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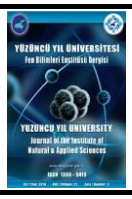

Research Article

In Vitro α-Glucosidase, α-Amylase Inhibitory and Antioxidant Activities of Root Crude Extract and Solvent Fractions of *Arbutus unedo* L. (Ericaceae)

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Abstract: Roots of *Arbutus unedo* (Ericaceae) have been used traditionally to treat diabetes in some countries. Conducting more experiments is needed to support this plant's potential antidiabetic properties, though. Ethanolic extract yielded by cold maceration was subjected into fractionation to get hexane, chloroform, ethyl acetate, butanol, and aqueous fractions. Total phenolic contents and antioxidant capacity have been estimated utilizing 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric ion reducing antioxidant potential (FRAP) assay models, respectively. Antidiabetic activity of *A. unedo* extracts were evaluated using *in vitro* α-glucosidase, α-amylase inhibition activity. The ethanol extract had the highest antioxidant activity among used extracts. While the results of both α-glucosidase, α-amylase enzyme inhibition activity was detected to be dose-dependent, the strongest inhibition activity for α-glucosidase and α-amylase was shown by ethanol extract (526.65 and 522.66 μg mL^{-1} respectively) compared to the standard acarbose. Results demonstrated the beneficial effects of *A. unedo* extracts by showing antioxidant and antidiabetic activities.

Arbutus unedo L. (Ericaceae) Kök Ham Ekstresinin ve Çözücü Fraksiyonlarının İn Vitro α-Glikosidaz, α-Amilaz İnhibitör ve Antioksidan Aktiviteleri

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Anahtar Kelimeler
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Öz: *Arbutus unedo* (Ericaceae) kökleri bazı ülkelerde geleneksel olarak diyabet tedavisinde kullanılmaktadır. Ancak bu bitkinin potansiyel antidiyabetik özelliklerini desteklemek için daha fazla çalışmalara ihtiyaç vardır. Soğuk maserasyonla elde edilen etanolik ekstreden, heksan, kloroform, etil asetat, bütanol ve sulu fraksiyonlar elde edilmiştir. Toplam fenolik içerik ve sırasıyla 2,2-difenil-1-pikrilhidrazil (DPPH), 2,2-azino-bis-3-etilbenzotiyazolin-6-sülfonik asit (ABTS) ve ferrik iyon indirgeyici antioksidan güç (FRAP) tahlil modelleri kullanılarak antioksidan kapasite değerlendirilmiştir. *A. unedo* ekstrelerinin antidiyabetik aktivitesi, *in vitro* α-glukosidaz, α-amilaz inhibisyon aktivitesi kullanılarak değerlendirilmiştir. Kullanılan ekstreler arasında etanol ekstresi en yüksek antioksidan aktiviteye sahiptir. Hem α-glukosidaz hem de α-amilaz enzim inhibisyon aktivitesinin sonuçları doza bağlı olarak bulunurken, standart Akarbozla karşılaştırıldığında α-glukosidaz ve α-amilaz için en güçlü inhibisyon aktivitesi etanol ekstresi ile gösterilmiştir (sırasıyla 526,65 ve 522,66 μg mL^{-1}). Sonuçlar *A. unedo* ekstrelerinin antioksidan ve antidiyabetik aktiviteler göstererek faydalı etkilerini ortaya koymuştur.

