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Original article

Antimicrobial Activity Evaluation of Black Currant (*Ribes nigrum L.*) Variety Čačanska Crna Juice and Extract

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SUMMARY

Aim. This study aimed to evaluate and quantify the antimicrobial activity of lyophilized fruit juice (BCLJ) and waste extract (BCLW) obtained from the black currant (*Ribes nigrum L.*) variety Čačanska crna.
Materials and method. The study was conducted using four Gram (+) (*Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*) and five Gram (-) bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Proteus mirabilis, Enterobacter aerogenes*) as well as one yeast (*Candida albicans*). Cyanidin-3-O-glucoside, delphinidin-3-O-rutinoside and delphinidin-3-O-glucoside present in black currant were used as standards, so the second aim was to determine their influence on the total antimicrobial activity.
Results. The tested samples showed moderate antimicrobial activity. The inhibitory effect of BCLJ was shown on all Gram (+) bacteria (*B. cereus*, *E. faecalis*, *S.aureus*), apart from *L. monocytogenes*, for which the extracts were not effective. It was noted that BCLJ did not suppress the growth of Gram (-) bacteria. Black currant waste extract on the other hand was shown to be efficient on Gram (+) as well as on Gram (-) bacteria. The results of minimum inhibitory concentrations MIC (MFC) of BCLJ and BCLW were 100 mg/mL and MBC was higher than 100 mg. The MIC/MBC (MFC) of standards were 0.13 - 0.5 mg/mL.
Conclusion. Results indicate that these black currant lyophilizates might be potentially used as antimicrobial agents.

Keywords: **black currant, berries, antimicrobial activity, anthocyans**

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INTRODUCTION

Berries are one of the most commonly used plant foods rich in antioxidants. Black currant (*Ribes nigrum L.*) belongs to the Ribes L. genus and the family Grossulariaceae. The plant itself is a small deciduous shrub that grows 1 – 2 m tall. The cultivation zones are more prominent in northern temperate Europe and parts of North America and Northern Asia, as well as mountain regions of South America and North Africa (1).

Black currant is a significant source of bioactive compounds such as anthocyanins, flavonols (myricetin, isorhamnetin, quercetin), phenolic acids, proanthocyanidins, as well as carotenoids and vitamin C (2, 3). The biological and chemical properties of black currants are found to be dependent on the cultivar and scion age of the plant (4). Black currants predominantly contain anthocyanins as well as phenolic acids and flavonols (5, 6). Anthocyanins can be found in the stem, leaves, and storage organs of plants. Anthocyanins can be present in the form of glycoside, in which one or more saccharides are bonded with the aglycone structure, as well as the aglycone anthocyanin itself (7, 8). According to Nour et al., nine individual anthocyanins in ethanolic extracts of black currants were found, such as cyanidin-3-rutinoside, cyanidin-3-glucoside, delphinidin-3-glucoside, delphinidin-3-rutinoside, pelargonidin-3-rutinoside, peonidin-3-O-rutinoside, petunidin-3-rutinoside, petunidin-3-(6-coumaroyl)-glucoside, and cyanidin-3-(6-coumaroyl) -glucoside (9). The most prominent were 3-O-glucosides and the 3-O-rutinosides of delphinidin and cyanidin (10, 11). Results of the study conducted by Slimestad and Solheim (2002) showed the presence of fifteen anthocyanin structures. Here, a glucoside cyanidin-3-O-arabinosid was found along with already mentioned 3-O-glucosides and the 3-O-rutinosides of delphinidin and cyanidin. These antioxidants are correlated with the genotype and ripeness of the fruits (12).

