# Antinociceptive and anti-inflammatory activity of extracts and α-pyrones isolated from Cantinoa stricta

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# Abstract

The present study aimed to evaluate the composition of the crude extracts obtained from the leaves of *Cantinoa stricta* (Lamiaceae) and explore the antinociceptive and anti-inflammatory activity of these extracts and two isolated compounds: anamarine (ANA) and 10-*epi*-olguine (eOL). Different extracts were obtained from the leaves of *C. stricta* and compared by NMR data. Crude ethanolic extract (EEt) and dichloromethane extract (DCM) were selected for the pharmacological tests in mice. The oral administration of EEt and DCM in male Swiss mice significantly reduced the second phase of formalin-induced nociception, lipopolysaccharide (LPS)-induced mechanical hyperalgesia, and carrageenan (Cg)-induced edema. ANA and eOL, the major compounds in EEt and DCM extracts, administered orally or locally (in the paw), also reduced the LPS-induced mechanical hyperalgesia and the Cg-induced edema without changing the thermal acute nociception or the motor performance of the animals. These isolated compounds did not change the mechanical hyperalgesia induced by tumor necrosis factor-α, interleukin-1β, prostaglandin E2, dibutyryl cyclic AMP, or forskolin but reversed the hyperalgesia induced by dopamine, epinephrine, and phorbol 12-myristate 13-acetate. The mechanical hyperalgesia induced by epinephrine was reversed in male but not in female mice, in which this response is not dependent on protein kinase C. These results suggest that *C. stricta* extracts possess antinociceptive and anti-inflammatory activity which is, at least partially related to the presence of ANA and eOL. Differently from the known analgesics, these substances seem to exert their action mainly interfering with the sympathetic component of pain, possibly with protein kinase C.


# Introduction

The search for new compounds with anti-inflammatory and antinociceptive activity is still in evidence nowadays since the available drugs show some important side effects. For instance, non-steroidal anti-inflammatory drugs (NSAID) are often related to serious gastrointestinal disturbances and cardiovascular effects (Grosser et al, 2017), while opioids are related to drug abuse and even deaths due to respiratory depression (Montandon & Slutsky, 2019).

Lamiaceae is the sixth largest plant family, with 236 genera and around 7300 species. In Brazil, the family is represented by 71 genera and 595 species, of which the majority belong to the subtribe Hyptidinae, including the genus *Cantinoa*, which comprises 25 species (Antar et al, 2020; Bridi et al, 2021; Buchoski, 2020). Plants of this genus have been used in folk medicine for the treatment of stomach disorders, diarrhea, liver disorders, malaria, wounds, infectious diseases, inflammations, and also as insect repellent. Essential oils, diterpenes, triterpenes, and α-pyrones have been reported for *Cantinoa* species. The essential oil of *C. mutabilis* showed antiviral and antiprotozoal activities, while antinociceptive, anti-ulcer, and anti-inflammatory activities were reported for *C. americana* extract, which also showed sedative and anti-convulsant properties (Bridi et al, 2021). Therefore, the chemical and pharmacological investigation of *Cantinoa* species can provide new insights for therapeutics, including regarding the antinociceptive and anti-inflammatory activity of these plants.

*Cantinoa stricta* (Benth.) Harley & J.F.B. Pastore, formerly known as *Hyptis stricta* (Lamiaceae) is an aromatic herb, native to Brazil, where it is distributed in the South region of the country (Buchoski, 2020). In an early study, the cytotoxic activity of ethanolic extract of this plant against human tumor cell lines HT-29 (colon) and NCI-H460 (lung, no small cells) was reported, but the active compounds were not identified (Monks et al, 2002). Subsequently, it was demonstrated that the essential oils from the leaves and flowers of *C. stricta* exhibited cytotoxic activity against several human tumor cell lines. The major components of the essential oil leaves were identified as caryophyllene oxide (31.6%) and *cis*-pinane (15.4%), while the flower oil contained mainly caryophyllene oxide (21.7%), *cis*-pinane (9.7%), α-pinene (9.4%) and β-pinene (9.1%) (Scharf et al, 2016). Conversely, the essential oil from the leaves of other population of *C. stricta* showed *E*-caryophyllene (24.1%), bicyclogermacrene (22.3%), and germacrene D (10.8%) as main constituents, and the diterpene kaurenoic acid was detected in this exudate (Bridi et al, 2021). The present study reports, for the first time, the phytochemical composition of extracts of *C. stricta* leaves. Additionally, the antinociceptive and anti-inflammatory effects of extracts and isolated compounds were evaluated as well as their mechanisms of action were investigated.

## Materials and methods

# General procedures in phytochemical analysis

Optical rotation was measured in $CHCl_3$ on a JASCO PTC-203 polarimeter at a temperature of 20 °C and a wavelength of 589 nm. One-dimensional ($^1H$, $^{13}C$) and two-dimensional (HSQC, HMBC) NMR spectra were recorded on Bruker spectrometers (AC 200 and/or Avance 600) observing $^1H$ at 200, or 600 MHz, and $^{13}C$ at 50 or 150 MHz. Deuterated chloroform ($CDCl_3$) was used as the solvent, and the chemical shifts are given in ppm (δ), with coupling constants (*J*) in Hz. TMS (tetramethylsilane) was used as the internal reference. Vacuum liquid chromatographic (VLC) and open column chromatographic separations (CC) were carried on silica gel 60 (Merck, 230–400 mesh). Silica gel 60 $GF_{254}$ plates (Macherey-Nagel®) were used for analytical TLC (0.25 mm), while silica gel 60 $PF_{254}$ (Macherey-Nagel®) was used for preparative TLC (PTLC, 1.0 mm). Compounds were visualized by exposure under $UV_{254/365}$ light and spraying with 5% (v/v) $H_2SO_4$ in EtOH solution, followed by heating on a hot plate. All solvents were analytical or spectroscopic grade, and the mixtures of solvents were prepared as v/v.

# Plant material

*Cantinoa stricta* (Benth.) Harley & J. F. B. Pastore was collected in Curitiba, Paraná State, Brazil (25º30'22" S; 49º18'30" W), in February/2011. The plant was collected and identified by Elide P. Santos, who deposited a voucher specimen in the Herbarium of Jardim Botânico do Rio de Janeiro (RB 01363056). The study of this plant was registered on the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under number A19F875.

# Extract preparation and isolation of anamarine and 10-epi-olguine

For the preliminary biological assays, dried and powdered leaves (102.0 g) were extracted with ethanol, at room temperature (three times, 500 mL each extraction). The solvent was removed under reduced pressure to give the crude ethanol extract (EEt, 7.18 g). For the isolation of chemical constituents, dried and powdered leaves (375.0 g) were extracted, in Soxhlet, with petroleum ether (30–60°C), dichloromethane (DCM), and ethanol, sequentially, for six hours, each solvent (1.5 L, each one). After the solvent removal, the extracts yielded 6.20 g (petroleum ether), 13.01 g (DCM), and 6.09 g (ethanol). Since the comparison among the obtained spectra in $^{1}$H NMR revealed that EEt and DCM had the same profile, containing two secondary metabolites as major components, DCM was used for further analysis. An aliquot of DCM (10.0 g) was submitted to VLC eluted with hexanes (Hex), followed by mixtures of Hex-acetone (98:2, 9:1, 8:2, 1:1), and finally pure acetone, to give seven fractions ($DCM_{1-7}$). An aliquot (1.0 g) of $DCM_7$ (1.69 g) was submitted to CC, eluted with mixtures of Hex-acetone (7:3, 1:1), yielding nine subfractions after TLC analyses ($DCM_{7.1-9}$). Fraction $DCM_{7.2}$ (114.0 mg) was purified by PTLC (Hex:acetone 3:2), followed by recrystallization in Hex-dichloromethane, to give 10-*epi*-olguine (eOL) (32.2 mg). $DCM_{7.3}$ (560.8 mg) furnished impure anamarine (ANA). An aliquot (80.5 mg) was recrystallized in Hex-dichloromethane, yielding pure ANA (53.1 mg) (Fig. 1).

