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Determination of Phenolic Compounds, Organic Volatile Molecules and Anti-Cancer Properties in *Inula Viscosa* L., *Viscum Album* L. and *Raphanus Sativus* L.

Hafize DİLEK TEPE*[1], Aslı UĞURLU[2], İdris YAZGAN[2]

Abstract

The plants elecampane (*Inula Viscosa* L.), mistel (*Viscum album* L.) and black radish seed (*Raphanus Sativus* L.) have been used in the treatment of common diseases worldwide as part of traditional medicine for many years. Especially in Turkey, elecampane plant is commonly used as remedy of cancer. In this study, phytochemical components of these three plants were analyzed using liquid chromatography-mass spectrometer/mass spectrometer and gas chromatography-mass spectrometer techniques. Antioxidant activity of the characterized extracts were evaluated using DPPH assay, followed by biological properties were studied using MDA-MB-231 breast cancer line. Differences in the chemical compositions of the extracts resulted in alteration in antioxidant potentials, where elecampane gave the highest antioxidant activity while black radish seed extracts did not provide any meaningful results within the test period. Cytotoxicity studies showed that chemical composition is of the most prominent factor that defined the IC_{50} value of each extract, where pro-oxidant and antioxidant affects were observed in relation to presence of flavonoids. Mistel extract was further tested for wound healing and apoptosis tests, and the extract was obtained as a trigger for both apoptosis and wound-healing. The findings can be a basis for refinement as fractionation of the mistel and elecampane extracts so as to obtain the best mixture that can serve as strong anticancer agent mixture.

Keywords: Elecampene, mistel, phenolic compounds, LC-MS/MS, GC-MS, anticancer.

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1. INTRODUCTION

Cancer is a multifactorial and genetically complex disease. Biological mechanisms such as genetic/epigenetic mutations, continuous proliferative signals, overexpression of oncogenes, inactivation of tumor suppressor genes, inhibition of apoptosis, increased angiogenesis, and metastasis cause cancer development and progression [1]. Among the cancer types, breast cancer is one of the most common types of cancer that affects more than 1 million women worldwide. Breast cancer includes a heterogeneous population of cells. Based on histological and molecular analyzes, five different breast cancer subtypes have been identified as Luminal A (estrogen receptor ER+, progesterone receptor PR+, human epidermal growth factor receptor 2 Her2-), Luminal B (ER+, PR+, HER2+), TNBC (ER-, PR-, HER2-), Her2+ (ER-, PR-, Her2+) and normal breast-like [2]. These different subtypes of breast cancer respond differently to treatment. Nowadays, surgery, chemotherapy, targeted chemotherapy, hormonal therapy and radiotherapy are used in breast cancer treatment and these treatments provide a significant decrease in mortality rates. However, the heterogeneous nature of breast cancer is an obstacle for the treatment. Furthermore, cancer recurrence, metastasis and drug resistance may develop [3,4]. This reveals the need for more effective treatment strategies with less side effects for breast cancer. Bioactive components isolated from natural sources are capable of inhibiting DNA damage, cell proliferation, angiogenesis and metastasis, and stimulating apoptosis and autophagy [5]. Some preclinical studies showed that natural components increase the susceptibility of resistant cancers to existing chemotherapy drugs [6]. In addition, existing chemotherapy drugs usually have a single target effect, while natural components can target many signaling pathways altered in cancer cells [7]. Natural compounds are of great interest because they are less toxic than synthetic drugs and can also affect cancer stem cells. *In vitro* and in vivo studies have shown that a large number of plants and their bioactive compounds exert an inhibitory effect on breast cancer by reducing estrogen receptor expression, inhibiting cell cycle and proliferation, stimulating caspase-mediated apoptosis, reducing anti-apoptotic factors, inhibiting angiogenesis and metastasis [2], [8-13].

The genus *Inula* (Elecampane) belongs to the chamomile family and has different species (*Inula viscosa* L., *I. racemosa, I. helenium* L., *and I. Britannica* L.*)* with very high medicinal value. *Inula* grows in Africa, Asia and Europe, especially in the Mediterranean region. Traditional uses of *Inula* plant species were first used in Roman and Greek medicine. It is also used in traditional Chinese medicine in Ayurveda and Tibetan medicine to treat various diseases such as bronchitis, diabetes, fever, hypertension and inflammation [14]. In Turkey, flowers, leaves and roots of *I. viscosa* are consumed as food raw or cooked [15]. *I. viscosa* has attracted great interest in recent years as a natural source of bioactive compounds in its structure. The anti-inflammatory [16], anti-cancer [17] and antimicrobial [18] studies of this plant have been conducted. Due to the polyphenols in the structure has been shown to have strong antioxidant properties [19]. A complex mixture of secondary metabolites was already identified in *I. viscosa*, as flavonoids, sesquiterpenes lactones and acids, phenolic acids derivatives, glycolipids and triterpenoids [16], [20-23].