Considering the preventive role of berries regarding oxidative stress, black currants were found to help prevent hypertension and other cardiovascular-associated illnesses as well as neoplasms and neurodegenerative diseases (6). Black currant preparations were found to be helpful in improving serum lipid profile since an elevation of HDL cholesterol level was noted, as well as reducing triglycerides and total cholesterol (13). Juices obtained from berries such as black currant, cranberry and raspberry were shown to significantly decrease the proliferation rate of certain cancer cell lines (14, 15). Also, the antimicrobial action of berry extracts has been thoroughly investigated, since berry phenols were found to selectively inhibit the growth of different human pathogenic bacteria. Thus, these constituents act as bacteriostatic agents, but the potential for bactericidic effect has not yet been proven (9, 16, 17).

Therefore, the purpose of this study was to evaluate and quantify the antimicrobial activity of lyophilized waste extract and lyophilized juice of black currant (*R. nigrum L.*) variety Čačanska crna on four G(+) and five G(-) bacteria, as well as on one yeast (*Candida albicans*). Also, the activity of standard compounds (delphinidine-3-O-rutinoside, delphinidine-3-O-glucoside and cyanidin-3-O-glucoside), dominant anthocyanins in black currants, was evaluated under the same conditions to evaluate their antimicrobial effect and see if antimicrobial activity stem from these compounds. The results would contribute to the food industry since black currant extracts might have an important role as potential natural antimicrobial agents.

MATERIALS AND METHODS

Plant material and sample preparation

Black currant variety Čačanska crna was bred and harvested from Radmilovac, an experimental field of the Faculty of Agriculture University of Belgrade. Berries at their full ripeness were collected from the end of June until the beginning of July 2020. After being collected, the fruits were stored frozen in the freezer, until use. At the beginning, the fruits were defrosted and pressed for squeezing fruit to obtain the juice for further analysis. The waste (residue after straining) was air-dried on filter paper for 24 hours and was subsequently dried in a laboratory dryer (Instrumentaria ST 01/02, Zagreb, Croatia) at 40°C for 48 hours. The waste was then grinded in a mill to fine powder. In order to extract the plant material, the method used was maceration. For this purpose, 60% ethanol was used, with a material to solvent ratio of 1:20. The extraction of the sample was performed using a laboratory shaker. Extraction was done at room temperature and lasted one hour. The ethanol was removed from the sample in a rotary evaporator (Büchi CH, Switzerland). The obtained samples of juice and waste were used for lyophilization. Extracts were firstly frozen at -80° and then lyophilized at -60°C (at a pressure of 0.011 mbar) for 24 hours and at -60°C (at a pressure of 0.0012 mbar) for an additional hour, to remove the capillary water residues.

Antimicrobial activity evaluation

The evaluation of the antimicrobial activity of black currant Čačanska crna lyophilized fruit juice (BCLJ) and black currant Čačanska crna lyophilized waste (BCLW) was done via standardized bacterial isolates produced by the American Type Culture Collection (ATCC). For the needs of our investigations, the Gram (+) bacteria included *Staphylococcus aureus* ATCC 6538, *Bacillus cereus* ATCC 10876, *Listeria monocytogenes* ATCC 15313 and *Enterococcus faecalis* ATCC 19433. The Gram (−) bacteria included *Salmonella enteritidis* ATCC 13076, *Proteus mirabilis* ATCC, *Escherichia coli* ATCC 25922, Pseudomonas aeruginosa ATCC 27853, 12453, and *Enterobacter aerogenes* ATCC 13048. Evaluation of antifungal activity was done using *Candida albicans* ATCC 15313 yeast.

