*FABAD J. Pharm. Sci., 49, 3, 525-538, 2024*
*Doi: 10.55262/fabadeczacilik.1511498*
*RESEARCH ARTICLE*

# Evaluation of Antioxidant, Cytotoxic Effects and Phytochemical Profiles of Galls Caused by Eriophyidae mite in *Juglans regia* Leaves

Semih BULUT[*°], Burçin ÖZÜPEK[**], Sultan PEKACAR[***], Aysun ÖZDEMİR[****], Didem DELİORMAN ORHAN[*****]

***Evaluation of Antioxidant, Cytotoxic Effects and Phytochemical Profiles of Galls Caused by Eriophyidae mite in Juglans regia Leaves***

***SUMMARY***

*This research presents the first study findings on the phytochemical contents and anticancer and antioxidant activities of galls caused by Eriophyidae mites on the leaves of Juglans regia L. Gall extracts collected from different localities in Turkey and prepared with solvents of different polarity were investigated for both antioxidant and cytotoxic activity. Cytotoxic activity studies showed that MCF-7 cancer cells were more sensitive to WLAA extract at a concentration of 100 µg/mL compared to healthy HUVEC cell lines. LC-QTOF-MS analysis results showed that all extracts contain chlorogenic acid, quercetin 4'-O-glucoside/quercetin 3-O-galactoside, quercetin 7-xyloside/quercetin 3-O-arabinoside, quercetin 7-O-rhamnoside, kaempferol 3-O-xyloside/kaempferol 3-O-arabinoside, and kaempferol derivatives. It was concluded that polyphenolic extracts obtained from galls formed in J. regia leaves can be considered as a new potential natural source for drug development studies due to their antioxidant and cytotoxic effects.*

***Key Words:*** *Antioxidant, cytotoxicity, galls, Juglans regia, phytochemical profile*

***Juglans regia Yapraklarında Eriophyidae Akarının Neden Olduğu Gallerin Antioksidan, Sitotoksik Etkileri ve Fitokimyasal Profillerinin Değerlendirilmesi***

***ÖZ***

*Bu araştırma Juglans regia L. yapraklarında Eriophyidae akarlarının neden olduğu gallerin fitokimyasal içerikleri ile antikanser ve antioksidan aktivitelerine ilişkin ilk çalışma bulgularını sunmaktadır. Türkiye'nin farklı bölgelerinden toplanan ve farklı polaritedeki çözücüler ile hazırlanan gal ekstrelerinin hem antioksidan hem de sitotoksik aktiviteleri araştırılmıştır. Sitotoksik aktivite çalışmaları, MCF-7 kanser hücrelerinin, sağlıklı HUVEC hücre hatlarına kıyasla 100 µg/mL konsantrasyondaki WLAA ekstresine daha duyarlı olduğunu göstermiştir. LC-QTOF-MS analiz sonuçları tüm ekstrelerin klorojenik asit, kersetin 4'-O-glukozit/kersetin 3-O-galaktozit, kersetin 7-ksilozit/kersetin 3-O-arabinozit, kersetin 7-O-ramnozit, kemferol 3-O-ksilozit/kemferol 3-O-arabinozit ve kemferol türevleri içerdiğini göstermiştir. J. regia yapraklarında oluşan gallerden elde edilen polifenolik ekstrelerin, antioksidan ve sitotoksik etkileri nedeniyle ilaç geliştirme çalışmaları için yeni bir potansiyel doğal kaynak olarak değerlendirilebileceği sonucuna varılmıştır.*

***Anahtar Kelimeler:*** *Antioksidan, sitotoksisite, galler, Juglans regia, fitokimyasal profil*

*Received: 06.07.2024*
*Revised: 10.09.2024*
*Accepted: 03.10.2024*

[*] ORCID: 0000-0002-4098-0221, Department of Pharmacognosy, Faculty of Pharmacy, Suleyman Demirel University, Isparta, Turkiye
[**] ORCID: 0000-0003-2159-9860, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkiye
[***] ORCID: 0000-0002-7782-9832, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkiye
[****] ORCID: 0000-0003-0311-0507, Department of Pharmacology, Faculty of Pharmacy, Gazi University, Ankara, Turkiye
[*****] ORCID: 0000-0003-3916-4048, Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara, Turkiye

[°] Corresponding Author; Semih BULUT
E-mail: semihbulut@sdu.edu.tr

## INTRODUCTION

Cancer is a disease that occurs when normal cells in the body transform into tumor cells in a multi-stage process and multiply uncontrollably. In the next stage, these cells, which multiply uncontrollably, can spread to the surrounding and distant organs. Authorities reported that 9.6 million people died from cancer worldwide in 2018. Lung, breast, stomach, prostate, colorectal, liver, and cervical cancers are common today (WHO, 2024). The majority (90-95%) of all cancer cases are related to environmental factors, while a small amount (5-10%) is related to genetic causes (Anand et al., 2008). Approximately 33.3% of cancer deaths are due to behavioral and nutritional risk factors. These risk factors include obesity, insufficient physical activity, and tobacco use (Republic of Türkiye Ministry of Health, 2024).

Antioxidants interact with free radicals by eliminating free radicals in the body and reduce oxidative stress. In addition, they prevent cancer development by stopping uncontrolled cell division. Therefore, endogenous and exogenous antioxidants are important in cancer prevention. Medicinal plants and foods are among the sources of exogenous antioxidants (Alzeer et al., 2017). Numerous studies have shown that natural polyphenols (apigenin, luteolin, quercetin, kaempferol, resveratrol, etc.) can be used for the prevention and treatment of cancer through their antioxidant and anti-inflammatory effects (Zhou et al., 2016). The main methods used in cancer treatment today are radiotherapy, surgery, and chemotherapy. Chemotherapy has unpleasant side effects such as vomiting, nausea, diarrhea, hair loss, loss of appetite, fever, constipation, pain, fatigue, mouth sores, the formation of bruises on the skin, and bleeding. Medicinal plants, traditional folk medicines, and natural compounds are being evaluated for new opportunities in cancer prophylaxis and treatment (Greenwell & Rahman 2015; Altun & Sonkaya 2018).

*Juglans regia* L., a species belonging to the Juglandaceae family, is known as walnut in Anatolia. It is a tree that naturally spreads throughout the world from the Balkans to the Himalayas and Southwest China. Its fruits are a strong source of nutrients owing to their high amount of fixed fat, as well as protein, carbohydrate, and mineral content. On the other hand, leaves of the plant contain carbohydrates, fatty acids, naphthalene derivatives (juglone), tannin, phenolic compounds, and ascorbic acid (Delaviz et al., 2017). It has been determined by researchers that *J. regia* leaf extracts show strong cytotoxic activities in colon, lung, breast adenocarcinoma, prostate, and human oral cancer cells (Salimi et al., 2012; Delaviz et al., 2017; Ara et al., 2023).

