

Artículo Original | Original Article

Comparative study of anti-inflammatory activity and qualitative-quantitative composition of triterpenoids from ten genotypes of *Ugni molinae*

[Estudio comparativo de la actividad antiinflamatoria y composición química cualitativa y cuantitativa de triterpenoides de diez genotipos de *Ugni molinae*]

Jorge Arancibia-Radich¹, Marcelo Peña-Cerda¹, Daniela Jara¹, Paula Valenzuela-Bustamante¹, León Goity¹, Gabriela Valenzuela-Barra¹, Ximena Silva², Gabino Garrido³, Carla Delporte¹ & Ivette Seguel⁴

¹Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile

²Unidad de Pruebas Biológicas, Instituto de Salud Pública de Chile, Santiago, Chile

³Departamento de Ciencias Farmacéuticas, Facultad de Ciencias, Universidad Católica del Norte, Antofagasta, Chile

⁴Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación Carillanca, Temuco, Chile

Contactos / Contacts: Carla DELPORTE - E-mail address: cdelpor@uchile.cl

Abstract: The aim of this study was to assess the differences in qualitative-quantitative composition of triterpenoids and total phenolic contents, together with anti-inflammatory activity of *Ugni molinae* leaves obtained from ten genotypes. The ethyl acetate (EAE) and ethanol extracts (ETE) were obtained and analyzed. The plant genotypes were grown under same soil and climate conditions and under same agronomic management; the leaves were also harvested under the same conditions. Anti-inflammatory activity was evaluated by mice ear edema induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) at a single dose of 200 mg/kg BW of each extract. Composition of triterpenoids and total phenolic contents was determined by HPLC-DAD and Folin-Ciocalteu method, respectively. *Ugni molinae* leaves of different plant genotypes exhibited significant differences in regard to their anti-inflammatory activity, as well as in qualitative-quantitative composition of triterpenoids and total phenolic content.

Keywords: *Ugni molinae* genotypes; anti-inflammatory activity; pentacyclic triterpenoids; mouse ear edema; HPLC-DAD; TPA (12-O-tetradecanoylphorbol-13-acetate)

Resumen: El objetivo de este estudio fue establecer las diferencias en la composición cualitativa y cuantitativa de triterpenoides y en los contenidos totales de fenoles, junto con la actividad antiinflamatoria de las hojas de *Ugni molinae* provenientes de diez genotipos. Los extractos de acetato de etilo (EAE) y etanólicos (ETE) fueron obtenidos y analizados. Los genotipos fueron cultivados bajo las mismas condiciones edafo-climáticas y con el mismo manejo agronómico; las hojas fueron cosechadas bajo las mismas condiciones. La actividad antiinflamatoria fue evaluada en ratones a los que se les indujo un edema en la oreja mediante la aplicación del 12-O-tetradecanoilforbol-13 acetato (TPA) y los extractos fueron evaluados a una dosis única de 200 mg/kg de peso corporal. La composición en triterpenoides y los contenidos de fenoles totales fueron determinados por CLAE-DAD y por el método de Folin-Ciocalteu, respectivamente. Las hojas provenientes de los diferentes genotipos de *U. molinae*, exhibieron significativas diferencias en sus actividades antiinflamatorias, así como, en el contenido cualitativo y cuantitativo de triterpenoides y en el contenido de fenoles totales.

Palabras clave: genotipos de *Ugni molinae*; actividad antiinflamatoria; triterpenoides pentacíclicos; edema en oreja de ratón; CLAE-DAD; TPA (12-O-tetradecanoilforbol-13-acetato)

Recibido | Received: January 11, 2016

Aceptado | Accepted: February 21, 2016

Aceptado en versión corregida | Accepted in revised form: March 24, 2016

Publicado en línea | Published online: September 30, 2016

Declaración de intereses | Declaration of interests: This work was supported by FONDECYT, Chile [Grant N° 1130155] and CONICYT, Chile [Grants N°21130672 and N°21120377].

Este artículo puede ser citado como / This article must be cited as: J Arancibia-Radich, M Peña-Cerda, D Jara, L Goity, G Valenzuela-Barra, X Silva, G Garrido, C Delporte, I Seguel. 2016. Comparative study of anti-inflammatory activity and qualitative-quantitative composition of triterpenoids from ten genotypes of *Ugni molinae*. *Bol Latinoam Caribe Plant Med Aromat* 15 (5): 274 – 287.

INTRODUCTION

Ugni molinae Turcz. Myrtaceae (*Myrtus ugni* Mol.) is a Chilean native species commonly known as murtilla, murta, uñi or Chilean guava that grows wild in the South of Chile. *Ugni molinae* is a 1.5 to 2 m height bush. Its edible red and aromatic fruits are commonly used for the preparation of hand-made jams, syrups, desserts and liquors (Muñoz *et al.*, 1981; Wilhelm de Mosbach, 1999). In Southern Chile, the indigenous people of the area have been using this fruit as produce and to brew alcoholic beverages long before the Spanish arrived. According to popular tradition, murtilla leaves have anti-inflammatory properties (Muñoz *et al.*, 1981; Montenegro, 2000).

Our previous murtilla leaf studies, which were carried out with wild murtilla leaves, have shown that both ethyl acetate extract (EAE) and ethanolic extract (ETE) have topical anti-inflammatory and analgesic activity, as well as intraperitoneal analgesic activity. In addition, we have previously reported several triterpenoid acids present in wild murtilla leaves, which have significant anti-inflammatory and analgesic activity; namely, oleanolic, ursolic, betulinic, aliphatic, corosolic, maslinic, asiatic and madecassic acids (Aguirre *et al.*, 2006; Delporte *et al.*, 2007; Goity *et al.*, 2013).

Several studies have shown that the berries and leaves of *U. molinae* present antioxidant activity and contain different kinds of phenolic compounds such as caffeic acid-3-glucoside, quercetin-3-glucoside, quercetin, rutin, gallic acid, quercitrin, luteolin, kaempferol, kaempferol-3-glucoside, luteolin-3-glucoside, p-coumaric acid and myricetin (Rubilar *et al.*, 2006; Rubilar *et al.*, 2011; Arancibia-Avila *et al.*, 2011; Avello *et al.*, 2014; Junqueira-Gonçalves *et al.*, 2015).