# Pharmacological studies

# Animals

Male and female Swiss mice (25–45 g) were housed under a 12 h light/dark cycle with controlled humidity (60–80%) and temperature (22 ± 1°C), with food and water provided *ad libitum*. All procedures were approved by the institution's Ethics Committee for Animal Use (no. 937) and are following the Brazilian and International regulations for animal use. All efforts were made to optimize the number of animals used and to reduce their stress and suffering.

# Drugs

Carrageenan (Cg, Fraction V), lipopolysaccharide from *E. coli* 0111:B4 (LPS), indomethacin (IND), dexamethasone (DEX), dipyrone (DIP), prostaglandin $E_2$ ($PGE_2$), dopamine (DOP), epinephrine (EPI), forskolin (FSK), dibutyryl cyclic adenosine monophosphate (dbcAMP), and phorbol 12-myristate13-acetate (PMA) were purchased from Sigma Chemicals (St. Louis, MO, USA). Tumor necrosis factor α (TNF-α) and Interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN, USA). Fentanyl (FEN) and Diazepam (DZP) were of commercial grade. All the other reagents were of analytical grade.

# Formalin-induced nociception

The procedure was described previously (Lomba et al, 2017). A formalin solution (2.5% in 0.9% sterile saline, 20 µL) was injected into the sub-plantar region of the right hind paw of the animals. The time that the animals spent licking or elevating the formalin-injected paw in seconds was measured from 0–5 min (first phase, neurogenic pain) and 15–40 min (second phase, inflammatory pain) after formalin injection.

# Mechanical hyperalgesia

The mechanical threshold was measured using an electronic von Frey anesthesiometer (Bonther, Ribeirão Preto, SP, Brazil) as described previously (Lomba et al, 2021; Soares et al, 2017). The mice were first acclimated for 1 h in individual clear Plexiglas boxes (9 × 7 × 11 cm) on an elevated wire mesh platform to allow access to the plantar surface of the hindpaws. Pressure was applied to the hind paw of each animal and the mechanical threshold was determined as the corresponding pressure (in g) when the animal exhibited a stereotype flinching response and attempted to remove the foot from the apparatus. The basal threshold was then calculated by the mean of three similar measurements taken in 15 s intervals, from the maximum of 5 measurements. The threshold was measured again as described above 1, 3, and 5 h after an injection of the algogenic stimulus. For further analysis, the intensity of hyperalgesia was calculated by the area under the curve from the change in the mechanical threshold over time and was expressed in arbitrary units (A.U.).

# Carrageenan-induced paw edema

The procedure was described previously (Lomba et al, 2017; Soares et al, 2017). Carrageenan (300 µg, 20µl) was injected into the sub-plantar region in the right hind paw of the mice. The contralateral paw received only saline and was used as a control. Paw thickness (in micrometers) was measured using a digital micrometer (MT-045B, Metal Great Tools Co., Shanghai, China) before any treatment and at 1, 3, and 5 h after the injection of Cg. For further analysis, the intensity of edema was calculated by the area under the curve from the change in the paw thickness over time and was expressed in arbitrary units (A.U.).

# Acute thermal nociception

The procedure was described previously (Lomba et al, 2017; Soares et al, 2017) using a hot plate apparatus (Bonther, Ribeirão Preto, SP, Brazil). This test consisted of exposing the mice to a 54±1 °C metal plate in an acrylic box. The temperature threshold was reached when the animal withdrew or licked its hind paws, and the time (in seconds) of exposure to the hot plate was recorded. The cutoff time was 30s. Hot plate latencies were then converted to a percentage of maximal possible effect (%MPE): %MPE = (postdrug latency – basal latency)/(cutoff time – basal latency) × 100.

# Motor performance

A rota-rod apparatus (Ugo Basile, Gemonio, VA, Italy) was used to evaluate the possible muscle relaxation or motor incoordination effects produced by the extracts and compounds in the animals (Lomba et al, 2017; Soares et al, 2017). The animals were selected based on the ability to remain on the device's rotating bar for more than 180 seconds at 22 rpm. Subsequently, animals received the treatments and were submitted to rota-rod again. The time that the animals remained on the rota-rod, till a maximum of 180 s, was recorded.

# Experimental protocols

# Antinociceptive and anti-inflammatory effects of extracts

The antinociceptive effect of the EEt and DCM were evaluated by formalin-induced nociception and mechanical hyperalgesia. For formalin-induced nociception, the animals were treated with EEt (10, 30, or 100 mg/kg), DCM (50 mg/kg), the positive control IND (5 mg/kg), or the same volume of vehicle (Tween 80 0.1% in water) by oral route. After 1 h, the animals received an Intraplantar (i.pl.) injection of formalin into the hind paw, and the nociceptive behavior was evaluated. For LPS-induced hyperalgesia, the animals were treated with EEt (10, 18, or 30 mg/kg), DCM (15 mg/kg), the positive control IND (5 mg/kg), or the same volume of vehicle (Tween 80 0.1% in water) by oral route. After 1 h, the animals received LPS *E.coli* 0111:B4 (100 ng, 20 µl, i.pl.) or vehicle (saline). The mechanical threshold was evaluated as described before.

The anti-inflammatory effect of the EEt and DCM were evaluated by Cg-induced edema. The animals were treated with EEt (10, 30, or 100 mg/kg), DCM (50 mg/kg), the positive control DEX (1 mg/kg), or the same volume of vehicle by oral route. After 1 h, the animals received Cg (300 µg, 20µl, i.pl.) into the right hind paw, and paw thickness was evaluated as described before.

# Antinociceptive and anti-inflammatory systemic effects of isolated compounds

The antinociceptive effect of the isolated compounds ANA and eOL administered systemically was evaluated by mechanical hyperalgesia. The animals were treated with ANA (1, 1.8, or 3 mg/kg), eOL (1, 3, or 10 mg/kg), the positive control IND (5 mg/kg), or the same volume of vehicle (Tween 80 0.1% in water) by oral route. After 1 h, the animals received an i.pl. injection of LPS (100 ng) or vehicle (saline). The mechanical threshold was evaluated as described before. To further investigate the antinociceptive effects of ANA e eOL, the highest doses were also tested in the acute thermal nociception. The animals were treated with ANA (3 mg/kg), eOL (10 mg/kg), the positive control FEN (100 µg/kg, s.c.), or the same volume of vehicle by oral route. Three hours after ANA or eOL administration or 15 min after FEN administration, the animals were tested in the hot plate apparatus. Since both experiments are based on motor-associated behavior, the motor performance was also tested using the highest doses. The positive control for this experiment was DZP (5 mg/kg, s.c.). Three hours after ANA or eOL administration or 15 min after DZP administration, the animals were tested in the rota rod apparatus.