Pests that infest trees such as walnuts, pears, pistachios, almonds, and figs can have negative effects on the fruit shape, fruit productivity, and leaves of these species. Agricultural control methods are applied against this type of pest. The Eriophyidae mites, which infest walnut leaves and fruits, feed by sucking the sap of these parts, and the harmful substances they secrete during this time cause deterioration in the plant tissues and the formation of blisters. These blisters formed on the leaf are light green at first, then gradually become darker, turning from red to brown and finally to black. These blisters are known as galls. This pathological formation causes early shedding of leaves and deformation of fruits. Research should be carried out for the use of these galls in the development of some products with added value (medicine, cosmetics, etc.) (Denizhan & Çobanoğlu 2009; T.C. Gıda Tarım ve Hayvancılık Bakanlığı, 2017). Based on traditional knowledge, the ideas that form the basis for discovering pharmaceutical raw materials have emerged from the principles of sometimes similarities and sometimes contrasts. Semi-parasitic plants such as *Viscum album* L. (European mistletoe) damage the tree by absorbing all the minerals and water of the host tree with their haustorium. In this way, the tree, which dries up day by day, is likened to the spread of cancer in the human body. For this reason, European mistletoe has been included in cancer research for many years. Today,

fermented extracts of European mistletoe are used as an anticancer drug in anthroposophical medicine (Davis, 1982; Tennakoon & Pate 1996; Deliorman et al., 2000; Dela et al., 2015; Delebinski et al., 2015).

With the same approach, the polyphenolic extracts prepared from these pathological structures (gall) formed by Eriophyidae mites from the leaves of walnut trees in two different localities (Sinop and Ankara) in Turkey were investigated for cytotoxic activity in MCF-7 and HUVEC cell lines in this study. In addition to antioxidant activities of these polyphenolic extracts, analyses of polyphenolic substances were carried out by LC-QTOF/MS. The current research is original as it is the first report of phytochemical analysis and activity screening studies on galls caused by Eriophyidae mites on walnut leaves.

## MATERIAL AND METHODS

### Plant material

Walnut leaves were collected from Boyabat, Sinop, Turkey and Çankaya, Ankara, Turkey in July and August 2019. The plant materials were identified by Gülsen Kendir (Department of Pharmaceutical Botany, Suleyman Demirel University, Isparta, Turkey). Voucher specimens were stored in the GUL Herbarium, Suleyman Demirel University (GUL 111/1/1-2 and GUL 111/1/1-3).

### Extraction

The galls on the walnut leaves were carefully removed with a scalpel and dried at 25ºC. 50 mL of 80% acetone (WLSA) and 80% ethanol (WLSET) were added to the separately weighed two gall samples (1.32 g and 1.37 g, respectively) collected from Sinop and macerated at room temperature. 50 mL of 80% acetone (WLAA) was added to 0.51 g of gall sample collected from Ankara. Since the sample amount was insuffcient, an 80% ethanol extract could not be prepared. These samples were extracted for 14-18 hours on a shaker at 25ºC, and then the extracts were filtered. The same procedures were repeated three times by adding solvents again. All solvents were evaporated using a rotary evaporator.

### Chemical composition analysis of plant extracts

### Total phenolic content

Folin-Ciocalteu reagent (10% v/v) was first added to the 20 µL extracts and kept at 25°C for 5 minutes. Then sodium carbonate ($Na_2CO_3$) solution (7.5% w/v) was mixed to the extract-folin mixture. The absorbance of the resulting mixture was measured at a wavelength of 735 nm. Total phenolic content was shown as gallic acid equivalent (GAE) mg/g extract (Zongo et al., 2010). The calibration equation was found as $y = 6.1419x$ and calculated as $r^2 = 0.9982$.

### Analysis of phenolic compounds by LC-QTOF-MS method

LC-QTOF-MS was used for qualitative analysis. Analyses were performed on Agilent 1260 series HPLC system and Agilent 6550 iFunnel High Resolution Mass Spectrometer device connected to this system. Analyses were made in negative mode. MS operating mode is 2 GHz Extended Dynamic Range. Agilent Zorbax Extend C-18 column was used in the analysis. Agilent MassHunter Software B06.00 and Metlin Metabolite database were used for analysis and data evaluation. LC-QTOF-MS analysis parameters of phenolic compounds: Column: Agilent Zorbax Extend C-18 (4.6 mm x 150 mm x 3.5 µm); Column oven: 35°C; Injection volume: 5 µL; Analysis Time: 25 min.; Mobile phase A: water-acetic acid (0.1%); Mobile phase B: Acetonitrile; Flow: 0.65 mL/min; Method; At the beginning of the analysis, the ratio of solvent B was 5% and isocratic flow was applied for one minute. Between 1-4 minutes, the solvent B rate has a 10% gradient flow. Between 4-10 minutes, solvent B 70% gradient flow was reached. Solvent B 90% gradient flow was applied between 10-11 minutes. 90% isocratic flow was applied between 11-16 minutes. Solvent B was decreased gradually to 5% between 16-16.1 minutes. Solvent B reached 55% with gradient flow between 16.1-20 minutes.

### Antioxidant activity

In antioxidant activity studies, extracts and reference compounds in all methods were dissolved in methanol.

**DPPH radical scavenging activity**

The mechanism of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity method is based on the hydrogen atom and electron donating capacity of the extract to bleach the purple color of DPPH solution (Orhan et al., 2011). DPPH solution (1 mM) was added to the 80 µL extracts. The resulting mixture was left for 30 minutes. The absorbance values of the extracts and ascorbic acid were measured at 520 nm. The standard compound in the experiment was ascorbic acid (Jung et al., 2011).

**Metal chelating capacity**

The mechanism of action of metal chelating capacity is based on the inhibition of $Fe^{2+}$-ferrozine complex formation after reaction of the extracts with $Fe^{2+}$ (Gülçin, 2010). The first $FeCl_2$ solution (2 mM) was added to the extracts, then ferrozine solution (5 mM) was added. The experiment was performed with a final volume of 130 µL. After this process, the absorbance of the extracts at 562 nm wavelength was measured with a microplate reader. Ethylene diamine tetra acetic acid (EDTA) was used as the standard compound in the experiment (Dinis et al., 1994).

