

Azorella compacta Infusion Activates Human Immune Cells and Scavenges Free Radicals *In vitro*

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ABSTRACT

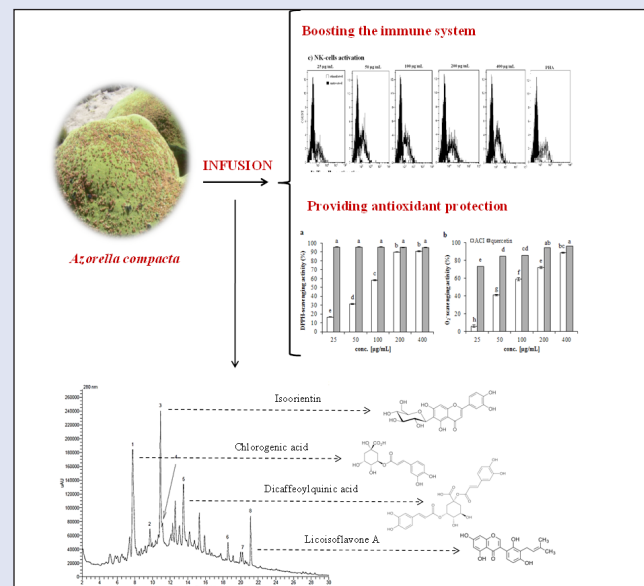
Background: *Azorella compacta* is traditionally used in the form of tea (infusion), in the Andean region of South America, to treat various chronic diseases. However, the health-promoting properties of this herbal tea have not yet been extensively explored. **Materials and Methods:** The free radical scavenging activity of *A. compacta* infusion (ACI) was evaluated by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical and superoxide anion radical assays. The activation of immune cells by ACI, as determined by cell surface cluster of differentiation 69 expression, was measured by flow cytometry. The qualitative polyphenolic composition of ACI was investigated by HPLC/PDA/ESI-MS, (High-performance liquid chromatography coupled with photodiode array detection and electrospray ionization - mass spectrometry) and the total content of polyphenols was estimated by spectrophotometric methods. **Results:** Eight polyphenols including chlorogenic acid, 6,8-di-C-hexosyl apigenin, isoorientin, orientin, dicaffeoylquinic acid, biochanin A-O-glucoside, biochanin A-O-(malonyl)-glucoside, and licoisoflavone A were tentatively identified in ACI. The total contents of phenols, flavonoids, and tannins in lyophilized ACI were 5.40 mg/100 mg ACI, 1.79 mg/100 mg ACI, and 1.76 mg/100 mg ACI, respectively. ACI, within the range of 25-400 µg/mL, scavenged DPPH and O₂⁻ by 15-90% and 20-88%, respectively. The human natural killer (NK) cells were substantially activated by ACI, whereas T cells and granulocytes were slightly stimulated. **Conclusion:** Overall, the results demonstrate the free radical scavenging and immune-stimulating properties of ACI, and support, at least in part, its potential utilization as a functional herbal tea for preventing chronic diseases and as a nonspecific immune stimulator during human immunosenescence.

Key words: *Azorella compacta*, immune cells, CD69 expression, phenolic, free radicals scavenging activity

SUMMARY

- The total contents of phenols, flavonoids, and tannins in *Azorella compacta* infusion (ACI) were 5.40 mg/100 mg ACI, 1.79 mg/100 mg ACI, and 1.76 mg/100 mg ACI, respectively.
- Eight polyphenols including chlorogenic acid, 6,8-di-C-hexosyl apigenin, isoorientin, orientin, dicaffeoylquinic acid, biochanin A-O-glucoside, biochanin A-O-(malonyl)-glucoside, and licoisoflavone A were tentatively identified in ACI by HPLC/PDA/ESI-MS.
- ACI, within the range of 25-400 µg/ml, scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) and O₂⁻ by 15-90% and 20-88%, respectively.

- The human natural killer (NK) cells were substantially activated by ACI, whereas T cells and granulocytes were slightly stimulated.



