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

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Abstract

BACKGROUND: Crude extracts obtained from the edible shoots of *Cicerbita alpina* using microwave assisted extraction have been qualitatively profiled by liquid chromatography coupled with an Ion Trap Mass Spectrometry detector and an ESI interface (LC/ESI-MS^3) for their phenolic content. The main challenge of the present investigation was to create a working strategy designed to obtain a rich phenolic profile despite the limited amount of starting plant material and phytochemical data available.

RESULTS: The best extraction conditions (temperature 90°C; time 5 min; solvent methanol:water 50:50; sample weight 3 g) were achieved using a full factorial 2^4 experimental design. Fifteen compounds, including flavonoid conjugates and phenolic acid derivatives were detected and tentatively identified. The total phenolic content varied from 93.58 mg g^{-1} GAE, for the cultivated plant to 10.54 mg g^{-1} GAE for the wild one whereas the total flavonoid content varied from 145.00 mg g^{-1} rutin for the cultivated plant to 25.22 mg g^{-1} rutin for the wild one.

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

Keywords: *Cicerbita alpina*; full 2^4 factorial design; microwave assisted extraction; LC/ESI-MS; phenolics.

INTRODUCTION

Cicerbita alpina (L.) Wallroth. (Blue sow thistle) is a perennial alpine herbaceous plant (*Asteraceae* family) which is itself common throughout the Alps. [1] The traditional culinary use of the edible shoots of wild plant is well known in the northeastern part of Italy. Moreover, this species is of commercial value due to its limited diffusion which is safeguarded by two Friuli-Venezia Giulia regional laws. [2] As a result, a great deal of attention has recently been dedicated to the cultivation of this herb. Having carried out an up-to-date literature survey of the chemical composition of *C. alpina* it is clear that there is a lack of data regarding the aerial part of the plant, but more detailed data on the composition of the roots is, however, available. Table 1 [3-7] sums up the secondary metabolites isolated and identified in *C. alpina* which have been reported in the literature so far and the chemical structures of the phenolic compounds are shown in Fig. 1. Nowadays, phenolic compounds are one of the most common groups of phytochemicals in the vegetal kingdom to play an important role in human and animal diet. They exhibit a wide range of physiological properties including anti-allergenic, antiatherogenic, anti-inflammatory, anti-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-15β-Dglucopyranosyllactucin Sonchuside A	3,4
Air Dried Root	EtOH	8-Acetyl-lactucin	5

		8-Acetyl-11β,13-dihydrolactucin Lactucin 11β,13-Dihydrolactucin	
Freeze-dried shoot	MeOH, (CH3)2 CO H2O	8-O-Acetyl-15-β- D-glucopyranosyllactucin Chlorogenic acid 3,5- Dicaffeoylquinic acid Caffeoyltartric acid Cichoric acid	6
Dried roots Dried leaves	EtOH	Imperatorin Isoimperatorin Oxypeucedanin Ostruthol 11β,13- Dihydrolactucin 8-Acetyl-15-β-Dglucopyranosyllactucin	7

