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Original Article

RP-HPLC and LC–MS–MS determination of a bioactive artefact from *Ipomoea pes-caprae* extract

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ABSTRACT

Solvents play important and critical role in natural product chemistry and could generate artefacts during the extraction and purification of metabolites from a biological matrix. This study aimed to correlate the chromatographic profile with biological activity of *Ipomoea pes-caprae* (L.) R. Br., Convolvulaceae, extracts obtained with hydroethanolic extraction. Thus, aerial parts of *I. pes-caprae* were extracted with different concentration of ethanol (50, 70 and 90°GL) and the obtained extracts were analysed by HPLC–UV. HPLC data were studied employing chemometrics to discriminate the samples. Moreover these samples were further characterized by using UPLC–QTOF/MS data. The extracts were also biomonitored through the paw-oedema and spontaneous nociception induced by trypsin in mice. Different chromatographic profiles were obtained and the exploratory analysis clearly revealed higher level of ethyl caffeate in extracts of lower strength of ethanol (50°GL). This compound was suggested to be an artefact formed by transesterification of caffeoylquinic acid derivatives present in the plant, once it was not observed when other solvents were employed. During the biological assay, only the extract obtained with ethanol 50°GL presented significant inhibition of inflammation (45 ± 9%) and nociception (24 ± 3%). Ethyl caffeate seems to be linked to the anti-inflammatory effect since it reduced 86 ± 5% of paw-oedema induced by trypsin. Artefacts could contribute to the biological activity of herbal preparations and consequently lead to misinterpretation of the results.

Introduction

A large number of solvents are being used to extract selectively the natural products from herbal matrices. The interaction solute-solvent is mainly based on solubility and polarity (Maltese et al., 2009). However, there are many examples of artefacts in natural products, due to the solvent and extractions conditions used.

As example, lawsone is an artefact formed during the extraction process produced when leaf powder of *Lawsonia inermis* L. (henna) is added to water made slightly acidic with lemon juice or tea during henna dye preparation (Ashnagar and Shiri, 2011); eleutherobin, from soft coral *Erythropodium caribaeorum*, is present only when using methanol but not ethanol in the extraction (Britton et al., 2001). Alcohols can react with carboxylic groups to form esters, and with hemiacetals to form acetal (Maltese et al., 2009). Therefore, considering the wide range of biological activity of plant extracts, which of these activities could be attributed to artefacts generated during the steps of obtaining these preparations? It is important to consider the protocol used for the herbal material and compounds extraction, because it can interfere in the final herbal product.

Ipomoea pes-caprae (L.) R. Br., Convolvulaceae, in Brazil is known as "salsa", "salsa-brava" and "salsa-pé-de-cabra" (Simão-Bianchini and Ferreira, 2015), and in other countries, as bayhops, beach morning glory or goat's foot. The leaves have been used as a first aid to treat jellyfish stings and also in ritual baths to alleviate evil spells and Portuguese man-of-war stings-induced

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dermatitis (Manigaunha et al., 2010). Recently our research group demonstrated that the soft extract of *I. pes-caprae* aerial parts (7.5% of aerial parts, ethanol 50°GL as solvent), when topically applied in mice paw (2% in a semisolid), blocked the mechanical hypersensitivity induced by the toxin of *Physalia physalis*. The intraplantar injection of the major component, 3,5-di-*O*-caffeoylquinic acid, also abolished the mechanical hypersensitization (Barth et al., 2017). Previous studies reported the antinociceptive and anti-inflammatory topical effects of this plant, relating with apolar herbal derivatives (oil extract by steam-distillation with petroleum ether), which do not mimic popular use of the species (Sunthonpalin and Wasuwat, 1985).

The active components reported for this species were 2-hydroxy-4,4,7-trimethyl-1(4*H*)-naphthalenone, (-)-mellein, eugenol, 4-vinylguaiacol (Pongprayoon et al., 1991), glycosides of 2-methylpropanoic, (2*S*)-methylbutyric, *n*-hexanoic, *n*-decanoic, and *n*-dodecanoic acids, as well as pentasaccharides of jalapinolic acid, pescaproside A, pescapreins I-IV, stoloniferin III (Pereda-Miranda et al., 2005), and actinidol 1a and 1b (Pongprayoon et al., 1992a). Further metabolites, such as *trans*-β-damascenone, *E*-phytol (Pongprayoon et al., 1992b), glochidone, betulinic acid, α-and β-amyrin acetate, isoquercitrin (ISQ) (Krogh et al., 1999), 3,5-di-*O*-caffeoyl-quinic acid (isochlorogenic acid a, denominated ISA), 3,4-di-*O*-caffeoyl-quinic acid (isochlorogenic acid b, denominated ISB), 4,5-di-*O*-caffeoyl-quinic acid (isochlorogenic acid c, denominated ISC), among other caffeic acid derivatives were also identified (Teramachi et al., 2005).

Thus, this study aimed to investigate if solvent extraction of aerial parts of *I. pes-caprae* affects topical antinociceptive and anti-inflammatory activities using preclinical models of spontaneous nociception and paw oedema induced by trypsin in mice, regarding the chemical composition.

