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Effects of *Euphorbia characias subsp. characias* flower extracts on nociceptive pain and acute inflammatory models in mice.

Amalia Di Petrillo[a*], Francesca Pintus[b], Sonia Floris[b], Carlo Ignazio Giovanni Tuberoso[b], Ana Beatriz Alcantara Bérenger Samarcos de Almeida[c], Thais Biondino Sardella Giorno[c], Patricia Dias Fernandes[d] and Fabio Boylan[d]

[a] Department of Medical Science and Public Health, Gastroenterology Unit, University of Cagliari, Cittadella Universitaria di Monserrato, 09042 Monserrato, Cagliari, Italy. Electronic address: amalia.dip@unica.it.

[b] Department of Life and Environmental Sciences, University of Cagliari, SS 554-Bivio per Sestu, Monserrato, 09042 Cagliari, Italy. Electronic address: fpintus@unica.it; s.floris@unica.it; tuberoso@unica.it.

[c] Laboratório de Farmacologia da Dor e da Inflamação, Institute of Biomedical Sciences, Federal University of Rio de Janeiro, Brasil. Electronic address: bealcantara@gmail.com; thaissardella.ufrj@gmail.com.

[d] School of Pharmacy and Pharmaceutical Sciences, Panoz Institute and Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland. Electronic address: patricia.dias.icbufrj@gmail.com; fabio.boylan@tcd.ie.

* Corresponding Author:

Amalia Di Petrillo, PhD

Dep. of Medical Science and Public Health

ss554 bivio per Sestu – Cittadella Universitaria

09042, Monserrato, Italy

Email: amalia.dip@unica.it

Highlights

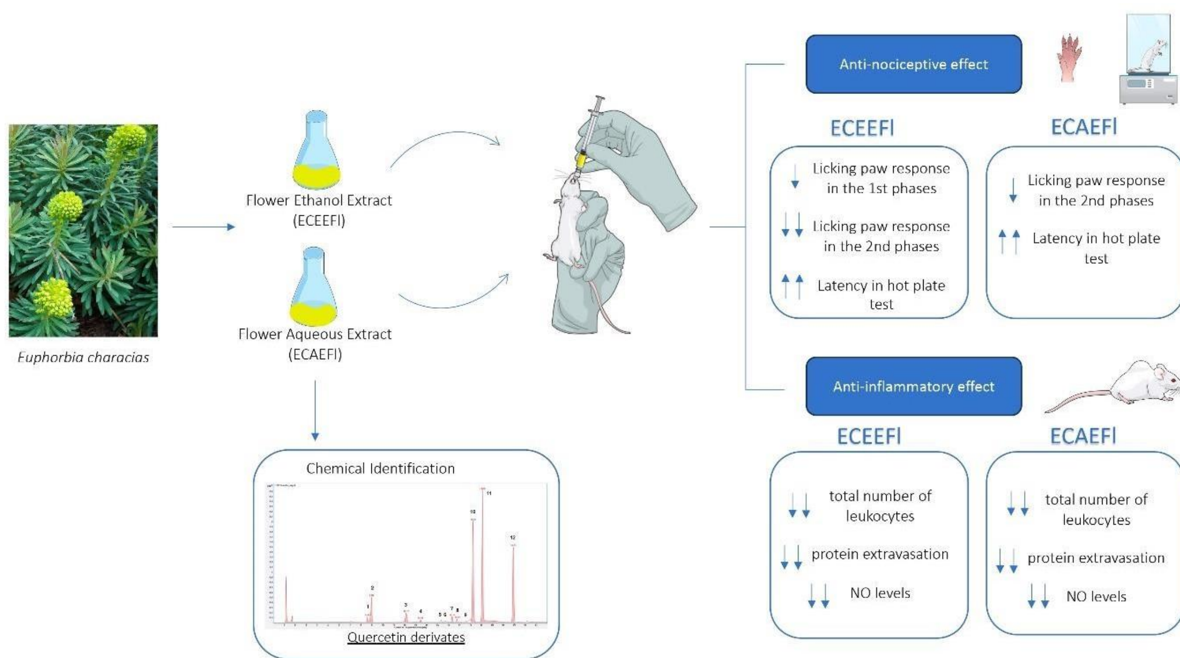
1. Euphorbiaceae family members have shown to possess analgesic, antinociceptive and anti-inflammatory properties.
2. *Euphorbia characias subsp.characias* flower extracts, rich in quercetin and glycosylated derivatives, offer valuable resources for natural product research.

3. These extracts demonstrate therapeutic potential by mitigating pain and inflammation through mechanisms involving primary afferent fiber sensitization and modulation of inflammatory mediators.

Abstract

Pain and inflammation are major health issues worldwide, leading to negative consequences. Despite several drugs being available to manage these conditions, their effectiveness can be limited by cost, adverse reactions, and potential tolerance and dependence with long-term use. *Euphorbia characias* traditionally used in folk medicine for its diverse biological activities - including antiproliferative, antimicrobial, and anti-inflammatory effects - has not been extensively studied *in vivo* for its analgesic and anti-inflammatory properties. In this study, the antinociceptive and anti-inflammatory properties of the water and ethanolic extracts of *E. characias* flowers (ECAEFl and ECEEFl) were evaluated using various models. Both extracts significantly reduced paw licking time in a formalin-induced paw licking model, with ECAEFl specifically targeting and ECEEFl affecting both the neurogenic and inflammatory phases. Additionally, in the carrageenan-induced cell migration model, both extracts showed a significant decrease in leukocyte migration, protein extravasation and nitric oxide levels, further demostrating their anti-inflammatory activity. High-Resolution HPLC-ESI-QTOF-MS-MS and HPLC-PDA analysis characterized the chemical composition of the extracts, identifying a significant presence of phenolic compounds, particularly quercetin and its derivatives, which likely contribute to the observed biological activities. These findings highlight the potential of *E. characias* extracts as natural sources of compounds with antinociceptive and anti-inflammatory properties. Further investigations are needed to elucidate the underlying mechanisms and explore their therapeutic potential in pain and inflammation-related disorders.

Graphical Abstract



Keywords

Euphorbia characias, anti-nociceptive, anti-inflammatory, identification

Abbreviations

AA	Antinociception
ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
AUC	Area under the curve
BCA	Bicinchoninic acid
BL	Baseline
CNS	Central nervous system
COX	Cyclooxygenase
Dex	Dexamethasone
DMSO	Dimethyl sulfoxide

ECAEFl	*E. characias* aqueous extracts of flowers
ECEEFl	*E. characias* ethanol extract of flowers
ESI	Electrospray ionization
HPLC-DAD	High-performance liquid chromatography coupled to diode-array detection
HPLC-ESI-QTOF-MS-MS	High- performance liquid chromatography with quadrupole time-of-flight mass spectrometry
HPLC-PDA	high- performance liquid chromatography/photodiode array detector
IB	Increase from the baseline
IM-QTOF	Ion Mobility Quadrupole Time-of-Flight
LC-DAD	Liquid chromatography -photodiode array
LC-MS	Liquid chromatography – Mass Spectrometry
LPH	Lactase-phlorizin hydrolase
NO	Nitric Oxide
SAP	Subcutaneous Air Pouch
SGLT1	Sodium-GLUcose Transporter 1
TCC	Total compound chromatogram
UPLC	Ultra-performance liquid chromatography

1. INTRODUCTION

Euphorbia characias is a plant species widely distributed throughout the Mediterranean region. This plant belongs to the family Euphorbiaceae and is commonly referred to as the "Mediterranean spurge"[1]. Members of the Euphorbiaceae family have long been utilized in traditional medicine for their various medicinal properties, particularly as analgesics, antinociceptives, and sedatives[2,3].

Moreover, they have demonstrated other biological activities, such as include antiproliferative, antitumour [4], antibacterial [5], anti-inflammatory[6], and antiviral properties [7].

E. characias, in particular, has shown antimicrobial, antifungal, and antioxidant properties in its aerial part extracts [1,8]. Latex, on the other hand, has been traditionally used for wound healing [6], and recent research has shown that it can stimulate the growth of new blood vessels, a crucial factor in promoting wound healing[6]. The diverse biological actions of *E. characias* are believed to be due to the presence of different bioactive constituents, such as diterpenoids, triterpenoids[9], volatile compounds[10], tannins, phytosterols, and flavonoids[11]. Among flavonoids, quercetin and its derivatives are the most abundant in the aerial part of *E. characias* [1,8].

