



Phenolic Contents, Antioxidant Activities, LCMS Profiles of *Mespilus germanica* Leaf Extract and Effects on mRNA Transcription Levels of Apoptotic, Autophagic, and Necrotic Genes in MCF7 and A549 Cancer Cell Lines

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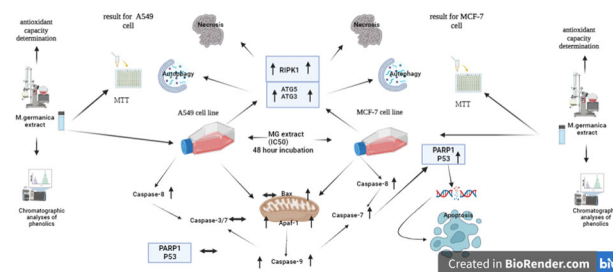
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Abstract

Cancer, defined by the continuous, uncontrollable proliferation of cells in the human body, is a disease with a rapidly increasing incidence and mortality rate. Scientists are looking for novel ways to cure and prevent this sneaky disease because of the toxicity of contemporary chemotherapy and the cancer cells' resilience to anticancer drugs. Determining the effect of herbal medicines, which do not have as harmful side effects as synthetic drugs, on cancer cell lines is an essential preliminary study in the production of effective drugs against cancer. In this study, the phenolic acid profile, antioxidant capacity, and cytotoxicity of the medicinal plant *Mespilus germanica* (MG) leaf extract were determined, and its effects on the expression of some apoptotic, necrotic, and autophagic pathway genes of MCF7 (Human breast cancer line) and A549 (Human lung cancer line) and healthy HDF (Human Dermal Fibroblasts) cells were investigated for the first time. The LCMS device detected many important phenolic compounds previously reported to act against cancer cells in *Mespilus germanica* leaf extract. DPPH and total phenolic content showed high antioxidant capacity. The cytotoxicity of MG was determined by the MTT method. The levels of mRNA transcription for Atg5, Atg3, Ripk1, Bcl2, Bax, Apaf1, Caspase-8, Caspase-7, Caspase-3, and Caspase-9, as well as the expression patterns of the DNA damage markers P53 and Parp-1 genes, were assessed. MG leaf extract did not cause significant toxicity against healthy HDF cells. However, it had a cytotoxic effect on A549 and MCF7 cancer cell lines, increasing the transcription levels of essential genes involved in cell death mechanisms. This research is the first to analyze the phenolic components and antioxidant capabilities of leaf extracts from *Mespilus germanica*. Additionally, it investigates the impact of these extracts on crucial genes involved in cell death pathways of A549 lung cancer, MCF7 breast cancer, and non-cancerous HDF (Human Dermal Fibroblasts) cells.

Graphical Abstract



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Keywords *Mespilus germanica* L.(MG) · Medicinal plant · Phenolic acids · A549 cell line · MCF7 cell line · Cell death genes

Introduction

Cancer is a pathological condition distinguished by unregulated growth and division of cells, invasion into neighboring tissues, and suppression of programmed cell death due to genetic or environmental determinants. According to the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), the global incidence of cancer in 2020 was approximately 19.3 million cases, accounting for one-sixth of all recorded deaths. While lung cancer is the leading cause of cancer-related deaths worldwide, breast cancer is the leading cause of cancer-related deaths in women. So far, no effective treatment method has been developed against either cancer. Because they have fewer side effects, medicinal plants constitute an important research area for cancer treatment today [1].

The efficient management of cancer has not yet been accomplished despite significant advancements in understanding disease biology and the development of traditional therapies [2]. The cost of anticancer treatments is relatively high, and many of these drugs have harmful side effects, especially on healthy cells that proliferate rapidly. The most current treatments for the disease are chemotherapy, radiotherapy, and chemical drugs. However, the fact that chemotherapy causes many side effects in patients and causes more harm to their health directs scientists to alternative treatments and plant-based therapies against cancer [3]. Determining the impact of herbal drugs on cancer cell lines is considered the most effective preliminary study against cancer worldwide. Over 3,000 plant species have been researched for their possible anticancer properties to minimize these unfavorable consequences [4].

Phenolics are a class of plant secondary metabolites with remarkable antioxidant properties due to their redox properties. These compounds can effectively adsorb and counteract the harmful effects of free radicals, neutralize reactive oxygen species, and chelate iron and copper cations [5]. They have also been observed to exert a significant inhibitory effect on all stages of tumor growth and metastasis [6].

Apoptosis is a regulated cellular mechanism of self-destruction that serves a crucial function in preserving tissue equilibrium by eliminating surplus or aberrant cells. Atg5, Atg3, Ripk1, Bcl2, Bax, Apaf1, Caspase-8, Caspase-9, Caspase-3, Caspase-7, Tp53, and Parp-1 play essential roles in both apoptosis, necrosis, autophagy and DNA damage mechanisms. Manipulating these pathways with more harmless phytochemicals than chemotherapeutics is a crucial strategy for cancer treatment. Therefore, it is highly desirable to develop novel medications that can be

implemented as alternative approaches to cancer treatment. Plants are highly valued in this context due to their significant potential as sources of chemicals with diverse medicinal applications. Most anticancer medications are currently derived from botanical sources [7].

Mespilus germanica L. (Medlar), a member of the Amelanchier-Crataegus sister group [8], is a shrub plant of the Rosaceae family [9] rich in phytochemicals with potent antioxidant activities [10, 11]. Its fruits and leaves are also used in the treatment of enteritis [12], constipation, diuretics, kidney and bladder stones [13], wounds, mouth abscesses, diabetes, and microbial infections [14]. In general, leaf extracts of MG have been reported to show higher antioxidant activity than fruit extracts [15].

Although studies examine the effects of MG extract on different cancer cell lines [16], no study investigates its cytotoxic effects on A549 lung cancer, MCF7 breast cancer, and healthy fibroblast cells. Therefore, this study is the first to both detect the phenolic acids and antioxidant capacity of *Mespilus germanica* leaf extract and examine its effects on the transcription levels of cell death mechanisms' key genes in A549, MCF7, and non-cancerous (healthy) HDF cells (Human Dermal Fibroblasts, adult).

Methods

Chemicals

Chemicals and phenolics standards used in this study are analytical and/or HPLC grade and purchased from Sigma-Aldrich (Steinheim, Germany).

Plant Material

The leaves of *Mespilus germanica* L. were collected in July from the province of Van in Turkey (latitude: N38°34'33" and longitude: E43°16'10 ") in 2021. Specialist Prof. Dr. Fevzi Özgökçe identified the *Mespilus germanica* plant. The voucher specimen is stored in VANF Herbarium with a 165247 voucher number and an F15537 collector number. The leaves were dried in the dark at room temperature (22 ± 2 °C), ground with a grinding mill (IKA, A 11 primary Analytical mill), and stored at -20 °C until the analyses.

