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Digestive and gastroprotective effects of *Achillea moschata* Wulfen: from traditional uses to preclinical studies

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Abstract: *Achillea moschata* Wulfen is an alpine endemic plant harvested by locals mainly for its digestive properties. Our purpose was to validate this prerogative documented from an ethnobotanical point of view with in vitro studies as a first step. To this end, the release of free fatty acids (+ 13% compared to the control) from the emulsified lipids as a result of the lipase activity was monitored using the pH-stat method. Moreover, other possible effects of *A. moschata* on the gastrointestinal system were evaluated by testing various extracts, whose volatile and non-volatile content was characterized by different techniques. All samples were considered active against the growth of both antimicrobial susceptible and resistant strains of *Helicobacter pylori* with MIC values starting from 16 μg/mL. They also showed interesting results when assessed in gastric, oesophageal and colorectal cell models. In particular, the dichloromethane extract distinctly inhibited their viability by MTT test, with IC_{50} equal to 168 μg/mL, 281 μg/mL and 96 μg/mL against NCI-N87, OE21 and Caco-2 cell lines, respectively. The obtained data are encouraging although they need to be further corroborated by studies involving the investigation of both the in vivo activities and the role of the compounds detected in the extracts.

Keywords: Asteraceae; antibacterial activity; antiproliferative activity; anti-inflammatory activity; lipid digestion; medicinal plants; traditional food; phenols; terpenoids; VOCs.

1. Introduction

The genus *Achillea* (Asteraceae) has been extensively studied from a pharmacological point of view due to the wide spectrum of disorders for which it is indicated in traditional medicine [1]. The countless data obtained are largely difficult to evaluate due to the variability of the taxonomic nomenclature used by researchers to identify the numerous species (more than 100) [2]. It is considered sufficiently demonstrated the aperitif and anti-dyspeptic action and, for external use, the anti-inflammatory and antibacterial potential [3–5]. Among the *Achillea* species, *A. millefolium* L. is the most widespread and one of the

most widely used medicinal plants in the world [2,6]. *A. moschata* Wulfen is one of the 23 species present in Italy. It is an endemic herbaceous perennial plant up to 20 cm tall with a strong camphor odor growing on the siliceous Alps starting from 1,800 meters above sea level (Figure 1A,B). Its stems, whose upper part is glandular, are woody and creeping with sterile shoots and erect flowering branches. The leaves are deeply 1-pinnatifid and their peduncles some-what glandular, the cauline ones are shortly petiolate or sessile. Flowers are arranged in white corymbs which develop between June and August (Figure 1C-E) [7]. *A. moschata* is a species collected for a long time and locally known as a powerful digestive, but traditionally used also for other properties (e.g., antineuralgic, balsamic, diuretic, hypotensive, sedative) including aromatic qualities [8–11]. In our ethnobotanical researches, *A. moschata* was the most quoted plant species (by more than 95% of the informants). In the study areas, the preference ranking put this species at first place, both for citations and for quantitative measures of relative importance [10,11]. The biological properties of *A. moschata* have so far been only marginally probed. The published works substantially indicate data on antioxidant and antibacterial activity [12–14]. This project aimed to investigate the aspects most closely related to its most frequent medicinal use reported by local people, trying to validate the putative digestive power of the infusion with in vitro studies as a first step. Moreover, the activity of different extracts (infusion, AE; ether petroleum, PET; dichloromethane, DCM; methanol, MeOH; essential oil, EO) against *Helicobacter pylori* was evaluated. *H. pylori* is a spiral-shaped, Gram-negative bacterium which infects about 50% of the world population. It is responsible for development of gastric ulcer, gastritis, MALT lymphoma and gastric carcinoma. So, cytotoxic effects of extracts on gastric and colorectal cell lines, were also investigated.

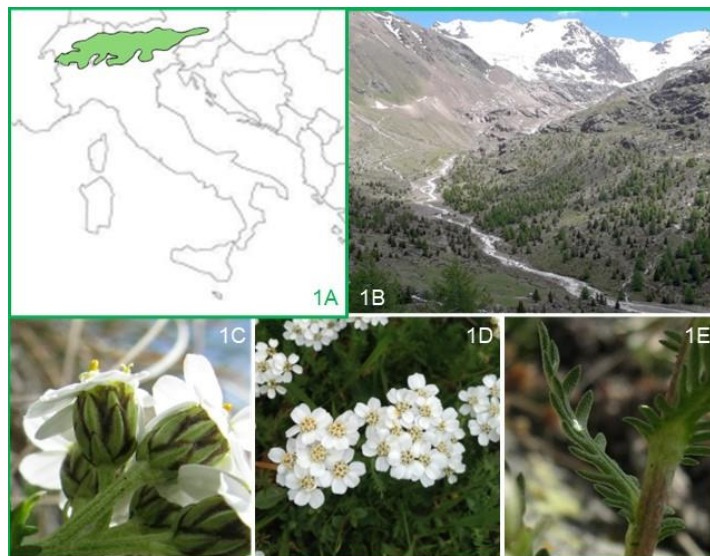

Figure 1. (**A**) Distribution range of *A. moschata.* [Adapted from 7]; (**B**) Growing environment; (C) Hemispherical floral involucre with subglabrous bracts characterized by brown cartilaginous margins; (D) Corymbs consisting of flower heads with tubular and ligulate flowers; (E) Leaves pinnatifid divided into acute lacinia.

2. Results and Discussion

2.1. SPME-GC/MS analysis of A. moschata aerial parts and extracts

To describe the volatile chemical profile of *A. moschata*, the SPME-GC/MS technique was performed. In the dried and powdered aerial parts (Table 1), 33 and 29 compounds were found, respectively. Both matrices were rich in camphor (45.91% and 39.20%) and in 1,8-cineole (11.43% and 12.68%). Other compounds such as camphene, *p*-cymene, *β*-thujone and borneol showed similar percentage values while *α*-thujene (0.43% and 9.04%), *β*-caryophyllene (3.70% and 0.98%) and sesquicineole (4.70% and 8.89%) were the detected compounds with major quantitative differences.

Table 1. Percentage (%) volatile composition of dried and powdered aerial parts analyzed by SPME-GC/MS.

