Life Sciences 216 (2019) 215–226

Contents lists available at ScienceDirect

Life Sciences

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

Diosmetin as a novel transient receptor potential vanilloid 1 antagonist with antinociceptive activity in mice

Gabriela Adamante[a,1], Amanda Spring de Almeida[b,1], Flávia Karine Rigo[a], Edinara da Silva Silveira[a], Yanka Oliveira Coelho[a], Samira Dal-Toé De Prá[a], Alessandra Marcone Milioli[a], Camila Camponogara[c], Rosana Casoti[d], Fernando Bellinaso[b], Alexandre Vinhal Desideri[a], Mario Ferreira Conceição Santos[e], Juliano Ferreira[f], Sara Marchesan Oliveira[c], Gabriela Trevisan[a,b,*]

[a] Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense (UNESC), 88006-000 Criciúma, SC, Brazil
[b] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil
[c] Programa de Pós-Graduação em Ciências Biológicas: Bioquímica Toxicológica, Universidade Federal de Santa Maria (UFSM), 97105-900 Santa Maria, RS, Brazil
[d] Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto – Universidade de São Paulo (FCFRP-USP), 14040-903 Ribeirão Preto, SP, Brazil
[e] Programa de Pós-Graduação em Química, Instituto de Química de São Carlos - Universidade de São Paulo (IQSC-USP), 13560-970 São Carlos, SP, Brazil
[f] Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Catarina (UFSC), 88049-900 Florianópolis, SC, Brazil

ARTICLE INFO

Keywords:
Pain
Capsaicin
Resiniferatoxin
Diosmin
Inflammation
Neuropathy

ABSTRACT

Diosmetin is an *O*-methylated flavone found naturally in citrus fruit, and it was identified in *Amphilophium crucigerum* (L.), a plant popularly used as an analgesic. This compound had different pharmacological effects and presented a chemical structure like the flavonoid eriodyctiol that exhibited antinociceptive effects by TRPV1 antagonism. However, the possible antinociceptive effect of this compound was not well documented. Thus, the goal of the present study was to evaluate the antinociceptive effect of diosmetin and its mechanism of action. The diosmetin effect on different pain models and its possible adverse effects were assessed on adult Swiss male mice (25–30 g). Mice spinal cord samples were used on calcium influx and binding assays using TRPV1 agonists. First, it was observed that the diosmetin reduced calcium influx mediated by capsaicin in synaptosomes and displace the specific binding to [^{3}H]-resiniferatoxin in membrane fractions from the spinal cord of mice. Diosmetin (0.15 to 1.5 mg/kg, intragastric, i.g.) presented antinociceptive and antiedematogenic effect in the capsaicin intraplantar test and induced antinociception in a noxious heat test (48 °C). Also, treatment with diosmetin reduced mechanical and heat hypersensitivity observed in a model of inflammatory or neuropathic pain. Acute diosmetin administration in mice did not induce locomotor or body temperature changes, or cause liver enzyme abnormalities or alter renal function. Moreover, there were no observed changes in gastrointestinal transit or induction of ulcerogenic activity after diosmetin administration. In conclusion, our results support the antinociceptive properties of diosmetin which seems to occur via TRPV1 antagonist in mice.

1. Introduction

Diosmetin ([3′,5,7-trihydroxy-4′-methoxyflavone]) belongs to the flavonoid family; this compound can be found in citrus fruit, *Olea europaea* L. leaves, legume *Acacia farnesiana* Wild, and medicinal plants [1–3]. Different studies showed the pharmacological effects of this

Abbreviations: ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; BSA, bovine serum albumin; Ca^{2+}, calcium; CCI, chronic constriction injury; CFA, complete Freund's adjuvant; CGRP, peptide related to the calcitonin gene; Crd, crude extract; Dcm, dichloromethane fraction; DMSO, dimethyl sulfoxide; ED_{50}, effective dose 50%; i.g., intragastric; i.p., intraperitoneal; i.pl., intraplantar; IC_{50}, inhibitory concentration 50%; I_{max}, maximal inhibition; NSAIDs, nonsteroidal anti-inflammatory drugs; PBS, phosphate buffer solution; [^{3}H]-RTX, [^{3}H]-resiniferatoxin; TRP, transient potential receptor; TRPV1, transient potential receptor vanilloid 1

* Corresponding author at: Programa de Pós-Graduação em Farmacologia, Universidade Federal de Santa Maria (UFSM), Avenida Roraima, 1000, building 21, room 5207, 97105-900 Santa Maria, RS, Brazil.
E-mail addresses: gabrielatrevisansantos@gmail.com, gabriela.trevisan-santos@ufsm.br (G. Trevisan).
[1] Both authors contributed equally to this manuscript.

https://doi.org/10.1016/j.lfs.2018.11.029
Received 3 August 2018; Received in revised form 1 November 2018; Accepted 12 November 2018
Available online 14 November 2018
0024-3205/

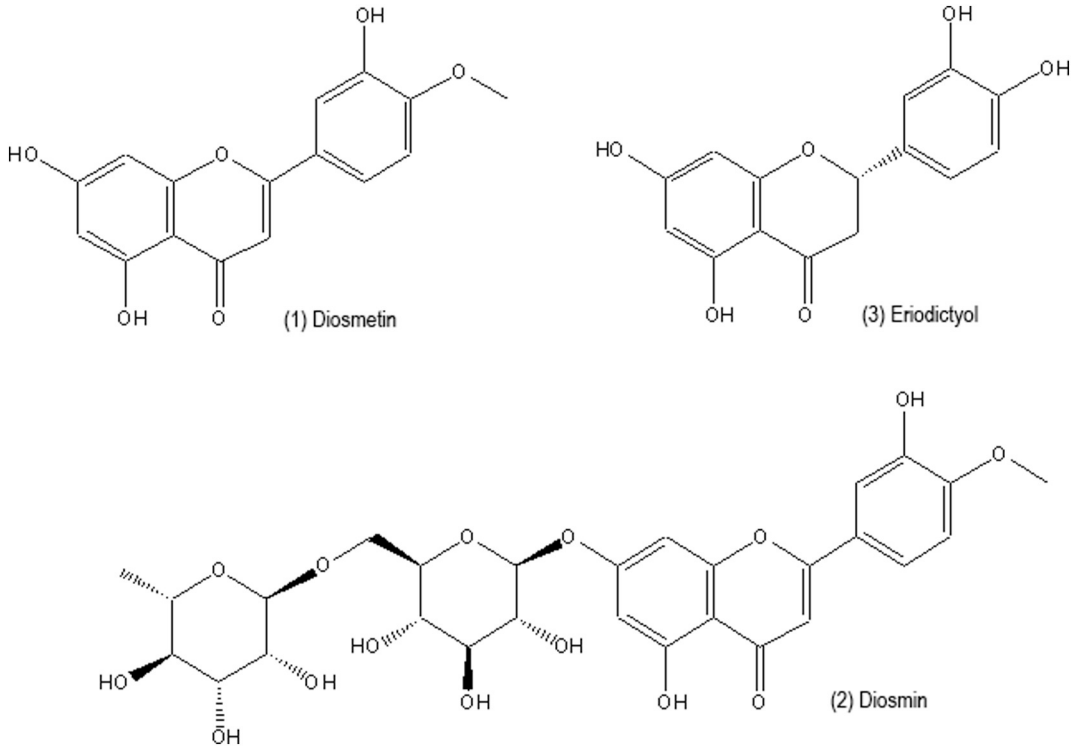

Fig. 1. Chemical structure of (1) diosmetin, (2) diosmin, and (3) eriodyctiol.

compound, including anti-inflammatory, antioxidant, and anticancer activities [1,2,4]. Moreover, diosmetin is the main bioactive metabolite of diosmin (Fig. 1), which is a flavonoid commonly used to treat chronic venous insufficiency [5]. Also, it is worth mentioning that the chemical structure of diosmetin is very similar to that of eriodyctiol, which was described previously as a transient receptor potential vanilloid 1 (TRPV1) antagonist with antinociceptive and antiinflammatory effects [6].

The TRPV1 channel is expressed in nociceptors and has been associated with the detection of heat stimuli (> 43 °C), and it is also activated by irritants compounds, such as capsaicin and resiniferatoxin [7–10]. Moreover, it is well investigated that TRPV1 receptor activation is related to the mechanisms of pain development, mainly using models of inflammatory and neuropathic pain in rodents [11–13]. However, the antinociceptive and antiedematogenic effect of diosmetin and its possible capacity to antagonize the TRPV1 receptor were not evaluated. Thus, the discovery of new compounds that could antagonize or even desensitize this receptor would be of great value for the development of novel analgesics that may be safer and more effective in the control of persistent pain [9,10].

Moreover, diosmetin was previously identified on the dichloromethane fraction of the plant popularly known as monkey's comb (*Amphilophium crucigerum*) by our research group [3]. This plant has a description for popular use as an analgesic for the treatment of neuropathic pain and headache and thus appears to be an interesting alternative for the treatment of pain. Previously, our research group performed a preclinical study in experimental pain models in mice, using the crude extract and the dichloromethane fraction of the seeds of this plant, and these preparations showed antinociceptive activity, and the mechanism of action seems to be associated to the antagonism of TRPV1 [3].

In this view, this study aimed first to test using in vitro assays if diosmetin could act as a TRPV1 antagonist. After, we evaluated diosmetin antinociceptive activity in different pain models in mice that are sensitive to TRPV1 antagonists. Finally, we assessed if diosmetin may induce adverse effects in mice after acute administration, this was done to exclude the possibility of hyperthermia, sedation, constipation or gastric damage caused by this compound.

2. Material and methods

2.1. Plant material, extraction, and isolation

The seeds of *Amphilophium crucigerum* (L.) L.G. Lohmann were collected in southern Brazil (at 29°41′02″S and 53°48′25″W). The species was authenticated, and a voucher specimen was deposited in the herbarium of the Federal University of de Santa Maria (record #12872/2010 SMDB). The seeds powder (400 g) was macerated with EtOH:H_2O ratio of 70:30 (v/v). The crude extract yielded 92 g, after removal of the solvent by rotary-evaporation on rotavapor (RII Buchi) and drying in lyophilizer. Then a portion of crude extract was subjected to a liquid-liquid partitioning with solvents: *n*-hexane and CH_2Cl_2. The compounds present on CH_2Cl_2 fraction (Dcm) were separated by chromatography using 10 g of sample on C_{18} cartridge (Strata, Phenomenex) under MeCN:H_2O gradient (from 1:9 to 100% MeCN), which generated seven fractions. Subfractions 3 to 6 were pooled (148.4 mg) and separated by semi-preparative HPLC (Waters Corporation, Milford, Massachusetts). The chromatographic separation was carried out on Inertsil ODS-SP C_{18} column (250 mm × 4.6 mm, 5 μm) in gradient elution of H_2O/MeOH/MeCN/CHO_2H (from 44:28:28:0.1 to 20:40:40:0.1) at 20 min with monitoring at 254 nm by UV detector. HPLC-MS analyses were performed using a system that utilized a Waters Alliance 2695 controller and pump, connected to Micromass Quattro LC triple-quadrupole detector with a high-flow electrospray ionization probe, operating in negative mode. The analyses were performed using a Waters ODS2 column (125 mm × 4.0 mm, 5 μm), with a 0.8 mL min^{-1} flow rate in gradient of H_2O/MeCN/MeOH, starting at 60:15:25 until 40:15:45 during 8 min, and then to 30:15:55 during 7 min. A post-column split was used to direct 0.25 mL min^{-1} of the effluent toward the ionization source. Nitrogen was used as the nebulizing (34 L h^{-1}) and desolvation gas (834 L h^{-1}). The ESI capillary probe voltage was set to 3.57 kV, and the sampling and extraction cones were set to 46 and 4 V, respectively.

