



# Investigation of Enzyme Inhibitory Activities, Antioxidant Activities, and Chemical Properties of *Pistacia vera* Leaves Using LC-QTOF-MS and RP-HPLC

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## Abstract

Since the leaves of some *Pistacia* species are used in traditional folk medicine for diabetes, this study investigated the in vitro antidiabetic effect ( $\alpha$ -glucosidase and  $\alpha$ -amylase) of *Pistacia vera* leaves. Additionally, the current study investigated the antihypercholesterolemic (cholesterol esterase), antiobesity (pancreatic lipase), and antioxidant activities (i.e., total antioxidant capacity, DPPH (2,2-Diphenyl-1-picrylhydrazyl) radical scavenging activity, metal chelating activity, and ferric-reducing antioxidant power) of *P. vera* leaves. The aqueous-alcoholic leaf extract inhibited  $\alpha$ -amylase,  $\alpha$ -glucosidase, and pancreatic lipase with the half-maximal inhibitory concentration values of  $7.74 \pm 0.72$ ,  $11.08 \pm 3.96$ , and  $168.43 \pm 26.10$   $\mu\text{g/mL}$ , respectively. It was determined that the crude extract had high DPPH radical scavenging activity, ferric-reducing power, and moderate metal chelating activity. The ethyl acetate (EtOAc) subextract obtained by the liquid-liquid fractionation of the crude extract showed potent  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities. The EtOAc subextract ( $5.794 \pm 0.027$  g/100 g subextract) was standardized by reversed-phase high-performance liquid chromatography based on  $\beta$ -pentagalloyl glucose, which showed inhibitory effects on both amylase and glucosidase enzymes. Fifteen compounds, seven of which are organic acid derivatives and eight of which are flavonoids, were identified by liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) analysis in the crude extract of *P. vera* leaves. Seven of the fifteen phenolic compounds detected in the crude extract by LC-QTOF-MS have both glucosidase and amylase inhibitory effects. As a result, *P. vera* leaves can be a potential source for compounds with high antioxidant effects that show inhibitory effects on enzymes involved in carbohydrate digestion in the prevention and treatment of diabetes or can be evaluated as a standardized extract.

**Keywords:** Antioxidant, Antidiabetic, RP-HPLC, LC-QTOF-MS, Phytochemistry, *Pistacia vera*

## 1. Background

Diabetes mellitus is a disease that occurs due to impaired insulin secretion or effect. As a result of the defect in carbohydrate, fat, and protein metabolism, blood sugar rises, and damage and dysfunctions develop in various organs in the long term (1). Insulin resistance occurs in type 2 diabetes and is associated with various disorders, such as obesity, hypertension, and hyperlipidemia (2). The inhibition of enzymes that metabolize carbohydrates is one of the treatment approaches for diabetes mellitus. Alpha-amylase, one of these enzymes, enables the breakdown of complex carbohydrates into smaller oligosaccharides; however, the  $\alpha$ -glucosidase enzyme causes these oligosaccharides to break down into sugars with even smaller molecules. The inhibition of these enzymes pre-

vents blood sugar from rising.

Diabetes mellitus also causes disorders in lipid metabolism. For this reason, it is important that these molecules act on enzymes that play a role in lipid metabolism in discovering candidate molecules that affect diabetes complications. Lipase plays a role in the breakdown of lipids and acts in their digestion and transport (3). Pancreatic cholesterol esterase is a serine hydrolase enzyme that plays an important role in the absorption of dietary cholesterol into the blood. Cholesterol esterase inhibition plays a role in the treatment of hypercholesterolemia and atherosclerosis by reducing the bioavailability of dietary cholesterol (4). It might be possible to treat these diseases or alleviate their complications by inhibiting such key enzymes that play a role in the pathophysiology of diabetes, obesity, and hyperlipidemia.

Medicinal plants are important sources for discovering effective and safe new natural drug molecules in the treatment of diabetes mellitus.

*Pistacia vera* L. is a tree belonging to the Anacardiaceae family. The fruits and seeds of *P. vera* L. are traditionally used for skin conditions, such as abdominal ailments, abscesses, bruises, itching and sores, chest ailments, gynecological ailments, amenorrhea, dysentery, rheumatism, and trauma (5). The seeds inside the ripe fruit are edible and used in desserts, meats, sauces, and cakes. The fruits are known by the name “pistachio”. Since it is widely consumed as roasted, it is also grown in different countries, such as Turkey, Iran, the USA, the West of Asia, and the Middle East (6). In 2017, it was reported that pistachio production was 1,115,066 tons in 770,861 ha in the world, and the export rate of pistachio reached approximately 2.811 billion dollars (7). It is also interesting that although the fruits and seeds of the plant are widely consumed as food, its leaves have no economic or commercial importance in the field of food and medicine. The literature has shown that the leaves of *P. terebinthus* L. ssp. *palaestina* (Boiss.) Engler in Anatolia and *P. atlantica* Desf. in Jordan are traditionally used in folk medicine against diabetes (8, 9).

With the above-mentioned background in mind, the present study aimed to investigate the in vitro antidiabetic potential of *P. vera* leaves, on which no antidiabetic activity study has been conducted to date. For this purpose, the aqueous-alcoholic extract of *P. vera* leaves and its subextracts (i.e., petroleum ether [PE], chloroform [ $\text{CHCl}_3$ ], ethyl acetate [EtOAc], and n-butanol) were tested against enzymes involved in diabetes mellitus (i.e.,  $\alpha$ -glucosidase and  $\alpha$ -amylase), obesity (i.e., pancreatic lipase), and hypercholesterolemia (i.e., cholesterol esterase). In addition, this study determined antioxidant activities (i.e., total antioxidant capacity, 2,2-diphenyl-1-picrylhydrazyl [DPPH] radical scavenging activity, metal chelating activity, and ferric-reducing capacity) and total flavonoid and phenolic contents of the leaf extract. The phytochemical profile of the aqueous-alcoholic extract of the leaves and the most active subextract were elucidated by tandem liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). The amount of 1,2,3,4,6-penta-O-galloyl-D-glucopyranose (PGG), which was determined to be the major compound in both the aqueous-alcoholic extract and the most active subextract, was measured by reversed-phase high-performance liquid chromatography (RP-HPLC).

