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ORIGINAL ARTICLE

Bioactivity-Guided Isolation of Cytotoxic and Antioxidant Phytochemicals from Four *Cousinia* Species from Stenocephala Bunge Section

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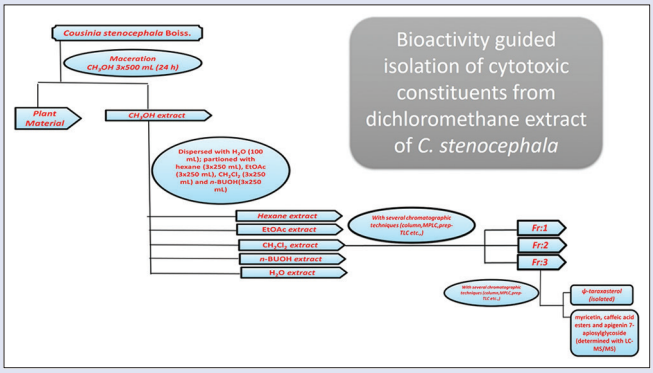
ABSTRACT

Background: Asteraceae family contains several cytotoxic compounds bearing genus. *Cousinia* genus is included in the Asteraceae; it has not been studied phytochemically in detail. **Objective:** In this study, chemical compositions of four *Cousinia* species (*Cousinia davisiana* [CD], *Cousinia foliosa*, *Cousinia ramosissima*, and *Cousinia stenocephala* [CS]) were evaluated according to their cytotoxic and antioxidant effects using bioactivity-guided isolation. **Materials and Methods:** The cytotoxic effect was investigated with Sulphorhodamine B method against Colo205 (human colon carcinoma), A549 (human non-small cell lung cancer) cell lines, and antioxidant activity tested with 1,1-diphenyl-2-picrylhydrazyl, 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid scavenging tests, and β-carotene/linoleic acid co-oxidation test. Purified compounds were elucidated by one-dimensional and two-dimensional nuclear magnetic resonance and mass spectroscopic techniques. The quantitative and qualitative determination of unpurified compounds within the extracts was carried out by liquid chromatography-mass spectrometry/mass spectrometry. **Results:** CS methanol extract, dichloromethane subextract, and FR-3 showed more cytotoxicity; isolated compound (ψ-taraxasterol) showed no cytotoxic activity. CD methanol extract and *n*-butanol subextract showed significant antioxidant activity. **Conclusion:** This is the first report that these phytochemical compounds were identified in *Cousinia* genus, and it is thought that these compounds could contribute to the chemotaxonomy of the genus.

Key words: Antioxidant, *Cousinia,* cytotoxicity, liquid chromatography-mass spectrometry/mass spectrometry, nuclear magnetic resonance

SUMMARY

- *Cousinia stenocephala* methanol extract, dichloromethane subextract, and FR-3 showed more cytotoxicity against human colon adenocarcinoma cell line, but isolated compound (ψ-taraxasterol) showed no cytotoxic activity
- *Cousinia davisiana* methanol extract and *n*-butanol sub-extract showed significant antioxidant activity, and the highest content of antioxidant compounds were detected in this extract and subextract.



Abbreviations used: NMR: Nuclear magnetic resonance; LC-MS/MS: Liquid chromatography-mass spectrometry; Colo 205: Human colon adenocarcinoma cell line; A549: Human non-small lung cancer cell line; DPPH•: 1,1-diphenyl-2-picrylhydrazyl; ABTS •+: 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid; CS: *C. stenocephala*; CD: *C. davisiana*; CR: *C. ramosissima*; CF: *C. foliosa*; CSH: *C.* stenocephala *n*-hexane subextract; CSD: *C. stenocephala* Dichloromethane subextract; CSE: *C. stenocephala* Ethyl acetate subextract; CSB: *C. stenocephala* n-butanol subextract; CSS: *C. stenocephala* water subextract, CDE: *C. davisiana* ethyl acetate subextract; CDB: *C. davisiana* n-butanol subextract; CDS: *C. davisiana* water subextract

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INTRODUCTION

Free radicals are linked to pathology of various diseases such as diabetes, cancer, and cirrhosis. Reactive oxygen species (ROS) can react with fatty acids in the cell membrane and with sulfhydryl bonds in nucleotides and proteins, leading to cell damage. Natural antioxidants can scavenge these free radicals that are responsible from the pathology of ROS-related diseases. Because of their natural antioxidants compositions, the plants are rich sources and are being widely investigated in such diseases.[1]

Cancer is a result of abnormal cell proliferation. Some difficulties such as severe side effects and interactions in using conventional drugs in cancer therapy are well-defined. On the other hand, because of their wide application, therapeutic efficacy and low toxicity medicinal plants remain an important source that can be used as potential drugs in the

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treatment of various diseases including cancer.[2-4] The novel compounds which isolated from various plants are being used for treatment of different tumor types today. There are about 250.000–500.000 plant species on the earth, but only about 20% of them have been studied with biological and phytochemical screening assays. However, therapeutic efficacy of many plants has not been evaluated yet.[5]

The Asteraceae family (syn. Compositae) comprises several members that are used in folk medicine for treatment of various diseases. A screening of the antitumor effects of 538 extracts representing 34 different families revealed that the Asteraceae family contains the majority of active species with cytotoxic activity.[2]

Cousinia Cass. is one of the most diverse genera of Asteraceae family with 600–700 species distributed in Central and South-West Asia. There are 38 species and six section of *Cousinia* genus in Turkey. One important section is Stenocephalae Bunge. The species of this section are *Cousinia davisiana* (CD) Hub.-Mor., *Cousinia foliosa* (CF) Boiss. and Bal., *Cousinia ramosissima* (CR) DC. and *Cousinia stenocephala* (CS) Boiss. The two of species, CD and CF, are endemic to Turkey.[6] In the literature, taxonomic and systematic studies are generally performed on the genus of *Cousinia*, but phytochemical and activity studies are rarely seen. Phytochemical studies have shown that plants are rich in triterpenes, sesquiterpenes, flavonoids, and steroids. In addition, effects of ethanol extracts of some *Cousinia* species on different cancer cell lines and matrix metalloproteinase protein inhibitor effects were examined.[7-15] In this study, we aimed to investigate the cytotoxic effect of four *Cousinia* species against human colon adenocarcinoma (Colo 205) and on human non-small cell lung cancer (A549) cell lines and antioxidant effects with 1,1-diphenyl-2-picrylhydrazyl (DPPH•), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS $^{\bullet+}$) scavenging tests and β-carotene/linoleic acid co-oxidation test, which has not been evaluated previously. We carried out bioactivity-guided fractionation of methanol extracts and we elucidated/characterized the bioactive compounds by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) and nuclear magnetic resonance (NMR) spectroscopy. Phytochemical profiles of extracts and fractions and quantitative analyses of bioactive compounds were determined by LC-MS/MS.

MATERIALS AND METHODS

Chemicals

All chemicals and reagents were analytical or high performance liquid chromatography grade and purchased from Sigma-Aldrich.

Plant material

Localities and collection periods of plant materials used in this study are as follows:

1. CD from East Ermenek, Karaman, Turkey; July, 2013
2. CF from Ahir Dagı, Kahramanmaraş, Turkey; June, 2013
3. CR from around Birecik, Şanlıurfa, Turkey; May, 2013
4. CS from Ceylanpınar, around Şanlıurfa, Turkey; July, 2013.

