https://doi.org/10.31925/farmacia.2020.3.18 **ORIGINAL ARTICLE**

CYTOTOXIC AND PHYTOCHEMICAL INVESTIGATION OF *COUSINIA ERMENEKENSIS* HUB.-MOR.

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Abstract

Cousinia is one of the widespread genera of *Asteraceae* family. There are numerous studies on this genus and it has not been examined phytochemically in detail. Thus, we aimed to evaluate the phytochemical composition and the cytotoxic effect of *Cousinia ermenekensis* on human lung cancer (A549), human colon adenocarcinoma (Colo 205), hepatocellular carcinoma (HepG2) cell lines and human bronchial epithelial (Beas-2b) cell line. *C. ermenekensis* methanol extract showed higher cytotoxic activity against Colo 205 cell line (IC_{50} = 69 µg/mL). A preliminary examination of the mass spectrums revealed the presence of 13 compounds in the extract. According to the quantitative analyses, the highest content of chlorogenic acid, malic acid, quinic acid and, rutin were detected in extract (28.496, 18.264, 15.115 and 12.506 µg/mg extract respectively).

Rezumat

Cousinia este unul dintre genurile cele mai răspândite din familia *Asteraceae*, existând numeroase studii asupra acestui gen, dar fără a prezenta detalii fitochimice. Studiul de față prezintă compoziția fitochimică și efectul citotoxic al speciei *Cousinia ermenekensis* asupra cancerului pulmonar uman (A549), adenocarcinomului de colon uman (Colo 205), carcinomului hepatocelular (HepG2) și liniilor de celule epiteliale bronșice umane (Beas-2b). Extractul metanolic de *C. ermenekensis* a prezentat activitate citotoxică superioară asupra liniei celulare Colo 205 (IC_{50} = 69 µg/mL). O evaluare preliminară a spectrelor de masă a evidențiat prezența a 13 compuși în extract. Conform analizelor cantitative, au fost detectate, în concentrație ridicată, acid clorogenic, acid malic, acid chinic și rutină (28,496; 18,264; 15,115 și, respectiv, 12,506 µg/mg extract).

Keywords: *Cousinia*, *Asteraceae*, cytotoxicity, LC-MS/MS

Introduction

Cancer is a disease that starts when cells grow out of control and crowd out normal cells in any place in the body. There are many types of cancer, that take place in the lungs, the breast, the colon or in the blood. Cytotoxicity is the inhibition of the uncontrolled growth of cells [7]. Because of their side effects and interactions, synthetic drugs are not preferred in the treatment of different cancer types. However, in the recent years, the use of herbal medicines has been increasing due to their therapeutic activity and low toxicity [5].

The members of the *Asteraceae* family (syn. *Compositae*) were used in the treatment of various cancer types [4]. *Cousinia* Cass. is one of the most diverse genera of the *Asteraceae* family. This genus consists of 600 - 700 species distributed in Central and South-West Asia and is represented by 38 species and 6 sections in Turkey. *C. ermenekensis* Hub.-Mor. is endemic in Turkey and could be seen in open areas and scrublands in Karaman. This a biennial plant, with spiny-dentate leaves and pink, purple or rose flowers. The flowering period of the plant is July [9].

In the literature, taxonomic and systematic studies are generally performed on the genus of *Cousinia*, but phytochemical and activity studies are rarely seen. Thus, we aimed to investigate the phytochemical and biological properties of the species. In this study, the cytotoxic effect of this species was screened on human lung cancer (A549), human colon adenocarcinoma (Colo 205), hepatocellular carcinoma (HepG2) cell lines and human bronchial epithelial (Beas-2b) cell line and phytochemical profile of extract and quantitative analyses of phenolic compounds were determined by LC-MS/MS.