Material and methods

Drugs and reagents

Trypsin was purchased from Sigma–Aldrich (St. Louis, MO, USA), indomethacin from Deg (São Paulo, Brazil). For LC analysis, all solvents used were LC grade (Tedia, Fairfield, Ohio, USA). The water was purified using a Milli-Q system (Millipore, Massachusetts, USA). All solutions were filtered through RC 0.45 μm membrane (Macherey-Nagel, D age, Germany). ISQ, ISA, ISB and ISC (>95% of purity by HPLC-UV) were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Ethyl caffeate was obtained from the esterification of caffeic acid according with Campos-Buzzi et al. (2009), with purity >90% (by HPLC–UV). The structural characterization was confirmed based on the spectral data (MS and ^{1}H and ^{13}C NMR) and direct comparison with the literature data (Jaikang et al., 2011).

For the extract and cream preparation all solvents were analytical grade. Hostacerin® CG, Phenonip®, EDTA, propyleneglycol and BHT were purchased from PharmaSpecial (Santana de Parnaíba, São Paulo, Brazil). Polymols (triglycerides of caprylic and capricacid) was purchased from All Chemistry (São Paulo, Brazil).

Plant material

The aerial parts of *Ipomoea pes-caprae* (L.) R. Br., Convolvulaceae, were collected in the Esplanada Beach (Jaguaruna, Santa Catarina, Brazil, 28°36′55″S and 49°01′31″W), in January (summer) of 2013. An exsiccate was deposited in the Universidade Estadual de Maringá Herbarium (Maringá, PR, Brazil) under number HUEM 23566. The activity of access to the Genetic Patrimony/Associated Traditional Knowledge was registered in the *Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado* (SisGen), in compliance with Law 13.123/2015 and its regulations under number AFB43EB. The leaves and stems were dried in room temperature for seven days, powdered in a mill of hammers (Marconi® 600) and sieved (10 mesh).

Extracts preparation

The hydroethanolic extracts was obtained from aerial parts (leaves:stem at 60:40 *w*/*w*) and prepared by dynamic maceration with 50, 70 and 90°GL ethanol at a plant:solvent ratio of 7.5% (w/v), followed by turbo extraction for 10 min (5 cycles with 2 min intervals). Leaves were also used to obtain an extract with 50°GL ethanol in view of comparison with aerial parts. Thereafter, the extract was filtered and the extractive solution was reduced by heating at 50 °C to obtain the soft extract with syrupy appearance (dried residue of 93%, determined on 2 g) (Farmacopeia Brasileira, 2017). These extracts were characterized by dry residue (and quantification of secondary metabolites by HPLC-UV. Methanol 50% (v/v) and acetone 50% (v/v) soft extracts were also elaborated, using analytical grade solvents, in the same way of above described.

HPLC-UV analysis

A Shimadzu LC-10AD LC system (Shimadzu, Tokyo, Japan) consisting of a binary pump and a Shimadzu SPD-M10A photo diode array detector were used. Injections (20 μl) were carried out on a Phenomenex® (Torrance, California, USA) Luna C18, 5 μm (250 × 4.6 mm) column at 30 °C with detection at 338 nm, adapting the methodology previously developed (Dutra et al., 2014; Barth et al., 2017). The mobile phase, at 1 ml min^{-1}, consisted of acetonitrile (A) and pH 3 water acidified with sulphuric acid (B) according to the following gradient (A:B *v*/*v*): 10:90 to 15:85 (0–7 min); 15:85 to 20:80 (7–18 min); 20:80 (isocratic 18–25 min); 25:75 to 60:40 (25–40 min); 60:40 to 70:30 (40–43 min); 70:30 to 10:90 (47–50 min), 10:90 (isocratic until 60 min).

Soft extracts were dissolved in 1:1 v/v of methanol:water pH 3 with sulphuric acid and a diluted to 2.5 mg ml^{-1} solution. Reference standards of 3,5-di-*O*-caffeoylquinic acid (ISA), 3,4-di-*O*-caffeoylquinic acid (ISB), 4,5-di-*O*-caffeoylquinic acid (ISC) and isoquercitrin (ISQ) were used to identify these substances in the extract. In addition, other unknown peaks were also quantified, expressed in ISQ (peaks 1-4,8–4) or ISA (peaks 5–-7), depending on the UV absorption profile, typical of flavonoid or phenolic, respectively (Fig. 1).

Multivariate analysis

Principal component (PCA) analysis of chemical data were performed with the MINITAB® 15 software (State College, PA, USA). A correlation matrix was employed, and the number of components was determined from Kaiser's eigenvalue greater than 1.0 rule.17. All data were processed using MATLAB software, version 7.13 (MathWorks, Natick, USA), together with the PLS_Toolbox, version 6.5 (Eigenvector Technologies, Manson, USA). The number of principal components (PC) was determined by observing the percentage of variance explained.

