ACTA MICROBIOLOGICA BULGARICA

In Vitro Antimicrobial and Antioxidant Activity of Extracts from Wild Small Fruits Spread in Bulgaria

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Abstract

The antimicrobial and antifungal activities of the methanol extracts from 5 different wild small berries fruits originating from Bulgaria and collected in 2012 were evaluated against selected Gram-positive and Gram-negative bacteria as well as the fungus *Candida albicans* by agar diffusion method. Microbial growth was measured by the microdilution test. The antioxidant potential of the same extracts was determined by two complementary methods - DPPH-radical scavenging test and superoxide-anion scavenging assay. Different bacterial species exhibit varying sensitivities towards total extracts, but all extracts exhibited a strong antioxidant capacity. No antifungal activity was found in all examined berry extracts.

In general, the wild Bulgarian berry fruits are a good source of antioxidant compounds with defined antimicrobial properties and can be used as a potential value-added ingredient in the food, cosmetic and pharmaceutical industry.

Ключови думи: диви дребни горски плодове, антимикробна активност, антиоксидантна активност

Резюме

Антимикробната и антимикотична активности на метанолни екстракти от 5 различни вида диворастящи дребни горски плодове, събрани през 2012 г. на територията на България, бяха изследвани върху избрани Грам положителни и Грам отрицателни бактерии и фунги от рода *Candida albicans* с помощта на агар-дифузионен метод. Микробният растеж беше измерен с микротитърен тест. Антиоксидантния потенциал беше определен по два комплементарни метода - DPPH-радикал и супероксид-анйон улавяща активност. Различните бактериални видове показаха различна чувствителност спрямо тоталните екстракти, всички от които имаха силна антиоксидантна активност. Не беше установена антимикотична активност при нито един от изследваните екстракти.

В заключение, диворастящите български горски плодове са добър източник на антиоксиданти с дефинирани антимикробни свойства и могат да бъдат използвани като потенциални продукти с висока добавена стойност във фармацевтичната индустрия.

Introduction

Berries are traditional part of the European diet and represent good sources of beneficial biologically active compounds, which may play an important role in the maintenance of the human health (Howell, 2002; Puupponen-Pimia *et al.*, 2001; Rauha *et al.*, 2000). Wild berries are distinguished by a wide spectrum of pharmacologically active substances like vitamins (ascorbic acid, vitamins A and E), phenolic compounds (flavonoids, phenolic acids, lignans, and polymeric tannins), titratable acids, sugars, anthocyanins, essential oils, carotenoids, minerals, fibres etc. (Lee *et al.*, 2012; Nile and Park, 2014; Nohynek *et al.*, 2006; Paredes-Lopez and Valverde, 2012). The beneficial preventive and therapeutic effects of these compounds for human health have been known from at least the 16th century (Cisowska *et al.*, 2011). Nowadays, their antimicrobial, antitumor, antiulcer, antioxidant and anti-inflammatory activities are being intensively

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investigated and documented (Ayachi *et al.*, 2009; Kähkönen *et al.*, 2001; Nakajima *et al.*, 2004; Panico *et al.*, 2009; Ribera *et al.*, 2010; Rufino *et al.*, 2010; Vasco *et al.*, 2008) due to their high potential for use in the food, cosmetic and pharmaceutical industry. Recently, there is an increasing interest to measure the antimicrobial and antioxidant capacity of different berries (Ayachi *et al.*, 2009; Panico *et al.*, 2009; Vasco *et al.*, 2008).

