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BIOLOGICAL ACTIVITIES AND PHENOLIC-FLAVONOID CONTENTS OF BILBERRY EXTRACTS

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SUMMARY

In this study, the petroleum ether, dichloromethane, methanol and hot water extracts were prepared from bilberry blooms. Total phenolic and flavonoid contents of these extracts were determined as pyrocatechol and quercetin equivalents, respectively. The antioxidant activity of the extracts was investigated by using four methods including DPPH free radical and ABTS cation radical scavenging, *β*-carotene bleaching and cupric reducing antioxidant capacity (CUPRAC) assays. The methanol extract showed the highest antioxidant activity in two assays. The anticholinesterase effect of the extracts was determined by using Ellman method via inhibition of acetyl- (AChE) and butyryl-cholinesterase (BChE) enzymes. The strongest inhibition against BChE was observed for the methanol extract. The antioxidant and anticholinesterase potential of bilberry bloom extracts was determined for the first time.

ÖZET

Bu çalışmada, yaban mersini bitkisinin çiçek kısımlarının petrol eteri, diklorometan, metanol ve sıcak su ekstreleri hazırlandı. Bu ekstrelerin toplam fenolik madde miktarları pirokatekole eşdeğer, toplam flavonoit madde miktarları kersetine eşdeğer olarak belirlendi. Antioksidan tayin yöntemleri olarak, DPPH serbest radikal ve ABTS katyon radikali giderim, *β*-karoten renk açılım ve CUPRAC yöntemleri kullanıldı. Metanol ekstresi bu yöntemlerin ikisinde en yüksek antioksidan aktiviteyi gösterdi. Ekstrelerin antikolinesteraz aktiviteleri, asetilkolinesteraz ve bütirilkolinesteraz enzimlerinin inhibisyonuna dayanan Ellman yöntemi ile belirlendi. Metanol ekstresi bütirilkolinesteraza karşı en yüksek aktiviteyi gösterdi. Yaban mersini bitkisinin çiçek kısımlarının ekstrelerinin antioksidan ve antikolinesteraz aktiviteleri ilk kez bu çalışma ile belirlendi.

Key words: Bilberry; Antioxidant activity; Anticholinesterase activity.

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INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) (Ericaceae) is a perennial dwarf shrub native to Europe and Northern America, widely known for its tasty fruit of high nutritive value and its use in folk medicine (1). The berries contain high levels of phenolics – mainly anthocyanins (2) – which, due to their antioxidative properties, are considered to be the pharmacologically active and health-promoting constituents (3).

Bilberry is one of the best natural sources of anthocyanins; however, other compounds, such as stilbenes and iridoid glycosides, are also found in its berries. The leaves of this plant, traditionally used as a folk medicine for the treatment of diabetes, have recently been proposed as a potential source of phenolic compounds with many prohealth properties. The less explored organs of bilberry, such as the stems and rhizomes, have also been found to contain phenolics with various biological activities. Flavonoids and other phenolic compounds are reported to have multiple biological effects including antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory, antiproliferative and antimicrobial activities (4).

The common mechanism underlying these different macroscopic effects might be related to the antioxidant properties of dietary phenolics, including anthocyanins. Indeed, extracts of berries, including bilberries and blueberries, have shown high antioxidant potential determined in vitro by several basic antioxidant tests based on quenching free radicals in cell-free systems; e. g. radical scavenging capacity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) or 2,20-azinobis-(3-ethylbenzothiazoline-6 sulphonic acid (ABTS), ferric reducing antioxidant activity (FRAP), oxygen radical absorbance activity (ORAC), and inhibition test for lipid peroxidation (5). Furthermore, by implementing a cellbased antioxidant assay, fruit extracts have been shown to have an intracellular antioxidant activity also (6).

Because of the wide range of anthocyanin concentrations reported (7), the antioxidant capacity varied accordingly. The purpose of this study was to determine total phenolic and total flavonoid contents, antioxidant and ancholinesterase capacity of bilberry bloom extracts for the first time.

EXPERIMENTAL

Plant material

Dried bilberry blooms were purchased from a local market in Istanbul in August 2011.

Preparation of the extracts

Dried bilberry blooms (144.4 g) were powdered and ground in a grinder. They were sequentially macerated, for 24 h at room temperature, with petroleum ether (3x500 mL), dichloromethane (3x500 mL), methanol (3x500 mL) and hot water (3x500 mL). After filtration, the solvents were evaporated to dryness in vacuo. After evaporization of the solvents, all crude extracts were directly used for the activity assays.

