Journal of Ethnopharmacology 216 (2018) 283–294

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Tetraglochin andina Ciald.: A medicinal plant from the Argentinean highlands with potential use in vaginal candidiasis

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ARTICLE INFO

Keywords:
Argentine Puna
Tetraglochin andina
T. cristatum
Anti-*Candida*
Anti-inflammatory
Antioxidant

ABSTRACT

Ethnopharmacological relevance: The Argentinean medicinal plant *Tetraglochin andina* Ciald, formerly classified as *T. cristatum* (Britton) Rothm is used in traditional medicine by inhabitants from Argentinean northwestern highlands (Puna) to treat candidiasis and as anti-inflammatory.

Aim of the study: To assess the potential of the crude drug as an anti-Candida agent with anti-inflammatory properties. The bioactivity and phytochemical composition of a dry extract of the plant was investigated.

Material and methods: The pharmacognostic description of the crude drug is carried out for the first time, including macroscopic and microscopic examinations of the different organs, physicochemical and extractive values (petroleum ether-, ethanol- and water-soluble). The dry extract from *T. andina* was evaluated as antifungal against pathogenic *Candida* sp. and *Saccharomyces cerevisiae* isolated from vaginal infections and reference strains, by the macrodilution and microdilution assays. The normal vaginal microbiome in women is characterized by the dominance of lactic acid-producing bacteria, mainly *Lactobacillus* spp. The effect of *T. andina* extract on *Lactobacillus* strains was also assayed. The inhibitory effect on proinflammatory enzymes (cyclooxygenase, lipoxygenase and phospholipase A_2) and antioxidant capacity was studied. The chemical profile was analyzed by HPLC-ESI-MS.

Results: The hydroalcoholic extract inhibited the growth of all yeasts with Minimal Inhibitory Concentration (MIC) values between 12.5 and 400 μg GAE/mL and the MIC values on *Lactobacillus* were higher than the MIC values against *Candida* isolates (> 400 μg GAE/mL). These results indicate that the hydroalcoholic extract could be used without affecting the normal microbiota of vaginal fluid. The extract showed antioxidant activity and could modulate the inflammatory process by three pathways ($sPLA_2$, COX-2, LOX). The plant extract contained high total phenolic levels (386.9 ± 1.7 mg GAE/g dry extract) and flavonoid levels (260.4 ± 2.7 mg GAE/g dry extract). Fifty phenolic compounds were identified by HPLC-ESI-MS. They were mainly hydrolysable and condensed tannins. The dry extract was chemically and biologically stable during one year at room temperature or 4 °C.

Conclusions: The presence of anti-Candida and anti-inflammatory activities in *Tetraglochin andina* extracts give support to their traditional use for treating conditions associated with microorganism infections and inflammatory process in humans. This plant preparation could be used to design phytopharmaceutical preparations to inhibit yeast growth and moderate the inflammatory and oxidative process.

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https://doi.org/10.1016/j.jep.2018.01.001
Received 29 June 2017; Received in revised form 29 December 2017; Accepted 2 January 2018
Available online 04 January 2018
0378-8741/

1. Introduction

The Argentine Puna is a cold and arid region characterized by high radiation levels, and strong winds. In this region, the diversity of plant species is lower than in other regions and the flora that grows is mostly herbaceous (grasslands), and bush (shrublands) with xerophite and halophyte species that have chemical, morphological and physiological adaptations to grow in extreme conditions (Lentz, 2013; Solbrig, 1976). The plants are used by the Puna inhabitants as a source of food, medicine, forage and building (García and Beck, 2006; Barbarán, 2008). The popular knowledge about medicinal uses of plant species has been transmitted orally throughout generations by Puna inhabitants and several of them are actually marketed in northwestern Argentina markets (Barbarán, 2008). In previous works we validate some popular uses of native Argentine Puna plants as antibacterial against antibiotic-resistant human pathogenic bacteria and as anti-inflammatory (Zampini et al., 2007a, 2007b, 2008, 2009a, 2009b; Cuello et al., 2011; D'Almeida et al., 2012; D'Almeida et al., 2013; Torres Carro et al., 2015, 2017).

The native plant *Tetraglochin andina* according to Acosta et al. (2016) and Cialdella and Pometti (2017) was formerly classified as *Tetraglochin cristatum* (Britton) Rothm (common names: horizonte, canguia, rancha-rancha, kailla, añahuaya). It is a species of the Rosaceae family that grows in northern Argentina highlands between 3000 and 4500 m above sea level (masl) (Fig. 1). The plant is a food for camelids (lama, alpaca) and domestic livestock. Due to their indiscriminate use as firewood and forage tend to disappear. Its fruits are also employed in human food and the aerial parts as medicinal to treat inflammatory processes and infections by the inhabitants of this region (García and Beck, 2006; Zampini et al., 2009b; Thomas et al., 2009; Torres Carro et al., 2015, 2017). In a previous paper, we reported the antibacterial activity of ethanolic extracts (ethanol 80°) of *T. andina* (*T. cristatum*) aerial parts on *Staphylococcus aureus* (Zampini et al., 2009b) and the anti-inflammatory activity of 17° ethanol extract (Torres Carro et al., 2015, 2017). There are no reports that scientifically validate the use of this species as antifungal.

Vaginal infections are one of the most frequent reasons for women to search for medical consultation (Donders, 2007; Mardh et al., 2002). Several surveys have estimated that more than 70% of adult women have had a vaginal infection requiring the application of vaginal therapies or products (Nappi et al., 2006; Sobel, 2007). Although they are not associated with high mortality rates, vaginal infections are a cause of considerable morbidity. The most frequent vaginal infections are caused by bacteria (such as vaginal bacteriosis, VB- also known as bacterial vaginosis- and aerobic vaginitis, AV), by fungi (vulvovaginal candidosis, VVC), and by protozoa (trichomoniasis)(Nappi et al., 2006). VVC results from the overgrowth of different *Candida* species, usually present in vagina as commensals, sometimes as part of the indigenous microbiota of healthy women (Sobel, 2007). The VVC is mainly caused by *Candida albicans* (85–95% of cases) followed by non- *C.albicans* species (Sobel, 2007) in women with recurrent VVC, post-menopausal and with uncontrolled diabetes and HIV-infected (Achka and Fries, 2010). It has been estimated that VVC affects 70–75% of women at least once in their lifetime and that by 25 years old about half of women have had at least one episode of VVC (Sobel, 2007). Furthermore, it has been reported that 40–50% of women will experience a recurrence, and 5–8% of adult women will develop recurrent VVC, as defined by the occurrence of four or more episodes within one year (Hurley and De Louvois, 1979). VVC can exhibit inflammatory signs and symptoms.

Particularly, VVC is not a life-threatening condition, but has a significant impact on the quality of life of the affected women (Hurley and De Louvois, 1979). The standard treatment of uncomplicated VVC is mainly based on oral or topically applied azoles that are effective in more than 90% of *C. albicans* infections (Hurley and De Louvois, 1979). On the other side, treatment of complicated VVC such as recurrent VVC and those caused by non-*C. albicans* species are still challenging mainly due to reduced azole susceptibility of non-*C. albicans* species and to the persistence of *C. albicans* on the external vulva in patients with recurrent VVC after the cessation of treatment (Hurley and De Louvois, 1979; Beikert et al., 2011). Consequently, the use of natural products to treat vaginal infections could be drive as complementary/alternative medicine therapies or to replace conventional therapies in recurrent drug-resistant cases.

In the present study a dry extract of *T. andina* aerial parts was biologically and phytochemically characterized to validate its traditional use as antifungal and anti-inflammatory crude drug.

2. Materials and methods

2.1. Materials and reagents

Soybean lipooxygenase-1 (9029-60-1), nimesulide (N1016), naproxen (N8280), dimethylaminocinnamaldehyde (6203-18-5), 2,2-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (30931-67-0) and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (2997-92-4) were purchased from Sigma-Aldrich (St. Louis, MO, USA), catalogue numbers

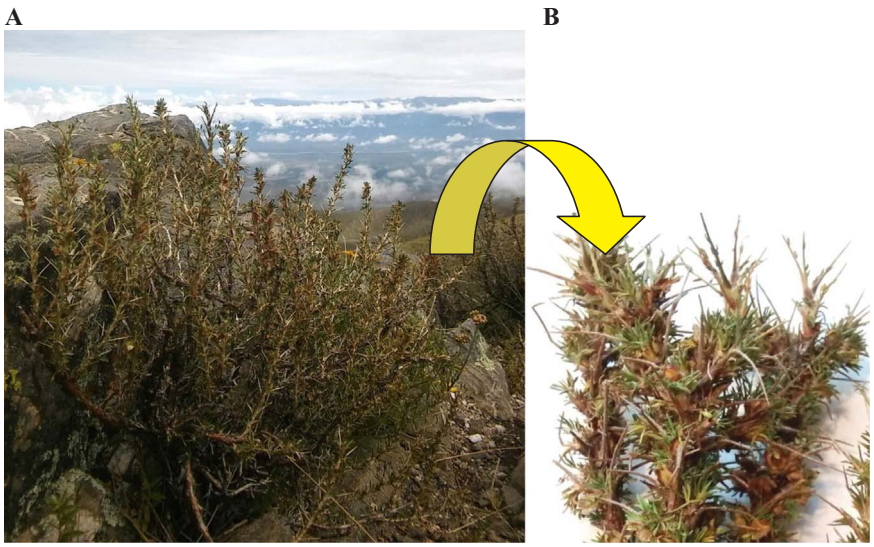

Fig. 1. The highland shrub *Tetraglochin andina* Ciald. (Rosaceae), formerly classified as *T. cristatum* (Britton) Rothm, growing in the Argentinian Puna (A) and detail of the aerial parts (B).

are given in parentheses. Linoleic acid (L-5900) was obtained from Fluka (Germany). COX kit assay (560131), secretory phospholipase A2 (sPLA2) (0424023-1), 1,2-diheptanoylthio-glycerophosphocholine (1,2 dHGPC), (89019-63-6) and 5,5-dithiobis-2- nitrobenzoic acid (DTNB) were from Cayman Chemical Co. (MI, USA). Other chemicals were purchased from local commercial sources and they were of analytical grade quality.