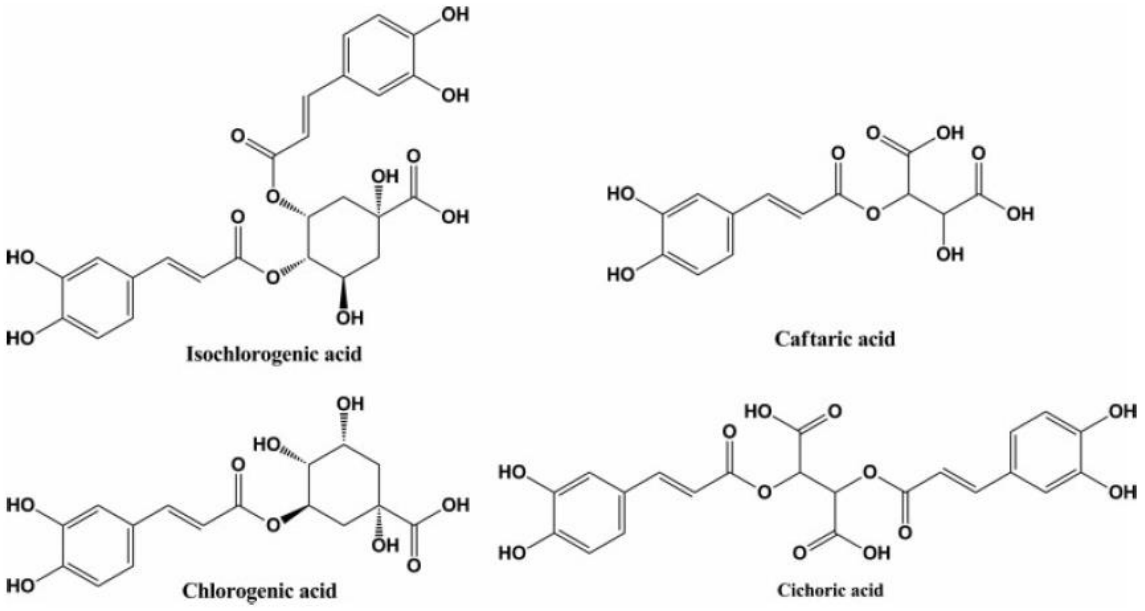

Fig. 1. Chemical structures of phenolic compounds found in the edible *Cicerbita alpina* shoots.

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

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

Experimental

Chemicals

The following flavonoids and phenolic acids were used as standards: acacetin (MW 284), apigenin (MW 270), diosmin (MW 608), eriodictyol (MW 288), hesperetin (MW 302), kaempferol (MW 286), luteolin (MW 286), caffeic acid, ferulic acid, gallic acid, para hydroxybenzoic acid, syringic acid and two flavanone glicosides, apigetrin (apigenin 7-*O*-glucoside MW 432) and hesperidin (hesperetin 7-rhamnoglucoside MW 610). HPLC-grade methanol, HPLC-grade formic acid and Milli-Q grade water (Milli-Q Plus system, Milipore, Bedford, USA) were used as solvents. All the chemicals were purchased from Sigma Aldrich (Milan, Italy). 1mg mL^{-1} stock solutions were prepared using flavonoid standards dissolved in dimethylsulfoxide (DMSO) and phenolic acids in H_2O: MeOH (5:95, v/v).

Plant material

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

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

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

A MAE set was designed for the extraction of phenolics from the cultivated *C. alpina* shoots. MAE experiments were performed with a professional microwave oven (Mars 1200 W, 2450 MHz, CEM Corporation, Matthews, North Carolina, USA). The instrument has an internal temperature control system with an optical fiber thermometer and a pressure control system. 30 mL of solvent (MeOH/H_2O mixture) were added to 1.0-3.0 g of plant material which has been placed in an inner vessel. The extraction was carried out under different extraction conditions at 300 W irradiation power. The extracts were filtered and the filtrates evaporated under vacuum.

The variables of the study include methanol/water ratio, sample quantity, extraction temperature and time, whereas the response is expressed in the total chromatographic area of the most intense characteristic peaks monitored at 280 nm. The coded values which correspond to the upper (+1), 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_2O)	50	75	100
X4: Sample quantity (g)	1	2	3

2^4 Full factorial design

Modde procedures for design of experiments (Modde ver. 8.02, MKS Umetrics AB, Sweden) were employed to design and analyze the experimental data. 19 experiments were used to optimize the four aforementioned factors in the 2^4 full factorial design applied to the MAE of phenols from *C. alpina* edible shoots. The most important factors and their interactions are depicted as contour plots of the MAE process. All runs were randomly performed in triplicate and the chromatographic area averages of the most characteristic peaks at 280 nm were taken as the response.

The model fit was evaluated by examining the summary of the fit, R^2 (measures fit), Q^2 (measures predictive power), model validity (indicates if the model is appropriate) and reproducibility, coefficients, ANOVA and the effect plots for screening designs. Model adequacy was further assessed using a plot of residuals against predicted values.

Total phenolic content micro-assay

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

weight DW). All measurements were performed in triplicate and expressed as averages ± standard deviation.