The regular intake of murtilla leaves infusions has proved to increase plasmatic antioxidant capacity due to the high amount of polyphenols identified (Avello & Pastene, 2005; Rubilar *et al.*, 2006; Rubilar *et al.*, 2011). Other reported properties of murtilla leaves are their antimicrobial activity (Avello *et al.*, 2009; Shene *et al.*, 2012) and their inhibitory effect on enzymes involved in the control of glycemia such as α -amylase and α -glucosidase (Rubilar *et al.*, 2011).

Due to the increasing commercial value of *U. molinae* in the national market, in 1996, the Instituto Nacional de Investigaciones Agropecuarias (INIA, Spanish acronym for National Institute of Agricultural Research), located in Carillanca, Temuco, Chile, began a program of systematic investigation leading to the domestication of this species. Nowadays, the INIA-Carillanca has murtilla crops of different plant genotypes and at least fifteen years of agronomic evaluation of their fruits (Seguel *et al.*, 2000; Ramos *et al.*, 2012).

The aim of this study was to assess the differences in qualitative-quantitative composition of triterpenoids, total phenolic content and anti-inflammatory activity of EAEs and ETEs from the leaves of ten genotypes of *Ugni molinae*, which were grown under same soil and climate conditions and under same agronomic management; the leaves were also harvested under the same conditions.

MATERIALS AND METHODS

Chemicals

Asiatic, maslinic, betulinic, ursolic acids and TPA were obtained from Sigma (MO, US); madecassic acid from Phytolab, (DE); and Folin-Ciocalteu reagent, hexane, dichloromethane, ethyl acetate, ethanol and methanol were purchased from Merck SA. In HPLC analyses, Milli-Q Water was used for the mobile phase in all measurements, and Hibar Purospher Star RP-18 column was purchased from Merck S.A. Indomethacin was donated by Laboratorio Chile (Santiago, Chile).

Plant material and extraction process

Genotypes of *U. molinae* leaves were obtained from the INIA germplasm bank, in Carillanca, Temuco, Chile, in May 2013. These were grown under the same soil and climate conditions and same agronomic management and were harvested under the same conditions. A voucher sample of each plant genotype is kept at the Herbarium of the Facultad de Ciencias Químicas y Farmacéuticas (SQF, for its Spanish acronym) of Universidad de Chile (14-4 SQF 22549; ZF-18 SQF 22550; 31-1 SQF 22551; 22-1 SQF 22552; 19-1^{ha} SQF 22553; 19-1 SQF 22554; 27-1 SQF 22555; 2 3-2 SQF 22556; 19-2 SQF 22557; 8-2 SQF 22571). The leaves from a group of five plant genotypes with higher relative leaf yield and the

leaves from another group of five plant genotypes with higher relative fruit yield, provided by INIA, were used in this study (Seguel *et al.*, 1999; Seguel *et al.*, 2000).

The plant genotypes from the INIA germplasm bank exhibiting a higher relative leaf yield were labelled using an L superscript: 19-1^L; 19-1ha^L; 14-4^L; 8-2^L; and ZF-18^L, whereas those selected for a higher relative fruit yield were labelled in plain numbers: 19-2; 31-1; 23-2; 27-1 and 22-1. The study's design into these two groups, eventually, led us to demonstrate that a plant genotype with fruit agronomic potential has leaves with important medicinal properties, providing added value to the crops of this species, since both fruits and leaves can be exploited. Normally, fruits are collected in April/May and, posteriorly, leaves are pruned and discarded. In contrast, the results could indicate that the leaves from plant genotypes selected for producing a higher relative amount of leaves are the ones with a higher anti-inflammatory activity, and in this way the production of *U. molinae* could have a beneficial impact for medicinal purposes.

Dried and ground leaves (2 kg) of each plant genotype were successively extracted by maceration at room temperature with hexane, dichloromethane, ethyl acetate and ethanol (6 L of each solvent); after removing the solvents, the extracts were completely dried at 30°C, the yields of dry extracts (HE, DME, EAE and ETE, respectively) expressed as g/100 g dried leaves were: a) **HEs** 14-4^L: 1.3%, 19-1^L: 1.0%, 19-1ha^L: 1.1%, 22-1: 1.1%, 27-1: 1.1%, 23-2: 1.2%, 8-2^L: 1.2%, 31-1: 1.5%, 19-2: 1.2% and ZF-18^L: 1.1%; b) **DMEs** 14-4^L: 1.9%, 19-1^L: 1.5%, 19-1ha: 2.9%, 22-1: 1.6%, 27-1: 1.7%, 23-2: 1.3%, 8-2^L: 1.3%, 31-1: 1.7%, 19-2: 1.9% and ZF-18^L: 1.7%; c) **EAEs** 14-4^L: 5.2%, 19-1^L: 5.6%, 19-1ha^L: 4.4%, 22-1: 5.4%, 27-1: 4.8%, 23-2: 7.0%, 8-2^L: 5.6%, 31-1: 5.1%, 19-2: 4.7% and ZF-18^L: 3.1%; and d) **ETEs** 14-4^L: 11.3%, 19-1^L: 16.4%, 19-1ha^L: 14.8%, 22-1: 13.0%, 27-1: 12.5%, 23-2: 12.1%, 8-2^L: 9.5%, 31-1: 8.5%, 19-2: 10.2% and ZF-18^L: 27.7%.

Since HEs and DMEs from wild *Ugni molinae* did not exhibit *per os* activity (data not shown), only topical and intraperitoneal activity, both EAEs and ETEs were the ones selected for chemical and pharmacological study.

Chemical studies

Total phenolic content

The total phenolic content of EAEs and ETEs in the leaves of plant genotypes was determined by the Folin-Ciocalteu method described by Cicco *et al.* (2009). Extracts were dissolved in methanol/water (8:2) at a concentration of 1.0 mg/mL (EAEs) and 0.5 mg/mL (ETEs); afterwards, 30 µL of each sample or blank (hydromethanolic solutions only, without samples) were mixed with 30 µL of Folin-Ciocalteu reagent and 240 µL of 5% sodium carbonate solution. Then, the mixture was heated in a water bath at 40°C for 20 min and absorbance was measured in a microplate reader (Thermo Scientific Multiskan GO) at 765 nm.