**Ferric-reducing antioxidant power**

The mechanism of action of the reducing power of the extracts is based primarily on the reduction of $Fe^{3+}(CN)^6$ to $Fe^{2+}(CN)^6$ and then on the absorbance measurement of the blue-colored complex formed after the addition of excess $Fe^{3+}$ (Gülçin, 2010). All test samples (50 µL) and quercetin (50 µL) were mixed with sodium phosphate buffer (pH = 7.2, 0.1 mol/L). Then, potassium ferricyanide solution (1% w/v) was added to the mixtures and the microplate was placed in an oven at 37°C. Then, trichloroacetic acid solution (10% w/v) was added to the mixture. The experiment was performed with a final volume of 210 µL. Results were measured at a wavelength of 700 nm. $FeCl_3$ (0.1% w/v) was added to the mixture, and the wavelength was measured again (Orhan et al., 2017).

**Cytotoxic Activity**

**Cell culture**

Breast cancer cells (MCF-7) and human umbilical vein endothelial cells (HUVEC) were cultured in an incubator in Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal bovine serum and 100 U/mL penicillin/streptomycin. Incubation conditions are set at 37 °C, with 5% $CO_2$. The extracts were dissolved in dimethyl sulfoxide (DMSO) and applied to the cells in increasing logarithmic concentrations (18, 30, 56, 100, and 180 µg/mL). Cells were treated with 0.2% DMSO.

**Cytotoxic activity**

The cytotoxic activity of the extracts was determined against MCF-7 cancer cells and HUVEC cell lines using the MTT assay. 10000 MCF-7 and HUVEC cells were transferred to each well of 96-well plates. The next day, cells were replaced with fresh medium in DMEM without phenol red (200 µL), and then extracts were added. After 72 hours of incubation, media containing 0.5 g/l MTT 50 µL (Life Technologies) was added to each well. Subsequently, formazan crystals were dissolved with DMSO (160 µL). Absorbances were measured at a wavelength of 570 nm. The cell viability (CV) percentage was calculated as follows (Özdemir et al., 2017). CV% = (Absorbance of extract group/absorbance of control group) x 100.

**Statistical analysis**

In cytotoxic activity studies, all values were evaluated using a one-way analysis of variance (ANOVA), followed by the Tukey post hoc test to analyze multi-group comparisons. The GraphPad Software Instat program was used to calculate the standard errors from the values found in other studies.

**RESULTS AND DISCUSSION**

The yields of acetone and ethanol extracts prepared from galls in walnut leaves collected from two different localities (Sinop and Ankara) in Turkey are

given in Table 1. Since the amount of gall formed on the walnut tree grown in Ankara is low and acetone is the solvent that can extract polyphenolic compounds from plants, especially tannins, only acetone extract was prepared from this plant sample. The extract yields of all three extracts were found to be quite close to each other. The chemical profile of the tested extracts was analyzed by the Folin-Ciocalteu method and LC-QTOF-MS methods. According to the results of the total phenol content determination using UV spectroscopy, it was determined that the acetone extract (WLSA; 177.31 ± 6.99 GAE mg/g extract) of the sample collected from Sinop had the highest total phenol content (Table 1).

**Table 1**. Yields and total phenolic contents of extracts

| Extracts | Yield% | Total phenol content (GAE) mg/g extract ± SD[d] |
|---|---|---|
| WLSA[a] | 14.67 | 177.31 ± 6.99 |
| WLAA[b] | 14.82 | 109.47 ± 1.24 |
| WLSET[c] | 15.29 | 157.12 ± 13.14 |

[a]WLSA: walnut leaves acetone Sinop, [b]WLAA: walnut leaves acetone Ankara,

[c]WLSET: walnut leaves ethyl alcohol Sinop, [d]SD: Standard deviation

The extracts were analyzed by LC-QTOF-MS in the negative mode. Total ion chromatograms of the extracts are given in Figure 1.

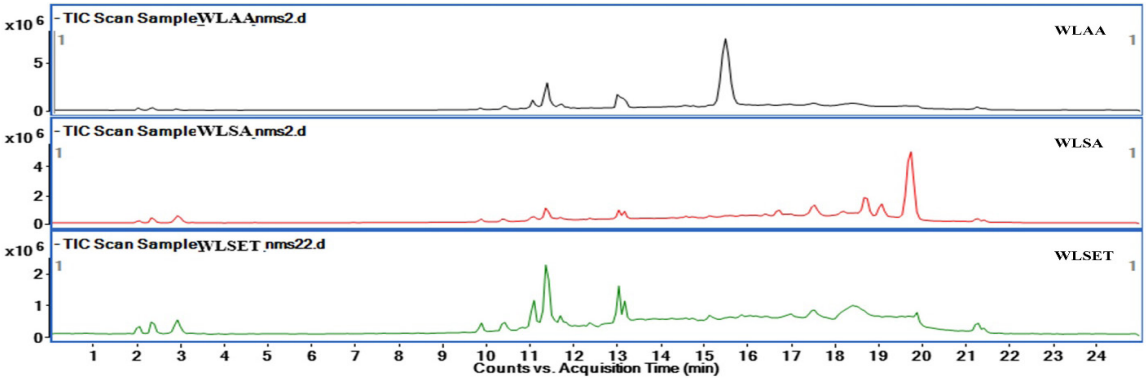


**Figure 1**. Total ion chromatograms of the extracts

Compounds with molecular weights of the chlorogenic acid 354.09508; - Quercetin 4'-*O*-glucoside / Quercetin 3-*O*-galactoside 464.09548; - Quercetin 7-xyloside / quercetin 3-*O*-arabinoside 434.08491; - Quercetin 7-*O*-rhamnoside 448.10056; - Kaempferol 3-*O*-xyloside / Kaempferol 3-*O*-arabinoside 418.09000; - Kaempferol derivative 578.14243 and - Kaempferol derivative 578.14243 of phenolic compounds with retention times of 9.85, 11.06, 11.33, 11.46, 11.73, 13.05 and 13.20 minutes in the chromatogram of the three extracts (Table 2, Figure 2).