1. Introduction

Hyperglycemia caused by abnormalities in insulin production, action, or both characterizes a group of metabolic illnesses known as diabetes mellitus (DM). According to reports, diabetes now affects about 463 million people globally and may increase to 578 and 700 million by the years 2030 and 2045, respectively (Saeedi et al., 2019). There are two recognized kinds of DM: Type 1, which results in an absolute lack of insulin secretion, and Type 2, which results in both resistance to the effect of insulin and insufficient insulin production (American Diabetes Association, 2008). Beside the main two types there are gestational DM which is the most common metabolic complication for pregnant women, it positively correlates with Type 2 DM later in life (Zhu & Zhang, 2016). Reactive oxygen species (ROS) were produced because of chronic hyperglycemia. Three key processes that result in the production of ROS-glucose autooxidation, protein glycation, and activation of the polyol pathway-can be outlined. These processes are accompanied by weakened antioxidant defenses that cause oxidative stress (Bonnefont-Rousselot, 2002). Oxidative stress causes damage to DNA, protein, lipid, carbohydrate, and enzyme components (Halliwell & Gutteridge, 2015). Numerous pharmacological methods have been employed to reduce hyperglycemia, primarily by promoting insulin release, raising glucose transport activity, obstructing gluconeogenesis, and lowering gastrointestinal glucose absorption. To improve glycemic control, currently available medications can be used singly or in combination (Jung et al., 2006; Zhao et al., 2018). Side effects, such as gastrointestinal symptoms, weight gain, edema, heart failure, impaired kidney function, genital infections, and pancreatitis, etc., are associated with the existing conventional antidiabetic agents. Treatments with less side effects are necessary, and plant extracts may be a useful therapeutic strategy (Lee et al., 2021). Recently, several antioxidants, particularly those derived from plants (e.g. *Punica granatum*, *Quillaja saponaria* and *Yucca schidigera*), demonstrated benefits against diabetes and associated complications (Fidan et al., 2009; Okumuş, 2023).

The Ericaceae family includes the *Arbutus* genus, which has 12 accepted species. Mediterranean to the Macaronesia and Western Europe; and Central America to Western Canada make up this species' natural distribution (POWO, 2023). One of the important species of this genus is *A. unedo*, commonly known as Kocayemiş (Turkish), Strawberry tree (English), the most well-known and most researched species among them because of its industrial, therapeutic, and nutritional use. This species is an evergreen shrub or small tree with a height reach to 3-6 m. Spherical red fruits are 1-2 cm in diameter and it ripens in autumn (Baytop, 1999; Morales, 2022). Various classes of phytochemicals, including phenolic compounds, terpenoids, anthocyanins, flavonoids, and tannins, have been found in diverse plant components, according to the study conducted by El Haouari and colleagues (El Haouari et al., 2021). Various parts of *A. unedo* (e.g., flower, root, fruit, and leaf) are used traditionally against different diseases (Bebek Markovinović et al., 2022). Results of research studies conducted both *in vivo* and *in vitro* indicate antioxidant, antibacterial, anti-inflammatory, cytotoxic, anti-hypertensive, and antidiabetic actions of *A. unedo* (Koyu et al., 2019; Mrabti et al., 2021).

Although numerous studies have been conducted to examine this plant's antidiabetic activity using various extraction techniques in various experimental models, additional research is still required on this species that is widely distributed in our country to support the findings from the earlier studies (Mrabti et al., 2018; Tenuta et al., 2020). The goal of the current investigation was to ascertain whether the ethnopharmacological relevance of using *A. unedo* roots to treat diabetes had any scientific backing using *in vitro* antioxidant activity, α-glucosidase, and α-amylase inhibition of crude extract and solvent fractions obtained from the plant.

2. Material and Methods

2.1. Materials

The α-glucosidase, α-amylase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2, 4, 6-tripyridyl-s-triazine (TPTZ), Trolox, gallic acid and Folin-Ciocalteu's reagent were from Sigma–Aldrich (St. Louis, Missouri, USA). Ethanol, methanol, butanol, ethyl acetate, and hexane were purchased from Merck (Darmstadt, Germany). Acarbose (tablets 50 mg Glucobay®, Türkiye) was obtained from a local pharmacy.