## 2. Methods

### 2.1. Plant Material

The leaves of the *P. vera* were collected from Tillo, Siirt, Turkey, in July 2014 (Alt. 1100 m). The plant material was identified by M. Ufuk Özbek (Department of Biology, Gazi University, Ankara, Turkey). Herbarium materials were stored in the Herbarium of Faculty of Pharmacy, Gazi University (GUEF 3437).

### 2.2. Extraction of Plant Material

The leaves were dried in the shade at room temperature and ground. The dried leaves (190 g) were extracted with 3 L 80% ethanol for 24 hours, twice at room temperature. The filtrates were combined and concentrated using a rotary evaporator at 45°C (80% ethanol extract: 31.38 % w/w dry plant). The extract (45 g) was suspended in distilled water, and then fractionation was carried out using solvents with increasing polarity (PE,  $\text{CHCl}_3$ , EtOAc, and n-butanol/saturated with water [n-BuOH]). All the subextracts were evaporated under reduced pressure at a temperature not exceeding 45°C. After this fractionation process, the remaining aqueous (R-H<sub>2</sub>O) phase was dried in a lyophilizer at -80°C. The yields of the five subextracts were calculated (PE: 15.43 w/w,  $\text{CHCl}_3$ : 1.262 w/w, EtOAc: 16.407 w/w, n-BuOH: 13.02 w/w, and R-H<sub>2</sub>O: 43.794 w/w).

### 2.3. Total Phenolic Content of Crude Extract

The total phenolic content of the crude extract was determined by adding 10% (w/v) Folin-Ciocalteu reagent and  $\text{Na}_2\text{CO}_3$  solution to the extract. After waiting 30 minutes in the dark, the absorbance of the mixture was read at 735 nm. The total phenol content of the extract was expressed in terms of gallic acid equivalent (GAE) mg/g of the extract (10).

### 2.4. Total Flavonoid Content of Crude Extract

The total flavonoid content of the crude extract was determined by adding 1 M sodium acetate and 10% (w/v)  $\text{AlCl}_3$  solutions to the extract. After waiting 30 minutes at room temperature, the absorbance of the mixture was read at 415 nm. The total flavonoid content of the extract was expressed in terms of quercetin equivalent (QE) mg/g of the extract (11).

### 2.5. Antioxidant Activities

#### 2.5.1. DPPH Radical Scavenging Activity

In this study, 1 mM DPPH was added to the samples and left in the dark at room temperature for 30 minutes. The absorbance of the samples was read at 520 nm. Ascorbic acid was used as the reference compound (12).

### 2.5.2. Metal Chelating Activity

In this study, 2 mM FeCl<sub>2</sub> solution was added to the samples and incubated for 5 minutes at room temperature. Then, 5 mM ferrozine solution was added. After 10 minutes, the absorbance of the samples was measured at 562 nm. Ethylenediaminetetraacetic acid (EDTA) was used as the reference compound (13).

### 2.5.3. Ferric-Reducing Antioxidant Power

In a 0.1 mol/L sodium phosphate buffer (pH = 7.2) 1% (w/v), potassium ferricyanide solution was added to the samples and incubated at 37°C for 60 minutes. After incubation, 10% (w/v) trichloroacetic acid was added, and the absorbance was measured at 700 nm. After the measurement, 0.1% FeCl<sub>3</sub> solution was added, and the measurement was made again; the difference between the two absorbance values was calculated. The QE was used as the reference compound (14).

### 2.5.4. Total Antioxidant Capacity

Molybdate reagent solution was mixed with the samples in the test tubes. The tubes were incubated at 90°C for 90 minutes. Then, the tubes were cooled immediately to room temperature, and the absorbance was measured at 695 nm. The results were expressed as ascorbic acid equivalent (AAE) (15). The equation of calibration was  $y = 1.5828x + 0.1354$ ,  $r^2 = 0.9961$

## 2.6. Enzyme Inhibitory Activities

### 2.6.1. Alpha-Glucosidase Inhibitory Activity

Alpha-glucosidase (Sigma Co., St. Louis, USA) Type IV prepared in 0.5 M phosphate buffer (pH 6.5) was incubated with the samples for 15 minutes, and the p-nitrophenyl- $\alpha$ -D-glucopyranoside solution (20 mM) was added as a substrate to the mixtures. After 35 minutes, the absorbance of the formed p-nitrophenol was read at 405 nm. The incubations were conducted at 37°C. Acarbose (Bayer) was used as the reference compound (16).

### 2.6.2. Alpha-Amylase Inhibitory Activity

Alpha-amylase (Sigma-Aldrich, Zwijndrecht, Netherlands) Type I (4 U/mL) was incubated with the samples for 5 minutes, and the starch solution (0.5% w/v, in phosphate buffer pH 6.9) was added as a substrate to the mixtures. The incubations were conducted at 37°C. After 3 minutes, 3,5-dinitrosalicylic acid solution (96 mM) prepared in 5.31 M sodium potassium tartrate (in 2 M NaOH) was added as a color reagent, and keep the mixtures were at 85°C for 40 minutes. The tubes were immediately cooled, and the absorbance of the mixtures was read at 540 nm. The formed maltose with the degradation of the starch was

calculated using the maltose calibration equation ( $y = 0.4373x - 0.024$ ,  $r^2 = 0.9959$ ) acarbose (Bayer) was used as the reference compound (17).