E. characias, widely recognized in traditional medicine, has been extensively studied *in vitro*, revealing a broad range of bioactive properties. However, *in vivo* investigations into its analgesic and anti-inflammatory effects are notably sparse. *In vitro* research has shown significant anti-inflammatory activity by reducing increased capillary permeability, a common model for assessing anti-inflammatory responses, with the methanol extract of the plant demonstrating a notable inhibition rate of 34.74%. This anti-inflammatory effect has been partially attributed to the presence of quercetin derivatives such as quercetin-3-O-rhamnoside, quercetin-3-O-galactoside, and quercetin-3-O-arabinoside, isolated from the plant's extracts [6]. Additionally, bioactive compounds in *E. characias* have demonstrated the ability to modulate key enzymatic pathways involved in inflammatory processes. Notably, some constituents have been found to inhibit cyclooxygenase-2 (COX-2), an enzyme directly linked to the inflammation cascade and pain perception. Similarly, the plant extracts have shown potential in down-regulating matrix metalloproteinases (MMPs), enzymes that break down collagen and other matrix components during inflammation. [12]. Despite this, there remains a gap in the systematic exploration of its biochemical interactions *in vivo*, particularly concerning the comparative efficacy of different solvent extracts in established pain and inflammation models.

Pain and inflammation are major health issues worldwide, leading to various negative consequences, such as limitations in daily activities, decreased quality of life, and increased risk of mortality [13,14]. Despite several drugs being available to manage these conditions, their effectiveness can be limited by cost, accessibility, adverse reactions, and potential tolerance and dependence with long-term use[15,16]. New classes of potential analgesic drugs have been developed using chemical compounds from natural sources, such as some phenolics, known for their strong analgesic effects [17]. Additionally, phytochemicals such as quercetin and its derivatives have potent anti-inflammatory and antinociceptive effects[18,19], making them promising candidates with unique physiological and pharmacological properties.

Given the rich tradition of utilizing the Euphorbiaceae family for their analgesic, antinociceptive, and sedative properties, the aim of this study is to investigate the analgesic and anti-inflammatory properties of water and ethanol extracts from *E. characias* flowers, specifically assessing their bioactive compounds and mechanisms of action in nociceptive and acute inflammatory models in mice.

2. MATERIALS AND METHODS

2.1. Reagents, standards and drugs

All the chemicals used were of analytical grade. Ethanol, methanol, and 85% w/w phosphoric acid were procured from Sigma-Aldrich (Steinheim, Germany). LC-MS grade acetonitrile, formic acid, and water were obtained from Merck (Darmstadt, Germany). Standards of gallic acid, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, kaempferol-3-O-glucoside, and chlorogenic acid (5-O-caffeoylquinic acid) were sourced from Extrasynthese (Genay Cedex, France) and TransMIT (Giessen, Germany). Ultrapure water with a resistance of 18 MΩ·cm was generated using a Milli-Q Advantage A10 System apparatus (Millipore, Milan, Italy). Carrageenan (type IV), dexamethasone, and acetylsalicylic acid (ASA) were obtained from Sigma (St. Louis, MO, USA), while morphine sulfate was supplied by Cristália (São Paulo, Brazil).

2.2. Plant material

Flowers of *E. characias* were gathered in the southern region of Sardinia specifically in Dolianova, CA. The precise geographical coordinates of the collection site were 39° 24' 19.0'' N latitude and 9° 12' 57.6'' E longitude. A voucher specimen, identified as number 1216/16, was formally deposited in the Herbarium CAG, which is located within the Department of Life and Environmental Sciences at the University of Cagliari, Italy. Collected flowers were immediately frozen at −80 °C and then lyophilized. The lyophilized plant material (1 g) was extracted in water or ethanol (10 mL) in a dark environment and involved continuous stirring for a duration of 24 hours, all conducted at room temperature. The choice of water and ethanol as extraction solvents was informed by their differing polarities. Water, a polar solvent, efficiently extracts hydrophilic compounds, while ethanol, with its medium polarity, effectively extracts both hydrophilic and lipophilic compounds, ensuring a comprehensive extraction of the plant's bioactive constituents. To obtain a dry extract, the water extract was further subjected to lyophilization while the ethanol extract was evaporated in a rotary evaporator operating at 45 °C under a vacuum. Before use, stock solutions of the *E. characias* aqueous extracts of flowers (ECAEFl) and *E. characias* ethanol extract of flowers (ECEEFl) were prepared in water and dimethyl sulfoxide (DMSO), respectively, at a concentration of 100 mg/mL, and stored at -20° C.

2.3. High-Resolution HPLC-ESI-QTOF-MS-MS and HPLC-PDA Analysis

To qualitatively investigate the plant extracts, we employed an ion mobility Q-TOF LC/MS system, following the methodology described by De Luca et al.[20]. Our setup comprised a 1290 Infinity II UPLC equipped with a 6560 IM-QTOF instrument from Agilent Technologies Inc. (Palo Alto, CA, USA). We conducted these experiments using an electrospray ionization (ESI) source in negative ion mode, acquiring mass spectra in the m/z range of 40-1300. For chromatographic separation, we utilized a Kinetex EVO C18 column (150 × 2.1 mm, 1.7 µm 100Å, Phenomenex, Castel Maggiore, BO, Italy) and a mobile phase consisting of 0.1% formic acid and acetonitrile with 0.1% formic acid. The elution gradient was adjusted appropriately, with a flow rate of 0.3 mL/min, and 4 µL injections were

made. Data acquisition and processing were conducted using Agilent MassHunter Workstation Acquisition software v. B.09.00. The ESI/QTOF MS data were subjected to analysis using the molecular feature extraction algorithm of the MassHunter Workstation Qualitative Analysis software v. 10.0 (Agilent Technologies). Tentative identification of metabolites, along with the prediction of fragmentation patterns and molecular formulae, was performed using the MassHunter METLIN metabolite PCDL database B.08.00 (Agilent Technologies) and Sirius® software version 4.7.4 [21,22].

For the quantitative analysis of specific phenolic compounds, we employed an HPLC-DAD method as described by De Luca et al.[23]. This analysis utilized an Agilent 1260 Infinity II HPLC system equipped with an Agilent G4212B photodiode array detector (Agilent Technologies). Chromatographic separation was achieved using a Kinetex EVO C18 column (150 × 4.60 mm, 2.6 µm, Phenomenex) with a mobile phase consisting of 0.22 M phosphoric acid and acetonitrile, mixed in a gradient elution scheme, and maintained at a constant flow rate of 0.8 mL/min. Injections of 10 µL were made, and chromatograms and spectra were processed using the OpenLab V. 2.51 data system (Agilent Technologies). The quantification of phenolic compounds was categorized into main classes, including flavonols (detected at 360 nm), hydroxycinnamic acids (detected at 313 nm), and hydroxybenzoic acids (detected at 280 nm). For quantitative analysis, plant extracts were initially dissolved in a MeOH: H2O 80:20 v/v mixture, with a plant-to-solvent ratio of 1:50 w/v. These solutions were then diluted 1:1 v/v with 0.22 M H3PO4 and filtered through a 0.22 µm CA syringe filter before injection. The amounts of phenolic compounds were expressed as mg/g dry extract mass (dm).

2.4. Animals and treatments

Female Swiss Webster mice (28 - 35 g, 8 - 10 weeks), donated by the Animal Production Centre Laboratory of the Vital Brazil Institute (Niterói, RJ, Brazil) were kept in a well-ventilated room with a 12-hour light/dark cycle, controlled room temperature (22±2°C) and free access to water and feed. The approval of the ethics committee on Animal Use (UFRJ) was obtained before the start of the experiments (CEUA/UFRJ 31/19, 34/19 and 35/19).

Solutions of the ECAEFl and ECEEFl were administered to the animals orally (p.o.), 60 or 30 min before the pharmacological tests, at doses of 10, 30 or 100 mg/kg, in a final volume of 0.1 mL. The reference drugs used in the models were acetylsalicylic acid (ASA, 200 mg/kg, p.o.) and morphine (2.5 mg/kg), administered 60 minutes before or dexamethasone (2.5 mg/kg, i.p.) administered 15 minutes before experiments.

2.5. Acute Toxicity

The animals received oral treatment with ECAEFl or ECEEFl at the highest dose (100 mg/kg) while the control received distilled water (10 mL/kg). After 24 hours of treatment, the animals were euthanized with an overdose of ketamine (50 mg/kg) and xylazine (10 mg/kg). Their bone marrow was washed, to quantify total leukocytes, and the blood was collected to evaluate the hematological parameters.

Behavioral parameters (i.e. convulsion, hyperactivity, sedation, grooming, loss of righting reflexes, or increased or decreased respiration), as well as food and water intake, were observed over a 5-day period after a single oral dose of extracts.

2.6. Evaluation of antinociceptive activity

2.6.1. Formalin-induced licking model

The formalin test is characterized by being a model where peripheral nociception can be evaluated. This model is characterized by a biphasic response, where an initial phase can be identified shortly after the injection lasting the first 5 minutes (first phase, pain due to neurogenic response) and a late phase lasting from 15 to 30 minutes (second phase, pain due to inflammatory response) [24,25].

