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12 **Microwave-assisted Extraction of edible *Cicerbita alpina* Shoots and its LC-**
13 **MS Phenolic Profile.**

14

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21

22 **Abstract**

23 BACKGROUND: Crude extracts obtained from the edible shoots of *Cicerbita alpina* using
24 microwave assisted extraction have been qualitatively profiled by liquid chromatography
25 coupled with an Ion Trap Mass Spectrometry detector and an ESI interface (LC/ESI-MS³) for
26 their phenolic content. The main challenge of the present investigation was to create a working
27 strategy designed to obtain a rich phenolic profile despite the limited amount of starting plant
28 material and phytochemical data available.

29 RESULTS: The best extraction conditions (temperature 90°C; time 5 min; solvent
30 methanol:water 50:50; sample weight 3 g) were achieved using a full factorial 2⁴ experimental
31 design. Fifteen compounds, including flavonoid conjugates and phenolic acid derivatives were
32 detected and tentatively identified. The total phenolic content varied from 93.58 mg g⁻¹ GAE, for
33 the cultivated plant to 10.54 mg g⁻¹ GAE for the wild one whereas the total flavonoid content
34 varied from 145.00 mg g⁻¹ rutin for the cultivated plant to 25.22 mg g⁻¹ rutin for the wild one.

35 CONCLUSION: A total of eleven compounds are herein reported, for the first time, as coming
36 from this plant source.

37

38 *Keywords: Cicerbita alpina*; full 2⁴ factorial design; microwave assisted extraction; LC/ESI-MS;
39 phenolics.

40

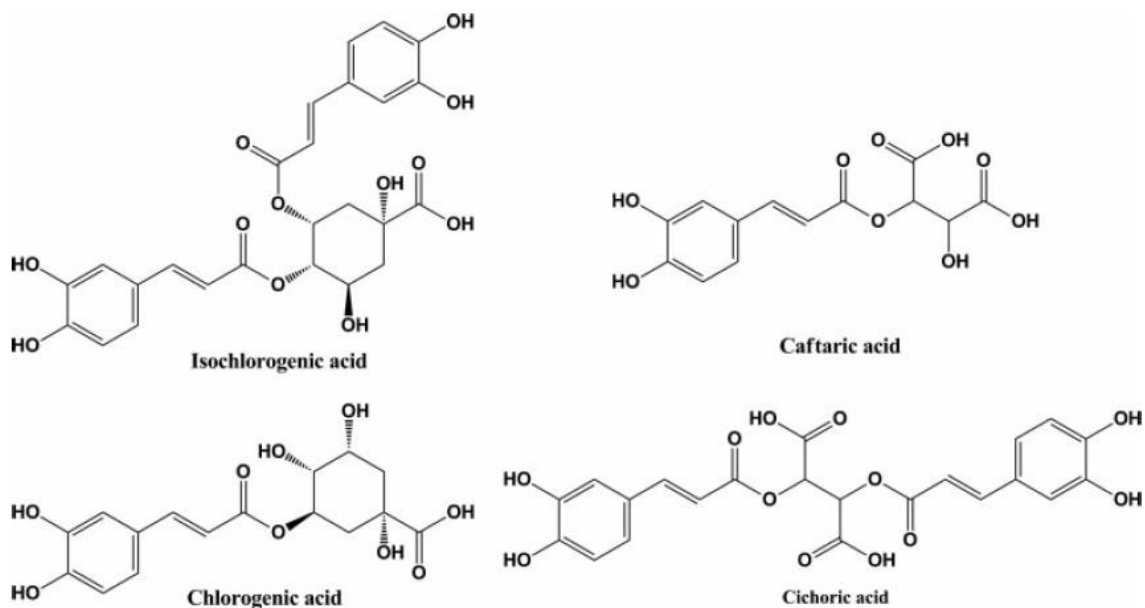
41 INTRODUCTION

42 *Cicerbita alpina* (L.) Wallroth. (Blue sow thistle) is a perennial alpine herbaceous plant
43 (*Asteraceae* family) which is itself common throughout the Alps. ¹ The traditional culinary use
44 of the edible shoots of wild plant is well known in the northeastern part of Italy. Moreover, this
45 species is of commercial value due to its limited diffusion which is safeguarded by two Friuli-
46 Venezia Giulia regional laws. ² As a result, a great deal of attention has recently been dedicated
47 to the cultivation of this herb. Having carried out an up-to-date literature survey of the chemical
48 composition of *C. alpina* it is clear that there is a lack of data regarding the aerial part of the
49 plant, but more detailed data on the composition of the roots is, however, available. Table 1 ³⁻⁷
50 sums up the secondary metabolites isolated and identified in *C. alpina* which have been reported
51 in the literature so far and the chemical structures of the phenolic compounds are shown in Fig.
52 1. Nowadays, phenolic compounds are one of the most common groups of phytochemicals in the
53 vegetal kingdom to play an important role in human and animal diet. They exhibit a wide range
54 of physiological properties including anti-allergenic, antiatherogenic, anti-inflammatory, anti-
55 microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects.⁸⁻¹⁰

Table 1. Secondary metabolites occurring in *C. alpina*

Plant organ	Extraction solvent	Isolated compounds	References
Air-dried subaerial parts	MeOH	8-Acetyl-15 β - Dglucopyranosyllactucin Sonchuside A	3,4
Air Dried Root	EtOH	8-Acetyl-lactucin	5

			8-Acetyl-11 β ,13-		
			dihydrolactucin		
			Lactucin		
			11 β ,13-Dihydrolactucin		
Freeze-dried shoot	MeOH, (CH ₃) ₂ CO		8-O-Acetyl-15- β -	D-	6
	H ₂ O		glucopyranosyllactucin		
			Chlorogenic acid		
			3,5- Dicaffeoylquinic acid		
			Caffeoyltartric acid		
			Cichoric acid		
Dried roots	EtOH		Imperatorin		7
Dried leaves			Isoimperatorin		
			Oxypeucedanin		
			Ostruthol		
			11 β ,13- Dihydrolactucin		
			8-Acetyl-15- β -		
			Dglucopyranosyllactucin		



57

58 **Fig. 1.** Chemical structures of phenolic compounds found in the edible *Cicerbita alpina* shoots.

