

Cytotoxic evaluation and LC-MS/MS analysis of aerial parts of *Eryngium kotschy* Boiss. grown in Turkey

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Increasing biological activity and phytochemical investigations on *Eryngium* species showed its potential as pharmaceutical approach. *Eryngium kotschy* Boiss. is one of the species of *Eryngium* genus and is endemic to Turkey. It is known that this plant is traditionally used in the South-western part of Turkey for the treatment of various diseases. This study focuses on cytotoxic activities of methanol extract and ethyl acetate, *n*-butanol and water sub-extracts from *E. kotschy* in A549, COLO 205 and MDA-MB-231 cell lines by Sulforhodamin B assay and qualitative and quantitative determination of phytochemical constituents in active extract by LC-MS/MS. From the result of the study, it was seen that *E. kotschy* ethyl acetate (EKE) sub-extract showed the strongest cytotoxic effect with the low IC₅₀ values (50.00; 31.96 and 22.26 µg/mL in A549; COLO 205 and MDA-MB-231 cells at 48 h, respectively). Preliminary examination of the mass spectrums revealed the presence of 15 phytochemical compounds in active sub-extract and 7 of them was quantified. According to quantitative analyses the main compounds of EKE sub-extract were rosmarinic acid (485.603 µg/mg_{extract}), chlorogenic acid (62.355 µg/mg_{extract}) and caffeic acid (59.266 µg/mg_{extract}). Moreover, this preliminary study on inhibitory activity of EKE sub-extract suggests further toxicologic investigations and detailed investigation on cytotoxic effect of various combinations of determined compounds.

Keywords: *Eryngium kotschy*. LC-MS/MS. A549. COLO 205. MDA-MB-231. SRB.

INTRODUCTION

Cancer is one of the leading causes of mortality among the world today. Due to be an exceedingly complex disease, treating cancer has become a major challenge. Moreover, incidence and death rates are still important for several cancer types, including lung, colon and breast. Advancing the fight against cancer will require continued clinical and basic research (Siegel, Miller, Jemal, 2016). Herbal drugs have been used for the treatment of many diseases, including cancer and plants have recently been identified as the best source for clinically useful cytotoxic agents (Safarzadeh, Shotorbani, Baradaran, 2014).

The genus of *Eryngium* L. is widely distributed in the world and used in traditional medicine for different

therapeutic purposes. In Turkish folk medicine, various species of the plant are used for a wide range of ailments; particularly, roots are used against various inflammatory disorders, edema, sinusitis, urinary infections or inflammations and snake or scorpion bites or goiter; roots and leaves for infertility and herbs for wound healing (Küpeli *et al.*, 2006).

Eryngium (Apiaceae, Saniculoideae) genus comprises about 250 species, growing in Eurasia, North and South America, North Africa, and Australia. It is the most species-rich genus of the Apiaceae (Pimenov, Leonov, 1993). The most recent monograph of *Eryngium* is now over 90 years old (Wolff, 1913) and outdated. Many regional treatments in “Florae” were subsequently published, among them Davis (1972) (P.H., 1972) for Turkey, Pimenov, M. & S. Tamamschian (1987) (Pimenov, Tamamschian, 1987) for the Flora Iranica area and Mathias & Constance (1941) (Mathias, Constance, 1941)

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for North America (Wörz, Duman, 2004). There are 23 taxa in Turkey according to Türkiye Bitkileri Listesi (Güner, Aslan, 2012).

It was reported that some species of *Eryngium* have different biological activities such as cytotoxic (Kartal, *et al.*, 2005, Bogucka-Kocka, Smolarz, Kocki, 2008, Zhang, *et al.*, 2008, Vukic, *et al.*, 2018), anti-inflammatory and anti-nociceptive (Küpeli, *et al.*, 2006), anti-amebicide (Derda, *et al.*, 2013), anti-snake and scorpion venom (Alkofahi, Sallal, Disi, 1997), anti-leishmanicidal (Rojas-Silva, *et al.*, 2014), anti-malarial (Fokialakis, *et al.*, 2007), antioxidant (Thomas, *et al.*, 2017), antibacterial (Çelik, Aydınlık, Arslan, 2011), antifungal (Cavaleiro, *et al.*, 2011) and anti-diabetic (Pereira, *et al.*, 2018). These pharmacological effects are mainly related to the terpenoids, triterpenoid saponins, flavonoids, coumarins, polyacetylenes and steroids (Küpeli, *et al.*, 2006, Çelik, Aydınlık, Arslan, 2011, Wang, *et al.*, 2012). To date, terpenoids, triterpenoid saponins, flavonoids, coumarins, polyacetylenes, steroids, and essential oils have been reported in the genus *Eryngium* (Drake, Lam, 1972, Erdelmeier, Sticher, 1985).

In the literature, there are a few studies concerning the cytotoxic activity and chemical composition of *E. kotschyi*. In the light of this, the present study is mainly designed to evaluate the cytotoxic effect of *E. kotschyi* methanol, ethyl acetate, *n*-butanol and water extract on human lung cancer (A549), human colon adenocarcinoma (COLO 205), human breast adenocarcinoma (MDA-MB-231) cell lines. Additionally, the phytochemical profile of the most active extract was established by LC-MS/MS, for the first time.

MATERIAL AND METHODS

Plant material

Eryngium kotschyi Boiss. was harvested from Konya; South Hadim at 1600 m altitude of steppe areas in 2015 year. Plant samples was deposited in the Herbarium of Science Faculty at Selcuk University (Herbarium No: KNYA 26907). In this study dried flowering aerial parts of plants have been used.

Preparation of extracts

The aerial parts of *E. kotschyi* (500 g) were dried in well ventilated rooms and 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. *E. kotschyi* methanol extract (EKM) was dispersed with water and partitioned with ethyl acetate (EKE) and *n*-butanol (EKB) sequentially. The crude extracts were stored in dark at -20°C. A total 3 sub-extracts were obtained from EKM extract. Yields of extract and sub-extracts were given in Table I.

TABLE I - Yields of extracts and sub-extracts (%)

Extracts	Extract code	Yields (%)
Methanol	EKM	10
Ethyl acetate	EKE	25
<i>n</i> -butanol	EKB	30
Water	EKW	30

EKM: *E. kotschyi* methanol extract, **EKE:** *E. kotschyi* ethyl acetate extract, **EKB:** *E. kotschyi* *n*-butanol extract, **EKW:** *E. kotschyi* water extract.

LC-MS/MS Analyses

Compounds in active sub-extract were determined by using liquid chromatography-electrospray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS, Shimadzu 8040). Mass spectrometry was conducted using a Shimadzu LCMS-8040 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface. The mass spectrometric behavior of compounds 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.