Analyses by ^{1}H and ^{13}C NMR were recorded on Bruker DRX spectrometer operating either at 400 MHz (^{1}H) or 100 MHz (^{13}C), using samples diluted in $CDCl_3$. All chemicals were of analytical grade. MeOH and CHO_2H were purchased from Merck (Darmstadt, Germany). The isolated compound (diosmetin, Fig. 1) yielded 6.3 mg corresponding to 0.01% of the crude extract. As the amount of diosmetin isolated from this plant was just 6.3 mg, we obtained diosmetin from the company Sigma Aldrich Chemical Co. (St. Louis, USA) to perform the experiments.

2.2. Animals

In this study male albino Swiss mice (25–35 g) bred in-house were used. The animals were accommodated in a controlled temperature (22 ± 2 °C) with a 12-h light/dark cycle (lights on 6:00 a.m. to 6:00 p.m.). It was provided the laboratory standard animal's food (Puro Lab 22 PB pelleted form, Puro Trato, Rio Grande do Sul, Brazil) and tap water ad libitum. Animals were accommodated in ventilated cages (10 per cage) with wood shaving bedding and nesting material. The animals were taken away to get used to the experimental room for at least 1 h before each experience. Each animal was used in just one experiment between 7:00 a.m. and 5:00 p.m. The experiments reported in this study were realized according to the ethical guidelines to investigate pain in conscious animals [14], and they were approved by the Ethics Committee of the Universidade do Extremo Sul Catarinense (CEUA, protocols numbers 072-2014-01 and 010/2015-1). The behavioral studies followed the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines [15]. All experiments were performed by an operator blindly concerning drug administration or in vitro treatment. Also, experimenters were blinded to the experimental group when performing the analysis. No animal or sample was excluded from the study. The group size for each experiment was determined by sample size estimation for each test based on previous results obtained in our laboratory. For neuropathy and inflammatory pain model, and acute pain model caused by heat noxious stimuli, allocation concealment was not performed because we had allocated the animals in different groups to yield groups with similar basal values in the initial phase of the experiment. However, for the other tests, we have randomized the groups. Each experiment was repeated at least two to three times in different experimental days to complete the total number of animals required for each test, this was done to confirm our results with a distinct experimental group of animals in different experimental days.

2.3. Drug treatments for in vivo experiments

To determine the possible systemic antinociceptive effect of diosmetin the drug was used in animals by intragastric administration (0.0015–1.5 mg/kg, i.g.). We used these doses of diosmetin because in a previous study we tested the antinociceptive effect of the *Amphilophium crucigerum* seeds using a dose of 100 mg/kg of crude extract (i.g.) [3]. Thus, we selected to test lower doses of the isolated compound observing that crude extract possesses 0.01% of diosmetin. Diosmetin was dissolved in 1% dimethyl sulfoxide (DMSO) in hypotonic saline (0.9% NaCl). The TRPV1 antagonists AMG-9810 (or 2E-*N*-(2,3-dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-Propenamide) and SB-366791 (or *N*-3-methylphenyl 4-chlorocinamide) were diluted in the same vehicle and used i.g. If not specified all reagents were obtained from the company Sigma Aldrich Chemical Co. (St. Louis, USA). Morphine sulfate was acquired from Cristália (São Paulo, Brazil), and was diluted in the same vehicle (1% DMSO in hypotonic saline solution). Also, indomethacin was diluted in 1% DMSO in hypotonic saline solution.

2.4. Calcium (Ca^{2+}) influx assay

First, we assessed the diosmetin capacity to change calcium influx and after evaluated its ability to bind to the TRPV1 receptor. To observe the calcium influx promoted by the TRPV1 agonist (capsaicin) we used synaptosomes prepared from mice spinal cord [6,16]. Synaptosomes were prepared from mice spinal cord samples and incubated with Fura 2-AM (10 μM) for 30 min at 37 °C. The mixture was diluted to 1.5 ml (5 mg/mL of protein) with Krebs-Ringer medium (Ca^{2+} free) and incubated for 30 min at 37 °C. The reaction was stopped by centrifugation (30 s at 12,000 × *g*), and the product was resuspended in 1.5 mL Krebs-Ringer medium (Ca^{2+} free). After that, 1.5 μL of 1 M $CaCl_2$ (1 mM) plus different concentrations of diosmetin (0.0001–1 μM), SB-366791 (1 μM), eriodyctiol (a TRPV1 antagonist, 0,1 μM) or vehicle (0.1% DMSO) were added followed by the addition of capsaicin (20 μM) to start the reaction. The results were expressed as the percentage of capsaicin-induced Ca^{2+} influx as described previously [6,16]. Total protein was measured with Coomassie blue dye, and bovine serum albumin (BSA) was used as a standard [17].

2.5. [^{3}H]-resiniferatoxin binding assay

To evaluate the capacity of diosmetin to bind to the TRPV1 receptor, then we used the [^{3}H]-resiniferatoxin (RTX, a TRPV1 agonist) binding assay as described previously [6,16]. For that, mouse spinal cords were homogenized in buffer A (5 mM KCl, 5.8 mM NaCl, 2 mM $MgCl_2$, 0.75 mM $CaCl_2$, and 137 mM sucrose, pH 7.4) with 10 mM HEPES and centrifuged for 10 min at 1000 × *g* at 4 °C. Then the supernatant was centrifuged for 30 min at 16,000 × *g* at 4 °C. The resulting pellets were resuspended in buffer A and frozen until analysis. To start the experiment, the binding mixture containing buffer A with 0.25 mg/mL BSA, spinal cord membranes (0.5 mg/mL of protein), and [^{3}H]-RTX (2 nM) in the presence or absence of the diosmetin (0.01 μM), eriodyctiol (0.1 μM) or vehicle (0.1% DMSO) with a final volume of 500 μl. For the measurement of nonspecific binding 100 μM nonradioactive RTX was included in different tubes. The reaction was initiated by incubating the tubes at 37 °C for 60 min and stopped by transferring the tubes to an ice bath and adding 100 μg of bovine α_1-acid glycoprotein to allow the detection of specific binding. Finally, [^{3}H]-RTX in the bound and free membranes were separated by centrifugation for 30 min at 16,000 × *g* at 4 °C. Radioactivity in the pellet was quantified by scintillation. The pellets were suspended in 1 ml of scintillation fluid, and radioactivity was counted in a scintillator apparatus (Tri-Carb 2100TR; PerkinElmer Life and Analytical Sciences, Waltham, MA). Specific binding was calculated as the difference of the total and nonspecific binding, and the results are reported as a percentage of specific binding. Total protein was measured with Coomassie blue dye, and BSA was used as a standard [17].

2.6. Pain models

2.6.1. Capsaicin-induced acute nociception and paw edema test

To observe the effect of diosmetin in the capsaicin intraplantar test [18,19] animals were taken away to get used to the observation place, which consisted of a glass clamber, for at least 30 min before the experiment. Then, diosmetin (0.0015–1.5 mg/kg, i.g.), SB-366791 (3 mg/kg, i.g.), or vehicle (10 mL/kg DMSO 1% in saline 0.9%) were administered. Then, after 1 h of administration of compounds 20 μL of capsaicin (1 nmol/paw) was injected into the right hind paw intraplantar (i.pl.). Also, we determine the diosmetin (0.15 mg/kg, i.g.) effect in different time points (0.5, 1, 2, and 4 h) after injection. The time spent flinching and licking the injected paw after capsaicin i.pl. treatment was measured for 5 min as a nociception index. We also evaluated the paw edema that developed 15 min after capsaicin injection as described previously. Paw edema was described as the difference between the tested paw thickness and basal paw thickness using a digital caliper [3,20]. Capsaicin-induced nociception and edema were observed in the same group of animals. The dose of the TRPV1 antagonist (SB-366791) used was described in a previous study

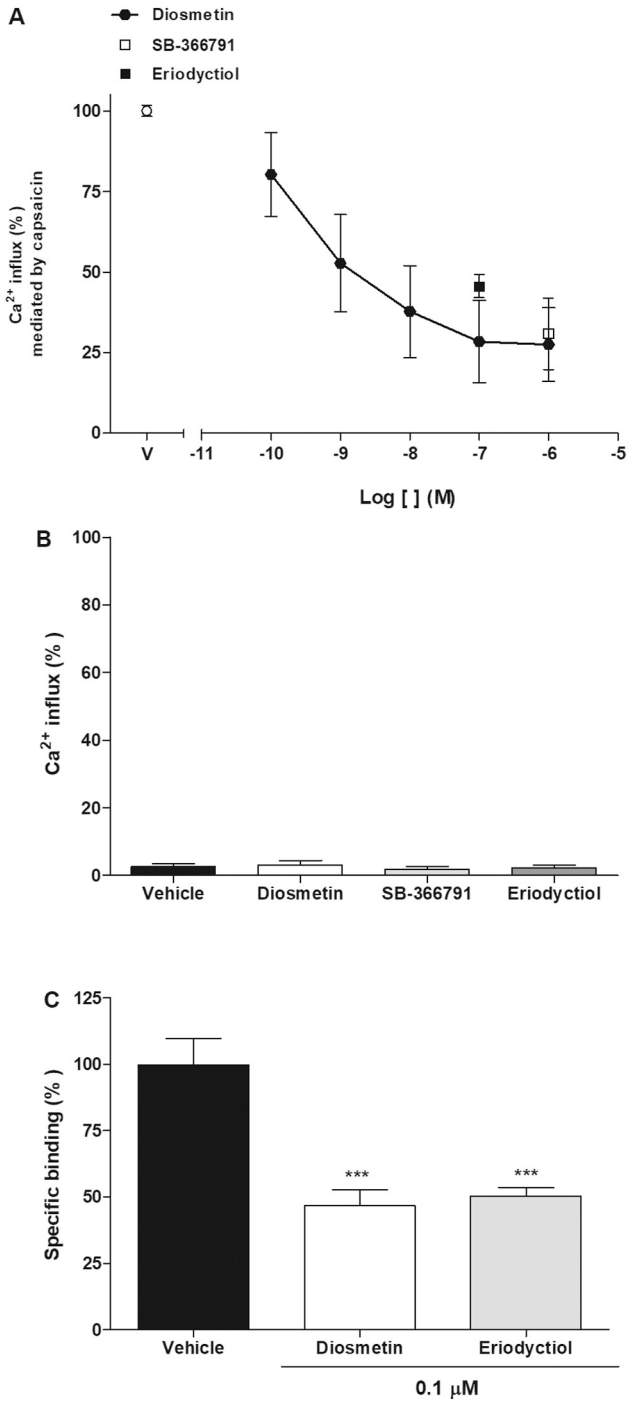

Fig. 2. The diosmetin functions as a TRPV1 antagonist. (A) Calcium influx caused by capsaicin (20 µM) in spinal cord synaptosomes of mice. The diosmetin was used at concentrations of 0.0001–1 µM, eriodyctiol (0.1 µM) and SB-366791 (1 µM) was used as controls. Vehicle (V, 1% DMSO in buffer) (n = 5–8). (B) Calcium influx was observed only in the presence of vehicle (1% DMSO in buffer), diosmetin (1 µM), eriodyctiol (0.1 µM) or SB-363791 (1 µM) (n = 5). (C) Specific binding assay on spinal cord membrane of mice using [^{3}H]-RTX (n = 5). The diosmetin (0.1 µM) and eriodyctiol (0.1 µM) were able to reduce specific binding. Data were plotted as mean ± S.E.M. (panel A) or mean + S.E.M. (panels B and C). ***P < 0.001 compared to vehicle (graph B and C, one-way ANOVA followed by Bonferroni post hoc test).

to induce antinociceptive and antiedematogenic effect in the capsaicin test, or antinociceptive effect on the other pain models used in this study [3].

