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Determination of Phytochemical Profile of *Allium tuncelianum* and Evaluation of Its Antiproliferative Effect on *Various Human Cell Lines*

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ABSTRACT
In this study, *Allium tuncelianum*, one of the endemic garlic species growing in Anatolia, was investigated for its phytochemical content. LC-MS/MS, HPLC, GC-MS, GC-FID and ICP-OES techniques were used for this purpose. Major phenolic components of *Allium tuncelianum* were found to be malic acid (3322.6 µg/g), kainic acid (626.8 µg/g), cinnamic acid (69.15 µg/g), fumaric acid (13.02 µg/g) and catechin (3933.3 µg/g). The main components of volatile oil, on the other hand, were detected as diallyl disulfide (28.30%), diallyl trisulfide (30.90%) and allyl methyl trisulfide (9.44%). The fatty acid composition of the plant was revealed as oleic acid (27.19%), linoleic acid (19.46%) and elaidic acid (3%) whereas high content of potassium (4207± 67 mg/kg), calcium (518± 35 mg/kg) and magnesium (376 ± 20 mg/kg) was determined as the minerals. Moreover, cytotoxic effects of ethanol/water and hexane/chloroform extracts of the plant were evaluated in prostate, colon, cervical and breast cancer cell lines and cytotoxicity of both extracts were detected for all the cell lines studied. Therefore, we conclude that *Allium tuncelianum* may be a new phytotherapy agent with its rich phytochemical content and anticancer activity.

Research Article

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Allium tuncelianum' un Fitokimyasal Profilinin Belirlenmesi ve Çeşitli İnsan Hücre Hatlarında Antiproliferatif Etkilerinin Değerlendirilmesi

ÖZET
Bu çalışmada, Anadoluda yetişen endemik sarımsak türlerinden biri olan Tunceli Dağ Sarımsağı (*Allium tuncelianum*) incelenmiştir. Fitokimyasal içeriği belirlemek için LC-MS / MS, HPLC, GC-MS, GC-FID ve ICP-OES teknikleri kullanılmıştır. *Allium tuncelianum*'un başlıca fenolik bileşenleri; malik asit (3322,6 µg / g), kainik asit (626.8 µg / g), sinamik asit (69.15 µg / g), fumarik asit (13.02 µg / g) ve kateşin (3933.3 µg / g) olduğu belirlendi. Uçucu yağın ana bileşenleri, dialil disülfit (% 28.30), dialil trisülfit (% 30.90) ve allil metil trisülfit (% 9.44) idi. Yağ asidi bileşimi; oleik asit (% 27.19), linoleik asit (% 19.46) ve elaidik asit (% 3) iken, mineral içeriğindeki major elementler; potasyum (4207 ± 67 mg / kg), kalsiyum (518 ± 35 mg / kg) ve magnezyum (376 ± 20) mg / kg) olarak belirlenmiştir. Prostat, kolon, servikal ve meme kanseri hücre dizilerinde sitotoksik etki de incelenmiştir. Kolon, Meme, Servikal ve Prostat kanseri hücre hatlarında sitotoksik etki tespit edildi. Bu sonuçlar göstermektedir ki; *Allium tuncelianum*, zengin fitokimyasal içeriği ve antikanser aktivitesi ile yeni bir fitoterapi ajanı olabilir.

Araştırma Makalesi

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Anahtar Kelimeler
Sarımsak
Yağ asitleri
Uçucu yağ
Fenolik
Sitotoksik aktivite

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INTRODUCTION
The species of Allium, especially *A. cepa* and *A. sativum* of Alliaceae are important vegetable crops worldwide. There are nearly 500 *Allium* species in the world and 164 of these originated in Turkey. Forty percent of *Allium* species found in Turkey is endemic

(Özhatay, N. 2002). Tunceli Rural Garlic (*A. tuncelianum*) is an endemic and edible garlic species and is used as a trade Product (Yanmaz et al. 2010). It is sold under the names of 'Tunceli Rural Garlic' and 'Ovacik Garlic' and is used as a trade product. Tunceli garlic has a good chance of being used in industry and being consumed as fresh. Because it is a single tooth, the number of shells is less than the known culture garlic, and heads can be stored at 18-20 °C for a long time.

Natural phenolic compounds play an important role in cancer prevention and treatment. Phenolic compounds from medicinal herbs and dietary plants include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones, and others. Various bioactivities of phenolic compounds are responsible for their chemo preventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects) and also contribute to their inducing apoptosis by arresting cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways. This review covers the most recent literature to summarize structural categories and molecular anticancer mechanisms of phenolic compounds from medicinal herbs and dietary plants.

Phytochemical compounds obtained from plants have been used for medicinal purposes, especially against cancer in recent years. Various bioactivities of phenolic compounds are responsible for their chemopreventive properties (e.g., antioxidant, anticarcinogenic, or antimutagenic and anti-inflammatory effects). Also, Phenolic compounds contribute inducing apoptosis by arresting the cell cycle, regulating carcinogen metabolism and ontogenesis expression, inhibiting DNA binding and cell adhesion, migration, proliferation or differentiation, and blocking signaling pathways (Huang et al. 2009).

In this study, various techniques are used to identify phytochemical compounds in plants. The main techniques are Liquid Chromatography-Mass Spectrometry (LC-MS), High-Performance Liquid Chromatography (HPLC), and gas chromatography (GC). Conjoined with Flame Ionization Detector (GC-FID) and Mass Spectrometry (GC-MS), Gas Chromatography technique is used to determine the fatty acid esters and essential oil components of the plants (Pandey et al. 2011). Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) is widely used for trace element analysis. Enzyme based water-soluble tetrazolium salt (WST-1) method was used for cytotoxicity analysis. *Allium tuncelianum* ethanol/water (1/1) (ATEpEW) and *Allium tuncelianum* hexane/chloroform (1/1) (ATEpHC) extract were used for this purpose.

