

Original Research Article

Phytochemical profile and some biological activities of three *Centaurea* species from Turkey

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Abstract

Purpose: To characterise the phytochemical profile of whole plants of *Centaurea balsamita*, *C. depressa* and *C. lycopifolia* with LC-ESI-MS/MS, and as well as their antioxidant, anticholinesterase and antimicrobial activities.

Methods: Organic and aqueous extracts of the three *Centaurea* species were evaluated for DPPH free radical, ABTS cation radical scavenging and cupric reducing antioxidant capacity (CUPRAC). Acetyl- and butyryl-cholinesterase enzyme inhibition abilities of the extracts using petroleum ether, acetone, methanol and water were studied to determine anticholinesterase activity, while antimicrobial activity was determined by disc diffusion method using appropriate antimicrobial standards and organisms. The phytochemical components of the methanol extracts were assessed by LC-MS/MS.

Results: The methanol extract of *C. balsamita* exhibited much higher DPPH free and ABTS cation radicals scavenging activities (with IC_{50} of 62.65 ± 0.97 and 24.21 ± 0.70 mg/ml, respectively) than the other extracts. The petroleum ether extracts of the plant species exhibited moderate inhibitory activity against butyrylcholinesterase enzymes while the acetone extract of *C. balsamita* showed good antifungal activity against *Candida albicans*. Quinic acid (17513 ± 813 μ g/g, 63874 ± 3066 μ g/g and 108234 ± 5195 μ g/g) was the major compound found in the methanol extracts of *C. balsamita*, *C. depressa* and *C. Lycopifolia*, respectively.

Conclusion: These results indicate quinic acid is the major compound in the three plant species and that *Centaurea balsamita* has significant antioxidant, anticholinesterase and antimicrobial properties. Further studies to identify the compounds in the extracts responsible for the activities are required.

Keywords: *Centaurea*, LC-ESI-MS/MS, Anticholinesterase, Antioxidant, Antimicrobial

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INTRODUCTION

The genus *Centaurea* L. belongs to Asteraceae family which comprises of more than 500-600 species that are widespread all over the world, particularly around the Mediterranean and

western Asia [1]. In Anatolia, the genus comprises about 207 taxa including 134 endemic species classified in 4 sections with endemism 64 % [1]. *Centaurea* species are often called zerdali diken, timur diken and peygamber çiçeği in Anatolia [2]. In Turkish traditional medicine, these

species are widely used as expectorant, antidiabetic, antipyretic and antidiarrhoeal [2]. *Centaurea* species are known to have various biological activities such as antimicrobial [4], antifungal [5], antiinflammatory [6], antiulcerogenic [7], antioxidant [8], antiplasmodial [9], antiprotozoal [4], cytotoxic [9,10] and anticancer [10].

In previous studies, sesquiterpene lactones, flavonoids and phenolic compounds have been isolated from the plants [3]. These isolates include 10 flavonoids (myricetin, fisetin, quercetin, naringenin, hesperetin, luteolin, kaempferol, apigenin, rhamnetin, chrysin), 3 flavonoid glycosides (rutin, hesperidin, hyperoside), 9 phenolic acids (gallic, chlorogenic, protocatechuic, tannic, tr-caffeic, p-coumaric, rosmarinic, 4-OH benzoic and salicylic acids), one phenolic aldehyde (vanillin), one coumarin and other 3 organic acids (quinic, malic and traconitic acids). Some of the compounds obtained from other *Centaurea* species include quercetin from *C. omphalotricha* [11], luteolin and apigenin and kaempferol from *C. urvillei* subsp. *urvillei* [12], rutin and chlorogenic acid from *C. calolepis* [13], protocatechuic acid from *C. isaurica* [14], chlorogenic acid from *C. cadmea* [15], and *C. isaurica* [14], vanillin from *C. sadleriana* [16]. To the best of our knowledge, none of these earlier studies used LC-MS/MS in determination of phytochemical compositions of the plant species.

Therefore the purpose of this study was to determine the chemical profiles of *Centaurea* species using liquid chromatography coupled to tandem electrospray mass spectrometry and as well as the biological activities of the plants.

EXPERIMENTAL

Chemicals

Butylated hydrox-toluene (BHT) and 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Merck (Darmstadt, Germany). Malic acid, quercetin, protocatechuic acid, chrysin, rutin, hesperetin, naringenin, rosmarinic acid, vanillin, p-coumaric acid, caffeic acid, chlorogenic acid, hyperoside, myricetin, coumarin, kaempferol, formic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 5,5-dithiobis-(2-nitro benzoic acid) (DTNB), copper (II) chloride dihydrate ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$), neocuproine (2,9-dimethyl-1,10-phenanthroline), EDTA (Ethylenedi-aminetetraacetic acid), acetylcholinesterase (AChE: from electric eel) (Type-VI-S, EC 3.1.1.7, 425.84 U/mg), and butyryl-cholinesterase (BChE: from horse serum) (EC 3.1.1.8, 11.4 U/mg) were obtained from

Sigma (Steinheim, Germany). Quinic acid, traconitic acid, 4-hydroxybenzoic acid, fisetin, α -tocopherol and acetylthiocholine iodide were from Aldrich (Steinheim, Germany). Gallic acid, tannic acid, salicylic acid, and galanthamine hydrobromide were purchased from Sigma-Aldrich (Steinheim, Germany). Folin Ciocalteu Phenol reagent was from Applichem (Darmstadt, Germany) while hesperidin, luteolin, apigenin, rhamnetin and butyrylthiocholine iodide were from Fluka (Steinheim, Germany). All solvents were of LC-MS and analytical grade.

