



# Gas Chromatography/Mass Spectrometry characterization and antinociceptive effects of the ethanolic extract of the leaves from *Clusia minor* L.

[Caracterización por Cromatografía Gaseosa/Espectrometría de Masas y actividad antinociceptiva del extracto etanólico de las hojas de *Clusia minor* L.]

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## Abstract

**Context:** The search of new substances with analgesic properties has grown in the last years. Brazil and Cuba have a big biodiversity allowing the study of several plants with potential pharmacological activities.

**Aims:** To evaluate the chemical composition and potential antinociceptive effect of the ethanolic extract from *Clusia minor* L. leaves (*Clusiaceae*) in mice.

**Methods:** Phytochemical characterization was performed by Gas Chromatography/Mass Spectrometry. Antinociceptive effect was evaluated using acetic acid, formalin, hot plate, and capsaicin models. Mechanical hypernociception was induced by intraplantar carrageenan, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and responses were measured after 3 h of injection.

**Results:** Mass Spectrometry analysis allowed the identification of 16 compounds. Fatty acid derivatives, steroids, triterpenoids, and vitamin E were the main findings. The most abundant sterol was  $\beta$ -sitosterol (14.04%); followed by the triterpenes  $\alpha$ -amyirin (11.94%), and  $\beta$ -amyirin (7.82%). Vitamin E represented the 8.44% of the total identified compounds. The evaluation of the acetic acid-induced nociception model showed that the extract was effective in reducing pain in a dose-dependent manner. This resulted in a maximal inhibition of  $53 \pm 4\%$ . The extract was also effective in other pain models. Additionally, the extract presented a considerable inhibition of paw mechanical hypernociception.

**Conclusions:** The data suggest that the antinociceptive effect of *Clusia minor* occurs by interaction of various mechanisms; which probably take places via central and peripheral pathway. Therefore, modulating the inflammatory and neurogenic pain.

**Keywords:** acute mechanical hypernociception; acute nociceptive models; antinociceptive effect; *Clusia minor* L.; GC/MS; triterpenoids.

## Resumen

**Contexto:** La búsqueda de nuevas sustancias con propiedades analgésicas se ha incrementado en los últimos años. Brasil y Cuba cuentan con una gran biodiversidad permitiendo el estudio de varias plantas con actividades farmacológicas potenciales.

**Objetivos:** Evaluar la composición química y el efecto antinociceptivo del extracto etanólico de las hojas de *Clusia minor* L. (*Clusiaceae*) en ratones.

**Métodos:** La caracterización fitoquímica fue realizada por Cromatografía Gaseosa-Espectrometría de Masas. El efecto antinociceptivo fue evaluado utilizando los modelos de ácido acético, formalina, plato caliente y capsaicina. La hipernocicepción mecánica fue inducida por carragenina, factor de necrosis tumoral (TNF $\alpha$ ) y prostaglandina E<sub>2</sub> (PGE<sub>2</sub>) intraplantares y la respuesta se midió después de 3 h de inyección.

**Resultados:** El análisis por Cromatografía Gaseosa-Espectrometría de Masas permitió la identificación de 16 compuestos, principalmente derivados de ácidos grasos, esteroides, triterpenos y vitamina E. El esteroil más abundante fue el  $\beta$ -sitosterol (14,04%) seguido por los triterpenos  $\alpha$ -amirina (11,94%) y  $\beta$ -amirina (7,82%). La vitamina E representó el 8,44% del total de los compuestos identificados. El modelo de nocicepción inducido por ácido acético mostró la efectividad del extracto para reducir el dolor vía dosis dependiente con una inhibición máxima de  $53 \pm 4\%$ . El extracto también resultó efectivo en los otros modelos de dolor empleados. Además, se observó una considerable inhibición de la hipernocicepción mecánica de la pata.

**Conclusiones:** Los resultados sugieren que el efecto antinociceptivo de *Clusia minor* ocurre por la interacción de varios mecanismos, probablemente vía central y periférica modulando el dolor inflamatorio y neurogénico.

**Palabras Clave:** *Clusia minor* L.; GC/MS; efecto antinociceptivo; hipernocicepción mecánica aguda; modelos de nocicepción aguda; triterpenoides.