The voucher specimens were deposited at the Herbarium Unit of the Science Faculty, Selcuk University, Konya, Turkey (Voucher No. 1, KNYA 26.976; Voucher No. 2, KNYA 26.977; Voucher No. 3, KNYA 26.978; Voucher No. 4, KNYA 26.979, respectively). In this study, dried flowering aerial parts of plants have been used.

Extraction, fractionation, and isolation

Air-dried aerial parts of CD (1 kg), CF (500 g), CR (500 g), and CS (1 kg) were powdered and extracted three times with methanol by maceration, at room temperature [Figure 1]. Combined macerates were filtered and evaporated to dryness under reduced pressure at 37°C using a rotary evaporator. The crude extracts were stored in dark at −20°C. Among the methanol extracts, CS showed more cytotoxic activity and CD had more antioxidant activity. Therefore, the CS and CD extracts were used in bioactivity guided fractionation assay for isolation of the active compounds. For cytotoxic activity, CS extract dispersed with water and partitioned with *n*-hexane (CSH), dichloromethane (CSD), ethyl acetate (CSE) and *n*-butanol (CSB) sequentially. But CD extract partitioned with ethylacetate (CDE) and *n*-butanol (CDB) sequentially for antioxidant assays. A total five sub-extracts were obtained from CS extract and three subextracts from CD extract. Yields of extract and subextracts were given in Table 1.

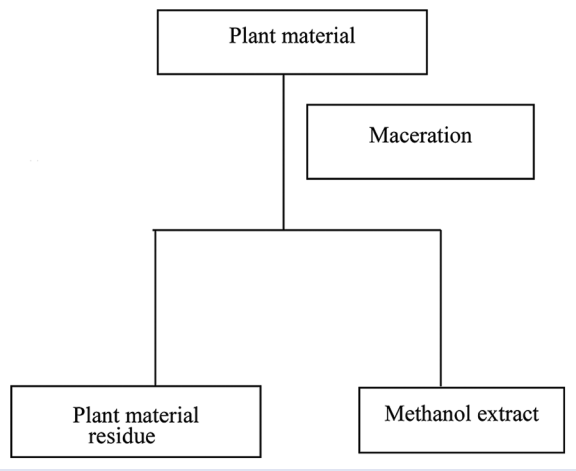

Figure 1: Extraction scheme of plants

The most active CSD subextract (10 g) was fractioned on a silica gel column, eluting with gradient mixtures of petroleum ether and ethyl acetate (100/0; 99/1; 99/5; 90/10; 80/20; 70/30; 50/50; 0/100) to afford three main fractions (FR:1–3). Most active fraction FR:3 (3 g) was subjected to silica gel column (SC) and eluent was hexane/EtOAc mixture (99/1 and 5/1), yielded ψ-taraxasterol (50 mg) compound. This compound was purified by Sephadex LH-20 column chromatography using methanol as eluent [Figure 2].

CDB subextract (30 g) was subjected to silica gel column chromatography using $CHCl_3/CH_3OH/H_2O$ (90/10/1; 80/20/2; 70/30/3; 61/32/7, each 200 mL) solvent mixture, and rutin (60 mg) and isorhamnetin 3-O-rutinoside (80 mg) were obtained. These compounds were purified by Sephadex LH-20 column chromatography and preparative layer chromatography [Figure 3].

Nuclear magnetic resonance analyses

The structures of isolated compounds were determined on the basis of their one-dimensional (1D) and two-dimensional (2D) NMR analyses in combination with mass spectroscopic data and comparison with literature data. NMR spectrums were obtained from Bruker AVANCE III HD 600 MHz spectrometer using CD_3OD as a solvent. The chemical shifts were in ppm, and coupling constants were in Hz.

Liquid chromatography-mass spectrometry/mass spectrometry instrumentation

Compounds in subextracts and fraction were evaluated using LC-electrospray ionization–MS/MS (ESI, Shimadzu 8040). The liquid chromatograph was a Shimadzu (Kyoto, Japan) Nexera XR system with an SIL-20AC autosampler, an LC-20AD high-pressure gradient

Table 1: Yields of extracts and subextracts (%)

Plant	Extract code					
	Methanol	*n*-hexan	Dichloromethane	Ethyl acetate	*n*-butanol	Water
C. ramosissima	10 (CR)	-	-	-	-	-
C. foliosa	8,7 (CF)	-	-	-	-	-
C. davisiana	10 (CD)	-	-	35 (CDE)	30 (CDB)	35 (CDS)
C. stenocephala	10 (CS)	35 (CSH)	10 (CSD)	10 (CSE)	20 (CSB)	25 (CSS)

C: *Cousinia*; CD: *C. davisiana* methanol extract; CDB: C. *davisiana n*-butanol extract; CDE: *C. davisiana* ethyl acetate extract; CDS: *C. davisiana* water extract; CF: *C. foliosa* methanol extract; CR: *C. ramosissima* methanol extract; CS: *C. stenocephala* methanol extract; CSB: *C. stenocephala n*-butanol subextract; CSD: *C. stenocephala* dichloromethane subextract; CSE: *C. stenocephala* ethyl acetate subextract; CSH: *C. stenocephala n*-hexane subextract; CSS: *C. stenocephala* water subextract

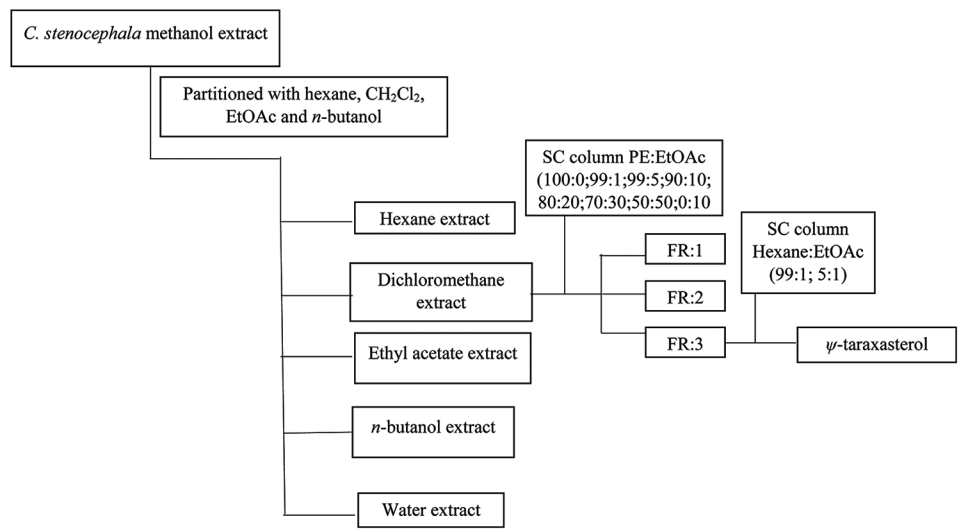

Figure 2: Isolation scheme of ψ-taraxasterol from *Cousinia stenocephala*

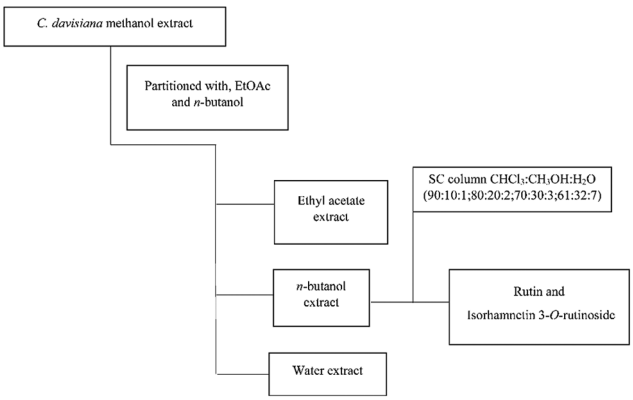

Figure 3: Isolation scheme of rutin and isorhamnetin 3-O-rutinoside from *Cousinia davisiana*

pump system (20-µL mixer), a DGU-20A3R vacuum degasser, and a CTO-10AS VP column oven. Mass spectrometry was conducted using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer equipped with an ESI interface.