MATERIALS and METHODS

Plant Material and Extraction Procedure

The plant of *Allium tuncelianum* (Ozhatay and Mathew 2007) was collected from Tunceli province and Munzur mountain at the altitude of 1100-1200 m, Turkey, in August of 2012. These specimens were identified at the Herbarium of Inonu University. The underground parts of the *A. tuncelianum* plant to be used in the study were stored in the freezer at +4°C until the extraction. The extraction process has proceeded as follows; ten milliliters of solvent (ethanol/water 1/1) were added to approximately 1 g of fresh homogenized garlic sample. Stirred for 24 h shaker and the water were removed by use lyophilizator and organic solvents (ethanol, hexane, chloroform) were removed by using a rotary evaporator and kept in refrigerator + 4^0C for 48 h. The dry samples were resolved in appropriate solvents and filtered using Whatman (2200-070) for phenolic analysis. ATEpEW and ATEpHC extracts prepared similarly.

Method of preparing hydrolyzed *A. tuncelianum* extract (HAT): The method used by Hertog was adapted for Tunceli Mountain Garlic in order to prepare the hydrolyzed *A. tuncelianum* extract (HAT) (Hertog et al. 1992). The samples of *Allium tuncelianum* were thoroughly shredded in a mortar; 10 g were weighed. Overall, 40 mL of 62.5% methanol and 10 mL of 6 M HCl were added (1.2 M HCl in 50% methanol). It was kept closed in a water bath at 80 ℃ for 2 h. After the sample was cooled to room temperature and filtered through the paper (Whatman No.4 0.45 µm).

20 µL of the filtrate was injected into the LC-MS/MS.

Identification and Quantification of EAT Phenolic Compounds by LC-MS / MS

The analytical method used in this analysis was developed by Dr. Mustafa Abdullah Yilmaz (2015). The LC-MS/MS system consists of a combination of UHPLC (Shimadzu Nexera model) and LC-MS (Shimadzu 8040 model triple quadrupole mass spectrometer) device. The liquid chromatography system used consists of the LC-30 AD model gradient pump, the DGU-20A3R model degasser, the CTO-10ASvp model column oven and the SIL-30AC model autosampler. Chromatographic separation was performed on Inertsil ODS-4 modern C18 (100 mm × 2.1 mm, 2 µm) column. Ultrapure water was used as mobile phase A for the elution gradient and acetonitrile was the mobile phase B. In addition, 10 mM ammonium formate and 0.1% formic acid were added to the water phase to facilitate better chromatographic separation and ionization. The mobile phase flow rate was 0.25 mL/min and the injection volume was set at 4 µL. The triple quadrupole mass spectrometer is equipped with an Electrospray

ionization (ESI) source operating in both negative and positive mode (Ertas et al. 2015). The LC-ESI-MS / MS data were processed collecting the registered by LabSolutions (Shimadzu, Kyoto, Japan) software. The quantitative analysis of the analytes was carried out in the multiple reaction monitoring (MRM) and the parent ions have been integrated with one or two cleavage products. Other parameters that are optimized in the mass spectrometer are: interface temperature; 350 ºC, DL temperature; 250 ºC, heat block temperature; 400 ºC, nebulizer gas (N_2) flow; 3 L / min and drying gas (N_2) flow rate; 15 L/ min (Ertas et al. 2015).

Identification and Qualification of EAT Phenolic Compounds by HPLC

Liquid chromatography of the *A. tuncelianum* extracts was carried out on a Shimadzu HPLC system with the LC-20AD pumping system and an SPD-20A UV detector. The wavelength used to detect the phenolic compounds was 278 nm. The column used was Waters Spheris orb 5 µm 4.6*250 mm analytical column (Soares et al. 2001). The elution was performed with 0.1% of phosphoric acid (A) and acetonitrile (B). The gradient profile was as follows: 0 min, 8% B; 35 min, 22% B; 45 min 8% B and then held for 5 min to initial conditions. The flow rate was 0.8 mL/min. The chromatographic column was washed with the initial conditions to stabilize for 10 minutes. The concentrations of each compound were reported as mg/kg on the basis of the total weight of oven-dry *A. tuncelianum* and percent amount on the basis of total compounds determined by HPLC. Detection wavelength for all reference compounds was studied according to the maximum adsorption wavelength between 200-400 nm wavelength and 278 nm wavelength was found suitable. The desired components form the RQC were identified by comparing both the retention times and UV spectra with those of the authentic standard. A perfect agreement between standard and sample spectra was found in all analyzed samples. All the calibration curves were marked based on linear regression analysis of the integrated peak areas (x) versus concentrations (y, mg/kg) of the 10 marker constituents in the reference solution at four different concentrations. In this study, the analysis of condensed tannins (catechin) and hydrolyzable tannins (Gallic acid) found in A*. tuncelianum* samples were performed under the same chromatographic conditions. The ethanol extract of *A. tuncelianum* was injected into the device and analyzed. In addition, the glycoside bonds of the phenolics in the plant extract have been hydrolyzed to be able to identify more phenolic compounds. After the samples are thoroughly shredded for this process, 10 g of Allium tuncelianum are kept in 40 mL of 62.5% methanol and 6M HCl (final concentration 1.2 M HCl, 50% methanol) at 80 ° C for 2 hours. After the sample was cooled to room temperature and filtered through the paper (Whatman No.4 0.45 µm). 20 µL of the filtrate was injected into the HPLC apparatus).

Extraction and identification of fatty acid composition by GC-FID

This study was carried out on the basis of Özkaya et al. (2013) method with minor changes In order to be able to perform GC analysis, the fatty acids in the lipids must be converted to their methyl ester derivatives. Lipids were extracted with hexane/isopropanol (3/2 v/v). The samples (5 g) were homogenized for 30 seconds in a mixture of hexane and isopropanol. The mixture was taken into centrifuge tubes and centrifuged at 10,000 rpm for 10 min. The supernatant was removed and placed in a test tube with a cap. 5 mL of 2% methanolic sulphuric acid was added and thoroughly mixed with the vortex. This mixture was allowed to methylate in a 50 ºC water bath for 12 hours. The tubes were removed from the water bath, cooled to room temperature, and 5 mL of 5% sodium chloride was added and mixed thoroughly. The fatty acid methyl esters consisted in the test tubes were ejected with 5 mL of hexane, the hexane phase was taken out by pipetting, processed with 5 mL of 2% $KHCO_3$ and were left to stand for 4 hours to separate the phases. The methyl esters were isolated and quantified by gas chromatography and flame ionization detection (Shimadzu GC, 17 Ver.3) linked to a glass GC 10 software computing recorder (Kokten et al. 2011).

