Research article

Guven L., et al., Evaluation of LC-MS/MS Analysis and In Vitro Biological Activities of *Rosa pimpinellifolia* Root, Pseudo-fruit, and Seed extracts**.** International Journal of Life Sciences and Biotechnology, 2022. 5(3): p. 480-503. DOI: 10.38001/ijlsb.1108547

Evaluation of LC-MS/MS Analysis and *In Vitro* Biological Activities of *Rosa pimpinellifolia* Root, Pseudo-fruit, and Seed extracts

Leyla Güven[1]*, Ufuk Özgen[2], Handan Gökben Sevindik[3], İclal Ağan[4], Mehmet Koca[5], İbrahim Turan[6], Selim Demir[7], Yüksel Aliyazıcıoğlu[8]

ABSTRACT

In this study, various extracts of *Rosa pimpinellifolia* antioxidant, anticholinesterase, and antityrosinase properties were determined with the total phenolic and flavonoid contents spectrophotometrically. The phytochemical composition of the methanol extract was analyzed using LC-MS/MS. In addition, the extracts of *R. pimpinellifolia* antimicrobial activity by disc diffusion and microdilution method, and antigenotoxic activities by comet assay were explored. The ethyl acetate extract of the root (EAR) had higher antioxidant activities at 10 µg/mL with inhibition of 39.7, 91.2, and 39.5% respectively in the $DPPH^{\cdot}$, $ABTS^{\cdot+}$, and superoxide anion radical scavenging activity assay than standard antioxidant molecules. The polyphenolic contents of the EAR and the ethyl acetate extract of the seed (EAS) were found to be 378.2 ± 0.477 and 305.39 ± 0.568 µg gallic acid equivalent GAE/mg respectively. The EAR showed butyrylcholinesterase activity with 19% inhibition at 100 µg/mL concentration and higher activity at 500 µg/mL with inhibition of 50% in the tyrosinase inhibitory assay than the other *R. pimpinellifolia* extracts. The extracts of *R. pimpinellifolia* exhibited antimicrobial activity against *Staphylococcus aureus* and *Candida albicans*. The extracts of *R. pimpinellifolia* did not show any antigenotoxic effect up to the concentration of 1000 µg/mL. In LC-MS/MS analysis, cyanidin-3-*O*-Glucoside and isoquercetin in the pseudo-fruit; procyanidin B2 and catechin in the root were the major phenolic compounds.

ARTICLE HISTORY
Received
25 April 2022
Accepted
09 August 2022

KEYWORDS
Rosa pimpinellifolia, Rosaceae, biological activities, LC-MS/MS

[1] Department of Pharmaceutical Botany, Faculty of Pharmacy, Atatürk University, Erzurum, 25240, Turkey
[2] Department of Pharmacognosy, Faculty of Pharmacy, Karadeniz Technical University, Trabzon, 61080, Turkey
[3] Department of Pharmacognosy, Faculty of Pharmacy, Atatürk University, Erzurum, 25240, Turkey
[4] Department of Medical Microbiology, Giresun University Prof. Dr. A. Ilhan Özdemir Training and Research Hospital, Giresun, 28100, Turkey
[5] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Atatürk University, Erzurum, 25240, Turkey
[6] Department of Biochemistry, Faculty of Pharmacy, University of Health Sciences, Ankara, Turkey
[7] Department of Nutrition and Dietetics, Faculty of Health Sciences, Karadeniz Technical University, Trabzon, Turkey
[8] Department of Medical Biochemistry, Faculty of Medicine, Karadeniz Technical University, 61080 Trabzon, Turkey
*Corresponding Author: Leyla Guven, leyla.guven@atauni.edu.tr

Introduction

Rosa pimpinellifolia belonging to the Rosaceae family is presented by 27 species in Turkish flora. It is a deciduous shrub that grows well on calcium soils. It is known as "Karakuşburnu, Koyungözü" in Turkey, "Shrub Rose" in Europe. It is used in the treatments of hemorrhoids, infections, flu, abdominal pain, and anemia in Turkish folk medicine by decoction of the roots and pseudo-fruits of the species [1-3].

Flavonoids [4], aurons [5], phenylethanoids [6], saponosides [7], steroids [8], sesquiterpenes [9], carotenoids [10], tannins [4], fatty acids [11], and volatile compounds [12] were identified in *Rosa* species. Furthermore, according to some studies, *R. pimpinellifolia* has condensed tannins (catechin, epigallocatechin, procyanidin B2) [13], anthocyanidins (cyanidin-3-*O* glucosides) [14,15], phenolic acids (ascorbic acid, caffeic acid) [15], and essential fatty acids (linoleic, oleic, linolenic acids) [16].

With regard to some studies also, *Rosa* species have antibacterial [17], anti-inflammatory [18], antioxidant [19], anticancer [20], antidiabetic [21], hepatoprotective [22], anxiolytic [23], antiobesity [24], anti-conflict [25], purgative [26], kidney stone reducer [27], antidiarrheal [28], antiallergic [29], antiproliferative [30], antinociceptive [31], antiulcerogenic [32], antihypertensive [33], antifungal [34], anti-HIV, and antitussive [35] activities. It is also reported that *Rosa* species are used in skin disorders [36], atherosclerosis, arthritis, brain dysfunction immunodeficiency [21], and hemorrhoids [37].

Medicinal plants are consumed, because of having thousands of different phenolic components such as reductive agents, free radical scavengers, and quenchers of singlet oxygen formation. It also plays an important role in the control of cancer and other diseases [38]. Oxidative stress is defined as the deterioration of the oxidative balance resulting from the deficiency of antioxidants, which is caused by the increase of reactive oxygen species (ROS) such as hydroxyl radicals, superoxide radicals, and hydrogen peroxides formed during cellular metabolism. ROS causes cell damage and the death of intracellular macromolecules. Moreover, ROS-induced-DNA damage is associated with many diseases. Polyphenolic compounds in plants protect cells against the harmful effects of ROS by donating electrons, chelating metal ions, and stimulating antioxidant enzymes [39].

Many medical professionals seek natural and safe antibiotics due to the increase in antibiotic use and the resistance of bacterial strains [40]. Therefore, the antimicrobial effects of medicinal plants are extensively studied in recent years. In addition, plants are used as an additive in the preservation of raw and processed foods and medicines by making use of their antimicrobial properties [41].

Tyrosinase, commonly found in plants, microorganisms, and animals, is a copper-containing enzyme involved in the synthesis of melanin in the skin, hair, and eyes. The enzyme has a key role in melanization [42, 43]. Recently, the search for safe and effective tyrosinase inhibitors has gained importance in the treatment of hyperpigmentation problems such as skin scarring caused by extreme melanin synthesis in the body and hypopigmentation problems caused by insufficient synthesis of melanin such as psoriasis and vitiligo. These enzyme inhibiting agents can be used in the treatment of hyperpigmentation problems. [44-46]. Tyrosinase inhibitors are utilized in the cosmetics industry due to their skin whitening effect [47] and in the food industry due to their ability to inhibit the enzymatic browning of food products [48]. It has also been reported that tyrosinase may cause dopamine neurotoxicity and that neurodegeneration is associated with Parkinson's disease [49]. Therefore, inhibition of tyrosinase is a popular target in drug development and research for Parkinson's disease [50].

