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Activity of Serbian *Aronia prunifolia* against *Prototheca wickerhamii* and *Prototheca zopfii*

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ABSTRACT

***Background*:** Beneficial effects of berries have been known from 16th century, but their antimicrobial effects have been explained scientifically only recently. The two most common aronia species are black chokeberry, *Aronia melanocarpa* [Michx.] Elliot and red chokeberry, *Aronia arbutifolia* [L.] Elliot. Purple chokeberry (*Aronia prunifolia*) is a hybrid of these two species. Protothecosis is a disease caused by achlorophyllous algae *Prototheca* species. Infections with *Prototheca* species are more common in veterinary medicine. The purpose of this study was to investigate the activity of *Aronia prunifolia* berries against *Prototheca zopfii* (*P. zopfii)* and *Prototheca wickerhamii* (*P. wickerhamii)*.

***Materials, Methods & Results*:** Purple chokeberry juice was made by squeezing the fruits and evaporated to dryness. Extracts of purple aronia were obtained by maceration with ethanol 80 % (v/v) for 24 h. *Prototheca zopfii* was obtained from udder of cow with mastitis and *Prototheca wickerhamii* was isolated from human oral cavity. Total phenolic and flavonoid contents were analized using spectrophotometric methods. The chemical composition of the tested substances was determined by a high performance liquid chromatography (HPLC) method. The examination was conducted by a micro-dilution method according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Microsoft Excel program 2007 was used for statistical analysis. The antialgal activity was expressed as minimal inhibitory concentrations MIC_{99}, MIC_{90} and MIC_{80}, the lowest concentration which kills 99%, 90% and 80% of organisms, respectively. Furthermore, minimal algaecide concentration (MAC), the lowest concentration which kills 99.9% of organisms is determined, as well as break point, the lowest concentration at which there is no algal growth.

***Discussion*:** The content of ascorbic acid was twice as high in the ethanolic extract as in the juice. Content of polyphenolic compounds was high in both juice and ethanolic extract. The quantity of phenocarbonic acids in juice and ethanolic extract was relatively low. Some of them were found only in juice (ellagic, coumaric and gentisic acids) as opposed to others found only in ethanolic extract (chlorogenic acid). Flavonoids were also detected in juice and ethanolic extract. Extract was much richer in flavonoid content when compared to aronia juice. Catechin was present in concentration of 186.3 mg/ 100 g of dry matter in the aronia juice, and 680.65 mg/ 100 g of dry matter in the ethanolic extract, which was more than 3.6 times higher. Quercetin was found only in the extract. The rutin content was 12 times and the chrysin content was 2.5 times higher in the aronia extract. The biggest difference could be noted in the quantitative contents of anthocyanins, 26 times higher concentration in extract than in juice. In general, higher content of bioactive compounds could be observed in the extract than in the juice. The results showed that the ethanolic extract of aronia fruits exhibited antialgal activity against both *Prototheca* species, while the juice showed no antialgal activity. This difference in antialgal effect is presumably related to the high content of several groups of biocompounds, especially catechin and anthocyanins, present in the ethanolic extract, and probably their synergistic action. There is no comparable data of antialgal effects of aronia in literature.

Keywords: *Aronia prunifolia*, chokeberry, chemical composition, *Prototheca*, antialgal.

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INTRODUCTION

Purple chokeberry (*Aronia prunifolia*) is a hybrid of *Aronia melanocarpa* [Michx.] Elliot and red chokeberry, *Aronia arbutifolia* [L.] Elliot. It contains high concentration of antioxidants (tannins, biphenols, flavonoids, anthocyanins, catechins), scientifically proven to prevent various disease [6,8,31]. Besides its antioxidant, antiinflammatory, anticancer, lipid-lowering, gastro- and hepatoprotective, antiaggregatory, antihypertensive and antidiabetic activity [12,16], this plant also showed antimicrobial effects *in vitro* against some bacteria [17] and against influenzae A virus [24]. Extracts of aronia may also prevent biofilm formation of *Escherichia coli* and *Bacillus cereus* [4], while the juice reduced urinary tract infections [11]. However, there is no data about the antimicrobial effects of aronia on microalgae.

