An Acad Bras Cienc (2020) 92(1): e20180793 DOI 10.1590/0001-3765202020180793
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

HEALTH SCIENCES

Anti-hyperalgesic properties of ethanolic crude extract from the peels of *Citrus reticulata* (Rutaceae)

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Abstract: The therapeutic effects from *Citrus reticulata* on painful inflammatory ailments are associated to its flavonoids constituent and phytochemical studies with Citrus genus affirm that the peels have important amounts of it. These bioactive compounds have been a considerable therapeutic source and evaluate potential application of the peel extract is significant. This research aims to investigate the influence of ethanolic crude extract from the peels of Citrus reticulata and its possible mechanism of action in different animal models of pain. The extract reduced hyperalgesia in the second phase of formalin test (vehicle: 501.5 ± 40.0 s; *C. reticulata* extract 300 mg/kg: 161.8 ± 41.1 s), in the carrageenan model (vehicle at 4th h: 82.5 ± 9.6 %; *C. reticulata* extract 300 mg/kg at 4th h: 47.5 ± 6.5 %) and in Complete Freund's Adjuvant model (vehicle: 501.5 ± 40.0 s; *C. reticulata* extract 300 mg/kg: 161.8 ± 41.1 s). The possible contribution of opioidergic and adenosinergic systems in the anti-hyperalgesic effect of *C. reticulata* extract was observed after treatment, with non-selective antagonists for both systems, which produced reversal effects. In conclusion, these properties of *C. reticulata* extract suggest a potential therapeutic benefit in treating painful conditions.

Key words: Citrus reticulata, medicinal plants, pain, opioids, adenosine.

INTRODUCTION

For many populations, relations based on herbal medicines is being groundwork for treating many inflammatory and painful disorders, given that conventional medicines are associated with many adverse effects, toxicity and drug interactions (Chan et al. 2017). Development of safe alternatives for treating such diseases from compounds derived from plants have been studied for medicinal purposes and some of them have shown anti-inflammatory and analgesic properties, including *Achillea millefolium* L., *Arnica montana* L., *Curcuma longa* L., *Salix alba* L., *Citrullus colocynthis* (L.) Schrad, *Conium maculatum* L. and *Cyperus rotundus* L. amonh others from Citrus species (Al-Snafi 2016).

These properties are associated with naturally occurring bioactive compounds that have been a considerable therapeutic source. Within these, flavonoids are unquestionably a class of agents with potential applications (Brodowska 2017). Flavonones are the preponderance flavonoids in Citrus species (Waheed et al. 2009) and around the world, citrus plants like tangerines are widely consumed by the population, mainly as food supplements

and nutraceuticals for many physiological, pharmacological and medicinal activities (Ye 2017). China, Spain and Brazil account for 60.3% of world tangerine production, while Brazil was the second world leading citrus producer in 2014 (19 million tons) after China (33 million tons) (FAO 2016).

For Brazilian folk medicine, *Citrus aurantium* L., *Citrus sinensis* (L.) Osbeck, *Citrus limon* (L.) Osbeck, *Citrus aurantiifolia* (Christm.) Swingle and *Citrus reticulata* Blanco, have been commonly used in the treatment of different conditions that feature inflammatory components such as bronchitis, stomach problems including gastritis, throat pain, among others (De Medeiros et al. 2013).

These therapeutic effects from *Citrus reticulata* on painful inflammatory ailments are associated to its large spectrum of flavonoids constituent (Zhang et al. 2014, Barreca et al. 2017) and phenolic acids, both with recognized anti-inflammatory and antinociceptive actions, as mentioned by Ambriz-Pérez et al. (2016). Additionally, there are phytochemical studies with Citrus genus that affirm that is in the peels that are found important amounts of flavonoids (Nogata et al. 2006, Bermejo et al. 2011). Nevertheless, the consumption of Citrus occurs mainly in the form of juice, and the peels become unusable products. Even though it has been characterized for its valuable organic compounds, it is still a major environmental problem and product waste derived from the industrial processing of Citrus fruits (Mahato et al. 2017).