The following instrument settings were used for analysis: column RestEKM (150 x 4.6 mm x 3 µm); column heat, 40°C; heat block temperature, 400°C; DL temperature, 250°C; nebulizing gas (N₂), 3 L/min; drying gas (N₂), 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 μ L.

Preparation of standard and sample solutions

Stock solutions of the malic acid, caffeic acid, quinic acid, chlorogenic acid, rutin, isorhamnetin 3-*O*-rutinoside and rosmarinic acid were prepared in methanol at 8 μ g/mL concentrations. The extract and sub-extracts solutions were prepared in methanol at 10 μ g/mL.

Calibration curve

Linearity of the methods was established by triplicate injections of each concentration (0.01-8 μ g/mL) of standard solutions. Response function of the standards calibration curve was $y=2842x+54.151$ for rosmarinic acid, $y=10074x + 994.36$ for malic acid, $y=33716x - 2152.2$ for chlorogenic acid, $y=16535x + 275.47$ for quinic acid, $y=181197x + 9999$ for caffeic acid, $y=511143x - 4056$ for rutin and $y=18006x + 928.47$ for isorhamnetin 3-*O*-rutinoside. The correlation coefficient (r^2) of the calibration curves was 0.9989, 0.9988, 0.9995, 0.9994, 0.9991, 0.9997 and 0.9996, respectively.

Cell lines, culture and reagents

The cytotoxic activity of the extracts was determined against different cell lines; namely, A549 (human lung carcinoma) (CCL-185TM, ATCC Manassas, VA, USA), COLO 205 (human colon adenocarcinoma) (CCL-222TM, ATCC Manassas, VA, USA) and MDA-MB-231 (human breast adenocarcinoma) (HTB-26TM, ATCC Manassas, VA, USA). A549 cells maintained in F12 Kaighn's medium and both of COLO 205 and MDA-MB-231 maintained RPMI-1640. Complete medium was supplemented with 2 mM l-glutamine (Sigma Chemical Co., Saint Louis, MO, USA), 100 IU/mL penicillin, 100 μ g/mL streptomycin and 10% (v/v) fetal bovine serum (FBS) at 37 °C under humidified air with 5% CO₂. Stock solutions of the extracts were prepared in

dimethyl sulfoxide (DMSO). DMSO (Cat No: A3672) and phosphate-buffered saline (PBS) (Cat No: A9177) were purchased from AppliChem. Trypsin-EDTA (T3924) were purchased from Sigma. Sulforhodamine B (SRB) sodium salt (Cat No: sc-253615A) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Determination of cell viability

The effect of the extracts on the viability of A549, COLO 205 and MDA-MB-231 cells was determined by Sulforhodamine B (SRB) assay as described previously (Vichai, Kirtikara, 2006). The extracts were dissolved in DMSO (10 mg/mL). The final DMSO concentration in the medium was less than 0.1%. The cells were seeded 12,500 (for A549); 25,000 (for COLO 205) and 1000 (for MDA-MB-231) cells/wells and after 24 h incubation cells treated for different final concentrations (1; 2; 4; 8; 16; 65; 125; 250 and 500 μ g/mL) of the extracts for 24 h and 48 h, followed by fixing the cells in 10% (v/v) of trichloroacetic acid (TCA) for 1 h at 4°C. After washing 5 times, cells were exposed to 0.5% (w/v) SRB solution for 30 min in a dark place and subsequently washed with 1% (v/v) acetic acid. After drying, 10 mM (pH 10.5) Tris base solution was used to dissolve the SRB-stained cells using a plate-shaker (PST-60 HL plus Biosan) and the absorbance was measured at 510 nm using a microplate reader (BiotEKM Synergy HT). Data are represented as a percentage of control cells. The measurement of the "half maximal inhibitory concentration" (IC₅₀) values were calculated with GraphPad Prism Software Version 7.01 (La Jolla, CA, USA). Mean values were calculated in three experiments using 4 wells per condition. Results were given as the mean \pm SD of independent experiments.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism Software Version 7.01 (La Jolla, CA, USA) to compare differences in values between the control and experimental group. The results are expressed as the mean \pm standard deviation (S.D.). Statistically significant values were compared using two-way ANOVA with Tukey Multiple Comparison Test to compare all

columns vs. control and p -values of less than 0.05 were considered statistically significant. $*p < 0.05$, $**p < 0.001$ and $***p < 0.0001$ were considered as compared to the untreated control.

RESULTS

Cell viability is decreased by *E. kotschy*

In order to determine the cytotoxic efficacy of EKB, EKE, EKM and EKW extracts, the SRB assay was carried out using A549, COLO 205 and MDA-MB-231 cells. All the cell lines were treated with extracts of *E. kotschy* at different concentrations ranging from 1 to 500 $\mu\text{g/mL}$ for 24 and 48 h.

Viability of A549, COLO 205 and MDA-MB-231 cells is presented in Figure 1, 2 and 3.

Results show that EKB exhibited statistically significant ($p < 0.001$) decrease in cell viability of A549

cells at 500 $\mu\text{g/mL}$ for 48 h (Figure 1a). The A549 cells exposed to EKE at 65 $\mu\text{g/mL}$ and 500 $\mu\text{g/mL}$ concentrations for 48 h were found to be cytotoxic (Figure 1b). The percent cell viability was recorded as 43.81%, 17.06%, 14.45% and 16.97% at 65, 125, 250 and 500 $\mu\text{g/mL}$ of EKE, respectively (Figure 1b) and 73%, 48%, and 23% at 0.25, 0.5, and 1 mg/mL of EKM, respectively (Figure 1c). Extract of EKW at 500 $\mu\text{g/mL}$ and lower concentrations did not show any cytotoxic effect on A549 cells (Figure 1d).

As shown in Figure 1b and 1c, the viability of treated A549 cancer cells with EKE and EKM was significantly reduced in a time- and dose-dependent manner compared to untreated cancer cells. Also, the IC_{50} values (minimum concentration of extract to reduce cell viability to 50 %) of ethyl acetate and methanol extracts of *E. kotschy* after incubation for 24 and 48 h are reported in Table II. The inhibitory effect of the EKE extract on cell proliferation was significantly superior to that of EKM.

