T.C.
YEDITEPE UNIVERSITY
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DEPARTMENT OF NUTRITION AND DIETETICS

DETERMINATION of ANTIOXIDANT, PHENOLIC CONTENT and ANTIMICROBIAL ACTIVITY of BILBERIES (*Vaccinium myrtillus L.*)

MASTER'S THESIS

EMİNE DENİZ ÖZDEMİR

İSTANBUL, 2019

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ONAY

Bu tez Yeditepe Üniversitesi Lisansüstü Eğitim-Öğretim ve Sınav Yönetmeliğinin ilgili maddeleri uyarınca yukarıdaki jüri tarafından uygun görülmüş ve Enstitü Yönetim Kurulu'nun 22./11./2019.. tarih ve 2019/18-01 sayılı kararı ile onaylanmıştır.

Prof. Dr. Bayram YILMAZ
Sağlık Bilimleri Enstitüsü Müdürü

DECLARATION

I hereby declare that this thesis in my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgement has been made in text.

Emine Deniz ÖZDEMİR

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1ABSTRACT

Özdemir E.D (2019). Determination of antioxidant, phenolic content and antimicrobial activity of bilberies (*Vaccinium myrtillus L.*). Yeditepe University, Institute of Health Sciences, Department of Nutrition and Dietetics, MSc Thesis. İstanbul.

Today, there is a growing interest in nutritional awareness and a selective preference towards a healthy and balanced diet. A number of varieties of vegetables are good sources of essential components in human nutrition, providing vitamins, minerals, and fibre in general. *Vaccinium myrtillus L.* is a well- known bilberry shrub belonging to the *Ericaceae* family. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to participate in the pathogenesis of various human diseases and may be involved in the conditions which *Vaccinium myrtillus* is used to treat. According to above mentioned it is important and reasonable to get to know and to investigate the antioxidant action and beneficial properties of billberry fruits and leaves grown in Turkey. In this study, dried fruit and leaves extracts of bilberies (*V. myrtillus* L.) growed in Turkey have been investigated in two different solvents (methanol, and water) and antimicrobial activity.In the present study, antioxidan activity of water extract from *V. Myrtillus* leaves revealed 949 μMTE/g. *V. Myrtillus* leaves had the highest antioxidan activity(949 μMTE/g) followed by water extract from *V. Myrtillus* fruits(151.98 μMTE/g), while the lowest value was observed in methanol extract *from V. Myrtillus*(60.85 μMTE/g) followed leaves followed by methanol extract from *V. Myrtillus* fruits(85.21 μMTE/g). The phenolic compound of *V. Myrtillus* varied from 0.081 to 1.037 mMGAE/g. The highest phenolic compound was determined by methanol extract from *V. Myrtillus* leaves (1.037 mMGAE/g), and the lowest phenolic compound was found by methanol extract *V. Myrtillus* fruits (0.081 mMGAE/g).

Extracts of leaves and fruits of blueberries (*Vaccinum myrtllus*) prepared with two different solvents, indicated maximum antimicrobial activity on *Escherichia coli* (10 mm) and *Bacillus subtilis* (10 mm). Fruit ethyl alcohol extract indicated antimicrobial activity against *Escherichia coli*, *Bacillus subtilis* (10 mm), *Staphylococcus aureus* (9 mm), *Pseudomonas aeruginosa* (7 mm).

Keyword: Antioxidan, *Vaccinium myrtillus L.*, antimicrobial activity

ÖZET

Bilberies (*Vaccinium myrtillus L.*)' in antioksidan, fenolik içerik ve antimikrobiyal aktivitesinin belirlenmesi. Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Beslenme ve Diyetetik Anabilim Dalı, Master Tezi. İstanbul.

Günümüzde beslenme bilincine artan bir ilgi ve sağlıklı ve dengeli bir diyete doğru seçici bir tercih vardır. Bir dizi sebze türü, genel olarak vitamin, mineral ve lif sağlayan, insan beslenmesinde temel bileşenlerin iyi kaynaklarıdır.

Vaccinium myrtillus L., *Ericaceae* familyasına ait bir çalıdır. Reaktif oksijen türlerinin (ROS) ve reaktif azot türlerinin (RNS) çeşitli insan hastalıklarının patogenezine katıldığı bilinmektedir ve *Vaccinium myrtillus* tedavide kullanılan koşullarda yer alabilir. Yukarıda belirtilenlere göre, Türkiye'de yetişen yaban mersini meyvelerinin ve yapraklarının antioksidan etkilerini araştırmak önemli ve makuldür. Bu çalışmada, Türkiye'de yetişen kurutulmuş meyve ve yaprakları (*V. myrtillus* L.) iki farklı çözücüde (metanol ve su) ve antimikrobiyal aktivitesi incelenmiştir. Bu çalışmada, *V. myrtillus* yapraklarından su ekstraktının antioksidan aktivitesi 949 µMTE / g olarak ortaya çıkmıştır.

V. myrtillus yaprakları en yüksek antioksidan aktivitesine (949 µMTE / g) ve ardından *V. myrtillus* meyvelerinden su ekstresi (151.98 µMTE / g) sahipken, en düşük değer *V. myrtillus*'tan (60.85 µMTE / g) metanol ekstresinde gözlendi. *V. myrtillus*'un fenolik bileşiği 0.081 ila 1.037 mMGAE / g arasında değişmiştir. En yüksek fenolik bileşik, *V. myrtillus* yapraklarından (1.037 mMGAE / g) metanol ekstresi ile belirlendi ve en düşük fenolik bileşik, metanol özü *V. myrtillus* meyvelerinde (0.081 mMGAE / g) bulundu.

İki farklı çözücüyle hazırlanan yaban mersini yapraklarının ve meyvelerinin özleri (*Vaccinum myrtllus*), *Escherichia coli* (10 mm) ve *Bacillus subtilis* (10 mm) üzerinde yüksek antimikrobiyal aktivite göstermiştir. Meyve etil alkolü ekstresi, *Escherichia coli*, *Bacillus subtilis* (10 mm), *Staphylococcus aureus* (9 mm), *Pseudomonas aeruginosa*'ya (7 mm) karşı antimikrobiyal aktivite göstermiştir.

Anahtar Kelimeler: Antioksidant, *Vaccinium myrtillus L.*, antimikrobial

1. INTRODUCTION

Reactive oxygen species [ROS], sometimes called as active oxygen species, are various forms of activated oxygen, which include free radicals such as superoxide ions (O2-.) and hydroxyl radicals (OH.) as well as non-free radical species such as hydrogen peroxide (H2O2) [1]. These ROS play an important role in degenerative or pathological processes, such as aging, cancers, coronary heart diseases, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, cataracts and inflammations [2]. Living Living organisms have antioxidant defence systems that protects against oxidative damage by removal or repair of damaged molecules [3]. The term 'antioxidant' refers to the activity of numerous vitamins, minerals and phytochemicals which provide protection against the damage caused by ROS [4]. Antioxidants interfere with the oxidative processes by scavenging free radicals, chelating free catalytic metals and by acting as electron donors [5]. The natural antioxidant mechanisms maybe insufficient in variety of conditions and hence dietary intake of antioxidant compounds are important [6]. The therapeutic effects of several medicinal plants are usually attributed to their antioxidant phytochemicals. It has been suggested that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases [1]. Plant based antioxidants are preferred to the synthetic ones because of their multiple mechanisms of actions and non-toxic nature. These facts have inspired screening of plants for possible medicinal and antioxidant properties; the isolation and characterization of diverse phytochemicals and the utilization to antioxidants of natural origin to prevent the diseases [7]. *Vaccinium myrtillus L*. is a well- known bilberry shrub belonging to the *Ericaceae* family. It has been reported to have multiple pharmacological activities from its anthocyanosidic fraction: ophthalmic, vasoprotective, antiinflammatory, wound- healing, antiulcer and antiatherosclerosis. It is to be expected that several activities might be related to a possible antioxidant action from anthocyanosides. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to participate in the pathogenesis of various human diseases and may be involved in the conditions which *Vaccinium myrtillus* is used to treat. According to above mentioned it is important and reasonable to get to know and to investigate the antioxidant action and beneficial properties of billberry fruits and leaves grown in Turkey. In this study, dried fruit and leaves extracts of bilberies (*V. myrtillus* L.) growed in Turkey have been investigated in two different solvents (methanol, and water) and antimicrobial activity.

