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Research Paper

Chemical study and anti-inflammatory, analgesic and antioxidant activities of the leaves of Aristotelia chilensis (Mol.) Stuntz, Elaeocarpaceae

Orlando Muñoz[a], Philippe Christen[d], Sylvian Cretton[d], Nadine Backhouse[b], Vanessa Torres[b], Olosmira Correa[b], Edda Costa[b], Hugo Miranda[c] and Carla Delporte[b]

[a]Departamento de Química, Facultad de Ciencias, [b]Departamento de Química, Farmacológica y Toxicológica, Facultad de Ciencias Químicas y Farmacéuticas, [c]Programa de Farmacología, ICBM, Facultad de Medicina, Universidad de Chile, Casilla, Santiago, Chile and [d]School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland

Abstract

Objectives *Aristotelia chilensis* leaves (Elaeocarpaceae) are used in Chilean folk medicine to treat pain and inflammation. A bioguided study was carried out on serial extracts (hexane, dichloromethane, methanol, aqueous extract (INFU) and a crude mixture of alkaloids (ALK-MIX). All extracts were evaluated for (1) topical administration against both arachidonic acid and 12-deoxyphorbol-13-decanoate (TPA)-induced inflammation in mice and (2) per-os administration against inflammation by λ-carrageenan-induced paw oedema in guinea-pigs and (3) topical analgesia in tail flick and formalin models and per-os writhing test in mice.

Methods Greater anti-inflammatory effects were obtained against TPA with dichloromethane extract and methanol extract (63.9 and 66.0%, respectively). INFU showed the most potent effect (56.2%) against arachidonic acid. Greater effects were obtained in the writhing test with hexane and dichloromethane extracts (89.2% both). In the topical analgesia models, all the extracts and ALK-MIX were active with exception of the hexane extract in the formalin assay. In tail flick test, ALK-MIX and the methanol extract were the most active (58.2 and 55.2%, respectively). In relation to the tail formalin assay, the methanol extract (74.1%) was the most active. Concerning antioxidant activity, both INFU and the methanol extract were the most active either in the inhibition of xanthine oxidase (52.9 and 62.7%, respectively) or in the DPPH free radical scavenging activity (EC50 (concentration that produced 50% of activity) = 12.1 and 9.7 μg/ml, respectively).

Key findings Aristoteline, aristone, serratoline and hobartinol were isolated from ALK-MIX. Ursolic acid, friedelin and quercetin 5,3′-dimethyl ether were present in the dichloromethane extract while quercetin 3-O-β-D-glucoside and kaempferol were present in the methanol extract. From INFU were isolated protopine, aristoteline and caffeic and ferulic acids.

Conclusions The effects of *A. chilensis* are herein demonstrated, validating its use in traditional medicine. Protopine is reported for the first time in Elaeocarpaceae.

Keywords alkaloids; *Aristotelia chilensis*; flavonoids; triterpenes

Introduction

Aristotelia chilensis (Mol.) Stuntz (Elaeocaepaceae), a 4–6 m evergreen tree with yellow flowers and edible black-coloured fruits, grows in central and southern Chile and in south-western Argentina. Chilean folk medicine attributes various properties to the leaves of this species (aka maqui), such as astringent and febrifuge properties,[1] anti-diarrhoeal,[2] anti-inflammatory, analgesic and anti-haemorrhagic activity[3] and antioxidant and cardioprotective activity.[4] Crushed fresh leaves are used as a poultice for treating burns and counteracting fever.[5] This species has a high concentration of anthocyanin pigments, giving the characteristic dark violet colour to its berries.[6] *A. chilensis* is rich in polyglycosylated derivatives with high antioxidant capacity, which suggests anti-atherogenic properties.[7] Reports show that the aqueous leaf extract both alters human erythrocytes[8] and contains

Correspondence: Carla Delporte, Departamento de Química, Farmacológica y Toxicológica. Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile. Casilla 233, Santiago-1, Chile.
E-mail address: cdelpor@uchile.cl

indolic alkaloids – aristoteline, aristotelone, aristotelinine, aristone – together with a quinoline alkaloid.[9,10]

This species is widely known in Chile and extensively used in folk medicine by the Mapuche Indians; however, the pharmacological actions of its metabolites are still to be studied.[5]

As part of an ongoing research program devoted to secondary metabolites in Chilean species used in folk medicine, we report here on a chemical and pharmacological study of the leaves of *A. chilensis*. The main aim was the determination of the analgesic, anti-inflammatory and antioxidant activity of *Aristotelia chilensis* through a bioguided in-vivo assessment both to identify its main chemical constituents and for the first time to scientifically validate its medicinal use. The unusual presence of protopine, an isoquinoline alkaloid, was isolated from leaves of *A. chilensis* for the first time. This alkaloid is a novel finding for Elaeocarpaceae.

Materials and Methods

General experimental procedures

Xanthine and xanthine oxidase from cow's milk were purchased from Sigma (Poole, UK) and the standard inhibitor allopurinol from Laboratorios Saval (Santiago, Chile).

Unless otherwise indicated, all starting materials were obtained from commercial suppliers and used without further purification. Column chromatography was carried out using silica gel 60 (Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was performed on silica gel GF254 (Merck) with (1) CH_2Cl_2–MeOH (3 : 1), (2) AcOEt–MeOH (4 : 1) and aluminum oxide 60 F254 (Merck) with (3) CH_2Cl_2–MeOH (10 : 1). Spots were detected by UV light and Dragendorff or Liebermann–Burchard, anisaldehyde and NP/PEG reagents. Preparative TLC was performed on 2-mm thick silica gel F254 plates (Merck). Melting points are uncorrected. Optical rotations were measured with a Perkin Elmer 241 MC polarimeter at 25°C.

High-performance liquid chromatography–diode array detector (HPLC-DAD) measurements were carried out on a Hewlett Packard series 1100 apparatus (Waldbronn, Germany). Two columns were used. (1) Nova-Pak C18 (300 × 3.9 mm, 4 µm) column (Waters). The mobile phase was a gradient of 2% aqueous AcOH, MeCN–AcOH–water (20 : 2 : 78) and 100% MeOH. The flow rate was set at 1 ml/min and the UV detector at 280 nm. The injection volume was 20 µl. (2) Symmetry C18 (150 × 3.9 mm, 5 µm) column (Waters). The mobile phase was a gradient of 0.1% formic acid in H_2O (A), 0.1% formic acid in MeOH (B). The gradient was 5–35% (B) in 10 min, 35–70% (B) in 25 min and then 5 min with 100% (B). The flow rate was 1 ml/min. The injection volume was 20 µl. The detector was set at 210, 254, 280 and 350 nm. HPLC-ESI-MS was performed on an HP 1100 apparatus equipped with a binary pump. MS spectra were recorded with an ion trap MS detector LCQ equipped with an electrospray interface Finnigan MAT (San Jose, USA) in the negative mode with an Symmetry C18 (150 × 3.9 mm, 5.0 µm) column (Waters) at 25°C. The mobile phase was a gradient of MeOH (+0.1% formic acid) (A), H_2O (+0.1% formic acid) (B). The gradient was 5% (A) to 100% (A) in 30 min and then 10 min with 100% (A). The injection volume was 20 µl and the flow rate 1.0 ml/min. ESI conditions: capillary temperature 200°C, source voltage 5 kV, source current 80 µA, corona needle current 5 µA, collision sheath gas pressure 80 psi, collision energy 15 eV.

