only an endothermic event following decomposition.

The analysis of the TG/DTG curves evidences the complexation. The curves show 18.6% and 11.2% of mass loss up to 130 °C for PM and SC (Table 1), respectively. That can be attributed mainly to the water loss and to the release of surface oil for PM, but the weight loss of the SC is related to the release of a small amount of guest compound from the sample. Between 130 and 280 °C, the PM and SC lost 3.4% and 8.7%, respectively. That means that through the slurry method, in this range, carvacrol strongly encapsulated is released, and at ≈280 °C, the decomposition of β-CD molecules began to appear.

3.2. Scanning electron microscopy (SEM)

The Fig. 3 shows SEM images obtained for β-CD (A1 and A2), PM (B1 and B2) and inclusion complex (C1 and C2) powders at different magnifications (1000 and 2000×, respectively). The β-CD was composed by different sizes of rectangular-shaped crystals (14.8 ± 4.9 μm). Furthermore, they presented drastic changes in particle shapes and original morphologies of the inclusion complex products. The complexation between CARV and β-CD appeared as agglomerates of size and smaller crystals (7.5 ± 2.6 μm). In contrast, the particle shapes and morphologies of the corresponding PMs were similar to those of β-CD. The particle sizes of the PMs (80.2 ± 28.3 μm) were much larger than those of the inclusion complex product and β-CD alone.

3.3. Time-effect curve

CARV in free form, when administered orally, showed a significant effect only at a dose of 100 mg/kg, which was continued until 9 h after treatment. On the other hand, CARV/β-CD complex (50 mg/kg) was capable of promoting the same effect until 24 h after treatment. Thus, it was observed that the complexation increased the anti-hyperalgesic effect for 15 more hours (Fig. 4A).

3.4. Mechanical hyperalgesia

Subsequently, three increasing doses of the CARV/β-CD complex were tested (Fig. 4B). All doses of the complex (12.5 and 50 mg/kg: $p < 0.01$; 25 mg/kg: $p < 0.05$) promoted a significant decrease in hyperalgesia induced by S180. After 24 h, mice were reevaluated regarding the mechanical sensitivity and it was observed that they were still under the effect of the complex (12.5 mg/kg: $p < 0.01$; 25 and 50 mg/kg: $p < 0.05$) on the 11th day. However, after 72 h, mice were again sensitive towards mechanical stimulation, similarly to mice treated with vehicle alone (data not shown), requiring a new treatment for the effective control of pain. For this reason, a treatment interval for mice on alternate days was established.

This anti-hyperalgesic effect was also observed from the 12th to the 15th day, when mice were treated on alternate days with the complex at the doses of 12.5 ($p < 0.001$ or 0.01), 25 ($p < 0.05$), and 50 mg/kg ($p < 0.01$ or 0.05). On the other hand, free CARV (50 mg/kg) orally administered was not able to promote statistically significant changes in hyperalgesic responses.

3.5. Spontaneous and palpation-induced nociception

All doses of the complex were able to reduce spontaneous and palpation-induced nociception in animals with S180 (Fig. 5A and B). Between the 10th and 15th days of the experiment, the animals were treated on alternate days with the complex at the doses of 12.5, 25 and 50 mg/kg of the CARV/β-CD complex; they showed a statistically significant decrease in the number of spontaneous and post-palpation flinches ($p < 0.001$ or 0.05). Free CARV

