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ÜMİT YIRTICI

FATİH GÖGER

MEHMET SARIMAHMUT

AYSUN ERGENE

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Cytotoxic and apoptotic effects of endemic *Centaurea fenzlii* Reichardt on the MCF-7 breast cancer cell line

Ümit YIRTICI¹, Fatih GÖGER², Mehmet SARIMAHMUT³, Aysun ERGENE^{4,*}

¹Department of Medical Laboratory, School of Health Care, Kırıkkale University, Kırıkkale, Turkey

²Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskişehir, Turkey

³Department of Biology, Faculty of Science and Arts, Uludağ University, Bursa, Turkey

⁴Department of Biology, Faculty of Science and Art, Kırıkkale University, Kırıkkale, Turkey

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Abstract: The main purpose of this study was to analyze the cytotoxic activity of an extract obtained from *Centaurea fenzlii* Reichardt, and the fractions eluted from this extract, in breast cancer cells. After isolation and structural analysis of the fractions were conducted, a meaningful cytotoxic effect was indicated. The goal of the analysis was to reveal the mechanism by which this effect occurs through researching the apoptotic side of these fractions and determining the amount of several proteins that are the products of the genes. Test substances were applied to breast cancer cells and the inhibitory concentration value 50 (IC₅₀) that caused a cytotoxic effect was determined using MTT and ATP assays. The *Centaurea fenzlii* Reichardt dichloromethane extracts-ethyl acetate fractions (CFDCM-EAF) exhibited a stronger growth-inhibitory effect on MCF-7 cells (45.771 µg/mL). The apoptotic effect was studied using double staining and flow cytometry. The death rate in the cells treated with the CFDCM-EAF IC₅₀ dose was approximately 90%: 9.2% living cells, 22.8% necrotic cells, 62.3% late apoptotic cells, and 5.8% early apoptotic cells. Structural analysis of the CFDCM-EAF, which indicated significant cytotoxic effects, was performed using chromatographic methods. Hispidulin was the major component of the CFDCM-EAF by LC-APCI-MS/MS analysis.

Key words: Endemic *Centaurea fenzlii* Reichardt, MCF-7, dichloromethane extract- ethyl acetate fraction, cytotoxicity, apoptotic effect

1. Introduction

The genus *Centaurea* L. (Asteraceae) is represented in the Turkish flora by 181 species, of which 112 are endemic. This genus is among the richest in endemic species (Candan et al., 2016). Turkey is the main genetic center for *Centaurea*. Many *Centaurea* species are used traditionally for medicinal purposes, including *Centaurea pulchella*, *Centaurea drabifolia*, *Centaurea triumfettii*, *Centaurea depressa*, and *Centaurea virgate*. Various biological activities of the members of the genus *Centaurea* L. have been reported previously, such as cytogenetic (Radic et al., 2005), antiinflammatory (Garbacki et al., 1999; Erel et al., 2011, 2014), anticancer (Shoeb et al., 2006), and cytotoxic activities (Seghiri et al., 2009; Ahmed and Kamel, 2014). The chemical composition of the members of this genus was also reported in other studies (Dural et al., 2003; Beltagy, 2015).

Breast cancer is the most common form of cancer found in women and ranks second among cancer deaths in women, with a 15% fatality rate. Despite the progress and new therapeutic approaches for metastatic breast cancer,

there is still not a perfect success rate in its treatment. Therefore, novel therapeutic approaches are required for better treatment of metastatic breast cancer (Torre et al., 2015).

In the present study, the aim was to investigate the cytotoxic and apoptotic effects of the ethyl acetate fractions (EAFs) obtained from *Centaurea fenzlii* Reichardt dichloromethane extracts (CFDCM) on the breast cancer cell line MCF-7. To date, not only have the effects of specific components of endemic *Centaurea fenzlii* Reichardt extracts not been investigated, but also the cytotoxic and apoptotic effects of these extracts are unknown.

2. Materials and methods

2.1. Cell culture and chemicals

The MCF-7 breast cancer cell line was provided by Prof Dr Engin Ulukaya (Uludağ University, Turkey). MCF-7 cells were cultured in DMEM with penicillin G (100 U/mL), streptomycin (100 mg/mL), L-glutamine, and 5% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO₂.

