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IN-VITRO ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF AERIAL PARTS OF *PRUNUS SPINOSA* L. GROWING WILD IN BOSNIA AND HERZEGOVINA

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Keywords:

Prunus spinosa L., Extraction, Antioxidant activity, Antimicrobial activity, Mineral content

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ABSTRACT: Phytotherapeutical properties of blackthorn (*Prunus spinosa* L.) are well known and used in Bosnia and Herzegovina traditional medicine. This study aimed to investigate total phenolic content, total anthocyanin content, total flavonoid content, antioxidant and antibacterial activities of different ethanolic extracts from wild blackthorn flowers, leaves and fruits, obtained by microwave-assisted extraction and ultrasound-assisted maceration. The mineral profile has been identified using flame atomic absorption spectrophotometry. Plant samples were collected from three areas in Bosnia and Herzegovina. In order to detect antioxidant activity of extracts, DPPH assay was carried out. DPPH radical scavenging activity was higher for flower and fruit extract than leaf extracts, regardless of the extraction method used. Ethanolic extracts show significant antimicrobial activity against all analyzed bacterial strains (*Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Salmonella enterica*, *Escherichia coli*, *Pseudomonas aeruginosa* and antifungal activity against *Candida albicans*). Blackthorn fruits contained high mass fractions of some essential minerals on dry mass basis, including K, Ca, Mg, Na, Fe, and Cu. Blackthorn flower, leaf and fruit extracts exhibit a high phenolic content and a high antioxidant activity and can be used as a potent antioxidant in food and pharmaceuticals.

INTRODUCTION: Fruits of wild-grown bushes have been used for a long time in traditional medicine, and fruits are also used in the food industry for the production of jams, tea or juices. In many societies, consumers are interested in nutritional quality, especially in healthy food [1]. They prefer to choose products that may have a particularly positive impact on their health, rather than those with desired taste.

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The food manufacturers try to develop new food products that will be attractive to the widest potential consumers and become competitive in the market. Such materials are traditional products that are least processed like wild grown fruits, which are often a good source of healthy compounds, as well as antioxidants [2].

One such plant might be *Prunus spinosa* L. (blackthorn or sloe); a traditional medicinal plant from the Rosaceae family. It is one of the rarely applied sources of healthy food. In European tradition, blackthorn has been known for over 7000 years, at first as a source of edible fruits and then also as a medicinal plant [3]. In traditional medicine of Bosnia and Herzegovina, it has also been used to treat various diseases.

It contains substantial quantities of phenolic compounds [4, 5], including specifically, flavonol heterosides (quercetin and kaempferol), phenolic acids (neochlorogenic and caffeic derivatives), coumarin derivatives as aescu-letin, umbelliferone and scopoletin, anthocyanins and type A proantho-cyanidins, class of secondary metabolites consisting of units of flavan-3-ol bound together by one or two interflavan bonds, which are considered to be one of the strongest natural antioxidants. Anthocyanins belong to the group of phenolic compounds and are responsible for color in fruits and vegetables [2].

In literature, there are results of phytotherapeutic activities of *Prunus spinosa* L. for the treatment of many diseases related to various forms of cough; it is a mild laxative, diuretic, spasmolytic and anti-inflammatory agent [6]. It has an anti-septic effect because of the presence of tannins and shows activity against inflammation of the mucosal layer of the digestive system. Marchelak group [6] wrote about flower extracts of *Prunus spinosa* L. indicated for the treatment of urinary tract disorders, inflammation, and adjunctive therapy of cardiovascular diseases. Phenolic compounds, as free radical scavengers, are very important and they are present almost in all fruits and vegetables in varying quantities [7]. Fresh fruit extracts are an excellent source of polyphenolic compounds, which can significantly alleviate the negative effect of free radicals in the organism. Therefore, they have an important role in the prevention of neurodegenerative diseases, cardiovascular diseases, and cancer [8].