Number	Compounds	LRI[2]	LRI[3]	Dried aerial parts (%)	Powdered aerial parts (%)
1	santolina triene	900	905.3	tr	0.14
2	*α*-thujene	918	923	9.04	0.43
3	*α*-pinene	940	945	1.19	1.39
4	camphene	945	948	4.46	4.59
5	*β*-pinene	988	986	0.79	0.77
6	*α*-terpinene	1014	1010	0.24	Tr
7	*p*-cymene	1020	1016	2.67	3.15
8	1,8-cineole	1028	1025	12.68	11.43
9	cis-*β*-ocimene	1035	1033	0.49	1.15
10	*β*-terpinene	1042	1036	-	1.97
11	*γ*-terpinene	1059	1054	-	0.49
12	terpinolene	1084	1080	1.75	0.90
13	butanoic acid, 2-methyl-, 3-methyl butyl ester	1090	1094	-	0.15
14	*β*-thujone	1101	1099	4.88	5.00
15	chrysanthenone	1106	1103	0.14	1.00
16	camphor	1130	1125	39.20	45.61
17	pinocarvone	1139	1140	0.24	0.39
18	cosmene	1138	1130*	-	0.12
19	*α*-campholenal	1140	1132*	0.36	0.83
20	chrysanthenol	1146	1143	0.31	0.34
21	borneol	1157	1155	3.74	2.51
22	*α*-terpineol	1183	1182	1.18	1.08
23	carvone	1218	1215	0.31	0.17
24	bornyl acetate	1265	1262	2.15	5.72
25	*p*-cymene-7-ol	1275	1270	0.24	-
26	*β*-caryophyllene	1428	1424	0.98	3.70
27	cis-*β*-farnesene	1447	1444	-	0.34
28	humulene	1470	1473	0.40	0.32
29	*α*-curcumene	1481	1478	0.15	0.12
30	*β*-eudesmene	1485	1483	2.02	0.56
31	zingiberene	1489	1490	0.15	-
32	sesquicineole	1507	1503	8.89	4.70
33	*α*-farnesene	1511	1506	0.53	0.32
34	caryophyllene oxide	1590	1586	0.69	1.01
	Total			99.87	100.00
	Monoterpenoids			86.06	88.52
	Sesquiterpenoids			13.78	11.07
	Others			-	0.41

[1]The components are reported according to their elution order on apolar column; [2]Linear Retention indices measured on apolar column; [3]Linear Retention indices from literature; [4]Percentage values of *A. moschata* powder components; [5]Percentage values of *A. moschata* inflorescence components; *Normal Alkane retention index; - Not detected; tr: traces <0.1.

As for the extracts, 11 compounds were found in AE, 10 in PET, 6 in DCM and 5 in MeOH one (Table 2). All samples showed a prevalence of the monoterpene component characterized by the presence of camphor, bornyl acetate and sesquicineole, although with different percentage values. Some qualitative differences were also found. 1,8-Cineole was missing in the MeOH extract, camphene and β-caryophyllene were present only in PET and DCM extracts. Otherwise, *p*-cymene, β-terpinene and β-thujone were detected only in PET and AE extracts. Regarding the vapor phase chemical composition of EO, 17 compounds were identified among which camphor (39.06%) was the principal constituent followed by 1,8-cineole (12.08%) and bornyl acetate (11.01%). β-Caryophyllene (6.34%) and sesquicineole (4.84%) were the most abundant sesquiterpene compounds (Table 2).

Table 2. Percentage (%) volatile composition of *A. moschata* extracts analyzed by SPME-GC/MS.

Number	Compounds	LRI[2]	LRI[3]	AE[4] (%)	PET[5] (%)	DCM[6] (%)	MeOH[7] (%)	EO[8] (%)
1	camphene	945	948	-	8.14	0.73	-	1.43
	β-pinene	981	986	-	-	-	-	0.84
2 3	2(3H)-furanone, dihydro-5-methyl- 5-vinyl-	995	996.8	-	-	-	-	0.61
4	α-terpinene	1008	1010	0.55	-	-	-	0.84
5	*p*-cymene	1020	1016	0.55	1.96	-	-	8.70
6	1,8-cineole	1028	1025	13.77	14.38	18.35	-	12.08
7	β-phellandrene	1038	1034	-	0.49	-	-	-
8	β-terpinene	1042	1036	1.93	1.19	-	-	-
9	γ-terpinene	1059	1054	1.02	-	-	-	3.03
10	isoterpinolene	1080	1088	-	-	-	-	0.72
11	β-thujone	1101	1099	17.10	2.23	-	-	2.18
12	camphor	1130	1125	53.55	63.59	71.36	33.73	39.06
13	terpinen-4-ol	1160	1161	3.01	-	-	-	5.29
14	α-terpineol	1183	1182	2.16	-	-	-	0.84
15	bornyl acetate	1265	1262	4.12	3.22	4.01	9.07	11.01
16	β-caryophyllene	1428	1424	-	3.18	2.88	-	6.34
17	humulene	1460	1465	-	-	-	-	0.61
18	sesquicineole	1507	1503	2.01	1.62	2.67	22.95	4.84
19	α-farnesene	1511	1506	-	-	-	9.13	0.25
20	δ-selinene	1514	1509	-	-	-	25.12	-
	Total			99.77	100.00	100.00	100.00	98.67
	Monoterpenoids			97.76	95.20	94.45	42.80	86.63
	Sesquiterpenoids			2.01	4.80	5.55	57.20	12.04
	Others			-	-	-	-	-

[1]The components are reported according to their elution order on apolar column; [2]Linear Retention indices measured on apolar column; [3]Linear Retention indices from literature; [4]Percentage values of *A. moschata* aqueous extract components; [5]Percentage values of *A. moschata* PET extract components; [6]Percentage values of *A. moschata* dichloromethane extract components; [7]Percentage values of *A. moschata* methanolic extract components; [8]Percentage values of *A. moschata* essential oil components; - Not detected.

2.2. GC-MS analysis of A. moschata EO

The yield of *A. moschata* EO obtained by hydrodistillation was 1.08% (v/w, relative to dry weight material). Its chemical composition was characterized by 41 compounds of which caryophyllene oxide (20.41%) followed by camphor (11.62%) were the most abundant components. Nevertheless, other terpene derivatives such as α-bisabolol (8.03%), γ-eudesmol (7.67%), caryophylladienol II (6.98%), β-eudesmol (5.34%), corymbolone (5.13%) and bornyl acetate (4.88%) reached relevant percentage values. A series of minor compounds, ranging from 0.21% to 3.52%, completed the chemical profile of EO.

Table 3. Percentage (%) chemical composition of *A. moschata* essential oil analyzed by GC/MS.