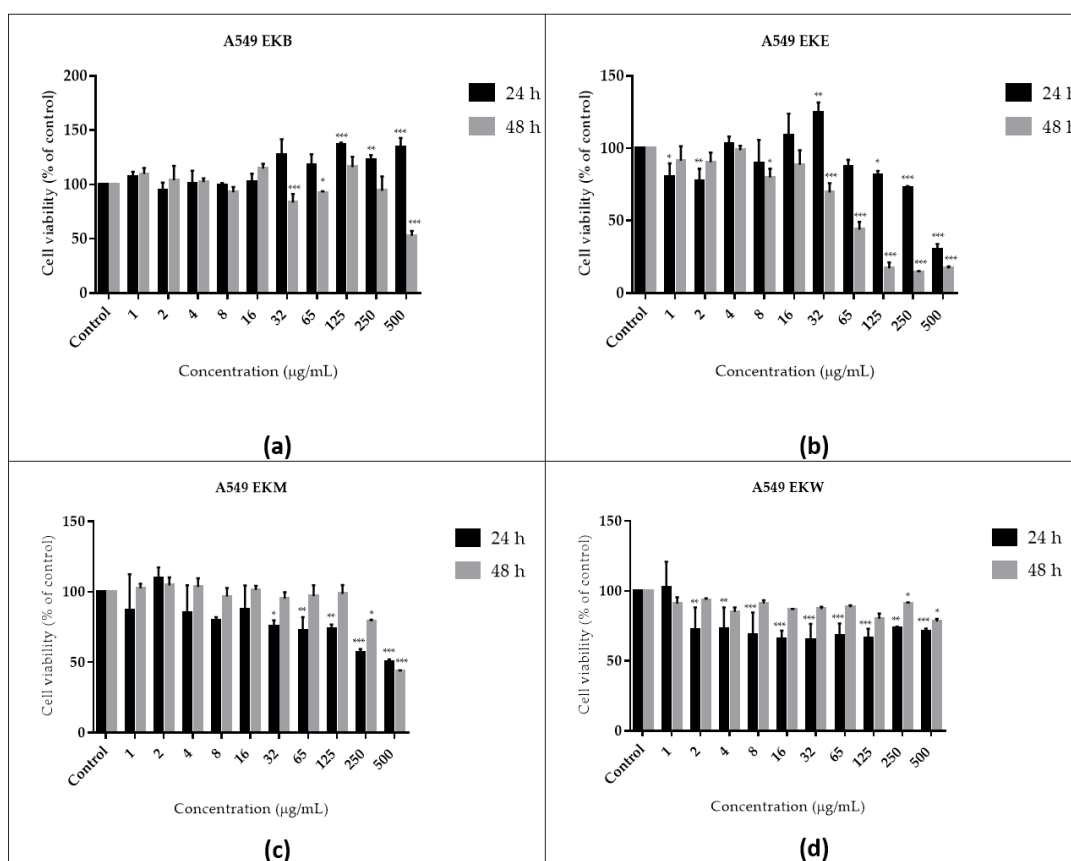


FIGURE 1 - SRB assay in A549 cells after 24 h and 48 h of treatment with EKB (a), EKE (b), EKM (c) and EKW (d) extracts.

TABLE II - The IC₅₀ values of *E. kotschyi* extracts on three cell lines after incubation for 24 and 48 h

Cell line	Extract code	24 h IC ₅₀ (µg/mL)	48 h IC ₅₀ (µg/mL)
A549	EKB	N.D.	N.D.
	EKE	N.D.	50.00±0.061
	EKM	N.D.	308.80±0.042
	EKW	N.D.	N.D.
COLO 205	EKB	N.D.	N.D.
	EKE	86.93±0.041	31.96±0.023
	EKM	N.D.	138.00±0.134
	EKW	N.D.	N.D.
MDA-MB-231	EKB	N.D.	N.D.
	EKE	15.35±0.112	22.26±0.120
	EKM	249.90±124	34.59±0.041
	EKW	N.D.	N.D.

The IC₅₀ of all the extracts were obtained based on the dose–response curves at 24 h and 48 h exposure in and calculated from repeated experiments (n=4) with SRB assay. Values are expressed as mean ± S.D. of three determinants. Symbols N.D. represents not determined.

The concentrations of EKB at 250 µg/mL and lower did not show decrease in the cell viability on COLO 205 cells (Figure 2a). The COLO 205 cells exposed to EKE at 65 µg/mL and above concentrations for 24 and 48 h were found to be cytotoxic (Figure 2b). COLO 205 cells exposed to EKE for 24 and 48 h also showed the statistically significant ($p < 0.001$) decrease in the cell

viability (Figure 2b). The cell viability at 65, 125, 250, 500 µg/mL was recorded to be 70.84%, 39.22%, 16.84% and 32.65% (Figure 2b), respectively for 24 h and 36.17%, 34.69%, 36.86% and 43.46%, respectively for 48 h (Figure 2b). The concentrations of EKM and EKW at both of times did not show dose- or time- dependent cell viability of COLO 205 cells (Figure 2c and d).