The animals were orally treated with vehicle (distilled water), ECAEFl or ECEEFl (10, 30 or 100 mg/kg), acetylsalicylic acid (200 mg/kg) or morphine (2.5 mg/kg) 60 minutes before formalin injection into the left hind paw of each mouse. The time spent in licking response of the injected paw was recorded an indicative pain.

2.6.2. Hot Plate Test

The hot plate test was performed through the thermal nociceptive stimulus aiming to identify central effect analgesic substances [26,27]. In this model, two control measurements (C1 and C2) were taken with a 30-minute interval, and their average referred to as baseline (BL), was calculated. Thirty minutes after the second control measurement, vehicle (distilled water), ECAEFl or ECEEFl (10, 30 or 100 mg/kg) or morphine (2.5 mg/kg) were orally administered to mice. Animals were then placed on a hot plate (55 ± 1°C) and observed at 30-minute intervals for 180 minutes post-treatment. Reaction time, indicated by paw licking, was recorded.

Antinociceptive effect was evaluated as % antinociception (% AA) using the formula: $A(\%)$ = (Experimental latency – Control latency/$cut{-}off-$ Control latency) x 100. Antinociception was also quantified as the area under the curve (AUC) from 30 minutes post-drug administration until the end of the experiment. AUC was calculated using the trapezoid rule: AUC=30×BL [(min 30)+(min 60)+…+(min 180)/2], where IB is the increase from the baseline (in %).

2.7. Evaluation of anti-inflammatory activity

2.7.1. Carrageenan-induced Cellular Migration into the Subcutaneous Air Pouch (SAP)

The SAP model was adapted according to Raymundo et al.[28]. Ten mL of sterile air was injected subcutaneously into the back of the animals to form a sterile cavity. After three days, 8 mL of sterile air were injected to maintain the preformed cavity. On the sixth day, the animals were orally treated with the vehicle (distilled water), ECAEFl or ECEEFl (10, 30 or 100 mg/kg) or dexamethasone (2.5 mg/kg, intraperitoneally) and one hour after treatment, the animals were divided into two groups: one received injection of 1 mL of sterile saline into the cavity while the other received injection of 1 mL of carrageenan (0.5%).

After 24 hours of injection into the SAP, the animals were anaesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) intraperitoneally, and the cavity was washed with 1 mL of sterile saline.

From the exudates collected, the total leukocyte count was performed with subsequent centrifugation at 1,500 rpm, for 8 minutes, at 4° C. The supernatants were collected and stored at -20 ºC for dosages.

2.7.2. Nitrate and Protein Measurement

The production of nitrate in the exudate was evaluated followed by the Griess reaction[29], according to the method described by Bartholomew [30] and adapted by Raymundo et al.[28].

Protein quantification in exudate was through the colorimetric method with bicinchoninic acid (BCA) using the commercial protein kit BCA™ Protein Assay (ThermoScientific, NY, USA). Absorbance was measured using a microplate reader (FlexStation 3, Molecular Devices), at 562 nm.

2.8. Statistical analysis

The data were analyzed using a one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test, utilizing GraphPad Prism® version 8.0 (GraphPad Software, San Diego, CA, USA). The results were subsequently presented as mean values with standard deviation (SD). Statistical significance was defined as p-values less than 0.05 ($^{*}p < 0.05$).

3. RESULTS

3.1. Quali-quantitative determination of phenolic compounds in *E. characias* flower extracts

In a previous study [31], we have already characterized phenolic compounds in ECEEFl using (HR) LC-ESI-QToF MS/MS in negative ion mode. Additionally, targeted phenolic compounds were quantified using LC-photodiode array (LC-DAD) analysis.

In ECAEFl, the negative LC-MS profile highlighted the presence of different phenolic derivatives, namely hydroxybenzoic and hydroxycinnamic acids and flavonoid derivatives (**Figure 1**). Compounds were identified by comparison of their *m/z* values in the total compound chromatogram (TCC) profile with fragmentation patterns reported in the literature for the same analytes or with the fragmentation patterns and spectra reported in a public repository of mass spectral data [22,32]. **Table 1** reports the 12 identified compounds, listed according to their retention times, the chemical formula derived by accurate mass measurement, MS/MS results, mass error (Δ ppm), the references used for

identification and the identification confidence levels [33]. **Table 1** also reports the quantitative analysis performed by LC-DAD analysis, and polyphenols were quantified at 360 nm for flavonols, 313 nm for hydroxycinnamic acids and 280 for hydroxybenzoic acid.

Compounds **1** and **4** were tentatively identified as gallotannin, gallic acid derivatives. Peak **1** was identified as digalloyl glucose due to the $[M-H]^-$ at *m/z* 483.0780 and fragments at *m/z* 271.0477, 211.0251 and 169.0107 (acid gallic unit)[34]. Peak **4** was identified as trigalloyl glucose due to the $[M-H]^-$ at *m/z* 635.0876 and a fragment at *m/z* 313.0560. Gallic acid derivatives were previously reported in *Euphorbia hirta* and *Euphorbia heterophylla* aerial parts studies[34]. Trigalloyl glucose amount resulted to be more than 15 times higher than digalloyl glucose (3.59 ± 0.12 and 0.23 ± 0.01 mg/g dm, respectively). Peak **2** was attributed to chlorogenic acid due to the $[M-H]^-$ at *m/z* 353.0878 and a fragment at *m/z* 191.0557 (quinic acid unit). This compound was confirmed by comparison with pure standard; its amount was 7.02 ± 0.41 mg/g dm. Peaks **1**, **2**, and **4** were not previously detected in *E. characias* flowers. Peak **3** with $[M-H]^-$ *m/z* 951.0724 was attributed to an unknown compound with the molecular formula $C_{23}H_{36}O_{40}$, confirming what was previously reported[31].

The most representative class of compounds was found to be flavonols (compounds **5** to **12**), accounting for 70% of dosed target compounds (**Table 1**). Compound **5** was tentatively attributed to isorhamnetin diglucoside with $[M-H]^-$ *m/z* 639.1564 and a fragment at *m/z* 315.1264 [35]. Compound **6** was tentatively identified as kaempferol diglucoside due to $[M-H]^-$ *m/z* 609.1459 and a fragment at *m/z* 285.0412 [35]. Peaks **7-12** were identified as quercetin derivatives by the diagnostic $[M-H]^-$ ions shown in HR ESI-MS analysis and the comparison of their fragmentation profiles with literature. Compounds **7** corresponding to $C_{21}H_{20}O_{12}$ with $[M-H]^-$ at *m/z* 463.0897 and fragments at *m/z* 300.0272, 301.0333 was identified as quercetin hexoside [8,31]. Peak **8** was attributed to quercetin-3-*O*-glucoside due to $[M-H]^-$ at *m/z* 463.0897 and fragments at *m/z* 301.07, 300.0239 [31,36] , and it was confirmed through the comparison with a pure standard.

Peaks **9** and **10** corresponding to $C_{20}H_{18}O_{11}$ were identified as quercetin pentosides due to $[M-H]^-$ at *m/z* 433 and fragments at *m/z* 301 and 300. Compounds **9** and **10** were tentatively attributed to quercetin xyloside and quercetin arabinoside, respectively, by comparisons with literature data. Compound **11** was attributed to quercetin-3-*O*-rhamnoside due to $[M-H]^-$ at *m/z* 447.0937, fragments at *m/z* 301.0347, 300.0283 [31,36]. This compound was confirmed by comparison with pure standard and its amount was 10.02 ± 0.49 mg/g dm, resulting the most abundant phenolic compound in *E. characias* flower extracts. Peak **12** was identified as a quercetin pentoxide derivative due to $[M-H]^-$ at *m/z* 475.0883 and fragment at *m/z* 300.0279. From the comparison with literature data [31,36] the compound was tentatively identified as quercetin acetyl-arabinoside.

We compared the compounds found in ECAEFl with those in ECEEFl found by Fais et al. [31]., as shown in **Table 2**. The results demonstrate that the most abundant compounds in both extracts are derivatives of quercetin, in fact, both extracts contain quercetin-3-*O*-rhamnoside and quercetin arabinoside. In the ECAEFl extract, the predominant compound is quercetin-3-*O*-rhamnoside, followed by quercetin-3-*O*-arabinoside. Their concentrations were 10.02 ± 0.49 and 9.12 ± 0.37 mg/g dm, respectively.

On the other hand, in the ECEEFl extract, the most abundant compound is quercetin-3-(2-*O*-acetyl)-arabinoside, followed by quercetin-3-*O*-rhamnoside and quercetin-3-*O*-arabinoside. These compounds exhibited concentrations of 47.89 ± 1.30, 36.62 ± 0.94, and 21.70 ± 0.68 units, respectively.

