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Research article

The influence of environmental variations on the phenolic compound profiles and antioxidant activity of two medicinal Patagonian valerians (*Valeriana carnosa* Sm. and *V. clarionifolia* Phil.)

Nicolas Nagahama[1,2,3,*], Bruno Gastaldi[2,3], Michael N. Clifford[4], María M. Manifesto[5] and Renée H. Fortunato[2,5]

[1] Estación Experimental Agroforestal Esquel, Instituto Nacional de Tecnología Agropecuaria (INTA), Esquel, Chubut, Argentina
[2] Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET)
[3] Laboratorio de Investigación de Plantas Aromáticas y Medicinales Nativas (LIPAM), FCNyCS, Universidad Nacional de la Patagonia San Juan Bosco, Esquel, Chubut, Argentina
[4] School of Bioscience and Medicine, University of Surrey, Guildford, United Kingdom and Department of Nutrition, Dietetics and Food, Monash University, Notting Hill, Victoria, Australia
[5] Instituto de Recursos Biológicos, CIRN- INTA, Hurlingham, Buenos Aires, Argentina

*** Correspondence:** Email: nagahama.nicolas@inta.gob.ar; Tel: (+54) 2945451558.

Abstract: *Valeriana carnosa* and *V. clarionifolia* stand out as principal elements in the indigenous pharmacopeias of Patagonia; however, their phytochemical characterization is unknown. This study constitutes the starting point of a general project that aims to characterize secondary metabolites in these species. The variability of phenolic compounds in root ethanolic extracts was analyzed and compared for thirteen populations of *V. carnosa* and two of *V. clarionifolia* from the south of Argentinean Patagonia. Phenolic content was quantified by the Folin-Ciocalteu method and the putative phenolic compound profiles were investigated using HPLC-UV-MS. Antioxidant activity was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assays. Total phenolic content values ranged from 5.6 to 16.6 mg GAE/g in *V. carnosa* and 7.3 to 9.7 mg GAE/g in *V. clarionifolia*. Antioxidant evaluation results evidenced that the percentage of neutralized DPPH varied between 26% and 85% in *V. carnosa* and 39% and 58% in *V. clarionifolia*. A positive correlation between total phenolic content and antioxidant activity ($r = 0.90$) was observed. In *V. carnosa* total phenolic content was not correlated with

altitude or latitude ($p > 0.05$), and chemical variability seems to be associated with genetic variability and/or different growing habitats (microclimatic conditions). However, the presence of some specific phenolic compounds was associated with latitude. In *V. carnosa* and *V. clarionifolia* 15 and 10 phenolic compounds were tentatively identified, respectively, and several of these are reported to have beneficial attributes from a phytomedical viewpoint. This study contributes to the phytochemical characterization project of these two medicinal valerians from Patagonia.

Keywords: antioxidant activity; ethanolic extracts; HPLC-UV-MS analysis; inter-population variability; phenols

1. Introduction

Approximately 250 species of the genus *Valeriana* L. (Valerianoideae, Caprifoliaceae) are distributed widely throughout the world, except for Australia and New Zealand. In Argentinean Patagonia there are 20 *Valeriana* species [1–3], of which *V. carnosa* and *V. clarionifolia* are important medicinal plants in regional folk medicine [4]. These species are similar in geographical distribution but grow in different environments. *V. carnosa* grows from −35°36' to −54°17' latitude, mainly in the Patagonian-Andean forest-steppe ecotone (west Patagonia) and in the highest mountains of the steppe region, from 0 to 2500 m a.sl. [1,2,5], while *V. clarionifolia* grows between −35°03' and −50°30' latitude, generally associated with steppe environments from 0 to 2000 m a.sl [1,6].

Argentinean Patagonia is characterized by strong climatic gradients of latitude, longitude, and altitude, ranging from relatively colder and humid environments in the west (Andean Patagonia) to more arid and warmer environments in the east (Patagonian steppe) [7]. The climatic variation determined by the latitudinal amplitude in Patagonia and the large thermal variations detected across its longitude, which are mostly related to differences in annual and daily thermal ranges, radiation, and the influence of the winds from the Pacific Ocean, generate a great variety of environments in this region [8].

Plants synthesize different chemical compounds in response to different stress conditions, such as herbalism, pathogens, UV radiation, altitude, drought and frost [9,10]. Environmental factors are known to influence the quality and quantity of these metabolites [11,12]. In consequence, environmental factors influence phytochemical production in plants, generating variation in the regulation and accumulation of these compounds [13–15], which also vary among populations of the same species [16–19]. Extreme plant environments (i.e. high UV-B radiation related to high altitude) stimulate the synthesis of free radicals, which cause oxidative stress and simultaneously affect the general structural and functional properties of plants [20]. Oxidative stress causes damage in proteins, lipids and DNA, being able to stimulate mutagenic processes. A defense mechanism of plants to counteract oxidative stress is to synthesize antioxidative defense components such as polyphenolic compounds, terpenes, carotenoids and alkaloids [21].

Phenolic compounds are secondary metabolites characterized by an aromatic backbone with one or more hydroxyl groups, derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants [22]. These compounds are of physiological and morphological significance in plants, with an important role in growth and reproduction, providing protection against adverse environmental

conditions, pathogens, and predators [20,23]. They have also become of interest to humans due to their implications for health, particularly (but not restricted to), their antioxidant properties [24,25], whereby free radicals are captured and dangerous reactive oxygen species and other potentially damaging species are neutralized either directly or indirectly [26]. Several medicinal properties are attributed to phenolic compounds, such as a protective effect against cardiovascular diseases, as some have moderate vasodilator activity [27], and a potential anticancer effect, particularly associated with the phenolic acids [28]. The total phenolic content and antioxidant potential of some species of the Valerianaceae family have been reported [19,29–31]; however, information on the Patagonian valerians is limited.