Plant material

The whole plants of *Centaurea balsamita* Lam., *Centaurea depressa* Bieb. and *Centaurea lycopifolia* Boiss et Kotschy were collected from southeastern Turkey (Diyarbakır, Malatya and Maraş, respectively) in July 2012 by Dr A Ertaş, and identified by Dr Y Yeşil. These specimens were stored at the Herbarium of Istanbul University (ISTE 97140, ISTE 97664 and ISTE 97138).

Preparation of plant extracts for LC-ESI-MS/MS

The plants were dried, powdered and 10 g of each was extracted with MeOH for 24 h at room temperature. Resultant extracts were filtered and evaporated under vacuum. Dry filtrate was diluted to 250 mg/L and passed through the microfiber filter (0.2 μm) for LC-ESI MS/MS.

Preparation of plant extracts for biological activities

Whole plant materials were dried, powdered and 100 g of each was sequentially macerated three times with petroleum ether, acetone, methanol and water (250 mL) for 24 h at room temperature. After filtration, the solvents were evaporated to obtain the crude extracts. The yields of the petroleum ether extracts of the three plants studied were obtained as: CBP (*Centaurea balsamita* petroleum ether extract) 0.7 %, CDP (*Centaurea depressa* petroleum ether extract) 0.6 %, CLP (*Centaurea lycopifolia* petroleum ether extract) 0.8 %; the acetone extracts as CBA (*Centaurea balsamita* acetone extract) 2.1 %, CDA (*Centaurea depressa* acetone extract) 1.3 %, CLA (*Centaurea lycopifolia* acetone extract) 2.6 %, the methanol extracts as CBM (*Centaurea balsamita* methanol extract) 7.2 %, CDM (*Centaurea depressa* methanol extract) 5.2 %, CLM (*Centaurea lycopifolia* methanol extract) 8.5 %, and the water extracts as CBW (*Centaurea balsamita* water extract) 2.3 %, CDW (*Centaurea*

depressa water extract) 1.8 %, CLW (*Centaurea lycopifolia* water extract) 3.1% (w/w).

Phenolic compound identification and quantification

LC-MS/MS analysis of the phenolic compounds were performed using a Shimadzu Nexera model UHPLC instrument coupled to a tandem MS instrument. The liquid chromatograph was equipped with LC-30AD binary pumps, DGU-20A3R degasser, CTO-10ASvp column oven and SIL-30AC autosampler. Chromatographic separation of plant extract samples was performed on a C18 reversed-phase Inertsil ODS-4 (150 mm × 4.6 mm, 3µm) analytical column with the column temperature fixed at 40 °C. The elution gradient consisted of mobile phase (A) water (5mM ammonium formate and 0.1 % formic acid) and (B) methanol (5mM ammonium formate and 0.1 % formic acid). Gradient elution using the proportions of solvent B of 40 %, 90 %, 90 %, 40 % and 40 % at 0, 20, 23.99, 24 and 29 min, respectively was applied. The solvent flow rate was maintained at 0.5 mL/min and injection volume was 4 µL. MS detection was performed using Shimadzu LCMS 8040 model triple quadrupole mass spectrometer equipped with an ESI source operating in both positive and negative ionization mode (Shimadzu, Kyoto, Japan). LC-ESI-MS/MS data were collected and processed using LabSolutions software (Shimadzu, Kyoto, Japan). The multiple reaction monitoring (MRM) mode was used to quantify the analytes: the assay of phenolic compounds was performed following two or three transitions per compound, the first one for quantitative purposes and the second and/or the third one for confirmation.

Optimization of LC-ESI-MS/MS method

Subsequent to several combinations of trials, a gradient of methanol (5 mM ammonium formate and 0.1 % formic acid) and water (5 mM ammonium formate and 0.1 % formic acid) system was concluded to be the best mobile phase solution. ESI source was chosen instead of APCI (Atmospheric Pressure Chemical Ionization) and APPI (Atmospheric Pressure Photoionization) sources as the phenolic compounds were small and relatively polar molecules. Tandem mass spectrometry was used for the current study since this system is commonly used for its fragmented ion stability [18]. The working conditions were determined as interface temperature of 350 °C, DL temperature of 250 °C, heat block temperature of 400 °C, nebulizing gas flow using Nitrogen, 3 L/min and drying gas flow (nitrogen) of 15 L/min.