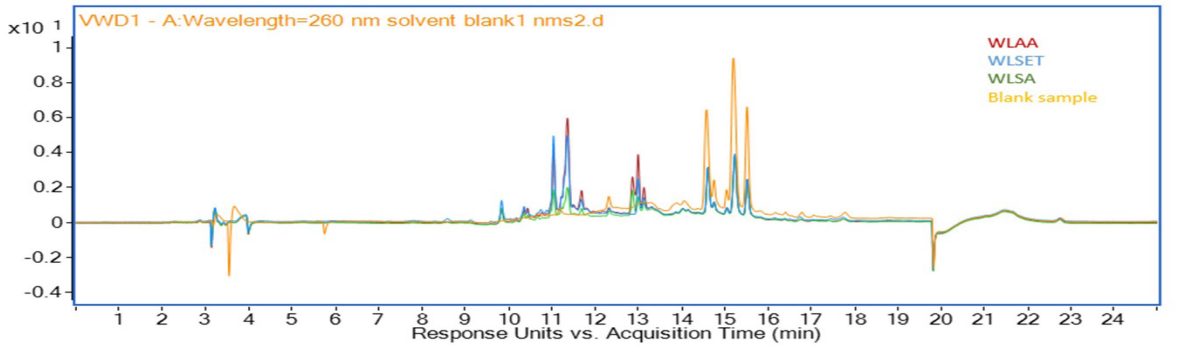


**Figure 2.** RP-HPLC chromatogram connected to LC-QTOF (260 nm) of the extracts and solvent. (Solvent and sample signals overlapped)

In addition, when the fragment ions of the compounds thought to have this molecular weight and the fragment ions belonging to the peaks thought to belong to these compounds in the extracts were compared, it was predicted that these compounds could be chlorogenic acid, quercetin 4'-*O*-glucoside/quercetin 3-*O*-galactoside, quercetin 7-xyloside/quercetin 3-*O*-arabinoside, quercetin 7-*O*-rhamnoside, kaempferol 3-*O*-xyloside/ kaempferol 3-*O*-arabinoside, and kaempferol derivatives, respectively.

Interestingly, in the DPPH free radical scavenging activity method, it was observed that the effect was stronger in all extracts with decreasing doses. Similarly, in a different study, *J. regia* dichloramethane leaf extract showed stronger DPPH radical scavenging activity at 0.5 mg/mL concentration compared to 1 mg/mL (Erdogan Orhan et al., 2011). In another study, DPPH radical scavenging activity increased as the concentration of leaf essential oil of *J. regia* increased, but **β**-pinene, the major constituent of the essential oil, showed higher activity at 80 µg/mL concentration than at 100 µg/mL concentration (Rather et al., 2012). In this respect, there are differences in the literature, and our research results are similar to those of Erdoğan Orhan et al. WLSA, WLAA and WLSET extracts at 0.5 mg/mL concentration (85.90 ± 0.62, 86.95 ± 0.77 and 87.76 ± 0.21%, respectively) showed as potent radical scavenging effects as the reference compound ascorbic acid (91.80 ± 0.31%). The ferric reducing power was 2.517 ± 0.090, 1.925 ± 0.010 and 1.969 ± 0.070 in the extracts respectively, at a concentration of 2 mg/mL, with an absorbance value of 1.874 ± 0.030 for the reference compound quercetin. In the ferric reducing power test, on the contrary to the extracts, a decrease was observed in the absorbance values of the reference compound quercetin, on the contrary to the increase in the dose. It was determined that only WLAA extract had a very high metal chelating capacity (74.17 ± 11.06%) at 2 mg/mL concentration. On the other hand, the metal binding capacity of EDTA used as a reference at 2 mg/mL was found to be 100 ±0.00% (Table 3).

MCF-7 and HUVEC cell lines were used to test the cytotoxic activities of the extracts. The extracts were incubated with the cells for 72 hours. As a result of subsequent colorimetric assays, none of the WLSET and WLSA extracts changed the viability of MCF-7 cancer cells, while WLAA extracts reduced cell viability at concentrations of 100 and 180 µg/mL compared to the control group. WLAA extract reduced cell viability to 83.66 ± 3.84% at a concentration of 100 µg/mL, and the same extract reduced cell viability to 81.45±2.75% ($p < 0.05$) at a concentration of 180 µg/mL (Table 4, Figure 3).

In order to understand whether the induced cytotoxic effect is specific to cancer cells, the effects of the extracts on the healthy cell line HUVEC were also evaluated. It was determined that only WLSET and WLSA extracts cause a significant cytotoxic effect on HUVEC cell lines at a concentration of 180 µg/mL (Figure 4).

All these results showed that MCF-7 cancer cells were more sensitive to WLAA extract at a concentration of 100 µg/mL compared to healthy HUVEC cell lines.

**Table 2.** Mass fragments of phenolic compounds detected in extracts by LC-QTOF-MS

| Retention time (min) | Molecular formula | Molecular weight | $[M-H]^-$ | Theoretical ion | Fragment ions | Ppm (Δ) | Compound | Determination methods |
|---|---|---|---|---|---|---|---|---|
| 9.85 | $C_{16}H_{18}O_9$ | 354.0951 | 353.0908 | 353.0881 | 191, 135, 173, 85 | 2.7 | Chlorogenic acid | PubChem |
| 11.06 | $C_{21}H_{20}O_{12}$ | 464.0955 | 463.0914 | 463.0000 | 301, 300, 271, 255, 179, 151 | 9.1 | Quercetin 4'-*O*-glucoside / Quercetin 3-*O*-galactoside | PubChem |
| 11.33 | $C_{20}H_{18}O_{11}$ | 434.0849 | 433.0812 | 433.0800 | 301, 300, 271, 179, 151 | 1.2 | Quercetin 7-xyloside / Quercetin 3-*O*-arabinoside | PubChem |
| 11.46 | $C_{21}H_{20}O_{11}$ | 448.1006 | 447.0968 | 447.0927 | 301, 300, 271, 255, 151 | 4.1 | Quercetin 7-*O*-rhamnoside | PubChem |
| 11.73 | $C_{20}H_{18}O_{10}$ | 418.090 | 417.0856 | 417.0899 | 285, 255, 227 | 4.3 | Kaempferol 3-*O*-xyloside / Kaempferol 3-*O*-arabinoside | Metlin |
| 13.05 | $C_{30}H_{26}O_{12}$ | 578.1424 | 577.1402 | 577.1424 | 413, 285, 255, 227, 163 | 2.2 | Kaempferol derivative | Metlin |
| 13.20 | $C_{30}H_{26}O_{12}$ | 578.1424 | 577.1402 | 577.1424 | 413, 285, 255, 227, 163 | 2.2 | Kaempferol derivative | Metlin |

**Table 3**. Antioxidant activity results of the extracts

| Extracts | Metal chelating capacity inhibition% ± S.D[b]. | | | Ferric-reducing power absorbance ± S.D. | | | DPPH radical scavenging activity inhibition% ± S.D. | | |
|---|---|---|---|---|---|---|---|---|---|
| | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** |
| WLSA | 8.80 ± 2.23 | - | - | 2.517 ± 0.090 | 1.681 ± 0.260 | 1.336 ± 0.030 | 75.39 ± 0.44 | 82.67 ± 0.32 | 85.90 ± 0.62 |
| WLAA | 74.17 ± 11.06 | 31.06 ± 8.10 | 16.16 ± 4.17 | 1.925 ± 0.010 | 1.446 ± 0.060 | 0.756 ± 0.010 | 73.05 ± 2.09 | 83.11 ± 2.49 | 86.95 ± 0.77 |
| WLSET | 54.58 ± 9.66 | 32.13 ± 8.17 | - | 1.969 ± 0.070 | 1.594 ± 0.100 | 1.134 ± 0.060 | 80.53 ± 1.21 | 85.86 ± 0.71 | 87.76 ± 0.21 |
| **Reference** | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** | **2 mg/mL** | **1 mg/mL** | **0.5 mg/mL** |
| EDTA | 100 ± 0.00 | 99.86 ± 0.48 | 99.68 ± 0.08 | NT | NT | NT | NT | NT | NT |
| Quercetin | NT[a] | NT | NT | 1.874 ± 0.030 | 2.296 ± 0.010 | 2.931 ± 0.150 | NT | NT | NT |
| Ascorbic acid | NT | NT | NT | NT | NT | NT | 94.75 ± 0.71 | 95.23 ± 0.14 | 91.80 ± 0.31 |