V. carnosa is one of the plants most used by Patagonian ethnic groups (Mapuche-Tehuelche) to counteract different ailments, since anxiolytic, analgesic, antitumor, antitussive, circulatory, digestive and urinary-liver properties are attributed to it [4,32]. This species is one of the most prominent medicinal plants in the Mapuche pharmacopeia, and from an ethnopharmacological viewpoint, one of the most promising, versatile medicinal plants in Patagonia [4]. *V. clarionifolia* also has a record of medicinal use; it is used for its analgesic, antitussive, urinary and healing properties [33]. Both species are collected from natural populations, and due to the demand for *V. carnosa*, *in situ* cultivation programs for this species are currently being initiated in Patagonia [5,34].

In this study we compared the phenolic compounds (qualitatively and quantitatively) and antioxidant activity of root ethanolic extracts from thirteen populations of *V. carnosa* and two of *V. clarionifolia* from the south of Argentinean Patagonia. The objectives of this study were to (1) compare the total phenolic compound content and antioxidant activity among different populations of *V. carnosa* and *V. clarionifolia*; (2) establish whether total phenolic content and phenolic profiles in *V. carnosa* are related to latitudinal and altitudinal variation; and (3) report a preliminary characterization/identification of phenolic compounds of phytomedicinal interest in these two Patagonian valerians by HPLC-UV-MS. The certainty of these identifications are graded from 1 (most certain) to 4 (least certain) according to the criteria and recommendations of Sumner et al. [35].

2. Material and methods

2.1. Plant material

One hundred and twelve *V. carnosa* plants were collected in January 2017 (fruiting stage) from thirteen populations in Southern Patagonia, Argentina (Figure 1; Table 1). Additionally, sixteen plants from two populations of *V. clarionifolia* were collected; one from the Andean region (west) and the other from the Atlantic coast region (east). Specimens of this species were collected for comparison with *V. carnosa* (Figure 1; Table 1). The botanical identity of the plants was authenticated by Dr. Nagahama and herbarium voucher checks (BAB and CORD). Voucher specimens were deposited in the BAB herbarium. From each population of *V. carnosa* and *V. clarionifolia* we selected eight adult plants located at least 20 m distant from each other. The roots of collected specimens were cut and stored in paper bags and protected from light and moisture for 45 days.