The linearity of the phenolic standards was affirmed in the range of: 0.025 to 10 mg/L (Table 1). Regression coefficient of each calibration graph was found to be higher than 0.99. Limit of detection (LOD) and limit of quantitation (LOQ) of the method reported in this study were dependent on the calibration curve established from six measurements. LOD and LOQ were determined using the equations, $3S/N$ and $10S/N$, respectively where S/N is the signal (S) to noise (N) ratio (Table 1). For different compounds, LOD ranged from 0.05 to 25.8 µg/L and LOQ ranged from 0.17 to 85.9 µg/L (Table 1). Furthermore, the recovery of the phenolic compounds standards ranged from 96.9 % to 106.2 %.

Determination of total phenolic and flavonoid contents

Total phenolic and flavonoid amounts in the crude methanol extracts were determined as previously described and expressed as pyrocatechol and quercetin equivalents using the following equations [19]:

$$\text{Absorbance} = 0.0251 \text{ pyrocatechol } (\mu\text{g}) + 0.0445 \text{ (R}^2 = 0.9945\text{)}$$

$$\text{Absorbance} = 0.0301 \text{ quercetin } (\mu\text{g}) + 0.0553 \text{ (R}^2 = 0.9984\text{)}$$

Antioxidant activity of the extracts

We used the DPPH free radical, ABTS cation radical scavenging activity and cupric reducing antioxidant capacity (CUPRAC) methods to determine the antioxidant activity [19].

Anticholinesterase activity of the extracts

A spectrophotometric method developed by Ellman *et al* [19] was used to test the acetyl- and butyryl-cholinesterase inhibitory activities.

Antimicrobial activity of the extracts

Five different microorganisms including Gram positive bacteria (*Streptococcus pyogenes* ATCC19615 and *Staphylococcus aureus* ATCC 25923), Gram negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922) and yeast (*Candida albicans* ATCC10231) which were purchased from Refik Saydam Sanitation Center (Turkey) were used for detecting the antimicrobial activity of the extracts. The disc diffusion method was employed for this purpose and the minimum inhibitory concentrations (MICs) were determined

by the broth macrodilution method according to NCCLS [20]. Ampicillin and fluconazole were used as positive controls for bacteria and yeast, respectively.

Statistical analysis

The results of the antioxidant and anticholinesterase activity assays were expressed as means \pm SD. Unpaired Student's t-test and ANOVA were used for data comparison. A 2-tailed *p* values less than 0.05 was considered to be significant.

RESULTS

Quantitative analysis of phenolic compounds

Methanolic extracts of *C. balsamita*, *C. depressa* and *C. lycopifolia* were found to contain 27 compounds including 10 flavonoids, 3 flavonoid glycosides, 9 phenolic acids, 1 phenolic aldehyde, 1 coumarin and 3 other organic acids (Figure 1A). These compounds were monitored by the transition from the specific deprotonated molecular ions $[M-H]^+$ to the corresponding fragment ions $[M-H^+-X]$. The molecular ions and fragments observed in MS/MS, related collision energies for these fragments and the quantified results for the three *Centaurea* species are shown in Table 1.

Fisetin and kaempferol were not detected by this method from *C. balsamita*. Similarly, fisetin and hesperetin were not detected from *C. depressa*. Myricetin, fisetin, hesperetin, quercetin, naringenin and kaempferol were equally detected from the extracts of *C. lycopifolia*. However, the quantities of quinic acid found in *C. balsamita* ($17513.16 \pm 813.48 \mu\text{g/g}$), *C. depressa* ($63874.33 \pm 3065.97 \mu\text{g/g}$) and *C. lycopifolia* ($108233.80 \pm 5195.22 \mu\text{g/g}$) were more than the quantities of other compounds. About 10 % of the methanol extract of *C. lycopifolia* was quinic acid. *C. balsamita* and *C. lycopifolia* were found to be rich in chlorogenic acid ($10770.70 \pm 365.66 \mu\text{g/g}$ and $15782.64 \pm 463.45 \mu\text{g/g}$, respectively) while *C. depressa* was found to have high amount of malic acid ($22587.95 \pm 1524.52 \mu\text{g/g}$) (Table 1, Figure 1B-D).

Antioxidant activity assays and total phenolic and flavonoid content

The methanol extract of *C. balsamita* had the highest amount of total phenolic while the water extract of the same plant species had the highest content of total flavonoid content (Table 2).

As shown in Table 3, CBM, CDM and CLM extracts had 62.65 ± 0.97 , 82.21 ± 0.92 and $81.45 \pm 1.32 \mu\text{g/mL}$ IC_{50} values in the DPPH free radical scavenging activity assay, respectively. The other extracts showed weak or no activity.