2.2. Plant material

Tetraglochin andina according to Acosta et al. (2016) and Cialdella and Pometti (2017), formerly *Tetraglochin cristatum* (Britton) Rothm (www.theplantlist.org) was collected from January to February 2015 in Huaca Huasi, Tucumán, Argentina (4300 masl, 65°44.23'W, 26°39.35'S). The plant was identified by Dra. Ana Soledad Cuello, INBIOFIV (CONICET) and a voucher specimen was included in the "Fundación Miguel Lillo" herbarium (voucher specimen number 610669/LIL). The parts used were leaves and stems (aerial parts), according to the traditional use. The samples were dried in a forced air oven at 40 °C.

2.3. Pharmacognostic characterization

2.3.1. Micrographic analysis

Leaves, leaflets, brachyblast and macroblast of three *T. andina* specimens were fixed in FAA (100 mL of 37% formaldehyde, 300 mL of 96% ethanol, 50% of 100% glacial acetic acid and 35 mL of distilled water). Entire leaves, spines, medium and terminal leaflets and fragments of brachyblast and macroblast were mounted in dental wax supports and sectioned with a rotary microtome (Microm HM315). The cuts (thickness range 10–25 μm) were cleared with 50% NaClO solution, washed with distilled water, colored in two successive steps with Astra blue-safranin dye and then mounted in 50% glycerol solution. Epidermal tissues were diaphonized following the technique of Dizeo de Strittmatter (1973). Leaves, leaflets and petiole-rachis samples were clarified with 10% KOH solution and then with 50% NaClO solution. The diaphonized material was colored with metachromatic 1% cresyl violet stain (Zarlavsky, 2014). Maceration of stems and thorny leaves was carried out following the Jeffreys method according to Zarlavsky (2014). All tissues were visualized with a Zeiss Axiolab optic microscope equipped with a Zeiss Axiocam ERc 5 s digital camera. Measurements were made using the AxioVision version 4.8.2 software (Carl Zeiss Ltd, Herts, UK). Palisade index was calculated as the average number of palisade cells beneath each epidermal cell. Stomatal density was calculated as number of stomata per unit area (mm^2), only considering the crypt area were stomata were located.

2.3.2. Dry extract preparation and physicochemical values determination

The powdered air-dried plant material (10 g) was macerated in 200 mL of 60° ethanol, petroleum ether or water for 1 h with ultrasonic application (10 min, five times). Then, each extract were filtered through Whatman N° 1 paper, evaporated at 40 °C in a rotatory evaporator and then lyophilized to determine the w/w extraction yield (dry weight). The dry extract was placed in oxygen barrier bags, vacuum packed (Multivac, D-8941, Germany). The percentage of total ash, acid insoluble ash, acid soluble ash and extractive values were determined according to Farmacopea Argentina, VII Edition (2003).

2.3.3. Phytochemical characterization

2.3.3.1. Qualitative phytochemical screening. Qualitative screening was carried out to determine the occurrence of cardiac glycosides (Teke et al., 2010), coumarins (Teke et al., 2010), flavonoids (Mojab et al., 2003), tannins (Adegboye et al., 2008), free anthraquinones (Onwukaeme et al., 2007), saponins (Ayoola et al., 2008), terpenoids, steroids and alkaloids (Adegboye et al., 2008).

2.3.3.2. Quantitative analysis. Total phenolic content (TPC) was determined by the Folin–Ciocalteau method (Cuello et al., 2011). Non-flavonoid phenols (NF-PC) were determined according to Isla et al. (2014). Flavonoid phenolic (FPC) content was calculated by difference between TPC and NF-PC. Flavone and flavanone content was determined according to Cuello et al. (2011). The total condensed tannin (proanthocyanidins) and hydrolized tannins content was quantified according to Torres Carro et al. (2015). All the determinations were carried out at different times during one year storage.

2.4. Biological activity studies

2.4.1. Antimicrobial assays

2.4.1.1. Candida strain and inoculum preparation. Candida strains were provided by Instituto Dr. Carlos G. Malbrán, Buenos Aires, Argentina. The strains used were *Candida albicans* (144783, 134333, 2089); *C. glabrata* (031646, 042030, 031982); *C. tropicalis* (1841); *S. cerevisiae* (134528, 134544, 124263), *C. parapsilopsis* (DMic 134410) and *C. krusei* (DMic 134409). All the microorganisms were maintained in brain–heart infusion containing 30% (v/v) glycerol at −20 °C. Before testing, the suspensions were transferred to Yeast Medium Agar (malt extract 0.3%, yeast extract 0.3%, peptone 0.5%, glucose 1%, agar 2%) and aerobically grown at 37 °C during 24 h. Individual colonies were isolated and suspended in 2 mL of 0.9% NaCl solution. The inocula were prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland scale in order to achieve the adequate inoculum in each case. The cell number was estimated using a serial dilution technique according to the recommendations of the M27-A3-S4 reference document of CLSI (Clinical and Laboratory Standards Institute, 2012a) for each assay.

2.4.1.2. Lactobacillus strain and inoculum preparation. Lactobacillus casei CRL 1267, L. *paracasei* CRL 1291 and L. *johnsonii* CRL 1292 strains isolated from healthy vagina were provided by CERELA (Centro de Referencia de Lactobacilos, Tucumán, Argentina) (Ocaña et al., 1999). The bacteria were growth in MRS broth during 24 h at 37 °C. The inoculum was adjusted with 0.9% NaCl to an OD corresponding to 0.5 McFarland scale, containing $1–5 \times 10^8$ CFU/mL, which was diluted 1:10, resulting in a viable cell number between 1 to 5×10^7CFU/mL.

2.4.1.3. MIC and MFC determination for yeast. MIC values of aqueous, 60° ethanol (hydroalcoholic) and petroleum ether extracts of *T. andina* against *Candida* and *Saccharomyces* were determined by the macrodilution method (Clinical and Laboratory Standards Institute, 2012b) using different concentrations of extracts (5–800 μg/mL). The MIC of the active extract was confirmed by broth microdilution method (Clinical and Laboratory Standards Institute, 2012b). The extract was transferred to each microplate well in order to obtain two-fold serial dilutions of the original extracts (5.25–400 μg/mL) with ethanol 60°. The inoculum (200 μL) containing $0.5–2.5 \times 10^3$ CFU/mL was added to each well. A number of wells were used in each plate as control: sterility (no inoculum added), microorganism viability (no extract added) and solvent effect (ethanol 60°). Plates were aerobically incubated at 37 °C. After incubation for 48 h, fungal growth was evidenced by the presence of turbidity and a pellet on the well bottom. The lowest concentration of extract without macroscopically visible growth was determined as MIC values. Then, an aliquot of 10 μL was removed from each well with no visible growth and inoculated in Sabouraud agar plates. After 16–20 h of aerobic incubation at 37 °C, the number of surviving organisms was determined to determine MFC values.

2.4.1.4. MIC determination for Lactobacillus. MIC values of the extracts against *Lactobacillus* were performed by agar macrodilution method (Ocaña et al., 1999). Two-fold serial dilutions of the original extracts. (5.25–400 μg/mL) was included in MRS-agar. The inoculum (2 μL)

containing 0.5×10^4 CFU/mL was seeded on the agar plates aerobically incubated during 24 h at 37 °C. MIC values were determined from the extract concentration that did not allow macroscopic growth of the cultures. All controls were performed.

2.4.2. Inhibition of inflammation-related enzymes

2.4.2.1. Effect on lipoxygenase (LOX). LOX activity was determined according to Torres Carro et al. (2015) using different concentrations of extract (between 12.5 and 70 μg GAE/mL). Caffeic acid (25–75 μg/mL) and sodium naproxen (5–25 μg/mL) were used as reference compounds. The enzyme inhibition effect was expressed as IC_{50} value, defined as the test extract or compound concentration able to produce 50% inhibition of LOX enzyme.