2.6.2. Acute pain model caused by heat noxious stimuli

In this assay, the sensitivity of mice to a thermal stimulus was evaluated by observing the tail-flick response when mice tail was exposed to a 48 °C bath [3,21]. First, we determine 2 basal latencies before the test, the basal latency in the graph is an average of these values (one basal latency was measured on the day before the test, and the other on the day of testing, 7–9 s). Then, diosmetin, SB-366791 or vehicle was injected and at 0.5, 1, 2 and 4 h after the administration latency to noxious heat was determined again. The maximum latency for this test was 18 s to avoid tissue damage. The increase in time latency was considered as an antinociceptive effect.

2.6.3. Inflammatory pain model

To induce inflammatory thermal and mechanical hypersensitivity we used the CFA-induced inflammatory pain model [6,16]. For that, animals were anesthetized with halothane, and 20 µL of CFA (1 mg/mL suspension of heat-killed *Mycobacterium tuberculosis* in liquid paraffin) was injected into the right hind paw. Forty-eight hours later, the nociceptive tests were evaluated. Control animals were administered i.pl. with phosphate buffer solution (PBS, 50 mM, pH 7.4, 20 µl).

2.6.4. Mechanical allodynia measurement

Mechanical allodynia was calculated with the up-and-down method [6,16,22] using von Frey filaments with increasing intensity (0.02–4 g). Briefly, the animals were set in the experimental site, consisting of elevated chambers with metal screen floor, for 1 h. After this period, stimulation of the right hind paw of each animal with von Frey filaments was performed by the up-and-down method. The first filament used promoted a pressure of 0.6 g, if the paw was removed, a filament with a lower pressure was applied. When no withdrawal occurred, a filament with a higher pressure was used. In total, six measures were performed, using filaments of 0.02; 0.07; 0.16; 0.40; 0.60; 1.4; 2.0 and 4.0 g. With the results obtained, the value corresponding to 50% of the threshold, in grams, that each animal supports (threshold 50%) was calculated. A decrease in this value was considered as mechanical allodynia and a reversal in this fall as an antinociceptive (anti-allodynic) effect.

2.6.5. Thermal hyperalgesia

Thermal hyperalgesia was observed as previously described [6,16]. In this protocol, the right hind paw of animals was immersed in a bath, with the temperature of 48 °C and the time of reaction to the stimulus was timed (normal between 6 and 7 s). Baseline measurements of the threshold were made to the mechanical or thermal stimuli, then CFA or PBS was administered, and 48 h later, further measurements were taken. Also, after the administration of treatments (diosmetin, SB-366791 or vehicle), new measures were taken at different times points (0.5, 1, 2 and 4 h).

2.6.6. Neuropathic pain model

In this model, first mice were anesthetized by an intraperitoneal (i.p.) injection of 90 mg/kg off ketamine plus 3 mg/kg of xylazine hydrochloride. Neuropathy was promoted by of chronic constriction injury (CCI) of the sciatic nerve using a similar procedure previously described for rats [23] and adapted for mice [24]. Three loosely constrictive ligatures were placed around the right sciatic nerve under anesthesia. Sham surgery was performed in animals that were anesthetized, and the sciatic nerve was uncovered without performing constriction. Sham-operated animals were used as neuropathy control. Nociceptive tests were performed seven days after the procedures by the mechanical and thermal threshold were evaluated as described above.

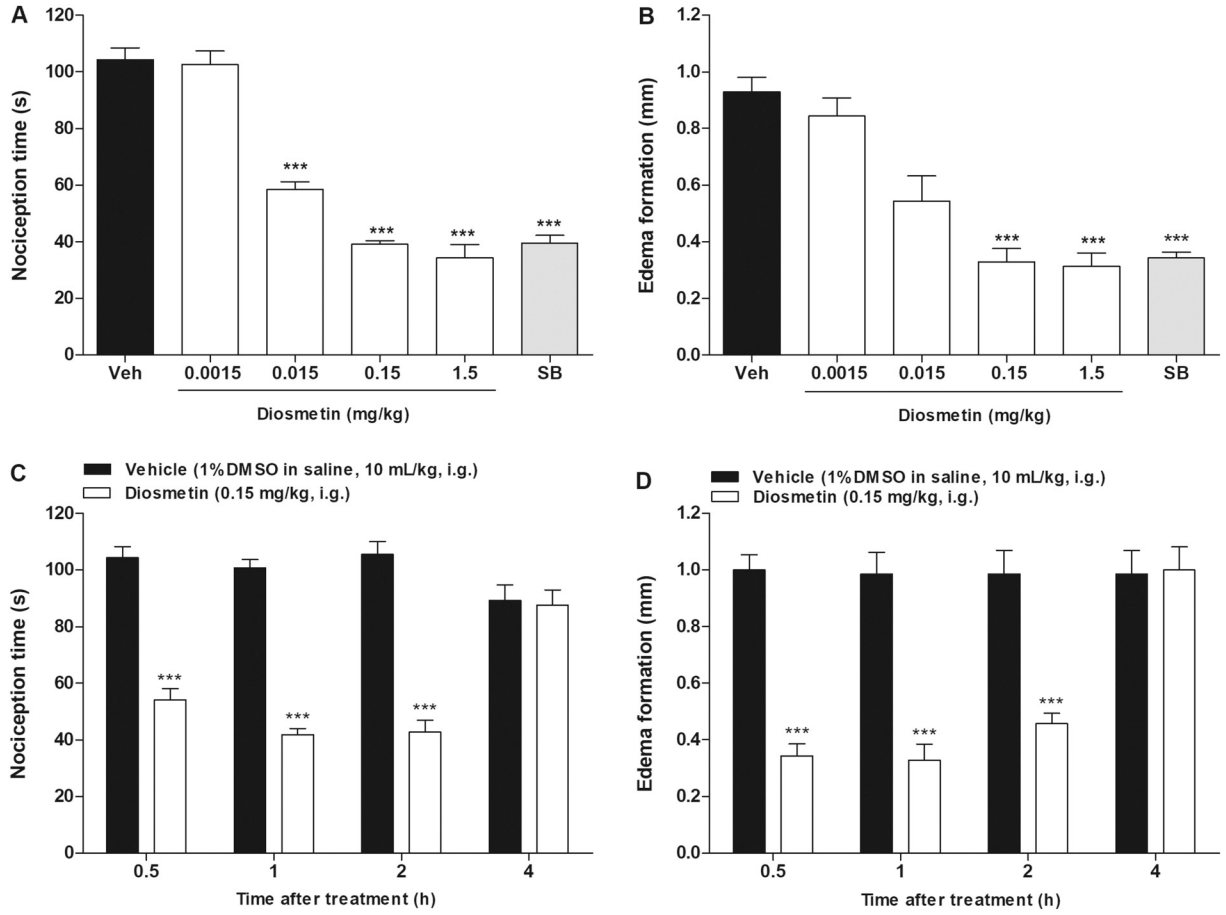

Fig. 3. The diosmetin caused antinociceptive and antiedematogenic effect on the acute pain model induced by the administration of a TRPV1 agonist (capsaicin, intraplantar) in mice. (A) Antinociceptive or (B) antiedematogenic effects of diosmetin at different doses (0.0015 to 1.5 mg/kg, i.g.) or SB-366791 (SB, 3 mg/kg, a selective antagonist of the TRPV1 receptor used as positive control) 1 h after the intragastric administration. Time curve for measurement of the effect of (C) the antinociceptive or (D) antiedematogenic of diosmetin (0.15 mg/kg) from 0.5 to 4 h after intragastric administration. Data were plotted as mean + S.E.M. (n = 7 animals). ***P < 0.001; when compared to the vehicle-treated group (graphs A and B, one-way ANOVA followed by Bonferroni post hoc test; C and D, two-way ANOVA followed Bonferroni post hoc pathways test).

2.7. Adverse effects assessment

2.7.1. Locomotor activity

To grade possible non-specific muscle relaxant or sedative effects of the diosmetin, mice were subjected to motor impairment evaluation [6,16]. We first inspected spontaneous motor coordination in the open-field test. The supplied consisted of a Plexiglas box measuring 40 cm × 60 cm × 50 cm, the floor of which was divided into 12 equal squares. The number of squares crossed with all paws was measured in a 5 minute session. Forced motor activity was also graded using the rotarod test. Twenty-four hours before the experiment, all animals were trained on the rotarod (3.7 cm in diameter; 8 rpm) until they could prevail on the apparatus for 60 s without falling. On the day of the experiment, animals were subjected to the rotarod test 1 h after the application of diosmetin or vehicle. The total number of falls that occurred over a 240 second period and the latency for the first fall from the apparatus was recorded.

2.7.2. Body temperature

Considering some TRPV1 ligands may inflict in body temperature, we researched the effect of diosmetin on body temperature [25]. First, the basal rectal temperature was determined; they were then given vehicle, diosmetin or AMG9810 (10 mg/kg) orally. New temperature measurements were taken at various time points following drug administration, and the difference between pre-injection and post-injection values was calculated as previously mentioned [6,16]. The dose of the TRPV1 antagonist (AMG9810, 10 mg/kg) used was described in a previous study to induce hyperthermia [3,6,26]. We tested this TRPV1 antagonist and not SB-366791, because we have not observed an increase in the body temperature caused by SB-366791 previously [6].

2.7.3. Biochemical markers assessment

For the determination of urea and creatinine levels and the activity of enzymes alanine transaminase (ALT) and aspartate transaminase (AST), the serum was withdrawn from the animals 6 h after administration of the treatments (diosmetin - 0.15 mg/kg or vehicle 10 mL/kg 1% DMSO in 0.9% saline, i.g.). Whole blood was withdrawn using an insulin syringe following administration with sodium thiopental (50 mg/kg, i.p.) and after, centrifuged for 5 min, 3000 ×*g*. For determination of urea and creatinine levels and enzyme activity ALT and AST, the following commercial kits were used: Urea - Enzymatic kinetic method by Biotechnical Kit, Creatinine - Kinetic method by the Labtest Kit, Transaminase AST (TGO) - Enzymatic kinetic method by the Bioclin Kit, Transaminase ALT (TGP) - Enzymatic kinetic method by the Bioclin kit. Dosed by the Cobas MIRA® automated system (Roche Diagnostics, Basel, Switzerland).