In the ABTS cation radical scavenging assay, CBA, CBM, CBW and CLW extracts exhibited 47.50 ± 0.63 , 24.21 ± 0.70 , 44.89 ± 1.09 and $35.03 \pm 0.80 \mu\text{g/mL}$ IC_{50} values, respectively. The CBM, and CLW extracts exhibited strong ABTS cation radical scavenging activity. CBA and CBW extracts showed moderate activity with 70.96, 65.91 and 79.49 % inhibitions, respectively (Table 3).

Anticholinesterase and antimicrobial activities

As provided in Table 2, all the extracts had no activity against acetylcholinesterase enzyme, while CBP, CBA, CDP, CLP and CLA extracts showed moderate activity (56.69, 57.46, 48.15, 53.11 and 47.19 % inhibition, respectively) against butyrylcholinesterase enzyme at 200 $\mu\text{g/mL}$. The acetone extracts of the plants were active against all microorganisms tested with different levels of inhibition: weak (inhibition zone <12) and moderate (inhibition zone $<20-12$) (Table 4). Among the tested microorganisms, *C. albicans* was the most susceptible microorganism against acetone extracts. The extracts exhibited moderate antimicrobial activity against *C. albicans* and *S. pyogenes*. For a more reliable assessment of antimicrobial activity, a broth dilution assay was carried out. The susceptibility of the test microorganisms against active extracts was evaluated and results are shown as MIC (Table 4). The lowest MIC value was recorded by *C. balsamita* against *C. albicans* ($45 \mu\text{g mL}^{-1}$) in conformity with the result of disc diffusion method. MIC values of *C. balsamita* against *E. coli* and other Gram (-) bacteria were more than $1000 \mu\text{g mL}^{-1}$.

DISCUSSION

Present study has revealed that *C. balsamita* has antioxidant, anticholinesterase and antimicrobial properties. Several studies are present in literature reporting the use of liquid chromatography electrospray ionization tandem mass spectrometry to perform quantitative analyses [18,19]. In this study, an accurate method on a mass spectrometer equipped with a

Table 1: Analytical parameters, identification and quantification of phenolic compounds of methanol extract of *C. balsamita*, *C. depressa* and *C. lycopifolia* by LC ESI MS/MS^a

Analyte no	Analytes	RT ^a	RSD% ^b	Linearity Range (µg/L)	LOD/LOQ (µg/L) ^c	Recovery (%)	U ^d	M-H ⁺ (m/z) ^e	MS ² (CE) ^f	Quantification (µg analyte/g extract) ^g		
										<i>C. balsamita</i>	<i>C. depressa</i>	<i>C. lycopifolia</i>
1	Quinic acid	3.36	0.0388	250-10000	22.3 / 74.5	103.3	4.8	190.95	85 (22), 93 (22)	17513.16±813.48	63874.33±3065.97	108233.80±5195.22
2	Malic acid	3.60	0.1214	250-10000	19.2 / 64.1	101.4	5.3	133.05	115 (14), 71 (17)	3370.64±178.64	22587.95±1197.16	8534.11±452.31
3	tr-Aconitic acid	4.13	0.3908	250-10000	15.6 / 51.9	102.8	4.9	172.85	85 (12), 129 (9)	198.99±9.75	551.47±27.02	217.05±10.64
4	Gallic acid	4.25	0.4734	25-1000	4.8 / 15.9	102.3	5.1	169.05	125 (14), 79 (25)	16.03±3.10	346.09±17.65	550.79±28.09
5	Chlorogenic acid	5.29	0.1882	250-10000	7.3 / 24.3	99.7	4.9	353	191 (17)	10770.07±549.27	1552.63±76.08	15782.64±773.35
6	Protocatechuic acid	5.51	0.5958	100-4000	25.8 / 85.9	100.2	5.1	152.95	109 (16), 108 (26)	179.45±9.15	1872.96±95.52	3749.46±191.22
7	Tannic acid	6.30	0.9075	100-4000	10.2 / 34.2	97.8	5.1	182.95	124 (22), 78 (34)	67.70±3.45	3413.75±174.10	35.96±1.83
8	tr- caffeic acid	7.11	1.0080	25-1000	4.4 / 14.7	98.6	5.2	178.95	135 (15), 134 (24), 89 (31)	54.79±2.85	96.92±5.03	138.31±7.19
9	Vanillin	8.57	0.4094	250-10000	10.1 / 33.7	99.2	4.9	151.05	136 (17), 92 (21)	61.69±3.02	55.34±2.71	49.58±2.43
10	p-Coumaric acid	9.17	1.1358	100-4000	15.2 / 50.8	98.4	5.1	162.95	119 (15), 93 (31)	70.68±3.60	702.78±34.44	246.46±12.57
11	Rosmarinic acid	9.19	0.5220	250-10000	10.4 / 34.8	101.7	4.9	358.9	161 (17), 133 (42)	110.36±5.40	180.53±8.85	160.70±8.19
12	Rutin	9.67	0.8146	250-10000	17.0 / 56.6	102.2	5.0	609.1	300 (37), 271 (51), 301 (38)	40.87±2.04	133.87±6.69	90.17±4.51
13	Hesperidin	9.69	0.1363	250-10000	21.6 / 71.9	100.2	4.9	611.1	303, 465	N.D.	238.13±11.67	87.24±4.27
14	Hyperoside	9.96	0.2135	100-4000	12.4 / 41.4	98.5	4.9	463.1	300, 301	1688.96±82.76	1272.65±62.36	1998.14±97.91
15	4-OH Benzoic acid	11.38	1.4013	25-1000	3.0 / 10.0	106.2	5.2	136.95	93, 65	50.24±2.61	290.55±15.11	347.92±18.09
16	Salicylic acid	11.39	0.6619	25-1000	4 / 13.3	106.2	5.0	136.95	93, 65, 75	52.23±2.60	267.83±13.39	308.89±15.44
17	Myricetin	11.42	2.8247	100-4000	9.9 / 32.9	106.0	5.9	317	179, 151, 137	189.38±11.17	31.32±1.85	N.D.
18	Fisetin	12.10	2.4262	100-4000	10.7 / 35.6	96.9	5.5	284.95	135, 121	N.D.	N.D.	N.D.
19	Coumarin	12.18	0.4203	100-4000	9.1 / 30.4	104.4	4.9	146.95	103, 91, 77	D.	26.20±1.28	12.75±0.62