Alzheimer's disease (AD), a neurodegenerative disease, is characterized by a low level of the neurotransmitter acetylcholine (ACh). In the treatment of this disease, it is often aimed to prolong the availability of ACh by stimulating the cholinergic receptors or using agents that increase or improve acetylcholine levels. Therefore, inhibition of acetylcholine degrading enzymes (acetylcholinesterase (AChE) and butyrylcholinesterase (BChE)) is preferred in AD therapy. Potential sources of AChE and BChE inhibitors in natural products, provide for the development of drug molecules. Cheap, safe, alternative AChE and BChE inhibitory plants are researched in this publication [51].

The aim of this study is to reveal the chemical characterization of the root, pseudo-fruit, and seed extracts of *R. pimpinellifolia* by LC-MS/MS and to investigate their *in vitro* antioxidant, antityrosinase, anticholinesterase, antimicrobial, antigenotoxic, and anticancer activities.

Materials and Methods

Chemicals and reagents

Gallic acid monohydrate, sodium carbonate, Folin-Ciocalteu phenol Reagent (FCR) (Sigma-Aldrich) for determination of total phenolic compound quantity. Aluminum chloride; hydrochloric acid (37%), routine hydrate (Sigma-Aldrich) for determination of total flavonoids. Ammonium thiocyanate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS), 1,1 diphenyl-2-picryl-hydrazole (DPPH), gallic acid monohydrate, (±) 6- hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), D-methionine, nitrotetrazolium blue chloride (NBT), potassium persulfate, riboflavin, sodium phosphate monobasic (NaH_2PO4), (±) α-tocopherol (Sigma-Aldrich) for determination of antioxidant activity. AChE, acetylthiocholine iodide (ATCI), BChE, butyrylthioline iodide (BTCI), 5,5-dithiobis- (2-nitrobenzoic acid) (DTNB), donepezil, tris hydrochloride (Sigma-Aldrich) for determination of anticholinesterase activities. Tyrosinase, 3,4-dihydroxy-L-phenylalanine (L-DOPA), L-tyrosine, α-kojic acid, sodium phosphate monobasic (NaH_2PO4) (Sigma-Aldrich) for determination of antityrosinase activity.

Fetal bovine serum (Biochrom), penicillin/streptomycin (Gibco), eagle's minimal essential medium (Lonza), polylysine solution (Biochrom), normal boiling point agarose (Lonza), dimethyl sulfoxide (Amresco), low boiling point agarose, NaOH, phosphate buffer tablet, NaCl triton X-100, ethylenediamine tetraacetic acid, H_2O_2, tris, trypan blue solution, ethidium bromide (Sigma-Aldrich) for investigation of antigenotoxic effects.

1,3-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, 7-acetyl sideroxol, apigenin, caffeic acid, catechin, chlorogenic acid, cyanidin-3-*O*-glucoside, emodin, (-)-epicatechin, epigallocatechin, epigallocatechin gallate, eubotriol, fumaric acid, gallic acid, isoquercetin, kaempferol, luteolin, luteolin-5-*O*-glu, luteolin-7-*O*-glu, pelargonin chloride, punicalagin, procyanidin B2, pyrogallol, quercetin, quercetin-3-*O*-arabinoside, quercitrin, ursolic acid, vannilin, p-hydoxybenzoic acid, t-ferulic acid were purchased from Sigma-Aldrich for determination of phenolic compound via LC-MS/MS.

Plant materials

The pseudo-fruits, seeds, and roots of *R. pimpinellifolia* were collected from Erzurum Kosk village in August 2013 (1900 m) and identified by Ufuk Özgen. The voucher specimen (ATA 9876) has been deposited in the herbarium of the Faculty of Sciences, Atatürk University, Erzurum, Turkey.

Preparation of the extracts

20 g pseudo-fruits, seeds, and roots of the plant were extracted separately with 300 mL methanol, ethyl acetate, and water for 12 h at 25°C. The solvents were removed under vacuum.

Determination of the total phenolic content

The Folin-Ciocalteau method, modified by Singleton and Rossi, was used to determine the total phenol content [52-54]. The plant extract was dissolved in distilled water to 1000 μg/mL. Then, 1000 μL of each sample was taken and distilled water was added to 23 mL. After completion, 0.5 mL of Folin-Ciocalteau reagent and after 3 min 1.5 mL of 2% Na_2CO_3 solution were added. After the mixture was stirred for 2 hrs on a magnetic stirrer at room temperature, the absorbances were read on a spectrophotometer against the blindly used distilled water at a wavelength of 765 nm. On the other hand, gallic acid dilutions were prepared at concentrations of 100, 200, 400, and 600 μg/mL to create the calibration curve, and gallic acid dilutions were added to the sample and other solutions were added exactly. The method is based on electron transfer from phenolic compounds to molybdenum in an alkaline environment. The reduced molybdenum turns into a blue complex, which can be measured spectrometrically at a wavelength of 760 nm.

Determination of the total flavonoid determination

The total flavonoid amounts of the plant samples were determined according to the method developed by Lar'kina et al 2009 and were calculated as rutin equivalent (RE) [55]. For this purpose, 30 mL of 70% ethanol was added to the 1 g powdered drug of both parts of the plant and heated at 60 °C for 1 hr under reflux. The process was repeated three times. The extracts were filtered into a 100 mL graduated flask and the volumes were made up to 100 mL with ethanol (solution A).

Sample solution: 2 mL of solution A, 4 mL of 10% ethanolic $AlCl_3$, and 0.1 mL of dilute HCl were placed in a flask and the volume was completed to 50 mL with 95% ethanol. Absorbances were measured against reference solutions at 410 nm after 20 min.

Reference solution: 2 mL of solution A and 0.1 mL of dilute HCl was placed in a flask and the volume was made up to 50 mL with 95% ethanol.

Rutin sample solution: 0.05 g routinely weighed and placed in a volumetric flask. 10 mL of 95% ethanol was added and dissolved by heating in an 80 °C water bath. After complete dissolution, the volume was made up to 50 mL with 95% ethanol (solution A).

Rutin test solution: 1 mL of solution A was taken into a volumetric flask, 4 mL of 10% $AlCl_3$ solution was added and the volume was completed to 50 mL with 95% ethanol. Absorbance at 410 nm was measured. The levels of total flavonoid contents in extracts were determined in triplicates and the averages were taken. Calculations were made according to the following equation.

X= [D (Sample)xM (Rutin)/ D (Rutin)xM (Material)] x100

D (Sample): Absorbance of the sample solution

D (Rutin): Absorbance of rutin test solution

M (Material): Weight of raw material (g)

M (Rutin): Weight of the rutin (g)

X: Total amount of flavonoids calculated over rutin (%)

Evaluation of the antioxidant capacity

DPPH radical scavenging assay

The free radical scavenging activity of the extract was established by DPPH assay according to the method developed by Marsden S. Blois [56]. The extracts were prepared in concentrations of 10, 50, 250, 500, and 1000 µg/mL and the DPPH stock solution at a concentration of 1 mM and dissolved in ethanol. 210 µL of stock solution and 70 µL of DPPH solution were added from each sample. The samples were shaken for approximately 1 min and stored at 37 °C in the dark for 30 min. At the end of the period, the absorbances of the samples were read in a spectrophotometer against Ethanol used blindly at 517 nm wavelength. For the control sample, 210 µL of Ethanol and 70 µL of DPPH solution were used, and tocopherol was used as the standard antioxidant. The % inhibition of the samples

against DPPH free radical was calculated according to the formula given below and tocopherol was used as a reference.