Abbreviations used: ESI: electrospray ionization, HPLC: high performance liquid chromatography, PDA: photodiode array detector, MS: mass spectrometry, MS/MS: tandem mass spectrometry, MW: molecular weight, m/z: mass-to-charge ratio, FITC: fluorescent isothiocyanate, PE: phycoerythrin.

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INTRODUCTION

Azorella compacta, a yellow-green resinous cushion shrub grown in the Andean region of South America, is traditionally used in the form of herbal tea (aqueous infusion) by the indigenous population. *A. compacta* infusion (ACI) is traditionally used as gastric stimuli, diuretics, analgesics in case of cold, and in the treatment of diabetes, migraine, neuralgia, pneumonia, and rheumatism.^[1] Previous chemical investigations of the genera *Azorella* have been focused mainly on its nonaqueous extracts,

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which have been shown to accumulate diterpenes.^[2] There is growing understanding that reactive oxygen species (ROS) such as superoxide radical, hydrogen peroxide, singlet oxygen, and hydroxyl radical are implicated in the cause or progression of several human diseases, including diabetes, atherosclerosis, chronic inflammation, viral infection, myocardial infarction, and ischemia-reperfusion injury.^[3] Since ACI is traditionally used for the treatment of some of these conditions, the investigation of its free radical-scavenging potential and chemical profile is warranted. Concomitantly, given that traditional medicine uses ACI to treat symptoms that are commonly present in viral infections; the potential immune-stimulating properties of this herbal tea deserved to be validated. The present study aimed to evaluate the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide radical-scavenging activities of ACI. Moreover, we investigated its effects on natural killer (NK) cells, T cells, and granulocytes activation. The chemical profile of the ACI was determined by HPLC/PDA/ESI-MS and spectrophotometric methods.

MATERIALS AND METHODS

Plant collection and extract preparation

Plants were collected at Cerro el Potro (3500 m above sea level in Copiapo, III Region, Chile) and the botanical identity of the plant specimen was confirmed as *A. compacta* by Dr. Garcia O. (Biology Department, University of Chile), voucher specimen (no. 140111). Plants were dried in an oven at 40°C and ground to fine powders. For preparing the infusion, 3.0 g of powdered plant was added to 300 ml of distilled water (95-100°C), allowed to stand for 20 minutes, and then filtered through a Whatman No. 1 filter paper. The resulting infusion was lyophilized and the extraction yield was calculated on the basis of weight of the used dried plant. Lyophilized ACI was assessed for its biological activities, and its chemical profile was determined.

Determination of total content of polyphenols and free radical-scavenging activity

The total content of phenols, tannins, and flavonoids was determined as previously described.^[4] The free radical-scavenging capacity of the sample against DPPH free radical and superoxide anion radical ($O_2^{\cdot-}$) was spectrophotometrically evaluated according to a previously reported procedure.^[4]

Analysis of immune cells

The activation of immune cells was analyzed by flow cytometry, as measured by cell surface cluster of differentiation 69 (CD69) expression.^[5] Peripheral blood was drawn from three healthy volunteers into sodium heparin. Samples of ACI were dissolved in *ex-vivo* medium (Bio-Wittaker, USA) at different concentrations and then filtered