[a]NT: Not tested, [b]SD: Standard deviation, -: No activity

**Table 4**. Cytotoxic activity of the extracts

| Cell Line | Concentration (µg/mL) | Cell viability% ± SEM | | |
|---|---|---|---|---|
| | | WLSET | WLSA | WLAA |
| MCF- 7 | 18 | 94.41±1.29 | 99.94±3.08 | 95.64±2.79 |
| | 30 | 98.48 ± 2.53 | 104.40±2.00 | 100.20±2.36 |
| | 56 | 96.68±1.03 | 100.50±2.21 | 89.32±0.95 |
| | 100 | 97.76±1.33 | 106.40±1.77 | 83.66±3.84* |
| | 180 | 108.30±2.97 | 112.40±6.14 | 81.45±2.75* |
| HUVEC | 18 | 99.05 ± 5.90 | 98.27±2.95 | 103.40±2.12 |
| | 30 | 106.80±3.01 | 100.70±2.94 | 103.60±2.51 |
| | 56 | 94.95±1.58 | 99.29±1.83 | 95.69±3.21 |
| | 100 | 97.76±2.21 | 102.60±1.60 | 97.46±1.99 |
| | 180 | 64.11±1.65* | 84.23±1.19* | 92.81±3.43 |

n = 4; * p < 0.05; SEM: Standard error meaning

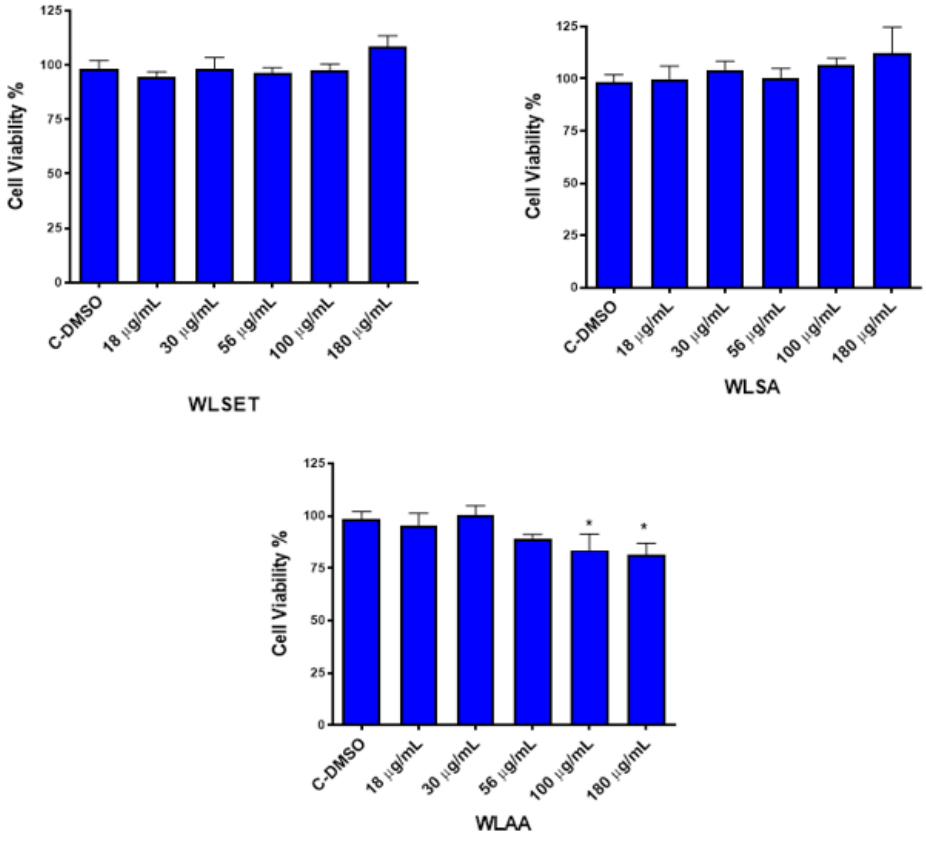


**Figure 3**. Cytotoxic effects of WLSET, WLAA, and WLSA extracts in MCF- 7 cell lines (n = 4 tested, One-Way ANOVA, * p < 0.05 vs. control DMSO)

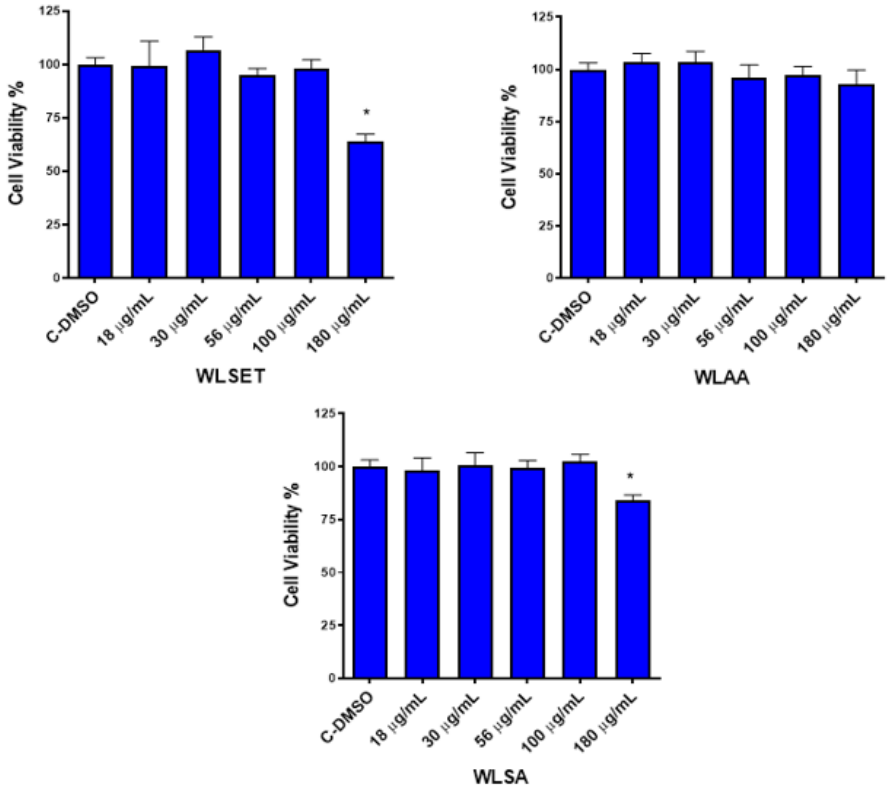


**Figure 4.** Cytotoxic effects of WLSET, WLAA, and WLSA extracts in HUVEC cell lines (n= 4 tested, One-Way ANOVA, * $p < 0.05$ vs. control DMSO).