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## INTRODUCTION

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The genus *Clusia* with approximately 250 species, is one of the most studied in the Clusiaceae family. A wide diversity of bioactive metabolites has been identified in this genus, (Silva et al., 2013; Ferreira et al., 2015; 2016) demonstrating several pharmacological activities. These activities include; anti-inflammatory, antinociceptive, and antioxidant activity (de Souza et al., 2013; Ferreira et al., 2016; Lins et al., 2016).

*Clusia minor* L. is a wild bush that is usually found in the forests of western Cuba. It is characterized by white and pink flowers and green fruits. It can also be found in other areas of Antillas, except Jamaica, and North of South America (Mesa and Freixas, 1992). In spite of its potential as bioactive phytochemical source, there are relatively few reports about the phytochemical and pharmacological characterizations of *C. minor* L. Previous phytochemical study of the *C. minor* fresh fruits revealed the presence of three polyisoprenylated benzophenones derivatives: garcinielliptone I, propolone D, and hyperibone B (Mangas et al., 2008a). Also, in the GC/MS analysis of the non-polar extract from *C. minor* L. leaves 25 compounds were identified. The two most abundant triterpenoids were lupeol and  $\alpha$ -amyryn (Mangas et al., 2008b).

The aim of this investigation was to analyze by GC/MS the ethanolic extract of the leaves from *C. minor* and to evaluate its antinociceptive potential using classical models of acute nociception and mechanical hypernociception.

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## MATERIAL AND METHODS

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### Preparation of leaves extract

The selected plant material for the present study was *C. minor* L. leaves from the National Botanical Garden, Cuba (23° 07' 59.8800" N 82° 21' 59.7600" O) collected in September 2013. The identification of this species was done by Dra. Cristina Panfet. A voucher specimen (No. 482) was deposited in the Herbarium of the Fundamental Investi-

gations of Tropical Plants Institute for further identification. The drying leaves process was done in an oven at 40°C for 15 days and subsequently fragmented. The dried powdered leaves (32 g) was successively extracted by static maceration at room temperature with ethanol absolute (3 X 500 mL). The extract was filtered using filter paper and concentrated to dryness under reduced pressure at 45°C using a rotary vacuum evaporator.

### Gas Chromatography (GC)/Mass Spectrometry (MS) analysis

The dried residue (1 mg) was dissolved in 1 mL of absolute ethanol, and 500  $\mu$ L of tridecanoic acid solution (50 ng/mL in chloroform (Sigma, USA)) used as internal standard. Gas chromatography analysis was performed using a gas chromatograph Hewlett-Packard 6890 (Palo Alto, CA, USA) coupled with a mass spectrometer Hewlett-Packard 5973. Analytes separation was performed on a capillary column (SPB-5, 15 m X 0.25 mm i.d., film thickness 0.10  $\mu$ m; Supelco, PA, USA). Helium was the carrier gas at a flow rate of 1 mL/min using a constant flow mode. Oven temperature started at 60°C for 2 minutes. Temperature was increased to 100°C at a rate of 4°C/min and later increased to 290°C at 10°C/min and held for 5 min. For GC/MS analysis, 1  $\mu$ L volume was injected without derivatization in split injection mode (1:10). The mass spectrometer was operated in electron impact (EI) mode using an ionization voltage of 70 eV. The injection port, ion source, and interface temperatures were: 280, 280 and 150°C, respectively. The mass spectra were recorded in full scan mode with a scan range from m/z 40-700. Identification was accomplished by comparison of retention times (RT) and spectra from available commercial standards and similar compound spectra at the libraries NIST98 and Wiley275 MS. Some reference compounds were analyzed to confirm retention times and mass spectra (triterpenes, sterols). The semiquantification process of the identified substances was performed by internal normalization with the area of each compound.

## Animals and experimental protocol

Adult male Swiss mice (weight, 25–30 g) from the Central Biotery, University of Vale do Itajaí were used and housed under optimum light (12 h light-dark), temperature ( $22 \pm 1^\circ\text{C}$ ) and humidity conditions (50–60%) with free access to tap water and food. Before the experiments, the animals were acclimatized to the environment laboratory for at least 1 h. All behavioral experiments were conducted between 8 a.m. - 1 p.m. The procedures used in this investigation were accomplished in concordance with the Ethical Committee for Animal Care, University of Vale do Itajaí regulations (Process number 001/08) and in accordance with the Federal Government guidelines on animal care. The minimum number of animals and the duration of observation required to obtain reliable data were used. Standard drugs, vehicle, and extract were administered 30 min prior to the experiment. The extract was dissolved in Tween 80 (0.1%) and saline (NaCl, 0.9%), and administered by oral route (100, 150 and 300 mg/kg).