Total flavonoid content

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

LC/ESI-MS analyses

5 mg of freeze-dried crude cultivated herb extract were dissolved in 5% MeOH (2 mL). Prior to the analysis, the sample was filtered through a 0.45 µm syringe filter. LC/ESI-MS analyses and MS^n experiments were performed on a Finnigan LXQ linear ion trap operating in negative ion mode coupled with a Finnigan Surveyor LC Pump Plus equipped with a Finnigan Surveyor Autosampler Plus (Thermo Scientific, San Jose, CA, USA). The LC separations were performed on a C18 Novapack 4 µm analytical column, 150 x 4.6 mm, operating at 30°C, 1 mL min^{-1} flow after 1:20 flow splitting (accurate flow splitter, LC Packings, Amsterdam, The Netherlands) and with an acidified mobile phase composed of H_2O (0.1% HCOOH, v/v) and MeOH ((0.1% HCOOH, v/v). The injection volume was 10µL. Collision-induced dissociation (CID) multiple MS spectra (MS^n experiments) were acquired using helium as the collision damping gas in the ion trap at a pressure of 1 mTorr. The gradient elution steps were (time, % B): 0.01, 5; 5, 5; 20, 30; 30, 40; 40, 45; 50, 50; 60, 100; 70, 100. Mass selection of the analyte by *m/z* was followed by

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

RESULTS AND DISCUSSION

Optimization of MAE conditions

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

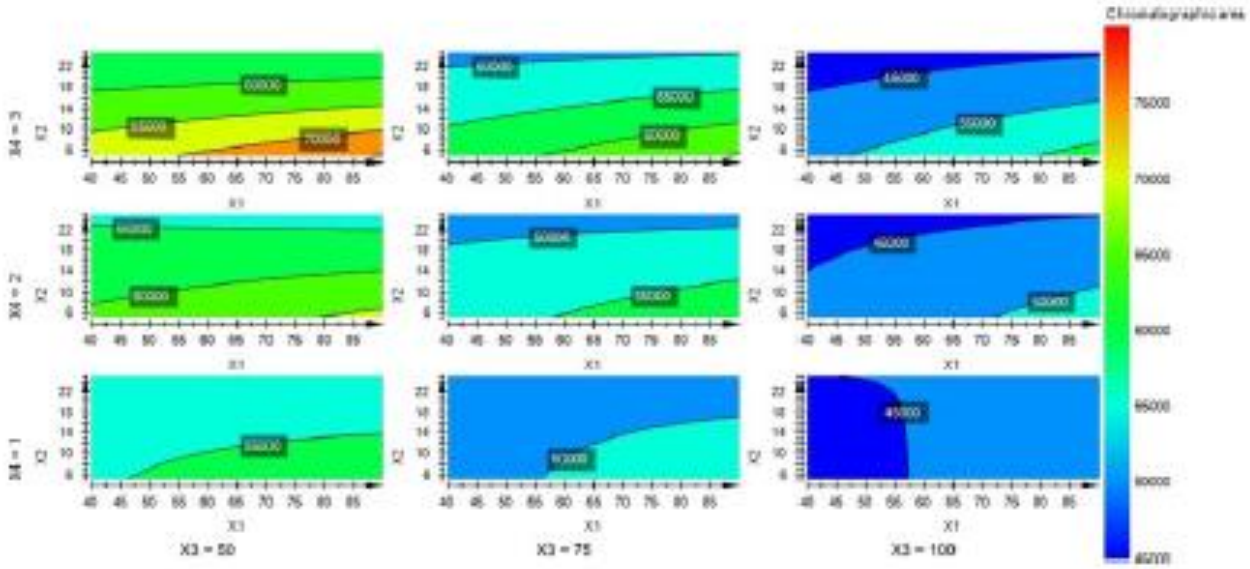

Fig. 2. UV-chromatograms of the crude extracts of the cultivated (**A**) and wild (**B**) edible shoots of *C. alpina* recorded at 280 nm.