Viscum album L. is generally known as mistel and grows as a semi-parasite plant on different host trees in Europe and Asia. Mistel, with the help of the fringe roots, is coniferous like fir, pine, spruce; apple, plum, apricot, cherry-like fruit and poplar, chestnut, alder, oak, willow shed leaves in winter grows as semi-parasites on trees or shrubs [24]. Mistel is used in many different diseases in traditional medicine. These are cardiac diseases, hemorrhoid, diabetes, anxiety, headache, epilepsy, hyperactivity in children, anticancer, toothache, tonsillitis and throat ache, headache, asthma, prostatitis, hypercholesterolemia, hypertension, tachycardia, bronchitis, ulcer, gastritis, cough, hypercholesterolemia, atherosclerosis, splenopancreases diseases, sterility (woman) and brain tumor. *V. album* has remarkable popular usage in Turkish folk medicine as a remedy for cardiac diseases [25-

35]. To date, numerous studies have been carried out to determine the biological potential of the plant [36-38]. Various clinical studies reported the improvement in survival and quality of life, after using mistel extracts, underling the ability of the plant to support the conventional medicine [39]. It has been shown that the mistel extracts reduce the harmful and mutagenic effects of free oxygen radicals produced during radio and chemotherapy [40,41]. Extracts from subspecies have been shown to contain viscotoxins [42], phenylpropanoids [43] and flavonoids [44] in different concentrations depending on the subspecies.

Raphanus sativus L. (Brassicaceae), commonly known as radish, is used in the world as a vegetable or spice in human nutrition [45]. The roots, seeds and leaves of the radish plant show a variety of medicinal properties. In traditional Korean medicine, the seeds are used as degassing, diuretic, expectorant, laxative, stomach-strengthening anti-cancer and anti-inflammatory agents [46,47]. Radish has anti-carcinogenic activity because it contains phytochemicals, glucosinolates (GLS), phenolics, vitamins and their metabolites. In addition, vitamin C, a powerful antioxidant in its structure, prevents DNA and tissue damage caused by free radicals [48,49]. Many studies have been made in different species of radish. However, there are not many publications related to black radish seeds.

These three plant species mentioned above are used in similar diseases in Turkish and world traditional medicine. Therefore, in these three plants volatile biological molecules were scanned with gas chromatography- mass spectrometer (GC-MS) and 32 phenolic compounds were screened with liquid chromatography- mass spectrometer / mass spectrometer (LC-MS/MS) in this study. These scans were identified as some plant-specific phyto-component molecules. Thus, the antioxidant and anti-cancer properties of these three plants could be compared with each other.

2. MATERIAL AND METHODS

2.1. Extraction Process

Before the extraction process, elecampane (1), mistel (2), and black radish seeds (3) samples were washed in pure water and dried in an oven at 80 °C. For analysis, dried samples were pulverized in high-speed plant mill and prepared for extraction. 2 g of powdered plant samples were extracted in 40 mL of 80% methanol and ethanol in ultrasonic bath for 30 min then left at room temperature. The final extraction concentrate was then adjusted to 50 mg / mL.

2.2. Determination of volatile organic molecules by GC-MS

Volatile molecules in the extract were qualitatively analyzed in electron ionization (EI) mode with Agilent Technology 7890A Gas Chromatography (GC) Mass spectrometer (MS). Chromatographic column Agilent HP-5MS, capillary column (30 m* 0.25 mm, film thickness of 0.25 mm). The furnace temperature was started at 40 ^{0}C, followed by standing for 5 min, then at 5 ^{0}C. min^{-1} at 280 ^{0}C. and held for 5 min. Helium gas (99.999%) was used as the carrier gas. The constant flow rate was 1.5 mL min^{-1} and the injector temperature was 250 ^{0}C. The extract was injected in splitless mode with 1 mL. Interpretation of the mass spectrum was performed according to the National Institute of Standards and Technology (NIST) database.

2.3. Determination of phenolic compounds by LC-MS/MS

Determination of phenolic profiles of plants, high performance liquid chromatography mass spectrometer - mass spectrometer (Agilent 1260 Triple Quadrupole MSMS) was used. Each analysis was performed with 3 replications. HPLC column C18 ODS used in the analyzes (25 x 4.6 mm x 5µ) was used. Injection amount for analysis: 2 µL. Water / 0.1% formic acid (A), methyl alcohol (B) was used as carrier phase. The gradient method as follows: 3 min 2% B, 6 min 25% B, 10 min 50% B, 14 min 95% B, 17.5 min 2% B. Flow rate: 0.4 mL / min. In the identification of compounds was performed in positive and negative modes [50]. The chromatogram peaks of the standards and sample were shown in figure 1.