Table 1. Identification of polyphenolic compounds in *E. characias* flower extracts using (HR) LC-ESI-QToF MS/MS in negative ion mode and quantification by LC-DAD (mg/g dry extract mass, mean ± SD; $n = 3$)

	Putative identification	**RT (min)**	**mg/g dm (mean ± SD)**	**[M-H]$^-$ *m/z***	**Molecular formula**	**Δ ppm**	**MS/MS * *m/z***	**References**	**Level #**
1	Digalloyl glucose [a]	8.59	0.23 ± 0.01	483.0780	$C_{20}H_{20}O_{14}$	0.32	271.0477(80)/211.0251(100)/ 169.0107(78)	[34]	2
2	Chlorogenic acid	8.96	7.02 ± 0.41	353.0878	$C_{16}H_{18}O_9$	0.23	191.0557(100)	[21]	1
3	Unknown	12.12	NQ	951.0724	$C_{23}H_{36}O_{40}$	-0.17	300.9981(100)	[31]	3
4	Trigalloyl glucose [a]	13.44	3.59 ± 0.12	635.0876	$C_{27}H_{24}O_{18}$	-1.33	313.0560(40)/169.0122(9)	[34]	2
5	Isoramnetindiglucoside [b]	15.31	0.14 ± 0.02	639.1564	$C_{28}H_{32}O_{17}$	-0.50	315.1264(11)	[35]	2
6	Kaempferol diglucoside [c]	15.72	0.22 ± 0.02	609.1459	$C_{27}H_{30}O_{16}$	-0.79	285.0412(9)	[35]	2
7	Quercetin hexoside[b]	16.31	0.36 ± 0.01	463.0897	$C_{21}H_{20}O_{12}$	-0.68	301.0333(13)/300.0272(100)	[8,31]	2
8	Quercetin-3-*O*-glucoside	16.76	0.19 ± 0.01	463.0873	$C_{21}H_{20}O_{12}$	-0.23	301.0369(46)/300.0239(91)	[36]	1
9	Quercetin xyloside[b]	17.61	0.92 ± 0.05	433.0779	$C_{20}H_{18}O_{11}$	1.19	301.0327(64)/300.0263(77)	[36]	2
10	Quercetin-3-*O*-arabinoside [b]	18.22	9.12 ± 0.37	433.0778	$C_{20}H_{18}O_{11}$	0.63	301.0344(82)/300.0286(100)	[36]	2
11	Quercetin-3-*O*-rhamnoside	19.10	10.02 ± 0.49	447.0937	$C_{21}H_{20}O_{11}$	0.86	301.0347(88)/300.0283(100)	[36]	1
12	Quercetin acetyl-arabinoside [b]	21.90	4.12 ± 0.28	475.0883	$C_{22}H_{20}O_{12}$	0.37	300.0279(100)	[36]	2

[a] Quantified as gallic acid equivalents; [b] Quantified as quercetin-3-*O*-glucoside equivalents; [c] Quantified as kaempferol-3-*O*-glucoside equivalents;NQ: not quantified. * in parentheses the relative intensity; # according to Blaženović [33].

Figure 1. LC-ESI-QToF MS/MS total compound chromatogram of *E. characias* flowers aqueous extract acquired in negative ion mode. Chromatographic conditions are described in the text. Peak identification is given in Table1.

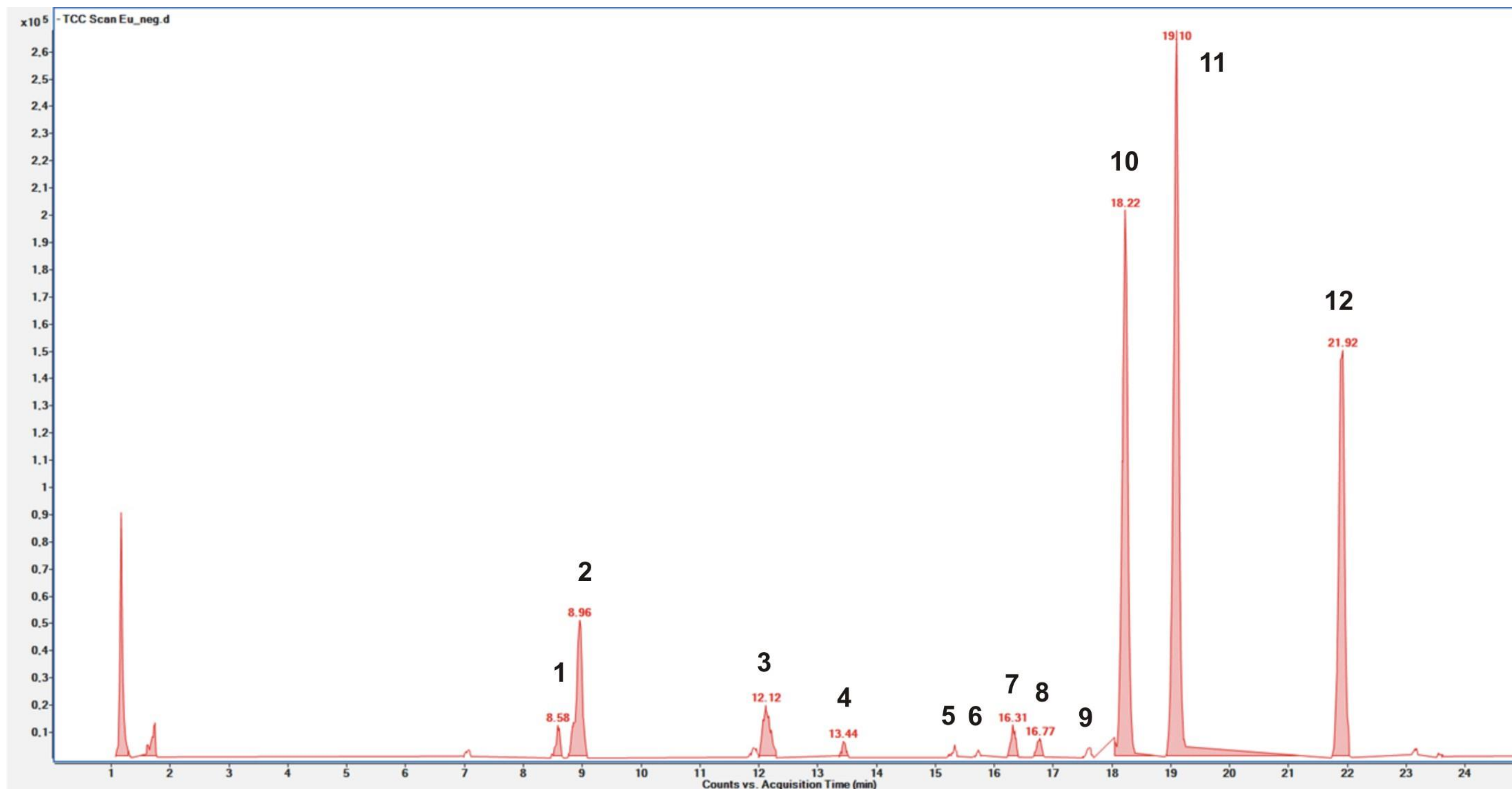


Table 2. Comparative analysis of phenolic compounds in ECAEFl and ECEEFl.

Putative identification	Extracts mg/g dm (mean ± SD)	
	ECAEFl	**ECEEFl**
Digalloyl glucose	0.23 ± 0.01	ND
Chlorogenic acid	7.02 ± 0.41	ND
Trigalloyl glucose	3.59 ± 0.12	ND
Isoramnetin diglucoside	0.14 ± 0.02	ND
Kaempferol diglucoside	0.22 ± 0.02	ND
Quercetin hexoside	0.36 ± 0.01	ND
Quercetin-glucoside (Isomer)	ND	3.95 ± 0.04
Quercetin-3-*O*-glucoside	0.19 ± 0.01	5.87 ± 0.10
Ellagic acid	ND	0.57 ± 0.01
Quercetin-3-*O*-xyloside	0.92 ± 0.05	6.09 ± 0.02
Quercetin-3-*O*-arabinoside	9.12 ± 0.37	21.70 ± 0.68
Quercetin-3-*O*-rhamnoside	10.02 ± 0.49	36.62 ± 0.94
di-*O*-Caffeoylquinic acid	ND	0.03 ± 0.00
Kaempferol-3-*O*-arabinoside	ND	0.02 ± 0.00
Kaempferol-3-*O*-rhamnosidec	ND	0.04 ± 0.01
Quercetin-3-(2-*O*-acetyl)-arabinoside	4.12 ± 0.28	47.89 ± 1.30
Acacetin glucuronide	ND	0.89 ± 0.07
Quercetin	ND	0.31 ± 0.04

3.2. Antinociceptive effect of ECAEFl and ECEEFl

3.2.1. Effect of ECAEFl and ECEEFl in formalin-induced paw licking

The orally treated animals with the vehicle spent 66.1 ± 6.3 seconds and 210.8 ± 14.7 seconds licking their paws in the 1st and 2nd phases, respectively.

