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Phenolic Composition, Antioxidant and Antimicrobial Activity of *Cotoneaster* Medik. Species from Bosnia and Herzegovina

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Abstract: Although the genus *Cotoneaster* Medik. includes mainly ornamental species, there are some data regarding its biological activity. The purpose of this study was to analyze the content of phenolic compounds, acetylcholinesterase inhibition, antioxidant and antimicrobial activity of methanolic extracts of leaf and bark of *C. integerrimus* Medik., *C. tomentosus* (Aiton) Lindl. and *C. horizontalis* Decne. The *C. tomentosus* leaf extract exhibited the highest content of total phenols (135.86 mg GAE/g) and flavonoids (18.17 mgQE/g), and also the most potent antioxidant activity against nonbiogenic free radicals, while the highest inhibition of acetylcholinesterase had the leaf extract of *C. horizontalis* (IC_{50} 0.34 mg/mL). All extracts showed a significant level of antibacterial and antifungal activity against tested microbial strains. The largest inhibition zones were observed against *Candida albicans* treated with *C. integerrimus* leaf extract (30.50±0.50 mm). Furthermore, *C. integerrimus* extract was the most effective in the majority of bacterial strains tested. The results indicated that methanolic extracts of the investigated *Cotoneaster* species have promising bioactive and therapeutic potentials.

INTRODUCTION

Many medicinal plants are so far recognized as valuable resources of natural antimicrobial compounds, and wide range of phytochemicals in plants have potential to inhibit microbial pathogens (Romero *et al*., 2005). These biomolecules are mainly secondary metabolites, such as: alkaloids, glycosides, terpenoids, saponins, steroids, flavonoids, tannins, quinones and coumarins (Das, Tiwari and Shrivastava, 2010). Despite the fact that numerous plant species have been used in traditional medicine for centuries to treat infectious diseases, their bioactive compounds need to be determined. Furthermore, discovering the new potential antimicrobial resources from plants overcomes the domain of folk medicine and could have a promising impact in modern phytopharmacy, especially in the case of multidrug-resistant (MDR) pathogens (Agarwal *et al*., 2016). Usage of plant products have significantly increased in last decades, since synthetically derived compounds are often expensive, and could have unfavorable health effects (Uysal *et al*., 2016). The genus *Cotoneaster* Medik. belongs to the family Rosaceae, and in Bosnia and Herzegovina is represented by two species: *C. integerrimus* Medik. and *C. tomentosus* (Aiton) Lindl. (Beck-Mannagetta, 1927; Euro+Med PlantBase). Many *Cotoneaster* species are cultivated in Europe as ornamental plants in urban areas (Fryer and Hylm, 2009), and *C. horizontalis* Decne. is one of the most famous cultivars widely used in B&H. Data suggest that *Cotoneaster* species have traditionally been used for medicinal purposes. *Cotoneaster racemiflorus* (Desf.) K. Koch is known as an aperient, expectorant and stomachic, as well as a treatment for reducing jaundice (Chopra and Nayar, 1956). Khan *et al*. (2008) isolated two bioactive compounds from this species, and these new aromatic esters were named cotonoates A and B. Furthermore, specific bioactive compounds are recognized in other *Cotoneaster* species, e.g. phenolic glycosides in *C. orbicularis* Schltdl. (El-Mousallamy *et al*., 2000). Phytoalexin named cotonefuran with bactericidal activity was primarily isolated from *C.*

lacteus W. W. Sm. (Burden *et al*., 1984), and about a decade later, confirmed as well in *C. acutifolius* Turcz., with prominent antifungal properties (Kokubun *et al*., 1995). Antimicrobial effects have also been reported in *C. nummularius* Fisch. & C. A. Mey. (Zengin *et al*., 2014) and *C. nummularioides* Pojark. (Kanaani, Sani, and Yaghooti, 2015; Siami, Sani and Branch, 2016). Data on the chemical composition and antimicrobial activity of investigated *Cotoneaster* species are scarce (Mohamed *et al*., 2012; Sokkar *et al*., 2013; Sytar *et al*., 2016; Uysal *et al*., 2016), or non-existent. The aim of this study was to investigate: phenolic compounds content, acetylcholinesterase (AChE) inhibition, antioxidant and antimicrobial activity of three *Cotoneaster* species: *C. integerrimus* Medik., *C. tomentosus* (Aiton) Lindl. and *C. horizontalis* Decne.