2.4. Antioxidant Test

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay is based on electron-transfer from anti-oxidant molecule to the free radical DPPH molecule that produces a violet solution in ethanol, whose intensity is monitored optically [51], [52]. 0.1, 0.5,1.0 and 5.0 mg/mL DPPH solutions in ethanol, and 1.0, 5.0 and 10.0 mg/mL ascorbic acid prepared in ethanol were used to draw standard graphic. All the tested extracts were used as 1/10th and 1/100th dilutions of the stock to reveal antioxidant capability.

2.5. Cell Culture and Cell Viability Assay

Triple negative MDA-MB-231 breast cancer cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% non-essential amino acid (NEAA), 0.1% penicillin/streptomycin, and 0.01 mg/mL human insulin. The cells were incubated at 37°C in a humidified 5% CO_2 incubator. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to analyze cytotoxic effects of the extracts. 10^4 MDA-MB-231 cells/well were seeded in 96-well plates and grown for 24 h. The cells were exposed to each test extract at different concentrations (0.25, 0.5, 1, 2.5, 6.25, 12.5, 25 mg/mL) and incubated for 24 h. After extract treatment, 0.5% FBS and 0.5 mg/mL MTT containing DMEM was added and incubated for 4 h at 37°C. Formazan crystals were dissolved in 40 mM HCl/isopropanol and 3% SDS, and the optical density was measured at 570 nm using Multiskan Go (Thermo Scientific). IC_{50} values were calculated using GraphPad Prism software.

2.6. Gene Expression Analysis

Subconfluent MDA-MB-231 cells were treated with Mistel extract at doses of IC_{50} values for 24 h. GeneJET RNA Purification Kit (Thermo Scientific) was used for RNA isolation and DNase treatment was performed for 30 min at 37°C. RevertAid First Strand cDNA synthesis kit (Thermo Scientific) was used to convert total RNA (2 µg) into cDNA according to instructions. qPCR reaction mixture was prepared with 10 µL of SYBR Green (Biorad), 1 µL of forward and reverse primer (5 µM), 1 µL of cDNA and 7 µL of dH2O were. Primer sequences were as follows: human Bax: forward 5'-CCCGAGAGGTCTTTTTCCGAG-3', reverse 5'-CCAGCCCATGATGGTTCTGAT-3', human Bcl-2: forward 5'-GGTGGGGTCATGTGTGTGG-3', reverse 5'-CGGTTCAGGTACTCAGTCATCC-3', human GAPDH (internal reference gene): forward 5'-GGAAGGTGAAGGTCGGAGTC-3'; reverse 5'-AACATGTAAACCATGTAGTTGAGGT-3'. Amplifications were carried out in Rotor Gene-Q (Qiagen) with denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 10 sec, annealing and elongation at 57°C for 30 sec. Relative transcript levels were analyzed using ΔΔCt method.

2.7. Wound Healing Assay

$2.5x10^5$ MDA-MB-231 cells were seeded into 6-well plates and grown until confluency. The next day, the cells were treated with 10 µg/mL mitomycin for 2 h to inhibit cell proliferation and then a wound was formed with a 200 µL pipette tip on the monolayer. The cells were treated with Mistel extract at IC_{50} value. The images of the wound were obtained after 24 h and 48 h incubation periods using an inverted microscope (Leica). Wound width was measured for analysis.

Statistical analysis

Data were subjected to Analysis of Two-way ANOVA using GarphPad Prism. Means were separated from each other by Bonferroni post-tests ($p<0.05$). The analysis was performed in triplicate.

3. RESULTS AND DISCUSSION

Table 1 GC-MS characterization of the extracts. Molecules given in the table were chosen based on 90% or higher similarity.

Extract	Identified molecule (similarity%)
Elecampane	1-Dodecene (97); ylangene (98); Copaene (98); Caryophyllene (99); Neoisolongifolene (95); 2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl- (98); trans-Caryophyllene (95); Hexadecane (96); 1-Hexacosene (95); Squalene (99); 3-Hexenoic acid, (E)- (91); Nerolidol 2 (91); Caryophyllene oxide (91); Selina-6-en-4-ol (91); Cycloheptane,4-methylene-1-methyl-2-(2-methyl-1-propen-1-yl)-1-vinyl (91); 11,11-Dimethyl-spiro[2,9]dodeca-3,7-dien (90); 4',5-Dihydroxy-7-methoxyflavanone (91)
Mistel	*1-Dodecene* (96); methyl linoleate (99); alpha-linolenic acid (95); 9-Tricosene, (Z)- (96); Heptadecane (97); 9-Octadecenamide, (Z)- (98); Eicosane (96); Pentacosane (96); Octadecane (96); Octacosane (98); Nonacosane (99); Vitamin E (99); Beta.-Sitosterol (96); 1-Di(t-butyl)silyloxypropane (90); 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (90); 3,5,7-Trimethyl [1,2,4] triazolo [1,5-a]pyrimidinium-2-thiolate (90); Phytol (91); 3,10,10-trimethyl-6-methyliden-1-oxa-spiro(4.5)dec-3-ene (91); 6-fluoro-4,6-cholestadien-3-ol (91);
Black Radish Seeds	*1-Dodecene* (98); 1-Hexadecene (95); Oleic Acid (95); 2(1H)-Naphthalenone, octahydro-4a- (95); Squalene (98); Gamma-Tocopherol (95); Gamma-Sitosterol (97); 2,6-dimethyl-3-(methoxymethyl)-p-benzoquinone (90); Dimethyl-(1,2,3,4,5,6,7,8-octahydro-carbazol-9-yl)-amine (90); 1-Oxaspiro[2.5]octane, 5,5-dimethyl-4-(3-methyl-1,3-butadienyl)- (93); 1,2-Longidione (91); Diepicedrene-1-oxide (91); Lup-20(29)-en-3-ol, acetate, (3.beta.) (91); Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl) (90); Campesterol (91)