Extraction Procedure

Phenolic compounds were extracted using the previous method [17] with some modifications. The ground leaves

were extracted separately with a ratio of 1/20 (w:v) acetone, methanol, ethanol, and water. 5 g dry herb +100 ml 80% acidic acetone (80 ml acetone +19.9% purified water +0.1% acetic acid) was added. It was kept in an ultrasonic water bath (IsoLab-Laborgerate GmbH) for 1 h at 45 °C. Then, it was centrifuged (Hitachi-High speed refrigerated centrifuge-CR22N) at 15.320 g (10,000 rpm) for 10 min at +4 °C. The supernatant (upper liquid) was filtered with a 0.45 µm syringe tip filter and taken into an amber Eppendorf tube to store at +4 °C. After filtration, 80% acidic methanol (80 ml methanol +19.9% purified water +0.1% acetic acid) was added to the remaining pellet, and the same procedures were repeated. The liquid portion was taken, and the process was repeated by adding acidic ethanol (80 ml ethanol +19.9% purified water +0.1% acetic acid) to the remaining pellet. The previous processes were repeated by adding 100 ml of pure water to the remaining pellet for the last time. After centrifugation, it was filtered. The liquid obtained after filtration was combined with the supernatants obtained with acetone, methanol, and ethanol solvents. The resulting supernatant was evaporated (Heildolph) at 45 °C, 100 rpm for 45 min. Ultrapure Milli-Q water (18.2 MΩ cm resistivity) (from Milli-Q synthesis-Millipore) was added to the extract obtained after evaporation to come out of the flask. The residue was wholly removed from the flask and lyophilized (LyoQuerst-Telstar) at −80 °C at 0.05 psi pressure for 48 h to remove the contained water. The resulting extract was then stored at −20 °C for further use.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) Analyses for Antioxidant Capacity

Using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging method (Sigma-Aldrich, Germany), the antioxidant activity of the extracts was determined [18]. Methanol was utilized as a solubilizing medium. 10 ml of solvent was added to 8 mg of dry extract and vortexed. Then, 3.9 ml of DPPH solution was added to 0.1 ml of extract according to the DPPH procedure. After incubation for 1 h in the dark, the absorbance of the samples was measured at 515 nm by the Multiskan® SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA). DPPH % inhibition value and IC₅₀ of the extract were determined while α tocopherol was used as a control.

Determination of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method [19]. The samples were measured at 760 nm by the Multiskan® SkyHigh microplate spectrophotometer (Thermo Fisher Scientific, USA), and the results were reported as Gallic acid equivalent (GAE) in milligrams per 100 grams of dry weight (Dw).

Liquid Chromatography Mass Spectrometry (Lcms) Analysis for Phenolic Acid Detection in *Mespilus germanica* Leaf Extract

LCMS was performed using a high-resolution MS composite of the Dionex UltiMate 3000 RS pump, the Dionex UltiMate 3000 RS autosampler, and the LC system with the Dionex UltiMate 3000 RS column furnace and Exactive Plus Orbitrap (Thermo Fisher Scientific) with a heated electrospray ionization interface. The Orbitrap-MS equipment was calibrated using positive (Pierce™ L_q Velos Esi Positive Ion Calibration Solution) and negative (Pierce™ Negative Ion Calibration Solution) calibration solutions. This calibration process was carried out utilizing an automatic syringe injector manufactured by Thermo Fisher Scientific. The LC-HRMS analyses involved operating the LC and MS components concurrently using the TraceFinder 3.2 software (Thermo Scientific) installed on the computer system. The data acquisition and recording were performed using the Xcalibur software version 2.1.0.1140 (Thermo Fisher Scientific).

The analyses were conducted with a Phenomenex® Gemini® 3 µm Nx-C18 110 Å (100 mm × 2 mm) column. The temperature of the column furnace was set to 30 °C. A solution containing 2% (v/v) glacial acetic acid was created using ultrapure water from the Ultrapure water system (Gfl 2004/ Human power 1). This solution was utilized as the mobile A phase in an elution gradient. The mobile B phase comprised 99.9% pure methanol purchased from Sigma and was employed in LC-MS analysis.

The sample was injected with a volume of 20.0 µL, and the elution process followed a gradient profile at a flow rate of 0.3 mL/min. The allocated time for analysis was established as a cumulative duration of 20 min.

Equipped with a heated electrospray ionization interface, the Orbitrap LCMS operated in positive (Full Ms/Aif) and negative (Full Ms/Aif) modes. Ionization interface sheath gas flow rate 35; auxiliary gas flow rate 7; spray voltage 3.5 kV; capillary temperature 350 °C; additional gas temperature 350 °C; S-lens RF level was set to 50. MS scan range 60–800 m/z; resolution 17,500; Acg target 3.106; maximum IT 2 ms; CE (collision energy)/ step CE was carried out under 25 V conditions.

All standards were prepared as 10ppb-20ppb-40ppb-60ppb-80ppb-100ppb-200ppb-300ppb-400ppb-500 ppb concentrations for the phytochemical compounds of the LC-Orbitrap HRMS analysis method, and each was injected in triplicate.

Ten milligrams of the sample were measured and dissolved in a 10-milliliter solution consisting of a 50% mixture of methanol and pure water. The resulting solution was then introduced into a vial with a volume of 1.5 milliliters using a polytetrafluoroethylene syringe filter with a pore size of 0.22 micrometers.

Cell Culture

The study materials comprised breast cancer MCF-7 cell line (Atcc® Crl-3435™) and lung cancer A549 cell line (Atcc® Ccl-185™) and, non-cancerous (healthy) HDF cells (Human Dermal Fibroblasts, Atcc® Pcs-201-012) were purchased from the American Type Culture Collection. The cells were cultured in vitro using regular passages in Dulbecco's modified Eagle medium (DMEM high glucose, Cat-No: 11965092) supplemented with 10% fetal bovine serum (Biological Industries, Certified FBS), 1% penicillin/streptomycin (Capricorn, Cat-No: PSG-B), 1% L-glutamine (Capricorn, Cat-No: GLN-B). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ using an Esco CelCulture CO₂ incubator (Changi, Singapore). All the other chemicals and reagents used were of high-purity grade and were obtained from commercial sources.

Preparation of Plant Extract Solutions Applied to Cell Culture

M. germanica plant extract was dissolved in the stock solution DMSO (Dimethyl sulfoxide). Final concentrations (100, 200, 400, 600, 800, and 1000 µg/ml) were prepared by dilution with the medium. In this dilution, the DMSO ratio was ready to have a nontoxic (≤ 0.005) effect.

2.9. Cytotoxicity (MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) Cell Viability) Test

The in vitro cytotoxicity tests were done using the method explained by Meerle et al. [20]. This method involves checking the oxidoreductase activity to see how quickly the tetrazolium salts change form. The MTT cell viability assay was used to find the IC₅₀ values for the *Mespilus germanica* (MG) plant extract after 48 h. The A549 and MCF7 cell lines were incubated with MG extract for 48 h at final concentrations of 0 (control), 100, 200, 400, 600, 800, and 1000 µg/ml. Subsequently, an MTT assay was conducted to determine the concentration of MG extract that resulted in the highest level of cell growth and cell death. The study was carried out in 3 groups in each cell line: control group (control), IC₅₀ group (IC₅₀), and Proliferative Group (Pro) group. Cell lines without extract application were considered the control group.