## Drugs and chemicals

The solvents and reagents used for the GC/MS analysis, formaldehyde and acetic acid were obtained from Merck (Darmstadt, Germany). Indomethacin, sodium diclofenac, morphine, capsaicin, and carrageenan were obtained from Sigma-Aldrich. The drugs were dissolved in saline solution, except capsaicin that was dissolved in absolute ethanol. The final concentration of ethanol or Tween 80 did not exceed 5%, so, they did not produce any effect *per se*.

## Assessing antinociceptive activity

### *Rota-rod testing*

Motor performance was measured as the latency to fall as a previous report of Dunham and Miya (1957). The rota-rod consisted of a 3 cm diameter bar rotating at 12 rpm. Mice were submitted to a 24 h trial before experiment for three consecutive periods of 60 s. Thirty minutes before, mice were placed on the rotating rod over 2 min, and then they were administered with *C. minor* extract or

saline (control). Results were expressed as the time (s) that mice stayed on the rota-rod and the cut off time was 60 s.

### *Open field test*

The apparatus used for the experiments with mice was made of wood (30 x 30 x 15 cm) with a front glass. The inside of the apparatus was subdivided into nine quadrants. The following parameters were observed: number of quadrant crossings and number of rearing attempts, as described by de Oliveira et al. (2006). The observation time was 6 minutes.

### *Acetic acid-induced writhing*

Abdominal constriction was induced by an intraperitoneal (i.p.) injection of acetic acid (0.6 %, 0.01 mL/kg) according to the previous report of Collier et al. (1968) with minor modifications. After treatment with the extract and sodium diclofenac (10 mg/kg, i.p.) animals received the acetic acid injection while control animals received a similar volume of saline. The number of abdominal constrictions was cumulatively counted over a 30 min period.

### *Formalin-induced nociception*

The selected procedure was same as the one initially described by Hunskaar and Hole (1987). Sixty minutes before formalin injection, mice were administered with the extract, sodium diclofenac (10 mg/kg, i.p.) and vehicle. Then, animals received a 20  $\mu\text{L}$  intraplantar (i.pl.) injection of 2.5% formalin solution into the right hind paw. Immediately, mice were located in a glass cylinder. The time spent licking the injected paw in a 30 min period was considered indicative of nociception.

### *Hot-plate test*

Response latencies (licking or jumping) in hot plate test ( $56 \pm 1^\circ\text{C}$ ) were done according to the method described by Santos et al. (1999). A cut off period of 30 s was defined as the complete latency period. Positive controls were administered with morphine (10 mg/kg, s.c.) and control animals received the vehicle. Twenty-four hours before the

experiment, mice were selected using the same algescic stimulation for a cut off period of 8 s.

#### *Capsaicin-induced acute hind paw nociception*

Thirty minutes after treatment animals received a volume of 20  $\mu$ L of capsaicin (1.6  $\mu$ g/paw) (Sakurada et al., 1993) by i.pl. injection into the right hind paw. Mice were examined over a 5 min period. Positive controls were administrated with morphine (5 mg/kg, s.c.). Also, a separate control group that received a subplantar injection of the only vehicle was included. The amount of time spent licking the injected paw was registered with a chronometer and it was considered indicative of nociception.

#### *Evaluation of the mechanical hypernociception induced by carrageenan, TNF $\alpha$ , and PGE $_2$*

Mechanical hypernociception was induced by i.pl. injection of carrageenan (300  $\mu$ g/paw), TNF $\alpha$  (50 pg/paw) and PGE $_2$  (100 ng/paw). Indomethacin (10 mg/kg, p.o) was used as a standard positive control. Mice received the extract or vehicle and after three hours of receiving the inflammatory stimulus (or PBS), the hypernociceptive response was measured. The withdrawal response frequency was calculated following ten applications of von Frey hairs (VFH, Stoelting). Previous investigations demonstrated that 0.6 g VFH produced a mean withdrawal frequency of about 15% (Quintao et al. 2011). This result was considered adequate for the measurement of mechanical hypernociception.