Among the berries, lingonberries, blackcurrant, cloudberries, red raspberries, strawberries, blackberry, and bilberries possess clear antimicrobial effects against various human pathogens. Berry ellagitannins (ETs) are strong antimicrobial agents acting as possible anti-adherence compounds in preventing the colonization and infection by many pathogens (Nile and Park, 2014). Several studies have shown the capacity of berry fruits' extracts to inhibit the growth of food-borne, urinary tract and skin pathogens such as *Salmonella, Enterococcus, Staphylococcus* and *Streptococcus* sp., *E. coli, Mycobacterium phlei* and *Neisseria meningitidis*, as well as pathogenic fungi such as *Candida albicans* (Cavanagh *et al.*, 2003; Toivanen *et al.*, 2011). Lingonberries (*Vaccinium vitis-idaea*) products are most commonly applied for prevention and treatment of urinary tract infections caused by uropathogenic *E. coli* being included in the composition of multiple dietary supplements (Česonienė *et al.*, 2009; Wojnicz *et al.*, 2012). They possessed antimicrobial activity also on a wide range of other human pathogenic bacteria, both Gram-negative (*Salmonella typhimurium*) and Gram-positive (*Enterococcus faecalis, Listeria monocytogenes, Staphylococcus aureus,* and *Bacillus subtilis*) (Česonienė *et al.*, 2009). In addition, purified flavonol glycosides and procyanidins isolated from *V. vitis-idaea* inhibited biofilm formation of *Streptococcus* mutans, a human oral pathogen routinely associated with the formation of human dental caries (Riihinen *et al.*, 2014). Berry juices from *Ribes* species demonstrated antifungal properties against human pathogenic *Candida* species (Krisch *et al.*, 2009). Crude extracts from *Ribes nigrum* fruits displayed antiviral activity against herpes and influenza viruses (Krisch *et al.*, 2009; Roschek *et al.*, 2009). *Rubus idaeus* L. extracts are traditionally used in eastern parts of Europe as herbal remedy in common cold, fever and flu-like infections. Recent studies revealed that young shoots of *R. idaeus* stand out as a valuable source of sanguiin H-6 and ellagic acid, thus possessing antioxidative, antimicrobial and cytotoxic properties (Denev *et al.*, 2014; Krauze-Baranowska *et al.*, 2014).

Phytochemicals such as flavonoids and other phenolics may have antioxidant activity that helps protect cells against the oxidative damage caused by free radicals (Nile and Park, 2014). Due to a high content and wide diversity of phenolic compounds, berries exhibit high biological activity against free radicals, including superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen (Panico *et al.*, 2009) reducing the oxidative damage of human cells (Bazzano *et al.*, 2003). The major contribution to the antioxidant capacity of berries of the genus *Rubus* and *Fragaria* are the ellagitannins, which represent between 51% and 88% of all phenolic compounds depending on the growth and stress conditions (Jimenez-Garcia *et al.*, 2012). Blackberry and strawberry phenolic compounds are known for their protective effects on age-related neurodegenerative diseases and bone loss *in vivo* and can inhibit low-density lipoprotein and liposomal oxidation *in vitro* (Giampieri *et al.*, 2012; Kaume *et al.*, 2012). Strawberry fruits also demonstrate scavenging effect against radicals thus preventing lipid peroxidation in *in vitro* experiments (Giampieri *et al.*, 2012; Ozsahin *et al.*, 2012).

The antimicrobial and antioxidant properties of cultivated plants are usually well documented in difference to the wild plants. However, the literature data indicate that wild berry fruits possess higher concentration of sugar, organic and phenolic compounds than the cultivated and domesticated cultivars of the same species or genus (Bunea *et al.*, 2011; Mikulic-Petkovsek *et al.*, 2012). Differences between investigated cultivars and wild plants are usually based on geographic region, genotype, growing season, maturity, period of harvesting, etc. Giovanelli and Buratti (2009) reported that the antioxidant activity of wild Italian blueberries is much higher as compared to cultivated ones. Unfortunately, the beneficial biological properties of wild small fruits are poorly investigated by now and there are only few scientific reports available about their antimicrobial and antioxidant activities. Further detailed studies are required in order to elucidate their pharmacological potential.

The consumption of small wild berries has been a long time tradition in Bulgaria, especially in the mountain areas where they have been used in folk medicine as water extracts and dried fruits (Dimkov, 2001). It should be noted that the information of the antimicrobial and antioxidant properties of the Bulgarian berries is scare in general. Therefore, the principal objective of this study was to provide data about the *in vitro* antimicrobial and antioxidant activity of the total extracts from 5 different wild berries fruits (blackberries, strawberries, raspberries, lingonberries, and bilberries) naturally growing in Bulgaria.