2.2. Plant material collection and extraction

Roots of *A. unedo* were collected near Armutlu District, Yalova (40°31'35.7"N 28°47'23.3"E) in June 2022. Plant authenticated by Dr. Genç from Istanbul University's Faculty of Pharmacy's Department of Pharmaceutical Botany (voucher number: ISTE-118582) (Fig. 1). The *A. unedo* root was cleaned to remove undesired particles, and then dried under the shade for 20 days at room temperature (25-27°C). After the dried plant was powdered, 250 g of the material was maintained in a medium containing 80% (v/v) ethanol for 72 hours. After being filtered using filter paper, the solvent was then eliminated in a rot-evaporator at 40°C. After being dissolved in dH_2O, the residue was partitioned with hexane, chloroform, ethyl acetate, and butanol. The fractions and remaining aqueous fraction were concentrated in a rotary evaporator (Akuodor et al., 2010; Hossain et al., 2014).

Figure 1. The photos of *A. unedo* by Ahmet Beyatlı.

2.3. Phytochemicals screening

The preliminary qualitative screening of different phytochemicals for *A. unedo* root fractions has been done on the ethanolic extract and solvent fractions using standard methods as described by the Tyler (Tyler, 1993) and Harborne (Harborne, 1998) methods. Results were qualitatively shown as positive (+) or negative (−).

2.4. Determination of total phenolic content

In summary, the reaction mixture included 50 µL of sodium carbonate (7%), 2.5 µL of the Folin-Ciocalteu reagent, 195 µL of dH_2O, and 2.5 µL of extracts. The samples and standards absorbances were measured after 30 minutes in the dark at 765 nm. Absorbance versus gallic acid concentration was plotted and a calibration graph was drawn. Then, samples absorbance was substituted in the graphic equation and the determination of the total phenolic substance was determined in mg gallic acid g^{-1} sample (Zhou et al., 2004).

2.5. *In vitro* antioxidant activity

Trolox was utilized as the reference substance in tests to determine the DPPH (2,2-diphenyl-1-picrylhydrazyl) antioxidant capacity. 0.1 mL of sample/standard, 0.18 mL of 1 mM DPPH solution, and 3 mL of methanol in total were added to tubes. After 30 minutes in darkness, the absorbance of analysis tubes was measured at 515 nm (sample). Following measurements, percentage inhibition values were calculated using the formula:

$$\% \text{ Inhibition} = \frac{\text{A blank} - \text{A sample}}{\text{A blank}} \times 100 \quad (1)$$

The extracts' antioxidant activity levels were determined as "mg Trolox g^{-1} sample" using the calibration table for the standard (Şahin et al., 2012).

In 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) antioxidant activity assessment Trolox was utilized as the reference material in investigations. It was combined in water with 0.245 mM K2S2O8 and 0.746 mM ABTS. The ABTS solution was diluted at 1:10 after being left in the dark for

between 24 and 48 hours. After adding the sample and standard, 3.9 mL of ethanol, and 1 mL of the diluted ABTS solution to the analysis tubes, absorbance was measured at 734 nm after 6 minutes (Sample). Then samples' percentage inhibition values were computed using the same formula used for DPPH calculation.

Extract levels of antioxidant activity were determined as "mg Trolox g^{-1} sample" using the calibration chart (Benzie & Strain, 1996; Şahin et al., 2012).

The ferric ion reducing antioxidant potential (FRAP) method, based on the reduction of Fe^{3+}-TPTZ (ferrictripyridyltriazine) complex to Fe^{2+} in the presence of antioxidants in an acidic medium, was utilized to measure the antioxidant activity using Trolox as a standard (Benzie & Strain, 1996). The analysis tubes were filled with 0.1 mL of sample/standard, and 2.9 mL of FRAP reagent, and maintained in the dark for 30 minutes before the sample/standard absorbances were measured at 593 nm. The antioxidant capacity values of the extracts were calculated in mg Trolox/g sample using the derived line equation.