Number	Compounds[1]	LRI[2]	LRI[3]	EO (%)[4]
1	α-pinene	940	945	tr
2	camphene	945	948	0.22
3	β-pinene	988	986	0.29
4	*p*-cymene	1020	1016	1.16
5	1,8-cineole	1028	1025	2.28
6	γ-terpinene	1059	1054	0.56
7	α-ocimene	1060	1057	0.47
8	β-thujone	1101	1099	0.70
9	hotrienol	1117	1114	0.53
10	camphor	1130	1125	11.62
11	trans-verbenol	1148	1144	0.33
12	borneol	1157	1155	1.41
13	terpinen-4-ol	1160	1161	2.44
14	α-terpineol	1183	1182	1.11
15	carveol	1204	1201	tr
16	fragranol	1215	1212	0.22
17	carvone	1218	1215	0.11
18	eucarvone	1251	1248	0.12
19	camphostene	1257	1255.8	0.23
20	bornyl acetate	1265	1262	4.88
21	2,5-bornanedione	1269	1264	0.30
22	cis-pulegone oxide	1278	1275	0.70
23	decanoic acid	1357	1353	3.52
24	β-caryophyllene	1428	1424	2.01
25	cis-β-farnesene	1447	1444	0.21
26	cabreuva oxide B	1461	1457	0.19
27	humulene	1469	1465	0.29
28	cabreuva oxide D	1477	1473	0.21
29	α-curcumene	1481	1478	0.39
30	sesquicineole	1507	1503	2.30
31	elemol	1546	1543	1.38
32	longipinocarvone	1405	1398*	0.75
33	caryophyllene oxide	1590	1586	20.41
34	γ-eudesmol	1631	1630	7.67
35	β-eudesmol	1663	1665	5.34
36	germacra-4(15),5,10(14)-trien-1α-ol	1685	1680*	1.13
37	α-bisabolol	1693	1689	8.03
38	corymbolone	1902	1898.7	5.13
39	palmitoleic acid	1938	1930	0.93
40	palmitic acid	1960	1951	3.44
41	caryophylladienol II	2330	2324*	6.98
	Total			99.99
	Monoterpenoids			29.15
	Sesquiterpenoids			62.42
	Others			8.42

[1]The components are reported according to their elution order on apolar column; [2]Linear Retention indices measured on apolar column; [3]Linear Retention indices from literature; *: Normal Alkane retention index; [4]Percentage values of *A. moschata* EO components; tr: traces <0.1.

2.3. GC/MS analysis of the apolar extracts

Apolar constituents in PET and DCM extracts were identified by means of chromatography-mass spectroscopy (GC/MS) analyses. Average percentages from each component peak in each replicate GC analysis are reported in Table 4. PET extract was characterized by a higher number of compounds (28) than DCM extract (18). Monoterpenes made up 44.9% of the total content in the PET extract, 36.06% in the DCM extract. Among them, oxygenated monoterpenes were the main compounds with

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camphor as the most abundant component (19.55% and 24.25%, respectively) followed by borneol (4.41% and 6.44%) (Table 4). Sesquiterpenes were present in smaller amounts in both extracts (9.87% and 11.48%), mainly represented by oxygenated sesquiterpenes (8.83% and 11.48%) (Table 4). The PET and DCM extracts were also characterized by the presence of triterpenoids representing respectively 16.56% and 24.99% of the total composition. In the first, *α*-amirine was the most abundant equal to 8.8% while in the second *β*-amirine prevailed (13.7%). Furthermore, both samples were characterized by the presence of sterols, in particular *β*-sitosterol, whose amount was halved in the PET extract compared to the DCM extract (4.5% vs 8.2%).

182 183 184 185 186 187 188 189 190

Table 4. Percentage (%) chemical composition of petroleum ether and dichloromethane extracts analyzed by GC/MS.

191 192 193

Number	Compounds[1]	LRI[2]	LRI[3]	PET (%)[4]	DCM (%)[5]
1	santolina triene	901	909	0.44	-
2	*α*-pinene	938	942	0.38	-
3	camphene	951	947	0.76	-
4	Sabinene	972	977	0.22	-
5	*β*-pinene	981	985	0.24	-
6	2-carene	1000	1002	0.39	-
7	*p*-cymene	1023	1024	0.31	-
8	1,8-cineole	1032	1036	4.37	0.34
9	cis-sabinene hydrate	1100	1102	3.88	1.21
10	trans-sabinene hydrate	1105	1087*	1.28	-
11	thujone	1117	1115	2.36	-
12	chrysantenone	1130	1126	0.90	0.36
13	terpin-1-ol	1131	1134	0.95	-
14	camphor	1140	1146	19.55	24.25
15	trans-pinene hydrate	1148	1151	-	0.34
16	cis-chrysanthenol	1155	1160	0.10	-
17	borneol	1160	1164	4.41	6.44
18	terpinen-4-ol	1172	1180	0.53	-
19	*α*-terpineol	1180	1189	1.43	0.70
20	isobornyl formate	1221	1227	-	0.48
21	isobornyl acetate	1283	1286	-	1.94
22	bornyl acetate	1285	1291	2.35	-
23	thymol	1287	1292	tr	-
24	*β*-caryophyllene	1422	1419	0.71	-
25	*β*-longipinene	1433	1430	tr	-
26	*α*-humulene	1461	1456	tr	-
27	*α*-curcumene	1490	1488	tr	-
28	*β*-bisabolene	1508	1511	0.18	-
29	sesquicineole	1510	1514	1.95	3.46
30	caryophyllene oxide	1477	1581	3.63	-
31	humulene epoxide	1604	1608	0.11	-
32	*γ*-eudesmol	1628	1632	3.14	3.23
33	caryophylla 4(12), 8(13)-dien-5α-ol	1631	1634*	-	1.70
34	*β*-eudesmol	1640	1649	-	2.01
35	*β*-sitosterol	2980	*	4.54	8.20
36	*β*-amyrin	3342	3337	7.77	13.75
37	*α*-amyrin	3368	3376	8.79	11.24
	Unidentified compounds			22.30	19.28
	Total			99.77	98.93
	Monoterpenoids			44.41	36.06
	Sesquiterpenoids			9.72	10.4
	Triterpenoids			16.56	24.99
	Others			0.44	-

[1]The components are reported according to their elution order on apolar column; [2]Linear Retention indices measured on apolar column; [3]Linear Retention indices from literature; *: Normal Alkane retention index; [4]Percentage values of *A. moschata* PET, petroleum ether extract components; [5]Percentage values of *A. moschata* DCM, dichloromethane extract components; tr: traces <0.1.

2.4. HPLC/MS analysis of the polar extracts

The chemical profile of *A. moschata* MeOH extract revealed that caffeic acid derivatives were the main metabolite group (27.83 µg/mg). In particular, 4,5-*O*-dicaffeoylquinic acid was the predominant compound (13.70 µg/mg) followed by 5-*O*-caffeoylquinic acid (7.91 µg/mg) (Table 5). About its flavonoidic composition (21.89 µg/mg), the UV spectra showed the presence of flavones, mainly luteolin and apigenin derivatives. Apigenin-7-*O*-glucoside (11.19 µg/mg) was the predominant followed by apigenin (5.38 µg/mg), luteolin-7-*O*-glucoside (3.93 µg/mg) and luteolin (1.39 µg/mg) (Table 5).