### 2.6.3. Pancreatic Lipase Inhibitory Activity

Pancreatic lipase (Sigma Co., St. Louis, USA) Type II prepared in a solution (pH 6.8) containing 10 mM 4-morpholinepropanesulfonic acid and 1 mM EDTA was incubated with the samples in a Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl) buffer (pH 7.0) containing 100 mM Tris-HCl and 5 mM CaCl<sub>2</sub> for 15 minutes. A substrate solution containing p-nitrophenyl butyrate (10 mM) in acetonitrile was added to the mixture. After 30 minutes, the absorbance of the formed p-nitrophenol was read at 405 nm. The incubations were conducted at 37°C. Orlistat (F. Hoffmann-La Roche AG, Basel, Switzerland) was used as the reference compound (18).

### 2.6.4. Cholesterol Esterase Inhibitory Activity

Cholesterol esterase (Sigma-Aldrich, Zwijndrecht, Netherlands) from the porcine pancreas was dissolved in 100 mM phosphate buffer (pH = 7) containing 100 mM NaCl. Furthermore, 12 mM taurocholic acid and substrate (p-nitrophenyl butyrate) solution were added to the samples in the phosphate buffer, respectively. The mixture was incubated for 5 minutes at 25°C. Then, the enzyme was added, and the differences in the absorbance were determined using kinetic measurement at 405 nm for 6 minutes. Simvastatin was used as the reference compound (19).

## 2.7. Characterization of Crude Extract by LC-QTOF-MS

The analyses were carried out on Agilent 1260 series HPLC system and Agilent 6550 iFunnel High-Resolution Mass Spectrometer device connected to this system (Agilent Technologies, Inc., CA, USA). The analyses were made in the negative mode, and MS (mass spectrometry) system was utilized in the double spray Agilent Jet Stream Electrospray ionization technique. The MS operating mode is the 2 GHz extended dynamic range. Agilent MassHunter Software B06.00 and Metlin Metabolite database were used for data evaluation and analysis. For the separation, TC-C18 column (170 Å, 4.6 mm × 150 mm × 5  $\mu$ m) (Agilent technologies 6410, USA) was used. The column temperature was 30°C; the injection volume was 5.00  $\mu$ L; the flow rate was 0.650 mL/minute; the analysis time was 38 minutes. The mobile phase was set using solvent A (10 mM ammonia acetate) and solvent B (acetonitrile). The flow program was as follows:

0 minute 4% B, to 10 minutes 10% B, to 22 minutes 30% B, to 24 minutes 70% B, to 25 minutes 90% B, to 29 minutes 90% B, to 29.10 minutes 4% B, to 30 minutes 4% B.

UV: 280 nm (20 Hz); Ionization mode: Negative; Drying gas flow, nitrogen: 14 L/minutes; Drying gas temperature: 200°C; Nebulizer: 40 psi Sheath gas flow, nitrogen: 11 L/minutes; Sheath gas temperature: 350°C; Nozzle voltage: 1000 V; Capillary voltage: 1400 V; Mass reading range: 40 - 1700 amu; Reference ions: 966.0007, 980.01637.

### 2.8. PGG Analysis in Crude Extract and EtOAc Subextract by RP-HPLC

The PGG determined by RP-HPLC in the crude extract and EtOAc subextract was isolated from *Rhus coriaria* by Gok et al. 2020 (20). The extract was prepared in 25% (v/v) acetonitrile solution at a concentration of 1 mg/mL and was transferred to vials by filtering through membrane filters. The PGG was also prepared in a 25% aqueous acetonitrile solution as a standard. The HP Agilent 1260 series LC System and ACE 5 C18 (5  $\mu$ m, 150 mm  $\times$  4.6 mm) column were used. The column temperature was set as 25°C. The mobile phase was set using Solvent A (acetonitrile: H<sub>2</sub>O: formic acid [50:50:0.5]) and Solvent B (H<sub>2</sub>O: formic acid [100:0.5]). The gradient system was used for the separation of the peaks. The flow program was as follows:

0 minute 5% A, to 10 minutes 15% A, to 17 minutes 15% A, to 22 minutes 20% A, to 32 minutes 30% A, to 50 minutes 100% A, to 53 minutes 100% A.

The analysis time was set at 53 minutes. The flow rate was 0.8 mL/minute, and the injection volume was 20  $\mu$ L. The calibration equation and correlation coefficient determined for PGG were obtained ( $y = 43.936x + 4.7902$ ,  $r^2 = 0.9998$ ).

### 2.9. Statistical Analysis

Statistical analyses were conducted using the one-way analysis of variance and Dunnett's Multiple Comparison Test. A P-value less than 0.05 was considered statistically significant (\*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001).

## 3. Results

### 3.1. Total Flavonoid and Phenolic Contents of Crude Extract

The total flavonoid content of the aqueous-alcoholic extract was obtained as 33.48  $\pm$  2.07 mg/g QE. The equation of calibration of the total flavonoid content was  $y = 2.216x - 0.0042$ ,  $r^2 = 1$ . The phenolic content of the extract was determined at 220.30  $\pm$  8.13 mg/g GAE. The equation of calibration graph of total phenolic content was  $y = 6.2614x + 0.0346$ ,  $r^2 = 0.9993$ .

### 3.2. Antioxidant Activity

The DPPH scavenging activity of the aqueous-alcoholic extract of *P. vera* leaf showed as much activity as the reference compound ascorbic acid at all tested concentrations. The DPPH radical scavenging activity of the crude extract was slightly increased at low concentrations. It was concluded that the metal chelating capacity of the extract was significantly lower than EDTA at all concentrations. On the other hand, the increased absorption values in the ferric-reducing power assay indicated that the tested compounds had a high reducing ability. It was observed that the extract (3.928 - 3.915) had the same absorbance values as quercetin (3.940 - 3.941), especially at concentrations of 1 and 2 mg/mL. Again, the same extract gave a high absorbance value (2.961  $\pm$  0.050) at 0.5 mg/mL concentration (Table 1). In addition, the total antioxidant capacity of the extract was determined as 97.68  $\pm$  2.68 mg/g AAE.