^1H and ^{13}C NMR were recorded in $CDCl_3$ at 400 and 500 MHz for ^1H and 125 MHz for ^{13}C; measurements of NMR spectra of all compounds, except for protopine, were made on a Bruker Avance AM-400 spectrometer (Karlsruhe, Germany) equipped with 5-mm probes. For protopine, the ^1H spectrum used for the measurements of the coupling constants and HMBC spectrum used for the substituents connectivity were recorded in $CDCl_3$ on a Bruker-DRX 500 MHz ^1H Larmor frequency using a 5-mm quadruple nuclei probe (QNP). Chemical shifts (δ) are in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants (J) are in Hz. For protopine, GC-MS analyses were carried out using a Hewlett-Packard 5890 series II chromatograph coupled to a HP 5972 mass-selective detector (Agilent Technologies, Palo Alto, USA). The MS detector was used in the electron impact (EI) ionization mode with ionization voltage of 70 eV. Mass spectra were recorded in the range m/z 30–600 at 1.3 scan/s and the MS transfer line was set at 280°C. The capillary column (HP5–MS, 30 m × 0.25 mm i.d., 0.25 µm film thickness) was used with He as carrier gas under the following conditions: an initial oven temperature of 70°C was maintained for 1 min then linearly increased at 5°C/min to a final temperature of 285°C, and held at this temperature for 15 min. A sample volume of 1 µl was injected in the splitless mode into laminar liner at 250°C using a fast HP 6890 series autosampler. High resolution mass spectra for analysis of protopine were obtained on a QStar XL TOF mass spectrometer (MDS Sciex, Concord, Canada) by direct infusion (5 ng/ml MeCN + 0.1% HCO_2H) using NanoMate 100 (Advion Biosciences, Ithaca, USA).

Plant material

The leaves of *Aristotelia chilensis* (Mol.) Stuntz (Elaeocarpaceae) were collected in October 2005 by Dr Orlando Muñoz, in Juan Gómez Milla Campus, University of Chile, Region Metropolitana, Santiago, Chile, and identified by Prof. Dr Carla Delporte. A voucher specimen was kept at the Herbarium of the Escuela de Química y Farmacia (SQF 22257), University of Chile.

Extraction and isolation of alkaloids

Air-dried powdered leaves of *A. chilensis* (1.1 kg) were extracted exhaustively at room temperature with (6.0 l) n-hexane, dichloromethane (CH_2Cl_2) and methanol (MeOH), yielding, after filtration and evaporation of the solvent *in vacuo*, 61.2 g, 76.0 g and 100.0 g of crude extracts, respectively (HE, DCM, ME). After concentration *in vacuo*, parts of extracts and fractions were used in bioassays.

After TLC analysis, the HE did not show any positive spots with Dragendorff reagent. Since DCM and ME showed a similar alkaloid profile, they were mixed together (35 g each), filtered and the solvent evaporated to leave about 35 ml of a gummy residue that was taken up in 250 ml 0.5 M HCl. The solution was extracted successively with Et_2O (6 × 75 ml) and CH_2Cl_2 (6 × 50 ml) to remove nonbasic material. The solution was then basified to pH 11 with NH_4OH and extracted with

CH_2Cl_2 (6 × 50 ml). The dried (Na_2SO_4) organic solution was evaporated to yield 5.0 g (7.1%) of a crude alkaloid residue (ALK-MIX). The basic material (3.5 g) was subjected to repeated column chromatography on silica gel (5–40 μm) and aluminium oxide using gradients of n-hexane : EtOAc and EtOAc : MeOH, respectively, to afford three fractions: Alk-M1, 300 mg; Alk-M2, 270 mg; and Alk-M3, 100 mg.

Additionally other fractions were also obtained containing known triterpenes, ursolic acid and friedelin, and a complex mixture of flavonoids, F-MIX (25 mg). Ursolic acid (71 mg) and friedelin (50 mg) were identified by comparison with authentic compounds isolated previously in our laboratory and the structures were confirmed by ^{1}H NMR with literature data.[11,12]

Fractions Alk-M1, Alk-M2 and Alk-M3 were dissolved in 1 ml of MeOH and submitted to preparative TLC on silica gel plate and developed successively with AcOEt : MeOH (4 : 0.5) and AcOEt. Alk-M1 afforded aristoteline (**1**) 3.1 mg; Alk-M2, aristone (**2**) 2.5 mg and Alk-M3, serratoline (**3**) 1.5 mg and hobartinol (**5**) 2.1 mg (Figure 1). From the latter, other minor alkaloids were obtained but the small amount isolated precluded their identification.

Further purification of F-MIX by column chromatography on silica gel (gradient of AcOEt : MeOH) followed by preparative TLC on silica gel (AcOEt : MeOH 10 : 2) resulted in the isolation of quercetin 5,3′-dimethyl ether (5 mg), quercetin 3-O-β-D-glucoside (8 mg) and kaempferol (21 mg). Their structures were confirmed by ^{1}H NMR and literature data.[13–15]

Infusion

An aqueous extract (INFU) was prepared from 200 g dried powdered leaves of *A. chilensis* with 2 l boiling water. The mixture was macerated for 30 min, filtered and evaporated to yield 50 g of crude extract (25%). Using the extraction method described above (4.5 g of a crude alkaloid residue was obtained) and preparative TLC on silica gel (AcOEt : Et_2O 9 : 0.5), 1.2 mg of **1** and 1.2 mg of **4** were isolated. For **4** HPLC/ESI-MS analyses were carried out (Figure 2).

Phenolic compounds were also obtained from this aqueous extract, by preparative TLC on silica gel (EtOAc–H_2O–MeOH–HOAc 1 : 3 : 3 : 3) and visualized by spraying with vanillin and hydrochloric acid. Caffeic (1.2 mg) and ferulic (1.2 mg) acids were obtained and identified with authentic compounds isolated previously in our laboratory and the structures were confirmed by ^{1}H NMR with literature data.[16]

Animals for in-vivo assays

All animal experiments were performed according to the ethical guidelines suggested by the 'International Norms for the Biomedical Investigation with Animals', elaborated by the Council of International Organizations (1990) and the bioethics norms of the Commission of the Chilean Public Health Institute and Faculty of Chemical and Pharmaceutical Sciences.