2.6. Inhibition of α-glucosidase activity

The mixture of reaction containing 10 µl α-glucosidase (1 U mL^{-1}), 50 µl phosphate buffer (100 mM, pH = 7.0), and 20 µl of *A. unedo* extract, fractions and acarbose dissolved in DMSO at multiple concentrations (200, 400, 600, 800, and 1000 µg mL^{-1}) was preincubated at 37°C for 15 min. Then, 20 µL of PNPG (*p*-nitrophenyl-glucoside) (5 mM) was added, and the reaction was incubated for 15 minutes. 80 µL of sodium carbonate solution (0.10 M) was used in finishing up the reaction. The experiment's absorbances were measured at 405 nm. Acarbose has been used as a standard drug (Telagari & Hullatti, 2015).

2.7. Inhibition of α-amylase activity

The phosphate buffer was used in the preparation of the amylase solution. The amylase solution was mixed with *A. unedo* extract, fractions and acarbose dissolved in DMSO at multiple concentrations (200, 400, 600, 800, and 1000 µg mL^{-1}). After the reaction was finished, the starch solution was added. After incubation, the dinitro-salicylic acid solution was applied to control and test groups. Heated solution absorbances were measured at 650 nm. As a positive control, acarbose was utilized (Shabab et al., 2021; Huneif et al., 2022). By plotting the inhibitions against the measured extract solution concentrations, both α-glucosidase and α-amylase IC50 (half inhibitory concentration) values were determined.

2.8. Statistical analysis

The Statistical Package for the Social Sciences (SPSS) (version 13.0) has been utilized for the statistical analyses, experiments were carried out in triplicate. Results were reported as means and standard errors of the means (SEM). One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were used to examine the variations. *p*-value of 0.05, differences were deemed significant. Plotting % inhibition against concentration curves allowed us to obtain the values of IC50 (half-maximal inhibitory concentration).

3. Results and Discussion

Members of the genus *Arbutus*, e.g. *andrachne*, *pavarii* and *unedo*, can be a good source for phenolics and flavonoids. These phytochemicals widely recognized to have substantial antioxidant and antidiabetic properties, making them an excellent alternative for chemical drugs at the future (Tenuta et al., 2019; Buzgaia et al., 2021).

Qualitative phytochemical analysis of *A. unedo* root extract and fractions showed the presence of different bioactive components (Table 1). Earlier reports of plants showed the presence of tannins and flavonoids, while saponins and alkaloids were not detected (Dib et al., 2013). However, a variety of factors, such as the type of extraction solvents used and its polarity, the plant habitat, the maturity stage of harvested plant, genetic variability, and post-harvest management, all affect the extraction yield

(Siddhuraju & Becker, 2003). The major driver of biological activity may be the presence of these components.

Table 1. Phytochemical analysis of *A. unedo* extract and fractions. Key: (−) absent, (+) present

Phytochemicals	Ethanol Extract	Hexane Fraction	Chloroform Fraction	Ethyl acetate Fraction	Butanol Fraction	Aqueous Fraction
Alkaloids	–	–	–	+	+	–
Flavonoids	+	–	–	+	–	+
Saponins	–	+	+	–	–	–
Terpenoids	+	+	+	+	–	–
Steroids	+	–	–	–	–	–
Tannins	+	–	+	+	+	+
Glycosides	+	–	–	–	–	+

The chemical structure and redox characteristics of phenolic compounds, enable them to function as reducing agents, singlet quenchers, hydrogen donators (Babbar et al., 2011), and chelators of transitional metals, as well as their ability to inhibit lipoxygenase and scavenge free radicals, are primarily responsible for their antioxidant action. Total polyphenols in the samples were determined using the Folin-Ciocalteu reagent (Boivin et al., 2009). Gallic acid is used in the construction of the standard curve. Various plant groups contain gallic acid, a polyhydroxy phenolic molecule that is water soluble. The result of total phenolic content determined spectrophotometrically is shown in Table. 2. The top value was found in the ethanol extract (851.64 ± 2.16 mg gallic acid g-1). Other studies showed that ethyl acetate extract had the highest concentration (590.31±17.62) of total phenolic content (Mrabti et al., 2021). In another study, total phenolic content of *A. unedo* from Portugal was 172.21±6.29 mg GAE/g for water and 149.28±5.33 mg GAE/g for methanol leaf extracts (Oliveira et al., 2009). The total amount of phenolic substances of plant extracts and fractions can be affected by a number of variables, including the used solvent type, extraction temperature and duration, solvent-solid ratio (Pinelo et al., 2005).