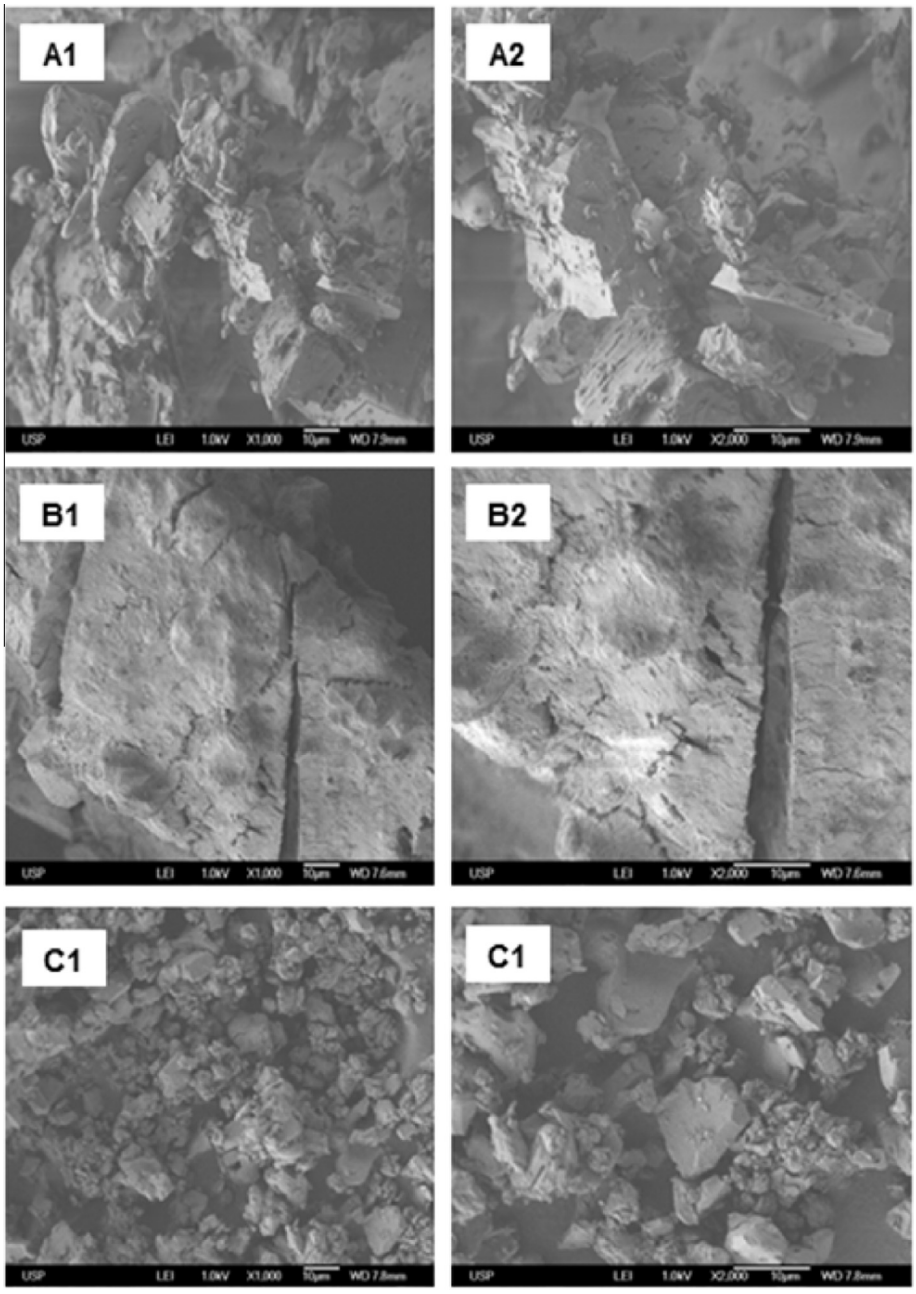

Fig. 3. SEM micrographs of cross-sections (1000 and 2000 μm) of (A) β-CD, (B) physical mixture (PM), and (C) slurry complex (SC).

(50 mg/kg) did not promote significant changes in nociceptive responses.

3.6. Movement-evoked pain and measurement of forelimb grip strength

CARV/β-CD complex did not promote significant changes in the limb use. Acute treatment of healthy animals with CARV/β-CD complex at the doses tested was not able to change the grip strength of animals at 30 min and 24 h after treatment, ruling out the hypothesis of a myorelaxing activity of this complex (data not shown).

4. Discussion

Cyclodextrins (CDs) are included in the group of pharmaceutical excipients, and represent one of the complexing agents most commonly used by the pharmaceutical industry because they are inexpensive, friendly to humans and also capable of improving the biological, chemical and physical properties of bioactive molecules, especially those extracted from plants [30]. Now, we demonstrated that the complexation of carvacrol (CARV) with β-CD was able to improve the pharmacological response on cancer pain protocols.

DSC and TG/DTG curves demonstrated profiles similar to those already described in literature for CARV and β-CD [31–34]. The difference in the DSC curves of the PM and the SC (or complex of CARV/β-CD) clearly indicates a complex formation between the components. The curve of the PM was a superposition of the guest and host curves, which indicates a lower evidence of inclusion and significant interaction between the host and guest molecules. SEM indicated morphological differences in the products obtained, which is in agreement with the observations made by other authors [35–38]. The drastic change of the particle shapes and