2.4.2.2. Effect on cyclooxygenase. The inhibitory activity of *T. andina* extract on cyclooxygenase 2 (COX-2) was measured using a COX inhibitor screening assay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer instructions, based on measuring prostaglandin (PG) by ELISA. A human recombinant COX-2 enzyme was used to form PG from arachidonic acid. The inhibitory assays were developed in the presence of different phenolic compound concentrations. Enzyme control was performed with COX that had been inactivated by placing it in boiling water for 3 min. The intra- and inter-assay coefficients of variations were 5% and 10%, respectively. A commercial anti-inflammatory drug (nimesulide) was used as reference. The effect of the phenolic extracts (25–75 μg GAE/mL) on pro-inflammatory mediators was evaluated by calculating inhibition percentage of PGE2 production.

2.4.2.3. Effect on phospholipase A_2. The $sPLA_2$ activity was determined using 1,2-diheptanoylthio-glycerophosphocholine (1,2dHGPC) and Triton X-100 as substrates (D'Almeida et al., 2013). The buffer Tris–HCl (10 mM pH 8) with $CaCl_2$ (10 mM), KCl (100 mM) and Triton X-100 (0.3 mM) (reaction buffer) was used for reconstitution of substrate to achieve a final concentration of 1.25 mM. The mixture contained 50 μL reaction buffer, 10 μL DTNB (5,5′ dithiobis-2-nitrobenzoic acid, 10 mM), 10 μL enzyme sPLA2 (1 mg/mL) and the hydroalcoholic extract dissolved in DMSO (25–75 μg GAE/mL) or commercial anti-inflammatory drug (acetylsalicylic acid, 20–80 μg/mL). The action was initiated by the addition of 150 μL of 1,2dHGPC (1.66 mM) and maintained during 40 min at 25 °C. Absorbance was read at 414 nm in a microplate reader (BiotekELx 808™).

2.4.3. Antioxidant activity

2.4.3.1. Total antioxidant capacity assay. The antioxidant capacity assay of the extract (concentration range between 0.1 and 11 μg GAE/mL) was carried out by the improved ABTS radical cation ($ABTS^{\bullet+}$) method as described by Re et al. (1999). Results were expressed as the concentration of extract required to scavenge 50% of $ABTS^{\bullet+}$ (SC_{50}). The negative control was performed with the vehicle. BHT and quercetin were used as reference compounds.

2.4.3.2. Protection against oxidative hemolysis. The protection of oxidative hemolysis of red blood cells (RBC) by the extract (concentration range between 0.12 and 2 μg GAE/mL) was determined according to Mendes et al. (2011), using azo compound solution [2,2′-azo-bis(2-amidinopropane) dihydrochloride (AAPH). The extent of hemolysis was quantified spectrophotometrically. The reaction mixture was incubated during 1 h at 37 °C and then was centrifuged (4000 × g for 3 min). The absorption of the supernatant at 545 nm was read. Percentage hemolysis was calculated and the IC_{50} values were determined as the concentration needed to protect the RBC from oxidative hemolysis by 50%. For the 100% hemolysis control, ethanol 60° was used as solvent control instead of the extracts. BHT and quercetin were used as reference compounds. All assays were carried out at different times during 1 year.

2.5. Identification of phenolic compounds

The identification of phenolic compounds in the extract was carried out by HPLC-ESI-MS/MS. The mass spectra were recorded employing an Agilent 1100 (Agilent Techno logies Inc., CA, USA) liquid chromatography system connected through a split to an Esquire 4000 Ion Trap LC/MS(n) system (Bruker Daltoniks, Germany). The ionization was performed at 3000 V. Nitrogen was used as nebulizing and drying gas (50 psi at 365 °C) at a flow rate of 10 L/min. Negative ions were detected using full scan (*m/z* 20–2200) and normal resolution (scan speed 10,300 *m/z*/s; peak with 0.6 FWHM/*m/z*). The trap parameters were set in ion charge control (ICC) using manufacturer default parameters, and maximum accumulation time of 200 ms. The conditions for mass spectrometric analysis were as follows. Electrospray needle, 4000 V; end plate offset, −500 V; skimmer 1, 56.0 V; skimmer 2, 6.0 V; capillary exit offset, 84.6 V; capillary exit, 140.6 V. The collision induced dissociation (CID) spectra were obtained with fragmentation amplitude of 1.00 V (MS/MS). Helium was used as the collision gas and was automatically controlled through SmartFrag option. The chromatography column used was a MultoHigh 100 RP 18–5 μ (250 × 4.6 mm) column (CS-Chromatographie Service GmbH, Langerwehe, Germany) maintained at 25 °C. The solvents used for HPLC-MS analyses were 1% formic acid in water (A) and acetonitrile (B). The linear gradient solvent system was as follow: 92% A from 0 to 10 min, 92–90% A from 10 to 15 min, 90–82% A from 15 to 35 min, 82–75%A from 35 to 60 min, 75–20% A from 60 to 65 min, changing to 92% A from 65 to 70 min and isocratic (92% A) from 70 to 80 min. The volume injected was 20 μL. The flow rate was 0.5 mL/min. The detection of phenolics by UV was at 280 nm.

3. Results

3.1. Pharmacognostic characterization of Tetraglochin andina

3.1.1. Macroscopic characters

The crude drug is aerial part of *Tetraglochin andina*, a perennial shrub up to 1.5 m high (Fig. 1). The branches are erect with macroblasts and brachyblasts covered by the sheathing base of the petioles. The sheaths are glabrous at the central region to densely villous at the margin (Fig. 2A and D). The leaf are imparipinnate, with 2–3 pairs of opposite to sub-opposite leaflets that easily come off, rachis glabrous at the mid region to pubescent at the apical end, 10–16 (28) mm length (Fig. 2E). Leaves of the macroblasts and basal leaf of the brachyblast shows petiole-rachis axis persistent, indurated to slightly hard, thorny, brown, glabrous at maturity, straight or slightly curved, leaflets easily deciduous; leptophyll to nanophyll (0.5) 1.5–2.8 (3.3) cm long. Leaves of the brachyblasts are leptophyll 7–15 mm long (Fig. 2D-E). Leaflets are sessile, (2.1) 3.5–5.5 (7.2) × (0.4) 0.6–1 mm, oblong, with acuminate acute apex and obtuse base, margin entire revolute, glabrous, rarely with few hairs near the margin of the upper (adaxial) side (Fig. 2F-H). The venation pattern of the terminal leaflet presented primary vein pinnate, massive with straight course. Secondary veins were alternated, cladodromous to reticulodromous, with irregular spacing gradually increasing towards the base, forming acute to straight, rare obtuse angles, with the main vein. Dichotomizing to random reticulated tertiary veins form ultimate venation medially ramified with freely ending veinlets (Fig. 2F–H). The flowers are solitary and axillary on the brachyblasts. Sepals are 1.2 mm long, ovate-elliptic, acute, glabrous. Flowers are apetalous and show three stamens. Stigma fimbriate (Fig. 2B). Fruits covered by the hypanthium, 4–9 × 2.5–6 mm, winged, with three wings 0.5–2 mm wide, papery, hard, glabrous, with entire to slightly erose margin, all of them equally developed, rarely one of them narrower or irregular (Fig. 2C).