3.1. Phytochemical profile (Volatile compounds content)

GC-MS results revealed that all three extracts do not have many molecules in common while characteristic molecules for each extract were more common. Elecampane extract gave sesquiterpenoids and furanone that show strong biological activities. For example, copaene shows strong anti-oxidant and anti-genotoxic effect [52] while caryophyllene can alter PI3K/Akt/mTOR/S6K1 and STAT3 to inhibit cancer growth and increase apoptosis in different cancers [53]. Similarly, presence of squalene, ylangene, β-caryophyllene oxide and trans-nerolidol are strong resource for antioxidant [54] and anti-cancer source [55] including breast cancer [56]. Besides, presence of benzofuranone derivatives [57] gave strong anticancer capability of elecampane extract. A similar chemical content from guava leaves extract showed regulatory effect on AKT/mTOR/ribosomal p70 S6 kinase (S6K1) and MAPK to suppress cancer growth for prostate cancer [58]. Extracts containing 4',5-Dihydroxy-7-methoxyflavanone gave potent anti-proliferation activity for human breast, colon and melanoma cell lines [59].

3.2. Phenolic Compouns Content

According to the data obtained from the determination of phenolic compounds, chloregic acid, hyperoside and protocathuic acid were found high in elecampene and mistel plants. In

addition, quercetin ratio (125.73 µg/g DW) in elecampene plant was higher than mistel plant and black radish seed. The reason why quercetin has various biological activities is that it has antioxidant properties [60]. With the depletion of this molecule, a link has been found between reducing the risk of cancer and cardiovascular diseases. These findings indicate that this phenolic compound can be used as a protective nutraceutical compound [61]. The amount of sinapic acid (43.70 µg/g DW) in black radish seed is higher than elecampene and mistel (Table 2).