DPPH radical scavenging capacity (%) = $[(A_1 - A_2) / A_1] \times 100$

A_1 = absorbance value of control sample containing DPPH and Ethanol

A_2 = absorbance value found after sample addition to DPPH solution

ABTS radical cation decolorization assay

ABTS cation radical scavenging capacity was determined according to the method made by Re et al [57]. Firstly, 2 mM ABTS solution was prepared. ABTS $\bullet^+$ was obtained by adding 2.45 mM potassium persulfate solution to this solution in a ratio of 1:1. The extracts were transferred to the wells with 140 µL of stock solutions at concentrations of 10, 50, 250, 500, and 1000 µg/mL, and 100 µL of the ABTS $\bullet^+$ solution and shaken for 1 min. 140 µL of ABTS $\bullet^+$ solution and 100 µL (0.1 M pH = 7.4) phosphate buffer were used as control samples. The absorbance value of the control sample at 734 nm should be 0.700 ± 0.025. At the end of the 30 min waiting period, the absorbances at 734 nm were recorded against the buffer-formed blank. Trolox was used as the standard compound. Calculations of ABTS cation radical radical scavenging capacities in the percent of the extracts and compounds were made according to the following equation. ABTS $\bullet^+$ sweeping capacity (%) = $[(A_1 - A_2) / A_1] \times 100$

A_1 = Absorbance value of control sample containing only ABTS $\bullet^+$ and buffer solution

A_2 = absorbance value found after addition of samples to ABTS $\bullet^+$ solution

Superoxide anion radical scavenging assay

Superoxide anion radical scavenging tests were performed using the method developed by Zhishen et al [58]. 10, 50, 250, 500, and 1000 µg/mL stock solutions of the extracts and compounds at different concentrations were prepared. 0.5 mL each of riboflavin (1.33×10^{-5} M), methionine (4.46×10^{-5} M) and NBT (8.15×10^{-5} M) solutions were added onto 0.5 mL of sample solution. The resulting reaction mixture was stimulated with 20 W fluorescent light for 40 min at room temperature. At the end of the period, the absorbance of each sample was recorded at 560 nm against a water-based blank. Phosphate buffer (0.05 M, pH=7.8) was used in the control sample instead of the stock solution. The calculations of the superoxide anion radical scavenging capacities in percent of the samples were made according to the following equation.

$O_2^{\bullet+}$ sweeping capacity (%) = [(A_1 - A_2) / A_1] x 100

A_1 = Absorbance value of the control sample

A_2 = Absorbance value found after addition of samples

Anticholinesterase activity

AChE inhibitory activity was determined using a modified spectrophotometric Ellman's method [59]. The prepared extracts were dissolved in methanol and diluted to concentrations of 1000, 750, and 500 µg/mL. However, with the solutions to be added to the wells later, final concentrations will be 100, 75, and 50 µg/mL. 50 µL of tris buffer solution, 125 µL of 3 mM DTNB solution, 25 µL of AChE enzyme solution at a concentration of 0.2 U/mL, and 25 µL of dilutions of the samples prepared at concentrations of 500, 750 and 1000 µg/mL were added to the wells. The resulting mixture was allowed to incubate for 15 min at 37 °C. After the 15 min incubation period, 25 µL of 15 mM ATCI solution was added to each well. As controls, 25 µL methanol, 125 µL DTNB, 50 µL tris buffer, 25 µL AChE, and after 15 min 25 µL ATCI were added again with a micropipette. For the blind, 25 µL of methanol, 75 µL of Tris buffer, 125 µL of DTNB, and 25 µL of AChE were added using a micropipette. The absorbances of the reaction mixtures were recorded at 412 nm for 20 min with a microplate reader. Calculations were made using the following formula:

Enzyme inhibition (%) = [(A_1 - A_2) / A_1] x 100

A_1: Absorbance value of the control sample

A_2: Absorbance value measured in the presence of samples

Antityrosinase activity

The tyrosinase inhibition capacity of the methanol, ethyl acetate, and aqueous extracts of the root and aqueous extracts of the pseudo-fruits of *R. pimpinellifolia* were determined spectrophotometrically with the method modified by Likhitwitayawuid and Sritularak using L-DOPA as substrate [43, 60]. Dilutions of the methanol extracts were prepared as 10 mg/mL in concentrations of 25, 50, 100, and 500 µg/mL in %5 DMSO with potassium phosphate buffer (pH = 6.8). α-Kojic acid (25, 50, 100, and 500 µg/mL) was used for positive control. The concentrations were determined with ELISA. Absorbances of the samples and the control were read at a wavelength of 490 nm and the inhibitions of tyrosinase were calculated according to the formula given. % Inhibition = [[(A-B)-(C-D)]/(A-B)]x 100.

Antimicrobial activity

The antimicrobial effect of pseudo fruit and root extracts was determined by using Disk diffusion and micro-dilution methods [40].

Microorganisms

Escherichia coli, Klebsiella pneumoniae, and *Pseudomonas aeruginosa* from Gram-negative bacteria; *Enterococcus faecalis, Staphylococcus aureus*, and *S. epidemidis* strains from Gram-positive bacteria were used to determine the antibacterial activity. *Candida albicans* and *C. tropicalis* strains were used to investigate the antifungal activity. *Staphylococcus aureus* (ATCC BAA977) and *Candida albicans* (ATCC 14053) standard strains were studied in the microdilution method. Oxacillin, standard antibiotic discs; fluconazole, standard antifungal discs were used to determine the sensitivity of each microbial species tested and to control.

Disc diffusion assay

Suspensions were prepared according to 0.5 McFarland (108 CFU/μL for bacteria, 106 CFU/μL for yeasts) from bacteria and yeast strains were grown on solid media. Sterile swabs were dipped into each of the suspensions, mixed by dipping and the suspensions were spread over the surface of the media with the swab. 15 μL of extracts at 100 mg/mL concentrations dissolved in DMSO under aseptic conditions were absorbed into sterile discs of 6 mm diameter. After this process, discs impregnated from the extracts and standard antibiotic discs were placed in Petri dishes at regular intervals. The Petri dishes in which bacteria were inoculated were incubated at 37 °C for 24 hrs, and the Petri dishes in which fungi were inoculated at 37 °C for 48 hrs. At the end of the incubation, the diameters of the inhibition zones occurring around the discs were measured. The zone diameters of standard antibiotic discs and only DMSO impregnated discs were used as controls. Only DMSO impregnated disk was used as a control for yeasts.

Broth dilution assay

MIC (Minimum Inhibitory Concentration) values were used to measure the sensitivity of the disk diffusion method. This method was made in accordance with the CLS (Clinical and Laboratory Standards Institute) criteria and Aliyazicioglu et al method [40].

Anti-genotoxic Activity

Cell culture

Human foreskin fibroblast (BJ) cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The cells were grown in EMEM supplemented with 10% FBS, 2 mM glutamine, 1% penicillin, and streptomycin at 37°C.

Determination of H_2O_2 concentration

A total of $2x10^5$ BJ cells were cultured in a T-25 flask. After 24 h, cells were handled with 10-30 μM for 5 min to determine the concentration resulting in DNA damage, but not toxicity. After incubation, cells were trypsinized and centrifuged for comet assay protocol.

Determination of extract concentration

BJ cells were pre-incubated with various concentrations of *R. pimpinellifolia* extracts (25-1000 μg/mL) for 60 min. After that, flasks were washed with PBS, and cells were handled with 20 μM H_2O_2 for 5 min. Then, flasks were washed, trypsinized, and centrifuged for comet assay protocol.