One of the cells in the cancer microenvironment is the endothelial cell and endothelium plays important roles in cancer diseases (Sobierajska et al., 2020). Thus we also evaluated the cytotoxic effects of walnut leaves extracts on endothelial cells. Among the extracts examined in this study, it was determined that only WLAA extract exerted cytotoxic activity in MCF-7 cancer cell lines, on the other hand, it did not show any cytotoxic effect on healthy cell lines. A compound and/or extract evaluated for cytotoxic effect should not be cytotoxic to healthy cell lines. In addition, it is important that the extract is effective in terms of both cytotoxic and antioxidant effects, and when the WLAA extract is evaluated from this point of view, it is considered extremely promising.

Due to of the differences in the phenolic content of the extracts and LC-QTOF-MS analyses were performed to determine the phenolic content of the extracts. In LC-QTOF-MS analysis, fragmentation ions belonging to molecules thought to be derivatives of chlorogenic acid, quercetin 4'-*O*-glucoside/quercetin 3-*O*-galactoside, quercetin 7-xyloside/quercetin 3-*O*-arabinoside, quercetin 7-*O*-rhamnoside, kaempferol 3-*O*-xyloside/ kaempferol 3-*O*-arabinoside, and kaempferol derivatives were detected in all extracts. As seen in the chromatograms, it was not determined which phenolic compounds belonged to the main peaks observed at the 15th and 16th minutes in the WLAA extract, which was thought to be particularly effective.

As a result of our literature survey, it was concluded that scientific studies were also carried out on the galls formed in different plants. Gall, which is formed by an insect named *Adleria gallae* tinctoria on the branches, leaves, and buds of *Quercus species* (Oak), contains secondary metabolites in

tannin, phenolic acid, flavonoid, triterpenoid, and steroid structure, and depending on these contents, cholinesterase and monoamine oxidase inhibitor, antitumor, antihypertensive has been determined that they show antioxidant, antimicrobial, insecticidal, anti-inflammatory, and antiparasitic effects (Mirpour et al., 2015; Arina & Harisun 2019; Mahboubi 2020; Sukor et al., 2020; Elham et al., 2021).

Similarly, in the galls formed by a Pemphigus insect on the leaves and petioles of *Pistacia integerrima* Stewart from the Anacardiacae family; it has been reported that flavonoids, monoterpene, triterpenoid, sterol, triterpenic acid, fatty esters, ketoalcohol structured compounds, and dihydromalvalic acid are present. Scientific studies have also been published on the fact that the extracts obtained from these galls have antihyperalgesic, anti-inflammatory, antidepressant, and antihyperuricemic effects (Ahmad et al., 2010; Rauf et al., 2016).

It has been reported that the galls formed by the aphid *Schlechtendalia chinensis* on the leaves of *Rhus chinensis* Mill. are also rich in hydrolyzed tannins and gallotannins and display alpha glucosidase enzyme inhibitory, anticancer, antiviral, antimicrobial, and anti-inflammatory activities (Shim et al., 2003; Liu et al., 2014; Kwak et al., 2014).

In a study on oak galls caused by Eriophyidae, galls parts of the plant were found to be rich in gallotanene and gallic acid (Patni et al., 2012). In this context, it is thought that the biological activities of galls belonging to different plant species with different phytochemical content may also be different.

As a result of our literature studies, no phytochemical or activity studies were found on galls caused by Eriophyidae mites on *J. regia* leaves. For the first time in this study, the chemical compositions of polyphenol extracts obtained from galls were analyzed, and their antioxidant and cytotoxic activities were investigated.

Hakimuddin et al., evaluated the cytotoxic effects of the flavonoid fraction obtained from red wine on MCF-7 cell lines. Due to the strong selective cytotoxic effect of the fractions, the effects of some flavonoids (catechin, quercetin, and naringenin) in these fractions were tested again in the same cell line. All three flavonoids showed dose-dependent cytotoxic effects on the proliferation of MCF-7 cells. The $IC_{50}$ values of quercetin, naringenin and catechin are listed as follows; 13, 51, and 150 µg/mL (Hakimuddin et al., 2004). In a study by Silva et al., it was reported that the ethanol extract of *Mimosa caesalpiniifolia* leaves had a cytotoxic effect on MCF-7 cell lines and that this extract was rich in flavonoids (Silva et al., 2014). These literature data showed that the cytotoxic effect of WLAA extract on MCF-7 cells may be due to the fact that it contains more catechins than other extracts. On the other hand, it can be predicted that the identified/unidentified phenolic compounds in this extract may also cause synergistic effects in both antioxidant and cytotoxic activities. LC-QTOF-MS analyses showed that some secondary metabolites in walnut leaves are also present in galls, and some compounds identified in galls have not been identified in walnut leaves so far. In other words, these findings suggest that walnut leaves contain some phenolic compounds (quercetin 4'-*O*-glucoside, quercetin 7-xyloside, quercetin 7-*O*-rhamnoside, kaempherol 3-*O*-xyloside, and kaempherol 3-*O*-arabinoside) that have not been detected until now.

## CONCLUSIONS

In this report, the phytochemical contents, antioxidant and anticancer effect potentials of galls caused by *Eriophyidae* mites on *J. regia* leaves were investigated for the first time. While eight phenolic compounds were defined by LC-QTOF-MS. While it was observed that MCF-7 breast cancer cell lines were more sensitive to the polyphenolic extract of galls collected from the Ankara region, it was also concluded that this extract had a high antioxidant potential. When approached from a different perspective, the galls formed on the leaves of the trees both damage the trees and cause negative economic effects in terms of affecting fruit productivity.

Therefore, the results obtained in this study showed that these galls are worth examining in terms of anticancer, antioxidant, and many other activities, and in this way, a pathogenic condition for the tree can be a source for the discovery of new and natural drug raw materials. Future studies will continue to test the cytotoxic activities of the fractions obtained from the polyphenolic extracts of these galls formed on walnut leaves in different cancer cell lines and to determine the active compound or compounds.