Comparing the MeOH extract with the AE, no difference was found from the qualitative point of view, with caffeic acid derivatives as the most abundant metabolites (Table 5). As regards the quantitative profile, a similar trend was observed. The total phenolic content was comparable between the two extract (49.72 µg/mg and 42.90 µg/mg, respectively) although apigenin-7-*O*-glucoside (12.77 µg/mg) was the predominant constituent of AE followed by 5-*O*-caffeoylquinic acid (10.73 µg/mg).

Table 5. Phenolic composition of methanolic and aqueous extracts analyzed by HPLC.

Number	Compounds	µg/mg Extract ± SD	
		MeOH	AE
1	5-*O*-caffeoylquinic acid	7.91 ± 0.04	10.73 ± 0.06
2	caffeic acid	0.22 ± 0.01	2.50 ± 0.20
3	luteolin-7-*O*-glucoside	3.93 ± 0.01	1.68 ± 0.02
4	disuccinylcaffeoylquinic acid	1.58 ± 0.00	2.16 ± 0.03
5	4,5-*O*-dicaffeoylquinic acid	13.70 ± 0.04	8.78 ± 0.07
6	apigenin-7-*O*-glucoside	11.19 ± 0.12	12.77 ± 0.11
7	3,5-dicaffeoylquinic acid	4.42 ± 0.00	2.19 ± 0.11
8	kaempferol-3-*O*-glucoside	tr	Tr
9	apigenin-7-*O*-rutinoside	tr	0.81 ± 0.05
10	luteolin	1.39 ± 0.00	0.09 ± 0.03
11	apigenin	5.38 ± 0.06	1.19 ± 0.00
12	isorhamnetin-3-*O*-glucoside	tr	Tr
13	isorhamnetin-3-*O*-rutinoside	tr	Tr
	Total	49.72 ± 0.28	42.90 ± 0.68
	Caffeic compounds	27.83 ± 0.09	26.36 ± 0.47
	Flavonoidic compounds	21.89 ± 0.19	16.54 ± 0.21

SD, standard deviation; MeOH, methanol extract; AE, aqueous extract.

In this work the untreated dried and powdered aerial parts of *A. moschata*, EO and AE, PET, DCM, MeOH extracts were investigated, for the first time, using the SPME-GC-MS technique. Further, EO, PET and DCM extracts were also characterized by direct injection in GC-MS apparatus.

In the literature, there are a small number of works conducted to study the chemical content of *A. moschata* extracts and, among these, none concerning their volatile profile through the SPME sampling technique [12–14]. Differently, the number of references reporting information on the composition of EOs [15–19] and solvent extracts [5,20,21] obtained from other *Achillea* species is much higher. They are rich sources of many classes of compounds including flavonoids, phenolic glycosides and sesquiterpenoids [22]. In particular, regarding EO, our results showed a sesquiterpene rich composition (62.42%) with caryophyllene oxide as the main component. This datum was in agreement with that

both of Afsharypuor and co-authors [23] for *A. wilhelmsii* Koch. and Turkmenoglu et al. [24] for *A. schischkinii* Sosn. On the contrary, previous works on *A. moschata* EO reported a prevalence of camphor and 1,8-cineole [12,14] or of terpinen-4-ol [25]. However, differences (o discrepancies) in the chemical composition could be due to factors such as the geographical area of growth, the climatic conditions, the development stage, the plant part, the plant storages and above all the method used for extraction [26–29]. Some of these causes could also justify the differences, especially quantitative, observed in the composition of the MeOH extract compared to those previously indicated [12,13]. As for apolar extracts, their characterization provided information on the presence of terpenoids including triterpenoids in common with other *Achillea* species such as *A. collina* Becker or *A. tenuifolia* Lam. [30,31].

2.4. Lipid digestion

The in vitro lipid digestion model was set to test the effectiveness of *A. moschata* AE under simulated small intestinal conditions. It was monitored for 20 min using the volume of NaOH added to the final emulsion in order to calculate the amount of free fatty acids (FFAs) generated by lipolysis (pH-stat method) (Figure 2).

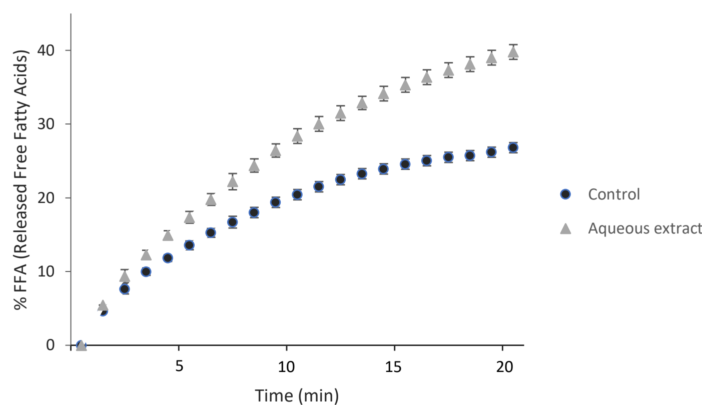

Figure 2. Free fatty acid release (%) during 20 min of in vitro intestinal digestion of extra virgin olive oil due to the effect of the infusion (AE) measured by pH-stat method.

Although the digestion of lipids in the small intestine is known to depend on various factors including health status, genetics, food consumed (type and quantity), time of day [32] and that the phytochemicals undergo modifications in the digestive system after intake [33], making the evaluation of the tested biological activity necessarily prudent, the results obtained showed a significant impact of *A. moschata* AE on this process. The percentage of released FFAs increased by 13.0% compared to that determined by the control sample. Pancreatic lipase is a key enzyme for the digestion and absorption of dietary triglycerides [34]. Therefore, it can be hypothesized a synergistic and/or additive action of *A. moschata* having a relevant effect on its activity by improving it and consequently influencing the lipid metabolism.

2.5. Growth inhibition of Helicobacter pylori by A. moschata extracts

The antimicrobial activity of *A. moschata* was also investigated against eight *H. pylori* clinical isolates and the reference strain ATCC 43504 by using the microdilution method. As reported in Table 6, PET, DCM and MeOH extracts showed a MIC_{50} of 32 μg/mL whereas EO showed a MIC_{50} of 64 μg/mL. Kuete [35] defined value for natural products

as follows: MIC below 100 μg/mL (significant activity), 100 ≤ MIC ≤ 625 μg/mL (moderate activity) and MIC > 625 μg/mL (low activity). Thus, all the extracts tested can be considered active against the growth on both antimicrobial susceptible and resistant *H. pylori* strains.