through 0.2- μ m filters, before use. Phytohemagglutinin (PHA) (Sigma-Aldrich, Steinheim, Germany), a nonspecific activator of lymphocytes, was used as a positive control at a concentration of 10 μ g/ml. Some 100 μ L of blood suspension was incubated with 100 μ L of test samples into a sterile 96-well flat-bottomed plate at 37°C with 5% CO_2 for 24 h. The final concentrations of ACI in the assay media were in the range of 25-400 μ g/ml. After incubation, 40 μ L of each suspension was labeled with a cocktail of fluorescently-labeled monoclonal antibodies (CD69 PE, CD56 FITC, and CD3 APC) (Immunotech, France and Dako, Denmark). The PE-labeled antibody specific for CD69 was used to detect activated immune cells. The FITC-labeled antibody to CD56 was specifically used to identify NK cells, whereas antibody to CD3 is used to detect T cells. Because of the lack of a monoclonal antibody specific to granulocytes, these cells were gated on the basis of their characteristic forward scatter (FSC) and side scatter (SSC) profiles, which represent size and granularity, respectively. The effect of ACI on the percentage of activated NK cells and T cells at 24 h is presented as dot plots. The CD69 expressions on activated NK and T cells (identified by gating on CD69/CD56 or CD3), and on activated granulocytes (identified by gating based on FSC/SSC) are shown as mean fluorescent intensity (MFI) values. The activation index (AI), which presents the values of activation, was calculated by dividing the MFI of cells treated with test samples by that of control. Values higher as $AI \geq 2$ were defined as a positive immune cell response. Analyses were performed on Cytomics FC500 flow cytometer (Beckman Coulter, USA) and data were analyzed by CXP analysis software (Coulter Electronic, USA). Fluorescence signals from 10,000 events were obtained and presented as logarithmically amplified signals.

HPLC/PDA/ESI-MS analysis of *Azorella compacta* infusion

Analyses were performed on a Thermo Finnigan HPLC/MS system (San Jose, CA, USA) consisting of Surveyor MS pump, Surveyor autosampler, Surveyor PDA, and LCQ Advantage ion trap mass spectrometer as a detector. For separations, a Titan C18 column (100 \times 2.1 mm, 1.9 μ m; Supelco Analytical, Bellefonte, PA, USA) fitted with guard column Titan C18 (5 \times 2.1 mm, 1.9 μ m; Supelco Analytical, Bellefonte, PA, USA) was used. The column temperature was set to 40°C. The mobile phase consisted of a combination of A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) at a flow rate of 180 μ L/min with the following gradient elution: 0 min: 5% B; 45 min: 80% B; 45.1 min: 95% B; 52.9 min: 95% B; 53 min: 5% B; 63 min: 5% B. A volume of 10 μ L of the sample was injected for each analysis. The UV chromatograms were

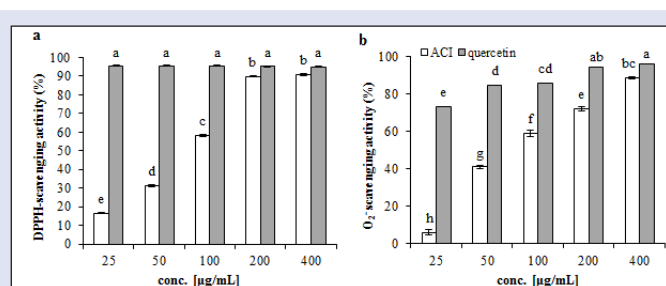


Figure 1: DPPH radical (a) and superoxide radical (b) scavenging activity of *Azorella compacta* infusion (ACI). Data are means \pm SD. Values within columns showing the same letters are not significantly different (Tukey's test, $P < 0.05$).

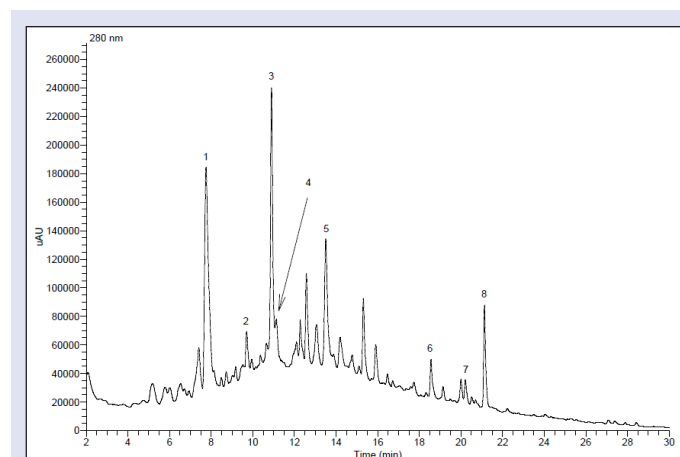


Figure 2: HPLC-UV chromatogram at 280 nm of *Azorella compacta* infusion (ACI).