Material and Methods

Plant Collection

Fruits of blackberries (*Rubus ursinus*), strawberries *(Fragaria vesca)*, raspberries (*Rubus idaeus* L.), lingonberries (*Vaccinium vitis – idaea)* and bilberries (*Vaccinium myrtillus)* were collected in 2012, in west Rhodope mountain (nearby Bozyova GPS: 41°55‘51.73“N; 23°46‘14.98“E; altitude: 1600 m). The samples were freeze-dried, ground and stored at –80°C prior to extraction.

Chemicals

DPPH, nitro blue tetrazolium (NBT), methionine, riboflavin, NaCN, superoxide dismutase (SOD) from bovine erytrocytes and caffeic acid were provided by Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany.

Extraction Procedure

Frozen wild berry fruits were used for preparation of total methanol extracts. The samples of wild berry fruits were placed in 1 L round bottom flasks and lyophilized. Determined standard quantity of lyophilized powder of each sample was moistened with methanol in ratio 1:20, stirred and kept in the dark at a room temperature 24 h. Then a second extraction under the same conditions was carried out.

Individual extracts of wild berry fruits were filtered through a filter paper and both filtrates of each sample were mixed and concentrated under vacuum at 40°C to a defined volume. The concentration of each of filtrates used in the next biological tests was determined and calculated.

Bacterial strains and growth conditions

The test bacteria *Staphylococcus aureus* 209, *Streptococcus pyogenes* 10535, (Gram-positive) and *Escherichia coli* WF+ and *Salmonella typhimurium* 1923, (Gram-negative) were obtained from the Bulgarian Type Culture Collection. All bacteria were cultivated on Tryptic Soy Agar (Difco, USA). *Candida albicans* 562 grown on Sabouraud agar (Difco, USA) was used for screening antifungal activity.

Antibacterial and antifungal activity

Antibacterial and antifungal activity was checked by the agar diffusion method of Spooner and Sykes (Spooner and Sykes, 1972) encountering the diameter of the inhibitory zone in a soft agar layer. Briefly, 0.2 ml of bacterial/fungal suspension (~1.0×10^5 CFU ml^{-1}) was plated on the agar layer into Petri dishes (10 cm d). After preparing 6 wells per dish with a diameter of 9 mm, 0.1 ml of each sample was dropped into each well. The Petri dishes were placed for pre-diffusion at 4°C for 2 h for improvement the sensitivity of the test system. Antimicrobial activity was measured by the diameter of inhibitory zones in the agar layer after 48 h incubation at 37 °C. Extracts giving an inhibitory zone with a diameter (*d*) less than 13 mm indicated a lack of activity. Experiments with methanol as a respective solvent were carried out as negative control. Ceftazidime (Biochemie, Austria) and Nistatin (Actavis, Bulgaria) were used as reference substances (positive control). The assays were performed in triplicates for each berry extract.

Minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of all samples was determined by the microdilution method described by Andrews (Andrews, 2001) by using 96-well standard microtiter plates. Briefly, 50 µl of twofold serial dilutions of examined samples were added to 50 µl of microbial suspension adjusted to yield approximately 1.0×10^5 CFU ml^{-1}. MIC was encountered as the lowest concentration of examined sample that inhibits the visible microbial growth after 24 h incubation at 37 °C. Negative controls were included too.

DPPH radical scavenging assay

The scavenging of DPPH radicals was determined following the procedure described by Kovatcheva-Apostolova et al. (Kovatcheva-Apostolova *et al.*, 2008). Two milliliters of 100 µM DPPH solution were mixed with 0.1 mL solution, containing enhanced concentration of total extract of above mentioned small fruits (from 0 to 120 µg/mL). The inhibition of DPPH radicals (in %) was determined after 5 min by reading the absorbance at 516 nm (Shimadzu 1240 UV/vis spectrophotometer). Inhibition of free radical DPPH in percent was calculated according the formula:

$$\text{Inhibition } \% = (A_{blank} – A_{sample}/A_{blank}) \times 100,$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. Further the IC_{50} values, which represent the necessary concentration of sample for 50% reduction of the absorbance of DPPH radicals, were calculated. Tests were carried out in triplicate. The synthetic antioxidant buthylated hydroxy toluene (BHT) was included in the experiments as positive control.