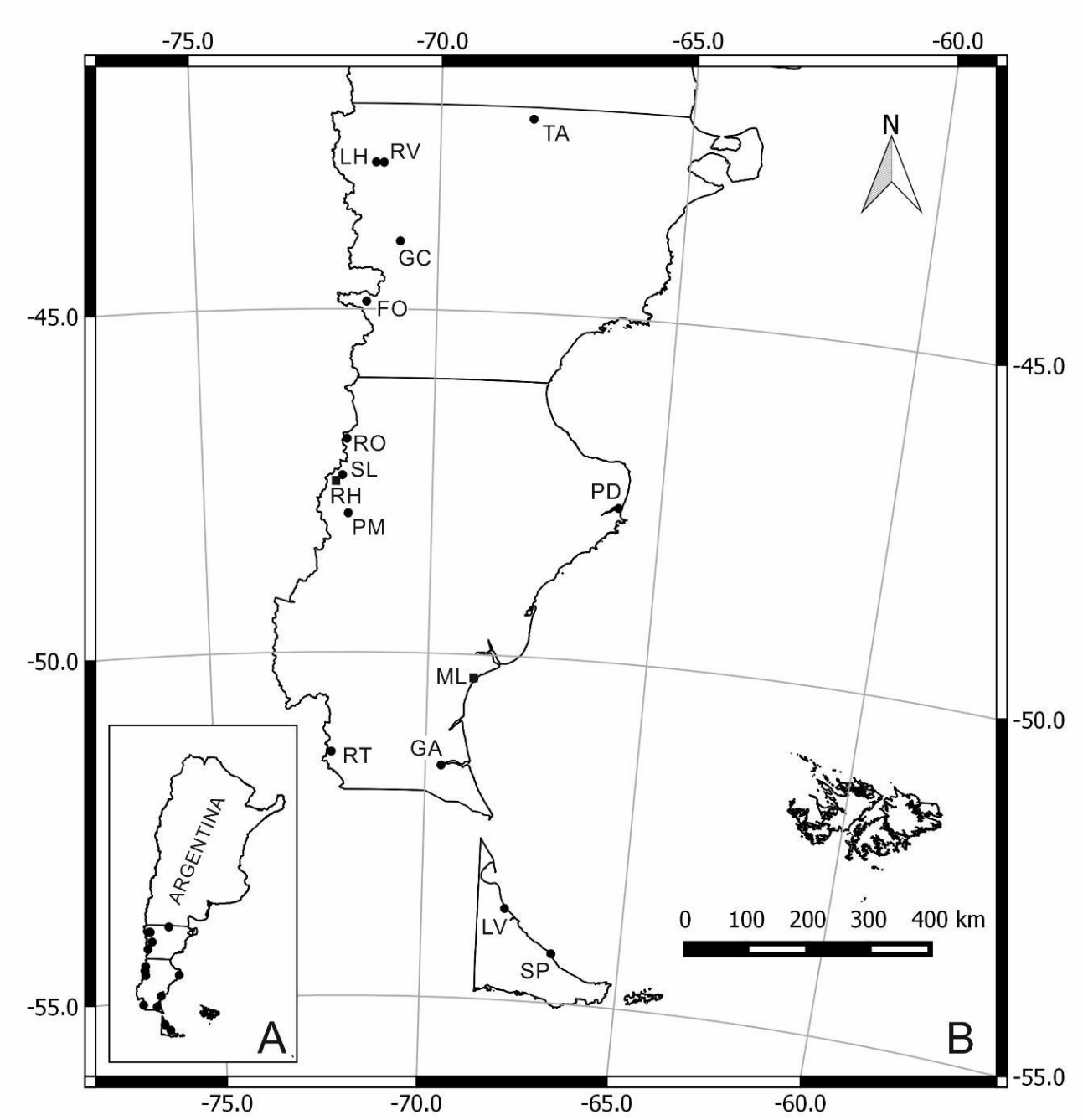

Figure 1. Distribution of analyzed populations of *Valeriana*. A. Location of the surveyed area. B. Detail of southern Patagonia. *V. carnosa* populations (circles): TA, Talagapa; RV, Río Viejo; LH, La Hoya; GC, Gobernador Costa; FO, Fontana; RO, Río Roballos; SL, San Lorenzo; PD, Puerto Deseado; PM, Perito Moreno; RT, Río Turbio; GA, Güer Aike; LV, Las Violetas; SP, San Pablo. *V. clarionifolia* (squares): RH, Río Hielo; ML, Monte León.

Table 1. Details of plant material collection sites. TA, Talagapa. RV, Río Viejo. LH, La Hoya. GC, Gobernador Costa. FO, Fontana. RO, Río Roballos. SL, San Lorenzo. PD, Puerto Deseado. PM, Perito Moreno. RT, Río Turbio. GA, Güer Aike. LV, Las Violetas. SP, San Pablo. RH, Río Hielo. ML, Monte León.

	Species	Population code	Province	Latitude	Longitude	Altitude (m a.sl)
1	*V. carnosa*	TA	Chubut	42°10′17′′S	68°09′40′′W	1863
2	*V. carnosa*	RV	Chubut	42°51′30′′S	71°07′40′′W	823
3	*V. carnosa*	LH	Chubut	42°51′22′′S	71°17′10′′W	848
4	*V. carnosa*	GC	Chubut	44°00′17′′S	70°46′51′′W	754
5	*V. carnosa*	FO	Chubut	44°53′12′′S	71°27′51′′W	1119
6	*V. carnosa*	RO	Santa Cruz	46°53′16′′S	71°51′56′′W	922
7	*V. carnosa*	SL	Santa Cruz	47°25′10′′S	71°57′51′′W	431
8	*V. carnosa*	PD	Santa Cruz	47°45′02′′S	65°55′04′′W	10
9	*V. carnosa*	PM	Santa Cruz	47°58′31′′S	71°50′12′′W	824
10	*V. carnosa*	RT	Santa Cruz	51°26′49′′S	72°13′35′′W	345
11	*V. carnosa*	GA	Santa Cruz	51°37′31′′S	69°37′31′′W	14
12	*V. carnosa*	LV	Tierra del Fuego	53°40′13′′S	67°56′02′′W	5
13	*V. carnosa*	SP	Tierra del Fuego	54°17′01′′S	66°42′18′′W	25
14	*V. clarionifolia*	RH	Santa Cruz	47°25′08′′S	71°57′53′′W	431
15	*V. clarionifolia*	ML	Santa Cruz	50°20′49′′S	68°55′49′′W	58

2.2. Preparation of ethanolic extracts

The roots of each specimen were milled to a fine texture in a grinding machine and 1 g of the powdered plant material was macerated with 10 mL of ethanol at room temperature for 7 days (root ethanolic extract). Each extract was filtered using Whatman's No. 1 filter paper to eliminate residues. Following this, composite samples from eight specimens of each population (population ethanolic extract) were prepared and stored at 4 ℃ for different analyses.

2.3. Determination of total phenols

Total phenolic content was determined by the Folin-Ciocalteu method (April 2017) according to Chaisri & Laoprom [36] as follows: 2 mL of distilled water was mixed with 20 μL of extract solution, followed by the addition of 0.2 mL of Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis USA) and 0.8 mL of Na_2CO_3. After 20 min of incubation at room temperature, absorbance was measured at 765 nm by spectrophotometer (Metrolab 324). All measurements were made in triplicate and the average value was used for quantification. A calibration curve was made with gallic acid to express the total phenolic content in mg of gallic acid equivalent per g (mg GAE/g) of dry weight (DW).

2.4. DPPH radical scavenging activity

In vitro antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) protocol, according to Gastaldi et al. [37]. We mixed 3.9 mL of DPPH ethanol solution (15 mg DPPH/500 mL ethanol), at a concentration of 30 mg/L, with 50 μL of ethanolic extracts from the populations. A cuvette containing only DPPH ethanol solution was used as a control. Absorbance was measured at 517 nm using a spectrophotometer. All measurements were made in triplicate and the average values were used to estimate antioxidant activity. The reduced DPPH percentage of each cuvette was calculated according to the following equation:

$$\% \text{ inhibition of DPPH} = [(\text{Abs control} - \text{Abs samples}) \div \text{Abs control}] \times 100$$

where "Abs control" is the absorbance of DPPH solution without extract and "Abs samples" is the sample absorbance with DPPH solution. Thus, a higher percentage of inhibition of DPPH indicates greater antioxidant activity.