Table 1: HPLC/PDA/ESI-MS mass measurements of compounds identified in *Azorella compacta* infusion (ACI)

Peak	t_R (min)	UV max	MW	Experimental m/z values		Important product ions of [M+H] ⁺ , m/z , (relative intensity)	Important product ions of [M-H] ⁻ , m/z , (relative intensity)	Identification	Ref.
				[M+H] ⁺	[M-H] ⁻				
1	7,81	286, 321	354	355	353	145(3), 163(100), 193 (3)	135(5), 173(37), 179(24), 191(100)	Chlorogenic acid	[14–16]
2	9,79	269, 315sh	594	595	593	457(56), 475(18), 481(13), 499(9), 511(30), 523(11), 528(25), 541(24), 559(32), 577(100)	353(63), 383(29), 413(5) 455(5), 473(100), 503 (35), 515 (7), 533 (5), 575(12)	6,8-di-C- symmetricglycosyl flavon	[17]
3	11,02	266, 337	448	449	447	329(27), 353(56), 383(100), 395(31), 413(37), 431(72)	327(81), 357(100), 369(4), 387(3), 411(3), 429(25)	Isoorientin	[18,19]
4	11,27	265, 340	448	449	447	329(29), 353(14), 383(48), 395(19), 413(53), 431(100)	327(100), 357(46), 369(5), 393(3), 411(1), 429(3)	Orientin	[20]
5	13,63	295, 325	516	517	515	n.d.	173(8), 179(18), 203(10), 255(4), 299(9), 335(8), 353(100)	Dicaffeoylquinic acid	[14,15]
6	18,56	255, 320sh	446	447	491*	285(100)	283(100), 445(16)	Biochanin A –O-glucoside	[18]
7	20,21	<250**, 320sh	532	533	n.d.	285(100), 447(9)	n.d.	Biochanin A –O-(malonyl)- glucoside	[18]
8	21,27	282, 335	354	355	353	319(8), 337(100)	309(100)	Licoisoflavone A	[8]

*formic acid adduct; ** exact measurement was not possible due to mobile phase background; n.d.: not detected

Table 2: The cell activation shown as activation index (AI) by *Azorella compacta* infusion (ACI)

Immune cells	Activation Index (AI)					
	ACI concentration ($\mu\text{g mL}^{-1}$)					PHA
	25	50	100	200	400	
NK cells	20.33±12.97 ^a	29.45±13.57 ^a	33.04±10.84 ^a	31.54±13.61 ^a	34.79±8.59 ^a	43.09±3.03 ^a
T-cells	1.53±1.02 ^a	2.12±0.64 ^a	2.57±1.11 ^a	2.56±1.65 ^a	2.53±1.01 ^a	43.09±3.03 ^b
Granulocytes	1.58±0.36 ^a	2.56±0.34 ^a	4.03±1.47 ^a	3.71±0.66 ^a	5.87±3.03 ^a	48.92±14.20 ^b

The AI values were calculated by dividing the mean fluorescent intensity (MFI) of ACI-treated cells by that of untreated cells (control). A positive immune cell response was defined as an AI ≥ 2 . Values are presented as means \pm SD of three determinations. Means in each row with the same letter superscript are not significantly different ($P < 0.05$). NK: natural killer; PHA: phytohemagglutinin.

monitored at 260, 280, and 330 nm for peak detection. Ultraviolet (UV) spectra from 210 to 800 nm (scan bandwidth 1 nm) were also recorded for contributing to a better peak characterization. Mass spectrometer equipped with ESI ion source was set to monitor spectra in the range of 100–1000 m/z . The measurements in positive and negative ion mode were carried out separately. The mass spectrometer parameters were tuned up using a solution of two compounds expected in samples, daidzein and genistein (10 $\mu\text{g/mL}$ solutions in mobile phase). Final optimized parameters were as follows: capillary temperature ($^{\circ}\text{C}$): 250; gas flow (arbitrary units): sheath 18, auxiliary 38; source voltage (kV): 5.30 (4.30 in negative ion mode); capillary voltage (V): 12.00 (–10.00 in negative ion mode). Nitrogen was used as both sheath and auxiliary gas, and helium was used as the damping gas. For MS/MS analysis, collision energy was set to 40%. Mass range varied for each MS/MS analysis. Xcalibur software (version 1.4 SR1 SUR3) was used to control the HPLC/MS system and to process data.

