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Investigation of antibiofilm and biological activities of *Vaccinium arctostaphylos* L.

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Abstract

This study investigated the total phenolic and flavonoid contents and the antioxidant, antimicrobial, antibiofilm and α-glucosidase inhibitory activities of the methanol extract from *Vaccinium arctostaphylos* L. leaf and fruit parts. The highest antioxidant activity determined in leaf part with 53 µM TEAC and 8.4 µg/mL SC_{50} using the ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH•) radical scavenging assays, respectively. The data indicated that the leaf of the plant had the higher total phenolic content (49 µg/mL GAE) and total flavonoid content (0.071 µg/mL QAE) compared to fruit. The α-glucosidase enzyme activity of the leaves (IC_{50}; 0.179 mg/mL) was observed to be higher than that of the fruits (IC_{50}; 0.386 mg/mL). The MIC values of the leaf and fruit parts of *V. arctostaphylos* were 6.25 mg/mL and 3.125 mg/mL, respectively. The results of this study indicate that the leaf extract was found to significantly reduce the biofilm-forming capacity of the *Acinetobacter baumannii* isolate by approximately 3-fold, whereas the fruit extract was observed to have only a marginal effect, reducing the biofilm-forming capacity by approximately 1.4-fold. The effects of plant extracts on microbial biofilms may be examined with a view to combating antibiotic resistance. Also, results suggesting that it might be an effective medical plant to prevent or treat diseases associated with oxidative damage and bacterial infections.

Keywords: Antibiofilm, antioxidant activity, *Vaccinium arctostaphylos* L., α-glucosidase

1. Introduction

Medicinal plants contain bioactive compounds that are used instead of drugs in traditional treatment methods. The use of plants containing secondary metabolites, including phenolics and flavonoids, in alternative therapies is a growing area of research. These metabolites have been shown to have therapeutic effects in the treatment or suppression of a number of diseases [1]. Secondary metabolites which have antioxidant activity considered able to scavenging and prevent free radicals that cause oxidative damage in biomolecules [2]. In addition, natural alternatives with much fewer side effects are preferred instead of synthetic antioxidants in the treatment of diseases [3]. Therefore, there is a growing interest in the identification of natural compounds that can prevent oxidative damage and the harmful effects of free radicals.

The genus *Vaccinium* L., which belongs to the family Ericaceae, includes nearly 450 species worldwide [4]. Türkiye, which has an important flora in terms of medicinal plants, is home to several of the *Vaccinium* species. The literature contains numerous reports of the biological activities of *Vaccinium* [5,6]. Previous studies have indicated that *Vaccinium* species exhibit bioactivity properties, including antioxidant, antimicrobial and antidiabetic effects [7–9].

Vaccinium arctostaphylos L. is a compact shrub with a height of approximately 1.5-2.5 m, found between 1600-1800 m above sea level. Fruits of the *V. arctostaphylos* contain approximately levels of 30% sugar, 15% protein, and 2% fat. Phenolics with antioxidant properties are the highly important metabolites in the leaves and fruits of *V. arctostaphylos* [10]. The plant is widely have been used as an antidiabetic agent for a long time in traditional medicine [11].

It has been reported that the fruit and leaves of this plant have serum glucose and lipid level lowering

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activities and are also used in the treatment of hypertension [4]. Moreover, previous studies have reported that caffeic acid is the main phenolic compound of *V. arctostaphylos*, which contains various phenolic compounds [12].

One of the most important public health problems in the world is the high resistance of gram-negative bacteria to antibiotics. Infections caused by these bacteria are associated with high morbidity and mortality due to limited treatment options [13]. It is essential to research and develop more effective natural antibacterial agents to combat bacterial infections caused by pathogens.

To date, there have been few studies investigating the antimicrobial activity of *V. arctostaphylos* fruit and leaf extracts. Furthermore, to the best of our knowledge, no study has been conducted on the antibiofilm activity of these extracts. Thus, the present study aimed to evaluate the antioxidant, antidiabetic, antimicrobial and antibiofilm activities of the fruit and leaf methanolic extracts of *V. arctostaphylos*.

