CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITY OF POPULATIONS OF VALERIANA CARNOSA SM (CAPRIFOLIACEAE) DISTRIBUTED IN THE SOUTH-CENTRAL ANDES

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ABSTRACT

Valeriana carsona SM (Caprifoliaceae), a native species to Chile, is distributed from the Maule Region to Tierra del Fuego. The rhizomes and roots have been used to treat different diseases as an infusion, mainly due to the wide spectrum of chemical compounds with biological concern. This research consisted in carrying out a chemical characterization and evaluation of the antioxidant activity of V. carsona in natural populations distributed in the central-southern part of Chile. The characterization of the extracts was carried out by GC-MS, showing that the rhizome extracts for both Laguna del Laja National Park and Malalcahuello National Reserve showed a higher percentage of sesquiterpenes, fatty acids and alkanes. On the other hand, leaf extracts for the Antuco site presented a higher abundance of alkanes and fatty acids and for Malalcahuello, alkanes and phytosterols. Significant differences were demonstrated in the rhizome extracts according to the IC50 values (157.6 and 154.8 μg/ml) at both sites, however, for the leaf extracts, Antuco presented a higher IC50 (450.33 μg/ml). ABTS, showed that Antuco had a higher activity for both extracts, and the same trend was demonstrated in the polyphenol content, evaluated by the Folin-Ciocalteu method. In conclusion, the distribution of the species and, indirectly, the site conditions are considered relevant in this research, showing a direct incidence on the biological activity and chemical profile of V. carsona.

Keywords: Antioxidant activity, polyphenols, geographical distribution, environmental conditions.

1. INTRODUCTION

Plants synthesize a diverse of secondary metabolites as a protective mechanism against oxidative compounds produced in response to stress conditions in the environment, being able to generate damage on membranes, organelles and macromolecules [1]. In general, secondary metabolism in plants can be seen as a mechanism of adaptation and survival to various stimuli during the growth and development of the species [2], and also plays an important role at the ecological level, participating in the defense against pathogens in general, attracting pollinators or conferring protection against various abiotic conditions of the external environment [3]. Much of the intraspecific variation of secondary metabolites in plants is mainly attributed to genetic, environmental, ontogenetic and phenological aspects [4]. Environmental variables (temperature, precipitation, radiation etc.) are among the predominant factors that generate significant quantitative and qualitative variations in the synthesis of secondary metabolites in plants [5], they can even cause the absence of the active compounds of the same species collected in different geographical regions and under completely different environmental conditions [6-7], such factors have also demonstrated their relevance on the specific accumulation in the different vegetative organs of plants [7].

The knowledge of the fluctuation of metabolites during physiological development, allows to a large extent to establish an optimal collection design, involving minimal disruption of the normal cycle of plants under natural growth conditions [8]. In this way it is possible to predict the dynamic behavior of the species with the objective of making a good use and adequate management of the species without affecting the populations and the community.

Currently, the study of secondary metabolites in plants has been widely used for practical purposes, mainly in the area of pharmacology and medicine [9]. They are commonly used for their antioxidant activity, as they are able to neutralize free radicals, preventing the process of oxidation and cell damage. Liu et al. [10] investigated the influence of geography on the chemical composition and antioxidant activity in leaf extracts of Cyclocarya paliurus. Their results showed that there is a significant variation in the chemical composition and antioxidant activity investigated in five geographical locations with different environmental variables, where the site called JZS presented the highest antioxidant activity, influenced by phenolic compounds. With such results, they conclude that considering geographic location as a variable is relevant for a better use of the species for industrial purposes. Han et al. [11] investigated the chemical composition and antioxidant activity in the different organs of Origanum vulgare, showing that flower and leaf oils presented the highest antioxidant activity with low IC50 values. From this research, we infer the importance of not only considering an organ of the species to be used for ethnomedical purposes, since it is demonstrated that although the IC50 values are higher in roots, they are also effective to be used for this purpose.

