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Polyphenol content and bioactivity of *Achillea moschata* from the Italian and Swiss Alps

https://doi.org/10.1515/znc-2019-0214
Received November 29, 2019; revised January 14, 2020; accepted January 21, 2020

Abstract: *Achillea moschata* Wulfen, which grows in the Alps, is extensively used by local people for its medicinal properties. Two studied samples were collected, at the flowering stage, in Val Mustair (Switzerland) and Valchiavenna (Italy), respectively. The aerial parts were defatted with petroleum ether (PET) and successively extracted with dichloromethane (DCM) and methanol (MeOH). High-performance liquid chromatography and electrospray ionization-tandem mass spectrometry analyses of the methanolic extracts evidenced that flavonoids were the predominant compounds compared to phenolic acids in both samples (89.5 vs. 33.0 μg/mg DW in *A. moschata* Valchiavenna and 82.5 vs. 40.0 μg/mg DW in *A. moschata* Val Mustair). Among flavonoid derivatives, luteolin and apigenin were the predominant aglycones, free and glycosilated. The *A. moschata* Valchiavenna extract was characterized by apigenin as the main compound (60.4 μg/mg DW), while *A. moschata* Val Mustair was characterized by its derivative apigenin 7-*O*-glucoside (44.7 μg/mg DW). The antioxidant activity of all the obtained extracts was tested by the DPPH (2,2-diphenyl-picryl hydrazyl) and ABTS (2,21-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods, which showed their increasing scavenger capacity in relation to extract polarity (PET extract < DCM extract < MeOH extract). The extracts were also investigated against three Gram-positive (*Bacillus cereus*, *Enterococcus faecalis* and *Staphylococcus aureus*) and three Gram-negative (*Escherichia coli*, *Proteus mirabilis* and *Pseudomonas aeruginosa*) bacterial species using the disc diffusion assay. DMC and PET were the most active extracts (inhibition diameter: ≥12 mm).

Keywords: alpine species; antibacterial activity; antiradical activity; Asteraceae; flavonoids; musk milfoil; officinal plants; phenolic acids.

1 Introduction

The genus *Achillea* (Asteraceae) consists of about 140 species worldwide [1]. Among the 23 Italian species, *A. moschata* Wulfen is an endemic plant growing on siliceous rocks, screes and stony pastures along the Alps up to 3400 m a.s.l. and reaching on average 1–2 dm in height characterized by leaves deeply 1-pinnatifid and white flowers arranged in corymbs [2, 3]. In some alpine areas, their characteristic fragrance and flavor are exploited in the preparations of local dishes and beverages (e.g. soups, meats, dessert, grappa, liqueurs). *A. moschata* is also used in folk remedies for various purposes both in human and veterinary fields. It is collected to mainly treat disorders affecting the intestine, stomach and, in general, the digestive system and administered as an infusion against dyspepsia, abdominal bloating, flatulence and gastric pains [4–7]. Anti-inflammatory, antimicrobial, antineuralgic, diuretic, hypotensive and sedative effects are also recognized as properties of *A. moschata* [6, 7].

Various properties of *A. moschata* may be due to the presence of secondary active metabolites including polyphenols, a category of chemicals whose health benefits are well documented. Many beneficial effects may be related to their antioxidant and anti-inflammatory properties that provide a significant protection against the development of several chronic diseases such as cardiovascular and neurogenerative disorders, cancer, diabetes and obesity, as documented by a large number of animal, human and epidemiological studies [8]. In the human body, polyphenols also play osteoprotective, antiasthmatic, antihypertensive, antiageing, cholesterol-lowering, hepatoprotective and antimicrobial roles [9]. More than 8000 polyphenols have been identified and broadly divided into four classes – phenolic acids, flavonoids, stilbenes and lignans. They are classified according to the number of phenol rings present in their molecule and the structural elements that bind these rings to each other [10, 11].

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A. moschata has been harvested and used for a long time, but the related scientific studies are very limited [12]. Therefore, we decided to investigate the composition of the secondary metabolites of this species, starting from the phenolic content. Moreover, antioxidant and antibacterial properties were evaluated to confirm the few previous data [12].