UPLC-ESI-QToF-MS/MS

An Acquity UPLC system H-class (Waters, São Paulo) equipped with a sample manager and a quaternary solvent manager was used. The separation was carried out in a BEH C18 column: 50 mm, 1.0 mm, particle size 1.7 μm (Waters, São Paulo). The column and the sample tray were kept at temperatures of 40 °C and

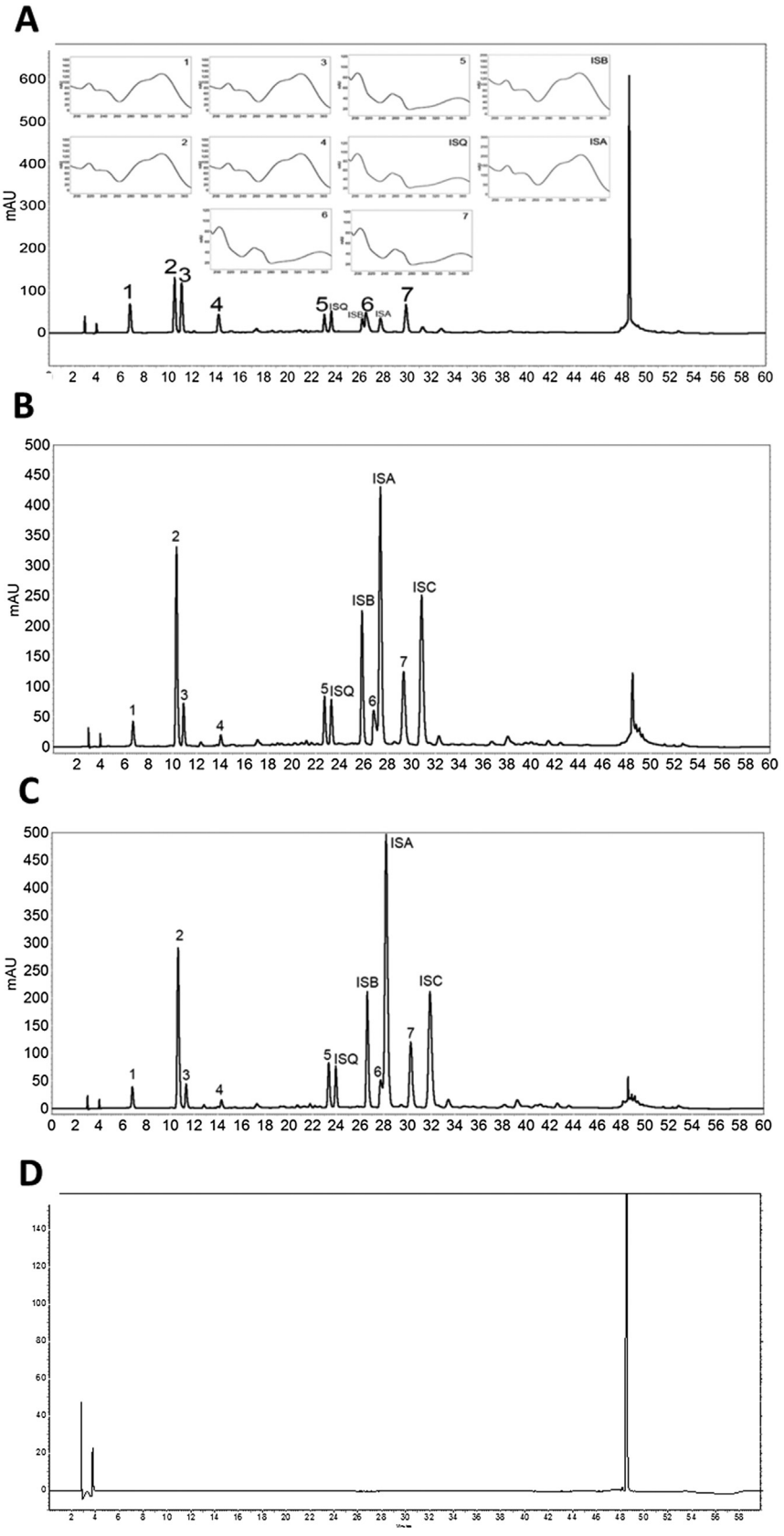

Fig. 1. Chromatographical profile, at 338 nm, of *Ipomoe pes-caprae* extracts obtained with ethanol in different strength: (A) 50°GL, (B) 70°GL, (C) 90°GL, (D) ethyl caffeate.

20 °C, respectively. The mobile phase, at 0.3 ml min^{-1}, consisted of water:formic acid, 99.9:0.1 *v*/*v* (A) and acetonitrile (B), according to the following gradient (A:B *v*:*v*): 95:5 to 80:20 (0–5 min), 80:20 to 50:50 (5–8 min), 50:50 to 45:35 (8–10 min), 45:35 to 10:90 (10–13 min), 10:90 (13–14.5 min), 10:90 to 95:5 (14.5–15 min), 95:5 (15–20 min). The injection volume was 2 μl.

Mass data were recorded with a Xevo G2-S QTof (Waters) mass spectrometer equipped with an electrospray probe operating in positive (ESI^+) and negative (ESI^-) ionization modes. The nebulizer gas was: nitrogen; cone gas flow 100 l/h; desolvation gas flow 900 l/h; sampling cone 40 V; source offset 80 V; collision gas: argon; Lockspray reference sample: Leucine enkephalin. Lock masses *m/z* 556.2771 (ESI^+) and *m/z* 554.2615 (ESI^-).