Extraction and analysis of volatile oil components by GC-MS

Volatile oils were obtained from Clevenger using the water vapor distillation method. For this process, 200 g of the sample was stripped and crushed in the mill, then stirred with 1000 mL pure water and kept for 30 minutes at room temperature. Subsequently, the essential oils were obtained by applying heat treatment in the Clevenger for about 3 hours. The resulting essential oil was stored in a refrigerator autoclave vial (flask) at +4 °C for GC-MS analysis. Separation of volatile components was used Shimadzu GC-2010; QP-2010 mass spectrometry system; Shimadzu Corp., Kyoto, Japan DB-FFAP capillary columns (60 m x 0.25 mm x 0.25 µm; J & W Scientific, Folsom, CA, USA). The furnace temperature was set from 60° to 280°C at 4°C / min. The Helium gas was used as carrier atcarrier at a flow rate of 2of 2 mL/min. For evaluation of the chromatograms, Wiley 7 and NIST 147 mass spectral libraries were utilized to identify the peaks. To confirm retention indices, n-alkane (C10-C26) series were used by the same conditions and compared with the literature data

(Hayaloglu and Demir 2016).

Trace element analysis in *A. tuncelianum*

In this study, the ICP-OES analyzer (Perkin-Elmer 3100, Norwalk, USA) was used for the determination of trace elements. ICP-OES analyzer was combined with a Gem Cone nebulizer on a cyclonic spray chamber and an autosampler (AS 91, Perkin-Elmer). *A. tuncelianum* was thoroughly crushed in the mortar. The crushed garlic was made ash by using the Ash Furnace (Protherm PLF 110/8). The residue was dissolved by adding 2 mL of concentrated acid mixture [HNO_3 (65%, w/w) and 1 mL of $HCIO_4$ (60%, w/w)]. The solution was then transferred to the ICP-OES analyzer's pumping system and analyzed (Ozkaya et al. 2013).

Determination of Total Phenolic Content

The determination of total phenolic content was done according to a previous method with minor alteration by Yildirim et al. (2015). Sample extracts (40 µL) of *A. Tuncelianum* (1 mg/mL) was stirred with 1160 µL of distilled water and 200 µL Folin Ciocalteau flavor, followed by 600 µL 20 % Na_2CO_3 3 min later. The mixture was swashed for 2 h at room temperature and absorbance was measured at 765 nm. Gallic acid was used as a standard. All tests were performed in three replicates. The calibration graph was done with Gallic acid, and the results were expressed as 1 g of Gallic acid equivalents (l g GAE / g d w) as suggested by Emen et al. (2009).

Condensed tannin analysis

Condensed tannin analysis was applied to *A. tuncelianum* by changing the method of Sarneckis et al (2006). To prepare a standard curve, we put 1 mL of 2, 4, 6, 8, 10, 12 µg / mL tannin in a test tube. Later we added 500 µL of Folin Ciocalteau flavor on top and mixed the tube for 3 minutes. Then 2500 µL Na_2CO_3 is added and the glass tube is shaken. 1 hour after the shake the absorbance is read at 580 nm. The obtained data is processed and a standard graphic is drawn. 0.05 g of $FeSO_4$ is placed in a tube and 0.015 g of the milled plant sample is added. Then 2 mL of 0.55 M Butanol-HCl buffer is added to the vortex. The mouth of the test tube is tightly closed and kept at 97-100 ° C for 1 hour. After being cooled, the absorbance value is read at 580 nm. When the absorbance value obtained from this is substituted in the form obtained from the graph, the result corresponds to µg of tannic acid.

Antioxidant capacity was determined by cuprac method

The antioxidant capacity assay which uses Trolox as a standard was carried out as described by Çelik et al. (2010) using cupric reducing antioxidant power (CUPRAC). A sample of 10 mL was taken from the extracts. A series of solutions were prepared at 50, 100, 250 and 500 ppm concentrations to determine the antioxidant capacity. To each test tube; 1 mL of Cu (II), neocuproine (NC) and $(NH_4)_2SO_4$ buffer solutions were added. To make the final volume as 4.1 mL, antioxidant sample (or standard) solution (x mL) and H_2O (1.1-x) mL were added to the first mixture. The tubes were stopped and after half an hour the absorbance at 450 nm (A_{450}) was read against the reagent blank. The standard calibration curve of each antioxidant compound was made in this manner, relative to the absorbance concentration. The result of the CUPRAC method for each antioxidant was found in the corresponding calibration equation.

Determination of Cytotoxic Activity

In this study, the water-soluble tetrazolium (WST-1) method was used. The WST method is an enzyme-based method. It is based on a reductive coloring reagent and dehydrogenase enzyme activity in a viable cell by a colorimetric method to determine cell viability. WST takes two electrons from living cells to produce purple Formosan dye.

To determine cytotoxic activity in this study; Human prostate carcinoma (PC-3), endometrial carcinoma (ECC-1), colon adenocarcinoma (DLD-1), prostate (PNT-1A), normal mammary cell (CRL-4010), and cervical carcinoma (HeLa) cell lines were incubated in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (*RPMI*) *1640* medium. The cell lines, free of pathogenic contamination, were cultivated as monolayers in their suitable medium reinforced with the inclusion of 100 mg/mL streptomycin and 100 µg/mL penicillin, 10% fetal bovine serum (FBS) and L-glutamine (2 mM). The cell lines were protected in an incubator with a humidified atmosphere (5% CO_2 in air at 37°C). Cells were passaged three times a week to maintain a logarithmic growth stage (Kilic et al. 2018).

Cell proliferation was determined by the dye reduction method as described according to Mosmann's (1983) method with minor alterations. The WST-1 assay was performed to analyze the proliferation of normal and cancer cells. Cells (1×10^5/ml) were seeded in 96 well plates and maintained for 24 h under standard conditions (37 °C, and 5% of O_2). The compound 5-Fu (5-fluorouracil) used as a reference to be positive control. ATEpEW and ATEpHC were applied three times in different concentrations between ranges of 0-200 µM on wells of cells grown. After 48 h of incubation, the medium was removed and then WST-1 assay was performed. Cytotoxicity data were fitted to a sigmoidal curve and a four-parameter logistic model was used to calculate the IC_{50}, which is the concentration of material causing 50% inhibition in comparison to the untreated controls.

