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Determination of antibacterial, antioxidant, antiplatelet and inhibition of cholinesterase activities from the methanolic extracts of *Azorella* species (Apiaceae)

[Determinación de la actividad antibacteriana, antioxidante, antiagregante plaquetaria e inhibición de la colinesterasa a partir de los extractos metanólicos de especies *Azorella* (Apiaceae)]

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Abstract
In this study, we investigated the potential antibacterial, antioxidant and anti-platelet activities and the inhibition of cholinesterase from the methanolic extracts obtained from aerial parts of the two species of *Azorella*: *A. spinosa* (Constitution, Chile) and *A. monantha* (Torres del Paine, Enladrillado and Paso Vergara). All extracts showed only moderate inhibitory activity on acetylcholinesterase (AChE), the most active extract with IC50 = 27µg/mL was *A. spinosa*. Inhibition of platelet aggregation induced by ADP presented maximal aggregation to 70 and 57% on extracts of *A. spinosa* and *A. monantha* (Paso Vergara), respectively. The most active extract with antioxidant effect was *A. spinosa* with IC_{50} of 28.72µg/mL. Antibacterial activity of the extract on *Escherichia coli*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* was not present. The extracts of *A. spinosa* and *A. monantha* (Paso Vergara) presented the best results on the activities that were evaluated.

Keywords: antibacterial, antioxidant, antiplatelet, cholinesterase, total phenolics, *Azorella*.

Resumen
En este estudio se investigó las potenciales actividades antibacteriana, antioxidante, antiplaquetaria e inhibición de la colinesterasa de extractos metanólicos a partir de las partes aéreas de dos especies de *Azorella: A. spinosa* (Constitución, Chile) *y A. monantha* (Torres del Paine, Enladrillado y Paso Vergara). Todos los extractos mostraron actividad inhibidora moderada solamente sobre acetilcolinesterasa (AChE), siendo el más activo el extracto de *A. spinosa* con IC_{50}= 27µg/mL. La inhibición de la agregación plaquetaria inducida por ADP presentó máxima agregación al 70 y 57% sobre los extractos de *A. spinosa y A. monantha* (Paso Vergara) respectivamente. El extracto más activo con efecto antioxidante fue el de *A. spinosa* con un IC_{50} de 28,72µg/mL,). No se presentó actividad antibacteriana de ningún extracto sobre *Escherichia* coli, *Pseudomonas aeuroginosa* y *Acinetobacter baumannii*. Los extractos de *A. spinosa* y *A. monantha* (Paso Vergara) presentan los mejores resultados sobre las actividades evaluadas, lo que permite el estudio bioguiado de los metabolitos presentes en estos extractos.

Palabras Clave: Antibacteriano, antioxidante, antiagregante, colinesterasa, fenoles totales, *Azorella*.

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INTRODUCTION

Azorella (Apiaceae) is a genus of 70 species of Central America, west temperate South America, and the Southern Ocean (Wachter *et al.*, 1998). The common name "llareta" is used for several species in the genera *Azorella* and *Laretia* (Apiaceae). These species are distributed from the high Andes Mountains, northeastern Chile. In folk medicine, infusions made of llareta are employed as stomach stimulants and the extracts obtained from vegetable oils are used for the treatment of wounds, asthma, colds and bronchitis, and illnesses with inflammation and pain as the main symptoms. These species are recognized for producing unique diterpenoid structures having the novel mulinane and azorellane skeletons with a wide variety of interesting biological activities, including 13β-hydroxy-azorellane (trichomonicidal) (Loyola *et al.*, 2001), azorellanone (spermicidal) (Morales *et al*., 2003), mulin-12,14-dien-11-on-20-oic acid (antibacterial) (Wachter *et al.*, 1999), azorellanol (antihyperglycemic) (Fuentes *et al.*, 2005), 13-epiazorellanol (antiinflamma-tory) (Borquez *et al.*, 2007), and 9,12-Cyclomulin-13-ol (antituberculosis) (Molina-Salinas *et al.*, 2010). There are some publications about the composition of isoflavonoids and biological activities alpinumisoflavone, licoisoflavone A, angustone C and isolupalbigenin (antibacterial and gastroprotective) (Quesada *et al.*, 2012) and triterpenoids lanosta-7, 24-dien-3β-yl acetate and 28-acetoxycycloartenyl acetate (acetylcholinesterase) (Areche *et al.*, 2009).