Superoxide anion-scavenging activity

The inhibition of NBT-reduction by photochemically generated $^{\bullet}O_2^-$ was used to determine the superoxide anion-scavenging activity of the test samples(Beauchamp and Fridovich, 1971). The reaction mixture contained 56 μM (NBT), 0.01 M methionine, 1.17 μM riboflavin, 20 μM NaCN and 0.05 M phosphate buffer pH 7.8. Superoxide was measured by the increasing amount of the absorbance at 560 nm at 30° C after 6 min incubation from the beginning of illumination. The plant extracts and the reference substance caffeic acid were assayed at varying concentrations with three repetitions (inhibition between 20 and 80%). IC_{50} (concentrations, required to inhibit NBT reduction by 50%) values were calculated from dose-inhibition curves. The influence of superoxide dismutase (SOD from bovine erythrocytes, 4870 U/mg) on non-enzymatic reduction of NBT was determined by addition of varying amounts of SOD to the above reaction mixture.

Results

The antimicrobial and antifungal activities of five berry extracts were first measured by agar diffusion which is a suitable for semi-quantitative estimation followed by MIC evaluation as a valuable quantitative and reproducible assay. For agar diffusion and microdilution assays all total extracts from blackberry, strawberry, raspberry bilberry and lingonberry were dissolved in methanol in concentration varying from 225.2 to 264.8 mg/ml. Their antimicrobial and antifungal activities are examined against the Gram-positive bacterial pathogens *S. pyogenes* and *S. aureus*, the Gram-negative enteric pathogens *E. coli* and *S. typhimurium*, and the fungus *C. albicans*. The highest antibacterial activity against the Gram-positive species *S. aureus* and *S. pyogenes* demonstrated the extract from strawberry with inhibitory zones of 17.3±1.2, respectively 20.7±1.1 mm (Table 1). Similar high activity against *S. pyogenes* was found by extracts from raspberry and lingonberry (inhibitory zones: 20.3±0.6, respectively 18.7±1.2 mm). Interesting finding was the antibacterial activity of lingonberry's extract not only against the Gram-positive species *S. pyogenes* (inhibitory zone 18.7±1.2), but against the Gram-negative enteropathogen *S. typhimurium* (inhibitory zone 17.0±1.0 mm). Bacteria of the species *E. coli* were not inhibited by any of the tested extract in difference to the applied positive control ceftazidime (34.3±3.1). Similarly, the antifungal drug nistatin was applied against the fungus *C. albicans* (Table 1). No antifungal activity was found in all examined berry extracts.

Total methanol extracts of strawberry, raspberry, and lingonberry were subjected also to broth microdilution assay. Only the Gram-positive bacteria (*S. aureus* and *S. pyogenes*) were inhibited by the extract of strawberry in a concentration of 15 mg ml^{-1} (Table 1). A weaker activity was found for the extracts of raspberry and lingonberry against *S. pyogenes* in concentrations of 28.1 mg ml^{-1}, respectively 33.1 mg ml^{-1}. No activity was found against the Gram-positive *S. aureus* and the Gram-negative pathogens *E. coli* and *S. typhimurium,* as well as against *C. albicans.*

The antioxidant potential of total extracts was determined by two complementary methods. The free radical scavenging activity of all extracts,