3.1.2. Microscopic characters

Anatomically, the sheathing base of the petioles in paradermal view

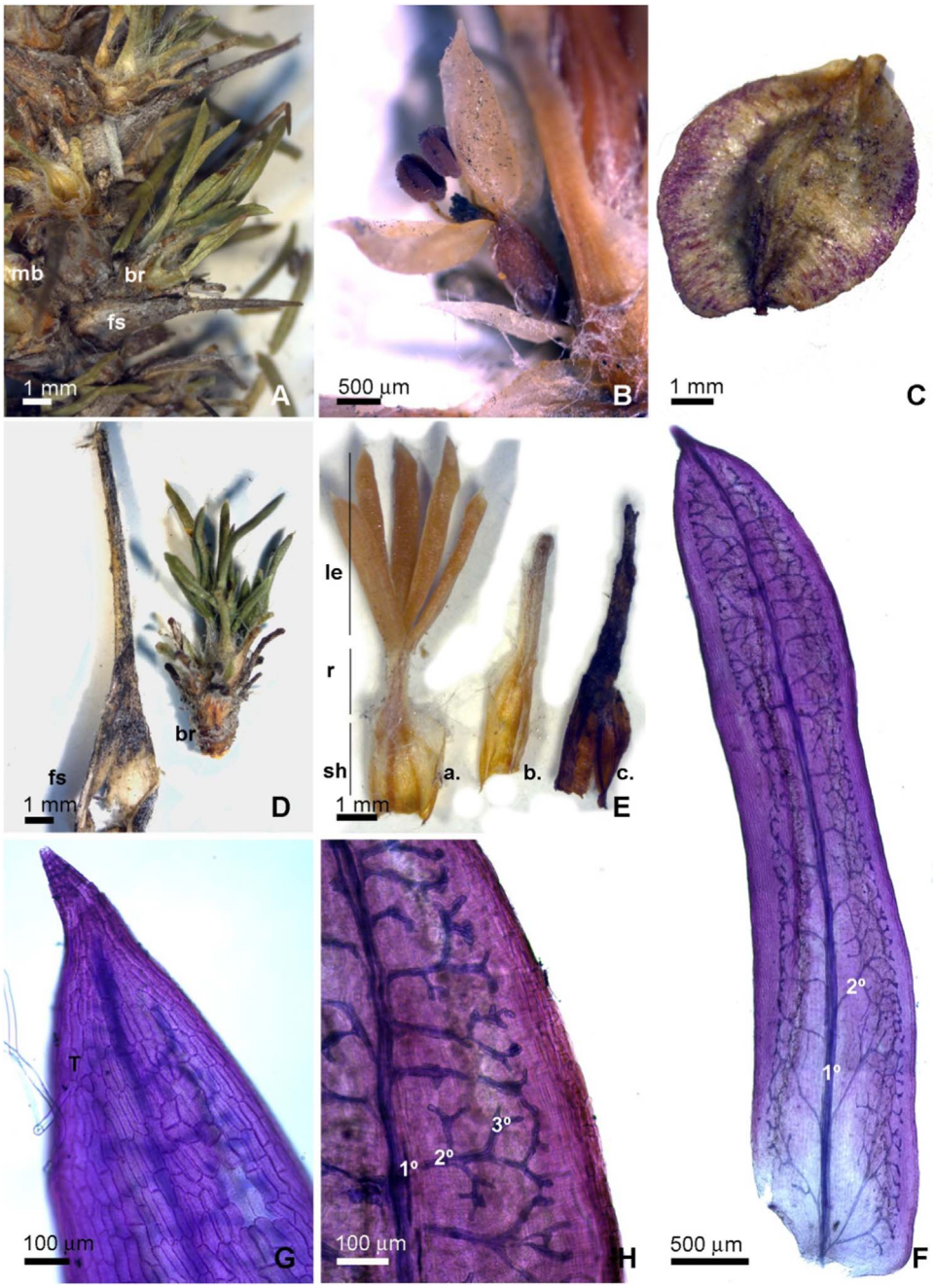

Fig. 2. General morphological aspects of *Tetraglochin andina*. **(A)** Macroblast and brachyblast with leaflets; **(B)** solitary and axillary flower on the brachyblasts; **(C)** hypanthium with three wings with erose margin; **(D)** macroblast hardened old thorny leaf as foliar spine and brachyblast aspect; **(E)** brachyblast leaf stages: (a) apical leaf with leaflets; (b) thorny leaf which has lost its leaflets; (c) hardened old thorny leaf; **(F)** general aspects of leaf architecture; **(G)** leaflet acuminate acute apex; **(H)** detail of venation, tertiary veins forms ultimate venation with freely ending veinlets. Abbreviations: mb, macroblast; br, brachyblast; fs, foliar spine; sh, sheath; r, petiole-rachis; le, leaflets; 1°: primary vein; 2°: secondary vein, 3°: tertiary vein.

presented polyhedral elongated epidermal cells, with thick, straight anticlinal walls (Fig. 3A-B). The lower epidermis showed smaller cells and rarely anomocytic or ciclocytic stomata types (Fig. 3B). The margin presented crenate aspect with unicellular non-glandular trichomes (231.26 ± 83.01 µm) and glandular capitate trichomes with uniseriate stalk of four or five cells and globose multicellular head (135.27 ± 67.82 µm) (Fig. 3C). The same arrangement is observed at the petiole-rachis with the difference that it presented abundant glandular trichomes at the apical end and stomata on both epidermal surfaces (Fig. 3D-E). In section the sheath presented wings and three prominent nerves conformed by single collateral vascular bundles with reinforcements of fibers at the phloem pole, the surrounding parenchyma was homogeneous and sclerified (Fig. 4A). Towards the petiole-rachis the wings decrease, forming reinforcements of laminar collenchyma in marginal and sub-epidermal position, until disappearing and present a circular shape. Calcium oxalate druses were observed in the parenchyma surrounding the vascular bundles. The vascular bundles approach each other to form a single nerve formed by three collateral vascular bundles with strong adaxial and abaxial fiber caps (Fig. 4B-C).

In frontal view, the leaflets were hypostomatic with polyhedral elongated epidermal cells, with straight anticlinal walls, rarely non-glandular or glandular trichomes as previously described were observed (Fig. 4F-G). The palisade index ranged between 13.75 and 14.05. The lower epidermis showed smaller cells under the revolute margin and anomocytic stomata (25.84 ± 2.36 µm long x 21.67 ± 1.85 µm lat. for

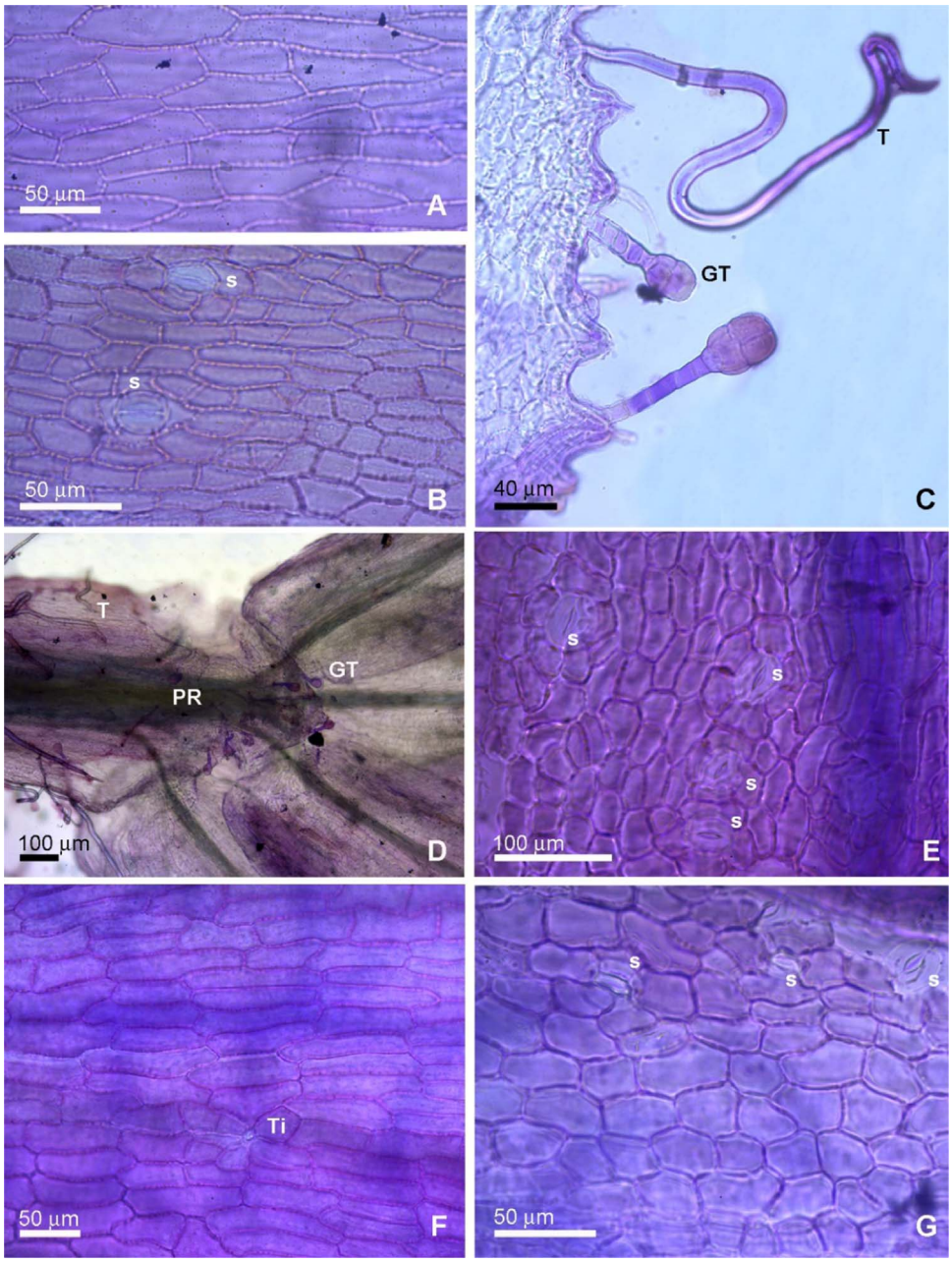

Fig. 3. Leaf morphological features. (**A**) upper (adaxial) epidermis; (**B**) lower (abaxial) epidermis; (**C**) leaf sheath hirsute margin with unicellular non-glandular and capitated glandular trichomes; (**D**) details of the petiole-rachis; (**E**) petiole-rachis adaxial epidermis with stomata; (**F**) leaflet adaxial epidermis; (**G**) leaflet abaxial epidermis. **Abbreviations:** GT, glandular trichome; s, stoma; T, non-glandular trichome; Ti, trichome insertion. PR, petiole-rachis.

the occlusive cells) sunken and distributed along crypts form by the revolute margin of the leaf, density 130.16 ± 36.81 stomata per mm^2 (Fig. 3G). In transverse section, the leaflet was isolateral with thick cuticle (4 ± 0.7 µm for both epidermal surfaces), uniseriate epidermis with tick external periclinal walls (39.36 ± 4.13 µm and 38.72 ± 10.72 µm for the adaxial and abaxial surfaces, respectively), 2–3 layers of adaxial palisade parenchyma (69.57 ± 12.34 µm) and 1–2 layers of abaxial palisade parenchyma (55.35 ± 9.23 µm). Primary, secondary and tertiary veins presented collateral vascular bundles surrounded by parenchymatous sheaths. Calcium oxalate druses were observed in the sub epidermal layer of the middle vein (Fig. 4E). Macroblast and brachyblast sections were similar with circular shape, revealing secondary growth. Persistent periderm with growth rings contained by the presence of the petiole sheathing bases was observed. Parenchymatous cortex showed 6–10 layers of quadrangular cells with a highly ordered radial disposition. A continuous eustele, with calcium oxalate druses at the phloem and abundant xylem fibers was observed. The pith, which tends to be collapsed, was formed by thick walled parenchymatic cells, which become sclerified centrifugally (Fig. 4F-G). Maceration of macroblast, blachyblast and thorny leaves revealed abundant fibers (Fig. 4H–J), parenchyma cells and vessel elements (not shown).