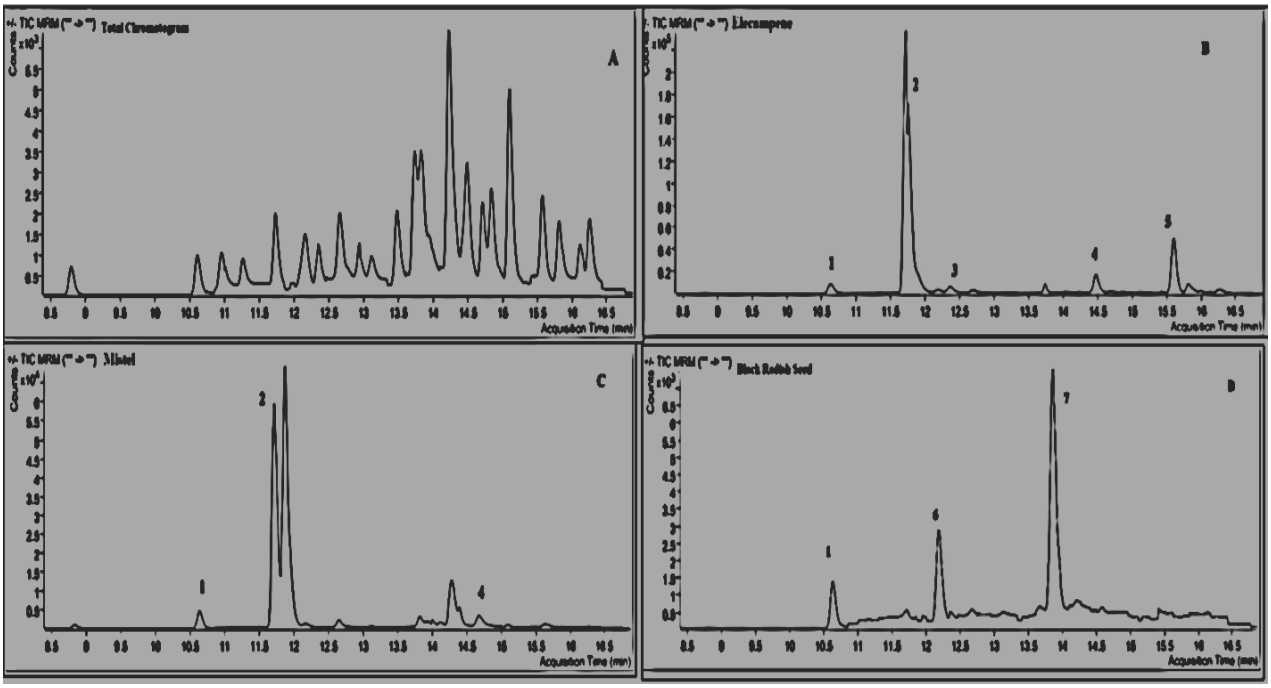

Figure 1 LC-MS/MS chromatograms of phenolic compounds. A: Total chromatogram, B: Elecampene, C: Mistel, D: Black radish seed. Chromatogram peaks:1:Protocathuic acid, 2:Chlorogenic acid, 3:2,5 Dihydroxybenzoic acid, 4: Hyperoside, 5: Quercetin, 6: 4-Hydroxybenzoic acid, 7: Sinapic acid.

Table 2 Phenolic compound content of Elecampane, Mistel and Black Radish Seed
Data represents the means ± SE, *, p < 0.05, nd:not detected., Rt: retention time of phenolic compounds, DW: Dry weight

Phenolic compound	**Elecampene**		**Mistel**		**Black Radish Seed**	
	Rt	**µg/g DW**	**Rt**	**µg/g DW**	**RT**	**µg/g DW**
Gallic acid	8.816	1.23±0.08	8.833	7.29±0.18*	8.825	0.12±0.00
Protocatechuic acid	10.643	46.21±1.18**	10.643	22.45±0.38**	10,634	9.24±0.50*
3,4-Dihydroxyphenylacetic acid	10.981	0.54±0.03	10.981	0.32±0.03	10.914	0.07±0.05
Pyrocatechol	11,124	0.27±0.03	11.074	0.23±0.06	11.049	0.22±0.01
(+)-Catechin	11.286	0.06±0.02	11.444	0.23±0.02	11.319	0.27±0.02
Chlorogenic acid	11.735	844.82±15***	11.727	173.79±7.11***	11.727	1.05±0.01
4-Hydroxybenzoic acid	12.198	13.86±0.06*	12.181	4.12±0.08	12.189	17.53±0.25**
2,5-Dihydroxybenzoic acid	12.366	64.11±0.6***	12.391	1.34±0.12	11.954	0.36±0.03
(-)-Epicatechin	12.428	0.02±0.01	12.512	0.03±0.00	12.369	1.08±0.07
Vanillic acid	12.608	4.48±.0.09	12.583	2.58±0.08	12.617	1.23±0.11
Caffeic acid	12.699	9.90±0.20*	12,658	5.56±0.25*	12.683	0.68±0.06
Syringic acid	12.806	0.84±0.01	12.790	2.51±0.05	12.748	0.20±0.01
3-Hydroxybenzoic acid	12.97	0.24±0.01	12.97	0.14±0.02	12.962	0.15±0.00
Vanillin	13.148	0.25±0.02	13.131	1.33±0.02	13.148	1.26±0.17
Verbascoside	13.492	0.07±0.00	13.492	0.07±0.00	13.459	0.04±0.00
Taxifolin	13.745	7.80±0.18*	13.745	0.04±0.01	13.628	nd
p-Coumaric acid	13.859	0.14±0.02	13.834	7.00±0.30*	13.859	12.49±0.6**
Ferulic acid	13.998	0.21±0.01	13.958	3.15±0.11	13.973	3.65±0.12*
Sinapic acid	14.039	0.07±0.00	13.873	0.31±0.02	13.881	43.70±08**
Luteolin 7-glucoside	14.247	0.27±0.06	14.23	0.12±0.02	14.304	nd
Hesperidin	14.452	21.97±0.18**	14.411	23.23±0.42**	14.444	0.22±0.01
Hyperoside	14.487	59.49±3.20***	14.687	20.73±0.38**	14.512	0.07±0.00
Rosmarinic acid	14.498	2.12±0.05	14.632	0.42±0.03	14.514	0.11±0.00
Apigenin 7-glucoside	14.705	4.67±0.15	14,81	0.15±0.00	14.738	0.16±0.00
Pinoresinol	15.001	1.24±0.02	14.984	6.28±0.40*	14.825	0.44±0.02
Eriodictyol	15.127	1.05±0.11	15,103	0.72±±0.01	15.12	0.01±0.00
2-Hydroxycinnamic acid	15.171	0.06±0.02	14.76	0.06±0.00	15.222	0.09±0.01
Quercetin	15.611	125.73±6.00***	15.635	3.26±0.02	15.603	0.23±0.01
Luteolin	15.825	29.43±0.33**	15.816	0.41±0.03	15.833	nd
Kaempferol1	16.138	6.54±0.30*	16.155	0.10±0.01	16.138	0.46±0.03
Kaempferol	16.138	15.90±0.09**	16.262	nd	16.138	nd
Apigenin	16.251	4.50±0.09	16,276	0.40±0.02	16.276	nd