Materials and Methods

Plant material and preparation of extracts

The flowering aerial parts of *C. ermenekensis* were harvested from East Ermenek, Karaman in July 2013 and identified by Prof. Dr. Osman Tugay. This specimen was stored at the Herbarium of Selçuk University (Voucher No. 1, KNYA 26.976).

Air-dried aerial parts of *C. ermenekensis* (500 g) were powdered and extracted three times with methanol by maceration, at room temperature. 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. The yield of extract was 10%.

Qualitative and quantitative LC-MS/MS assay

LC-MS/MS instrumentation

The quantitative and qualitative analyses were performed by using liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS, Shimadzu 8040). The mass spectrometric behaviour of extract was studied using the negative-ion mode. The sample was prepared in methanol and was injected (1 µL) into the LC-MS/MS equipped with a C18 reverse-phase column (150 x 4.6 mm x 3 µm; Restek). A mixture of methanol: formic acid (99:1 v/v) (A) and water: formic acid (99:1, v/v) (B) was selected as mobile phase. The mobile phase consisted of 90% solvent A and 10% solvent B at a flow rate of 0.4 mL/min.

In vitro cytotoxic activity assay

A549, Colo205, HepG2, and Beas-2b were cultured in RPMI medium (Sigma R8758) supplemented with 10% FBS (Biochrome S0415) and 100 U/mL penicillin-100 ug/mL streptomycin (Sigma P4333). 10.000 cells were dispensed to 96 well plates in 100 µL medium and incubated overnight. The extract was dissolved in dimethylsulfoxide (DMSO, Applichem A3672) in order to obtain 3 mg/mL stock solutions of extracts which contains 1.5% DMSO. 125, 250, 500, 750, 1000 µg/mL extract concentrations were prepared two times by diluting stock solutions with the medium. Then, extracts were added to cultures in 100 µL volume. To obtain a homogenous solution, plates were incubated on a plate shaker for 5 minutes and further incubated at 37ºC under a humidified atmosphere of 5% CO_2. 24 h later, cell viability was determined by SRB assay [25].

Statistical Analysis

All data were calculated by using Systat Sigma Plot (ver. 12.0) software and a One-way ANOVA post hoc Dunnett test was used to determine the statistical significance (*$p < 0.05$) in cytotoxicity tests.

Results and Discussion

Qualitative LC-MS/MS analyses of phenolic compounds

The identification of phenolic compounds in the CE extract was evaluated based on the accurate mass, the registered mass spectra fragmentation patterns, and literature data. Compounds were studied in the negative ion mode and total ion chromatograms (TIC) of the extract are presented in Figure 1. A preliminary examination of the mass spectrums revealed the presence of 13 compounds in the methanolic extract (Table I). The mass spectra of CE extract are presented in Figure 2.

Table I

Mass spectral characteristics and identity of compounds in *Cousinia ermenekensis* extract

Pik No	RT t_R (min)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Compounds
1	3.2	461	285	Luteolin 7-*O*-glucuronide [17]
2	3.8	269	269, 183, 159, 151, 149, 117, 107, 83, 65	Apigenin [20]
3	4.3	377	341, 215, 179, 161, 119	Caffeic acid derivative [18]
4	4.9	341	179, 161, 683	Dicaffeic acid [3]
5	5.1	191	191, 93, 85	Quinic acid [8]
6	5.3	353	191, 179, 173, 135	Chlorogenic acid [16]
7	5.6	179	135, 87	Caffeic acid [12]
8	7.2	133	115, 71	Malic acid [11]
9	8.1	609	301	Rutin [14]
10	10.2	623	315	Isorhamnetin 3-*O*-rutinoside [16]
11	14.1	317	-	Myricetin [10]
12	14.2	387	223, 191, 179	5-Sinapoylquinic acid [16]
13	17.5	615	469, 393, 317, 169	Myricitrin-*O*-gallate [1]