2. Materials and methods

2.1. Chemicals and reagents

Methanol, ethanol, NaOH, NaCl, HCl, Na_2CO_3, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DPPH• (2,2-diphenyl-1-picrylhydrazyl), $FeCl_3.6H_2O$, acetic acid, gallic acid, quercetin, ammonium acetate, ammonium nitrate, α-Glucosidase (*Saccharomyces cerevisiae*, lyophilized powder, ≥ 10 units/mg protein), 4-nitrophenyl-α-D-glucopyranoside, Folin-Ciocalteu's phenol reagent and yeast extract were purchased from Sigma Aldrich (St. Louis, MO, USA). TPTZ (2,4,6-tris(2-pyridyl)-s-triazine), tryptone and crystal violet were purchased from Merck (Darmstadt, Germany).

2.2. Plant material and sample preparation

V. arctostaphylos leaves and fruits were collected from Sürmene, Trabzon, Türkiye, in September 2023. The leaves and fruits were subjected to a drying process at room temperature over a period of four months. Subsequently, the dried samples were pulverized using a grinder, after which the pulverized samples were extracted with methanol solvent in a shaker for a period of two hours. Following the shaking process, the extracts were filtered through 0.45 µm syringe filters (Whatman) to produce clear solutions. Methanol, used as solvent was evaporated with a rotary evaporator under low pressure. Both the fruit and leaf extracts were dissolved in methanol to the desired concentration. The extracts were stored at a temperature of 4 °C until further use in subsequent experiments.

2.3. Determination of antioxidant activities

2.3.1. DPPH radical scavenging activity

The DPPH• radical scavenging activities of methanol extracts of the leaf and fruit parts were investigated using the method described by Brand-Williams et al. [14]. The method was applied by mixing the extracts with DPPH• solution and keeping them at ambient temperature and in the dark for 50 minutes. The changes in the absorbance of the DPPH• treated with extracts and standard antioxidant were measured at a wavelength of 517 nm. A graph was drawn according to the concentrations that corresponded to these absorbance values. In this graph, the amount of sample required to halve the concentration of DPPH• was determined in µg/mL and expressed as the SC_{50} value. The SC_{50} values were compared with the standard antioxidant Trolox.

2.3.2. Ferric reducing antioxidant power (FRAP)

The FRAP values of extracts were evaluated using the method described by Benzie and Strain [15], whereby the total reduction capacity determined indirectly. Each extract and standard solution were mixed newly prepared FRAP reagent. Then solutions were vortexed and kept for 20 min incubation period at ambient temperature and the absorbance values were read at 595 nm. The FRAP activities of the extracts were expressed as µM TEAC (Trolox Equivalent Antioxidant Capacity), which was obtained by using the calibration graph of Trolox.

2.4. Determination of total phenolic and flavonoid contents

2.4.1. Total phenolic content (TPC)

The total phenolic content of the extracts was evaluated *in vitro* using the Folin-Ciocalteu reagent method, as described by Slinkard and Singleton [16]. Each extract and standard solution were mixed Folin reagent. Then, the Na_2CO_3 (7.5%) was added to the solution and it was vortexed. The reaction solutions were incubated for a period of two hours at a temperature of ambient. The total phenolic content was calculated based on absorbance measurement at 765 nm, with the values expressed as µg of gallic acid equivalent per mL of extract (µg/mL GAE) using calibration graph.

2.4.2. Total flavonoid content (TFC)

The total flavonoid content of the extracts was determined in accordance with the method of Fukumoto and Mazza [17]. The calibration curve was obtained with quercetin (QAE) and the results expressed as quercetin equivalent (µg/mL QAE).

2.5. Determination of antimicrobial activity

2.5.1. Determination of minimum inhibitory concentrations of extracts

Both the leaf and fruit parts of the *V. arctostaphylos* were extracted. The final concentrations were determined as 50 mg/mL, and these concentrations were used as the starting concentration (50-0.097 mg/mL) in the liquid microdilution method to determine minimum inhibitory concentrations (MIC). MIC values of the extracts were investigated against the previously determined clinical antibiotic-resistant *Acinetobacter baumannii* isolate with biofilm-forming capacity. All experiments were performed in 96 well plates and in triplicate [18].