3.1.3. Physical constants

The air dried aerial parts of *T. andina* showed 13.32% moisture, 5.27% w/w total ash, 0.76% w/w acid insoluble ash and the extractive yields were 14.1, 14.3 and 1.13 g dry extract/100 g plant material for water, 60° ethanol and petroleum ether extracts, respectively.

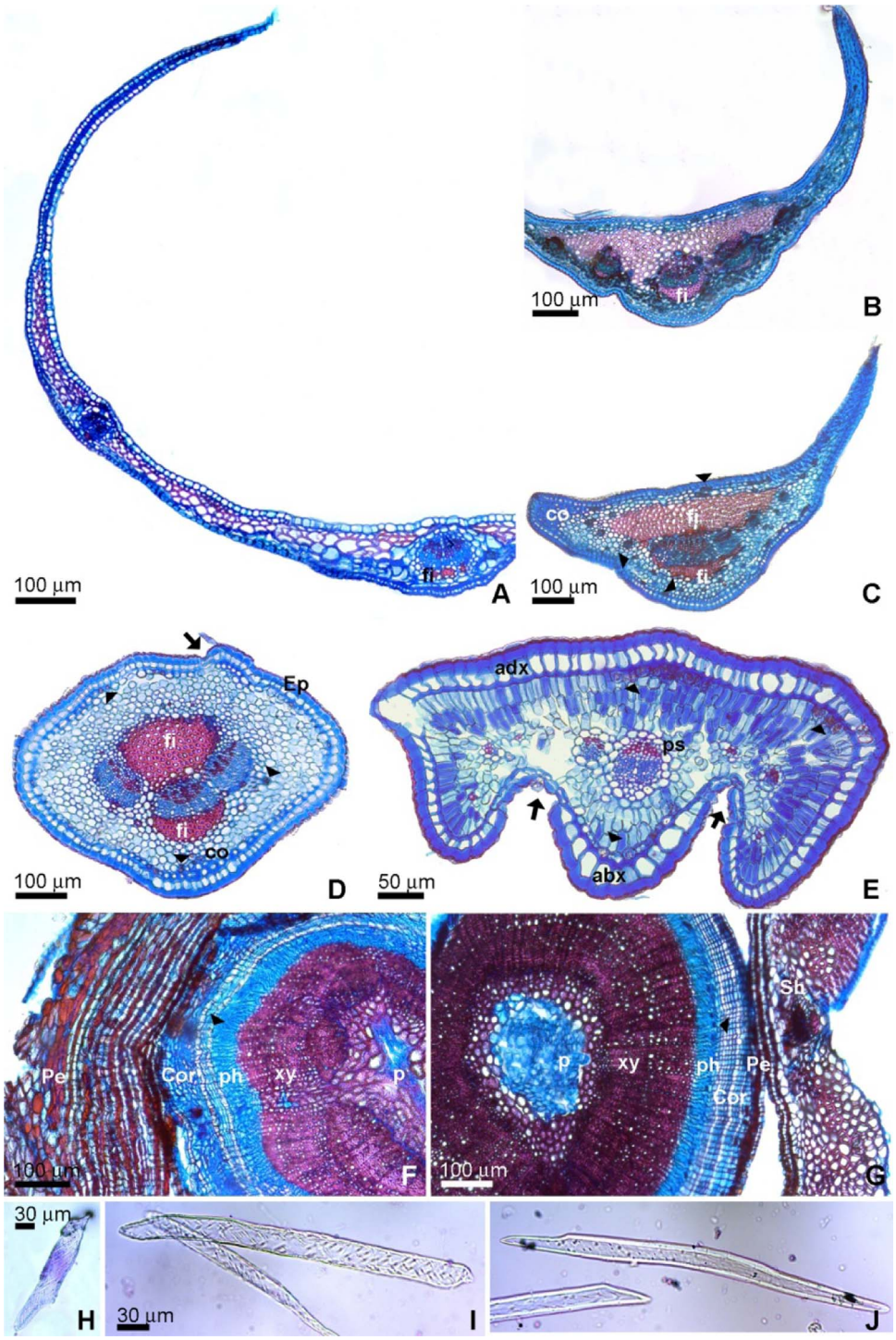

Fig. 4. Histological leaf details: (**A**) sheath transversal section at its proximal end near the stem; (**B**) cross sheath section at its middle region; (**C**) cross sheath section at its distal end near the petiole-rachis; (**D**) petiole-rachis transversal section; (**E**) cross section of the leaflet; (**F**) brachyblast cross section; (**G**) macroblast cross section; (**H-J**) fibers observed in macerated tissues. **Abbreviations**: Arrow: stoma; arrow head: druses; fi: fibers; Ep: epidermis; co: collenchyma; adx: adaxial epidermis; abx: abaxial epidermis; ps: parenchyma; Pe: peridem; Cor: cortex; ph: phloem; xy: xylem; p: pith (medulla, parenchima).

3.1.4. Qualitative phytochemical screening

The extracts were screened for different phytoconstituents. The aqueous extract was positive for triterpenoids/steroids, tannins and flavonoids. The ethanolic extract showed positive reaction to the same compounds as well as for cardiotonic glycosides and saponins. The petroleum ether extract was positive for cardiatonic glycoside and triterpenes/steroids.

3.2. Characterization of the hydroalcoholic extract from Tetraglochin andina

The phytochemical composition of the dry hydroalcoholic extract was determined. The extract contained a high level of total phenolic (TPC: 386.9 ± 1.7 mg GAE/g dry extract), flavonoid phenolic (FPC: 260.4 ± 2.7 mg GAE/g dry extract) and non-flavonoid phenolic compounds (NFPC: 126.5 ± 2.3 mg GAE/g dry extract). Condensed tannin (60.4 ± 3.5 mg PB2E/g dry extract) and hydrolyzed tannin (26 ± 2 mg

Table 1
Effect of dry extract of *Tetraglochin andina* on different yeast strains isolated from vaginal infections.

Strain	Strain collection number	Phenotype of clinical isolates	MIC 90/50[a] µgGAE/mL	MFC µgGAE/mL
C. albicans	144783	Flu^S,Am^S,Ny^S	400/12.5	> 400
	134333	Flu^R,Am^S,Ny^S	400/12.5	> 400
	2089	Flu^S,Am^S,Ny^S	400/12.5	> 400
C. glabrata	031646	Flu^S,Am^S,Ny^S	400/12.5	400
	042030	Flu^S,Am^S,Ny^S	400/12.5	400
	031982	Flu^R,Am^S,Ny^S	400/12.5	400
C. tropicalis	1841	Flu^S,$Am^S$$Ny^S$	400/25	> 400
S. cerevisiae	134528	Flu^S,$Am^S$$Ny^S$	25/12.5	> 400
	124263	Flu^S,$Am^S$$Ny^S$	400/12.5	> 400
	134544	Flu^R,Am^S,Ny^S	> 400/400	> 400
C. parapsilosis	134410	Flu^S,$Am^S$$Ny^S$	400/12.5	> 400
C. krusei	134409	Flu^R,$Am^S$$Ny^S$	100/12.5	> 400

[a] The MIC values represent the 90% and 50% of growth inhibition compared with control growth. Fluconazole: Flu; Amphotericin B (Am); Nystatin (Ny). Resistent: R; sensitive: S.

GAE/g dry extract) were also detected. The content of TPC, NFPC and FPC remained unchanged during one year of storage at room temperature and 4 °C in the dark.