#### **Statistical analysis**

For GC/MS analysis five samples were measured on three independent times. Mann-Whitney U test was used for statistical analysis. Data of antinociceptive effect are represented by the mean  $\pm$  S.E.M. values and were analyzed by means of Analysis of Variance (ANOVA). Whenever ANOVA was significant, Dunnet's Multiple Comparison Test was used additionally for multiple comparisons as the post hoc test. All analyses were carried out using GraphPad Prism (version 5. GraphPad Software, Inc). The levels of statistical significance ranged from  $p < 0.05$  to  $p < 0.001$ .

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## **RESULTS AND DISCUSSION**

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GC/MS analysis of the ethanolic extract based on a comparison of retention indexes and mass spectra allowed the identification of 16 chemical compounds between 10 and 25 minutes (Fig. 1, Table 1). The major constituents were: 7,10,13 hexadecatrienoic acid, methyl ester (17.27%);  $\beta$ -sitosterol (14.04%);  $\alpha$ -amyrin (11.94%); 9,12,15 octadecatrienoic acid, methyl ester (10.14%);  $\beta$ -amyrin (7.82%) and vitamin E (8.44%). Also, the study revealed the presence of stigmastan-3,5-dien (2.25%), friedelin (4.15%), stigmasterol (3.86%) and various fatty acid derivatives.

To discard possible nonspecific effects of the extract on locomotor activity and motor coordination or on muscle relaxation the animals were tested on the rota-rod and open field. Mice treated with the extract failed to show any detectable alteration (data not shown) when compared to control vehicle-treated animals. Hence, any nociceptive behavior observed should not be directly associated with nonspecific actions.

The first selected test to assess the antinociceptive effects of *C. minor* L. extract was the acetic acid-induced writhing test that has been commonly used as a screening model for evaluating the analgesic properties of new agents (Gupta et al., 2015). The oral pretreatment with *C. minor* extract (100, 150 and 300 mg/kg) significantly decreased the number of writhing induced by the i.p. injection of acetic acid by 28.6, 46.8 and 53.4%, respectively (Table 2). This antinociceptive effect was dose-dependent and was not comparable with sodium diclofenac (75.5%). Abdominal pain induced by acetic acid injection has been attributed to several events. Protons can depolarize sensory neurons by directly activating a non-selective cationic channel localized on cutaneous, visceral and other types of peripheral afferent C fibers. Acetic acid also stimulates the release of endogenous mediators including cytokines, serotonin, prostaglandins, bradykinin and lipoxygenases products (Davies et al., 1984; Le Bars et al., 2001) that acting together stimulates the nociceptive neurons transmitting the pain and injury messages to the brain. An efficient antinociceptive mechanism in visceral pain is the inhibi-

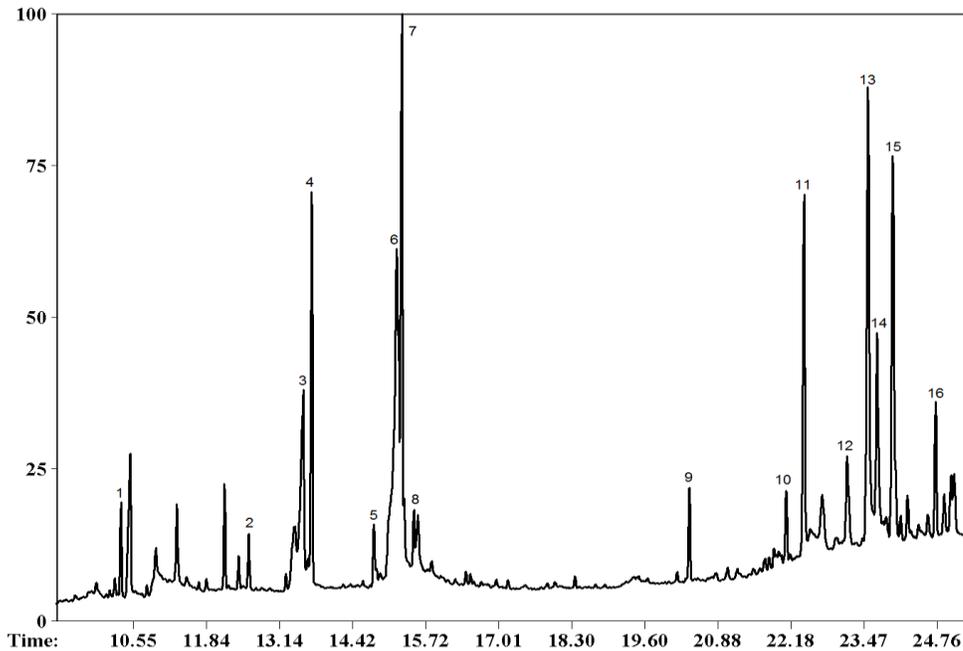
tion of prostaglandin synthesis (Collier et al., 1968), however, substances possessing a variety of mechanism including central or peripheral action inhibit writhing reaction (Bertollo et al., 2006; Tang et al., 2007; Miranda et al., 2014; Wu et al., 2014). Thus, the antinociceptive effect demonstrated by *C. minor* extract in this model does not determine whether or not the antinociceptive activity was mediated by peripheral or central action.