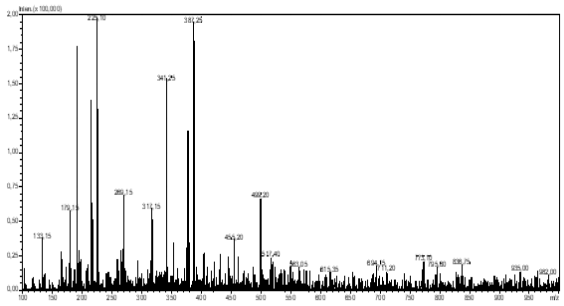

Figure 1.
TIC profile of *Cousinia ermenekensis* extract

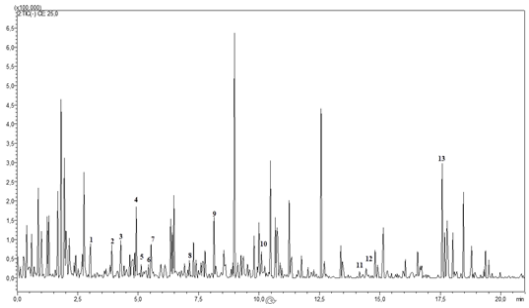

Figure 2.
Mass spectra of *Cousinia ermenekensis* extract

This is the first report about the phenolic composition of *C. ermenekensis.* Based on chemical reports on some *Cousinia* species, various chemical compounds including steroids, triterpenes, sesquiterpene lactones and flavonoids were declared [13, 19, 23, 24]. To the best of our knowledge, only one study reported data about the phenolic composition of *Cousinia verbascifolia* identifying apigenin and caffeic acid as the phenolic compounds [22].

Quantitative Analyses of Compounds

Optimization of LC-MS/MS condition

The mass spectrometric behaviour of compounds was studied using both positive-ion and negative-ion mode. Negative-ion mode provided a superior sensitivity for these compounds due to more efficient ionization, simpler fragmentation, and lower baseline noise.

These compounds were subsequently analysed 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. The obtained LC-MS/MS chromatogram and mass spectrum of compounds are presented in Figure 3.

Preparation of standard and sample solutions

Stock solutions of compounds were prepared in methanol at 8 µg/mL concentrations. The extract solutions were prepared in methanol at 10 µg/mL.

The quantitative results of the compounds are given in Table II. As shown in the table, the main compounds of CE were chlorogenic acid, malic acid, quinic acid and rutin with the highest content (28.496**,** 18.264**,** 15.115 and 12.506 µg/mg extract respectively). This is the first report regarding the quantitative analyses of phenolic compounds in *Cousinia* species.

Table II

Compounds in *Cousinia ermenekensis* extract

Constituent	**RT (min)**	**Content[a]** (µg/mg extract)
Malic acid	7.2	18.264 ± 0.058
Caffeic acid	5.6	1.248 ± 0.004
Quinic acid	5.1	15.115 ± 0.027
Chlorogenic acid	5.3	28.496 ± 0.055
Rutin	8.1	12.506 ± 0.012
Isorhamnetin 3-*O*-rutinoside	10.2	1.727 ± 0.112

RT-retention time, [a]Mean ± SD (n = 3)