The anti-inflammatory effect of the ANA and eOL were evaluated by Cg-induced edema. The animals were treated with ANA (3 mg/kg), eOL (10 mg/kg), the positive control DEX (1 mg/kg), or the same volume of vehicle by oral route. After 1 h, the animals received Cg (300 µg, 20µl, i.pl.), into the right hind paw. Paw thickness was evaluated as described before.

# Antinociceptive and anti-inflammatory local effects of isolated compounds

The antinociceptive effect of ANA and eOL administered locally was evaluated by mechanical hyperalgesia. The animals were treated with ANA (30, 90, or 300 ng), eOL (100, 300, or 1000 ng), the positive control IND (150 ng), or the same volume of vehicle (20 µl, Tween 80 0.1% in saline) injected directly in the paw. After 15 minutes, the animals received an additional i.pl. injection of LPS (100 ng) or vehicle (saline). The mechanical threshold was evaluated for 5 h. To confirm the local effect, in another group of animals, the highest doses of ANA and eOL were injected in the contralateral paw to the one where LPS was injected. The mechanical threshold was evaluated in the LPS-injected paw for 5 h.

The local anti-inflammatory effect of the ANA and eOL were evaluated by Cg-induced edema. The animals were treated with ANA (300 ng), eOL (1000 ng), DEX (130 ng), or the same volume of vehicle (Tween 80 0.1% in saline) injected in the paw. After 15 minutes, the animals received Cg (300 µg, 20µl, i.pl.), into the sub-plantar region of the right hind paw. Paw thickness was evaluated for 5 h as described before.

To clarify possible mechanisms for the antinociceptive effects of ANA and eOL, the animals were treated with the highest doses of isolated compounds, or the adequate positive control IND (150 ng) or DIP (320 µg), or the same volume of vehicle (20 µl) directly in the paw. After 15 min, the animals received an i.pl. injection of TNF-α (1 pg), IL-1β (100 pg), $PGE_2$ (100 ng), dbcAMP (5 µg), DOP (3 µg), EPI (100 ng), FSK (1 µg), PMA (50 pmol), or the same volume of vehicle (saline, 20 µl). The mechanical threshold was evaluated as described before. Since these experiments suggested a mechanism affected by sexual dimorphism, an additional experiment using EPI was also done in male and female mice for comparison.

# Statistical analysis

The results are presented as mean ± standard error of the mean (s.e.mean) of the time spent expressing the nociceptive behavior induced by formalin (s), the mechanical threshold (g), the intensity of hyperalgesia (A.U.), the paw thickness (µm), the intensity of edema (A.U.), the % MPE, or the motor performance (s). The data were evaluated by repeated measures two-way analysis of variance (ANOVA) when the time course was evaluated or by one-way ANOVA for the remaining data using GraphPad Prism version 10.01 (San Diego, CA, USA). All of these analyses were followed by the *post-hoc* Bonferroni test. In all cases, differences were considered significant when $p < 0.05$. $IC_{50}$ was also calculated using GraphPad Prism, by using the log of the dose vs. % of the response (intensity of hyperalgesia).

## Results

# Phytochemical study

Crude EEt and DCM, petroleum ether, and ethanol extracts obtained sequentially were analyzed by $^1H$ NMR. The obtained spectra revealed that EEt and DCM had the same profile, containing two major compounds (Fig. S1). These compounds were lacking in the other extracts, whose $^1H$ NMR spectra showed only signals typical of long-chain aliphatic compounds (Fig. S1). Therefore, DCM extract was

selected for chromatographic fractionation, and the purified compounds were analyzed by NMR 1D ($^{1}H$, $^{13}C$) and 2D (HSQC, HMBC) techniques. These procedures led to the isolation of two known pyrones, identified as ANA and eOL (Fig. 1) by comparison of their NMR data with literature (Gao & O'Doherty, 2005; Lu et al, 1997). Figures S2 to S8 show the NMR 1D ($^{1}H$, $^{13}C$) and 2D (HSQC, HMBC) spectra for ANA and eOL identification.

# Antinociceptive and anti-inflammatory activity of EEt and DCM

Oral treatment with EEt, in all doses, and DCM, similarly to the positive control IND, did not change the first phase but reduced by around 60% the second phase of the formalin-induced nociception (Fig. 2A). The reduction in phase II, suggested an effect over inflammatory responses and has prompt us to test the effect of EEt and DCM on mechanical hyperalgesia and edema. The administration of vehicles did not change the mechanical threshold or the thickness of the paws of the animals (Fig. S9). Conversely, the i.pl. injection of LPS induced a significant reduction in the mechanical threshold (mechanical hyperalgesia) from 3 to 5 h after the injection (Fig. S9A). EEt (18 and 30 mg/kg) and DCM (15 mg/kg) and the positive control IND, but not EEt 10 mg/kg, reduced the LPS-induced mechanical hyperalgesia (Fig. S9A). The analysis of the intensity of hyperalgesia was used for a comparison of the overall change in the mechanical hyperalgesia and showed that the treatment with EEt at doses of 10, 18, and 30 mg/kg produced a dose-dependent effect (0, 40, and 90% reduction, respectively, Fig. 2B). DCM also abolished the mechanical hyperalgesia (Fig. 2B). The i.pl. injection of Cg induced an increase in the paw thickness 1 h after the injection that remained significantly higher compared to control until 5 h (Fig. S9B). The oral treatment with EEt (10, 30, or 100 mg/kg), DCM (50 mg/kg), or DEX, significantly reduced the Cg-induced edema from the third hour after the injection of Cg until the end of the experiment (Fig. S9B). The overall edema reduction for the extracts and positive control was around 25–40% (Fig. 2C). The oral treatment with EEt and DCM at the highest doses (100 and 50 mg/kg, respectively) did not increase the latency time in the hot plate test or reduce the motor performance in the rota-rod test (data not shown).

# Antinociceptive and anti-inflammatory activity of isolated compounds

Initially, ANA and eOL were given by oral route. The administration of vehicles did not change the mechanical threshold of the animals (Fig. 3A-D). The doses of the isolated compounds to be given by oral route were based on the yielding. ANA at doses of 1.8 and 3 mg/kg (Fig. 3A and B) and eOL at doses of 3 and 10 mg/kg (Fig. 3C and D) reduced the LPS-induced mechanical hyperalgesia while the lower dose of 1 mg/kg of both compounds did not cause significant changes in this response. Similarly, the administration of IND abolished the LPS-induced hyperalgesia (Fig. 3A-D). Based on the intensity of hyperalgesia shown in Figs. 2B and 2D, doses response curves were built and the $IC_{50}$ was calculated (Fig. 3E). ANA showed an $IC_{50}$ of 1.9 mg/kg and eOL showed a two times higher $IC_{50}$ of 3.9 mg/kg, when given orally. The oral treatment with ANA and eOL at the highest effective antinociceptive dose in the

mechanical hyperalgesia test (3 and 10 mg/kg, respectively) did not increase the latency time in the hot plate test (Fig. 2F) or reduce the motor performance in the rota-rod test (Fig. 2G).