59 One of the main characteristics required of any biological or analytical test is a systematic
 60 approach to the experiments. Design of experiment (DOE, ¹¹⁻¹³) is a statistical methodology that
 61 aims to plan and conduct experiments to extract the maximum amount of information in the
 62 fewest number of experimental runs. The basic idea is that of simultaneously varying all the
 63 relevant factors within a set of planned experiments. The result is a causal predictive model that
 64 shows the importance of all the factors and their interactions. These models can be summarized
 65 as informative contour plots that highlight the optimum combination of factor settings.¹⁴
 66 Screening is the first stage of such a process and the goal is simply to establish the most
 67 important factors together with their interactions. If the combinations of k factors are
 68 investigated at two levels, a factorial design will consist of 2^k experiments.¹¹ A factorial design
 69 explores the influence of all experimental factors and interaction effects on the response. In the
 70 present study, a design of experiments approach using microwave assisted extraction (MAE) has
 71 been developed for the extraction of phenols from the cultivated edible *C. alpina* shoots. The
 72 factors considered in MAE included: methanol/water ratio, sample quantity, extraction

73 temperature and time, whereas the response is expressed in total chromatographic peak area of
74 the most intense characteristic peaks monitored at 280 nm. MAE is widely recognized as a very
75 efficient technique for this application. ¹⁵⁻²⁰ LC/ESI-MS was used as the tools for phenol
76 investigation in the crude extract. Multistage MS analyses provided useful information about the
77 phenolic profile of *C. alpina*. Moreover, this is the first report where the preliminary results
78 obtained combined an improved MAE strategy with the LC/ESI-MS analysis for limited amounts
79 of starting plant material.

80

81 **Experimental**

82 **Chemicals**

83 The following flavonoids and phenolic acids were used as standards: acacetin (MW 284),
84 apigenin (MW 270), diosmin (MW 608), eriodictyol (MW 288), hesperetin (MW 302),
85 kaempferol (MW 286), luteolin (MW 286), caffeic acid, ferulic acid, gallic acid, para
86 hydroxybenzoic acid, syringic acid and two flavanone glycosides, apigetrin (apigenin 7-*O*-
87 glucoside MW 432) and hesperidin (hesperetin 7-rhamnoglucoside MW 610). HPLC-grade
88 methanol, HPLC-grade formic acid and Milli-Q grade water (Milli-Q Plus system, Milipore,
89 Bedford, USA) were used as solvents. All the chemicals were purchased from Sigma Aldrich
90 (Milan, Italy). 1mg mL⁻¹ stock solutions were prepared using flavonoid standards dissolved in
91 dimethylsulfoxide (DMSO) and phenolic acids in H₂O: MeOH (5:95, v/v).

92

93 **Plant material**

94 Edible shoots of wild *C. alpina* were collected on the 27th of May, 2010 from the area of
95 Collina/Forni Avoltri in the Udine province of Italy at 1800 m, whereas samples of the cultivated
96 *C. alpina* were collected on the 15th of May, 2010 from Piani di Vas/Rigolato, in the same
97 province, at an altitude of 1400 m. The cleaned fresh samples were stored at -20°C until they

98 were freeze-dried. Prior to extraction, the samples were reduced to a fine powder using a
99 grinding mixer (Analytical grinder A10 3250000, Ika, Germany).

100

101 **Design of experiment of microwave-assisted extraction (MAE)**

102 A MAE set was designed for the extraction of phenolics from the cultivated *C. alpina* shoots.

103 MAE experiments were performed with a professional microwave oven (Mars 1200 W, 2450

104 MHz, CEM Corporation, Matthews, North Carolina, USA). The instrument has an internal

105 temperature control system with an optical fiber thermometer and a pressure control system. 30

106 mL of solvent (MeOH/H₂O mixture) were added to 1.0-3.0 g of plant material which has been

107 placed in an inner vessel. The extraction was carried out under different extraction conditions at

108 300 W irradiation power. The extracts were filtered and the filtrates evaporated under vacuum.

109 The variables of the study include methanol/water ratio, sample quantity, extraction temperature

110 and time, whereas the response is expressed in the total chromatographic area of the most intense

111 characteristic peaks monitored at 280 nm. The coded values which correspond to the upper (+1),

112 intermediate (0) and the lower level (-1) ranges for each variable, are listed in Table 2.