Valeriana carsona is a native species popularly known as Ñancolahuen. In Chile, V. carsona populations are widely distributed from the Libertador General Bernardo O’Higgins Region to Magallanes [12]. It corresponds to a perennial herb up to 80 cm tall that inhabits open places in forested areas, in the steppe and in the forest-steppe transition associated with an elevation of 0-2,500 m.a.s.l [13-14]. The root system of this species is commonly used in traditional Mapuche medicine to treat various diseases [15], mainly used for hepatic, respiratory, circulatory, urinary, digestive, analgesic, anti-inflammatory and other conditions [16-17]. Valerencic acids and valepotriates are estimated to be the active compounds to which such therapeutic properties are attributed [15]. Given its wide distribution, both in Chile and Argentina, it is shown that this species develops under different climatic conditions, thus presenting a great ecological amplitude. However, there is no evidence to report on the variation of chemical constituents and biological activity considering as variable the geographical distribution of V. carsona populations under natural growing conditions.

Against this background, we posed the following research questions: Does geographic distribution influence the chemical composition and biological activity in populations of V. carsona, and are there differences in the chemical profile and antioxidant activity among the organs of this species? Thus, the research aimed to qualitatively characterize the chemical composition and evaluate the antioxidant effect of V. carsona from the different vegetative organs (rhizomes and leaves) and to identify if this composition varies according to its geographic distribution.

2. EXPERIMENTAL

2.1 General information

Solvents such as methanol and hexane were used in analytical grade, were obtained from Merck (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), and acid gallic were purchased from Sigma-Aldrich. Potassium persulfate (K₂S₂O₈) was purchased from Merck (Darmstadt, Germany).

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2.2 Plant material and study sites

Populations of *V. carnosa* were collected in Laguna del Laja National Park (37°32’5.55”S 71°14’34.46”W, 1562 m.a.s.l) and Malalcahuello National Reserve (38°25’19”S 71°32’31”W, 1635 m.a.s.l); hereafter referred to as Antuco and Malalcahuello respectively. The plant material was cleaned and dried in the shade for 14 days.

2.3 Total extracts obtention of *V. carnosa*

Extractions were carried out using the protocol described by Sermukhamedova et al. [18], with modifications. The vegetative organs of *V. carnosa* (rhizomes and leaves) were crushed and used to carry out the maceration with a duration of 7 days under constant agitation. The extracts obtained were concentrated in a rotary evaporator at constant temperature and pressure and stored at 4°C for subsequent analysis. For the identification of compounds present in the extracts, hexane p.a. was used in the technique of gas chromatography coupled to mass spectrometry (GC-MS).

2.4 DPPH radical inhibitory activity

For the measurement of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibitory activity, the methodology described by Morales et al. [19] with modifications was used. Concentrations of the methanolic extracts of *V. carnosa* (leaves and rhizomes) were prepared in the range of 25 to 800 µg/ml from a stock concentration of 2000 µg/ml. 270 µl of the methanolic DPPH solution with the different concentrations of the extracts for the two populations were mixed independently (30 µl). The reaction was allowed to incubate for 60 min in the dark and the decrease in DPPH radical was measured at 515 nm using an ELX800 microplate reader (BioTek Instruments, Inc., Winooski, VT). The percentage decrease of the DPPH radical was determined by the following equation:

\[
\text{DPPH} \% = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Where *A* corresponds to the absorbance of sample and *A* corresponds to the absorbance of the sample. The results were expressed as IC<sub>50</sub> values calculated from a linear regression curve. All assays were performed in triplicates and Trolox (2,6-di-tert-butyl-4-methylphenol) was used as a standard control.

2.5 ABTS radical inhibitory activity

The inhibitory activity of the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid radical (ABTS) was determined using the methodology described by Re et al. [20]. The ABTS radical was obtained by mixing ABTS (7.35 mM) with potassium persulfate (1.25 mM). The aqueous mixture was allowed to stand at room temperature (25°C) for 16 hours in the dark for subsequent use. Initially, a stock concentration of each methanolic extract of *V. carnosa* of 2000 µg/ml was prepared, from which dilutions were prepared in the range of 25-800 µg/ml. Each concentration of the methanolic extract (20 µl) was seeded in microplates together with the aqueous solution containing the radical (180 µl). The reaction was allowed to incubate for 60 min in the dark and the decrease in ABTS radical was measured at 750 nm using ELX800 microplate reader (BioTek Instruments, Inc., Winooski, VT). The percentage decrease of the ABTS radical was determined by the following equation:

\[
\text{ABTS} \% = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Where, *A* corresponds to the absorbance of sample and *A* corresponds to the absorbance of the sample. The results were expressed as IC<sub>50</sub> values estimated from regression analysis. All concentrations were assayed in triplicate and trolox was used as the standard curve.