3.3. Biological activities of T. andina dry extract

3.3.1. Antifungal activity

The antifungal activity of the *T. andina* dry extracts was assayed in vitro against 10 yeast strains obtained from vaginal exudates of patients with vaginal yeast infection. They included three strains of *Saccharomyces cerevisiae*, three strains of *C. albicans*, three strains of *Candida glabrata* and one strain of *Candida tropicalis* (Table 1). Some *S. cerevisiae* and *C. albicans* and non-*albicans* strains are azole-susceptible as fluconazole, voriconazole, itraconazole (85%) or resistant (R) (15%). The *Candida* species and *Saccharomyces* were also susceptible to nystatin and amphotericin B. The *T. andina* dry water and petroleum ether extracts were not active against any yeast. The ethanolic extract was effective at low concentration against *C. albicans* and non-*albicans*. The MIC_{50} values against most yeast strains were between 12.5 and 25 µg GAE/mL (Table 1). The ethanolic extract showed comparable potencies against two strains of *S. cerevisiae* but was less active against *S. cerevisiae* 134544 strain (Table 1). The MIC values did not significantly change after one year storage of dry extract at room temperature or at 4 °C (MIC_{50} values between 12.5 and 25 µg GAE/mL). The dry extract activity was also examined in terms of minimum fungicidal concentration (MFC), to assess whether the antifungal susceptibility results correlates with the killing capacity. For all *C. glabrata* isolates, MFC values of extract were equal to MIC_{90}values (Table 1), consistent with powerful candidacidal activity. The dry extract of *T. andina* produced growth inhibition of L. *casei* CRL1267, L. *paracasei* CRL1291 and L. *johnsonii* CRL1292 isolated from human vagina, with MIC values higher than against *Candida* (> 400 µg/mL).

3.3.2. Antioxidant activity

The *T. andina* ethanolic extract showed $ABTS^{\bullet+}$ radical scavenging activity with SC_{50} value of 1.67 ± 0.12 µg GAE/mL, similar to the commercial natural antioxidant quercetin (Table 2). In the assay of the oxidative hemolysis, the dry extract exhibited an inhibitory effect on lipoperoxidation of human red blood membranes with IC_{50} values of 0.23 ± 0.02 µg GAE/mL (Table 2).

3.3.3. Effect on proinflammatory enzymes

The first enzyme of the arachidonic acid (AA) pathway is sPLA2. In response to intracellular cytokines or to an increase of intracellular levels of calcium, sPLA2 releases AA from the plasmatic membrane,

Table 2
Antioxidant activity of dry extract of *Tetraglochin andina* and commercial reference drugs.

Sample	$ABTS^{\bullet+}$ scavenging activity SC_{50} (µgGAE/mL)	AAPH assay IC_{50} (µgGAE/mL)
Dry extract (T-0 month)	1.67 ± 0.12[a]	0.23 ± 0.02[a]
Dry extract (T-12 month)	1.69 ± 0.14[a]	0.27 ± 0.02[a]
BHT	3.52 ± 0.2[b]	1.2 ± 0.1[c]
Quercetin	1.41 ± 0.08[a]	0.9 ± 0.08[b]

$IC_{50\ AAPH}$ and $SC_{50\ ABTS^{\bullet+}}$ average values of stored dry extract analyzed each month during one year. No significant difference was observed between each month value.

Table 3
Inhibition of the inflammation related enzymes LOX, COX-2 and $sPLA_2$ by dry extract of *Tetraglochin andina* and commercial reference drugs.

Sample	LOX IC_{50} (µg/mL)	COX-2 IC_{50} (µg/mL)	$sPLA_2$ IC_{50} (µg/mL)
Dry extract (T-0 month)	55.6 ± 2.2[a]	54.92 ± 1.8[a]	72.42 ± 0.8[a]
Dry extract (T-12 month)	54.8 ± 2.1[a]	50.90 ± 1.0[a]	82.42 ± 0.5[a]
Naproxene	14.0 ± 0.2[b]	–	–
Caffeic acid	57.0 ± 0.4[a]	–	–
Nimesulide	–	0.39 ± 0.1[b]	–
Acetyl salicylic acid	–	–	65.0 ± 1.0[a]

$IC_{50\ LOX}$, $IC_{50\ COX\text{-}2}$ and $IC_{50\ sPLA2}$ are average values of stored dry extract analyzed each month during one year. No significant difference was observed between each month value.

which is then oxidized by COX or LOX to produce eicosanoids. Thus, inhibition of the production of these inflammatory mediators may prevent or suppress a variety of inflammatory diseases. The IC_{50} values of *T. andina* ethanolic extract for $sPLA_2$ and COX-2 was 72.42 ± 0.8 µg GAE/mL (186.84 ± 1.8 µg DW/mL) and 54.92 ± 1.8 µg GAE/mL, respectively. The extract showed also inhibitory effect on LOX with IC_{50} values of 55.6 ± 2.2 µg GAE/mL similar to caffeic acid, a phenolic acid used as reference (Table 3).

3.4. HPLC-ESI-MS/MS analysis of the T. andina extract

The HPLC-ESI-MS/MS analysis of the *T. andina* extract allowed the tentative identification of 50 compounds, including tannins, flavonoids, simple phenolics and phenylpropanoids (Table 4, Fig. 5). Selected ion chromatograms were used to identify the main constituents and related compounds in the extracts. The ion at *m/z* 301 was used to detect ellagic acid and quercetin derivatives while the ion at *m/z* 289 allowed the identification of catechin/epicatechin monomers and oligomers.

The compound 1 exhibited a $[M\text{-}H]^-$ ion at *m/z* 481 and showed a neutral loss of 180 amu leading to a MS^2 base peak at *m/z* 301, in agreement with an ellagic acid moiety. According to Fischer et al. (2011) and Sandhu and Gu (2010), this compound was assigned as HHDP-hexoside. In the same way, the compounds 3, 10, 14, 15, 17, 24 and 30 showed a molecular ion of 633 amu. These ions exhibited a neutral loss of 332 amu (galloyl glucose), leading to an intense fragment ion at *m/z* 301, in agreement with galloyl-HHDP-hexoside derivatives (Fischer et al., 2011). The compounds 4, 5, 7, 9, 13, 16, 36, 37, 39 and 41 were identified as bis-HHDP hexosides (pedunculagins), based on the [M-H]- ion at *m/z* 783, with fragmentation ions at *m/z* 633, 615, 481 and 301 characteristic of galloyl-HHDP-hexoside, its dehydrated derivative, HHDP-hexoside and ellagic acid, respectively (Fischer et al., 2011). An $[M\text{-}H]^-$ ion at *m/z* 785 was observed for the compounds 20, 33 and 35. They exhibited fragment ions of 633, 483 and 301 amu, in accordance with galloyl-HHDP-hexoside, digalloyl-hexoside and ellagic acid, respectively. The compounds 20, 33 and 35 were identified as digalloyl-HHDP hexoside isomers (pedunculagin II isomers). Two galloyl di-HHDP hexoside isomers (casuarinins) were

Table 4
Identification of phenolic compounds in *Tetraglochin andina* extract by HPLC-ESI-MS/MS.

Peak	Rt	[M-H]$^-$	MS/MS	Identification
1	8.0	481.4	301(100)	HHDP hexoside
2	8.5	331.3	271(74), 169(100)	Galloyl hexoside
3	10.1	633.5	463(8), 301(100)	Galloyl HHDP hexoside
4	12.7	783.2	481(65), 301(100)	bis-HHDP hexoside (pedunculagin)
5	14.1	783.2	481(71), 301(100)	bis-HHDP hexoside (pedunculagin) 2
6	15.6	483.1	331(100), 169(24)	Digalloyl hexoside
7	16.6	783.4	481(73), 301(100)	bis-HHDP hexoside (pedunculagin) 3
8	17.5	316.5	152(100)	Protocatechuic acid hexoside
9	19.2	783.2	481(60), 301(100)	bis-HHDP hexoside (pedunculagin) 4
10	19.6	633.7	301(100)	Galloyl HHDP hexoside 2
11	20.3	483.5	313(100)	Digalloyl hexoside 2
12	23.0	353.7	191(100), 179(32)	3-caffeoylquinic acid[a]
13	23.3	783.1	481(51), 301(100)	bis-HHDP hexoside (pedunculagin) 5
14	23.9	633.7	481(21), 301(100)	Galloyl HHDP hexoside 3
15	24.9	633.7	421(27), 301(100)	Galloyl HHDP hexoside 4
16	25.7	783.4	481(43), 301(100)	bis-HHDP hexoside (pedunculagin) 6
17	26.2	633.5	301(100)	Galloyl HHDP hexoside 5
18	28.2	576.9	451(84), 425(100), 289(35)	(epi)catechin-(epi)catechin dimer
19	29.6	577.4	451(66), 425(100),289(6)	(epi)catechin-(epi)catechin dimer 2
20	29.7	785.1	633(49), 483(88), 301(100)	Digalloyl-HHDP hexoside (pedunculagin II)
21	30.7	865.0	695(41), 577(100), 520(7)	(epi)catechin-(epi)catechin-(epi)catechin trimer
22	30.9	454.8	291(100), 246.7(19)	Brevifolin carboxylic acid hexoside[b]
23	31.9	483.0	331(20), 313(18), 271(100)	Digalloyl hexoside 3
24	31.9	632.8	613(100), 301(68)	Galloyl HHDP hexoside 6
25	32.7	658.9	329(100)	2[M-H] vanillic acid hexoside pseudomolecular ion
26	32.9	329.2	167(100)	Vanillic acid hexoside[b]
27	33.0	409.2	329(16), 241(100), 167(8)	Vanillic acid hexoside sulfate
28	33.6	289.0	245(100), 205(36)	(epi)catechin[c]
29	33.8	367.2	193(100)	3-feruloylquinic acid[a]
30	34.4	633.2	463(11), 301(100)	Galloyl HHDP hexoside 7
31	34.5	455.3	291(100), 247(24)	Brevifolin carboxylic acid hexoside[b]
32	34.7	625.8	463(100), 301(8)	Ellagic acid dihexoside
33	35.2	785.2	633(23), 483(83), 301(100)	Digalloyl HHDP hexoside (pedunculagin II) 2
34	36.4	639.4	463(100), 301(10)	Ellagic acid glucuronide hexoside
35	37.6	785.5	633(19), 483(100), 302(23)	Digalloyl HHDP hexoside (pedunculagin II) 3
36	40.4	783.7	633(100), 301(44)	bis-HHDP hexoside (pedunculagin) 7
37	43.9	783.7	615(39), 419(53), 301(100)	bis-HHDP hexoside (pedunculagin) 8
38	44.7	463.3	301(100)	Ellagic acid hexoside
39	45.7	783.7	613(95), 481(35), 301(100)	bis-HHDP hexoside (pedunculagin) 9
40	46.5	935.0	633(100), 301(29)	Galloyl di HHDP hexoside (casuarinin)
41	46.9	783.5	613(100), 481(17), 301(85)	bis-HHDP hexoside (pedunculagin) 10
42	50.3	481.4	439(100), 313(85)	Digalloyl hexoside 4
43	52.5	934.5	783(20), 633(19), 301(100)	Galloyl di HHDP hexoside (casuarinin) 2
44	52.5	615.7	463(100), 301(2)	Ellagic acid galloyl hexoside
45	54.7	477.2	315(100), 301(4)	Isorhamnetin hexoside
46	55.4	609.3	301(100)	Quercetin rutinoside
47	57.3	463.1	301(100)	Quercetin hexoside
48	58.6	477.1	301(100)	Quercetin glucuronide
49	61.5	433.6	301(100)	Quercetin pentoside 1
50	64.7	433.6	301(100)	Quercetin pentoside 2