The mass spectrometric behavior of active subextract and fraction was studied using both positive-ion and negative-ion mode. The samples were prepared in methanol. The following instrument settings were used for analysis: Column Restek (150 mm × 4.6 mm, 3 µm); column heat, 40°C; heat block temperature, 400°C; DL temperature, 250°C; nebulizing gas (N_2), 3 L/min; drying gas (N_2), 15 L/min; collision energy, 25.0, 12.0, 9; dwell time, 100 msec. A mixture of methanol: formic acid (99:1 v/v) (A) and water:formic acid (99:1, v/v) (B) was selected as the mobile phase. The mobile phase consisted of 50% solvent A and 50% solvent B at a flow rate of 0.4 mL/min, and injection volume was 1 µL.

In vitro cytotoxic activity assay

12500 cells were seeded to 96 wells and incubated in 100 µL RPMI medium supplemented with %10 fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. After an overnight incubation, cells were treated with extracts, subextracts, fractions (1000, 750, 500, 250, 125 µg/ml), and compound (100, 50, 25, 12.5, 6.25 µg/ml) for 24 h. Cell viability was measured using Sulphorhodamine B (SRB) assay. All extract groups were normalized to control group which was treated with complete medium. Besides, cell viability were measured for vehicle of the extracts (max 1% ethanol) whether to see the solution was toxic to A549 (human lung carcinoma) and Colo 205 (human colorectal adenocarcinoma) cell lines. After 24 h, IC_{50} values of the extracts were calculated. In this study, a known cytotoxic compound cisplatin was used as a positive control.

The total phenol and flavonoid contents

Total phenol contents (TPC) were estimated as gallic acid equivalents (GAE) per gram of extract. This test was carried out

according to the Folin–Ciocalteu method in triplicate.[16] The absorbance was measured and compared to a gallic acid calibration curve. Total flavonoid content (TFC) was carried out according to the Zhishen *et al.*[17] Catechin was used for the construction of a standard curve. All tests were carried out in triplicate. TFC of extracts was measured as milligram of catechin.

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl scavenging activity

DPPH• Radical scavenging abilities of samples were determined using the method of Gyamfi *et al.*[18] In this study, butylated hydroxyanisole (BHA) was the reference standard, and all tests carried out in triplicate. The % inhibition was calculated using equation (1).

$$\% \text{ inhibition} = ([Abs_{control} - Abs_{sample}]/Abs_{control}) \times 100 \quad (1)$$

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging activity

Synthetic $ABTS^{+\bullet}$ radical model was used as an alternative radical scavenging activity. Trolox was chosen as a reference compound. Absorbance was measured on a ultraviolet spectrophotometer at 734 nm, and results are given in terms of trolox equivalent antioxidant capacity (TEAC).[19]

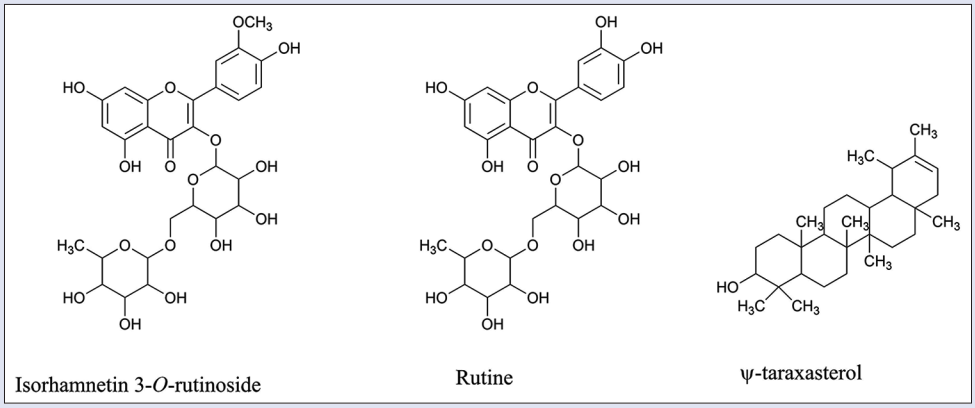

Figure 4: Chemical structures of isolated compounds

Table 2: Nuclear magnetic resonance spectral data of ψ-taraxasterol ($CDCl_3$, ^{13}C: 150 MHz; ^1H: 600 MHz)

n	DEPT	δ_C (ppm)	δ_H (ppm), J (Hz)	H→C HMBC	C→H HMBC	H→H COSY
1	CH_2	38.90	1.66/0.88			
2	CH_2	27.54	1.16			
3	CH	79.17	3.14 m	C4		H2
4	C	39.01	-			
5	CH	55.43	0.63			
6	CH_2	18.45	1.31/1.46			
7	CH_2	34.37	1.33			
8	C	41.21	-			
9	CH	50.56	1.22			
10	C	37.25	-			
11	CH_2	21.76	1.48/1.56			
12	CH_2	27.78	1.55			
13	CH	39.36	1.54	C14, C18		H14
14	C	42.48	1.48			
15	CH_2	27.18	1.70			
16	CH_2	36.85	1.22/1.55			
17	C	34.54	-			
18	CH	48.83	0.98			
19	CH	36.46	1.50			
20	C	140.01	-		H19, H29, H30	
21	CH	119.02	5.19 d (8)	C19, C22	H19, H30	
22	CH_2	42.32	1.48/1.64			
23	CH_3	28.14	0.92 s			
24	CH_3	15.55	0.70 s			
25	CH_3	16.45	0.78 s			
26	CH_3	16.19	0.98 s			
27	CH_3	14.89	0.88 s			
28	CH_3	17.86	0.66 s			
29	CH_3	22.70	0.92 d (6.8)			
30	CH_3	21.79	1.57 s	C20	H21, H22	

S: Singlet; d: Doublet; m: Multiplet; DEPT: Distortionless enhancement by polarization transfer; HMBC: Heteronuclear multiple bond correlation; COSY: ^1H-^1H correlation spectroscopy

Table 3: Nuclear magnetic resonance spectral data of rutin and isorhamnetin 3-*O*-rutinoside ($CDCl_3$, ^{13}C: 150 MHz; ^{1}H: 600 MHz)