Statistical Analysis

In this study, Graph Phad Prism 5.01 program was used for statistical analysis. A comparison of data was conducted by using One Way ANOVA Tukey's Post Hoc test. Group averages were communicated as ± standard deviation, and between-group comparisons were given as ± standard error. For parametric data analysis, One-Way ANOVA was used. The statistical importance of between-group differences was examined through the Tukey HSD test. In graphics, while the groups were being compared, $p<0.05$ was regarded to be the value of statistical significance. Any statistical significance difference was communicated via letters on the data in Tables.

RESULTS and DISCUSSION

Identification and Quantification of Phenolic Compounds by LC-MS/MS

Thirty-seven phenolic compounds (flavonoid glycosides, flavonoids, hydroxybenzoic acids and hydroxycinnamic) besides that three non-phenolic organic acids which are widespread in plant materials were analyzed by an LC-MS/MS method. The method was developed by Yılmaz (2015). This method was used to look for the phenolic compound in ethanol extract of *A. tuncelianium*.

Table 1. Analytical parameters of LC-MS/MS method, and identification and quantification of phenolic compounds in ethanol extract of *A. tuncelianum* (EAT)

Çizelge 1. LC-MS/MS yönteminin analitik parametreleri ve *A. tuncelianum*'un etanol ekstresindeki fenolik bileşiklerin tanımlanması ve nicelenmesi.

No (pick)	Analyte	RT [c]	Parent Ion(m/z) [a]	Regression Equation	R^2 [d]	RSD % [e]	LOD/LOQ (µg/L) [f]	U [g]	Quantification (µg Analyte/g EAT) [h]
1	Coumarin	17.40	147.05	y=33.64×-89700	0.994	0.01306	208.4/228.4	0.0237	11.25±0.0026
2	Hesperidin	12.67	610.90	y=1340.27×-43769	0.998	0.00945	3.4/4.2	0.0262	9.73±0.0025
3	P- Coumaric acid	11.53	162.95	y=3199.20×+13002	0.992	0.01820	7.3/9.1	0.0516	2.61±0.0013
4	O- Coumaric acid	15.45	162.95	y=1219.34×-10915	0.999	0.02730	24.4/31.1	0.0513	N.D
5	Gallic acid	3.00	168.85	y=226.76×+38152	0.998	0.01601	95.5/106.9	0.0282	1.75±0.0015
6	Caffeic acid	8.80	178.95	y=3963.32×+178156	0.998	0.01454	18.4/22.4	0.0354	N.D
7	Vanilic acid	8.57	166.90	y=35.84×-12097	0.999	0.00528	122.2/139.7	0.0508	22.91±0.0125
8	Salicylic acid	11.16	136.95	y=5286.26×+309192	0.989	0.01016	5.0/6.5	0.0329	N.D
9	Kainic acid	1.13	190.95	y=41.06×+10671	0.996	0.00259	75.8/79.4	0.0082	626.8±0.0513
10	4-OH-benzoic acid	7.39	136.95	y=409.03×+112079	0.998	0.01284	33.2/38.1	0.0289	N.D
11	Ferulic acid	12.62	192.95	y=80.45×-31782	0.997	0.00708	36.6/42.0	0.0494	3.8±0.0018
12	Chlorogenic acid	7.13	353.15	y=781.36×-18697	0.998	0.00058	6.2/8.1	0.0069	N.D
13	Rosmarinic acid	14.54	359.00	y=909.67×-201692	0.994	0.02014	6.6/8.8	0.0713	N.D
14	Protocatechuicacid	4.93	152.95	y=297.75×+30590	0.995	0.01236	28.2/31.4	0.0411	N.D
15	Cinnamic acid	25.61	147.00	y=9.06×-12403	0.996	0.00648	821.8/859.7	0.0143	69.15±0.0098
16	Sinapinic acid	12.66	222.95	y=141.96×-73294	0.992	0.01446	78.7/86.1	0.0281	N.D
17	Fumaric acid	1.48	115.00	y=64.99×-11592	0.997	0.00536	28.1/34.5	0.0124	13.02±0.0016
18	Vanillin	10.87	151.00	y=446.10×+70934	0.998	0.00696	44.3/53.1	0.0280	N.D
19	Pyrocatechol	6.48	109.00	y=30.61×+14735	0.996	0.01313	261.1/278.4	0.0235	N.D
20	Malic acid	1.23	133.00	y=316.95×-42041	0.999	0.00477	55.3/67.5	0.0113	3322.66±0.3754
21	Syringic acid	9.02	196.95	y=42.33×-52547	0.996	0.01049	212.5/233.3	0.0238	2.4±0.0006
22	Hesperetin	31.76	300.95	y=876.67×+48916	0.997	0.03209	5.6/6.9	0.0562	N.D
23	Naringenin	30.68	270.95	y=4315.1×+178410	0.995	0.02054	5.4/6.4	0.0521	N.D
24	Rutin	12.61	609.05	y=561.91×-16879	0.997	0.00473	5.5/6.5	0.0159	N.D
25	Quercetin	28.17	300.90	y=1198.48×+480562	0.990	0.01589	23.3/28.9	0.0543	N.D
26	Quercitrin	16.41	447.15	y=339.39×+38910	0.999	0.01528	22.0/25.2	2.0079	N.D
27	Apigenin	31.43	268.95	y=4548.36×+295252	0.990	0.02304	5.4/6.3	0.0650	N.D
28	Chrysin	36.65	252.95	y=2032.13×+95593	0.993	0.00490	5.4/6.2	2.0083	N.D
29	Liquiritigenin	25.62	254.95	y=2384.96×+59141	0.996	0.01849	5.5/6.6	0.0341	N.D
30	Isoquercitrin	13.42	463.00	y=803.23×+4981	0.999	0.00682	5.4/6.3	0.0133	N.D
31	Apigetrin	16.59	431.00	y=1775.55×+91121	0.993	0.01797	5.4/6.1	0.0597	N.D
32	Rhoifolin	16.11	577.05	y=237.15×+11887	0.999	0.00747	23.1/27.9	0.0941	N.D
33	Nicotiflorin	14.68	593.05	y=498.38×+79274	0.991	0.00737	22.4/25.5	0.0276	0.15±0.0001
34	Fisetin	19.30	284.95	y=547.46×+274791	0.991	0.00557	54.4/61.4	0.0148	N.D
35	Luteolin	28.27	284.75	y=3272.65×+150557	0.997	0.00575	5.4/6.5	0.0174	N.D
36	Myricetin	18.72	317.00	y=583.55×+205727	0.999	0.00652	53.2/57.2	0.0126	N.D
37	Kaempferol	31.88	284.75	y=26.29×+87558	0.992	0.01436	206.6/214.3	0.0209	N.D

[a]: Parent ion (*m/z):* Molecular ions of the standard compounds (mass to charge ratio); [b]: MS^2 (CE): MRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions); [c]: RT; Retention time; [d]: R^2; coefficient of determination; [e]: RSD; relative standard deviation; [f]; LOD/LOQ (µg/L); Limit of detection/Limit of quantification; [g]: U (%); 95% Relative standard uncertainty at confidence level, [h]: Values in µg/g (*w/w*) of plant extract; **N.D.**: not detected.