The constituents of the peels of *Citrus reticulata* emerge as a possible alternative to conventional medicines because of their effectiveness and low cytotoxicity (Lim 2012) thus it seems especially significant to evaluate new possibilities for clinical use of the peel extract as phytomedicine. Moreover, some properties related to the compounds present in it have already been studied and they suggest important anti-inflammatory and anti-oxidant role (Amorim et al. 2016, Zhang et al. 2018). Despite this, no register was found about the potential antinociceptive and/or anti-hyperalgesic activities obtained with substances derived from the peels of *Citrus reticulata* fruits.

The present study is justified because this plant was selected for being widely cultivated in the southern region of Santa Catarina, as well as knowing that the peels are currently a by-product of the plant and that *Citrus reticulata* specie reveal higher content of flavonoid in their peels when compared to other Citrus fruits (Nogata et al. 2006, Huang & Ho 2010). Taking the above discussed into account, the aim of this study was to investigate antinociceptive and anti-hyperalgesic properties of ethanolic crude extract from peels of *Citrus reticulata* (ECE-CR) in different animal models of neurogenic and inflammatory pain (Santos et al. 1998) and preliminarily explore possible contribution of adenosinergic and opioidergic systems for this effect.

MATERIALS AND METHODS

Animals

In this preclinical study, experiments were conducted using male Swiss mice (n= 8/group) weighting 25-30 g. They were maintained under standardized conditions of 12 h light/12 h dark and room temperature at 22 ± 2 °C, with access to food and water *ad libitum*. After acclimatization of the animals to the Laboratory of Experimental Neuroscience (Lanex), at UNISUL/Brazil-SC, the animals where homogenously distributed between different treatment groups and experiments were conducted from 8:00 a.m. to 5:00 p.m. Animal care and the experimental protocols were approved by the Ethics

Committee for Animal Use (CEUA)-UNISUL, under protocol number 13.039.4.03.IV. The number of animals and intensity of noxious stimuli used were the minimum necessary to demonstrate the consistent effects of treatments.

Preparation of the ethanolic crude extract of *Citrus reticulata* (ECE-CR)

The plant was collected at a cultivar in Araranguá, state of Santa Catarina, Brazil (latitude 28º56'05" south and longitude 49º29'09" west). Professor Jasper Zanco, faculty of the Agronomy course at University Southern Santa Catarina (UNISUL), identified the plant as belonging to the Rutaceae family, by comparing it directly with a voucher specimen (SRS 00050047) deposited at the *Laelia Purpurata* (SRS), herbarium of UNISUL.

Peels of the plant were placed to dry in an oven at 40°C for 48 h and the dried material was milled and standardized in particle sizes ranging from 250 µm to 850 µm.

The extract was produced at a ratio of 1:8 of peels/solvent to ethanol under dynamic maceration, the fluid remaining in contact with the peels for 10 days, under constant stirring in a closed vessel at room temperature. At the end, the ECE-CR was concentrated in rotavapor under reduced pressure to evaporate the solvent, in a water bath at a temperature of 35°C to 40°C until the total evaporation of the ethanol.

Evaluation of anti-nociceptive and anti-hyperalgesic activities of ECE-CR

Formalin test

Swiss mice were orally treated with sterile saline (0.1 ml/10 g weight) or different doses (30, 100 and 300 mg/kg) of ECE-CR, based on previous works performed in our laboratory in which this range of doses were antinociceptive for hydroalcoolic crude extraxt of other plant (De Mattos et al. 2007, Piovezan et al. 2017). One hour after this treatment, the animals received an intraplantar (i.pl.) injection of 20 µL of 2.5% formalin. Nociceptive responses were determined by the time the animal spent licking the treated paw during the periods between 0-5 min (acute phase; phase I) and 15-30 min (inflammatory phase; phase II) after the injection of this agent (Martins et al. 2013, López-Cano et al. 2017).