A calibration curve was obtained with gallic acid standard (2.0 – 10.5 µg/mL) under the same conditions mentioned for the samples ($y = 0.062x + 0.052$), $R^2 = 0.998$ and F calculated $0.3445 < F$ table 3.7083.

Results were reported as mean \pm SD of three independent replicates and expressed as mg of gallic acid equivalents (GAE) /g dry extract.

Qualitative-quantitative composition in triterpenoids

The triterpenoid composition of EAEs and ETEs obtained in the process of serial extraction was established by high performance liquid chromatography with photodiode array (Waters 600C chromatograph coupled to a photodiode array detector Waters 996, Waters quaternary pump 600 and automated injector equipped with a Waters 717 plus controlled by Empower Pro 2 software). Triterpenoids were previously identified by RMN and HPLC-ESI-MS (Aguirre *et al.*, 2006; Goity *et al.*, 2013); in this study, they were identified by comparing the retention time at 201 nm and the UV spectrum obtained between 200 and 400 nm with the samples (EAEs and ETEs) and the standard substances. Triterpenoid quantification was performed by drawing calibration curves with reference standards. For the analysis of EAEs, these were injected into the HPLC-DAD in a volume of 20 µL of a methanolic solution of 5 mg/mL, and for the ETEs analysis, these were injected a volume 20 µL of a methanolic solution of 10 mg/mL.

The determination of the content of madecassic, asiatic, maslinic, alipholic and corosolic acids was performed under the following conditions:

20 μ L sample volume; Hibar Purospher Star RP-18 (250 mm x 4 mm, 5 μ m) column was used at room temperature; using 0.1% acetic acid in acetonitrile (75:25) as mobile phase; flow rate of 0.6 mL/min and UV detection at 201 nm. Primary standards of madecassic, asiatic, maslinic, betulinic and ursolic acids were used. Concentration of corosolic and aliphatic acids was expressed in ursolic and betulinic acids, respectively; in the absence of primary standards commercially available and the concentration of oleanolic and ursolic acid mixture it

was expressed in ursolic acid. The retention time (RT) and UV spectrum of corosolic and aliphatic acids (12.9 min \pm 0.09 and 12.0 \pm 0.07 respectively) were determined by using as standards both compounds which had been previously isolated and identified in our laboratory (Aguirre *et al.*, 2006).

Two HPLC chromatograms of *Ugni molinae* leaves, those of EAE (genotype 8-2^L) and ETE (genotype 19-1^L), are provided as Figure 1a and Figure 1b, respectively. Triterpenoid structures are shown in Figure 2.

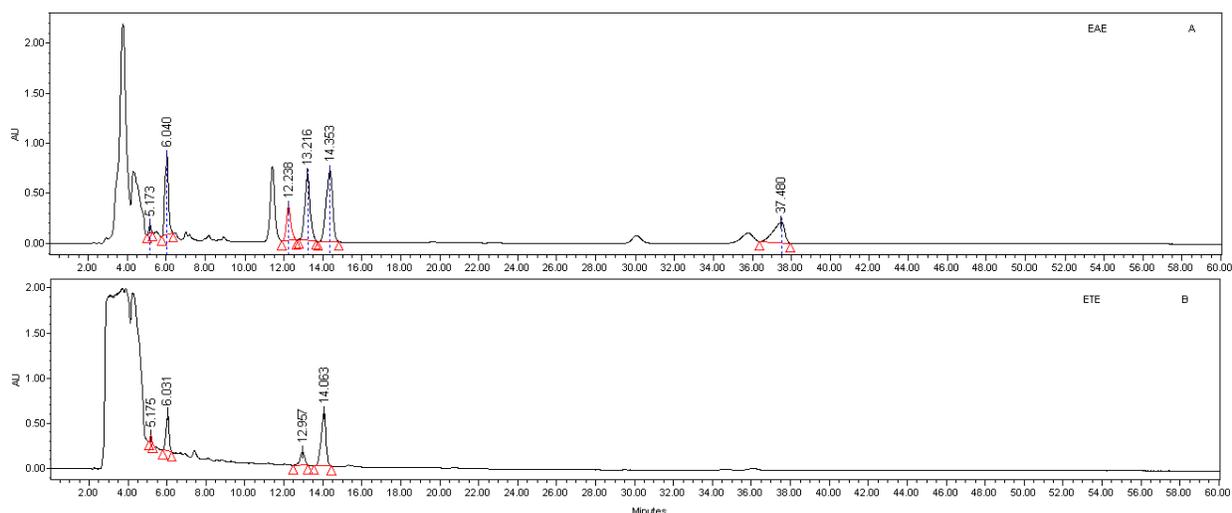


Figure 1

HPLC chromatograms of *Ugni molinae* leaves at 201 nm. (A): EAE (ethyl acetate extract) 5 mg/mL of genotype 8-2L; (B): ETE (ethanolic extract) 10 mg/mL of genotype 19-1L; Compounds: RT = 5.2 min madecassic acid; RT = 6.0 min asiatic acid; RT = 12.0 min aliphatic acid; RT = 12.9 min corosolic acid; RT = 14.0 min maslinic acid; RT = 37.1 min ursolic and oleanolic acids.

Calibration curves were obtained in the same conditions mentioned for the samples with a) asiatic acid standard (RT = 6.0 min \pm 0.01) in a concentration range 10 - 200 μ g/mL ($y = 18909x + 6218$), $R^2 = 0.9985$; b) madecassic acid standard (RT = 5.2 min \pm 0.01) in a concentration range 10 - 200 μ g/mL ($y = 18779x + 14633$), $R^2 = 0.9987$; c) ursolic acid standard (RT = 36.9 \pm 0.14) in a concentration range 10 - 200 μ g/mL ($y = 18806x - 29823$), $R^2 = 0.9990$; d) betulinic acid standard (RT = 32.0 \pm 0.08) in a concentration range 10 - 200 μ g/mL ($y = 23177x - 15435$), $R^2 = 0.9941$ and e) maslinic acid standard (RT = 14.0 \pm 0.25) in a concentration range 10 - 200 μ g/mL ($y = 27699x - 160125$), $R^2 = 0.9949$.