2 Materials and methods

2.1 Plant material

The two analyzed samples were collected, at the flowering stage, in Val Mustair (Switzerland, 2300 m a.s.l.) (VMS) and in Valchiavenna (Italy, 2200 m a.s.l.) (VCI), air dried exsiccated and kept in paper bags until analyses. Voucher specimens were deposited at the Department of Agricultural and Environmental Sciences of the Milan State University (Milan, Italy) after the identification according to *Flora d'Italia* [2].

2.2 Extraction

Powdered aerial parts of *A. moschata* VMS and VCI (26.5 g each) were defatted with petroleum ether (PET) and successively extracted with dichloromethane (DCM) and methanol (MeOH) in a Soxhlet apparatus. The extracts were evaporated at 30 °C (rotary evaporator) to dryness and then stored at 4 °C until analyses.

2.3 High-performance liquid chromatography (HPLC)

The HPLC analysis was performed on a Waters HPLC 600 liquid chromatograph (Milan, Italy) system equipped with a photo diode array detector, DAD 2828 Waters. Data were processed with the Empower™ 2 Waters Software. The analyses were running on a Gemini C18 (Phenomenex) column (250 × 4.6 mm internal diameter; 5 µm particle size). The mobile phase was water with 0.1% (v/v) formic acid (A) and acetonitrile with 0.1% (v/v) of formic acid (B). The linear gradient started from 10% B and reached to 60% B in 60 min. The flow rate was 1 mL/min. UV spectra of the samples were conventionally recorded at 210, 270, 310 and 350 nm. All analyses were run in triplicate. An aliquot of 20 µL of each polar extract was injected for each run. For quantitative analysis, a five-level calibration curve was obtained by injection of 10–250 µg/mL concentrations of different standards such as luteolin-6-*C*-glucoside (Extrasynthese, 99% purity; $y = 44{,}625x + 119{,}464$; $R^2 = 0.9992$) and 5-*O*-caffeoylquinic acid ($y = 32{,}345x - 58{,}693$; $R^2 = 0.9999$).

2.4 Electrospray ionization-tandem mass spectrometry (ESI-MS/MS)

Flow injection MS analyses were performed on a 1100 Series Agilent LC/MSD Trap System VL. An Agilent Chemstation (LC/MSD TrapSoftware 4.1, Agilent Technologies, Santa Clara, CA, USA, 2002) was used for the data acquisition and processing. All analyses were carried out using an ESI ion source in the negative mode with the following settings: capillary voltage, 4000 V; nebulizer gas (N_2), 15 psi; drying gas (N_2), 350″C, 5 L/min. Full-scan spectra were acquired over the range of 100–2200 *m*/*z* with a scan time of 13,000 *m*/*z*/s. Automated ESI-MS/MS was performed by isolating the base peaks (molecular ions) using an isolation width of 4.0 *m*/*z*, threshold set at 100 and ion charge control on, with a max acquire time set at 300 ms. Different collision energies were conventionally used (1.0, 10 and 30 V) for MS/MS fragmentation. Polar samples were dissolved in MeOH at the concentration of 20–30 ppm and injected at a flow rate of 10 µL/min (KD Scientific Syringe Pump).

2.5 Antiradical activity

2.5.1 DPPH (2,2-diphenyl-picryl hydrazyl) assay

The radical-scavenging potential of the extracts toward DPPH$^{\cdot}$ was carried out according to Iriti et al. [13], with some modifications. Briefly, aliquots of each sample, at five different concentrations (1–100 µM), were added to DPPH$^{\cdot}$ MeOH solution (0.07 mM) to reach a final volume of 2 mL. After 30 min in the dark (23 °C), the mixtures were read at 517 nm to measure their absorbance decrease. The IC_{50} was calculated by employing Prism®4 as a software (GraphPad Software Inc., La Jolla, CA, USA).