Several reports have described different biological activities, a study published by *A. compacta* (Rojo *et al.*, 2009) refers to the antioxidant activity of tea and they found a protective effect against oxidative damage in human erythrocytes and the Infusions of *A. compacta* (llareta) have been used by folk medicine in the highlands of Chile and antidiabetic (Fuentes *et al.*, 2005).

The purpose of this study was to evaluate the biological activity of methanol extracts from aerial parts of *A. spinosa* and *A. monantha*; therefore, the results of this study will be useful for carrying out a new study in the search for compounds that are responsible for positive activities in this research work.

MATERIALS AND METHODS

Plant Material

The plant aerial parts of a population of *A. spinosa*, near Constitución, three populations of *A. monantha*, one at Paso Vergara (Curicó) and two at Enladrillado (Talca, San Clemente), and lakefront Grey (Última Esperanza, Torres del Paine), were respectively collected in December 2010 and January 2011. Voucher specimens were deposited in the Herbarium of University of Talca, Chile with Herbarium Number 3360, 3361, 3362, 3363, respectively.

Preparation of the Extracts

The aereal parts of the *Azorella* were extracted with methanol and left for 15 days on percolation. The extracts were filtered, concentrated on and kept in darkness at 4°C until analysis.

Determination of Antibacterial Activity

The microorganisms used in the study were the Gram-positive bacteria *Staphylococcus aureus* and Gram-negative bacteria *Pseudomonas aeruginosa* and *Acinetobacter baumanni*, clinically isolated strains. Antibacterial assays were carried out by the doubling dilution method using the procedure reported by Gutierrez (Gutierrez *et al*., 2005), in 96-well microtiter plates. Bacterial suspensions were obtained from overnight cultures in luria broth base nutrient broth (Gibco BRL, Scotland) cultured at 25°C and diluted to approximately 10^5 colony-forming units (CFU)/well in fresh medium. The compounds were dissolved to give 5 mg/mL in methanol (MeOH) as a stock solution. Stock solutions of compounds were diluted to give serial 2-fold dilutions that were added to each medium resulting in concentrations ranging from 1.25 to 0.078125 mg/mL. The final concentration of MeOH in the assays did not exceed 2%. The plates were kept at 25 °C overnight (12 h). After incubation, 20 μL of 0.5 mg/mL aqueous 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO) was added in each well and re-incubated for 30 minutes to detect living bacteria. The absorbance was read in a universal microplate reader (Multiskan EX Thermo, Finland) at 405 nm. The results were transformed to the percentage of controls, and the median inhibitory concentration (IC_{50}) values were graphically obtained from the dose-response curves. Penicillin G (Sigma-Aldrich, St. Louis, MO) and streptomycin (Laboratorio Chile, Santiago, Chile) were used as standard antibacterials.

Determination of Total Phenolics

The total phenolic concentration was determined using the Folin-Ciocalteu reagent according to Singleton (Singleton *et al*., 1965). To 60 µL of each sample, 3 mL of 1/5 dilution of Folin-Ciocalteus reagent and 2 mL of Na_2CO_3 (8 %, w/v) were incubated at 40 °C for 20 min. the absorbance of all samples was measured at 760 nm using a UV-Vis spectrophotometer. Results were expressed as gallic acid equivalents (GAE). Every determination was performed in triplicate.

Antioxidant activity:

(2,2-diphenyl-1-picrylhydrazyl (DPPH) essay

The free radical scavenging activity of the extracts was measured by the decrease in absorbance (AD) of methanolic DPPH solution at 517 nm in the presence of the extract. Crude extracts were assayed at final concentration ranging from 200 to 25 μg/mL. A freshly prepared DPPH solution (20 mg/L) was used for the assays. Samples were dissolved in water and the methanolic solution of DPPH served as a control. A mixture of methanol and water (2:1) was used as blank. The degree of decoloration indicates the free radical scavenging efficiency of the substances. Catechin and quercetin were used as a free radical scavengers, reference table 3. The percentage of DPPH decoloration was calculated as: (1-[AD of sample/AD of control]) x100 (Schmeda-Hirschmann *et al.*, 2003). This formula eliminates the effect of the extract absorbance. Assays were carried out in triplicate.