The concentrations of identified compounds analyzed in *EAT* are given in Table 1. As a result of the study, 3322.66 ± 0.37 µg Analyte / g EAT was found to be malic acid. The malic acid concentration is very high and the retention time (RT) is about 1.23 minutes, causing it to appear as the first peak and affect the visibility of the other peaks. The second major component of our study was kainic acid (626.8 ± 0.05

µg Analyte / g EAT). Then, the amount of component (µg Analyte / g EAT) respectively; Cinnamic acid (69.15 ± 0.01), Vanilic acid (22.91 ± 0.01), Fumaric acid (13.02 ± 0.0016), Coumarin (11.25 ± 0.0026), Hesperidin (9.73 ± 0.0025), Ferulic acid (3.8 ± 0.0018) P-coumaric acid (2.61 ± 0.0013), Syringic acid (2.4 ± 0.0006), Gallic acid (1.75 ± 0.0015), Nicotiflorin (0.15 ± 0.0001) a total of 12 compounds were identified.

In LC-MS / MS studies for *A. sativum* (Farag et al. 2017) found Citric acid / Isocitric acid, Phthalic acid, Caffeic acid, Ferulic acid, Quercetin, Caffeic acid dimethyl ether, Kaempferol and Isorhamnetin. A Content analysis study was performed by Izol (2016) with LC-MS / MS on 12 Allium species. Approximately 15 different phytochemicals have been identified. In general, although it differs for some species, the highest component in species is found to be the dominant component, while malic acid in 7 species is the dominant component. The amount of malic acid in the species is µg Analyte / g extract; *A. scorodoprasum* (787.6), *A. shatakiense* (292.66), *A. shirnakiense* (771.89), A. *vineale* (3304.12), *A. chrysantherum* (889.21), *A. rhetoreanum* (235.8), *A. schoenoprasum*. The highest components in other species were expressed as hesperidin, rutin, p-coumaric acid and vanillin. In general, it has been determined that phenolic contents of all species studied are poor in diversity and quantity based on LC-MS/MS results.

Identification and Quantification of Phenolic Compounds by HPLC

In this step, the analyzed ethanol extract of *A. tuncelianum* (EAT) and hydrolyzed *A. tuncelianum* extract (HAT) were analyzed. Seven phenolic compounds were investigated by using HPLC. The concentrations of identified compounds analyzed in *EAT and HAT* are given in Table 2. It was also found that about 21-26% of the total compounds in the EAT roots were determined by HPLC for all the extractions. Four active compounds (fumaric acid, Gallic acid, catechin and 4-hydroxybenzoic acid) were found in the EAT and HAT extract Table 2). HPLC chromatograms of standard compounds and *A. tuncelianum* extracts are presented in Figures 3-5 respectively.

Table 2. Analytical parameters of HPLC, their concentrations (mg/kg and percent) determined by HPLC study in ethanol extracts of *A. tuncelianum* (EAT) and hydrolyzed *A. tuncelianum* extract (HAT)

Çizelge 2. HPLC'nin analitik parametreleri, *A. tuncelianum* etanol ekstraktı (EAT) ve hidrolize edilmiş *A. tuncelianum* ekstraktında (HAT); fenolik bileşiklerin konsantrasyonları (mg/kg ve yüzde) HPLC çalışması ile belirlenmiştir.

No (Pick)	Analit	R.T (min)	LOD/LOQ (mg/kg)	Regression equation	R^2	Quantification EAT mg/kg (ppm) %	Quantification mg/kg (ppm)	HAT %
1	Fumaric acid	5.25	0-5000	y=0.0015x	0.9985	449.24 18.86	752.33	0.63
2	Gallic acid	6.38	0-300	Y=5E-05X	0.9988	1.61 2.04	13.69	0.34
3	4-Hydroxy benzoic acid	21.01	0-700	Y=5E-05X	0.9988	N.D	20.83	0.32
4	Catechin	27.29	0-2500	Y=0,0002X	0.9972	N.D	3933.3	24.63
5	Vanilic acid	29.42	0-800	Y=8E-05X	0.9973	N.D	N.D	
6	Cafeic acid	36.91	0-700	Y=5E-05X	0.9986	N.D	N.D	
7	Syringic acid	39.07	0-700	Y=4E-05X	0.999	N.D	N.D	
	Total					20.90		25.92

According to the results of the study, fumaric acid (18.86%) and catechin (24.63%) were found as the main components in the extracts. "Vlase et al. (2013) have identified phenolic substances by HPLC on ethanol extracts and hydrolyzed extracts of some Allium species grown in Romania and they identified p-Coumaric acid and ferulic acid in all ethanolic extracts. In the HPLC study on *A. cepa* and *A. sativum* (Yunlu 2011), the authors determined that the phenolic compounds are phenolic acids such as Gallic acid, p-hydroxybenzoic acid, protocatechuic acid, caffeic acid, chlorogenic acid, ellagic acid and ferulic acid. Fratianni et al. (2016) investigated the polyphenolic contents of some endemic Italian garlic species (Bulbs of *A. sativum* varieties " *Schiacciato, Bianco*, *Uvita Flumeri*, *Salomone* and *Torella*) and high levels of Gallic acid and chlorogenic acid. In addition to the differences between the species, trace amounts of caffeic acid, hyperoside, epicatechin, apigenin, p-coumaric acid and ferulic acid were detected in the components. These results were obtained as a phenolic component in garlic species; Gallic acid, fumaric acid, 4-hydroxybenzoic acid, p-Coumaric acid, chlorogenic acid, caffeic acid and quercetin. Our work, while paralleling these results, revealed that *A. tuncelianum* had a higher level of catechin than these species.