Both bacterial and yeast populations of microorganisms were obtained from overnight nutrient media used to grow bacteria. The microorganism suspensions were set to 0.5 turbidity of McFarland standard (optical density comparable to 10^7 - 10^8 colony forming units (CFU/mL) for bacteria depending on genera and 0.4×10^5 to 5×10^5 spore/mL for fungal strains, standardized following the National Committee for Clinical Laboratory Standards (NCCLS)) (18). The minimum inhibitory concentration (MIC) and minimum bactericidal/ fungicidal concentration (MBC/MFC) were evaluated using the microwell dilution method, recommended by NCCLS (2003). The samples were dissolved in 10% dimethyl sulfoxide (DMSO). The final concentrations of the standard substances delphinidine-3-O-rutinoside (D3R), delphinidine-3-O-glucoside (D3G) and cyanidin-3-O-glucoside (C3G) were 1 mg/mL, 0.6 mg/mL and 0.5 mg/mL, respectively, in 10% DMSO. Each bacterial suspension in the adequate growth medium was added to the wells of a sterile 96-well microtiter plate and the samples were serially diluted with nutrient broth. In each well, the final volume was 100 μL, whilst the final bacterial concentration was 2×10^6 CFU/mL and fungal 2×10^5. The bacterial microtiter plates were then incubated for 24 h at 37^0C in dark, and the yeast microtiter plates were incubated for 48 h at 25^0C. In order to evaluate microbial growth, 20 μL of 0.5% triphenyltetrazoliumchloride aqueous solution was added (19). The minimal inhibitory concentration (MIC) was defined as the minimal concentration of samples/standards needed to inhibit visible *in vitro* microbial growth. On the other hand, the minimal bactericidal/fungicidal concentration (MBC/MFC) was defined as the lowest concentration of samples/ standards that showed efficacy to eliminate 99.9% of microbial cells. The evaluation of MBC/MFC was done by taking the liquid medium from each well and inoculating it into Mueller Hinton agar at room temperature for 24 h for bacteria. Fungi growth inhibition was tested using malt extract agar at 25^0C for 48 h (18). The antibacterial drug doxycycline and the antifungal drug nystatin were used to compare the antimicrobial activity.

RESULTS AND DISCUSSION

The results that indicate the antimicrobial activity of black currant Čačanska crna lyophilized fruit juice and waste and standards against selected microbes are shown in Table 1. Results showed that the MIC of BCLJ and BCLW against pathogenic bacteria was moderate. Antifungal activity was noted amongst all samples and standards. The inhibitory effect of BCLJ was shown on all Gram (+) bacteria (*B. cereus*, *E. faecalis*, *S. aureus*), apart from *L. monocytogenes*, for which the extracts were not effective. It was noted that BCLJ did not suppress the growth of Gram (-) bacteria (*P. mirabilis*, *P. aeruginosa, S. enteritidis*, *E. aerogenes* and *E. coli*). The results are in line with the data obtained by Puupponen-Pimiä et al. (11). This researcher evaluated lyophilized black currant berry extract that did not suppress the growth of *L. monocytogenes* (11).

Black currant waste, on the other hand, was shown to be efficient on Gram (+) as well as Gram (-) bacteria. The minimum inhibitory concentrations, MIC (MFC) of BCLJ and BCLW were 100 mg/ml, respectively. More powerful antimicrobial effects were expressed by BCWL extracts compared to BCLJ, especially since BCWL inhibited the growth of Gram (-) bacteria, which was not shown by BCLJ. The best MIC exhibited the BCLW against *S. aureus* and *C. albicans* (25 mg/mL).

Table 1. *Antimicrobial activity of blackcurrant lyophilized juice and waste and standard anthocyanins against the investigated pathogenic microbes (MIC/MBC in mg/ml)*