Cell viability and Comet assay

The determination of the possible protective effect of different extracts of *R. pimpinellifolia* on DNA damage induced by hydrogen peroxide (H_2O_2) in BJ cells was carried out using the comet assay. The alkali protocol of the Comet method, it is aimed to determine chain breaks at low levels with high sensitivity. The alkaline comet protocol developed by Singh et al. includes 7 steps: Preparation of microscopic slides, lysis of cells to release DNA, alkaline treatment (pH>13) to reveal lesions such as chain breakage, electrophoresis under alkaline conditions (pH>13), neutralization of alkaline conditions, DNA staining and comet imaging, and comet scoring. For each treatment condition, 100 randomly selected cells from each slide were evaluated for DNA damage visually using a 40x objective on a fluorescent microscope (Nikon Eclipse E800, Tokyo, Japan). The selected cells were classified between 0 and 3, from non-damaged to most damaged, according to tail length. Excessively long tails and DNA spectra scored 4 were not included. All slides were scored with the following formula with a maximum damage possibility of 300 [61, 62].

Comet score = $(1 \times n_1) + (2 \times n_2) + (3 \times n_3)$ (n: cell number for every score)

LC-MS/MS analysis

Preparing standard solutions

Stock solutions of secondary metabolites were prepared in methanol solvent at 100 ppm concentrations. Before starting the study, methanol-water (v:v, 60:40) solutions at different concentrations between 5, 2.5, 1, 0.5, 0.25, and 0.1 ppm were prepared from 10 ppm intermediate stock for each standard compound and prepared in LC-MS / MS. It was kept at + 4 °C before being analyzed.

Determination of the phenolic compounds

The extracts were weighed about 3 mg in weight and placed in Eppendorf tubes. 4000 ppm master stock solutions were prepared in 0.75 mL of methanol solvent. Curcumin was used as the internal standard (IS), a 100 ppm IS stock solution was prepared in methanol solvent and added to the injection vial at a concentration of 5 ppm. All samples were kept at + 4°C before being analyzed. The calibration curve of phenolic compounds was obtained by plotting working standard solutions with LC-MS/MS data.

Instrumental analysis and chromatographic conditions

Zivak Tandem Gold Triple Quadrupole mass spectrometer (Istanbul, Turkey) device was used in the analysis. The Synergy Max C18 column (250 x 2 mm i.d, 5 µm particle size) was used as the chromatography column. The gradient elution steps indicated in Table 1 below were applied.

Table 1 Gradient elution steps for the detection of secondary metabolites

Minute	Mobile Phase A (%)	Mobile Phase B (%)
0.00-1.00	55	45
1.00-20.00	0	100
20.01-23.00	55	40

Mobile phase A **:** % 0.05 formic acid -HPLC grade water
Mobile phase B **:** % 0.05 formic acid: methanol
Flow rate **:** 0.25 mL/min
Injection volume **:** 10 µL
Detector **:** DAD 280 nm
Column **:** RP-C18
Column temperature **:** 30 °C

LC-MS/MS Procedure

Zivak Tandem Gold Triple Quadrupole mass spectrometer device was used in the analysis of the samples. During optimization experiments, the best mobile phase composition was determined after satisfactory results were obtained with high ionization abundance and peaks were separated successfully. Optimum electrospray ionization (ESI) parameters were used: CID gas pressure 2.0 mTorr, 4000 V ESI needle voltage, 600 V spray protection voltage, 300 °C drying gas temperature, 55 psi nebulizer gas pressure, and 35 psi drying gas pressure. The measurement method was established by determining the LC-MS/MS characteristics of standard compounds.

Results

Total flavonoid contents of the extracts

The total flavonoid contents of the root and pseudo-fruits of the *R. pimpinellifolia* were calculated as % of the rutin (mg RE/dry drug) and the results are given in Table 2.

Table 2 Total flavonoid contents of the extracts

	Root	Pseudo-fruit
Total Flavonoid Amounts Calculated Over Rutin (%)	0.12	0.21

Total phenolic contents of the extracts

Total phenolic contents of the various extracts of *R. pimpinellifolia* plant were determined as gallic acid equivalents. As shown in Table 3, the phenolic contents of the extracts are higher than flavonoid contents. Among the extracts of *R. pimpinellifolia*, the EAR has the highest total phenolic content (378.2 ± 0.477 μg GAE/mg extract).

Antioxidant activities

The DPPH radical scavenging activity

The DPPH radical scavenging capacities of *R. pimpinellifolia* extracts and the standard antioxidant compound α-tocopherol at a concentration of 10 μg/mL are shown in % inhibition in Table 3.

$ABTS^+$ cation radical scavenging capacity

The ABTS radical scavenging capacities of *R. pimpinellifolia* extracts and the standard antioxidant compound trolox at a concentration of 10 µg/mL are shown in % inhibition in Table 3.

Superoxide anion radical scavenging capacity

Superoxide Anion radical scavenging capacities of *R. pimpinellifolia* extracts and the standard antioxidant compound Trolox at a concentration of 10 µg/mL are shown in % inhibition in Table 3.

Table 3 Total phenolic contents and antioxidant activities of *R. pimpinellifolia* various extracts at 10 µg/mL

Samples*	Total Phenolic Compound (µg GAE/mg extract)	DPPH Radical Scavenging Activity	$ABTS^+$ Cation Radical Scavenging Capacity	Superoxide Anion Radical Scavenging Capacity
MR	236.36 ± 0.4	30.9	57.9	21.9
EAR	378.2 ± 0.4	39.7	91.2	39.5
WR	237.087 ± 0.1	15.6	40.2	25.2
WPF	195.6 ± 0.2	30.4	32.9	31.2
MS	224.12 ± 0.2	11.1	56.7	21.1
EAS	305.39 ± 0.5	21.2	39.1	30.0
α-tocopherol		12.5		
trolox			68.4	16.3

(*MR: methanol extract of *R. pimpinellifolia* root, EAR: ethyl acetate extract of *R. pimpinellifolia* root, WR: water extract of *R. pimpinellifolia* root, WPF: water extract of *R. pimpinellifolia* pseudo-fruit, MS: methanol extract of *R. pimpinellifolia* seed, EAS: ethyl acetate extract of *R. pimpinellifolia* seed)

Anticholinesterase activity

The AChE inhibitory activities of the extracts of *R. pimpinellifolia* and the standard donepezil at a concentration of 100 µg/mL are shown in % inhibition in Table 4. The extracts of *R. pimpinellifolia* did not show anticholinesterase activity against AChE but showed BChE with 19% inhibition at 100 µg/mL concentration.

Antityrosinase activity

The α-Kojic acid used as a standard antioxidant shows 92.7% inhibition at 500 µg/mL. the concentration range to be studied has been determined as 25-500 µg/mL. The % inhibition values of the extracts and *α*-Kojic acid in each dose range are shown in Table 4.

Table 4 BChE and tyrosinase inhibitory activities of *R. pimpinellifolia* extracts

	BChE % Inhibition (100 µg/mL)	Tyrosinase % Inhibition (500 µg/mL)
MR	14	61.5
EAR	19	50
WR	0	37.7
WPF	9	37.9
MS	16	31.9
EAS	18	32.9
Donepezil	97	
α-Kojic acid		92.7

Antimicrobial activity

Extracts of the root, pseudo-fruits, and seeds of *R. pimpinellifolia* plant made by disc diffusion method were found to be effective against *Staphylococcus aureus* and *Candida albicans* in the determination of antimicrobial activity. On the other hand, it was found to be ineffective against bacteria and yeasts such as *Escherichia coli, Enterococcus faecalis, Candida tropicalis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus epidermidis.*

The MIC (Minimum Inhibitory Concentration) values in the Microdilution method used to measure the sensitivity in the disk diffusion method were compared with the disk diffusion method. The results of both experiments confirm each other.