Glutamate test

Acute nociception was induced by glutamate in mice that were initially orally treated with vehicle (saline, 0.1 ml/10 g weight) or different doses (30, 100 and 300 mg/kg) of ECE-CR. One hour after this treatment, the animals received an i.pl. injection of 20 µL of glutamate (10 µmol/paw) dissolved in a vehicle. The injection of glutamate in the paw of the animal induces direct stimulation of nociceptive neurons, as well as the release of various inflammatory mediators and neuropeptides involved in nociception, promoting immediately nociceptive activity, which was determined by the behavior of biting or licking the treated paw (Beirith et al. 2002, Martins et al. 2011). This index was recorded within 15 min after the administration of the compound (Sousa et al. 2017).

Carrageenan-induced mechanical hyperalgesia

The protocol was developed according to previously use by Albano et al. (2013), for the induction of mechanical hyperalgesia by i.pl. administration of carrageenan (300 µg/paw) in the right hind paw of mice. The mechanical hyperalgesia induced by sensibilization of paw was evaluated using von Frey monofilaments (VFH, Stoelting, Chicago, USA). The percentage values for paw withdrawal frequency to 10 applications of von Frey filament of 0.6 g for each animal was used as an indicator of response. This

pressure was selected to evaluate mechanical hyperalgesia from previous study (Martins et al. 2011). The test was performed using a platform measuring 70 x 40 cm, which consists of a wire screen with a mesh size of 6 mm to facilitate the application of the filament on the ventral surface of the hind paw. Mice were placed individually in an observation acrylic bottomless chamber (9 x 7 x 11 cm) and covered with a lid. The criteria for the application of mechanical stimulus were: a) application made perpendicularly to the plantar surface, to provide enough pressure to bend the filament, thereby obtaining total pressure; b) animals were evaluated when all four paws were accommodated on the screen; c) the withdrawal response was considered positive when the animal completely removed the paw from the surface of the support screen.

Anti-hyperalgesic effect for ECE-CR was investigated in different groups of animals treated orally with vehicle (sterile saline, 0.1 ml/10 g weight) or different doses of ECE-CR (30, 100 and 300 mg/kg) at intervals of 1 h before (prophylactic treatment) or 1 h after the administration of carrageenan (therapeutic treatment). Basal values were registered before carrageenan injection.

CFA-induced hyperalgesia and possible mechanism of action

Since CFA model is adequate for study of artrithis, a higly prevalent painfull disease in the world in which the pathophysiology relays on inflammatory mechanisms. Confirmation of the effects of ECE-CR and preliminary exploration of its mechanism of action were evaluated with different groups of animals injected i.pl. with 20 µL of the vehicle (sterile saline, 0.1 ml/10 g weight, negative control) or a 30% solution of CFA for the induction of inflammatory pain, as used by Nascimento et al. (2010). To confirm the anti-hyperalgesic effect of ECE-CR, different groups of mice were orally treated with ECE-CR (30, 100 and 300 mg/kg), 24 h after the administration of CFA. The development of mechanical hyperalgesia was assessed as described in the previous section. For the dose with greater activity in these first 24 h (30 mg/kg), the anti-hyperalgesic activity of the plant was also investigated up to 5 days by its long-term effect (always 1h after daily treatment, once a day).

To explore the possible contribution of adenosinergic and opioidergic systems upon the anti-hyperalgesic effect of ECE-CR in a CFA-induced model, in the 3rd day after induction, different groups of animals were treated with vehicle (sterile saline, 0.1 ml/10 g weight, via i.pl.) or CFA (same conditions); prior to this (15 min before), these two groups were subdivided into two groups treated as follows: a) vehicle group: naloxone (non-selective opioidergic receptor's antagonist, 1 mg/kg, subcutaneously [s.c.]) or caffeine (non-selective adenosinergic receptor's antagonist, 10 mg/kg, intraperitoneally [i.p.]); b) CFA group: naloxone (non-selective opioidergic receptor's antagonist, 1 mg/kg, s.c.) or caffeine (non-selective adenosinergic receptor's antagonist, 10 mg/kg, i.p.). One hour after i.pl. treatment, mice were evaluated for mechanical hyperalgesia, as previously described.