The desolvation and source temperatures were set at 200 °C and 90 °C, respectively. The capillary voltage was operating at 3 kV. Data was collected between 100 and 1500 Da, with a scan time of 1 s over an analysis time of 20 min. LC–MS/MS analyses were performed with collision energies of 25 eV (for mass values <400 Da) and 40 eV (for mass value >400). The mass data were acquired and processed with the *MassLynxV4.1* software (Waters)

Ipomoea pes-caprae extract semisolid preparation

A cream based on: 12% Hostacerin® CG, 0.7% Phenonip®, 0.1% EDTA, 0.9% Polymol®, 0.003% BHT, 5% propyleneglycol and purified water. This formulation was prepared with or without the soft extract at 1% (w/w). The semisolids were packed in aluminium tubes, with a seal and polypropylene cover.

Pharmacological assays

Male Swiss mice (25–30 g; 8-week-old) were used for this study (6–10 Swiss mice per group). Animals were housed under conditions of optimum light, temperature, and humidity (12-h light-dark cycle, 22 ± 1 °C), with food and water *ad libitum*. All the procedures used in the present study followed the "Principles of Laboratory Animal Care" of National Institutes of Health publication nº 85-23 and were approved by the Animal Ethics Committee of UNIVALI (protocol numbers 024/2012).

For spontaneous nociception induced by trypsin, the mices were topically pre-treated with the semisolid containing *I. pes-caprae* extract (1%), ethyl caffeate (0.15 mg/g) or placebo 30 min before the test. Then, they received a 20 μl-injection containing trypsin (300 μg/paw) (Paszcuk et al., 2008) into the plantar surface of the right hind paw. Followed the injection, they were individually observed for a period of 10 min. The amount of time spent by the mouse licking or biting the injected paw was obtained with a chronometer and considered indicative of nociception.

For paw oedema induced by trypsin, the animals were pre-treated with *I. pes-caprae* semisolid (1%) and, 30 min later, they received a 50 μl-intraplantar-injection of saline 0.9% containing trypsin (30 μg/paw) into the right hind paw (Paszcuk et al., 2008). The contralateral hind paw (left paw) received 50 μl of saline and was used as control. Paw oedema was measured using a plethysmometer (Ugo Basile) at different time points after the phlogistic-agent injection and was expressed in microliter as the difference between the right and left paws.

The results are presented as the mean ± SEM of 6–12 animals in each group. The percentages of inhibition were based on AUC (area under curve) for the line graphs, calculated using the entire time course of each experiment and reported as the mean ± SEM of inhibitions obtained for each individual experiment. Statistical comparison of the data was performed by two-way analysis of variance (ANOVA) followed by Bonferroni's post-test and one-way ANOVA followed by Dunnett's post-test. *p*-values less than 0.05 ($p < 0.05$) were considered significant.

Table 1
Quantification (average ± standard deviation) of secondary metabolites of *Ipomoea pes-caprae* soft extracts obtained with different strengths of ethanol.

Markers (mg/g)	Ethanol (°GL)		
	50	70	90
Peak 1 *ISA	5.17± 0.22	3.67 ± 0.16	3.35 ± 0.26
Peak 2 *ISA	9.51 ± 0.32	25.10 ± 0.81	24.30 ± 1.42
Peak 3 *ISA	8.21 ± 0.53	5.22 ± 0.06	3.39 ± 0.08
Peak 4 *ISA	3.44 ± 0.32	1.61 ± 0.05	1.32 ± 0.09
Peak 5 **ISQ	5.54 ± 0.55	12.08 ± 0.60	12.44 ± 0.73
ISQ	6.55 ± 0.59	11.29 ± 0.16	10.95 ± 0.26
ISB	2.46 ± 0.97	13.03 ± 0.29	13.27 ± 0.52
ISA	3.64 ± 0.67	44.15 ± 2.54	61.61 ± 2.32
Peak 6 **ISQ	11.13 ± 1.50	13.44 ± 0.41	15.92 ± 1.74
Peak 7 **ISQ	12.28 ± 1.08	26.94 ± 1.40	43.43 ± 0.93
ISC	nd	12.65 ± 1.46	11.07 ± 0.53
Peak 8 *ISA (mg/g)	11.83 ± 0.24	3.32 ± 0.13	2.04 ± 0.20

nd: undetected; *ISA: assay of peak with UV absorption profile typical of phenolic compounds, expressed in ISA; **ISQ: assay of peak with UV absorption profile typical of flavonoid compounds, expressed in ISQ.

Results and discussion

In this study, we obtained different extracts from *I. pes-caprae* aerial parts by employing three different concentration of ethanol (50, 70 and 90°GL) for extraction. The choice of a polar solvent such as ethanol aimed to the future establishment of a phytomedicine as well as the proximity to the popular use that employs the juice of the leaves (Manigaunha et al., 2010).