Table 1: Analytical parameters, identification and quantification of phenolic compounds of methanol extract of *C. balsamita*, *C. depressa* and *C. lycopifolia* by LC ESI MS/MS^a

Analyte no	Analytes	RT ^a	RSD% ^b	Linearity Range (µg/L)	LOD/LOQ (µg/L) ^c	Recovery (%)	U ^d	M-H ⁺ (m/z) ^e	MS ² (CE) ^f	Quantification (µg analyte/g extract) ^g		
										<i>C. balsamita</i>	<i>C. depressa</i>	<i>C. lycopifolia</i>
20	Quercetin	13.93	4.3149	25-1000	2.0 / 6.8	98.9	7.1	300.9	179, 151, 121	3731.86±264.96	67.66±4.80	N.D.
21	Naringenin	14.15	2.0200	25-1000	2.6 / 8.8	97.0	5.5	270.95	151, 119, 107	40.15±2.21	37.19±2.05	N.D.
22	Hesperetin	14.80	1.0164	25-1000	3.3 / 11.0	102.4	5.3	300.95	164, 136, 108	410.57±21.76	N.D.	N.D.
23	Luteolin	14.84	3.9487	25-1000	5.8 / 19.4	105.4	6.9	284.95	175, 151, 133	97.11±6.70	2019.30±139.33	43.53±3.00
24	Kaempferol	14.85	0.5885	25-1000	2.0 / 6.6	99.1	5.2	284.95	217, 133, 151	N.D.	1887.07±98.13	N.D.
25	Apigenin	16.73	0.6782	25-1000	0.1 / 0.3	98.9	5.3	268.95	151, 117	52.48±2.78	3958.61±209.80	2.25±0.12
26	Rhamnetin	18.41	2.5678	25-1000	0.2 / 0.7	100.8	6.1	314.95	165, 121, 300	56.69±3.46	45.69±2.79	12.06±0.74
27	Chrysin	20.60	1.5530	25-1000	0.05 / 0.17	102.2	5.3	253	143, 119, 107	7.75±0.41	7.88±0.42	5.74±0.30

^aRT: Retention time, ^bRSD: Relative standard deviation, ^cLOD/LOQ(µg/L): Limit of detection/Limit of quantification, ^dU (%): Percent relative uncertainty at 95% confidence level (k=2), ^eM-H⁺(m/z): Molecular ions of the standard compounds (mass to charge ratio), ^fMS²(CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions), ^gValues in µg/g (w/w) of plant methanol extract, ^hN.D: not detected, ⁱD: peak observed, concentration is lower than the LOQ but higher than the LOD

Table 2: Total phenolic and flavonoid contents and anticholinesterase activity of *Centaurea* extracts[†]

