Antimicrobial and Antioxidant Activities of *Gentianella multicaulis* Collected on the Andean Slopes of San Juan Province, Argentina

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The infusion of the aerial parts of *Gentianella multicaulis* (Gillies ex Griseb.) Fabris (Gentianaceae), locally known as 'nencia', is used in San Juan Province, Argentina, as stomachic and as a bitter tonic against digestive and liver problems. The bioassay-guided isolation of *G. multicaulis* extracts and structural elucidation of the main compounds responsible for the antifungal and free radical scavenging activities were performed. The extracts had strong free radical scavenging effects in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (45–93% at 10 μg/mL) and ferric-reducing antioxidant power (FRAP) assay at 200 μg/mL. Demethylbellidifolin (**4**) had high antioxidant activity in the DPPH and FRAP assay. The dermatophytes *Microsporum gypseum*, *Trichophyton mentagrophytes*, and *T. rubrum* were moderately inhibited by the different extracts (MIC values of 125–250 μg/mL). Demethylbellidifolin (**4**), bellidifolin (**5**), and isobellidifolin (**6**) showed an antifungal effect (MIC values of 50 μg/mL), while swerchirin (**3**) was less active with a MIC value of 100 μg/mL. In addition, oleanolic acid (**1**) and ursolic acid (**2**) were also isolated. These findings demonstrate that *Gentianella multicaulis* collected in the mountains of the Province of San Juan, Argentina, is an important source of compounds with antifungal and antioxidant activities.

Key words: *Gentianella multicaulis*, *Trichophyton* spp., Antioxidant Activity, Antifungal Activity

Introduction

The continuing increase in the incidence of fungal infections together with the gradual rise in resistance mainly to azoles in the last two decades highlight the need to search for novel compounds not tested previously in antifungal assays. Fungal infections pose a continuous and serious threat to human health and life especially to immunocompromised patients (Pfaller and Diekema, 2007). In this respect, plants provide unlimited opportunities for the isolation of new antifungal compounds because of their high chemical diversity (Cos *et al*., 2006; Maregesi *et al*., 2008). On the other hand, natural products with antioxidant activity are used to strengthen the endogenous protective system. This fact has led to increasing interest in the antioxidative role of nutraceutic products, and nowadays antioxidants in human diet have a leading role as possible protective agents to help the human body to reduce oxidative damage.

The Province of San Juan in Argentina has an important tradition in folk medicine. Its flora comprises a large number of species distributed in different ecosystems which have particular edaphic and climatic conditions. Plants of the high

Andes mountains have been used as medicines since pre-hispanic times and are still being used for their renowned therapeutic properties.

Gentianella multicaulis (Gillies ex Griseb.) Fabris (Gentianaceae) is a small herb widely distributed in northwestern and central Argentina and growing at an altitude between 1500 and 2700 m above sea level in the Andes region. The infusion of the aerial parts of *G. multicaulis*, locally known as 'nencia', is used in San Juan Province, Argentina, as stomachic and as a bitter tonic to treat digestive and liver problems (Bustos *et al.*, 1996).

No previous reports were found in the literature concerning antimicrobial and antioxidant activities of *G. multicaulis*. Herein, we report the bioassay-guided isolation and structural elucidation of the main compounds responsible for the antifungal and free radical scavenging activities.

Material and Methods

General reagents

All solvents used were of analytical grade. Chloroform was purchased from Fisher Scientific (Pittsburgh, PA, USA) and methanol was obtained from J. T. Baker (Phillipsburg, NJ, USA). Acetonitrile from Caledon Lab. Ltd. (Georgetown, Canada) and formic acid from Merck (Darmstadt, Germany) were used. Potassium ferricyanide, trichloroacetic acid (TCA), $FeCl_3$, and standards of gallic acid and quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TLC analysis was carried out on aluminum coated silica gel F_{254} plates from Merck. Folin-Ciocalteu phenol reagent, aluminium chloride hexahydrate, and sodium carbonate were obtained from Merck. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. Sephadex LH-20 was obtained from Pharmacia Inc. (Uppsala, Sweden).

Plant material

Gentianella multicaulis (Gillies ex Griseb.) Fabris (vernacular name 'nencia') was collected at 2750 m above sea level, in the mountains of Bauchaceta and Chita districts, Departamento de Iglesia, San Juan Province, Argentina, during March 2008, and was identified by one of us (E. F.). A voucher specimen was deposited at the Museo Botánico de Córdoba (CORD 1092), Córdoba, Argentina.