EXPERIMENTAL

Chemicals and plant material

All reagents and solvents were purchased commercially and were of analytical grade. The plant material (leaves and bark) of three *Cotoneaster* species*: C. integerrimus*, *C. tomentosus*, and *C. horizontalis* was collected in September 2017. Ten individuals of each species were analyzed. *Cotoneaster integerrimus* and *C. tomentosus* were collected on Mountain Ozren near Sarajevo, Bosnia and Herzegovina, while *C. horizontalis* was sampled in urban Sarajevo area. Determination of plant material was carried out in Laboratory for Plant Systematics, Department of Biology, Faculty of Science, University of Sarajevo. Voucher specimens were deposited in the Herbarium of the Faculty of Science, University of Sarajevo. Separation of plant material into leaves and bark was performed, and such separated plant material was dried in dark in ventilated room at the ambient temperature.

Preparation of extracts

Dried plant material was grounded to fine dust and then extracted for 24 hours using 80% methanol. The extracts were filtered, evaporated to dryness under reduced pressure on a rotary evaporator and dissolved in dimethyl sulfoxide (DMSO) to the final concentration of 6 mg/mL. All extracts were stored at +4°C until use.

Determination of total phenolic content

The modified Folin-Ciocalteu method was used to determine the total phenolic content of the extracts (Singleton and Rossi, 1965). Folin-Ciocalteu reagent (1.0 mL) was reacted with 0.2 mL of diluted sample, and then 0.8 mL saturated sodium carbonate solution was added into the reaction mixture. After 30 minutes, the absorbance of the mixture was measured using the UV-Vis spectrophotometer at 765 nm. The total phenolic contents were determined from the standard curve prepared with gallic acid and the content of total phenolic compounds are expressed as gallic acid equivalents (mg GAE/g).

Determination of total flavonoid content

The Dowd method, which is based on the reaction between flavonoids and $AlCl_3$, was used to determine the total flavonoid content in the plant extracts (Dowd, 1959). The diluted extract solution (0.5 mL) was mixed with 2% $AlCl_3$ (0.5 mL). After standing for 10 minutes at room temperature the absorbance was measured at 415 nm. Total flavonoid content of the extracts was calculated from the regression equation of the quercetin calibration curve, and the results were expressed as quercetin equivalents (mg QE/g).

Acetylcholinesterase inhibition

The tests were conducted based on the Ellman´s spectrophotometric method with slight modification (Ellman *et al*., 1961). Galantamine was applied as the standard compound. In a 1.5 mL cuvette, 0.1 mL of sodium phosphate buffer (100 mM, pH 8), 0.1 mL of sample, and 0.1 mL AChE solution containing 0.54 U/mL, were mixed and allowed to incubate for 15 min at 37°C. After that, 0.1 mL of a solution of acetylcholine iodide (15 mM) and 0.5 mL of 3 mM Ellman's reagent were added and the absorbance at 405 nm was read after 5 min of the reaction. The percentage of AChE inhibition was calculated based on the absorbance value as follows:

$$\%\ Inhibition = (1 - A_t/A_0) \times 100 \qquad (1)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. The IC_{50} value was determined by non-linear regression of the log inhibitor concentration versus the percentage of inhibition.

Evaluation of antioxidant activity against DPPH$^{\bullet}$

The antioxidant activity of analyzed extracts was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Blois, 1958). An aliquot of plant extracts (0.1 mL) was added to the DPPH$^{\bullet}$ solution (1 mL, 55 mM) and left to stand in the dark at room temperature for 30 min. After that, the absorbance of each mixture was measured at 517 nm. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\%\ DPPH^{\bullet} = (1 - A_t/A_0) \times 100 \qquad (2)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. The results are expressed as mg/mL of plant extract needed to reduce DPPH radical signal by 50% (IC_{50}).

Evaluation of antioxidant activity against ABTS$^{\bullet+}$

The 2,2´-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) radical cation (ABTS$^{\bullet+}$) was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to react in a dark at room temperature over night (Re *et al*., 1999). Different concentrations of the plant extracts (0.1 mL) were mixed to 1 mL ABTS$^{\bullet+}$ solution. After mixing and incubating at room temperature for 7 min, the absorbance was recorded at 734 nm. The ability to scavenge the ABTS$^{\bullet+}$ was calculated using the following equation:

$$\%\ ABTS^{\bullet+} = (1 - A_t/A_0) \times 100 \qquad (3)$$

where A_0 is the absorbance of the control and A_t is the absorbance of the tested plant extract. From the percentage of the scavenging activity at different tested extracts concentrations, IC_{50} values were calculated.