The results of the Microdilution and Disk diffusion method applied to determine the antimicrobial activity of the extracts are shown in Table 5.

Table 5 Antimicrobial activity (MIC values and zones of inhibition) of extracts of *R. pimpinellifolia* plant against bacteria and yeast species

MIC (µg/mL) and Zone Inhibition (mm), values	MR	EAR	WR	WPF	MS	EAS	Standard Drog (µg/mL)	Negative Control (DMSO)	Positive Control Standard antibiotic disc µg/mL
S. aureus (ATCC BAA977)	1000 7	1000 11	1000 10	1000 8	>1000 8	1000 11		-	0.25 (OKS)
C. albicans (ATCC 14053)	250 20	500 18	250 17	250 15	1000 20	1000 20		-	1 (FLU)

NT: Not tested, OKS: Oxacillin, FLU: Fluconazole

Antigenotoxic Activities

In this study, visual analysis and Comet analysis were performed to reveal the DNA damage created in fibroblast cells with H_2O_2 at a concentration range of 10-30 μM. Comet scoring was made using the scale with 0 for no damage and 3 for the greatest damage. Since the highest comet score according to this scale can be 300 and 20 μM H_2O_2 exhibiting the closest Comet score to this value was determined as the optimum damaging concentration in the next trials. (Table 6, 7)

Table 6 Comet scores (n=3) versus H_2O_2 given to fibroblast cells at increasing concentrations

H_2O_2 Concentration	**Comet Score**
10 μM H_2O_2	185.7±9.1
20 μM H_2O_2	300±5
30 μM H_2O_2	300+

Table 7 Comet scores (n = 4) showing the potential of different extracts of *R. pimpinellifolia* to inhibit H_2O_2-induced DNA damage

		25 μg/mL	50 μg/mL	100 μg/mL	250 μg/mL	500 μg/mL	1000 μg/mL
Negative Control	24.2±2.3						
Positive Control (20 μM H_2O_2)	300±5						
WPF→DMSO		300	300	300	300	300	300+
MPF→DMSO		300	300	300	300	300	300+
WPF→ water		300	300	300	300	300	300+
MR→DMSO		300	300	300	300	300	300+

LC-MS/MS analysis

Validation of experiments and uncertainty evaluation

The validation parameters were determined to be LOD (limit of detection), LOQ (limit of quantification), linearity, recovery, and repeatability. The LOD and LOQ values of standards for the LC-MS/MS method are given in Table 8. LOD and LOQ were determined by using the signal-to-noise method. A signal-to-noise ratio of three was accepted for estimation of LOD and signal-to-noise ratio of 10 was used for estimation of LOQ [63]. The repeatability in the intra-day (RSD%) values phenolics ranged between 0.1 – 5 mg/kg were obtained using

the corresponding peak area of 3 replicate analyses at approximately 2.5 mg/kg concentration level.

Table 8 Method validation and uncertainty parameters for phenolic compounds

	Compounds	Linear regression equation	R^2	LOD (mg/L)	LOQ (mg/L)	RSD (%)
1	7-acetylsideroxol	y=0.105x+0.0224	0.9834	0.46	1.55	5.61
2	Apigenin	y=0.182x+0.072	0.9940	0.15	0.50	4.01
3	Catechin	y=0.067x+0.035	0.9941	0.06	0.21	6.49
4	Chlorogenic acid	y=0.262x-0.004	0.9981	0.44	1.48	5.45
5	Cyanidin-3-*O*-glucoside	y=0.35x+0.01	0.9912	0.09	0.29	1.37
6	Emodin	y=0.15x+0.09	0.9806	0.58	1.95	2.46
7	Epigallocatechin	y=0.034x+0.022	0.9876	0.04	0.13	5.53
8	Epigallocatechin gallate	y=0.1175x-0.007	0.9957	0.09	0.32	4.79
9	Eubotriol	y=0.13x+0.09	0.9931	0.31	1.03	3.33
10	Fumaric acid	y=0.056x+0.015	0.9944	0.07	0.23	5.44
11	Gallic acid	y=0.040x-0.020	0.9986	0.54	1.82	7.23
12	Isoquercetin	y=0.323x+0.054	0.9974	0.56	1.87	9.42
13	Pelargonin chloride	y=0.189x+0.0041	0.9928	0.17	0.56	4.45
14	Procyanidin B2	y=0.049x+0.0067	0.9902	0.09	0.31	10.36
15	Pyrogallol	y=0.039x+0.014	0.9875	0.04	0.15	5.47
16	Quercetin	y=0.115x+0.027	0.9901	0.303	1.01	1.14
17	Quercetin-3-arabinoside	y=0.33x+0.04	0.9958	0.56	1.87	9.42
18	Quercitrin	y=0.02x-0.004	0.9976	0.02	0.06	4.28
19	Ursolic acid	y=0.015x+0.0044	0.9948	0.07	0.23	3.04

The concentration of each analyte was within the linear range and the concentration of the reported method was obtained from the calibration curve. In conclusion, the calculated concentrations were converted to μg/g of the crude extract sample, by using Equation (X)

$$Amount\ (\mu/g) = \left(\frac{(C_a\ x\ V\ x\ F)/1000}{m/1000}\right) \qquad (X)$$

Where Ca is the analyte concentration obtained by calibration curve (in mg/kg), V is the final diluted volume (in grams) before the analysis, m is the amount of the extract (in milligrams), F is the dilution factor, 1000 is the conversion factor. LC-MS/MS parameters of selected compounds were shown in Table 9.

Table 9 LC-MS/MS parameters of selected compounds

Compounds	Parent ion	Daughter ion	Capillary	Collision energy (V)	ESI mode
7-acetyl sideroxol	385.4	325	100	20	Positive
Apigenin	269	151	100	22	Negative
Catechin	289	245	50	15	Negative
Chlorogenic acid	353	191	80	14	Negative
Cyanidin-3-O-glucoside	449	287	90	19	Positive
Emodin	268.9	224.3	100	30	Negative
Epigallocatechin	305	125	80	18	Negative
Epigallocatechin gallate	457	169	100	14	Negative
Eubotriol	343	343	100	20	Positive
Fumaric acid	115	71	80	8	Negative
Gallic acid	168.6	124	50	13	Negative
Isoquercetin	463.3	300	90	25	Negative
Pelargonin chloride	595	271	80	30	Positive
Procyanidin B2	577.4	288.5	80	20	Negative
Pyrogallol	125	80	100	16	Negative
Quercetin	301	178,5	120	16	Negative
Quercetin-3-arabinoside	463.3	300	80	21	Negative
Quercitrin	471.9	309.9	60	16	Positive
Ursolic acid	455.6	455.1	50	10	Negative

The EURACHEM/CITAC guide was used for the evaluation of sources and quantification of uncertainty of the LC-MS/MS method [64]. The maximum contribution comes from the calibration curve. Detailed procedures of uncertainty evaluation were reported previously in the literature [65]. To obtain expanded uncertainty, combined standard measurement uncertainty has to be multiplied by 2 (coverage factor) at 95% confidence level. The expanded relative uncertainties for all the compounds in each plant sample are given in Table 8. In LC/MS/MS analysis, Cyanidin-3-*O*-Glucoside and isoquercetin in the pseudo-fruit; procyanidin B2 and catechin in the root were the major phenolic compounds. The results of the chemical compounds were given in Table 10.