Pirbright guinea-pigs, 220–300 g, of both sexes were used for the per-os anti-inflammatory study. CF-1 mice of either sex, 20–25 g, were used to assess the analgesic and topical anti-inflammatory effects.

Per-os anti-inflammatory activity

The anti-inflammatory activity was evaluated in guinea-pigs, using the λ-carrageenan-induced paw oedema test. Paw volume was measured with an Ugo Basile plethysmometer (model 7150) 3 h after injecting 0.1 ml of 1% sterile saline λ-carrageenan (Sigma). Anti-inflammatory activity ($A_{carrageenan}$) was evaluated as:

$A_{carrageenan}$ % = [(% Ic − % Is)/% Ic] × 100; where % Ic is the median inflammatory response reached in the control group receiving only the vehicle (34.0 ± 2.3% paw volume increase), and % Is corresponds to the median inflammation response in the sample-treated guinea-pigs, expressed as: I % = [Vf − Vi/Vi] × 100, where Vf and Vi are the final and initial paw volumes, respectively. Sodium naproxen (Laboratorios Saval, Santiago, Chile) was used as reference drug at 1, 2, 4 and 6 mg/kg with 54.6% of maximum anti-inflammatory effect at 4 mg/kg.[17]

Groups of eight guinea-pigs for a single dose and groups of 16 for control were used and treated similarly. Another group of eight guinea-pigs was pre-treated with reference drug.

Topical anti-inflammatory activity

The anti-inflammatory activity was evaluated in mice. Mice were treated with the sample and 5 min later they received 2 mg arachidonic acid or 5 μg phorbol 12-myristate 13-acetate (TPA) dissolved in 20 μl acetone. Control mice received only arachidonic acid or TPA at the same concentration. Both the sample and the arachidonic acid or TPA were applied to the inner (10 μl) and outer (10 μl) surfaces of the right ear. The left ear received only the acetone. Mice were sacrificed (1 h after applying arachidonic acid and 4.5 h after TPA) by cervical dislocation and a 6-mm diameter section of the right and left ears were cut and weighed. Dermal anti-inflammatory activity (TAE) was evaluated according to the following equation: TAE% = [Wc − Ws/Wc] × 100, where Wc and Ws are the difference between the median weight of the right and the left ear sections of the control and the treated mice, respectively.[17] Nimesulide (Laboratorio, Santiago, Chile) (arachidonic acid-induced oedema) and indometacin (Laboratorio Madex, Santiago, Chile) (TPA-induced oedema) were used as reference drugs (at a dose of 0.25, 0.50, 1.0 and 2.0 mg/20 μl/ear and 0.063, 0.125, 0.25, 0.5 and 1.1 mg/20 μl/ear, respectively) with 48.8% (at 1 mg/ear for nimesulide) and 92.9% (at 0.5 mg/ear for indometacin) of maximum anti-inflammatory effect.

Groups of eight mice for a single dose and groups of 16 for control were used and treated similarly. Another group of eight mice was pre-treated with reference drug.

Topical analgesic activity

Tail flick test

A radiant heat automatic tail flick algesiometer (Ugo Basile, Comerio, Italy) has been reported to measure response latency.[18] The intensity of the light beam was focused on mice tails 2–2.5 cm from the tip and adjusted for baseline readings between 2 and 3 s. An 8-s cut-off was imposed to avoid tail damage by heat. Control reaction was recorded twice with 20-min intervals between readings. After topical administration of the extracts, the reaction time

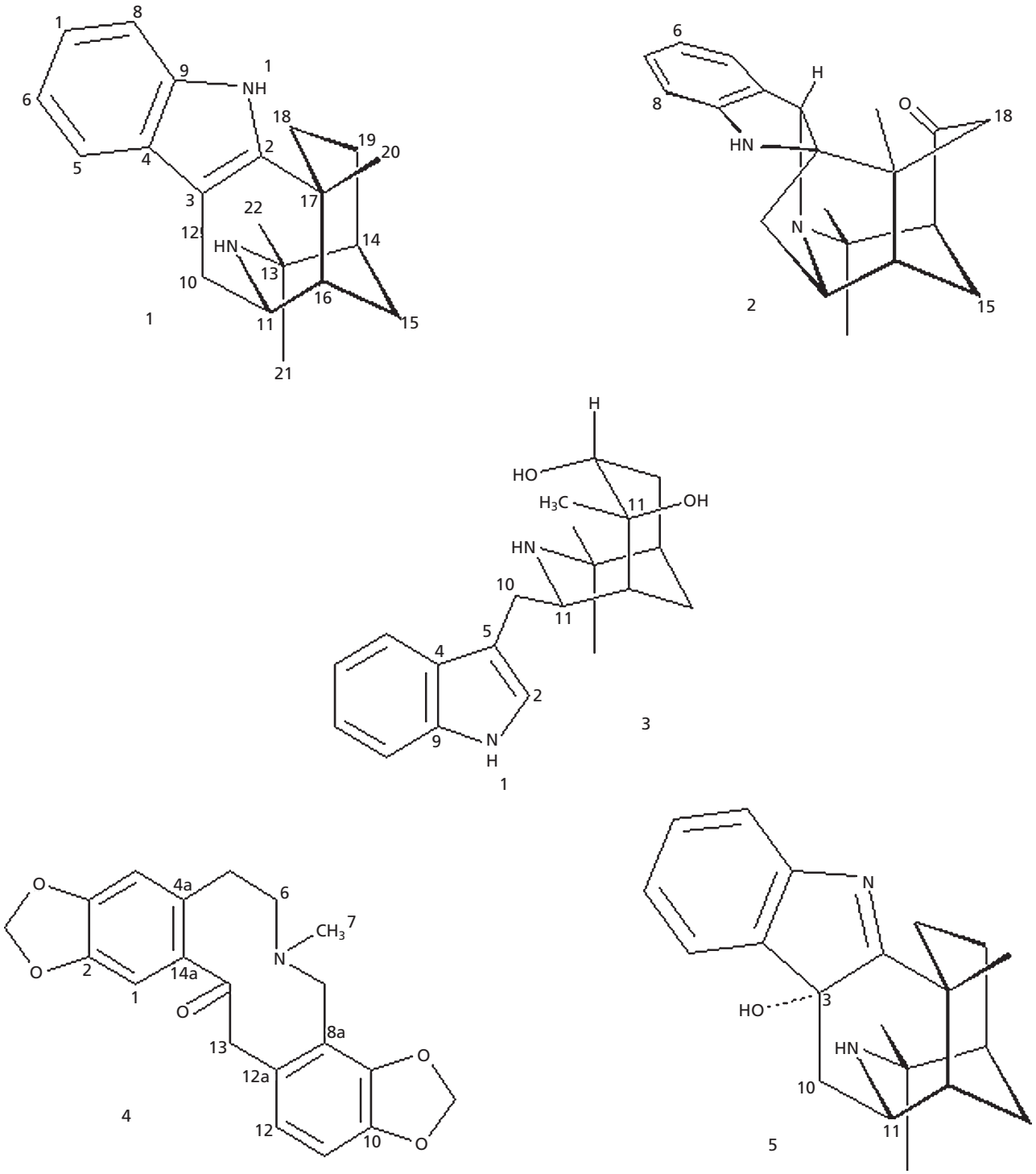

Figure 1 Alkaloids isolated from *Aristotelia chilensis* leaves.