10⁴ (10000) cells/mL were seeded into each well of a 96-well culture plate, and the cells were supplied with suitable growth conditions. The cells were incubated in a 5% CO₂ incubator at 37 °C for 24 h. After removing the medium from the cells, a new medium containing MG extract at varying concentrations was introduced. Four wells were used for every concentration of the MG extract. Following

the completion of the 48-h incubation periods, the medium was removed from the cells. The MTT solution was produced in phosphate-buffered saline (Pbs P4417; Sigma Aldrich) with a 5 mg/mL concentration. The cell media mixture, which consisted of 10% MTT (M5655; Sigma Aldrich), was applied to each well. The plate was incubated at 37 °C for 3 h to transform the MTT dye into water-insoluble formazan crystals. MTT, a tetrazolium salt, transforms into formazan when reduced and gains electrons, causing it to change color. It turns out that the formazan is purple, while the MTT complex is yellow. The quantity of viable cells is directly proportional to the amount of formazan produced. Active mitochondria are the only ones that can break the tetrazolium ring, which means that the color change reaction can only happen in live cells. Dimethyl sulfoxide was used as a solvent to dissolve the purple formazan product, which was not soluble. We used an enzyme-linked immunosorbent assay reader (Biochrom, Anthos Zenyth 200) with a wavelength of 570 nm to measure the optical density values of the cells [20]. The Microsoft Office Excel program was used to figure out the data of the MTT assay. When calculating the cell viability rate, the control group's viability was 100%. Each dose's impact on cell viability was evaluated using the formula [21]:

$$\text{cell viability}(\%) = (\text{sample absorbance}/\text{control absorbance}) \times 100.$$

Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

The real-time qPCR method was used to evaluate the messenger RNA (mRNA) expression levels of some critical genes that encode proteins involved in apoptosis, autophagy, necrosis, and DNA repair. After the treatment, the cells were removed from the surface of the flask using the trypsinization technique. Centrifugation was used to separate the collected cells from the supernatant. The cells were centrifuged a second time following a wash in sterile phosphate-buffered saline (Pbs). Using the Trizol (Life Technologies) reagent, the RNA of these cells was manually extracted [22].

The concentration and purity of RNA were measured using a nanodrop spectrophotometer (BioDrop; Biochrom). The acquired images revealed the presence of 28S, 18S, and 5S RNA. To obtain complementary DNA (cDNA) from RNA, a high-capacity cDNA reverse transcription isolation kit (Applied Biosystems™ Cat: 4368814, Lithuania) was utilized. After cDNAs were produced, PCR was carried out in the RT-qPCR device (Rotor-Gene Q; Qiagen) using Sybr Green master mix (Enzo Life Science cat: Enz-Nuc104-0200). The PCR program followed the kit procedure's instructions (Table 1). Each sample was evaluated in triplicate.

Table 1 Reaction program for RT-qPCR

Reaction content	For a sample	Reaction cycle
Buffer (2X)	10 μ l	95°C 2' denaturation
Primers	Forward : 0,5 μ l	40 cycles
	Reverse : 0,5 μ l	
dH ₂ O	8,4 μ l	*Binding temperature varied according to primers.
cDNA	0,6 μ l	Melting Curve
Total	20 μ l	Ramp: 50-99 (1 degree increment) 90°C 5 seconds

cDNA complementary DNA, RT-qPCR reverse transcription-quantitative polymerase chain reaction

The control gene utilized in this study was Actin Beta (Actb). The cycle at which the fluorescence levels of the gene products surpassed the cycle threshold (CT) value was identified. The calculation of gene expression levels was performed using the $2^{-\Delta\Delta CT}$ method [23]. The expression levels of the target genes were compared using the fold change number and subjected to statistical analysis. The levels of mRNA expression were checked for the Ripk1 gene, which is linked to necrotic cell death; the Atg3 and Atg5 genes, which are linked to autophagic cell death; the Bcl2, Bax, Apaf1, Caspase-8, Caspase-9, Caspase-3, and Caspase-7 genes, which are linked to apoptotic cell death, and the P53 and Parp1 genes, which repair DNA damage. The nucleotide sequences of the primer bases for the target genes can be found in Table 2.

Statistical Analysis

The statistical analyses were conducted using the Spss version 22.0 software package. The Kruskal–Wallis test was employed to achieve a variance analysis across multiple groups. The descriptive statistics were analyzed using a one-way Anova and a post-hoc Bonferroni test. The parameters are reported in the form of mean \pm standard deviation. The “ $2^{-\Delta\Delta CT}$ ” of delta C_T formulation was used to calculate the temporal variations between expressions to determine their difference. Statistical significance was determined for all intergroup analyses at a threshold of $p < 0.05$.

Results

LCMS and Total Phenolic Content Results

The chromatogram of standart used in LCMS device is given in Fig. 1. Possible phenolic compounds, retention time, m/z charge determined by LCMS are given in Table 3. The phenolic components in the MG extract were identified by comparing the peak areas with the curve of the relevant calibration standard [24].

The quantification of total phenolic content was conducted using the Folin-Ciocalteu method. The outcomes regarding gallic acid equivalent (mg GAE/kg dw) were quantified. The total phenolic content was measured as 420.31 ± 6.71 mg GAE/ml. The DPPH assay was carried out on the extract using a spectrophotometric technique [18] and equation :

$$\%RSA(\text{Radical scavenging activity}) = [(A_0 - A_1)/A_0] \times 100$$

A₀: Absorbance of DPPH and dH₂O mixture

A₁: Absorbance of the sample with DPPH

Table 2 Sequences of primers used for gene expression analysis with RT-qPCR

Name of the gene	Sequencing of the primary sequence	
	F: 5'-3'	R: 5'-3'
Actin Beta (Actb)	ACTCTTCCAGCCTTCCTTC	ATCTCCTTCTGCATCCTGTC
Atg3	GAGATCACCTAGTCCACCAC	GCTTCCGTTATTCCTGTAATACC
Atg5	GAGACAAGAAGACATTAGTGAG	GATATTCCATGAGTTTCCGA
Ripk1	TGAGCTTCCGCTAGACA	CTGAAGCTCAACGCCCA
Caspase-8	GATGTTATTCCAGAGACTCCAG	GGTAGGTAATCAGCAAATCCA
Bcl2	GTGGTGGAGGAACTTTCAG	GTTCCACAAAGGCATCCCAG
Caspase-3	ATGGAAGCGAATCAATGGAC	AAACATCACGCATCAATTCC
Caspase-9	GGCTCTTCCTTTGTTTCATCTCC	TCACCAAATCCTCCAGAACCA
Bax	AGCAAAGTGGTGTCAAGGC	CCACAAAGATGGTCACTGTC
Kaspaz-7	GGCTTGTATTGAAGAGCAGGG	CTGATCTTGTATCGAGGATTAGCA
Apaf-1	CCCTTTGTGTCCAGTAGTGGG	CTCTGTCTCGCCACATACCC
Caspase-3	CCATGCAGGTGAGCTTCGT	GAATCTGCGAGAGACACCATC
Parp-1	CACCAAAAAGGAGGTGGAAA	CAACTCCTGAAGGCTCTTGG
P53	CCCAGGTCCAGATGAAGCTC	CATGTAGTTGTAGTGGATGGTGGT