The great interest in phenolic compounds has been rapidly grown in recent years, due to the increased evidence of their attractive nutritional properties on human health. Phenolic compounds have high antioxidant activity, and the consumption of food with abundant phenolic compounds might reduce the risk of coronary heart disease, certain types of cardiovascular disease and cancer [62].

3.3. Antioxidant Test Results

Ascorbic acid (AA) at 10 mg/mL concentrations were used as a reference reducing agent in DPPH assay as described elsewhere [63]. Antioxidant activity of the extracts were calculated as percentage decrease in DPPH absorbance (Equation 1).

$$\%\ Reduction = [\frac{(Abs0-Absi)}{Abs0}]X100 \qquad (1)$$

where Abs0 refers to the absorbance of DPPH at 0 µM vitamin C concentration and Absi is the absorbance of remaining DPPH (unreduced) absorbance at the tested vitamin C concentration.

Elacampane extract revealed the highest antioxidant activity as 0.5 mg/mL extract reduced DPPH that is equivalent to 1 mg/mL AA mediated DPPH reduction. Mistel extract at 10 mg/mL reduced DPPH that is equivalent to 1 mg/mL AA mediated DPPH reduction while black radish seed extract at 10 mg/mL did not provide a meaningful reduction of DPPH within 3-min assay period.

3.4. Cell Viability

MTT analysis was performed in order to determine the cytotoxic effects of elecampane, mistel and black radish seed extracts on MDA-MB-231 cells. There was an inverse correlation between elecampane concentration and cytotoxic effect on MDA-MB-231 cells. The highest cytotoxic activity of elecampane extract was observed at the lowest tested concentration (Figure 2). For detailed analysis of elecampane extract, 0.25, 0,5 and 1 mg/mL concentrations were also evaluated with MTT analysis. The highest cytotoxic activity of elecampane extract was detected at 1 mg/mL concentration. The cytotoxic activity has decreased whether the elecampane concentration is lower or higher than 1mg/mL (Figure 3). This trend has been overwhelmingly reported for flavonoid extracts, where they behave as antioxidants and pro-oxidants depending on the concentration and physiological conditions [64]. It is possible that the elecampane extract behaved like flavanoids. Mistel extract showed the highest reduction in MDA-MB-231 cell viability (92%) at 12.5 mg/mL concentration (Figure 2). IC_{50} values of mistel treatment were calculated as 4.84 mg/m L for 24 h and 3.5 mg/mL for 48 h. Mistel extracts had higher amount of fatty acid and fatty acid esters as shown in Table 1. Methyl linoleate and 9-octadecenamide were reported as antioxidants [65,66]. Beta-sitosterol has anticancer properties against wide range of cancers such as breast cancer, prostate cancer, colon cancer, lung cancer, stomach cancer, ovarian cancer, and leukemia [67]. Vitamin E has also anticarcinogenic activities [68]. These compounds present in mistel extract might synergistically reduce MDA-BM-231 cell viability. Radish extract did not have cytotoxic effect against MDA-MB-231 cells (Figure 2).