* Correspondence: ayergene@yahoo.com

2.2. Collection and extraction of the plant material

Centaurea fenzlii Reichardt was collected from the Muş–Elazığ road at the 7th km, from an altitude of 1270 m, in the East Anatolian region of Turkey and was previously authenticated. A voucher specimen (ADO 3) has been maintained in the Kırıkkale University Herbarium. The plant was identified immediately after collection and air-dried at room temperature for analysis.

The air-dried and powdered aerial parts of *C. fenzlii* Reichardt (43 g in 344 mL) were extracted successively with n-hexane, dichloromethane, and methanol in a Soxhlet apparatus until the last portion of the extract became colorless. The solvents were removed from all the extracts under low vacuum pressure using rotary evaporation.

The dichloromethane extract was taken in a round bottomed flask of a simple condenser and further fractionated using various solvents. Fractions were collected as ethyl acetate–methanol (1:1), ethyl acetate, methanol, formic acid–methanol 2% (1:1), and n-hexane–ethyl–acetate (1:1). Stock solutions of the structural extracts and fractions were prepared with DMSO.

2.3. Determination of cytotoxic activity by MTT assay

Cell viability was assessed by MTT assay (Mosmann, 1983), which determines the metabolically active mitochondria of intact cells. MCF-7 cells were seeded in 96-well plates (Greiner; Frickenhausen, Germany) with 10×10^3 cell/100 μ L medium and incubated for 48 h at 37 °C. Cells were treated with media alone as a negative control and hydrogen peroxide (1 mM) as a positive control (Ari et al., 2010). The assay was performed by the addition of a premixed MTT reagent, to a final concentration of 10% total volume, to the culture wells containing various concentrations (each test substance was applied in a series of 8 two-fold dilutions) of the test substance and incubated for an additional 4 h. During the 4 h incubation, living cells converted the tetrazolium component of the dye solution into a formazan

product. To solubilize the formazan product, 100 μ L of the solubilization solution (10% SDS) was then added to the cell culture wells and the absorbance at 570 nm was recorded using a 96-well plate reader (Biotek, PowerWave XS2; Winoski, VT, USA). The concentration of DMSO did not exceed 0.1% in the cell culture wells. The experiments were performed in triplicate. Cell viability of treated cells was measured by MTT assay. To find the inhibition % values of the samples applied to MCF-7 cells, a microplate reader was used to read optical density (OD) values calculated by the following formula:

$$\text{Inhibition \%} = [1 - (\text{sample OD} - \text{positive control OD}) / (\text{negative control OD} - \text{positive control OD})] \times 100.$$

The concentrations of the test substance (MTT results) in the first wells for each condition are given in Table 1.

2.4. Determination of cytotoxic activity by ATP assay

The ATP assay uses a highly sensitive reaction to determine the level of cellular ATP used as a surrogate marker for the number of viable (ATP-producing) cells (Andreotti et al., 1995). This assay was also performed to confirm the results of the MTT assay, since the ATP assay is more reliable and sensitive than the MTT assay (Ulukaya et al., 2008). MCF-7 cells were seeded in 96-well plates (Greiner) with 10×10^3 cell/100 μ L medium and incubated for 48 h at 37 °C. The cells were treated with media alone as a negative control and hydrogen peroxide as a positive control (Ari et al., 2010). The experiments were performed in triplicate. Cellular ATP content was then determined according to the manufacturer's recommendations (ATP Bioluminescence Assay, Sigma; St. Louis, MO, USA). Following the treatment, ATP was extracted from cells and a luciferin–luciferase solution was added. The resulting luminescence was then determined in a luminometer (BioTek).

2.5. Double staining of apoptotic cells

Determination of the mode of cell death was made on the basis of both nuclear morphology and cell membrane

Table 1. The concentrations of the test substance in the first wells.