Rotarod for evaluation of the influence of ECE-CR on motor coordination

To evaluate the possible occurrence of non-specific effects of ECE-CR on locomotor performance, the mice were submitted to the rotarod test (De Mattos et al. 2007). For that purpose, only animals which remained successfully on the revolving bar of the apparatus for two consecutive periods of 60 s were selected to receive orally either ECE-CR (30–300 mg/kg) or vehicle (2% Tween 80) on the following day. At 30, 60, 90 and 120 min after treatment, the animals were placed on the apparatus for up to

90 s at a time, and the amount of time that each animal remained on the revolving bar during each trial was recorded (in s).

Chemicals and drugs

Morphine hydrochloride was purchased from Merck A.G. (Darmstadt, Germany). Formalin was prepared by dilution of formaldehyde (LAFAN Química Fina, SP, Brasil) in saline. Glutamate, carrageenan, CFA, naloxone hydrochloride and caffeine were purchased from Sigma Chemical Co. (Porto Alegre, Brasil). Ethanol was acquired from Vetec Quimica Fina (Duque de Caxias, RJ, Brasil).

Statistical analysis

Results are expressed as media ± standard error of the mean (S.E.M) standard deviation and $p < 0.05$ value was considered statistically significant. The comparison between groups was assessed by using one-way analysis of variance (ANOVA) followed by Tukey's test or by two-way ANOVA followed by Bonferroni's test when appropriated. The GraphPad InStat® software was used for data analysis.

RESULTS

As it can be seen in Figure 1, the ECE-CR effect was more pronounced in pain of inflammatory origin, according to the results presented in the second phase of formalin-induced nociception and mechanical hyperalgesia on the carrageenan and CFA models. Moreover, a change in nociceptive response of neurogenic origin was not observed, neither in the 1st phase of the formalin test nor in the glutamate test.

Concerning to the anti-hyperalgesic effect of ECE-CR in the carrageenan model, different results were obtained depending on the type of treatment, whether it was prophylactic or therapeutic. Whereas the prophylactic treatment failed to alter this response in any of the evaluated doses, the therapeutic treatment of the animals at doses ranging from 100 to 300 mg/kg reduced hyperalgesic response, even in the 1st hour after treatment (data not shown).

In the evaluation of anti-hyperalgesic activity of ECE-CR significant change was observed in mechanical hyperalgesia in the hind paw of mice, 24 h after i.pl. injection of CFA (Figure 2).