### 3.3. Enzyme Inhibitory Activities

This study evaluated the  $\alpha$ -amylase,  $\alpha$ -glucosidase, pancreatic lipase, and cholesterol esterase inhibitory activities of the aqueous-alcoholic extract of *P. vera* leaf. The crude extract specifically inhibited  $\alpha$ -amylase and  $\alpha$ -glucosidase with the half-maximal inhibitory concentration (IC<sub>50</sub>) values of 7.74  $\pm$  0.72 and 11.08  $\pm$  3.96  $\mu$ g/mL, respectively; however, the reference compound acarbose inhibited these enzymes with IC<sub>50</sub> values of 1.35  $\pm$  0.10 and 0.95  $\pm$  0.27  $\mu$ g/mL, respectively. The crude extract (168.43  $\pm$  26.10  $\mu$ g/mL) displayed a very weak inhibitory effect on the pancreatic lipase enzyme, compared to orlistat (4.09  $\pm$  1.08  $\mu$ g/mL), and no effect on the cholesterol esterase enzyme. The subextracts obtained (i.e., PE, CHCl<sub>3</sub>, EtOAc, n-BuOH, and R-H<sub>2</sub>O) by activity-directed fractionation were also tested due to the strong inhibitory activity observed on the  $\alpha$ -amylase and  $\alpha$ -glucosidase of the crude extract. Especially when the EtOAc subextract was compared to the crude extract, it was determined that it caused stronger inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase with IC<sub>50</sub> values of 7.31  $\pm$  1.36  $\mu$ g/mL and 7.69  $\pm$  0.05  $\mu$ g/mL, respectively (Figure 1). Additionally, none of the subextracts showed significant inhibitory effects on pancreatic lipase and cholesterol esterase enzymes.

### 3.4. LC-QTOF-MS Analysis of Crude Extract

Seven organic acid derivative compounds were identified by LC-QTOF-MS in the aqueous-alcoholic extract of *P. vera* leaves. Gallic acid m/z 169.0151 [M-H]<sup>-</sup>, gentisic acid-O-hexoside m/z 315.1096 [M-H]<sup>-</sup>, methyl gallate m/z 183.0316 [M-H]<sup>-</sup>, ethyl gallate m/z 197.0462 [M-H]<sup>-</sup>, methyl digallate m/z 335.0416 [M-H]<sup>-</sup>, PGG m/z 939.1113 [M-H]<sup>-</sup>, and ethyl 2,4-dihydroxy-3-((3,4,5-trihydroxybenzoyl)oxy)benzoate m/z

**Table 1.** Antioxidant Activity Results of *Pistacia vera* Crude Extract

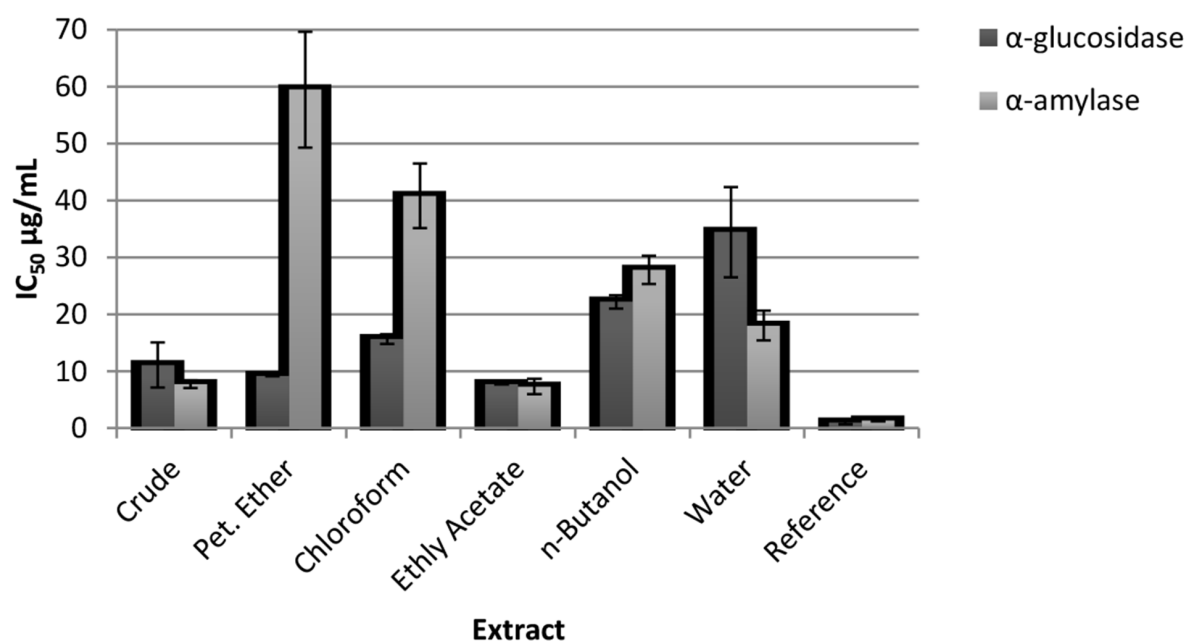
Samples Concentration ( $\mu\text{g/mL}$ )	DPPH Radical Scavenging Activity (Mean% $\pm$ Standard Deviation)	Metal Chelating Capacity (Mean% $\pm$ Standard Deviation)	Ferric-Reducing Power (Absorbance $\pm$ Standard Deviation)
<b><i>Pistacia vera</i> extract</b>			
250	90.51 $\pm$ 0.40***	6.54 $\pm$ 1.26 <sup>ns</sup>	1.597 $\pm$ 0.090***
500	90.34 $\pm$ 0.77***	13.51 $\pm$ 1.27***	2.961 $\pm$ 0.050***
1000	88.93 $\pm$ 1.38***	22.76 $\pm$ 3.02***	3.928 $\pm$ 0.000***
2000	87.44 $\pm$ 0.55***	34.51 $\pm$ 1.34***	3.915 $\pm$ 0.000***
<b>References</b>			
250	91.61 $\pm$ 0.15 <sup>a***</sup>	99.69 $\pm$ 0.18 <sup>b***</sup>	3.774 $\pm$ 0.170 <sup>c***</sup>
500	90.34 $\pm$ 0.77 <sup>a***</sup>	99.88 $\pm$ 0.36 <sup>b***</sup>	3.934 $\pm$ 0.000 <sup>c***</sup>
1000	91.35 $\pm$ 0.20 <sup>a***</sup>	100.03 $\pm$ 0.24 <sup>b***</sup>	3.940 $\pm$ 0.000 <sup>c***</sup>
2000	91.17 $\pm$ 0.57 <sup>a***</sup>	100.00 $\pm$ 0.13 <sup>b***</sup>	3.941 $\pm$ 0.000 <sup>c***</sup>