Statistical analysis

Significant differences between values were determined by a one-way analysis of variance (ANOVA). For determining pair-wise differences of means, the Tukey's test was performed ($P < 0.05$). Data are presented as the means \pm standard deviation (SD) of the three experiments.

RESULTS AND DISCUSSION

The total extraction yield of ACI was 13.1%. The total phenolic content in lyophilized ACI was 5.40 mg gallic acid equivalents (GAEs)/100 mg ACI. An earlier study with *A. compacta* infusion reported 0.028 mg GAEs/100 mg dry weight.^[6] Studies with other *Azorella* species informed total phenolic content ranged from 3.71 to 19.88 mg GAEs/100 mg dry

weight.^[7] The total content of tannins and flavonoids in lyophilized ACI were 1.76 mg GAEs/100 mg ACI and 1.79 mg quercetin equivalents/100 mg ACI, respectively. A similar total content of flavonoids was found in *Azorella madreporica*.^[8] The total content of tannins in *A. compacta* is informed for the first time in the present study. ACI, within the concentration range from 25 to 400 $\mu\text{g/mL}$, scavenged DPPH [Figure 1a] and O_2^- [Figure 1b] by 15–90% and 20–88%, respectively. The superoxide anion radical is one of the most common ROS formed *in vivo*, and it is known to be deleterious to cellular components and consequently may contribute to tissue damage and progression of diseases.^[9] The O_2^- -scavenging capacity of ACI might contribute significantly to its health-promoting properties. CD69 is a human transmembrane glycoprotein that is expressed on the surface of activated immune cells upon stimulation. Once CD69 is expressed on T cells, it co-stimulates T-cell activation and proliferation. CD69 is also inducibly expressed by B cells, NK cells, monocytes, neutrophils, and eosinophils, whereas it is constitutively expressed on mature thymocytes and platelets.^[10] The CD69 expression, as an indicator of immune cell activation, has been used to ascertain the immune-stimulating properties of medicinal herbs using *in-vitro*^[4,11–13] and *in-vivo* studies.^[5] As seen in Figure 3a, ACI led to a substantial increase in the number of activated NK cells, but only afforded a slight increase in the number of activated T cells [Figure 3b] at 24 h. The CD69 expression on activated NK cells, T cells, and granulocytes in response to ACI treatment was shown as a shift to the right in the histograms [Figure 3c–Figure 3e]. ACI at the range of 25–400 $\mu\text{g/mL}$ substantially stimulated the CD69 expression on NK [Figure 3c], whereas T cells [Figure 3d] and granulocytes [Figure 3e] were activated on a lower scale within the concentration range from 25 to 400 $\mu\text{g/mL}$. As shown in Table 2, the AI on the tested immune cells confirmed these observations. As judged by the results of the present study, ACI might be a potential