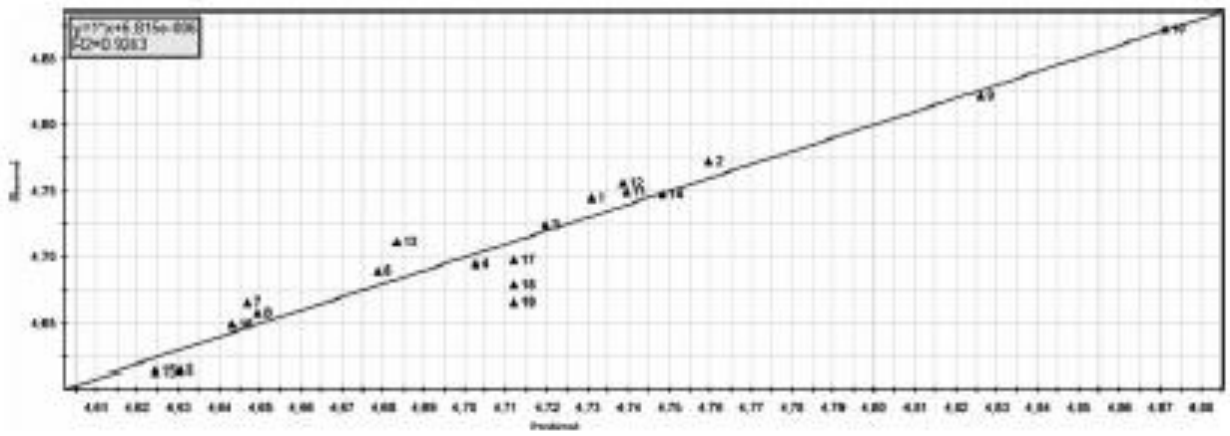

Fig. 3. Plot of residuals against predicted values.

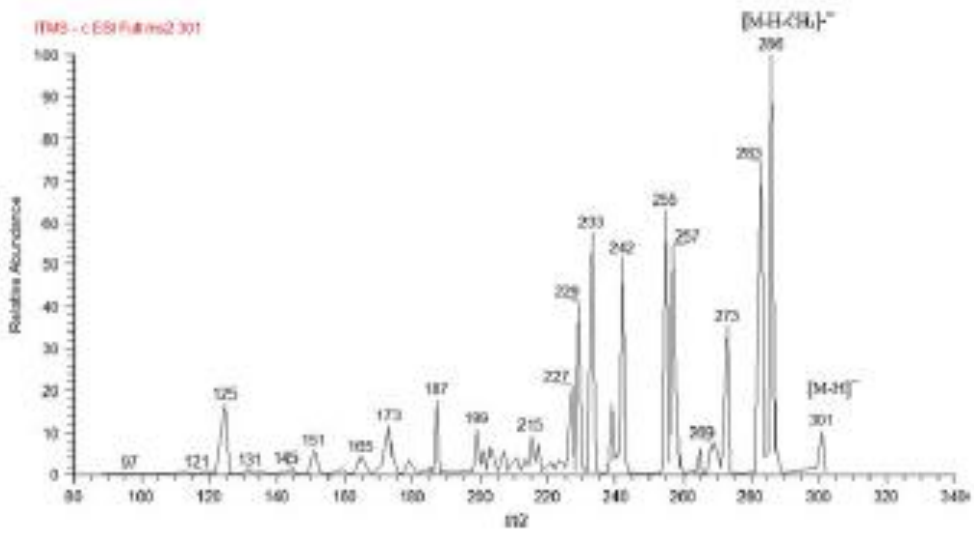

Fig. 4. Product-ion spectra of hesperitin (parent ion *m/z* 301).

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

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

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

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

Colorimetric analyses

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

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

LC/ESI-MS analyses

The analyses of the crude extracts by LC-MS were preceded by the analysis of a mixture of common phenolic standards, which are available in our laboratory, in order to evaluate the fragmentation behavior present in the product-ion spectra. According to Justensen [30], the product-ion spectra of the standard aglycones (for flavonoids) provided information about their specific A-ring and B-ring fragmentation. The A-ring fragments *m/z* 151 and *m/z* 107 were present as common fragments in the product-ion spectra of the main flavanone (eriodyctiol, hesperitin) and flavone (apigenin, luteolin, acacetin) standard compounds. The specific B-ring fragments observed for flavones and flavanones were: *m/z* 117 for apigenin, *m/z* 133 for luteolin and *m/z* 135 for eriodyctiol. Mass spectra of the methoxylated flavonoids, acacetin and hesperitin, revealed base peaks whose *m/z* values correspond to the loss of $\bullet CH_3$ from the deprotonated ion $[M-H-15]^{\bullet-}$ and did not provide B-ring fragments (see example in Fig. 5).