The compounds were then injected directly into the animal's hind paw. The administration of vehicles did not change the mechanical threshold of the animals (Fig. 4A-D). ANA at doses of 30, 90, and 300 ng/paw dose-dependently reduced the mechanical hyperalgesia induced by LPS (Fig. 4A and B). eOL at doses of 300 and 1000 ng/paw reduced the mechanical hyperalgesia induced by LPS while the lower dose of 100 ng did not cause a significant change in this response (Fig. 4C and D). Similarly, the administration of IND abolished the LPS-induced hyperalgesia (Fig. 4A-D). Based on the intensity of hyperalgesia shown in Figs. 3B and 3D, ANA showed an $IC_{50}$ of 93.4 ng and eOL showed a seven times higher $IC_{50}$ of 677.3 ng when injected directly in the inflammatory site (Fig. 4E). To confirm that ANA and eOL at these doses were not having a systemic effect, the highest doses of the compounds were injected in the contralateral paw (left hind paw). As observed in Fig. 3F, while the injection of the compounds in the same paw as LPS reduced the hyperalgesia, the injection of ANA and eOL in the contralateral paw did not change this response. The local treatment with the highest doses of ANA, eOL, or DEX, significantly reduced the Cg-induced edema from the third hour after the injection of Cg until the end of the experiment (Fig. S10A). Fig S10B shows that ANA, similarly to DEX, reduced the intensity of edema by 40% while eOL significantly reduced 23% of the edema.

Despite being very effective in reducing LPS-induced hyperalgesia, both substances did not modify the hyperalgesia induced by TNF-α (Fig. 5A) or IL-1β (Fig. 5B), two of the cytokines most commonly related to the nociceptive effect of LPS. ANA and eOL also did not modify the hyperalgesia induced by $PGE_2$ (Fig. 5C), FSK (Fig. 5F), and dbcAMP (Fig. 5G) but were effective in abolishing hyperalgesia induced by the sympathomimetic amines DOP and EPI (Fig. 5D and 5E, respectively). Since previous studies demonstrated an essential difference between males and females regarding EPI-induced hyperalgesia, we also evaluated the antinociceptive effect of ANA and eOL in this response in male and female mice. While the treatment with ANA and eOL abolished EPI-induced mechanical hyperalgesia in males these were ineffective in mechanical hyperalgesia in females (Fig. 6).

# Discussion

Plants of the genus *Cantinoa* have been used in inflammatory and infectious diseases and it has been shown that some species possess antinociceptive and anti-inflammatory activity (Bridi et al, 2021). In this study, we show the isolation of two pyrones ANA and eOL from *C. stricta* and the anti-nociceptive and anti-inflammatory activity of the extracts and isolated compounds.

EEt and DCM extracts obtained from *C. stricta* had the same profile in NMR, containing two secondary metabolites as major components. Our initial studies had shown that the EEt and DCM extracts had shown pharmacological activity, particularly reducing formalin-induced nociception, LPS-induced hyperalgesia, and Cg-induced edema. Considering these two sets of results, DCM extract was selected for chromatographic fractionation. These two major compounds were purified and identified as two known

pyrones: ANA (Gao & O'Doherby, 2005) and eOL (Lu et al., 1997). This is the first time that these pyrones were identified in *C. stricta*.

EEt showed a similar antinociceptive effect in all the doses tested in formalin-induced nociception suggesting that the maximum effect had been reached at the lower dose (10 mg/kg) in this model. This is a quite low dose for a crude extract in the formalin-induced nociception test compared to other plants (Hajhashemi et al, 2018; Kassuya et al, 2009; Moniruzzaman et al, 2015; Santos et al, 2017) suggesting the presence of substances with potent antinociceptive activity in both EEt and DCM. Since EEt and DCM only reduced the inflammatory phase of formalin-induced nociception the effect on mechanical hyperalgesia was tested. Differently from the formalin test, in the mechanical hyperalgesia, EEt showed a clear dose-response effect, while DCM, at the dose used, abolished the LPS-induced hyperalgesia indicating a potent effect of these extracts in reducing the sensitization of nociceptive neurons. Additionally, none of the extracts changed the latency time in the hot plate test or affected the motor performance in the rota-rod test suggesting that the antinociceptive effect was not related to an effect in the central nervous system, nor the responses observed in the nociceptive tests were due to a motor impairment. Both EEt and DCM extracts significantly reduced the edema induced by Cg, even though this reduction was less pronounced than that observed for nociceptive responses. This result suggests that the main compounds in these extracts are acting on pain-related mechanisms. Taken together these results confirmed that the EEt and DCM possess an important anti-inflammatory and antinociceptive activity, mainly related to inflammatory pain and by blocking the sensitization of the nociceptive terminal.

As mentioned before, from DCM extract two compounds were isolated, ANA and eOL, which were evaluated for antinociceptive and anti-inflammatory activity. For eOL, the doses were increased because the proportion between ANA and eOL was 4:1, as calculated by integration in the $^{1}$H NMR spectrum of DCM extract. Both compounds showed a dose-dependent anti-nociceptive effect when administered by oral route with ANA being two times more potent in reducing mechanical hyperalgesia than eOL ($IC_{50}$ of 1.9 mg/kg and 3.9 mg/kg, respectively), also without affecting the motor performance in the rota-rod test. These results suggest that ANA and eOL are, at least in part, the active compounds responsible for the anti-nociceptive activity observed in the EEt and DCM and they are reducing the sensitization of nociceptive fibers. The same compounds also seem to be responsible for the anti-inflammatory activity (reduction of edema) since the reduction in the Cg-induced edema promoted by these compounds was similar to the one observed for EEt and DCM.

When these compounds were injected directly in the animal´s hind paw they also showed anti-nociceptive activity with lower $IC_{50}$ ($IC_{50}$ of 93.4 ng for ANA and $IC_{50}$ of 677.3 ng for eOL) and anti-inflammatory activity. This effect was confirmed to be local since, when the treatment was done in the contralateral paw, no antinociceptive activity was observed.

The identification of the local effect of ANA and eOL allowed us to study in more detail its mode of action. Therefore, the effect of local administration of these compounds was evaluated in the mechanically induced hyperalgesia by different algogenic stimuli. It is known that IL-1β and TNF-α are

two of the cytokines most commonly related to the nociceptive effect of LPS, particularly in inducing mechanical hyperalgesia (Cunha et al, 2000; Cunha et al, 2005). Surprisingly, despite being effective in reducing LPS-induced hyperalgesia, both compounds ANA and eOL did not modify the hyperalgesia induced by TNF-α and IL-1β. Several studies demonstrate that these cytokines induce the synthesis of COX-2 (Basbaum et al, 2009; Samad et al, 2001; Schäfers et al, 2004) with consequent generation of prostaglandins that are among the main inducers of inflammatory hyperalgesia (Ferreira et al, 1973; Samad et al, 2001). The antinociceptive effect of NSAID such as aspirin and IND, is related to the blockade of this enzyme and therefore the synthesis of prostaglandins (Ferreira et al, 1973). Our positive control IND was effective in blocking the hyperalgesia induced by both cytokines. Therefore, these data suggested that ANA and eOL have a different mechanism of action than classic NSAID. On the other hand, some studies suggest that LPS-induced hyperalgesia is not dependent only on prostaglandin synthesis but has another important component, the sympathetic component (Calil et al, 2014; Cunha et al, 2005). Both components are capable, among other mechanisms, of activating adenylate cyclase and promoting the increase of cAMP within the nociceptive terminal. This increase in cAMP promotes, mainly through the activation of protein kinase A (PKA), activation of sodium channels Nav1.8 and Nav1.9, responsible for ultimately the entry of $Na^+$ into the terminal and consequent reduction in the threshold of excitement that characterizes the hyperalgesic state (Cunha et al, 1999; Dina et al, 2001; Kawabata, 2011). Indeed, our results demonstrate that the administration of $PGE_2$, of the sympathomimetic amines DOP and EPI, from the adenylate cyclase activator FSK and of the stable form of cAMP, dbcAMP induced hyperalgesia in the animals. In these experiments, we used as a positive control the DIP, which although classified as a NSAID, has additional mechanisms of action. It has already been demonstrated that the local administration of DIP promotes the opening of ATP-sensitive potassium channels, allowing the $K^+$ output from sensitized cells. This output of positive charges is responsible for restoring the nociceptor threshold to levels before sensitization (Alves & Duarte, 2002). Thus, as shown in our experiments, DIP abolished the hyperalgesia induced by all these stimuli. Unlike DIP, ANA and eOL did not modify the hyperalgesia induced by $PGE_2$, FSK, and dbcAMP but were effective in abolishing hyperalgesia induced by the sympathomimetic amines DOP and EPI. These data suggest that the antinociceptive effect of these substances is not related to the prostaglandin component of mechanical hyperalgesia, but rather to the sympathetic component of this response.