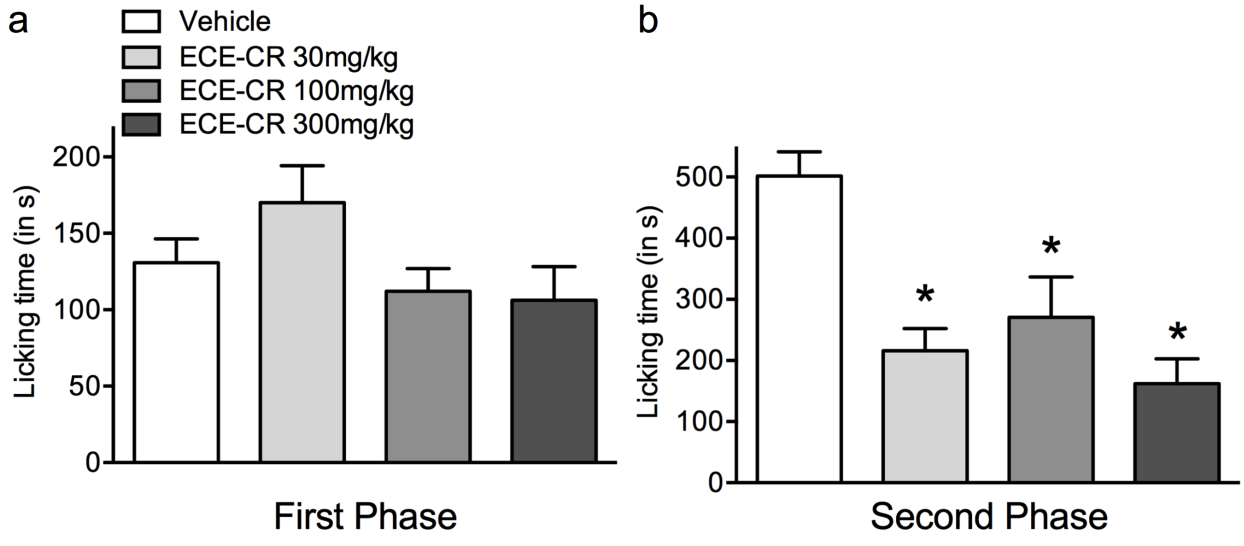

Figure 1. Influence of ECE-CR on neurogenic (First phase) or inflammatory (Second Phase) nociception in the formalin test in mice. Animals were evaluated 1 h after treatment with the extract. Data are presented as mean ± S.E.M. for 8 animals. (*)$p \leq 0.05$; One-way ANOVA followed by Tukey's test.

The evaluation on the time course of action of this extract demonstrated greater effectiveness in the first hour and lasted up to 3 h after its administration (Fig. 1a). Moreover, the effect on mechanical hyperalgesia was maintained until the 5th day after the induction of CFA model when the animals received daily treatment (Fig. 1b), even though no cumulative effect of the extract was registered.

Regarding the involvement of the opioidergic and adenosinergic systems on the effects of ECE-CR, on the 5th day after the i.pl. injection of CFA in the paw of mice, pre-treatment (1 h earlier) with naloxone or caffeine reversed the anti-hyperalgesic effect caused by ECE-CR (vehicle: 87.5 ± 3.7%; ECE-CR 30 mg/kg orally: 27.5 ± 5.2%), 55.0% ± 6.3 and 65.0 ± 3.3%, respectively (Figure 3).

The results of the rotarod, for evaluation of the influence of ECE-CR on motor coordination, presented in Table I showed that the treatment of the animals with the extract (30 mg/kg, orally), selected as the most effective dose in this study, did not alter their resistance time on rotarod (in s), used as an index of evaluation for motor coordination of the animals at 22 r.p.m. (De Mattos et al. 2007) in any of the time frames chosen for observation in this study.