In consequence, formalin-induced nociception test was used due to this test can distinguish pain in its peripheral and central components. The early phase is caused by the direct activation of primary afferent sensory neurons, while the second phase is originated by local inflammation due to the release of inflammatory mediators such as cytokines eicosanoids and nitric oxide (Abbott et al., 1995; Bertollo et al., 2006). Both phases are sensitive to centrally acting drugs, like opioid; however, the

second phase is also inhibited by corticosteroids and non-steroid anti-inflammatory drugs (NSAID) particularly when the formalin is injected at high concentrations (Shibata et al., 1989). These results showed that the extract had a significant antinociceptive activity in both neurogenic (Fig. 2A) and inflammatory (Fig. 2B) phases. However, the first phase was inhibited only by the highest dose of the extract (300 mg/kg, 52.4%;  $p < 0.01$ ), whereas the second phase was inhibited by all the doses tested, although higher doses appeared to be most effective when they were compared to control (decrease 58.5%;  $p < 0.001$ ). The reference drug, sodium diclofenac was only effective in the second phase. Hence, it seemed likely that the antinociceptive activity of *C. minor* extract could be partly associated with the inhibition of different inflammatory mediators and the involvement at the peripheral level.

**Table 1.** Chemical constituents identified in ethanolic extract from leaves of *C. minor* L. by GC/MS.

Peak	Constituents	RT (min)	Relative abundance (% $\pm$ SD)
1	Ethyl citrate	10.36	1.35 $\pm$ 0.032
2	Neophytadiene	12.59	0.85 $\pm$ 0.029
3	Hexadecanoic acid	13.50	6.99 $\pm$ 0.053
4	Hexadecanoic acid, ethyl ester	13.70	6.7 $\pm$ 0.016
5	Phytol	14.80	1.15 $\pm$ 0.035
6	7,10,13 Hexadecatrienoic acid, methyl ester	15.19	17.27 $\pm$ 0.018
7	9,12,15 Octadecatrienoic acid, methyl ester	15.31	10.14 $\pm$ 0.041
8	Octadecanoic acid, ethyl ester	15.51	1.46 $\pm$ 0.050
9	Squalene	20.38	1.59 $\pm$ 0.018
10	Stigmast-3,5-dien	24.731	2.25 $\pm$ 0.008
11	Vitamin E	22.11	8.44 $\pm$ 0.030
12	Stigmasterol	22.41	3.86 $\pm$ 0.011
13	$\beta$ -Sitosterol	23.18	14.04 $\pm$ 0.007
14	$\beta$ -Amyrin	23.55	7.82 $\pm$ 0.033
15	$\alpha$ -Amyrin	23.70	11.94 $\pm$ 0.044
16	Friedelin	24.74	4.15 $\pm$ 0.024



**Figure 1.** Chromatogram obtained by GC/MS analysis of ethanolic extract of *C. minor* L. leaves.

Numbers represent identified constituents in the extract (see Table 1).

**Table 2.** Effects of single oral administration of ethanolic extract of *C. minor* L. leaves on acetic acid, hot plate and capsaicin-induced nociception in mice.