Although both prostaglandins and sympathomimetic amines share a common mechanism for generating hyperalgesia involving increased cAMP in the nociceptive terminal, there are studies suggesting that hyperalgesia can be generated through other second messengers. In 2001, Dina et al. demonstrated that in male mice, while $PGE_2$ uses only PKA activation to generate hyperalgesia, EPI, through the activation of $\beta_2$ adrenergic receptors, activates PKA, mitogen-activated protein kinase/extracellular-signal related kinase kinase (MEK) and protein kinase Cε (PKCε) (Dina et al, 2001). PMA is a known PKC activator and previous studies have demonstrated that i.pl. injection of PMA induces hyperalgesia (Robilotto et al, 2022; Wandji et al, 2018). Our data corroborate this observation as the administration of PMA promoted a reduction in the mechanical threshold of the animals. The treatment of male mice with ANA and eOL abolished this hyperalgesic response. These data suggest that the effect antinociceptive activity of these

compounds is related to PKC activation during hyperalgesia. Therefore, it is not surprising that ANA and eOL do not affect pathways essentially related to PKA activation such as those activated after administration of PGE2, FSK, and dbcAMP. Interestingly, Dina et al. (2001) demonstrated a critical difference between males and females regarding the hyperalgesia-inducing pathways concerning EPI. While in males, as previously mentioned, EPI activated 3 pathways of signal transduction (PKA, MEK, and PKCε), in females, this agonist activates only the signal transduction pathway through MEK. Therefore, considering that the activation of PKCε would be the main target of ANA and eOL, these substances should not be effective in EPI-induced hyperalgesia in females. Corroborating this hypothesis, while the treatment of male mice with ANA and eOL abolished EPI-induced mechanical hyperalgesia in males these were ineffective in mechanical hyperalgesia in females. It is interesting to emphasize that PKC seems to be important to sensitize TRPV1 channels in inflammatory and chronic pain conditions (Pan et al, 2010; Robilotto et al, 2022) and therefore, an important target for new treatments for pain. It has been also shown that PKC is important in the upregulation of bradykinin B1 receptor induced by IL-1β and TNFα (Campos et al, 1999), suggesting that the inhibition of Cg-induced edema may also be related to these two compounds targeting PKC.

Taken together, the results presented here show that the EEt and DCM from *Cantinoa stricta* leaves possess an important antinociceptive and antiedematogenic activity. This antinociceptive activity is related to the reduction of inflammatory mechanical hyperalgesia, not being effective in reducing non-inflammatory acute pain, particularly of thermal origin. Two pharmacologically active α-pyrones ANA and eOL present in the extracts are related to the described activities and both, when administered orally or directly on the inflammatory site, were effective at particularly low doses. ANA is about 3 times more potent than eOL considering the tests used in this study. These substances do not act by mechanisms similar to other known anti-inflammatory drugs/analgesics such as NSAID, DIP, or opioids but the results suggest that they act by modulating the activity/action of PKCε involved in the sensitization of nociceptors and edema formation.

# Declarations


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### *Conflicts of interest*

None of the authors has any conflicts of interest to declare.

### *Data availability*

The datasets generated during the current study are available from the corresponding author on reasonable request.


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# Figures

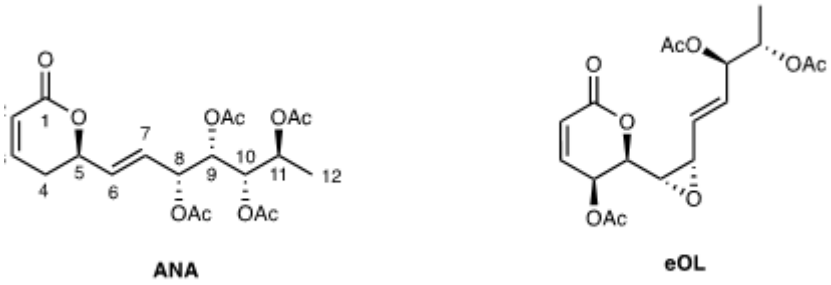
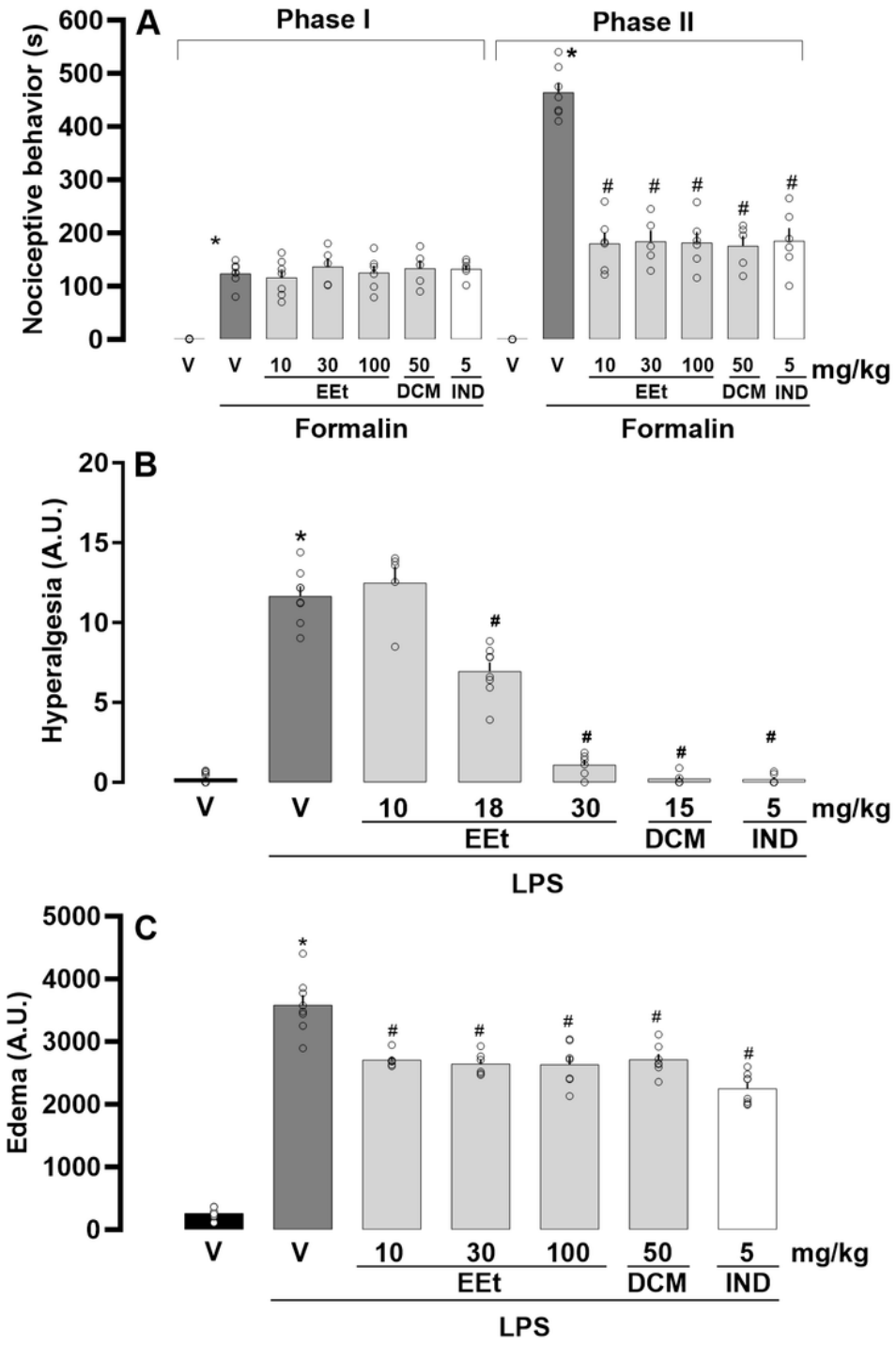



