ORIGINAL ARTICLE

SYNERGIC BENEFITS OF *ARONIA MELANOCARPA* ANTHOCYANIN – RICH EXTRACTS AND ANTIBIOTICS USED FOR URINARY TRACT INFECTIONS

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Abstract

Anthocyanins are well known for their powerful antioxidative potential, however new insights are discovered every year regarding their health benefits. For the present study, we investigated several anthocyanin-rich extracts in terms of their antimicrobial synergistic effect with commonly used antibiotics in urinary tract infections (UTI). The selected samples, lyophilized powders from *Aronia melanocarpa* L., were obtained from mature fruits cultivated in ecological environment in the Eastern Carpathians in Romania. The optimized extracts were obtained by selective liquid-liquid extraction in ethanol and acetone and then submitted to analysis. The chemical profile of the polyphenols was established by spectrophotometric and ultra-performance liquid chromatography (UPLC) techniques. To further confirm the value of the extracts we submitted them to some antioxidant tests: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$) radical cation scavenging assay and lipoxygenase inhibition test. The minimal inhibitory concentration (MIC) and minimal biofilm eradication concentration (MBEC) of the extracts against several standard and clinical isolated Gram-positive and Gram-negative strains were determined. Our results indicated that the differences induced by the type of solvent used for extraction has a significant impact on the biological activity intensity of each sample.

Rezumat

Antocianii sunt bine cunoscuți pentru acțiunea lor antioxidantă deosebită. Cu toate acestea, în fiecare an sunt descoperite noi perspective cu privire la beneficiile lor pentru sănătate. În prezentul studiu, am investigat două extracte bogate în antociani, în ceea ce privește efectul antimicrobian sinergic cu antibiotice utilizate frecvent în infecțiile tractului urinar (UTI). Probele selectate, pulberi liofilizate din *Aronia melanocarpa* L., au fost obținute din fructe mature cultivate în mediu ecologic în regiunea județului Neamț, România. Extractele optimizate au fost obținute prin extracție selectivă lichid-lichid în etanol și acetonă și apoi supuse analizei. Profilul chimic al polifenolilor a fost stabilit prin tehnici spectrofotometrice și de cromatografie de lichide de ultra performanță (UPLC). În plus, s-a demonstrat că extractele au efect antioxidant (testul de *scavenger* față de cationul acid 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonic) ($ABTS^{\cdot+}$) și testul de inhibare a lipoxigenazei). Au fost determinate concentrațiile minime inhibitoare a microorganismelor (MIC) și concentrația antibiofilm minimă (MBEC) a extractelor față de mai multe tulpini gram-pozitive și gram-negative standard și izolate clinic. Rezultatele noastre indică faptul că diferențele induse de tipul de solvent utilizat pentru extracție au un impact semnificativ asupra activității biologice a fiecărei probe.

Keywords: anthocyanins, *Aronia melanocarpa*, antimicrobial, antioxidant

Introduction

Urinary tract infections (UTI) annually affect over 150 million people worldwide, with immense medical costs and causing significant recurring and chronicling morbidity [10, 11, 14]. The penetration and multiplication of bacteria in the urinary tract is manifested by symptomatic or asymptomatic bacteriuria. Therefore, the need for an effective treatment implies that antimicrobial drugs should be used to stop the multiplication of pathogens. Although, with the discovery of penicillin such therapeutic approaches were possible, soon enough (as early as 1944) resistant strains were discovered. Moreover, depending on the type of resistance there are natural and acquired resistant strains. Especially the last type is very problematic due to its properties that allow the bacteria to adapt and become resistant to a drug to which it had been sensitive before. Also, in the last decade multi resistant strains have spread all over the world due to irrational use of antibiotics [5, 6, 10, 11, 14]. Recently, clinical researchers have found that approximately 24% of *Escherichia coli* isolates and 22% of *Klebsiella pneumoniae* isolates were resistant to fluoro-

quinolones, whereas third-generation cephalosporin-resistant pathogens were significantly more common in cases of patient fatality [5, 10].

On the attempt to decrease the numbers of resistant bacteria researchers have looked in nature to find compounds that possess antimicrobial properties and could be used as antibacterial agents on their own or in combination with commonly used chemotherapeutics [5, 7]. Polyphenols such as anthocyanin derivatives have been proven to possess antioxidant, good antiseptic and cancer preventive activities [3, 13, 15, 17]. Among the richest sources of anthocyanins are the intensely coloured berries (red, blue, black) usually used as foods.