2.5.2. Investigation of antibiofilm properties of extracts

After determining the MIC values of *V. arctostaphylos* leaves and fruit parts against the antibiotic-resistant *A.baumannii* isolate, 1/2 MIC values were used as reference values for the antibiofilm experiment. The experiment was carried out in 96 well plates and in triplicate. *A. baumannii* was incubated in 3 mL of LB medium at 37 °C overnight. After incubation, 1/100 diluted *A. bumannii* isolate along with 1/2 MIC leaf and fruit extracts were added to 96 well plates and incubated again overnight. Then, the suspension in the plate was poured and the plate was washed three times with distilled water. 200 µL of 1% crystal violet dye was added to each well and left at room temperature for 20 minutes. Crystal violet was removed from the plate and the washing process was repeated with distilled water. After washing the plate, it was left to dry for 15 minutes at room temperature. Then, 200 µL of 95% ethanol was added to the wells. *Escherichia coli* Dh5@ was used as a negative control (Ac). Optical absorbance (A) value was measured at 620 nm in the spectrometer. The evaluation was made according to four different criteria: 1. A≤Ac Negative, 2. Ac<A≤2Ac Weak positive, 3. 2Ac<A≤4Ac Moderately positive, 4. A>4Ac Strong positive [18].

2.6. Determination of enzyme inhibition

2.6.1. α-Glucosidase inhibition assay

The α-glucosidase enzyme activity of fruit and leaf extracts of *V. arctostaphylos* was investigated in a modified method of Yu et al. [19]. Initially, 650 µL of buffer solution (pH 6.8, concentration 0.1 M), 20 µL sample and 30 µL enzyme (*Saccharomyces cerevisiae*, lyophilized powder, ≥ 10 units/mg protein) were mixed. The mixture was maintained at a temperature of 37 °C for a period of 10 minutes, after which 75 µL of the substrate (4-nitrophenyl-α-D-glucopyranoside) was added. Following a 20-minute incubation period at 37 °C, 650 µL of 1 M Na_2CO_3 was added to halt the reaction. The absorbance values were determined by measuring them at a wavelength of 405 nm using a UV/VIS spectrophotometer.

Acarbose was employed as the positive control, with different concentrations employed as the standard inhibitor. The study was conducted in triplicate and in reagent-sample blinds. IC_{50} values (the concentration of the sample that halves the enzyme activity) were calculated for both acarbose and the samples.

3. Results and discussion

3.1. Evaluation of antioxidant activity

In this study, antioxidant activities of methanolic extracts of *V. arctostaphylos* leaf and fruit were determined using DPPH and FRAP methods (Table 1). DPPH•, a stable free radical, is a widely used method to evaluate the antioxidant activity of extracts. The FRAP test, which measures the reducing capacity of an extract, is an indirect method for determining its antioxidant activity.