Extracts	Phenolic content ($\mu\text{g PEs/mg extract}$) ^a	Flavonoid content ($\mu\text{g QEs/mg extract}$) ^b	Inhibition % against AChE	Inhibition % against BChE
CBP	28.88 \pm 0.82 ^a	4.00 \pm 0.51 ^a	NA	56.69 \pm 0.92 ^a
CBA	30.88 \pm 1.82 ^a	20.39 \pm 1.64 ^b	NA	57.46 \pm 0.79 ^a
CBM	174.30 \pm 1.90^b	22.60 \pm 1.87 ^b	NA	30.87 \pm 1.46 ^b
CBW	45.48 \pm 1.05 ^c	41.43 \pm 0.33 ^c	NA	NA
CDP	23.38 \pm 0.92 ^d	5.39 \pm 0.41 ^a	NA	48.15 \pm 1.03 ^c
CDA	36.68 \pm 0.83 ^e	18.04 \pm 1.18 ^b	NA	34.21 \pm 0.64 ^b
CDM	67.75 \pm 1.24 ^f	25.82 \pm 1.06 ^b	NA	NA
CDW	46.34 \pm 0.74 ^c	22.71 \pm 0.98 ^b	NA	NA
CLP	38.84 \pm 1.45 ^e	1.89 \pm 0.33 ^a	NA	53.11 \pm 0.97 ^a
CLA	41.87 \pm 1.41 ^c	32.57 \pm 1.19 ^d	NA	47.19 \pm 1.56 ^c
CLM	52.24 \pm 1.15 ^g	10.97 \pm 1.70 ^e	NA	NA
CLW	37.85 \pm 1.14 ^e	11.75 \pm 2.52 ^e	NA	NA
Galanthamine[†]	-	-	75.11 \pm 0.69	82.49 \pm 0.32

^aValues expressed are means \pm S.D. of three parallel measurements, different letters in the same column indicate a significant difference ($p < 0.05$), [†]Standard drug, NA: Not active, ^aPEs, pyrocatechol equivalents ($y = 0.0251x + 0.0445$ $R^2 = 0.9945$), ^bQEs, quercetin equivalents ($y = 0.0301x - 0.0553$ $R^2 = 0.9984$).

Table 3: Antioxidant activity of the extracts and standards^a

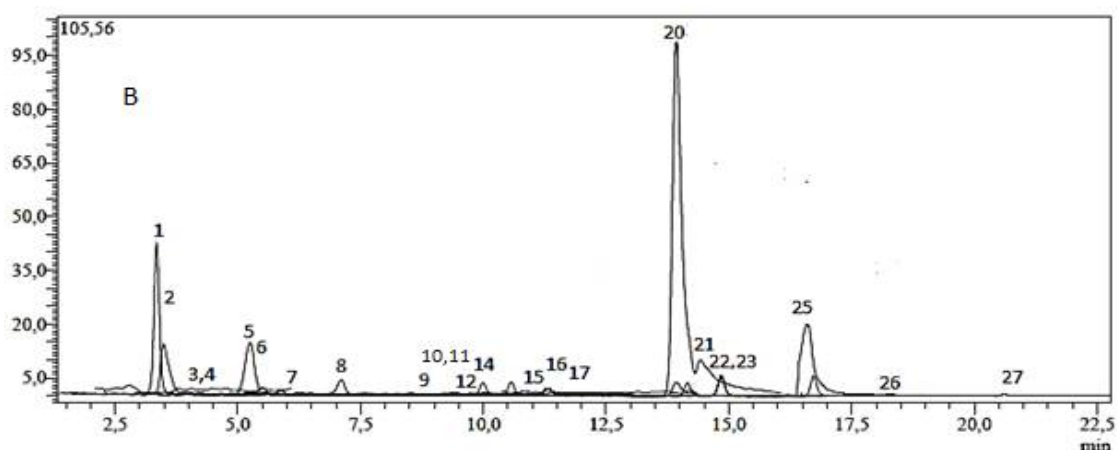
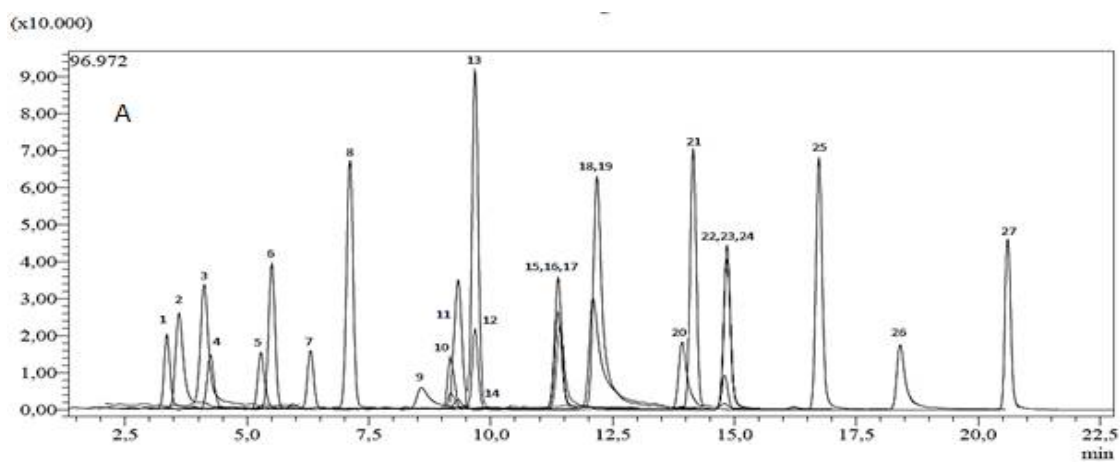
Samples	IC ₅₀ values	
	DPPH Free Radical	ABTS Cation Radical
CBP	>200	>200
CBA	139.19 \pm 0.85	47.50 \pm 0.63
CBM	62.65 \pm 0.97	24.21 \pm 0.70
CBW	105.12 \pm 0.74	44.89 \pm 1.09
CDP	>200	>200
CDA	>200	96.29 \pm 1.06
CDM	82.21 \pm 0.92	70.08 \pm 0.52
CDW	107.06 \pm 1.27	97.39 \pm 0.87
CLP	>200	>200
CLA	>200	>200
CLM	81.45 \pm 1.32	89.05 \pm 0.56
CLW	100.88 \pm 1.21	35.03 \pm 0.80
α -TOC	18.76 \pm 0.41	9.88 \pm 0.23
BHT	48.86 \pm 0.50	10.67 \pm 0.11