Table 10 LC-MS/MS Quantitative Analysis Results of Secondary Metabolites (μg/g)

Compounds	Pseudo-fruit	Root
7-acetylsideroxol	18,80±3,65	83,60±16,25
Apigenin	37,39±3,67	41,11±4,03
Catechin	48,77±5,71	2211,13±258,77
Cyanidin-3-*O*-Glu	2102,35±232,57	-
Emodin	38,21±8,29	44,33±9,62
Epigallocatechin	-	1196,35±133,88
Epigallocatechin Gallate	-	78,75±7,09
Eubotriol	-	202,60±21,93
Fumaric acid	368,22±34,14	85,06±7,89
Gallic acid	64,92±10,36	63,04±10,06
Isoquercetin	1096,88±317,66	-
Pelargonin Chloride	8,579±0,24	88,79±2,51
Procyanidin B2	382,63±60,93	5401,88±860,26
Pyrogallol	-	-
Quercetin	72,57±11,59	-
Quercetin-3-Arabinoside	188,28±54,32	-
Quercitrin	85,38±6,08	-
Ursolic acid	204,54±18,37	-

Amounts of the samples in μg of secondary compounds per g extract with the expanded (k = 2) uncertainty

Discussion

Numerous studies have been carried out on the fruits of the *Rosa* species [66,10,67]. However, in this study, no biological activity studies were conducted on the roots of *R. pimpinellifolia*. Therefore, it can be said that this study is original. Previously, anti-inflammatory and anticancer studies have been conducted on different *Rosa* roots, and it has been observed that the roots are effective in these studies [68, 69].

Previously, total phenolic contents of *R. spinosissima, R. canina,* and *R. rugosa* fruits were examined. It has been reported that *R. spinosissima* has the highest total phenolic content and antioxidant activity [70].

In our study, we determined that the roots and fruits were not rich in flavonoids. There are mostly made of catechic and triterpene substances [71].

Antioxidant activities of *Rosa* species's fruits and roots are high [72]. It was reported that methanol extract of *R. davurica* roots showed strong antioxidant activity in DPPH radical scavenging activity assay [73]. The relationship between the rich polyphenol content and antioxidant mechanisms in *R. canina* was investigated, and good antioxidant activity was

demonstrated by H_2O_2 and superoxide anion radical scavenging activity experiments at all concentrations [74].

In our study, we found that *R. pimpinellifolia* roots and fruits did not have cholinesterase activity. In another study, it was reported that *R. canina* fruits are also inactive [75].

In our study, it was observed that the roots and fruits of *R. pimpinellifolia* had moderate antityrosinase activity. Tyrosinase enzyme inhibition values in *R. pimpinellifolia* extracts were the highest at 500 µg/mL. 37.7% in root water extract; 50% in root ethyl acetate extract, 61.5% in root methanol extract, 37.9% in pseudo-fruit extract. At a concentration of 500 µg/mL, α-Kojic acid Showed an inhibition of 90%. Inhibitory activity on tyrosinase enzyme at 500 µg/mL. Natural compounds such as kojic acid, arbutin are preferred than chemical substances such as hydroquinone and azelaic acid because they have no inflammatory effects on the skin. Results have shown that *R. pimpinellifolia* extracts can be used as an alternative in the therapy treatment of hyperpigmentation and may be involved in the formulations of cosmetic products used for hyperpigmentation. In our previous study, the antityrosinase activity of *R. pimpinellifolia* petals was shown to be promising [76]

In our study, a weak antimicrobial activity of *Rosa* roots and fruits against bacteria and fungi was determined. In certain studies, *R. rugosa* root extracts were found to exhibit antimicrobial activity against *S. epidermidis, S. aureus, B. subtilis, Micrococcus luteus, E. coli, Klebsiella pneumoniae, Ps. aeruginosa, Proteus mirabilis,* and *Candida albicans* [77].

Furthermore, in another study, the activity of alcohol and aqueous extracts of R. damascena was determined against 10 pathogenic microorganisms (*S. aureus* ATCC 25923, *Ps. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, *S. pneumoniae* ATCC 55143, *Acinetobacter calcaoceuticus, Salmonella enteritidis* and *Aspergillus niger* ATCC 1640) MIC, MBC and inhibition area diameter were determined by *in vitro* disc diffusion and microdilution method. While hexane extracts showed very low activity against the microorganisms, it was stated that the aqueous extract inhibited the growth of Gram (+) and Gram (-) bacteria, as well as *A. niger*. With regards to its ethanol extract, it showed antibacterial activity against *P. aeruginosa* ATCC 27853 bacteria (MIC and MBC 62.5 µg/mL, DIZ = 34 mm), *E. coli* ATCC 25922 bacteria (MIC and MBC 62.5 µg/mL, DIZ = 30 mm) according to some studies was reported [78].

According to our results, it was observed that 4 different extracts did not show antigenotoxic effect in the concentration range of 25-500 µg/mL, while extracts at a concentration of 1000 µg/mL showed toxic effects and increased the existing damage. In further studies, we believe that *R. pimpinellifolia* extracts to be prepared using different solvent and extraction techniques should be studied both in different treatment types (simultaneous or post) and different cell types. With this study, antigenotoxic data were obtained for the first time in *Rosa* roots and fruits.

In an LC/MS/MS study, it was reported that Catechin, epicatechin, quercetin-3-glucoside, protocatechuic acid, chlorogenic acid, quercetin, rutin, fumaric acid, and gallic acid are major phenolic compounds in *R. pimpinellifolia* fruits.

In other studies, it has been reported that catechin [68, 79], Cyanidin-3-O-Glucoside [80], and procyanidin [81], are present in different *Rosa* species. In this study, phenolic compounds in *R. pimpinellifolia* roots were first elucidated by LC/MS/MS analysis.

Conclusion

With this study, a start was provided for the studies on the biological activity studies and chemical content of *R. pimpinellifolia* root, pseudo-fruits, and seeds in the literature. By investigating their possible antioxidant, antityrosinase, anticholinesterase, antimicrobial, anti-genotoxic, and anti-cancer properties and determining the chemical content of *R. pimpinellifolia* root, pseudo-fruit, and seed and the deficiency of work in the scientific world was eliminated. Determination of medicinal properties of *R. pimpinellifolia*, production of active substances with biological properties, and application of these substances in the pharmacological field will provide meaningful support. In future studies, *in vitro* and *in vivo* studies of active ingredients to be obtained by plant isolation should be carried out.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study is part of Leyla GÜVEN's PhD thesis. The authors thank Abdulkayyum İdrisu proofreading.

Competing Interests

Authors have declared that no competing interests exist.