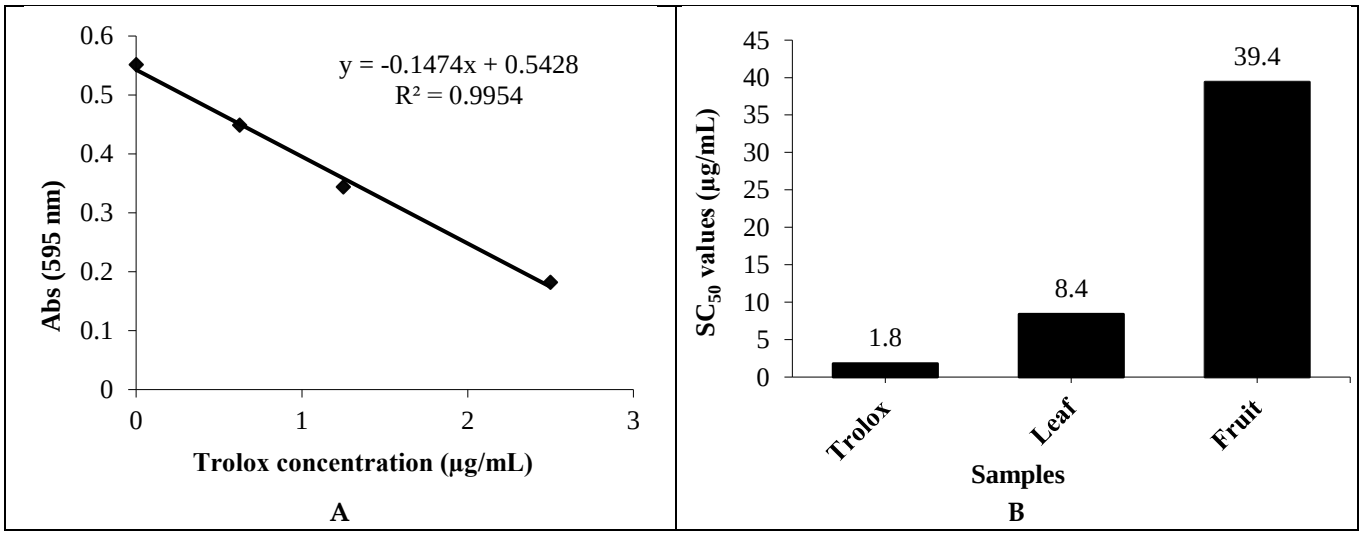

Figure 1. A: The graph depicts the changes in absorbance of the DPPH radical in the presence of varying concentrations of Trolox, B: SC_{50} values of *V. arctostaphylos* leaf and fruit extracts and Trolox

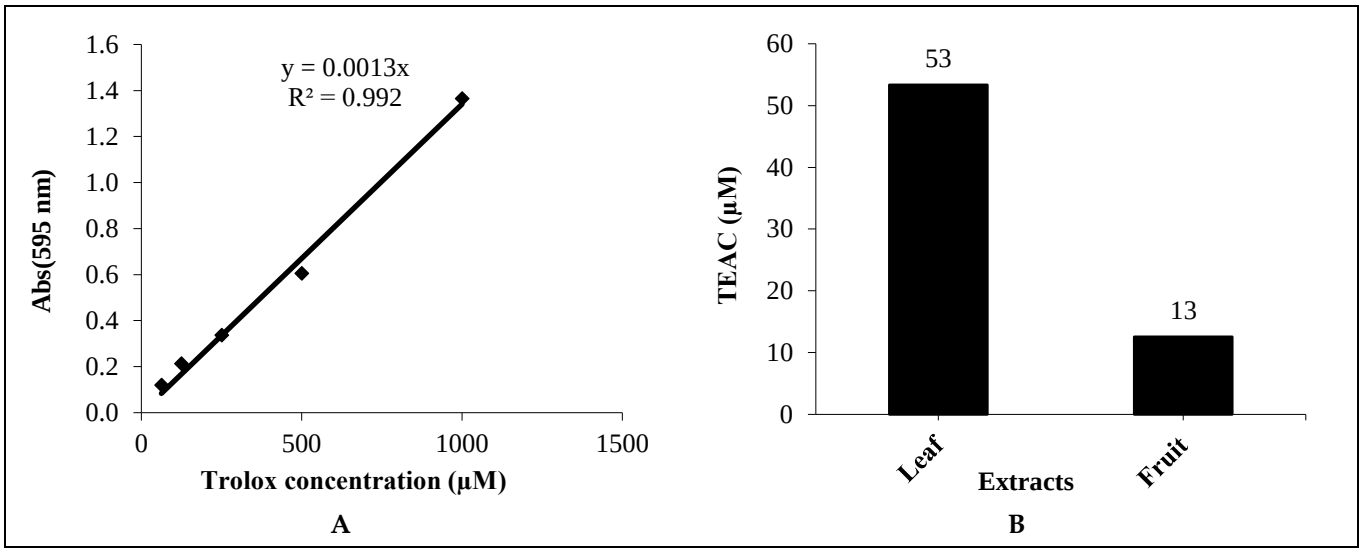

Figure 2. A: Graph of calibration corresponding to changes in absorbance of iron (III) reduction potential of the with different concentrations of Trolox, B: TEAC (µM) values of the leaf and fruit parts of the *V. arctostaphylos* extracts