Morphine and acetylsalicylic acid (AAS) were used as reference drugs in this model with paw licking values of 30.2 ± 5.6 seconds and 60.8 ± 5.7 seconds in the 1st phase, respectively, when compared to

the vehicle group that received only distilled water orally. In the 2nd phase, values of 102.2 ± 15.8 seconds and 139. ± 18.5 seconds were obtained for morphine and AAS, respectively. After oral treatment with ECAEFl, there was no reduction in licking time in the 1st phase for all three doses used. In the 2nd phase, pre-treatment with a dose of 10, 30 or 100 mg/kg significantly reduced the response time of the animals (118.3 ± 13.1, 108.4 ± 9.3, 100.9 ± 5.9 seconds, respectively) (**Figure 2A**).

On the other hand, oral treatment with ECEEFl at the doses of 10, 30 or 100 mg/kg was able to reduce the licking paw response in the 1st and 2nd phases significantly when compared with vehicle treated group (1st phase: 42.4 ± 5.6, 40.2 ± 5.4, 38.4 ± 6.0 seconds; 2nd phase: 93.6 ± 6.1, 91.0 ± 11.6, 90.7± 6.0 seconds, respectively (**Figure 2B**).

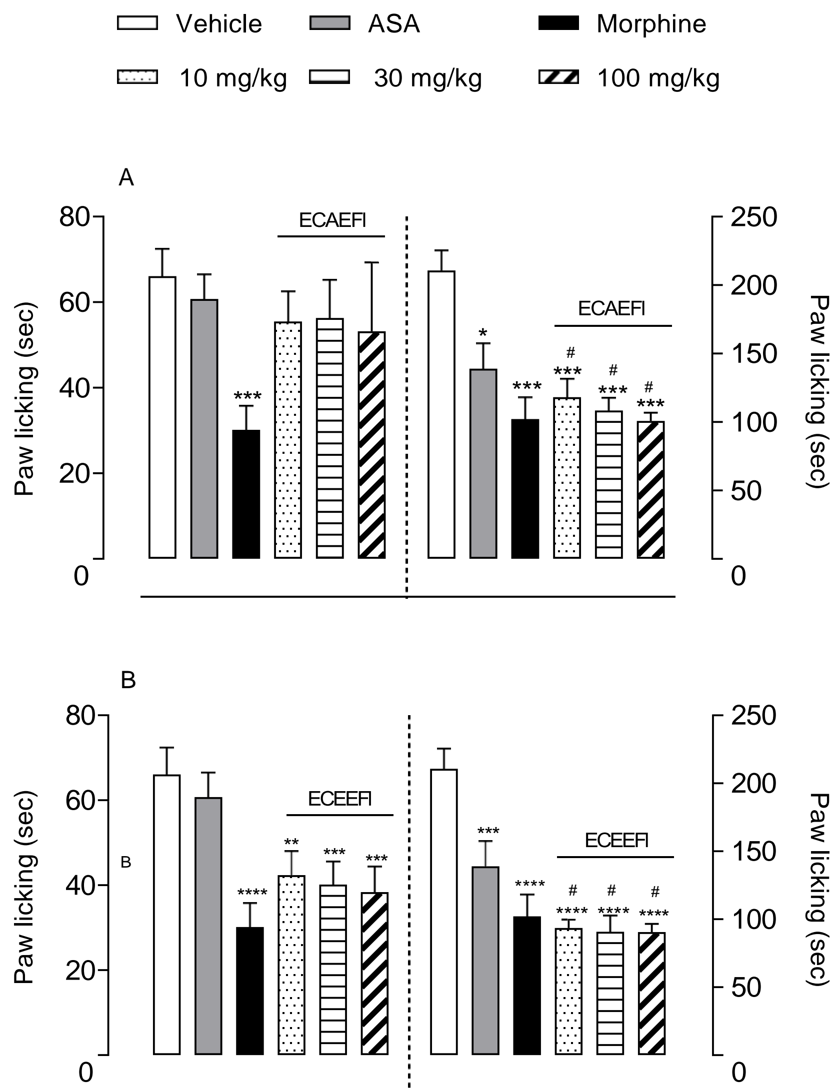


Figure 2. Effects of ECAEFl (A) and ECEEFl (B) on the formalin-induced paw licking model in mice. Results are expressed as mean ± S.D. of the time that the animals remained licking the paw injected with formalin (n = 6-8). ****p <0.0001; ***p <0.001; **p <0.01; compared to vehicle-treated group; #p <0.05 compared to ASA-treated group.

3.2.2. Effect of ECAEFl and ECEEFl in hot plate test

After observing a peripheral antinociceptive effect of ECAEFl and ECEEFl in a formalin-induced paw licking model, we sought to investigate whether they also had a pharmacology modulation at the level of the central nervous system using the hot plate test. The antinociceptive effects were compared with the vehicle group, which presented a percentage of antinociception of 0% (%AA) in the evaluated times.

The animals treated with ECAEFl (**Figure 3A**) at a dose of 10 mg/kg presented, within 90 minutes after its administration, values of antinociception of 38.1% compared to those treated with a vehicle. The group treated with a dose of 30 mg/kg showed, at 90 and 120 minutes, antinociception values of 49.7% and 35.1%, respectively. The group of animals treated with a dose of 100 mg/kg showed, at times of 90, 120 and 150 minutes after its administration, antinociceptive values of 62.0%, 56.8% and 47.9%, respectively.

By analyzing the area under the curve, it is possible to observe that only the 100 mg/kg dose causes a significant increase (p value < 0.05) of this parameter in relation to the group treated with the vehicle (**Figure3B**).

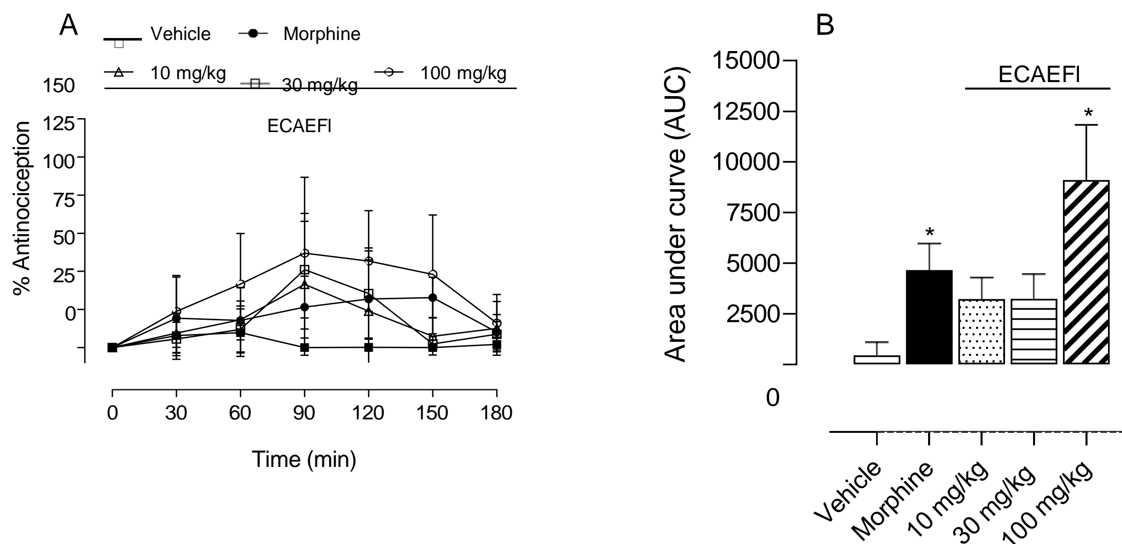


Figure 3. Antinociceptive effect of ECAEFl in mice in the hot plate test on the time course curve (A) and calculated as area under the time course curve (B). *p <0.05 compared to vehicle-treated group.

The animals treated with ECEEFl (**Figure 4A**) at a dose of 10 mg/kg presented, within 90 minutes after its administration, values of antinociception of 53.8% compared to the group treated with vehicle. The group treated with a dose of 30 mg/kg showed, at 90 and 120 minutes, antinociception values of 30.7% and 41.5%, respectively. The group of animals treated with a dose of 100 mg/kg showed, at times of 120 and 150 minutes after its administration antinociceptive values of 35.8% and 50.1%, respectively.

By analyzing the area under the curve, it is possible to observe that 10, 30 and 100 mg/kg doses increase this parameter in relation to the group treated with the vehicle (**Figure 4B**).