2.7.4. Gastrointestinal transit

The possible effect of diosmetin (0.15 mg/kg) on gastrointestinal transit was also tested. In this study, the mice were fasted for 16 h (water ad libitum) before analysis of gastrointestinal transit, as previously described [20]. After, animals were treated with diosmetin

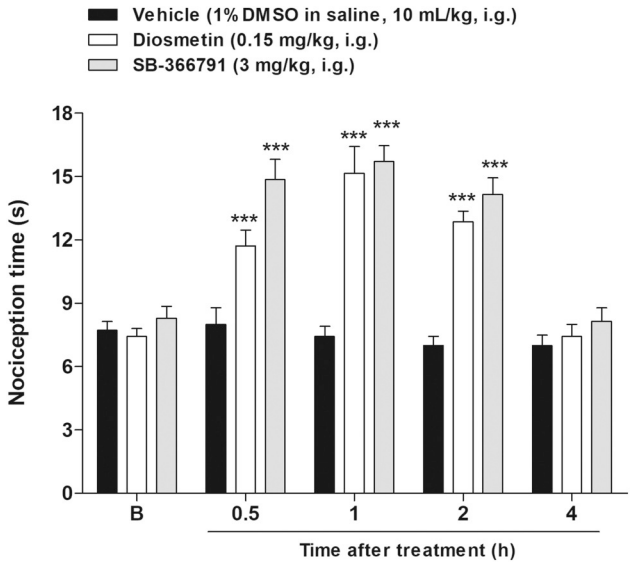

Fig. 4. Diosmetin showed antinociceptive effect in an acute pain model caused by heat exposure in mice. Time-curve for measurement of antinociceptive effect of diosmetin (0.15 mg/kg) or SB-366791 (3 mg/kg) from 0.5 to 4 h after intragastric administration. Basal measurement (B, 7–9 s) was performed before administration. Data were plotted as mean + S.E.M. (n = 7 animals). ***P < 0.001 compared to vehicle-treated group (two-way ANOVA followed by the Bonferroni post hoc test).

(0.15 mg/kg), morphine (10 mg/kg, positive control test) or vehicle (10 mL/kg, 1% DMSO in 0.9% saline, i.g.) and 40 min later 0.3 mL of a standard activated charcoal meal (5% activated charcoal, 20% Arabic gum i.g.) was administered. Twenty minutes after administration of the activated carbon blend, the animals were sacrificed, and their stomachs and small and large intestines were removed to measure the length of the intestine (from the pyloric sphincter to the ileocecal junctions, the entire length of the intestine) and the distance covered by activated charcoal in the gastrointestinal tract. The propulsive activity of the intestine was determined by the percentage of charcoal in the gastrointestinal tract, calculated as % distance traveled = 100 × (distance traveled by coal / total length of intestine). The dose of the morphine (10 mg/kg) used was described in a previous study to reduce gastrointestinal transit [3].

2.7.5. Ulcerogenic activity

To evaluate the effect on the gastric mucosa of the different treatments the animals were euthanized 6 h after the administration of diosmetin (0.15 mg/kg), indomethacin (100 mg/kg, test positive control) or vehicle (10 mL/kg, 1% DMSO in 0.9% saline, i.g.) and their stomach was removed for evaluation of the mucosa. The lesions were analyzed with the support of a magnifying glass. The lesions were quantified according to the number of lesions and the size using a scale of 0 to 5 points: (0) without damage, (1) color modification, (2) few petechiae and villous changes, (3) 1–3 small lesions (< 1 mm in length), (3) 1–3 large lesions (> 1 mm), (4), > 3 small lesions, (5) > 3 large lesions [27,28]. The dose of indomethacin (100 mg/kg) used was described in a previous study to reduce gastrointestinal damage [3].

2.8. Statistical analysis

The results are conferred as the mean + S.E.M., except for the effective dose 50% values (ED_{50}) and 50% inhibitory concentration (IC_{50}) values, which are announced as geometric means accompanied by their respective 95% confidence limits. The ED_{50} and IC_{50} values were established by nonlinear regression analyses with a sigmoid dose-response equation using GraphPad 5.0 (GraphPad Software, Inc., San Diego, CA). The percentages of maximal inhibition (I_{max}) are reported as the mean ± S.E.M. or mean + S.E.M. of inhibition obtained in each individual experiment in relation to the control values (vehicle for the in vivo results, 100% specific binding for the [^{3}H]-RTX binding assay, and 100% response obtained for capsaicin for the Ca^{2+} influx assay). The significance level was set at $P < 0.05$. Data were considered by using Student's *t*-test, or one-way analysis of variance (ANOVA) and two-way ANOVA followed by Bonferroni's post hoc test.

3. Results

3.1. Diosmetin functions as a TRPV1 antagonist in calcium influx and binding assays

To observe the effect of diosmetin on TRPV1 receptor, we initially performed an in vitro assay to detect the calcium influx mediated by a TRPV1 agonist (capsaicin) and the specific binding test using [^{3}H]-RTX. First, it was identified that the diosmetin reduced calcium influx mediated by capsaicin in a concentration-dependent manner (Fig. 2A) and the positive controls eriodyctiol and SB-366791 also showed inhibitory effect. A maximal inhibition percentage of 73 ± 11% were detected (at concentration of 1 μM) for diosmetin, with a calculated value of IC_{50} of 2.7 nM (confidence interval 2.3 to 27 nM). SB-366791 (1 μM) and eriodyctiol (0.1 M) were also able to reduce the calcium influx mediated by capsaicin (inhibition of 69 ± 11 and 54 ± 4%, respectively). However, there was no calcium influx mediated only by diosmetin (1 μM), eriodyctiol (0.1 μM) or compound SB-366791 (1 μM) (Fig. 2B). Also, it was observed that diosmetin (0.1 μM), and eriodyctiol (0.1 μM) reduced the specific binding to [^{3}H]-RTX in the membranes of the spinal cords of mice, and calculated values of inhibition were 47 ± 5 and 50 ± 3%, respectively (Fig. 2C) [$F(2, 12) = 18.72$, $P < 0.001$; Fig. 2C].

3.2. The diosmetin reduced nociception and edema induced by intraplantar administration of a TRPV1 agonist in mice

The intragastric administration of diosmetin at different doses showed an antinociceptive [$F(5, 36) = 80.34$, $P < 0.001$; Fig. 3A] and antiedematogenic [$F(5, 36) = 22.91$, $P < 0.001$; Fig. 3B] effect after 1 h of the treatment in the acute pain model caused by administration of capsaicin a selective TRPV1 agonist. The value of maximum inhibition was 67% ± 5 and 65 ± 5% at the dose of 1.5 mg/kg for antinociceptive and antiedematogenic effects, respectively (Fig. 3A and B). The calculated ED_{50} values were 0.12 mg/kg (with a confidence interval of 0.05–0.16 mg/kg) and 0.17 mg/kg (with a confidence interval from 0.07 to 0.43 mg/kg), for the antinociceptive and antiedematogenic effects, respectively. The compound SB-366791 were used as a positive control test and had antinociceptive (62 ± 3% inhibition) and antiedematogenic effects (63 ± 2% inhibition) at 1 h after the treatment (Fig. 3A and B). Also, the antinociceptive effect of diosmetin was tested at different times after administration, and this compound showed the antinociceptive [$F(1, 36) = 22.91$, $P < 0.001$; Fig. 3C] and antiedematgenic [$F(1, 36) = 12.58$, $P < 0.001$; Fig. 3D] effects from 0.5 to 2 h after the treatment, inhibition values of 58 ± 2 and 67 ± 6% at 1 h after the treatment, respectively (Fig. 3C and D). We choose to test only one dose of diosmetin (0.15 mg/kg) in the other pain models to reduce the number of animals used in this research project, and this dose presented an inhibition value similar to 1.5 mg/kg in the capsaicin test.

3.3. The diosmetin showed antinociceptive effect in an acute pain model caused by exposure to noxious heat

The intragastric administration of diosmetin produced an increase in the tail flick latency to noxious heat 0.5 to 2 h after treatment (inhibition of 84 ± 7%, 1 h after the treatment) [$F(1, 72) = 9.53$,

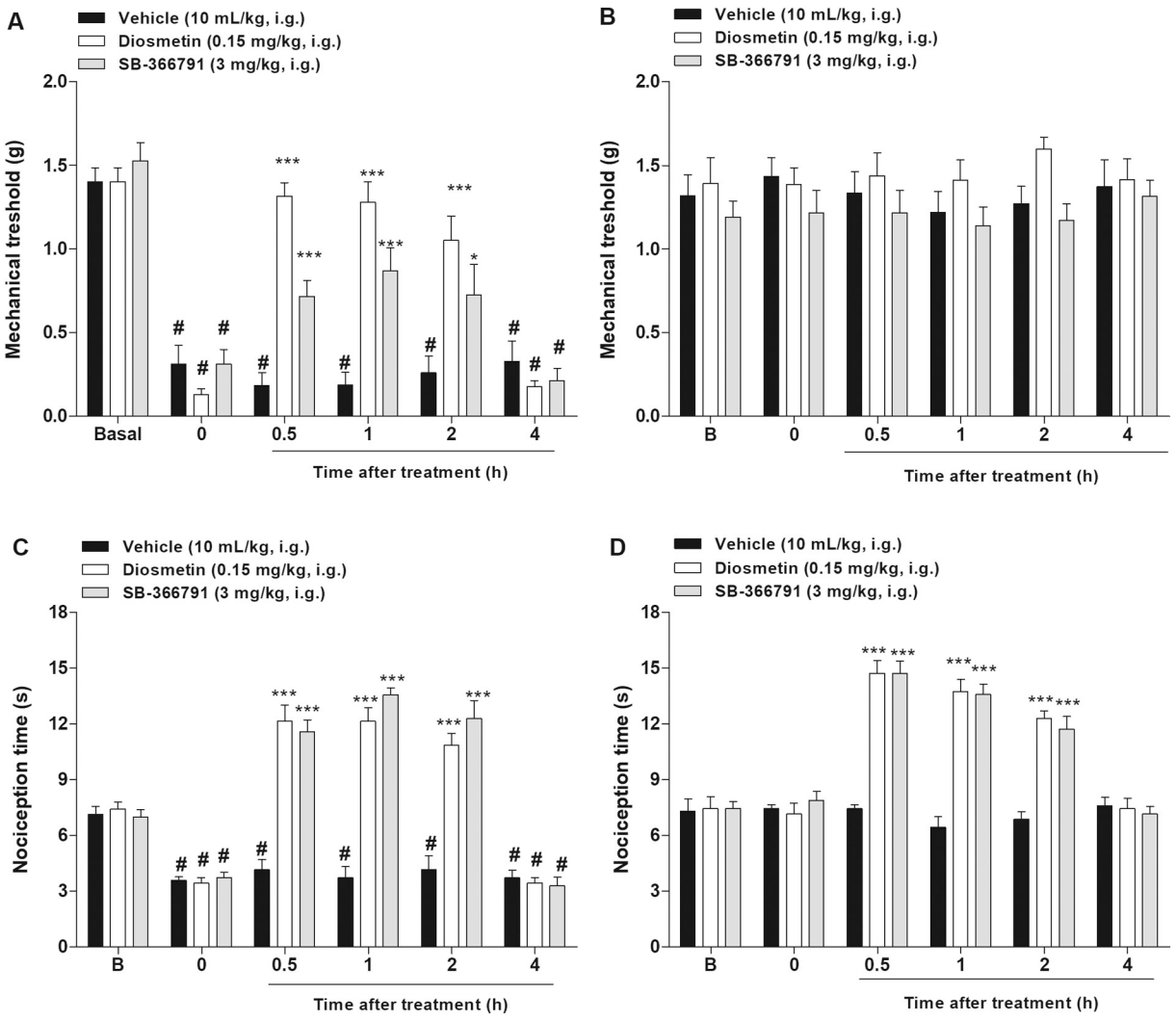

Fig. 5. Diosmetin administration induced antiallodynic and antihyperalgesic effect in an inflammatory pain model induced by administration of complete Freund's adjuvant (CFA) in mice. (A) Antiallodynic effect against mechanical stimulation or (C) antihyperalgesic effect against noxious heat stimulus of diosmetin (0.15 mg/kg) evaluated 0.5 to 4 h after intragastric administration (i.g.). Diosmetin effect on (B) threshold to mechanical stimulation or (D) latency to thermal stimulus in control animals. SB-366791 (3 mg/kg, a selective antagonist of TRPV1 receptor was used as a positive control). Baseline measurements were represented as B on the graph, and 0 represents the time measurements performed 48 h after the administration of CFA or PBS i.pl. in mice. Data were plotted as mean + S.E.M. (n = 7 animals). $^{\#}P < 0.001$ compared to baseline; $^{*}P < 0.05$; $^{***}P < 0.001$ compared to vehicle-treated group (two-way ANOVA followed by the Bonferroni post hoc test).