2.5. Phenolic compound analysis by HPLC-UV-MS

Phenolic compound profile determination was performed by high-performance liquid chromatography coupled to diode array UV detection and tandem mass spectrometry (HPLC-UV-MS [38–40], as follows: 10 μL of ethanolic extracts were filtered and injected. Reference standards of 5-caffeoylquinic acid (IUPAC 1976) which is commonly referred to as chlorogenic acid, caffeic acid, ferulic acid, and gallic acid (Sigma-Aldrich, St. Louis, USA) were solubilized in methanol, filtered, and injected. The chromatographic equipment was an Ultimate 3000 RSLC Dionex model from Thermo Scientific, with a UV-Vis detector model VWD-3400 RS, and a triple quadrupole mass detector TSQ Quantum Access Max. The separation was performed on a C18 Hypersil-GOLD column (50 × 2.1 mm; 1.9 um particle size) kept at 30 ℃, at a flow rate of 0.20 mL/min for 50 minutes. Gradient elution: solvent (A) H_2O (containing 2.0% AcOH), solvent (B) MeOH; 85%–60% from A to 30 minutes, 60%–25% to 40 minutes, 25%–15% to 45 minutes, ending isocratic to 50 minutes. The analysis was monitored at 254, 280, 330, and 365 nm by ESI in the positive mode at a probe temperature of 360 ℃, and probe voltage of 4.5 kV. The tentative identifications proposed are based on retention time (RT), UV spectral maxima, and MS fragmentation (*m/z*) in combination with the examination of commercial reference standards, database and bibliographic data [27,31,38–44], and these assignments are graded from 1 to 4 (see Table 3) according to the recommendations and criteria of Sumner et al. [35].

2.6. Statistical analyses

Considering the number of populations of each species, statistical analyses were performed only with *V. carnosa* populations. Total phenolic content and antioxidant activity, total phenolic content and latitude, and total phenolic content and altitude were tested by Pearson correlation analysis. To determine whether there were significant differences in total phenolic content and antioxidant activity in relation to latitude and altitude, populations of *V. carnosa* were grouped in: 1- northern populations, including populations from Chubut province from −42 ℃10' to −44 ℃53' latitude (TA, RV, LH, GC, and FO), 2- southern populations, from Santa Cruz and Tierra del Fuego provinces from −46 ℃53' to −54 ℃17'

latitude (RO, SL, PD, PM, RT, GA, LV, and SP), 3- a low altitude (0–800 m asl) population group (GC, SL, PD, RT, GA, LV, and SP), and 4- a high altitude (801–1900 m asl) population group (TA, RV, LH, FO, RO, and PM). The normality of distribution of the data was analyzed by Shapiro–Wilks test [45], as well as diagram boxes. A significant deviation from a normal distribution was observed for each of the studied variables. Therefore, for statistical evaluation of differences in the total phenolic content and antioxidant activity among population groups, multiple comparisons after a Kruskal–Wallis one-way analysis of variance test were employed at $p \leq 0.05$ level [46]. Additionally, cluster analysis (CA) was performed to reveal the structure residing in a dataset. Sample similarities using the identified compound data matrix (HPLC-UV-MS) were calculated on the basis of the Sokal Sneath 1 distance (recommended for binary data) and the average linkage hierarchical agglomerative method was used to establish clusters.

All the statistical analyses were performed using the Infostat v. 2015 software [47].

3. Results

3.1. Total phenol quantification and antioxidant activity

Quantitative variability in phenolic compounds was observed at both intraspecific and interspecific levels between different populations of *V. carnosa* and *V. clarionifolia*. Among species, the total phenolic content for *V. carnosa* was 5.6–16.2 mg GAE/g, with an average of 10.9 mg GAE/g, and for *V. clarionifolia* the values were 7.3–9.7 mg GAE/g, with an average of 8.5 mg GAE/g (Table 2; Figure 2). Regarding antioxidant activity, variability was observed at the intraspecific and interspecific levels in both species. In *V. carnosa* the percentage of neutralized DPPH varied between 26.3% and 84.7%, with an average of 62.2%, and in *V. clarionifolia* it was 39.2% and 58.4%, with an average of 48.8% (Table 2; Figure 2).

Table 2. Total phenolic content and antioxidant activity of *V. carnosa* and *V. clarionifolia* populations.

#	Species	Population	Total phenolic content (mg GAE/g DW)	% DPPH scavenging activity
1	*V. carnosa*	TA	14.68	71.37
2	*V. carnosa*	RV	11.12	64.58
3	*V. carnosa*	LH	8.57	82.57
4	*V. carnosa*	GC	5.59	26.27
5	*V. carnosa*	FO	13.61	84.71
6	*V. carnosa*	RO	16.22	75.29
7	*V. carnosa*	SL	12.23	80.00
8	*V. carnosa*	PD	10.09	45.49
9	*V. carnosa*	PM	10.19	47.45
10	*V. carnosa*	RT	11.72	69.41
11	*V. carnosa*	GA	9.07	36.86
12	*V. carnosa*	LV	14.38	81.18
13	*V. carnosa*	SP	7.84	29.41
14	*V. clarionifolia*	RH	9.68	58.04
15	*V. clarionifolia*	ML	7.33	39.22

Table 3. Summary of the assigned compounds in each of the samples analyzed by HPLC from different populations of *V. carnosa* and *V. clarionifolia*. The full circles represent the presence of the compound; empty circles the absence of the compound. TA. Talagapa. RV. Río Viejo. LH. La Hoya. GC. Gobernador Costa. FO. Fontana. RO. Río Roballos. SL. San Lorenzo. PD. Puerto Deseado. PM. Perito Moreno. RT. Río Turbio. GA. Güer Aike. LV. Las Violetas. SP. San Pablo. RH. Río Hielo. ML. Monte León. The standards used in the tests are shown in bold type. Level of certainty as defined by Sumner et al. [35].