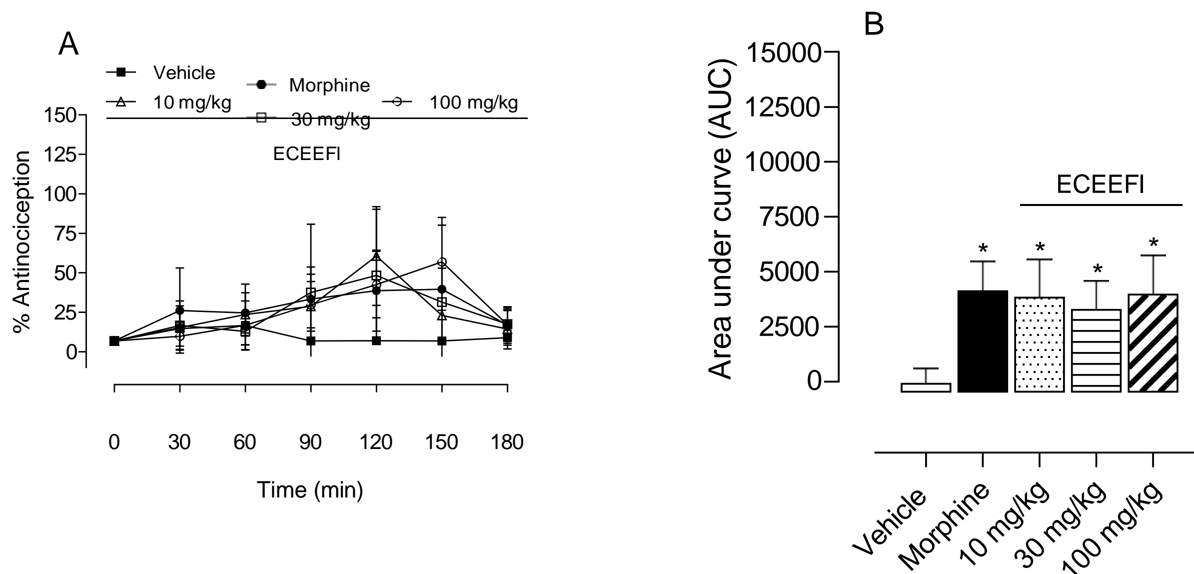


Figure 4. Antinociceptive effect of ECEEFl in mice in the hot plate test on the time course curve (A) and calculated as area under the time course curve (B). *p <0.05 compared to vehicle-treated group.

3.3. Anti-inflammatory effect of ECAEFl and ECEEFl in carrageenan-induced air pouch inflammation

3.3.1. Effect of ECAEFl and ECEEFl on total leucocytes (x10^{6}cells/mL), total proteins and NO quantification in carrageenan-induced exudate in the air pouch

As previously shown, treatment with ECAEFl and ECEEFl reduced formalin-induced paw licking time in the 2nd phase of the experiment (inflammatory pain phase), characterized by the local release of endogenous mediators, such as histamine, serotonin, prostaglandin and bradykinin [37]. Thus, we decided to assess whether ECAEFl and ECEEFl could influence some of the parameters involved in the inflammatory response, such as cell migration, protein leakage, and nitric oxide production. For that, we used the model of cell migration induced by carrageenan in the subcutaneous air pouch.

In **Figure 5A**, it is possible to observe that 24 hours after the injection of carrageenan into the SAP, the animals treated orally with the vehicle showed a significant increase in the total count of leukocytes that migrated to the SAP (72.8 ± 5.8 x 10^6 cells/mL) when compared to the group that received saline in the cavity (1.1 ± 0.3 x 10^6 cells/mL). In the animals that received dexamethasone

(2.5 mg/kg) intraperitoneally and carrageenan in the cavity, an 64.3% reduction in the total leukocyte count (26.0 ± 2.8 x 10^{6}* cells/mL) was observed when compared to the group that received the oral vehicle and carrageenan in the cavity.

The animals treated orally with ECAEFl, at doses of 30 and 100mg/kg, reduced the number of leukocytes that migrated into the cavity by 49.1% and 75.7%, respectively (**Figure 5A**).

One of the events during the inflammatory process due to tissue injury and release of inflammatory mediators is the increase in vascular permeability, which can lead to increase in protein extravasation and NO production [38]. To evaluate whether ECAEFl or ECEEFL could be modulating protein extravasation or NO production, quantification of total proteins and NO in the exudate collected from the SAP was performed. The reduction observed in the mice pretreated with dexamethasone (2.5 mg/kg, i.p.), was 51.8% and 68.2% to both parameters. The oral administration of ECAEFl (100 mg/kg) 1 h previous to carrageenan injection in the SAP caused a reduction of 53.5% in the amount of protein extravasated to the exudate (**Figure 5B**). ECAEFl at 10, 30 and 100 mg/kg also reduced NO levels of measured in the fluid (86.2%, 88.0%, and 91.0%, respectively) (**Figure 5C**).

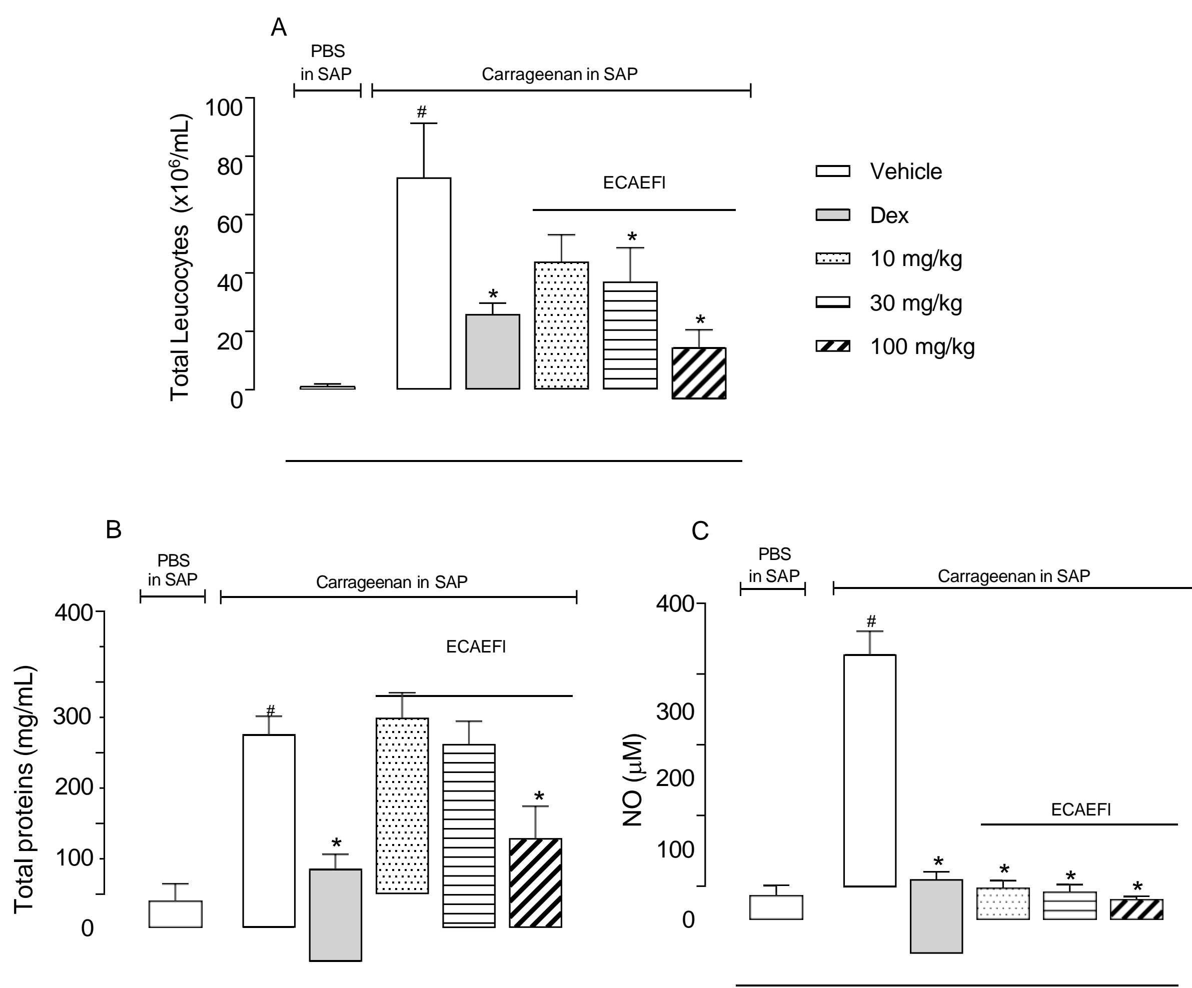


Figure 5. Effect of ECAEFl on model of carrageenan-induced cell migration into the subcutaneous air pouch. Results are expressed as mean ± S.D. of total leucocytes count (A), total proteins count (B) and NO quantification (C) (n=6-8). #$p < 0.05$ when comparing the vehicle-treated group thatreceived carrageenan in SAP with the vehicle-treated group that received PBS in SAP and *$p < 0.05$ when comparing the dexamethasone or ECAEFl pretreatedgroups that received carrageenan in SAP with the vehicle-treated group that received carrageenan in SAP.