The obtained extracts were then submitted to the chromatographic profile and secondary metabolites quantification by HPLC-UV (Fig. 1) using methodology previously validated (Dutra et al., 2014). The extract obtained with ethanol 50°GL (Fig. 1A) shows the presence of seven unknown peaks related to phenolic components (peaks 1–4 with similar UV absorption profile of chlorogenic acid derivatives ISA, ISB and ISC) or flavonoids (peaks 5, 6 and 7, with similar UV absorption profile of isoquercitrin), besides the presence of chlorogenic acid derivative (ISA, ISB) and isoquercitrin (ISQ). The maximum absorbance of the phenolic compounds was at 215, 240, 290 and 325 nm, while the flavonoids exhibit maximum of absorption at 210, 254 and 350 nm (inserts of Fig. 1A).

The extract obtained with 70°GL ethanol (Fig. 1B) also shows the presence of the above compounds, but in greater concentration, including the presence of ISC, as previously detected by Barth et al. (2017). Similar profile was observed for the 90°GL ethanol extract (Fig. 1C). However, an additional peak was observed at the end of the chromatographic run, at 48 min, especially in the 50°GL extract (Fig. 1A), and its chromatographic area decreases with the increase of ethanol strength (Fig. 1B and C).

The ethyl caffeate peak showed the same retention time and UV absorption profile similar to the additional peak that appeared in the extract chromatograms, more pronounced in the extract obtained with 50°GL ethanol (Fig. 1A–D).

Due to the large presence of caffeic acid derivatives in the plant, this latter peak was suspected to be an artefact related to caffeic acid. This presumption was checked by comparing the extract chromatogram with that of ethyl caffeate using the same LC gradient (Fig. 1D). Ethyl caffeate is presumably a product of transesterification of caffeic acid esters and ethanol, formed during the extraction process.

Each secondary metabolite in the different extracts was quantified, including the supplemental peak at 48 min (called peak 8, suspected to be ethyl caffeate) by external standardization, using the previous validated method (Dutra et al., 2014). The results are presented in Table 1. The 50°GL ethanol extract showed lower amount of caffeic acid derivatives and some peaks with phenolic-

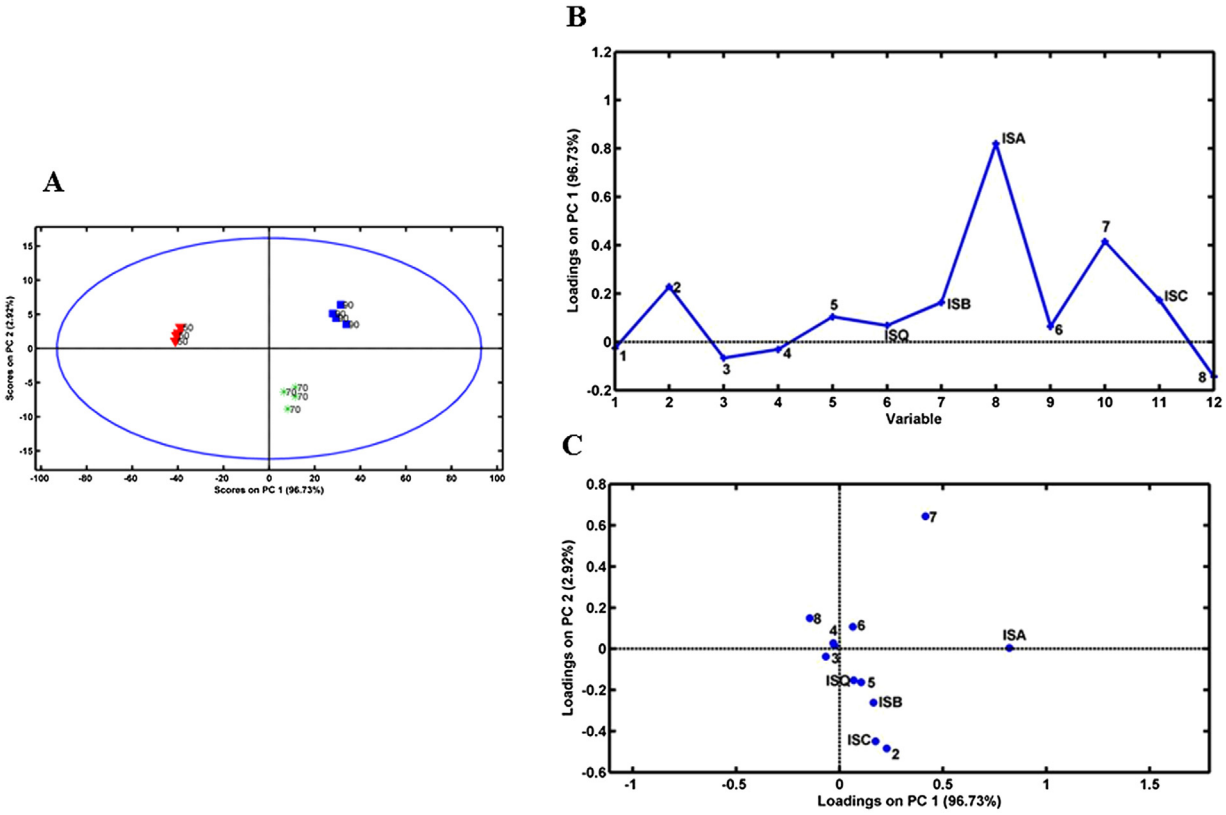

Fig. 2. (A) Score plot of the samples; (B) Loadings on PC1 per variable; (C) Loadings on PC1 versus Loadings on PC2.