Determination fatty acid (FA) composition of A. tuncelianum by GC-FID

The fatty acid levels of *A. tuncelianum* were presented in Table 3. A total of 29 fatty acid compounds were detected. *A. tuncelianum* major components has been identified as oleic acid, (27.19±1.17%), linoleic acid (19.46±1.09%), Elaidic Acid (7.13±0.19%) Palmitic Acid Pentadecanoic Acid (4.71±0.11%), Arachidic Acid (4.68±0.14%), alfa-Linolenic Acid (6.31±0.18%), cis-4,7,10,13,16,19-Docosahexaenoic acid (5.37±0.45%), cis-4,7,10,13,16,19-Docosa hexaenoic Acid 5.37±0.08 and Stearic Acid (3.12±0.09%).

Table 3. Fatty acid composition of *A. tuncelianum*
Çizelge 3. *A. tuncelianum* 'un yağ asit içerikleri

Name	Numeric Formula	R.T.	Area%
Lauric Acid	12:0	17.27	0.5579±0.02
Myristic Acid	14:0	22.40	0.5891±0.04
Pentadecanoic Acid	15:0	26.54	0.1341±0.01
Cis-10-Pentadecanoic Acid	15:1	27.37	4.7008±0.11
Palmitic Acid	16:0	28.41	6.3069±0.18
Palmiteloic Acid	16:1	29.77	1.2296±0.03
Heptadecanoic Acid	17:0	31.05	0.0815±0.01
Cis-10-Heptadecanoic Acid	17:1	31.89	0.9794±0.07
Stearic Acid	18:0	33,57	3,1254±0,09
Elaidic Acid	18:1	34.37	7.1344±0.19
Oleic Acid	18:1	34.65	27.1983±1.17
Linoleic Acid	18:2	36.46	19.4610±1.09
gama-Linolenic Acid	18:3 ω-3	38.14	0.2139±0.03
alfa-Linolenic Acid	18:3 ω-3	38.56	3.3549±0.08
Arachidic Acid	20:0	39.02	4.6874±0.14
Cis-11-Eicosenoic Acid	20:1	39.91	0.5064±0.02
Heneicosanoic Acid	20:0	41.18	3.0031±0.16
Cis-11,14-Eicosadienoic Acid	20:2	41.86	0.6327±0.09
Cis-8,11,14-Eicosatrienoic Acid	20:3	42.33	0.2781±0.02
Behenic Acid	22:0	42.74	0.2765±0.01
cis-11,14,17-Eicosatrienoic Acid	20:3	42.97	0.7592±0.03
Erucic Acid	22:1	43.27	2.9943±0.18
Arachidonic Acid	20:4	43.85	0.6866±0.31
Tricosanoic Acid	23:0	44.79	1.2584±0.15
cis-13.16-Docosadienoic Acid	22:2	45.27	2.1921±0.07
cis-5.8.11.14.17-Eicosapentaenoic Acid	20:5 ω-3	46.33	0.1174±0.01
Lignoceric Acid	24:0	47.21	1.7748±0.11
Nervonic Acid	24:1	48.77	0.3937±0.03
cis-4,7,10,13,16,19-Docosahexaenoic Acid	22:6 ω-3	49.44	5.3723±0.45
Total			100.0000

In a study done for Allium species in the literature; Tsiaganis et al. (2006) determined fatty acid in Allium species [onion (*A. cepa*), garlic (*A. sativum*) and leek (*A. porrum*)] by gas chromatography (GC). They determined eighty percent of the total lipids of all species consists of four FA: linoleic (46-53%), palmitic (20-23%), oleic (4-13%) and α-linolenic acid (3-7%). This result confirms the accuracy of our work. The fat profile of Tunisian garlic was conducted by Chekki et al. (2014), the main fatty acids identified were lauric acid (49.3%) and linoleic acid (20.4%). It was revealed in a study of Chinese garlic (*A. tuberosum*); that *A. Tuberosum* contained many important fatty acids with linoleic (57.0-71.6%) and palmitic (6.6-9.7%). This result shows that the fatty acid content and diversity of *A. tuncelianum* is richer than other garlic (*Allium sativum*).

Determination volatile oil components of A. tuncelianum by GC-MS

The volatile oil components of *A. tuncelianum* were presented in Figure 2. and Table 4. Twenty-five compounds were identified in *A. tuncianium* volatile oil. Diallyl trisulfide (DATS) (30.90%) and Diallyl disulfide (DADS) (28.30%) were identified as the major component in the essential oil of *A. tuncelianum.* Other components in the essential oil of *A. tuncelianum* were found as; allyl methyl trisulfide (9,44%), allyl methyl sulfide (8.66%), dimethyl trisulfide (3.72%), phthalic acid diethyl ester (2.90%) propyl disulfide (2.87%), isobutyl isothiocyanate (2.37%), methyl (methyl sulphinyl) methyl sulphite (2.30%), Diallyl sulfide (DAS) (2.12%) and linoleic acid methyl ester.

Table 4. Result of volatile oil components of *A. tuncelianum*
Çizelge 4. A. tuncelianum uçucu yağ bileşenleri sonucu