Abbreviations: DPPH, 2,2-diphenyl-1-picrylhydrazyl; ns, not significant.

<sup>a</sup> Ascorbic acid

<sup>b</sup> EDTA

<sup>c</sup> Quercetin

**Figure 1.** Enzyme inhibitory activities ( $\mu\text{g/mL}$  half-maximal inhibitory concentration [ $\text{IC}_{50}$ ]) of crude extract and subextracts of *Pistacia vera*.

349.0573 [M-H]<sup>-</sup> were tentatively annotated and identified using HRMS (high-resolution mass spectrometry) data and (MS/MS) (mass fragmentation) data and compared to fragmentation fingerprints given in the literature (20-25).

The presence of eight flavonoids in the same extract was tentatively annotated and identified using HRMS data and MS/MS data and compared to fragmentation

fingerprints given in the literature. The predicted compounds and molecular weights were Galocatechin m/z 305.0681 [M-H]<sup>-</sup>, myricetin 3-(6''-galloyl)hexoside m/z 631.0958 [M-H]<sup>-</sup>, myricetin-O-rutinoside m/z 625.1418 [M-H]<sup>-</sup>, myricetin 3-O-hexoside m/z 625.1418 [M-H]<sup>-</sup>, myricetin 3-O-glucuronide m/z 493.0638 [M-H]<sup>-</sup>, quercetin 3-O-(6''-galloyl)-hexoside m/z 615.1012 [M-H]<sup>-</sup>, myricetin-3-

O- $\alpha$ -pentoside m/z 449.0734 [M-H]<sup>-</sup>, and quercetin-3-O-glucuronide m/z 477.0690 [M-H]<sup>-</sup> (21, 24, 26-28). Table 2 shows detailed information about the data of the molecules and fragments.

### 3.5. RP-HPLC Analysis of PGG in Crude Extract and EtOAc Subextract

The PGG, which has an inhibitory effect on  $\alpha$ -glucosidase,  $\alpha$ -amylase, and pancreatic lipase enzymes isolated from *R. coriaria* leaves by Gok et al. (20), was also detected as a result of LC-QTOF-MS analysis of *P. vera* crude extract. For this reason, PGG was used to standardize both the crude extract and the EtOAc subextract, which is the most effective subextract. The retention time of PGG was 31.329 minutes. The amount of PGG was observed to be higher in the EtOAc subextract ( $5.794 \pm 0.027$  g/100 g) than in the crude extract ( $1.295 \pm 0.001$  g/100 g). Figure 2 illustrates the chromatograms of standard PGG, *P. vera* crude extract, and EtOAc subextract.

## 4. Discussion

The  $\alpha$ -amylase,  $\alpha$ -glucosidase, pancreatic lipase, and cholesterol esterase enzyme inhibitory activities of *P. vera* leaves were investigated in the present study. As a result, the hydroalcoholic extract of the leaves showed remarkable  $\alpha$ -amylase ( $77.36 \pm 7.13$   $\mu$ g/mL IC<sub>50</sub>) and  $\alpha$ -glucosidase ( $110.84 \pm 39.64$   $\mu$ g/mL IC<sub>50</sub>) activities. The EtOAc subextract obtained from the crude extract by liquid fractionation also showed the strong inhibition of  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. These enzymes play a role in carbohydrate digestion and cause the carbohydrates consumed to be broken down into glucose. By inhibiting these enzymes, carbohydrate digestion can be slowed down, thereby reducing glucose absorption and elevating blood glucose levels. This is considered an important treatment approach, especially in type II diabetes. On the other hand, since a metabolic disease, such as diabetes mellitus, might be a cause of oxidative stress in the body, it is important that *P. vera* extract has antioxidant activity.