2. LITERATURE REVIEW

2.1. Data on medicinal use

Dry bilberry fruit has been present as single active ingredient in 115 herbal teas on the German market for more than 30 years, traditionally used for unspecific acute diarrhoea, mild inflammation of the oropharyngeal mucosa [8].

Comminuted dry bilberry fruit has been on the Polish market as single active ingredient for more than 30 years and it is also registered as a herbal tea for unspecific acute diarrhoea in Austria. A bilberry methanolic dry extract prepared from fresh fruit (DER 153-76:1; extraction solvent methanol 70% v/v) containing 36% anthocyanosides, corresponding to 25% anthocyanidins has been on the Italian market for more than 30 years, at least since 1984, being the active substance of three medicinal products, in soft and hard capsules and as a granulate for oral solution [9].

Information on relevant combination medicinal products marketed in the EU/EEA:

Belgium

Pharmaceutical form> containing 100 mg *V. myrtillus* L., anthocyanosidic extract (no further detail available) + 5 mg beta-carotene (not authorized).

Indication: Capillary fragility, CVI, visual problems related to circulatory problems

Posology: 3 to 6 x 100 mg a day

On the market from 1965 to 1991,Italy

Soft capsules containing 70 mg *V. myrtillus* L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins + 40 mg d,l-alfa-Tocoferil-acetato + 10 mg beta-carotene

Indication: Conditions of capillary fragility

Posology: 4 to 6 capsules a day or according to medical prescription

On the market since 1993

Information on other products marketed in the EU/EEA (where relevant)

Five single active ingredient herbal teas on the Polish market as food supplements (for unspecific acute diarrhoea) [9].

Overall conclusions on medicinal use:

Long-standing medicinal use for at least 30 years within the European Community, is therefore demonstrated for the following preparations and indications:

1) *V. myrtillus* L., fructus siccus (dry bilberry fruit), whole or comminuted, as herbal tea for oral use as an adjuvant in unspecific acute diarrhoea. Traditional medicinal use of this preparation is substantiated by extensive bibliography and the presence on the German and

Polish market for more than 30 years. The daily dose in adults and adolescents over 12 years ranges from 15 to 60 g, divided in 3-4 single dose of 5 to 15 g in 250 ml as a 10 minutes decoction. (In Poland it is used also as an infusion (for 10 -20 min under cover): 4 g in 200 ml of boiling water, 2 - 3 times daily). [9]

2) *V. myrtillus* L., fructus siccus (dry bilberry fruit), whole or comminuted, as a decoction for oromocosal use for the topical treatment of mild inflammation of the mucous membranes of the mouth and throat. Traditional medicinal use of this preparation is substantiated by extensive bibliography and the presence on the German and Polish market for more than 30 years. It is used as a 10% decoction to rinse the mouth several times daily. 2) *V myrtillus* L., fructus recens dry extract; DER 153-76:1; extraction solvent methanol 70% v/v containing 36% anthocyanosides, corresponding to 25% anthocyanidins (BEM), in solid dosage forms for oral use for the treatment of symptoms of venous insufficiency and conditions of capillary fragility. Traditional medicinal use of this preparation is substantiated by the presence of medicinal products since 1984 in Italy. Single dose: 80 -160 mg; Daily dose: 160- 540 mg. [9]

2.2. Non-Clinical Data

Overview of available pharmacological data regarding the herbal substance(s), herbal preparation(s) and relevant constituents thereof

2.2.1. Primary pharmacodynamics

Vasoactive properties *In vitro* Experiments; Effect of the *V. myrtillus* fresh fruits extract, DER 153-76:1, extraction solvent methanol 70% v/v containing 36% anthocyanosides (corresponding to 25% of anthocyanidines) (BEM) on the venous smooth muscles to contraction response to the 5-HT was investigated *in vitro* by Bettini *et al.* (1984a)[10].

The study was performed on isolated thoracic vein calf preparations. *V. myrtillus* extract (25% of anthocyanosides) (25 – 100 μg/ml) alone caused a moderate decrease in tension as the response to the contractions induced by 5-HT (0.5 – 1 μg/ml). The effect was more pronounced after addition of ascorbic acid (1 – 4 μg/ml). The relaxation effect was nullified or highly decreased by the pre-treatment with indomethacin (1 μg/ml) or lysine acetylsalicylate (1 μg/ml) [11]. Contractility of the segments of internal thoracic vein calf preparations induced by barium chloride (50 μg/ml) was reduced by *V. myrtillus* extract concentration dependently (25, 50, 75, 100 μg/ml). Indomethacin (1 – 30 μg/ml) and lysine acetylsalicylate (1 – 30 μg/ml) reduced or completely suppressed the reduction of the venous muscle tone produced by BEM [12].

In other experiment the influence of BEM on contractility of the smooth muscles of the calf splenic arteries segments induced by 5-HT was investigated. *V. myrtillus* extract (25, 50, 75,

100 μg/ml) alone caused a concentration dependent decrease in tension of the arterial muscles as the response to the contractions induced by 5-HT (0.2 μg/ml). The effect was potentiated after addition of ascorbic acid (1–4 μg/ml). Indomethacin (1-30 μg/ml) and lysine acetylsalicylate (1–30 μg/ml) decreased the reduction of the arterial muscle tone produced by BEM. The results obtained by the authors indicate that the mechanism of the vasodilatating effect of BEM on vascular muscles is based on the local synthesis of prostacyclin [11].

In another study Bettini *et al.* (1991) investigated contractile responses of the isolated calf coronary vessels to acetylcholine (ACh) and methylene blue [13]. Experiments were carried out without removal of the endothelium. BEM (50 – 200 μg/ml) decreased both the tone and in higher concentrations the contractile response of the preparations to ACh (0.001 – 1 μg/ml). A small decrease of the tone was observed with BEM alone (50 – 200 μg/ml) and the effect was more marked with addition of the ascorbic acid (100 – 300). Indomethacin (40 μg/ml) and lysine acetylsalicylate (40 μg/ml) reduced or completely suppressed the reduction of the venous muscle tone produced by BEM. Pre-treatment with methylene blue (24 μg/ml) resulted in the partial reduction of the vasodilatatory effect of BEM. The authors concluded, that the vasodilator effect of BEM is related to the release of prostaglandins and to a facilitation of the endothelium-derived relaxing factor (EDRF) release, as the methylene blue is known to block the release of EDRF [13].

Continuing research of Bettini *et al.* (1993) found after use of BEM (5-100 μg/ml) a significant increase in contractility of endothelium-deprived isolated calf coronary arteries induced by ACh administration (0.001–1 μg/ml). The potentiating effect was completely suppressed by methylene blue (24 μg/ml) or haemoglobin (0.015–0.020 μg/ml) which block the release of EDRF [14]. The direct vasorelaxating activity of a lyophilized dry BE (no further detail) was tested *in vitro* by Bell and Gochenaur (2006) on coronary arterial rings isolated from pigs. BE contained 15 different anthocyanins including cyanidin, peonidin, delphinidin, petunidin, and malvidin. The total anthocyanin composition was 12.1 g/100 g and total phenolics 35.7 g/100 g. BE produced dose- and endothelium-dependent vasorelaxation in isolated rings with endothelium (% maximal relaxation at 5 mg/l total anthocyanins: 59 ± 10). The authors tested the role of nitric oxide (NO) in these relaxations and found that such relaxation could be abolished by the application of 100 μM NO2-L-arginine (*Nitric Oxide Complex* with *L-arginine*). These observations suggest that the endothelial NO system may be involved in the relaxation response of coronary arteries to the BE. At a concentration too low to directly alter coronary vascular tone, they did not alter coronary responses to endogenous or exogenous NO. However, this same low concentration (≅100 nM) had a considerable

potential to prevent loss of endothelial-dependent relaxation caused by exposure of arteries to exogenous ROS (*Reactive Oxygen Species*) as pyrogallol. As anthocyanins are absorbed intact across the gastrointestinal tract and such concentration roughly reflects that seen in several studies to exist even in human plasma after oral consumption of these products they may have vasoprotective properties [15].