2.5.2 ABTS$^{\cdot}$+ assay

The radical cation-scavenging capacity of the extracts was also determined against 2,21-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\cdot}$+) [13]. The ABTS$^{\cdot}$+ radical was produced by reacting 7 mM ABTS$^{\cdot}$+ with 2.45 mM potassium persulfate (final concentration) and

maintaining the mixture in the dark at room temperature for at least 6 h before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbance of 0.7 (±0.02) at 734 nm and equilibrated at 30 °C. Ten microliters of each sample, ethanol (negative control) and standard solution of the synthetic antioxidant 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid (Trolox, positive control) were mixed for 30 s with 1 mL of diluted $ABTS^{\cdot+}$ solution. Their absorbance was read at 734 nm, at room temperature, 50 s after the initial mixing. The results are expressed as Trolox equivalent antioxidant capacity (μmol eq Trolox mL^{-1}).

2.6 Antibacterial activity

The disk diffusion agar test was performed to assess antibacterial properties of the *A. moschata* extracts (VMS and VCI) according to Vitalini et al. [12]. Sterile paper disks (6 mm in diameter) impregnated with 10 μL of each plant extract (MeOH, DCM and PET), extraction solvents as negative controls or ceftazidime (30 μg) and erythromycin (5 μg) as references for Gram-negative and Gram-positive bacteria, respectively, were placed onto the surface of the media inoculated with *Bacillus cereus* ATCC 999091, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923, as well as *Escherichia coli* ATCC 35218, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853 strains. After18 h of incubation at 37 °C, the plates were read and the bacterial growth inhibition zones were measured according to the following scale: no activity (–); <10 mm, low activity (+); 10–20 mm, moderate activity (++); >20 mm, high activity (+++) [14]. Each extract or solvent or antibiotic/microorganism combination was tested in triplicate.

3 Results and discussion

3.1 Qualitative and quantitative analysis of the MeOH extracts

The phytochemical composition in the phenolic compounds of *A. moschata* VMS and VCI methanolic extracts was achieved by a combination of analytical data from HPLC-DAD (diode-array detection), ultraviolet (UV) and ESI-MS/MS and spiking with authentic available compounds. Both samples were characterized by the presence of a prevalent compound (peaks 9 and 4, respectively) (Figure 1A and B) with a UV spectrum typical of the isorhamnetin and apigenin derivatives. The HPLC profile of *A. moschata* VCI showed another remarkable phenolic compound, apigenin as a free aglycone (peak 8, Figure 1B).

The *A. moschata* VMS and VCI extracts showed different fragmentation patterns, suggesting the presence of flavonoids and phenolic acids (Figure 2A and B). This information was supported by the spectroscopic data (Figure 3A and B). The UV spectra of the HPLC eluted components show two major absorption bands: band I absorption in the 330–350 nm range due to the B-ring cinnamoyl system; band II in the range 240–280 due to the A-ring benzoyl system. A closer inspection of the spectroscopic data suggested that they were derivatives of four different aglycones (luteolin, apigenin, kaempferol and isorhamnetin). Compounds with absorption ranges at 324.7–329.3 and 246.1–250.0 nm plus a diagnostic sharp shoulder at 290–300 nm were unequivocally identified as chlorogenic acids.

Quantitative analyses (Tables 1 and 2) showed that the extracts of *A. moschata* VCI and VMS were characterized by flavonoids as major compounds (85.20 and 80.27 μg/mg DW, respectively) followed by phenolic acids (33.03 and 40.69 μg/mg DW, respectively). Among the flavonoid derivatives, apigenin was the predominant aglycone, free and glycosylated, followed by isorhamnetin, kaempferol and luteolin. The *A. moschata* VCI extract was characterized by apigenin as the main compound (60.36 μg/mg DW) followed by di-succinilcaffeoylquinic acid (25.23 μg/mg DW) and isorhamnetin-3-*O*-glucoside (12.29 μg/mg DW). Apigenin 7-*O*-glucoside was the principal metabolite of *A. moschata* VMS (44.66 μg/mg DW) followed by 4.5-*O*-di-caffeoylquinic acid (26.42 μg/mg DW), apigenin (19.22 μg/mg DW) and 5-*O*-caffeoylquinic acid (14.27 μg/mg DW).

In agreement with previous phytochemical studies, our data confirmed that in the genus *Achillea*, flavonoids mainly occur as flavones and flavonols and their derivatives. They are usually mono- and diglycosides of apigenin, luteolin and quercetin. Like *A. moschata*, many *Achillea* species also accumulate free flavonoid aglycons [15–17]. In the same way, in addition to flavonoids, phenolic acids including caffeoylquinic acids were also quantified in other *Achillea* species [18–21].