Number (Rutin)	DEPT	δ_C (ppm)	δ_H (ppm), J (Hz)	H→C HMBC	Number (Isorhamnetin 3-O-rutinoside)	DEPT	δ_C (ppm)	δ_H (ppm), J (Hz)	H→C HMBC
l	C	158.50			2	C	158.36		
3	C	135.61			3	C	135.41		
4	C	179.39			4	C	179.25		
5	C	162.97			5	C	162.84		
6	CH	99.94	6.20 d (2.0)	C-10	6	CH	100	6.20 d (2.02)	C-8, C-10
7	C	166.05			7	C	165.99		
8	CH	94.85	6.39 d (2.08)		8	CH	94.95	6.39 d (2.07)	C-6, C-10, C-7, C-9
9	C	159.31			9	C	158.83		
10	C	105.60			10	C	105.61		
1’	C	123.10			1’	C	122.93		
2’	CH	117.67	7.66 d (2.17)	C-6’	2’	CH	114.49	7.93 d (2.04)	C-2, C-6’, C-3’
3’	C	145.83			3’	C	148.25		
4’	C	149.80			4’	C	150.78		
5’	CH	116.04	6.86 d (8.48)	C-1’, C-3’	5’	CH	116.07	6.91 d (8.48)	C-1’, C-3’, C-4’
6’	CH	123.54	7.62 dd (8.44/2.20)	C-4’	6’	CH	123.95	7.60 dd (8.47/2.09)	C-2, C-2’
1”	CH	104.70	5.10 d (7.72)	C-3	1”	CH	104.39	5.22 d (7.31)	C-3
2”	CH	78.17	3.42**	C-5”’	2”	CH	75.86	3.75**	
3”	CH	75.72	3.44**		3”	CH	77.28	3.47**	
4”	CH	72.22	3.62 dd (3.48/1.68)		4”	CH	72.02	3.66**	
5”	CH	77.20	3.32**		5”	CH	78.09	3.41**	
6”	CH_2	68.53	3.79/3.39 dd/** (11.03, 1.42/**)	C-1”’	6”	CH_2	68.50	3.82/3.46 dd/** (8.22/2.26)	C-1”’
1”’	CH	102.41	4.51 d (1.63)	C-2”’	1”’	CH	102.47	4.53 d (1.61)	
2”’	CH	72.09	3.53 dd (9.52/3.42)		2”’	CH	71.56	3.26**	
3”’	CH	71.38	3.26**		3”’	CH	72.23	3.43**	
4”’	CH	73.92	3.28**		4”’	CH	73.79	3.26**	
5”’	CH	69.70	3.46**		5”’	CH	69.76	3.34**	
6”’	CH_3	17.89	1.11 d (6.22)	C-5”’	6”’	CH_3	17.87	1.10 d (6.2)	C-5”’
					OCH3	CH_3	56.74	3.94 s	C-3’

**Splitting pattern and J value cannot be determined due to overlapping. S: Singlet; d: Doublet; dd: Doublet-doublet; DEPT: Distortionless Enhancement by polarization transfer; HMBC: Heteronuclear multiple bond correlation

β-carotene/linoleic acid co-oxidation test

The β-carotene bleaching method was performed to determine the antioxidant activity of extracts.[20] In this study, butylated hydroxytoluene (BHT) was selected as a reference standard. Absorbance values were measured using a spectrophotometer at 470 nm. Antioxidant activity was calculated according to equation (2).

$$AAC = ([Abs^{120}{}_{sample} - Abs^{120}{}_{control}]/[Abs^{0}{}_{control} - Abs^{120}{}_{control}]) \times 100 \quad (2)$$

Statistical analysis

All data were calculated using SigmaPlot 12.0 (Systat Software, San Jose, CA, USA), and one-way ANOVA *post hoc* Tukey test was used to determine the statistical significance ($P < 0.05$) in cytotoxicity tests.

In antioxidant tests, all data were presented as mean values ± 95% confidence interval. Analyses of variants were performed using ANOVA procedures. Significantly differences between means were determined by Tukey test at a level of $P < 0.05$.

RESULTS

Extraction and bioactivity-guided isolation of chemical constituents

In this study, among the methanol extracts of *Cousinia species*, CS and CD extracts were found to be more potent in the cytotoxicity and antioxidant assay, respectively, that is why these two extracts were selected for further partitionation. Afterward, CSD subextract of CS extract showed more cytotoxic and CDB subextract of CD extract showed more antioxidant activity. Accordingly, CSD and CDB subextracts were subjected to further fractionation. In result, from CSD subextract, three fractions (FR: 1–3) and from CDB subextract, two major compounds (rutin and isorhamnetin 3-O-rutinoside) were obtained. Subsequently, among these fractions, FR:3 was found more cytotoxic and was subjected to further purification, yielded one major compound ψ-taraxasterol. In this way, the bioactivity-guided fractionation of CS and CD extracts led to isolation of three major compounds [Figure 4]. This is the first study for these compounds that reported to be present in *Cousinia* species.

Structure elucidation of the isolated compounds

CSD and CDB subextract were fractionated using bioactivity-guided isolation, and three major compounds, ψ-taraxasterol, rutin, and isorhamnetin 3-O-rutinoside, were isolated and then elucidated by 1D and 2D NMR techniques. ψ-taraxasterol was obtained as white amorphous powder. The molecular formula, $C_{30}H_{50}O$, was established on the basis of its MS ([M+H]+ ion peak at m/z 427) and NMR data. NMR data suggested the presence of seven quaternary carbon atoms (C), six methine carbon atoms (CH), nine methylene carbon atoms (CH_2), and eight methyl carbon atoms (CH_3) in the structure. The ^{1}H and ^{13}C NMR spectrums exhibited that structure has characteristic ethylenic moiety (δ_c 140.01 [C20] and 119.02 [C21]; δ_H 5.19 [H21] bonded to C21) bonded to C19, C22, and C30. Also, one aliphatic hydroxyl group was determined, bonded to C3 [Table 2]. Comparison of the spectral data with those published before allowed us to establish the structure of ψ-taraxasterol.[21,22]

Rutin was obtained as yellow powder. The molecular formula, $C_{27}H_{30}O_{16}$, was established on the basis of its MS ([M-H]$^-$ ion peak at m/z 609) and NMR data. Analysis of spectral data and comparison of the spectral

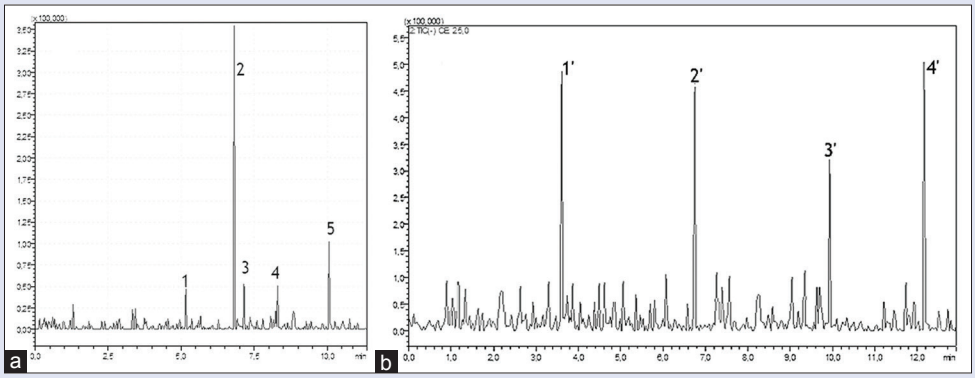

Figure 5: Total Ion Chromatogram (TIC) profile of the *n*-butanol extract subextract (a) and FR3 fraction (b)

data with those published before allowed us to establish the structure of rutin [Table 3].[23-25]