Peak	Rt (min)	m/z	λmax (nm)	Identification	Level of certainty	TA	RV	LH	GC	FO	RO	SL	PD	PM	RT	GA	LV	SP	RH	ML
1	2.22	355	330	**5-caffeoylquinic acid**	1	●	○	●	●	●	●	●	●	●	●	●	●	●	●	●
2	2.92	181	330	**Caffeic acid**	1	○	○	○	○	○	●	○	○	○	●	●	●	○	●	●
3	5.2	451	254	not identified	4	●	○	●	●	●	●	●	●	●	●	●	●	●	●	○
4	5.75	579	254	not identified	4	○	●	●	●	●	●	●	●	●	○	●	●	●	○	○
5	5.8/6.6	195	330	**Ferulic acid**	1	○	●	●	●	●	●	●	●	●	●	●	●	●	○	○
6	8.38	435	280	not identified	4	●	●	●	●	●	●	●	●	●	●	●	●	●	●	○
7	13.39	517	330	Dicaffeoylquinic acid isomer	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
8	13.75	595	330	not identified	4	○	○	○	○	○	○	○	●	●	○	●	●	●	●	●
9	13.8	403	330	not identified	4	●	●	●	●	●	○	●	●	●	○	●	●	●	○	○
10	19.05	553	330/254	not identified	4	○	○	○	○	●	●	●	○	○	○	○	○	○	●	○
11	19.63	539	330	not identified	4	●	○	●	●	●	●	●	●	●	●	●	●	●	●	○
12	23.32	545	330	Diferuloylquinic acid isomer	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
13	25.47	199	330	Syringic acid	2	○	○	○	●	○	●	○	○	●	○	○	○	○	○	○
14	28.6	465	330	not identified	4	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●
15	30.73	165	330	(o-/p-)Coumaric acid	2	●	●	●	●	●	●	●	●	●	○	●	●	●	○	○
16	31.1	369	330	Methyl caffeoylquinate	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
17	32.55	153	254	p-Hydroxy-phenylacetic acid	2	●	●	●	●	●	●	●	●	●	○	●	●	●	●	●
18	42.8	225	330	Sinapic acid	2	○	○	○	○	○	○	○	○	○	●	○	○	○	●	●
19	43.17	333	254	not identified	4	○	○	○	○	○	○	○	○	○	○	○	○	○	●	●
20	43.87	149	254	Cinnamic acid	2	●	●	●	●	●	●	●	●	●	●	●	●	●	○	●
21	44.18	167	254	Phloretic acid	2	○	○	○	○	○	○	○	●	●	●	●	●	●	○	○

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Peak	Rt (min)	m/z	λmax (nm)	Identification	level of certainty	TA	RV	LH	GC	FO	RO	SL	PD	PM	RT	GA	LV	SP	RH	ML
22	45.42	361	254	Rosmarinic acid	2	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
23	45.91	169	280	3,4-Dihydroxy-phenylacetic acid	2	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○
24	47.32	171	254	Gallic acid	1	●	●	●	●	●	●	●	●	●	●	●	●	●	●	●
Total number of identified compounds						14	13	16	17	17	18	17	18	19	15	20	19	18	15	13

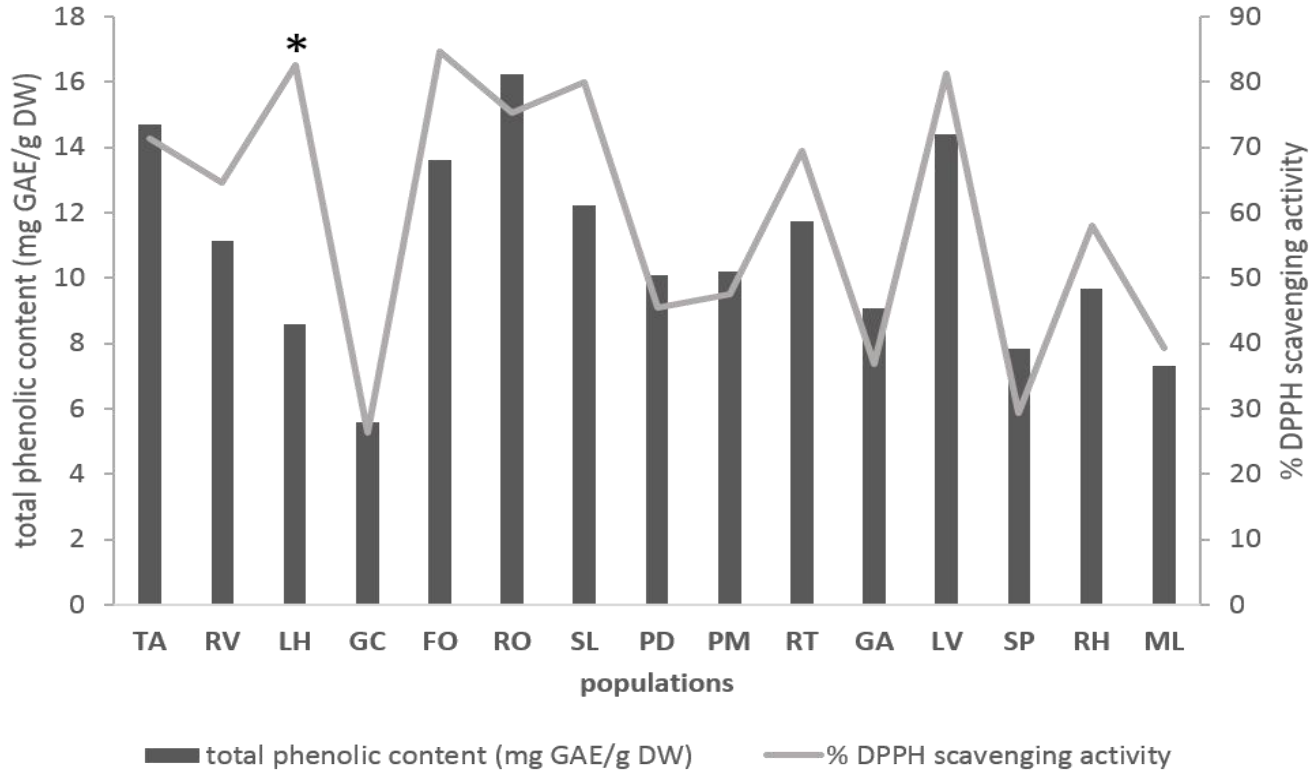

Figure 2. Total phenolic content and antioxidant activity among the populations of *V. carnosa* and *V. clarionifolia* studied. The asterisk indicates the population in which the antioxidant activity was high and the total phenolic content was relatively low.