2.2.2. Anti-inflammatory activity

In vitro Experiments; Triebel *et al.* (2012) studied the influence of a lyophilized BE (no further detail) and comprising anthocyanins on pro-inflammatory genes in IFN-γ/IL-1β/TNF-stimulated human colon epithelial cells (T84) by real-time polymerase chain reaction (qRT-PCR) and cytokine activity. Fifteen anthocyanins were detected in the BE by HPLC-DAD analysis, the most numerous being del-3-gal, del-3-glc, del-3-ara, cy-3-gal, and cy-3-glc, at concentrations (mg/g extract) which also shows the concentrations (μM) of the substances in 25 μg/ml BE extract (used in incubations with the cultured cells).

The authors studied the expression of inflammatory bowel diseases-associated pro-inflammatory marker genes (TNF-α, IP-10, IL-8) in the cultures of the human colon epithelial cells (T84) by quantitative real-time PCR. The cytotoxic effects of BE and the singular anthocyanins/anthocyanidins on T84 cells were determined using a resazurin reduction assay. Selected cytokines and chemokines were analyzed using the "Human Cytokine Array Panel A" antibody array.

After 4 and 24 h, 250 μg/ml BE reduced viability to 80 ± 5 and 60 ± 3% and 200 μM cyanidin reduced it to 83 ± 2 and 78 ± 3%, whereas 200 μM delphinidin reduced it to 79 ± 1 and 63 ± 2%, respectively. All investigated anthocyanins had only slight cytotoxic effects at 200 μM (cell viability > 95%) after 4 and 24 h of incubation.

Cytotoxic effects of the anthocyanidins were stronger than the corresponding anthocyanins declining in the order delphinidin > cyanidin > pelargonidin >peonidin > malvidin so that increasing with increases in hydroxylation.

BE significantly and dose-dependently inhibited expression of the pro-inflammatory marker genes TNF-α and IP-10 in CM stimulated T84 cells, at concentrations of 2.5 and 25 μg/ml, respectively.

Influence of chosen anthocyanins on expression of TNF-α, IL-8, IP-10 genes in T84 stimulated cells depended on both the aglycone and of sugar residues. IP-10 expression was significantly inhibited by cyanidin-3-ara, the most potent inhibitor, cyanidin-3-glc at 25 μM (the lowest concentration tested), and cyanidin-3-gal at 50 μM. Peonidin-3-*O*-glycosides were active concentration dependently only as glucose conjugates in the tested concentrations (25,

50, 100 μM). The investigation of activity of the corresponding anthocyanidins have shown that cyanidin, delphinidin, and petunidin significantly down-regulated IP-10 mRNA expression, but peonidin or malvidin did not (even at 100 μM). Cyanidin significantly reduced TNF-α transcript levels at ≥50 μM, but peonidin or malvidin did not have any effect. Pre-treatment with cyanidin (25 μM) and BE (25 μg/ml) completely inhibited synthesis of interferon gamma-induced protein 10 (IP-10), interferon-inducible T-cell alpha chemo-attractant (I-TAC), and soluble intercellular adhesion molecule 1 (sICAM-1).

Authors conclude that single anthocyanins from BE modulates inflammatory genes and protein secretion *in vitro* and thus may act as transcription-based inhibitors of the pro-inflammatory gene expression associated with inflammatory bowel diseases. Moreover, the anti-inflammatory activity of the investigated anthocyanins is strongly dependent on their aglycone structure and the attached sugar moieties [16].

2.2.3. Microcirculation injury

In vivo Experiments; In another study Bertuglia *et al.* (1995) tested activity of the fresh fruits BEM (100 mg per day/kg p.o. for 2 and 4 weeks) in the microcirculation ischemia model. Ischemia was induced by clamping the hamster cheek pouch for 30 min with subsequent reperfusion also lasting 30 minutes. Changes in the microcirculation were visualized by fluorescence method. Ischemia and reperfusion were associated with increased number of leukocytes sticking to venules, decreased number of perfused capillaries and increased permeability.

After treatment there was a significant reduction in ischemic symptoms ($p<0.01$) [17].

2.2.4. Impact on lipid peroxidation

In vitro Experiments; The comprehensive and extensive monograph by Upton *et al.* (2001) showed that an extract of bilberries protected microsomes from rat liver against oxidative damage and apolipoprotein B before brought about by UV radiation [18].

An anthocyanoside complex extract from *V. myrtillus* was tested for its ability to inhibit lipid peroxidation and to scavenge hydroxyl and superoxide radicals [19]. An antiperoxidative action of this *V. myrtillus* extract was assayed by the Fe^{3+} -ADP/NADPH method in rat liver microsomes. Superoxide anions were generated by preparing a mixture of hypoxanthine and xanthine oxidase. The results were expressed as percentage inhibition of cytochrome C reduction [18].

2.2.5. Antioxidant activity

In *in vitro* studies conducted by Cluzel *et al.* (1969), it was found that anthocyanins of *V. myrtillus* affected the activity of various enzymes of retina in the pig and in the rabbit

(inhibiting the activity of phosphoglucomutase, and increasing the activity of lactate dehydrogenase, α-hydroxybutyrate dehydrogenase, 6-phosphogluconate dehydrogenase and α-glycerophosphate dehydrogenase) [20]. However, in these studies, the authors used a complex formulation consisting, beyond of an extract of bilberry, of other components, including beta-carotene. Therefore the significant effect of beta-carotene contained in the preparation in large quantities cannot be excluded.

It was found, that the extract scavenged superoxide anion and inhibited microsomal lipid peroxidation at all concentrations (25, 50 75 and 100 μg/ml) ($p<0.01$) and a 50% inhibition of rate of reaction was observed with a final concentration of 25 μg/ml. The anthocyanoside complex extract was able to inhibit lipid peroxidation (IC 50 = 50.28 mg/ml) and to scavenge superoxide anion (IC 50 < 25 mg/ml). The ability to remove hydroxyl radical exerted by this extract was detectable from 50 mg/ml of extract in the reaction mixture [19]. According to Prior *et al.* (1998), comparison of the antioxidant capacity variety of *Vaccinium* species have shown high activity of *V. Myrtillus.* (Table 1)[21].

Table 1. Antioxidant activity of *V. myrtillus* L. [21].

V. myrtillus L.

Cultivar, state, and source	**ORAC ROO a μmol/g)**	**Anthocyanin b (mg/100 g)**	**Phenolics c (mg/100 g)**	**A/P d (mg/mg)**	**Ascorbate (mg/100 g)**
Bilberry	44.6 ± 2.3 (282.3)	299.6 ± 12.9	525.0 ± 5.0	0.571	1.3 ± 0.1

a-Expressed as micromole Trolox equivalents per gram of fresh fruit. Oxygen radical absorbance capacity (ORAC ROO). Data in parentheses expressed per gram of dry matter. Bilberry was harvested on 7/2/97.

b-Concentration based upon cyanidin-3-glucoside as standard.

c-Concentration based upon gallic acid as standard.

d-Anthocyanin/phenolics.

Bilberries were extracted with acetonitrile/acetic acid for the analysis of ORAC, total anthocyanins, and total phenolics.

In conclusion the increased maturity at harvest increased the ORAC, the anthocyanin, and the total phenolic content. A linear relationship existed between ORAC and anthocyanin ($rxy=0.77$) or total phenolic ($rxy = 0.92$) content [22].