Protothecosis is a disease caused by achlorophyllous algae *Prototheca zopfii* (*P. zopfii*), more frequent in veterinary medicine, and *Prototheca wickerhamii* (*P. wickerhamii*), common for human infections [18]. Dominant form of *P. zopfii* infections is granulomatous mastitis in dairy cows [3,21,22,37], but sporadic infections are also reported in deer, dogs, cats and humans [23,25,30]. There is no defined pharmacological treatment for protothecosis. Antimycotics, various antiseptics and disinfectants are usually given in practice [16,20,26,27]. Due to growing resistance of pathogens to conventionally used antimicrobial drugs [36], use of natural products is becoming a trend with many beneficial effects. The aim of this study was to examine the antialgal effect of juice and extract of purple chokeberry on two *Prototheca* species.

MATERIALS AND METHODS

Plant material

Aronia fruits were purchased in September 2015 from individual producers in Novi Sad, Serbia and processed fresh. Sample of plant material was identified as purple chokeberry, *Aronia prunifolia* and archived as voucher specimen in the Herbarium of the Department of Biology and Ecology (BUNS Herbarium), Faculty of Natural Sciences, University of Novi Sad by register number 2-2133. In order to evaluate the antimicrobial activity of this fruit, the juice and the extract obtained from pomace were separately investigated. Methods of preparation are previously described [13]. The juice was obtained by squeezing aronia fruits through sterile gauze in a sterile glass bottle and then evaporated to dry in the rotary evaporator. Extraction from pomace was performed at room temperature using a solvent mixture containing 80% ethanol, 19.95% sterile water and 0.05% acetic acid.

Microorganisms

The study was carried out on two autochthonous strains of *Prototheca* species, *Prototheca wickerhamii,* isolated from oral cavity [28], and *Prototheca zopfii*, isolated from the milk sample of dairy cow with granulomatous mastitis [22]. The identity of *Prototheca* was confirmed on the basis of their cultivation, microscopic and biochemical properties. The strains were recultivated before antimicrobial testing on plates of Sabouraud dextrose agar[1] at 37°C.

Determination of chemical composition

The standards of catechin, rutin, chrysin, quercetin, myricetin, ascorbic, protocatechuic, gallic, *p*-hydroxybenzoic, syringic, vanilic, ellagic, chlorogenic, coumaric, gentisic, caffeic, ferulic and sinapic acid and cyanidin chlorid were purchased from Sigma[2]. Acetonitrile, formic, acetic and metaphosphoric acid, ammonium acetate and methanol were obtained from Mallinckrodt Baker Inc[3]. Identification and quantification was carried out by HPLC analysis [33-35]. The samples were analyzed by a Shimadzu Prominence chromatographic system[4]. Chromatograms were recorded using different wavelengths: 265 nm for ascorbic acid, 280 nm for hydroxybenzoic acids (gallic, protocatechuic, *p*-hydroxybenzoic), catechin, and ellagic acid; 320 nm for hydroxycinnamic acids (chlorogenic, coumaric, gentisic, ferulic and sinapic acid); 520 nm for anthocyanins.

Antialgal analysis

Both the juice and the extract dry matter were reconstituted in sterile water. Concentrations for testings were from 100 mg/mL to 0.78 mg/mL. Antimicrobial effects were determined by modified micro-dilution broth method for yeasts according to CLSI Guidelines (2002) [7]. In short, the turbidity of microbial suspension was adjusted to 0.5 McFarland standard[5].

This suspension was further diluted 1:100 in saline. Diluted microbial suspension was inoculated in 96 wellmicrotiter plates[6] containing Sabouraud dextrose broth[1]. Nystatin[7] in concentration of 6.25 μg/

mL was used for comparison. Sterility control test and growth inhibition control test were also included. After incubation for 24 h at 37°C, 100 μL of contents of each well was transferred to separate Sabouraud dextrose agar plates[1], incubated for 72 h at 37°C and examined daily for colony forming. The percentage of killed microorganisms was calculated by comparison of the number of surviving microorganisms treated with different concentration of aronia with the number of microorganisms that were introduced into the well [29].

Statistical analysis

Graphs show the percentage of surviving microorganisms vs. concentrations of the tested extract and juice. The function switch were used to approximate the experimental results using the Trendline supplement from the Microsoft Excel[8] program 2007. The antimicrobial activity was expressed as following values: minimal inhibitory concentrations MIC_{99}, MIC_{90} and MIC_{80}: the lowest concentration which kills 99%, 90% and 80% of microorganisms introduced to the wells (pharmacological MIC), respectively; minimal algaecide concentration MAC: the lowest concentration which kills 99.9% of microorganisms introduced to wells and break point: the lowest concentration at which there is no algal growth.