Extracts and isolation of bioactive compounds from Gentianella multicaulis

Aerial parts (310 g) were extracted successively with petroleum ether (PE), dichloromethane (DCM), and methanol (MeOH) under reflux to afford PE, DCM, and MeOH extracts. The w/w yields in terms of dry starting material were 0.45%, 2.58%, and 4.34%, respectively. A representative portion of MeOHE (12 g) was dissolved in H_2O under sonication. Then the aqueous phase was successively extracted with diethyl ether (Et_2O; 3 x 300 mL) and ethyl acetate (EtOAc; 3 x 300 mL) to afford extracts: EEE (4.23 g, 35.25%) and EtOAcE (3.01 g, 25.08%), respectively.

The petroleum ether/methanol/chloroform (2:1:1 v/v/v)-soluble fraction of the bioactive DCM extract (5 g) was permeated through a Sephadex LH-20 column (column length, 47 cm; diameter, 3.8 cm) equilibrated with petroleum ether/methanol/chloroform (2:1:1 v/v/v), and 33 fractions of 25 mL each were obtained. After TLC comparison with ethyl acetate/petroleum ether (3:7 v/v) as mobile phase, detection under UV light, and subsequent spraying with *p*-anisaldehyde, fractions with similar patterns were pooled into sixteen groups, S1–S16.

From fraction S4 (221 mg), oleanolic acid (**1**) and ursolic acid (**2**), the main constituents of the DCM extract, were isolated by successive permeation of fraction S4 through a Sephadex LH-20 column (20 cm x 2.5 cm) using MeOH as eluant. Both compounds were identified by their ^1H and ^{13}C NMR spectra and TLC comparison with standard samples.

Fraction S12 (221.6 mg) was suspended in methanol, and the MeOH-soluble portion (13 mg) was purified by preparative HPLC using H_2O/MeOH (1:9 v/v) as mobile phase, to yield swerchirin or methylbellidifolin (**3**, 1,8-dihydroxy-3,5-dimethoxy-9*H*-xanthen-9-one, 5 mg) with R_t = 40 min, molecular formula $C_{15}H_{12}O_6$, M_r 288.2520, and NMR spectra in agreement with literature data (Hajimehdipour *et al.*, 2003).

The MeOH-soluble portion (30 mg) of S14 (38.8 mg) was purified by preparative HPLC with H_2O/MeOH (3:7 v/v) as eluent, to yield bellidin or demethylbellidifolin (**4**, 1,3,5,8-tetrahydroxy-9*H*-xanthen-9-one, 6 mg) with R_t = 50 min, molecular formula $C_{13}H_8O_6$, M_r 260.1990, and NMR spectroscopic data in agreement with reported ones (Markham, 1964).

Fraction S15 (103.3 mg) was purified by column flash chromatography on silica gel (column length, 23 cm; diameter, 2 cm) using a CH_2Cl_2/EtOAc/MeOH gradient. On the basis of TLC analysis of the fractions, six groups (I–VI) were obtained. Separation of the MeOH-soluble fraction of I (29.2 mg) by preparative HPLC with MeOH/H_2O (8:2 v/v) as eluant followed by a second step of HPLC purification using acetonitrile/H_2O (7:3 v/v) as mobile phase yielded bellidifolin (**5**, 1,5,8-trihydroxy-3-methoxy-9*H*-xanthen-9-one, 2.2 mg) with R_t = 25 min, molecular formula $C_{14}H_{10}O_6$, M_r 274.2296, and NMR data comparable to the literature (Markham, 1964).

Fraction S16 (30 mg) was successfully purified by preparative HPLC with acetonitrile/H_2O (7:3 v/v) as mobile phase to afford isobellidifolin or swertianol (**6**, 1,3,8-trihydroxy-5-methoxy-9*H*-xanthen-9-one, 3 mg) with R_t = 18 min, molecular formula $C_{14}H_{10}O_6$, M_r 274.2280, and NMR data similar to the literature (Markham, 1965).

The EEE (4 g) was applied to a Sephadex LH-20 column (column length, 43 cm; diameter, 3.5 cm) equilibrated with methanol, and 42 fractions of 25 mL each were eluted. After TLC comparison on silica gel [ethyl acetate/petroleum ether (3:7 v/v) as the mobile phase, UV detection, and then spraying with diphenylboric acid] fractions with similar TLC patterns were combined. The major fractions 10–14 (590 mg) were applied to a Sephadex LH-20 column (column length, 24 cm; diameter, 2.5 cm) equilibrated with methanol. Thirty fractions were obtained on the basis of TLC analysis: 1 (2 mg); 2 (30 mg); 3 (1 mg); 4 (1 mg); 5 (2 mg); 6 (6 mg); 7 (5.5 mg); 8 – 13 (136.3 mg); 14–22 (413.8 mg); 23–26 (72.3 mg); 27–30 (23.8 mg). Fractions 23–26 (72.3 mg) led to the isolation of 60 mg demethylbellidifolin (**4**).