RT-qPCR reverse transcription-quantitative polymerase chain reaction

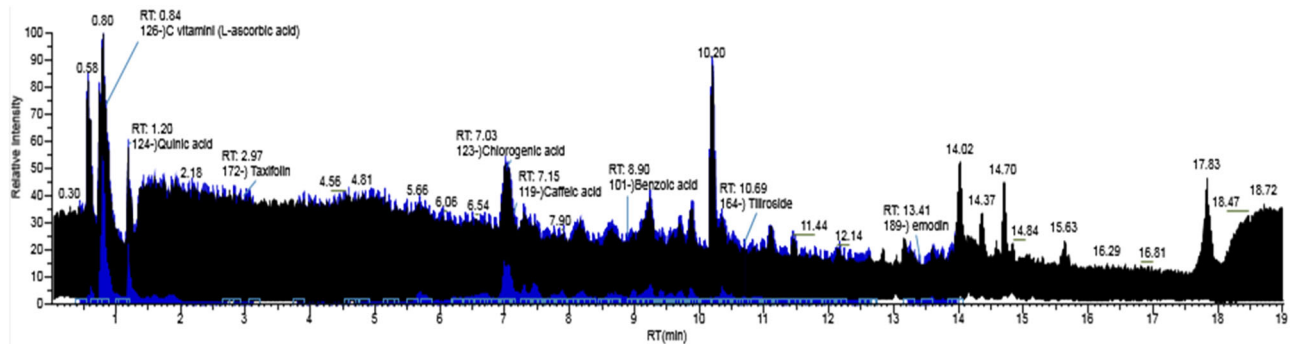


Fig. 1 Chromatogram of standarts detected by LCMS

The extract exhibited a DPPH % inhibition value of 55 ± 9.45 . The IC₅₀ value of the extract was determined to be 0.73 ± 0.14 , while the IC₅₀ value of α -tocopherol was found to be 0.54 ± 0.03 . The DPPH value exhibited a more remarkable similarity to the antioxidant capacity of α -tocopherol, which was utilized as the positive control in our study.

MTT Results

MTT was performed at various times and concentrations to understand the cytotoxicity mechanism better. The study compared the cell viability rates with the control group. The control group's viability rate was determined to be 100%. Every sample was tested three times. The parameter values are expressed as the mean \pm the standard deviation.

After 48 h of administration of various concentrations of *Mespilus germanica* leaf extracts on cell lines, the proliferative concentration (100 μ g/ml) was determined. MTT

% viability results for MCF7 (Fig. 2) and A549 (Fig. 3) are given, assuming the control group is 100% viable. IC₅₀ was determined as 770 μ g/ml in the MCF7 cell line (Fig. 2) and 800 μ g/ml in A549 cells (Fig. 3).

Real-time-qPCR Results

The levels of gene expression were compared to those of the control group. The data are presented as three repetitions' average values (\pm standard deviation). There are notable differences compared to the control group, which are statistically significant ($p < 0.05$). The following figures (Figs. 4–6) display the identified gene expression levels.

The levels of mRNA expression were checked for the Ripk1 gene, which is linked to necrotic cell death; the Atg3 and Atg5 genes, which are linked to autophagic cell death; the Bcl2, Bax, Apaf1, Caspase-8, Caspase-9, Caspase-3, and Caspase-7 genes, which are linked to apoptotic cell death, and the P53 and Parp1 genes, which repair DNA damage.

Table 3 Lc-Ms/Ms parameters of selected compounds and amount of antioxidants in MG leaf extract in ug/g concentration

Phenolic compounds	Retention Time (min)	Quantification Peak (m/z)	Amount of phenolic compounds inmean \pm std ($\mu\text{g/g}$)
3-(4-Hydroxyphenyl) propionic acid	7.6	165.05	53.59 \pm 0.006
4-Hydroxybenzoic acid	6.13	137.02	11.41 \pm 0.005
3,4-dihydroxybenzaldehyde (Protocatechuic aldehyde)	5.56	137.02	6.070 \pm 0.021
3,4Dihydroxyphenylaceticacid(Dopac, Homoprotocatechuic acid)	5.08	167.03	700.5 \pm 0.007
Afzelin (Kaempferol 3-rhamnoside)	10.26	431.09	32.96 \pm 0.008
Apigenin 7-glucuronide	10	445.07	27.44 \pm 0.110
Benzoic acid	8.9	121.02	2.440 \pm 0.005
C vitamin (L-ascorbic acid)	0.84	175.02	773.3 \pm 0.007
Caffeic acid	7.15	179.03	12.39 \pm 0.009
Caffeic acid phenyl ester (Cape)	11.95	283.09	1.190 \pm 0.006
Catechin (Cianidanol)-p	6.38	289.07	8.090 \pm 0.005
Eriodictyol (3,4,5,7-Tetrahydroxyflavanone)	9.29	287.05	37.52 \pm 0.013
Esculin hydrate	6.2	339.07	110.5 \pm 0.163
Gallic acid(3,4,5-trihydroxybenzoic acid)	0.69	169.01	6.190 \pm 0.008
Genistein (5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one)	10.74	269.04	1015 \pm 0.018
Kaempferitrin	9.61	579.17	475.2 \pm 0.012
Kaempferol	10.93	287.05	56.92 \pm 0.070
Liquiritigenin	9.76	255.06	765.1 \pm 0.020
Luteolin	10.73	285.04	1.420 \pm 0.006
Naringenin	10.48	271.06	817.9 \pm 0.008
Phloridzin	9.33	435.12	995.0 \pm 0.009
Protocatechuic acid (3,4-Dihydroxybenzoic acid)	4.19	153.01	9.590 \pm 0.009
Quercetin	10.45	301.03	5.360 \pm 0.007
Rhoifolin (Apigenin 7-O- neohesperidoside)	9.6	577.15	462.3 \pm 0.012
Rutin hydrate M-OH2	9.19	609.14	44.53 \pm 0.006
Rutin trihydrate M-3H2O	13.86	253.05	25.87 \pm 0.011
Schaftoside	8.31	563.14	50.35 \pm 0.008
Tilioside	10.69	593.13	301.9 \pm 0.010
Vanillic acid	7.32	167.03	22.75 \pm 0.011
Vicenin 2	7.82	593.15	13.95 \pm 0.007

After the administration of the IC₅₀ dose, a significant increase ($p < 0.05$) was determined in the transcription levels of autophagic genes Atg3, Atg5, and necrotic gene Ripk1 in both cell lines compared to the control group (Fig. 4). Data are presented as mean \pm standard deviation (SD). MG extract increased Atg3 transcription by 38.33fold, Atg5 transcription by 3.84fold, and Ripk1 transcription by 20fold in MCF7 cell lines compared to the control. In the A549 cell line, the transcription level of the Atg3 gene increased twofold, the Atg5 gene by 3.6fold, and the Ripk1 gene by 6.58fold compared to the control group. MG extract increased Atg3 transcription by 0.16fold, Atg5 gene 0.2fold and Ripk1 0.4fold compared to the control and in healthy

HDF cells (Fig. 4). While MG extract directed cancer cells to autophagy and necrosis, it did not affect healthy cells.