The AE activity in term of MIC_{50} and MBC_{50} was 3.125 mg/mL. By considering that the AE is an infusion, and all the components are more diluted respect to the extracts, the activity showed in this study is very interesting.

Table 6. Evaluation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of essential oil and extracts against clinical *H. pylori* strains with different antimicrobial susceptibility patterns.

***H. pylori* Strains**	**PET MIC/MBC (μg/mL)**	**DCM MIC/MBC (μg/mL)**	**MeOH MIC/MBC (μg/mL)**	**EO MIC/MBC (μg/mL)**	**AE MIC/MBC (mg/mL)**	**Antimicrobials susceptibility (μg/mL)**
F4	32/64	32/64	32/32	64/128	3.125/3.125	MNZ^R-CLR^R AMX^S-
190	32/64	32/64	32/32	64/64	3.125/3.125	MNZ^S-CLR^S AMX^S-
E17	32/64	32/32	32/32	64/64	6.25/6.25	MNZ^S-CLR^R AMX^S-
23	64/64	32/64	32/64	128/128	3.125/3.125	MNZ^S-CLR^S AMX^S-
110 R	32/64	32/32	64/64	64/64	6.25/6.25	MNZ^R-CLR^S AMX^S-
ATCC 43504	64/64	64/64	32/64	64/64	6.25/6.25	MNZ^R-CLR^S AMX^S-
F1	32/64	32/32	16/32	32/64	3.125/3.125	MNZ^S-$CLR^R$$AMX^S$-
F40/499	32/64	32/64	32/64	64/64	6.25/6.25	MNZ^R-CLR^R AMX^S
F40/442	64/64	32/32	32/32	64/64	6.25/6.25	MNZ^R-CLR^S AMX^S-

PET = petroleum ether extract; DCM = dicloromethane extract; MeOH = methanol extract; EO = essential oil. AE, aqueous extract.; MNZR = metronidazole resistant; MNZS = metronidazole susceptible; CLRR = clarithromycin resistant; CLRS = clarithromycin susceptible; AMXS = amoxicillin susceptible.

Mahady and coworkers [36] reported a study about the anti-*H. pylori* effect of MeOH extracts of different plants including *A. millefolium*. Although it is a different species, the MIC value from powdered aerial parts extracted with MeOH, was 50 μg/mL, a value very close to that we obtained (32 μg/mL) by considering the different concentration of starting MeOH solution. Therefore, both plants could have a similar antimicrobial mechanism of action. Because the predominant compound was apigenin-7-*O*-glucoside (12.77 μg/mg) followed by 5-*O*-caffeoylquinic acid (10.73 μg/mg), we can assume that these molecules are the active ones. The health beneficial effects of 5-*O*-caffeoylquinic acid that include also gastrointestinal protection are known [37]. So, other tests have to be performed in order to identify the antimicrobial compounds although the activity also depends on their structure and presence of other group of molecules.

2.6. BSL assay

The effects of the different extracts were tested in an invertebrate model, the brine shrimp (*Artemia salina*). The results showed that *A. moschata* were non-toxic for all lower concentrations of the tested extracts. Only higher concentrations (2 μg/mL for EO, 500 μg/mL for DCM, 1000 μg/mL for PET and MeOH, 500 μg/μL for AE) were able to decrease shrimp survival (Figure S1).

2.7. Cytotoxicity and clonogenic assay

The effects of the different extracts were tested in 3 cell line models, NCI-87, OE21 and Caco-2 (Figure 3). The IC_{50}s calculated for MTT tests were: a) for AE, 710±39 μg/mL in NCI-N87 cells, and 450±44 μg/μL in Caco-2 cells, (not reached for OE21 cells); b) for PET, 658±27 μg/mL in NCI-N87 cells, 612±41 μg/mL in OE21 cells, and 89±26 μg/mL in

Caco-2 cells; c) for DCM, 168±22 μg/mL in NCI-N87 cells, 281±15 μg/mL in OE21 cells, and 96±17 μg/mL in Caco-2 cells; d) for MeOH no IC_{50} was reached; e) for EO 1.48±0.25 μl/mL in NCI-N87 cells, 1.42±0.33 μl/mL in OE21 cells, and 3.44±0.51 μl/mL in Caco-2 cells. Likewise, the data obtained from SRB assay showed comparable results, even if with a slight different trend (Figure S2). Furthermore, clonogenic survival assay showed that ability to form colonies was significantly reduced in both cell lines when treated with DCM at 200 μg/mL (Figure S3).

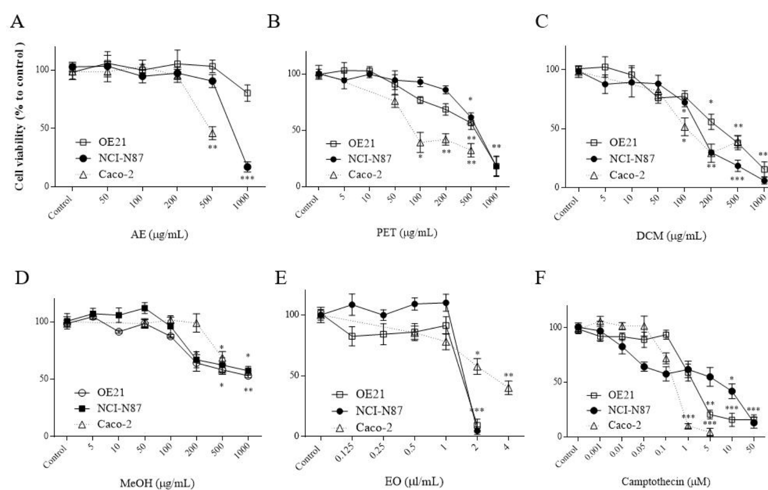

Figure 3. MTT assays for *A. moschata* extracts at different concentrations in NCI-N87 and OE21 cell lines. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Experiments were performed in triplicate and repeated three times. AE, aqueous extract; PET, ether petroleum extract; DCM, dichloromethane extract; MeOH, methanol extract; EO, essential oil. Camptothecin, positive control.

2.8. Anti-inflammatory activity

The results of the anti-inflammatory effect of *A. moschata* are shown in Figure 4. In general, IL-8 gene expression measured by RT-qPCR was reduced for higher concentrations of all samples. In particular, DCM and PET (50, 100, 200, 500 μg/mL) extracts demonstrated a significant capacity to reduce inflammation. Less effects were perceived with EO (all concentrations), while MeOH extract and AE possessed a minor anti-inflammatory role.