In a previous study, Mahboubi et al. [20] reported that the SC_{50} values of DPPH radical scavenging in leaf and fruit samples were 60 and 35 µg/mL, respectively. In another study Musavi et al. [21] reported that the SC_{50} values of DPPH radical scavenging activity in the methanolic extract of the leaf and fruit parts of *V. arctostaphylos* were 3.20 and 2.98 µg/mL, respectively. In contrast to these studies, the SC_{50} values of the DPPH radical scavenging of the extracts were 8.4 and 39.4 µg/mL in leaf and fruit, respectively in our study (Fig. 1). Trolox, which was evaluated as standard, showed 4.5 times higher the radical scavenging activity from the leaf extract with an SC_{50} value of 1.8 µg/mL (Fig. 1). Leaf extracts of *V. arctostaphylos* L. showed higher than fruit extract DPPH• scavenging activity. Moreover, the *in vitro* outcomes exhibited a positive correlation of the total phenolic and flavonoid content of the extracts (Table 1).

In a study conducted by Hasanloo et al. [22] it was found that the leaf and fruit of *V. arctostaphylos* exhibited remarkable reducing activities across a range of genotypes. In another study, Güder et al. [23] reported that ethanolic extract of *V. arctostaphylos* fruit exhibited notable FRAP activities at varying concentrations. In the present study, when the iron (III) reduction potential of the extracts was examined by the FRAP method, it was found that the antioxidant power of the leaf extract was 4 times higher than the fruit extract, with 53 ± 2.72 µM TEAC (Fig. 2).

Table 1. Antioxidant activity, TPC, and TFC values of the leaf and fruit parts of the *V. arctostaphylos* extracts

	DPPH SC_{50} (µg/mL)	FRAP TEAC (µM)	TPC GAE (µg/mL)	TFC QAE (µg/mL)
Leaf	8.4 ± 0.5	53 ± 2.72	49 ± 3.47	0.071 ± 0.0030
Fruit	39.4 ± 6.1	13 ± 1.08	12 ± 1.92	0.008 ± 0.0006

3.2. Evaluation of total phenolic and flavonoid contents

Phenolic and flavonoid compounds, which are secondary metabolites, are responsible for a multitude of biological activities, including antioxidant, antidiabetic, and antimicrobial effects. [22,24,25]. In this study, evaluation of total phenolic and flavonoid contents was