Table 2. Factors and coded levels applied in the MAE procedure of total phenols

Independent variable	Lower level (-1)	Intermediate level (0)	Upper level (1)
X1: Extraction temperature	40°C	65°C	90°C
X2: Extraction time (min)	5	15	25
X3: Extraction solvent (%) (binary mixtures MeOH-H ₂ O)	50	75	100
X4: Sample quantity (g)	1	2	3

113

114

115 **2⁴ Full factorial design**

116 Modde procedures for design of experiments (Modde ver. 8.02, MKS Umetrics AB, Sweden)
117 were employed to design and analyze the experimental data. 19 experiments were used to
118 optimize the four aforementioned factors in the 2⁴ full factorial design applied to the MAE of
119 phenols from *C. alpina* edible shoots. The most important factors and their interactions are
120 depicted as contour plots of the MAE process. All runs were randomly performed in triplicate
121 and the chromatographic area averages of the most characteristic peaks at 280 nm were taken as
122 the response.

123 The model fit was evaluated by examining the summary of the fit, R² (measures fit), Q²
124 (measures predictive power), model validity (indicates if the model is appropriate) and
125 reproducibility, coefficients, ANOVA and the effect plots for screening designs. Model adequacy
126 was further assessed using a plot of residuals against predicted values.

127

128 **Total phenolic content micro-assay**

129 Phenolic content was determined, according to the method developed by Cicco *et al.*²¹, on the
130 crude extracts obtained under the optimized MAE conditions. The proposed method is a
131 variation on the classical Folin-Ciocalteu method which uses a new combination among time,
132 temperature, alkali and alcohol for the spectrophotometric evaluation of low-concentration
133 phenolics in methanol extracts. The absorption of the final mixtures was measured at 740 nm, in
134 a 1 cm cuvette, on a UV/VIS Varian Cary 1E spectrophotometer (Agilent Technologies, Santa
135 Clara, CA, USA). The former conditions enabled a high accuracy and reproducibility of the
136 assay. Quantification was carried out on the basis of a standard curve which was prepared using
137 different dilutions of a 1 mg mL⁻¹ solution of gallic acid in methanol as the reference phenolic
138 compound. Total phenolics values are expressed as gallic acid equivalents (mg g⁻¹ GAE of dry

139 weight DW). All measurements were performed in triplicate and expressed as averages \pm
140 standard deviation.

141 **Total flavonoid content**

142 The aluminum chloride colorimetric method was used for the determination of flavonoid content
143 in the crude extracts obtained under optimized MAE conditions.²² Each plant extract (0.5 mL)
144 was mixed with MeOH (1.5 mL), 10% aluminum chloride (0.1 mL), 1 M sodium acetate (0.1
145 mL) and distilled water (2.8 mL). Absorbance at 415 nm was recorded after 30 min of incubation
146 at room temperature. A standard calibration curve was generated at 415 nm using known
147 concentrations of rutin. Total flavonoid values in the test samples were calculated from the
148 calibration curve and expressed as mg rutin equivalents per g of sample.

149

150 **LC/ESI-MS analyses**

151 5 mg of freeze-dried crude cultivated herb extract were dissolved in 5% MeOH (2 mL). Prior to
152 the analysis, the sample was filtered through a 0.45 μm syringe filter. LC/ESI-MS analyses and
153 MSⁿ experiments were performed on a Finnigan LXQ linear ion trap operating in negative ion
154 mode coupled with a Finnigan Surveyor LC Pump Plus equipped with a Finnigan Surveyor
155 Autosampler Plus (Thermo Scientific, San Jose, CA, USA). The LC separations were performed
156 on a C18 Novapack 4 μm analytical column, 150 x 4.6 mm, operating at 30°C, 1 mL min⁻¹ flow
157 after 1:20 flow splitting (accurate flow splitter, LC Packings, Amsterdam, The Netherlands) and
158 with an acidified mobile phase composed of H₂O (0.1% HCOOH, v/v) and MeOH ((0.1%
159 HCOOH, v/v). The injection volume was 10 μL . Collision-induced dissociation (CID) multiple
160 MS spectra (MSⁿ experiments) were acquired using helium as the collision damping gas in the
161 ion trap at a pressure of 1 mTorr. The gradient elution steps were (time, % B): 0.01, 5; 5, 5; 20,
162 30; 30, 40; 40, 45; 50, 50; 60, 100; 70, 100. Mass selection of the analyte by m/z was followed by

163 fragmentation and fragment analysis. The full mass spectra were recorded in the 100-1000 m/z
164 range.

165

166 RESULTS AND DISCUSSION

167 Optimization of MAE conditions

168 In view of the greater availability of the cultivated, as opposed to the collected edible shoots of
169 *C. alpina*, the experimental design was conducted on this source. In order to optimize the four
170 extraction parameters, 19 software generated runs were used to evaluate the total
171 chromatographic area at 280 nm. The response values together with the experimental
172 combinations are listed in Table 3. The MAE total chromatographic area of the most
173 characteristic peaks at 280 nm ranged from 41218 to 74554 A.U. The crude extract of the wild
174 edible *C. alpina* shoots was obtained with the optimized MAE method. Two representative
175 HPLC chromatograms recorded at 280 nm and that correspond to the crude extracts of the
176 cultivated (A) and the wild edible shoots of *C. alpina* (B), both obtained following the optimum
177 MAE conditions, are shown in Fig. S1. The response contour plots that show the different
178 interactions between the variables are shown in Fig.2.