Table 1. Antimicrobial activity of methanol berry extracts

Wild berry / Test pathogen	Inhibitory zone of methanol berry extracts					MIC* of methanol berry extracts	
	S. aureus	*S. pyogenes*	*E. coli*	*S. typhimurium*	*C. albicans*	*S. aureus*	*S. pyogenes*
Blackberry	15,5±1	14,6±1,2	13,5±1	12,2±1,4	11,8±1,2	0.0	0.0
Strawberry	17.3±1.2*	20.7±1.1	11,6±0,6	10,8±0,8	11,2±1	15.0§	15.0
Raspberry	10,6±0,6	20.3±0.6	12,4±0,8	11,4±1	11,0±0,8	0.0	28.1
Lingonberry	12,1±1	18.7±1.2	13,0±1,2	17.0±1.0	12,4±1	0.0	33.1
Bilberry	13,2±1,4	12,4±1	12,8±1,0	12,0±1,0	11,8±0,8	0.0	0.0
Methanol	0.0	0.0	0.0	0.0	0.0	n.t.	n.t.
Ceftazidime	29.3±2.5	27.0±2.0	34.3±3.1	37.0±3.6	0.0	n.t.	n.t.
Nistatin	n.t.	n.t.	n.t.	n.t.	38.5±5.0	n.t.	n.t.

Legend: * - minimal inhibitory concentration (MIC); & - inhibitory zone (mm); § - MIC (mg ml^{-1}); n.t. – not tested.

evaluated by the DPPH method, is presented in Fig. 1. All extracts tested exhibited a strong antioxidant capacity. They were able to reduce the stable free radical DPPH to yellow-colored diphenylpicrylhydrazine. The extracts can be divided into two groups according to the detected inhibitory activity. Strawberry, lingonberry and bilberry, demonstrated better radical scavenging activity than the blackberry and raspberry. The extracts from first group showed 100% inhibitions in variants with concentration above 80 μg/ml. IC_{50} values in DPPH method were calculated: blackberry - 91 μg/ml; strawberry - 56 μg/ml; raspberry - 91 μg/ml; lingonberry - 57 μg/ml, and bilberry - 52 μg/ml. The most promising antioxidant in terms of DPPH radicals was E5 (bilberry).

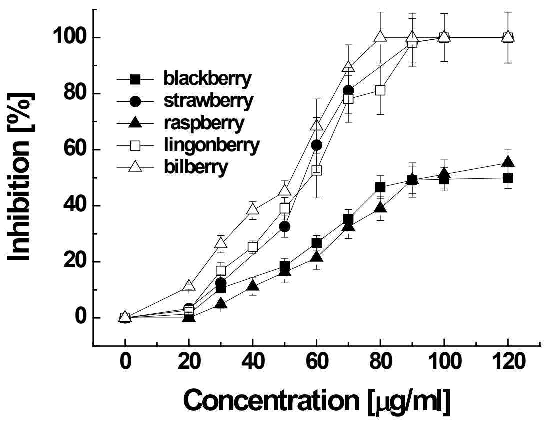

Fig. 1. Free radical scavenging effect of the total extracts from blackberry (■), strawberry (●), raspberry (▲), lingonberry (□), and bilberry (△) (DPPH assay). Error bars represent standard deviations.

Further, the antioxidant potential of total extracts was evaluated by suppression of the superoxide anion radicals generated in a photochemical system in the presence of the test samples. The results are shown in Fig. 2. All of the investigated extracts in concentration 50 μg/ml showed $^{\bullet}O_2^-$ radical scavenging activity. The order of scavenging effectiveness was: bilberry (62.5% inhibition) ≥ strawberry (56.3%) ≥ raspberry (40%) ≥ lingonberry (36.8) ≥ blackberry (25.5). These results were lower than those of positive controls, caffeic acid and commercial SOD, which were used in concentrations 6.5 and 3.5 μg/mL, respectively (data not shown).

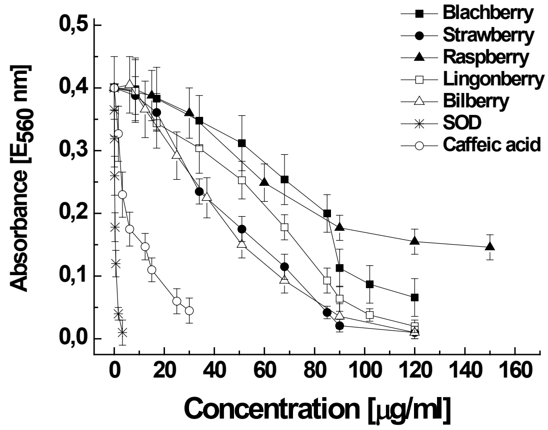

Fig. 2. Inhibitory effect of total extracts on the reduction of NBT by photochemically generated superoxide anion radicals. Samples contained 50 μg/mL of the corresponding preparations. Control does not contain any extract. Error bars represent standard deviations.