2.6 Determination of the total content of phenolic compounds.

According to the protocol described by Mongkolsilp et al. [21], the total polyphenol content of *V. carnosa* methanolic extracts was evaluated by the Folin-Ciocalteu colorimetric method. 10 µl of each extract (100 µg/ml) was homogenized with 20 µl of 1N Folin-Ciocalteu reagent and 200 µl of distilled water, the reaction was incubated for 5 minutes. After this time, 100 µl of 15% Na<sub>2</sub>CO<sub>3</sub> was added to neutralize the reaction and kept in the dark for 60 minutes. Finally, absorbance was measured at 750 nm using ELX800 microplate reader (BioTek Instruments, Inc., Winooski, VT).

The blank, on the other hand, contained all the reagents except for the methanolic extract of the different vegetative organs of the species under study. The assay was performed in triplicate and gallic acid was used as the standard curve. The concentration of total phenols was expressed as gallic acid equivalents (GAE) per 100 µg of dry extract.

2.7 Gas chromatographic analysis coupled to mass spectroscopy (GC-MS) for the extracts of *V. carnosa*

Analysis of the extracts by gas chromatography-mass spectrometry was based on the protocol previously described [22]. A gas chromatograph (Agilent 7890A) equipped with an agilent HP-5MS column (30 m × 0.25 mm × 0.25 µm) was used. The injector temperature was set to 250°C. The oven was programmed with an initial temperature program of 100°C for 3 minutes, then increasing at a rate of 10°C min<sup>-1</sup> until a constant temperature of 280°C was reached and maintained for 20 minutes, for a total time of 41 minutes of analysis for each sample. Helium was used as a carrier gas at a constant flow rate of 1.0 mL/min and an injection volume of 100 µl of each extract diluted in n-hexane p.a. of *V. carnosa* was used. The identification of the components of the different organs of *V. carnosa* was based on the comparison of their mass spectra with those stored in the NIST17 library.

2.8 Statistical analysis

The antioxidant activity assays and the determination of polyphenol content were performed in triplicate and the data recorded were expressed as the mean ± SD. An analysis of variance (ANOVA) was performed to determine the differences between study sites and organs with respect to the percentage of inhibition and polyphenol content by means of the aov function. The IC<sub>50</sub> values and polyphenol content were evaluated by means of a t-test in order to demonstrate differences between the two study sites (Antuco and Malalcahuello) and organs (leaves and rhizomes). The significance level was corrected with a p<0.05. Statistical analyses were performed using the Rstudio program.

3. RESULTS AND DISCUSSION

3.1 Antioxidant activity of the extracts of *V. carnosa* extracts for both sites.

Methods such as DPPH and ABTS are commonly used to determine the antioxidant activity of phenolic compounds by measuring the ability of plant extracts to neutralize reactive oxygen species by electron transfer [23-24]. The IC<sub>50</sub> values are inversely related to the antiradical activity of the compounds, that is, the lower the IC<sub>50</sub> value, the higher the antioxidant capacity of the extracts studied.

Analysis of variance (ANOVA) showed that site, organ, and concentration were significant categorical variables as a function of percent inhibition in both ABTS and DPPH methods (Table 1).

| Variables | gl | F    | p    |
|-----------|----|------|------|
| Concentration | 8 | 1462.828 | <0.001 |
| Site | 1 | 310.116 | <0.001 |
| Organ | 2 | 2756.251 | <0.001 |
| Concentration:Site:Organ | 16 | 6.601 | <0.001 |
| Concentration | 8 | 3622.17 | <0.001 |
| Site | 1 | 152.45 | <0.001 |
| Organ | 3 | 6742.90 | <0.001 |
| Concentration:Site:Organ | 24 | 13.41 | <0.001 |

The t-test showed that, for the ABTS method, the root extract from the Antuco site presented a significantly lower IC<sub>50</sub> value (126.25 ± 1.17 µg/ml) than the root extract for Malalcahuello (IC<sub>50</sub> of 213.76 ± 0.89 µg/ml).
Leaf extracts showed the same trend, being statistically significant for the Antuco site (Table 2, Figure 1). For the DPPH method, it was shown that the root extracts did not present significant differences in their IC50 value between both study sites. However, the leaf extract for the Malalcahuello site presented a lower IC50 value (154.68 ± 0.41 µg/ml) than Antuco (Table 2, Figure 1).