Table 2. Total phenolic content of *A. unedo* extracts

Extracts	mg gallic acid g^{-1} sample
Ethanol	851.64 ± 2.16
Hexane	95.54 ± 0.76
Chloroform	304.84 ± 3.26
Ethyl acetate	624.74 ± 0.26
Butanol	274.36 ± 1.41
Aqueous	454.64 ± 3.36

Three distinct techniques, including DPPH, ABTS, and FRAP, were used to assess *A. unedo* antioxidant activity. The DPPH radical is a frequently utilized method to assess antioxidant activity in a short amount of time which is based on the capacity of antioxidants to scavenge DPPH radicals. ABTS assays are among the most popular because they are simple, reproducible, and suitable. This test is based on the antioxidants in the analyzed plant extracts reducing ABTS+• radicals. Additionally, the FRAP test is a rapid and affordable technique that relies on the conversion of a colorless (Fe^{3+}-tripyridyltriazine) complex to a blue (Fe^{2+}-tripyridyltriazine) complex at low pH under the influence of antioxidants that donate electrons (Dudonne et al., 2009).

As shown in Table 3, the used antioxidant activity assays revealed that ethanol extract had the top activity (561.81 ± 6.12, 293.34 ± 6.00 and 387.71 ± 7.43 respectively), and that the hexane fraction had the lowest (307.24 ± 3.49, 11.33 ± 0.63 and 9.64 ± 0.31 respectively). Most bioactive components, including polyphenols (e.g., flavonoids and tannins), which were present in extract and fractions, are responsible for this antioxidant activity (El-Shemy et al., 2010).

Table 3. Results of antioxidant activity of *A. unedo* extracts

Extracts	DPPH (mg Trolox g^{-1})	ABTS (mg Trolox g^{-1})	FRAP (mg Trolox g^{-1})
Ethanol	561.81 ± 6.12	293.34 ± 6.00	387.71 ± 7.43
Hexane	307.24 ± 3.49	11.33 ± 0.63	9.64 ± 0.31
Chloroform	503.21 ± 5.81	122.14 ± 2.41	120.15 ± 4.34
Ethyl acetate	536.97 ± 2.80	91.57 ± 1.83	67.28 ± 2.80
Butanol	418.60 ± 5.86	21.01 ± 1.23	17.56 ± 0.75
Aqueous	419.89 ± 4.41	121.02 ± 2.68	376.60 ± 6.92

A study carried out by Macchioni and colleagues showed that *in vitro* α-glucosidase activity was suppressed by *A. unedo* fruit methanolic extract with a high level of phenolic components (Macchioni et al., 2020). Both ethanolic and hydroalcoholic extracts of fruit and leaf extracts of *A. unedo*, contained notable quantities of flavonoids and iridoids and inhibiting α-amylase. Both ethanolic and hydroalcoholic extracts of fruit and leaf extracts of *A. unedo*, contained notable quantities of flavonoids and iridoids and inhibiting α-amylase (Tenuta et al., 2020). In this study the *in vitro* antidiabetic effect of *A. unedo* on α-glucosidase is shown in Table 4. Extract, various fractions, and acarbose all shown increased inhibitory efficacy as the concentration increased**.** The utilized plant extracts, and acarbose each had an IC50 value of 526.65, 762.84, 586.97, 708.46, 809.00, and 472.92 µg mL^{-1}. Similarly, α-amylase inhibition levels of different *A. unedo* root extract, fractions and acarbose raised in a concentration-dependent manner. IC50 values of tested groups were 522.66, 867.18, 737.36, 557.84, 782.35, 699.43, and 519.33 µg mL^{-1} respectively. The most potent antioxidant and antidiabetic activities shown especially by ethanol and ethyl acetate, while other fractions demonstrated varying degrees of activity. The capacity to quench free radicals is a crucial component in preventing the oxidative stress associated with hyperglycemia (Oyeniran et al., 2020).