Direct *in vitro* influence of the bilberry fruit extracts on the oxidative phosphorylation of isolated rat heart mitochondria was tested by Trumbeckaitè *et al.* (2013). For testing two types of extracts were used: the hydroethanolic extract (BEE) of the crushed plant material was prepared by maceration with 50% ethanol at room temperature (1:10, v/v), initially for 48 h and thereafter until exhaustion; the aqueous extract (BAE) was prepared using repercolation method (1:10, v/v). The obtained hydroethanolic extract was filtered and concentrated under vacuum (at 50oC) and then subjected to freeze drying. Freeze dried bilberry powder was packed into a glass jar and dissolved before experiments. The levels of anthocyanidins, measured by use of

HPLC, varied in the two extracts (Table 2) [23].

Table 2. Anthocyanidins in the two extracts tested by Trumbeckaitè *et al.* (2013). Amount of anthocyanidins (ng/ml) in 1 μl) of bilberry fruit extracts [23].

Bilberry extract	**Delphinidin**	**Cyanidin**	**Petunidin**	**Peonidin**	**Malvidin**	**Total**
BAE	0.14±0.1	0.36±0.05	0.22±0.2	0.15±0.3	0.31±0.1	1.18±0.3
BEE	0.18±0.2	0.80±0.1	0.26±0.1	0.19±0.2	0.28±0.3	1.71±0.2

BAE, bilberry aqueous fruit extract; BEE, bilberry ethanolic fruit extract.

When measured the effects of BEs on complex I-dependent substrate pyruvate plus malate oxidation, mitochondrial respiratory rates only in the presence of 5–30 ml/1.5 ml of the BAE extract the mitochondrial state 3 respiration rate decreased from 33% to 61% ($p < 0.05$). BEE induced the decrease in state 3 respiration rate also starting from 5 ml/1.5 ml. High doses of BEE (15–30 ml/1.5 ml) induced a decrease in the state 3 respiration rate by 35%–56%, that is similar to BAE. In effect at higher concentrations, BAE and BEE induced significant uncoupling of oxidative phosphorylation and decrease in the state 3 respiration rate. The true mechanism of the diminishing of the state 3 respiration rate by BEs may be the inhibition of mitochondrial respiratory chain at complexes I and II.

Pure anthocyanins, the main components of used extracts, malvidin-3-glucoside, malvidin-3-galactoside, and cyanidin-3-galactoside, had no effect on oxidation of pyruvate plus malate. A statistically significant decrease in H2O2 production by mitochondria was found in the presence of bilberry fruit extracts. BAE at concentrations of 1.5 and 15 μl/1.5 ml clearly

suppressed this process and caused a 46% and 62% reduction, respectively, in the H2O2 generation as compared with that in the absence of BAE.

Similar effects (reduction by 50%) were obtained by BEE (15 ml/1.5 ml), whereas lower amounts of BEE (1.5 ml/1.5 ml) suppressed H2O2 generation by 16%, that is, less than BAE.

The results revealed that the effect of BAE and BEE on mitochondrial function is bivalent: lower concentrations (they correspond to 6–9 mg/l of total anthocyanins) had no effect on mitochondria, whereas at high concentrations (they would correspond to 18–52 mg/l of total anthocyanins), the extracts caused an obvious decrease in the state 3 respiration, but the radical scavenging activity remained increased. The effects of BAE and BEE on mitochondria were dose dependent c

A BE from dried fruits containing anthocyanins (25.0%, w/w) (no further detail) reduced UVA-induced oxidative stress in keratinocytes [24]. In the first experiment keratinocytes grown in culture medium were pre-treated with the BE (5–100 mg/l) in serum free medium at 37oC for 1 h, irradiated and incubated in serum-free medium at 37^{o}C for another 4 h. In the second experiment keratinocytes were irradiated and after UVA exposure the BE (5–100 mg/l) in the serum-free medium was added to the cells for 4 h. The effect of extract in the concentration range of 1–250 mg/l, various UVA doses (10–40 J/cm2) or combinations of the extract and UVA on keratinocytes cell viability was assessed after 4/24 h. Pre-treatment (1 h) or post-treatment (4 h) of keratinocytes with the BE resulted in attenuation of UVA-caused damage. Viability of the cells was determined photometrically. BE at the concentrations tested (1–250 mg/l) did not affect incorporation of water-soluble dyes into lysosomes, but decreased lactate dehydrogenase (LDH) activity in medium samples containing 100 and 250 mg/l of the extract after 24 h. The last finding evaluates activity a cytosolic enzyme, which reflects cell membrane integrity. Moreover application of the extract significantly reduced UVA-stimulated ROS (*Reactive Oxygen Species*) formation in keratinocytes: the maximal decline in ROS generation was at concentrations of 50 and 100 mg/l of the extract. Administration of BE also prevented/reduced UVA-caused peroxidation of membrane lipids: the maximal protection was observed in pre-treatment at a concentration of 50 mg/l (over 90%), post-treatment with the extract also markedly inhibited membrane lipid damage with maximum at concentrations of 25 and 50 mg/l (75–80%). The extract also induced depletion of intracellular GSH: pre-treatment with the extract significantly protected against UVA caused GSH depletion, especially at concentrations of 25 and 50 mg/l (55%). Post-treatment was the most effective in the concentration range of 50–100 mg/l (50%) [24].

In the other experiments of the same group HaCaT keratinocytes were used to assess the effects of pre-and post-treatment with BE phenolic fractions (5–50 mg/l) on keratinocyte damage induced UV radiation by a solar simulator (295–315 nm) [25]. For the assessment of UVB (photo) protective potency of phenolic fractions non-toxic concentrations (5, 10, 25 and 50 mg/l) of BE were used. BE efficiently reduced the extent of DNA breakage (especially at concentrations of 25 and 10 mg/l) together with caspase-3 and -9 activity. The effect of post-treatment on caspase-3 activity was similar for all concentrations tested. Pre-treatment of keratinocytes with BE also reduced caspase-9 activity. BE effect was concentration-dependent (maximal protection of 87%). BE was the most potent at a concentration of 5–10 mg/l (around 80%), which slightly decreased in higher concentrations.

Application of the extract before UVB exposure significantly prevented DNA fragmentation. BE effectiveness culminated at a concentration of 10 mg/l (70%) and at higher concentrations the protection diminished to 50% and 40% at a concentration of 25 mg/l.

The phenolic fraction of *Vaccinium myrtilli* berries significantly decreased generation of reactive oxygen and nitrogen species (RONS), of oxidizing lipids, proteins and DNA. Application of the BE (4 h) to non-irradiated HaCaT slightly reduced RONS generation compared to untreated non irradiated cells. The effectiveness of the extract showed 40% protection at the highest concentration. Supreme RONS elimination was found in post-treated cells. At concentrations of 25 and 50 mg/l phenolic fractions reduced RONS amount to control level.

The extract decreased IL-6 production in irradiated cells, when it was applied before UVB exposure. The effect of BE was concentration-dependent with maximal protection 35%. The maximal potency was found at the highest concentration approximately 33% [24].

Antioxidant activity of bilberry (*Vaccinium myrtillus* L.) and blueberry (*Vaccinium corymbosum* L.) was examined at the cellular level in different cell lines: human colon cancer (Caco-2), human hepatocarcinoma (HepG2), human endothelial (EA.hy926) and rat vascular smooth muscle (A7r5). The bilberry crude methanolic extract was further purified in order to obtain the anthocyanin fraction: [(crude BE: phenolic acids, proanthocyanidins, flavanols, flavonols) →(purified bilberry extract BE: anthocyanin fraction)]. Anthocyanins had intracellular antioxidant activity if applied at very low concentrations (<1 μg/l; nM range) (Table 3). Delphinidin and cyanidin glycosides were the predominant anthocyanins in BEs, whereas malvidin glycosides dominated in the blueberry extract [25].

Table 3. Half maximal effective concentrations (EC 50) of the extracts in 4 cell lines [26].