RESULTS

The results of identification and quantification of active compounds in aronia juice and its ethanolic extract are shown in Table 1, and representative chromatograms are presented in Figures 1 and 2. The content of vitamin C was twice as high in the extract as in the juice, 10.42 mg/ 100 g of dry matter (DM) in the juice, 20.82 mg/ 100 g DM in the extract (Table 1). The quantity of phenocarbonic acids (gallic, syringic, protocatechuic, vanilic, ferrulic and caffeic acid) in both juice and extract was relatively low (total of 45.05 mg/ 100 g DM and 55.86 mg/ 100 g DM, respectively). However, some of them were found only in juice (ellagic, coumaric and gentisic acids), while chlorogenic acid was found only in extract. As shown in Table 1 and Figure 1, aronia extract was much richer in flavonoid content when compared to juice. Catechin was present in concentration of 186.3 mg/ 100 g of DM in the juice, and 680.65 mg/ 100 g of DM in the extract. Quercetin was found only in the extract. The rutin content was 12 times and the chrysin content was 2.5 times higher in the aronia extract. The content of myricetin was two times higher in juice than in extract. The biggest difference could be noted in the content of anthocyanins, 20.91 mg/ 100 g DM in the juice, and 542.93 mg/ 100 g DM in the extract.

The ethanolic extract showed antialgal activity, in opposite to the lack of activity of aronia juice. Nystatin, as a control, also showed no antialgal effect on the tested microorganisms. The functions of antialgal activity of purple chokeberry ethanolic extract were $y = -44{,}753*\ln(x) + 125{,}116$ for *P. zopfii* and $y = -44{,}736*\ln(x) + 125{,}035$ for *P. wickerhamii*. The MIC and MAC values for the ethanolic extract are shown in Table 2.

DISCUSSION

The main aim of aronia cultivation is the increase of anthocyanins' presence which can represent up to 25% of polyphenols found in fruits [16]. The analysis of active compounds in the juice and extract showed that the content of polyphenolic compounds was high (Table 1). In general, higher content of bioactive compounds could be observed in the extract than in the juice. This is in accordance with the literature data. Review of Kokotkiewicz *et al.* [12] point out that the two most prominent groups of compounds are flavanols and anthocyanins. Research of Taheri *et al.* [31] found that 100 g of fresh aronia berries can provide up to aproximately 500 mg of anthocyanins. In the same research, species of red aronia had 0.63 mg/ g of dry weight, which is 8.62 times lower concentration than the one obtained in present study. Different method of extraction and sample preparation could have influence on the results. It is more likely that conditions of aronia cultivation in Serbia are promising.

Small range between MIC_{80} and breakpoint indicates an algaecide effect by active compounds in ethanolic extract. Concentrations of catechin and its derivatives and total anthocyanins are much lower in juice than in ethanolic extract, and chlorogenic acid is present in extract only. Since antialgal effect is presented only by extract, it can be assumed that these groups of compounds and their synergistic action could be responsible for the antialgal activity.

In available literature, no comparable data of antialgal effects of aronia was found, but some other fruits from Rosaceae family have been tested for antialgal effects. Krstić *et al.* [13] examined the antialgal activity of juice and extract of sour cherry

Table 1. Quantification of components in the juice and extract.

Compound	Wavelenth [nm]	Retention time [min]	Contents [mg/100 g DM*]	
			Juice	Ethanolic extract
catechin	280	9.93	186.30	680.65
total anthocyanins**	520	6.77; 7.68; 8.8; 9.39; 9.9; 10.58; 10.97; 11.37; 11.99; 13.16; 14.79	20.91	542.93
vitamin c	265	4.57	10.42	20.82
gallic acid	280	4.05	10.96	18.24
rutin	360	14.01	1.16	14.31
chlorogenic acid	320	6.76	-	10.5
chrysin	280	27.62	4.11	10.01
syringic acid	280	12.49	4.59	9.18
protocatechuic acid	280	8.56	3.92	8.07
ferrulic acid	320	16.54	7.20	6.16
quercetin	360	20.85	-	4.49
myricetin	360	17.06	1.48	3.52
caffeic acid	320	12.1	4.23	3.28
vanilic acid	280	13.34	1.02	0.43
gentisic acid	320	11.82	10.52	-
coumaric acid	320	11.31	1.84	-
ellagic acid	280	14.95	0.78	-
p-hydroxybenzoic acid	280	11.5	-	-
sinapic acid	320	16.86	-	-

*Compounds in *Aronia* juice measured by spectrophotometer and expressed as mg/100 g of dry matter (DM).** Data expressed as mg CyCE equivalence per 100 g of dry matter (DM).