Structural identification of the compounds and HPLC conditions

NMR experiments were performed on a Bruker Avance II (500 MHz) instrument (Karlsruhe, Germany) at 500.13 MHz for ^{1}H NMR and 125.13 MHz for ^{13}C NMR. All spectra were recorded in $CDCl_3$ using TMS as internal standard. Homonuclear ^{1}H connectivities were determined by COSY experiments. The edited reverse-detected single quantum heteronuclear correlation (DEPT-HSQC) experiment allowed the determination of carbon multiplicities, as well as one-bond proton-carbon connectivities, and the heteronuclear multiple bond correlation (HMBC) experiment allowed the determination of long-range proton-carbon connectivities. The determination of the substitution pattern of the aromatic rings was completed with the help of gradient-enhanced NOESY experiments. All 2D NMR experiments were performed using standard pulse sequences. HRESI mass spectra were recorded using a MicrOTOF QII Bruker mass spectrometer (Billerica, MA, USA). Dry column flash chromatography was carried out on silica gel (Aldrich Chemical Co., Milwaukee, WI, USA). HPLC separations were performed using a Thermo Separations Spectra Series P100 pump (Waltham, MA, USA), a Thermo Separations Refractomonitor IV RI detector, and a Thermo Separations Spectra Series UV100 detector, with simultaneous UV (220 nm) and RI detection. An YMC RP-18 (5 mm, 20 mm x 250 mm) column working at a flow rate of 5 mL/min was used for separations. All solvents were HPLC grade [MeOH/H_2O (9:1; 8:2; 7:3 v/v); acetonitrile/water (7:3 v/v)]. Typical retention times under the previous conditions were: swerchirin (**3**), 40 min; demethylbellidifolin (**4**), 50 min; bellidifolin (**5**), 25 min; and isobellidifolin (**6**), 18 min.

Microorganisms and media

For the antibacterial evaluation, strains from the American Type Culture Collection (ATCC) (Rockville, MD, USA), Malbrán Institute (MI) (Buenos Aires, Argentina), Pasteur Institute (PI) (Buenos Aires, Argentina), and from the Laboratorio de Microbiología (LM), (Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina) were used: *Escherichia coli* ATCC 25922, *Escherichia coli*-LM_1, *Escherichia coli*-LM_2, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enteritidis*-MI, *Salmonella* sp.-LM, *Yersinia enterocolitica*-PI, *Staphylococcus aureus*-LM, *Staphylococcus aureus* methicillin-sensitive ATCC 29213, and *Staphylococcus aureus* methicillin-resistant ATCC 43300. Bacteria were grown on Mueller Hinton agar medium. For the antifungal evaluation, strains from ATCC, and CEREMIC, Centro de Referencia Micológica, Facultad de Ciencias Bioquímicas y Farmacéuticas, Rosario, Argentina (CCC), were used: *Candida albicans* ATCC 10231, *Candida tropicalis* CCC 131, *Saccharomyces cerevisiae* ATCC 9763, *Cryptococcus neoformans* ATCC 32264, *Aspergillus flavus* ATCC 9170, *Aspergillus fumigatus* ATTC 26934,

Aspergillus niger ATCC 9029, *Trichophyton rubrum* CCC 110, *Trichophyton mentagrophytes* ATCC 9972, and *Microsporum gypseum* CCC 115.

Antibacterial susceptibility testing

Cultures less than 30 h old were transferred to sterile broth (Mueller Hinton broth), with the help of a loop, and incubated in the broth at 37 °C until the growth reached a turbidity equal to or greater than that of 0.5 McFarland standard. The culture was adjusted with sterile physiological solution to give a final organism density of $5 \cdot 10^5$ CFU/mL (CLSI, 2008; Jorgensen *et al.*, 1999). The antibacterial activity was evaluated with the agar dilution method using Mueller Hinton agar medium for Gram-positive and Gram-negative bacteria. Stock solutions of extracts in dimethylsulfoxide (DMSO) were diluted to give serial two-fold dilutions that were added to each medium resulting in concentrations ranging from 100 to 1000 μg/mL and for compounds from 10 to 100 μg/mL. The final content of DMSO in the assay did not exceed 1%. The antimicrobial agent cefotaxime (Argentia Pharmaceutica, Buenos Aries, Argentina) was included in the assays as positive control. Minimal inhibitory concentration (MIC) was defined as the lowest extract and/or compound concentration showing no visible bacterial growth after an incubation time of 24 h at 37 °C. Tests were done in triplicate.