For all the calibration curves, the value of F calculated was lower than the value of F table; therefore, the results adjust to a linear model.

Results were reported as mean \pm SD of nine independent replicates and expressed as g of triterpenoid/100 g dry extract.

HPLC reproducibility

Under the established conditions, intra- and inter-day reproducibility was assayed by injecting 20 μ L of EAEs or ETEs three times per day. In both EAEs and ETEs, intra-day relative standard deviations (RSDs) of retention time and peak height were less than 0.6% and 1.4%, respectively (n = 3); while inter-day RSDs

were less than 1.5% and 2.0%, respectively (n = 9). These results indicate that the method shows good stability and reproducibility. Madecassic, asiatic, maslinic, betulinic and ursolic acids standards also showed adequate reproducibility under their respective retention times.

Anti-inflammatory activity

Anti-inflammatory activity of oral administration of EAEs and ETEs was evaluated through TPA-induced

mice ear edema, CF-1 male mice weighing 25 to 30 g were housed under a 12 h light to dark cycle at $22 \pm 2^\circ \text{C}$ with *ad libitum* access to food and water. Experiments were performed in accordance with current "Guidelines on the care and use of animals for scientific purpose" and approved by the Animal Care and Use Committee of the Facultad de Ciencias Químicas y Farmacéuticas of Universidad de Chile and Instituto de Salud Pública de Chile (code of approval CBE-2012-16 in 2012).

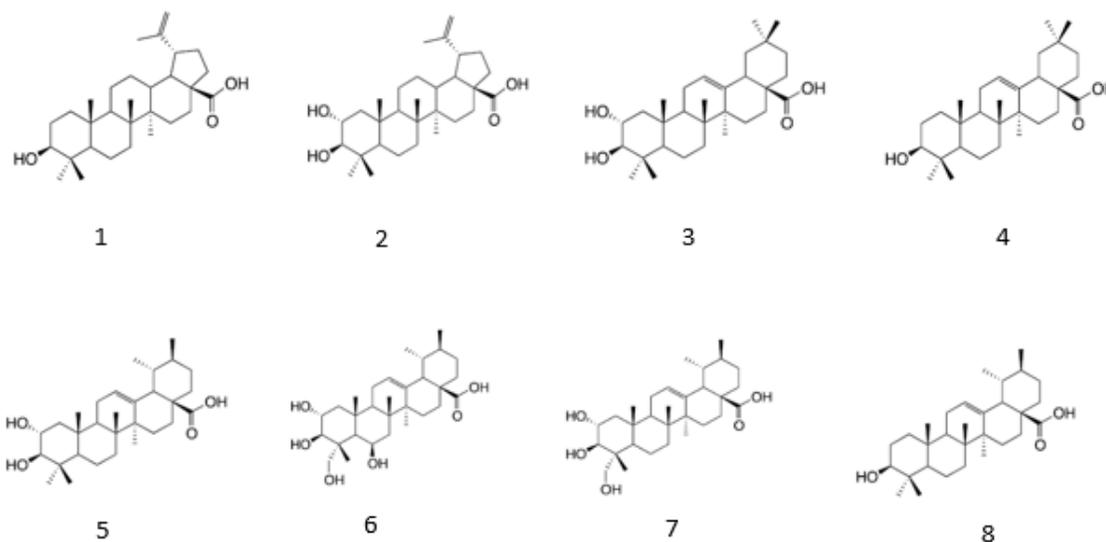


Figure 2

Structure of betulinic acid (1), alphitolic acid (2), maslinic acid (3), oleanolic acid (4), corosolic acid (5), madecassic acid (6), asiatic acid (7) and ursolic acid (8)

Animals were acclimatized to the laboratory environment for at least 24 h before testing, fasted overnight before the day of assay, with free access to water, and they were used only once in the experiment.

The assay was done on a group of 8 animals, to which a single dose of the sample was administered (treated group); the control group consisted of 16 animals (received vehicle of the sample).

Firstly, TPA (5 μg / 20 μL acetone) was topically administered on the right ear and acetone on the left ear as a solvent control (this solvent does not interfere with the assay) for mice in the treated and control groups. Immediately after this, the EAEs or EETs in Arabic gum were orally administered, at the

single dose of 200 mg/kg BW to the treated group. The control group only received Arabic gum. According with Xi *et al.* (2009), 6 h seemed to be suitable for triterpenoid absorption, said authors reported a pharmacokinetic study for oleanolic acid, indicating that it had delayed *in vivo* absorption (at least 5.2 h).

The animals were sacrificed by cervical dislocation 6 h after TPA application, and a section of 6 mm diameter of the right and left ears was punched out and weighed.

Anti-inflammatory effect (AE) was determined according to the following equation: $\%AE = [Wc - Ws / Wc] \times 100$; where Wc and Ws are the difference median values of the weights of the right and left ear sections of the control and treated

animals, respectively (Delporte *et al.*, 2003). A single 200 mg/kg BW dose was used as comparative test; due to low oral bioavailability of triterpenoids and phenolic compounds, we evaluated the highest dose of extracts that was possible to solubilize in Arabig gum (Xi *et al.*, 2009; Biasutto *et al.*, 2014). Indomethacin was used as reference drug and it was evaluated at the doses 5, 10, 20 and 40 mg/kg BW.

Statistical analysis

The statistical analysis of data was carried out using GraphPad Prism version 6.01. Lack-of-fit test (F), one-way ANOVA and Tukey post hoc multiple comparison tests were used to analyze data; p-values of ≤ 0.05 were considered statistically significant. *In vivo* assays: the results were presented as % AE \pm

SEM. Statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests. The criterion for statistical significance was set at $p \leq 0.05$. Anti-inflammatory activity and triterpenoid content were correlated by a median regression using Stata 13.0 statistical software.

RESULTS

Total phenolic content

Folin-Ciocalteu colorimetric method was carried out to determine the total phenolic content of EAEs and ETEs. Table 1 shows the quantification of total phenolic content in the leaves of plant genotypes of the species under study, statistically significant differences were detected between EAEs and ETEs.