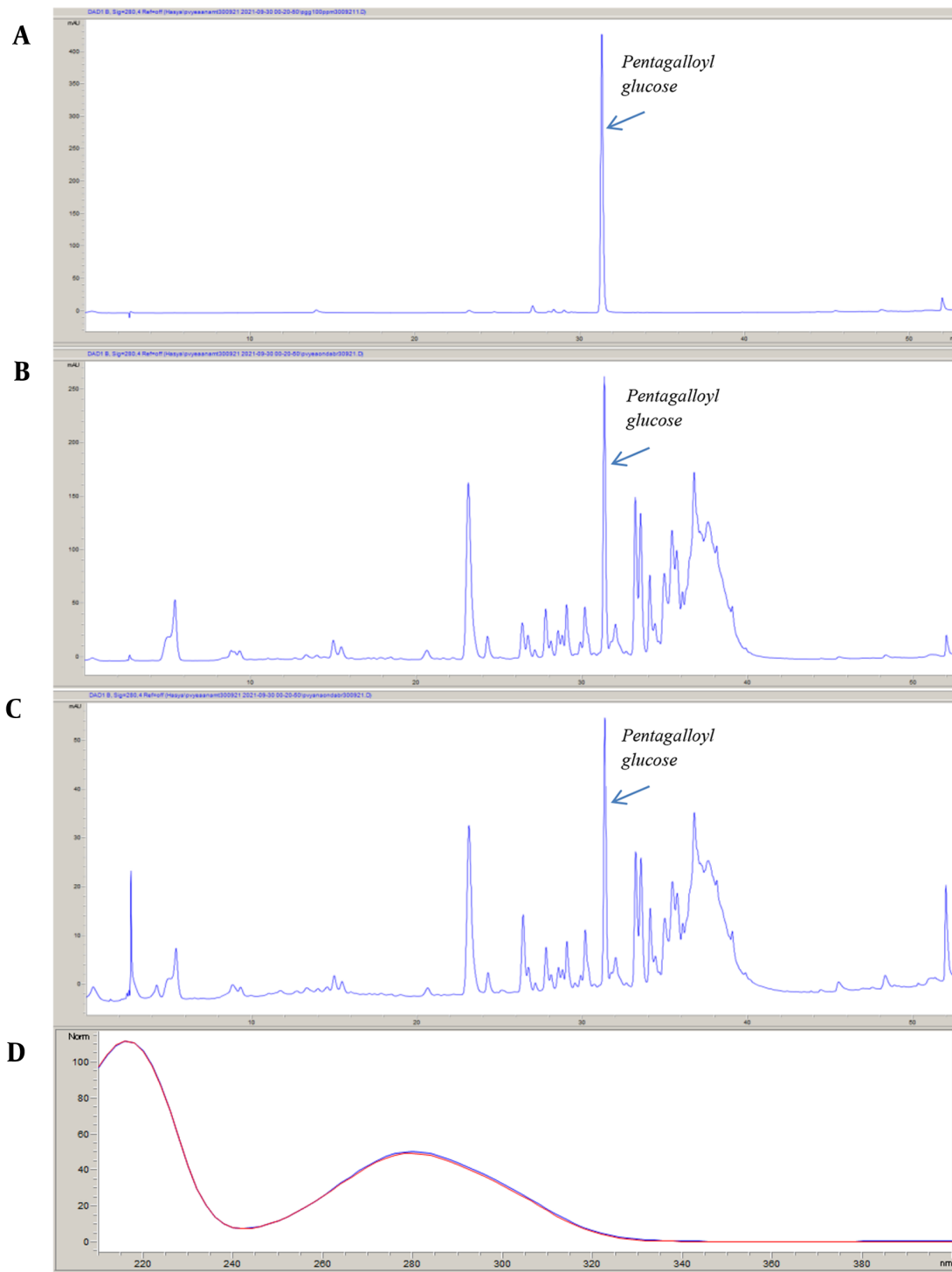
When the in vitro, in vivo, and clinical antidiabetic activity studies on *P. vera* were examined, it was concluded that these studies mostly evaluated the fruits of the plant in terms of this activity. Lalegani et al. (29) evaluated the  $\alpha$ -amylase and  $\alpha$ -glucosidase activities of polyphenol-rich extract from pistachio green hull (PGH). The PGH inhibited  $\alpha$ -amylase from human saliva, porcine pancreas, and *Bacillus* sp. with  $109.05 \pm 0.01$ ,  $174.14 \pm 0.06$ , and  $305.25 \pm 0.04$  IC<sub>50</sub> values ( $\mu$ g GAE/mL), respectively. The PGH also inhibited  $\alpha$ -glucosidase from *Saccharomyces cerevisiae* with a  $6.10 \pm 0.02$  IC<sub>50</sub> value ( $\mu$ g GAE/mL).

In addition, the antioxidant activity of PGH was assessed by DPPH and ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) radical scavenging methods. The PGH showed DPPH and ABTS radical scavenging activities with IC<sub>50</sub> values of  $44.08 \pm 0.01$  and  $139.84 \pm 0.04$   $\mu$ g/mL, respectively. Moreover, considering the results of both activity experiments, the antioxidant activity of PGH was higher than the reference compound Trolox. On the other hand, the phenolic compounds of PGH were analyzed by RP-HPLC; phloroglucinol ( $65.40 \pm 2.33$  mg/g), gallic acid ( $5.22 \pm 0.21$  mg/g), and vanillic acid ( $2.30 \pm 0.02$  mg/g) were determined as major compounds. In addition to the major compounds, protocatechuic acid, 4-hydroxybenzoic acid, catechin, eriodictyol-7-O-glucoside, and naringin were detected in PGH (29).

A randomized, crossover, controlled nutrition study on 30 adults with type II diabetes examined the effects of daily pistachio consumption on lipid/lipoprotein profile, glycemic control, inflammation markers, and endothelial function. There was no significant change in body weight, body mass index, waist-to-hip ratio, blood pressure, total cholesterol, triglycerides, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and fasting blood sugar levels at the end of 12 weeks in patients who consumed 42 g/day and 70 g/day pistachios. At the end of the 12th week, the blood glucose level of the patients in the second group (70 g/day) was observed to be lower than at the beginning; nevertheless, the triglyceride level of the patients in the first group (42 g/day) decreased significantly ( $P = 0.018$ ) (30).

In a double-blind, randomized, placebo-controlled, crossover study to examine the effects of *P. vera* fruits on blood glucose levels in 48 patients with type 2 diabetes, a group of patients consumed 25 g of these fruits twice daily for 12 weeks. At the end of the 12 weeks, the patients had an 8-week washout period. Then, the other group of patients consumed the same amount of *P. vera* fruits for 12 weeks. At the end of the 12 weeks, a decrease was observed in hemoglobin A1c (0.4%) and fasting blood glucose (16 mg/dL), compared to those of the control group ( $P < 0.001$ ) (31).