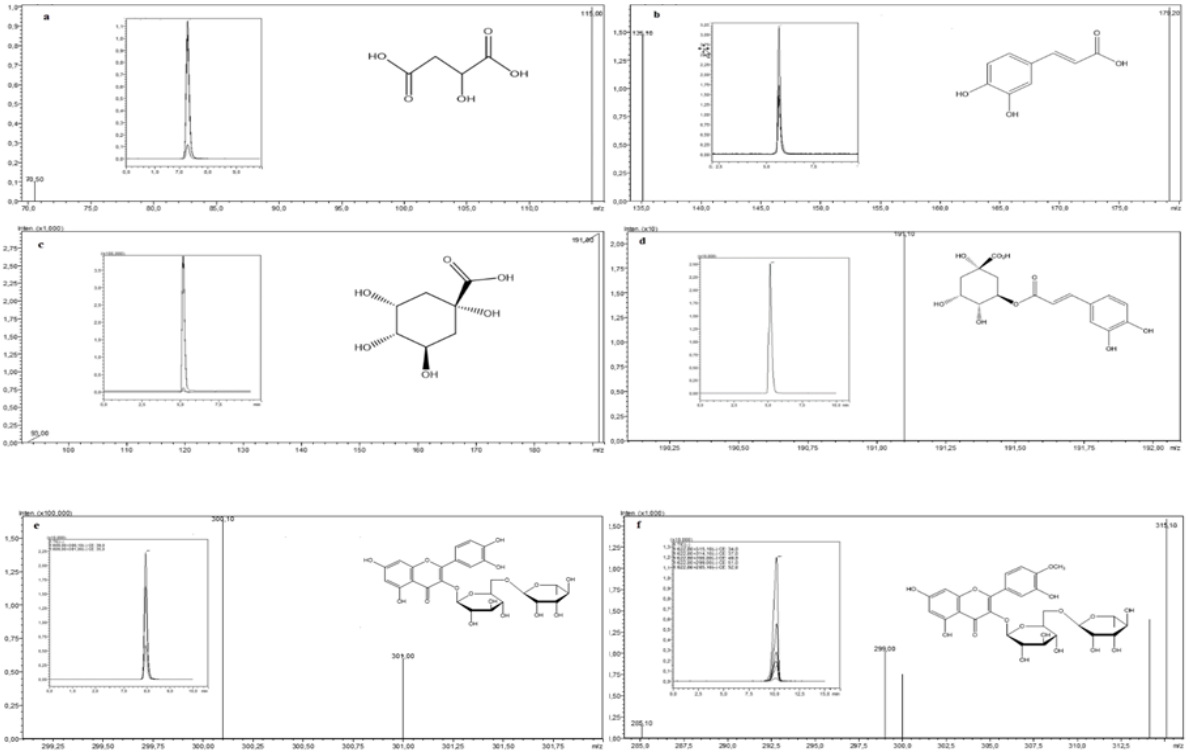

Figure 3.

LC-MS/MS chromatogram and mass spectra of malic acid (a), caffeic acid (b), quinic acid (c), chlorogenic acid (d), rutin (e) and isorhamnetin 3-O-rutinoside (f)

In vitro cytotoxic activity

The cytotoxicity of the methanolic extract of *C. ermenekensis* was investigated against A549, Colo205, HepG2 cancer cell lines and Beas-2b cell line by SRB method. Results showed that CE extract (1000, 750, 500, 250, 125 µg/mL) was more cytotoxic against particularly Colo205 cancer cell line with the 69 µg/mL IC_{50} value (Table III). As shown in the table, CE extract is not toxic to A549 and HepG2 cancer cell lines. Additionally, this extract was the least toxic to Beas-2b (Figure 4). In a previous study, the effects of ethanol extracts of some *Cousinia* species on

different cancer cell lines and matrix metalloproteinase protein (MMP) inhibitor effects were examined. The cytotoxic activity of ethanol extracts from different *Cousinia* species was investigated against fibrocarcinoma cell line and *C. verbasciflora* have shown higher cytotoxic activity with 18.4 ± 0.59 µg/mL IC_{50} value [23]. In another study, sesquiterpene compounds, namely desoxyjanerin and raserolit, obtained from the dichloromethane extract of *C. aitchisonii*, were subjected to the cytotoxic screening on five different cell lines. As a result, both compounds showed significant cytotoxic effect on breast cancer MCF-7 cell line (IC_{50} = 4.5 µg/mL and 4.6 µg/mL, respectively) [13]. Additionally, the MMP inhibitory effect of *C. shulabadensis* was investigated and reported to have a considerable inhibitory effect (IC_{50} = 49.2 ± 0.51 µg/mL) [23]. Our results are in agreement with the previously reported. So, we can affirm that *C. ermenekensis* has selective cytotoxic activity because it is not cytotoxic to Beas-2b and no IC_{50} can be detected. Some of the identified substances in the extract already were known for their anticancer properties. Numerous studies have reported anticancer properties for apigenin [15], caffeic acid, chlorogenic acid, quinic acid [21], luteolin-7-*O*-glucuronide, myricetin [2, 6] through various mechanisms. According to these results, we can consider that the potent cytotoxic activity of CE on the Colo205 cell line may be explained with the presence of these anticancer flavonoid compounds.