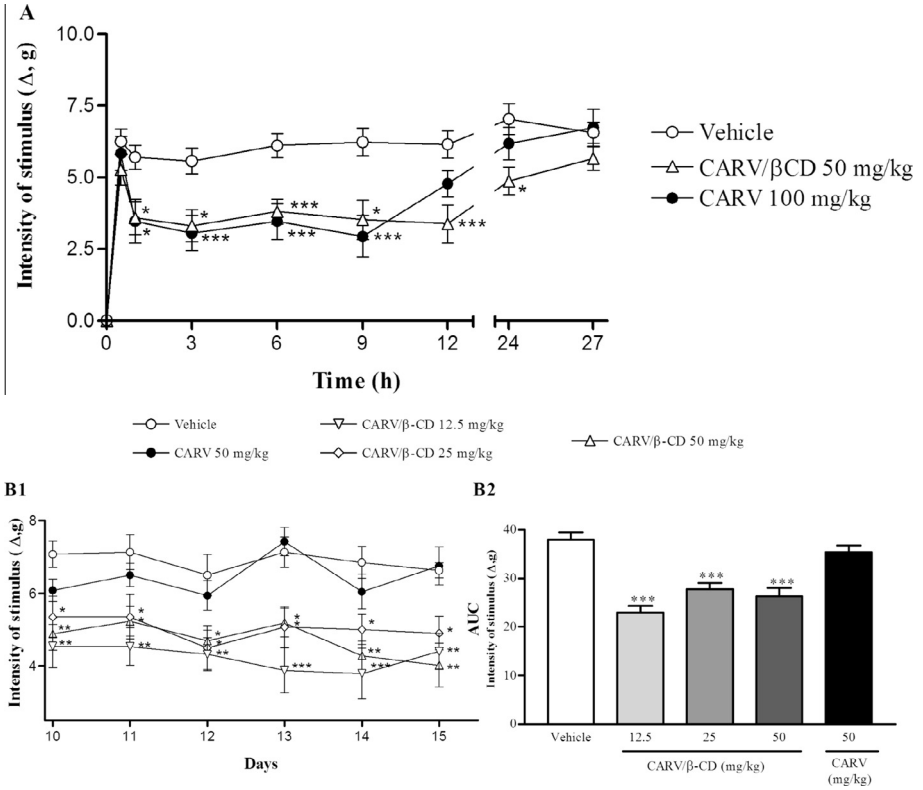

Fig. 4. Effect of carvacrol/β-cyclodextrin complex (CARV/β-CD) on the mechanical hyperalgesia induced by S180. (A) Time-effect curve of CARV/β-CD (50 mg/kg) and CARV (100 mg/kg). (B) Effect of CARV/β-CD (12.5–50 mg/kg, p.o.) and carvacrol (CARV, 50 mg/kg) from the 10 to 15 days. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. the control group (vehicle) (ANOVA followed by Tukey's test).