UV-absorption profile. On the other hand, this extract showed the highest area of peak 8, which was quantified and expressed in ISA (Table 1).

PCA with mean centering was employed on the dataset constituted of twelve samples described by twelve variables. According to Fig. 2, only two PC were necessary to separate the three different classes of alcoholic strength (50, 70 and 90°GL). Analysing the Fig. 2A (constructed based on the first two principal components, accounted for more than 99% of the total variance), PC1 succeeded in discriminate extracts obtained with 50°GL ethanol of others (negative scores correspond to samples of 50°GL ethanol). On the other hand, PC2 discriminated the extracts of 70 and 90°GL ethanol (positive scores correspond to extract 90 and negative ones to extract 70°GL ethanol).

Examining the loadings plot of PCA (Fig. 2B and C), it is clear that the variables directly related to the extract of 50°GL ethanol are the peak 8 and the peak 3. Thus, the extract of 50°GL ethanol contains high amount of peak 8 and peak 3 (Fig. 2B). On the other hand, the variables ISA, peak 7, peak 2 and ISC are also important to discriminate the samples, but, by having positive values of loadings, they are in small quantity in extracts of ethanol 50°GL. In this way, this latter extract is characterized by higher levels of peak 8 and 3 (compared to the others) and low levels of ISA, peak 7, peak 2 and ISC.

To discriminate the samples with 70 and 90°GL (Fig. 2C), the variables that are most distinctive are peak 7 and peak 2 (ISC is also important). Extract of 90°GL ethanol have higher amount of peak 7 and lower content of peak 2 (and ISC). On the other hand, extract of 70°GL ethanol has higher amount of peak 2 (and ISC) and lower content of peak 7. Thus, the peak 8 seems to be the more important compounds related to the biological effect observed.

With the aim to illustrate the difference among the chemical composition of the three extracts, UPLC-QTOF/MS analysis was performed. The extracts were exclusively sensitive to the negative mode ionization and the base peak ion (BPI) current chromatogram from the three extracts is show in Fig. 3. The diagnostic of the LC-ESI-MS data showed the presence of quinic acid, 4,5-di-*O*-caffeoylquinic acid, 3,5-di-*O*-caffeoylquinic acid, 3,4-di-*O*-caffeoylquinic acid, and isoquercitrin, corroborating with data obtained from the HPLC-UV analysis (Fig. 1). The caffeoylquinic acid derivatives revealed in their tandem mass spectra similar fragmentation pattern and the observed ions were *m/z* 353.0809 [M-H-caffeoyl]$^-$, *m/z* 191.0501 [quinic acid-H]$^-$ and 179.0285 [caffeic acid-H]$^-$. Similar MS^2 pattern were reported in the literature for the same metabolites (Ncube et al., 2014). Isoquercetrin was proposed as a constituent based on its fragments *m/z* 301.0354 and *m/z* 151.0037, which were in accordance to those reported in the literature (Simirgiotis, 2013). However, it is worth to highlight the presence of a unique peak at 7.32 min, with *m/z* 207.0492 [$C_{11}H_{12}O_4$-H]$^-$ that stands out in the extract obtained with 50°GL ethanol (Fig. 3A). These metabolites were lightly found in 70°GL ethanol extract (Fig. 3B), and absent in 90°GL ethanol extract (Fig. 3C). The peak at 7.32 min was identified as ethyl caffeate based on its MS^2 spectrum, which showed a fragment at *m/z* 161.0224 [M-H-ethanol]$^-$. The foregoing data confirm the qualitative analysis by HPLC and identified the peak 8 that distinguishes the extract of 50°GL ethanol from the 70 and 90°GL, being an artefact generated during the extraction/evaporation process (as suggested in Fig. 3F). This can be explained by the fact that esterification is already a well known reaction that occurs between a carboxylic acid and an alcohol, being favored by the increase in temperature. It is a typical nucleophilic substitution reaction on acyl carbon in which a nucleophile (ethanol) attacks the carbon of carbonyl group (caffeic