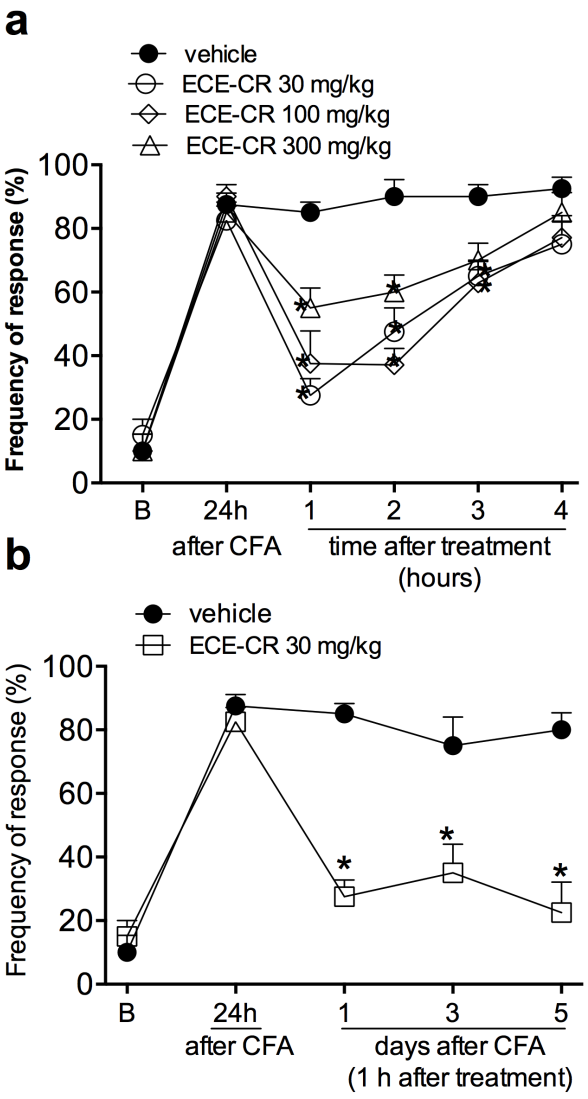

Figure 2. Influence of treatment with ECE-CR on mechanical allodynia in the CFA-induced arthritis model in mice. a) Treatment with ECE-CR (30, 100 and 300 mg/kg) was performed 24 h after induction of the inflammation by the i.pl. administration of CFA (30% solution, 20 µl). b Treatment with HECR (30 mg/kg) was performed on days 1, 3 and 5 after induction of the inflammation by the i.pl. administration of the agent. Data are presented as mean ± S.E.M. for 8 animals. (*)$p \leq 0.05$; Two-way ANOVA followed by Bonferroni test.

DISCUSSION

The present study evidenced anti-hyperalgesic properties of the ethanolic crude extract based on the fruit peels of *Citrus reticulata* in different animal models of nociception and hyperalgesia and provides some evidence on the mechanisms implicated in these effects.

Part of the results obtained in this study highlights that the antinociceptive activity of ECE-CR seem to be more efficient for inflammatory pain, because it has not changed the nociceptive response in animal models of neurogenic pain like in the glutamate test and the 1st phase of the formalin test. Therefore, the anti-hyperalgesic activity from the extract might occur without influence of central nervous system and possibly

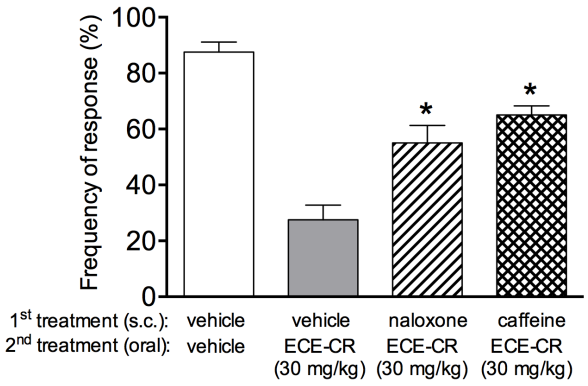

Figure 3. Contribution of opioidergic and adenosinergic systems on the effects of ECE-CR on mechanical allodynia in the CFA-induced arthritis model in mice. On the 5th day, after i.pl. treatment of the animals with vehicle (white bars) or CFA (30% solution, 20 µl grey bars), the mice received naloxone (1 mg/kg, s.c.) or caffeine (10 mg/kg, i.p.) and 15 min later they received ECE-CR (30 mg/kg; orally). One hour after this latest treatment, the animals were evaluated for mechanical allodynia. Data are presented as mean ± S.E.M. for 8 animals. (*)$p \leq 0.05$; One-way ANOVA followed by Tukey's test.

inhibits peripheral mechanisms of inflammatory mediators. In addition, this result could explain the primordial popular use of Citrus species as anti-inflammatory analgesic and antipyretic medication (Arias & Ramón-Laca 2005).

The activity of ECE-CR in models of inflammatory nociception as the 2nd phase of the formalin test and the carrageenan model reinforce the findings observed in the reduction of pain induced by CFA model, which comprises pivotal inflammatory components. This fact suggests that the phytochemicals present in ECE-CR may have important activity on relief of pain symptoms by peripheral mediated analgesic activity, possibly interfering with factors that cause peripheral and/or spinal cord sensitization observed in these models, such as histamine, serotonin, bradykinin, substance P, CGRP or PGE_2 (Granados-Soto et al. 2001, Morris 2003).

Another possible mechanism of action for ECE-CR on mechanical hyperalgesia observed in the CFA model, is that it's possibly affecting T cell-derived mediators, such as cytokines, as these are also activated by mycobacteria, that leads to an increased immune response (Huang et al. 2016). According to this, hesperidin, another Citrus flavanone compound has been the subject of various studies in the CFA-induced model, it's efficacy was specified by Sakr (2017), who characterized reduction in the infiltration of inflammatory cells by inhibition of pro-inflammatory cytokines production (IL-1, IL-6 and TNF-α) secreted by macrophages, suppression of T lymphocyte proliferation, as well as increased anti-inflammatory cytokines (IL-4 and IL-10).