$P < 0.001$; Fig. 4]. The compound SB-366791 was used as positive control test, and exhibited antinociceptive effect 0.5 to 2 h after the treatment during the tail flick test to the thermal stimulus, with maximal inhibition values of 87 ± 4%, 1 h after the treatment.

3.4. Diosmetin administration reduced the mechanical allodynia and heat hyperalgesia in an inflammatory model of pain

Administration of diosmetin and SB-366791 induced antiallodynic and antihyperalgesic effects in an inflammatory pain model caused by the administration of CFA in mice. The compounds possess antinociceptive effects on 0.5, 1 and 2 h after treatment. The inhibition values for the observed effect on mechanical stimulation were 98 ± 6 and 32 ± 8% for diosmetin (calculated 0.5 after treatment) or the compound SB-366791 (calculated 1 h after treatment) respectively, compared to the vehicle-treated group [$F(1, 90) = 8.70$, $P < 0.001$; Fig. 5A]. Animals that received the administration of PBS on the paw (i.pl., control group) and later were treated with diosmetin or SB-366791 showed no change of the threshold to mechanical stimulation, showing that the treatments did not influence the perception to this form of stimulus (Fig. 5B). The antihyperalgesic properties, against thermal stimulus of diosmetin or SB-366791 on the inflammatory pain model, produced 100% inhibition for all treatments compared with the group treated with vehicle [$F(1, 90) = 23.59$, $P < 0.001$; Fig. 5C]. But when the diosmetin or SB-366791 were administered to the control animals (PBS, i.pl.), a per se effect was obtained [$F(1, 90) = 16.59$, $P < 0.001$; Fig. 5D], since for all treatments there was an increase of the latency to a thermal stimulus.

3.5. Diosmetin produced antiallodynic and antihyperalgesic effect in a neuropathic pain model

The induction of CCI of the sciatic nerve caused mechanical allodynia and heat hyperalgesia, and the administration of diosmetin induced antinociception effect from 0.5 to 2 h after administration (Fig. 6A and C). The treatment with diosmetin induced inhibition of 90 ± 21 and 100% for mechanical allodynia and heat hyperalgesia caused by CCI in mice 1 h after the treatment, respectively [$F(1, 90) = 4.64$, $P < 0.001$; Fig. 6A] or [$F(1, 90) = 30.45$, $P < 0.001$; Fig. 6C]. The control group (non-operated) exposed to mechanical

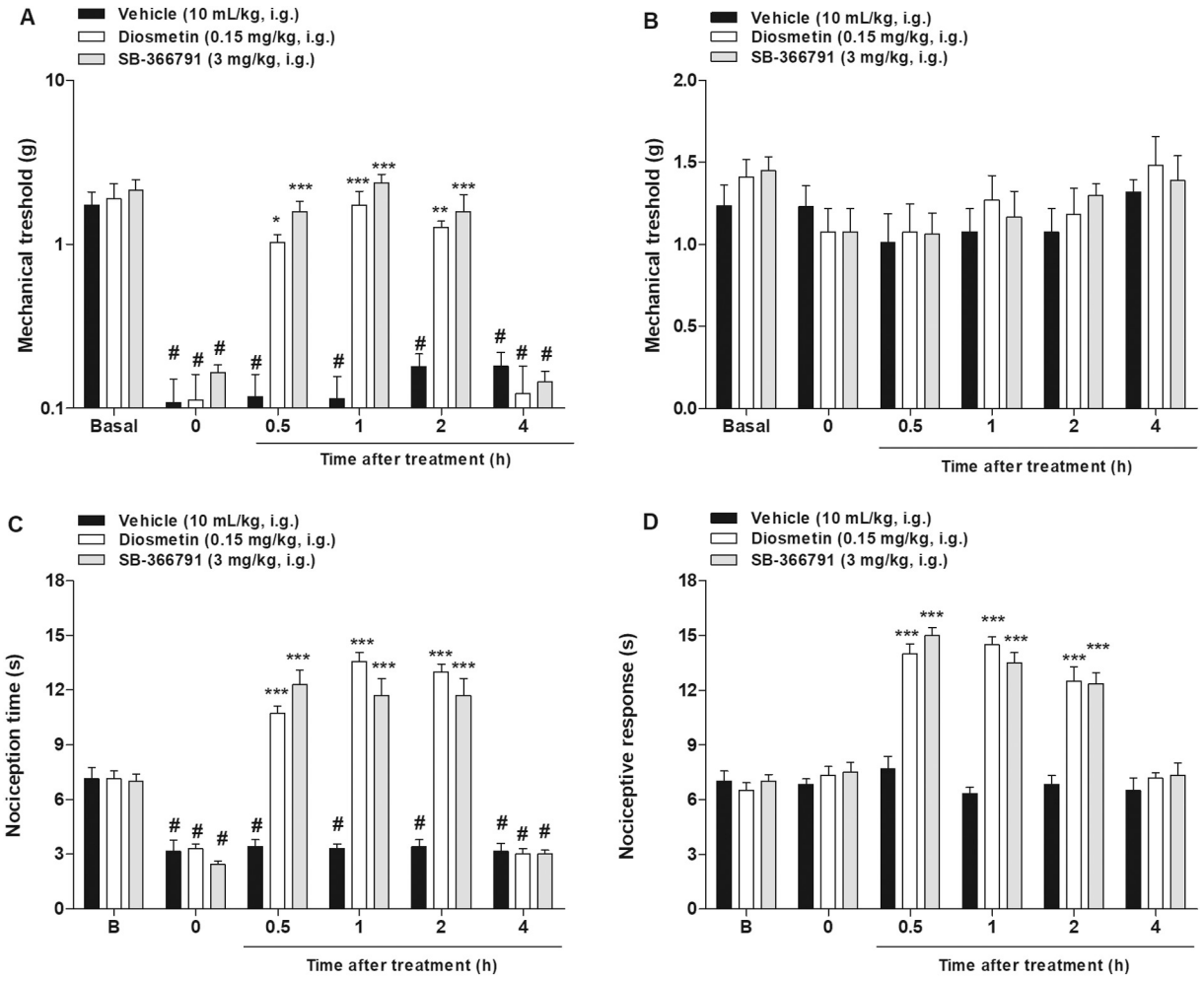

Fig. 6. The administration of diosmetin caused antiallodynic and antihyperalgesic effect in the neuropathic pain model induced by chronic constriction of the sciatic nerve (CCI) in mice. Evaluation of (A) antiallodynic effect against mechanical stimulation or (C) antihyperalgesic effect against noxious thermal stimulus of diosmetin (0.15 mg/kg) evaluated at 0.5 to 4 h after intragastric administration (i.g.) (n = 7 animals). Evaluation of diosmetin effect under (B) threshold to mechanical stimulation and (D) latency to thermal stimulus of control animals. SB-366791 (3 mg/kg, a selective antagonist of TRPV1 receptor was used as a positive control) (n = 6 animals). Nociceptive tests were performed 7 days after the induction of CCI, as control false operated animals were used. Baseline measurements were represented as B in the chart, and the time 0 represents the measurements 7 days after induction of the CCI or not (control animals, false operated). Data were represented as mean + S.E.M. $^{\#}P < 0.001$, when compared to baseline; $^{*}P < 0.05$, $^{***}P < 0.001$, when compared to the vehicle-treated group (two-way ANOVA followed by the Bonferroni post hoc test).

Table 1
Assessment of locomotor activity.

Treatment (i.g.)	Open field		Rotarod	
	Crossings	Rearings	Number of falls	Latency for the 1st fall
Vehicle (10 ml/kg)	70 ± 6	29 ± 3	0.3 ± 0.2	223 ± 17
Diosmetin (0.15 mg/kg)	70 ± 12	23 ± 3	0.1 ± 0.1	214 ± 26

The spontaneous locomotor activity (open field) and forced (rotarod) observed 1 h after administration of diosmetin (0.15 mg/kg, i.g.), or vehicle (1% DMSO in 0.9% NaCl; 10 ml/kg, i.g.). No significant differences were observed between the groups; Student "*t*" test. The results were expressed as mean ± S.E.M. (n = 7).

Table 2
Evaluation of body temperature.

Treatment (i.g.)	Body temperature (°C)	
	Basal	1 h after treatment
Vehicle (10 ml/kg)	36.2 ± 0.1	36.8 ± 0.1
Diosmetin (0.15 mg/kg)	36.0 ± 0.3	35.7 ± 0.3
AMG-9810 (10 mg/kg)	35.9 ± 0.2	37.2 ± 0.1*

The body temperature was measured 1 h after administration of diosmetin (0.15 mg/kg, i.g.), AMG-9810 (10 mg/kg, i.g.) or vehicle (1% DMSO in 0.9% NaCl; 10 ml/kg, i.g.). The results were expressed as mean ± S.E.M. (n = 7). $^{*}P < 0.05$; One-way ANOVA followed by post hoc Bonferroni, when compared to basal values.

stimulation and administered with diosmetin showed no change in detection of mechanical stimuli (Fig. 6B). However, the control group with diosmetin showed per se effect (increased latency time) for thermal stimulation [$F(1, 75) = 17.09$, $P < 0.001$; Fig. 6D]. The compound SB-366791 was used as positive control of the test and showed antiallodynic and antihyperalgesic effect 0.5 to 2 h after treatment, with inhibitions of 100 and 100% for the mechanical allodynia and heat hyperalgesia 1 h after the heat treatment, respectively (Fig. 6A and C). The treatment with SB-366791 did not alter the sensitivity of the control animals about mechanical stimuli (Fig. 6B), but this compound increased latency in non-operated animals to noxious thermal stimuli (Fig. 6D).

Table 3
Evaluation of the activity of liver enzymes (ALT and AST) and levels urea and creatinine in serum.