3.2. Phenolic compounds putatively identified by HPLC-UV-MS

IUPAC recommendations (IUPAC 1976) have been followed in describing acyl-quinic acid (chlorogenic acid) regio-isomers. Twenty-three compounds were observed in *V. carnosa* and seventeen in *V. clarionifolia* population ethanolic extracts by HPLC-UV-MS, according to retention time, UV absorption, and mass of the pseudomolecular ion. Five of the phenolic compounds were present in all analyzed populations of both species (compounds 7, 12, 16, 22 and 24). We observed 16 compounds common to both species, 10 phenols (1, 2, 7, 12, 16, 17, 18, 20, 22 and 24) and six unknowns (3, 6, 8, 10, 11 and 14), as shown in Table 3.

Seven compounds were exclusive to *V. carnosa*, five phenols (5, 13, 15, 21 and 23), and two unknowns (4 and 9), but the presence of these compounds varied between populations (Table 3). In this species, six phenolic compounds (7, 12, 16, 20, 22 and 24) and two unknowns (6 and 14), were observed in all populations. In addition, one phenol (1) and two unknowns (3 and 11) were present in twelve populations of *V. carnosa*, but not in RV. Ferulic acid (5) commonly present in *V. carnosa* and confirmed with a reference standard, was also observed in twelve populations, but not TA. In this species, two phenols (15 and 17) were absent only in the RT population. The compound 4 (unknown) was observed in eleven populations but was absent in TA and RT. The compound 9 (unknown) was present in eleven populations, but not in RO and RT. Syringic acid (13) was present only in three populations (GC, RO, and PM), as was unknown compound 10 (FO, RO, and SL). Phloretic acid (21) was observed only in the six populations of *V. carnosa* located in southernmost Patagonia (PD, PM, RT, GA, LV, and SP; Table 3). Sinapic acid (18) and 3,4-dihydroxyphenylacetic acid (23) were present in specific populations, RT and GA, respectively.

In *V. clarionifolia* we identified seventeen phenolic compounds, one of which was observed in both analyzed populations but was not observed in *V. carnosa* (unknown 19). Eleven compounds were observed in both populations of *V. clarionifolia*: nine phenols (1, 2, 7, 12, 16, 17, 18, 22 and 24) and two unknowns (8 and 19; Table 3). In the RH population we observed fifteen compounds, among which, unknowns 3, 6, 10 and 11 were observed only in this population. In the ML population thirteen phenolic compounds were observed, among which unknown 14 and cinnamic acid (20) were absent in the RH population.

3.3. Statistical analyses

The Pearson correlation analysis between total phenolic content and antioxidant activity was found to be significant ($p < 0.001$), with a correlation coefficient of 0.77. Since only the LH population showed a high percentage of neutralized DPPH and low phenolic content (see Figure 2), we also performed a Pearson correlation analysis excluding this population, and the correlation was found to be significant ($p < 0.001$) with a higher correlation coefficient ($r = 0.90$; Figure 3). The results of the correlation analyses between total phenolic content and population latitude ($p = 0.73$, $r = 0.1$) and altitude ($p = 0.12$, $r = 0.42$) were not significant. The Kruskal–Wallis one-way analysis of variance test showed non-significant differences between the northern and southern population groups with respect to total phenol content ($p = 0.833$) and antioxidant activity ($p = 0.524$). For the comparison of low altitude and high altitude population groups, non-significant differences were observed (total phenol content $p = 0.051$, antioxidant activity $p = 0.071$).

Following the CA, the populations were grouped in clusters in terms of their nearness or similarity according to the presence or absence of phenolic and unknown compounds. We observed two main groups: cluster I (*V. clarionifolia* populations) and cluster II (*V. carnosa* populations; Figure 4). In cluster II we observed four subgroups: cluster III (RT population), cluster IV (RV population), cluster V (southern populations), and cluster VI (northern populations; Figure 4).

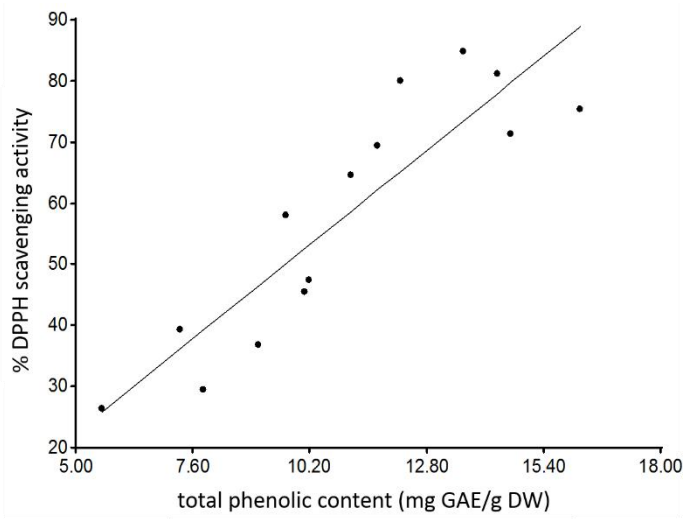

Figure 3. Correlation between total phenolic content and DPPH radical scavenging activity of the extracts, excluding LH population. Pearson's correlation coefficient r = 0.90, $p < 0.001$.