Aronia melanocarpa (*Maloideae* subfamily, *Rosaceae* family), also known as black chokeberry, was originally thought to have little medicinal importance, but lately has been proven to present antioxidant, hypoglycaemic, immunomodulatory and enzyme inhibitory properties [2, 4, 12, 15, 16, 17]. It is known that the chemical composition of a vegetal material is highly influenced by the environmental conditions and the processing methods. Therefore, in our study we used ripen berries from a controlled environment.

In the present study, we investigated the antimicrobial profile of two different types of extracts from *Aronia* berries grown in Romania. Some aspects regarding their chemical and antioxidant properties were also assessed.

Materials and Methods

Reagents: All chemical and reagents were of analytical grade or of chromatographic quality and were purchased from Sigma Aldrich (Seelze, Germany) or Fluka (Buchs, Switzerland).

Plant material

The samples were represented by ripe fruits of *Aronia melanocarpa* harvested in 2015 from the Eastern Carpathians in Romania. After sorting, they were frozen at -20^{o}C until extraction. One voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy, Iaşi, Romania, and a second specimen is kept at Department of Agriculture, University of Presov, Slovak Republic.

Extraction process and lyophilisation

The extraction consisted of maceration with acetone (1:1), followed by filtration and extraction in chloroform, and then extracted with water. Water fraction was first frozen and then freeze-dried. This extract was coded as sample 1. The lyophilized extract was obtained by GEA Lyophil SMART LYO SL2 system at the Research and Development Department, Medicproduct, Co., Lipany, Slovak Republic.

The hydralcoholic extract (coded as sample 2) was obtained in a comparable manner using ethanol 70% instead of water. The alcohol was eliminated in a Buchi rotavapour system and then the sample was lyophilized to a thin dark-violet powder.

The chemical analysis

RP-UPLC (reverse phase ultra-high-performance liquid chromatography) was used for the identification of compounds found in the extracts. The system consisted of UPLC Thermo Fischer Ultimate 3000, DAD detector and Accucore XL C18 (150 x 4.6 x 4) column. The temperature used was 35ºC and a debit flow of 0.45 mL/min. The solvent system was a mixture between 5% acetic acid (A) and acetonitril: methanol (5:3) (B) as follows 0 - 12 – 9% B, 13 - 30 – 50% B, 31 - 35 – 9% B. The obtained chromatograms were compared to the standard and the literature for further reference [2].

ABTS cation scavenging assay

ABTS cation ($ABTS^{\cdot+}$) was generated by incubation at room temperature of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic) acid stock solution (7 mmol/L) with potassium persulfate (2.45 mM) in the dark, for 12 h. The solution was then diluted with ethanol in order to obtain an absorbance of 0.68 - 0.72 absorption units (AU) (measured at 734 nm). An aliquot (0.02 mL) of lyophilized powder dissolved in dimethyl sulfoxide was mixed with $ABTS^{\cdot+}$ solution to a volume of 2 mL. The absorbance at 734 nm was recorded 6 min after continuous mixing. Quercetin and gallic acid were used as positive controls. The scavenging activity was calculated as follows: ABTS scavenging activity (%) = $100 \times (A_{control} - A_{sample})/(A_{control})$, where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance in the presence of extracts or positive controls [3, 10].

Trolox equivalent antioxidant capacity was used to calculate TEAC values and Trolox was used as standard. Three concentrations (μg/mL) were selected for extracts/positive controls corresponding to 20% - 80% activity (that decreased the absorbance in the linear region of Trolox curve). Absorbance decrease (%) of selected concentrations was plotted for each sample and positive control. TEAC (μM) was calculated as the ratio between the slopes of dose-response curves of samples/positive control and Trolox [5-7].

Determination of 15-lipoxygenase activity (Maltreud modified method)

Polyphenols have the ability to inhibit the activity of 15-lipoxygenase which catalyses the oxidation of linoleic acid, thus resulting a reduction of the absorbance measured at 234 nm. An aliquot (0.05 mL) of 15-lipoxygenase in borate buffer (pH 9) was mixed with 0.05 mL of the sample solution in DMSO (various concentrations); after 10 minutes 2 mL of 0.16 mM linoleic acid borate buffer (pH 9) were added and the absorbances were recorded at 234 nm

for 90 seconds. 15-lipoxygenase inhibition was calculated using the formula: % activity = (A_{EFI} - A_{ECI}) x 100/A_{EFI}. Where: A_{EFI} represents the difference between the absorbance of the enzyme solution without inhibitor at 90 seconds and second absorbance at 30 seconds; A_{ECI} equals the difference between the absorbance of the enzyme solution treated with inhibitor at 90 seconds and second absorbance at 30 seconds. The value of IC_{50} (mg extract/mL) was calculated for each sample and gallic acid was used as positive control [6, 12].