Isorhamnetin 3-O-rutinoside was obtained as yellow powder. The molecular formula, $C_{28}H_{32}O_{16}$, was established on the basis of its MS ([M-H]$^-$ ion peak at m/z 623) and NMR data. Analysis of spectral data and comparison of the spectral data with those published before allowed us to establish the structure of isorhamnetin 3-O-rutinoside [Table 3].[23,25]

Qualitative analyses of compounds

The structural characterizations of minor compounds in CDB subextract and FR: 3 were evaluated on the basis of the accurate mass, the registered mass spectra fragmentation patterns, and literature data. Compounds were studied in negative ion mode in CDB subextract and in positive ion mode in FR:3 fraction using MS/MS product ion scans [Figure 5]. Preliminary examination of the mass spectrums revealed the presence of luteolin-7-O-glucuronide,[26] kaempferol 7-O-neohesperidoside,[27] and myricetin 3-O-glucoside[28] in CDB subextract and myricetin,[29] caffeic acid ester and apigenin 7-apiosylglucoside[30] in FR:3. For flavonol and flavone O-glycosides, the spectra present both the deprotonated molecule [M-H]$^-$ of the glycosides and the deprotonated aglycone ion [A-H]$^-$. The latter ion is established by loss of the glycon residue from the glycosides. Fragmentation of aglycones yielded specific ions for each type of flavonoids.[31] Using ESI MS/MS in the positive ion mode, the protonated molecular ions of caffeic acid esters produced an abundant ion at m/z 163. The typical fragmentation pathway resulted from the positive ionization of the carbonyl oxygen.[32] The mass spectra of CDB subextract and FR: 3 fraction were shown in Figure 6. Molecular ion, retention time, MS/MS data, and molecular formulas of identified compounds are given in Tables 4 and 5.

Quantitative analyses of compounds

Optimization of liquid chromatography-mass spectrometry/mass spectrometry condition

The mass spectrometric behavior of rutin and isorhamnetin 3-O-rutinoside was studied using both positive-ion and negative-ion mode. Negative-ion mode provided a better sensitivity for these compounds due to more efficient ionization, simpler fragmentation, and lower baseline noise.

These compounds were subsequently analyzed in Q1Scan (product ion scan) mode, using [M−H]$^-$ ions as precursors. Obtained MS2 spectra were used to select the optimal product ions. The MRM parameters, such as the precursor ion m/z, collision energy, and product ion m/z for compounds, were optimized by an automatic MRM optimization function [Figure 7].

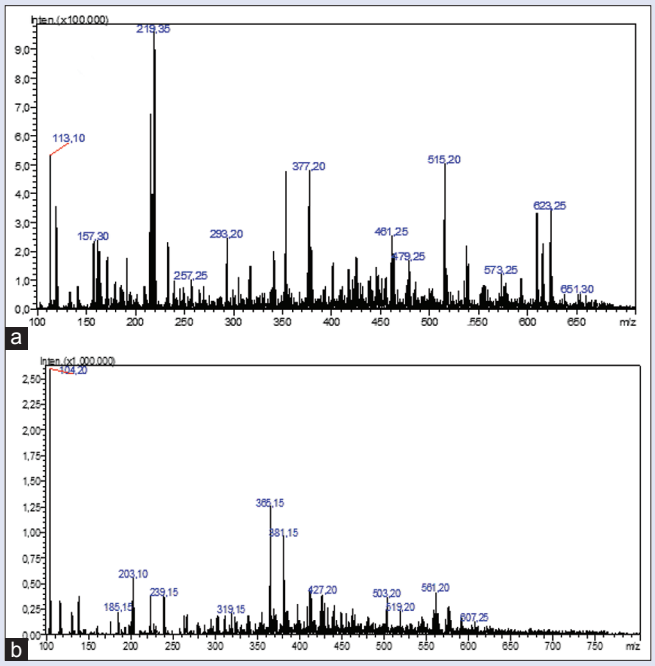

Figure 6: Mass spectra of *n*-butanol extract subextract (a) and FR3 fraction (b)

Fragmentation of [M-H]$^-$ ion (m/z 609) of rutin resulted in two major ions at m/z 300 and 301, showing the loss of rhamnose–glucose unit. The other flavonol diglycoside isorhamnetin 3-O-rutinoside is a 3'-methoxylated derivative of rutin. Fragmentation of this molecule [M-H]$^-$ ion (m/z 623) resulted ions m/z 285, 300, and 315. Isorhamnetin represents specific fragmentation with the loss of CH_3 radical from the deprotonated aglycone, thus giving m/z 315 → m/z 300 and the m/z 285 pattern as a result of fragmentation in C-ring. The obtained LC-MS/MS chromatogram and mass spectrum of compounds are presented in Figure 8.

Preparation of standard and sample solutions

Stock solutions of rutin and isorhamnetin 3-O-rutinoside were prepared in methanol at 4 μg/mL and 5 μg/mL concentrations, respectively. The extract and subextract solutions were prepared in methanol at 10 μg/mL.

Calibration curve

Linearity of the methods was established by triplicate injections of each concentration of standard solutions. Response function of the standards

Table 4: Mass spectral characteristics and identification of compounds in *Cousinia davisiana n*-butanol extract subextract

Peak number	$(M-H)^-$(m/z)	MS/MS fragments (m/z)	RT (t_R/min)	Formula	Compound
1	461	285	5.2	$C_{21}H_{18}O_{12}$	Luteolin-7-O-glucuronide
2	479	317, 316, 287, 271	6.7	$C_{21}H_{20}O_{13}$	Myricetin 3-glucoside
3	609	301	7.3	$C_{27}H_{30}O_{16}$	Rutin
4	593	285, 284, 331, 593	8.7	$C_{27}H_{30}O_{15}$	Kaempferol 7-O-neohesperidoside
5	623	285, 300, 315	10.1	$C_{28}H_{32}O_{16}$	Isorhamnetin 3-O-rutinoside

Table 5: Mass spectral characteristics and identify of compounds in FR: 3 fraction

Peak number	$(M-H)^+$(m/z)	MS/MS fragments (m/z)	RT (t_R/min)	Formula	Compound
1'	319	287, 271, 179, 151, 137	3.6	$C_{15}H_{10}O_8$	Myricetin
2'	519	325, 163	6.7	-	Caffeic acid ester
3'	561	271	9.9	$C_{26}H_{28}O_{14}$	Apigenin 7-apiosylglucoside
4'	427	409	12.2	$C_{30}H_{50}O$	ψ-taraxasterol

RT: Retention time

Table 6: Contents of rutin and isorhamnetin 3-*O*-rutinoside in extracts and subextracts (μg/$g_{extract}$±Standard deviation)

Constituent	RT (min)	Content[a] (*μg/mg* $_{extract}$)						
		CD	CF	CS	CR	CDB	CDE	CDS
Rutin	7.83	8.706±0.031	5.557±0.026	1.480±0.009	4.386±0.003	22.352±0.013	1.154±0.001	n.d.
Isorhamnetin 3-*O*-rutinoside	10.24	55.432±0.417	22.475±0.218	0.212±0.155	0.631±0.031	169.062±4.082	0.146±0.028	n.d.

