

# Antidiabetic and Antioxidant Properties of Bilberry (*Vaccinium myrtillus* Linn.) Fruit and Their Chemical Composition

A. Güder<sup>1\*</sup>, M. Gür<sup>2</sup>, and M. S. Engin<sup>3</sup>

## ABSTRACT

Bilberry [*Vaccinium myrtillus* Linn. (Ericaceae)] fruit (VMF) has been traditionally used for treatment of bladder stones, biliary disorders, scurvy, coughs, and lung tuberculosis. VMF may have some pharmaceutical properties owing to these uses, but *in vivo* and *in vitro* studies are limited for clarification of medicinal activity and its antidiabetic and antioxidant properties are not investigated in detail. Anti-amylase, anti-glucosidase, and antioxidant activities of methanol (ME), ethanol (EE), acetone (AE), and water (WE) extracts of VMF were investigated. In addition, some chemical compositions were determined by using spectrophotometric methods. Antidiabetic and antioxidant activities of extracts were studied by using different tests such as  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory, total antioxidant, DPPH scavenging activities according to *in vitro* methods at different concentrations (10-250  $\mu\text{g mL}^{-1}$ ). Compared with the standards, ME, WE, and EE showed strong total antioxidant activities with  $\text{IC}_{50}$  ( $\mu\text{g/mL}$ ) values of  $24.46 \pm 0.34$ ,  $25.24 \pm 0.78$ , and  $27.48 \pm 0.60$ , respectively. At the same time, ME ( $\text{IC}_{50}$   $61.38 \pm 1.40$   $\mu\text{g mL}^{-1}$ ) and EE ( $\text{IC}_{50}$   $65.52 \pm 1.19$   $\mu\text{g mL}^{-1}$ ) demonstrated very effective inhibitory activity against  $\alpha$ -amylase and moderate inhibitory activity against  $\alpha$ -glucosidase. All extracts also showed high reducing power, metal chelating activity, superoxide anion, DPPH radical, and  $\text{H}_2\text{O}_2$  scavenging activities. Important relationships were found between biological activity and chemical composition by statistical analyses. The VMF can be used as an antidiabetic and antioxidant source in medicinal and pharmaceutical areas due to its chemical composition. Anthocyanin contents may influence the anti-amylase inhibition activity more than phenolic and flavonoid contents.

**Keywords:** Alpha-amylase, Anthocyanin, Glucosidase inhibition, Flavonoid, Phenol.

## INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized as two types: i) Type I (insulin dependent diabetes mellitus/IDDM) is congenital. ii) Type II (noninsulin-dependent diabetes mellitus/NIDDM) is acquired later in life due to inability of glucose transport from the bloodstream into cells. The effects of diabetes mellitus can be long-term damage, dysfunction, and failure of various organs. The

type II diabetes accounts for approximately 90% of all diabetes worldwide (Nickavar *et al.*, 2008).

Inhibitors of  $\alpha$ -amylases, which are used to modify or control the activity of this enzyme, have medical applications such as the influence on blood glucose, serum insulin and starch loading tests in animals and man (Puls and Keup, 1973). Screening of  $\alpha$ -glucosidase inhibitors in plants and other synthetic sources are increased. For example, several plants that are commonly used to treat diabetes in the

<sup>1</sup> Department of Medical Services and Techniques, Vocational High School of Health Services, Giresun University, Giresun, Turkey.

\* Corresponding author; e-mail: aytac.guder@giresun.edu.tr

<sup>2</sup> Department of Forest Industry Engineering, Faculty of Forestry, Kastamonu University, Kastamonu, Turkey.

<sup>3</sup> Faculty of Engineering, Department of Food Engineering, Giresun University, Giresun, Turkey.



traditional systems of medicine have been explored by many research groups for investigating their chemical constituents, elemental analysis, and their role in diabetes management as well as pharmacological activities (Shukla *et al.*, 2011).

Antioxidant properties are majorly used for determination of medicinal bioactive components (Mansour *et al.*, 2013). Recently, the antioxidants that are used in food and medicinal industries gained considerable attention since the natural antioxidants are more beneficial to human health than the synthetic ones (Steffen *et al.*, 2003). Epidemiological studies suggest that a reduced risk of cancer is associated with higher consumption of a phytochemical-rich diet that includes fruits and vegetables (Sadeghi *et al.*, 2009). Natural plants have received much attention as sources of biologically active substances that have wide range of pharmacological activities such as antimicrobial, anti-inflammatory, antioxidant, and anticancer effects. Also, studies have focused on health functions of phenolic compounds, including flavonoids and anthocyanins (Salmanian *et al.*, 2014).

Bilberry [*Vaccinium myrtillus* Linn. (Ericaceae)] fruit (VMF) is known as Çalı Çileği and Çoban Üzüümü in Turkey and found in mountains and forests. The height of this shrubby perennial plant varies from 1 to 2 feet, its leaves are bright green and branches are alternating and elliptical. Its bell-shaped flowers are reddish or pink in color, bloom between the months of April and June producing blue-black or purple fruit. Even though it is encountered as a precious wild delicacy today, bilberry has been used as food for centuries in Turkey. More recently, bilberry fruit extracts have been used for the treatment of diarrhea, dysentery, and mouth and throat inflammations. Decocted bilberry leaves have been used to decrease blood sugar in diabetes (Baytop, 2000).

In this study, anti-amylase, anti-glucosidase, antioxidant activities, and chemical contents of VMF were investigated.

## MATERIALS AND METHODS

### Chemicals

All chemicals were analytical grade and obtained from either Sigma-Aldrich or Merck.

### Preparation of Fruit Materials and Extraction

VMF was collected by the corresponding author in June 2011 from a plateau in Giresun, Turkey, and the specie of fruit was authenticated by Prof. Dr. Hamdi Güray Kutbay, Department of Biology, Faculty of Science and Art, Ondokuz Mayıs University. After drying in an oven at 40°C, the samples were chopped by a hand blender. The small pieces were then subjected to extraction using Soxhlett apparatus for 24 hours, with absolute methanol, absolute ethanol, acetone, and water, successively. The extracts were filtered over Whatman No.1 paper. The extracts were evaporated to dryness; the filtrates were frozen and lyophilized in a lyophilizator (Christ Alpha 1–2 LD Plus) at -50°C and 10 µm Hg pressure. The residues were placed in a plastic flask, and kept at -30°C until used (Güder *et al.*, 2014).