Treatment	Doses (mg/kg)	Antinociceptive model		
		Acetic acid (number of writhings)	Hot plate (min)	Capsaicin (sec)
Control		37.50 ± 2.27	6.50 ± 1.47	101.8 ± 17.90
<i>Clusia minor</i> (p.o.)	100	26.83 ± 0.87*	11.04 ± 1.58	77.0 ± 4.60***
	150	19.17 ± 0.87**	10.62 ± 0.76	49.0 ± 5.26***
	300	15.67 ± 3.75***	14.62 ± 0.76***	17.5 ± 1.97***
Sodium diclofenac (i.p.)	10	9.16 ± 4.31***	-	-
	5	-	-	17.0 ± 3.48***
Morphine (s.c.)	10	-	19.15 ± 1.43***	-

Values are mean ± S.E.M., n = 6. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, significantly different from control. One-way ANOVA followed by Dunnett's Multiple Comparison Test. (-) no tested.

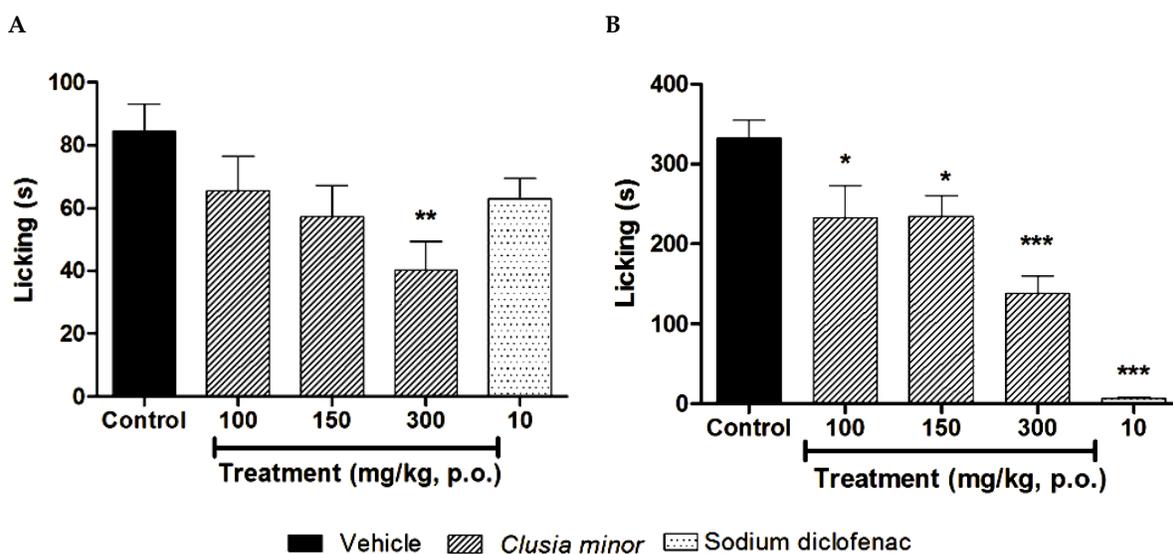
Hot plate test is a useful tool for analgesic drug screening as is largely insensitive to NSAID (Le Bars et al., 2001). Results demonstrated (Table 2) that only the higher dose of the extract increased the latency of the nociceptive behavior ( $p < 0.01$ ) as occurred with the opioid agonist morphine ( $p < 0.001$ ). Because the nociceptive response in the first phase of the hot plate and formalin test is

originated by direct activation of nociceptors by chemical and thermal stimuli (Tjølsen et al., 1992; Le Bars et al., 2001), these results suggest that *C. minor* extract possess antinociceptive activity probably of central origin at higher doses. This effect is similar than the opioid derived analgesics. However, the specific involvement of opioidergic pathway remains to be elucidated.