was tested at 30 min (time of peak effect) and the difference in reaction time was recorded (Δ latency). For topical administration, the mouse tail was immersed for 3 min in dimethyl sulfoxide (DMSO) containing the extracts at different concentrations. Tail flick latencies were converted to maximum possible effect ($MPE_{tail\ flick}$), according to the following formula: $MPE_{tail\ flick}\ \% = 100 \times$ (postextract latency – preextract latency)/(cut-off time – preextract latency). Ibuprofen was the reference drug at 5% w/v with 50% analgesic effect.

Groups of eight mice for a single dose were used. Another group of eight mice was pre-treated with reference drug.

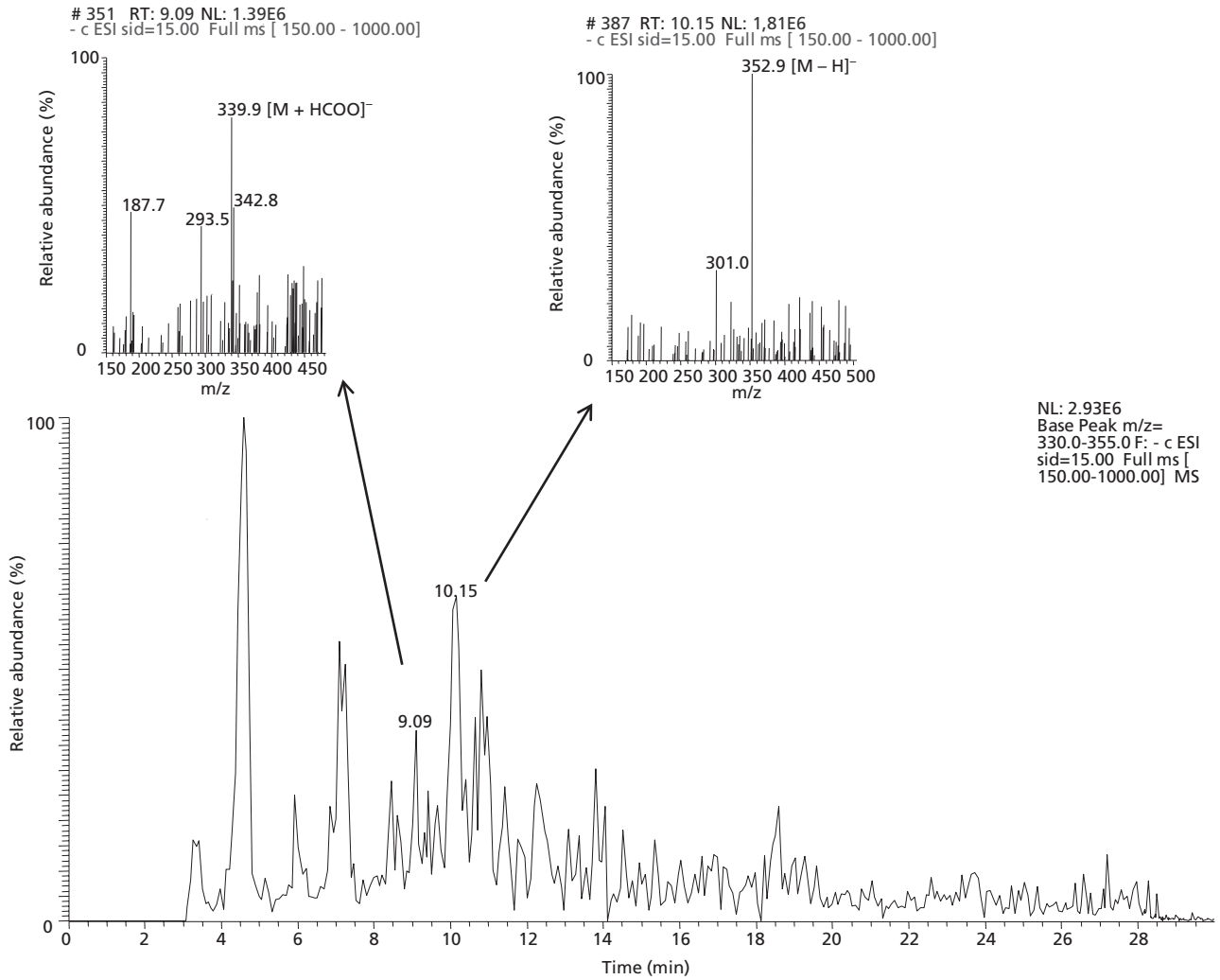

Figure 2 HPLC-ESI-MS chromatogram of *A. chilensis* aqueous extract (INFU) detected as base peak. Injection volume: 20 µl. Mass spectra were obtained with electrospray ionization (ESI) in negative mode. Symmetry C18 (150 × 3.9 mm, i.d.) column (Waters); MeOH-H_2O + formic acid 0.1% in gradient mode as mobile phase. Peak at 9.09 min: (+)-aristoteline; peak at 10.15 min: protopine.

Tail formalin test

A modified formalin test was used as described by Koleniskov *et al*.[19] Different extracts at 5% w/v concentration were applied by topical administration as described in the tail flick test, and mice were immediately intra-dermally injected with 20 µl of a 10% formaldehyde solution into the dorsal surface of their tail, using a tuberculin syringe. Then the mouse was enclosed in mirrored-wall chamber to enable clear observation of the tail for 5 min. The nociceptive behaviour is directly proportional to the licking time of the tail, which is a monophasic process.[19] The time-course observation was restricted to 5 min, the length of time during which pain occurs. Antinociceptive activity ($An_{formalin}$) was expressed according to the following formula:

$An_{formalin}\ \% = 100 - [(T1 \times 100)/T2]$, where T1 stands for the median licking time post-extract and T2 is the median licking time for control. Ibuprofen was the reference drug at 5% w/v with 76.5% maximum analgesic effect.

Groups of eight mice for a single dose and groups of 16 for control were used and treated similarly. Another group of eight mice was pre-treated with reference drug.