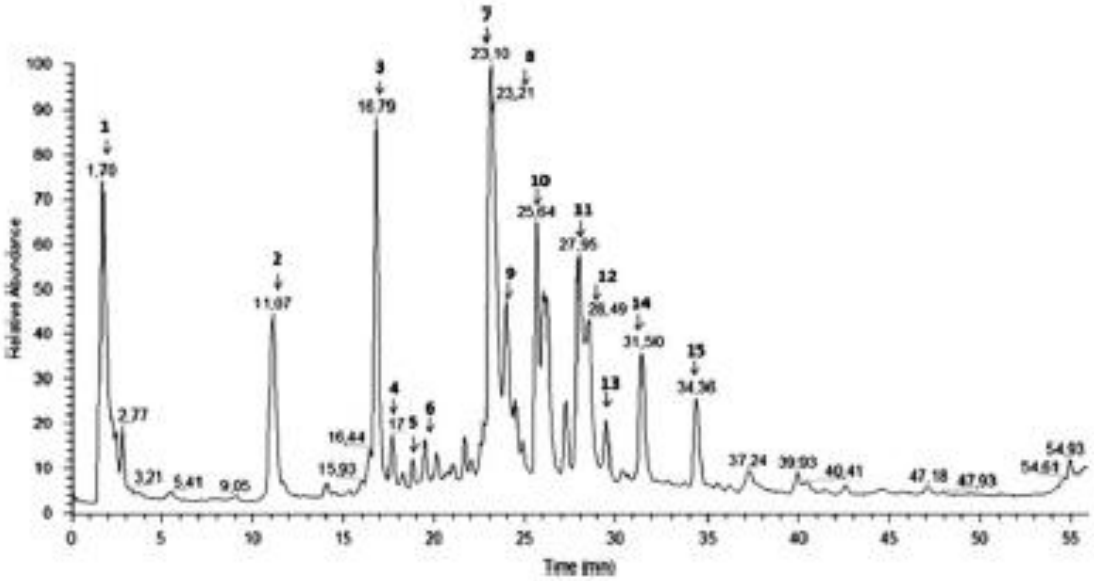

Fig. 5. HPLC-MS total ion chromatogram of the cultivated *C. alpina* crude extract

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

Peak	*t*R (min)	[M−H]− (m/z)	MS2 ions	MS3 ions	Tentative assignement
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-*O*-*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-*O*-glucoside
11	27.95	515	353 (100)	179 (53), 191 (100)	3_,5_-*O*-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-*O*-*β*-glucoside
15	34.36	533	489 (100)	285 (100)	Luteolin acetylglucoside

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

In negative MS analysis, the molecular ion occurs due to the breakdown of the *O*-glycosidic bond in the 7-position. 162 amu are lost in monohexosides and 324 in dihexosides. [39] The MS spectra of compounds **1-4** revealed specific fragments that characterize the presence of three free phenolic acids (**2-4**) and a diglucoside of quinic acid (**1**) which is highlighted by the fragment MS^2 191, the loss of two hexose units and a water molecule. Based on literature data [40], compounds **2** and **3** were tentatively assigned as caftaric acid ((m/z) 311→179→149) and caffeoylquinic acid ((m/z) 353→179→191). Caffeic acid ((m/z) 179→161→135→143) was identified by comparing its mass spectra with the one of a reference compound. Another mass fragmentation pattern, similar to the one found for compound **2,** can be seen in the case of compound **11**. In this case, the fragmentation pattern of 3', 5' caffeoylquinic acid is identical to the same compound reported in other studies. A caffeic acid derivative found in the mass spectra of compound **8**, is revealed by the presence of the fragment MS^2 179 which corresponds to caffeic acid and by the losses of 162 amu (hexose unit) and 132 amu (apiosyl moiety). Data previously reported in literature and the mass fragmentation of compound **9** revealed a derivative of caffeic acid (MW 180) and tartaric acid (MW 150), namely cichoric acid. The negative ESI-MS spectra of compounds **2, 5, 9** and **11** are shown in Fig. 7. On the basis of literature data [31-39] and of the mass spectra analyses, four compounds (**5**, **12**, **13 and 15**) were tentatively assigned as

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

Conclusions

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

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