To obtain more results that confirm the antinociceptive effect of this extract, the model of capsaicin induced licking in the mouse paw was used. It is an appropriate model to investigate central/peripheral sensitization following the activation of vanilloid receptor 1 (TRPV1) present in primary sensory neurons (Fattori et al., 2016). Table 2 shows the results in this assay for the extract. As expected, capsaicin evoked a nociceptive paw licking behavior in mice that appeared maximal during the first 5 min, whereas the vehicle induced only a minimal paw licking. Interestingly, the ethanol extract of *C. minor* (100, 150 and 300 mg/kg), provoked a marked and dose-dependent antinociception of 24.4%, 51.8%, and 82.9%, respectively. Morphine given orally (5 mg/kg) also caused significant suppression of nociception to a similar extent that the higher doses of the extract (83.3%). Therefore, it could be hypothesized that *C. minor* should behave as an antagonist on the TRPV1 receptors. However, recent investigations have demonstrated that TRPV1 antagonists abolished both phases of formalin test and thermal stimulated nociceptive behavior in tail-flick test (Tang et al., 2007). Thus, these current data suggest that the

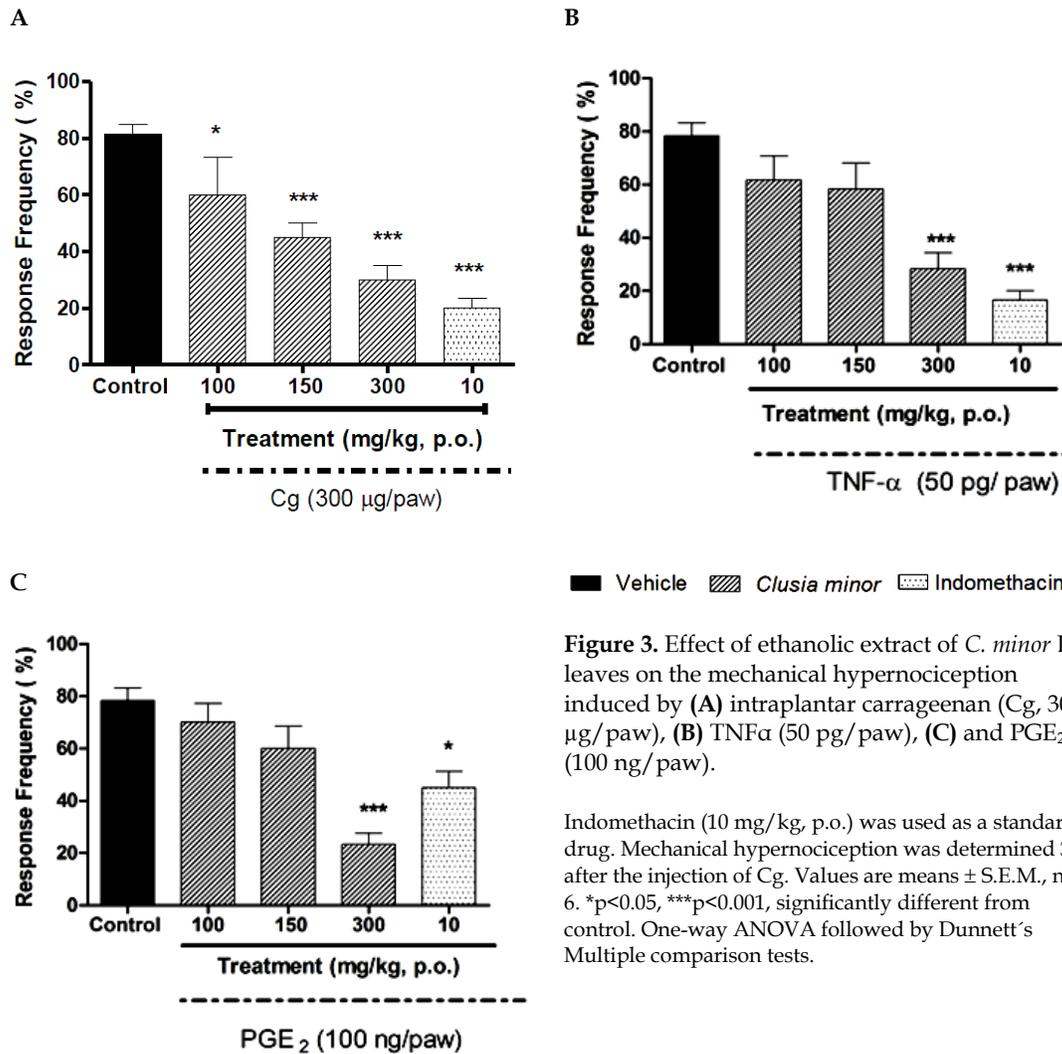
extract probably functions by inhibiting the cellular events that occurred after the activation of TRPV1 receptors rather than by blocking these receptors.