**ACKNOWLEDGEMENTS**

We would like to thank Ayhan İbrahim Aysal (Faculty of Pharmacy Central laboratory, Gazi University) for his support during LC-QTOF-MS analysis.

**AUTHOR CONTRIBUTION STATEMENT**

Concept: SB, SP, DDO; Design: SB, BÖ, DDO; Control: SB, BÖ, AÖ, SP, DDO; Sources: SB, SP; Materials: SB, BÖ, SP, AÖ, DDO; Data Collection and/or processing: SB, SP, BÖ, SP, AÖ, DDO; Analysis and/or interpretation: SB, SP, AÖ, DDO; Literature review: SB, BÖ, DDO; Manuscript writing: SB, BÖ, SP, DDO; Critical review: SB, DDO; Other: SB, BÖ, SP

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**REFERENCES**

Ahmad, S., Ali, M., Ansari, S. H., & Ahmed, F. (2010). Phytoconstituents from the galls of Pistacia integerrima Stewart. *Journal of Saudi Chemical Society, 14*(4), 409-412. https://doi.org/10.1016/j.jscs.2010.05.003

Altun, I., & Sonkaya, A. (2018). The most common side effects experienced by patients were receiving first cycle of chemotherapy. *Iranian Journal of Public Health*, *47*(8), 1218-1219.

Alzeer, J., Arafeh, R., & Al-Gubory, K. H. (2017). Antioxidants in the prevention and treatment of cancer. *Nutritional Antioxidant Therapies: Treatments and Perspectives* (pp. 493-521)

Anand, P., Kunnumakara, A. B., Sundaram, C., Harikumar, K. B., Tharakan, S. T., Lai, O. S., . . . Aggarwal, B. B. (2008). Cancer is a preventable disease that requires major lifestyle changes. *Pharmaceutical Research*, *25*, 2097-2116.

Ara, T., Shafi, S., Ghazwani, M., Mir, J. I., Shah, A. H., Qadri, R. A., . . . Wahab, S. (2023). In vitro potent anticancer, antifungal, and antioxidant efficacy of Walnut (Juglans regia L.) Genotypes. *Agronomy*, *13*(5), 1232. https://doi.org/10.3390/agronomy13051232

Arina, M. Z. I., & Harisun, Y. (2019). Effect of extraction temperatures on tannin content and antioxidant activity of Quercus infectoria (Manjakani). *Biocatalysis and Agricultural Biotechnology, 19*, 101104. https://doi.org/10.1016/j.bcab.2019.101104

Davis, P. H. (1982). Flora of Turkey and the East Aegean Islands 7. *Edinburgh University Press*, Edinburgh.

Dela, Cruz, J. F., Kim, Y. S., Lumbera, W. M., & Hwang, S. G. (2015). Viscum album var hot water extract mediates anti-cancer effects through G1 phase cell cycle arrest in SK-Hep1 human hepatocarcinoma cells. *Asian Pacific Journal of Cancer Prevention*, *16*, 6417-6421.http://dx.doi.org/10.7314/APJCP.2015.16.15.6417

Delaviz, H., Mohammadi, J., Ghalamfarsa, G., Mohammadi, B., & Farhadi, N. (2017). A review study on phytochemistry and pharmacology applications of Juglans regia plant. *Pharmacognosy Review*, *11*(22), 145-152. https://doi.org/10.4103/phrev.phrev_10_17

Delebinski, C. I., Twardziok, M., Kleinsimon, S., Hoff, F., Mulsow, K., Rolff, J., . . . Seifert, G. (2015). A natural combination extract of Viscum album L. containing both triterpene acids and lectins is highly effective against AML in vivo. *PLoS One*, *10*, e0133892. https://doi.org/10.1371/journal.pone.0133892

Deliorman, D., Caliş, I., Ergun, F., Doğan, B. S., Buharalioğlu, C. K., & Kanzik, I. (2000). Studies on the vascular effects of the fractions and phenolic compounds isolated from Viscum album ssp. album. *Journal of Ethnopharmacology*, *72*(1-2), 323-329. https://doi.org/10.1016/s0378-8741(00)00251-8

Denizhan, E., & Çobanoğlu, S. (2009) Eriophyid Mites of Walnut Trees (Juglans regia L.) and Their Predators in Ankara. *Yüzüncü Yil University Journal of Agricultural Sciences*, *19*(1), 33-37.

Dinis, T. C., Maderia, V. M., & Almeida, L. M. (1994). Action of phenolic derivatives (acetaminophen, salicylate, and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. *Archives of Biochemistry and Biophysics*, *315*(1), 161-169. https://doi.org/10.1006/abbi.1994.1485

Elham, A., Arken, M., Kalimanjan, G., Arkin, A., & Iminjan, M. (2021). A review of the phytochemical, pharmacological, pharmacokinetic, and toxicological evaluation of Quercus Infectoria galls. *Journal of Ethnopharmacology*, *273*, 113592. https://doi:10.1016/j.jep.2020.113592

Erdogan Orhan, I., Suntar, I. P., & Akkol, E. K. (2011). In vitro neuroprotective effects of the leaf and fruit extracts of Juglans regia L.(walnut) through enzymes linked to Alzheimer's disease and antioxidant activity. *International Journal of Food Sciences and Nutrition*, *62*(8), 781-786.

Greenwell, M., & Rahman, P. K. S. M. (2015). Medicinal plants: their use in anticancer treatment. *International Journal of Pharmaceutical Sciences and Research*, *6*(10), 4103-4112. https://doi: 10.13040/IJPSR.0975-8232.6(10).4103-12

Gülçin, I. (2010). Antioxidant properties of resveratrol: A structure–activity insight. *Innovative Food Science & Emerging Technologies*, *11*(1), 210-218.