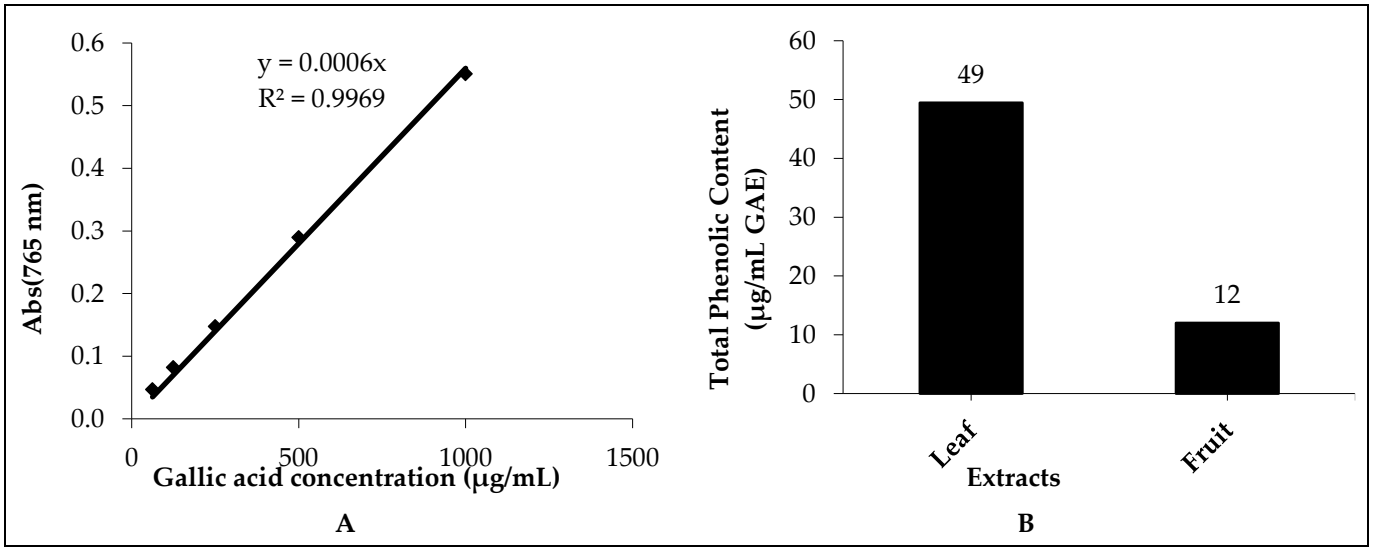

Figure 3. A: The standard curve of gallic acid, B: The TPC of leaf and fruit parts of the *V. arctostaphylos* expressed as µg/mL GAE

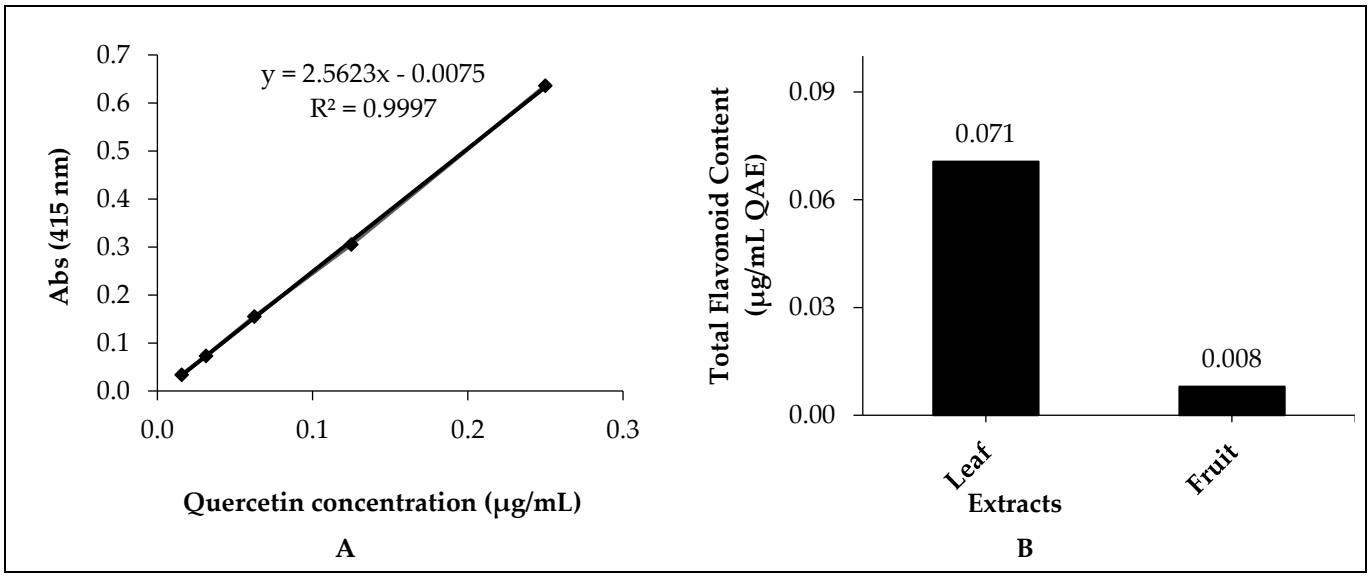

Figure 4. The standard curve of quercetin, B: The TFC of leaf and fruit parts of the *V. arctostaphylos* expressed as µg/mL QAE