Table 1
Total phenolic content in EAEs and ETEs from the leaves of different *Ugni molinae* genotypes assessed by the Folin-Ciocalteu method

Genotype	EAE	ETE
	mg GAE/g dry extract	mg GAE/g dry extract
27-1	92.3 \pm 4.0 ^a	225.8 \pm 1.3 ^a
19-1 ^L	89.9 \pm 4.5 ^a	191.7 \pm 0.6 ^b
ZF-18 ^L	85.6 \pm 1.8 ^a	249.9 \pm 3.7 ^c
14-4 ^L	62.4 \pm 5.8 ^b	212.8 \pm 2.7 ^d
23-2	57.1 \pm 0.6 ^{b,c}	195.6 \pm 2.5 ^b
22-1	55.2 \pm 1.9 ^{b,c}	222.5 \pm 4.1 ^a
31-1	53.1 \pm 3.2 ^{b,c}	184.8 \pm 2.1 ^b
19-1ha ^L	47.2 \pm 4.2 ^{c,d}	164.9 \pm 3.7 ^e
8-2 ^L	38.2 \pm 3.9 ^{d,e}	194.0 \pm 2.6 ^b
19-2	36.1 \pm 2.8 ^e	157.6 \pm 2.9 ^e

Each value is an average \pm SD of triplicate analysis. EAE: ethyl acetate extract; ETE: ethanolic extract; mg GAE: mg of gallic acid equivalents; same letter (a, b, c, d and e) in each column represents values that are not statistically different ($p \geq 0.05$); the statistical analysis was carried out using: lack-of-fit test (F), one-way ANOVA and Tukey post hoc multiple comparison tests; the codes of the plant genotypes with letter L correspond to the genotypes selected because they produce a higher quantity of leaves.

Regarding total phenolic content, EAE from leaves of plant genotype 19-2 has the lowest content while the EAE from leaves of plant genotype 27-1 has the highest concentration of these compounds, approximately three times higher in relation to plant genotypes of lower concentration. Note that the

leaves come from plant genotypes 27-1 and 19-2, which were selected because of their high fruit yield. In relation to the results obtained with ETEs, the lowest total phenolic content also corresponds to ETE from the leaves of plant genotype 19-2, and the highest concentration of phenolic compounds

corresponds to ETE from the leaves of plant genotype ZF-18^L, which reached the highest yield in the process of serial extraction. Plant genotype ZF-18^L was selected due to its ability to produce a high relative number of leaves. This study also showed that ETEs of the leaves of all plant genotypes have about three times more phenolic compounds than EAEs.

Qualitative-quantitative composition of triterpenoids
Pentacyclic triterpenoids in EAEs and ETEs from the leaves of ten plant genotypes under study were quantified using calibration curves obtained by their respective reference standard. The results are shown in Table 2 and Table 3.

Table 2

Content of pentacyclic triterpenoids in EAEs (g/100 g dry extract) from leaves of ten *Ugni molinae* genotypes

Genotype	Madecassic acid	Asiatic acid	Alphitolic acid*	Corosolic acid**	Maslinic acid	Ursolic/Oleanolic acids**
8-2 ^L	0.5 ± 0.1 ^a	8.5 ± 0.1 ^{a,c}	5.4 ± 1.3 ^a	12.9 ± 2.6 ^a	13.3 ± 2.0 ^a	15.4 ± 0.8 ^a
14-4 ^L	0.5 ± 0.0 ^a	9.1 ± 0.3 ^a	ND	18.1 ± 0.4 ^b	10.2 ± 0.4 ^{b,c}	12.8 ± 0.7 ^b
19-1 ^L	0.5 ± 0.0 ^a	7.2 ± 0.2 ^{b,d,e}	1.7 ± 0.2 ^b	8.0 ± 0.3 ^c	10.5 ± 0.6 ^c	17.4 ± 0.2 ^{c,d,e}
19-1 ha ^L	0.3 ± 0.0 ^b	9.5 ± 0.1 ^a	2.9 ± 0.1 ^{c,d,e}	12.2 ± 0.1 ^{a,d,e}	16.0 ± 0.4 ^{d,f,g}	15.3 ± 0.4 ^a
ZF-18 ^L	0.4 ± 0.0 ^b	6.2 ± 0.3 ^e	4.0 ± 0.1 ^f	11.8 ± 0.6 ^{a,d}	11.6 ± 0.8 ^{a,b,c}	18.3 ± 1.0 ^{d,g}
22-1	0.4 ± 0.0 ^{a,b}	7.9 ± 0.1 ^{c,d}	2.8 ± 0.1 ^d	10.5 ± 0.7 ^{d,e,f}	18.8 ± 1.8 ^e	19.2 ± 3.2 ^{e,f,g}
23-2	0.5 ± 0.0 ^a	9.2 ± 0.8 ^a	3.7 ± 0.3 ^{e,f}	10.1 ± 0.7 ^{c,d,e}	16.7 ± 1.2 ^{e,f}	15.7 ± 0.6 ^{a,c}
27-1	0.4 ± 0.0 ^{a,b}	6.5 ± 0.6 ^{b,e}	ND	8.4 ± 0.3 ^{c,f}	17.1 ± 1.2 ^{e,f,g}	21.1 ± 1.0 ^f
31-1	0.3 ± 0.0 ^b	8.4 ± 0.6 ^{a,d,f}	ND	13.9 ± 2.2 ^a	13.7 ± 2.1 ^{a,d}	17.1 ± 1.4 ^{a,c,d}
19-2	0.9 ± 0.2 ^c	9.7 ± 2.0 ^a	ND	12.0 ± 3.0 ^{a,d}	14.6 ± 3.6 ^{a,d,f}	18.4 ± 0.7 ^{d,e}

Each value corresponds to the average concentration obtained in nine determinations expressed as g of triterpenoid/100 g dry extract ± SD. * concentration expressed in betulinic acid; ** concentration expressed in ursolic acid; EAE: ethyl acetate extract; ND: not detectable; same letter (a, b, c, d, e, f and g) in each column represents values that are not statistically different ($p \geq 0.05$); the statistical analysis was carried out using: lack-of-fit test (F), one-way ANOVA and Tukey post hoc multiple comparison tests; the codes of the plant genotypes with letter L correspond to the genotypes selected because they produce a higher quantity of leaves.