The animals treated orally with ECAEFl, at doses of 10, 30 and 100mg/kg, reduced the number of leukocytes that migrated into the cavity by a 45.9%, 63.0% and 78.0%, respectively (**Figure 6A**). The oral administration of ECEEFl (10, 30 and 100 mg/kg) 1 h previous to carrageenan injection in the SAP caused a reduction in 27.4%, 39.5% and 49.4%, respectively in the amount of protein extravasated to the exudate (**Figure 6B**). ECAEFlat 10, 30 and 100 mg/kg also reduced the levels of

NO measured in the fluid 82.4.2%, 83.8%, and 91.6%, respectively) (**Figure 6C**).

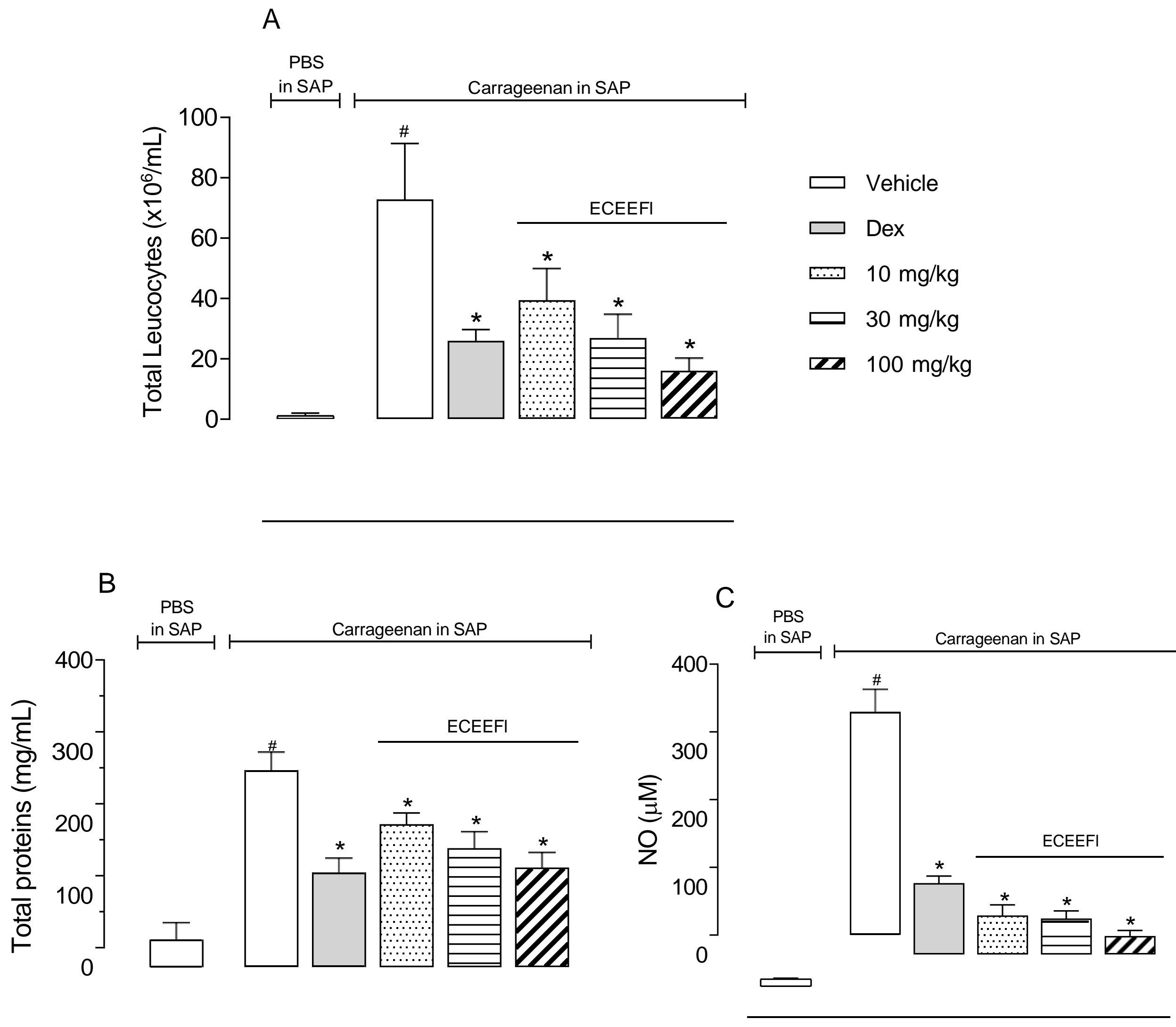


Figure 6. Effect of ECEEFl on model of carrageenan-induced cell migration into the subcutaneous air pouch. Results are expressed as mean ± S.D. of total leucocytes count (A), total proteins count (B) and NO quantification (C) (n=6-8). # $p < 0.05$ when comparing the vehicle-treated group that received carrageenan in SAP with thevehicle-treated group that received PBS in SAP and * $p < 0.05$ when comparing the dexamethasone or ECEEFl pretreated groups that received carrageenan in SAP with the vehicle-treated group that received carrageenan in SAP.

3.4. Acute toxicity

3.4.1. Effect of ECAEFl and ECEEFl in total blood leucocytes, total leucocytes in bone marrow and behavioral toxicity.

To evaluate if the acute treatment with ECAEFl or ECEEFl (100 mg/kg) could cause hematological or bone marrow alterations in the animals, some parameters were evaluated, such as total leukocytes present in the blood and bone marrow. bone. After 24 hours of oral treatment with ECAEFl or ECEEFl at a dose of 10 mg/kg, the animals were euthanised and peripheral blood was collected and bone marrow was washed. We observed that none of the extracts was able to alter any of these pa- rameters (**Figure 7A** and **7B**, **Figure 8A** and **8B**).

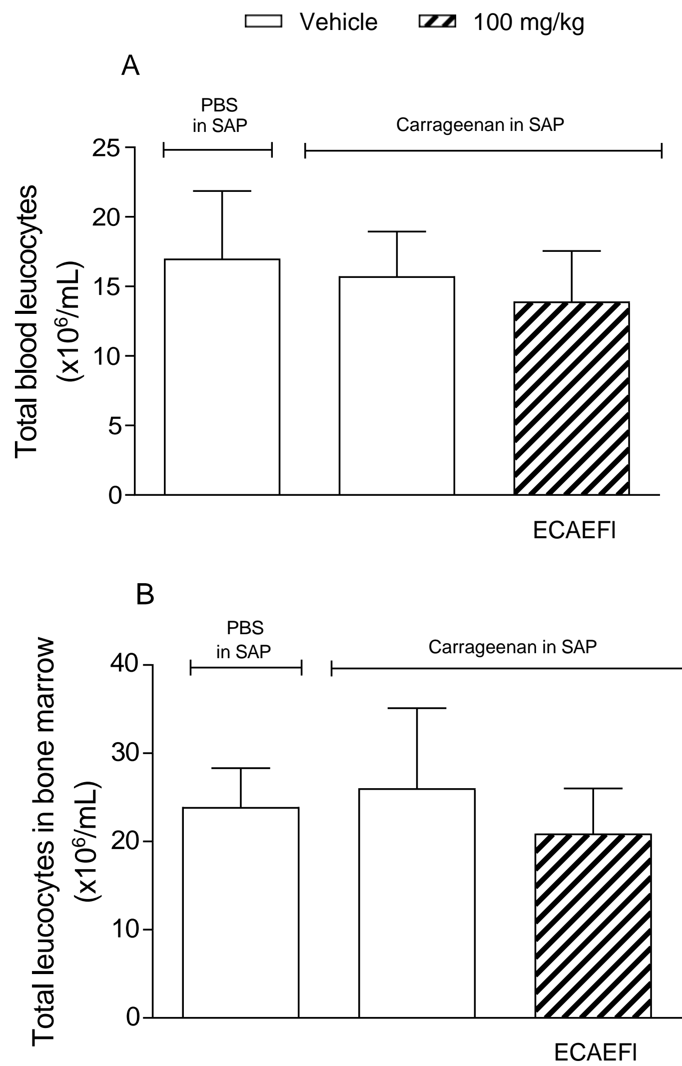


Figure 7. Effect of ECAEFl on the total blood leucocytes (A) and total leucocytes in bone marrow (B). Results are expressed as mean ± S.D. of total blood leucocytes and total leucocytes in bone marrow (n=6-8).