### Determination of Antidiabetic Activity

#### Assay of $\alpha$ -Amylase Inhibition

*In vitro*  $\alpha$ -amylase inhibition was studied by previously developed method (Bernfeld, 1955) with minor modifications. The extracts and acarbose (100 µL) were reacted with the  $\alpha$ -amylase enzyme (EC 3.2.1.1) solution (200 µL) and phosphate buffer solution (PBS) (100 µL, 20 mM, and pH 6.9). The enzyme solution (0.5 unit mL<sup>-1</sup>) was prepared by mixing 0.001 g of  $\alpha$ -amylase in PBS (100 mL, 20 mM, pH 6.9) containing 6.7 mM sodium chloride. After incubation for 20 minutes, starch solution (100 µL, 0.5%) was added. The starch solution was obtained by boiling 0.25 g potato starch in 50 mL of deionized water for 15 minutes while stirring.

The same procedures were performed for control and the enzyme solution (200  $\mu\text{L}$ ) was replaced by PBS. The color reagent was a solution containing 3,5-dinitrosalicylic acid (20 mL, 96 mM), sodium potassium tartrate (5.31M) in sodium hydroxide (8.0 mL, 2.0M) and deionized water (12 mL). After incubation for 5 min, color reagent (500  $\mu\text{L}$ ) was added to all tubes and kept in water bath 25°C for 30 minutes. The absorbance was recorded at 540 nm using a spectrophotometer and  $\alpha$ -amylase inhibition activities were expressed as  $\text{IC}_{50}$  (the concentration required to inhibit  $\alpha$ -amylase activity by 50%). The  $\text{IC}_{50}$  values were determined by linear regression analysis using four different concentrations in triplicate and presented as the mean of the data.

### Assay of $\alpha$ -Glucosidase Inhibition

Alpha-Glucosidase inhibition activity was determined according to the chromogenic method described by McCue *et al.* (2005), with minor modifications. The enzyme solution contained  $\alpha$ -glucosidase (EC 3.2.1.20) solution (20  $\mu\text{L}$ ) (0.5 unit  $\text{mL}^{-1}$ ) and PBS (120  $\mu\text{L}$ , 0.1 M, pH 6.9). Later, *p*-nitrophenyl- $\alpha$ -D-glucopyranoside (5.0 mM) in the PBS was used as the substrate solution. Test samples and acarbose (10  $\mu\text{L}$ ) were dissolved at various concentrations in DMSO. They were mixed with enzyme solution and incubated for 15 minutes at 37°C. Substrate solution (20  $\mu\text{L}$ ) was added to the solution and incubated for 15 minutes. The reaction was terminated by adding sodium carbonate solution (80  $\mu\text{L}$ , 0.2 M) and absorbance was measured at 405 nm. The  $\alpha$ -glucosidase inhibition activities were expressed as  $\text{IC}_{50}$ .

### Determination of Antioxidant Properties

#### Total Antioxidant Activity

Total antioxidant activities were determined via ferric thiocyanate (FTC) method (Mitsuda *et al.*, 1996). Extracts and standard solutions were prepared by diluting

the stock solution in PBS (2.5 mL, 0.04 M, pH 7.0) and these were added to linoleic acid emulsion (2.5 mL) in PBS (0.04M, pH 7.0). The linoleic acid emulsion was prepared by homogenizing linoleic acid (15.5  $\mu\text{L}$ ), Tween-20 (17.5 mg) as emulsifier, and PBS (5.0 mL, 0.04M, pH 7.0). Linoleic acid emulsion (2.5 mL) and PBS (2.5 mL, 0.04M, pH 7.0) were used for the control reaction. The reaction mixture (5.0 mL) was incubated at 37°C in polyethylene flasks. The peroxides, which were newly formed during linoleic acid peroxidation should oxidize  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , subsequently caused a complex formation with thiocyanate that has a maximum absorbance at 500 nm (Unicam UV2-100). The assay steps were repeated every 10 hours until reaching a maximum (80 hours). The inhibition values of lipid peroxidation were calculated as  $\text{IC}_{50}$ .

### Ferric Ions ( $\text{Fe}^{3+}$ ) Reducing Antioxidant Power Assay (FRAP)

The reducing powers of samples (extracts) or standards were determined by the colorimetric method (Oyaizu, 1986). Solutions of (2.5 mL, 25-100  $\mu\text{g mL}^{-1}$ ) were mixed with PBS (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide (2.5 mL, 1.0%) and incubated at 50°C for 20 minutes. Aliquots of trichloroacetic acid (2.5 mL, 10%) was added to obtain the final reagent. Then, 2.5 mL of it was mixed with distilled water (2.5 mL) and  $\text{FeCl}_3$  (0.5 mL, 0.1%). The  $\text{Fe}^{3+}/\text{Fe}^{2+}$  transformation was investigated in the presence of extract or standard and the absorbance values were measured at 700 nm.

$$\text{Reducing power (\%)} = (A_s/A_c) \times 100$$

Where,  $A_c$  is the absorbance of the control and  $A_s$  is the absorbance of samples (extracts) or standards.

### Superoxide Anion Radical Scavenging Activity

Measurement of superoxide anion radical ( $\text{O}_2^{\cdot-}$ ) scavenging activities were based on the previously developed method (Liu *et al.*,



1997). In these experiments, superoxide anion radicals were produced by NBT (1.0 mL, 50  $\mu\text{M}$ ) in PBS (0.1M, pH 7.4), NADH (1.0 mL, 78  $\mu\text{M}$ ) in PBS (0.1M, pH 7.4) and sample extracts (1.0 mL) were mixed. The reactions were started by adding PMS solutions (0.1 mL, 10  $\mu\text{M}$ ) in PBS (0.1M, pH 7.4) to the mixtures and incubated at 25°C for 5 minutes while measuring the absorbance at 560 nm by a spectrophotometer (Unicam UV2–100). The  $\text{SC}_{50}$  values (the concentration required for scavenging superoxide anion radical by 50%) were determined by linear regression analysis using five different concentrations in triplicate and presented as the mean of the data.

#### Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide scavenging assay was carried out by the spectrophotometric procedure (Ruch *et al.*, 1989). In this assay, a solution of  $\text{H}_2\text{O}_2$  (40 mM) was prepared in PBS (0.1M, pH 7.4). Sample solutions were prepared in a concentration range of 5-100  $\mu\text{g mL}^{-1}$  in PBS (3.4 mL, 0.1M, pH 7.4) and they were added to  $\text{H}_2\text{O}_2$  solution (0.6 mL) mentioned above. Absorbance of the reaction mixture was recorded at 230 nm (Unicam UV2–100) and the results were expressed as  $\text{SC}_{50}$  by linear regression analysis.

#### DPPH Free Radical Scavenging Activity

The DPPH radical (DPPH $\cdot$ ) scavenging capacities of extracts were studied by following a previous report (Blois, 1958). Serially diluted samples (200  $\mu\text{L}$ ) at the different concentrations (5-100  $\mu\text{g mL}^{-1}$ ) were added to DPPH $\cdot$  solutions (2.8 mL, 0.2 mM) in ethanol. The mixtures were shaken forcefully and allowed to sit at room temperature for 30 min. Then, absorbance was recorded at 517 nm (Unicam UV2–100) and the results were expressed as  $\text{SC}_{50}$  by linear regression analysis.

#### Ferrous Metal Ions Chelating Activity

By following the method of Dinis *et al.* (1994), ferrous ion ( $\text{Fe}^{2+}$ ) chelating activity was measured via inhibition of the  $\text{Fe}^{2+}$ -ferrozine complex which was formed after treating the test material with  $\text{Fe}^{2+}$  ions. Firstly, different concentrations of extract and standard solutions (25-100  $\mu\text{g mL}^{-1}$ , 0.4 mL) in ethanol were added to  $\text{FeCl}_2$  solution (0.1 mL, 2.0 mM). In order to initiate the reaction, ferrozine solution (0.1 mL, 5.0 mM) in ethanol was added. Then, the mixture was shaken vigorously and kept at room temperature for 10 min. The absorbance of the complex was measured by a spectrophotometer (Unicam UV2–100) at 562 nm. The percent inhibition of  $\text{Fe}^{2+}$ -ferrozine complex formation was calculated by using the formula given below:

$$\text{Ferrous ions chelating effect (\%)} = [1 - (A_s/A_c)] \times 100$$

Where,  $A_c$  is the absorbance of the control (contains only  $\text{FeCl}_2$  and ferrozine) and  $A_s$  is the absorbance of the samples (extracts) and standards.

#### Determination of Total Phenolic Compounds

The total phenolic contents were analyzed by the colorimetric method (Dewanto *et al.*, 2002). Stock solutions (1,000  $\mu\text{g mL}^{-1}$ ) were prepared from the extracts. Each solution (125  $\mu\text{L}$ ) was mixed with distilled water (0.5 mL) and subsequently with FC reagent (125  $\mu\text{L}$ ). After 6 minutes,  $\text{Na}_2\text{CO}_3$  solution (1.25 mL, 7.0%) was added into the mixture. Then, water was added to bring the total volume to 3.0 mL. The observed color during 90 minutes and the absorbance was measured by a spectrophotometer (Unicam UV2–100) at 760 nm. The total phenolic contents were expressed as microgram of catechin equivalent by using an equation that was obtained from standard catechin graph (0-250  $\mu\text{g mL}^{-1}$ ) ( $r^2 = 0.9988$ ).

### Determination of Total Flavonoid Contents

The total flavonoid contents were determined by using a modified colorimetric method described previously (Dewanto *et al.*, 2002). Stock solutions (250  $\mu\text{L}$ ) were added to test tubes containing distilled water (1.25 mL) and subsequently  $\text{NaNO}_2$  solutions (75  $\mu\text{L}$ , 5.0%) were added to each of them. After 6 min,  $\text{AlCl}_3$  solutions (150  $\mu\text{L}$ , 10%) were added to the tubes and the mixtures were allowed to stay for 5 minutes before further addition of  $\text{NaOH}$  solution (0.5 mL, 1.0M). The absorbance values were read at 510 nm (Unicam UV2-100) immediately after bringing the total volume up to 2.5 mL with distilled water. Total flavonoid contents were expressed as microgram of catechin equivalent that was obtained from standard graph (0-250  $\mu\text{g mL}^{-1}$ ) ( $r^2 = 0.9968$ ).

### Determination of Total Anthocyanin Contents

The total anthocyanin contents were determined according to pH differential method (Fuleki and Francis, 1968). The dried extracts (100 mg) were added to  $\text{HCl}$  (5.0 mL, 1.0%) centrifuged at 3,000 rpm for 10 minutes (MSE Mistral 2000, UK). Two supernatant tubes (0.2 mL) were prepared with buffer solutions having pH values of 1.0 (potassium chloride-HCl, 0.025M) and 4.5 (sodium acetate-HCl, 0.4M) respectively. Absorbance values were measured by using a spectrophotometer (Unicam UV2-100) at 520 and 700 nm. The following buffer solutions were used as blank tubes in this experiment.

Total anthocyanin contents in the extracts determined as  $\text{mg L}^{-1}$  of cyanidin-3-glucoside (cyd-3-glu) equivalent using the following equation:

Where,  $A$ :  $(A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH } 1.0} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH } 4.5}$ ,  $MW_{\text{cyd-3-glu}}$  (Molecular Weight of cyanidin-3-glucoside): 449.2  $\text{g mol}^{-1}$ ,  $DF$ : Dilution factor,  $l$ : Path length in cm,  $\epsilon = 26,900$  molar extinction coefficient for cyd-3-glu ( $\text{L mol}^{-1} \text{cm}^{-1}$ ), and  $10^3$ : factor for conversion from g to mg.