Analgesic activity per os

The analgesic activity was evaluated in mice using an intraperitoneal injection of 0.5 ml of 0.6% acetic acid.[17] The analgesic effect (An_{writhe}) was calculated by comparing the number of abdominal writhes of the treated mice with the control group, which only received the vehicle. The number of abdominal writhes of each mouse was counted for 5 min, beginning 5 min after acetic acid administration.

The following equation was used to calculate the pain percentage: $P\ \% = [(C_{sample}/C_{control}] \times 100$, where C_{sample} is the median writhes reached in sample-treated animals and $C_{control}$ (21.6 ± 3.8) is the median writhes reached in control animals which received only the vehicle.[17] The An_{writhe} was calculated according to the following equation: $An_{writhe}\ \% = 100 - \%\ P$.

Sodium naproxen (Laboratorios Saval, Santiago, Chile) was used as reference drug at 12.5 mg/kg with 70% maximum effect.

Groups of eight mice for a single dose and groups of 16 for control were used and treated similarly. Another group of eight mice was pre-treated with reference drug.

Determination of primary skin irritation

The hair from the backs of six rabbits (New Zealand Albino) was clipped, exposing two test areas on each side of the spine. The exposed areas were cleaned with ethyl alcohol (70°). One of the sides of the back was abraded using a sterile needle. Two cameras of the patch (Finn Chambers on Scanpor) were filled with the HE, DCM and ME (20 µl each) and two other cameras were filled with water (control). Then the patch was placed on the back. After 24 h, the patch was removed and the test sites were scored for erythema and oedema using the criteria of score 0 to 4 (no oedema, no erythema, up to severe oedema and severe erythema). The test sites were scored again after 48 h. The primary irritation index (PII) for each test rabbit and substance is the sum of the abraded and intact skin scores for erythema and oedema at 24 and 48 h, divided by six. The average primary irritation index is equal to the sum of the PIIs for each substance for all test rabbits divided by the number of test rabbits. The irritation potential was ranked according to the PII value, ranging from 0–0.4 (no irritation) up to 5–8 (severe irritation).[20]

General procedures and statistics

In per-os anti-inflammatory and analgesic assays, the dry extracts (HE, DCM, ME), ALK-MIX and reference drugs were orally administered 1 h before λ-carrageenan or acetic acid by means of an intragastric catheter, suspended in 5% w/v saline arabic gum. The oral doses used in each assay were selected according to previous work on the same biological activity: 200 and 100 mg/kg for extracts and ALK-MIX, respectively.[17] For topical administration, samples were dissolved in acetone, and the doses used for extracts or ALK-MIX were selected according to previous work on the same biological activity for anti-inflammatory assay: 3, 2 and 1 mg/20 µl/ear, respectively. Similarly, for topical administration, 30, 15, 7.5 and 3.75% w/v were used in analgesic assays and the samples were dissolved in DMSO. All experimental animals were fasted overnight before the day of the assays.

In all assays, the dose–response curves were obtained for HE, DCM, ME, ALK-MIX and reference drugs to determine their maximum effect (↑). Results are presented as E % ± SEM. For all pharmacological assays, the drug-induced changes were statistically estimated using a non-parametric method of analysis. Statistical significance of more than two groups were evaluated using the Kruskall–Wallis test, followed by Dunnett's multiple test for individual comparisons (free PRISM software was used).[21] The criterion for statistical significance was set at $P \leq 0.05$. Due to the low amount obtained, different isolated compounds were not assessed.

Xanthine oxidase activity

Different extracts solubilized in bi-distilled water (50 µg/ml) were evaluated. Inhibition of the xanthine oxidase activity was measured in relation to the amount of uric acid produced from xanthine.[22] This quantity was spectrophotometrically measured at 290 nm using a UNICAM spectrophotometer. Extract evaluation required a mixture of 1.0 ml of extract solution, 2.9 ml of phosphate buffer (Na_2HPO_4/KH_2PO_4; pH = 7.5) and 0.1 ml of the enzyme solution at the concentration of 0.042 U/ml. After pre-incubation of the mixture at 25°C for 15 min, the reaction was initiated by adding 2.0 ml of a 150 µM aqueous solution of xanthine as a substrate. This mixture was incubated at 25°C for 30 min. Reaction was stopped by the addition of 1.0 ml 1 N HCl and the absorbance was measured. The inhibition percentage of xanthine oxidase activity (I_{xo} %) was calculated as: $I_{xo}\ \% = (A - B) - (C - D)/(A - B) \times 100$, where A is the xanthine oxidase activity without extract (total uric acid), B, the blank of A without xanthine oxidase, C, the enzyme activity with the extract (residual uric acid) and D, the blank of C without the enzyme.[22]

The IC50 (concentration that produced 50% of inhibition) determination of allopurinol, used as reference drug, was 0.035 µg/ml (0.267 µM). The data are present as mean value ± SD. For xanthine oxidase activity (in triplicates), the extract-induced changes were statistically determined using the Kruskall–Wallis test, followed by Dunnett's multiple test for individual comparisons (free PRISM software was used).[21] The criterion for statistical significance was set at $P \leq 0.05$.

DPPH decolouration assay

Free radical scavenging by the extracts was spectrophotometrically evaluated at 517 nm through the residual absorbance (A) of the DPPH radical. The scavenging activity of extracts and fractions was assessed by the decolouration of a methanolic solution of DPPH.[23] A freshly prepared DPPH solution (20 mg/l) was used for the assays. Samples were dissolved in methanol and the DPPH solution served as a control. The decolouration degree indicates the free radical scavenging efficiency of the samples. Quercetin (Aldrich, Buchs, Switzerland) was used as reference free radical scavenger. The percentage of DPPH decolouration was calculated as follows:

$$\text{Decolouration (\%)} = 1 - (\text{Compound with DPPH} - \text{Blank sample})/(\text{DPPH control}) \times 100$$

Extracts were assayed at final concentrations of 400, 200, 100, 50, 10 and 1 µg/ml. The $EC_{50\text{-DPPH}}$ (concentration that produced 50% of activity) was calculated.

The reaction tubes, in triplicates, were wrapped in aluminium foil and kept at 30°C for 30 min in the dark. All measurements were done under dim light. Spectrophotometric measurements were done using a UNICAM spectrophotometer. The data are present as mean value ± SD.

(+)-Aristoteline (**1**): Crystallized (hexane-acetone-CH_2Cl_2) mp 161.7–162.0°C (lit.mp 160–162.5°C.[24] $[\alpha]_D + 18 \pm 6°$ (c 0.980, $CHCl_3$) ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.09 (s, 3 H, H-20); 1.31 (s, 3 H, H-22); 1.41 (q, 1 H, H-14); 1.47 (s, 3 H, H-21); 1.61–1.72 (m, 3 H, H-16, H-18, H-19′); 1.91–1.95 (m, 1 H, H-19); 1.98 (td, 1 H, H-15′); 2.07 (qd, 1 H, H-15); 2.31 (m, 1 H), 2.63 (d, 1 H, H-10′); 3.08 (dd, 1 H, H-10); 3.63 (md, 1 H, H-11); 7,08 (ddd, 1 H, H-7); 7.13 (ddd,1 H, H-6); 7.30 (d, 1 H, H-8); 7.47 (d, 1 H, H-5); 7.79 (br s, 1 H, N_a-H).