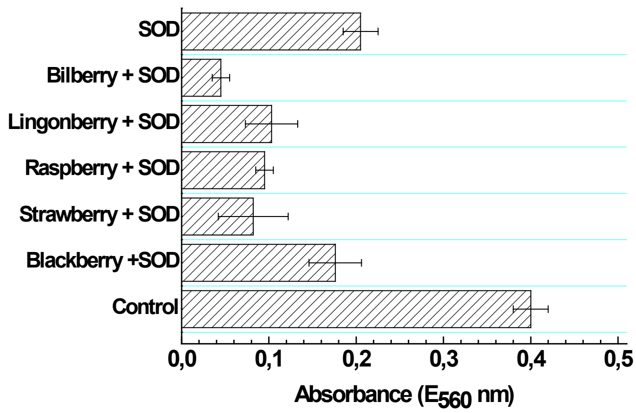

Fig. 3. Dose-dependence of the superoxide anion scavenging effect of the total extracts from blackberry (■), strawberry (●), raspberry (▲), lingonberry (□), and bilberry (△). Caffeic acid (○) and SOD (∗) were used as positive controls. Commercial SOD from bovine erythrocytes with 4870 U/mg protein and 4870 U/mg solid was used; the working solution was prepared as 1 mg/mL. Error bars represent standard deviations.

For a detailed study of radical scavenging capacity, we investigated the $O_2^{-\bullet}$ scavenging activities of samples at different concentrations, and dose-response curves were formed (Fig. 3). Caffeic acid and commercial SOD were used as positive controls. The results indicated that the best scavenging activity among tested extracts demonstrated E2 (strawberry) and E5 (bilberry). It is worthy to note that all extracts exhibited less scavenging activity than SOD and caffeic acid.

The 50% $^{\bullet}O_2^-$ scavenging concentrations (IC_{50}) of the total extracts were calculated from the dose-activity curves. The IC_{50} were found to be 85, 43.9, 80.5, 63 and 41.8 µg/ml for blackberry, strawberry, raspberry, lingonberry, and bilberry, respectively. As positive controls, 50% inhibitory concentrations of SOD and caffeic acid were detected as 1.04 and 4.9 µg/mL, respectively.

Further we studied the combined $^{\bullet}O_2^-$ scavenging effect of the fruit preparations in the dose 50 µg/mL, applied together with 5.5 U/mL of commercial SOD (Fig. 4). The combination resulted in 50% and 70% decrease in NBT-reduction respectively, compared to the inhibition caused by SOD alone. The results outlined the possibilities to use these alone or in combination with SOD effectively in cases, when the cells are exposed to oxidative stress.

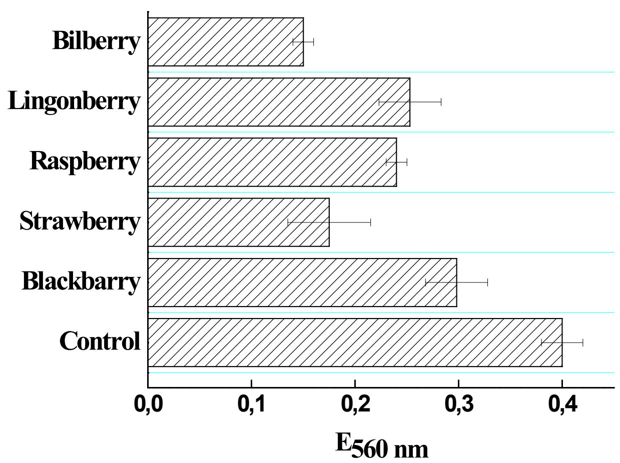

Fig. 4. Inhibitory effect of the total extracts on the NBT-reduction by photochemically generated superoxide anions in the presence of SOD. Samples contained 5.5 U/mL SOD from bovine erythrocytes and 50 µg/mL of the corresponding preparation. Control does not contain any extract. Error bars represent standard deviations.