Microbial strains		Sources ATCC	BCLJ	BCLW	D3R (1mg/mL)	D3G (0.6 mg/mL)	C3G (0.5 mg/mL)	Doxycyclin
			MIC/MBC (mg/ml)	MIC/MBC (mg/ml)	MIC/MBC (mg/ml)	MIC/MBC (mg/ml)	MIC/MBC (mg/ml)	MIC/MBC (µg/mL)
Gram (+) bacteria	*Staphylococcus aureus*	*6538*	100.0/>100.0	25.0/>100.0	0.25/>0.5	0.15/>0.3	0.13/>0.25	7.81/15.61
	Enterococcus faecalis	19433	100.0/>100.0	100.0/>100.0	0.25/>0.5	0.15/>0.3	0.25/>0.25	0.90/1.90
	Bacillus cereus	10876	100.0/>100.0	100.0/>100.0	0.5/>0.5	0.3/>0.3	0.25/>0.25	0.90/15.61
	Listeria monocytogenes	15313	na	100.0/>100.0	0.5/>0.5	0.3/>0.3	0.25/>0.25	7.81/15.61
Gram (-) bacteria	*Proteus mirabilis*	12453	na	100.0/>100.0	na	na	0.25/>0.25	7.81/15.61
	Salmonella enteritidis	13076	na	100.0/>100.0	0.5/>0.5	0.3/>0.3	0.13/>0.25	0.90/1.90
	Enterobacter aerogenes	13048	na	100.0/>100.0	0.5/>0.5	0.3/>0.3	0.25/>0.25	7.81/15.61
	Escherichia coli	25922	na	100.0/>100.0	0.5/>0.5	0.3/>0.3	0.25/>0.25	15.61/15.61
	Pseudomonas aeruginosa	27853	na	100.0/>100.0	na	na	na	15.61/15.61
Yeast	*Candida albicans*	15313	100.0/>100.0	25.0/>100.0	0.13/>0.5	0.08/>0.3	0.25/>0.25	na

na* - no activity

The antimicrobial activity of berry phenols was investigated on various bacteria, fungi and viruses (20, 21). Berry extracts with a high content of polyphenols were shown to act inhibitory to bacteria like *Salmonella, Escherichia, Staphylococcus, Helicobacter, Bacillus, Clostridium* and *Campylobacter*, and also to *S. aureus* strains. In comparison to other berry extracts, black currant had more potent antibacterial properties among several tested microorganisms (22, 23). The consequences of microbiological contamination of food and herbal preparations can be severe and they represent a significant risk to public health (24).

Bacterial contamination with *Salmonella* can be introduced through a variety of food such as chicken, beef, pork, eggs, fruits, vegetables, or cutting boards and utensils used for raw meat (11, 25). *Salmonella* contamination can be responsible for gastroenteritis and enterocolitis (26). *Pseudomonas* is one of the most diverse contaminants of water, soil and marine environments, thus also found in cosmetics, and medical products (27). It affects the quality of raw milk on prolonged storage, but data also shows that it can be found in pasteurized milk, as well, because of the production of thermotolerant lipolytic and proteolytic enzymes (28, 29). *Listeria* is the bacteria that can contaminate deli meats, hot dogs, and dairy products such as soft cheeses, but also celery, sprouts, and ice cream (30). Microbial contamination can occur during transport, storage, and marketing. Synthetic fungicides and antibiotics were developed to prevent microbial impact, however, their excessive use led to the development of resistant strains of microbial species. The significance of alternative antimicrobial agents is growing and various therapeutics are being used against a panel of microorganisms (31).

In a research conducted by Miladinović et al., there was no significant difference in the antimicrobial effect of the four black currant juices against Gram (+) and Gram (-) bacteria. Methanolic extracts of the black currants varieties exhibited their activity in lower concentrations than juices (9).

Another study examined the antimicrobial effects of black currant juice on *E. faecalis*, which was even shown to be higher than the effects of chlorhexidine, a widely used and powerful antiseptic. The black currant juice expressed complete suppression of the species *E. faecalis*. Black currant juice also showed lower cytotoxicity than chlorhexidine, which can be a result of the high polyphenolic content present in black currants (16).

Our study determined the antimicrobial activity of black currant anthocyanins C3G, D3R and D3G, as standard substances. The standards expressed significant antimicrobial activity at lower concentrations than the tested samples. MIC/MBC (MFC) of standards were 0.08/> 0.5 mg/mL. None of the standard substances were effective on Gram (-) bacteria *P. aeruginosa*, while the inhibition of *P. mirabilis* was expressed only by C3G, and MIC/MBC was 0.25/> 0.25 mg/mL. This might lead to the conclusion that the antimicrobial effect against *P. mirabilis* might stem from this compound. A potent antimicrobial activity of C3G was especially noted regarding *S. enteritidis* growth, where the MIC/MBC were 0.13/> 0.25 mg/mL. All standards were efficient in inhibiting Gram (+) bacteria, and the lowest MIC/MBC was 0.13/> 0.25 mg/mL, which was a result of C3G effectiveness against *S. aureus*. Antimicrobial effects of D3G have already been described against Gram (-) bacteria.