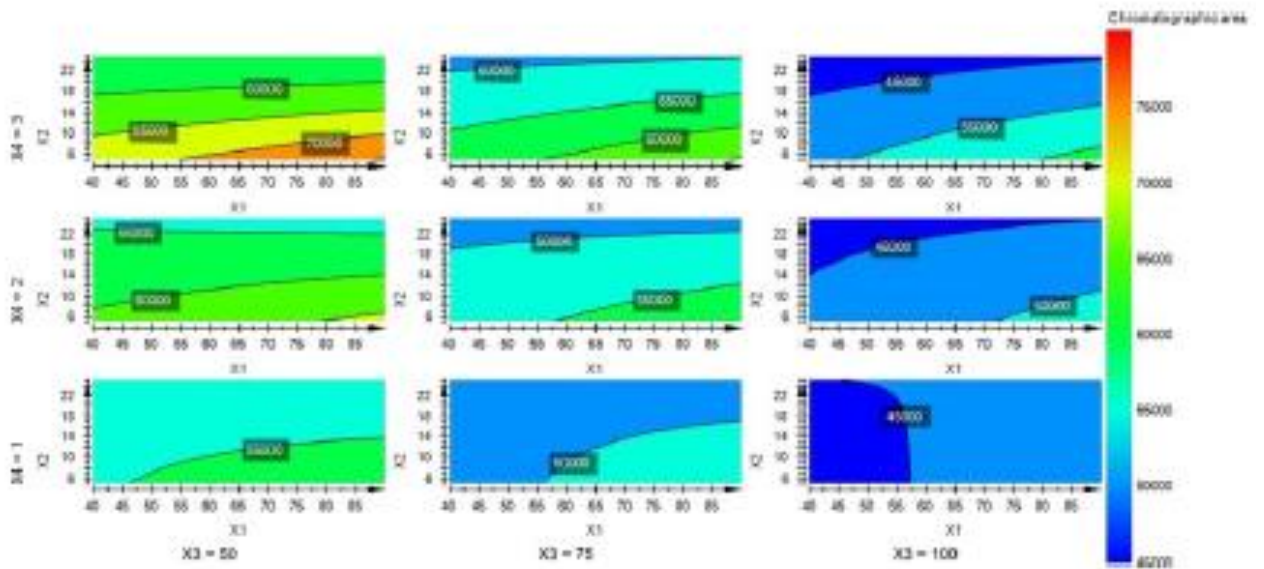
Table 3. Screening factorial design in terms of coded values and response values for total chromatographic area at 280 nm

Experiment no.	X1 (°C)	X2 (min)	X3 (%)	X4 (g)	Response (AU)
1	-1	-1	-1	-1	55 594
2	1	-1	-1	-1	59 216
3	-1	1	-1	-1	53 012
4	1	1	-1	-1	49 516
5	-1	-1	1	-1	41 344
6	1	-1	1	-1	48 943

7	-1	1	1	-1	46 335
8	1	1	1	-1	45 469
9	-1	-1	-1	1	66 283
10	1	-1	-1	1	74 554
11	-1	1	-1	1	56 078
12	1	1	-1	1	57 059
13	-1	-1	1	1	51 481
14	1	-1	1	1	55 857
15	-1	1	1	1	41 218
16	1	1	1	1	44 596
17	0	0	0	0	49 821
18	0	0	0	0	47 781
19	0	0	0	0	46 312

179

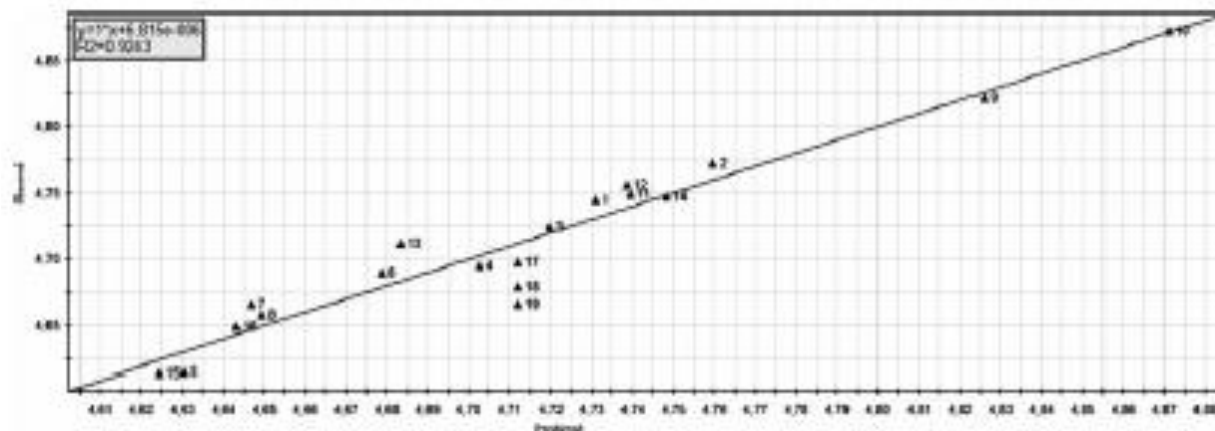
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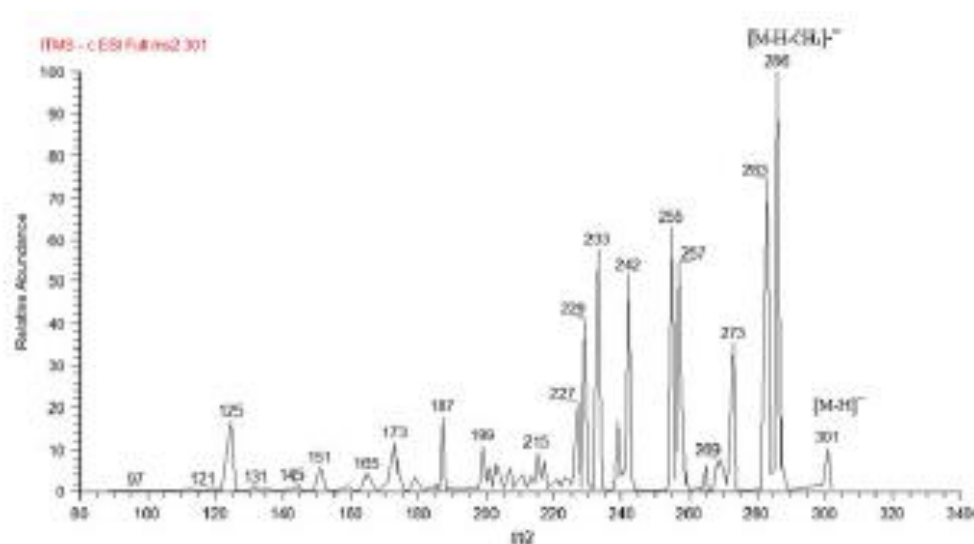
182 **Fig. 2.** UV-chromatograms of the crude extracts of the cultivated (A) and wild (B) edible shoots
183 of *C. alpina* recorded at 280 nm.