### Statistical Analysis

The data was analyzed using the statistical software program SPSS 15.0 for Windows. All results were given as mean  $\pm$  standard deviation (SD). The potential correlation among the  $\alpha$ -amylase and  $\alpha$ -glucosidase enzyme inhibitions, antioxidant capacity, total phenol, flavonoid and anthocyanin contents were analyzed and differences at  $P < 0.05$  were considered as "significant",  $P < 0.01$  "highly significant" and  $P > 0.05$  "non-significant".

## RESULTS AND DISCUSSION

### Alpha-amylase Inhibition Activity

Alpha-amylase is an enzyme which hydrolyzes starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units that cause hyperglycemia and development of type II diabetes mellitus (Shankaraiah and Reddy, 2011). In this study, the  $\alpha$ -amylase inhibitory activity of extracts were studied in a concentration range from 10 to 250  $\mu\text{g mL}^{-1}$  and compared with standard acarbose with an  $\text{IC}_{50}$  value of  $87.55 \pm 1.69 \mu\text{g mL}^{-1}$ . The  $\text{IC}_{50}$  values of ME, EE, WE, and AE were found to be  $61.38 \pm 1.40$ ,  $65.52 \pm 1.19$ ,  $96.64 \pm 1.73$ , and  $281.53 \pm 2.02 \mu\text{g mL}^{-1}$ , respectively (Table 1) ( $P < 0.05$ ). These

$$\text{Anthocyanin pigment (cyd-3-glu equivalent, } \frac{\text{mg}}{\text{L}}) = \frac{A \times MW_{\text{cyd-3-glu}} \times DF \times 10^3}{\epsilon \times l}$$



results made it clear that even if AE is not very effective  $\alpha$ -amylase inhibitor compared to acarbose, ME, EE, and WE show appreciable  $\alpha$ -amylase inhibitory effects. In a previous study, aqueous buffered extract solution of *V. myrtillus* leaf demonstrated very high  $\alpha$ -amylase inhibition (Sales *et al.*, 2012).

### Alpha-glucosidase Inhibition Activity

An effective way of controlling diabetes mellitus, especially the Type II diabetes, involves preventing excessive rise of the blood glucose level by inhibiting the starch digestive enzymes. Alpha-glucosidase, which is a membrane-bound enzyme located in the epithelium of the small intestines, catalyzes the cleavage of glucose from disaccharides before absorption. Recent studies have reported that the retardation of  $\alpha$ -glucosidase enzyme by inhibitors would be one of the most effective ways to control Type II diabetes (Chethan *et al.*, 2008; Kunyanga *et al.*, 2012). In this work,  $\alpha$ -glucosidase inhibitory activities of bilberry extracts were compared with standard acarbose with an  $IC_{50}$  value of  $81.05 \pm 0.51 \mu\text{g mL}^{-1}$ . The  $IC_{50}$  values of ME, EE, WE, and AE were found to be  $138.41 \pm 1.05$ ,  $128.94 \pm 1.26$ ,  $140.47 \pm 1.33$ , and  $313.18 \pm 3.94 \mu\text{g mL}^{-1}$ , respectively (Table 1) ( $P < 0.05$ ). These results show that ME, EE, and WE could be potential  $\alpha$ -glucosidase inhibitors for diabetic disorder. Alpha-glucosidase inhibition activity of this

plant material has been confirmed by researchers (Matsui *et al.*, 2001).

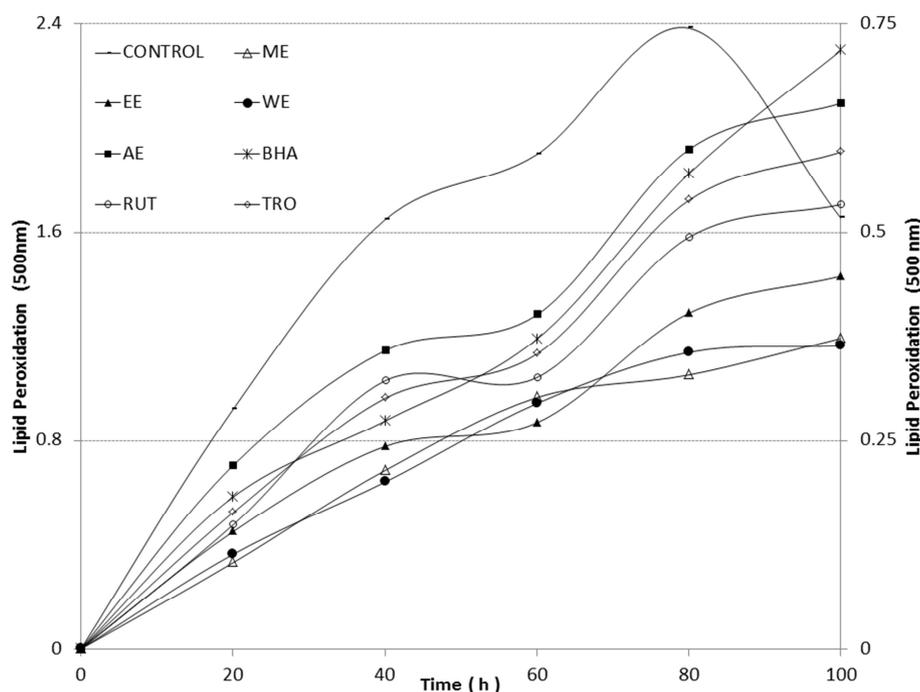
### Total Antioxidant Activity

Antioxidant activity of extracts was determined by ferric thiocyanate (FTC) method. This method indirectly measures the amount of peroxide, which is the primary product of lipid oxidation, produced during the initial stages of oxidation. Released peroxides cause oxidation of  $Fe^{+2}$  ions to  $Fe^{+3}$ , which later form a complex with  $SCN^-$  that has a maximum absorbance at 500 nm (Inatani *et al.*, 1983). In the presence of antioxidants,  $Fe^{3+}$  ion formation becomes more difficult and, therefore, the absorbance of  $SCN^-$  complex is expected to be lower. The results of activity assays of extracts after 100 h incubation with linoleic acid emulsion are summarized as inhibition % in Figure 1. The  $IC_{50}$  values of BHA, RUT, and TRO were found to be  $33.15 \pm 0.77$ ,  $30.47 \pm 0.85$ , and  $31.89 \pm 0.36 \mu\text{g mL}^{-1}$ , respectively ( $P < 0.05$ ). However, ME showed the most effective inhibition having the  $IC_{50}$  value of  $24.46 \pm 0.34 \mu\text{g mL}^{-1}$  and this difference was statistically highly significant ( $P < 0.01$ ). Similarly, EE, WE, and AE exhibited potential antioxidant activities with  $IC_{50}$  values of  $27.48 \pm 0.60$ ,  $25.24 \pm 0.78$ , and  $35.78 \pm 0.23 \mu\text{g mL}^{-1}$ , respectively (Table 1). The performed antioxidant activity parameters have been