Determination of total phenolic content

The concentrations of phenolic content in the crude extracts were expressed as micrograms of pyrocatechol equivalents (PEs), determined with FCR (Folin–Ciocalteu's Reagent) according to the method of Slinkard and Singleton (8). The solution of the crude extracts in methanol was added to 180 μL of distilled water and 4 μL of FCR and mixed thoroughly. After 3 min, 12 μL sodium carbonate (2 %) was added to the mixture and shaken intermittently for 2 h at room temperature. The absorbance was read at 760 nm. The concentration of phenolic compounds was calculated according to the following equation:

Absorbance = 0.0164 pyrocatechol (μg) + 0.0247 (R^2= 0.9954).

Determination of total flavonoid content

Measurement of flavonoid content of the crude extracts was based on the method described by Park et al. (9) with a slight modification and results were expressed as quercetin equivalents. An aliquot of the solution was added to test tubes containing 4 μL of 10 % aluminium nitrate, 4 μL of 1 M potassium acetate and 172 μL of methanol. After 40 min at room temperature, the absorbance was determined at 415 nm. The concentration of flavonoid compounds was calculated according to the following equation:

Absorbance = 0.1556 quercetin (μg) + 0.1366 (R^2= 0.9985).

DPPH free radical scavenging activity

The free radical scavenging activity was determined spectrophotometrically by the DPPH· assay (10) with slight modification. In its radical form, DPPH·

absorbs at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreases. Briefly, 120 µL of ethanol and 40 µL of sample solutions, dissolved in ethanol, at different concentrations were mixed. The reaction was then initiated by the addition of 40 µL of DPPH· (0.4 mM) prepared in ethanol. After thirty minutes, the absorbance was measured at 517 nm by using a 96-well microplate reader. Ethanol was used as a control. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability of scavenging the DPPH radical was calculated by using the following equation:

$$\text{DPPH radical scavenging effect (\%)} = \frac{[(A_{Control} - A_{Sample}) / A_{Control}]}{A_{Control}} \times 100$$

where $A_{Control}$ is the initial concentration of the DPPH· and A_{Sample} is the absorbance of the remaining concentration of DPPH· in the presence of the extract and positive controls. BHT and BHA were used as antioxidant standard for comparison of the activity.

ABTS cation radical decolorization assay

The spectrophotometric analysis of $ABTS^{\cdot+}$ scavenging activity was determined according to the method of Re et al. (11), with slight modifications. The $ABTS^{\cdot+}$ was produced by the reaction between 7 mM ABTS in water and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Oxidation of ABTS commenced immediately, but the absorbance was not maximal and stable until more than 6 h had elapsed. The radical cation was stable in this form for more than 2 days in storage in the dark at room temperature. Before usage, the $ABTS^{\cdot+}$ solution was diluted to get an absorbance of 0.708 ± 0.025 at 734 nm with ethanol. Then, 160 µL of $ABTS^{\cdot+}$ solution was added to 40 µL of sample solution in ethanol at different concentrations. After 10 min the absorbance was measured at 734 nm by using a 96-well microplate reader. The percentage inhibitions were calculated for each concentration relative to a blank absorbance (ethanol). The scavenging capability of $ABTS^{\cdot+}$ was calculated using the following equation:

$$ABTS^{\cdot+} \text{ scavenging effect (\%)} = \frac{[(A_{Control} - A_{Sample}) / A_{Control}]}{A_{Control}} \times 100$$

where $A_{Control}$ is the initial concentration of the $ABTS^{\cdot+}$ and A_{Sample} is the absorbance of the remaining concentration of $ABTS^{\cdot+}$ in the presence of sample. BHT and BHA were used as antioxidant standards for comparison of the activity.

Determination of the antioxidant activity with the β-carotene bleaching method

The antioxidant activity of the crude extracts was evaluated using the *β*-carotene-linoleic acid test system (12) with slight modifications. *β*-Carotene (1 mg) in 2 mL of chloroform was added to 100 µL of linoleic acid, and 800 µL of Tween-40 emulsifier mixture. After evaporation of chloroform under vacuum, 200 mL of distilled water saturated with oxygen, were added by vigorous shaking. Four thousand microliter of this mixture was transferred into different test tubes containing different concentrations of the sample. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a 96-well microplate reader. The emulsion system was incubated for 2 h at 50^0C. A blank, devoid of *β*-carotene, was prepared for background subtraction. BHT and BHA were used as standards. The bleaching rate (R) of β-carotene was calculated according to following equation:

$R = \ln(a/b)/t$

where ln: natural log; a: absorbance at time 0; and b: absorbance at time t (120 min).