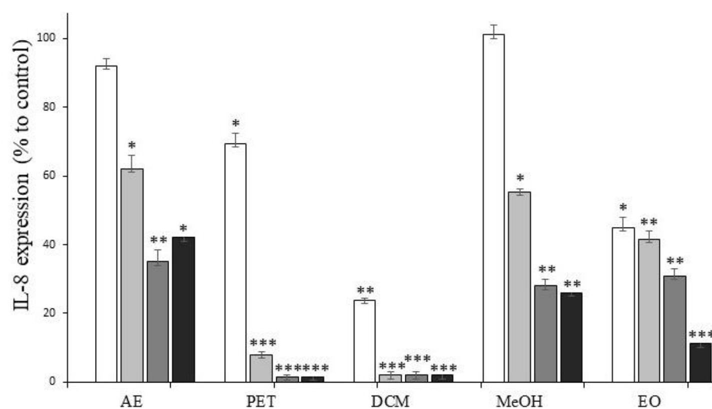

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Figure 4. The anti-inflammatory effect of *A. moschata* extracts in IL1β-elicited Caco-2 cells, determined by measuring IL-8 gene expression by RT-qPCR. Cells were incubated with various concentrations: white bars represent the lowest concentrations, dark bars represent max concentrations (see text for the concentrations used for each extract). The results are expressed as IL-8 expression percentage as compared to the maximum expression elicited by IL-1β in untreated cells, set to 100%. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. 325–330

To our knowledge, this is the first time that different *A. moschata* extracts were tested in gastric, oesophageal and colorectal cell models. Initially, we investigated whether these extracts could be lethal in a simple animal model. A screening test such as the BSL was applied. This reliable method uses an invertebrate to study a harmful compound or a mixture [38]. The BSL assay results demonstrated that IC_{50} doses (obtained by MTT) were non-toxic for animals (Figure S1), suggesting that these concentrations could be potentially used in higher animal models [39]. 331–337

Cytotoxicity assays showed that, among the different *A. moschata* extracts, the DCM extract markedly inhibited the cell viability by MTT test in all tested cell lines. These data suggest that the extraction in DCM preserves a bioactive mixture capable of impacting the cell viability of digestive system cell models (Figure 3), apparently more evident on colorectal cancer cell model. Of note that clonogenic assay revealed a decrease in cell colony formation at 200 μg/mL, remarking the considerable effect of this extract (Figure S3). Differently, PET extract was highly cytotoxic for Caco-2 cells if compared to the other two cell lines suggesting a cell-dependent effect [40,41]. It is unclear why colorectal cell model is more prone to the antiproliferative effects of PET extract. It can be speculated that the origin of the three cell lines is different, colorectal tissue for Caco-2 and gastroesophageal tissue for OE21 and NCI-N87. This difference is also reflected in the clinics for cancer type: colorectal cancer is the second most frequent with a high incidence and a 5-years survival rate of around 65% [42–44] while gastroesophageal cancer is less common, but with a reduced 5-years survival rate of approximately 46% [44–46]. Only novel experiments with the three cell models could lead to precisely uncover the biomolecular and genetic differences that lay at their basis. Moreover, we showed that SRB assay data were not always superimposable to MTT assay. Indeed, while MTT test analyses mitochondrial effects (measuring metabolic activity), SRB assay is based on the protein amount (measuring cell protein content). The two methods investigate different targets in cell models and, therefore, can give partially different results [47]. In addition, PET extract showed different behavior by not providing IC_{50} for the SRB test differently from what happened for the MTT one (Figure S2). 338–359

As IL-8 can mediate local tissue damage and spread the inflammatory response, we sought to understand whether *A. moschata* extracts could reduce inflammatory molecules. The results showed that, in different degrees, all samples could inhibit IL-1β-stimulated 360–362

IL-8 production in Caco-2 cells, suggesting that different compounds (alone or in synergic combination) of *A. moschata* could be responsible of this effect. Specifically, PET and DCM extracts possessed the strongest anti-inflammatory activity and similarly to MTT results, they could be called into question [48-50]. It is conceivable that these extracts may play a role in the prevention of intestinal inflammatory conditions, including cancer, nonetheless other research is needed to sustain this hypothesis.

4. Materials and Methods

4.1. Plant material

A. moschata aerial parts were collected at flowering stage on 12 August 2018 in Valle dei Forni, Sondrio, Italy (coordinates GPS: N 46°25'15'' E 10°33'22'') and identified according to Flora d'Italia (Pignatti, 1982). A voucher specimen was deposited at the Department of Agricultural and Environmental Sciences, Milan State University, Italy.

4.2. Sample preparation

The dried and subsequently powdered aerial parts of *A. moschata* (30 g) were sequentially extracted using PET, DMC and MeOH as solvents of increasing polarity. The extracts were evaporated to dryness in a rotary evaporator and stored at 4°C until analyses. Otherwise, the aqueous extract (AE) was obtained by adding 250 mL of boiling water to 2 g of plant material and leaving in infusion for 10 min to simulate the traditional herbal tea preparation. Then, the mixture was allowed to cool and filtered before use. The essential oil (EO) was repeatedly hydrodistillated for 3 h in a clevenger-type apparatus starting from 45 g of *A. moschata* each time, then preserved in airtight glass vials placed in the refrigerator.

For cytotoxic and anti-inflammatory tests, the extracts were dissolved in DMSO (w/v) to facilitate the solubility of the product. This solution was used for all experiments (unless otherwise indicated), aliquoted and stored at -80°C. After thawing, each aliquot was used only once.

4.3. Extract characterization

4.3.1. High Performance Liquid Cromatography (HPLC)

The HPLC analysis was performed on a Waters HPLC 600 Liquid Chromatograph (Milan, Italy) instrument equipped with a photo diode-array detector, DAD 2828 Waters. Data were processed with Empower™ 2 Waters Software. The analyses were running on a Gemini C18 (Phenomenex) column (250 x 4.6 mm i.d.; 5 μm particle size). The mobile phase was water containing 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 extract 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 (MeOH and AE) was injected for each run. For quantitative analysis, 5-level calibration curve was obtained by injection of known concentrations (10–250 μg/mL) of different standards compounds: luteolin-6-C-glucoside ($y = 44.625x + 119.464$; $R^2 = 0.9992$) and 5-*O*-caffeoylquinic acid ($y = 32.345x – 5.693$; $R^2 = 0.9999$).

4.3.2. Electrospray Ionization-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 acquisition and processing of the data. All analyses were carried out using a ESI ion source in the negative and positive 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 max acquire time set at 300 ms. Different collision energies were conventionally used (1.0, 10.0 and 30.0 V) for MS/MS fragmentation. Polar extracts 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).