Antifungal susceptibility testing

The MIC of extracts and compounds was determined using broth microdilution techniques following the guidelines of CLSI (2002) as described by the National Committee for Clinical Laboratory Standards for yeasts as well as for filamentous fungi in 96-well microtiter trays. MIC values were determined in RPMI-1640 medium (Sigma) buffered to pH 7.0 with MOPS [3-(*N*-morpholino) propanesulfonic acid] buffer. The starting inoculum was approximately $1-5 \cdot 10^3$ CFU/mL and was prepared according to the guidelines of CLSI. Microtiter trays were incubated at 35 °C for yeasts and hialohyphomycetes and at 28–30 °C for dermatophyte strains in a moist, dark chamber. MIC values were recorded after 48 h for yeasts and at a time according to the control fungus growth for the other fungi. The susceptibility of the standard drugs ketoconazole, terbinafine, and amphotericin B was defined as the lowest concentration of drug which resulted in the total inhibition of fungal growth. For the assay, stock solutions were two-fold diluted with RPMI medium from 1000 to 0.98 μg/mL (final volume, 100 μL) and a final DMSO content ≤ 1%. A volume of 100 μL of inoculum suspension was added to each well with the exception of the sterility control where sterile water was added to the well instead. The MIC was defined as the lowest concentration of the extract and/or compound which resulted in total inhibition of the fungal growth.

Determination of total phenolics content and flavonoids

The total phenolics content of the extracts was determined using the method described by Singleton *et al.* (1999). Briefly, the appropriate extract dilutions were oxidized with Folin-Ciocalteu reagent, and the mixture was neutralized with sodium carbonate. The absorbance of the resulting blue colour was measured at 765 nm after 30 min using a Spectronic Unicam spectrophotometer (Helios α UV-VIS) (Rochester, NY, USA). Quantification was done on the basis of a standard curve for gallic acid. Results were expressed as gallic acid equivalents (% GAE). Data are reported as mean ± standard deviation (SD) for at least three replicates.

The total flavonoids content in the samples was determined by the methodology of Chang *et al.* (2002). Quercetin was used as a reference for the calibration curve. The absorbance of the reaction mixture was measured at 415 nm. Results were expressed as quercetin equivalents (% QE). Data are reported as mean ± standard deviation (SD) for at least three replicates.

Free radical scavenging activity on DPPH

Free radical scavenging effects of extracts and compounds were assessed by the fading of a methanolic solution of DPPH radicals as reported by Tapia *et al.* (2004). Extracts were assayed at concentrations of 100, 50, 10, and 5 μg/mL. Scavenging activities were evaluated spectrophotometrically at 517 nm using the absorbance of the DPPH radical as reference. The loss of colour (fade percentage) was calculated as follows:

$$\text{fade percentage} = \left[1-\left(\frac{\text{absorbance of sample}-\text{absorbance of blank}}{\text{absorbance of DPPH}}\right)\right] \cdot 100.$$

The colour loss indicated the free radical scavenging efficiency of the substances. Values are reported as means ± standard deviations (SD) of three independent determinations.

Ferric-reducing antioxidant power (FRAP) assay

The iron-reducing power of the samples was tested using the assay of Oyaizu (1986). Two hundred μL of the extracts (0.1, 0.25, and 0.5 mg/mL) as well as gallic acid and 3-*tert*-butyl-4-hydroxyanisole (BHA) were added to 500 μL of phosphate buffer (0.1 M, pH 6.6) and 500 μL of potassium ferricyanide (1%, w/v). After the additions, the mixture was incubated at 50 °C for 20 min, and then 500 μL of 10% TCA were added and the mixture was shaken vigorously. One mL of this solution was mixed with 1 mL of distilled water and 200 μL of $FeCl_3$ (0.1%, w/v). After 30 min incubation, the absorbance was read at 700 nm. Analyses were made in triplicate. Increased absorbance of the mixture signified increased reducing power.

Statistical analysis

Values of total phenolics and flavonoids, DPPH and FRAP assays, respectively, are expressed as means ± standard deviations of triplicate determinations. Statistical analysis was done with one-way analysis of variance. The Pearson correlation coefficient (R) and p value were used to show correlations and their significance (SPSS 16.0 for Windows; SPSS Inc., Chicago, IL, USA). Probability values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and extremely significant, respectively.