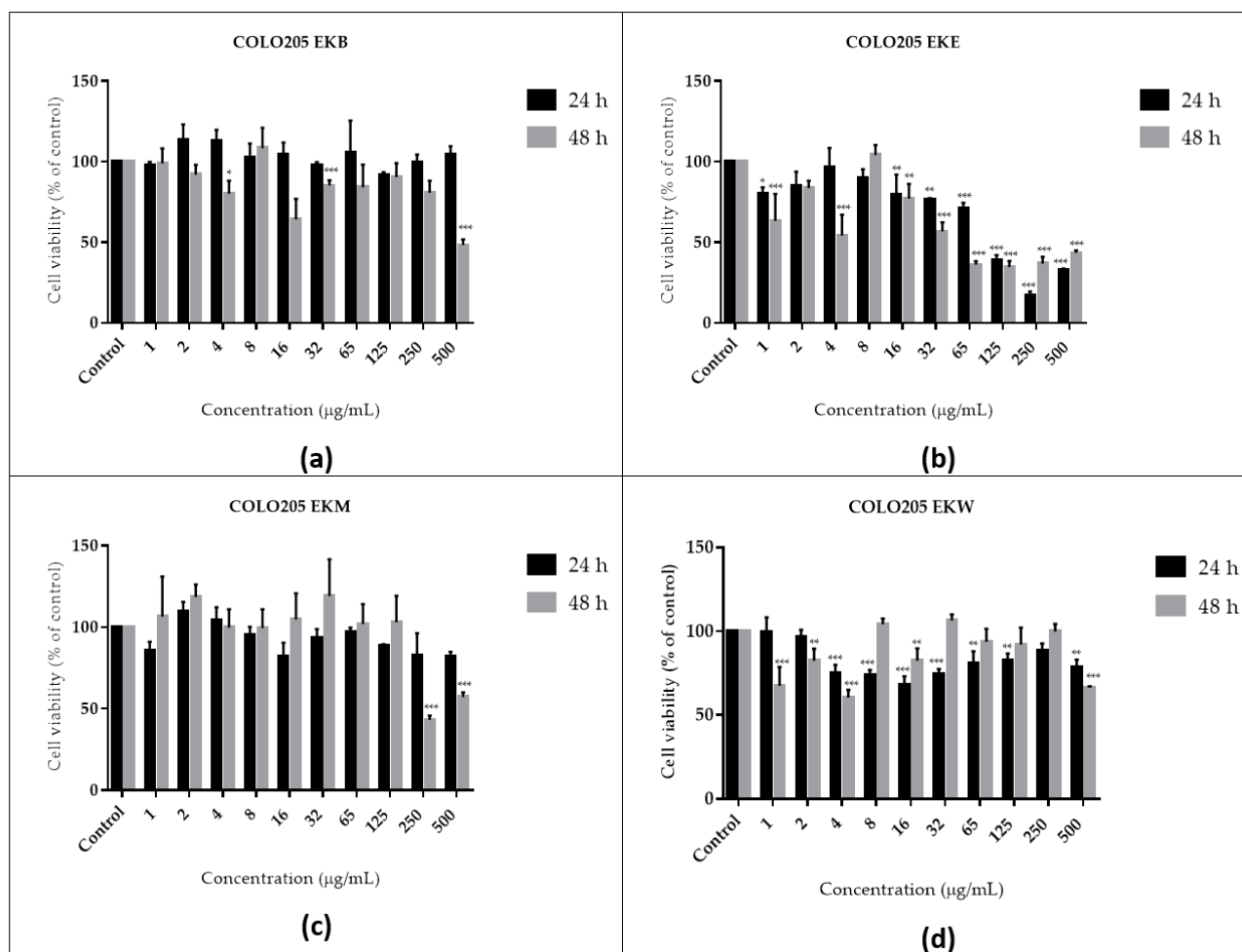


FIGURE 2 - SRB assay in COLO 205 cells after 24 h and 48 h of treatment with EKB (a), EKE (b), EKM (c) and EKW (d) extracts.

The concentrations of EKB at 65 µg/mL and higher proliferated COLO 205 cells (Figure 3a). The MDA-MB-231 cell line was more sensitive to EKE, which significantly reduced its viability (Figure 3b). The IC_{50} ($p < 0.001$) value of EKE is 15.35 and 22.26 µg/mL for 24 and 48 h, respectively. The cell viability at 16, 32, 65, 125, 250, 500 µg/mL was recorded to be 47.06%, 22.35%, 24.71%, 26.74%, 27.63% and 35.43%, respectively for

24 h and 88.59%, 42.75%, 37.49%, 42.37%, 27.95% and 20.71%, respectively for 48 h (Figure 3b). The MDA-MB-231 cells exposed to EKM at 16 µg/mL and above concentrations for 48 h were found to be cytotoxic (Figure 3c). The percent cell viability was recorded to be 66.82%, 74.79%, 10.48%, 11.75%, 16.25% and 15.69% at 16, 32, 65, 125, 250 and 500 µg/mL of EKE, respectively for 48 h (Figure 3c).

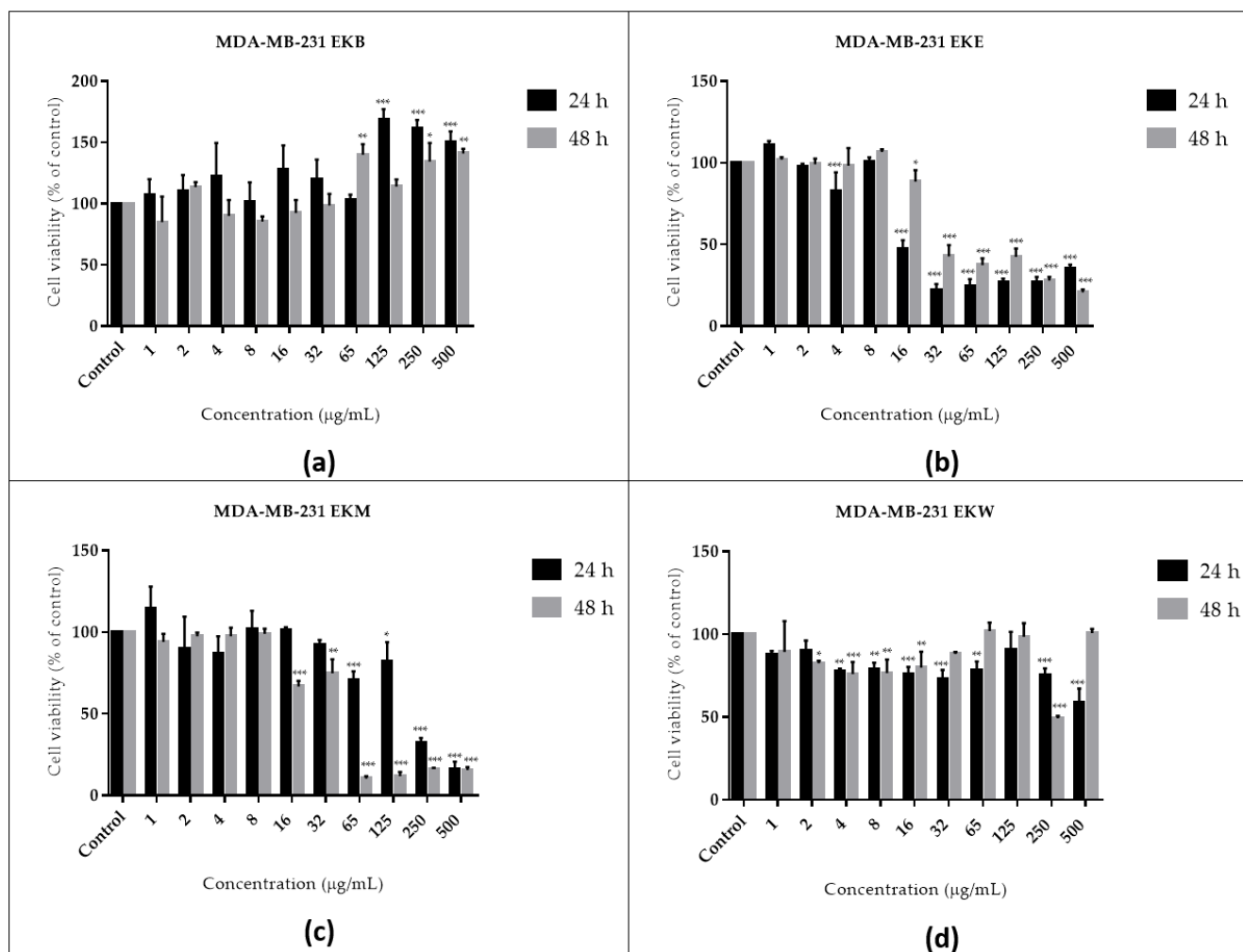


FIGURE 3 - SRB assay in MDA-MB-231 cells after 24 h and 48 h of treatment with EKB (a), EKE (b), EKM (c) and EKW (d) extracts.