Figure 5 shows the effect of MG extract on the mRNA expression of DNA repair genes in the A549 cell line and MCF7 cell lines and in healthy HDF cells after 48 h. No significant increase was observed in the mRNA transcription levels of Parp1 and P53 genes in the A549 cell line compared to the control group ($p < 0.05$). At the same time there was a significant increase ($p < 0.05$) in the mRNA transcription levels of Parp1 and P53 genes in the MCF7 cell lines compared to the control group. An approximately 26fold increase in the transcription level of p53 protein and a 15fold increase in the level of Parp genes in MCF 7 cell

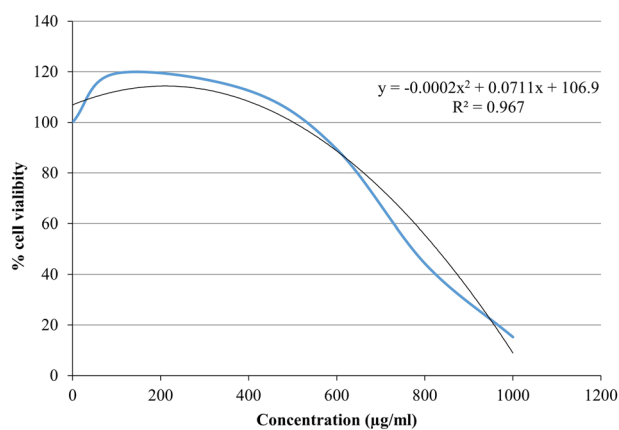


Fig. 2 Exel Slope graph of MCF7 cells obtained as a result of MTT viability test after different concentrations of extracts

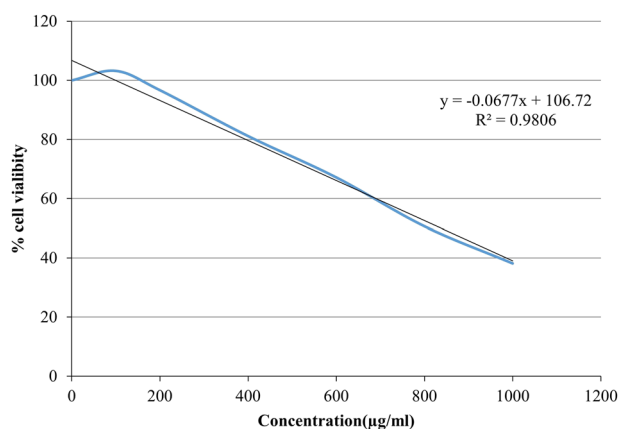


Fig. 3 Exel Slope graph obtained as a result of MTT viability test of A549 cells after different concentrations of extracts

lines were observed. But this in healthy HDF cells, MG extract increased the Parp gene by 3.80 times and the P53 gene by 1.6 times compared to the control (Fig. 5).

After 48 h of administering the extract, a statistically significant increase ($p < 0.05$) was observed in the mRNA transcription levels of Bcl2, Bax, Apaf-1, Caspase-8, and Caspase-9 genes in the MCF7 cell line, as indicated in Fig. 6. In comparison to untreated control cells, a significant increase in the expression levels of Caspase-8 (15-fold in A549 cells and 11-fold in MCF7 cells), and Caspase-9 (7-fold in A549 cells and 20-fold in MCF7 cells) (Fig. 6), and Bax (6-fold in A549 cells and 17-fold in MCF7 cells), Apaf-1 (5-fold in A549 cells and 10-fold in MCF7 cells), Caspase-7 (14-fold in MCF7 cells) (Fig. 7) was observed. However, only the mRNA levels of Apaf-1, Caspase-8, and Caspase-9 genes significantly increased ($p < 0.05$) compared to the control group in the A549 cell lines. After the application of MG extract, the Bcl2 gene increased 2.32 times in healthy HDF cells compared to the control, while Caspase9 increased at very low rates, such as 0.16 times,

Caspase3 0.06 times, and Caspase8, 1.17 times (Fig. 6). After the application of MG extract, the expression levels of Bax gene increased only 0.75 times, Caspase 7 only 0.03 times and Apaf1 only 0.13 times compared to the control in HDF cells (Fig. 7).

Discussion

This is the first study in which phenolic substances and antioxidant capacity of *Mespilus germanica* leaf extracts were determined, and their effects on essential genes involved in cell death pathways were investigated in A549 and MCF7 cancerous cell lines and in HDF (Human dermal fibroblasts:Pcs-201-012, ATTC) healthy cell line.

In this study, the total phenolic content of MG leaves was measured as 420.31 ± 6.71 mg GAE/ml, and the DPPH value was determined as $55 \pm 9.45\%$. Previous studies determined the total phenolic content in medlar fruits between 93 and 170 mg GAE/ml in five ripening periods [10]. Consistent with our research, it has been reported that leaves contain higher levels of phenolic substances and antioxidant capacity than fruits [25]. In previous studies it was reported that the leaf extract of *Mespilus germanica* naturally contains antioxidants, phenolic acids, flavonoids, fatty acids, and tannins [26, 27]. These rich phytochemicals indicate that *Mespilus germanica* leaves have antioxidant capabilities, which align with our discovery of a DPPH value of $55 \pm 9.45\%$ and a total phenolic content of 420.31 ± 6.71 mg GAE/ml.

Characterizing ingredients from plant extracts has always been extremely important for alternative medicinal uses. In this study, it has been suggested that a significant number of phenolic compounds, such as genistein, caffeic acid, 3,4-hydroxycinnamic acid, luteolin, protocatechuic acid, quercetin, vitamin C, kaempferol, and naringenin, were identified by LCMS in MG extract. These compounds have been reported to play an essential role in fighting cancer by affecting signaling pathways and triggering cell death. Genistein has documented anti-tumor effects by affecting signaling pathways like Ptk, Erk1/2, NF κ B, PI3K/Akt, Mapk, and Bax [28]. Thiliroside displays cancer properties against leukemia, lung, and breast cancer cells [29–31]. Caffeic acid plays a role in encouraging cell death and reducing cell growth through caspase activation [32]. Hydroxycinnamic acid derivates, which includes compounds like ferrulic acid shield against DNA damage and boost proteins linked to cell death in thyroid cancer cells [33, 34]. Quercetin, luteolin, protocatechuic acid, and naringenin are reported to induce apoptosis and inhibit metastasis in several cancers by modulating apoptosis-related pathways and targeting key oncogenes [35–43]. Similarly, kaempferol is potentially therapeutic against