Results and Discussion

Petroleum ether (PEE), dichloromethane (DCME), methanol (MeOHE), ethyl ether (EEE), and ethyl acetate (EtOAcE) extracts of *G. multicaulis* collected in the foot-hills of the Andes of San Juan Province, Argentina, were assessed for their antimicrobial and free radical scavenging activities, respectively.

Antimicrobial activity

PEE, DCME, and MeOHE were first assayed for their antifungal and antibacterial properties using the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2002, 2008; Jorgensen *et al.*, 1999). The panel of fungi included four yeasts, 3 filamentous fungi, and 3 dermatophytes. A panel of standardized bacteria and clinical isolates of Gram-positive and Gram-negative bacteria was included as well.

The results showed (Table I) that all dermatophytes were inhibited by PEE, DCME, and MeOHE with MIC values between 125 and 250 μg/mL. On the other hand, PEE, DCME, and MeOHE were less effective against bacteria, yeasts, and filamentous fungi with MIC values between 250 and 1000 μg/mL (data not shown).

Assay-guided fractionation of DCME and MeOHE led to the isolation of the two triterpenes **1** and **2** and the four xanthones **3**–**6** as antifungal compounds from *G. multicaulis* which are shown in Fig. 1: oleanolic acid (**1**), ursolic acid (**2**), swerchirin or methylbellidifolin (1,8-dihydroxy-3,5-dimethoxy-9*H*-xanthen-9-one) (**3**), demethylbellidifolin or bellidin (1,3,5,8-tetrahydroxy-9*H*-xanthen-9-one) (**4**), bellidifolin (1,5,8-trihydroxy-3-methoxy-9*H*-xanthen-9-one) (**5**), and isobellidifolin or swertianol (1,3,8-trihydroxy-5-methoxy-9*H*-xanthen-9-one) (**6**).

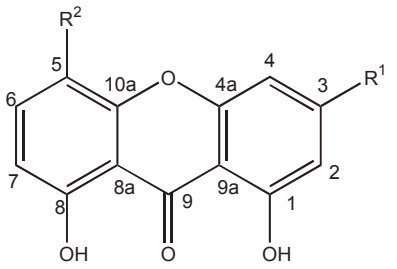
The spectroscopic data (^{1}H and ^{13}C NMR) of the isolated compounds **3**–**6** are in agreement with those previously reported in the literature. A full assignment of ^{1}H and ^{13}C signals was unambiguously accomplished using 2D COSY and HMBC techniques (Tables II and III).

Compounds **1**–**6** have been reported as constituents of *G. achalensis* collected in Córdoba Province, Argentina, at 1200 m above sea level

Table I. Antifungal activity of extracts and compounds isolated from *Gentianella multicaulis*.

Extracts and compounds	MIC [μg/mL]		
	Microsporum gypseum	*Trichophyton rubrum*	*Trichophyton mentagrophytes*
PEE	250	125	250
DCME	250	125	125
MeOHE	125	125	125
Swerchirin (**3**)	100	100	100
Demethylbellidifolin (**4**)	100	50	50
Bellidifolin (**5**)	50	50	50
Isobellidifolin (**6**)	50	50	50
Amphotericin B	0.125	0.075	0.075
Ketoconazole	0.05	0.025	0.025
Terbinafine	0.04	0.025	0.04

PEE, petroleum ether extract; DCME, dichloromethane extract; MeOHE, methanol extract.



	R^1	R^2
Swerchirin (**3**)	OCH_3	OCH_3
Demethylbellidifolin (**4**)	OH	OH
Bellidifolin (**5**)	OCH_3	OH
Isobellidifolin (**6**)	OH	OCH_3

Fig. 1. Xanthones isolated from bioactive extracts of *Gentianella multicaulis*.

and of *G. florida* (Nadinic *et al*., 1997). Rosella *et al*. (2007), compared two Argentinean *Gentianella* species (*G. parviflora* and *G. multicaulis*) by micrographic parameters and HPLC techniques, using standard compounds, and the main identified metabolites were compounds **3**–**6**.

The xanthones **4**–**6** exhibited the best antifungal activities against *M. gypseum* as well as both species of *Trichophyton* with MIC values of 50 μg/mL, while swerchirin (**3**) was less active with MIC values of 100 μg/mL (Table I). A study by Rojas *et al*. (2004) reported the antifungal activity of the ethanol extract and fractions of *Gentianella nitida* against *Candida albicans*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*. No previous reports were found in the literature concerning the antifungal activity of the xanthones identified in *Gentianella multicaulis*. This result is interesting since *T. rubrum* and *T. mentagrophytes* are responsible for approximately 80–93% of chronic and recurrent dermatophyte infections in humans. These fungi are the ethiological agents of tinea unguium (liable of invasive nail infections), tinea manum (palmar and interdigital areas of the hand infections), and tinea pedis, "athlete's foot", the latter being the most prevalent fungal infection in developed countries, and the first one accounting for 50% and 90% of all fingernail and toenail infections, respectively (Weitzman and Summerbell, 1995).