Antimicrobial assays

For the investigation of potential antimicrobial activity of three *Cotoneaster* species extracts, the following Gram-negative and Gram-positive bacteria, as well as fungi were tested: *Salmonella enterica* serovar Enteritidis ATCC 31194, *Pseudomonas aeruginosa* ATCC 9027, *Escherichia coli* ATCC 25922, Extended Spectrum Beta-Lactamase producing *E. coli* or ESBL *E. coli* ATCC 35218, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, Methicillin-resistant *Staphylococcus aureus* or MRSA ATCC 33591, *Bacillus subtilis* ATCC 6633 and *Candida albicans* ATCC 1023. Antimicrobial effects were evaluated through the agar well diffusion method (Balouiri, Sadiki and Ibnsouda, 2016). Standard antibiotic Ampicillin (10 µg; HiMedia Laboratories Pvt.Ltd., India) and antimycotic Nystatin (100 units; Oxoid Ltd., England) were used as positive controls, while DMSO was used as solvent control. Tested microbial species were cultured overnight at 37°C, in Mueller Hinton medium and Sabouraund Glucose Agar (*Fluka* Biochemica; Buchs, Switzerland). Inoculums were diluted in sterile saline solution and adjusted to the final density of 0.5 McFarland standard (~1.5×10^8 CFU/mL), according to Wayne (2007). In order to achieve a total absorption, after the spreading of inoculums over the plates, they are left for 15 minutes at ambient temperature. Investigated extracts and controls (100 µL) were transferred into the wells of inoculated plates and incubated for 18-24 hours at 37°C, and 24-48 hours at 37°C, for bacteria and fungi respectively.

Antimicrobial activity of investigated *Cotoneaster* extracts was evaluated on the basis of inhibition zones diameter (mm), which is the result of extract diffusion in the medium and inhibition of microbial growth.

Statistical analysis

All tests were performed in three replications and the mean values ± standard deviation (SD) were calculated. Descriptive statistical analyses were carried out by Microsoft Office 2013 Excel (Microsoft Corporation, Redmond, USA). Data were further analyzed by using one-way ANOVA and *post hoc* Newman-Keuls test (STATISTICA 10; StatSoft. Inc.), at the significance level of $p<0.05$.

RESULTS AND DISCUSSION

Phenolic compounds, which are widely found as secondary metabolites in plants, are known to act as biologically active molecules. Many phenolic compounds, including flavonoids are known to have potent antiviral, anticancer, antioxidant, antibacterial or anti-inflammatory activities (Aziz *et al*., 1998; Galati and O'Brien, 2004; Kicel *et al*., 2016). The content of total phenols and flavonoids in the analyzed extracts pointed that these species are rich source of polyphenolic compounds. The amount of total phenolics in extracts ranged between 65.04 and 135.86 mg GAE/g, while the content of flavonoids ranged from 2.76 to 18.17 mg QE/g (Table 1). The highest content of total phenols and flavonoids was found in the *C. tomentosus* leaf extract,followed by the leaf extract of *C. integerrimus*, 133.54 mg GAE/g and 16.42 mg QE/g respectively, while the corresponding bark extract of *C. tomentosus* showed the lowest content of total phenolics and flavonoids. It is noticeable that among all analyzed extracts, the content of total phenolic compounds and flavonoids in all samples is higher in the leaves. Results obtained in this study strongly suggest that phenolics are important components of these plants. The one-way ANOVA and Newman-Keuls test showed statistically significant differences in phenolic and flavonoid contents among all tested extracts.

Table 1: Phenolic compounds, antioxidant activity and acetylcholinesterase inhibition of investigated extracts

Sample	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)	DPPH - IC_{50} (mg/mL)	ABTS - IC_{50} (mg/mL)	AChE - IC_{50} (mg/mL)
C. integerrimus					
LE	133.54±2.81^a	16.42±0.35^b	1.23±0.02^e	0.20±0.01^e	0.88±0.03^b
BE	80.10±0.41^d	9.38±0.27^d	2.99±0.06^b	0.73±0.02^b	n.d.
C. tomentosus					
LE	135.86±1.29^a	18.17±0.30^a	1.22±0.04^e	0.12±0.01^f	0.73±0.01^c
BE	65.04±0.82^e	2.76±0.20^f	3.73±0.02^a	0.87±0.03^a	0.35±0.01^d
C. horizontalis					
LE	93.21±1.61^b	10.55±0.51^c	2.15±0.03^d	0.38±0.01^d	0.34±0.01^d
BE	82.97±1.57^c	8.73±0.28^e	2.50±0.03^c	0.42±0.01^c	0.94±0.03^a
Positive control	-	-	0.06±0.01^f	0.01±0.00^g	0.11±0.01^e

The results are the mean ± SD (n=3). LE = leaf extract. BE = bark extract. n.d. = not detected.
Values in the same column that don't share the same letters, differ significantly at $p<0.05$ after *post hoc* Neuman-Keuls test.