Antimicrobial assay

Four standard pathogens, *E. coli* ATCC 13202, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29212, and 18 clinical isolates *(E. coli* 2041, *E. coli* 1851, *E. coli* 1992, *Pseudomonas aeruginosa* 1908, *P. aeruginosa* 1128, *Klebsiella pneumoniae* 2110, *K. pneumoniae* 1074, *K. pneumoniae* 831, *Morganella morganii* 2520, *Acinetobacter baumanii* 1908, *A. baumanii* 2329, *Enterobacter cloacae* 2951, *E. faecalis* 2823, *E. faecium* 2862, *E. faecium* 2980, *E. faecium* 2027, *S. aureus* 14, *S. aureus* 17) from patients with urinary tract infections were used. All colonies were positively identified by MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time -of-Flight Mass Spectrometry). This method ascertains the unique molecular fingerprint of each microorganism by mass spectrometry. Then, the protein profile of each microorganism is compared to that existing in an extensive database.

Minimum inhibitory concentration quantification

Quantitative analysis was performed by the binary liquid serial microdilution method (single bacterial broth and Sabouraud for fungi) in 96-well plates using negative control of sterility and positive microbial growth control. The binary test series tested were: 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.039063, 0.019531, 0.009766. Dilutions were made in 200 µL broth and then each well was seeded with 50 µL of microbial inoculum. The plates were incubated for 24 hours at 37°C and the minimum inhibitory concentration (MIC) values were established as corresponding to the lowest concentration of the test extract which inhibited the growth of microbial cultures compared to the positive control [8].

Determination of minimum biofilm eradication concentration

The microtiter method was used to evaluate the influence of the tested extracts on the ability of microbial strains to form biofilms on the inert substrate. The microplates used for MIC testing were emptied and washed three times with saline phosphate buffer. The biofilm formed on the plastic well wall was fixed for 5 minutes with cold methanol, stained for 15 minutes with a purple crystal solution and resuspended with a 33% glacial acetic acid solution. The minimal biofilm eradication concentration (MBEC) was the lowest concentration of the test compound that inhibited the development of biofilm in plaque wells [1].

Synergy between the tested extracts and antibiotics against uropathogenic microbial strains

The principle of the method consists in sowing the microbial standardized inoculum liquid on the surface of an agarized medium. Then, at equal distances there are placed the standardized antibiotic disks, standard antibiotic disks along with 5 µL of stock solution from the investigated extracts, and the sterile filter paper disks only with 5 µL of extract stock solution. The active substances will diffuse in the medium, achieving a concentration gradient inversely proportional to the diameter of the diffusion zone, so with the distance from the disk. Standardized antibiotic disks were selected according to the International clinical laboratory standards recommendations for each microbial strain.

Results and Discussion

The results for the RP-UPLC analysis indicated that both extracts contain similar compounds (Figure 1), but the extraction method has an impact on the quantity of each aglycon and glycoside derivative. We observed that the use of ethanol in the extraction process decreases the quantity of saccharides and the consistency of the final extract is lighter, allowing an easier further processing.

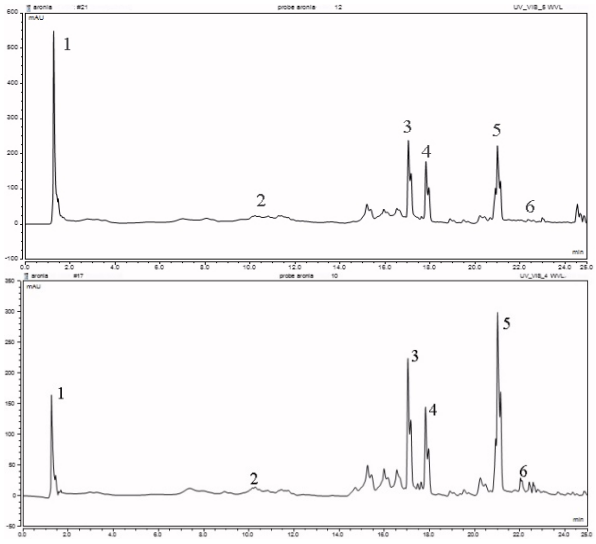

Figure 1.

UPLC chromatograms of the investigated *Aronia melanocarpa* acetone and ethanol extracts.