Molecular Formula	Name	R.T.	Area%
C_4H_8S	Allyl methyl sulfide (AMS)	7.25	0.71±0.001
C_4H_8S	1-methylthio-1-propene	7.26	0.77±0.003
$C_2H_6S_2$	Dimethyl disulfide (DMDS)	9.83	0.45±0.002
$C_6H_{10}S$	Diallyl sulfide (DAS)	11.69	2.12±0.011
$C_2H_6S_2$	2,3-dimercaptan	14.16	0.38±0.001
$C_4H_8S_2$	Methyl-trans-propenyl-disulfide (MPeDS)	15.11	0.39±0.002
C_4H_8S	Allyl methyl sulfide	15.64	8.66±0.021
$C_2H_6S_3$	Dimethyl trisulfide	19.32	3.72±0.013
$C_6H_{12}S_2$	Trans-propenyl propyl disulfide	19.86	2.87±0.012
$C_5H_{10}O_2S$	Propanoic acid	21.07	0.10±0.002
$C_6H_{10}S_2$	Diallyl disulfide (DADS)	21.43	28.30±0.31
$C_6H_{14}S_2$	Propyl disulfide (PeDS)	22.72	0.69±0.003
$C_{16}H_{24}O_2$	(Phenethyl octanoate)	22.96	0.21±0.014
$C_4H_8S_3$	Allyl methyl trisulfide (AMTS)	24.45	9.44±0.098
$C_3H_8S_3$	Methyl methyl thiomethyl disulfide	26.37	0.47±0.024
C_5H_9NS	Isobutyl isothiocyanate	27.85	2.37±0.025
$C_6H_{10}S_3$	Diallyl trisulfide (DATS)	29.49	30.90±1.56
$C_3H_8OS_2$	Methyl (methylsulfinyl) methyl sulfide	31.27	2.30±0.064
$C_5H_8O_2$	Methyl 3-butenoate	31.81	0.27±0.003
C_3HF_7	Heptafluoropropane	36.39	0.03±0.001
$C_{11}H_{18}$	Naphthalene	39.44	0.28±0.001
$C_{20}H_{38}$	Kauren (Cembrene)	40.80	0.17±0.002
$C_{16}H_{32}O_2$	Palmitic acid	41.17	0.39±0.003
$C_{12}H_{14}O_4$	Diethyl phthalate	41.65	2.90±0.015
$C_{19}H_{34}O_2$	Linoleic acid methyl ester	45.70	1.51±0.091

One of the studies about *A. sativum* was conducted by Lee et al. (2003), which determined Korean garlic flavor components by gas chromatography-mass spectrometry (GC-MS). Diallyl trisulfide (11.40%), allyl sulfide (23.59%) and diallyl disulfide (57.88%) were determined to be the dominant flavor components of garlic samples. Another study was conducted about essential oil of Egyptian garlic (*A. sativum* L.) by Jirovetz et al. (1992). They identified as sulfur-containing main constituents (concentration higher than 1%) of this oil, diallyl trisulfide (29.7%), diallyl tetra sulfide (4.4%), diallyl disulfide (3.2%), diallyl sulfide (2.5%) and methyl allyl trisulfide (2.1%). When compared our results; it was seen that the species diversity of the sulfur compounds in our work was higher than the previous studies. This might related to the garlic extraction method. Because, when the garlic is disintegrated, the allinase enzyme transforms the allin compound into various sulfur compounds. When the fractionation stage is extended and the extract is put on hold, these compounds are diversified and their quantities are increased (Banerjee et al. 2003)

Determination of trace element analysis with ICP-OES

The trace elements of *A. tuncelianum* were presented in Table 5. Alignment of mineral components found in *A. tuncelianum* as mg / kg (ppm) as followed; K (4207±67.77), Ca (518.1±35.14), Mg (376.5±20.83), Na (119.7±19.64), Fe (15.9±1.32), Zn (9.24±0.19), Mn (2.48±0.17), Se (1.03±0.38), Cu (0.54±0.04) and Ni (0.33±0.04). Heavy metals such as cadmium, cobalt, chromium and lead have not been encountered. These results are similar to those of other *Allium* species (Izol 2016; Waheed et al. 2003).

Determination of total phenolic content, antioxidant capacity and condensed tannin of EAT

Determining the total amount of phenolic content in food is important to give an idea of the hydroxyl groups that provide antioxidant activity. In this study, total phenolic compound were determined (Table 6). The total amount of phenolic compound of EAT was found as 406.51 ± 0.99 mg Gallic acid equivalents / g dry *A. tuncelianum*. The antioxidant capacity of EAT was found on 222.39 ± 2.38 mg Trolox equivalent / g dry *A. tuncelianum*. The amount of condensed tannin of EAT was found as; 53.50 ± 0.74 mg Tannic acid equivalent / g dry *A. tuncelianum*. It was determined that the total phenolic content was high, the total amount of tannin was low and the antioxidant capacity was strong. Narendhirakannan et al. (2010) found the total amount of phenolic compounds of *A. sativum* to be 44.58 ± 0.54 (GAE) / g dry *A. sativum*. The antioxidant capacity results of EAT are in line with those of other garlic species (Cai et al. 2004; Lachowicz et al. 2017).

Table 5. Result of trace elements in *A. tuncelianum*
Çizelge 5. A. tuncelianum içeriğindeki eser elementlerin sonucu

Element	Wavelength (nm)	Calibration equation	R^2	LOD / LOQ (mg/L)	Conc. in *A. tuncelianum* mg/kg (ppm)
Calcium (Ca)	318	Y=9158x – 164.12	0.9989	0-100	518.1±35.14
Copper (Cu)	327	Y=592.68x + 79.563	0.9895	0-5	0.54±0.04
Iron (Fe)	238	Y=201.14x + 92.204	0.9940	0-10	15.9±1.32
Potassium (K)	766	Y=1322.3x – 1007.5	0.9983	0-100	4207±67.77
Magnesium (Mg)	285	Y=357.43x + 1694.1	0.9604	0-100	376.5±20.83
Manganese (Mn)	258	Y=1204x + 207.38	0.9949	0-5	2.48±0.17
Sodium (Na)	589	Y=56.96x +56.24	0.9981	0-100	119.7±19.64
Nickel (Ni)	232	Y=199.58x + 2.8891	0.9815	0-5	0.33±0.04
Selenium (Se)	196	Y=514.12x + 15.23	0.9892	0-5	1.03±0.38
Zinc (Zn)	206	Y=68.178x + 2.8641	0.9967	0-5	9.24±0.19
Cadmium (Cd)	229	Y=151.37x + 25.951	0.9859	0-5	N.D
Cobalt (Co)	229	Y=317.34x – 21.515	0.9872	0-5	N.D
Chromium (Cr)	268	Y=659.71x – 66.7	0.9844	0-5	N.D
Lead (Pb)	220	Y=11.444x 19.158	0.9942	0-5	N.D

Table 6. Result of total phenolic, antioxidant capacity and condensed tannin in EAT
Çizelge 6. *A. tuncelianum* etanol ekstreleri 'nde toplam fenolik, antioksidan kapasite ve kondanse tanen sonucu