Samples		Concentration of first wells (μ g/mL)
Plant extracts	Dichloromethane	500
	n-Hexane	250
	Methanol	650
Dichloromethane extraction-fractions	Ethyl acetate–methanol (1:1)	1400
	Ethyl acetate	450
	Methanol	100
	Formic acid–methanol 2% (1:1)	16
	n-Hexane	1250
	n-Hexane–ethyl acetate (1:1)	10,000

integrity under a fluorescence microscope (Leica DM6000B, Leica; Lysakar, Norway). The fluorescent dye Hoechst 33342 was employed to stain the nucleus of normal or apoptotic cells, and propidium iodide (PI) was employed to stain the nucleus of necrotic cells. Mode of cell death was determined according to a previously published study (Ulukaya et al., 2001). For the imaging, cells were seeded in a 6-well plate at a density of 5×10^5 cell/2 mL per well, and then treated with an IC_{50} dose of CFDCM-EAF for 48 h. After the treatment, the cells were incubated with PI (2 mg/mL) and Hoechst dye 43332 (5 mg/mL) dyes for 30 min in the dark at 37 °C and then analyzed with fluorescence microscope. Briefly, the Hoechst 33342 dye stains live, primary necrotic, early stage apoptotic, or late-stage apoptotic (secondary necrotic) cells. PI stains both primary and secondary necrotic cells.

2.6. Flow cytometry

Flow cytometry was used to quantify the apoptotic, late apoptotic, live, and necrotic cells. Cells that express only annexin V give the percentage of early apoptotic cells. Cells that are positive for both annexin V and PI are the percentage of late apoptotic cells. The cells that express only PI show the percentage of necrotic cells (Vermees et al., 1995). In this study, the BD Pharmingen Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences; San Jose, CA, USA) was used. MCF-7 cells were seeded in a 6-well plate at a density of 2×10^6 cell/2 mL per well, and then treated with medium containing an IC_{50} dose of CFDCM-EAF. An active substance-free control group was used for each sample. The medium of cells was collected in separate tubes after 48 h of incubation. Wells were washed with cold PBS and collected in the same tube. The cells were washed 2 times with cold PBS at 1200 rpm for 5 min. Cell suspensions were then taken for flow cytometry tube and 5 μ L of annexin V-FITC (25 μ g/mL) or 5 μ L of PI (250 μ g/mL) was added. Following an incubation period, 400 μ L of the annexin V binding buffer was added and the cells were analyzed in flow cytometry (BD FACSAria II; BD Biosciences).

2.7. SDS-PAGE and western blotting

MCF-7 cells were seeded in 25 cm² flasks and treated with CFDCM-EAF. CFDCM-EAF were maintained for 48 h at an IC_{50} dose and the cells reached 70% confluency. Firstly, the cells were scraped and washed with ice-cold PBS. Then, the cells were lysed in RIPA buffer (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA, USA) containing protease inhibitors for 30 min at 4 °C. The samples were then centrifuged at 4 °C for 10 min at $10,000 \times g$ to separate proteins from other cellular particles. Equal amounts of protein (30 μ g protein/lane) were loaded on a 4%–12% gradient gel, subjected to SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was then probed with a rabbit anti-PARP monoclonal antibody

(1:1000 dilution; Cell Signaling; Danvers, MA, USA) and a rabbit anti- β -actin monoclonal antibody (1:1000 dilution; Cell Signaling). HRP-linked anti-rabbit-IgG antibodies (1:2000 dilution; Cell Signaling) and the LumiGLO reagent were used to detect primary antibodies according to the manufacturer's instructions. Proteins were visualized using the Fusion FX-7 imaging device (Vilber Lourmat; Torcy, France).