[a]Mean±SD (*n*=3). RT: Retention time; n.d.: Not detected- peak not observed; SD: Standard deviation; C: *Cousinia*; CD: *C. davisiana* methanol extract; CF: *C. foliosa* methanol extract; CS: *C. stenocephala* methanol extract; CR: *C. ramosissima* methanol extract; CDB: *C. davisiana n*-butanol extract; CDE: *C. davisiana* ethyl acetate extract; CDS: *C. davisiana* water extract

calibration curve was $y = 511143x - 4056$ for rutin and $y = 18006x + 928.47$ for isorhamnetin 3-O-rutinoside. The correlation coefficient (r^2) of the calibration curves was 0.9997 and 0.9996, respectively.

The quantitative results of rutin and isorhamnetin 3-O-rutinoside are given in Table 6. As shown in table, among the methanol extracts, the highest contents of rutin and isorhamnetin 3-O-rutinoside were detected in CD extract (8.706 and 55.432 μg/$mg_{extract}$, respectively). Furthermore, the lowest contents of compounds were detected in CS extract (1.480 and 0.212 μg/$mg_{extract}$, respectively). As seen in table, among the subextracts, the highest contents of rutin and isorhamnetin 3-O-rutinoside were found in CSB subextract (22.352 and 169.062 μg/$mg_{extract}$, respectively). None of these compounds were detected in CDS subextract.

In vitro cytotoxic activity

The cytotoxic effects of methanol extracts of *Cousinia* species were determined on Colo 205 and A549 cell lines by SRB method. Results showed that CS methanol extract (1000, 750, 500, 250, and 125 μg/mL) was more cytotoxic, particularly on the Colo 205 cell line, with the 130 μg/mL IC_{50} value. Therefore, CS methanol extract was partitioned, and subextracts were evaluated (1000, 750, 500, 250, and 125 μg/mL) on both cell lines. It was found that among the subextracts, CSD subextract possessed more cytotoxic activity than others (IC_{50} = 115 μg/mL, for Colo 205 cell line). Then, CSD subextract was fractionated and the cytotoxic effect of fractions (1000, 750, 500, 250, 125 μg/mL) also determined. In result, FR:3 was more cytotoxic with the 109 μg/mL IC_{50} value, against Colo 205 cell line. But, ψ-taraxasterol, a major compound isolated from FR:3 (100, 50, 25, 12.5, and 6.25 μg/mL), showed no cytotoxic effect against tested cancer cell lines. The IC_{50} values of all extracts and fractions are given in Table 7.

Table 7: *In vitro* 24 h cytotoxicity of the extracts, subextracts, fractions, and isolated compound from *Cousinia stenocephala*

Code	IC_{50} (μg/mL) Cell line	
	A549	Colo 205
CD	≥1000	306
CF	≥1000	≥1000
CR	≥1000	286
CS	990	130*
CSH	≥1000	≥1000
CSD	389	115*
CSE	708	141*
CSB	≥1000	210
CSS	≥1000	≥1000
FR: 1	≥1000	635
FR: 2	≥1000	≥1000
FR: 3	264*	109*
ψ-taraxasterol	≥100	≥100
Cisplatin	19	51

*P <0 .05, compared with the untreated control. Concentrations of extracts, sub-extracts, fractions (1000, 750, 500, 250, 125 μg/ml) and compound (100, 50, 25, 12.5, 6.25 μg/ml), A549: Human lung carcinoma; Colo 205: Human colorectal adenocarcinoma; C: *Cousinia*; CS: *C. stenocephala*; CR: *C. ramosissima*; CF: *C. foliosa*; CD: *C. davisiana*; CSH: *C. stenocephala* hexane extract; CSD: *C. stenocephala* dichloromethane extract; CSE: *C. stenocephala* ethyl acetate extract; CSB: *C. stenocephala n*-butanol extract; CSS: *C. stenocephala* water extract; FR: 3 fraction

The total phenol and flavonoid content

The TPC and TFC results of methanolic extracts of *Cousinia* species are shown in Table 8. The highest TPC was found in the ethyl acetate subextract, but the highest TFC was observed in *n*-butanol subextract of CD (TPC = 242.811 ± 12.89 mgGAE/$g_{extract}$, TFC = 131.265 ± 2.14 mgCA/$g_{extract}$).

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl scavenging activity

The DPPH radical scavenging capacity of methanol extracts of *Cousinia* species and subextracts of CD were determined and the

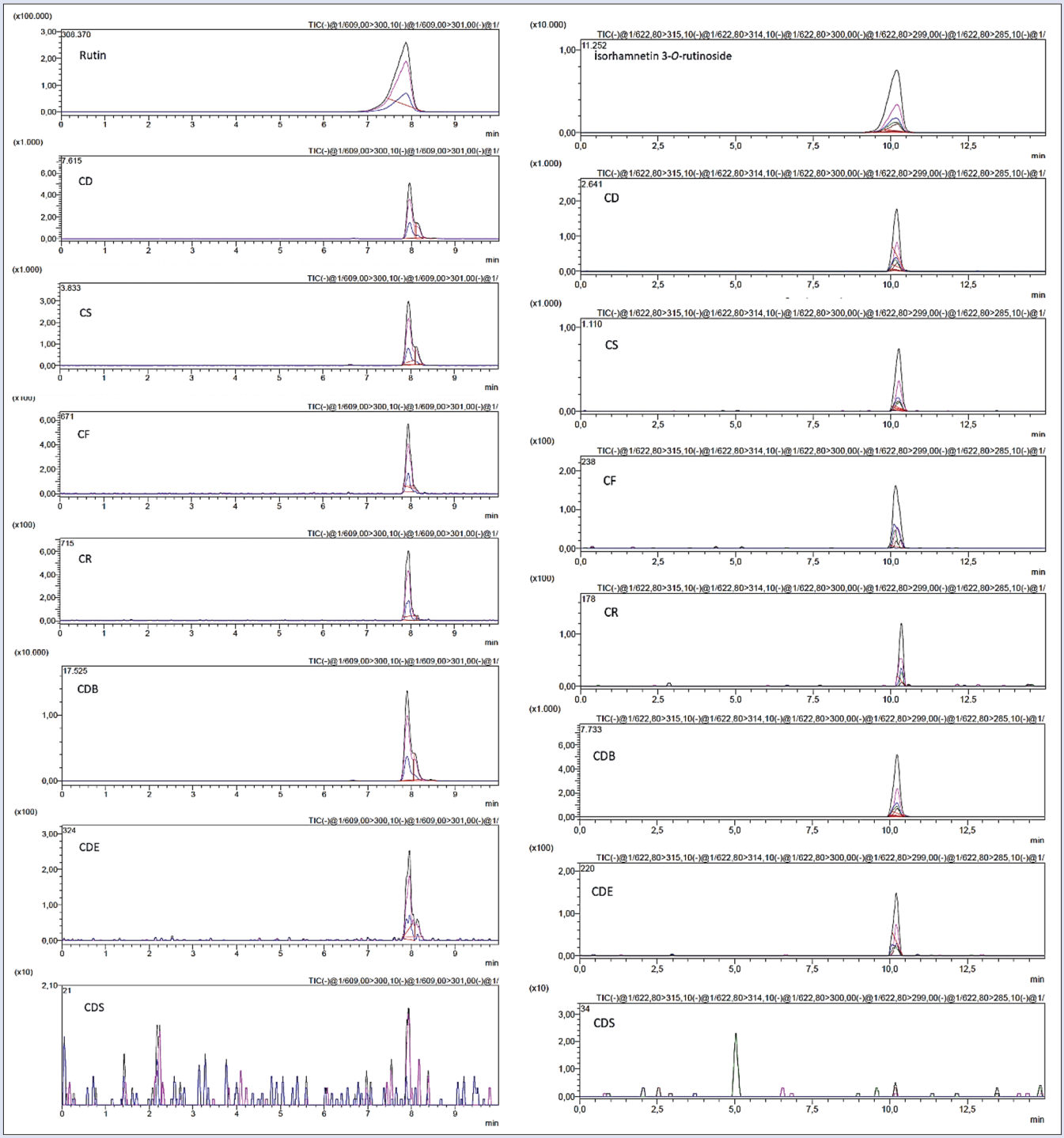

Figure 7: Representative MRM chromatograms of rutin and isorhamnetin 3-O-rutinoside