Abbreviations

EAR: Ethyl acetate extract of *R. pimpinellifolia* root; MR: Methanol extract of *R. pimpinellifolia* root; WR: Water extract of *R. pimpinellifolia* root; WPF: Water extract of *R. pimpinellifolia* pseudo-fruit; MS: Methanol extract of *R. pimpinellifolia* seed; EAS: Ethyl acetate extract of *R. pimpinellifolia* seed

References

1. Tetik, F., et al., Traditional uses of some medicinal plants in Malatya (Turkey). Journal of Ethnopharmacology, 2013. 146(1): p. 331-346.
2. Gürhan, G. and N. Ezer, Halk arasında hemoroit tedavisinde kullanılan bitkiler-I. Hacettepe Üniversitesi Eczacılık Fakültesi Dergisi, 2004. 24(1): p. 37-55.
3. Altundag, E. and M. Ozturk, Ethnomedicinal studies on the plant resources of east Anatolia, Turkey. Procedia-Social and Behavioral Sciences, 2011. 19: p. 756-777.
4. Kumar, N., et al., Reversed phase-HPLC for rapid determination of polyphenols in flowers of rose species. Journal of Separation Science, 2008. 31(2): p. 262-267.
5. Gao, X., et al., Aurone constituents from the flowers of *Rosa rugosa* and their biological activities. Heterocycles, 2012. 85(8): p. 1925-1931.
6. Gao, X.M., et al., Phenylethanoids from the flowers of *Rosa rugosa* and their biological activities. Bulletin of the Korean Chemical Society, 2013. 34(1): p. 246-248.
7. Liu, D.L., et al., Triterpenoids from the roots of *Rose odorata* var. gigantean. Chinese Journal of Natural Medicines, 2010. 8(1): p. 12-15.
8. Stoyanova-Ivanova, B., et al., Content and composition of neutral components in wax from *Rosa canina* flowers. Doklady Bolgarskoi Akademii Nauk, 1979. 32(11): p. 1503-1506.
9. Hashidoko, Y., The phytochemistry of *Rosa rugosa*. Phytochemistry, 1996. 43(3): p. 535-549.
10. Novruzov, E.N., Pigments of species in the genus *Rosa* and their chemotaxonomic value. Acta Horticulturae, 2005. 690(Proceedings of the 1st International Rose Hip Conference, 2004): p. 225-230.
11. Ghazghazi, H., et al., Phenols, essential oils and carotenoids of *Rosa canina* from Tunisia and their antioxidant activities. The African Journal of Biotechnology, 2010. 9(18): p. 2709-2716.
12. Al-Rehaily, A.J., et al., Essential oil of *Rosa abyssinica* R. Br. from Saudi Arabia. Journal of Essential Oil Research, 2003. 15(5): p. 344-345.
13. Elmastaş, M., et al., Changes in flavonoid and phenolic acid contents in some Rosa species during ripening. Food chemistry, 2017. 235: p. 154-159.
14. Odabaş, H.İ. and I. Koca, Simultaneous separation and preliminary purification of anthocyanins from *Rosa pimpinellifolia* L. fruits by microwave assisted aqueous two-phase extraction. Food and Bioproducts Processing. 125: p. 170-180.
15. Murathan, Z.T., et al., Characterization of bioactive compounds in rosehip species from East Anatolia region of Turkey. Italian Journal of Food Science, 2016. 28(2): p. 314.
16. Murathan, Z.T., et al., Determination of fatty acids and volatile compounds in fruits of rosehip (*Rosa* L.) species by HS-SPME/GC-MS and Im-SPME/GC-MS techniques. Turkish Journal of Agriculture and Forestry, 2016. 40(2): p. 269-279.
17. Nagaki, M., et al., Composition and antimicrobial activity of the essential oil and water extract from Japanese wild *Rosa rugosa*. Transactions of the Materials Research Society of Japan, 2011. 36(3): p. 517-521.
18. Lee, H.J., et al., Anti-inflammatory effects of hexane fraction from white rose flower extracts via inhibition of inflammatory repertoires. Biomolecules & Therapeutics, 2011. 19(3): p. 331-335.
19. Park, K.H., et al., Three new stereoisomers of condensed tannins from the roots of *Rosa multiflora*. Chemical and Pharmaceutical Bulletin, 2010. 58(9): p. 1227-1231.
20. Hsieh, T.C., et al., Effects of herbal preparation Equiguard on hormone-responsive and hormone-refractory prostate carcinoma cells: mechanistic studies. International Journal of Oncology, 2002. 20(4): p. 681-689.

21. Nagai, T., et al., Tea beverages made from Romanas rose (*Rosa rugosa* Thunb.) leaves possess strongly antioxidative activity by high contents of total phenols and vitamin C. Journal of Food, Agriculture and Environment, 2007. 5(3 & 4): p. 137-141.
22. Liu, Y.T., et al., Hepatoprotective activity of the total flavonoids from *Rosa laevigata* Michx fruit in mice treated by paracetamol. Food Chemistry, 2010. 125(2): p. 719-725.
23. Setzer, W.N., Essential oils and anxiolytic aromatherapy. Natural Product Communications, 2009. 4(9): p. 1305-1316.
24. Ninomiya, K., et al., Potent anti-obese principle from *Rosa canina*: Structural requirements and mode of action of trans-tiliroside. Bioorganic & Medicinal Chemistry Letters, 2007. 17(11): p. 3059-3064.
25. Umezu, T., et al., Anticonflict effects of rose oil and identification of its active constituents. Life Science, 2002. 72(1): p. 91-102.
26. Seto, T., et al., Purgative activity and principals of the fruits of *Rosa multiflora* and *R. wichuraiana*. Chemical and Pharmaceutical Bulletin, 1992. 40(8): p. 2080-2082.
27. Tayefi-Nasrabadi, H., et al., The effects of the hydroalcohol extract of *Rosa canina* L. Fruit on experimentally nephrolithiasic wistar rats. Phytotherapy Research, 2012. 26(1): p. 78-85.
28. Mandade, R.J., et al., Role of the *Rosa canina* L. leaf extract as an antidiarrheal drug in rodents. Indian Journal of Pharmacology, 2011. 43(3): p. 316-319.
29. Jeon, J.H., et al., Anti-allergic effects of white rose petal extract and anti-atopic properties of its hexane fraction. Archives of Pharmacal Research, 2009. 32(6): p. 823-830.
30. Tumbas, V.T., et al., Effect of rosehip (*Rosa canina* L.) phytochemicals on stable free radicals and human cancer cells. Journal of the Science of Food and Agriculture, 2012. 92(6): p. 1273-1281.
31. Jung, H.J., et al., 19α-hydroxyursane-type triterpenoids: antinociceptive anti-inflammatory principles of the roots of *Rosa rugosa*. Biological and Pharmaceutical Bulletin, 2005. 28(1): p. 101-104.
32. Gürbüz, İ., et al., Anti-ulcerogenic activity of some plants used as folk remedy in Turkey. Journal of Ethnopharmacology, 2003. 88(1): p. 93-97.
33. Yoshizawa Y., et al., Anticancer and anti-hypertensive effects of small fruit juices, *American Chemical Society*, 2001,2: 8-23.
34. Trovato, A., et al., In vitro anti-mycotic activity of some medicinal plants containing flavonoids. Bollettino Chimico Farmaceutico, 2000. 139(5): p. 225-227.
35. Boskabady, M.H., et al., Pharmacological effects of *Rosa damascena*. Iranian Journal of Basic Medical Sciences, 2011. 14(4): p. 295-307.
36. Vlachojannis JE, Duke RK, Tran VH, Duke CC, Chrubasik S. Medicinal properties of *Rosa canina*, *Studium Press*, 2010, 27: 441-463.
37. Vertuani, S., et al., Antioxidant herbal supplements for hemorrhoids developing a new formula. Nutrafoods, 2004. 3(3): p. 19-26.
38. Ghasemzadeh, A. and N. Ghasemzadeh, Flavonoids and phenolic acids: Role and biochemical activity in plants and human. Journal of medicinal plants research, 2011. 5(31): p. 6697-6703.
39. Park, H.-R., et al., Antioxidant activity of extracts from Acanthopanax senticosus. African Journal of Biotechnology, 2006. 5(23).
40. Aliyazicioglu, R., et al., Antioxidant, antigenotoxic, antimicrobial activities and phytochemical analysis of Dianthus carmelitarum. Rec. Nat. Prod., 2017. 11(3): p. 270-284.
41. Gyawali, R. and S.A. Ibrahim, Natural products as antimicrobial agents. Food Control, 2014. 46: p. 412-429.
42. Bao, K., et al., Design and synthesis of biphenyl derivatives as mushroom tyrosinase inhibitors. Bioorg. Med. Chem., 2010. 18(18): p. 6708-6714.