2.8. Liquid chromatography-atmospheric pressure chemical ionization/mass spectrometry (LC-APCI-MS/MS)

Experiments were performed with a Shimadzu 20A HPLC system coupled with an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument. Electrospray ionization was used in the negative ionization mode. Chromatographic separations were performed on a GL Science Intersil ODS 250 \times 4.6 mm, 5 μ m i.d. particle size, with an octadecyl silica gel analytical column operating at 40 °C at a flow rate of 1 mL/min (GL Sciences; Tokyo, Japan). Protein detection was carried out at 280 and 320 nm, and the elution was collected using a binary gradient of the solvent mixture composed of methanol:water:formic acid (10:89:1, v/v/v) (solvent A) and methanol:water:formic acid (89:10:1, v/v/v) (solvent B). The composition of solvent B was increased from 0% to 25% in 15 min, and from 25% to 50% in another 5 min. Solvent B was stable at 50% concentration for 12 min, increased from 50% to 75% in 5 min, and increased further from 75% to 100% at 40 min. For the enhanced mass scan, the MS was operated at a mass range of 150–800 amu. Enhanced product ion spectra were measured from m/z 100 up to m/z 800. Nitrogen was used as the collision gas, and the collision energy was set to 30. The measurement parameters were as follows: declustering potential, 20; entrance potential, 10; curtain gas, 20; gas source 1, 50; gas source 2, 50; CAD, medium; and temperature, 450. Analyst 1.5 (SCIEX; Framingham, MA, USA) was used for analysis.

2.9. Statistical analysis

The results were expressed as the mean \pm standard deviation. All statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc analysis. P-values less than or equal to 0.05 were considered statistically significant.

3. Results

3.1. Yield of extracts and fractions

The percent yields in the plant extracts are given in Table 2. The highest yield was obtained from the methanol extract at 7.02%.

The percent yields in the extracted fractions obtained from the dichloromethane extracts are given in Table 3.

3.2. Determination of cytotoxic activities of CFDCM-EAF via MTT and ATP assays

The effect of the CFDCM-EAF was investigated in the

Table 2. Percent yield of the extract of the plant.

	Dichloromethane	Methanol	n-Hexane
Yield (%)	1.02	7.02	1.40

MCF-7 human breast cancer cell line using both MTT and ATP assays. CFDCM-EAF exhibited an antigrowth effect in a dose-dependent manner ($P < 0.05$), and a stronger growth-inhibitory effect, on MCF-7 cells. Therefore, in this study, CFDCM-EAF were used. The IC_{50} value of CFDCM-EAF was calculated on the basis of the results from the MTT and ATP assays shown in Table 4.

According to the ATP assay results, the IC_{50} values for CFDCM-EAF complex were $45.771 \mu\text{g/mL}$ for MCF-7 cells. An antigrowth effect in this cell line was observed when using the ATP assay.

3.3. Double staining of apoptotic cells

The IC_{50} dose obtained from the ATP assay was used to determine the apoptotic effect. The CFDCM-EAF IC_{50} dose was applied to the MCF-7 breast cancer cell line and fluorescence imaging was used to determine the number of apoptotic and necrotic cells (Figure 1).

The death rate was approximately 1% in the control cells, and approximately 90% in the CFDCM-EAF treated cells. As the CFDCM-EAF dose and duration increased, the cells died with primary necrosis. In addition, the CFDCM-EAF-treated cells also underwent secondary necrosis.

3.4. Flow cytometry

The cells that did not label positive for the two dyes indicated living cells. The control group contained 100% living cells. In contrast, 9.2% living cells (annexin-PI⁻), 22.8% necrotic cells (annexin-PI⁺), 62.3% late apoptotic cells (annexin+PI⁺), and 5.8% early apoptotic cells (annexin+PI⁻) were found in the cells treated with the CFDCM-EAF IC_{50} dose. Generally, an increasing dose of CFDCM-EAF leads to an increasing percentage of apoptosis.

3.5. SDS-PAGE and western blotting

To further investigate the cell death mode employed by MCF-7 cells, we studied the cleavage of an apoptosis-related protein, PARP (Figure 2), catalyzed by activated caspase-3 or caspase-7. PARP cleavage is considered one

of the hallmarks of apoptosis (Kaufmann et al., 1993; Tewari et al., 1995). The 89 kDa cleaved PARP fragment appeared in MCF-7 cells treated with the CFDCM-EAF IC_{50} dose for 24 h, which suggests apoptotic cell death occurred. According to the literature and to the best of our knowledge, there is no information about the mechanism by which CFDCM-EAF leads to cell death.