**Figure 1**

Chemical structure of anamarine (ANA) and 10-*epi*-olguine (eOL)




## Figure 2

Effect of oral treatment with EEt and DCM from *C. stricta* on the formalin-induced nociceptive behavior, LPS-induced hyperalgesia and Cg-induced edema. Mice were orally treated with EEt, DCM, indomethacin (IND), dexamethasone (DEX) or vehicle (V, Tween 80 0.1%) at the indicated doses. After 1 h they received an i.pl. (20 µl) injection of formalin (2.5%, panel A), LPS (100 ng, panel B) or Cg (300 µg) or the same volume of vehicle (V, saline). The time (s) performing the nociceptive behavior (licking and paw elevation)

was registered as phase I (5 min) and II (15-40 min) (Painel A). Panels B and C show the intensity of hyperalgesia or edema, respectively calculated as the area under de curve (arbitrary units, A.U.) from 0 to 5 h after the injection of LPS or Cg. Bars show the mean ± s.e.mean of 5-8 animals. Data were analysed by one-way ANOVA, followed by the Bonferroni test. Symbols denote difference in comparison with the V/V-treated group (*p<0.05) or V/stimulus group (#p<0.05).

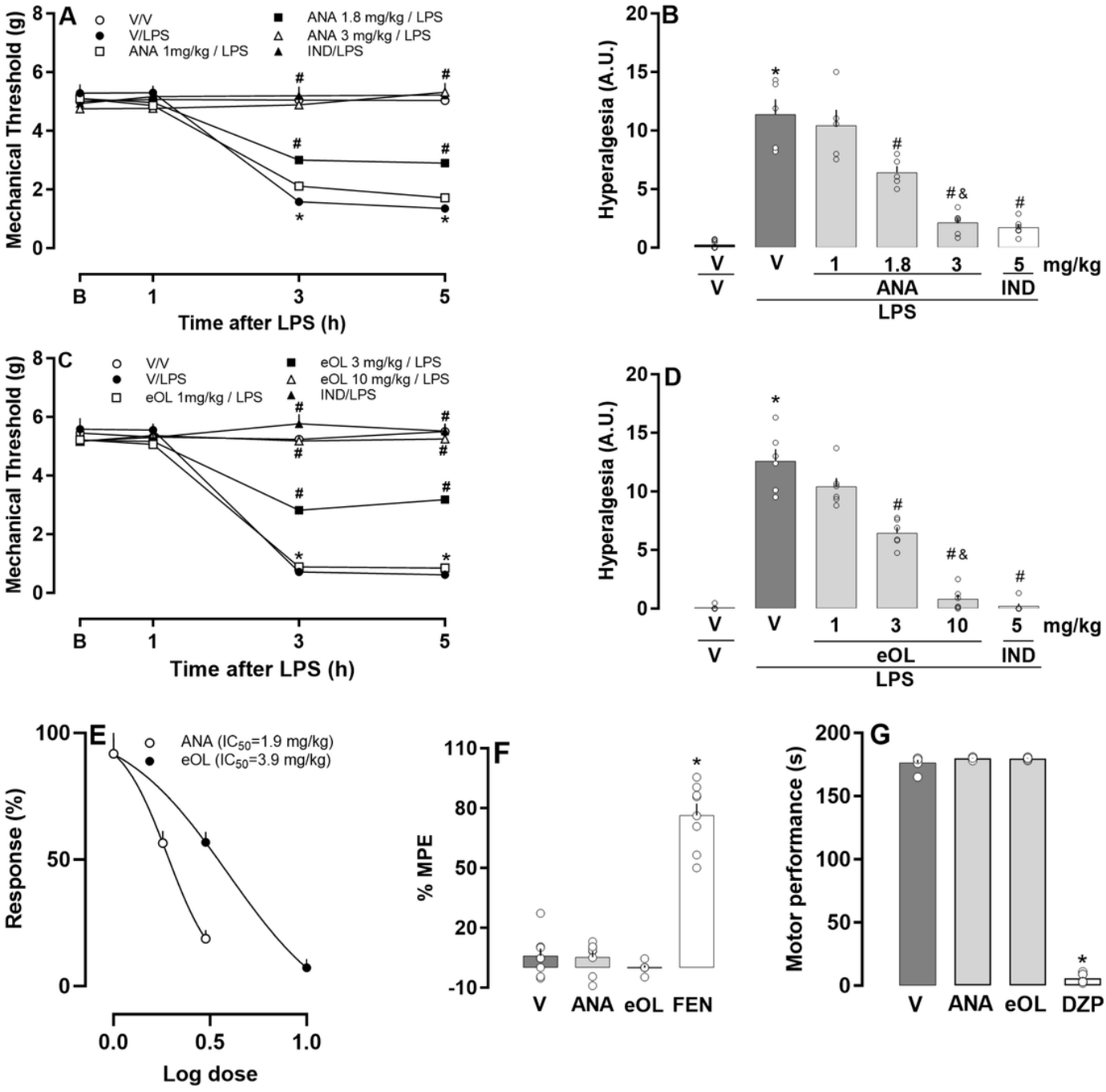
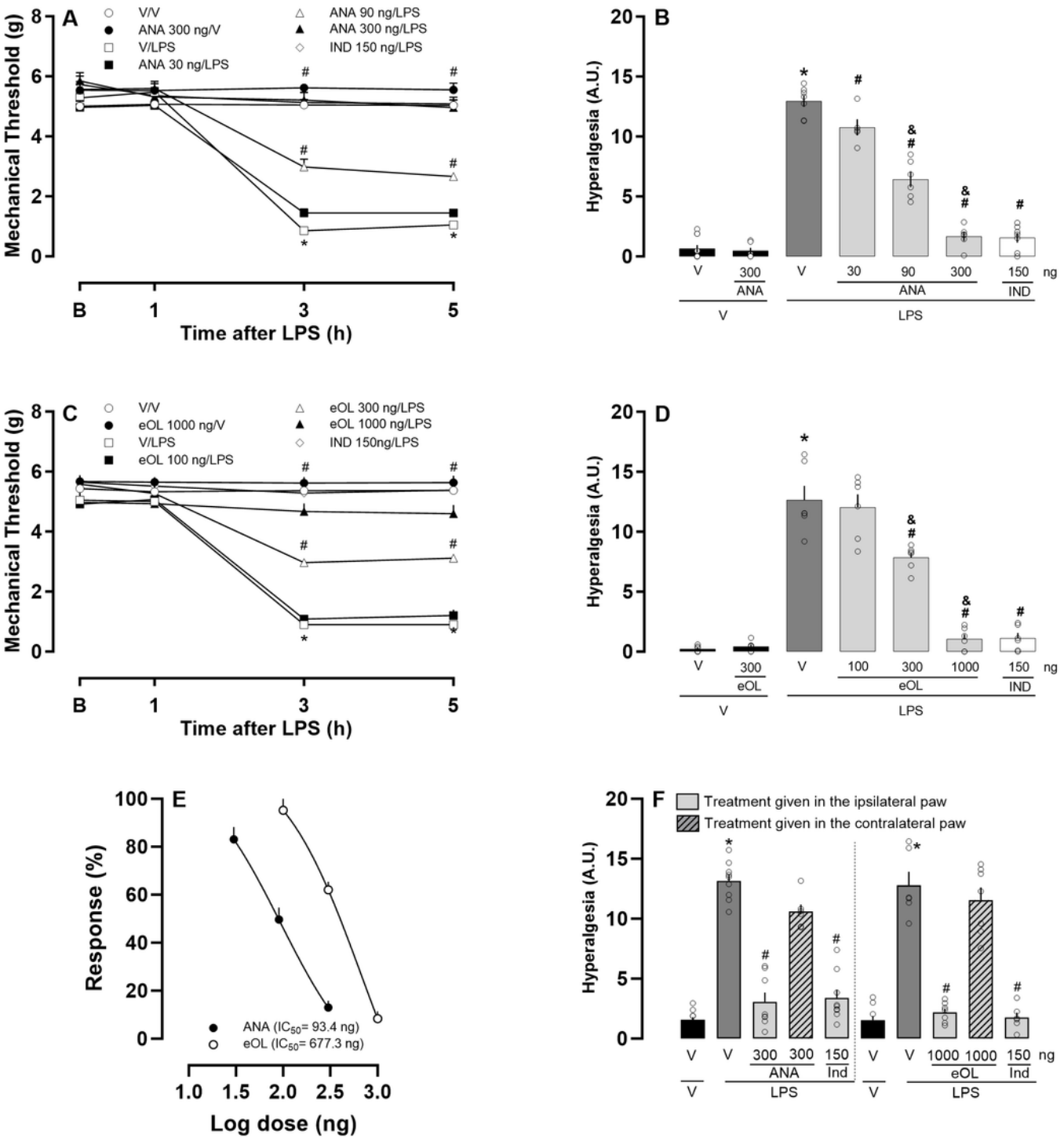