Treatment (i.g.)	Liver enzymes (U/L)		Renal function (mg/dL)	
	ALT	AST	Urea	Creatinine
Vehicle (10 ml/kg)	48 ± 10	26 ± 9	69 ± 1	8 ± 2
Diosmetin (0,15 mg/kg)	43 ± 5	36 ± 7	70 ± 6	12 ± 3

Effect of administration of diosmetin (0.15 mg/kg, i.g.) or vehicle (1% DMSO in 0.9% NaCl, 10 ml/kg, i.g.) on the activity of liver enzymes ALT and AST and levels urea and creatinine in the serum 6 h after administration. No significant differences were observed between the groups; one-way ANOVA followed by post hoc Bonferroni. The results were expressed as mean ± S.E.M. (n = 6).

3.6. The administration of diosmetin did not change the locomotion or body temperature

Diosmetin did not alter spontaneous or forced locomotion evaluated by the open field or rotarod tests, respectively (Table 1) when compared with vehicle-injected animals 1 h after administration.

Also, the treatment with diosmetin did not induce hyperthermia 1 h after intragastric administration. However, the administration of TRPV1 antagonist AMG-9810 induced a significant increase in rectal temperature 1 h after treatment [$F(5, 36) = 7.49$, $P < 0.001$; Table 2].

3.7. Effect of diosmetin administration on liver or kidney damage parameters

The diosmetin treatment did not induce any change in levels of creatinine or urea in the serum (an indicator of kidney damage) or the activity of enzymes ALT and AST in the serum of mice (liver damage parameters) (Table 3).

3.8. Diosmetin administration did not modify gastrointestinal transit or cause ulcerogenic activity

The treatment with diosmetin did not induce alteration of gastrointestinal transit (Fig. 7A). However, the administration of morphine caused significant reduction of gastrointestinal transit 1 h after treatment [$F(2, 15) = 31.83$, $P < 0.001$; Fig. 7A]. Also, administration of diosmetin did not produce ulcerogenic activity, while indomethacin increased markedly the appearance of gastric lesions (Fig. 7B) 6 h after the treatment [$F(2, 18) = 36$, $P < 0.001$; Fig. 7B].

4. Discussion

In the search for new TRPV1 antagonists the use of in vitro tests has great relevance, therefore, in vitro tests were performed to evaluate the ability of diosmetin to act as a TRPV1 antagonist, because this compound has a chemical structure like eriodyctiol, a flavonoid already described as an antagonist of this channel [6]. Initially, it was detected that diosmetin was able to reduce the calcium influx mediated by the TRPV1 agonist and presented a considerable IC_{50} value when compared to eriodyctiol, values of 2.7 and 44 nM, respectively [6]. Also, two positive controls, eriodyctiol, and SB-366791 showed similar inhibition values to those already described in the literature [6,16], and those calculated values for diosmetin. However, this assay cannot show that this compound can bind directly to the TRPV1 channel, so it was interesting to evaluate the effect of diosmetin on the specific binding of [^{3}H]-RTX. Through the specific binding assay, it was possible to observe that diosmetin can displace the binding of [^{3}H]-RTX in fractions of the spinal cord of mice, and it showed an inhibition value like the other compounds, such as α-spinasterol [16]. Also, the inhibition calculated for eriodyctiol in this study was comparable to that evaluated previously [6]. Thus, after obtaining the results of the in vitro assays, it is possible to suppose that diosmetin, as well as eriodyctiol and α-spinasterol [6,16], function as TRPV1 antagonists.

Knowing that diosmetin interacts with the TRPV1 receptor, evaluating its antinociceptive activity in different pain models becomes relevant. Capsaicin was the first agonist of the TRPV1 receptor to be described and it has been shown that subcutaneous administration of this vanilloid compound causes subcutaneous pain and edema in humans and animals [9,10,16,19,29,30]. Activation of the TRPV1 receptor generates the influx of cations, such as calcium, and this is important to give rise to action potentials for the perception of nociception. Also, with the activation of this receptor, the release of neuropeptides, such as substance P and the peptide related to the calcitonin gene (CGRP) may induce neurogenic inflammation [31–33]. It has previously been described that TRPV1 antagonists can reduce both nociception and edema caused by intraplantar administration of capsaicin [3,16]. It is worth noting that other compounds, such as opioids or NSAIDs, do not reduce intraplantar edema, only reduce nociception, indicating that there is no direct interaction with the TRPV1 receptor [18,34]. Intragastric administration of diosmetin reduced nociception and edema caused by capsaicin. When compared to other TRPV1 antagonists, diosmetin presented an ED_{50} value that was estimable for both antinociceptive and antidematogenic effects [6,16]. This finding is relevant for noting that a lower dose of the compound would be required to elicit analgesic effects, and thus it would not be necessary to

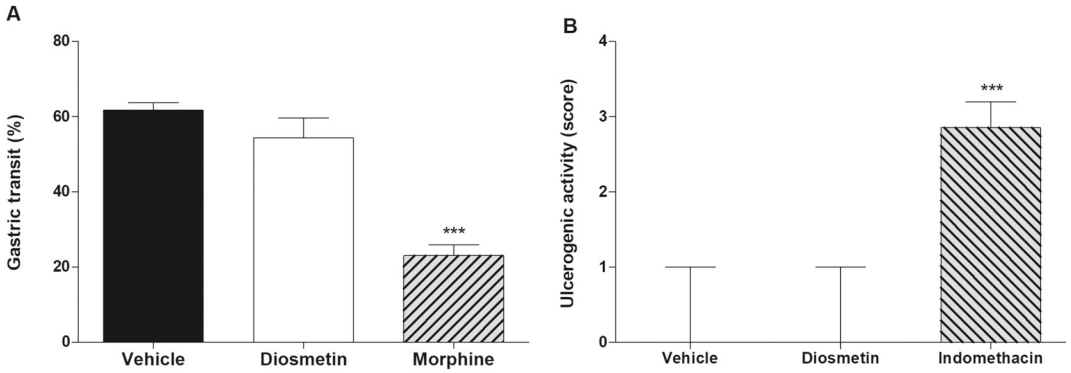

Fig. 7. The administration of diosmetin did not induce changes in gastrointestinal transit or ulcerogenic activity. (A) Evaluation of gastrointestinal transit after administration (1 h) of diosmetin (0.15 mg/kg, i.g.), morphine (10 mg/kg, i.g.) or vehicle (1% DMSO in 0.9% NaCl, i.g.). The gastrointestinal transit was expressed in % regarding the distance the mixture containing activated charcoal (5% activated charcoal, 20% Arabic gum, 0.3 ml) (n = 6 animals). (B) Determination of ulcerogenic activity caused by the administration of diosmetin, indomethacin (100 mg/kg, i.g.) or vehicle (1% DMSO in 0.9% NaCl, i.g.). The ulcerogenic activity was expressed in scores of gastric lesions (0 - no injury, 5 - maximum score of gastric lesions) (n = 7 animals). Data were expressed as mean + S.E.M. ***$P < 0.001$ when compared to the vehicle-treated group (one-way ANOVA of followed by Bonferroni post hoc test).

administer high doses which could be associated with adverse effects.

The involvement in detecting noxious temperatures beyond 43 °C is a characteristic of the TRPV1 receptor. Thus this channel is described as a thermoreceptor of sensorial afferents [9,35], because it can when activated protect the body from high temperatures that could lead to tissue damage. The diosmetin administration, as well as SB-366791, caused an increase in tail-withdrawal latency at a temperature of 48 °C, this result shows that these compounds can alter the perception of noxious heat, similarly to other TRPV1 antagonists [6,16].

Preclinical inflammatory pain models, such as that observed after administration of inflammatory substances (CFA) lead to mechanical allodynia development and thermal hyperalgesia at the site of administration [36–38]. This model can be considered as a way to mimic symptoms observed in the clinic in the case of tissue inflammation, where symptoms of allodynia and mechanical allodynia are observed in the patients [39,40]. In this study, it was observed that intragastric administration of diosmetin caused an antinociceptive effect in a model of inflammatory pain caused by intraplantar administration of CFA, either after thermal or mechanical stimulation. The positive control SB-366791 also showed antinociceptive effects, so it seems that the activation of TRPV1 receptor is relevant in this model of pain, as previously addressed [6,16,41–43].

The involvement of TRPV1 in inflammatory pain is explored because this channel can be sensitized by different inflammatory mediators produced after tissue injury, which leads to the possibility that the antagonism of this receptor is relevant to reduce the pain observed in the inflammatory process [44,45]. Intragastric administration of diosmetin was not able to alter the perception of mechanical stimuli in animals of the control group (administered with intraplantar PBS), a result which is expected for a TRPV1 antagonist [46]. The SB-366791 compound also did not cause alteration of the detection of mechanical stimuli; as described previously [16].

It was also observed that diosmetin reduced mechanical allodynia and thermal hyperalgesia in a neuropathic pain model caused by CCI, a model commonly used to test new compounds for the treatment of neuropathic pain [23,24,47,48]. The result obtained for diosmetin agrees with the indication for popular use of the plant *Amphilophium crucigerum*, in neuralgias, and also with the effects of the crude extract and the dichloromethane fraction on the neuropathic pain model in mice [3]. Also, TRPV1 antagonists reduce hypersensitivity in a the CCI model of neuropathy pain CCI [49]. In this study, it was found that SB-366791 diminished mechanical allodynia and thermal hyperalgesia observed after CCI, corroborating with those data in the literature, that TRPV1 antagonists may have antinociceptive effects in the neuropathy model caused by CCI [50,51].

A large reserve of polysaccharides and oils are present in *Amphilophium crucigerum* seeds since the seeds are known to be part of the plant that accumulates nutritive reserves [3,52]. Thus, the low yield of diosmetin about the crude extract (0.01%) is justifiable, since most of the extract is composed of sugars. Also, from the results obtained previously, it is possible to observe that the crude seed extract is formed of a small amount of different metabolites, but diosmetin is one of the major metabolites detected in the dichloromethane fraction by electrospray ionization mass spectrometry [3]. Then, as we observed that the dose of 0.015 mg/kg induced antinociceptive effect the capsaicin test, and the dose of 100 mg/kg of crude extract also induced effect in the same assay it is possible that *Amphilophium crucigerum* activity is due partially to diosmetin action, observing that 100 mg of crude extract may have in its composition at least 0.01 mg of diosmetin. However, as the dose of diosmetin that induced a more significant effect was the 0.15 mg/kg the antinociceptive effect of *Amphilophium crucigerum* could be also caused by other bioactive compounds, such as hesperitin. Previously it was described that hesperitin showed an antinociceptive effect in neuropathic and inflammatory pain models [53,54], and can antagonize the TRPM3 channel [55].

After observing the antinociceptive effect and mechanism of action of diosmetin, we also evaluated some adverse effects commonly found after the administration of analgesics available in the clinic, including opioids (such as morphine) and NSAIDs (including indomethacin), such as the development of constipation or gastric damage, respectively [56–59]. However, acute administration of diosmetin did not alter the gastrointestinal transit or induced ulcerogenic activity, effects that are also not induced by TRPV1 antagonists [25]. However, positive controls of the tests caused a reduction in gastrointestinal transit (morphine) or acute gastric mucosal injury (indomethacin).