^aValues were given as IC₅₀, Values are means \pm S.D., n = 3

Table 4: Zones of growth inhibition (mm) and MIC values showing the antimicrobial activities of the acetone extracts compared to positive controls

		Gram positive		Gram negative		Yeast
		<i>S. aureus</i>	<i>S. pyogenes</i>	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>C. albicans</i>
<i>Centaurea lycopifolia</i>	^a DD	11±0.2	12±0.1	11±0.5	11±0.3	16±0.3
	MIC	250±0.4	100±0.5	180±0.5	300±0.2	50±0.3
<i>Centaurea balsamita</i>	^a DD	11±0.2	12±0.3	10±0.3	8±0.6	17±0.2
	MIC	170±0.3	150±0.2	>1000	>1000	45±0.4
<i>Centaurea depressa</i>	^a DD	10±0.3	12±0.4	9±0.2	10±0.5	15±0.1
	MIC	240±0.6	180±0.5	>1000	500±0.1	50±0.2
Positive controls	^b DD	35±0.2	19±0.2	20±0.1	NA	30±0.3
	MIC	1.95±0.3	7.815±0.1	7.815±0.4	NA	3.125±0.2

^aDD: Inhibition zone in diameter (mm) around the discs (6 mm) impregnated with 30 mg mL⁻¹ of plant extracts, ^bDD: Inhibition zone in diameter (mm) of positive controls that are ampicillin for bacteria and fluconazole for yeast. Minimum inhibitory concentration (MIC) values are given as µg mL⁻¹, NA: Not active