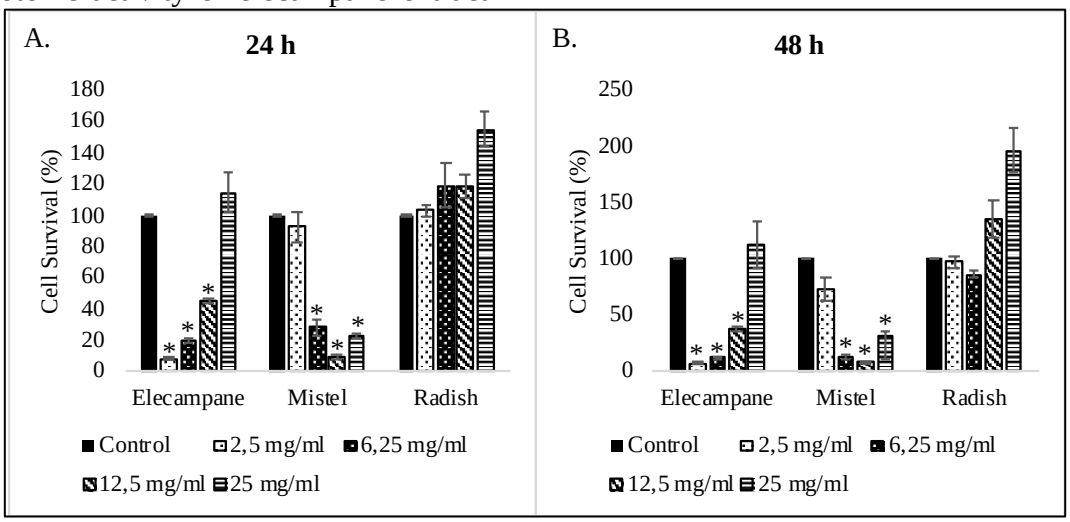

Figure 2 MTT results. MDA-MB-231 cells were treated with extracts at different concentrations for 24 h (A) and 48 h (B). Data represents the means ± SE, *, $p < 0.05$.

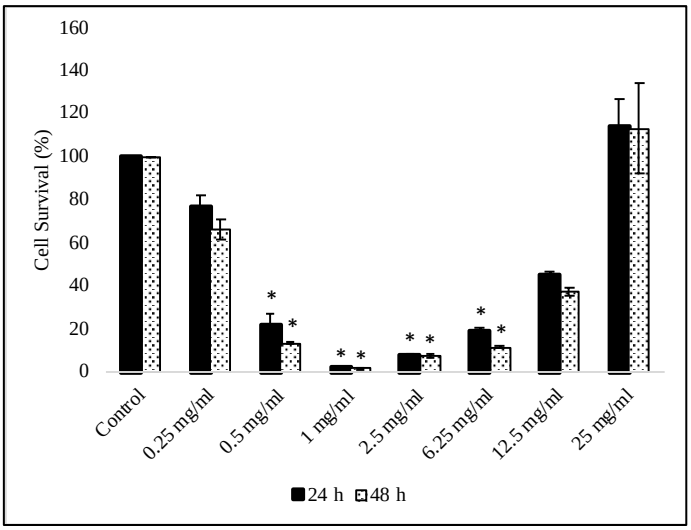

Figure 3 Concentration dependent cytotoxicity of Elacampane extract on MDA-MB-231 cells. The cells were treated with Elecampane extracts at different concentrations for 24 h and 48 h. Data represents the means ± SE, *, $p < 0.05$.

3.5. Apoptotic Marker Gene Expression

Apoptosis is a programmed cell death mechanism that eliminate dysfunctional cells. Evasion of apoptosis is one of the hallmarks of cancer cells [1]. Apoptosis can be triggered by various intracellular and extracellular stimuli, and many anticancer drugs target apoptosis. In mammals, there are two main apoptosis pathways, mitochondria-mediated (intrinsic) and death receptor-mediated (extrinsic). DNA damage, oxygen deficiency, and oxidative stress stimulate the initiation of the intrinsic pathway. When the intrinsic pathway is activated, the permeability of the mitochondria is increased and cytochrome-c is released into the cytoplasm. This pathway is regulated by B-cell lymphoma 2 (Bcl-2) family proteins. Bcl-2 family proteins are divided into two groups; pro-apoptotic and anti-apoptotic proteins. Pro-apoptotic proteins initiate apoptosis by inducing cytochrome-c release while anti-apoptotic proteins inhibit cytochrome-c release [69].

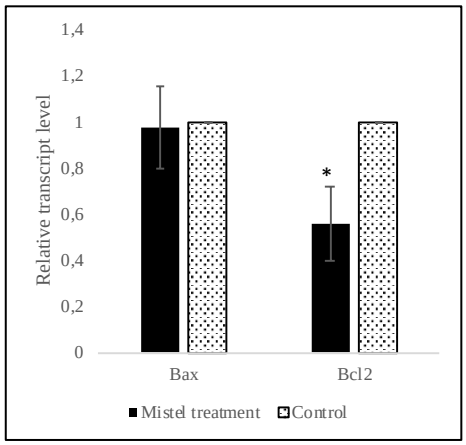

Figure 4 Apoptotic markers. MDA-MB-231 cells were treated with Mistel extract at IC_{50} concentration (3.5 mg/mL) for 48 h. Marker gene expressions were analyzed by qPCR. *, $p < 0.05$.