Achillea species including *A. moschata* are rich in polyphenols, which are partially responsible of their biological activities [16].

3.2 Antiradical activity

The scavenging ability of the different extracts against the $DPPH^{\cdot}$ stable radical, expressed as IC_{50}, is reported in

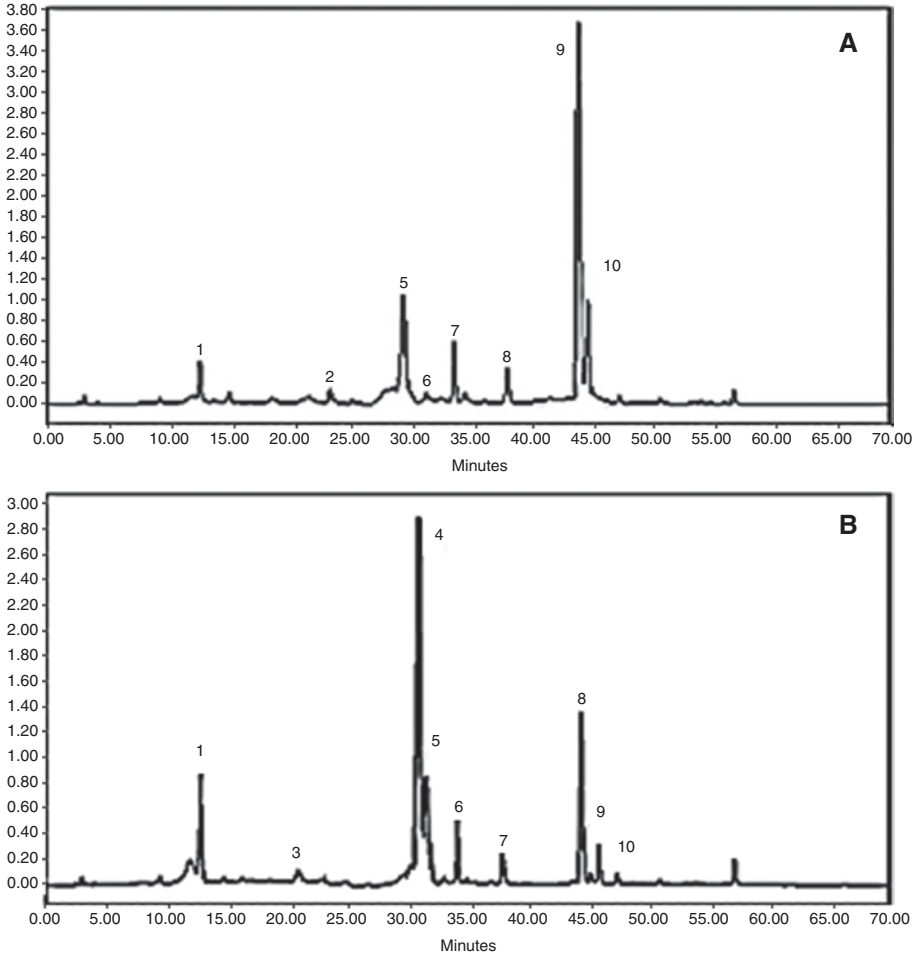

Figure 1: HPLC chromatograms (310 nm) of the methanolic extracts of (A) *A. moschata* VCI and (B) VMS: (1) 5-*O*-caffeoylquinic acid, (2) di-succinilcaffeoylquinic acid, (3) luteolin 7-*O*-glucoside, (4) apigenin 7-*O*-glucoside, (5) 4,5-*O*-di-caffeoylquinic acid, (6) kaempferol-3-*O*-glucoside, (7) luteolin, (8) apigenin, (9) isorhamnetin-3-*O*-glucoside and (10) isorhamnetin-3-*O*-rutinoside.

Table 3. In general, as expected, MeOH extracts from both samples – VCI and VMS – showed a higher activity in comparison with the DCM (~10-fold lower) and PET (40- to 50-fold lower) residues. Their removal action was similar to that of quercetin and four- to fivefold stronger than that of Trolox®, used as reference compounds (Table 3). Equally, the $ABTS^{\cdot+}$ assay demonstrated that the antiradical activity was noted only in the polar extracts (MeOH) (Table 3). Low values were obtained for the DCM and PET extracts (Table 3). No significant difference was reported in the two *A. moschata* samples. The obtained results are in agreement with those previously reported for *A. moschata* [12].