[a] According to Clifford et al. (2003).
[b] According to Fischer et al. (2011).
[c] Confirmed by comparing tR values with standard.

also identified. The compounds 40 and 43 showed a molecular ion of 935 amu with fragment ions at *m/z* 783, 633 and 301, characteristic of bis-HHDP-hexoside, galloyl-HHDP-hexoside and ellagic acid, respectively. Four ellagic acid derivatives were tentatively identified in the sample. The compounds 32, 34, 38 and 44 showed the characteristic ellagic acid ion at *m/z* 301. Compounds 32, 34 and 44 with molecular ions of 625, 639 and 615 amu, exhibited neutral losses of hexose, glucuronate and galloyl (162, 176 and 152 amu), respectively, leading to a MS^2 peak base at *m/z* 463, in agreement with ellagic acid hexoside. The compounds 32, 34 and 44 were tentatively identified as ellagic acid dihexoside (32), ellagic acid glucuronidehexoside (34) and ellagic acid galloylhexoside (44). Meanwhile, the compound 38 was identified as ellagic acid hexoside, based on the [M-H]$^-$ ion at *m/z* 463, exhibiting a neutral loss of 162 amu (hexose), leading to the MS^2 peak base of *m/z* 301, in accordance with ellagic acid.

The compound 2 was identified as galloylhexoside, based on its molecular ion of 331 amu and the neutral loss of a hexose (162 amu), leading to the characteristic gallic acid fragmentation ion at *m/z* 169. The compounds 6, 11 and 23 showed a [M-H]$^-$ at *m/z* 483. The compound 6 shows consecutive losses of 152 (galloyl) and 162 amu (hexose) leading to the ions at *m/z* 331 and 169. The fragmentation of compounds 11 and 23 shows a ion at *m/z* 313 (galloyl hexose). The compounds 6, 11 and 23 were identified as three different digalloylhexoside isomers (Fischer et al., 2011). The compound 42, with a molecular ion of 481 amu, showed a neutral loss of gallic acid (169 amu), leading to an ion at *m/z* 313 (galloylhexoside) and was tentatively identified as an additional digalloylhexoside isomer as well.

Hydroxybenzoic and hydroxycinnamic acids were detected in the extract. The compound 8 was tentatively identified as protocatechuic acid hexoside based on its molecular ion of 315 amu, whose fragmentation evidence a neutral loss of 162 (hexose) leading to an ion at *m/z* 153 in agreement with protocatechuic acid. Meanwhile, the compounds

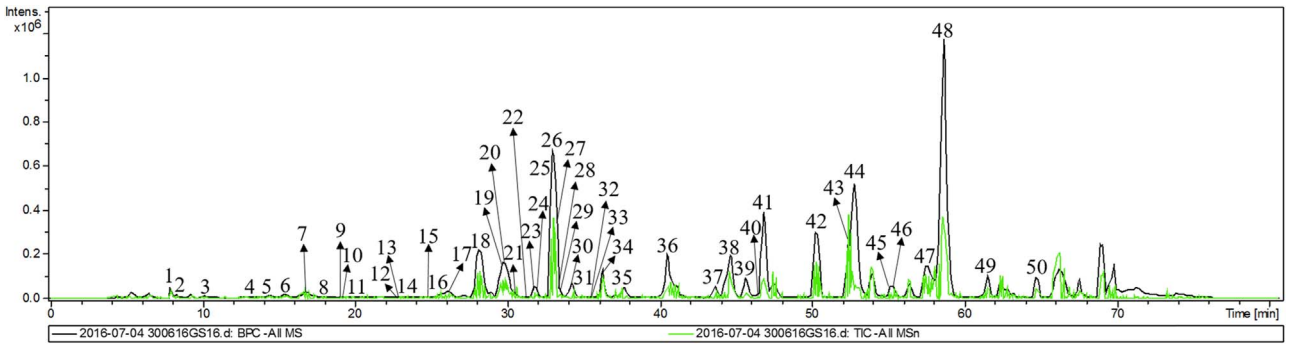

Fig. 5. HPLC-MS analysis of *Tetraglochin andina* extract. Detection: UV (280 nm, in black) TIC-all MS^n (in green). Compounds:**1**: HHDP hexoside; **2**: galloyl hexoside; **3**: galloyl HHDP hexoside; **4**: bis-HHDP hexoside (pedunculagin); **5**: bis-HHDP hexoside (pedunculagin) 2; **6**: digalloyl hexoside; **7**: bis-HHDP hexoside (pedunculagin) 3; **8**: protocatechuic acid hexoside; **9**: bis-HHDP hexoside (pedunculagin) 4; **10**: galloyl HHDP hexoside 2; **11**: digalloyl hexoside 2; **12**: caffeoylquinic acid; **13**: bis-HHDP hexoside (pedunculagin) 5; **14**: trigalloyl hexoside 1; **15**: trigalloyl hexoside 2; **16**: bis-HHDP hexoside (pedunculagin) 6; **17**: galloyl HHDP hexoside 3; **18**: (epi)catechin-(epi)catechin dimer; **19**: (epi)catechin-(epi)catechin dimer 2; **20**: digalloyl-HHDP hexoside (pedunculagin II); **21**: (epi)catechin-(epi)catechin-(epi)catechin trimer; **22**: brevifolin carboxylic acid hexoside; **23**: digalloyl hexoside 3; **24**: galloyl HHDP hexoside 4; **25**: vanillic acid hexoside; **26**: vanillic acid hexoside; **27**: vanillic acid hexoside sulfate; **28**: (epi)catechin; **29**: feruloylquinic acid; **30**: galloyl HHDP hexoside 5; **31**: brevifolin carboxylic acid hexoside; **32**: ellagic acid dihexoside; **33**: digalloyl HHDP hexoside (pedunculagin II) 2; **34**: ellagic acid glucuronide hexoside; **35**: digalloyl HHDP hexoside (pedunculagin II) 3; **36**: bis-HHDP hexoside (pedunculagin) 7; **37**: bis-HHDP hexoside (pedunculagin) 8; **38**: ellagic acid hexoside; **39**: bis-HHDP hexoside (pedunculagin) 9; **40**: galloyl di HHDP hexoside (casuarinin); **41**: bis-HHDP hexoside (pedunculagin) 10; **42**: HHDP hexoside 2;**43**: galloyl di HHDP hexoside (casuarinin) 2; **44**: ellagic acid galloyl hexoside; **45**: isorhamnetin hexoside; **46**: quercetin rutinoside; **47**: quercetin hexoside; **48**: quercetin glucuronide; **49**: quercetin pentoside 1; **50**: quercetin pentoside 2.

25, 26 and 27 were tentatively identified as vanillic acid hexoside derivatives. The compound 25, tentatively identified as 2[M-H]$^-$vanillic acid pseudomolecular ion, showed a molecular ion at *m/z* 659, whose fragmentation led to a MS^2 peak base of *m/z* 329, in agreement with vanillic acid hexoside. The compounds 26 and 27, with parent ions at *m/z* 329 and 409, fragments to *m/z* 167, characteristic of vanillic acid, after the loss of one hexose moiety (162 amu) in 26 and successive losses of sulfate and hexose (80 and 162 amu) in 27. The compounds were tentatively assigned as vanillic acid hexoside (26) and the hexoside sulfate derivative (27). The compounds 22 and 31 were in agreement with brevifolin carboxylic acid hexosideisomers, exhibiting [M-H]$^-$ ion at *m/z* 455 whose fragmentation led to the *m/z* ions at 291 and 247 amu (Fischer et al., 2011). Regarding hydroxycinnamic acids, the compound 12 and 29, with molecular ions of 353 and 367 amu, fragments to a base peak at *m/z* 191 and 193 amu, respectively, and were identified as 3-caffeoylquinic and 3-feruloylquinic acids following the hierarchical scheme of Clifford et al. (2003).