**Table 1.** Alpha-amylase inhibition (AIC),  $\alpha$ -glucosidase inhibition (GIC), total antioxidant activity (TAA), ferric reducing antioxidant power (FRAP) and ferrous ion chelating activities (FICA) of *Vaccinium myrtillus* fruit.

	AIC <sup>a</sup>	GIC <sup>a</sup>	TAA <sup>a</sup>	FRAP <sup>b</sup>	FICA <sup>b</sup>
ME <sup>c</sup>	$61.38 \pm 1.40$	$138.41 \pm 1.05$	$24.46 \pm 0.34$	$84.19 \pm 1.11$	$90.28 \pm 0.72$
EE <sup>d</sup>	$65.52 \pm 1.19$	$128.94 \pm 1.26$	$27.48 \pm 0.60$	$89.70 \pm 1.01$	$89.34 \pm 0.46$
WE <sup>e</sup>	$96.64 \pm 1.73$	$140.47 \pm 1.33$	$25.24 \pm 0.78$	$78.72 \pm 0.54$	$91.82 \pm 0.24$
AE <sup>f</sup>	$281.53 \pm 2.02$	$313.18 \pm 3.94$	$35.78 \pm 0.23$	$76.37 \pm 0.74$	$87.00 \pm 0.26$
Acarbose	$87.55 \pm 1.69$	$81.05 \pm 0.51$	–	–	–
BHA <sup>g</sup>	–	–	$33.15 \pm 0.77$	$71.97 \pm 0.27$	$84.45 \pm 0.99$
RUT <sup>h</sup>	–	–	$30.47 \pm 0.85$	$92.74 \pm 1.29$	$87.67 \pm 0.75$
TRO <sup>i</sup>	–	–	$31.89 \pm 0.36$	$53.76 \pm 0.92$	$69.77 \pm 0.92$

<sup>a</sup>  $IC_{50}$  values ( $\mu\text{g mL}^{-1}$ ); <sup>b</sup> Activity (%); <sup>c</sup> Methanol Extract; <sup>d</sup> Ethanol Extract; <sup>e</sup> Water Extract; <sup>f</sup> Acetone Extract; <sup>g</sup> Butylated HydroxyAnisole; <sup>h</sup> Rutin, <sup>i</sup> Trolox ( $P < 0.05$ ).



**Figure 1.** Total antioxidant activities of *Vaccinium myrtillus* fruit extracts and standards in the linoleic acid emulsion system at the same concentration ( $50 \mu\text{g mL}^{-1}$ ). ME: Methanol Extract; EE: Ethanol Extract; WE: Water Extract; AE: Acetone Extract; BHA: Butylated HydroxyAnisole; RUT: Rutin, and TRO: Trolox. Note: Left axis for control and right axis for samples and standards.

found higher than previous studies (Faria *et al.*, 2005; Burdulis *et al.*, 2009). This difference may be due to the chemical composition. However, further studies are needed on the isolation and characterization of individual phenolic compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

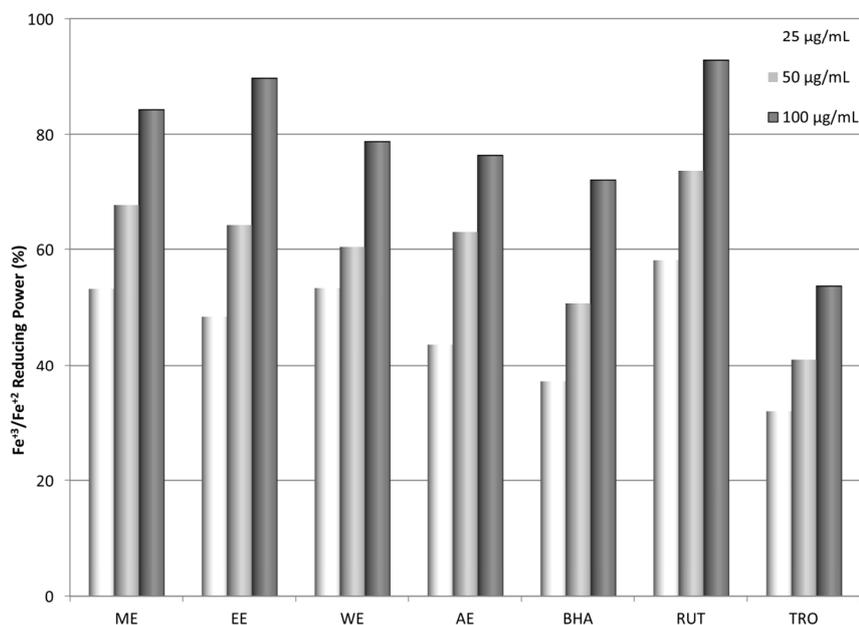
#### Ferric Reducing Antioxidant Power Capacity

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidants. The presence of reductants such as antioxidants causes the reduction of the  $[\text{Fe}(\text{CN})_6]^{3-}$  complex to the  $[\text{Fe}(\text{CN})_6]^{4-}$ . Therefore,  $\text{Fe}^{2+}$  can be monitored by measuring the formation of Perl's Prussian

Blue ( $\text{Fe}_4[\text{Fe}(\text{CN})_6]_3$ ) which has a maximum absorbance at 700 nm (Chung *et al.*, 2002; Güder and Korkmaz, 2012). The higher the absorbance of the reaction mixture, the bigger the reducing power. Figure 2 shows the reducing capability of extracts at the different concentrations compared to BHA, RUT, and TRO. Even though RUT had the highest reductive activity at the same concentration ( $100 \mu\text{g mL}^{-1}$ ), all extracts demonstrated more efficient reducing power than BHA and TRO ( $P < 0.05$ ). The reducing capacity (%) was in the following order: RUT ( $92.74 \pm 1.29$ ) > EE ( $89.70 \pm 1.01$ ) > ME ( $84.19 \pm 1.11$ ) > WE ( $78.72 \pm 0.54$ ) > AE ( $76.37 \pm 0.74$ ) > BHA ( $71.97 \pm 0.27$ ) > TRO ( $53.76 \pm 0.92$ ) (Table 1).