Table 2. IC50 values for methanolic extracts of V. carnosa at two study sites.

| Site         | Organ     | IC50 Leaf µg/ml | IC50 Rhizome µg/ml |
|--------------|-----------|-----------------|-------------------|
| DPPH Antuco  |           | 157.6 ± 0.89    | 450.33 ± 8.23     |
| Malalcahuello|           | 154.68 ± 0.41*  | 445.18 ± 5.34     |
| ABTS Antuco  |           | 157.48 ± 0.49*  | 126.25 ± 1.17*    |
| Malalcahuello|           | 233.46 ± 0.41   | 213.76 ± 0.89     |

The report by Bach et al. [14] shows that methanolic extracts of leaf (V. carnosa) evaluated at two phenological stages and for two study sites were significantly higher than root extracts at the final growth stage, only for Cerro Otto. Wojdyllo et al. [31] investigated the content of total polyphenols in 32 species of medicinal use in Poland. Among the species Valeriana officinalis, it was among the species with the highest content of polyphenols (11.1 mg GAE/100 g dw) over species that already had an important ethnomedical use. Bhattacharyya et al. [9] showed that the highest polyphenol content as in this study was led by the aboveground part (18.44 mg GAE/g dw), being significantly higher in the planted individuals than in the aerial parts of the wild (10.99 mg GAE/g dw). On the other hand, the polyphenol content in the aboveground parts was significantly higher than in the aerial parts in the wild source. The differences found in polyphenol content among the different vegetative organs tested may be associated with genetic and environmental factors, including, for example, geographic location, temperature, precipitation, soil characteristics, and interaction with pathogens [32]. Recently, Nagahama et al. [25] compared the phenol content in ethanolic extracts of V. carnosa and V. clarifolia, showing that, as well as the antioxidant activity, the highest polyphenol content corresponded to V. carnosa populations (5.6 to 16 mg GAE/g) over V. clarinifolia (7.3 to 9.7 mg GAE/g). In addition, they showed a positive correlation between total phenol content and antioxidant activity, suggesting that phenolic compounds are mainly responsible for antioxidant activity, influenced mainly by a microclimatic and genetic component. Although there are no findings that relate distribution to phytochemical aspects of the species, Zargooshi et al. [33] reported that the effect of site on polyphenol content in Scrophularia striata was significant, varying significantly in three regions of Ilam.

3.3 Chemical characterization from extracts of V. carnosa by means of GC-MS.

GC-MS analysis of V. carnosa rhizome extract at the Antuco site identified a total of 24 compounds, where the highest abundance was led by sesquiterpenes (29%), fatty acids (17%) and alkanes (17%); in lower percentages, triterpenes (4%), tetraterpenes (4%), aldehydes (4%) and others (Supporting Information, Table S1). For Malalcahuello, a total of 33 compounds were identified in the rhizome extract of V. carnosa, with the highest abundance of sesquiterpenes (39%), fatty acids (21%) and alkanes (21%); phytoestrogens (9%), iridoid monoterpenes (3%), triterpenes (3%) and others were found in lower percentages (Supporting Information, Table S1).

On the other hand, 24 compounds were identified in the methanolic leaf extract for Antuco, with the highest abundance of alkanes (38%), fatty acids (21%) and triterpenes (13%); however, diterpenes (8%), triterpenes (8%), ketones (4%), among others, were found in lower abundance in the sample (Supporting Information, Table S2). Finally, in the methanolic extract of leaves for Malalcahuello, a total of 18 compounds were identified, where the presence of alkanes (56%), phytoestrogens (11%) and triterpenes (11%) stood out in greater abundance; sesquiterpenes (6%), diterpenes (6%), triterpenes (6%) and others were found in lower abundance (Supporting Information, Table S2).

In general terms, it was shown that for rhizome extracts from both study sites the highest percentage was represented by the presence of sesquiterpenes (29-39%) and fatty acids (17-21%). About 49 sesquiterpenic compounds have been described for the genus Valeriana, and together with the iridoid-type compounds they are the predominant constituents in the species that make up the genus [34-35]. Similar to this research, Bach [14] characterized and quantified the essential oil of roots and rhizomes of V. carnosa and V. clarifolia in different natural study sites, showing a higher proportion of sesquiterpenes (32.8-49.1%) and a lower proportion of monoterpenes (16.7-29.7%).