Besides this, the possible activity of ECE-CR on neural nociceptive pathways cannot be completely ruled out, since the present analysis of the mechanism of action has demonstrated that its anti-hyperalgesic activity is affected by antagonists of opioid and adenosinergic systems, which exert modulatory role on the transmission of painful information by nociceptive ascendant or descendant pathways. The contribution of a peripheral activity of the opioid system in the reversal process of mechanical hyperalgesia in the CFA model should be considered, since the inflammatory process that occurs after CFA injection in the mouse hind paw triggers the recruitment of monocytes and macrophages, which are responsible for an endogenous analgesic activity in other models (Sauer et al. 2014).

Regarding the participation of the adenosinergic system in the activity of ECE-CR, it is known that opioids induce the release of adenosine in the spinal cord and that this mediator can act as a neuromodulator in promoting anti-hyperalgesic and anti-inflammatory effects, possibly mediated by

Table I. Influence of ECE-CR on locomotor activity of mice.

Treatment	Time of observation after treatment		
	1 h	2 h	24 h
Vehicle	90.0 s ± 0.0	90.0 s ± 0.0	90.0 s ± 0.0
ECE-CR 30 mg/Kg	85.5 s ± 4.5^{ns}	85.5 s ± 4.5^{ns}	90.0 s ± 0.0^{ns}

ECE-CR: ethanolic crude extract from peels of Citrus reticulata; vehicle (sterile saline 0.1 ml/10 g of weight, control group). Data are shown as media ± S.E.M. for 8 animals. Two-way ANOVA followed by Bonferroni tests ($p \leq 0.05$); $^{(ns)}$: no significant in relation to control group.

the activation of the descending pain control pathway. The adenosinergic system can also be effective in the control of peripheral inflammation, by helping reduce the harm caused by uncontrolled immune response (Sawynok & Liu 2003, Varani et al. 2017, Jacobson et al. 2017).

The rotarod test showed that the selected dose (30 mg/kg) for most of the experiments due to its high efficacy, did not affect the duration of time in which the animals stayed on the revolving bar, at the different time frames chosen as the representative of acute (1 or 2 h) or prolonged pre-treatment (24 h). Because of this, it seems not to be the case that a possible unspecific effect of ECE-CR on motor coordination might be responsible for the inhibition of painful behaviors registered in this study.

CONCLUSIONS

The results of the present study have demonstrated that systemic treatment with ECE-CR of *Citrus Reticulata* peel presents anti-hyperalgesic effects on inflammatory pain models in mice. The investigation of the participation of the adenosinergic and opioidergic systems, as possible mechanisms of action, is a preliminary achievement requiring more studies to identify new cellular targets such as transient receptor potential (TRP) channels, acid sensing ion channels (ASICs) and adenosinergic receptor subtypes (A1/A2), as well as their influence on intracellular mechanisms of nociceptive/hyperalgesic stimulus signaling such as protein kinase C, adenylyl cyclase and protein kinase C (Baggio et al. 2012, Nascimento et al. 2010, Marcon et al. 2009, review in: White et al. 2010,). These findings can also stimulate further investigation on the use of ECE-CR as a food supplement to people who suffer from different inflammatory disorders with painful symptoms.

Acknowledgments

This study was supported by Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC-CNPq). Adriele C. Schneider received a scholarship from CAPES, Brazil. Maria E.M. Lisbôa received a scholarship from PUIC-UNISUL.

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How to cite

SCHNEIDER ACA, BATISTI AP, TURNES BL, MARTINS TC, LISBOA MEM, CUSTÓDIO KM, ZANCO J, WILSON KSC, HEYMANNS AC, KANIS LA, MAGNAGO RF, MARTINS DF & PIOVEZAN AP. 2020. Anti-hyperalgesic properties of ethanolic crude extract from the peels of *Citrus reticulata* (Rutaceae). An Acad Bras Cienc 92: e20180793. DOI 10.1590/0001-3765202020180793.

Manuscript received on September 21, 2018;
accepted for publication on May 3, 2019

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