Free radical scavengers and phenolics content

The free radical scavenging effect was measured by the bleaching of DPPH radicals and by

Table II. ^{1}H, ^{13}C, HMBC, and NOESY spectroscopic data of compounds **3** and **4** (δ in ppm).

No.	Swerchirin (**3**)				Demethylbellidifolin (**4**)			
	^{13}C	^{1}H	HMBC	NOESY	^{13}C	^{1}H	HMBC	
1	162.5	-	-	-	162.7	-	-	
2	98.3	6.44 d (2.2)	167.7, 162.5, 102.8, 93.5	-	99.0	6.24 d (2.2)	162.7, 101.5, 94.8	
3	167.7	-	6.77, 6.70, 3.93	-	167.6	-	-	
4	93.5	6.70 d (2.2)	184.8, 167.7, 157.8, 102.8, 98.3	-	94.8	6.43 d (2.2)	167.6, 157.9, 101.5, 99.0	
4a	157.8	-	-	-	157.9	-	-	
5	140.1	-	-	-	137.7	-	-	
6	121.9	7.51 d (8.8)	153.5, 145.0, 140.1, 110.3, 108.1	-	124.0	7.27 d (8.8)	152.4, 143.2, 137.7, 107.9	
7	110.3	6.77 d (8.8)	184.3, 153.5, 145.0, 140.1, 108.1	-	109.8	6.66 d (8.8)	184.1, 152.4, 143.2, 137.7, 107.9	
8	153.5	-	-	-	152.4	-	-	
8a	108.1	-	6.77, 6.70	-	107.9	-	-	
9	184.8	-	-	-	184.1	-	-	
9a	102.8	-	-	-	101.5	-	-	
10	145.0	-	-	-	143.2	-	-	
3-OMe*	57.6	3.93 s	167.7	6.70, 6.44	-	-	-	
5-OMe*	57.6	3.92 s	140.1	7.51	-	-	-	

*Assignments may be interchanged.

Table III. ^{1}H, ^{13}C, HMBC, and NOESY spectroscopic data of compounds **5** and **6** in MeOD-d_4 (δ in ppm).

No.	Bellidifolin (**5**)				Isobellidifolin (**6**)			
	^{13}C	^{1}H	HMBC	NOESY	^{13}C	^{1}H	HMBC	NOESY
1	162.4	-	-	-	164.3	-	6.15	-
2	97.8	6.45 d (2.2)	167.3, 162.4, 102.1, 93.3	-	95.9	6.32 d (2.2)	-	-
3	167.3	-	-	-	171.2	-	6.32, 6.15	-
4	93.3	6.66 d (2.2)	167.3, 157.4, 102.1, 97.8	-	100.5	6.15 d (2.2)	-	-
4a	157.4	-	-	-	159.6	-	6.32	-
5	137.5	-	-	-	141.5	-	7.33, 6.65, 3.92	-
6	124.3	7.30 d (8.8)	152.2, 143.8, 137.5, 107.9	-	121.5	7.33 d (9.0)	-	6.65, 3.92
7	109.9	6.69 d (8.8)	184.5, 152.2, 143.8, 137.5, 107.9	-	109.8	6.65 d (9.0)	-	7.33
8	152.2	-	-	-	155.4	-	6.65, 6.32	-
8a	107.9	-	-	-	108.7	-	6.65, 6.32	-
9	184.5	-	6.69	-	185.6	-	6.65, 6.32	-
9a	102.1	-	-	-	102.1	-	6.32, 6.15	-
10	143.8	-	-	-	146.3	-	6.32, 6.15	-
3-OMe	56.9	3.94 s	167.3	6.66, 6.45	-	-	-	-
5-OMe	-	-	-	-	57.5	3.92 s	141.5	7.33

FRAP. The DCM and MeOH extracts as well as sub-extracts derived from the methanolic extract (EEE, EtOAcE) presented strong free radical scavenging effects in the DPPH assay with values ranging between 45 and 93%, at 10 μg/mL (Fig. 2). The same extracts showed the strongest reducing power in the FRAP assay at 200 μg/mL (Fig. 3), and the highest content of phenolics and flavonoids (Table IV).