The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using following equation:

$$AA = [(R_{Control} - R_{Sample})] / R_{Control}] \times 100$$

Cupric reducing antioxidant capacity (CUPRAC)

Cupric reducing antioxidant capacity of the extracts was determined according to the method described by Apak et al. (13). All crude extracts were dissolved in distilled water to prepare their stock solution at 1000 µg/mL concentration. Sixty-one microliter of 1.0×10^{-2} M copper (II) chloride, 61 µL of ammonium acetate buffer (1 M, pH 7.0), and 61 µL of 7.5×10^{-3} M neocuproine solution were mixed, x µL sample solution (2.5, 6.25, 12.5, and 25 µL) and (67- x) µL distilled water were added to make the final volume 250 µL. The tubes were stopped, and after 1 h, the absorbance at 450 nm was measured against a reagent blank, by using Bio Tek PowerWave XS. Absorbance was linearly correlated to antioxidant concentration. BHT and BHA were used as antioxidant standards for comparison of the activity.

Determination of anticholinesterase activity

Acetyl- and butyryl-cholinesterase inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman et

al. (14). Acetylthiocholine iodide and butyrylthiocholine iodide were used as substrates of the reaction and DTNB were used for the measurement of the anticholinesterase activity. All crude extracts were dissolved in ethanol to prepare their stock solution at 4000 µg/mL concentration. One hundred-fifty microliter of 100 mM sodium phosphate buffer (pH 8.0), 10 µL of sample solution and 20 µL AChE (or BChE) solution were mixed and incubated for 15 min at 25^0C, and 10 µL of DTNB is added. The reaction was then initiated by the addition of 10 µL acetylthiocholine iodide (or butyrylthiocholine iodide). Final concentration of the tested solutions was 200 µg/mL. The hydrolysis of these substrates was monitored using BioTek Power Wave XS by the formation of yellow 5-thio-2-nitrobenzoate anion as the result of the reaction of DTNB with thiocholine, released by the enzymatic hydrolysis of acetylthiocholine iodide or butyrylthiocholine iodide, at a wavelength of 412 nm. Ethanol was used as a solvent to dissolve the samples and controls. Galanthamine was used as standard drug.

RESULTS AND DISCUSSION

The extracts prepared from bilberry blooms were found to be rich in phenolic compounds but not in flavonoids (Table 1). The methanol extract showed the highest antioxidant activity in DPPH and ABTS assays (Figures 1 and 2). Also, the methanol extract had the highest phenolic content and exhibited the best inhibitory activity against the butyrylcholinesterase (BChE) among the tested extracts (Table 2).

Table 1. Total phenolic and flavonoid contents of the extracts[a]

Extracts	Phenolic content *(µg PEs/mg extract)*[b]	Flavonoid content *(µg QEs/mg extract)*[c]
BP	90.18 ± 0.00	9.18 ± 0.77
BD	88.66 ± 2.64	13.55 ± 4.25
BM	150.30 ± 0.30	24.09 ± 2.50
BW	97.03 ± 6.04	15.35 ± 0.77

[a] Values are means ±S.D.of three paralel measurements ($p<0.05$)
[b] PEs, pyrocatechol equivalents
[c] QEs, quercetin equivalents
BP: Bilberry petroleum ether extract
BD:Bilberry dicloromethane extract
BM:Bilberry methanol extract
BW:Bilberry hot water extract

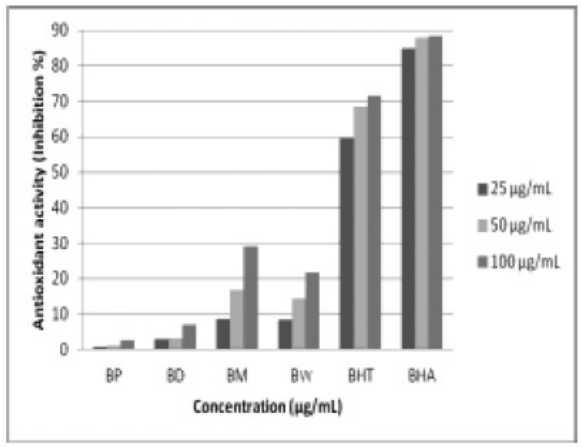

Figure 1. DPPH free radical scavenging activity of the extracts, BHT, BHA.