The IC_{50} values strongly indicated that the effective doses of EKE for COLO 205 were lower when compared to A549 cells after different incubation times (Table II). EKE extract also showed a marked cytotoxic activity against to the three cell lines (Figure 1b, 2b and 3b) and this cytotoxicity was cell-, dose- and time- dependent.

SRB results indicated that various concentrations of EKW had no cytotoxic effect on the tested cell lines after incubations of 24 and 48 h (Figure 1d, 2d and 3d).

Qualitative analyses of compounds

The structural characterizations of compounds in active EKE sub-extract were achieved based on the

accurate mass, the registered mass spectra fragmentation patterns and literature data. Compounds were studied in negative ion mode in using MS/MS product ion scans (Figure 4). Preliminary examination of the mass spectrums revealed the presence of apigenin-7-*O*-rutinoside (Lin, *et al.*, 2000), caffeic acid-3-glucoside (Gardana, *et al.*, 2007), caffeic acid derivative-I (Riethmüller, *et al.*, 2013), chlorogenic acid (Lin, *et al.*, 2000), epicatechin-3-*O*-(4-*O*-methyl) gallat (Kelebek, 2016), ferulic acid dimer (Bravo, Goya, Lecumberri, 2007), isorhamnetin 3-*O*-rutinoside (Lin, *et al.*, 2000), kaempferol cumaroil hexoside (Simirgiotis, Schmeda-Hirschmann, 2010), kaempferol-3-*O*-glucoside (Ribeiro, *et al.*, 2008), malic acid (Gonzalez, *et al.*, 2011), quinic

acid (Clifford, *et al.*, 2003) rosmarinic acid (Tang, *et al.*, 2016), rutin (Karaçelik, *et al.*, 2015) and 5-cynapoil quinic acid (Lin, *et al.*, 2000). The mass spectra of active sub-

extract was shown in Figure 5. Molecular ion, retention time (RT), MS/MS data of identified compounds were given in Table III.

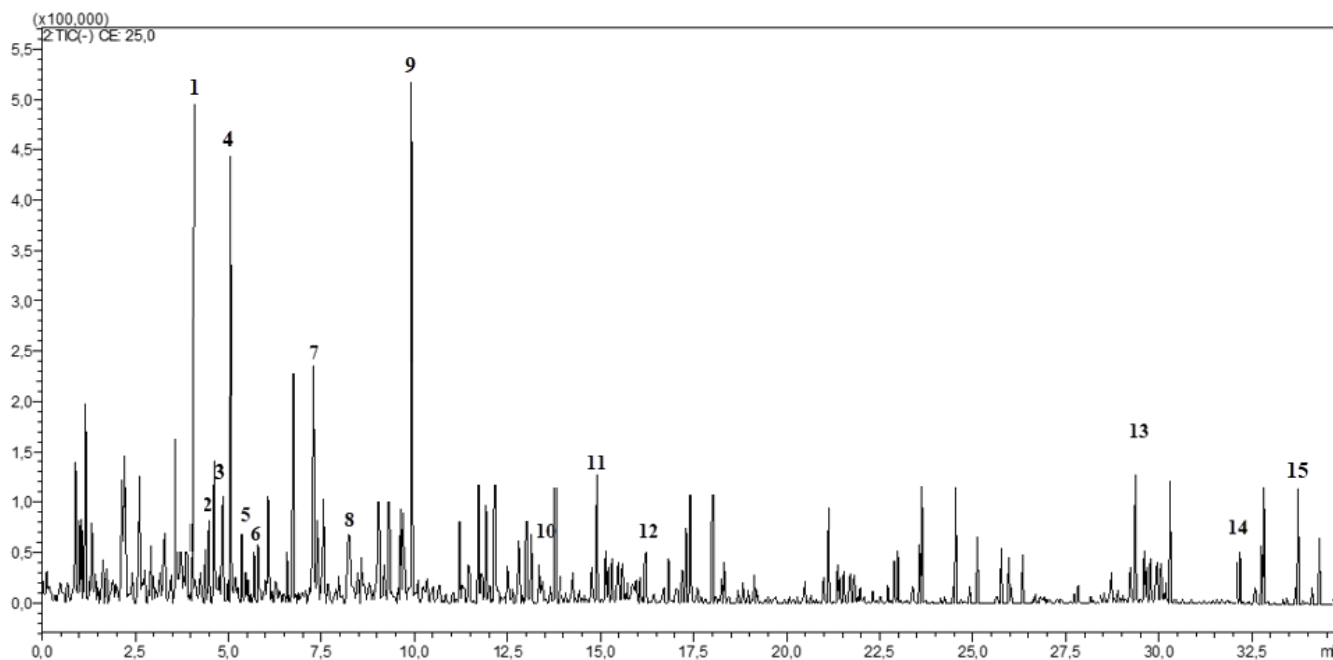


FIGURE 4 - TIC (Total Ion Chromatogram) profile of EKE sub-extract.