In order to analyze whether mistel extract treatment induce apoptosis on MDA-MB-231 cells, pro-apoptotic marker gene (Bax) and anti-apoptotic marker gene (Bcl-2) levels were analyzed by qPCR. Bax levels were not changed, however, there was a significant decrease in Bcl-2 level (45%) with mistel extract treatment (Figure 4). Increased Bax/Bcl-2 ratio (1.76) has demonstrated that apoptosis of MDA-MB-231 is stimulated with mistel extract treatment. Compounds found in mistel extract beta-sitosterol and vitamin E has been associated with apoptosis induction in several studies. Beta-sitosterol has been shown to inhibit proliferation and stimulate

apoptosis of MCF-7 and MDA-MB-231 breast cancer cells [67,70,71]. *In vitro* and *in vivo* studies have proven the pro-apoptotic effect of T3, a compound from vitamin E family, on several cancer types as breast, prostate, lung, bladder, liver, colorectal and pancreas [72,73]. The apoptosis induction observed with mistel extract treatment on MDA-MB-231 cells might be due to combined pro-apoptotic effect of beta-sitosterol and vitamin E.

3.6. Wound Healing Assay

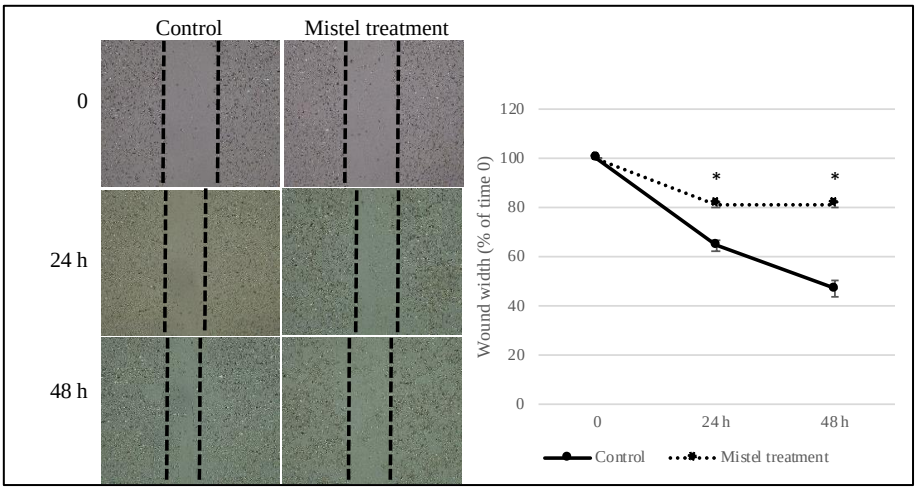

Figure 5 Effect of Mistel extract treatment on cell migration. MDA-MB-231 cells were treated with Mistel extract at IC_{50} concentration for 48 h. The images were obtained with inverted microscope. Cell migration was quantified according to relative distance in each wound.

Cancer cell migration is necessary for development of malignancies. Metastasis begins when cancer cells in the primary tumor site cross the extracellular matrix and join the blood vessels and lymph system and spreads out to the body. Wound healing assay was performed to analyze whether mistel extract treatment can inhibit MDA-MB-231 cell migration. For the control group, approximately 40% and 60% of wound closure was achieved after 24 h and 48 h respectively. Migration capacity of the MDA-MB-231 cells were decreased with mistel extract treatment compared to control group for both time points (Figure 5). Several studies have demonstrated that vitamin E family derivatives inhibit cancer cell migration. T3 has been reported to reduce migration of non-small-cell lung carcinoma cells and gastric cancer cells based on wound healing assay [73,74]. γ-Tocotrienol has significantly reduced migration and invasion of mammary cancer cells by inhibiting Rac1/WAVE2 signaling pathway [75]. It is suggested that vitamin E found in mistel extract might be responsible for the inhibition of MDA-MB-231 cell migration.

4. CONCLUSION

Natural compounds exert anticancer effect by regulating cell cycle, triggering apoptosis, and stimulating various signaling pathways. Current chemotherapy agents target both cancer and normal cells. Plant-derived natural compounds reduce the side effects of cancer treatment. In this study, we identified biochemical components of elecampane, mistel and black radish seed and investigated the anticancer activity of these extracts on MDA-MB-231 breast cancer cells. Mistel and elecampane extracts decreased the percentage of viable MDA-MB-231 cells. The increased Bax / Bcl2 ratio after mistel treatment has indicated that MDA-MB-231 cell death is induced by apoptosis. Besides, mistel extract treatment inhibit the MDA-MB- 231 cell migration. The observed anticarcinogenic

properties might be due to the presence of bioactive compounds such as methyl linoleate and 9-octadecenamide, beta-sitosterol and Vitamin E.

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the authors.

Authors' Contribution

The design and organization of this study and the characterization of the samples were done by Hafize DİLEK TEPE, cell culture experiments were carried out by Aslı UĞURLU and antioxidant activity measurements were carried out by İdris YAZGAN. In addition, all authors greatly supported the writing of the study.

The Declaration of Ethics Committee Approval

The authors declare that this document does not require an ethics committee approval or any special permission.

The Declaration of Research and Publication Ethics

The authors of the paper declare that they comply with the scientific, ethical and quotation rules of SAUJS in all processes of the article and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

These findings have suggested a new biological activity for mistel extracts.

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