According to the literature, phenolic content and antioxidant activity of the *Achillea* species are particularly investigated in relation to the traditional use of these plants in order to identify the active compounds responsible for the wide range of their pharmacological activities [17].

Although there may be significant inter- and intraspecific differences in the polyphenol content of the *Achillea* species due to factors such as their origin, the plant growth stage, the analyzed plant part, the extraction solvent and the extraction method, previous data suggested that phenolic acids and flavonoids may be the major contributors for the radical scavenging activity of the *Achillea* extracts [17–19, 22–24].

3.3 Antibacterial activity

The antibacterial results displayed as inhibition diameters are summarized in Table 4. MeOH extracts were inactive against almost all tested microorganisms. Only the Italian *A. moschata* VCI extract was weakly effective on *B. cereus* (inhibition diameter, 11 mm), and

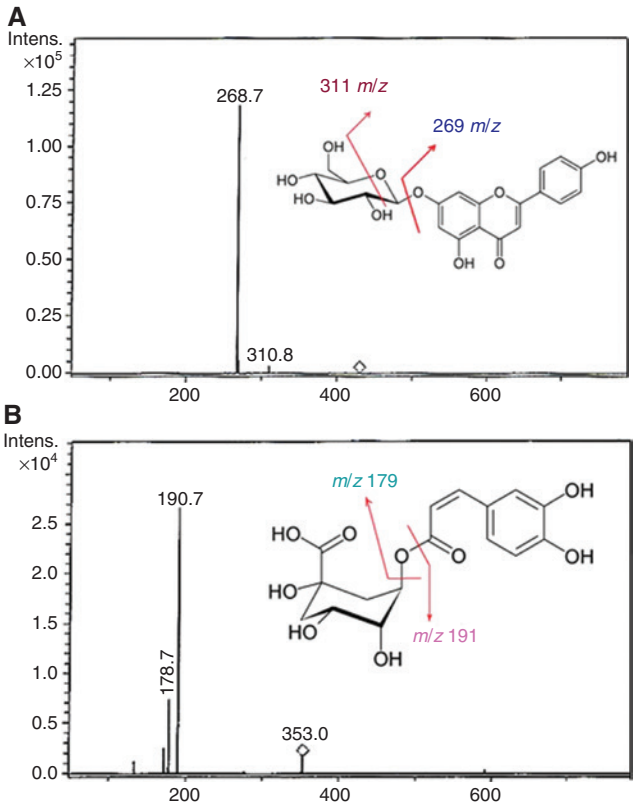

Figure 2: Typical fragmentation patterns of flavonoids (A) and phenolic acids (B).