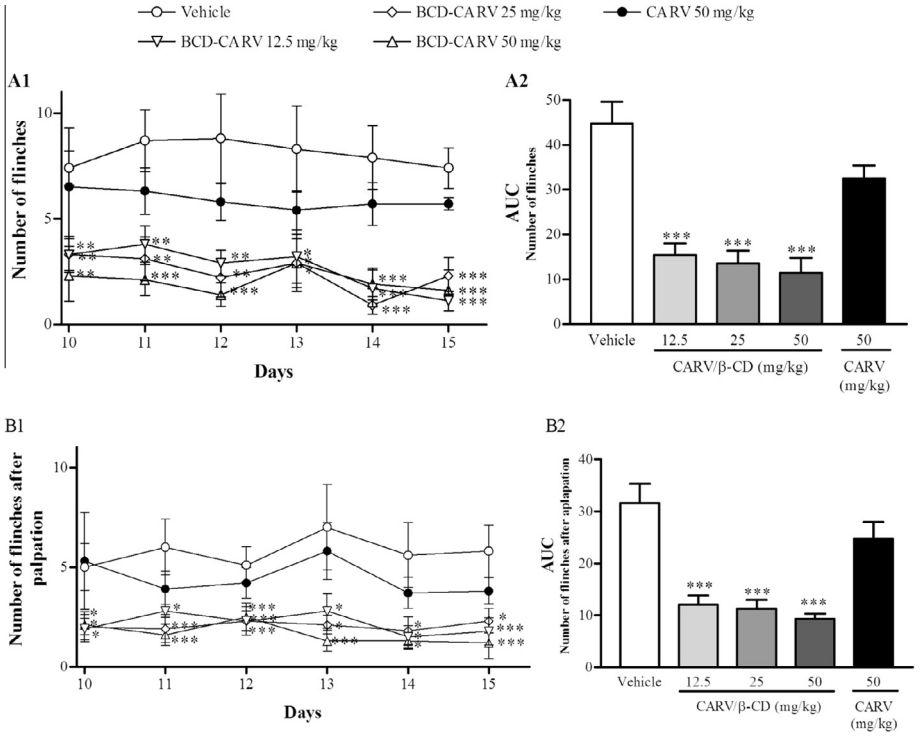

Fig. 5. Effect of carvacrol–β-cyclodextrin complex (CARV/β-CD, 12.5–50 mg/kg, p.o.) on the spontaneous (A) and palpation-induced nociception (B) induced by S180. Each point represents the mean ± SEM of the number of flinches (spontaneous lifting behavior and/or custody of the affected hind limb). $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. control group (ANOVA followed by Tukey's test).

aspects in the co-evaporation sample were indicative of the presence of a new solid phase.

Bethanis et al. [39] and Mulinacci et al. [40] demonstrated through different methods that the CARV is found fully immersed inside the hydrophobic cavity of β-CD. This complex, when prepared with the co-precipitation method in aqueous solution, gives a complex monomer of 1/1 stoichiometry, in which the isopropyl group of the CARV is located closer to the larger side of the hydrophobic cavity of β-CD. In aqueous medium, CDs are able to bind to organic molecules by non-covalent interactions and the complexation driving forces have been attributed to hydrophobic interactions, van der Waals-London dispersion forces, and hydrogen bonds [40].

Furthermore, Higueras et al. [41] recently developed a chitosan film in which the hydroxypropyl-β-cyclodextrins, a synthetic derivative of β-CD, was used to enhance: absorption of carvacrol by chitosan films, mechanical resistance and the properties of the barrier to the film water vapour. It also protected the preparation against thermal degradation. In this sense, chitosan films containing CDs are also employed as vehicle of active compounds in solid formulations, which can be applied topically for the treatment of skin disorders.

Carvacrol significantly reduced nociception induced by tumor cells and its complexation in β-CD improved this pharmacological profile. Some studies have demonstrated that after oral administration, CARV is rapidly absorbed in the stomach and proximal small intestine, yielding a first peak of plasma concentration (0.5 h), presenting a second increase in plasmatic concentration between 2–8 h, probably caused by re-uptake of CARV through enterohepatic recirculation [42–44].

Cyclodextrins are used in pharmaceutical applications for numerous purposes, including the improvement of the bioavailability of drugs, as well as the increase in the intensity or duration of therapeutic activity, especially for anti-inflammatory and antitumor drugs [17]. Furthermore, recent studies have shown that the encapsulation of plant bioactive compounds, such as essential oils and monoterpenes, promotes the increase in stability, solubility in aqueous environment, and also improves pharmacological activity [19–21,30]. In these cases, water-soluble CD complexes increase their diffusion to the mucosal surface of gastrointestinal tract leading to enhanced oral bioavailability [45].

This way, with the possible increase in CARV availability, there is a longer time to act on its action sites, as descending inhibitory pain pathway [13] through central neurotransmitters modulation, as dopamine and serotonin, and GABA and TRP receptors [10,46–50].

Encapsulation of CARV in β-CD also can contribute to the inflammatory response control on peripheral tissues associated to the presence of tumor, through decrease in inflammatory mediators release, such as TNF-α, IL-1β, IL-4 and PGE_2, endothelin, and suppression of the COX-2 and IL-1β expression [11,14–16,51].

Is also interesting to note that all doses of the complex CARV/β-CD tested were able to reduce nociception and hyperalgesia induced by Sarcoma 180, showing that the complexation of CARV in β-CD was able to improve the pharmacological effect, even at lower doses. Thus, the reduction of the dose required for optimum therapeutic effect is one of the most important benefits of cyclodextrins in drug development. This result has been attributed to an increase in drug solubility when complexated in CD, resulting in the increase of drug potency and reducing the drug toxicity [52].

5. Conclusions

Thus, it was possible to demonstrate that the slurry complexation method showed a better inclusion profile of the carvacrol when compared to physical mixture. Besides, our results produced evidence that the encapsulation of carvacrol in β-cyclodextrin represents an interesting alternative to the development of new drugs for the cancer pain management, since it masks its organoleptic properties, increases stability, solubility and improves their ability to modulate the painful responses associated with cancer.

Conflict of Interest

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

Transparency Document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

We thank Mr. Osvaldo Andrade Santos for the technical support. This work was supported by grants from the National Council of Technological and Scientific Development (CNPq/Brazil) and the Research Supporting Foundation of the State of Sergipe (FAPITEC-SE/Brazil).

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