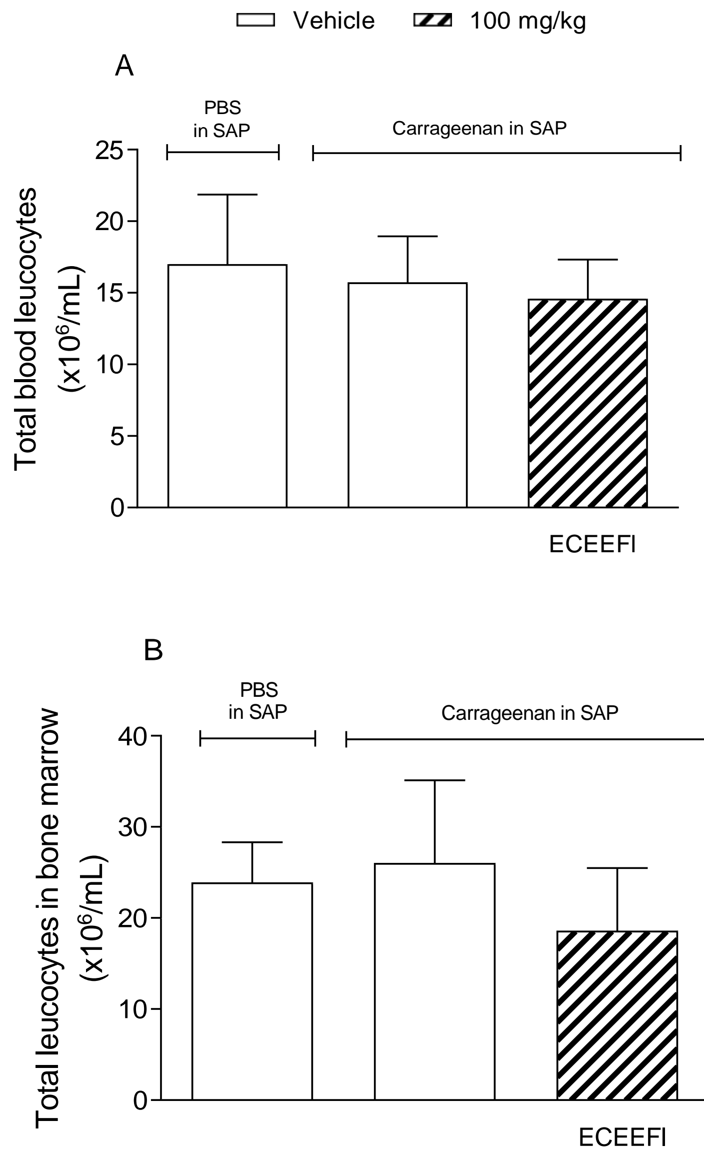


Figure 8. Effect of ECEEFl on the total blood leucocytes (A) and total leucocytes in bone marrow (B). Results are expressed as mean ± S.D. of total blood leucocytes and total leucocytes in bone marrow (n=6-8).

No alterations on respiration and no ulcers were observed in stomach after 5 days. Besides that, there were no alterations in normal activity, such as food and water intake, grooming, and loss of righting reflex (data not shown).

4. Discussion

In this study, both the water and ethanolic extracts of *E. characias* flowers (ECAEFl and ECEEFl) demonstrated significant antinociceptive effects in various nociceptive models, aligning with the known pharmacological profiles of quercetin and its derivatives identified within the extracts. Notably, ECEEFl, which contained higher concentrations of these glycosylated derivatives, exhibited enhanced efficacy in analgesic and anti-inflammatory effect, suggesting a dose-dependent effect of quercetin derivatives. which is consistent with their absorption dynamics; glycosylation generally enhances the bioavailability of quercetin, facilitating more effective antinociceptive outcomes [39].
To assess the antinociceptive activity, several models of nociception were employed. The first model used was the formalin-induced paw licking model, according to Dubuisson and Dennis[40]. This model is a useful tool for assessing analgesic substances and consists of the neurogenic and inflammatory pain phases. The first phase's results from the formalin test indicated a direct antinociceptive action possibly due to the inhibition of primary afferent neurotransmitters or modulation of their receptors [41]. Meanwhile, the reduction observed in the second phase underscores the extracts' anti-inflammatory potential by mitigating the synthesis or action of inflammatory mediators like cytokines, histamine, and prostaglandins [42].
ECEAFl significantly reduced paw licking time in the inflammatory phase (2nd phase), whereas ECEEFl reduced paw licking time in both the neurogenic phase (1st phase) and in the 2nd phase.
Quercetin has been reported in the literature to decrease sensitization and/or activation of primary afferent fibers by reducing the release of inflammatory mediators at the injury site[17]. This effect could be linked to a delay in the initiation of action potentials in the fibers, resulting in an antinociceptive effect. Moreover, quercetin could potentially interfere with ion channels or receptors involved in pain signal transmission, further reducing the generation of action potentials. [43,44]
In the second phase, both extracts demonstrated potential anti-inflammatory effects by decreasing the release of inflammatory mediators at the injury site. The higher concentration of compounds like

quercetin-3-O-rhamnoside and quercetin-3-O-arabinoside in ECEEFl, known inhibitors of COX [45,46], might explain the more pronounced inhibitory effect observed even at lower doses.

To assess the central antinociceptive effect, the hot plate model was employed. In this model, a thermal stimulus is applied, activating nociceptors that transmit acute nociceptive information to specific regions of the central nervous system (CNS), resulting in a motor response such as paw elevation or jumping [47]. Treatment with ECEEFl at various doses consistently increased the duration animals remained on the hot plate, suggesting significant supraspinal antinociceptive activity. This model highlights that flavonoids like quercetin not only affect peripheral pain mechanisms but also modulate central pain processing pathways.

Additionally, based on the results obtained in the second phase of the formalin-induced paw licking model, we also decided to investigate the anti-inflammatory activity of ECAEFl and ECEEFl using the carrageenan-induced cell migration model [49]. Post-treatment with ECAEFl or ECEEFl, a significant reduction in leukocyte migration to the inflamed site was observed, which may relate to the inhibition of chemokine-mediated leukocyte attraction or interference with leukocyte adhesion. Additionally, the extracts reduced protein extravasation and nitric oxide levels in the exudate, which aligns with their role in minimizing endothelial contraction, thereby mitigating vascular permeability. The distinctive phenolic profile of the extracts, particularly the high content of quercetin derivatives in ECEEFl, plays a pivotal role in modulating these responses. These compounds have been previously noted to inhibit the expression of adhesion molecules and ameliorate endothelial oxidative stress, contributing to the observed effects [45].

However, pain is a complex experience that cannot be fully captured by a single test, and even multiple tests may only partially represent how results might translate from animal models to humans [51]. In this context, our study carefully considers the methods used and their inherent limitations. While we elucidate potential mechanisms of the bioactive compounds in *Euphorbia c.* extracts, it is

crucial to recognize the constraints of animal models in predicting human physiological responses. Thus, interpretations of our findings should be made cautiously.

5. Conclusion

In this study, both water and ethanolic extracts of *E. characias* flowers (ECAEFl and ECEEFl) demonstrated significant antinociceptive effects in animal models of nociception. The presence of a higher concentration of quercetin derivatives in the ECEEFl extract may explain its enhanced effectiveness in reducing paw licking time during both neurogenic and inflammatory pain phases. Additionally, in the hot plate test, ECEEFl showed sustained supraspinal antinociceptive effects over time. The extracts also exhibited significant anti-inflammatory activity in the carrageenan-induced cell migration model. The observed decrease in leukocyte migration, protein extravasation, and nitric oxide (NO) levels suggests that these extracts inhibit the interaction of inflammatory mediators with their receptors on endothelial cells. This inhibition likely contributes to the observed reduction in inflammation.

Despite the traditional use of Euphorbia species as analgesics and antinociceptives in folk medicine, the specific antinociceptive properties of *E. characias* had not been thoroughly investigated prior to this study. Our research provides a crucial examination of these properties, marking a significant step in validating the traditional uses of this plant with scientific methods. These findings support the potential use of *E. characias* as a medicinal plant, providing natural compounds with minimal side effects and promising therapeutic potential for pain and inflammation-related disorders.

Future research should focus on isolating and characterizing individual bioactive compounds in E. characias extracts to better understand their roles and mechanisms in pain and inflammation modulation. Additionally, further studies are needed to evaluate the clinical relevance of these findings in human populations, taking into consideration the pharmacokinetics and pharmacodynamics of the active compounds.

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None

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

C.I.G.T., A.B.A.B.S.A., T.B.S.G., and P.D.F. conducted the experimental work and data analysis. F.P. and S.F. provided samples and reviewed the article. F.B. and A.D.P. critically reviewed the article and supervised the project. All authors contributed and approved the final version of the manuscript.

Data Availability Statement

The data underlying this article are available in the article.

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