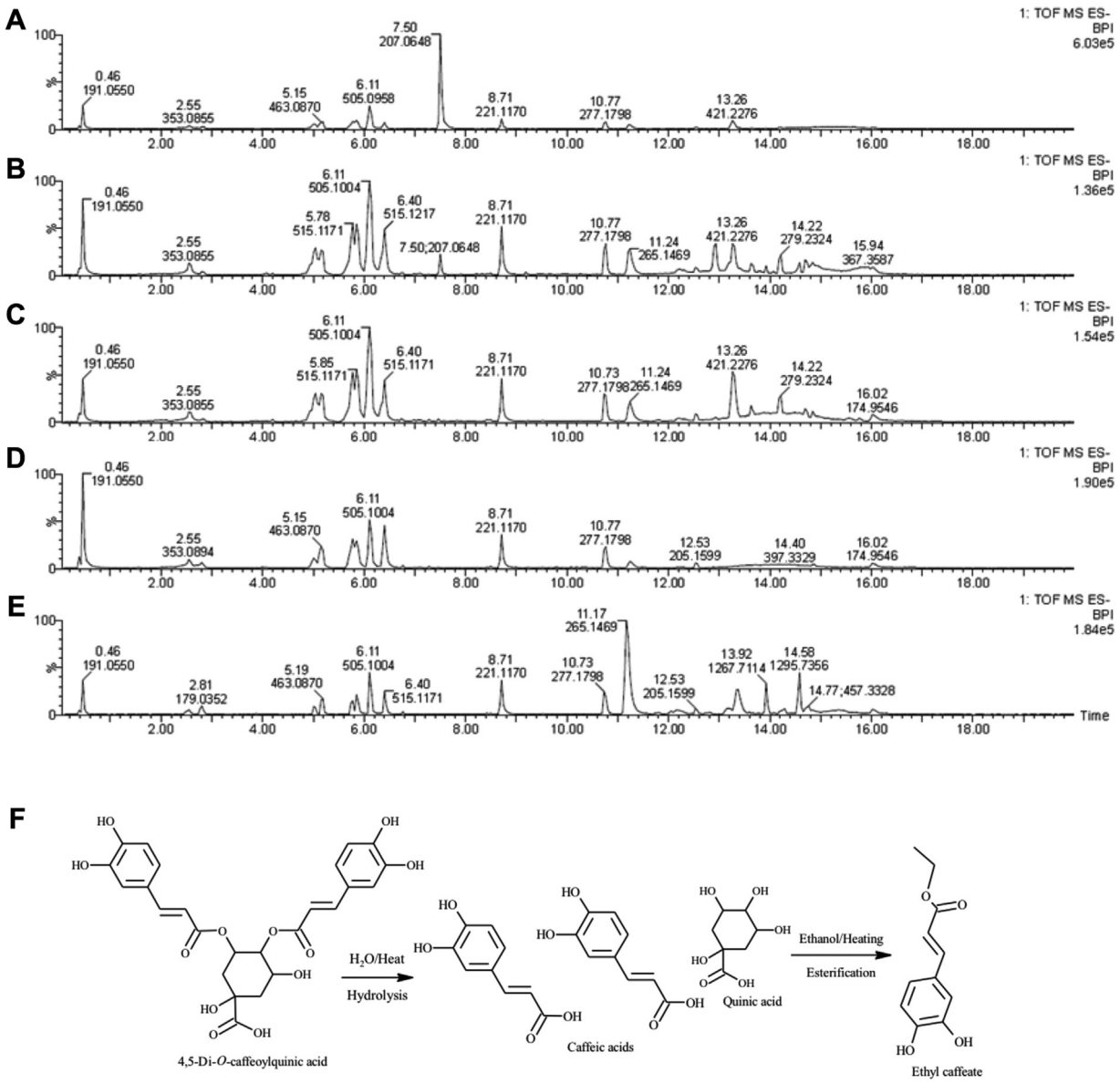

Fig. 3. Base peak chromatogram (negative ionization mode) of the extracts obtained with: (A) 90°GL ethanol, (B) 70°GL ethanol and (C) 50°GL ethanol, (D) methanol, (E) acetone, (F) ethyl caffeate formation.

acid) leading to the formation of the ester (Clayden et al., 2007; Campos-Buzzi et al., 2009).

To verify the hypothesis of artefact formation, the leaves and stems of *I. pes-caprae* were extracted with 50°GL methanol and acetone, once these solvents could not produce ethyl caffeate from caffeic acid derivatives. In fact, the presence of ethyl caffeate was not detected in these extracts (Fig. 3D and E).

However, it is important to understand how the phytochemical alteration in the extract affects its biological activity. As previously reported (da Cunha et al., 2004), caffeic acid derivatives (including ethyl caffeate) exerted *in vitro* and *in vivo* anti-inflammatory activities by scavenging NO and also modulating iNOS expression. In addition, antinociceptive effect was reported to caffeic acid derivatives in acetic acid-induced abdominal writhes in mice (Campos-Buzzi et al., 2009). Eventually, based on the hypothesis of that the presence of ethyl caffeate is the main difference between the three obtained extracts, they were incorporated in a semisolid formulation, including ethyl caffeate, to verify the biological effect.

In a previous work, we have described that the *I pes-caprae* extract obtained with ethanol 50°GL as solvent blocked the mechanical hypersensitivity induced by *Physalia physalis* venom (Barth et al., 2017). Furthermore, the same extract was effective in reducing the nociception and paw-oedema induced by trypsin, a protease capable of generating inflammatory responses by the activation of protease receptor 2 (PAR-2). As shown in Fig. 4(A–C), the extracts obtained with ethanol 70 and 90°GL did not present biological activity in both paw-oedema and spontaneous nociception induced by trypsin in mice.