In a randomized, crossover, controlled nutrition study of 30 adults (40 - 74 years) with type 2 diabetes, the participants consumed a pistachio or pistachio-free diet for 4 weeks. After the 2-week washout period, the patients changed groups and continued to be fed with pistachio or pistachio-free diet for 4 more weeks. Following the pistachio diet, total cholesterol and triglyceride levels were observed to be significantly reduced, compared to those of the control diet; nonetheless, no difference was observed



**Figure 2.** Chromatograms of 1,2,3,4,6-penta-O-galloyl-D-glucopyranose (PGG) (A), ethyl acetate subextract (B), and crude extract (C) and matching of spectra of the PGG peak in the crude extract and standard PGG (D) using reversed-phase high-performance liquid chromatography.

**Table 2.** Mass Spectrometric Identification of Compounds in *Pistacia vera* Leaf Extract

No.	T <sub>R</sub> [min]	[M-H] <sup>+</sup> <sub>exp</sub> [m/z]	[M-H] <sup>+</sup> <sub>calcd</sub> [m/z]	Δ [ppm]	Molecular Formula	MS/MS (-) [m/z]	Identification	References
1	7.769	169.0151	169.0142	5.05	C <sub>7</sub> H <sub>6</sub> O <sub>5</sub>	125.0252	Gallic acid	(24)
2	12.794	305.0681	305.0667	4.67	C <sub>15</sub> H <sub>14</sub> O <sub>7</sub>	261.0780; 219.0674; 125.0252	Gallocatechin	(26)
3	13.196	315.1096	315.0722	118.70	C <sub>13</sub> H <sub>16</sub> O <sub>9</sub>	153.0564; 109.0302	Gentisic acid-O-hexoside	(24, 25)
4	17.886	183.0316	183.0299	8.21	C <sub>8</sub> H <sub>8</sub> O <sub>5</sub>	124.0177; 78.0120	Methyl gallate	(24)
5	20.901	631.0958	631.0941	2.74	C <sub>28</sub> H <sub>24</sub> O <sub>17</sub>	479.0850; 457.0785; 316.0243; 167.0358	Myricetin 3-(6"-galloylhexoside)	(26)
6	21.454	625.1418	625.1410	1.28	C <sub>27</sub> H <sub>30</sub> O <sub>17</sub>	316.0236	Myricetin-O-rutinoside	(24)
7	21.789	479.0847	479.0831	3.34	C <sub>21</sub> H <sub>20</sub> O <sub>13</sub>	316.0237; 271.0255; 178.9994	Myricetin 3-O-hexoside	(26)
8	22.057	493.0638	493.0624	3.04	C <sub>21</sub> H <sub>18</sub> O <sub>14</sub>	317.0312; 178.9992	Myricetin 3-O-glucuronide	(21)
9	22.459	615.1012	615.0992	3.41	C <sub>28</sub> H <sub>24</sub> O <sub>16</sub>	463.0895; 300.0287; 169.0149	Quercetin 3-O-(6"-galloyl)-hexoside	(27)
10	22.710	197.0462	197.0455	3.55	C <sub>9</sub> H <sub>10</sub> O <sub>5</sub>	169.0148; 124.0170; 78.0115	Ethyl gallate	(22)
11	22.978	449.0734	449.0726	2.00	C <sub>20</sub> H <sub>18</sub> O <sub>12</sub>	316.0236; 271.0255; 197.0465	Myricetin-3-O-α-Pentoside	(28)
12	23.246	335.0416	335.0409	2.39	C <sub>15</sub> H <sub>12</sub> O <sub>9</sub>	183.0310; 124.0169	Methyl digallate	(23)
13	23.648	939.1113	939.1109	0.43	C <sub>41</sub> H <sub>32</sub> O <sub>26</sub>	769.0909; 617.0792; 169.0148	Pentagalloyl glucose	(20)
14	23.849	477.0690	477.0675	3.35	C <sub>21</sub> H <sub>18</sub> O <sub>13</sub>	301.0365	Quercetin-3-O-glucuronide	(24)
15	27.132	349.0573	349.0565	2.29	C <sub>16</sub> H <sub>14</sub> O <sub>9</sub>	197.0465; 124.0166	Ethyl 2,4-dihydroxy-3-(3,4,5-trihydroxybenzoyl)oxybenzoate	(23)

in fasting glucose and insulin levels (32).

In vivo antidiabetic activity studies were also conducted on other *Pistacia* species. The antidiabetic activities of *P. lentiscus* leaf and fruit extracts were analyzed in vivo on a streptozotocin (STZ)-induced diabetes mellitus model in rats. *Pistacia lentiscus* leaf extract was shown to be more active than the fruit extract. The leaf extract decreased serum glucose level at 125 mg/kg dose as nearly as the reference drug glibenclamide (33).

Behmanesh et al. aimed to evaluate the role of the hexane extract (200 mg/kg) of *P. atlantica* seeds in protecting against ovarian damage in an STZ-induced diabetes model in rats. In the aforementioned study, which continued for 4 weeks, the animals were given the extract every day. At the end of the experiment, blood sugar levels, oxidative stress parameters, and histological ovarian structure were evaluated. Blood glucose, malondialdehyde levels, and atretic follicle count were elevated in diabetic rats; catalase, superoxide dismutase levels, and the number of corpus lutea were significantly decreased. On the other hand, it was determined that the aforementioned values returned to nor-

mal or decreased in animals treated with *P. atlantica* extract and glibenclamide (34).

The α-amylase and α-glucosidase enzyme inhibitory activities of EtOAc subextract obtained from 70% acetone extract of *P. atlantica* leaves were examined in a study by using the Partial Least Squares regression and fingerprints correlation optimized warping. Based on the results of the study, only α-amylase was inhibited by glucogallin, quinic acid, and galloyl quinic acid in the leaves, thereby concluding that methyl gallate and tetragalloyl glucose inhibited only α-glucosidase; nevertheless, both α-amylase and α-glucosidase were inhibited by gallic acid, gentisic acid, and digalloyl quinic acid (35).