Table 4. Antidiabetic activity of *A. unedo* on α-glucosidase and α-amylase

Extracts	Concentration (µg mL^{-1})	α-glucosidase		α-amylase	
		% Inhibition	IC50 (µg mL^{-1})	% Inhibition	IC50 (µg mL^{-1})
Ethanol	200	21.93 ± 0.1ns	526.65	18.10 ± 0.15 ns	522.66
	400	38.66 ± 1.96*		36.73 ± 0.61 ns	
	600	56.70 ± 0.41*		50.56 ± 1.10*	
	800	70.83 ± 1.01*		72.43 ± 0.62 ns	
	1000	94.23 ± 0.24 ns		93.03 ± 1.29 ns	
Hexane	200	8.53 ± 0.14*	762.84	11.63 ±0.08*	867.18
	400	17.46 ± 0.17*		23.93 ±0.21*	
	600	33.26 ± 0.12*		34.16 ±0.99*	
	800	45.80 ± 0.43*		44.30 ±1.87*	
	1000	54.46 ± 0.64*		59.06 ±1.21*	
Chloroform	200	12.86 ± 0.64*	762.84	14.53 ± 0.20*	737.36
	400	26.10 ± 0.52*		28.26 ± 0.31*	
	600	39.80 ± 0.40*		40.53 ± 1.58*	
	800	54.36 ± 1.13*		53.96 ± 1.30*	
	1000	70.66 ± 1.26*		71.10 ± 0.79*	
Ethyl acetate	200	18.33 ± 0.54*	586.97	16.20 ± 0.23 ns	557.84
	400	31.10 ± 0.45*		33.73 ± 0.14*	
	600	49.03 ± 0.14*		45.16 ± 1.91*	
	800	66.40 ± 1.38*		61.80 ± 1.30*	
	1000	84.90 ± 1.19*		87.60 ± 2.37*	
Butanol	200	11.26 ± 0.06*	708.46	12.53 ± 0.20*	782.35
	400	20.73 ± 0.13*		23.60 ± 0.43*	
	600	34.36 ± 0.64*		37.76 ± 1.11*	
	800	48.80 ± 0.28*		47.30 ± 1.24*	
	1000	62.60 ± 0.55*		63.60 ± 2.22*	

Table 4. Antidiabetic activity of *A. unedo* on α-glucosidase and α-amylase (continued)

Extracts	Concentration (μg mL^{-1})	α-glucosidase		α-amylase	
		% Inhibition	IC50 (μg mL^{-1})	% Inhibition	IC50 (μg mL^{-1})
Aqueous	200	12.33 ± 0.40*	809.00	13.63 ± 0.14*	699.43
	400	24.13 ± 0.49*		27.16 ± 0.35*	
	600	39.03 ± 0.38*		41.03 ± 0.78*	
	800	47.36 ± 0.78*		53.83 ± 1.60*	
	1000	62.50 ± 1.73*		70.53 ± 1.10*	
Acarbose	200	24.50 ± 0.17	472.92	19.53 ± 0.08	519.33
	400	44.00 ± 0.40		39.70 ± 0.72	
	600	64.33 ± 0.76		56.53 ± 1.82	
	800	77.96 ± 1.53		75.66 ± 1.44	
	1000	94.30 ± 1.05		97.03 ± 1.13	

Values significantly different from those of reference drug (acarbose), i.e., * = $p< 0.05$ and ns: not significant.

4. Conclusion

The extract and fractions of *A. unedo* species obtained using different solvents were found to be high in terms of total phenolic substance and antioxidant activity. In terms of antidiabetic activity, Ethanolic extract of *A. unedo* roots has more inhibitory abilities on α-glucosidase and α-amylase. The presence of phenolics indicates that this plant has a variety of biological characteristics. More research is needed to separate, identify, and investigate the bioactivity of this plant.

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