Discussion

The recent study provides data on antibacterial and antioxidant activities of the most popular wild small berries naturally growing in Bulgaria like blackberries, strawberries, raspberries, lingonberries, and bilberries. We determined the antibacterial activity of crude methanol extracts against selected bacterial pathogens including also the fungus *C. albicans*. In general, all berry extracts tested were found to inhibit the growth of Gram-positive *S. aureus* and *S. pyogenes* but not the Gram-negative species, excepting the extract of lingonberries which was active against the virulent serovar *S. typhimurium*. The extracts were also more active against *S. pyogenes* in comparison with *S. aureus*. Notably, the strawberry extract was found to be highly active against both *S. aureus* and *S. pyogenes*. Highest activity against *S. pyogenes* showed the strawberry and raspberry extracts followed by the lingonberry extract. A decrease in the antimicrobial activity against *S. pyogenes* as compared to the untreated control was observed in the following order: strawberry > raspberry > lingonberry > blackberry > bilberry. As far as the various compounds available in the extract should be attributed to antimicrobial activities additional studies are required. Studies on Nordic berries reveal that strawberry extract contains ETs which may explain their moderate antimicrobial effects against *Staphylococcus* and *Salmonella* bacteria (Puupponen-Pimia *et al.*, 2005). Ellagitannins were detected in berries from the *Rosaceae* family (cloudberry, raspberry, rose hip, sea buckthorn, and strawberry), ranging from 21.7 to 83.2 mg/100 g (Giampieri *et al.*, 2012). Other studies discussed in the same review reported ET content in strawberries from 25 to 59 mg/100 g in fresh samples. The representative ET in strawberries and raspberries is sanguiin H-6. Other investigators have reported the presence of galloylbishexahydroxydiphenoyl-glucose, previously found in the *Rubus* berry (Mullen *et al.*, 2003). These findings support the results obtained in our study for the moderate to high activity of the strawberry extract in *S. aureus* and *S. pyogenes* (important causative agents of skin lesions) and of the raspberry extract against *S. pyogenes*. Different mechanisms of action proposed to explain tannin antimicrobial activities including inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation (Puupponen-Pimia *et al.*, 2005). The same authors found that freeze-dried lingonberry showed moderate inhibitory activity against *Salmonella* similarly to our finding with crude methanol extract against *S. typhimurium.* The antimicrobial properties of lingonberry juice are known for a long time. Moderate effect of lingonberry extract against *E. coli* was observed in our study too. *V. vitis-idaea* fruits' extracts have been found to inhibit the growth of uropathogenic *Escherichia coli* rods by influencing the virulence factors expression and biofilm formation (Česonienė *et al.*, 2009; Wojnicz *et al.*, 2012). The effect should be associated with inhibition of bacterial adherence to mucosal surfaces (Schmidt and Sobota, 1988). Evidently anti-adhesion might be one mechanism in action in antimicrobial activity of berry compounds in general but the mode of action of tannins probably depends on the individual microorganism.