Assay-guided fractionation of bioactive extracts led to the isolation of **3**, **4**, **5**, and **6** as the free radical scavengers and antioxidant compounds of *G. multicaulis*. Demethylbellidifolin (**4**) presented high antioxidant activity towards the DPPH radical (84.24% at 5 μg/mL) as well as in the FRAP assay at 100 μg/mL (Figs. 2 and 3). Rojas *et al.* (2004) reported that extracts of *G. nitida* displayed free radical scavenging activity towards DPPH.

Xanthones are a class of polyphenolics that exhibit well-documented pharmacological properties, mainly due to their oxygenated heterocyclic nature and diversity of functional groups. They

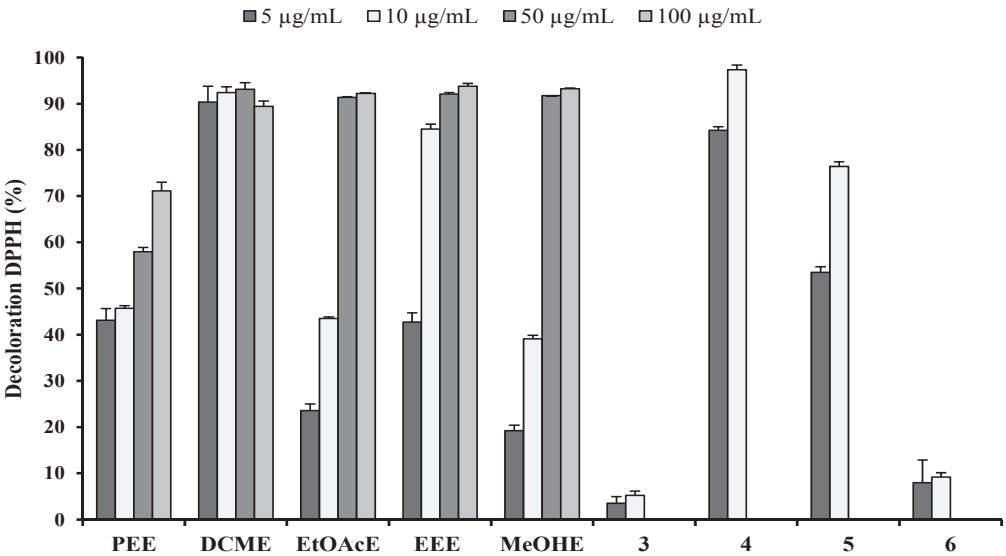


Fig. 2. DPPH radical scavenging activity of extracts and xanthones from *Gentianella multicaulis*. PEE, petroleum ether extract; DCME, dichloromethane extract; EtOAcE, ethyl acetate extract; EEE, diethyl ether extract; MeOHE, methanol extract; 3, swerchirin; 4, demethylbellidifolin; 5, bellidifolin; 6, isobellidifolin.

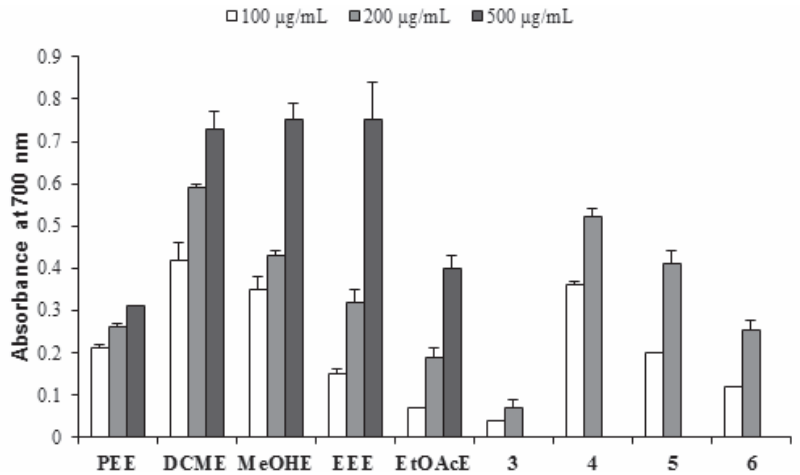


Fig. 3. Ferric-reducing antioxidant power of extracts and xanthones from *Gentianella multicaulis*. PEE, Petroleum ether extract; DCME, dichloromethane extract; EtOAcE, ethyl acetate extract; EEE, diethyl ether extract; MeOHE, methanol extract; 3, swerchirin; 4, demethylbellidifolin; 5, bellidifolin; 6, isobellidifolin.

have been described as strong scavengers of free radicals (Jiang *et al*., 2004). A review of the genus *Gentianella* Moench by Li *et al*. (2010) reported antioxidant, radioprotective, antimicrobial, anti-inflammatory, hypoglycemic, and anticholinergic activities for this genus, with xanthones as the bioactive compounds.