184



185

186 **Fig. 3.** Plot of residuals against predicted values.



187

188 **Fig. 4.** Product-ion spectra of hesperitin (parent ion m/z 301).

189 The ANOVA of the model showed that the values of the determination coefficient, R^2 , and the
190 adjusted determination coefficient, R^2 adj., were 0.9243 and 0.8295 respectively, which
191 demonstrates a high degree of correlation between the observed and the predicted values.

192 ANOVA also showed that the *p*-value of the model was 0.002 indicating that the model was
193 statistically significant at the 95% confidence level ($p < 0.05$).

194 In view of these results, the optimum MAE conditions were: extraction temperature (90°C),
195 extraction time (5 min), solvent ratio (50%) and sample quantity (3 g). Fig. 3 presents the
196 assessment of the model adequacy in the form of a plot of residuals against predicted values.
197 Under the optimal conditions, the model predicted a response of 76335 A.U. and a mean value of
198 74820 ± 1616 with RSD= 0.02% ($n=3$). The model adequacy was confirmed by the good
199 correlation between the results.

200 At higher temperature, solvent viscosity decreased enhancing the diffusivity and, thus, extraction
201 efficiency.^{23, 24} The reason for the higher extractability of phenols is the introduction of water to
202 methanol. The increase in plant tissue permeability can be explained by the presence of water
203 which enables better mass transfer via diffusion.²⁵ The volume of solvent used was indeed
204 important because a higher volume of solvent generally increases recovery in conventional
205 extraction techniques, but studies concerning recoveries in MAE show that in this case, lower
206 recoveries were achieved.^{15, 26, 27} The highest amount of phenolic compounds was achieved after
207 5 minutes of MAE irradiation, thus, it can be supposed that prolonged exposure to the irradiation
208 leads to the degradation of the phenols as the solute/solvent system overheats.²⁸

209

210 **Colorimetric analyses**

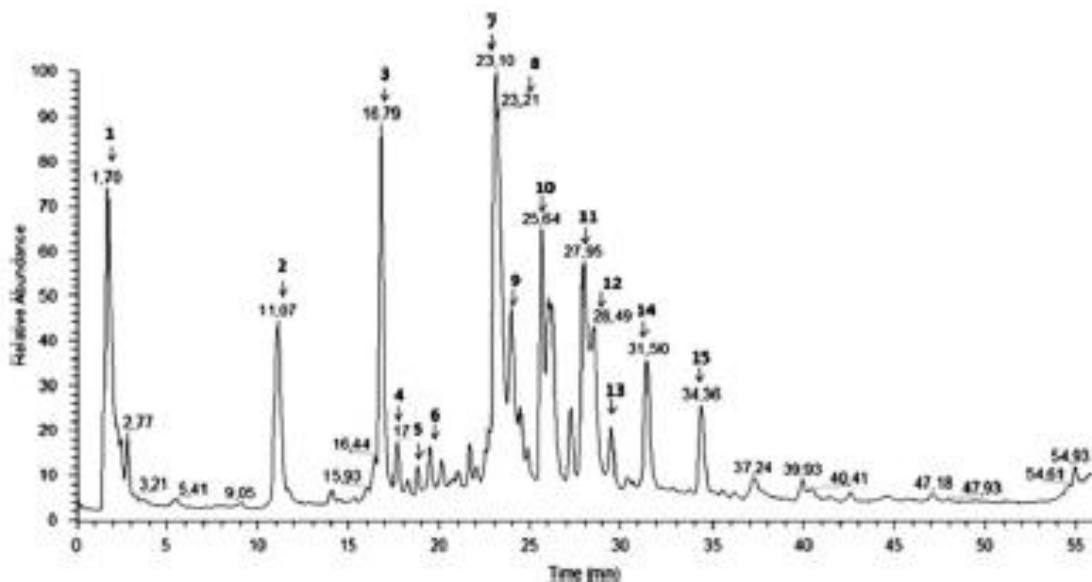
211 In order to roughly estimate the total phenolic and flavonoid content in the extracts, two basic
212 spectrophotometric assays were employed and preceded the LC-MS analyses. The total phenolic
213 content varied from 93.58 ± 0.47 mg g⁻¹ GAE, for the crude extract of the edible shoots of the
214 cultivated *C. alpina*, to 10.54 ± 0.41 mg g⁻¹ GAE for the wild one whereas the total flavonoid
215 content varied from 145.00 ± 0.59 mg g⁻¹ rutin for the crude extract of the cultivated *C. alpina*, to

216 25.22 ±0.21 mg g⁻¹ rutin for the wild one. According to Schieber *et al.* ²⁹, the Folin-Ciocalteu
217 method leads to an overestimation of the total phenolic content in plant extracts, due to
218 interference from reducing substances, so a chromatographic approach is essential for a better
219 estimation of the phenolic content.