Parameter	Cell line	Crude blueberry extract	Crude bilberry extract	Purified bilberry Extract
EC 50 (µg/l)	Caco-2	0.78 ± 0.15^{a}	0.29 ± 0.02^{b}	0.53 ± 0.04^{ab}
	HepG2	0.88 ± 0.10^{a}	0.59 ± 0.05^{b}	0.63 ± 0.03^{ab}
	Ea.hy926	0.17 ± 0.02^{a}	0.22 ± 0.02^{a}	0.59 ± 0.06^{b}
	A7r5	5.99 ± 0.81^{a}	0.36 ± 0.02^{b}	1.38 ± 0.10^{b}

Data were expressed as mean ± SEM, number of independent measurements was n = 6. Statistical analysis was performed using one-way ANOVA with post-Bonferroni test. Statistically significant differences ($p < 0.05$) are marked with letters (a,b,c) in the same row. [26]

The effective concentrations achievable after oral administration (27,28) are in the range of plasma anthocyanin concentrations in the presented experiments showing cellular antioxidant activity at very low concentrations in different human cell lines [25,26]. Such values in the range of 1nM are attained after consumption of ordinary servings of berries [29].

Cytoprotective effect of a fresh fruits BE against oxidative damage in primary cultures of rat hepatocytes was studied by Valentová *et al.* (2007). The BE analysed by HPLC contained 25.0% of total anthocyanins. Activity of BE against oxidative cell damage induced by tert-butyl hydroperoxide and allyl alcohol in primary cultures of rat hepatocytes was investigated. The hepatocyte monolayers were incubated with the tested extract for 4, 24 and 48 h and the viability of the cells was assessed by the MTT test. In the concentrations tested (100 and 500 µg/ml), no significant toxicity was registered. [29]

The extract showed significant dose-dependent protective activity against oxidative damage in rat hepatocytes primary cultures induced by tert-butyl hydroperoxide and allyl alcohol. Maximum cytoprotection (58.16%) was noted in the culture pre-incubated with 500 µg/ml of the extract [30].

Antiradical activity was evaluated spectrophotometrically as the ability of the tested substances to reduce 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The extract has shown scavenging activity; 50% inhibition was achieved at 3.99 ± 0.14 µg/ml (IC50). In the same experimental condition, IC50 of the synthetic analogue of vitamin E, trolox, was 2.15 ± 0.06 µg/ml (8.57 ± 0.25 µmol/l) [30].

Estimation of the antioxidant activity of the BE was also investigated in the xanthine/XOD superoxide generating system. The BE scavenged the superoxide radical and its activity was equivalent to 108 ± 7.2 units of SOD per mg of extract. In the same system, trolox had an activity equivalent to 16.4 ± 0.19 units of SOD/mg [30].

Ogawa *et al.* (2011) studied the lipid peroxidation and free radical scavenging activity of a BE (containing more than 25% anthocyanosides – no futher detail) in murine stomach tissue homogenates. BE in the concentration dependent range significantly induced decrease of activity of the lipid peroxide levels and revealed strong scavenging activity against superoxide and hydroxyl radicals. Table 4; Table 5) [31].

Table 4. Superoxide anion radical scavenging activity of the bilberry extract and its main anthocyanidins (delphinidin, cyanidin and malvidin) [31].

Compound	IC_{50}			
	µg/ml		µM	
Bilberry extract	1.2	(1.0-1.5)		
Delphinidin	1.2	(0.9-1.6)	3.5	(2.5-4.7)
Cyanidin	31.8	(21.8-50.9)	98.4	(67.5-157.8)
Malvidin	1.0	(0.7-1.4)	2.8	(2.0-3.8)
Trolox	130.8	(113.4-154.0)	522.4	(453.1-615.1)

IC50, 50% inhibitory concentration. The parentheses show 95% confidence limits

Table 5. Hydroxyl radical scavenging activity of the bilberry extract and its main anthocyanidins (delphinidin, cyaniding and malvidin) [31].

Compound	IC_{50}			
	µg/ml		µM	
Bilberry extract	116	(80-192)		
Delphinidin	237	(203-305)	0.7	(0.6-0.9)
Cyanidin	>323	>1.0		
Malvidin	>367	>1.0		
Trolox	325	(275-400)	1.3	(1.1-1.6)

IC50, 50% inhibitory concentration. The parentheses show 95% confidence limits.

The antioxidant activity of phenolics (at concentrations of 1.4, 4.2, and 8.4 µg of purified extracts/ml of liposome sample) such as anthocyanins, ellagitannins, and proanthocyanidins from bilberry was studied by Viljanen *et al.* (2004) in a lactalbumin-liposome system. Phenolic profile of BE determined using an analytical HPLC method is shown in Table 6.

The extent of protein oxidation was measured by determining the loss of tryptophan fluorescence and formation of protein carbonyl compounds and that of lipid oxidation by conjugated diene hydroperoxides and hexanal analyses (Table 6) [32].

Table 6. Inhibition of lipid and protein oxidation (after 6 days of oxidation) by bilberry phenolics incorporated into lactalbumin-lecithin liposomes [32]

Bilberry extract	Conjugated diene hydroperoxides			Hexanal		
	1.4 µg/ml	4.2 µg/ml	8.4	1.4 µg/ml	4.2 µg/ml	8.4 µg/ml
	19.4 ± 2.6^{b}	-13.1 ± 0.3^{c}	66.5 ± 1.5^{a}	38.8 ± 0.5^{b}	57.3 ± 3.4^{a}	98.4 ± 0.1^{a}
	Tryptophan fluorescence			Carbonyl gain		
	22.9 ± 0.5^{a}	6.1 ± 0.2^{a}	27.9 ± 0.6^{b}	67.6 ± 0.1^{b}	49.1 ± 0.1^{a}	79.9 ± 0.3^{a}

A SD, standard deviation. Negative values indicate pro-oxidant activity. Values in the same column at the same concentration followed by different letters are significantly different ($p < 0.05$).

Bilberry phenolics exhibited good overall antioxidant activity toward protein oxidation. The antioxidant effect toward lipid oxidation was more pronounced than the effect on protein oxidation [32].

2.2.6. Antimicrobial activity

It has long been known that several phenolic substances such as flavonoids, phenolic acids, tannins and lignans have antimicrobial activity[33]. It is believed that it is the flavonoid anthocyanins component in *V. myrtillus* that exerts such an effect. The mechanism of antimicrobial activity may include antiadhesion activity, destruction of the cytoplasmic phospholipid bilayer of the cell wall in microbes, damage of the outer membrane with disintegration of the liposaccharide (LPS) layer by phenolics, tannins complexation of metal ions and inhibition of plasma coagulation by bacteria. Another mechanism is the inhibition of antibacterial multidrug resistance (MDR) and impairment of the efflux pump activity in bacteria [34,35,36]. Rauha *et al.* (2000) evaluated the antimicrobial activity of a number of plants, including bilberry. To the *in vitro* studies, an aqueous solution of the dry extract prepared from the dry plant material (acetone/methanol 70% V/V – no further detail) was used to determine the diameter of the inhibition zones in the agar cultures of bacteria. Clear antimicrobial effect has been found for the BE (500 µg samples) against the *Micrococcus luteus* (inhibition zone (i.z.) of sample= 3 - 4 mm > i.z. of methanol and slight antimicrobial activity against *Staphylococcus epidermidis, Bacillus subtilis, Escherichia coli* and *Candida albicans* (i. z. of sample = 1 - 3 mm) > i.z. of methanol [37].

The antimicrobial activity of many plants, including *V. myrtillus* extract (acetone-water 70:30 V/V; elution with MeOH) prepared from fresh frozen berries was screened against the human pathogenic microbial strains on agar plates to estimate their growth and adherence of the bacterial cells to a berry material. The BE (1 mg/ml) revealed the death of the culture of *Helicobacter pylori*, very strong inhibition of growth of *Bacillus cereus* and strong inhibition of growth of *Clostridium perfringens* and *Staphylococcus aureus*. [38].