#### Superoxide Radical Anion Scavenging Activity

Superoxide anion radicals ( $\text{O}_2^{\cdot-}$ ) are precursors to active free radicals that have potential to react with biological



**Figure 2.** Reducing powers (%) of *Vaccinium myrtillus* fruit extracts and standards. ME: Methanol Extract; EE: Ethanol Extract; WE: Water Extract; AE: Acetone Extract; BHA: Butylated HydroxyAnisole; RUT: Rutin, and TRO: Trolox.

macromolecules, thereby inducing tissue damage (Halliwell and Gutteridge, 1984). Even if superoxide anion radical itself is quite unreactive compared to the other radicals, the biological systems convert it into more reactive species (Winterbourn and Kettle, 2003). In Table 2, the superoxide anion radical scavenging activity of extracts was compared with the known antioxidants BHA, RUT, and TRO. Superoxide anion radical scavenging activities ( $SC_{50}$ ) of ME, EE, and WE are stronger than BHA and

RUT ( $P < 0.05$ ).  $SC_{50}$  of those samples was in the following order: WE ( $16.83 \pm 0.29$ ) < TRO ( $18.42 \pm 0.27$ ) < EE ( $19.21 \pm 0.45$ ) < AE ( $19.75 \pm 0.44$ ) < ME ( $20.19 \pm 0.11$ ) < BHA ( $24.05 \pm 0.58$ ) < RUT ( $28.19 \pm 0.96$ ).

### Hydrogen Peroxide Scavenging Activity

It is known that hydrogen peroxide ( $H_2O_2$ ) itself is not very reactive, but, sometimes, it can be toxic to cells by giving rise to

**Table 2.** Superoxide anion radical, hydrogen peroxide and dpph radical scavenging activities ( $SC_{50}$ ) of *Vaccinium myrtillus* fruit.

	Superoxide Anion	Hydrogen Peroxide ( $\mu\text{g mL}^{-1}$ )	DPPH radical ( $\mu\text{g mL}^{-1}$ )
ME <sup>a</sup>	$20.19 \pm 0.11$	$64.73 \pm 0.43$	$14.87 \pm 0.52$
EE <sup>b</sup>	$19.21 \pm 0.45$	$48.47 \pm 0.57$	$15.14 \pm 0.30$
WE <sup>c</sup>	$16.83 \pm 0.29$	$46.46 \pm 0.26$	$13.59 \pm 0.55$
AE <sup>d</sup>	$19.75 \pm 0.44$	$67.21 \pm 0.89$	$25.40 \pm 0.13$
BHA <sup>e</sup>	$24.05 \pm 0.58$	$66.30 \pm 0.75$	$15.96 \pm 0.44$
RUT <sup>f</sup>	$28.19 \pm 0.96$	$47.35 \pm 0.69$	$13.96 \pm 0.49$
TRO <sup>g</sup>	$18.42 \pm 0.27$	$71.80 \pm 0.81$	$24.15 \pm 0.21$

<sup>a</sup> Methanol Extract; <sup>b</sup> Ethanol Extract; <sup>c</sup> Water Extract; <sup>d</sup> Acetone Extract; <sup>e</sup> Butylated HydroxyAnisole; <sup>f</sup> Rutin, <sup>g</sup> Trolox ( $P < 0.05$ ).

hydroxyl radicals ( $\cdot\text{OH}$ ).  $\text{H}_2\text{O}_2$  can cross membranes and slowly oxidize a number of biomolecules and compounds (Halliwell, 1991). Addition of  $\text{H}_2\text{O}_2$  to cells in culture can lead to formation of transition metal ion-dependent hydroxyl radical, mediating oxidative DNA damage. Therefore, removing hydrogen peroxide as well as superoxide anion radical is very important for protecting pharmaceuticals and food systems (Gülçin, 2010).  $\text{H}_2\text{O}_2$  scavenging ability of extracts is shown in Table 2 in comparison with that of BHA, RUT, and TRO ( $P < 0.05$ ). The  $\text{SC}_{50}$  values ( $\mu\text{g mL}^{-1}$ ) of all extracts and standards decreased in the order of TRO ( $71.80 \pm 0.81$ ) > AE ( $67.21 \pm 0.89$ ) > BHA ( $66.30 \pm 0.75$ ) > ME ( $64.73 \pm 0.43$ ) > EE ( $48.47 \pm 0.57$ ) > RUT ( $47.35 \pm 0.69$ ) > WE ( $46.46 \pm 0.26$ ).

#### DPPH Free Radical Scavenging Activity

DPPH $\cdot$  is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al.*, 1997). The concentration of DPPH $\cdot$  is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH $\cdot$  has a maximum absorbance at 517 nm, however, this absorbance decreases in the presence of antioxidants or other radical species due to the reaction between antioxidant molecules and/or the radicals with DPPH $\cdot$ . It is also visually noticeable as a color change from purple to yellow (Ancerewicz *et al.*, 1998; Elmastaş *et al.*, 2006). Table 2 shows that the  $\text{SC}_{50}$  values ( $\mu\text{g mL}^{-1}$ ) of extracts and standards on the DPPH $\cdot$  was in the following order: AE ( $25.40 \pm 0.13$ ) > TRO ( $24.15 \pm 0.21$ ) > BHA ( $15.96 \pm 0.44$ ) > EE ( $15.14 \pm 0.30$ ) > ME ( $14.87 \pm 0.52$ ) > RUT ( $13.96 \pm 0.49$ ) > WE ( $13.59 \pm 0.55$ ) ( $P < 0.05$ ).