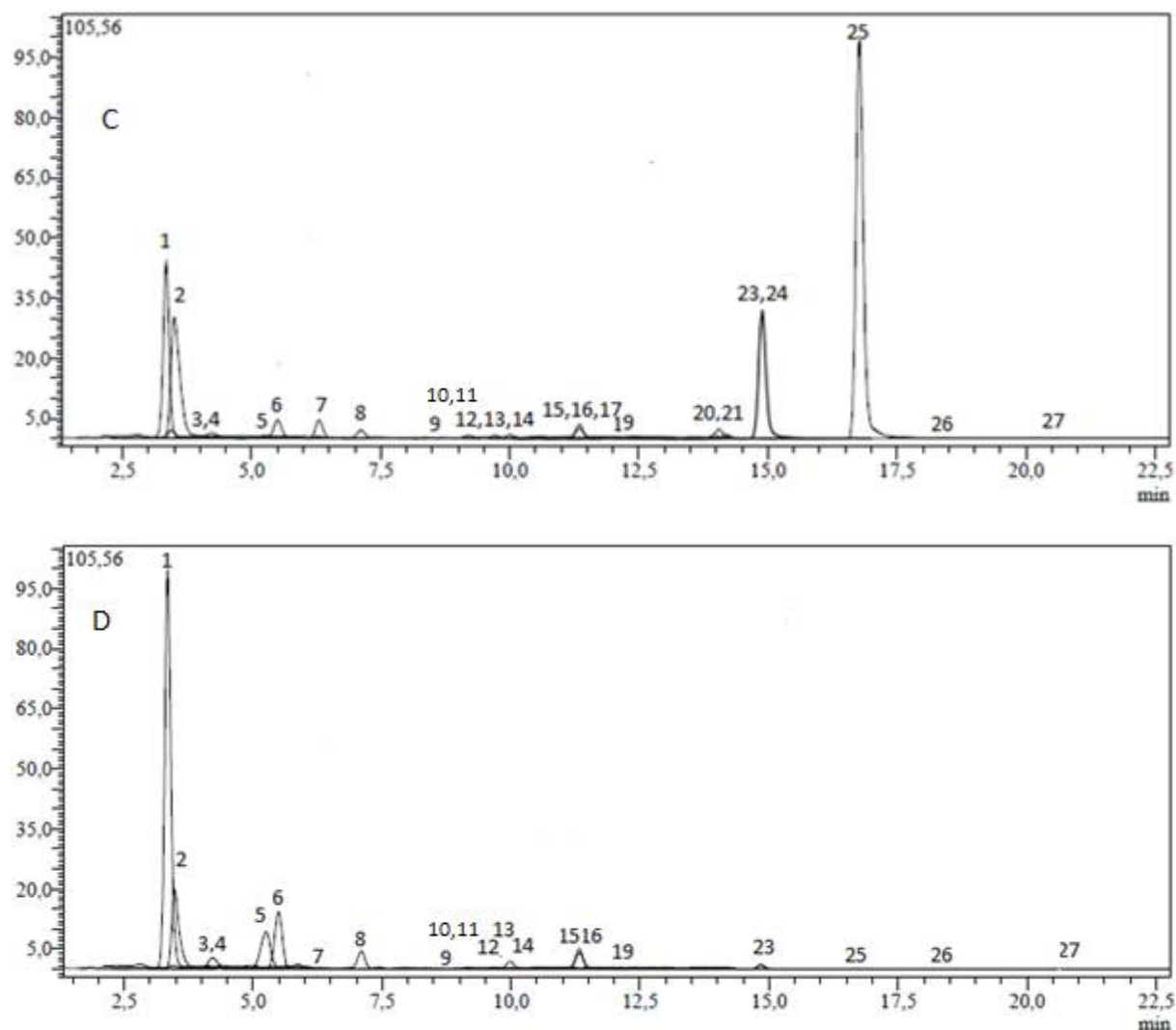


Figure 1: LC-MS/MS chromatograms of A: 250 ppb standard mix, B: CB methanol extract, C: CD methanol extract, D: CL methanol extract. 1: Quinic acid, 2: Malic acid, 3: tr-Aconitic acid, 4: Gallic acid, 5: Chlorogenic acid, 6: Protocatechuic acid, 7: Tannic acid, 8: tr-caffeic acid, 9: Vanillin, 10: p-Coumaric acid, 11: Rosmarinic acid, 12: Rutin, 13: Hesperidin, 14: Hyperoside, 15: 4-OH Benzoic acid, 16: Salicylic acid, 17: Myricetin, 18: Fisetin, 19: Coumarin, 20: Quercetin, 21: Naringenin, 22: Hesperetin, 23: Luteolin, 24: Kaempferol, 25: Apigenin, 26: Rhamnetin, 27: Chrysin

triple quadrupole analyzer was developed for the analyses of 27 compounds in *Centaurea* species. Quinic acid was found to be the most abundant among the compounds and about 10 % of methanol extract of *C. lycopifolia* was quinic acid. For the first time, myricetin, fisetin, naringenin, hesperetin, rhamnetin, chrysin, hesperidin, hyperoside, gallic acid, tannic acid, caffeic acid, p-coumaric acid, rosmarinic acid, 4-hydroxybenzoic acid, salicylic acid, malic acid and tr-aconitic acid were detected in the three plant species using LC-MS-MS. Also, flavonoids, flavonoid glycosides, phenolic acids and other organic acids constituents of *Centaurea* species have been detected with LC-MS-MS.

The antibacterial effect of *C. balsamita* seen in this study has been reported previously in eight *Centaurea* species [4,9]. Similarly, the antifungal activity of *C. balsamita* has been reported in *Candida krusei* [10]. As in an earlier report, the methanol extract of the three *Centaurea* species exhibited the highest effect in DPPH free and ABTS cation radicals scavenging activities just like *C. pulcherrima* var. *pulcherrima* [21]. The ABTS cation radical scavenging activity in our study may be caused by higher quercetin contents of *C. balsamita* than other investigated plants. In CUPRAC assay, *Centaurea* extracts showed very weak activity that verify the CUPRAC assay results of *Centaurea* species reported by Aktümsek et al [21].

In the report of Aktümsek *et al* [21], water extracts of *C. pyrrhoblephara* and *C. antalyense* were not active against AChE as in our results but their methanol extracts exhibited weak activity against AChE. However, all the tested extracts displayed more or less inhibition on BChE, except for methanolic extract of *C. pyrrhoblephara* [21] while our results of CBP, CBA, CDP, CLP and CLA extracts showed moderate anticholinesterase activity. Acetone extract of *Centaurea balsamita* has good antifungal activity against *Candida albicans*. Inhibition zones and MIC values of the acetone extracts of *Centaurea* species on the yeast *Candida albicans* compared to that of positive control (fluconazole) indicated their moderate antifungal properties.

CONCLUSION

Some compounds including mrycetin, fisetin, naringenin, hesperetin, rhamnetin, chrysin, hesperidin, hyperoside, gallic acid, tannic acid, caffeic acid, p-coumaric acid, rosmarinic acid, 4-hydroxybenzoic acid, salicylic acid, malic acid and tr-aconitic acid have been detected for the first time in *Centaurea* species using LC-MS-MS. *Centaurea balsamita* has antioxidant, anticholinesterase and antimicrobial properties. Further studies to identify the compounds in the extracts responsible for the activities are needed.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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