Also, several tests were performed that required the animals to be able to perform adequate locomotion, and it was investigated whether the administration of diosmetin could cause sedation or locomotive alterations [57,60]. After the administration of diosmetin, there was no change in forced or spontaneous locomotion, evaluated in rotarod or in the open field tests, respectively. Thus, this compound may not be altering the activity of the animals in the acute or chronic pain tests. The main adverse effect indicated for the TRPV1 antagonists was hyperthermia in both animals and humans. This was the main motive that impaired the possibility of these compounds entering for clinical use [25]. Thus, the possible change in body temperature was also evaluated for diosmetin, which did not show hyperthemia induction, unlike the AMG-9810, a TRPV1 antagonist, as previously published [16]. Moreover, diosmetin did not cause changes in creatinine or urea levels or change the activity of ALT and AST enzymes.

Our results showed that diosmetin possess antinociceptive effect in different pain models in mice, and we also detected the capacity of this compound to antagonize the TRPV1 receptor without showing any detectable adverse effect studied. However, more studies are needed to use this compound as an analgesic agent, especially it is interesting to investigate the diosmetin effect after repeated administration in models of chronic pain. These results are interesting, because diosmetin is present in citrus fruits and plants and possess anti-inflammatory and antioxidant effects [1,2,4]. Moreover, as diosmin is already used in the clinic on chronic venous insufficiency treatment [5] and diosmetin is a bioactive metabolite of diosmin, it is possible that also diosmetin could produce analgesic effect in humans as showed for diosmin [61,62]. Finally, diosmetin may act as a TRPV1 antagonist to induce antinociceptive effect in mice.

Acknowledgements

Fellowships from the Conselho Nacional de Desenvolvimento Científico (CNPq) and CAPES are also acknowledged.

Funding

This work was supported Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, protocol number 422376/2016-7).

Declarations of interest

The authors declare no conflict of interest.

References

[1] K. Patel, M. Gadewar, V. Tahilyani, D.K. Patel, A review on pharmacological and analytical aspects of diosmetin: a concise report, Chin. J. Integr. Med. 19 (2013) 792–800, https://doi.org/10.1007/s11655-013-1595-3.
[2] Y. Yang, X. Gong, L. Huang, Z. Wang, R. Wan, P. Zhang, Q.-Y. Zhang, Z. Chen, B.-S. Zhang, Diosmetin exerts anti-oxidative, anti-inflammatory and anti-apoptotic effects to protect against endotoxin-induced acute hepatic failure in mice, Oncotarget 8 (2017) 30723–30733, https://doi.org/10.18632/oncotarget.15413.
[3] S.D.T. De Prá, P.R. Ferro, A.M. Milioli, F.K. Rigo, O.J. Chipindo, G. Trevisan, C. Camponogara, S.M. de Oliveira, R. Casoti, R. Casoti, M.P. Manfron, J. Ferreira, G. Trevisan, Antinociceptive activity and mechanism of action of hydroalcoholic extract and dichloromethane fraction of *Amphilophium crucigerum* seeds in mice, J. Ethnopharmacol. 195 (2017) 283–297, https://doi.org/10.1016/j.jep.2016.11.032.
[4] K. Shanmugam, L. Holmquist, M. Steele, G. Stuchbury, K. Berbaum, O. Schulz, O. Benavente García, J. Castillo, J. Burnell, V. Garcia Rivas, G. Dobson, G. Münch,

Plant-derived polyphenols attenuate lipopolysaccharide-induced nitric oxide and tumour necrosis factor production in murine microglia and macrophages, Mol. Nutr. Food Res. 52 (2008) 427–438, https://doi.org/10.1002/mnfr.200700180.
[5] D. Sawmiller, A. Habib, S. Li, D. Darlington, H. Hou, J. Tian, R.D. Shytle, A. Smith, B. Giunta, T. Mori, J. Tan, Diosmin reduces cerebral Aβ levels, tau hyperphosphorylation, neuroinflammation, and cognitive impairment in the 3xTg-AD mice, J. Neuroimmunol. 299 (2016) 98–106, https://doi.org/10.1016/j.jneuroim.2016.08.018.
[6] M.F. Rossato, G. Trevisan, C.I.B. Walker, J.Z. Klafke, A.P. de Oliveira, J.G. Villarinho, R.B. Zanon, L.F.F. Royes, M.L. Athayde, M.V. Gomez, J. Ferreira, Eriodictyol: a flavonoid antagonist of the TRPV1 receptor with antioxidant activity, Biochem. Pharmacol. 81 (2011) 544–551, https://doi.org/10.1016/j.bcp.2010.11.004.
[7] D.N. Cortright, J.E. Krause, D.C. Broom, TRP channels and pain, Biochim. Biophys. Acta Mol. basis Dis. 1772 (2007) 978–988, https://doi.org/10.1016/j.bbadis.2007.03.003.
[8] C. Montell, The history of TRP channels, a commentary and reflection, Pflugers Arch. - Eur. J. Physiol. 461 (2011) 499–506, https://doi.org/10.1007/s00424-010-0920-3.
[9] D. Julius, TRP channels and pain, Annu. Rev. Cell Dev. Biol. 29 (2013) 355–384, https://doi.org/10.1146/annurev-cellbio-101011-155833.
[10] F.C. Meotti, E. Lemos de Andrade, J.B. Calixto, TRP modulation by natural compounds, in: B. Nilius, V. Flockerzi (Eds.), Mamm. Transient Recept. Potential Cation Channels, 1st ed., Springer International Publishing, Cham, 2014, pp. 1177–1238, , https://doi.org/10.1007/978-3-319-05161-1_19.
[11] P. Gopinath, E. Wan, A. Holdcroft, P. Facer, J.B. Davis, G.D. Smith, C. Bountra, P. Anand, Increased capsaicin receptor TRPV1 in skin nerve fibres and related vanilloid receptors TRPV3 and TRPV4 in keratinocytes in human breast pain, BMC Womens Health 5 (2005) 2, , https://doi.org/10.1186/1472-6874-5-2.
[12] T. Watabiki, T. Kiso, T. Kuramochi, K. Yonezawa, N. Tsuji, A. Kohara, S. Kakimoto, T. Aoki, N. Matsuoka, Amelioration of neuropathic pain by novel transient receptor potential vanilloid 1 antagonist AS1928370 in rats without hyperthermic effect, J. Pharmacol. Exp. Ther. 336 (2011) 743–750, https://doi.org/10.1124/jpet.110.175570.
[13] H. Urano, T. Ara, Y. Fujinami, B.Y. Hiraoka, Aberrant TRPV1 expression in heat hyperalgesia associated with trigeminal neuropathic pain, Int. J. Med. Sci. 9 (2012) 690–697, https://doi.org/10.7150/ijms.4706.
[14] M. Zimmermann, Ethical guidelines for investigations of experimental pain in conscious animals, Pain 16 (1983) 109–110, https://doi.org/10.1016/0304-3959(83)90201-4.
[15] J. McGrath, G. Drummond, E. McLachlan, C. Kilkenny, C. Wainwright, Guidelines for reporting experiments involving animals: the ARRIVE guidelines, Br. J. Pharmacol. 160 (2010) 1573–1576, https://doi.org/10.1111/j.1476-5381.2010.00873.x.
[16] G. Trevisan, M.F. Rossato, C.I.B. Walker, J.Z. Klafke, F. Rosa, S.M. Oliveira, R. Tonello, G.P. Guerra, A.A. Boligon, R.B. Zanon, M.L. Athayde, J. Ferreira, Identification of the plant steroid alfa-spinasterol as a novel transient receptor potential vanilloid 1 antagonist with antinociceptive properties, J. Pharmacol. Exp. Ther. 343 (2012) 258–269, https://doi.org/10.1124/jpet.112.195909.
[17] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[18] G. Trevisan, M.F. Rossato, C.I.B. Walker, S.M. Oliveira, F. Rosa, R. Tonello, C.R. Silva, P. Machado, A.A. Boligon, M.A.P. Martins, N. Zanatta, H.G. Bonacorso, M.L. Athayde, M.A. Rubin, J.B. Calixto, J. Ferreira, A novel, potent, oral active and safe antinociceptive pyrazole targeting kappa opioid receptors, Neuropharmacology 73 (2013) 261–273, https://doi.org/10.1016/j.neuropharm.2013.06.011.
[19] T. Sakurada, K. Katsumata, K. Tan-No, S. Sakurada, K. Kisara, The capsaicin test in mice for evaluating tachykinin antagonists in the spinal cord, Neuropharmacology 31 (1992) 1279–1285, https://doi.org/10.1016/0028-3908(92)90057-V.
[20] J. Milano, M.F. Rossato, S.M. Oliveira, C. Drewes, P. Machado, P. Beck, N. Zanatta, M.A.P. Martins, C.F. Mello, M.A. Rubin, J. Ferreira, H.G. Bonacorso, Antinociceptive action of 4-methyl-5-trifluoromethyl-5-hydroxy-4,5-dihydro-1H-pyrazole methyl ester in models of inflammatory pain in mice, Life Sci. 83 (2008) 739–746, https://doi.org/10.1016/j.lfs.2008.09.010.
[21] G. Trevisan, G. Maldaner, N.A. Velloso, G. da S. Sant'Anna, V. Ilha, C. de C. Velho Gewehr, M.A. Rubin, A.F. Morel, J. Ferreira, Antinociceptive effects of 14-membered cyclopeptide alkaloids, J. Nat. Prod. 72 (2009) 608–612, https://doi.org/10.1021/np800377y.
[22] W.J. Dixon, Efficient analysis of experimental observations, Annu. Rev. Pharmacol. Toxicol. 20 (1980) 441–462, https://doi.org/10.1146/annurev.pa.20.040180.002301.
[23] G.J. Bennett, Y.-K. Xie, A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man, Pain 33 (1988) 87–107, https://doi.org/10.1016/0304-3959(88)90209-6.
[24] C. Sommer, C. Schmidt, A. George, Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1, Exp. Neurol. 151 (1998) 138–142, https://doi.org/10.1006/exnr.1998.6797.
[25] N.R. Gavva, J.J.S. Treanor, A. Garami, L. Fang, S. Surapaneni, A. Akrami, F. Alvarez, A. Bak, M. Darling, A. Gore, G.R. Jang, J.P. Kesslak, L. Ni, M.H. Norman, G. Palluconi, M.J. Rose, M. Salfi, E. Tan, A.A. Romanovsky, C. Banfield, G. Davar, Pharmacological blockade of the vanilloid receptor TRPV1 elicits marked hyperthermia in humans, Pain 136 (2008) 202–210, https://doi.org/10.1016/j.pain.2008.01.024.
[26] G. Trevisan, M.F. Rossato, C.I.B. Walker, J.Z. Klafke, F. Rosa, S.M. Oliveira, R. Tonello, G.P. Guerra, A.A. Boligon, R.B. Zanon, M.L. Athayde, J. Ferreira, Identification of the plant steroid α-spinasterol as a novel transient receptor potential vanilloid 1 antagonist with antinociceptive properties, J. Pharmacol. Exp. Ther. 343 (2012) 258–269, https://doi.org/10.1124/jpet.112.195909.
[27] M.J. Magistretti, M. Conti, A. Cristoni, Antiulcer activity of an anthocyanidin from *Vaccinium myrtillus*, Arzneimittelforschung 38 (1988) 686–690.
[28] C.I.B. Walker, S.M. Oliveira, R. Tonello, M.F. Rossato, E. da Silva Brum, J. Ferreira, G. Trevisan, Anti-nociceptive effect of stigmasterol in mouse models of acute and chronic pain, Naunyn Schmiedeberg's Arch. Pharmacol. 390 (2017) 1163–1172, https://doi.org/10.1007/s00210-017-1416-x.
[29] D.A. Simone, T.K. Baumann, R.H. LaMotte, Dose-dependent pain and mechanical hyperalgesia in humans after intradermal injection of capsaicin, Pain 38 (1989) 99–107, https://doi.org/10.1016/0304-3959(89)90079-1.
[30] K. Walker, A.J. Fox, L. a Urban, Animal models for pain research, Mol. Med. Today 5 (1999) 319–321, https://doi.org/10.1016/S1357-4310(99)01493-8.
[31] A.E. Dubin, A. Patapoutian, Nociceptors: the sensors of the pain pathway, J. Clin. Invest. 120 (2010) 3760–3772, https://doi.org/10.1172/JCI42843.
[32] S.W. Mittelstadt, R.A. Nelson, J.F. Daanen, A.J. King, M.E. Kort, P.R. Kym, N.L. Lubbers, B.F. Cox, J.J. Lynch, Capsaicin-induced inhibition of platelet aggregation is not mediated by transient receptor potential vanilloid type 1, Blood Coagul. Fibrinolysis 23 (2012) 94–97, https://doi.org/10.1097/MBC.0b013e32834ddf18.
[33] S. Evangelista, Novel therapeutics in the field of capsaicin and pain, Expert. Rev. Clin. Pharmacol. 8 (2015) 373–375, https://doi.org/10.1586/17512433.2015.1044438.
[34] S.M. Oliveira, C. Gewehr, G.D. Dalmolin, C.A. Cechinel, A. Wentz, R.V. Lourega, R.C. Sehnem, N. Zanatta, M.A.P. Martins, M.A. Rubin, H.G. Bonacorso, J. Ferreira, Antinociceptive effect of a novel tosylpyrazole compound in mice, Basic Clin. Pharmacol. Toxicol. 104 (2009) 122–129, https://doi.org/10.1111/j.1742-7843.2008.00353.x.
[35] R.J. Laing, A. Dhaka, ThermoTRPs and pain, Neuroscientist 22 (2016) 171–187, https://doi.org/10.1177/1073858414567884.
[36] T.J. Coderre, P.D. Wall, Ankle joint urate arthritis (AJUA) in rats: an alternative animal model of arthritis to that produced by Freund's adjuvant, Pain 28 (1987) 379–393, https://doi.org/10.1016/0304-3959(87)90072-8.
[37] Z. Helyes, Á. Szabó, J. Németh, B. Jakab, E. Pintér, Á. Bánvölgyi, L. Kereskai, G. Kéri, J. Szolcsányi, Antiinflammatory and analgesic effects of somatostatin released from capsaicin-sensitive sensory nerve terminals in a Freund's adjuvant-induced chronic arthritis model in the rat, Arthritis Rheum. 50 (2004) 1677–1685, https://doi.org/10.1002/art.20184.
[38] C.I.B. Walker, G. Trevisan, M.F. Rossato, C.R. Silva, F.V. Pinheiro, C. Franciscato, E. Tatsch, M.B. Moretto, M.D. Silva, M.P. Manfron, R. Noal Moresco, A.R.S. Santos, M.E. Pereira, J. Ferreira, Antinociceptive effect of *Mirabilis jalapa* on acute and chronic pain models in mice, J. Ethnopharmacol. 149 (2013) 685–693, https://doi.org/10.1016/j.jep.2013.07.027.
[39] M. Peter-Szabo, G. Kekesi, E. Nagy, E. Sziver, G. Benedek, G. Horvath, Quantitative characterization of a repeated acute joint inflammation model in rats, Clin. Exp. Pharmacol. Physiol. 34 (2007) 520–526, https://doi.org/10.1111/j.1440-1681.2007.04606.x.
[40] N. Schlesinger, Anti-interleukin-1 therapy in the management of gout, Curr. Rheumatol. Rep. 16 (2014) 398, , https://doi.org/10.1007/s11926-013-0398-z.
[41] S. McGaraughty, K.L. Chu, C.R. Faltynek, M.F. Jarvis, Systemic and site-specific effects of A-425619, a selective TRPV1 receptor antagonist, on wide dynamic range neurons in CFA-treated and uninjured rats, J. Neurophysiol. 95 (2006) 18–25, https://doi.org/10.1152/jn.00560.2005.
[42] L. Yu, F. Yang, H. Luo, F.-Y. Liu, J.-S. Han, G.-G. Xing, Y. Wan, The role of TRPV1 in different subtypes of dorsal root ganglion neurons in rat chronic inflammatory nociception induced by complete Freund's adjuvant, Mol. Pain 4 (2008), https://doi.org/10.1186/1744-8069-4-61 (1744-8069-4–61).
[43] M.M. Moran, A. Szallasi, Targeting nociceptive transient receptor potential channels to treat chronic pain: current state of the field, Br. J. Pharmacol. (2017), https://doi.org/10.1111/bph.14044.
[44] R. Brito, S. Sheth, D. Mukherjea, L. Rybak, V. Ramkumar, TRPV1: a potential drug target for treating various diseases, Cell 3 (2014) 517–545, https://doi.org/10.3390/cells3020517.
[45] N. Malek, A. Pajak, N. Kolosowska, M. Kucharczyk, K. Starowicz, The importance of TRPV1-sensitisation factors for the development of neuropathic pain, Mol. Cell. Neurosci. 65 (2015) 1–10, https://doi.org/10.1016/j.mcn.2015.02.001.
[46] M.A. Schumacher, Transient receptor potential channels in pain and inflammation: therapeutic opportunities, Pain Pract. 10 (2010) 185–200, https://doi.org/10.1111/j.1533-2500.2010.00358.x.
[47] S.L. Casey, N. Atwal, C.W. Vaughan, Cannabis constituent synergy in a mouse neuropathic pain model, Pain 158 (2017) 2452–2460, https://doi.org/10.1097/j.pain.0000000000001051.
[48] N. Murai, H. Hiyama, T. Kiso, T. Sekizawa, T. Watabiki, H. Oka, T. Aoki, Analgesic effects of novel lysophosphatidic acid receptor 5 antagonist AS2717638 in rodents, Neuropharmacology 126 (2017) 97–107, https://doi.org/10.1016/j.neuropharm.2017.08.032.
[49] A.J. Culshaw, S. Bevan, M. Christiansen, P. Copp, A. Davis, C. Davis, A. Dyson, E.K. Dziadulewicz, L. Edwards, H. Eggelte, A. Fox, C. Gentry, A. Groarke, A. Hallett, T.W. Hart, G. a Hughes, S. Knights, P. Kotsonis, W. Lee, I. Lyothier, A. McBryde, P. McIntyre, G. Paloumbis, M. Panesar, S. Patel, M.-P. Seiler, M. Yaqoob, K. Zimmermann, Identification and biological characterization of 6-aryl-7-isopropylquinazolinones as novel TRPV1 antagonists that are effective in models of chronic pain, J. Med. Chem. 49 (2006) 471–474, https://doi.org/10.1021/jm051058x.