Binding of *Neisseria meningitidis* pili to *V. myrtillus* berries and juice polyphenolic fractions containing anthocyanins, proanthocyanidins and flavonols have been identified by Toivanen *et al.* [39]. Prevention of adhesion of pathogenic bacteria to host cell surfaces may constitute the protection from the activity of bacteria that use adhesins to colonize the host cells. [40]

Activity of bilberry against Gram positive and Gram negative intestinal pathogens was examined in *in vitro* cultures of *Salmonella*, *Staphylococcus*, *Listeria* and *Lactobacillus* bacteria [34,35]. The BEs (water/ethyl acetate/methanol) prepared from fresh frozen berries containing phenolic acids and fractions eluted with methanol (ellagitannins and anthocyanins) were tested. BE (2 mg/ml) inhibited the growth of *Staphylococcus aureus* for 12 and 24 hours (5 x 101 - 5 x 102) and *Salmonella enterica Typhimurium* (10- 5 x 101). BE fractions (10 mg/ml) exhibited stronger inhibition against *Staphylococcus aureus* (>5 x 104) for 12 and 24 hours compared with control. Stronger inhibition of growth was also seen against *Salmonella enterica Typhimurium* (5 x 102 - 5 x 103) compared with control.

Influence of various preparations of BE prepared from fresh berries, on trophozoites of *Giardia duodenalis* viability and spontaneous excystation of *Cryptosporidium parvum oocysts* was examined in *in vitro* experiments by Anthony *et al.* (2007, 2011) [41,42]. The water soluble extracts of bilberry containing polyphenols (167 μg/ml of gallic acid equivalents) killed 90.4 + 2.8% of *Giardia duodenalis trophozoites*. Increase of the spontaneous excystation of *Cryptosporidium parvum oocysts* observed after administration of the BE (equivalent to 213 μg/ml of the gallic acid). Because anthocyanins represent more than 70% of the polyphenols, it is believed that they are responsible for antiprotozoan activity of bilberry [41,42].

2.2.7. Antiulcer activity

Antiulcer activity of a BE (corresponding to 25% anthocyanidins) was tested *in vivo* in Wistar rats in experimental models of pyloric ligature induced ulcers, ulcers induced with the use of reserpine, phenylbutazone, ulcers caused by restraint and a local application of acetic acid to the gastric mucosa [43]. The results of experiments were compared with the control groups

and groups of rats receiving carbenoxolone and cimetidine [43]. The results of the performed experiments were analyzed by the Mann-Whitney U test or the Dunnett t test [44,45].

3. MATERIAL and METHOD

3.1. Plant Material: Aerial parts from *Vaccinum Myrtillus* (Bilberry) were randomly collected from Artvin, Turkey. Plant materials was harvested at the flowering period in March and April 2018. The taxonomic identification of plant materials was confirmed by a plant taxonomist. Collected plant materials were dried in the shade, then separated from the stem of the plant.

3.2. Preparation of extracts and their solutions

V. Myrtillus young shoots with leaves, fruits were harvested in March and April in Artvin. A dried sample (10 g) was chopped into small pieces using a blender. Metanol extraction was performed in a soxhlet apparatus until the refluxed solvent became colourless. Extraction was followed by filtration through Whatman No 1 filter paper and evaporation of the filtrate to dryness at 30^{o}C in the Büchi V-700 rotary vacuum evaporator. The dry residue was mixed with 150 ml of metanol in a screwcapped Erlenmeyer flask and placed on a Nüve SL 350 shaker (Nüve, Ankara, Turkey) to obtain an metanol extract. Extraction was repeared until the solvent became colourless; 200 ml of metanol was used in total. The combined extracts were filtered through Whatman No 1 filter paper and evaporated to dryness at 40^{o}C in the Büche V-700 rotary vacuum evaporator.

The residue obtained after filtration was left in a dark place at distilled water. This extract was filtered and the filtrate was freezedried in a Labconco 117 freeze-dryer at 5 m Hg and -50^{o}C. The dried samples of all the extracts were stored under nitrogen at 4^{o}C until use. For antioxidant activity measurements, dried extract solutions were prepared by dissolving 20 mg of dried exctract in 20 ml of solvent. Although the same solvent were used for all the assays, concentrations differed from assay to assay as described below. There was no detectable effect of the solvents on any meaured activity, as established by control experiments in which solvents containing no extract were used in the assays [46]. In all cases, three independent experiments, each with duplicate measurements, were performed. The results shown are the means of these measurements.

3.2.1. The 2,2-diphenyl-1-picrylhydrayl (DPPH) Free Radical Scavenging Assay

The antioxidant activity of the plants was evaluated by the 2,2-diphenyl-picrylhydrayl (DDPH) free radical scavenging assay, previously implemented [44]. Briefly, 10 μL of plants were diluted to a final volume 150 μL with methanol (HPLC-grade). Then, 4 mg of DPPH were diluted in 100 mL of methanol to obtain a working solution with an absorbance at 515 nm. Diluted plant s was mixed with 2.85 mL of DPPH and incubated 24 h at room

temperature in the dark. Finally, absorbance at 515 nm was measured in a visible light spectrophotometer. Methanol (HPLC-grade) was used as a blank, and trolox(6-hydroxy-2,5, 7,8-tetramethylchroman-2-carboxylic acid, Sigma) was used for the standard curve. Antioxidant activity was expressed as µg of trolox equivalents per 1 mL of extracts (µg trolox equiv./g DE) [44]. All determinations were performed in triplicate.

3.3.2. Total Phenolic Content

Total phenolic compound contents were determined by the Folin Ciocalteau method [47]. The extract samples (0.5 ml; 1; 10 diluted) were mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with distilled water) for 5 min and aqueous Na_2CO_3 (4 ml, 1 M) were then added. The mixture was allowed to stand for 15 min and the phenols were determined by colorimetric method at 765 nm. The standard curve was prepared using the standard solution of Gallic acid in methanol in the range 20-200µg/ml (R^2=0.987) which is a common reference compound. Total phenolic contents can be calculated from the formula: T = CV/M

Where, T=Total Phenolic concentration C= Concentration of gallic acid from caliberation curve (µg/ml) V= Volume of extract (ml) M= Wt. of extracts of plants [48].

3.3. Antimicrobial Activity

Disk diffusion susceptibility test was applied to determine the antimicrobial activity of plant extracts. From these extracts, 50 µL of these were impregnated to antibiotic disks in aseptic conditions using a micropipette with a diameter of 6 mm (Schleicher & Schül, Nr 2668, Germany). In our study, Mueller Hinton Agar (OXOID) was used to determine the antimicrobial activity of bacteria and yeast as medium. Plaques in which bacteria were inoculated were incubated for 24 hours at 35 ° C and plates inoculated with yeasts were incubated for 3 days at 30 ° C. When the time was over, the diameters of the inhibition zones formed around the disks were measured. The antimicrobial activity experiments against all the test microorganisms were repeated three times.

4. RESULTS and DISCUSSION

Natural products have always been a prefered choice of all as it plays a great role in discovering new medicines. There are many organic compounds which are capable of acting as antioxidants. Many natural substances with an antioxidant effect can protect particularly unsaturated fatty acids against oxidative damage, the process being a crucial step in the development of coronary heart diseases such as stroke and heart attack. The antioxidant compounds from food participate in the removal of reactive oxygen speice that is why a balanced diet rich in natural phenols and other antioxidants is required for the prevention of some lifestyle diseases. Furthermore, natural polyphenols have neuroprotective ability and maintain normal cognitive function in the process of brain ageing [50]Antioxidant activity (DPPH) results were given in Table 1. Standard curve is shown in Figure 1.