E. coli was shown to be the most resistant to treatment with anthocyanin-rich extracts (32, 33). The results of our study show that there is a moderate activity to *E. coli*, which makes the significance of the investigated anthocyanins even higher and may suggest a causal role in inhibiting this particular bacteria. Antifungal activity was more prominent observing standard solutions, as D3G had the lowest MIC value of 0.08/> 0.3 mg/mL, which made it more potent than the BCLJ and BCLW.

CONCLUSION

The results of our study showed that the BCLJ and BCLW expressed moderate antibacterial effects. The observed samples inhibit Gram (+) bacteria at the same MIC and MBC values. The BCLJ did not suppress the growth of Gram (-) bacteria. As for the standards, *P. aeruginosa* was not inhibited by any of the three standard substances, while *P. mirabilis* was inhibited only by cyanidin-3-O-glucoside. Also, delphinidin-3-O-glucoside had MIC value of 0.08/> 0.3 mg/mL against the yeast *Candida albicans*. Further investigation is required to establish the gained *in vitro* effects and to determine whether they could be transferred to the *in vivo* systems. Black currants have been found to be effective antimicrobial agents, which might be useful against rising antimicrobial resistance. Using black currants and their extracts as natural food additives could prevent microbial contamination.

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Procena antimikrobne aktivnosti soka i ekstrakta crne ribizle (*Ribes nigrum L.*) sorte Čačanska crna

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SAŽETAK

Cilj. Ovo istraživanje je imalo za cilj da proceni i kvantifikuje antimikrobnu aktivnost liofilizovanog voćnog soka (BCLJ) i ekstrakta ostalog nakon ceđenja soka (BCLW) dobijenog od crne ribizle (*Ribes nigrum L.*) sorte Čačanska crna.
Materijal i metoda. Studija je sprovedena korišćenjem četiri Gram (+) bakterije (*Bacillus cereus*, *Listeria monocitogenes*, *Staphilococcus aureus*, *Enterococcus faecalis*) i pet Gram (-) bakterija (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Proteus mirabilis*, *Enterobacter aerogenes*), kao i jedne gljive (*Candida albicans*). Kao standardi korišćeni su cijanidin-3-O-glukozid, delfinidin-3-O-rutinozid i delfinidin-3-O-glukozid prisutni u crnoj ribizli, pa je drugi cilj bio da se utvrdi njihov uticaj na ukupnu antimikrobnu aktivnost.
Rezultati. Ispitani uzorci su pokazali umerenu antimikrobnu aktivnost. Inhibitorni efekat BCLJ pokazao se na sve Gram (+) bakterije (*B. cereus*, *E. faecalis*, *S. aureus*), osim na L. monocitogenes, za koje ekstrakti nisu bili efikasni. Primećeno je da BCLJ nije delovao na rast Gram (-) bakterija. Ekstrakt ostatka crne ribizle, s druge strane, pokazao se efikasnim na Gram (+), kao i na Gram (-) bakterije. Rezultati minimalnih inhibitornih koncentracija (MIC (MFC) BCLJ i BCLW bili su 100 mg/mL, a rezultati minimalnih baktericidnih koncentracija (MBC) bili su veći od 100 mg. MIC/MBC (MFC) standarda bio je 0,13 – 0,5 mg/mL.
Zaključak. Rezultati pokazuju da se ovi liofilizati crne ribizle mogu potencijalno koristiti kao antimikrobni agensi.

Ključne reči: **crna ribizla, bobičasto voće, antimikrobna aktivnost, antocijani**