3.6. LC-APCI-MS/MS analysis

LC-APCI-MS/MS analysis of the active fraction led to a composition of cirsiolol, isorhamnetin, hispidulin, and cirsimaritin. Hispidulin was the major component of the CFDCM-EAF.

Compound 1 showed a pseudo molecular ion peak at m/z 217, which yielded several ions at m/z 202 ($-\text{CH}_3$), 189, 174, and 145. The ion fragmentation behavior was matched with bergapten and xanthotoxin, which are furocoumarins showing a molecular ion peak at m/z 216. According to spectral data, compound 1 may be a dehydro derivative of bergapten or xanthotoxin. Compound 2 presented a molecular ion peak at m/z 315 $[\text{M-H}]^-$ and additional other fragments at m/z 300, 243, 201, and 137. The compound, identified as isorhamnetin, is common in *Centaurea* and literature data confirm this compound (Pirvu et al., 2012; Kontogianni et al., 2013). Compound 3 was identified as hispidulin with a molecular ion peak at m/z 299 $[\text{M-H}]^-$. Further fragmentation was observed at m/z 283 due to the loss of methyl unit and a peak at m/z 137 (Figure 3). This compound was also found in previous studies of *Centaurea* and showed moderate antitumor activity (Forgo et al., 2012; Csupor et al., 2013). Compound 4 presented a molecular ion peak at m/z 313 $[\text{M-H}]^-$ and two fragmentation products of methyl groups at m/z 298 and 283. This compound was identified as cirsimaritin, which is common in *Centaurea*, and literature data confirm this compound (Hossain et al., 2010). Compound 5 had a molecular ion peak at m/z 329 $[\text{M-H}]^-$ and yielded several ions at m/z 314, 299, and 271. Fragmentation behavior of these compounds was determined with jaceosidin, cirsiolol, or 6-methoxykaempferol 3-methyl ether, which were previously isolated from *Centaurea* and showed moderate antitumor activity against MCF-7 cells (Forgo et al., 2012; Kitouni et al., 2015).

4. Discussion

In this study, the cytotoxic and apoptotic effects of *C. fenzlii* Reichardt, an endemic plant in Turkey, on the MCF-7 breast

Table 3. Percent yield in the extraction of the fractions obtained from the dichloromethane extracts.

	n-Hexane	n-Hexane-ethyl acetate	Ethyl acetate	Ethyl acetate-methanol	Methanol	2% Formic acid-methanol
Yield (%)	8.97	43.10	10.95	33.60	2.66	0.72

Table 4. Antigrowth parameters for CFDCM-EAF according to MTT and ATP assay after treatment for 48 h.

Cell line	MTT assay	ATP assay
	IC ₅₀ (µg/mL)	IC ₅₀ (µg/mL)
MCF-7	33.121	45.771

cancer cell line were analyzed using dichloromethane, methanol, and n-hexane extracts. According to the results of the conducted studies, this plant has an effect on cell death in the MCF-7 breast cancer cell line. Among the three extracts obtained from the plant, the maximum yield was obtained from methanol at a rate of 7.02%. Data obtained were compared with the literature. The n-hexane, chloroform, and methanol extracts were obtained from

the plants *C. calolepis*, *C. cariensis* subsp. *maculiceps*, *C. cariensis* subsp. *microlepis*, *C. hierapolitana*, *C. cadmea*, *C. ensiformis*, *C. depressa*, and *C. urvillei* subsp. *urvillei*. The highest yield was gained from methanol extracts (Karamenderes et al., 2007). This result is in agreement with that reported by Csupor-Loffler et al. (2009). Their group used chloroform, ethyl acetate, and butanol for the extraction of the plant *C. africana* in their study, and they obtained the highest yield from the butanol extract.