Figure 3

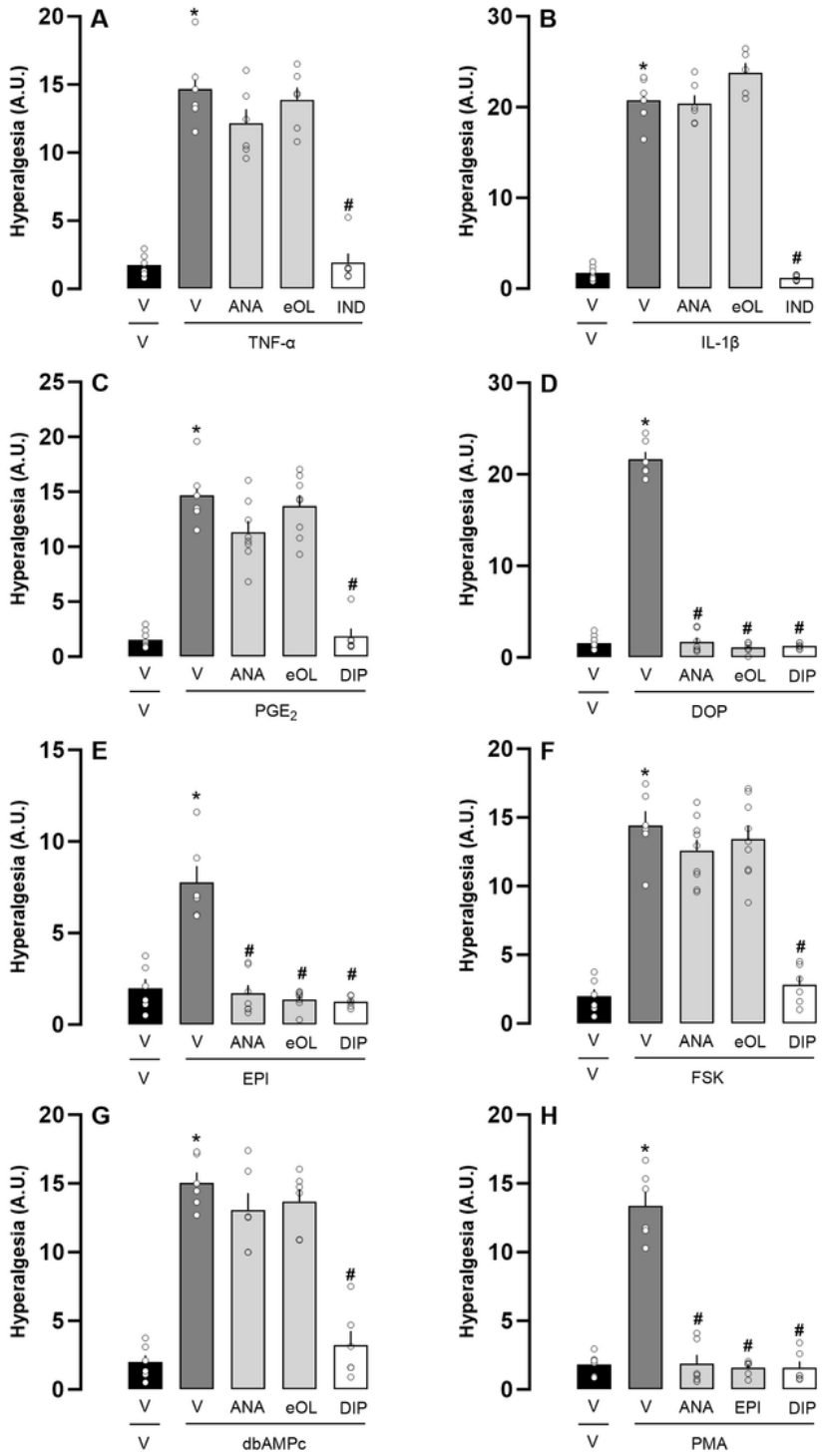
Effect of oral treatment with ANA and eOL from *C. stricta* on the LPS-induced hyperalgesia, acute nociceptive pain, and motor performance. Mice were orally treated with ANA, eOL at the indicated doses, indomethacin (IND, 5 mg/kg, v.o.), fentanyl (FEN, 100 µg/kg, s.c.), diazepam (DZP, 5 mg/kg, s.c) or vehicle (V, Tween 80 0.1%). In panels A to D, 1 h after the treatment animals received an i.pl. (20 µl) injection of lipopolysaccharide (LPS,100 ng) or the same volume of vehicle (V, saline). Panels A and C show the time course of the mechanical threshold and panels B and D the intensity of hyperalgesia calculated as the area under de curves from 0 to 5 h after the injection of LPS. Panel E shows the dose-response curve of the antinociceptive effect of ANA and eOL. Panes F and G show the effect of the highest doses of ANA and eOL on acute thermal nociception and motor performance. One hour after oral treatment with ANA or eOL or 15 min after subcutaneous FEN or DZP, the animals were submitted to the hot plate or to the motor performance test, respectively. Data show the mean + s.e.mean of mechanical threshold (g), intensity of hyperalgesia (arbitrary units, A.U.), response (%), the maximal possible effect (MPE, %) or the motor performance (s), (n = 6-10). Data were analysed by two-way repeated measures (A and C) or one-way (B, D, F, and G) ANOVA, followed by the Bonferroni test. Symbols denote difference in comparison with the V or V/V-treated group (*$p<0.05$), V/LPS group (#$p<0.05$) or1mg/kg/LPS group (&$p<0.05$).