Determination of AChE and BuChE inhibitory activities

The assay for measuring AChe and BuChE inhibitory activity was carried out according to Ellman (Ellman *et al*., 1961), and adapted to 96-well microtiter plates as described by Gutierrez (Gutierrez *et al*., 2005). In the 96-well plates 50 µL of the sample dissolved in phosphate buffer (8 mmol/L K_2HPO_4, 2.3 mmol/L NaH_2PO_4, 150 mmol/L NaCl, and 0.05% Tween 20 at pH 7.6) and 50 µL of the AChE/BuChE solution (0.25 unit/mL), from *Electroporus electricus* and equine serum were added respectively in the same phosphate buffer. The assay solutions, except substrate, were preincubated with the enzyme for 30 min at room temperature. After preincubation, the substrate was added. The solution substrate consists of Na_2HPO_4 (40 mmol/L), acetylthiocholine/butirilthiocholine (0.24 mmol/L) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (0.2 mmol/L, DTNB, Ellman´s reagent). The absorbance of the yellow anion product, due to the spontaneous hydrolysis of substrate, was measured at 405 nm for 5 min on a microtiter plate reader (Multiskan EX, Thermo, Finland). The AChE/BuChE inhibition was determined for each compound. The enzyme activity was calculated as a percentage compared to a control using only the buffer and enzyme solution. The compounds were assayed in the dilution interval of 625 - 78.125 µg/mL and the alkaloid galanthamine was used as the reference compound. Each assay was carried out in triplicate and each reaction was repeated at least three independent times. The IC_{50} values were calculated by means of regression analysis.

Both enzymes showed sigmoidal kinetics; therefore, the IC_{50} values were obtained by interpolation of dose-response curves considering a factor determined as: a = (% inhibition over 50% -% under 50% inhibition) / (Concentration inhibits over 50% - Concentration inhibited under 50%), and factor b given by: b = Inhibition under 50% - a x Concentration inhibited under 50%. Knowing these two factors IC_{50} = 50-b / ware calculated.

Platelet Aggregation Assay

Venous blood samples were taken from two volunteers (healthy university students), who previously signed an informed consent form, in 3.2% citrate tubes (9:1 v/v) by phlebotomy with vacuum tube system (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA). The protocol was authorized by the ethic committee of the Universidad de Talca in accordance with the Helsinki Declaration (approved by the 18th World Medical Assembly in Helsinki, Finland, in 1964).

Platelet aggregation was monitored by light transmission (turbidimetric method) by (Born and Cross., 1963) using a lumiagregometer (Chrono-Log, USA). Briefly, 480 µL of PRP in the reaction vessel were pre-incubated with 20 µL of extracts from *Azorella* (all at 500 µg/mL final reaction volume), negative control (0.9% saline) or positive control (acetylsalicylic acid 50 µM, Sigma Chemical Co., St. Louis, USA). After 5 minutes of incubation, 20 µL of agonist were added to initiate platelet aggregation, which was measured for 6 minutes. ADP (adenosine 5 '- diphosphate bis (cyclohexylammonium) salt, from bacterial source, Sigma Chemical Co., St. Louis, USA) was used as agonist. All measurements were performed in triplicate. The results of platelet aggregation were determined by the software

AGGRO/LINK (Chrono-Log, USA) as well as the percentage inhibition of the maximum platelet aggregation: 100-(% AgX * 100) /% AgC) (% AgX: percentage of aggregation of the component under study, % AgC: percentage of aggregation of control). The percentage of maximal platelet aggregation was expressed as mean±SD. To study platelet aggregation Student's t-test was used (samples v/s negative control) (SPSS 17.0; SPSS Inc., Chicago, Illinois, USA) and $p<0.05$ was considered as the significance limit.