determined of the both the leaf and fruit parts of the *V. arctostaphylos* (Table 1). A standard curve of gallic acid was used to determine total phenolic content (Fig. 3), with the results expressed in terms of µg/mL GAE. A standard curve of quercetin was used in order to determine the total flavonoid content (Fig. 4), and the results were expressed as µg/mL QAE. Leaf extract of *V. arctostaphylos* had the highest total phenolic and flavonoid content, with 49 ± 3.47 µg/mL GAE and 0.071 ± 0.0030 µg/mL QAE, respectively (Fig. 3 and Fig. 4). In the literature, Saral et al. [26] reported that the total phenolic and flavonoid contents of methanolic extracts of *V. arctostaphylos* fruits from different regions ranged from 20.74 to 11.54 mg GAE/g and 1.93 to 2.16 mg QAE/g dry weight of sample, respectively. Mahboubi et al. [20] found that the leaf parts of *V. arctostaphylos* methanolic extracts contained higher amounts of flavonoids compared to the fruit parts. In their study, Shamilov and colleagues identified 10 phenolic compounds in the leaves of *V. arctostaphylos* [6]. Also, the previous studies reported that caffeic acid is the major phenolic compound of *V. arctostaphylos* [12].

A scatter graph of the antioxidant activity, TPC and TFC values for the leaf and fruit extracts of *V. arctostaphylos* was created (Fig. 5). The results demonstrated that there was a positive correlation between DPPH, FRAP, TPC and TFC antioxidant activity results of the plant extracts leaf and fruit parts (R^2: 0.9999).

3.3. Evaluation of antimicrobial activity

Antimicrobial and antibiofilm activities of methanol extracts of leaves and fruit parts of *V. arctostaphylos* were investigated against the antibiotic-resistant clinical isolate *A. baumanii.* The MIC value of the leaf part of *V. arctostaphylos* was determined as 6.25 mg/mL, and the MIC value of the fruit part was determined as 3.125 mg/mL (Table 2).

After the MIC values were determined, the antibiofilm activities of the extracts were evaluated using 1/2 MIC values. *E. coli* Dh5@ isolate was used as a control. All evaluations were made based on the OD value (Ac: 0.1915) of the biofilm-forming capacity of the control isolate. The biofilm formation capacity of the control strain was taken as a reference point, and it was determined that the *A. baumannii* clinical isolate exhibited a moderate capacity for biofilm formation. In the experiment where the antibiofilm activity was investigated, it was determined that the biofilm-forming property of *A. baumannii* treated with the leaf extract decreased to negative, and in the experiments conducted with the fruit part, the biofilm-forming property of the isolate decreased from moderate to weak (Table 2). The findings demonstrate that the leaf part of *V. arctostaphylos* exhibits a higher antibiofilm activity than the fruit part.

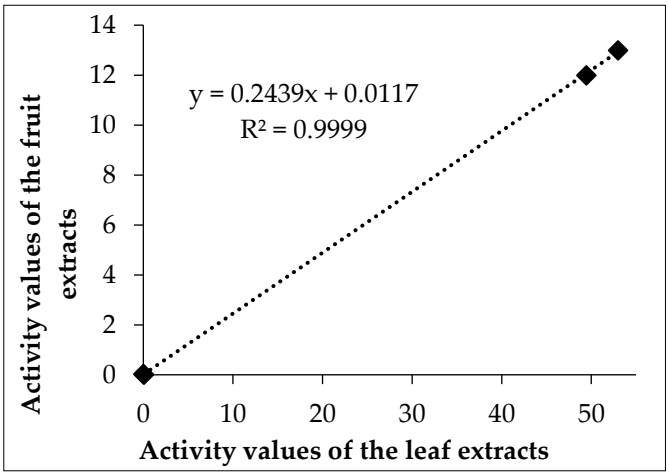

Figure 5. Correlations of total phenolics, FRAP, total flovanoids and DPPH antioxidant activity results of the leaf and fruit parts the *V. arctostaphylos* extracts