Aristone (**2**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.21, 1.37, 1.40 (s, 3 Me); 1.62 (dd, 1 H, H-8_a); 1.85 (ddd, 1 H, H-12); 1.89 (ddd, 1 H, H-13); 2.04 (dd, 1 H, H-8_b); 2.10 (dd,1 H, H-14); 2.13 (d, 1 H, H-16); 2.30 (ddd, 1 H, H-13); 2.80 (d, 1 H, H-16); 3.51 (ddd,1 H, H-9); 3.54 (s, 1 H, H-3) 3.68 (s, 1 H, NH); 6.61 (d, 1 H, H-7); 6.76 (t,1 H, H-5), 7.08 (t,1 H, H-6); 7.12 (d, 1 H, H-4).

Serratoline (**3**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (s, 3 H, H-22); 1.33 (s, 3H, H-21); 1.27 (dd, 1 H, H-18_a); 1.51 (dd, 1 H, H-10_a); 1.55 (m, 1 H, H-16); 1.56 (s, 3 H, H-20); 1.78 (tdd, 1 H, H-19); 1.98–2.04 (m, 3 H, H-15); 2.51 (dd, 1 H, H-10_b); 3.05 (dt, 1 H, H-18_b); 3.58 (q, 1 H, H-11); 7.19 (d, 1 H, H-6); 7.32 (d, 1 H, H-7); 7.36 (d, 1 H, H-5); 7.54 (d, 1 H, H-8).

Protopine (**4**): ^{1}H NMR (500 MHz, $CDCI_3$): δ 2.04 (s, 3 H, N-CH_3); 2. 35–2.70 (br s, 2 H, H-5); 2.35–2.70 (br s, 2 H, H-6); 3.5–3.7 (br s, 2 H, H-8); 3.74 (br s, 2 H, H-13); 5.92 (s, 2 H, O-CH_2-O); 5.93 (s, 2 H, O-CH_2-O); 6.62 (s, 1 H, H-4); 6.67 (m,1 H, H-11); 6.68 (m, 1 H, H-12); 6.90 (s, 1 H, H-1). ^{13}C NMR (125 MHz, $CDCI_3$): 30.9 (t, C-5); 41.6 (q, C-7); 45.6 (t, C-13); 51.3 (t, C-8); 57.4 (t, C-6); 101.2 (t, O-CH_2-O); 101.3 (t, O-CH_2-O); 106.9 (d, C-11); 107.9 (d, C-1); 110.2 (d, C-4); 116.9 (s, C-8_a); 124.7 (d, C-12); 128.3 (s, C-12_a); 131.7 (s, C-4_a); 135.2 (s, C-14_a), 146.1 (s, C-9); 146.1 (s, C-10); 146.1 (s, C-2); 148.1 (s, C-3); 184.1 (s, C-14). GC-EI-MS 70 eV: m/z 354 [M] + (1), 353(2), 267(7), 252(7), 190(12), 163(25), 149(12), 148(100), 134(10), 89(9). HRMS: m/z [M + H^+] calcd. for C_{20} H_{20} N_5 354.1341 found 354.1352.

Hobartinol (**5**): ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.15 (s, 3 H, H-22); 1.05 (s, 3 H, H-21); 1.48 (m, 1 H, H-14); 1.50 (s, 3 H, H-20); 1.80 (dq, 1 H, H-15_a); 1.85 (m, 1 H, H-16); 1.96 (dt, 1 H, H-19_a); 2.10 (dt,1 H, H-19_b), 2.39 (dt,1 H, H-15_b); 2.91 (dd,1 H, H-10_a); 3.05 (ddd,1 H, H-10_b); 3.30 (m,1H, H-18); 3.59 (ddd, 1H, H-11); 7.01 (ddd, 1 H, H-6); 7.10 (ddd,1 H, H-2); 7.31 (dt,1 H, H-8); 7.10 (s,1 H, H-7); 7.50 (dt, 1 H, H-5).

Results

Chemical results

Aristoteline (**1**), a pentacyclic alkaloid isolated from *A. chilensis*, *A. peduncularis*, *A. serrata* and *A. australasica*, is a compound widely found in four out of five *Aristotelia* species.[25,26] In our study, aristoteline was detected and obtained from both ALK-MIX and INFU; its presence in INFU was surprising and turned out to be significant since the aqueous extract is the form used in traditional medicine.

The observed ^{1}H NMR data were in good agreement with those reported earlier.[24,27,28] Aristoteline has been used as a marker (relay compound) within the *Aristotelia* genus and these correlations have served to establish the relative and absolute configuration of other related alkaloids such as makonine, aristotelinone, serratoline and others.[29]

Aristone (**2**), $C_{20}H_{24}N_2O$, the only hexacyclic *Aristotelia* alkaloid previously obtained from *A. chilensis* and *A. australasica*, was isolated by our group from ALK-MIX; the NMR data agreed with a previous report.[26,29] Aristone might biogenetically derive from aristotelinine.[29]

Both alkaloids, serratoline (**3**) and hobartinol (**5**) from ALK-MIX, have already been isolated from *A. chilensis* and *A. australasica*, respectively.[26,29,30]

In the INFU, aristoteline (**1**) and protopine (**4**) together with the phenolic compounds, caffeic and ferulic acids, were identified. Unexpectedly, no flavonoids were detected in this aqueous extract. Signal assignments to **4** correlated well with ^{1}H NMR, ^{13}C NMR and MS data reported elsewhere.[31,32]

Protopine and related alkaloids are widely distributed in Berberidaceae, Fumariaceae, Papaveraceae, Ranunculaceae and Rutaceae.[33] It should be noted that the isoquinoline alkaloid protopine is a novel finding for Elaeocarpaceae (Figure 1). To our knowledge, no experimental studies dealing with the biosynthesis of *A chilensis* have been reported.

Lastly, we want to emphasize that ursolic acid, friedelin and quercetin 5,3′-dimethyl ether are present in the DCM, while quercetin 3-O-β-D-glucoside and kaempferol are present in the ME. From the aqueous extract were isolated protopine, aristoteline and caffeic and ferulic acids. Aristoteline, aristone, serratoline and hobartinol are present in both DCM and ME.