4.3.3. Gas Chromatography (GC)

PET and DCM extracts were analyzed by gas-chromatography (GC) and gas-chromatography-mass spectrometry (GC-MS). A Trace GC Ultra Thermo Finnigan gas chromatograph equipped with a FID detector was used for the compositional analysis of the sample. It (1 µL) was injected in the cold on-column mode in a DB-5 (J&W Scientific) fused silica capillary column (30 m x 0.25 mm; 0.25 µm film thickness) and run at the following conditions: detector temperature, 300°C; column temperature was programmed from 60°C with 5 min hold up time to the final temperature of 270°C (30 min isothermal) at 4°C/min ramp. Each sample was analyzed in triplicate. Quantitative data of the extract constituents were expressed as a percentage composition from the total peak areas detected from GC-FID analyses without the use of correction factors. Hydrogen (H_2) was the carrier gas; air and H_2 were adjusted to obtain optimum separation. Data were processed using the Chrom-Card 32-bit computing software.

Commentato [mp2]: ok

4.3.4. Gas Chromatography-Mass Spectrometry (GC-MS) for apolar extract analysis

GC-MS analyses were carried out with a Hewlett Packard 6890-5973 mass spectrometer interfaced with a HP Chemstation. The chromatographic conditions were as follows: column oven program from 40°C (4 min isothermal) to 280°C (20 min isothermal) at 4°C/min; injector, 280°C. A HP-5 MS capillary column (30 m x 0.25 mm; 0.25 µm film thickness) was used. The carrier gas was helium at a flow rate of 1mL/min; ionization energy, 70 eV; ion source temperature, 230°C; electron current, 34.6 µA; vacuum 10-5 torr. Mass spectra were acquired over 40-550 amu range and a scan time of 1 s. The ion source was operating in the electron impact mode. Samples (1 µL) were injected using the splitless sampling technique.

4.3.5. Gas Chromatography-Mass Spectrometry (GC-MS) for EO analysis

For the chemical analysis of EO a gas chromatograph equipped with a FID (flame ionization detector) and coupled with a mass spectrometer was used (Clarus 500 model Perkin Elmer -Waltham, MA, USA). The analytical applied were as previously reported with some modifications [51]. The capillary column was a Varian Factor Four VF-1 (60m x 0.25 mm; 0.32 µm film thickness) and the optimized temperature program was the following: from 60°C to 170°C at 4°C/min; from 170°C to 220°C at 5°/min and held for 15 min. The components were identified by comparison their linear retention indices (LRIs) calculated and relative to a mix. Furthermore, the matching their mass spectra against commercial libraries (NIST) was performed. All analyses were conducted in triplicate and the results were expressed as average percentages calculated by peak area normalization from GC-FID chromatograms without the use of an internal standard or correction factors.

4.3.6. SPME/ GC-MS analysis

To analyze the volatile composition of the extracts, powder and inflorescences, a SPME device from Supelco (Bellefonte, PA) 311 with 1 cm fiber coated with 50/30µm DVB/CAR/PDMS (divinylbenzene/carboxen/polydimethylsiloxane) was used [52,53]. The operative conditions for the sampling were the following: equilibration time of 30 min, sampling time of 40 min. Lastly, the SPME fiber was inserted in the injector of the GC-MS system maintained at 250°C and operating in the same conditions above reported.

4.4. Bioassays

4.4.1. In vitro lipid digestion (pH-stat)

The influence of the presence of *A. moschata* AE on the in vitro lipid digestion was assessed by FFA titration during pancreatic lipase activity in a thermostatic reaction vessel maintained at 37°C following Capuano and co-authors [54], with some modifications. The digestion mixture consisted of 5 mL buffer phosphate 5 mM (pH 7.0) prepared with AE extract (instead of distilled water), 5 mL extravirgin olive oil, 2 mL NaCl 3 mM, 2 mL bile extract 1.5%, 1 mL $CaCl_2$ 75 mM. After 5 minutes, pH was adjusted at 7.7 with NaOH 200 mM and 1 mL pancreatin from porcine pancreas (P3292, Sigma-Aldrich, Saint Louis, MO, USA) solution 5 mg/mL was added to the suspension. Acidity, obtained from FFA released by triglycerides hydrolysis, was titrated with NaOH 5 mM and the volume used was recorded every min, for a total of 20 min. As control, the olive oil was digested without AE. The FFA release as a percentage of the initial total lipid content was calculated by applying the formula: % FFA = $V_{NaOH} \times m_{NaOH} \times M_{lipids} / w_{lipids} \times 2$, where V_{NaOH} corresponds to the volume of NaOH required to neutralise the FFA produced, m_{NaOH} is the concentration of the NaOH solution used (in M), w_{lipid} is the total mass (g) of lipids initially present in the reaction vessel, and M_{lipid} is the molecular weight of oil (in g mol^{-1}).

4.4.2. Bacterial strains culture

Eight clinical strains of *H. pylori* with different antimicrobial susceptibility, isolated from antral mucosal biopsies of patients with chronic gastritis or duodenal ulcer, were used for this study. These strains have been previously used in other research studies [55,56]. A reference strain (*H. pylori* ATCC 43504) was used as control. The strains were plated on Columbia agar base (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with 10% horse serum, 0.25% bacto yeast extract (Oxoid, and incubated for 72 h at 37°C in microaerophilic atmosphere.

4.4.3. Anti-*Helicobacter pylori* activity

The antimicrobial activity was evaluated by modified broth microdilution method as previously described [57]. The MeOH, PET, DCM and EO were dissolved in DMSO at concentration of 10 mg/mL (w/v); AE was dissolved in medium just before the use, at concentration of 100 mg/mL (v/v). Twofold serial dilutions of the test compounds were prepared in a 96-well microtiter plate. The final sample concentrations of each sample was: 128, 64, 32, 16, 8, 4, 2, and 1 μg/mL. Each well was inoculated to give a final concentration $5x10^5$ CFU/well. The plates were incubated for 3 days in a microaerophilic atmosphere at 37°C. After 72 h of incubation, the plates were examined visually, and the lowest concentration showing complete inhibition of growth was recorded as the Minimum Inhibitory Concentration (MIC). For minimum bactericidal concentration (MBC), 10 μL of suspensions without visible growth, were plated on Columbia agar plates, and incubated at 37°C for 72 h in microaerophily. The MBC was defined as the lowest concentration of drug, that killed ≥ 99.9% of initial inoculum. Experiments were performed in triplicate and repeated three times.