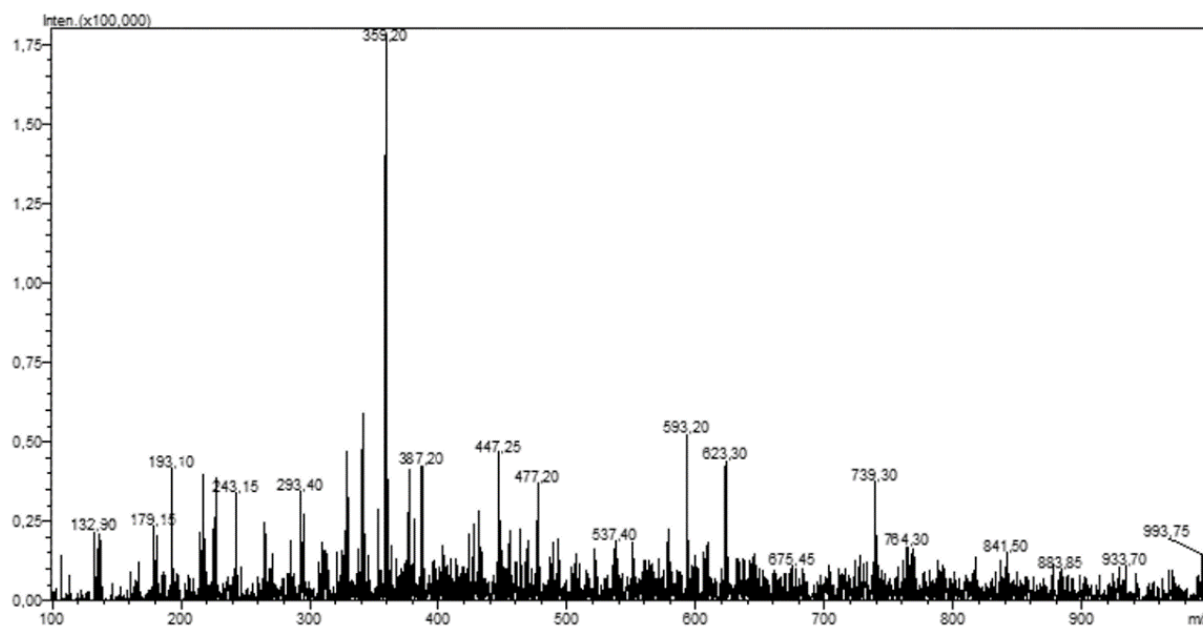


FIGURE 5 - Mass spectra of EKE sub-extract.

TABLE III - Mass spectral characteristics and identify of compounds in EKE sub-extract

Pik No	RT (min)	[M-H] ⁻ (m/z)	MS/MS (m/z)	Compounds	References
1	4.31	359	133,161,179	Rosmarinic acid	(Tang, <i>et al.</i> , 2016)
2	4.42	377	341, 215, 179, 161,119	Caffeic acid derivative-I	(Riethmüller, <i>et al.</i> , 2013)
3	5.15	191	191, 93, 85	Quinic acid	(Clifford, <i>et al.</i> , 2003)
4	5.16	353	191	Cholorogenic acid	(Lin, <i>et al.</i> , 2000)
5	5.21	341	179,135	Caffeic acid-3-glucoside	(Gardana, <i>et al.</i> , 2007)
6	5.61	179	135, 87	Caffeic acid	(Horai, <i>et al.</i> , 2010)
7	7.23	133	115	Malic acid	(Gonzalez, <i>et al.</i> , 2011)
8	8.10	609	301	Rutin	(Karaçelik, <i>et al.</i> , 2015)
9	10.22	623	315	Isorhamnetine 3- <i>O</i> -rutinoside	(Lin, <i>et al.</i> , 2000)
10	13.91	593	285, 255	Kaempferol cumaroil hexoside	(Simirgiotis, Schmeda-Hirschmann, 2010)
11	14.22	387	223,191,179	5-cynapoil quinic acid	(Lin, <i>et al.</i> , 2000)
12	16.31	447	273, 285, 257, 151	Kaempferol-3- <i>O</i> -glucoside	(Ribeiro, <i>et al.</i> , 2008)
13	29.3	577	269	Apigenin-7- <i>O</i> -rutinoside	(Lin, <i>et al.</i> , 2000)
14	32.12	455	289,183	Epicatchin-3- <i>O</i> -(4- <i>O</i> -methyl) gallat	(Kelebek, 2016)
15	34.79	735	191,193,367	Ferulic acid dimer	(Bravo, Goya, Lecumberri, 2007)

RT: Retention time

Quantitative analyses of compounds

The compounds were subsequently analyzed in Q1Scan (Product Ion Scan) mode, using [M-H]⁻ ions as precursors. Obtained MS2 spectras 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.

Malic acid (*m/z* 134) provided two fragment ions at *m/z* 115 with the loss of water [M-H-H₂O]⁻ and at *m/z* 71 with the loss of CO₂ (Fernández-Fernández, *et al.*, 2010).

The peak identified as a chlorogenic acid (*m/z* 353), yielded the fragment at *m/z* 191 (deprotonated quinic acid) with the loss of one of the caffeoyl moieties [M-H-caffeoyl]⁻, subsequent fragmentation yielded the fragment 179 [caffeic acid-H]⁻, 135 and the peak of the ion at *m/z* 173 (the absence of a C4 substituent) (Barros, *et al.*, 2013).

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 represents specific fragmentation with the loss of CH_3 radical from the deprotonated aglycone, thus giving m/z 315 \rightarrow m/z 300 and the m/z 285 pattern (Martucci, *et al.*, 2014).

The tentative mass spectrum for rosmarinic acid ($[M-H]^-$ ion at m/z 359.08) showed the caffeic acid at m/z 179.0 and m/z 161.0, m/z 135.0 corresponding to loss of water and carbon dioxide molecules respectively from the precursor ion (Hossain, *et al.*, 2010). The obtained LC-MS/MS chromatogram and mass spectrum of compounds are presented in Figure 6.