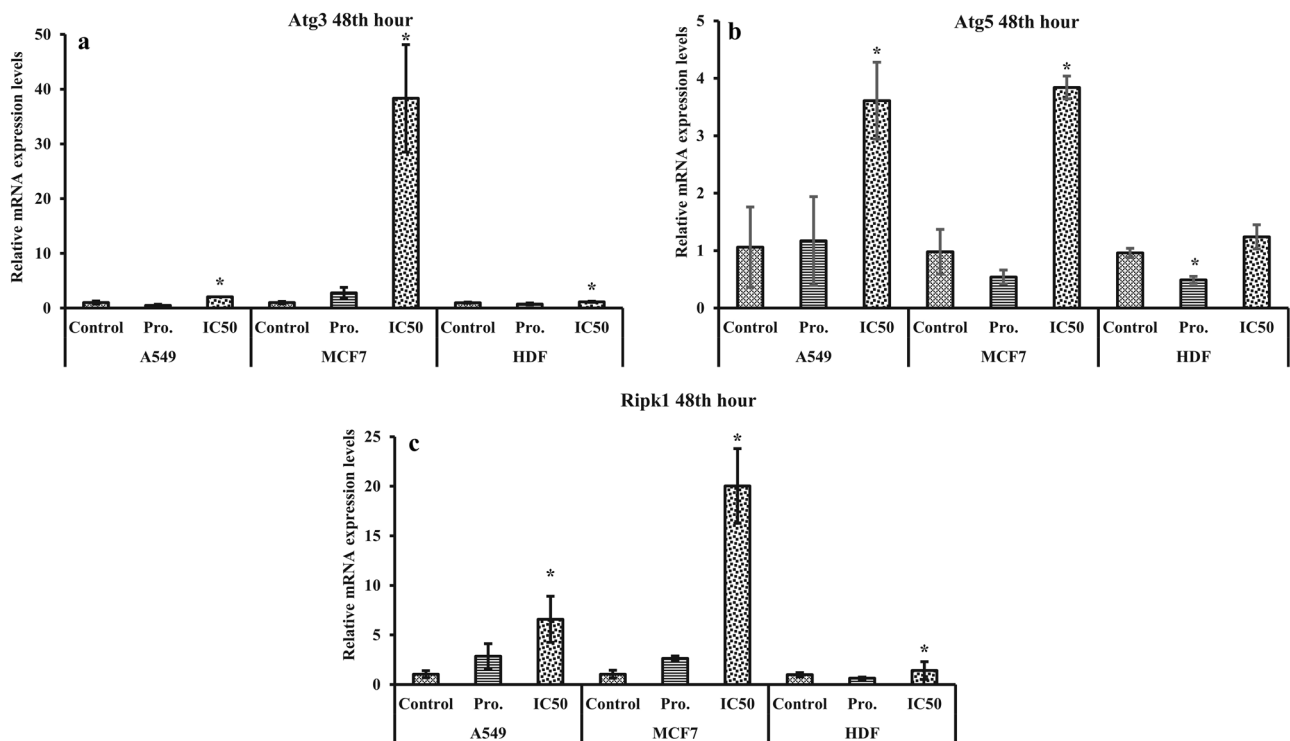


Fig. 4 The mRNA expression level variations in important autophagic genes (a- Atg3, b- Atg5) and necrotic gene (c- Ripk1) at 48 h, in A549, MCF7 and HDF cells. Standard deviations are shown by error bars.

Significant differences between control and treatment samples are indicated with an “*” ($p < 0.05$)

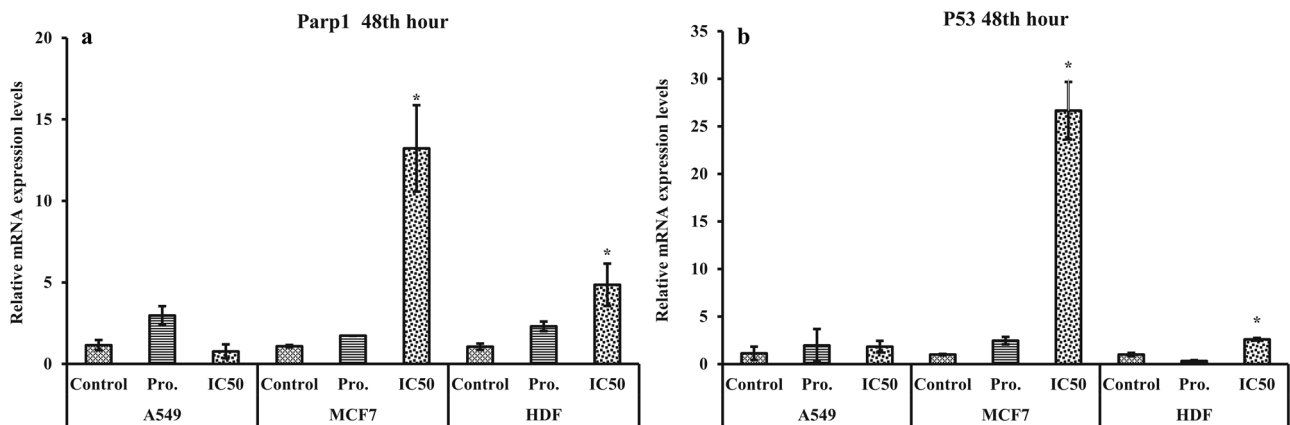


Fig. 5 The mRNA expression level variations in important DNA repair genes (a- Parp1, b- P53) at 48 h, in A549, MCF7 and HDF cells. Standard deviations are shown by error bars. Significant differences between control and treatment samples are indicated with an “*” ($p < 0.05$)

hormonally based, such as breast, ovarian, cervical, and ovarian cancers [44, 45]. We think that MG extract, which contains many phenolic substances with proven anti-cancer effects in previous studies, changes the levels of apoptotic, necrotic, and autophagic key genes by showing a synergistic cytotoxic effect on A549 and MCF7 cancer cell lines.

In this study, the dose-response relationship of the plant extract on cancer cell lines was examined; the findings showed that the 100 µg/ml dose had a proliferative effect, promoting the proliferation of cancer cells. The difference

between the proliferative dose and IC50 suggests that the plant extract may support cancer cells’ growth at low concentrations but may exhibit toxic effects at higher concentrations, killing cancer cells. This information provides an in-depth understanding of the extract’s proliferative and antiproliferative properties and potentially indicates its uses as a bidirectional therapeutic agent. Such findings are thought to effectively define the dose range by improving dosage recommendations for future drug researches. In cell lines MCF 7 and A549, the IC50 values were 770 µg/ml and