Table 2. Results of antialgal activity of ethanolic extract.

Microorganism	Breakpoint (mg/ mL)	MAC (mg/ mL)	MIC_{99} (mg/ mL)	MIC_{90} (mg/ mL)	MIC_{80} (mg/ mL)
Prototheca wickerhamii	16.36	16.32	16.00	13.08	10.46
Prototheca zopfii	16.38	16.34	16.02	13.10	10.48

on *P. wickerhamii*. Neither the juice nor the ethanolic extract inhibited the tested pathogen. The aronia extract had approximately 13 times higher content of catechin then sour cherry Epicatechin was present in extract of sour cherry only. No specific compounds were found in aronia extracts compared to sour cherry. This indicates that no single compound is crucial for the antialgal effect. Rather, this effect is presumably the result of synergistic action of various compounds found in fruit ethanolic extract. In a previous pilot study [14], juice of blackberry showed no effect on *P. zopfii*, while juice and ethanolic extract of raspberry showed moderate effect on *P. wickerhamii* [15]. Both investigations were performed by agar well diffusion method, so the results cannot be completely comparable, especially considering quantitative results.

The ethanolic extract of *Clematis vitalba* was investigated in agar well diffusion and micro-dilution methods [5]. Diameters of grow inhibition of investigated specimens obtained in the first method were