Pharmacological results of *A. chilensis* extracts

Topical and per-os anti-inflammatory effect

Table 1 shows results for the anti-inflammatory assays in mice of the various extracts and ALK-MIX. The higher effects against TPA-induced inflammation were similar for DCM and ME (63.9 and 66.0%, respectively). On the other hand, INFU showed the greatest effect (56.2%) against arachidonic acid-induced inflammation, and this aqueous extract exhibited a greater effect than the reference drug nimesulide, reaching almost double the effect exhibited for HE and DCM (30.0 and 31.5%, respectively). The topical anti-inflammatory effect of ME (20%) was not significant.

Since this crude alkaloid mixture was obtained from two highly bioactive extracts, we would expect anti-inflammatory activity; as this did not occur, we infer that the alkaloids do not contribute to the effects found for such extracts. In addition, no positive effects were observed in the anti-inflammatory per-os assays (carrageenan-induced paw oedema), where the different extracts and ALK-MIX seem to show a pro-inflammatory reaction.

Table 1 also shows the maximum topical anti-inflammatory effect of nimesulide, indometacin and the per-os anti-inflammatory activity of naproxen sodium.[17] These reference anti-inflammatory drugs exhibited a dose-related effect.

Analgesic effect

According to Table 2, in all the pharmacological models in mice, *A. chilensis* extracts proved to be more efficient in relieving pain than inflammation. Greater effects were obtained in the writhing test, when HE and DCM (89.2%, both) were administered *per os*, and were more potent than the maximum effect of the reference drug (naproxen sodium, 54.1%).

In the topical analgesic models (tail flick and formalin), all the extracts and ALK-MIX were active with exception of HE

Table 1 Anti-inflammatory effects of *A. chilensis* extracts, ALK-MIX and reference drugs

Sample	TAE_{AA} % Dose (mg/ear)	TAE_{TPA} % Dose (mg/ear)	$A_{carrageenan}$ % Dose (mg/kg)
INFU	↑56.2* ± 6.6 3	1^{b} ± 13.0 3	-8.8^{c} ± 4.39 200
HE	↑$30.0^{*,a}$ ± 6.8 3	↑28.3^{b} ± 5.4 3	-15.6^{c} ± 6.5 200
DCM	↑$31.5^{*,a}$ ± 8.7 3	↑$63.9^{*,b}$ ± 10.3 3	-23.6^{c} ± 9.2 200
ME	20.0^{a} ± 5.7 3	↑$66.0^{*,b}$ ± 12.1 0.5	1.7^{c} ± 10.4 200
ALK-MIX	-11.5^{a} ± 6.0 3	$-64.4^{*,b}$ ± 8.8 3	$-47.3^{*,c}$ ± 8.5 200
NM	↑48.8* ± 4 1	n.t.	n.t.
IND	n.t.	↑92.9* ± 3.2 0.5	n.t.
SN	n.t.	n.t.	↑54.6* ± 8 4

$A_{carrageenan}$, *per os* anti-inflammatory effect against λ-carrageenan-induced paw oedema in guinea-pigs; ALK-MIX, crude alkaloids obtained from mixture DCM and ME; DCM, dichloromethane extract; HE, n-hexane extract; IND, indometacin; INFU, aqueous extract; ME, methanol extract; n.t., non tested; NM, nimesulide; SN naproxen sodium; TAE_{AA}, topic anti-inflammatory effect against arachidonic acid in mice; TAE_{TPA}, topic anti-inflammatory effect against 12-deoxyphorbol-13-decanoate (TPA) in mice. Each value is presented as the median ± SEM of eight animals treated with samples or reference drugs; ↑ maximum effect; $^{*}P \leq 0.05$, comparing with control group; without asterisk, $P > 0.05$, comparing with control group; $^{a}P \leq 0.05$, comparing with NM; without a, $P > 0.05$, comparing with group treated with NM; $^{b}P \leq 0.05$, comparing with group treated with IND; $^{c}P \leq 0.05$, comparing with group treated with SN; without c, $P > 0.05$, comparing with group treated with SN.

in the formalin assay. In tail flick test, ALK-MIX and ME were the most active (58.2 and 55.2%, respectively).

In relation to tail formalin assay, ME (74.1%) was the most active analgesic extract, yielding a similar effect to that obtained with ibuprofen at the same concentration.

In summary, all the extracts showed dose-dependent antinociceptive activity in the tail flick and formalin test for topical administration and acetic acid writhing test for per-os administration. The exception was ALK-MIX, which was inactive in the writhing test for per-os administration.

Primary skin irritation

No irritation was observed with any tested bioactive extract from the leaves of *A. chilensis* in rabbits. The primary irritation index, PII, was zero for HE, DCM and ME.

Antioxidant activity

The polar extracts INFU and ME were the most active against the inhibition of xanthine oxidase (52.9 and 62.7%, respectively), close to the reference drug, and in the DPPH decoloration assay (EC50 = 12.1 and 9.7 μg/ml, respectively). DCM showed a weak activity against DPPH. The activity of HE was practically unnoticeable. The activity of all the tested extracts was lower than reference drugs (Table 3).

Discussion

The use of two topical inflammatory agents – TPA and arachidonic acid – has yielded data that provide some insight into the levels at which different extracts interfere with the inflammatory cascade. TPA acts primarily as an activator of protein kinase C and nuclear factor (NF)-κβ, promoting the enhanced expression of pro-inflammatory enzymes. On the other hand, arachidonic acid presumably acts as a precursor of inflammatory mediators such as prostaglandins and leukotrienes. In addition, neutrophils that quickly reach the inflamed site release myeloperoxidase and NADPH oxidase.[34,35]

The writhing test allows us to identify central and peripheral analgesic compounds.[36] The tail formalin test is a recent algesiometric assay in which the only suggestive behaviour of pain consists of the licking of the tail. The lack of the two distinct phases after formalin administration in the tail might correspond to a different pattern of the release of the chemical pain mediators at both the spinal and peripheral levels and this method mainly identifies peripheral analgesia.[19] The thermal model of the tail flick test is considered a spinal reflex, but could also involve higher neural structures, thus identifying mainly central analgesia.[36]

The topical anti-inflammatory effect in the TPA and arachidonic acid assays and the analgesic activity of DCM may be partly caused by the mixture of the pentacyclic triterpenoids, ursolic acid and friedelin, with quercetin 5,3′-dimethylether. This flavonoid has greater anti-inflammatory activity than the positive control mefenamic acid.[37]

Triterpenic acids with ursane, oleanane and lupane skeletons are well-known for their potent anti-inflammatory activity, which is similar to that of synthetic non-steroidal anti-inflammatory drugs.[38] Reports suggest that the topical anti-inflammatory activity of plant extracts is due to the presence of these compounds, mostly to the high content of ursolic acid.[39] In addition, reports have proved the antioxidant property of ursolic acid through a series of in-vitro tests such as DPPH radical scavenging assays.[40]

Friedelin from *Calophyllum brasiliense* exhibits considerable antinociceptive properties, particularly in the writhing test. This triterpenoid is even more potent than acetylsalicylic acid and paracetamol (acetaminophen).[41]

Quercetin 3-O-β-D-glucoside and kaempferol in ME may be responsible for the inhibition of both topical TPA-induced inflammation and analgesic activity. In-vivo assays show that kaempferol has a significant dose-dependent anti-inflammatory and analgesic activity.[42] The analgesic activity of kaempferol is mediated by the inhibition of nitric oxide production in activated peritoneal macrophages and by the reduction of tumour necrosis factor (TNF)-α and interleukin (IL)-12 production.[43] In addition to the anti-inflammatory activity, kaempferol shows apparent antioxidant activity; thus, Nagao *et al*.[44] demonstrated that kaempferol might suppress in-vivo formation of reactive oxygen species (ROS) and urate by inhibition of xanthine oxidase activity.