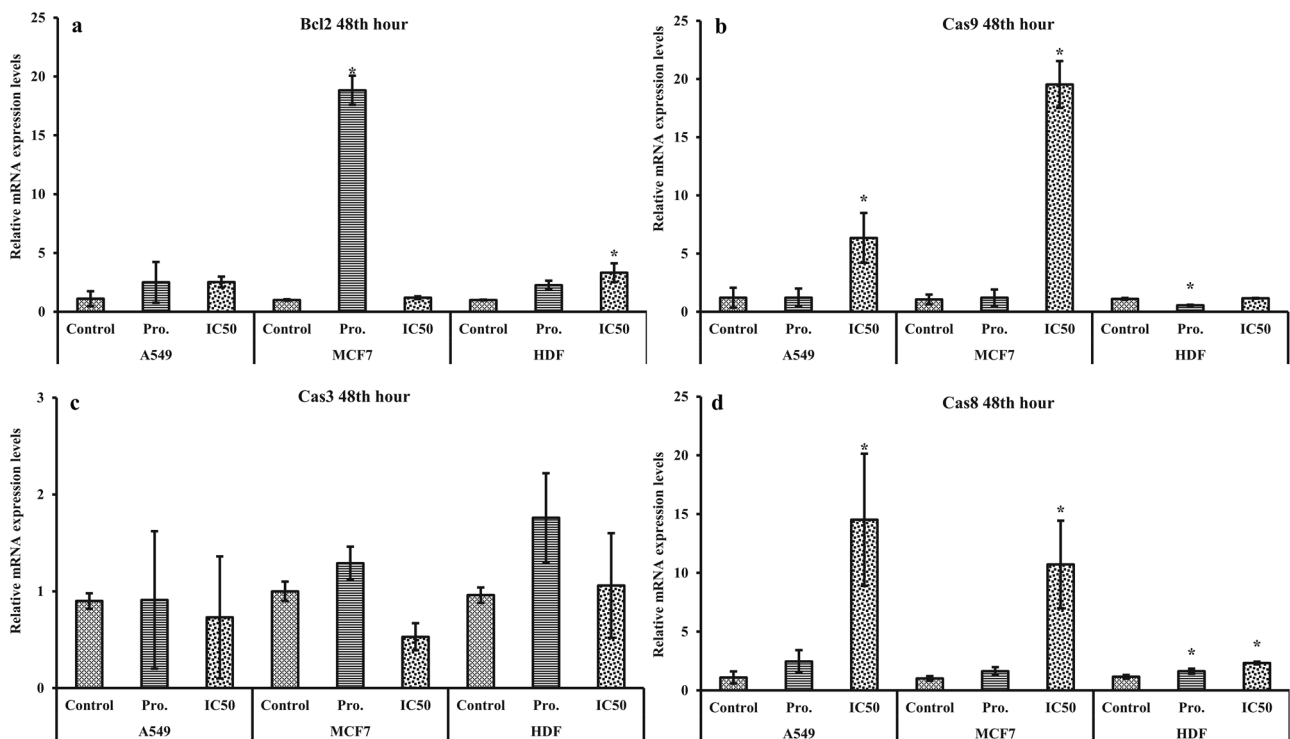


Fig. 6 The mRNA expression level variations in important apoptotic genes (a- Bcl2, b- Caspase 9, c- Caspase 3, d-Caspase 8) at 48 h, in A549, MCF7 and HDF cells. Standard deviations are shown by error

bars. Significant differences between control and treatment samples are indicated with an “*” ($p < 0.05$)

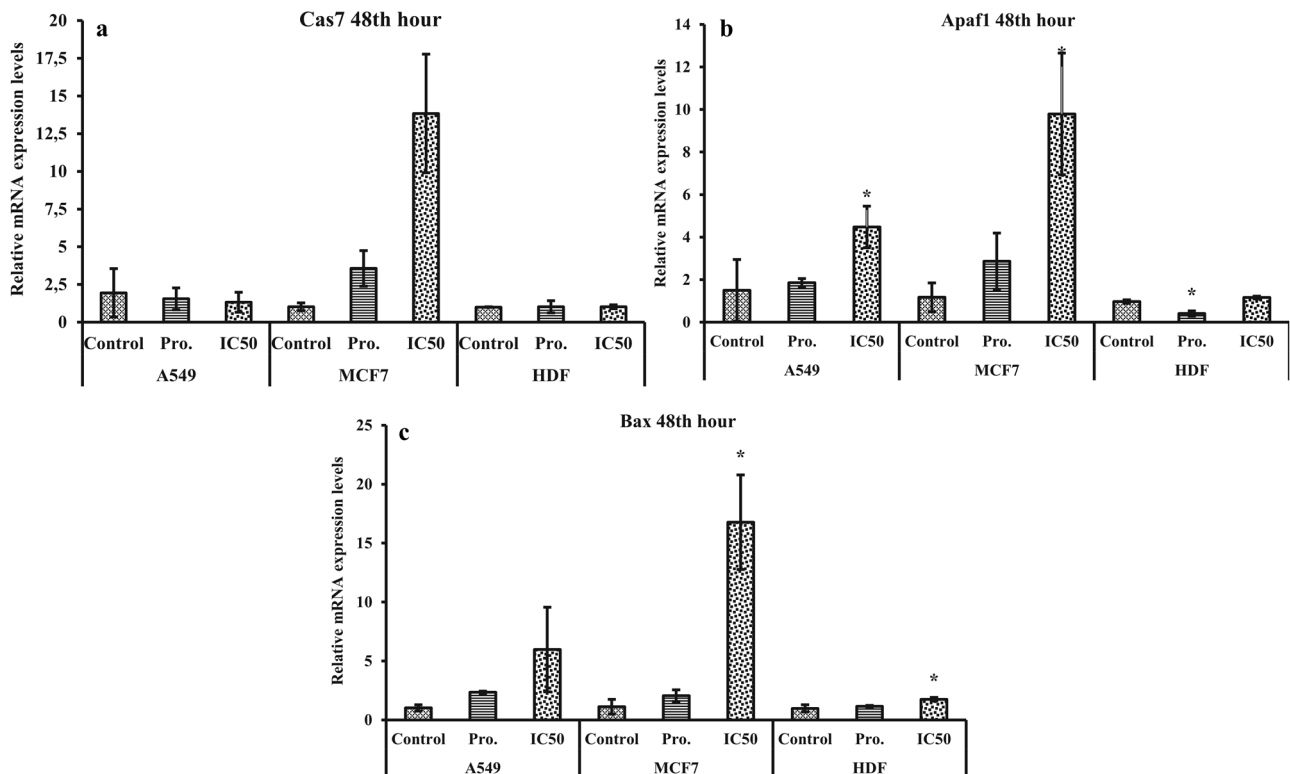


Fig. 7 The mRNA expression level variations in important apoptotic genes (a- Caspase 7, b- Apaf1, c- Bax) at 48 h, in A549, MCF7 and HDF cells. Standard deviations are shown by error

bars. Significant differences between control and treatment samples are indicated with an “*” ($p < 0.05$)

800 µg/ml, respectively. Consistent with our study, Stan-kovic et al. [16] reported that *Mespilus germanica* (*Rosa-ceae*) extract had a cytotoxic effect against HeLa cancer cell lines with an IC₅₀ value of approximately 625 µg/mL and *Crataegus monogyna* Jacq. (*Rosaceae*) extract had a cyto-toxic effect with an IC₅₀ value of 652 µg/mL [16]. As seen in the study conducted with *Rosaceae* family plants [16], the IC₅₀ value we determined in our research depends on the phytochemical and genetic structure of *Mespilus ger-manica*. Likewise, we think that the high IC₅₀ value depends on the genetic structure of the A549 and MCF7 cell lines and their resistance to the plant extract.

In addition, the IC₅₀ dose of MG extract effective against A549 and MCF7 cancer cells did not significantly affect apoptotic, necrotic, and autophagic gene regulation in healthy fibroblast cells compared to the control. This significant result suggests that MG extract is an active ingredi-ent that can potentially be a chemotherapy drug, killing cancer cells without harming healthy cells. Further in vivo and in vitro studies of MG extract in different cancers are planned.

MG extract significantly showed cytotoxic effects and affected the transcription levels of apoptotic, necrotic, autophagic, and DNA repair genes in A549 and MCF7 cell lines. Atg3 and Atg5 [46] are essential genes that play crucial roles in autophagy and Ripk1 in necrosis [47]. Ripk1 transcription level has decreased in cancerous tissues [48]. MG extract increased Atg3 in healthy fibroblast cells by 0.16 times, Atg5 by 0.24 times, and Ripk1 by 0.41 times compared to the control. These findings indicate that MG extract can induce necrosis and autophagy by increasing the transcription levels of Atg3, Atg5, and Ripk1 genes in MCF7 and A549 cell lines but does not damage healthy fibroblast cells. Although all three genes increased sig-nificantly in healthy HDF cells compared to the control, this increase is relatively insignificant compared to the increase in A549 and MCF7 cells. While MG extract directed cancer cells to autophagy and necrosis, it did not affect healthy cells. Consistent with our study, Nelson et al. [49] reported that *Eclipta alba* extract exhibited major cytotoxicity on colon cancer cells while demonstrating much lower toxicity towards normal lung fibroblast cells [49]. This indicates that the plant extract explicitly hinders the growth of cancer cells while having minimal harmful effects on normal cells, which is essential for reducing side effects in possible cancer treatments.