![Figure 1. IC50 values for leaf and rhizome extracts of V. carnosa at two study sites.](image)
In natural populations of *Valeriana officinalis* growing in mountainous areas in Serbia, it was demonstrated by GC-MS that the compounds in higher concentration in the sample corresponded to oxygenated sesquiterpenes (62.5%) with valerianol as the main constituent [36].

Only in rhizomes from the Malalcahuello site were compounds of iridoid nature typical of the *Valeriana* genus identified, highlighting the presence of valeric acid and dihydrovaltrate (Figure 2). The results were filtered over 90% confidence to establish the identity of the compounds analyzed by gas-mass chromatography. According to Villar del Fresno and Carretero [37], the total content of these compounds can vary from 0.5 to 1.2% in *V. officinalis*, being considered as marker compounds for the genus [38–34]. In addition, the presence of the baldriinal compound (RT 13.864) was observed with an abundance percentage of 5.77 % only for Antuco. This compound corresponds to an unsaturated aldehyde, which is the degradation product of valeropropionic acid [39], so it is inferred that the absence of these compounds for the Antuco site may be associated with their instability to experimental processing conditions, such as humidity, temperature fluctuations or an acidic environment [37–40]. In addition, it is important to mention that the finding of these compounds typical of the genus only in one site could be directly related to its geographical location, microclimatic conditions of each site, time of harvest and also to the type of vegetation present in the sites [41].

Most of the research described for the genus *Valeriana* is focused on the use of the root system, however, there is little research focused on the aerial part (leaves). However, Javidi et al. [43] investigated the chemical constituents of the essential oil of the aerial parts of *Valeriana sisyribrifolia* in Iran, showing that most of the constituents analyzed were oxygenated sesquiterpenes (39.1%) and valeric acid derivatives (11.2%). For the same species (*V. sisyribrifolia*) Pirbalouti et al. [32] showed that the essential oil of the aerial parts contained high levels of low molecular weight compounds derived from phenol and valeric acid, which was investigated in four populations found in different geographical areas. Based on this, they estimated that the difference in the components of the essential oil in leaves is due to the geographical origin and chemotype of the populations studied. Trakou et al. [44] investigated the composition of the aerial part of *V. discoridis* and showed a higher percentage content of sesquiterpene hydrocarbons (25.3%). In contrast to previous findings, our results indicated that for both sites the highest percentage of compounds was attributed to alkane compounds, 56% for Malalcahuello and 38% for Antuco. It is known that the synthesis of these compounds participate as important components in epicuticular waxes in plants, conferring them a crucial ecological and functional role, since they allow the safe interaction with the stressful environmental conditions of the habitat [45]. Similarly, the presence of phytosterols was observed, compounds probably synthesized as a preventive mechanism to maintain stable membrane fluidity and consequently a good cellular functioning [46–47–48], since the climatic conditions that characterize the habitat of *V. carnos* correspond to an alpine type environment, where it usually faces low temperature conditions and high photon flux density, which lead to the constant formation of reactive oxygen species leading to partial or total damage of plant cells.

CONCLUSION

With the results of the present study, we believe that ecological factors, such as the geographic distribution of *V. carnos* populations may have a considerable effect on the biological activity and chemical profile of the *V. carnos* extracts studied, assuming also that the populations are influenced by the climatic conditions of each site of origin. Antioxidant activity showed that IC50 values for both sites and organs varied according to the method tested. Antuco showed the lowest IC50 values for ABTS for both organs. On the other hand, methanolic extracts of *V. carnos* leaves showed low IC50 values and a higher polyphenol content than rhizome extracts. Although these results are related to the exposure of these organs to the environment, we also consider it important to investigate the leaves as an important component to be used in traditional medicine.

According to the chemical profile by GC-MS it was demonstrated that the predominant compounds in the root extracts in both sites, corresponded to sesquiterpenes and fatty acids, however, for the leaf extracts alkane and fatty acid compounds predominated for Antuco and for Malalcahuello there was a predominance of alkanes and phytosterols. In addition, we highlight the presence of marker compounds of the genus *Valeriana*, where two of them were only identified in Malalcahuello (valeric acid and dihydrovaltrate). Finally, we propose that considering the geographical distribution together with the climatic conditions that define the natural populations of *V. carnos*, are important variables when investigating its therapeutic effects, since they allow us to predict the chemical behavior of the species under natural growth conditions.

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**Figure 2.** Chromatogram of the root extract (n-hexane) of *V. carnos* for Malalcahuello.
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