## Figure 4

Effect of local treatment with ANA and eOL from *C. stricta* on the LPS-induced hyperalgesia. Mice received an i.pl. (20 µl) injection of ANA, eOL at the indicated doses, indomethacin (IND, 150 ng), or vehicle (V, Tween 80 0.1%). Fifteen min after the treatment they received an i.pl. (20 µl) injection of lipopolysaccharide (LPS,100 ng, panel B) or the same volume of vehicle (V, saline). Panels A and C show the time course of the mechanical threshold change and panels B and D the intensity of hyperalgesia

calculated as the area under de curves from 0 to 5 h after the injection of LPS. Panel E shows the dose-response curve of the antinociceptive effect of ANA and eOL. Panel F show the effect of the highest doses of ANA and eOL administered in the ipsilateral or in the contralateral paw or IND in the ipsilateral paw of the animals. Data show the mean + s.e.mean of mechanical threshold (g), intensity of hyperalgesia (arbitrary units, A.U.), or response (%), (n = 5-10). Data were analysed by two-way repeated measures (A and C) or one-way (B, D, and F) ANOVA, followed by the Bonferroni test. Symbols denote difference in comparison with the V or V/V-treated group (*p<0.05), V/LPS group (#p<0.05) or10 ng ANA/LPS or 100ng ng eOL/LPS group (&p<0.05).




## Figure 5

Effect of local treatment with ANA and eOL from *C. stricta* on the hyperalgesia induced by different stimuli. Mice received an i.pl. (20 µl) injection ANA (300 ng/paw), eOL (1000 ng/paw), indomethacin (IND, 150 ng/paw), dipyrone (DIP, 360 ng/paw) or vehicle (V, Tween 80 0.1%). Fifteen min after the treatment they received an i.pl. (20 µl) injection of TNF-α (1 pg, A), IL-1β (100 pg, B), $PGE_2$ (100 ng, C), DOP (3 µg, D), EPI (100 ng, E), FSK (1 µg, F) dbcAMP (5 µg), or PMA (50 pmol), or vehicle (saline). Data show the mean +

s.e.mean of the intensity of hyperalgesia (arbitrary units, A.U.), or response (%) (n = 6-9). Data were analysed by one-way ANOVA, followed by the Bonferroni test. Symbols denote difference in comparison with the V/V-treated group (*p<0.05) or V/stimuli group (#p<0.05).

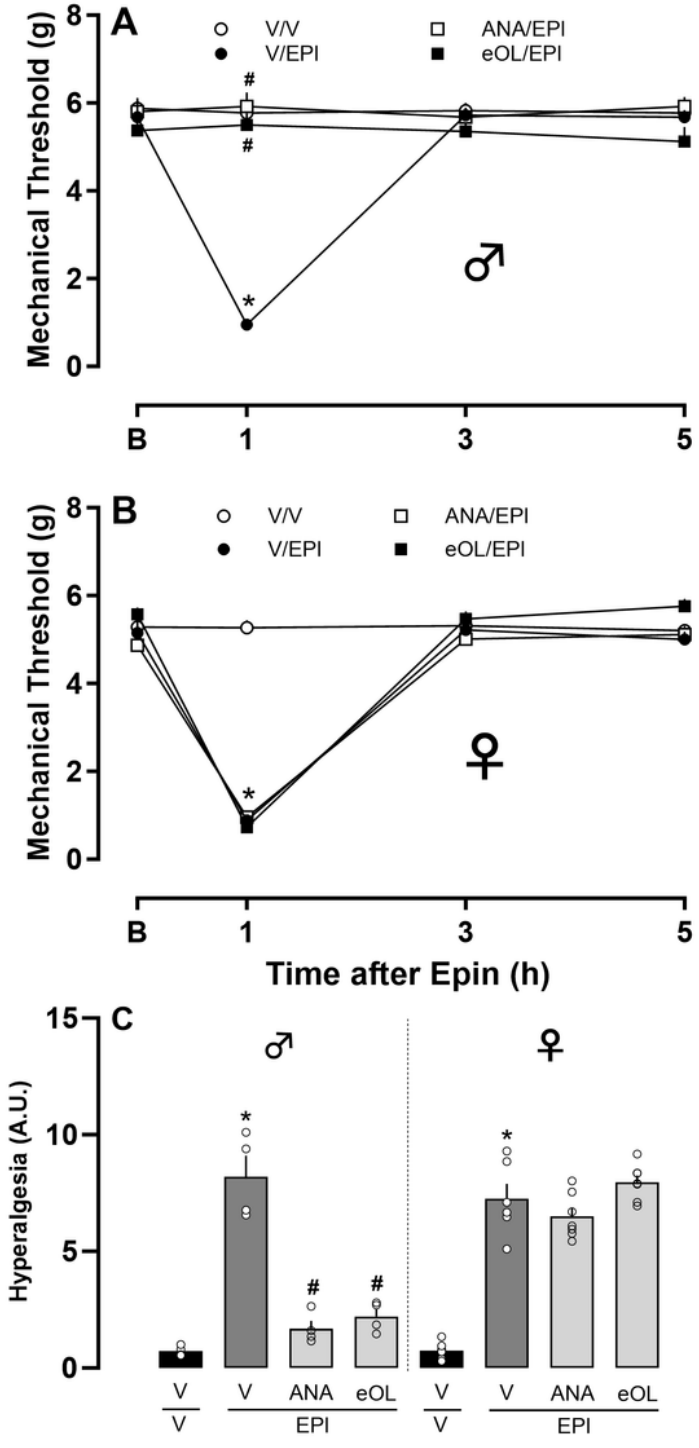



Figure 6

Effect of local treatment with ANA and eOL from *C. stricta* on the hyperalgesia induced by EPI in male and female mice. Male (A) and female (B) mice received an i.pl. (20 µl) injection of ANA (300 ng/paw), eOL (1000 ng/paw), or vehicle (V, Tween 80 0.1%). Fifteen min after the treatment they received an i.pl. (20 µl) injection of Epinephrine (EPI, 100 ng) or vehicle (V, saline) and the mechanical threshold was measured until 5 h. Data show the mean + s.e.mean of the mechanical threshold (g) or the intensity of hyperalgesia (arbitrary units, A.U.) (n = 4-6). Data were analysed by two-way repeated measures (A and B) or one-way (C) ANOVA, followed by the Bonferroni test. Symbols denote difference in comparison with the V/V-treated group (*p<0.05) or V/EPI group (#p<0.05).

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