[50] Y. Kanai, E. Nakazato, A. Fujiuchi, T. Hara, A. Imai, Involvement of an increased spinal TRPV1 sensitization through its up-regulation in mechanical allodynia of CCI rats, Neuropharmacology 49 (2005) 977–984, https://doi.org/10.1016/j.neuropharm.2005.05.003.

[51] D. Labuz, V. Spahn, M.Ö. Celik, H. Machelska, Opioids and TRPV1 in the peripheral control of neuropathic pain – defining a target site in the injured nerve, Neuropharmacology 101 (2016) 330–340, https://doi.org/10.1016/j.neuropharm.2015.10.003.

[52] R. Casoti, M.P. Manfron, J.M.S. de Oliveira, R. Casoti, M.P. Manfron, J.M.S. de Oliveira, Ovary and fruit morphology and anatomy of *Amphilophium crucigerum*, Rev. Bras. Farmacogn. 26 (2016) 15–22, https://doi.org/10.1016/j.bjp.2015.08.006.

[53] F.A. Pinho-Ribeiro, M.S.N. Hohmann, S.M. Borghi, A.C. Zarpelon, C.F.S. Guazelli, M.F. Manchope, R. Casagrande, W.A. Verri, Protective effects of the flavonoid hesperidin methyl chalcone in inflammation and pain in mice: role of TRPV1, oxidative stress, cytokines and NF-κB, Chem. Biol. Interact. 228 (2015) 88–99, https://doi.org/10.1016/j.cbi.2015.01.011.

[54] M. Aswar, P. Kute, S. Mahajan, U. Mahajan, G. Nerurkar, U. Aswar, Protective effect of hesperetin in rat model of partial sciatic nerve ligation induced painful neuropathic pain: an evidence of anti-inflammatory and anti-oxidative activity, Pharmacol. Biochem. Behav. 124 (2014) 101–107, https://doi.org/10.1016/j.pbb.2014.05.013.

[55] I. Straub, F. Mohr, J. Stab, M. Konrad, S.E. Philipp, J. Oberwinkler, M. Schaefer, Citrus fruit and fabacea secondary metabolites potently and selectively block TRPM3, Br. J. Pharmacol. 168 (2013) 1835–1850, https://doi.org/10.1111/bph.12076.

[56] G. Burgess, D. Williams, The discovery and development of analgesics: new mechanisms, new modalities, J. Clin. Invest. 120 (2010) 3753–3759, https://doi.org/10.1172/JCI43195.

[57] D. Labuz, H. Machelska, Stronger antinociceptive efficacy of opioids at the injured nerve trunk than at its peripheral terminals in neuropathic pain, J. Pharmacol. Exp. Ther. 346 (2013) 535–544, https://doi.org/10.1124/jpet.113.205344.

[58] C. Stein, Opioid receptors, Annu. Rev. Med. 67 (2016) 433–451, https://doi.org/10.1146/annurev-med-062613-093100.

[59] R. Ghosh, A. Alajbegovic, A.V. Gomes, NSAIDs and cardiovascular diseases: role of reactive oxygen species, Oxidative Med. Cell. Longev. 2015 (2015) 1–25, https://doi.org/10.1155/2015/536962.

[60] S.S. Negus, T.W. Vanderah, M.R. Brandt, E.J. Bilsky, L. Becerra, D. Borsook, Preclinical assessment of candidate analgesic drugs: recent advances and future challenges, J. Pharmacol. Exp. Ther. 319 (2006) 507–514, https://doi.org/10.1124/jpet.106.106377.

[61] Y. Wang, X. Fang, L. Ye, Y. Li, H. Shi, Y. Cao, A randomized controlled trial evaluating the effects of diosmin in the treatment of radicular pain, Biomed. Res. Int. 2017 (2017) 1–7, https://doi.org/10.1155/2017/6875968.

[62] P.H. Carpentier, M. Mathieu, Evaluation of clinical efficacy of a venotonic drug: lessons of a therapeutic trial with hemisynthesis diosmin in “heavy legs syndrome”, J. Mal. Vasc. 23 (1998) 106–112 http://www.ncbi.nlm.nih.gov/pubmed/9608923 , Accessed date: 31 October 2018.