The literature data on the phenolic and flavonoid content of *Cotoneaster* species are scarce and present highly variable results. The results by Uysal *et al*. (2016) showed that the total phenol content in the methanolic extract of twigs of *C. integerrimus* is 115.15 mg GAE/g, while the flavonoid content was 16.29 mg RE/g. In addition, the study of Mohamed *et al*. (2012) analysed the aerial parts of *C. horizontalis* for total phenols and flavonoids content

and obtained 14.00 mg GA/g and 6.80 mg RE/g respectively. The investigation of antioxidant activity was carried out to assess the ability of extracts of the three *Cotoneaster* species to scavenge free radicals by the ABTS and DPPH methods (Table 1). The obtained results were presented as IC_{50} values. In the DPPH method, IC_{50} values of extracts ranged from 1.22 to 3.73 mg/mL, while for the ABTS method values varied from 0.12 to 0.87 mg/mL. The best antioxidant activity showed the leaf extract of *C. tomentosus* and the lowest activity had the bark extract of the same species. Generally, extracts of leaves had better activity than bark extracts for both methods. Usually, the IC_{50} values for the DPPH method are higher than the value of ABTS (although they are based on the same reaction mechanism), due to possible steric hindrances in the case of DPPH. Also, some substances present in extracts can participate in the inactivation of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ (Bernatonienė *et al*., 2008). Previous studies are in lack of data on antioxidant activity of *Cotoneaster* species, and the published results showed divergence to our results (Sokkar *et al*., 2013; Kicel *et al*., 2016; Uysal *et al*., 2016). In addition, inhibition of AChE was investigated (Table 1). The obtained IC_{50} values were varied from 0.34 for the *C. horizontalis* leaf extract to 0.94 mg/mL for the corresponding bark extract. All extract showed significantly lower inhibition of AChE than galantamine (0,11±0.01) which was used as a positive control. Nevertheless, the *C. horizontalis* leaf sample showed moderate inhibitory activity (0.34±0.01) due to the presence of horizontoates A – C in this species which showed remarkable activity according to Khan *et al*. (2014).

The results regarding antimicrobial properties of the studied *Cotoneaster* leaf and bark extracts indicate that three investigated species exhibit antimicrobial activity against all microorganisms tested (Table 2).

Table 2: Zones of inhibition obtained through the agar well diffusion method

Strain / Extract	*C. integerrimus*		*C. tomentosus*		*C. horizontalis*		Positive control
	LE	BE	LE	BE	LE	BE	
S. enterica	20.12^{a}±0.71	17.24^{c}±0.63	18.01^{c}±0.21	$18.01^{c,b}$±0.53	16.82^{c}±1.50	$19.49^{b,a}$±0.86	16.03^{c}±0.07
P. aeruginosa	21.50^{a}±0.79	19.20^{b}±1.41	15.00^{d}±0.20	20.56^{b}±0.70	18.50^{c}±0.73	$18.50^{b,c}$±0.74	13.02^{e}±0.09
E. coli	$18.34^{a,b}$±0.59	19.27^{a}±0.46	12.10^{c}±0.22	18.67^{a}±0.28	16.56^{b}±2.02	18.21^{a}±0.23	8.96^{d}±0.16
ESBL *E. coli*	20.22^{a}±1.13	17.76^{b}±0.59	14.67^{d}±0.58	16.50^{c}±0.50	19.35^{a}±0.70	19.67^{a}±0.29	NI^{e}
E. faecalis	14.41^{b}±1.75	11.64^{c}±1.41	14.72^{b}±0.44	10.89^{c}±0.29	10.98^{c}±0.46	13.65^{b}±1.19	16.94^{a}±0.23
S. aureus	18.63^{b}±0.40	17.66^{c}±0.37	15.09^{e}±0.31	17.30^{c}±0.34	16.09^{d}±0.34	17.98^{c}±0.47	33.03^{a}±0.09
MRSA	$12.80^{b,c}$±1.93	14.74^{a}±0.45	$14.15^{a,b}$±0.14	$13.17^{a,b,c}$±0.50	12.22^{c}±0.23	12.03^{c}±0.50	NI^{d}
B. subtilis	20.48^{b}±0.45	18.77^{c}±0.52	19.38^{c}±0.56	19.18^{c}±0.55	17.73^{d}±0.38	20.97^{b}±0.49	47.98^{a}±0.23
C. albicans	30.50^{a}±0.50	27.66^{b}±1.33	21.83^{d}±0.35	24.36^{c}±0.71	30.40^{a}±0.53	25.04^{c}±0.83	21.12^{d}±0.24

The results are the mean ± SD (n=3). LE = leaf extract. BE = bark extract. NI = No inhibition zone.
Values in the same column that don't share the same letters, differ significantly at $p<0.05$ after *post hoc* Neuman-Keuls test.