Table 2. MIC and antibiofilm results of the leaf and fruit parts of the *V. arctostaphylos* extracts

	MIC Value (mg/mL)	Biofilm Value / OD
Leaf	6.250	0.1372
Fruit	3.125	0.2930
A73 (*A.baumannii*)	—	0.4132
E.coli Dh5@ (control)	—	0.1915

Despite the continuing global public health threat posed by antimicrobial resistance, there are also constraints on the discovery and development of new antimicrobial agents. Biofilm formation represents a significant mechanism of resistance employed by a multitude of pathogens, rendering them increasingly challenging to treat [27,28]. Microorganisms can form biofilms that protect them from the effects of natural host defenses and antimicrobial agents [27,29]. This is one of the mechanisms by which bacteria become resistant to antibiotics. Plants are a rich source of compounds with biological activity, making them an excellent resource for the discovery of useful and novel antimicrobial products [27,30,31]. Plant extracts may demonstrate antimicrobial activity when used in their own or may serve as a source of effective antimicrobial compounds that can act against biofilms of pathogens. Consequently, the investigation of the antibiofilm activities of plants against pathogenic bacteria may represent an important contribution towards the development of treatments for diseases [27]. Additionally, further research is needed to isolate compounds from plants with antibiofilm effects and determine the mechanism of activity.

3.4. Evaluation of α-glucosidase inhibitory effects of extracts

The α-glucosidase enzyme activity of fruit and leaf extracts of *V. arctostaphylos* was measured as IC_{50}; 0.386 and 0.179 mg/mL, respectively (Table 3). The α-glucosidase enzyme inhibition value of the leaf part of the plant is higher than that of the fruit part. The α-glucosidase enzyme plays a pivotal role in non-insulin-based treatments for diabetes, as it catalyzes the final step in the digestion of carbohydrates, which is a crucial aspect of the metabolic process [32]. In the literature, there are few studies on the α-glucosidase enzyme activity of the fruit parts of this plant. In a study, the fruit part of the plant was extracted with different solvents (Ethanol, Methanol and distilled water) and α-glucosidase enzyme inhibition values were determined. Inhibition was observed in all three solvents in the results obtained. The IC_{50} value of the methanol extract in this study was 0.477 mg/mL [33]. The results of this test demonstrated that the leaves of this plant, which are typically consumed as fruit, also possess significant enzyme activity.

Table 3. α-Glucosidase enzyme inhibition activity of *V. arctostaphylos*

Sample	IC50 (mg/mL)	R2
Acarbose	0.029 ± 0.02	0.9971
Leaf	0.179 ± 0.03	0.9912
Fruit	0.386 ± 0.05	0.9957

4. Conclusions

This study presented the total phenolic and flavonoid contents and the antioxidant, antimicrobial, antibiofilm, and α-glucosidase inhibitory activities of methanol extract from *V. arctostaphylos* leaf and fruit parts. The study data demonstrated that leaf extract of this plant had the higher biological activities compared to the fruit extracts. In addition, the similar results between antioxidant activity and total phenolic and flavonoid contents resulted in a very good correlation (R^2: 0.9999). The findings show that *V. arctostaphylos* has antibiofilm activity, and the leaf part exhibits a higher antibiofilm activity than the fruit part. The results of our study indicate that *V. arctostaphylos* may be a promising candidate for the prevention and treatment of diseases associated with oxidative damage and bacterial infections. Consequently, further research on these biological activities *V. arctostaphylos* may lead to the development of treatment options. Also, to be suggest for the treatment of human diseases, it must undergo thorough evaluation in various clinical trials.

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None.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' contribution

UK: Conducted the extraction, antioxidant activity experiments and wrote the article. ZA: Conducted the extraction, enzyme activity and antioxidant activity experiments. AÖD: Performed the antibiofilm experiments and contributed to the article writing.

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