43. Masuda, T., et al., Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from Garcinia subelliptica. Biosci., Biotechnol., Biochem., 2005. 69(1): p. 197-201.
44. Sezer Senol, F., et al., Memory-vitalizing effect of twenty-five medicinal and edible plants and their isolated compounds. S. Afr. J. Bot., 2016. 102: p. 102-109.
45. Tsong-Min, C., Tyrosinase and tyrosinase inhibitors. Journal of Biocatalysis & Biotransformation, 2012.
46. Gholamhoseinian, A. and Z. Razmi, Screening the methanolic extracts of some plants for tyrosinase inhibitory activity. Toxicological & Environmental Chemistry, 2012. 94(2): p. 310-318.
47. Shimizu, K., et al., Inhibition of tyrosinase by flavonoids, stilbenes, and related 4-substituted resorcinols. Structure-activity investigations. Planta Med., 2000. 66(1): p. 11-15.
48. Martinez, M.V. and J.R. Whitaker, The biochemistry and control of enzymic browning. Trends Food Sci. Technol., 1995. 6(6): p. 195-200.
49. Xu, Y., et al., Tyrosinase mRNA is expressed in human substantia nigra. Mol. Brain Res., 1997. 45(1): p. 159-162.
50. Asanuma, M., et al., Dopamine- or L-DOPA-induced neurotoxicity: the role of dopamine quinone formation and tyrosinase in a model of Parkinson's disease. Neurotox Res, 2003. 5(3): p. 165-76.
51. Adewusi, E.A., et al., Medicinal plants with cholinesterase inhibitory activity: a review. African Journal of Biotechnology, 2010. 9(49): p. 8257-8276.
52. Singleton, V. and J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture, 1965. 16(3): p. 144-158.
53. Folin, O. and W. Denis, Phosphotungstic-phosphomolybdic Compounds as Color Reagents. The Journal of Biological Chemistry, 1912. 12: p. 239-243.
54. Slinkard, K. and V.L. Singleton, Total phenol analysis: automation and comparison with manual methods. American Journal of Enology and Viticulture, 1977. 28(1): p. 49-55.
55. Lar'kina, M.S., et al., Quantitative determination of flavonoids from the aerial part of greater knapweed (*Centaurea scabiosa* L.). Pharmaceutical Chemistry Journal, 2009. 43(6): p. 320-323.
56. Blois, M.S., Antioxidant determinations by the use of a stable free radical. 1958.
57. Re, R., et al., Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology & Medicine, 1999. 26(9-10): p. 1231-1237.
58. Zhishen, J., et al., The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chemistry, 1999. 64(4): p. 555-559.
59. Ellman, G.L., et al., A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 1961. 7(2): p. 88-95.
60. Likhitwitayawuid, K. and B. Sritularak, A new dimeric stilbene with tyrosinase inhibitiory activity from *Artocarpus gomezianus*. Journal of Natural products, 2001. 64(11): p. 1457-1459.
61. Aliyazicioglu, Y., et al., Preventive and protective effects of Turkish propolis on H2O2-induced DNA damage in foreskin fibroblast cell lines. Acta Biologica Hungarica, 2011. 62(4): p. 388-396.
62. Tice, R.R., et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environmental and Molecular Mutagenesis 2000. 35(3): p. 206-221.
63. Ceylan, R., et al., GC-MS analysis and in vitro antioxidant and enzyme inhibitory activities of essential oil from aerial parts of endemic Thymus spathulifolius Hausskn. et Velen. Journal of enzyme inhibition and medicinal chemistry, 2016. 31(6): p. 983-990.
64. Gören, A.C., et al., HPLC and LC–MS/MS methods for determination of sodium benzoate and potassium sorbate in food and beverages: Performances of local accredited laboratories via proficiency tests in Turkey. Food chemistry, 2015. 175: p. 273-279.

65. Binici, B., et al., An efficient GC–IDMS method for determination of PBDEs and PBB in plastic materials. Talanta, 2013. 116: p. 417-426.
66. Feng-Zheng, C., et al., Chemical Constituents from Fruits of *Rosa davidii*. Acta Botanica Sinica, 2001. 1: p. 19.
67. Larsen, E., et al., An antiinflammatory galactolipid from rose hip (*Rosa canina*) that Inhibits chemotaxis of human peripheral blood neutrophils in vitro. Journal of Natural Products, 2003. 66(7): p. 994-995.
68. Çoruh, N. and N. Özdoğan, Identification and quantification of phenolic components of Rosa heckeliana Tratt roots. Journal of Liquid Chromatography & Related Technologies, 2015. 38(5): p. 569-578.
69. Yeşilada, E., et al., Inhibitory effects of Turkish folk remedies on inflammatory cytokines: interleukin-1α, interleukin-1β and tumor necrosis factor α. Journal of Ethnopharmacology, 1997. 58(1): p. 59-73.
70. Koczka, N., et al., Total polyphenol content and antioxidant capacity of rosehips of some *Rosa* species. Medicines, 2018. 5(3): p. 84.
71. Guven, L., et al., Phytochemical studies on the seeds, pseudofruits, and roots of Rosa pimpinellifolia. Journal of Research in Pharmacy, 2021. 25(2): p. 153-163.
72. Senol, F.S., et al., Memory-vitalizing effect of twenty-five medicinal and edible plants and their isolated compounds. South African Journal of Botany, 2016. 102: p. 102-109.
73. Hu, W., et al., Biological activity and inhibition of non-enzymatic glycation by methanolic extract of *Rosa davurica* Pall. roots. Journal of Food Sciences and Nutrition, 2011. 16(3): p. 242-247.
74. Kilicgun, H. and D. Altiner, Correlation between antioxidant effect mechanisms and polyphenol content of *Rosa canina*. Pharmacognosy Magazine, 2010. 6(23): p. 238-241.
75. Boğa, M., et al., Antioxidant and anticholinesterase activities of eleven edible plants. Pharm Biol, 2011. 49(3): p. 290-5.
76. Guven L., et al., Thyrozinase enzyme inhibitor activity of *Rosa pimpinelifolia* petals, in 5th International Medical and Health Sciences Research Congress (UTSAK). 12-13 December 2020: Ankara.
77. Olech, M., et al., Evaluation of rose roots, a post-harvest plantation residue as a source of phytochemicals with radical scavenging, cytotoxic, and antimicrobial activity. Industrial Crops and Products, 2015. 69: p. 129-136.
78. Halawani, E.M., et al., Antimicrobial activity of *Rosa damascena* petals extracts and chemical composition by gas chromatography-mass spectrometry (GC/MS) analysis. African Journal of Microbiology Research, 2014. 8(24): p. 2359-2367.
79. He, R.R., et al., Protective effects of radix *Rosa laevigata* against Propionibacterium acnes and lipopolysaccharide-induced liver injury. Bioscience, Biotechnology, and Biochemistry, 2009. 73(5): p. 1129-1136.
80. Odabaş, H.İ. and I. Koca, Simultaneous separation and preliminary purification of anthocyanins from *Rosa pimpinellifolia* L. fruits by microwave assisted aqueous two-phase extraction. Food and Bioproducts Processing, 2021. 125: p. 170-180.
81. Dabić Zagorac, D.Č., et al., Establishing the chromatographic fingerprints of flavan-3-ols and proanthocyanidins from rose hip (*Rosa* sp.) species. Journal of separation science, 2020. 43(8): p. 1431-1439.