In a study examining the antidiabetic activity of *P. terebinthus* extract in STZ-induced diabetic rats, it was determined that the extract reduced blood sugar, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, lactate dehydrogenase, glucose, total triglyceride, total cholesterol, HDL, and LDL levels (36). An in vitro study investigated the α-amylase, α-glucosidase, and pancreatic lipase inhibitory activities of aqueous extracts of



*P. lentiscus* and *P. terebinthus* fruits and leaves. It was determined that the most active extract against pancreatic lipase was *P. lentiscus* leaf extract ( $6.1 \pm 0.2 \mu\text{g/mL}$  IC<sub>50</sub>). *Pistacia terebinthus* leaf, *P. terebinthus*, and *P. lentiscus* fruit extracts inhibited the same enzyme system with IC<sub>50</sub> values of  $9.0 \pm 0.4$ ,  $125.2 \pm 12.1$ , and  $230.7 \pm 38.4 \mu\text{g/mL}$ , respectively. *Pistacia lentiscus* and *P. terebinthus* leaf and fruit aqueous extracts were observed to inhibit  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes with IC<sub>50</sub> values ranging from  $65.3 \pm 7.4 \mu\text{g/mL}$  to  $1.4 \pm 0.2 \text{ mg/mL}$ , respectively. The findings of the study exerted that Sardinian *Pistacia* species can be used as functional foods and nutraceuticals that modulate carbohydrate and lipid digestion and absorption as potential sources to prevent obesity and diabetes mellitus (37).

Studies carried out on the chemical profile of *P. vera* have mostly focused on nuts, kernels, and oleoresin. Gallic acid, catechin, epicatechin, eriodictyol-7-O-glucoside, genistein-7-O-glucoside, naringenin-7-O-neohesperidoside, quercetin-3-O-rutinoside, genistein, eriodictyol, daidzein, quercetin, naringenin, luteolin, kaempferol, apigenin, cyanidin-3-O-galactoside, and cyanidin-3-O-glucoside were detected by the HPLC analysis of pistachio seeds and skins. Among the quantified compounds, quercetin-3-O-rutinoside was observed to be the main compound in seeds ( $98.08 \pm 1.54 \mu\text{g/g}$ ) and cyanidin-3-O-galactoside in skins ( $5865.12 \pm 362.45 \mu\text{g/g}$  fresh weight) (38).

It has been determined by scientific studies that PGG has strong  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities. In a study performed on 14 ellagitannins, it was determined that the most active compounds against  $\alpha$ -glucosidase were  $\alpha$ -pentagalloyl glucose ( $1.2 \pm 0.3 \mu\text{M}$  IC<sub>50</sub>) and PGG ( $1.4 \pm 0.2 \mu\text{M}$  IC<sub>50</sub>), and both of these compounds ( $32.9 \pm 1.8$  and  $17.2 \pm 1.6 \mu\text{M}$  IC<sub>50</sub>, respectively) showed high inhibitory activity against  $\alpha$ -amylase (39). On the other hand, Gok et al. determined that pancreatic lipase was inhibited by 43.22% (200  $\mu\text{g/mL}$ ) and 48.68% (10  $\text{ng/mL}$ ) by PGG and orlistat, respectively, used as a reference (20). Since this compound has high enzyme inhibitory activity and is the main compound in both crude extract and EtOAc subextract as a result of RP-HPLC analysis, the standardization process was performed on this compound.

It has been reported in previous scientific studies that 7 (i.e., ethyl gallate, gallic acid, gallic acid, gallic acid, methyl gallate, myricetin 3-[6"-galloylhexoside], quercetin 3-O-(6"-galloyl)-hexoside, and quercetin-3-O-glucuronide) of 15 compounds detected in the crude extract by LC-QTOF-MS have  $\alpha$ -glucosidase enzyme inhibitory effect, and one (gallic acid) of them has  $\alpha$ -amylase enzyme inhibitory effect (20, 40-46). The compounds, including gallic acid, methyl gallate, ethyl gallate, gallic acid, gallic acid, myricetin 3-(6"-

galloylhexoside), quercetin 3-O-(6"-galloyl)-hexoside, and quercetin-3-O-glucuronide, that were observed to have  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory effects in previous studies were detected by LC-QTOF-MS in *P. vera* leaf extract. Apart from the aforementioned compounds, the presence of six phenolic compounds, namely gentisic acid-O-hexoside, methyl digallate, ethyl 2,4-dihydroxy-3-((3,4,5-trihydroxybenzoyl)oxy)benzoate, myricetin-O-rutinoside, myricetin 3-O-hexoside, myricetin 3-O-glucuronide, and myricetin-3-O- $\alpha$ -pentoside, in *P. vera* leaves was detected for the first time in this study, and they might be responsible for the activity through synergistic interactions.

Flavonoids and phenolic acids are secondary metabolites commonly detected in plants with numerous biological activities, such as antioxidant, antidiabetic, anticancer, antibacterial, cardioprotective, and anti-inflammatory activities (47). Different parts of *P. vera* contain various secondary metabolites, such as tannins, phenolic acids, flavonoids, and anthocyanins (48). In the present study, the high total phenol content of *P. vera* leaves was responsible for the antioxidant activity.

#### 4.1. Conclusions

In this study, the in vitro antidiabetic, antihypercholesterolemic, and antiobesity effects of *P. vera* leaves were evaluated for the first time. *Pistacia vera* leaf hydroalcoholic extract and EtOAc subextract showed potent  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme inhibitory activities. It has also been supported by literature information that especially seven phenolic compounds identified in the plant by LC-QTOF-MS have inhibitory activities on these enzymes. In the light of the aforementioned data, it was concluded that the plant can be used as a standardized medicinal herbal product for the prevention and treatment of diabetes or as a source for the isolation of bioactive compounds. It also suggested that the antioxidant effect of the plant might be beneficial for diabetes complications. Future studies should be performed on the evaluation of the leaves of the *P. vera* in terms of metabolic diseases with in vivo experimental models and the isolation of their active compounds.

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## Footnotes

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