This research showed significantly high inhibition of both Gram-positive and Gram-negative bacteria, as well as fungi, by tested extracts. Largest inhibition zones are noted in case of *C. albicans*, especially with *C. integerrimus* leaf extract (30.50±0.50 mm). This plant species caused the greatest zones of inhibition as well as in most investigated bacterial strains (Table 2). According to Uysal *et al*. (2016), methanolic extracts of *C. integerrimus* have significant antimicrobial potential and their study indicate phenolic components, especially epicatechin, responsible for antimicrobial activity. To our knowledge, this is the first report on antimicrobial properties of *C. tomentosus*. In addition to the antifungal effects observed against *C. albicans*, it is particularly interesting that *C. tomentosus* bark extract caused the greatest inhibition of *P. aeruginosa* growth (20.56±0.70 mm), which is known as a versatile pathogen with multidrug-resistance (Hirsch and Tam, 2010). *Cotoneaster horizontalis* proved to be most effective against *C. albicans* (30.40±0.52 mm), followed by *B. subtilis* (20.97±0.49 mm) and *P. aeruginosa* (20.00±0.74 mm). According to Mohamed *et al*. (2012), *C. horizontalis* is rich in polyphenols, flavonoids, hydrocarbons, phytosterols and different fatty acids, and it contain essential oil with many oxygenated compounds, as well as in phenolic acids (Mohamed *et al*., 2012; Sytar *et al*., 2016). Furthermore, this plant species has been recognized as an important source of antioxidative and anticancer compounds such as α-tocopherol and amygdalin (Sokkar *et al*., 2013). While previous studies have reported the resistance of only some Gram-negative strains to the *Cotoneaster* extracts (Zengin *et al*., 2014; Kanaani *et al*., 2015; Siami *et al*., 2016), this research showed that all investigated bacteria, including the multidrug-resistant pathogens were sensitive to the methanolic extracts of *C. integerrimus*, *C. tomentosus* and *C. horizontalis*.

CONCLUSIONS

This investigation shows that the leaf and bark extracts of three *Cotoneaster* species collected in Bosnia and Herzegovina possess significant and dose-dependent *in vitro* antioxidant activity, which positively correlate with their total phenolic content. Based on these facts, it is possible to justify the highest phenolics and flavonoid content for the *C. tomentosus* leaf extract. Different correlations were observed with AChE inhibition results. The *C. horizontalis* sample that showed an average content of total phenols and flavonoids simultaneously

shows the highest (leaves) and lowest (bark) activity by inhibition. In addition, this study shows a remarkable antimicrobial activity of the tested extracts against both Gram-positive and Gram-negative bacteria, as well as fungi. However, the compounds responsible for the bioactive properties of all three investigated *Cotoneaster* species remain unclear, so future research should be directed towards solving the detailed chemical composition and isolation of individual bioactive components.

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Summary/Sažetak

Iako rod *Cotoneaster* Medik. obuhvata uglavnom ukrasne vrste, postoje podaci i o njihovom bioaktivnom djelovanju. Cilj ovog istraživanja bio je analizirati sadržaj fenolskih spojeva, inhibiciju acetilholinesteraze, te antioksidativno i antimikrobno djelovanje metanolnih ekstrakata lista i kore vrsta *C. integerrimus* Medik, *C. tomentosus* (Aiton) Lindl. i *C. horizontalis* Decne. Dok ekstrakt lista *C. tomentosus* ima najveći sadržaj fenolskih spojeva (135.86 mgGAE/g) i flavonoida (18.17 mgQE/g), ali i najznačajniju antioksidativnu aktivnost spram nebiogenih slobodnih radikala, ekstrakt lista *C. horizontalis* je pokazao najveću inhibitornu aktivnost AChE (IC_{50} 0.34 mg/mL). Svi ekstrakti su pokazali značajan nivo antibakterijske i antifungalne aktivnosti spram testiranih mikrobnih sojeva. Najveće zone inhibicije su uočene kod vrste *Candida albicans* tretirane ekstraktom lista *C. integerrimus* (30.50±0.50 mm). Nadalje, ekstrakt *C. integerrimus* je bio najučinkovitiji i kod većine testiranih bakterijskih sojeva. Dobijeni rezultati sugerišu da metanolni ekstrakti analiziranih vrsta roda *Cotoneaster* posjeduju obećavajući bioaktivni i terapeutski potencijal.