Hakimuddin, F., Paliyath, G., & Meckling, K. (2004). Selective cytotoxicity of a red grape wine flavonoid fraction against MCF-7 cells. *Breast Cancer Research and Treatment*, *85*, 65-79. https://doi.org/10.1023/B:BREA.0000021048.52430.c0

Jung, H. A., Jin, S. E., Choi, R. J., Manh, H. T., Kim, Y. S., Min, B. S., . . . Choi, J. S. (2011). Anti-tumorigenic activity of sophoflavescenol against Lewis lung carcinoma in vitro and in vivo. *Archives of Pharmacal Research*, *34*, 2087-2099. https://doi:10.1007/s12272-011-1212-y

Kwak, H., Hegeman, A. D., & Park, S. (2014). Seasonal changes in metabolic profiles of galls and leaves of Rhus chinensis using gas chromatography mass spectrometry and liquid chromatography quadrupole time-of-flight mass spectrometry. *Journal of Plant Biology*, *57*, 127-135. https://doi:10.1007/s12374-013-0498-3

Liu, P., Yang, Z. X., Chen, X. M., & Foottit, R. G. (2014). The effect of the gall-forming aphid Schlechtendalia chinensis (Hemiptera: Aphididae) on leaf wing ontogenesis in Rhus chinensis (Sapindales: Anacardiaceae). *Annals of the Entomological Society of America*, *107*(1), 242-250. https://doi.org/10.1603/AN13118

Mahboubi, M. (2020). Quercus infectoria fruit hulls and galls and female genital disorders. *Clinical Phytoscience*, *6*, 44. https://doi.org/10.1186/s40816-020-00194-9

Mirpour, M., Gholizadeh Siahmazgi, Z., & Sharifi Kiasaraie, M. (2015). Antibacterial activity of clove, gall nut methanolic and ethanolic extracts on Streptococcus mutans PTCC 1683 and Streptococcus salivarius PTCC 1448. *Journal of Oral Biology and Craniofacial Research*, *5*(1), 7-10. https://doi:10.1016/j.jobcr.2015.02.002

Orhan, N., Deliorman Orhan, D., Gökbulut, A., Aslan, M., & Ergun, F. (2017). Comparative analysis of chemical profile, antioxidant, in-vitro and in-vivo antidiabetic activities of Juniperus foetidissima Willd. and Juniperus sabina L. *Iranian Journal of Pharmaceutical Research*, *16*, 64-74.

Orhan, N., Erdogan Orhan, I., & Ergun, F. (2011). Insights into cholinesterase inhibitory and antioxidant activities of five Juniperus species. *Food and Chemical Toxicology*, *49*(9), 2305-2312.

Özdemir, A., Yildiz, M., Senol, F. S., Şimay, Y. D., Ibişoglu, B., Gokbulut, A., … Ark, M. (2017). Promising anticancer activity of Cyclotrichium niveum L. extracts through induction of both apoptosis and necrosis. *Food and Chemical Toxicology*, *109*, 898-909. https://doi: 10.1016/j.fct.2017.03.062

Patni, V., Sharma, N., & Mishra, P. (2012). Oak (Quercus leucotrichophora) Galls, as an intense source of natural gallic acid. *LS International Journal of Life Sciences*, *1*(3), 186-191. https://doi: 10.5958/j.2319-118X.1.3.013

Rather, M. A., Dar, B. A., Dar, M. Y., Wani, B. A., Shah, W. A., Bhat, B. A., . . . Qurishi, M. A. (2012). Chemical composition, antioxidant and antibacterial activities of the leaf essential oil of Juglans regia L. and its constituents. *Phytomedicine*, *19*(13), 1185-1190.

Rauf, A., Uddin, G., Siddiqui, B. S., Khan, H., Shah, S. U., Ben, Hadda, T., . . . Khan, A. (2016). Antinociceptive and anti-inflammatory activities of flavonoids isolated from Pistacia integerrima galls. *Complementary Therapies in Medicine*, *25*, 132-138. https://doi: 10.1016/j.ctim.2016.02.002

Republic of Türkiye Ministry of Health, Early diagnosis saves lives. https://www.saglik.gov.tr/EN-102478/early-diagnosis-saves-lives.html, accessed 22 April 2024.

Salimi, M., Majd, A., Sepahdar, Z., Azadmanesh, K., Irian, S., Ardestaniyan, M. H., . . . Rastkari, N. (2012). Cytotoxicity effects of various Juglans regia (walnut) leaf extracts in human cancer cell lines. *Pharmaceutical Biology*, *50*(11), 1416-1422. https://doi.org/10.3109/13880209.2012.682118

Shim, Y. J., Doo, H. K., Ahn, S. Y., Kim, Y. S., Seong, J. K., Park, I. S., & Min, B. H. (2003). Inhibitory effect of aqueous extract from the gall of Rhus chinensis on alpha-glucosidase activity and postprandial blood glucose. *Journal of Ethnopharmacology* 2003, *85*(2-3), 283-287. https://doi: 10.1016/s0378-8741(02)00370-7

Silva, M. J. D., Carvalho, A. J. S., Rocha, C. Q., Vilegas, W., Silva, M. A., & Gouva, C. M. C. P. (2014). Ethanolic extract of Mimosa caesalpiniifolia leaves: Chemical characterization and cytotoxic effect on human breast cancer MCF-7 cell line. *South African Journal of Botany*, *93*, 64–69. https://doi.org/10.1016/j.sajb.2014.03.011

Sobierajska, K., Ciszewski, W. M., Sacewicz-Hofman, I., & Niewiarowska, J. (2020). Endothelial cells in the tumor microenvironment. *Advances in Experimental Medicine and Biology*, *1234*, 71-86. https://doi.org/10.1007/978-3-030-37184-5_6

Sukor, N. F., Jusoh, R., Kamarudin, N. S., Abdul Halim, N. A., Sulaiman, A. Z., & Abdullah, S. B. (2020). Synergistic effect of probe sonication and ionic liquid for extraction of phenolic acids from oak galls. *Ultrasonics Sonochemistry*, *62*, 104876. https://doi: 10.1016/j.ultsonch.2019.104876

T.C. Gıda Tarım ve Hayvancılık Bakanlığı. (2017). Ceviz entegre mücadele teknik talimatı. *Tarımsal Araştırmalar ve Politikalar Genel Müdürlüğü Gıda ve Kontrol Genel Müdürlüğü*, Ankara (in Turkish).

Tennakoon, K. U., & Pate, J. S. (1996). Effects of parasitism by a mistletoe on the structure and functioning of branches of its host. *Plant, Cell Environment*, *19*(5), 517-528. https://doi.org/10.1111/j.1365-3040.1996.tb00385.x

WHO, World Health Organization Cancer. https://www.who.int/health-topics/cancer#tab=tab_1, accessed 15 March 2024.

Zhou, Y., Zheng, J., Li, Y., Xu, D. P., Li, S., Chen, Y. M., & Li, H. B. (2016). Natural polyphenols for prevention and treatment of cancer. *Nutrients*, *8*(8), 515. https://doi.org/10.3390/nu8080515

Zongo, C., Savadogo, A., Ouattara, L., Bassole, I. H. N., Ouattara, C. A. T., Ouattara, A. S., . . . TraoreI, A. S. (2010). Polyphenols content, antioxidant and antimicrobial activities of Ampelocissus grantii (Baker) Planch.(Vitaceae): a medicinal plant from Burkina Faso. *International Journal of Pharmacology*, *6*(6), 880–887. https://doi: 10.3923/ijp.2010.880.887