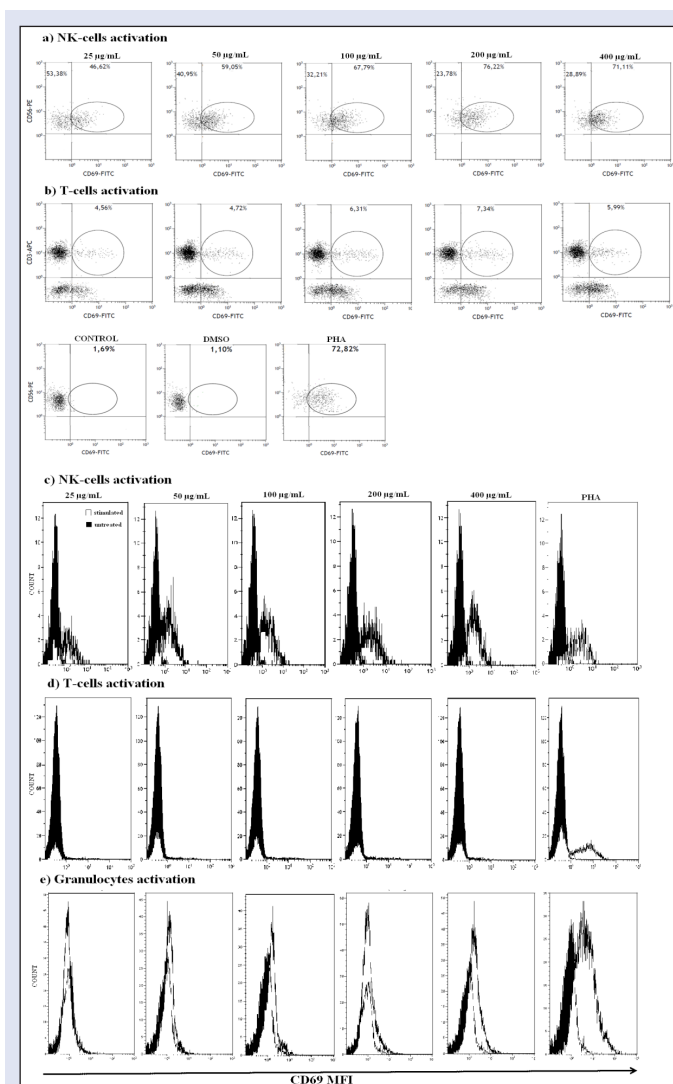


Figure 3: Percentage of activated NK cells (a) and T cells (b) in human peripheral blood in response to treatment with *Azorella compacta* infusion (ACI) for 24 h is shown on dot plots. Cells that stain both for CD69 and a cell marker appear in the upper right quadrant of a dot plot. Numbers in each quadrant are the percent of cells found in each quadrant. Mean fluorescent intensity (MFI) of CD69 expression on activated NK cells (c), T cells (d), and granulocytes (e) from human peripheral blood in response to treatment with ACI for 24 h is shown as histograms. The filled histograms (black color) represent the group control (untreated) and the open histograms the stimulated (pretreated) group. The mitogen, phytohemagglutinin (PHA), was used as a positive control. The histograms are representative of three separate experiments using cells from three different donors.

alternative to prevent viral and bacterial infections, and to face age-associated immune deficiency. The identities of the eight most abundant compounds in ACI [Figure 2] were determined on the basis of their UV and MS spectra after comparison with data reported in literature. The MS data and literature references^[14-20] are presented in Table 1. A description on the HPLC/PDA/ESI-MS characterization of the ACI polyphenolic profile, and also figures of MS/MS spectra corresponding to the molecular ions of compounds 1-8 are given in Appendix. The compounds 1 and 3, the major constituents of ACI, have been shown to exhibit free radical scavenging, and anti-inflammatory and antidiabetic properties.^[21,22] Therefore, it is

conceivable that the claimed benefits of this herbal tea might be attributed, at least in part, to these compounds.

CONCLUSIONS

In this study, the health-promoting properties of ACI were determined by evaluating its free radical scavenging and immune-stimulating effects. The total contents of polyphenols were quantified and individual phenolic acids and flavonoids were tentatively identified in ACI. Chlorogenic acid and iso-orientin, two powerful antioxidants, were identified as the major polyphenols in ACI. The infusion showed a potent scavenging effect against DPPH free radical and superoxide anion radical, which can be attributed to the presence of chlorogenic acid and iso-orientin. The *ex-vivo* activation of human immune cells by ACI, as determined by cell surface CD69 expression, was reported for the first time. ACI was shown to substantially stimulate NK cells, whereas T cells and granulocytes were activated on a lower scale. All together, these data support the traditional use of ACI to treat and prevent diseases in which free radicals are implicated, and suggest that this functional herbal tea could be used as a potential nonspecific immune stimulator.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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