The berry phenolics attribute to many diverse biological functions. In parallel to their antimicrobial and antifungal activities, they provide also ultraviolet radiation protection, chelation of toxic heavy metals, and antioxidant quenching of free radicals generated during photosynthesis. Anthocyanins, catechin, ellagic acid, gallic acid and quercetin are potent antioxidants with possible health effects in humans due to their reported positive effect on blood vessels, as well as hepatoprotective, antirheumatic and anticancer activities (Lee *et al.*, 2012; Nile and Park, 2014). All these observations provoked us to investigate the capacity of those 5 total extracts to scavenge the reactive oxygen species (ROS). Generally, ROS in the body are generated by exogenous agents (e.g., radiation, cigarette smoke, atmospheric pollutants, toxic chemicals, over nutrition, changing food habits, etc.) and/or endogenous sources (inflammation, mitochondria, *cytochrome P-450 metabolism, microsomes, and peroxisomes) (Pham-Huy et al., 2008)*. When ROS level reaches above threshold, an imbalance between oxidants and antioxidants take place in the cells. This situation results in oxidative stress that causes various degenerative diseases (Fridovich, 1998). In this context, antioxidants may play an important role in preventing and repairing ROS-induced damages by scavenging free radicals and repairing the enzymes involved in the process of cellular development. Present investigations confirmed the antioxidant potential of all tested extracts. The scavenging of the stable free radical 1,1-Diphenyl-2-picrylhydrazyl by the DPPH method gave reliable information concerning the antioxidant activity defined as the mean of free radical scavenging capacity (Fig. 1). The highest *in vitro* antioxidant capacity was determined for bilberry extract. A decrease in intracellular oxidation compared to control was observed in the following order: bilberry > strawberry > lingonberry > blackberry = raspberry. High scavenging capacity was also found for juices of chokeberry, blueberry and bilberry (Slatnar *et al.*, 2012) and extracts from strawberry, blackberry and raspberry leaves (Buřičová *et al.*, 2011). In small fruits (Panico *et al.*, 2009) the antioxidant capacity has been correlated to a significant degree with anthocyanin content. Moreover, phenolic phytochemicals with antioxidant properties are now believed to be an important component in fruits and vegetables responsible for beneficial health effects (Moyer *et al.*, 2001).

Further investigation of the total extracts was performed in a non-enzymatic system - NBT, methionine and riboflavin. There $^{\bullet}O_2^-$ were generated photochemically and SOD inhibited the reduction of NBT. Although the superoxide anion radical is a weak oxidant, it gives rise to the generation of more powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to the oxidative stress (Fridovich, 1998). Our results showed that the best $^{\bullet}O_2^-$ scavenging activity was determined by the bilberry extract, followed by the strawberry extract (Fig. 2). These preparations inhibited the development of the color, produced during the reaction of $^{\bullet}O_2^-$ with NBT by 62 and 56% respectively. On the other hand, the lowest activity showed blackberry and lingonberry. Moreover, the samples of total extracts suppressed $^{\bullet}O_2^-$ release in a dose-dependent manner (Fig. 3). The 50% $^{\bullet}O_2^-$ scavenging concentrations were between 85.0 and 41.8 μg/ml respectively. IC_{50} values of bilberry (41.8 μg/ml) and strawberry (43.9 μg/ml) demonstrated better inhibition of superoxide level compared to the other extracts. These values were comparable to the total extracts from other berries (Kim *et al.*, 2009; Palíková *et al.*, 2008).

The $^{\bullet}O_2^-$ scavenging effect of the extracts, obtained from wild Bulgarian berries was confirmed by measurement of inhibition of NBT-reduction in presence of commercial SOD (Fig. 4). The combination of SOD (5.5 U/mL) with the total extracts (50 μg/ml) resulted in 50% and 70% decrease in NBT-reduction respectively, compared to the inhibition by SOD alone. These results demonstrated synergistic effect between the antioxidant enzyme and the tested preparations suggesting SOD-like activity of the extracts from berries.

The obtained results show that the wild Bulgarian berry fruits are a good source of antioxidant compounds and can be used as a potential value-added ingredient in the food, cosmetic and in the pharmaceutical industry.

Conclusion

The results from the present experiments demonstrated that the total extracts from wild Bulgarian small berry fruits manifested strong antioxidant activities in model systems and moderate to high activity against the Gram-positive pathogens *S. aureus* and *S. pyogenes*, as well as the fungus *C. albicans*. The *in vitro* antioxidant properties of these preparations might be contributing to the overall antimicrobial effect on the selected bacterial pathogens. The demonstrated antibacterial activity is a good prerequisite for further studies on skin infection model systems *in vitro* and *in vivo*.

Acknowledgments

We are acknowledged Iva Tzvetkova for the excellent technical assistance. This study was supported by a research grant DO02-334/08 from the Bulgarian Scientific Fund.

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