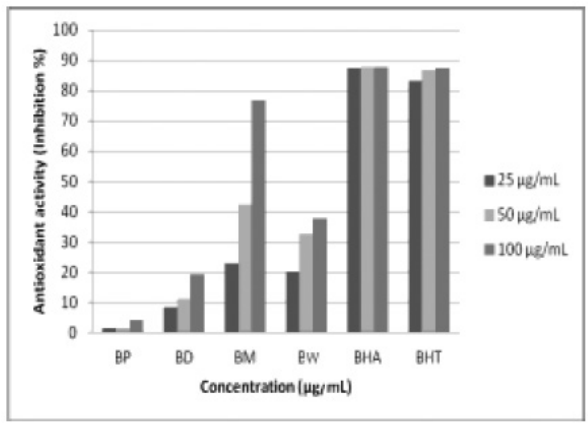

Figure 2. ABTS cation radical scavenging activity of the extracts, BHT, BHA.

The petroleum ether extract showed antioxidant activity only in CUPRAC method and the hot water extract was active only in β-carotene bleaching method (Figures 3 and 4). The dichloromethane and hot water extracts possessed no acetylcholinesterase inhibitory activity. This study indicated that there was a relationship between the antioxidant, phenolic content and anticholinesterase effects. Further in vitro and in vivo investigations will provide to indicate the antioxidant and anticholinesterase potential of the methanol extract of bilberry blooms and its active compounds.

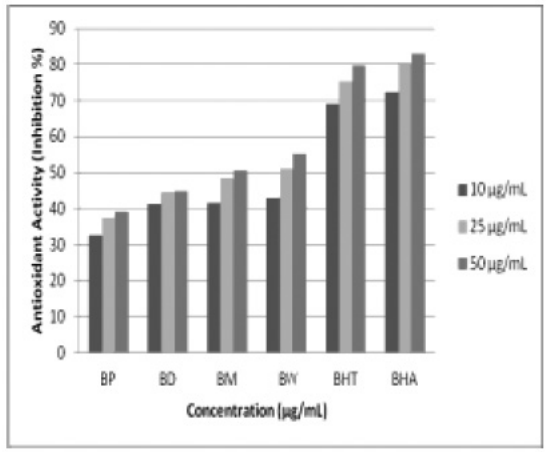

Figure 3. *β*-carotene bleaching activity of the extracts, BHT, BHA.

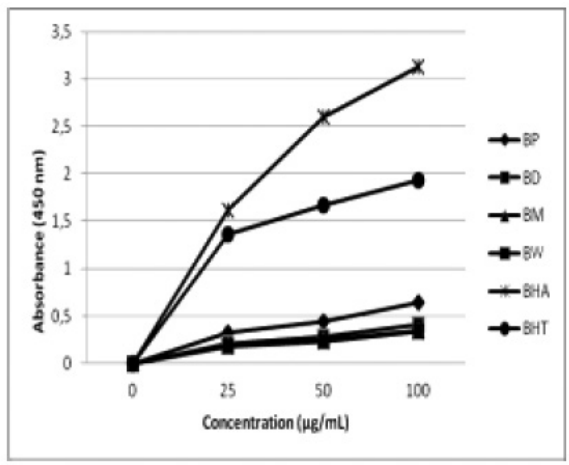

Figure 4. Cupric reducing antioxidant capacity of the extracts, BHT, BHA.

Table 2. Anticholinesterase capacity of bilberry extracts at 200 µg/mL[a]

Extracts	AChE (Inhibition %)	BChE (Inhibition %)
BP	23.21 ± 0.58	31.03 ± 0.59
BD	NA	19.13 ± 2.35
BM	12.41 ± 0.37	51.49 ± 0.71
BW	NA	21.21 ± 1.06
Galanthamine[b]	89.98 ± 0.61	92.47 ± 0.63

[a]Values expressed are mean ± SD of three parallel measurements ($p < 0.05$).
[b]Standard drug.

Statistical analysis

The results were mean ± SD of three parallel measurements. All statistical comparisons were made by means of Student's t-test, p values < 0.05 were regarded as significant.

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