RESULTS

Plant Material

Azorella plants were collected in different parts of Chile. A population of *A. spinosa* (Fig.1) was found at the roadside to Constitución (Pantanillos), Maule Region, in December 2010. It has cushion-shaped features and open branches. The plant material of *A. monantha*, a plant in cushion shape, comes from three different populations, which were found at the following areas: a) Torres del Paine, at the edge of Lake Grey near Puerto Natales in the South Chile (Fig.2) in December 2010; b) the Enladrillado mountains in Talca Andes (Altos de Lircay National Reserve) in January 2011 (Fig.3); and c) the Paso Vergara mountains in Curicó Andes in January 2011 (Fig. 4). The specimens were identified by the botanist Patricio Peñailillo from Universidad de Talca. After being dried, they were percolated in methanol during two weeks and the concentrated was filtered to obtain extracts. The quantity of plant material that was collected for each species is described in Table 1.

Table 1
Mass of extract of *Azorella*

Species	Mass (Kg)	Methanol Extract (g)
Azorella spinosa	8.12	180.60
Azorella monantha[1]	2.11	5.51
Azorella monantha[2]	3.34	41.10
Azorella monantha[3]	4.09	31.11

Location: [1]Paso Vergara, [2]Torres del Paine and [3] Enladrillado

Antibaterial activity of the extracts.

Antibacterial activity of the extracts prepared from *A. monantha* from Paso Vergara, *A. monantha* from Torres Del Paine, *A. monantha* from Enladrillado *and A. spinosa* were tested at 1250-78.125 µg/mL. All extracts showed $IC_{50} > 1250$ µg/mL.

These results show that the antibacterial activity reported for metabolites derived from *Azorella* species does not act by modifying the cellular metabolism, specifically it does not affect the activity of the succinate dehydrogenase enzyme, responsible for metabolizing MTT and transforming it into formazan.

Amount of Total Phenolics

The amount of total phenolics was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalents (GAE) using the standard curve equation: $y = 0.0784x + 0.0118$, $R^2 = 0.9989$. Concentrations used to achieve convergence were 0.6, 1.25, 2.5, 5 and 10 µg / mL. Where y is absorbance at 760 nm and x is total phenolic content in the different extracts of *Azorella* expressed in mg/g. Table 2 shows the contents of total phenols that were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent. The total phenol varied from 8.95 ± 0.02 to 3.05 ± 0.03 mg/g in the extracts. The maximum phenolic content was found in the *A. spinosa* extract (8.95 ± 0.02).

Figure 1
Habitat of *A.spinosa* (Pantanillo, Voucher 3360)

Figure 2
Habitat of *A.monantha* (Torres del Paine, Voucher 3363)

Fig 3
Habitat of *A.monantha* (Enladrillado, Voucher 3362)

Figure 4
Habitat of *A.monantha* (Paso Vergara, Voucher 3361)

Table 2
Total Phenolic Content in different extracts of *Azorella*

Sample	Concentration (µg/mL)	Mean±SD
Azorella spinosa	100	8.95±0.02
Azorella monantha[1]	100	3.79±0.001
Azorella monantha[2]	100	3.05±0.039
Azorella monantha[3]	100	4.86±0.002

Location: [1] Paso Vergara, [2] Torres del Paine and [3] Enladrillado

Antioxidant Activity

DPPH is a stable free radical in aqueous or methanol solution and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Kale *et al*., 2011). In order to evaluate antioxidant potency through free radical scavenging with the test samples, the change in the optical density of DPPH radicals is monitored. *Azorella* extracts showed a concentration dependent on antiradical activity by inhibiting the DPPH radical with an IC_{50} value of 28.72 to 152.18 µg/mL (Table 3).

A. spinosa showed the lowest activity against the DPPH stable radical, but the standard showed IC_{50} at 6.7 and 5.5 µg/mL respectively.