220

221 **LC/ESI-MS analyses**

222 The analyses of the crude extracts by LC-MS were preceded by the analysis of a mixture of
223 common phenolic standards, which are available in our laboratory, in order to evaluate the
224 fragmentation behavior present in the product-ion spectra. According to Justensen ³⁰, the
225 product-ion spectra of the standard aglycones (for flavonoids) provided information about their
226 specific A-ring and B-ring fragmentation. The A-ring fragments *m/z* 151 and *m/z* 107 were
227 present as common fragments in the product-ion spectra of the main flavanone (eriodyctiol,
228 hesperitin) and flavone (apigenin, luteolin, acacetin) standard compounds. The specific B-ring
229 fragments observed for flavones and flavanones were: *m/z* 117 for apigenin, *m/z* 133 for luteolin
230 and *m/z* 135 for eriodyctiol. Mass spectra of the methoxylated flavonoids, acacetin and
231 hesperitin, revealed base peaks whose *m/z* values correspond to the loss of •CH₃ from the
232 deprotonated ion [M-H-15]^{•-} and did not provide B-ring fragments (see example in Fig. 5).



233

234 **Fig. 5.** HPLC-MS total ion chromatogram of the cultivated *C. alpina* crude extract

Table 4 Tentative peak assignments of *C. alpina* crude extract obtained by negative LC/ESI-MS

Peak	<i>t</i> R (min)	[M-H] ⁻ (m/z)	MS2 ions	MS3 ions	Tentative assignment
1	1.7	533	191 (21)	—	Quinic acid derivative
2	11.07	311	179 (65)	149 (30)	Caftaric acid
3	16.79	353	179 (9), 191 (100)	—	Caffeoylquinic acid
4	17.01	179	161 (54), 135 (100), 143 (69)	—	Caffeic acid
5	18.81	447	401 (2)	285 (100)	Luteolin derivative
6	19.38	431	385 (100)	—	1- <i>O</i> -β-D-Glucopyranosyl sinapate
7	23.1	463	287 (100)	—	Eriodyctiol glucuronide
8	23.21	473	311 (100), 293 (92), 179 (2)	—	Caffeic acid apiosyl glucoside
9	24.07	473	311 (100)	179 (58)	Cichoric acid
10	25.64	609	447 (7), 285 (100)	—	Kaempferol-3,7-di- <i>O</i> -glucoside
11	27.95	515	353 (100)	179 (53), 191 (100)	3,5- <i>O</i> - Dicaffeoylquinic acid
12	28.49	461	285 (100)	—	Luteolin glucuronide
13	29.33	593	447 (100)	—	Luteolin rhamnoglucoside
14	31.5	431	269 (57)	—	Apigenin-7- <i>O</i> -β- glucoside
15	34.36	533	489 (100)	285 (100)	Luteolin acetylglucoside

235

236 In this first stage of our study we analyzed the crude extract of the cultivated herb, rather than the
237 wild type, due to the higher number of compounds that absorb at 280 nm present in it. Fifteen
238 peaks, numbered as **1-15**, were detected and tentatively assigned as belonging to both flavonoid
239 and phenolic acid classes. Characterization of the phenolic compounds was based on the mass
240 spectra obtained under ESI and multistage MS, comparison with reference compounds and with
241 literature data.³⁰⁻³⁸ The HPLC-MS profile of the *C. alpina* extract is presented in Fig 5. MSⁿ
242 analyses in negative mode were performed on each molecule and the data obtained are
243 summarized in Table 4.

244 In negative MS analysis, the molecular ion occurs due to the breakdown of the *O*-glycosidic
245 bond in the 7-position. 162 amu are lost in monohexosides and 324 in dihexosides.³⁹ The MS
246 spectra of compounds **1-4** revealed specific fragments that characterize the presence of three free
247 phenolic acids (**2-4**) and a diglucoside of quinic acid (**1**) which is highlighted by the fragment
248 MS² 191, the loss of two hexose units and a water molecule. Based on literature data⁴⁰,
249 compounds **2** and **3** were tentatively assigned as caftaric acid ((m/z) 311→179→149) and
250 caffeoylquinic acid ((m/z) 353→179→191). Caffeic acid ((m/z) 179→161→135→143) was
251 identified by comparing its mass spectra with the one of a reference compound. Another mass
252 fragmentation pattern, similar to the one found for compound **2**, can be seen in the case of
253 compound **11**. In this case, the fragmentation pattern of 3', 5' caffeoylquinic acid is identical to
254 the same compound reported in other studies. A caffeic acid derivative found in the mass spectra
255 of compound **8**, is revealed by the presence of the fragment MS² 179 which corresponds to
256 caffeic acid and by the losses of 162 amu (hexose unit) and 132 amu (apiosyl moiety). Data
257 previously reported in literature and the mass fragmentation of compound **9** revealed a derivative
258 of caffeic acid (MW 180) and tartaric acid (MW 150), namely cichoric acid. The negative ESI-
259 MS spectra of compounds **2, 5, 9** and **11** are shown in Fig. 7. On the basis of literature data³¹⁻³⁹
260 and of the mass spectra analyses, four compounds (**5, 12, 13 and 15**) were tentatively assigned as

261 luteolin derivatives. In all cases, the fragmentation of the deprotonated ion resulted in fragments
262 having in common the aglycone m/z 285. Luteolin was identified by comparison its mass spectra
263 with an authentic standard. The assignment of compound **14** as apigenin-7-*O*- β -glucoside was
264 done via its comparison with a standard compound. Kaempferol 3'-7'-di-*O*-glucoside was
265 unambiguously assigned to compound **10**, on the basis of literature data and comparison with the
266 aglycone kaempferol reference standard.