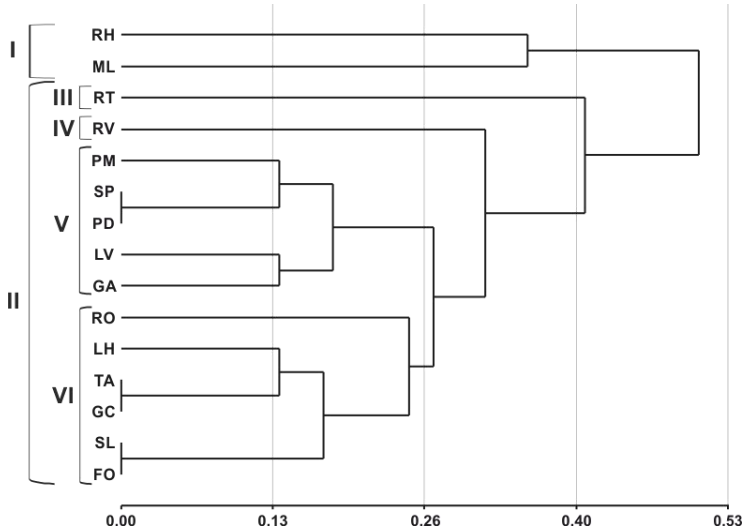

Figure 4. Dendogram resulting from CA obtained from the tentatively identified phenolic compound data of *V. carnosa* and *V. clarionifolia* populations, selecting the Sokal Sneath 1 as similarity measurement and the average linkage hierarchical agglomerative method. I, *V. clarionifolia*. II, *V. carnosa*. III, RT population. IV, RV population. V, southern populations. VI, northern populations.

4. Discussion

In this study we found differences in total phenol content, antioxidant activity, and profiles of phenolic and unknown compounds in root ethanolic extracts from different populations of *V. carnosa* and *V. clarionifolia*. In *V. carnosa* populations we observed a higher number of phenolic compounds and unknown compounds, higher total phenolic content, and higher antioxidant activity values than in *V. clarionifolia*. However, since in this study we analyzed only two populations of *V. clarionifolia*, the values obtained for this species should be corroborated by analyzing a greater number of populations.

In both species we observed a high positive correlation in the content of total phenols and antioxidant activity, suggesting that the predominant source of antioxidant activity could be the phenolic compounds. However, the LH population (*V. carnosa*) shows high antioxidant activity and relatively low total phenolic content. This could be due to the presence of other non-phenolic compounds with high antioxidant activity in the ethanolic extract, the presence of a greater amount of particular phenolic compounds, or a sample/assay problem. It is important to highlight that some interference compounds could affect the Folin-Ciocalteu method, leading to overestimation or underestimation of the results [48–50], but the present study did not detect any unique component(s) in the LC–MS profile. A positive correlation between total phenol content and antioxidant activity has previously been reported in two populations of *V. carnosa* from north Patagonia [19]. Moreover, a linear correlation between these variables was also observed in edible plants [51,52] and other medicinal plants [53–55].

Environmental factors affect the production of secondary metabolites in plants in different habitat conditions [56–58], and in this study, we observed quantitative and qualitative variation in phenolic and unknown compounds among the studied populations. Some authors suggest that this variability can be attributed to latitudinal variation [59] and the altitude at which the plant grows [60]. However, in *V. carnosa* we did not observe a correlation between total phenolic content and the altitude or latitude at which populations grow. Moreover, non-significant differences in total phenol content and antioxidant activity were found among populations in relation to latitude and altitude. Other causes of such variation in plants are attributed either to the microclimatic condition of the area [61] and the genotypes of populations/plants [62], among other factors.

Based on the results obtained we observed that the values for the total phenolic content of *V. carnosa* (5.6–16.2 mg GAE/g) were higher than those reported for root ethanolic extracts in other *V. carnosa* populations (3.6–11.7 mg GAE/g) from north Patagonia [19]. It should be noted that the values for total phenols observed in three populations of *V. carnosa* (LV, TA, and RO) were higher than those recorded in root ethanolic extracts of *V. officinalis* L. (14.2 mg GAE/g), a Eurasian valerian used worldwide for treating anxiety and mild sleep disorders, and *Nardostachys jatamansi* (Jones) DC (3.4 mg GAE/g), another medicinal plant belonging to the Valerianaceae family from India [29].

The tentative identification of phenolic compounds by HPLC-UV-MS showed that seven compounds were exclusive to *V. carnosa* and only one to *V. clarionifolia*. Based on the number of populations analyzed for each species, we can suggest that unknown 19 (RT = 43.17 min) is probably exclusive to *V. clarionifolia*, but more populations of this species should be studied to confirm the seven compounds that were observed only in *V. carnosa* (unknowns 4 and 9, phenols compounds 5, 13, 15, 21 and 23). At least seventeen phenolic and unknown compounds (of the twenty-four detected) were common to *V. carnosa* and *V. clarionifolia*; this similarity in chemical profiles is not surprising because these two species are phylogenetically closely related [63].