Method	Standard equivalent compound	Calibration equation	R^2	LOD / LOQ (µg/L)	EAT (mg equivalent /g dry plant)
Total phenolic	Gallic acid	Y=257.81x + 34.55	0.9974	0-6000	406.51±0.99
Antioxidant capacity	Trolox	Y=0.0058x	0.9958	0-12000	222.39±2.38
Condensed tannin	Tannic acid	Y=0.053x + 0.0067	0.9987	0-12	53.50±0.74

Determination of the cytotoxic activity of ATEpEW and ATEpHC

The cytotoxic effects of ATEpEW on DLD-1, PC-3, HELA, ECC-1, HGC-27, HEK-293 and MCF-7 cell lines were examined by WST-1 method. IC_{50} values are shown in Table 7. According to these results, Allium tuncelianum extract prepared with (1/1) ethanol/water (ATEpEW) showed little efficacy compared to the standard drug (5-Fu). Although ATEpHC showed the cytotoxic effect on Human prostate Carcinoma cells (PC-3) better cytotoxic effect in all cancer cell lines. On normal Human Embryonic Kidney Cell (HEK293); is the lowest cytotoxicity.

The results of our antiproliferative activity study for *A. tuncelianum* were found similar with previous studies. In a study, Aqueous garlic (*Allium sativum*) extract showed that has a significant effect against the HeLa cell line because 95% of cancer cells were found to be dead after 24 h incubation with a dose of 375 µg/mL (Islam et al. 2011). Oomen et al (2004) study results indicated that allicin inhibits the proliferation of HeLa cancer cells in a concentration-and time depended manner. Prakash et al. (2016) study show that cytotoxic activity by the ethanolic extract of *Allium sativum* against Human Cancer Cell Lines SF-295 (central nervous system cell line), Colon 502713, and Colo-205 (colon cancer line), were 0 %, 54 %, 6 %, respectively. The maximum cytotoxic activity shown against Human Cancer Cell Lines Colon 502713 was 54 % at concentration 100 µg/mL. In the other study cytotoxic activity of A tuncelianum solid-phase extracts was determined on HeLa cells originating from a human cervical carcinoma and it was found that 72 h, 0,5 mg/mL(500µg/mL)concentration led 30 percent of the cells to apoptosis (Gerçek et al.2017).

Recently, the identification of qualitative and quantitative phytochemical compounds in natural plants has become increasingly important. Epidemiological studies indicate that there is an inverse relationship between garlic consumption and the reduction of disease risk, such as cancer and cardiovascular disease (Banerjee et al. 2003). It is possible to explain the results obtained in this study with high amounts of DATS, DADS, other sulfur compounds and fatty acid components such as Alpha-Linolenic Acid (ALA), Gamma Linolenic Acid (GLA), oleic acid. These compounds were found by GC-MS and GC-FID analyzes on volatile and non-volatile oil extracts of *A. tuncelianum*. The literature relating to these compounds is summarized as follows. Diallyl sulfide, allyl methyl disulfide and diallyl trisulfide, which are present in the content of garlic, have been reported to accelerate the detoxification of carcinogenic substances (Singh and Shukla 1998). In a study investigating the mechanism of action of apoptosis; DADS, one of the active components of garlic, has been reported to increase apoptosis in the T24 cell line. DADS treatment resulted in apoptosis by increasing

caspase-3 and caspase-9 activities (Lu et al. 2004). In a study conducted by Menendez et al. (2005) was reported that oleic acid be able to suppress the proliferation of breast cancer cells by increasing intracellular ROS production or caspase-3 activity. In addition, antiproliferative effects of gamma-linolenic acid on BT-474, SK-Br3, MDA-MB-453, MDA-MB-231, SK-OV3 and NCI-N87 were also investigated. Respectively; 35%, 54%, 26%, 21%, 38% and 80% of the inhibition was observed.

Table 7. IC-50 values of *A. tuncelianum* extract prepared with (1/1) ethanol / water (ATEpEW) and Allium tuncelianum extract prepared with (1/1) hexane/chloroform (ATEpHC) in 7 different cell lines
Çizelge 7. (1/1) Etanol/su (ATEpEW) ve (1/1) heksan/kloroform (ATEpHC) çözgenleri ile hazırlanan *Allium tuncelianum* ekstraktlarının, 7 farklı hücre hattında, IC-50 değerleri.

Cell		IC-50 (5-Fu) (µg/mL)	IC-50 (ATEpEW) (µg/mL)	IC-50 (ATEpHC) (µg/mL)
HGC-27	Human Gastric Carcinoma	15.84±0.11	415.11±13.25	335.44±17.19
DLD-1	Human Colon adenocarcinoma	29.64±0.23	335.55±10.65	150.44±4.62
MCF-7	Human Breast adenocarcinoma	27.45±0.18	335.65±8.99	157.55±3.25
ECC-1	Human Cervics Carcinoma	46.38±0.31	290.5458±11.42	145.58±4.16
HELA	Human Cervical Carcinoma	37.67±0.27	285.44±12.32	135.44±5.24
PC-3	Human Prostate Carcinoma	24.14±0.16	347.5558±15.64	121.27±3.55
HEK-293	Human Embryonic Kidney Cell	39.48±0.34	436.1615±13.19	449.41±21.23

CONCLUSIONS

As a result of the evaluation of all findings, *A. Tuncelianum* contains K, Ca, Mg, Na, Fe, Zn, Mn, Se, Cu, and Ni. However, no heavy metals are present. The total phenolic substance is richer than *A. sativum* species. *A. tuncelianum* DATS DADS contains high amounts of DAS and other sulfurous compounds. In addition, oleic, linoleic alpha-linoleic, palmitic acids in terms of other garlic varieties are richer. In addition, *A tuncelianum* contains more catechin than other garlic species. High antiproliferative activity could not be seen in the *A. tuncelianum* extract prepared with (1/1) ethanol/water (ATEpEW). Carbohydrates and proteins abundant in ATEpEW may have contributed to the proliferation of cells. Because the antiproliferative activity results of hexane and chloroform extracts (ATEpHC) are quite good. Therefore, *A. tuncelianum* extracts can be tested using advanced purification techniques, free of constituents such as carbohydrates to increase the proliferation of cancer cells. In addition, in vivo anticancer activity studies in experimental animals can be evaluated.

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Conflict of interest statement

There are no conflicts to declare.

Author's Contributions

The contribution of the authors is equal.

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