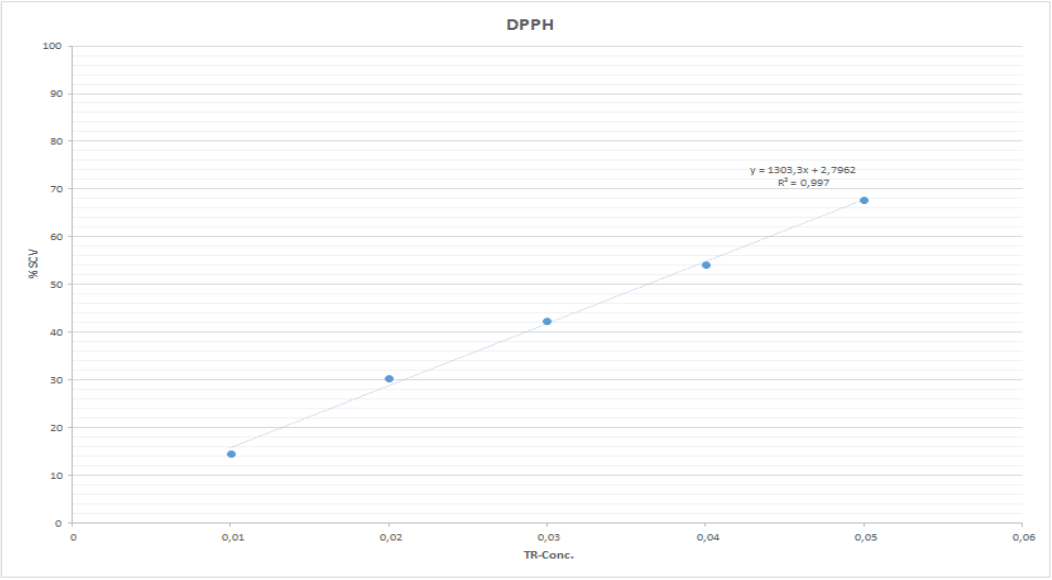

Figure 1. Trolox standard graphic for antioxidant activity(DPPH)

Gallic acid standard curve was used to calculate total phenol contents(Fig 2) and total phenolic content of the samples defined as mMGAE/g(Table 1).

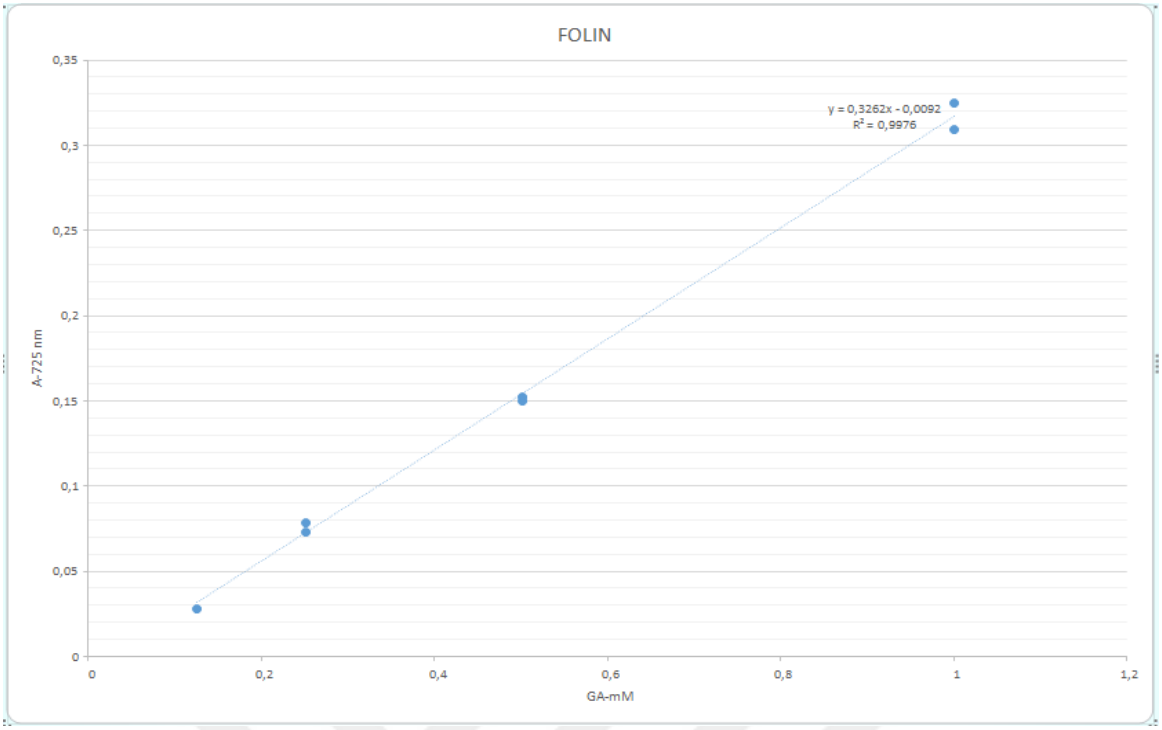

Figure 2. Standard graphic prepared with gallic acid for total phenol content

Table 7. Antioxidan activity(DPPH)µMTE/g, phenolic compound(Folin) mMGAE/g of plant extracts

Plant Extracts	**Antioxidan activity(DPPH)µMTE/g**	**Phenolic compound(Folin) mMGAE/g**
Vaccinum Myrtillus leaves(ETOH)	60.85	1.037
Vaccinum Myrtillus fruits (ETOH)	85.21	0.081
Vaccinum Myrtillus leaves (H_2O)	949.49	0.673
Vaccinum Myrtillus fruits (H_2O)	151.98	0.099

In the present study, the antioxidan activity and phenolic compound of *Vaccinum Myrtillus* extracts were investigated. Many reports had described the chemical constituents of different extracts of *V. Myrtillus* including antioxidan activity and phenolic compounds. In the present study, antioxidan activity of water extract from *V. Myrtillus* leaves revealed 949 µMTE/g. *V. Myrtillus* leaves had the highest antioxidan activity(949 µMTE/g) followed by water extract from *V. Myrtillus* fruits(151.98 µMTE/g), while the lowest value was observed in methanol

extract *from V. Myrtillus*(60.85 μMTE/g) followed leaves followed by methanol extract from *V. Myrtillus* fruits(85.21 μMTE/g).

The phenolic compound of *V. Myrtillus* varied from 0.081 to 1.037 mMGAE/g. The highest phenolic compound was determined by methanol extract from *V. Myrtillus* leaves (1.037 mMGAE/g), and the lowest phenolic compound was found by methanol extract *V. Myrtillus* fruits (0.081 mMGAE/g).

Phenolic compounds such as flavonoids, phenolic acids and tannins are widely distributed in plants and have gained much attention due to their antioxidant activities and free radical scavenging abilities, which potentially have benefit for human health [51].

Results obtained in the present study revealed that level of these phenolic compounds in the various extracts of the *V. Myrtillus* leaves and fruits were considerebly higher in methanol extract than that in other aqueous, and this could be due to different degree of polarity of the solvents used for the extraction of polyphenolic compounds. Morever, the phenolic content of *V. Myrtillus* leaves observed in this study corroborated with the findings of Gardeli *et al*. [52] and Nassar *et al*. [53] on different fractions of this plant. In italy, Giovanelli and Buratti(2009), collected *V. Myrtillus* fruits and made total polyphenols, DPPH analyses. They reported that the *V. Myrtillus* had a high antioxidant capacity [54]. In addition to this, they found that the fruit of *V. Myrtillus*. Patriot showed the highest activity among the all cultivars. It can be said that the similar results were determined from our study for *V. Myrtillus.* According to results of Burdulis et al. (2009) , study, *V. Myrtillus* had high radical scavenging and antioxidant activity [55].

In other study showed that *V. Myrtillus* had high total polyphenols 11.539-20.742 mgGAE/g dry sample. The antioxidant activities found with DPPH, expressed as trolox equivalent antioxidant capacity ranged from 0.143 to 0.297 mmol TEAC/g dry sample [56]. According to the study of Tumbas phenolic compound in bilberry (V. *Myrtillus* L.)observed 494.31 μg/gdry sample [57].