IC_{50} values are given in Table 8. Among the methanol extracts, CD extract showed remarkable DPPH radical scavenging activity with the 0.21 ± 0.01 mg/mL IC_{50} value. The *n*-butanol subextract of CD showed highest activity (IC_{50} = 0.13 ± 0.02 mg/mL) and showed statistically similar activity ($P > 0.05$) with BHA (IC_{50} = 0.08 ± 0.00 mg/mL). None of the extracts showed activity similar to BHA except for CDB ($P < 0.05$).

2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) scavenging activity

The ABTS radical scavenging activity of the extracts and subextracts were determined at concentration of 0.50 and 1 mg/mL. The concentrations of standards were 0.25 and 0.5 mg/mL. All extracts revealed the highest activity at 1 mg/mL. None of extracts and subextracts surpassed the activity of BHA. The TEAC value of extracts was determined, and CD

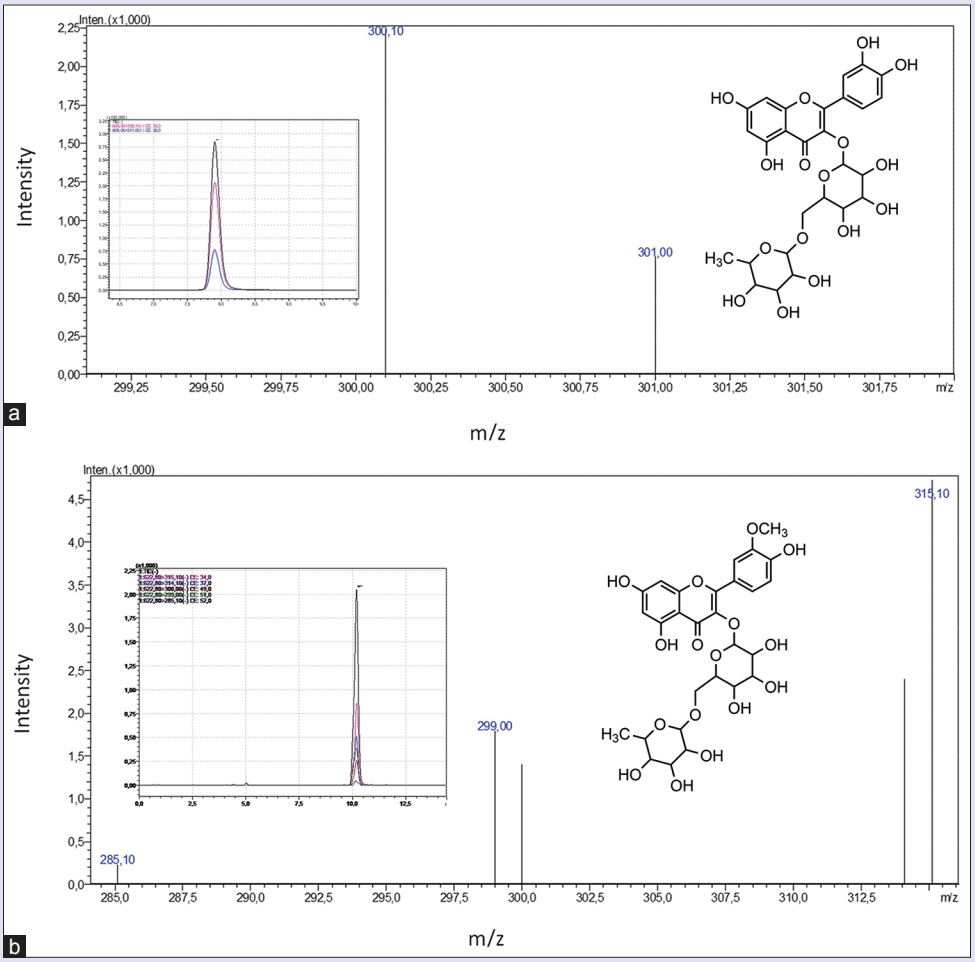

Figure 8: Liquid chromatography-mass spectrometry/mass spectrometry chromatogram and mass spectra of rutin (a) and isorhamnetin 3-O rutinoside (b)

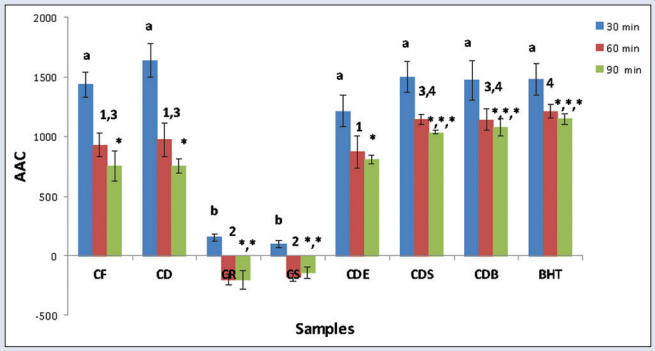

Figure 9: The effect of extracts and sub-extracts on beta-carotene linoleic acid co-oxidation

extract was more active with 0.79 ± 0.05 mmol/L Trolox at a 1 mg/mL than others. CDB subextract was found more active at 1 mg/mL concentration than water and ethyl acetate subextracts (TEAC: 1.42 ± 0.08 mmol/L Trolox) [Table 8]. The activity of the CDB at a concentration of 1 mg/mL was found statistically the same as the activity at a concentration of 0.25 mg/mL of BHA ($P > 0.05$).

β-carotene/linoleic acid co-oxidation test

The oxidation-inhibiting effect of extracts and sub-extracts was observed in a time-dependent manner. The time-dependent alteration was determined, and the inhibition values are given in Figure 9. According to the results, the CD extract was found more active than other methanol extracts (CS, CF, and CR) and positive control BHT after 30 min. But, CD and CF extracts showed statistically similar activities ($P > 0.05$) and were more active than the CS and CR extracts after 90 min. CDB and CDS extracts were found to inhibit oxidation to a statistically equivalent degree ($P > 0.05$) after 90 min with the BHT.