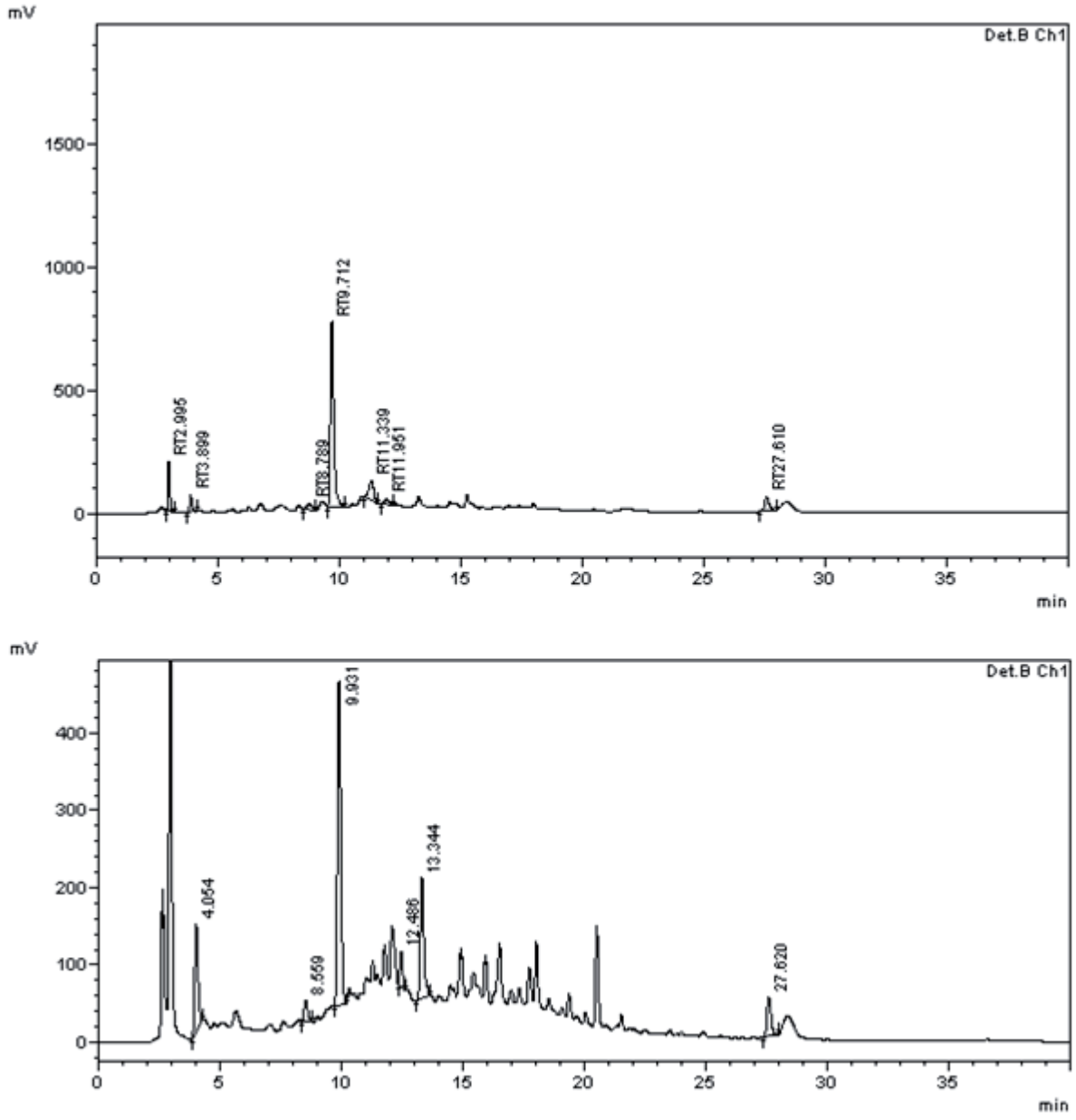

Figure 1. Chromatograms of (A) *Aronia* extract and (B) juice analysis at wavelength 280 nm.

13.2 mm for *P. wickerhamii* and 17 and 15.5 mm for two strains of *P. zopfii*. MIC values were 11.6 μg/mL for *P. wickerhamii* and 1.4 and 4.8 μg/mL for *P. zopfii*, significantly lower than MIC values in our study. Although the results are not completely comparable, it seems that extract of *Clematis vitalba* has a better antialgal effect than *Aronia prunifolia*.

Ethanolic and dichloromethanic extracts from *Senecio desiderabilis* were tested on *P. zopfii* [10]. While the CH_2Cl_2 extract showed no effect on *P. zopfii*, the ethanolic extract achieved the MIC effect at 2.5 mg/mL. Authors used solvents (DMSO and Tween 80) which themselves have antimicrobial activity questioning the results. Nevertheless, it seems that ethanolic extract of *Senecio desiderabilis* could have better antialgal effect than *Aronia prunifolia*.

Essential oils (EO) of *Satureja hortensis* and *Abies alba* were tested on ten strains of *P. zopfii* and one strain of *P. wickerhamii* [1]. Most of the examined strains of *P. zopfii* had a MIC value of 1 μL/mL for *S. hortensis* EO and MIC value 2 μL/mL for *A. alba* EO. For *P. wickerhamii*, these values were 0.25 μL/mL and 0.125 μL/mL, respectively. These values were much lower than the values obtained with our examined extract. Similar MIC values were noted in another study of protothecal susceptibility to antimycotics and