Table 3
Antioxidant activity of *Azorella* extracts

Species	200 (µg/mL)	100(µg/mL)	50(µg/mL)	25(µg/mL)	IC50(µg/mL)
A. spinosa	44.88 ± 0.7	78.17 ± 0.5	62.22 ± 0.7	47.22 ± 0.7	28.72 ±0.8
A. monantha[1]	82.18 ±0.5	73.19 ± 0.8	46.24 ± 0.4	22.53 ± 0.4	53.75 ±0.7
A. monantha[2]	64.15 ± 0.3	42.14 ± 0.9	17.08 ± 0.5	5.12 ± 0.3	107.15 ±0.5
A. monantha[3]	78.63 ±0.5	37.66 ± 0.5	23.44 ± 0.6	12.25 ±0.4	152.18 ±0.5

Location: [1] Paso Vergara, [2] Torres del Paine and [3] Enladrillado

AChE and BChE Inhibitory Activity.
The extracts were evaluated against cholinesterase enzyme in the range of 600 to 18.75 µg/mL. The IC_{50} value was calculated by means of regression analysis from three individual determinations. The most active extract was *A. spinosa* with IC_{50} of 27 µg/mL against AChE; however, no extract was active against BuChE, showing the species selectivity for the active site of the acethylcholinesterase enzyme. The values obtained are compared with the galanthamine alkaloid which was used as the reference compound (table 4)

Table 4
Inhibitory activity of *Azorella* species extracts against Cholinesterase enzyme, Values expressed as IC_{50} in µg/mL

Species	AChE (µg/mL)	BuChE (µg/mL)
A. spinosa	27 ± 0.7	> 600 ± 0.5
A. monantha[1]	32 ± 0.6	> 600 ± 0.5
A. monantha[2]	275 ± 0.5	> 600 ± 0.5
A. monantha[3]	160 ± 0.3	> 600 ± 0.5
Galanthamine	1.1 ± 0.5	10.6 ± 0.4

Location: [1] Paso Vergara, [2] Torres del Paine and [3] Enladrillado

Antiplatelet Activity
Table 5 shows the results from *A. spinosa*, *A. monantha* from Paso Vergara, *A. monantha* from Torres del Paine and *A. monantha* from Enladrillado extracts on maximum aggregation, slope, area under and lag time of platelet aggregation induced by the ADP agonist. Platelet aggregation results showed that the *A. monantha* from Paso Vergara and *A. spinosa* inhibit platelet aggregation induced by ADP, but in different percentages. The inhibition of platelet aggregation induced by ADP compared to negative control was in the following order: *A. spinosa* (21 ± 0.3%, $p < 0.05$) and *A monantha* (36 ± 0.2%, $p < 0.05$) (Figure 5).

DISCUSSION
We have found that the extracts showed low activity on bacteria at the concentrations tested; however, we cannot rule out this activity, it may be an antagonistic effect of the mixture of metabolites present in the extracts on the microorganisms used or it may act through another mechanism.

The literature reports metabolites isolated from *Azorella* with antibacterial activity over these and other strains (Areche *et al.*, 2010); however, we think that the mechanism of action presented by these may be independent from changes in energy metabolism, mainly by acting on specific receptors other than the ones assessed in this study. Other tests that include new receivers would help establish the antimicrobial potential of the extracts studied.

Table 5

Inhibition of platelet aggregation induced by ADP

Species	Maximum aggregation (%)	Slope	Area under	Lag time (s)
Azorella spinosa	70±0.3*	75±3*	330±12	28±0.1
Azorella monantha[1]	57±0.2*	45±2*	240±13*	24±0.1
Azorella monantha[2]	83±0.2	82±5	380±5	24±0.1
Azorella monantha[3]	87±0.1	86±2*	345±9	27±0.1
Negative control	89±0.2	94±5	389±15	27±0.1

Location: [1] Paso Vergara, [2] Torres del Paine and [3] Enladrillado
***p < 0.05, statistically significant.**