267

268 **Conclusions**

269 A multitask approach was developed for the extraction and the analysis of the phenolic profile of
270 the edible shoots of cultivated *C. alpina*. The best extraction conditions were achieved using a
271 full factorial 2^4 experimental design that covered all the main extraction parameters. The
272 analytical approach was carried out by an original way to overcome the limitations deriving from
273 the scarce availability of the plant material. The developed microwave-assisted extraction of
274 phenolic compounds was followed by a qualitative LC/ESI-MS analysis which enabled their
275 identification. Research is in course to set up a comprehensive metabolic profile of the crude
276 extracts derived from this culinary herb.

277 **Acknowledgement**

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281 **References**

- 282 1 Funk VA, Susanna A, Stuessy TF, Robinson H, Classification of Compositae. Systematics,
283 Evolution, and Biogeography of Compositae, International Association for Plant
284 Taxonomy (IAPT) Vienna. 171-189 (2009).
- 285 2 BioInnovErbe project. La coltivazione biologica delle erbe spontanee: una nuova filiera
286 produttiva di qualità a salvaguardia dell'ambiente. Legge regionale sull'innovazione
287 n°26/05 art 17, 8: June the 3rd 1981, N.34 integrated by August the 19th 1996 N. 32 and
288 subsequent modifications (2008).
- 289 3 Zidorn C, Sesquiterpenes lactones and their precursors as chemosystematic markers in the
290 tribe Cichorieae of the Asteraceae. *Phytochemistry* **69**: 2270–2296 (2008).
- 291 4 Zidorn C, Schawha R, Ellmerer E, Stuppner H, On the occurrence of sonchuside A in
292 *Cicerbita alpina* and its chemosystematic significance. *J Serb Chem Soc* **70**:171–175
293 (2005).
- 294 5 Djordjević I, Tešević V, Janacković P, Milosavljević S, Vajs V, Sesquiterpene lactones from
295 *Cicerbita alpina*. *Biochem Sys Ecol* **32**: 209-210 (2004).
- 296 6 Fusani P, Zidorn C, Phenolics and a sesquiterpene lactone in the edible shoots of *Cicerbita*
297 *alpina* (L.) Wallroth., *J Food Comp Anal* **23**: 658-663 (2010).
- 298 7 Appendino G, Tettamanzi P, Gariboldi P, Sesquiterpene lactones and furanocoumarins from
299 *Cicerbita alpina*, *Phytochemistry* **30**: 1319-1320 (1991).
- 300 8 Benavente G. Uses and properties of Citrus flavonoids (Reviews). *J Agric Food Chem* **45**:
301 4505-4515 (1997).
- 302 9 Manach C, Williamson G, Morand C, Bioavailability and bioefficacy of polyphenols in
303 humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr* **81**: 2309-2425 (2005).

- 304 10 Middleton E, Kandaswami C, Theoharides TC, The Effects of Plant Flavonoids on
305 Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer, *Pharmacol*
306 *Rev*, **52**: 673-751 (2000).
- 307 11 Lundstedt T, Seifert E, Abramo L, Thelin B, Nyström A, Pettersen J, Bergman R
308 Experimental design and optimization. *Chemometrics and Intelligent Laboratory*
309 *Systems*, **42**:3-40 (1998).
- 310 12 Carlson R, Carlson JE, *Design and Optimization in Organic Synthesis*, Elsevier Science,
311 Amsterdam, pp. 596 (2005).
- 312 13 Riter LS, Vitek O, Gooding KM, Hodge BD, Julian RK, Statistical design of experiments as a
313 tool in mass spectrometry, *J Mass Spectrom* **40**: 565-579 (2005).
- 314 14 Box GEP, Hunter WG, Hunter J S, *Statistics for Experimenters* Wiley, pp. 100 (1978).
- 315 15 Eskilsson CS, Bjorklund E, Analytical-scale microwave-assisted extraction, *J Chromatogr A*
316 **902**:227-250 (2000).
- 317 16 Escribano-Bailon MT, Santos-Buelga C, *Methods in Polyphenol analysis*, London: RSC
318 Publishing, pp. 1-16 (2003).
- 319 17 Cheng L, Ding L, Yu AM, Wang XP Li JT, Continuous determination of total flavonoids in
320 *Platycladus orientalis* (L.) Franco by dynamic microwave-assisted extraction coupled
321 with on-line derivatization and ultraviolet-visible detection, *Anal Chim Acta* **596**: 164-
322 170 (2007).
- 323 18 Abourashed EA, Vanderplank JR., Khan IA, High-speed extraction and HPLC
324 fingerprinting of medicinal plants, *Pharm Biol* **40**:81-91 (2002).