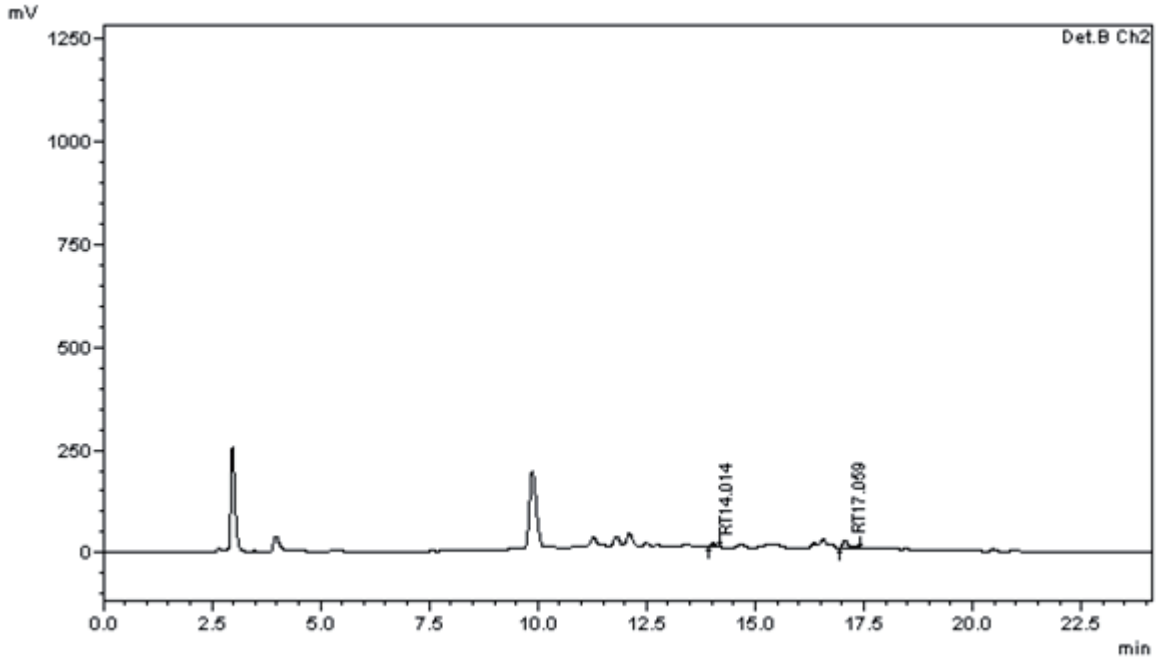

Figure 2. Chromatogram of aronia juice analysis at wavelength 360 nm.

essential oils [32]. A mean MIC value for 27 different strains of *P. zopfii* was 0.05% of essential oil (range 0.03-0.12%) for *Melaleuca alternifolia* EO and 1.45% (range 0.15-5%) for *Citrus bergamia* EO. For the single examined strain of *P. wickerhamii*, MIC values were 0.06% for *M. alternifolia* EO and 0.3% for *C. bergamia*. The efficiency of EO was confirmed in later *in vivo* studies [2].

Antibacterial and antifungal activity of aqueous and ethanolic extracts of aronia berries were tested [17]. In diffusion method, these extracts showed approximately one third of activity when compared to gentamicin. The ethanolic extract showed activity on *Staphylococcus aureus*, *Bacilus cereus* and partially on *Pseudomonas aeruginosa*. The aqueous extract showed an effect on the same microorganisms with smaller inhibition zone. This is in accordance with our findings that ethanolic extract has stronger antimicrobial activity. Antibacterial activity of aronia acetonic extract were demonstrated against 11 foodborne pathogens [8,9]. It showed weak to medium strong activity for majority of tested strains. Research on the effect of aronia extract on the prevention of biofilm forming [4] showed that a concentration of 1 mg/mL can achieve this effect. The component of the aronia extract supposed to be responsible for the prevention of *Escherichia coli* biofilm forming was epicatehin. Even though the crude extracts of various berries have been examined for antimicrobial effects, further studies of single compounds present in those extracts are needed [6].

There is no defined treatment for protothecosis. Unspecific measures, such as different buffer and NaCl solutions, have been tested against *Prototheca* species [19]. Several antiseptics and disinfectants were tested: copper sulfate, silver nitrate [20], sodium [26,27], alkyldiaminoethylglycine hydrochloride, dioxide chlorine, povidon iodine [27], iodine [26] and chlorhexidine, [20,27]. In the tested strains, predominantly isolated from mastitic cows, concentrations necessary for the algaecide effect were very low but these concentrations should not be compared with the antimicrobial effects of natural substances.

CONCLUSIONS

Identification and quantification of active compounds in aronia juice and its ethanolic extract were conducted. Furthermore, aronia juice and ethanolic extracts were tested for antialgal effects. Higher concentrations of bioactive compounds were detected in the extract. The most significant difference in concentration was noticed for catechin and its derivatives and total anthocyanins, which showed much higher concentration in ethanolic extract than in juice. Since antialgal effect is present only by extract, it can be assumed that these groups of compounds and presu-

mably their synergistic action could be responsible for the antialgal activity. We tested the effects of *Aronia prunifolia* extracts on a rare pathogen, *Prototheca*, which is one of two known plants (microalgae) able to cause diseases in humans and animals. The favorable content of polyphenols in Serbian aronia suggests that this berry should be recommended for daily intake. It would be rationally to think about the design of pharmaceutical formulations of concentrated extract as dietary supplements and creams, gels, ointments or pomades for external use.

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***Declaration of interest*.** The authors declare that there is no conflict of interest.

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