On the other hand, when ethanol 50°GL was used to obtain the extract, it was observed significant inhibition of the paw-oedema and nociception, with inhibition values of 45 ± 9% and 24 ± 3%, respectively. As expected, and accordingly with literature data (Campos-Buzzi et al., 2009), ethyl caffeate presented prominent anti-inflammatory effect, with inhibition of 86 ± 5%. However, it seems not to be related with the anti-nociception exerted by the extract (50°GL ethanol).

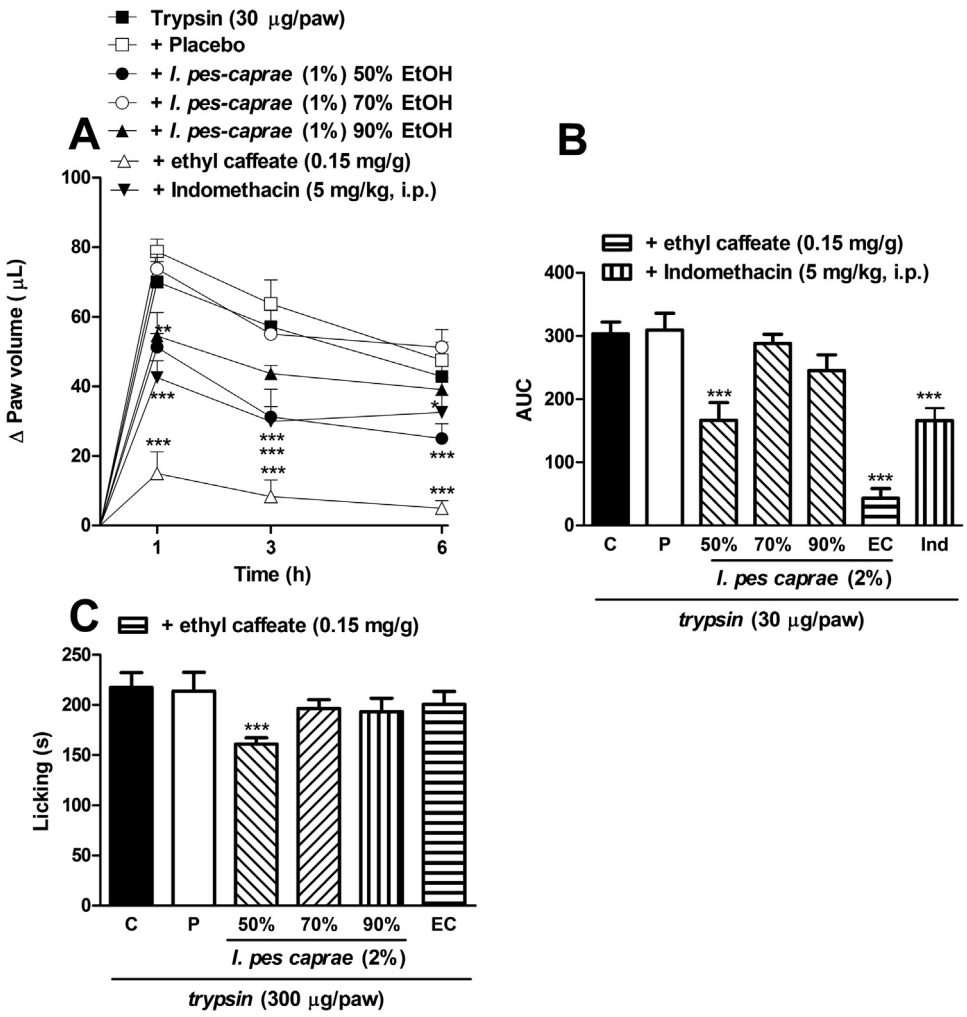

Fig. 4. Influence of extraction protocol in the biological activity of the *I. pes-caprae* extract. (A and B) Anti-inflammatory and (C) anti-nociceptive effects of *I. pes- caprae* semisolids (1%), obtained with etanol 50°GL, 70°GL or 90°GL, or semisolid containing ethyl caffeate (0.15 mg/g) on trypsin induced paw-oedema and spontaneous nociception in mice. Each group represents the mean of 6–12 mice and the vertical lines indicate the S.E.M. Significantly different from control values $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. (twe-way ANOVA followed by the Bonferroni's post hoc test and one-way ANOVA followed by the Dunnett's post hoc test for column graph). Panels 'B' represents the AUC of the respective line graph 'A'.

Conclusions

The high amount of the artefact in the extract of ethanol 50°GL could be, at least in part, the responsible for the topical anti-inflammatory activity of *I. pes-caprae* ethanol extract. Together, the herein results demonstrate that the extraction methodology is extremely important to determine the biological activity of the final herbal product and the chromatographic fingerprints have been used as an important tool to quality control of herbal medicines.

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Authors'contributions

The authors contribution was as follow: CSB, HGTS and LWR performed the pharmacological and technological experiments, CRSM and CA performed the chemometric analysis, TB, AM and LPS performed the LC analysis, RN, VCF and TMBB advised the chemical studies, AGC advised the technological studies, JRS and NLMQ advised the pharmacological studies. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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