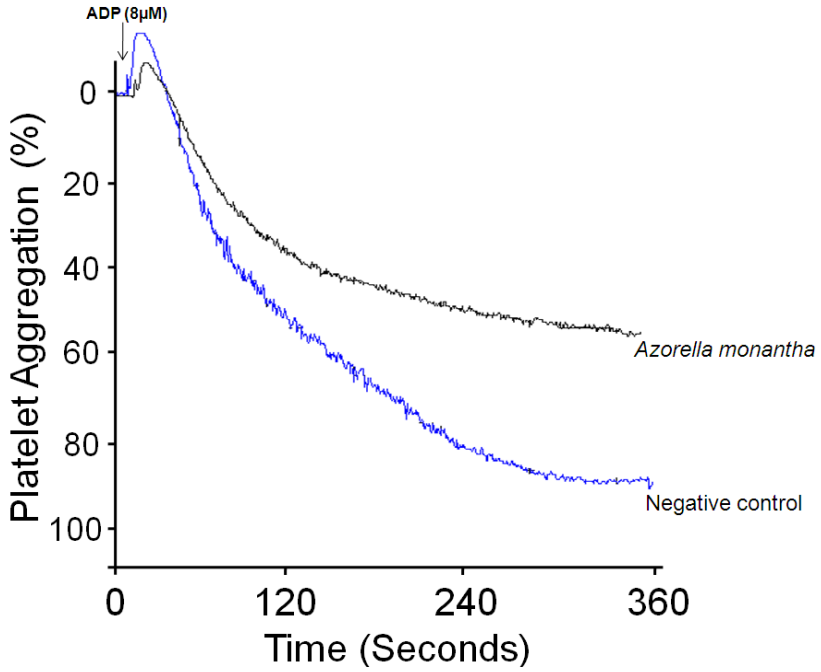

Figure 5
Effect of *Azorrella monantha* extract on platelet aggregation ADP-induced.

Natural antioxidants from plant extracts have attracted increasing interest due to consumer concern about the safety of the synthetic antioxidants in food. DPPH is a free radical, stable at room temperature, which produces a violet solution in methanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored methanol solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants. The antioxidant activity was concentration dependent, showing a greater ability to trap free radicals at high concentration, as shown in Table 3. The most active extract was *A. spinosa* with IC_{50} of 28.72 mg / mL; this may be related to the concentration of total phenols found in these extracts in accordance with the previous report (Velioglu *et al.*, 1998; Amin *et al.*, 2004). Several reports have

described different types of biological activity, a study published by *A. compacta* (Rojo *et al.*, 2009) refers to the antioxidant activity of tea and they found a protective effect against oxidative damage in human erythrocytes. However, no references to the antioxidant activity of its major metabolites have been reported to date.

Alzheimer´s disease (AD) is a late-onset neurodegenerative pathology that affects the memory, motor coordination, and cognition in a progressive and eventually lethal Manner (Whitehouse *et al*, 1982). It has been postulated that at least some of the cognitive impairment experienced by AD patients results from a deficit in acetylcholine levels and consequent reduction in cholinergic neurotransmission. Consequently, the key approach employed in the development of drugs for using in the symptomatic treatment of AD has targeted the cholinergic deficit. The screening of numerous plant species that were typically selected based on their ethnobotanical data or the report of their popular uses has been carried out in order to discover anticholinesterasic compounds with novel structural entities (Ma X and Gang DR, 2008; Yan T *et al.*, 2012, Rollinger *et al.*, 2006). A previous study in search of active compounds on AChE was conducted with *A. trifurcata* (Areche *et al*., 2009) where some isolated triterpenes with inhibitory activity against AChE enzymbioge were found, these results may be related to the assessed activity of *Azorella* species extracts because more assets could contain this type of metabolites.

Antiplatelet activity has been associated with a decrease in the prevalence of cardiovascular disease described by (O'Kennedy *et al*., 2006), note that the *A. monantha* (Paso Vergara) extract had a potent inhibitory effect on platelet aggregation. The possible action mechanism may correspond to inhibition of ADP receptor: P2Y1 and P2Y12. Hence, it is possible to establish that different extracts present more than one compound with platelet antiaggregant activity, with variations in their chemical structures, and therefore with a different degree of platelet aggregation inhibition. Finally, the inhibition of platelet aggregation may be specified for certain types of *Azorella* species.

CONCLUSION

The FC method for the determination of phenolic compounds is, such as the methods for antioxidant activity determination, based on redox properties of the compounds; thus, the values could partially express the antioxidant activity. This confirms a highly significant correlation between the values of FC method and the values of individual methods for antioxidant activity. The total phenolic content presented in the *A. spinosa* based extract increased the antioxidant activity.

The present study confirms the antiplatelet activity of methanolic extract of *A. monantha* (Paso Vergara) and the activity AChE in the extracts of all evaluated *Azorella.*

The biological activity of the different *Azorella* extracts that were assessed is modified by the species from which it comes, geographic location and date of collection, which implies a change in the content and type of metabolites generated by the species.

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