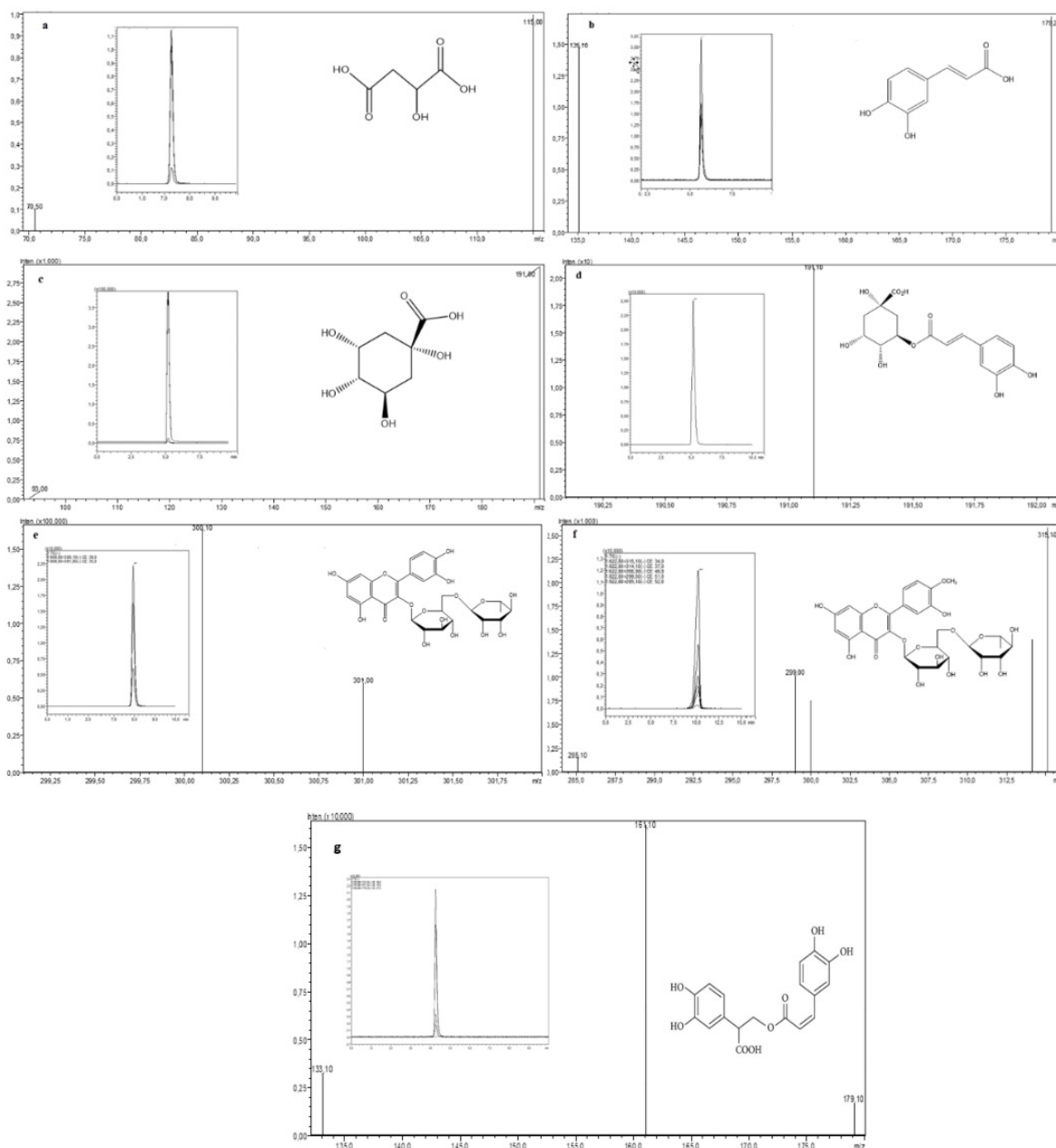


FIGURE 6 - LC-MS/MS chromatogram and mass spectra of malic acid (a), caffeic acid (b), quinic acid (c), chlorogenic acid (d), rutin (e), isorhamnetin 3-*O*-rutinoside (f) and rosmarinic acid (g).

The quantitative results of compounds were given in Table IV. As seen in the table, the main constituents of EKE extract were rosmarinic acid (485.603 $\mu\text{g}/\text{mg}_{\text{extract}}$),

chlorogenic acid (62.355 $\mu\text{g}/\text{mg}_{\text{extract}}$) and caffeic acid (59.266 $\mu\text{g}/\text{mg}_{\text{extract}}$).

TABLE IV - Contents of compounds in extracts and sub-extracts ($\mu\text{g}/\text{mg}_{\text{extract}} \pm \text{S.D.}$)

Constituent	Content ($\mu\text{g}/\text{mg}_{\text{extract}}$)			
	EKM	EKB	EKE	EKW
Malic acid	33.604 \pm 1.646	N.D.	N.D.	51.551 \pm 1.580
Caffeic acid	1.294 \pm 0.857	N.D.	59.266 \pm 2.838	2.018 \pm 0.542
Quinic acid	1.747 \pm 0.216	1.072 \pm 0.387	N.D.	1.753 \pm 0.566
Chlorogenic acid	36.850 \pm 0.340	30.717 \pm 0.188	62.355 \pm 0.270	17.735 \pm 0.240
Rutin	3.538 \pm 0.091	5.297 \pm 3.896	3.654 \pm 0.033	1.325 \pm 0.14
Isorhamnetin 3- <i>O</i> -rutinoside	34.957 \pm 3.468	92.559 \pm 1.030	2.770 \pm 0.676	N.D.
Rosmarinic acid	64.690 \pm 0.123	14.092 \pm 0.630	485.603 \pm 0.809	N.D.

Symbols N.D. represents not determined. **EKM:** *E. kotschy* methanol extract, **EKE:** *E. kotschy* ethyl acetate extract, **EKB:** *E. kotschy* *n*-butanol extract, **EKW:** *E. kotschy* water extract.

DISCUSSION

Cancer is one of the main causes of death worldwide. Natural products and their secondary metabolites have a considerable significance to be investigated for possible anticancer agents considering their major toxicity to cancer cells (Cabral, *et al.*, 2018). Assessment of the toxic effect of plant extracts is indispensable in cancer research. It allows identification of the intrinsic toxicity of the plant (Lagarto Parra, *et al.*, 2001).

Previous studies revealed that different *Eryngium* species have demonstrated various biological activities including cytotoxic, apoptotic, antifungal, antimicrobial, anti-amebicide, anti-snake and scorpion venom, anti-leishmanicidal, anti-malarial, antioxidant, anti-diabetic and anti-inflammatory (Cavaleiro, *et al.*, 2011, Yurdakök, Baydan, 2013, Yurdakök, 2014, Toktas, *et al.*, 2017, Derda, *et al.*, 2013, Alkofahi, Sallal, Disi, 1997, Rojas-Silva, *et al.*, 2014, Fokialakis, *et al.*, 2007, Thomas, *et al.*, 2017, Pereira, *et al.*, 2018, Roshanravan, *et al.*, 2018).

Some *Eryngium* species have previously shown cytotoxic activity such as *E. campestre* crude extract moderate antitrypanosomal (*Trypanosoma brucei brucei*), antileishmanial (*Leishmania mexicana mexicana*) and anticancer (cancerous macrophage-like murine cells) activities (Medbouhi, *et al.*, 2018). Recently, Toktas *et al.*, (2017) evaluated that *E. creticum* has cytotoxic effect on A549, CaCo2, HEKM293, HeLa and MCF7 cell lines. It was shown that *n*-hexane extract of this species had the highest cytotoxic activity against HeLa cell lines with 4.6185 \pm 0.12 $\mu\text{g}/\text{mL}$ and chloroform extract against HEKM293 cell line with 15.95 \pm 4.36 $\mu\text{g}/\text{mL}$ IC₅₀ value (Toktas, *et al.*, 2017). According to another study, *E. billardieri* was found toxic to MCF-7, HT-29, HepG2 and A549 cell lines with low IC₅₀ values at 6.5, 6.7, 59.9, 37.6 $\mu\text{g}/\text{mL}$, respectively (Esmaeili, *et al.*, 2016). In a study, *E. billardieri* extracts had cytotoxic effects on PANC-1 cancer cell lines. The results of that study demonstrated that dichloromethane and *n*-hexane extracts of *E. billardieri* significantly induce apoptosis by increasing Bax and decreasing cyclin D1 mRNA