- 325 19 Stalikas CD, Extraction, separation and detection methods for phenolic acids and flavonoids,
326 *J Sep Science* **30**: 3268-3295 (2007).
- 327 20 Casazza A, Aliakbarian B, Mantegna S, Cravotto G, Perego P, Extraction of phenolics from
328 *Vitis vinifera* wastes using non-conventional techniques, *J Food Eng* **100**: 50-55 (2010).
- 329 21 Cicco N, Lanorte MT, Paraggio M, Viggiano M, Lattanzio V, A reproducible, rapid and
330 inexpensive Folin–Ciocalteu micro-method in determining phenolics of plant methanol
331 extracts, *J Microchem.* **91**: 107-110 (2009).
- 332 22 Chang C, Yang M, Wen H, Chern J, Estimation of total flavonoid content in propolis by two
333 complementary colorimetric methods, *J Food Drug Anal* **10**: 178-182 (2002).
- 334 23 Camel V, Microwave-assisted solvent extraction of environmental samples, *Trends Anal*
335 *Chem* **19**: 229-248 (2000).
- 336 24 Pan X, Liu H, Jia G, Shu YY, Microwave-assisted extraction of glycyrrhizic acid from
337 licorice root, *J Biochem Eng* **5**: 173–177 (2000).
- 338 25 Arimboor R, Sarin R, Kumar K, Arumughan C, Simultaneous estimation of phenolic acids in
339 sea buckthorn (*Hippophaë rhamnoides*) using RP-HPLC with DAD, *J Pharm Biomed*
340 *Anal* **47**: 31-38 (2008).
- 341 26 Guo ZK, Jin QH, Fan GQ, Duan YP, Qin C, Wen MJ, Microwave-assisted extraction of
342 effective constituents from a Chinese herbal medicine *Radix puerariae*, *Anal Chim Acta*
343 **436**: 41–47 (2001).
- 344 27 Xiao, WH, Han LJ, Shi B, Microwave-assisted extraction of flavonoids from *Radix Astragali*,
345 *Separat Purif Technol* **56**: 397-400 (2008).

- 346 28 Huie CW A review of modern sample-preparation techniques for the extraction and analysis
347 of medicinal plants, *Anal Bioanal Chem* **373**: 23-30 (2002).
- 348 29. Schieber A, Keller P, Carle R, Determination of phenolic acids and flavonoids of apple and
349 pear by high-performance liquid chromatography, *J Chromatogr A* **910**: 265-273 (2001).
- 350 30 Justensen U, Negative atmospheric pressure chemical ionization low-energy collision
351 activation mass spectrometry for the characterization of flavonoids in extracts of fresh
352 herbs, *J Chromatogr A* **902**: 369-379 (2000).
- 353 31 Olsen H, Aaby K, Borge GIA, Characterization and Quantification of Flavonoids and
354 Hydroxycinnamic Acids in Curly Kale (*Brassica oleracea* L. convar. acephala var.
355 sabellica) by HPLC-DAD-ESI-MSⁿ, *J Agric Food Chem* **57**: 2816-2825 (2009).
- 356 32 Exarchou V, Fiamegos, YC, van Beek TA, Nanos C, Vervoort J, Hyphenated
357 chromatographic techniques for the rapid screening and identification of antioxidants in
358 methanolic extracts of pharmaceutically used plants, *J Chromatogr A* **1112**: 293-302
359 (2006).
- 360 33 Clifford MN, Wu W, Kirkpatrick J, Kuhnert N, Profiling the chlorogenic acids and other
361 caffeic acid derivatives of herbal chrysanthemum by LC/MSⁿ, *J Agric Food Chem* **55**:
362 929-936 (2007).
- 363 34 Krasteva I, Nikolov S, Flavonoids in *Astragalus corniculatus*, *Quim Nova* **31**: 59-60 (2008).
- 364 35 Lai JP, Lim YH, Su J Shen HM, Ong CN, Identification and characterization of major
365 flavonoids and caffeoylquinic acids in three Compositae plants by LC/DAD-APCI/MS,
366 *JChromatogr B* **848**: 215-225 (2007).

- 367 36 Liu G, Xu Z, Chen J Lang G, Tian Q, Shen, Y, Chen B, Yao S, On-line strategies for the
368 identification of unknown flavone glycosides in *Dracocephalum tanguticum* Maxim, *J*
369 *Chromatogr B* **877**: 2545-2550 (2009).
- 370 37 Lin LZ, Mukhopadhyay S, Robbins RJ, Harnly RJ, Identification and quantification of
371 flavanoids of Mexican oregano (*Lippia graveolens*) by LD-DAD-ESI/MS analysis, *J*
372 *Food Comp Anal* **20**: 361-369 (2007).
- 373 38 Tóth BH, Blazics B, Kéry A, Polyphenol composition and antioxidant capacity of *Epilobium*
374 species, *J Pharm Biomed Anal* **49**: 26-31 (2009).
- 375 39 Plazonić A, Bucar F, Maleš Ž Mornar A, Nigović B, Kujundžić N, Identification and
376 Quantification of Flavonoids and Phenolic Acids in Burr Parsley (*Caucalis platycarpus* L.),
377 Using High-Performance Liquid Chromatography with Diode Array Detection and
378 Electrospray Ionization Mass Spectrometry, *Molecules* **14**: 2466-2490 (2009).
- 379 40 Papetti A, Daglia M, Aceti C, Sordelli B, Spini V, Carazzone C, Gazzani G,
380 Hydroxycinnamic acid derivatives occurring in *Cichorium endivia* vegetables, *J Pharm*
381 *Biomed Anal* **48**: 472-476 (2008).