ROS play a role in signal transduction; whereas excessive ROS production lead to oxidative stress which has been involved in the pathophysiology of many cardiovascular diseases such as endothelial dysfunction, atherosclerosis and hypertension [58,59]. .Among all the extracts, water extract from *V. Myrtillus* leaves was shown to possess significant radical scavenging activity against DPPH, evidencing its ability of having extracted a considerable amount of polyphenols and flavonoids with specific structure with many hydroxyl groups. According to many reports, there is a highly positive correlation between polyphenols, flavonoids and

antioxidant activities in many plant species, and this is mainly due to their redox properties, which allowa them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers [60]. Furthermore, they scavenge free radicals and have a metal chelating potential [61].

DPPH radical scavenging methods are common spectrophotometric procedures for determining antioxidant capacities of components, and they are based on the ability of DPPH radical to decolorize in the presence of antioxidants by accepting an elecron or hydrogen donated by an antioxidant compound.Thus, the strong scavenging capacity of *V. Myrtillus* extract as possibly due to the hydrogen/electron donating ability of the polyphenolic compounds present in these extracts, which made them good antioxidants acting as free radical inhibitors or scavengers. Our results is in agreement with that obtained by Hayder et al., who studied the effect of extraction solvent on DPPH scavenging activity of myrtle leaf extract from Tunisia [62].

Myrtle extracts showed potent antioxidant activity mainly due to their richness in phenolic compounds. Therefore, these polyphenols should be considered to contain conjugated ring structures and hydroxyl groups that have the potential fo function as antioxidant in vitro by scavenging free radicals involved in this oxidative processes.

Phenolic compounds are the most abundant and potent antioxidants in plants, there is also a numbe of non-phenolic compounds that contribute to the overall antioxidant activity of plant extracts [63]. The antioxidant activity of phenolic compounds critically depends on the number and position of phenolic hydroxyls in the aromatic ring moieties. Generally, monophenols are less effective than catecholic phenols, and phenolic aglicons have higher antioxidant activity than their respective glycosides [64].

Although the leaves and fruits of *V. Myrtillus* are commonly used in traditional medicine as antihypertensive therapy, the pharmacological evidences of their activity are lacking. Our data reveal that aqueous extract showed a significant antioxidant activity than methanol extract, and this is most possibly due to its high total contents of phenolic compounds.

In general, bluberry fruits have a high antioxidant content. Its fresh and dried fruits are sold in many markets. In addition, leaves of bluberries can be found in markets as tea. According to the results of some previous researches, fruits of blueberries prevent memory lossing and aging since they includes antiageing features [65].

Anthocyanins, phenolic compounds and flavonoids have the ability to neutralize free radiacals. Blueberries contain high amounts of phenolic compounds and have a high antioxidant activity [65].

In the present study, our data highlight the good antioxidant proprieties of different extracts from *V. Myrtillus.*This antioxidant potential is probably attributed to the presence of polyphenolic compounds which may have many benefits in treating exidative stress related diseases. These results lay the ground work for further studies on the molecular mechanisms underlying the biologycal profile of the extracts and isolation and purification of more active principles in each extract as well as clarification of their mode of action.

Tablo 8. Result of antimicrobial activity

Mikroorganims	Leaf H2O extract	Fruit H2O extract	Leaf ETOH extract	Fruit ETOH extract
Escherichia coli ATCC 10536	7 mm	0 mm	7 mm	10 mm
Staphylococcus aureus ATCC 6538	7 mm	0 mm	0 mm	9 mm
Pseudomonas aeruginosa ATCC 15442	0 mm	0 mm	0 mm	7 mm
Bacillus subtilis ATCC 6633	8 mm	0 mm	6 mm	10 mm
Candida albicans ATCC 10231	0 mm	0 mm	0 mm	0 mm
Aspergillus niger ATCC 16404	0 mm	0 mm	0 mm	0 mm

In our study, extracts of leaves and fruits of blueberries (*Vaccinum myrtllus*) prepared with two different solvents, indicated maximum antimicrobial activity on *Escherichia coli* (10 mm) and *Bacillus subtilis* (10 mm). Fruit and leaf extracts were found to be more effective in gram-positive bacteria. This may be since gram-negative bacteria has a multi-layered structure which consists of a lipopolysaccharide layer on the outermost wall of the cell. This structure ensures gram-negative bacteria to be more resistant. In the antimicrobial activity of the plant's ethyl alcohol extracts on the test organisms, fruit extracts showed antimicrobial activity against microorganisms in the range of 7-10 mm and leaf extracts between 6-7 mm. Fruit ethyl alcohol extract indicated antimicrobial activity against *Escherichia coli*, *Bacillus subtilis* (10 mm), *Staphylococcus aureus* (9 mm), *Pseudomonas aeruginosa* (7 mm). No antimicrobial activity was detected on *Candida albicans* and *Aspergillus niger*. An antimicrobial activity was observed in leaf ethyl alcohol extract against *Escherichia coli* (7 mm), *Bacillus subtilis* (6 mm) while no inhibition zone was formed in *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger*.

Today, continuous exposure of people to substances with toxic activity, increase in diseases such as nutrition-related cardiovascular diseases, cancer, and lack of reach to sufficient and

quality food increases the significance of quality nutrition. Striving to produce foods with high nutritional value and long shelf life has also increased the importance of the quality of the produced foods and the ingredients used. Due to the devastating effects of synthetic antimicrobials on the body, the search for natural preservatives that can replace synthetic substances continues swiftly. Blueberry extracts with high antimicrobial activity should be determined through these types of studies and continuity of the studies should be ensured for industrial application by examining their protective effects on food systems.

In this study, the antifungal properties of fruit and leaf extracts are weaker than those of antibacterial properties. It can be asserted that this is due to sterols in the eukaryotic cell membrane. While antimicrobial agents must bind to sterols in the cell membrane to inhibit eukaryotic fungal cells, such binding is not necessary for prokaryotic bacterial cells that do not carry sterols. [66].

In leaf extracts prepared with water, *Bacillus subtilis* (8 mm), *Staphylococcus aureus* and *Escherichia coli* (7 mm) showed antimicrobial activity but *Pseudomonas aeruginosa*, *Candida albicans*, *Aspergillus niger* did not form an inhibition zone. Antimicrobial activity was not observed in fruit extracts. Ethanol extract of dried fruit and leaves of blueberries was found to be very effective against gram positive bacteria Escherichia coli which causes diseases such as urinary tract infections, meningitis, peritonitis and even showed better activity than ampicillin used as standard drug. In a study, it was observed that the natural species of blueberries, dried fruits and dry leaves showed more antimicrobial activity than wet fruits and fresh leaves. Among the extracts examined, the activity of the extract of blueberry dried fruit in ethanol was found to be highest [67].In a study by Burdulis *et al*. (2009), it was found that the antimicrobial activity of the blueberry fruit grown in Lithuania showed the highest activity among the test microorganisms against one of the gram positive bacteria, *Escherichia coli*. [68].

Today, continuous exposure of people to substances with toxic activity, increase in diseases such as nutrition-related cardiovascular diseases, cancer, and lack of reach to sufficient and quality food increases the significance of quality nutrition. Striving to produce foods with high nutritional value and long shelf life has also increased the importance of the quality of the produced foods and the ingredients used. Due to the devastating effects of synthetic antimicrobials on the body, the search for natural preservatives that can replace synthetic substances continues swiftly. Blueberry extracts with high antimicrobial activity should be

determined through these types of studies and continuity of the studies should be ensured for industrial application by examining their protective effects on food systems.

5. CONCLUSIONS

Antimicrobial resistance in bacteria is increasing rapidly. In contrast, bacteria do not gain resistance to plant and plant products that show antimicrobial properties. The reason for this is that synthetically produced medicines are made by isolating any active substance in plants. Bacteria can neutralize medicines by creating resistant breeds against synthetic drugs containing a single structure in time.

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