1) catechin, 2) catechin dimers, 3) cyanidin 3-galactoside, 4) cyanidin 3-glucoside, 5) cyanidin 3-arabinoside, 6) cyanidin 3-xyloside

Various parameters such as temperature, pressure and the time required for the operation have been checked to eliminate any changes that might occur during lyophilisation, to test its efficiency and possible

losses incurred during processing. At the same time, the losses were tested and the results were satisfactory, the established technique allowed the obtaining of stable fractions over time with a minimum of loss and high yield (minimum 45% anthocyanins).
The main anthocyanins identified in both lyophilized extracts were cyanidin 3-galactoside, cyanidin 3-glucoside, cyanidin 3-arabinoside, and cyanidin 3-xyloside. Ten times higher amounts (almost equal) were detected for cyanidin 3-galactoside and cyanidin 3-arabinoside. Epicatechin and its dimers and trimers were also present in the investigated extracts.

This chemical data are consistent with scientific literature that reported the presence of polymeric procyanidins, composed mainly of epicatechin units, as the major class of polyphenolic compounds found in *Aronia* berries and the corresponding extracts [2, 4].
Our results indicated that all samples have a good antioxidant activity that depends on their concentration. The IC_{50} values and the Trolox equivalent antioxidant capacity (TEAC) values were the highest for *Aronia melanocarpa* ethanolic extract, while 9 times less active proved to be the acetone sample, as indicated in the table below.

Table I

Systematic results for the antioxidant assays of the investigated samples

Assays		**Samples**		**Standard**	
		1 (acetone)	**2** (ethanol)	**gallic acid**	**ascorbic acid**
ABTS radical scavenging	IC_{50}*	18.2 ± 0.1	1.7 ± 0.1	0.6 ± 0.02	-
	TEAC**	0.55 ± 0.02	4.22 ± 0.1	20.48 ± 0.14	-
LOX inhibition	IC_{50}	20.83 ± 0.51	12.28 ± 0.1	-	29.91 ± 0.62

*IC_{50} values expressed in µg/mL; **TEAC values calculated as µM Trolox equivalent to 1 µg/mL extract

The LOX inhibition assay indicated that both investigated samples have an average potential comparable to ascorbic acid used as positive control. These activities were in close relation to the anthocyanidin concentration of each sample.
Previous research on black chokeberry revealed that *Aronia melanocarpa* juice can reduce different oxidative stress biomarkers [4, 13]. Our results come to complete such data, the antioxidant tests we used involved other mechanisms, thus leading to the conclusion that *Aronia* is a rich source of anti-oxidants not only as food or in its raw juice, but also as a great vegetal material to obtain selective extracts for therapeutic use.
Regarding the antimicrobial activity, both extracts showed a variable potential depending on the used concentration and the tested strains. All registered values are included in Table II.
The results indicate a good antibacterial activity for the ethanolic extract (sample 2) obtained from *Aronia* berries, whereas an average potential was noted for the acetone extract. The lowest MIC values corresponding to the most intense antimicrobial activity were obtained for sample 2, which proved to be most active against one strain of *E. coli* and one of *Morganella morganii* (5 mg/mL MIC), three strains of *P. aeruginosa* (2.5 mg/mL MIC), one *E. faecalis* and two *E. faecium* (5 mg/mL MIC) strains, and also against three strains of *S. aureus* (1.5 - 2.5 mg/mL MIC). Good activity against non-fermenting Gram-negative bacteria is extremely important for further studies considering that such microbial strains are ubiquitous in the environment and can adapt to acquire multiple resistance to antibiotics.

Table II

MIC values for the antimicrobial test for *Aronia* extracts

Microbial strain	**Tested extract**	
	1	**2**
Enterobacteria	**MIC (mg/mL)**	
E. coli ATCC	10	10
E. coli 2041	10	10
E. coli 1851	10	10
E. coli 1992	10	5
E. cloacae 2951	10	10
K. pneumoniae 2110	10	10
K. pneumoniae 1074	10	10
K. pneumoniae 831	10	10
Morganella morganii 2520	10	5
non-fermenting Gram-negative bacteria		
Pseudomonas aeruginosa ATCC	10	2.5
A. baumanii 1908	10	10
A. baumanii 2329	10	10
P. aerug. 1908	10	2.5
P. aerug.1128	10	2.5
Gram-positive, catalase-positive cocci		
E. fecalis ATCC	10	5
E. fecalis 2823	10	10
E. faecium2862	10	5
E. faecium 2980	10	10
E. faecium 2027	10	5
Gram-positive, catalase-negative cocci		
S. aureus ATCC	10	2.5
S. aureus 14	10	2.5
S. aureus 17	10	5