4.4.4. Brine shrimp lethality (BSL) assay

Brine shrimp lethality (BSL) assay was performed following the method published elsewhere [58]. The concentrations of the extracts tested were: 1, 10, 50, 100, 500 μg/mL for DCM; 1, 10, 100, 1000 μg/mL for PET and MeOH; 0.05, 0.1, 0.2, 0.5, 1, 10, 100, 500 μg/μL for AE; 0.125, 0.25, 0.5, 1, 2 μl/mL for EO. Brine shrimps were hatched in a round shaped vessel (1 L), with sterile artificial seawater and continuous oxygen supply. After 48 h, the active nauplii were transferred to a 96 well plate. Twenty nauplii were added to each well and kept them in room temperature for 24 h under light. After that time, dead larvae were counted. The mean percentage mortality was plotted against the logarithm of concentrations and LC_{50} (concentration that kills fifty percent of the nauplii) was

determined using the probit analysis described by Finney [59] as well as linear regression equation using the software "Microsoft Excel 2010". Plant samples were considered toxic when mortality rates were > 50% [60]. The experiments were performed in quadruplicates and repeated 3 times.

4.4.5. Cell lines

NCI-N87, human gastric tubular adenocarcinoma (from ATCC), OE21, human oesophageal squamous cell carcinoma (from Sigma-Aldrich, Saint Louis, MO, USA) cells were used as a gastroesophageal model, while Caco2 cells, human colorectal adenocarcinoma (from ATCC) were used as a colorectal model. All cell lines were routinely grown in RPMI-1640 or DMEM medium and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin plus 100 μg/mL streptomycin, at 37°C in a humidified atmosphere of 5% CO_2.

4.4.6. Cytotoxicity assays

NCI-N87, OE21, Caco-2 cells were seeded in 96-well tissue plates at a density of 1 × 104 cells/wells in 100 μL medium and incubated for 24-48 h. Then, cells were treated with the tested extracts at various concentrations for 48 h. During all experiments, the vehicle (DMSO) used to dissolve the extracts in the medium never exceeded 0.1% (v/v) and did not show inhibitory effects on the cell viability. The concentrations of the extracts for NCI-N87 and OE21 were: 50, 100, 200, 500, 1000 μg/mL for AE; 5, 10, 50, 100, 200, 500, 1000 μg/mL for PET, DCM and MeOH; 0.125, 0.25, 0.5, 1, 2 μL/mL for EO. For Caco-2 cells, only 4 concentrations were used (50, 100, 200, 500 μg/mL for AE, PET, DCM and MeOH; 0.5, 1, 2, 4 μL/mL for EO). Camptothecin was also used as a cytotoxic drug (positive control) in all cell lines: 0.001, 0.01, 0.05, 0.1, 1, 5, 10, 50 μM. Experiments were performed in triplicate and repeated three times.

MTT assay and SRB assay were performed as already described [61,62]. Moreover, the IC_{50} value, which is defined as 50% of the maximum observed inhibitory effect on cell viability, was calculated. Experiments were performed in triplicate and repeated three times.

4.4.7. Clonogenic assay

NCI-N87 and OE21 cells were seeded in 6-well plates at a low density (1000 cells per well) and treated with DCM 0.5, 5, 50, 200 μg/mL for 24 h. Then cell medium was replaced with extract free medium and cell cultured for at least 1 week. Cells were then fixed and stained with crystal violet. Only colonies >50 cells were counted. Each experiment was performed in triplicate and repeated 2 times.

4.4.8. Anti-inflammatory assay

The anti-inflammatory potential of the extracts was determined on cultivated Caco-2 cells and by assessing the modulation of interleukine IL8 expression as previously described [63]. Caco-2 cells were seeded in 12-well plates and incubated in complete DMEM to confluence. Cells were treated with extracts (50, 100, 200, 500 mg/mL) for 1 h in the presence of IL-1β (20 ng/mL). Total RNA was extracted with the Aurum Total RNA Tissue Kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions, and one μg of RNA was reverse transcribed with the iScript Reverse Transcription Supermix for RT-qPCR kit (Bio-Rad, Hercules, CA, USA). Reaction conditions were: 5 min at 25 °C and 20 min at 46 °C. Reverse transcriptase were denatured for 1 min at 95°C. cDNAs were then diluted 1:100 with sterile water and 2 μL used as templates in quantitative PCR, using SsoAdvanced Universal SYBR Green Supermix and a CFX Connect Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Primers for IL-8 were: 5′-ATGACTTCCAAGCTGGCCGTGGCT- 3′ and 5′-TCTCAGCCCTCTTCAAAAACTTCTC-3′.The GAPDH reference gene was amplified with the following primers: 5′-

GGAAGGTGAAGGTCGGAGTC-3′ and 5′-CACAAGCTTCCCGTTCTCAG-3′. Cycling conditions were: 3 min at 95°C, then 40 cycles of denaturation (20 s at 95°C), annealing (30 s at 55°C), and extension (30 s at 72°C). cDNA from unstimulated Caco-2 cells was used as the calibrator. Negative controls were performed without cDNA. Relative amounts of target genes compared to the GAPDH reference gene were calculated according to Livak [64]. Results were expressed as fold changes of target genes expression with respect to the untreated control sample. Each individual treatment was performed in triplicate triplicate and two biological replicas.

5. Conclusions

There are many studies on the bioactivity of plant extracts in vitro, but very few have considered gastrointestinal digestion. This study showed that *A. moschata* was effective in gastroprotection, confirming its bioactive potential. Our findings provided a scientific basis for its traditional use as a digestive agent. However, further studies on animal models should be carried out to establish this property in vivo. Moreover, it could be interesting to perform other tests with the pure compounds in order to identify the molecules responsible for *A. moschata* activity and their mechanism of action.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: BSL assay; Figure S2: SRB assay; Figure S3: Clonogenic assay.

Author Contributions: Conceptualization, M.I. and S.V.; methodology, M.P.A., S.C., S.G., R.P., A.S. and F.S.; validation, M.P.A., S.G., R.P., J.C., F.S. and S.V.; investigation, M.P.A., J.C., D.C.; S.G., R.P., F.S., S.V., M.Z.; resources, M.P.A., S.C., S.G., M.I., R.P., A.S., F.S., S.V.; data curation, M.P.A., S.G., R.P., A.S., F.S., S.V.; writing—original draft preparation, M.P.A., S.G., R.P., A.S., F.S., S.V.; writing—review and editing, S.C., M.I., S.G., R.P. and S.V.; visualization, M.P.A., S.G., R.P., F.S. and S.V.; supervision, M.P.A., S.C., S.G., R.P., A.S., F.S., M.I. and S.V.; project administration, M.I. and S.V.; funding acquisition, M.I. and S.V. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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