Nadinic *et al*. (1997) reported the isolation and identification of four 1,3,5,8-tetraoxygenated xanthones: swerchirin, bellidifolin, isobellidifolin, and demethylbellidifolin, in addition to the triterpenes ursolic and oleanolic acids of *G. florida* (Griseb.) Holub (Gentianaceae). The xanthones demethylbellidifolin, bellidifolin, isobellidifolin, and swertianin were also isolated from *G. achalensis* (Nadinic *et al*., 1999, 2002). Recently, Ŝavikin *et al*. (2009) reported the antibacterial activity of methanolic extracts and compounds isolated from *Gentiana lutea.* Urbain *et al.* (2008) informed about xanthones from *Gentianella amarella* ssp. *acuta* with acetylcholinesterase and monoamine oxidase inhibitory activities, respectively. Bellidifolin was reported as a potent hypoglycemic agent in streptozotocin-induced diabetic rats by both oral and intraperitoneal administration (Basnet *et al*., 1994). Demethylbellidifolin inhibited proliferation and activation of hepatic stellate cells (Li *et al*., 2011). These compounds also showed interesting inhibitory activity of monoamine oxidase (MAO) and were weakly active against acetylcholinesterase (Urbain *et al*., 2008). Recently, Singh *et al*. (2011) reported that bellidifolin and isobellidifolin were detected in the extracts of *Swertia chirayita* by antioxidant activity-guided isolation.

The identification of the xanthones **3**–**6** and the triterpenoids **1** and **2** in *G. multicaulis* collected at 2750 m above sea level in the Province of San Juan, Argentina is consistent with reports of these compounds in *G. achalensis* collected in central western Argentina at 1200 m above sea level (Nadinic *et al*., 1999, 2002).

In the past decade, antioxidants and free radical scavengers have gained importance in the prevention of several human pathologies in which reactive oxygen species are involved, including atherosclerosis, heart diseases, cancer, Down's syndrome, Friedreich's ataxia, rheumatoid arthritis, autoimmune diseases, and AIDS (Middleton *et al*., 2000; Halliwell and Gutteridge, 2003). Flavonoids and phenolics from plants have been reported as potent free radical scavengers and frequently occur in medicinal and aromatic plants. A high content of flavonoids and phenolic compounds has been detected in different extracts obtained from aerial parts of *G. multicaulis* (Table IV). A highly significant positive correlation between the antioxidant activity and the contents of total flavonoids, total phenolics, and DPPH assay were observed in this study.

Phytochemical research based on ethno-pharmacological information is considered an effective approach in the discovery of new agents from higher plants (Chen *et al*., 2008). *G. multicaulis*, in

Table IV. Total contents of phenolics and flavonoids in *Gentianella multicaulis* extracts.

Extract	Phenolics[a] (% GAE)	Flavonoids[b] (% QE)
PEE	11.65 ± 0.49	3.50 ± 0.13
DCME	27.82 ± 2.21	12.39 ± 0.81
MeOHE	18.09 ± 0.17	5.15 ± 0.24
EEE	11.54 ± 1.05	4.09 ± 0.48
EtOAcE	5.53 ± 0.66	1.88 ± 0.06

[a] Total phenolic content is expressed as grams of gallic acid equivalents (GAE) per 100 g of extract.
[b] Flavonoid content is expressed as grams of quercetin equivalents (QE) per 100 g of extract.
Values are shown as means ± standard deviations (SD).

San Juan Province, is consumed as a bitter tonic to treat digestive and liver problems. Its ethnobotanical use together with the results obtained on the antioxidant activity are consistent with the relationship between liver damage and redox imbalance and oxidative stress (Vrba and Modriansky, 2002).

The level of total flavonoids and phenolics significantly correlated with the values from the DPPH assay with R of 0.984 and 0.831, respectively ($p < 0.01$). This result suggests that both flavonoids and phenolic compounds could contribute to the antioxidant capacities of *G. multicaulis*.

Conclusions

The strongest antioxidant activity displayed by DCME and MeOHE in addition to a high phenolics content in both extracts, plus the identification of the xanthones **3**–**6** and the triterpenoids **1** and **2** suggest that beneficial effects could be expected by regular consumption of the infusion of *Gentianella multicaulis*, collected in the mountains of the Province of San Juan, Argentina. Since the extracts and main compounds isolated from *G. multicaulis* showed antifungal activity against dermatophytes of clinical relevance, their use in the treatment of fungal infections could also be beneficial.

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