To corroborate the antinociceptive activity of *C. minor* extract, the effect in the model of mechanical hypernociception induced by carrageenan was evaluated using a model largely used to evaluate inflammatory hyperalgesia in mice. The inflammatory hypernociception triggered by carrageenan in mice is believed to be produced by a sequential cytokine cascade release, which is stimulated by activated neutrophils (Cunha et al., 2005). This provokes the liberation of TNF- $\alpha$  which triggers the production of IL- $\beta$  that play a decisive role in the release of prostanoids. Results showed (Fig. 3) that the oral administration of this extract (150 and 300 mg/kg) produced a significant inhibition of mechanical response by 55.0 and 62.3%, respectively. Treatment with indomethacin also produced a significant inhibition (75.5%). Oral administration of the extract (300 mg/kg) significantly antagonized the mechanical hypernociception induced by TNF- $\alpha$  (65.4%) and PGE<sub>2</sub> (71.5%).



**Figure 2.** Effects of acute oral administration of ethanolic extract of *C. minor* L. leaves on formalin test first phase (A) and second phase (B).

Sodium diclofenac (10 mg/kg, i.p.) was used as a positive control. Values are mean  $\pm$  S.E.M., n = 6. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, significantly different from control. One-way ANOVA followed by Dunnett's Multiple Comparison Test.



**Figure 3.** Effect of ethanolic extract of *C. minor* L. leaves on the mechanical hypernociception induced by (A) intraplantar carrageenan (Cg, 300 µg/paw), (B) TNF $\alpha$  (50 pg/paw), (C) and PGE $_2$  (100 ng/paw).

Indomethacin (10 mg/kg, p.o.) was used as a standard drug. Mechanical hypernociception was determined 3 h after the injection of Cg. Values are means  $\pm$  S.E.M., n = 6. \*p<0.05, \*\*\*p<0.001, significantly different from control. One-way ANOVA followed by Dunnett's Multiple comparison tests.

Taken together, the effect of this extract in the aforementioned experiment suggest that the antinociceptive effects of *C. minor* extract on inflammatory pain may be due to interference with the cytokine TNF $\alpha$  and PGE $_2$  synthesis or nociceptive action. Also, as neutrophil activation and migration participates in the cascade of events leading to mechanical hypernociception, the blockade of neutrophil migration by the extract should not be ruled out. Some of the identified constituents might contribute to the antinociceptive effects so far observed. For instance, triterpenoids including  $\alpha$  and  $\beta$ -amyrin and friedelin are known to exhibit antinociceptive and anti-inflammatory properties (Antonisamy et al., 2011; Quintans et al., 2014; da Silva et al., 2017). Besides, vitamin E may contrib-

ute to the antinociceptive action due to their anti-inflammatory and antioxidative effects (Tahan et al., 2011). These compounds may act reflecting possible synergistic actions to the antinociceptive activity.

## CONCLUSIONS

The present study demonstrated that the antinociceptive effects of *C. minor* leaves extract could take place due to the interaction of different mechanisms and possibly happens via peripheral and central pathways modulating the neurogenic and inflammatory pain. However, because of its complex composition, these effects appear to depend on the dose administered. The exact mecha-

nisms by which the extract produced these effects are not completely understood. Additional assays designed to assess the involvement of the bioactive compounds are required to determine the precise mechanism of action and which metabolites are responsible for these pharmacological properties.

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## CONFLICT OF INTEREST

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The authors declare no conflict of interest.

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## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTION:

Contribution	Mangas R	Reynaldo G	Dalla Vecchia MT	Aver K	Piovesan LG	Bello A	Rodeiro I	Malheiros A	De Souza MM	Menéndez R
Concepts or ideas						x	x	x	x	
Design						x	x	x	x	
Definition of intellectual content						x	x	x	x	
Literature search	x									x
Experimental studies	x	x	x	x	x				x	
Data acquisition	x	x	x	x	x				x	
Data analysis	x	x				x	x	x	x	x
Statistical analysis	x	x							x	x
Manuscript preparation	x	x				x	x	x	x	x
Manuscript editing	x	x							x	x
Manuscript review	x	x	x	x	x	x	x	x	x	x

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