expression (Roshanravan, *et al.*, 2018). In a study the methanolic extract of *E. serbicum* possessed a prominent cytotoxic and antiproliferative effects on HCT-116, SW-480 and MDA-MB-231 cell lines after 72 h of exposure. In this study the extract obtained from flowers displayed a remarkable cytotoxic activity on HCT-116 and SW-480 cell lines (IC_{50} : 17.96 $\mu\text{g/mL}$ and 23.03 $\mu\text{g/mL}$, respectively) and moderate activity on MDA-MB-231 cells (IC_{50} : 54.23 $\mu\text{g/mL}$). Also, 72 h treatment with leaf extract significantly decreased viability of SW-480 and MDA-MB-231 cells (IC_{50} : 12.96 $\mu\text{g/mL}$ and 15.93 $\mu\text{g/mL}$, respectively). Obtained results indicated that SW-480 cell line was most sensitive to treatment with stems extract (IC_{50} 20.25 $\mu\text{g/mL}$) after 72 h of exposure. All investigated *E. serbicum* methanol extracts, except root extract, showed a significant cytotoxic activity on tested colon and breast cancer cells, considering their non-cytotoxic activity on healthy fibroblasts. Leaf and root extracts expressed higher cytotoxicity on MDA-MB-231 cells (Vukic, *et al.*, 2018).

Based on previous phytochemical reports on *Eryngium* genus various phytochemical compounds including terpenoids, triterpenoid saponins, flavonoids, coumarins, polyacetylenes, steroids and essential oils were declared, which can be correlated to the various biological activities (Drake, Lam, 1972, Erdelmeier, Sticher, 1985). Besides, from various *Eryngium* species different phenolic compounds such as flavonoids (apigenin, quercetin, quercitrin, luteolin and derivatives, kaempferol and derivatives, catechin and derivatives) (Suleiman, 1994, Ikramov, Bandyukova, Khalmatov, 1971, Kartnig, Wolf, 1971, Hiller, Pohl, Franke, 1981, de la Luz Cádiz-Gurrea, *et al.*, 2013, Marčetić, *et al.*, 2014) and flavonoid glycosides (rutin) (Ikramov, Bandyukova, Khalmatov, 1971, Kartnig, Wolf, 1971, Marčetić, *et al.*, 2014, Bimakr, Ganjloo, Noroozi, 2019) phenolic acids (rosmarinic acid, chlorogenic acid, caffeic acid and derivatives, ferulic acid and derivatives) (Le Claire, *et al.*, 2005, Zhang, *et al.*, 2008, Wang *et al.*, 2012, Paul, Seaforth, Tikasingh, *et al.*, 2011, de la Luz Cádiz-Gurrea, *et al.*, 2013), organic acids (malic acid, quinic acid and derivatives) and isorhamnetin derivatives (de la Luz Cádiz-Gurrea, *et al.*, 2013) were isolated and identified.

It is well known that the phenolic compounds are potential substances against oxidative and DNA damage, apoptosis induction in transformed cells or tumors (Chen, *et al.*, 2008, Duthie, Duthie, Kyle, *et al.*, 2000). These compounds are important because most of them have proven antitumor activity and may also act synergistically.

In this study, the initial cytotoxicity screening showed that EKE was effective against all the cell lines but especially MDA-MB-231. IC_{50} values is relatively low among all the cell lines for EKE extract especially in MDA-MB-231 cells. EKE also showed a marked cytotoxic activity against to the three cell lines and this cytotoxicity was cell-, dose- and time- dependent. The results indicated that EKE was generally more effective than EKM. The results demonstrated that these extracts do not have the same effects exactly due to the properties of cell lines and the presence of various potential bioactive molecules in extracts. Since the cell line has a well-known high aggressive and drug-resistant phenotype (Pillé, *et al.*, 2005), the following experiments could be performed with MDA-MB-231 cancer cells. Therefore, the effective inhibition of MDA-MB-231 cancer cells suggests that ethyl acetate extract from *E. kotschyi* may be potentially promising anticancer agent for the effective treatment of breast cancer cells. The anti-proliferative effect of *E. kotschyi* was recognized in some cancer cell lines including hepatocellular, laryngeal epidermoid and glioma (Yurdakök, Baydan, 2013) but its molecular mechanisms of action on cancer cells are not yet established.

The criterion of cytotoxicity established by the US National Cancer Institute (NCI) for crude extracts was determined as $IC_{50} < 30 \mu\text{g/mL}$ (Suffness 1990). Following this fact, extracts examined in this study with observed IC_{50} values lower than 30 $\mu\text{g/mL}$ were considered to have significant activity. EKE has more potential to be explored as novel anticancer agent. In contrast, the EKW did not show cytotoxicity against cancer cell lines. Furthermore, we also found that the selective cytotoxicity of these extracts against cancer cell lines was closely related to their chemical content. The cytotoxic effect of EKE may be related to its higher content of caffeic acid, chlorogenic acid and rosmarinic acid components. According to the literature, caffeic acid (Rzepecka-Stojko, *et al.*, 2015), chlorogenic acid

(Sadeghi Ekbatan, *et al.*, 2018) and rosmarinic acid (Jang, Hwang, Choi, 2018) presented inhibitory activities against different type of cancer cells.

To the best of our knowledge, this is the first report of the phytochemical analysis and *in vitro* cytotoxic activities of the aerial parts of *E. kotschy*. In conclusion, our data illustrated that various cytotoxic effect of EKM and EKE related to different amounts of phytochemical compounds. The EKE exerted a higher level of cytotoxic compounds seems to be more effective against proliferation of cancer cells. *In vitro* SRB assay indicated that EKE exhibited the strongest inhibitory effect on A549, COLO 205 and MDA-MB-231 cancer cell lines. According to these results we can consider that the potent cytotoxic activity of EKE on A549, COLO 205 and MDA-MB-231 cells may be explained as the presence of anticancer compounds. As our results agree with previously reported studies, we can say that this work has revealed further potentials of this plant in the area of pharmacology for cancer research. Furthermore, the identified compounds are the possible contributors to the antiproliferative and cytotoxic effects of EKE and EKM suggesting an interesting potential for the pharmacotherapy of cancer. Based on these results, it is suggested that further toxicologic investigations with EKE and EKM should be carried out. On the other hand, this preliminary research suggests detailed investigations on cytotoxic effect of various combinations of determined compounds.

CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest associated with this publication.

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