Compounds 18, 19, 21 and 28 were identified as proanthocyanidins. The compounds 18 and 19 exhibited a [M-H]$^-$ ion at *m/z* 577 and the characteristic signal of (epi)catechin units at *m/z* 289. Both compounds showed a neutral loss of 152 amu leading to a MS^2 base peak at *m/z* 425, in agreement with an additional (epi)catechin unit (Lin et al., 2014). The compounds were identified as (epi)catechin-(epi)catechin dimer isomers. The compound 21, showed a [M-H]$^-$ ion at *m/z* 865 amu, with a neutral loss of 289 amu leading to a MS^2 base peak at *m/z* 577 amu, suggesting an (epi)catechin unit linked to and (epi)catechin dimer (Lin et al., 2014). Thus, compound 21 was assigned as (epi)catechin-(epi)catechin-(epi)catechin trimer. The compound 28 with a molecular mass of 289 amu, showed neutral loss of 44 amu leading to a MS^2 base peak of 245, according with (epi)catechin. The identity of (epi)catechin was corroborated by injection of a catechin standard in the same chromatographic conditions.

Six flavonol glycosides were detected by the characteristic UV absorption maxima around 350 nm. The compound 45 exhibited [M-H]$^-$ ion at *m/z* 477 which presented consecutive neutrallosses of 162 amu (hexose) and 15 amu (methyl), in accordance with rhamnetin/ isorhamnetin hexoside. The compounds 46–50 were tentatively identified as quercetin rutinoside, hexoside, glucuronide and two pentoside isomers, respectively. The assignments were based on the neutral loss of 308 amu (rutinose, compound 46), 162 (hexose, compound 47), 176 (glucuronide, compound 48) and 132 amu (pentose, compounds 49 and 50), leading to the MS^2 peak base at *m/z* 301, characteristic of quercetin.

4. Discussion

According to the World Health Organization, the macroscopic and microscopic description of a medicinal plant is the first step to establish the botanical identity and degree of purity of such plant material and should be carried out in an early research stage. The macroscopic and micrographics characters of *T. andina* were described to allow characterization of the crude drug. The aqueous and hydroalcoholic extracts from the aerial parts of *T. andina* were assessed for qualitative chemical profile and antifungal activity to verify the traditional indications of use. The extraction with petroleum ether (PE) was carried out for comparison. The soluble content was higher in ethanol and water. The polar extracts contained mainly tannins and flavonoids while the PE extract contained mainly terpenoids. The content of acid soluble ash was higher than acid insoluble ash.

The composition of vaginal bacterial and fungal communities is dynamics. Previous papers showed that some communities changes markedly over short time periods, whereas others are relatively stable (Gajer et al., 2012). The fluctuation or constancy is mainly affected by time in the menstrual cycle, community class, and to a certain extent by sexual activity, and by other unknown factors. In this paper we studied the activity of *T. andina* extracts against *Candida* species isolated from vaginal communities. The aqueous and PE extracts did not showed antifungal activity while ethanolic extract showed strong antifungal activity according to the Tangarife-Castaño et al. (2011) classification (MIC < 0.5 mg/mL). The potency of *T. andina* ethanolic extract on vaginal yeast species was higher than reported by Zampini et al., (2009a, 2009b) on aerobic Gram positive bacteria (MIC values: 600–1200 μg GAE/mL) and on Gram negative bacteria (600–2400 μg GAE/mL). The activity on human fungi was also higher than the reported for aqueous and ethanolic extracts from other extremophile plant species *(Chuquiraga atacamensis, Parastrephia phyliciformis* and *Parastrephia lepidophylla)* on micelial phytopathogenic fungi (MIC values around 300 μg/mL) (Sayago et al., 2012; Palavecino Ruiz et al., 2016).

The normal vaginal microbiome in women of reproductive age is characterized by the dominance of lactic acid-producing bacteria, mainly *Lactobacillus* spp., which, consequently, maintain the acidic pH of vaginal fluids (3.5–4.5) (Das Neves et al., 2014). Therefore, the evaluation of the effect of plant extracts on vaginal lactic bacteria is essential, in order to avoid an imbalance in the vaginal microbiome, and allow the restoration of the ecological equilibrium of the tract after its administration. According to our results, the local use of *T. andina*

dry extract in the concentration range of the MIC values on *Candida* species does not affect the *Lactobacillus* normal vaginal microbiota. Our results stimulate further research on therapies using *T. andina* extract and *Lactobacillus* for vaginal applications (Nader-Macias and Juarez Tomás, 2015). This is the first report on antifungal activity and activity on *Lactobacillus* of *T. andina* hydroalcoholic extract. *Candida*-vaginal epithelial cell interactions in VVC promote the liberation of free radicals and a non-protective inflammatory response that results in mucosal damage (Fisher, 2012). This may explain why a treatment with a drug with multiple effects, anti-*Candida*, antioxidant and anti-inflammatory is required. For this reason this activities were evaluated in the ethanolic extract. The extract was able to reduce ABTS and prevent and limit the release of lysosomal enzymes from human red blood cell to the extracellular matrix indicating that could be able to prevent damage on neutrophils membrane reducing the inflammatory response. The *T. andina* extract showed similar antioxidant capacity than *Baccharis incarum* infusion (Zampini et al., 2009a) and was more active than ethanolic extracts of other Puna plant species (SC_{50} values between 25.7–101 μg/mL) such as *Parasthephia, Chilliotrichiopsis, Chuquiraga, Nardophyllum, Tessaria, Ephedra, Junellia,* and *Acantholipia* (D'Almeida et al., 2013; Torres Carro et al., 2017). Its potency was higher than BHT and quercetin, two commercial antioxidants.

The *T. andina* dry ethanolic extract was a strong inhibitor on three proinflammatory enzymes. The ethanol 60° extract was more active on $sPLA_2$ than *Tetraglochin* ethanol 17° extract (Torres Carro et al., 2017) and similar to the inhibitory activity found for Asteraceae species from the same Argentinean Puna environment such as *P. lepidophylla, P. phyliciformis, B. boliviensis* and *B. incarum* (around 200 μg DW/mL, Torres Carro et al., 2017). *Frankenia triandra* was not able to inhibit $sPLA_2$ (Torres Carro et al., 2016). The activity of dry hydroalcoholic extract of *T. andina* on COX-2 and LOX was higher than that reported for Asteraceae, Verbenaceae and Ephedraceae species from the Argentinean Puna (D'Almeida et al., 2012; Torres Carro et al., 2015, 2016, 2017).

According to the HPLC-MS/MS analysis, the main chemical components of the extract are gallotannins (galloylhexoside, digalloylhexoside and trigalloylhexoside), ellagitannins (pedunculangin), gallo-ellagitannin, condensed tannins (epi)catechin-(epi)catechin dimer, (epi)catechin-(epi)catechin-(epi)catechin trimer and phenolic acids derived from benzoic and phenylpropanoid acids. Hydrolizable tannin-rich plant materials are commonly used in the traditional medicine as anti-inflammatory, antioxidant and antimicrobial agents (Granica et al., 2015; Orabi et al., 2015). Especially, ellagitannins have various important medicinal effects including antioxidant, antiviral, antibacterial, antifungal, immunomodulatory, anti-inflammatory, antitumor, and hepatoprotective activities (Feldman, 2005; Quideau, 2008; Granica et al., 2015; Orabi et al., 2015). The activity of T. *andina* ethanolic extract on *Candida* species could be attributed to the content of caffeoyl quinic acid, HHDP, bis HDDP hexosides (pedunculangins) and galloyl HHDP hexosides (casuarinas) (Serrano et al., 2009; Bajko et al., 2016; Sieniawska and Baj, 2017). The inhibitory effect of proinflammatory enzymes could be attributed to hydrolysable and condensed tannins as well as epicatechin (Bettaieb et al., 2016; Sieniawska and Baj, 2017).

5. Conclusions

The information obtained in this work allowed a better understanding of the ethnopharmacological background of *Tetraglochin andina* (formerly *T. cristatum*) as antifungal and anti-inflammatory agent in the traditional medicine of highlands regions (Puna) from northwestern Argentina.

Acknowledgments

The authors acknowledge the financial support from Secretaría de Ciencia, Arte e Innovación Tecnológica (SCAIT-UNT, PIUNT G533), Argentina, Agencia Nacional de Promoción Científica y Técnica (ANPCyT, PICT 2014, Nº 3136), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 00590 CO), Argentina, CONICYT-PCHA/Doctorado Nacional/2015–21151561 (A. Burgos Edwards) and PIEI-QUIM-BIO, Universidad de Talca, Chile.

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