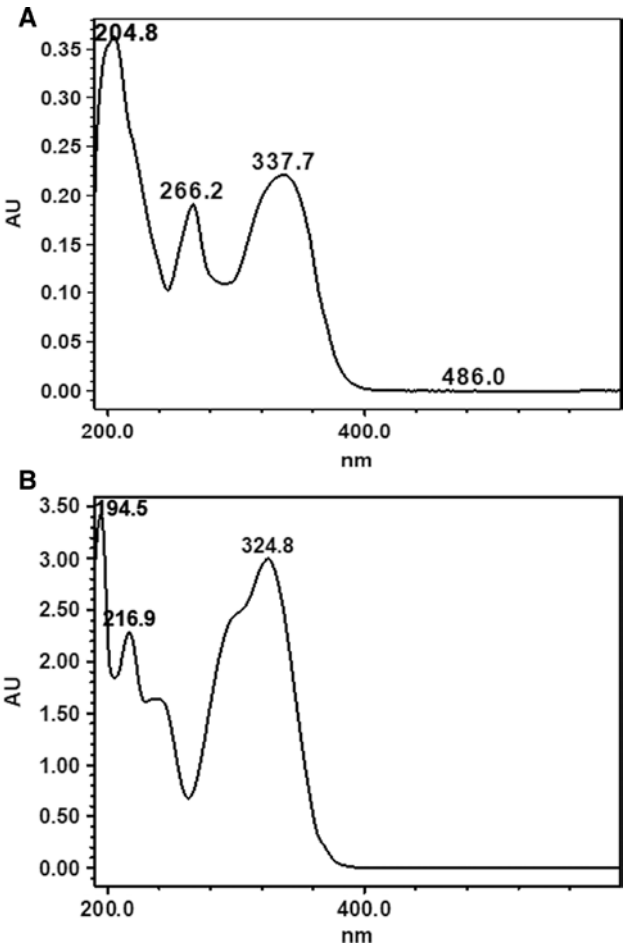

Figure 3: UV spectra showing typical behavior of flavonoids (A) and chlorogenic acids (B).

A. moschata VMS on *B. cereus* and *S. aureus* (8 mm, respectively). Otherwise, both DCM extracts showed different moderate activity against all three Gram-positive bacteria (*B. cereus*, *E. faecalis* and *S. aureus*) and *P. mirabilis* (Gram-negative), with inhibition diameters ranging from 10 to 16 mm. In particular, DCM extracts inhibited the growth of *B. cereus* similarly to the reference antibiotic erythromycin (16-mm diameter). Moreover, the DCM extract from *A. moschata* VMS was slightly active against *P. aeruginosa* (8 mm).

PET extracts were found to be active against all tested bacteria (inhibition diameters ranging from 8 to 15 mm), with the exception of *P. aeruginosa*, which was not inhibited by the extract of the VCI sample. On the other hand, it is a type of bacterium – Gram-negative – against which, in general, *A. moschata* was less effective (Table 4), which is in agreement with previous results from other *Achillea* species [12, 20]. Disks only impregnated with extraction solvents used as negative controls showed no bacterial growth inhibition. The ceftazidime reference disks showed inhibition diameters ranging from 17 to 23 mm against *E. coli*, *P. mirabilis* and *P. aeruginosa*, while erythromycin disks inhibited *B. cereus* and *S. aureus* growth with diameters ranging from 16 to 20 mm. Erythromycin was not able to be effective toward *E. faecalis*.

A. moschata, as other *Achillea* species (e.g. *A. millefolium* L.), is traditionally used against bacterial infections [7, 12, 25]. Some in vitro studies demonstrated sufficient evidence for their ethnopharmacological use. The extracts from aerial parts of different *Achillea* species were found to possess a broad spectrum of antimicrobial activity against several pathogenic organisms, including specific strains both of Gram-positive and Gram-negative bacteria [25, 26]. In some cases, the reported data confirmed our results showing the greater efficacy of the less polar extracts than the methanolic extracts [12, 26].

Table 1: Quantification results for phenolic compounds in the methanolic extract from *A. moschata* VCI and VMS.

Peak (#)	Compounds	VCI μg/mg DW	VMS (±SD)
1	5-*O*-caffeoylquinic acid	5.87 (0.06)	14.27 (0.23)
2	Di-succinilcaffeoylquinic acid	25.23 (0.39)	–
3	Luteolin 7-*O*-glucoside	–	2.67 (0.18)
4	Apigenin 7-*O*-glucoside	–	44.66 (0.64)
5	4,5-*O*-di-caffeoylquinic acid	1.93 (0.72)	26.42 (0.84)
6	Kaempferol-3-*O*-glucoside	6.05 (0.27)	5.04 (0.26)
7	Luteolin	4.91 (0.15)	3.73 (0.18)
8	Apigenin	60.36 (0.14)	19.22 (0.66)
9	Isorhamnetin-3-*O*-glucoside	12.29 (0.14)	3.46 (0.40)
10	Isorhamnetin-3-*O*-rutinoside	1.59 (0.60)	1.49 (0.08)
	Phenolic acids	**33.03**	**40.69**
	Flavonoids	**85.20**	**80.27**

Table 2: Summary of the phenolic compounds identified in the methanolic extract from *A. moschata* samples.