Quantification of pentacyclic triterpenoids in EAEs showed a higher relative content than in ETEs. EAEs showed significant differences in their triterpenoid contents. All EAEs exhibited madecassic, asiatic, corosolic and maslinic acids and a mixture of ursolic and oleanolic acids. However, EAEs did not contain betulinic acid, a triterpenoid described in previous studies on wild *U. molinae* (Aguirre et al., 2006; Goity et al., 2013). EAEs from leaves of plant genotypes 8-2^L, 19-1^L, 19-1ha^L, ZF-18^L, 22-1 and 23-2 contain alphitolic acid. The

triterpenoid with the lowest concentration proved to correspond to madecassic acid, while the highest relative concentration corresponded to a mixture of ursolic and oleanolic acids.

EETs showed significant differences in their triterpenoid contents. Pentacyclic triterpenoids in ETEs were asiatic, maslinic and corosolic acids; however, corosolic acid was undetected in plant genotype 19-2 and madecassic acid is present only in plant genotypes 19-1^L, 19-1ha^L, 31-1 and 19-2; on the other hand, ETEs contain neither alphitolic nor

betulinic acids nor the mixture of ursolic and oleanolic acids. Furthermore, madecassic acid occurs at lower relative concentrations and maslinic and

asiatic acids are present at higher relative concentrations.

Table 3
Pentacyclic triterpenoid contents in ETEs (g/100 g dry extract) from the leaves of ten *Ugni molinae* genotypes

Genotype	Madecassic acid	Asiatic acid	Corosolic acid**	Maslinic acid
8-2 ^L	ND	3.1 ± 0.2 ^{a,f}	1.1 ± 0.0 ^{a,f,g,h}	2.6 ± 0.1 ^a
14-4 ^L	ND	3.4 ± 0.0 ^{a,e}	1.5 ± 0.1 ^{a,c,d}	3.2 ± 0.1 ^{a,c,d}
19-1 ^L	1.1 ± 0.3 ^a	3.7 ± 1.8 ^{a,d,e}	2.0 ± 0.8 ^b	5.4 ± 1.8 ^{b,e,f}
19-1ha ^L	0.2 ± 0.0 ^{b,c}	3.5 ± 0.4 ^{a,e}	1.7 ± 0.1 ^{b,c}	4.2 ± 0.5 ^{b,c}
ZF-18 ^L	ND	3.3 ± 0.0 ^{a,e,f}	1.7 ± 0.0 ^{b,d}	4.1 ± 0.1 ^{b,d}
22-1	ND	1.2 ± 0.1 ^{b,c,f}	0.6 ± 0.0 ^{f,g,h}	2.2 ± 0.1 ^a
23-2	ND	2.3 ± 0.3 ^{c,f}	0.8 ± 0.0 ^{g,h}	3.0 ± 0.2 ^{a,c,d}
27-1	ND	1.6 ± 0.0 ^c	0.6 ± 0.0 ^h	2.2 ± 0.2 ^a
31-1	0.2 ± 0.0 ^c	4.6 ± 0.4 ^d	2.9 ± 0.5 ^e	5.8 ± 1.3 ^{e,f}
19-2	0.7 ± 0.0 ^d	4.4 ± 1.4 ^{d,e}	ND	5.9 ± 1.3 ^f

Each value corresponds to the average concentration obtained in nine determinations expressed as g of triterpenoid/100 g dry extract ± SD; ** concentration expressed in ursolic acid; ETE: ethanolic extract; ND: not detectable. Same letter (a, b, c, d, e, f and g) in each column represents values that are not statistically different ($p \geq 0.05$). The statistical analysis was carried out using: lack-of-fit test (F), one-way ANOVA and Tukey post hoc multiple comparison tests; the codes of the accessions with letter L correspond to the genotypes selected because they produce a higher quantity of leaves

Anti-inflammatory activity

Indomethacin, used as reference anti-inflammatory drug, presented a dose-dependent effect with a maximum effect of 63.2% at the dose of 20 mg/kg BW (Table 4). Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) used to reduce inflammation and pain by inhibiting COX-1 and COX-2, it can also inhibit mechanism related to nitric oxide (Summ et al., 2010).

According to our results, significant differences arise in the anti-inflammatory activity of EAEs and EETs from leaves of plant genotypes (Table 4).

In relation to EAEs, the most active extracts were obtained from the leaves of plant genotypes 19-2, 31-1 and 19-1ha^L. Conversely, plant genotypes 22-

1 and ZF-18^L showed EAEs with the lowest anti-inflammatory effect.

Among ETEs from the leaves of plant genotypes selected according to higher relative fruit yield, some showed a significant and higher relative anti-inflammatory effect (31-1, 22-1 and 23-2). However, ETEs from the leaves of plant genotypes that presented the lowest anti-inflammatory effect were those chosen due to their higher relative leaf amount (8-2^L, 19-1^L and ZF-18^L).

From genotype 31-1, both EAE and ETE were the extracts with the best anti-inflammatory activity at the single dose evaluated (200 mg/kg BW). Both in EAEs and ETEs, no statistically significant correlation was observed for the median anti-inflammatory activity vs. total triterpenoid contents (Figure 3; $p=0.896$ for EAEs and $p=0.332$ for ETEs).

However, a slightly directly proportional tendency was observed for EAEs and inversely proportional

for ETEs. On the other hand, a positive but non-significant correlation was found for asiatic acid.