DISCUSSION

It is well-known that there are some difficulties in use of conventional drugs in cancer therapy because of side effects and interactions. On the other hand, because of their wide application, therapeutic efficacy, and low toxicity, increasing attention has been paid to natural products for evaluation of their anticancer activities.[33,34]

In spite of the large investigation on taxonomy, systematics, and phylogeny of *Cousinia* species, their biological and phytochemical studies are very limited. Based on previous studies on cytotoxic activity of ethanol extracts of seven *Cousinia* species against fibrosarcoma WEHI 164 cancer cell line, *Cousinia verbascifolia* showed high activity (IC_{50} = 18.4 ± 0.59 μg/mL).[35] In another study, *C. verbascifolia* fractions were observed against ovarian cancer (OVCAR-3) and colorectal cancer (HT-29) cell lines and apigenin and caffeic acid were isolated as a bioactive compounds from this species.[35,36]

Investigation on C. *aitchisonii* led to isolation of sesquiterpene compounds namely desoxyjanerin and rhaserolide, and these compounds

Table 8: The antioxidant values of methanol extracts of *Cousinia* species and subextracts of *Cousinia davisiana*

Sample	DPPH.scavenging activity IC_{50} (mg/mL)	ABTS.scavenging activity (TEAC, mmol/L Trolox)*	TPC ($mg_{GAE}/g_{extract}$)	TFC ($mg_{CA}/g_{extract}$)
BHA	0.08 ± 0.00^{d}	$1.02\pm0.05^{+,a}$	-	-
CD	0.21 ± 0.01^{b}	$2.26\pm0.09^{++,b}$ $0.54\pm0.03^{++,c,d}$ $0.79\pm0.05^{+++,c,d}$	146.036±5.36	88.700±1.083
CR	0.83 ± 0.05^{a}	$0.50\pm0.03^{++,c,d}$ $0.61\pm0.06^{+++,c,d}$	66.230±4.90	25.230±4.66
CF	0.71 ± 0.06^{a}	$0.33\pm0.04^{++,c}$ $0.58\pm0.06^{+++,c,d}$	109.630±11.44	62.305±0.68
CS	0.88 ± 0.10^{a}	$0.48\pm0.04^{++,c,d}$ $0.64\pm0.05^{+++,c,d}$	128.142±2.86	82.346±1.26
CDB	$0.13\pm0.02^{b,d}$	$0.79\pm0.03^{++,c,d}$ $1.42\pm0.08^{+++,a}$	118.577±3.97	131.265±2.14
CDE	1.73 ± 0.05^{c}	$0.70\pm0.01^{++,c,d}$ $1.77\pm0.04^{+++,c,d}$	242.811±12.89	115.924±1.01
CDS	0.67 ± 0.15^{a}	$0.54\pm0.01^{++,c,d}$ $0.70\pm0.02^{+++,c,d}$	22.151±5.7	10.191±2.3

*scavenging activity equal to; +0.25 mg/mL, ++0.5 mg/mL, +++1 mg/mL sample dilution. Values expressed as mean±standard errors (n=3). Bars with the same lower-case letter and number (a-d) are not significantly (P>0.05) different. C: *Cousinia*; CD: *C. davisiana* methanol extract; CF: *C. foliosa* methanol extract; CR: *C. ramosissima* methanol extract; CS: *C. stenocephala* methanol extract; CDB: *C. davisiana n-* butanol extract; CDE: *C. davisiana* ethyl acetate extract; CDS: *C. davisiana* water extract. TEAC is defined as the concentration of Trolox (mmol/L) having the ABTS; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid; TPC: Total phenol contents; BHA: Butylated hydroxyanisole; TEAC: Trolox equivalent antioxidant capacity; TFC: Total flavonoid content; IC_{50}: Inhibitory concentration

showed significant cytotoxic effects on breast cancer (MCF-7) cell line (IC_{50} = 4.5 µg/mL and 4.6 µg/mL, respectively).[7]

To date, sesquiterpene lactones (*Cousinia picheriana, Cousinia piptocephala, Cousinia canescens*), triterpenes (*Cousinia adenostica*), steroids (*Cousinia canescens*) and flavonoids (*Cousinia verbascifolia*) have been isolated from *Cousinia* genus.[7-15]

The main purpose of this study is to identify and quantify the major bioactive compounds through the execution of bioactivity-guided isolation of *Cousinia* extracts for cytotoxic and antioxidant activity.

The cytotoxic effect of four *Cousinia* methanol extracts was determined against two cancer cell lines (Colo 205 and A549) by SRB method. Among these extracts, CS extract showed higher cytotoxic effect, particularly against Colo 205 cell line and selected as guide. Among the subextracts prepared from this extract, CSD subextract was found to be more active than others. Then, this subextract was fractionated, and obtained three fractions were subjected to cytotoxicity test also. In result, FR:3 was found to be more cytotoxic and led to bioassay-guided isolation of ψ-taraxasterol. Phytochemical assessments in *Cousinia* species revealed the presence of sesquiterpene lactones, triterpenes, lignans, and phenolics which have a wide range of cytotoxic and antitumor effects.[35] However, this compound did not show any cytotoxic effect alone at the tested concentrations. Hence, it was thought that the cytotoxicity of FR: 3 may be due to synergistic effect of purified compound (ψ-taraxasterol) and identified compounds (myricetin, caffeic acid ester, apigenin 7-apiosylglucoside). In literature, the cytotoxic effect of these identified compounds was reported previously.[37-39]

In this study, the antioxidant activity of four *Cousinia* species was measured with free radical (DPPH, ABTS) scavenging and lipid peroxidation inhibitory activity tests. Furthermore, TPC and TFC of plants were investigated. Among the methanol extracts, TPC and TFC were found higher in CD extract than others. But, among the subextracts of CD, TPC was higher in CDE and TFC was higher in CDB. Due to the presence of high amount of flavonoids, CDB subextract showed higher antioxidant activity. In a result of three antioxidant activity tests, CD extract showed higher activity. Among three subextracts, CDB showed higher activity and two major compound rutin and isorhamnetin 3-O-rutinoside were isolated and minor compounds (luteolin-7-O-glucuronide, kaempferol 7-O-neohesperidoside myricetin 3-O-glucoside) were identified by LC-MS/MS. The higher antioxidant activity of CDB subextract is correlated to the presence of these flavonoids. It is well known that antioxidant effect of flavonoid compounds is related to structure conformation of these compounds. The free radical scavenging activity of flavonoids is linked presence of -OH groups, 2,3-double bond in conjunction with 4-carbonyl group in ring C, allowing for delocalization of the phenoxyl radical electron to the flavonoid nucleus.[30] Antioxidant effect of purified compounds has previously been reported.[40-43] The results showed that amount of purified compounds – rutin and isorhamnetin 3-O-rutinoside were higher in CD extract and CDB subextract. In this way, the relationship of content of these compounds in extracts and antioxidant activity was verified.

Hence, determination of the cytotoxic and antioxidant properties and identification and quantification of active constituents of *Cousinia* species will promote advanced studies that may help to protect against free radical damage and oxidative stress-related diseases. Moreover, this is the first report that these major and minor compounds identified in these genera, and it was thought that these compounds could represent a chemical marker of the Stenocephalae section as contributing to the chemotaxonomy of the genus. It is thought that the fraction-based activity could be explained by the synergistic effect of ψ-taraxasterol and minor compounds, and in our view, this eventual synergism may be a new approach in natural product research. Furthermore, by regarding the potential antioxidant activity and rich content of flavonoids, CD plant may be used as a good source of antioxidant and could help protect against the diseases.

CONCLUSION

This is the first report on the bioactivity-guided isolation of species from Stenocephalae section of *Cousinia* genus. The results showed that these *Cousinia* species have cytotoxic and antioxidant activity. Identification and quantification of the antioxidant constituents of these plants were evaluated in this study, and their protective effect with other minor compounds may be investigated. Moreover, because of the limited studies on *Cousinia* species and identified compounds, this qualitative

and quantitative study combined with activity evaluation will shed new lights to the advanced studies.

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Conflicts of interest

There are no conflicts of interest.

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