Compounds	UV (λmax), nm	ms1 v	ms2
5-*O*-caffeoylquinic acid	216.6, 246.5 *sh*, 301.5 *sh,* 325.7	353 $[M-H]^-$	191 (100) $[(M-H)-162]^-$; 179 (27) $[(M-H)-174]^-$
Luteolin 7-*O*-glucoside	256.7, 348.5	447 $[M-H]^-$	285 (100) $[(M-H)-162]^-$
Apigenin 7-*O*-glucoside	267.4, 338.5	431$[M-H]^-$	311 (3) $[(M-120)-H]^-$; 269 (100) $[(M-H)-162]^-$
4,5-*O*-di-caffeoylquinic acid	217.7, 299.5 *sh*, 329.1	515 $[M-H]^-$	353 (100) $[(M-162)-H]^-$; 191 (16) $[(M-324)-H]^-$
Disuccinyl-caffeoylquinic acid	267.4, 330.5	623 $[M-H]^-$	315 (100) $[(M-H)-162-146]^-$; 299 (29) $[(M-324)-H]^-$
Kaempferol-3-*O*-glucoside	267.4, 336.5	447 $[M-H]^-$	285 (100) $[(M-H)-162]^-$
Luteolin	252.2, 348.5	285 $[M-H]^-$	270 (100) $[(M-15)-H]^-$; 255 (33) $[(M-30)-H]^-$; 243(22) $[(M-42)-H]^-$
Apigenin	267.4, 336.5	269 $[M-H]^-$	225 (48) $[(M-44)-H]^-$
Isorhamnetin-3-*O*-glucoside	255.5; 349.7	477 [M-H]	315 (100) $[(M-162)-H]^-$
Isorhamnetin-3-*O*-rutinoside	256.8; 352.6	623 $[M-H]^-$	315 (100) $[(M-H)-162-146]^-$; 299 (29) $[(M-H)-324]^-$

Table 3: In vitro antiradical activity of *A. moschata* extracts measured by $ABTS^{\cdot+}$ radical cation and DPPH · free radical assays.

Samples	ABTS, μmol eq Trolox/mL	DPPH IC_{50}, mM
MeOH		
VMS	4.77 (0.01)	2.61 (0.24)
VCI	3.91 (0.07)	3.52 (0.21)
DCM		
VMS	0.99 (0.01)	25.9 (0.56)
VCI	0.87 (0.01)	31.5 (0.49)
PET		
VMS	0.47 (0.01)	109 (1.33)
VCI	0.53 (0.02)	177 (1.94)
Quercetin	n.d.	4.39 (0.12)
Trolox	n.d.	12.9 (0.28)

Tests were performed in triplicate; means (±SD).

4 Conclusions

This comparative study showed some differences in the polyphenol content of the methanolic extracts of the *A. moschata* aerial parts, probably due to the different pedoclimatic conditions and biotic and abiotic stresses in the alpine valleys of origin. Nevertheless, *A. moschata* VMS and VCI showed similar biological activity both in terms of antiradical and antibacterial effectiveness. The obtained results could be connected to its medicinal properties recognized by the local populations. Moreover, the analyzed data add to the knowledge about *A. moschata*, confirming that this species may be considered as a source of compounds to be exploited from a pharmaceutical point of view.

Table 4: In vitro antibacterial activity of *A. moschata* extracts measured by disk diffusion method against Gram-positive and Gram-negative bacterial strains.

Samples	Inhibition zone diameters, mm					
	B. cereus	*E. faecalis*	*S. aureus*	*E. coli*	*P. mirabilis*	*P. aeuriginosa*
MeOH						
VMS	+	–	+	–	–	–
VCI	++	–	–	–	–	–
DCM						
VMS	++	++	++	–	++	+
VCI	++	++	++	–	++	–
PET						
VMS	++	++	++	++	++	+
VCI	++	+	++	++	++	–

Experiment were done in triplicate. No activity (–); low activity (+): inhibition diameter <10 mm; moderate activity (++): inhibition diameter between 10 and 20 mm; high activity (+++): inhibition diameter >20 mm.

Acknowledgments: Authors gratefully thank ERSAF (Ente Regionale per i Servizi all'Agricoltura e alle Foreste – Regione Lombardia) – Direzione Parco Nazionale dello Stelvio for its support. The authors are also grateful to Mrs. Iole Pedroncelli for the collection of *Achillea moschata*.

Conflict of interest: The authors report no declarations of interest.

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