At first, the cytotoxic effects of the extracts were tested using the MTT assay and compared to other extracts. The dichloromethane extract had a higher cytotoxic effect on the MCF-7 breast cancer cell line than the other extracts. Shoeb et al. (2006) obtained three different extracts from the plant *C. gigantea* in one of their studies. In their studies, among the other extracts, methanol had the highest and most significant cytotoxic effect on the CaCo-2 colon

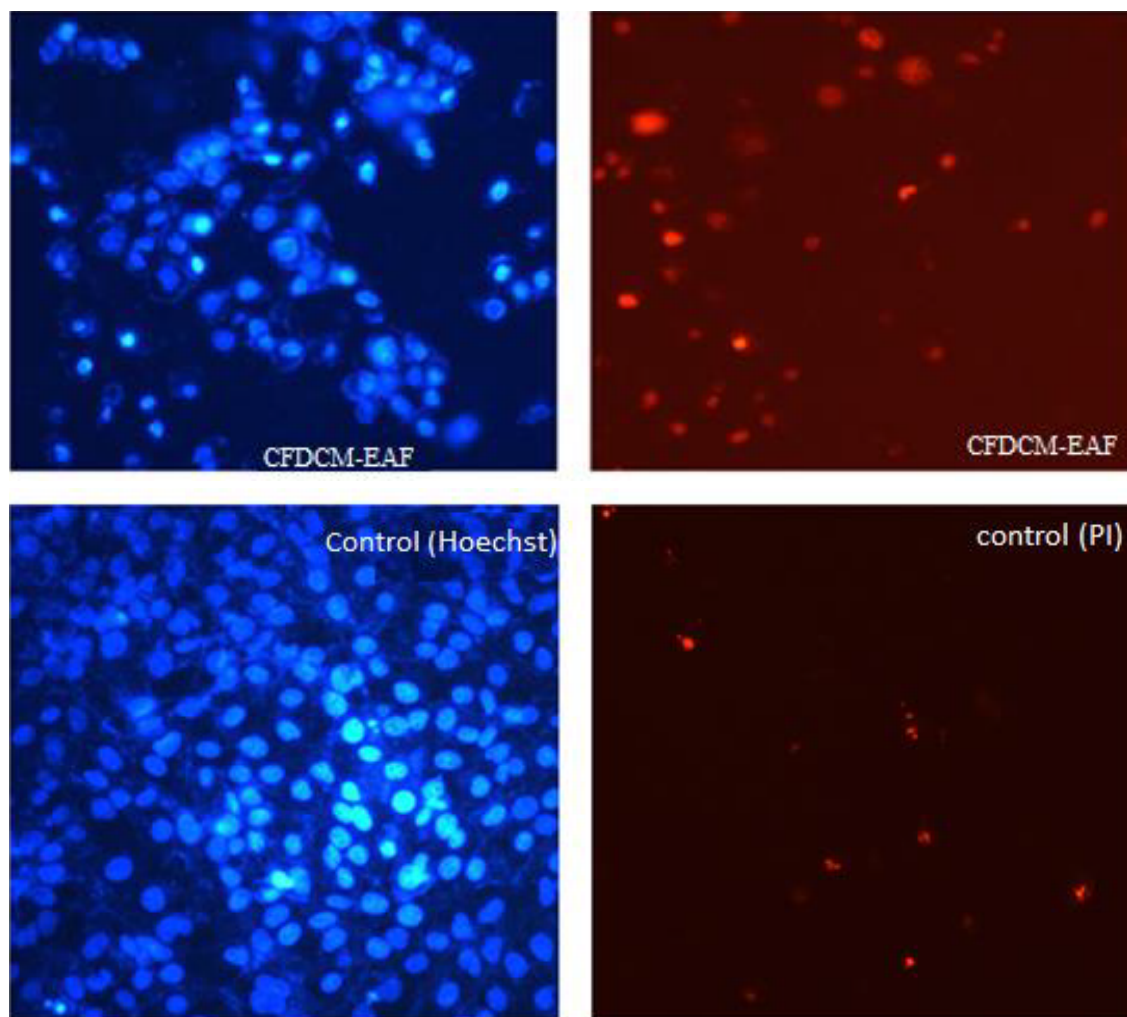


Figure 1. Fluorescence imaging for determination of cell death mode in MCF-7. The left panel shows Hoechst 33342 staining and the right panel shows PI staining. Cells were treated with 45.771 µg/mL CFDCM-EAF for 48 h.