In relation to the anti-inflammatory and antioxidant activity of quercetin-3-O-β-D-glucoside, Kong *et al*.[45] demonstrated that this flavonoid led to the reduction of the expression level and activity of matrix metalloproteinase (MMP)-9 and MMP-2. Monocyte-derived MMP-9 may be

Table 2 Maximum possible topical analgesic effect ($MPE_{tail\ flick}$ and $An_{formalin}$) and maximum *per os* analgesic (An_{writhe}) effect of *A. chilensis* extracts, ALK-MIX and reference drugs

Sample	$MPE_{tail\ flick}$ % ± SEM [% w/v]	$An_{formalin}$ % ± SEM [% w/v]	An_{writhe} % ± SEM dose (mg/kg)
INFU	↑50.0* ± 9.9	↑55.2*,a ± 5.3	↑43.2*,b ± 9.4
	10	5	200
HE	↑46.5* ± 6.5	↑27.8a ± 5.8*	↑89.2*,b ± 5.3
	10	5	200
DCM	↑37.8* ± 3.8	↑59.7*,a ± 11.3	↑89.2*,b ± 4.9
	10	5	200
ME	↑55.2* ± 8.3	↑74.1* ± 9.6	↑54.1*,b ± 10.2
	10	5	200
ALK-MIX	↑58.2* ± 9.9	↑39.1*,a ± 7.2	2.1b ± 8.7
	10	10	200
SN	n.t.	n.t	↑70.0* ± 4.0*
			12.5
IB	50.0* ± 0.1	↑76.5* ± 6.3	n.t.
	5	5	

ALK-MIX, crude alkaloids obtained from mixture DCM and ME; $An_{formalin}$, analgesic effect in tail formalin assay for topical administration in mice; An_{writhe}, antinociception in acetic acid writhing test for *per os* administration in mice; DCM, dichloromethane extract; HE, n-hexane extract; IB, ibuprofen; INFU, Infused; ME, methanol extract; $MPE_{tail\ flick}$, maximum possible analgesic effect in tail flick assay for topical administration in mice; n.t., non tested; SN, naproxen sodium. Each value represents the median ± SEM of eight animals treated with samples or reference drugs; ↑ maximum effect; $*P \leq 0.05$, comparing with control group; without asterisk, $P > 0.05$, comparing with control group; $^aP \leq 0.05$, comparing with group treated with IB; without a, $P > 0.05$, comparing with group treated with IB; $^bP \leq 0.05$, comparing with group treated with SN; without b, $P > 0.05$, comparing with group treated with SN.

Table 3 Antioxidant activity of *A. chilensis* extracts and reference drugs against xanthine-oxidase and DPPH free radical scavenging activity

Sample	I_{xo} % (μg/mL)	$EC_{50\text{-}DPPH}$ (μg/mL)
INFU	52.9* ± 1.4 [50]	12.1 ± 4.5
HE	28.6* ± 4.5 [50]	410.3 ± 0.8
DCM	8.0 ± 1 [50]	142.4 ± 4.4
ME	62.7*,a ± 4.4 [50]	9.7 ± 3.8
Allopurinol (0.37 mM)	50.0* ± 0.5 [0.035]	n.t
Quercetin (0.17 mM)	n.t	1.1 ± 0.2

DCM, dichloromethane extract; $EC_{50\text{-}DPPH}$, concentration that produces 50% DPPH free radical scavenging activity; HE, n-hexane extract; INFU, Infused; I_{xo}, inhibition of xanthine oxidase activity; ME, methanol extract. Each value stands for the mean ± standard deviation of three determinations; $*P \leq 0.05$, comparing with control; without asterisk, $P > 0.05$, comparing with control; $^aP \leq 0.05$, comparing with allopurinol; without a, $P > 0.05$, comparing with allopurinol.

important in facilitating cellular influx and MMP-2 plays a role in endometrial menstrual breakdown, regulation of vascularization, and the inflammatory response.

The caffeic and ferulic acids in INFU could be responsible for the analgesic effect in all assays and the topical arachidonic acid-induced inflammation.[46] Huang *et al*.[47] showed that these phenolic acids exhibited inhibitory effects on TPA-induced tumour promotion in mouse epidermis parallel to their inhibitory effect on TPA-induced epidermal inflammation, epidermal lipoxygenase and cyclooxygenase activity.

The analgesic effect of INFU could also be due to the presence of protopine.[48] In relation to its analgesia, the effect of protopine was blocked completely by naloxone, a specific blocker of opioid receptors, indicating the participation of the opioid system in the production of analgesia. Protopine showed an inhibitory effect against the contraction of rabbit blood vessels and guinea-pig intestines induced by various factors through inhibiting the release of Ca^{+2} in cells without affecting the generation of prostaglandin (PG)I_2. Such characteristics of protopine may be relevant to its analgesic effect.[49]

The phenolic compounds of INFU and ME are partly responsible for antioxidant activity.[47] Quercetin-3-O-glucoside in ME has been reported to have free radical scavenging properties.[50]

As to the analgesic activity, this study shows that systemic, per-os and/or topical administration of the HE, DCM, ME, INFU and ALK-MIX of *A. chilensis* leaves produce dose-dependent antinociceptive activity according to two chemical and one thermal models for nociception.

The fact that different extracts and ALK-MIX, at the tested doses, produced analgesia in different topic nociceptive models is indicative that they possess both central and peripheral antinociceptive properties and the mechanism of action of the active principles of these extracts could partially be related to lipoxygenase or cyclooxygenase of the arachidonic acid cascade or opioid receptors.[36] The topical tail flick assay allowed us to detect an ALK-MIX maximum effect greater than that of ibuprofen, though at double the concentration of the latter.

Conclusion

This report demonstrates the analgesic and anti-inflammatory properties of the leaves of *Aristotelia chilensis* in addition to their antioxidant effect and validates their use in Chilean traditional medicine. Protopine isolated from a bio-active aqueous extract is an isoquinoline alkaloid with analgesic properties and is reported for the first time in Elaeocarpaceae.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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