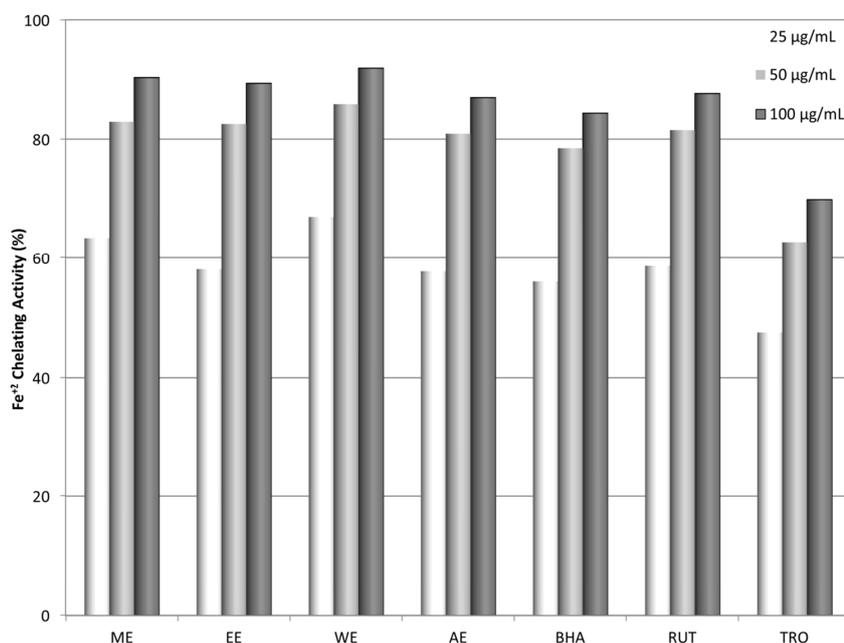
#### Ferrous Ion Chelating Activity

Ferrozine can quantitatively form from complexes with  $\text{Fe}^{2+}$  in this method. Since the intensity of the red color of this

complex decreases in the presence of other chelating agents, one can assume that the complex formation is disrupted as a result of chelating. Therefore, monitoring the intensity of the color allows us to estimate the metal chelating activity of the coexisting chelators (Güder and Korkmaz, 2012). In this assay, the samples containing  $\text{Fe}^{2+}$ -ferrozine complex are treated with the extracts which have chelating activity and, therefore, capable of capturing ferrous ion from this complex. As seen in Figure 3, the formation of the  $\text{Fe}^{2+}$ -ferrozine complex is not complete in the presence of the extracts, indicating all of the extracts chelate with the  $\text{Fe}^{2+}$ . At the concentration of  $100 \mu\text{g mL}^{-1}$ , the metal chelating activity (%) of all extracts and standards decreased ( $P < 0.05$ ) in the following order: WE ( $91.82 \pm 0.24$ ) > ME ( $90.28 \pm 0.72$ ) > EE ( $89.34 \pm 0.46$ ) > RUT ( $87.67 \pm 0.75$ ) > AE ( $87.00 \pm 0.26$ ) > BHA ( $84.45 \pm 0.99$ ) > TRO ( $69.77 \pm 0.92$ ) (Table 1).

#### Total Phenolic Contents

Phenolic compounds have antioxidant properties due to their ability of scavenging free radicals and active oxygen species such as singlet oxygen (Hall and Cuppett, 1997). According to our findings (shown in Table 3), the catechin equivalents of total phenolic contents of extracts ranged from  $116.67 \pm 1.37$  to  $182.33 \pm 1.40 \mu\text{g}$  in 1 mg of dried extracts ( $P < 0.05$ ). These phenolic compounds may directly contribute to the antioxidative activity (Elmastaş *et al.*, 2006). On the other hand, some others found no such relationship between antioxidant activity and total phenolic content since other compounds can be responsible for the antioxidant activity (Maillard and Berset, 1995; Yang *et al.*, 2002). Our findings also suggest that there is no correlation between antioxidant activity and total phenolic contents. The phenolic and flavonoid contents of VMF are compatible with literature data (Vučić *et al.*, 2013).



**Figure 3.** Metal chelating activities of extracts and standards on ferrous ions at different concentrations (25-100 µg mL<sup>-1</sup>). ME: Methanol Extract; EE: Ethanol Extract; WE: Water Extract; AE: Acetone Extract; BHA: Butylated HydroxyAnisole; RUT: Rutin, and TRO: Trolox.

**Table 3.** Total phenol, flavonoid and anthocyanins contents of *Vaccinium myrtillus* fruit.

	Total phenols <sup>a</sup>	Total flavonoids <sup>a</sup>	Total anthocyanins <sup>b</sup>
ME <sup>c</sup>	137.07 ± 1.45	26.51 ± 0.25	16.87 ± 0.24
EE <sup>d</sup>	139.27 ± 1.37	29.45 ± 0.13	13.69 ± 0.38
WE <sup>e</sup>	182.33 ± 1.40	37.49 ± 0.59	14.86 ± 0.29
AE <sup>f</sup>	116.67 ± 1.37	23.94 ± 0.11	10.52 ± 0.13

<sup>a</sup> µg mg<sup>-1</sup> catechin equivalent; <sup>b</sup> mg L<sup>-1</sup> cyanidin-3-glucoside equivalent; <sup>c</sup> Methanol Extract; <sup>d</sup> Ethanol Extract; <sup>e</sup> Water Extract, <sup>f</sup> Acetone Extract (P < 0.05).

### Total Flavonoid Contents

Flavonoids are very important plant constituents because of having active hydroxyl groups and, therefore, antioxidant activity (Kumar *et al.*, 2008). Flavonoids are not only responsible for giving fruits and vegetables various red, blue, or violet colors, but also are related to a group of bioactive compounds called stilbenes (Hui, 2006). In this work, the total flavonoid contents were expressed as µg of catechin equivalents mg<sup>-1</sup> of the dried extracts. As shown in the Table 3, the highest flavonoid content of 37.49±0.59 µg of

catechin equivalents/mg of dried extracts was observed in the WE and the lowest flavonoid content was observed in the AE i.e. 23.94±0.11 µg of catechin equivalents/mg of dried extracts. The order of total flavonoid content was found to be: WE (37.49±0.59) > EE (29.45±0.13) > ME (26.51±0.25) > AE (23.94±0.11) (P < 0.05).