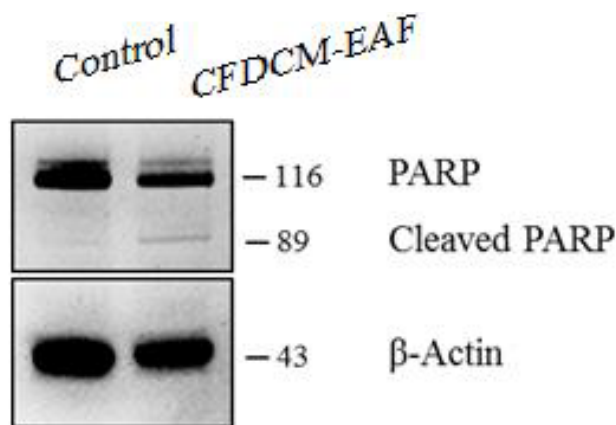


Figure 2. Cleaved PARP levels in MCF-7 cell line after 48 h of CFDCM-EAF IC_{50} dose examined by western blotting. *Denotes significant change from control ($P < 0.05$).

cancer cell line. Csupor-Loffler et al. (2009) confirmed that the chloroform extract from *C. biebersteinii*, *C. jacea*, and *C. spinulosa* had the highest cytotoxic effect on the HeLa cervical cancer cell line, the MCF-7 breast cancer cell line, and the A431 epidermal cancer cell line. In the present study, only the MCF-7 breast cancer cell line was used, and different solvent systems and extracts were used. The different solvent systems or different cancer cell lines used in the various studies examined might explain the

discrepancies between different extracts causing cytotoxic effects.

From the dichloromethane extract, whose cytotoxic effect was found to be the highest, the fractions ethyl acetate-methanol (1:1), ethyl acetate, methanol, methanol with 2% formic acid, n-hexane, and n-hexane-ethyl acetate (1:1) were obtained, and their yield percentages were specified. Among these fractions, the highest yield was obtained from the n-hexane-ethyl acetate (1:1) and ethyl acetate-methanol (1:1) fractions. The data obtained were compared with the literature. Kolli et al. (2012) obtained 25 fractions from the plant *C. omphalotricha*, and the petroleum ether-ethyl acetate (65:35) fraction obtained from a chloroform extract was the highest-yielding fraction. Out of 4 different fractions using the plant *C. bruguierana* subsp. *belangerana*, the chloroform extract obtained from the methanol fraction was found to be the fraction with the highest yield. In the present study, fractions were obtained after the most appropriate solvent system was created for our plant samples.

The cytotoxic effects of fractions gathered from the dichloromethane extract on the MCF-7 breast cancer cell line were analyzed. The highest cytotoxic effect was obtained from the ethyl acetate fraction. In their study using the plant *C. arenaria*, following advanced chromatographic experiments, Hammoud et al. (2012) performed a structural analysis of the samples gathered using NMR and researched the cytotoxic effects of 16 materials on the HeLa cervical cancer cell line, the MCF-