Table III

In vitro 24 h cytotoxicity of the methanol extract of *Cousinia ermenekensis*

	IC_{50} (µg/mL) Cell line			
CE	**A549**	**Colo 205**	**HepG2**	**Beas-2b**
	In	69	780	*In*

A549, human lung carcinoma; Colo 205, human colorectal adenocarcinoma; HepG2, human hepatocarcinoma; Beas-2b bronchial epithelial cell line; *In*, inactive; CE: *C. ermenekensis*

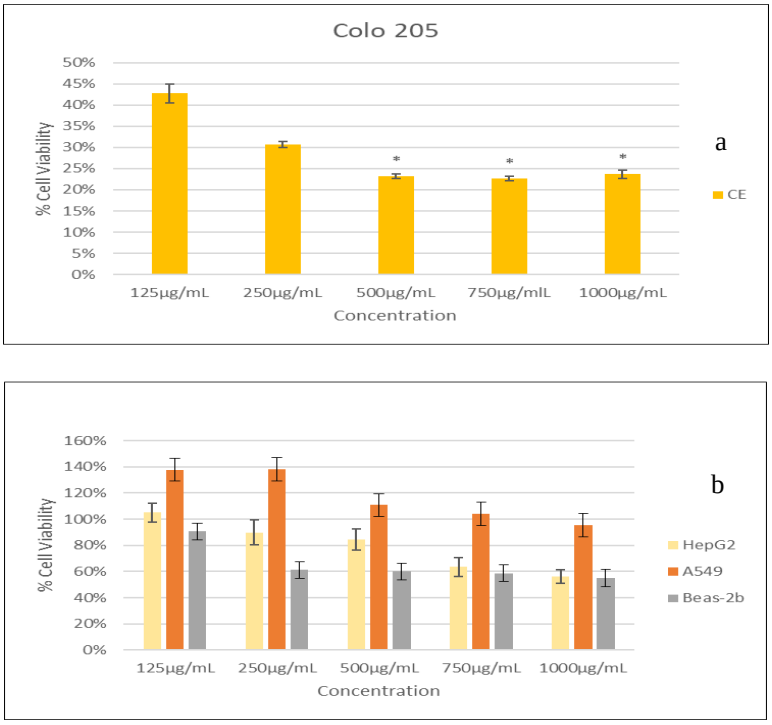

Figure 4.

Cell viability at the end of 24 h incubation with *Cousinia ermenekensis* extract a. Colo 205; b. HepG2, A549, Beas-2b

Mean values of groups are statistically different ($p < 0.001$). Mean values of the treatment groups are statistically different than the control group. (*$p < 0.05$). A549, human lung carcinoma; Colo 205, human colorectal adenocarcinoma; HepG2, human hepatocarcinoma; Beas-2b bronchial epithelial cell line; CE: *C. ermenekensis*

Conclusions

This is the first report on the phytochemical characterization and cytotoxic activity of *C. ermenekensis*. Moreover, investigation of the cytotoxic properties and identification and quantification of some phenolics in this species will promote advanced studies that may help to protect against free radical damage and oxidative stress-related diseases. Furthermore, this preliminary research suggests a detailed investigation to isolate and elucidate structures of the bioactive compounds, which are responsible for cytotoxic activity.

Conflict of interest

The authors declare no conflict of interest.

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