### Total Anthocyanin Contents

Anthocyanins and other flavonoids are regarded as important nutraceuticals mainly due to their antioxidant effects, which play a potential role in preventing various diseases associated with oxidative stress. Moreover,

flavonoids have also been used to modulate the activity of a wide range of enzymes and cell receptors (Andersen and Markham, 2006). Total anthocyanin contents in extracts found to be in a range from  $10.52 \pm 0.13$  to  $16.87 \pm 0.24$  mg L<sup>-1</sup> as cyanidin 3-glucoside equivalents (Table 3).

### Correlations between Different Compounds

The correlation coefficient between antioxidant capacity and total anthocyanin contents was statistically significant ( $r = 0.960$ ,  $P < 0.05$ ), whereas the one between antioxidant capacity and total phenol contents was non-significant ( $r = 0.656$ ,  $P < 0.05$ ). Likewise, the correlation between antioxidant capacity and total flavonoid contents was non-significant ( $r = 0.573$ ,  $P < 0.05$ ). On the other hand, a positive correlation was found between total phenol and flavonoid contents ( $r = 0.984$ ,  $P < 0.05$ ).

The correlation between  $\alpha$ -amylase inhibition activity and total anthocyanin content was statistically significant ( $r = 0.991$ ,  $P < 0.05$ ), while the ones between  $\alpha$ -amylase inhibition and total phenol ( $r = 0.480$ ,  $P < 0.05$ ), flavonoid ( $r = 0.427$ ,  $P < 0.05$ ) were non-significant. Similarly, those between  $\alpha$ -glucosidase inhibition activity and total phenol ( $r = 0.570$ ,  $P < 0.05$ ), flavonoid ( $r = 0.538$ ,  $P < 0.05$ ), anthocyanin ( $r = 0.838$ ,  $P < 0.05$ ) contents were non-significant.

### CONCLUSION

VMF could be used as antidiabetic by inhibiting the amylase and glucosidase enzyme activities due to the presence of anthocyanins contents. VMF extracts have highly significant inhibitory effects on  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, therefore, they can be used as natural therapeutic food for diabetes. All extracts of VMF showed strong antioxidant properties compared to different standards such as

BHA, RUT, and TRO. There is a significant and linear relationship between the antioxidant activity and anthocyanin contents. As a result, VMF can be used as an easily accessible source of natural antioxidant and a possible supplement in food, pharmaceutical, and medical industry. Therefore, our future work is focused on isolation and characterization of the active biomolecules from VMF to evaluate their *in vivo* effects.

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## خاصیت های ضد دیابتی و آنتی اکسیدانی میوه قره قاط یا بیل بری (*Vaccinium myrtillus* Linn.) و ترکیبات شیمیایی آنها

۱. گودر، م. گور، م. س. انگین

### چکیده

میوه بیل بری (*Vaccinium myrtillus* Linn. (Ericaceae)) به طور سنتی برای درمان سنگ مثانه، بی نظمی های صفراوی، اسکوربوت (نوعی بیماری خونی)، سرفه، و سل ریوی به کار می رود. به دلیل این مصارف، این میوه (VMF) ممکن است دارای خاصیت های دارویی باشد اما بررسی ها برای روشن کردن اثرات پزشکی آن در شرایط طبیعی زنده و آزمایشگاهی محدود بوده و خاصیت های ضد دیابتی و آنتی اکسیدانی آن به جزئیات بررسی نشده اند. در این پژوهش فعالیت های ضد آمیلاز، آنتی گلوکوسیداز و آنتی اکسیدانی VMF عصاره گیری شده با متانول (ME)، اتانول (EE) استون (AE) و آب (WE) بررسی شدند. نیز، بعضی ترکیبات آن هم با استفاده از روش اسپکتروفتومتر تعیین شد. اثرات ضد دیابتی و آنتی اکسیدانی عصاره ها با استفاده از آزمون هایی مانند باز دارندگی آلفا آمیلاز و آلفا گلوکوسیداز و آنتی اکسیدانت کل، و فعالیت خنثی سازی DPPH (scavenging) مطابق روش های آزمایشگاهی (درون شیشه ای) در غلظت های مختلف (۲۵۰-۱۰ میکروگرم در میلی لیتر) بررسی شد. در مقایسه با استانداردها، ME، WE، و EE فعالیت شدید آنتی اکسیدانی کل نشان دادند که مقادیر  $IC_{50}$  ( $\mu\text{g/mL}$ ) آن به ترتیب برابر بود با  $24.46 \pm 0.34$ ،  $25.24 \pm 0.78$  و  $27.48 \pm 0.60$  همزمان، ME ( $IC_{50}$   $61.38 \pm 1.40 \mu\text{g/mL}$ ) و EE ( $IC_{50}$   $65.52 \pm 1.19 \mu\text{g/mL}$ ) فعالیت بازدارندگی بسیار موثری بر علیه آلفا آمیلاز و فعالیت بازدارندگی متوسطی علیه آلفا گلوکوسیداز نشان دادند. همچنین همه عصاره ها قدرت کاهندگی زیاد و فعالیت قوی شلات کردن فلزات، آنیون ابر اکسید (superoxide anion)، رادیکال DPPH، و فعالیت خنثی سازی توسط  $H_2O_2$  بروز دادند. نیز، با تجزیه و تحلیل آماری روابط مهمی بین فعالیت زیستی و ترکیبات شیمیایی به دست آمد. بر این اساس، میتوان از VMF به عنوان داروی ضد دیابت و آنتی اکسیدان استفاده کرد. نتیجه اینکه از نظر پزشکی و دارویی، VMF به لحاظ ترکیب شیمیایی منبعی است ضد دیابت و آنتی اکسیدان. همچنین، محتوی آنتی سیانین ممکن است فعالیت بازدارندگی آنتی آمیلاز را بیشتر از محتوی فنولی و فلاونویدی تحت تاثیر قرار دهد.