Determination of the antimicrobial activity of the extracts was performed by determining the minimum biofilm eradication concentration (MBEC). Also in this case, similar to the previous test, the most active extract was sample 2, which inhibited the development of biofilm at lower CMEB values in the case of an *E. coli* and a *Morganella morganii*

strain (CMEB of 5 mg/mL), three strains of *P. aeruginosa* (2.5 mg/mL CMEB), one *E. faecalis* and two *E. faecium* (5 mg/mL CMEB) strains. Moreover, the same sample was active at concentrations range between 3.5 and 5 mg/mL against three strains of *S. aureus*.

The synergistic effect for both extracts was modest, but an increase in the growth inhibition zone diameter for the antibiotic disks supplemented with the investigated extracts (Figures 2 and 3) was noted when amikacin, tetracycline, nitrofurantoin, imipenem and norfloxacin were used against *P. aeruginosa*, *E. coli* and *M. morganii*.

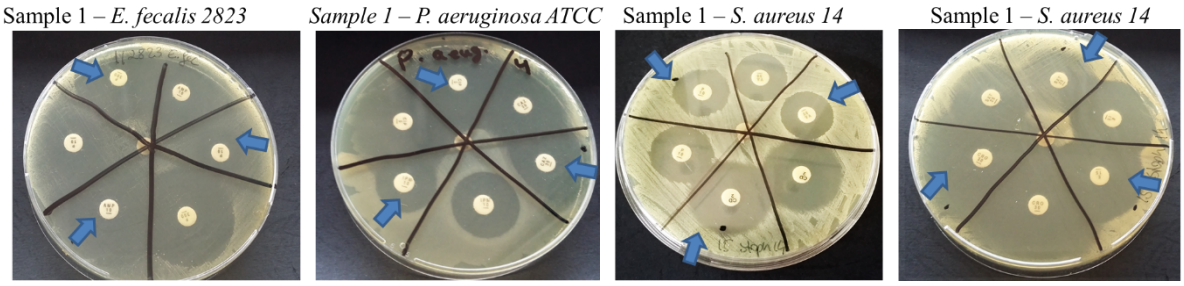

Figure 2.
Synergism testing of the acetone extract (the arrow indicates the presence of the extract on the disk)

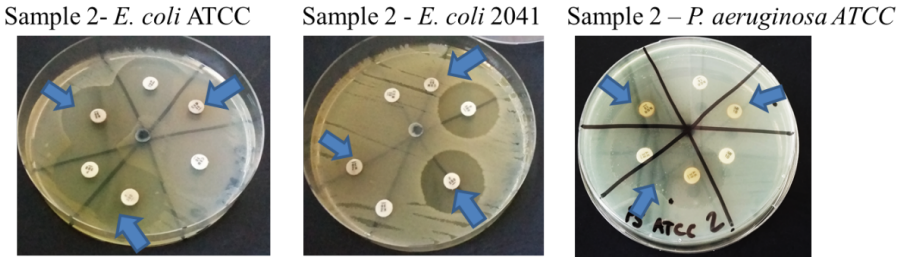

Figure 3.
Synergism testing of the ethanolic extract (the arrow indicates the presence of the extract on the disk)

Given all data, we can state that the antimicrobial activity observed in the initial tests is relevant when extracts are used on their own although, a modest to moderate synergy is observed when antibiotics are used. Yet, this study is only the starting point for investigating the correlation between the chemical composition and the mechanism in bacterial antibiotic susceptibility in the presence of *Aronia* extracts. Further studies are necessary to draw a concluding concept regarding synergism-based antibiotic treatment of anthocyanidin-rich extracts and its importance for therapy.

Conclusions

The present study indicates that ethanol favours the extractability of anthocyanins from chokeberry fruits and the lyophilisation is a good method to preserve the biologic activity of these compounds. The ethanolic extracts proved to be active against 10 out of the 18 tested strains at doses that amounted 2.5 - 5 mg/mL (MIC). The same sample, at doses of 2.5 - 5 mg/mL, showed significant inhibition of monoculture biofilm formation of 11 out of all tested strains. Therefore, well obtained and standardized extracts should be taken into consideration for expanding the preventing measures and treatment in case of drug resistant microbes. *In vivo* testing should be the next step towards the use of such berry extracts in patients with urinary tract infections.

Conflict of interest

The authors declare that they have no potential conflicts of interest to disclose.

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