The presence of some phenolic compounds seems to be associated with specific geographical areas and/or taxa. For example, compound 21 (RT = 44.18 min, probably phloretic acid) is only present in the southernmost *V. carnosa* populations (PD, PM, RT, GA, LV, and SP) and was not registered in *V. clarionifolia*. Phloretic acid (3-(4'-hydroxyphenyl)propanoic acid) has a role as a plant metabolite [64–66]. Unknown 8 (RT = 13.75 min), possibly kaempferol-3-rutinoside, was also present in five of these six southern populations (not observed in RT). Unknown 10 (RT = 19.05 min) was observed only in three neighboring populations of *V. carnosa* (FO, RO, and SL), located between −44 ° and −47 ° latitude in the Andean region (see Figure 1 and Table 1). Some phenolic compounds were only present in specific populations; for example, sinapic acid (18) in RH and ML (*V. clarionifolia*) and only in RT (*V. carnosa*), and 3,4-dihydroxyphenylacetic acid (23) only in the GA population (*V. carnosa*). Sinapic acid is one of the most common hydroxycinnamic acids in plants, showing potent antioxidant activity [67,68]. The antioxidant activity of sinapic acid is comparable to that of caffeic acid [69], another compound observed in only four southern populations of *V. carnosa* (RO, RT, GA, and LV) and in both populations of *V. clarionifolia*. In phytomedicine the attributes of sinapic acid are anti-inflammatory [70], antimicrobial [71,72], anticancer [73], and anti-anxiety [74]. Ferulic acid (5), another hydroxycinnamic acid identified only in *V. carnosa*, with the exception of TA population, is used as a natural antioxidant in foods, beverages, and cosmetics [75] and has lower antioxidant capacity than sinapic acid [76]. Other compounds in some populations of *V. carnosa* did not show a defined geographical pattern, for example: syringic acid (13) observed in GC, RO, and PM, and caffeic acid (20) observed in RO, RT, GA, and LV, a compound with high antioxidant activity [77]. In *V. carnosa* we observed two main groups of populations according to the HPLC-UV-MS results after CA analysis. Populations from the northern and the southern regions of southern Patagonia were separated according to their phenolic and unknown compound profiles (Figure 4). It is interesting to note that syringic acid (13) and sinapic acid (18) did not occur together despite both having the 4-hydroxy-3,5-dimethoxyphenyl substitution pattern.

One of the most interesting groups of phenolic compounds observed in this study are four acyl-quinic acids which are a family of esters formed between quinic acid and 1−4 residues of certain trans-cinnamic acids, most commonly caffeic, ferulic, p-coumaric and sinapic acids [78,79], but it is now recognized that *cis*-isomers form in tissues exposed to UV irradiation [80–83].

5-Caffeoylquinic acid (5-CQA) (1) was observed in all populations of both species except RV (*V. carnosa*). An incompletely characterized dicaffeoylquinic acid (diCQA) (7), incompletely characterized diferuloylquinic acid (diFQA) (12) and an incompletely characterized methyl caffeoylquinate (MeCQ) (16) were found in all samples, and it is almost certain that other regio-isomers would be found in at least some samples if more sensitive analyses were performed. The methyl caffeoylquinate can be distinguished from an isobaric feruloylquinic acid (FQA) by its comparatively late elution [84,85]. Such methyl esters can be produced easily as artefacts when methanol is used in extraction, but ethanol was used in this study thus eliminating this possibility.

There is a growing body of evidence that these acyl-quinic acids and their human metabolites at commonplace dietary levels may have modest health promoting properties which never the less could be important long term through effects on vascular health, and glucose and lipid metabolism, although detailed mechanisms have yet to be elucidated [86,87].

Acyl-quinic acids and other phenolic compounds have been found in other *Valeriana* species, i.e. 3-CQA, 4-CQA, 5-CQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA in *V. officinalis* [41,42], 5-CQA in roots of

V. prionophylla Standl. from Guatemala [27], 5-CQA, gallic acid, caffeic acid and *p*-coumaric acid in roots and aerial parts of *V. jatamansi* Jones from India [31,41], 5-CQA, gallic acid and 3,4-dihydroxyphenylacetic in aerial parts of *V. dioscoridis* Sibth. et. Sm. from Turkey [44], and 5-CQA in roots of *V. alliarifolia* Adams from Turkey [43], and *V. procera* Kunth and *V. edulis* Nutt. Ex Torr. & A. Gray. from Mexico [41]. It is intended to more fully characterize and quantify the compounds tentatively identified in this study.

5. Conclusion

In heterogeneous macro-environments such as Patagonia it is possible to find plant ecotypes which have differentiated by natural selection. Phytochemical investigation into root ethanolic extracts of *V. carnosa* and *V. clarionifolia* from Patagonian populations have revealed variability in phenolic compound content (quantitatively and qualitatively) and antioxidant activity. In *V. carnosa* total phenolic content was not correlated with altitude and latitude and this variation could probably be associated with the influence of genetic variability and/or different growing habitats (microclimatic condition). In both species there is a positive correlation between antioxidant activity and total phenolic content, suggesting that the phenolic compounds are the predominant source of antioxidant activity. Despite the fact that the number and amount of total phenolic compounds is not correlated with latitude, the presence of some of these compounds (and unknown compounds) can be associated with latitude or a particular region/population. Among the species analyzed, *V. carnosa* showed a greater number of phenolic compounds and some of these populations (LV, TA, and RO) showed higher values for total phenolic content, these values being higher than reported in *V. officinalis*. Finally, in the roots of these two Patagonian valerians, we find 5-caffeoylquinic acid, caffeic acid, ferulic acid and gallic acid with a level 1 of certainty as described by Sumner *et al*. [35]; a dicaffeoylquinic acid, a diferuloylquinic acid, a methyl caffeoylquinate and eight other phenolic acids have been identified tentatively at level 2. Several of these are reported to have beneficial attributes from a phytomedical viewpoint.

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Conflict of interest

The authors declare no conflict of interest.

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