Table 4

Oral anti-inflammatory effects of EAEs and ETEs from the leaves of ten *Ugni molinae* genotypes and reference drug (indomethacin) in inflammation

Genotype/ dose 200 mg extract/kg BW	%AE _{EAE} ± SEM	%AE _{ETE} ± SEM
19-2	32.1 ^{*,a} ± 3.1	18.0 ^{*,b} ± 2.1
31-1	30.8 ^{*,a} ± 3.8	35.5 ^{*,a} ± 3.6
19-1ha ^L	30.5 ^{*,a} ± 3.3	5.6 ^c ± 1.9
23-2	28.4 ^{*,a,b} ± 3.0	28.9 ^{*,a} ± 3.0
27-1	25.5 ^{*,a,b} ± 3.0	25.7 ^{*,a,b} ± 3.0
14-4 ^L	22.5 ^{*,b} ± 3.8	19.9 ^{*,a,b} ± 2.3
8-2 ^L	22.5 ^{*,b} ± 2.9	2.9 ^c ± 3.4
19-1 ^L	22.0 ^{*,b} ± 2.9	8.8 ^c ± 2.0
ZF-18 ^L	11.9 ^{*,c} ± 1.7	11.1 ^{b,c} ± 2.0
22-1	11.1 ^{*,c} ± 1.9	35.3 ^{*,a} ± 3.5
Indomethacin 40 mg/kg BW	57.8 [*] ± 7.5	
Indomethacin 20 mg/kg BW	↑63.2 [*] ± 7.3	
Indomethacin 10 mg/kg BW	23.8 [*] ± 6.3	
Indomethacin 5 mg/kg BW	9.2 ± 3.9	

* $p \leq 0.05$ significant differences between control group (n=16) and treated group (n=8); the same letter (a, b, and c) in each column represents values that are not statistically different ($p \geq 0.05$). AE_{EAE}: anti-inflammatory effect of ethyl acetate extract; AE_{ETE}: anti-inflammatory effect of ethanolic extract; TPA: phorbol 12-myristate 13-acetate; statistical significance was evaluated using the Kruskal-Wallis test followed by Dunn's multiple comparison tests; the criterion for statistical significance was set at $p \leq 0.05$; ↑ maximum effect; the codes of the accessions with L letter correspond to genotypes selected because they produce a higher leaf quantity. Indomethacin was the reference drug.

DISCUSSION

The content of active substances in a plant species is genetically controlled, but its phenotypic expression is conditioned by environmental variables such as light, temperature, humidity and level of fertilization (Suzuki & Iwai, 1984).

According to Rejeb *et al.* (2014), the synthesis of secondary metabolites is presented as a defense mechanism against stressful situations, either as the result of biotic stress (i.e. the stress caused by microorganisms such as fungi and bacteria) or abiotic stress (such as hydric stress, ultraviolet radiation, or

temperature). Plants react to environmental variations depending on the season of the year, their fertilization and also the damage caused by plagues and diseases (Chavez & Escudero, 1999), which have an impact on the production of secondary metabolites that regulate metabolic activity (Watterman & Mole,

1989; Laitinen *et al.*, 2000; Muzitano *et al.*, 2011). Therefore, the season, environmental variations, stressful situations, and agronomic management can affect secondary metabolism in plants that promotes the synthesis of bioactive compounds.

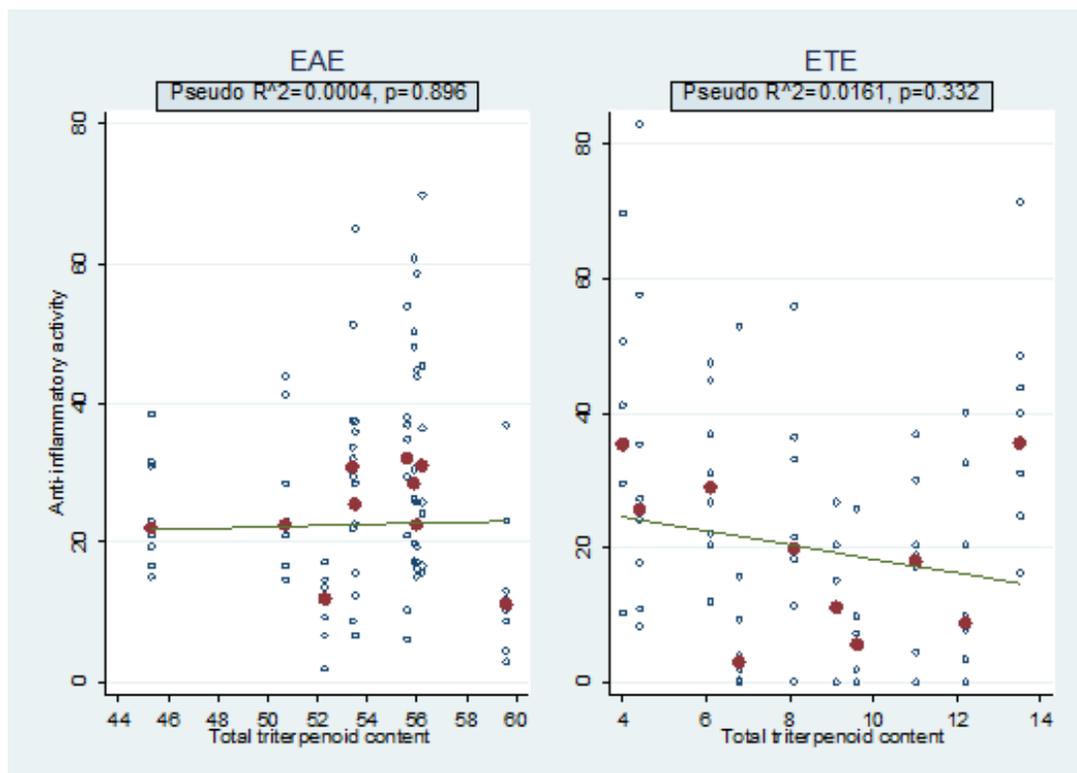


Figure 3

EAEs/ETEs correlation between total triterpenoid content and anti-inflammatory activity of each genotype

- Corresponds to the anti-inflammatory activity of each genotype associated to total triterpenoid content.
- Corresponds to the linear correlation between the median of the anti-inflammatory activity of each genotype and total triterpenoid content.

Recently, Avello *et al.* (2014) demonstrated the chemical differences between continental and Juan Fernandez Archipelago populations of *U. molinae*, this latter population presents greater content and variety of phenolic and triterpenoid compounds, probably due to the geoclimatic conditions of the archipelago.

In order to assess anti-inflammatory activity, qualitative-quantitative composition of triterpenoids and total phenolic content of the leaves of *U. molinae*

genotypes, the samples were grown and harvested under the same conditions.