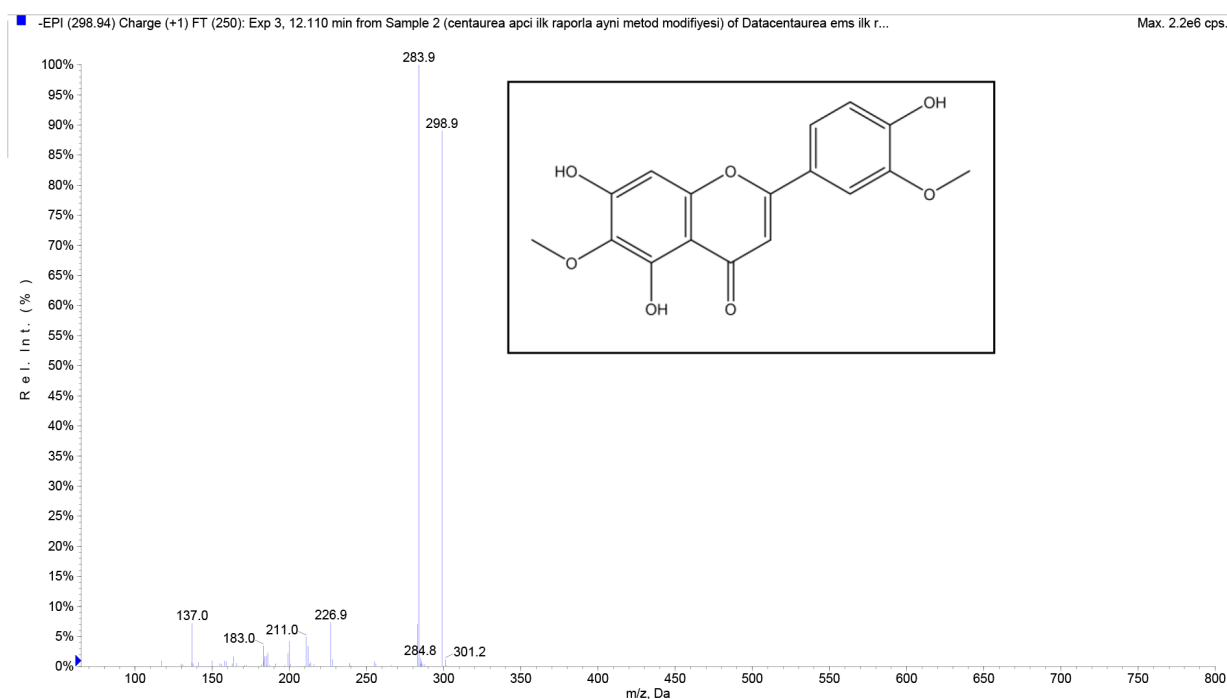


Figure 3. Mass fragmentation of hispidulin determined in active extract.

7 breast cancer cell line, and the A431 epidermal cancer cell line. The ethyl acetate fraction obtained in this study had a cytotoxic effect on the MCF-7 breast cancer cell line, according to advanced chromatographic technologies. The samples had significant cytotoxic activity, showing the validity of using NMR in subsequent studies.

An ATP test was utilized to confirm the cytotoxic activity of the dichloromethane extract, which had a significant cytotoxic effect according to the MTT assay. In a study conducted by Wang et al. (2010), the cytotoxic activity of the phenolic compounds in green tea on LNCaP prostate cancer cells and the MCF-7 breast cancer cell line was analyzed using MTT, MTS, and ATP assays. The cell viability percentages obtained from MTT and MTS assays were higher than those obtained from the ATP assay. Some limitations of the MTT assay include changes in the activity of succinate dehydrogenase. Due to the direct interaction of this enzyme with MTT, ATP assay was used to analyze the cytotoxic activity of CFDCM-EAF on cells (Hsu et al., 2003; Devika and Stanely Mainzen Prince, 2008).

In the literature, there has been one study investigating the apoptotic effects of *Centaurea* sp. on a cancer cell line (Ghantous et al., 2008). This study showed, through flow cytometry analysis and DNA Hoechst staining, that *Centaurea ainetensis* induces apoptosis. Western blot analysis showed that crude extracts apoptotic regulators, as well as NF- κ B signaling, in squamous cell carcinoma cell lines. In the present study, the apoptotic effect of the IC₅₀ dose of CFDCM-EAF on the MCF-7 breast cancer cell line

was explored via double staining. According to the flow cytometry results, the higher the dose of CFDCM-EAF, the higher the percentage of cells that die of apoptosis. The western blotting results suggest apoptotic cell death occurred.

In this study, CFDCM-EAF showed antigrowth activity against breast cancer cell lines by inducing apoptosis. The findings suggest that *C. fenzlii* can be a potential anticancer agent with potential effects for cancer chemotherapy, which could be of vital importance to human health.

The genus *Centaurea* is an important plant in Turkey. This plant is commonly used in folk medicine. Considering the positive results obtained from many national and international bioactivity studies in the literature, additional studies are needed to obtain medicinal drugs from this plant. Utilizing the diverse species of this plant is undoubtedly a great opportunity for a new, cost-effective pharmaceutical research area. The development of effective drugs using this plant can be easily achieved.

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