As a vital transcription factor, p53 regulates the tran-scriptional activity of specific genes and responds to DNA damage by inducing cell cycle arrest or apoptosis. The presence of p53 in the cytoplasm at elevated concentrations results in a significant inhibitory impact on Caspase-9. The regulation of target gene expression by p53 in response to cellular stress and DNA damage aligns with its recognized

function as the guardian of the genome [50, 51]. As a result, one of the cancer treatment methods could be to activate p53 in cancer cells. In this study, an approximately 26-fold increase in the transcription level of p53 protein was detected in MCF7 cancer cell lines applied with MG extract compared to the control group. Consistent with our study, Zhang and Huang [52] reported that the plant extract of *Astragalus membranaceus* inhibits the proliferation of endometrial cancer cells by activating the P53/P21 signaling pathway [52]. In another study by *Juniperus communis* L. was found to induce cell death and sensitize cancer cells to cytostatic drugs through the P53 and P13K/Akt pathways [53]. This findings support the notion that plant extracts can modulate P53 activity in cancer cells, potentially inhibiting cell proliferation and promoting apoptosis.

Parps are a family of nuclear enzymes that are effective in DNA repair and involved in necrosis and apoptosis. The extract applied in this study increased the transcription levels of Parp genes approximately 15-fold in MCF 7 cell lines. MG extract increased the levels of Parp1 and P53 genes by approximately 13 and 27-fold, respectively, in MCF7 cells and approximately four and 3-fold, respec-tively, compared to the control in healthy fibroblast cells. This shows that MG extract activates the Parp1 and P53 genes to repair damaged DNA in cancer cells and induces these genes in less effective amounts in healthy cells to protect the healthy cells.

The Bax protein, a member of the Bcl-2 family, is situ-ated within the cytosol and is responsible for triggering apoptosis through the augmentation of apoptosis-inducing factor and cytochrome-c release. The inhibition of apoptosis occurs by preventing the release of cytochrome-c and apoptosis-inducing substances by precursor forms of cas-pases [54]. Both Caspase 9, a mitochondrial pathway pro-tein, and Caspase 8, a receptor pathway protein, were triggered, increasing the expression of Caspase 7. The increase in Caspase 7 induced apoptosis. Consistent with our study, it has been reported that *Cararia draba* leaf extracts have shown significant cytotoxicity against cancer cell lines, particularly HepG2, with observed changes in Caspase 9 gene expression [55]. Similarly, extracts from *Anastatica hierochuntica* have been found to induce apop-tosis in breast cancer cells by upregulating genes related to apoptosis [56]. Additionally, research on *Ephedra major* and *Momordica charantia* extracts revealed their influence on cell viability in breast cancer cells through increased cytotoxicity and Caspase 3 gene expression [57]. Consistent with the above mentioned publications MG extract was effective inducing important genes in cell death pathways.

In this study, we observed that the MG extract induces cancer cell death by activating apoptotic, autophagic, and necrotic pathways, a phenomenon corroborated by previous research. Mahassni and Al-Reemi [58] reported that an

aqueous extract from garden cress seeds induced apoptosis and necrosis in human breast cancer cells [58]. The researchers highlighted the capability of plant extracts to engage cell death pathways simultaneously. Research by Zhang et al. [59] delved into how chemotherapeutic agents induce pyroptosis in A549 lung cancer cells through caspase 3/Gsdme activation [59]. Pyroptosis, a form of programmed cell death, shares features with both apoptosis and necrosis, involving responses and cell lysis akin to necrosis but demonstrating apoptotic characteristics. In another study, Yu et al. [60] showed that arsenite combined with tetrandrine triggered cell death processes in human breast cancer cells, including apoptosis, necrosis, S phase arrest, and autophagy [60]. This study underscores how natural compounds from plants can concurrently influence diverse cell death pathways.

The findings obtained in this study showed that the phytochemicals present in *Mespilus germanica* leaves could simultaneously trigger multiple cell death mechanisms in A549 and MCF7 cancer cell lines. In particular, its effects on genes regulating apoptosis, necrosis, and autophagy pathways in these cell lines indicate the potential therapeutic benefits of MG extract. On the other hand, no significant change in the expression of these genes was observed in normal human skin fibroblast (HDF) cells, providing substantial evidence that the extract exerts a selective effect.

This selectivity highlights the importance of targeting cancer cells without harming healthy cells in cancer treatment. This feature of MG extract can potentially reduce side effects in cancer treatment. However, further studies are needed to confirm the clinical significance of these results and the impact of MG extract on rats and humans. Future studies should further examine the effectiveness of this extract against different types of cancer, its dose-response relationship, and its role in the treatment process. These studies should also evaluate the extract's long-term effects and possible toxicity on healthy cells to establish a solid basis for the safe and effective use of this natural product in cancer treatment.

Conclusion

With this study, we report for the first time that, while MG extract kills A549 and MCF7 cancer cells, it does not affect HDFa cells (Human Dermal Fibroblasts). This significant result suggests that MG extract selectively inhibits cancer cell growth without causing significant toxicity to normal cells, which is crucial to minimizing side effects in potential cancer treatments. It is thought that such a result was achieved due to the antioxidant properties of *Mespilus*

germanica leaf extract and the variety of phenolic acids it contains.

Findings of this study are limited to in vitro conditions; therefore, extrapolation to in vivo contexts, including studies with rats and humans, is being considered to confirm these effects and explore clinical applications. Further studies are planned to examine the effects of MG extract on protein level changes and their impact on cellular mechanisms. Also studies involving a more detailed biochemical analysis are intended to identify the specific bioactive components within the MG extract. This study we have completed, and the studies we plan to do in the future will contribute to the design of medicinal-plant based cancer drugs with fewer side effects.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Abbreviations

<i>APAF-1</i>	Apoptosis Protease Activating Factor-1
<i>ATG3</i>	autophagy related 3
<i>ATG5</i>	autophagy related 5
<i>BAX</i>	BCL -2-Associated X Protein
<i>BCL-2</i>	B-cell lymphoma 2
<i>CASPASE</i>	Cysteine Aspartate Specific ProteASEs-CASPASE
<i>C DNA</i>	Complementary DNA
<i>DNA</i>	Deoksiribonucleic acid
<i>DMSO</i>	Dimethyl sulfoxide
<i>ER</i>	Endoplasmic Reticulum
<i>FADD</i>	Fas-Associated Protein with Death
<i>HDFa</i>	Human Dermal Fibroblasts, adult
<i>IAP</i>	Inhibitors of Apoptosis Proteins
<i>IC50</i>	The half maximal inhibitory concentration
<i>MTT</i>	(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)
<i>NF-κB</i>	Nuclear Factor Kappa Beta Pathway
<i>PARP</i>	Poly (ADP-Ribose) Polymerase
<i>PCR</i>	Polimer Chain reaction
<i>RIPK 1</i>	receptor interacting serine/threonine kinase 1
<i>ROS</i>	Reactive Oxygen Species
<i>TNF</i>	Tumor Necrosis Factor
<i>TP53</i>	tumor protein p53

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