In relation to the anti-inflammatory activity, the best model corresponds to *in vivo* assays, as they permit to extrapolate the effects to the human being. An *in vivo per os* assay would allow to infer that the active principles were actually absorbed in the gastrointestinal tract (Smith *et al.*, 2001; Brunton *et al.*, 2013). TPA was the inflammatory agent used topically to provoke an acute edema with leukocyte

infiltration, activating protein kinase C (PKC), which is Ca²⁺ and phospholipid-dependent protein. Active PKC acts at different levels, activating the nuclear factor kappa B (NF- κ B). This transcription factor promotes the expression of several pro-inflammatory agents, such as COX-2 and inducible nitric oxide synthase (iNOS), inflammatory cytokines such as interleukin-1 (IL-1), IL-2, IL-6, IL-8 and the tumor necrosis factor-alpha (TNF- α), which is another pro-inflammatory and host defense cytokine. Its excessive production leads to chronic pro-inflammatory diseases, leading to formation of prostanoids and increased production of free radicals (Ban *et al.*, 2009).

In the last fifteen years, interest in pentacyclic triterpenoids has increased. Several different studies provide abundant information revealing the anti-inflammatory properties of these compounds (Banno *et al.*, 2004; Yamaguchi *et al.*, 2006; Dzubak *et al.*, 2006).

Formerly, we have contributed to the knowledge accrual on the topical anti-inflammatory activity of various 2 α -hydroxy triterpene acids; namely, madecassic, asiatic, alphitolic, and corosolic acids, which are present in murtilla leaves (Aguirre *et al.*, 2006; Goity *et al.*, 2013).

In the current study, we have detected differences in the content of triterpenoids of the leaves of different *U. molinae* genotypes in relation to madecassic, asiatic, corosolic, maslinic and alphitolic acids and the mixture of ursolic and oleanolic acids. These triterpenoids reduced the production of IL-6, IL-1 β and TNF- α and the activation of NF- κ B (Ahn *et al.*, 1998; Yun *et al.*, 2008). Aguirre *et al.* (2006) and Huang *et al.* (2011) demonstrated the anti-inflammatory effect of asiatic, alphitolic and corosolic acids using the TPA-induced mice ear edema and the carrageenan-induced paw edema, respectively. Ursolic acid suppresses the expression of pro-inflammatory mediators in macrophages; these functions are the result of the inhibition of NF- κ B activation. Moreover, it suppresses TPA-mediated induction of COX-2 and the synthesis of prostaglandin E₂ (PGE₂) in epithelial cells (Ikeda *et al.*, 2008). On the other hand, oleanolic acid exerts anti-inflammatory effects via the decrease of pro-inflammatory factors, such as TNF- α , IL-1 β and IL-6 (Wang *et al.*, 2013).

Regarding maslinic acid, this triterpenoid

mitigates intracellular oxidative stress and reduces release of pro-inflammatory cytokines in macrophages (Marquez *et al.*, 2006).

In relation with total phenolic content, our results proved significant differences among EAEs and ETEs from the leaves of plant genotypes. In general, phenolic compounds show antioxidant activity, and as anti-inflammatory activity is closely related to antioxidant activity, the difference in total phenolic content could partly account for the difference in anti-inflammatory activity, since an increase in reactive oxygen and nitrogen species is generated during the inflammatory process (Leopoldina *et al.*, 2011; Brunton *et al.*, 2013; López-Alarcón & Denicola, 2013). In the leaves of wild *U. molinae*, anti-inflammatory phenolic compounds have been identified, compounds which could be present in the leaves of the plant genotypes studied in this investigation (Middleton *et al.*, 2000; Rubilar *et al.*, 2011; Arancibia-Avila *et al.*, 2011; Avello *et al.*, 2014; Junqueira-Gonçalves *et al.*, 2015). We are currently studying the identification of phenolic compounds in the leaves of plant genotypes and their antioxidant/anti-inflammatory properties for a better understanding of their contribution to anti-inflammatory activity.

Generally, triterpenoids and phenolic compounds showed low bioavailability *per os*, which could explain lower anti-inflammatory activity by oral route when compared to topical administration of *Ugni molinae*. It is worth to note that new formulations to improve the oral bioavailability of these compounds have been developed (Xi *et al.*, 2009; Tran *et al.*, 2014).

As previously mentioned, the increase of the commercial value of *U. molinae* in the national market has led researchers from the Instituto Nacional de Investigaciones Agropecuarias (INIA, Temuco, CL) to start a systematic investigation leading to the domestication of this species, which involved the collection of wild germplasms and the development of protocols for regeneration and multiplication of plants, plus the evaluation of the fruits' agronomic potential. INIA researchers constructed the molecular characterization of the collected wild germplasm to study the genetic diversity of this species and to complete its phenotypic and agronomic characterization. This genotyping was made through microsatellite markers

or simple sequence repeat (SSR). The INIA of Carillanca has murtilla crops of different plant genotypes and, at least, fifteen years of agronomic evaluation of their fruits (Seguel *et al.*, 1999; Seguel *et al.*, 2000; Ramos *et al.*, 2012).

Since we provide evidence of the different anti-inflammatory effect of the leaves of ten *Ugni molinae* plant genotypes, as well as the differences in qualitative-quantitative composition of triterpenoids and total phenolic contents, we consider that our study may be a contribution for INIA, promoting not only fruit but also leaf harvesting, thus improving commercial exploitation. In addition, INIA and other associations might become interested in cultivating the *Ugni molinae* leaf genotypes under study with the most optimal anti-inflammatory activity, such as 31-1.

Finally, we should mention that this is the first comparative study of the anti-inflammatory activity of *U. molinae* leaf genotypes and of their composition of pentacyclic triterpenoid acids and total phenolic content.

CONCLUSIONS

As the most relevant results of this study, we would like to mention that our data indicates that differences in the oral anti-inflammatory effects, total phenolic contents and triterpenoid compositions between different ethyl acetate and ethanolic extracts from *U. molinae* leaves could be explained by the corresponding plant genotype.

Additionally, it should be noted that significant differences were found in the qualitative-quantitative composition of oleanolic, ursolic, aliphatic, corosolic, maslinic, asiatic and madecassic acids.

Funding

This work was supported by FONDECYT, Chile [Grant N° 1130155] and CONICYT, Chile [Grants N°21130672 and N°21120377]. We thank the *Instituto de Salud Pública de Chile* for providing the experimental animals and to INIA (Carillanca, Temuco) for the leaves of plant genotypes.

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