It has been known that metals such as magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), iron (Fe), manganese (Mn), cobalt (Co), copper (Cu), chromium (Cr), nickel (Ni), zinc (Zn), molybdenum (Mo), selenium (Se) are essential nutrients that are required for various biochemical and physiological functions in the human organism. Inadequate supply of these essential elements results in a variety of deficiency diseases (WHO). For normal growth and completion of their life cycle, plants are in need of at least sixteen elements. They need a relatively large amount of nitrogen, potassium, and phosphorus [9]. In this paper, due to their importance, the effects of mineral elements were determined in fruit samples of blackthorn.

Blackthorn grows wild in various regions in Bosnia and Herzegovina. All plants, especially fruits, are used in traditional medicine for different purposes. Flowers in the form of tea are most commonly used for gastrointestinal problems such as vomiting and diarrhea and for high fiver treatment. Usage of fruit extracts is associated with cardiovascular problems. Furthermore, the usage of fresh fruits as food is also used for the treatment of erectile activity problems [8]. The aim of this study was to investigate the influence of extraction methods of blackthorn's aerial parts on the antioxidant and antimicrobial activity as well as mineral content.

MATERIAL AND METHODS: All the solvents, reagents, and standards used were of analytical grade. Folin-Ciocâlteu reagent and anhydrous sodium carbonate were obtained from Kemika (Zagreb, Croatia), Gallic acid, acetic acid, potassium chloride, sodium acetate, cyanidine-3-galactoside were purchased from Fluka Chemika (Switzerland), ethanol was obtained from Merck (Darmstadt, Germany), 1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid (Trolox), hydrochloric acid and $AlCl_3$ were obtained from Sigma Aldrich (St. Louis, USA). Mueller Hinton Agar from HiMedia laboratories, India. Chemicals and reagents Mineral standards were purchased from Merck (Darmstadt, Germany).

Plant Material: Fresh flowers, leaves and fruits of wild blackthorn were collected from three different areas in Bosnia and Herzegovina (Borije, Trnovo and Vareš) between April and September of 2018 **Fig. 1**.

FIG. 1: BLACKTHORN FRUITS

The plant was identified in the Department of Biology, Faculty of Sciences, and the University of Sarajevo, Bosnia, and Herzegovina, and deposited in the herbarium. Samples are vouchered in the laboratory of Organic Chemistry at the Faculty of Pharmacy, University of Sarajevo.

Microwave-Assisted Extraction (MAE) and Ultrasound-Assisted Maceration (UAM) of Blackthorn Flowers, Leaves and Fruits: Various extraction methods, including conventional and non-conventional extraction methods, can be used to extract molecules responsible for antioxidant and antimicrobial activity in food and medicinal plants [10, 11]. Ultrasound, microwave, pressurized liquid, enzyme hydrolysis, supercritical fluids, high hydrostatic pressure, pulsed electric field, and high voltage electrical discharges have been studied as non-conventional extraction methods to obtain antioxidant compounds from plants in an energy-efficient and economically sustainable way [12]. In this study, two non-conventional methods were used: MAE and UAM.

Ethanolic extracts were prepared from air-dried plant material (dried at room temperature in the dark). Using a single-mode focused microwave reactor Merck, MW 500 MAE experiments were performed. A mass (1.0±0.001) g of fine powdered blackthorn flowers, leaves, and fruits was mixed with 20 mL of ethanol and placed in a round-bottom PTFE flask. The duration of extraction was one minute at the temperature of 60 °C. The extracts were centrifuged at 4000 rpm for 10 minutes, and the supernatant was collected.

Ultrasonic Homogenizer Iskra, UZ 4R was used for UAM. The extraction temperature was kept constant at 30±1 °C using a water bath. Air-dried flowers, leaves, and fruits (2.0±0.001) g were ground into fine powder and extracted with 20 mL of ethanol by ultrasound maceration for 20 min.

The supernatant was separated after UAM extracts were exposed to centrifugation at 4000 rpm for 10 minutes. All MAE and UAM extracts were stored in glass vials at 4 °C and used for the spectrophotometric determination of TPC, TAC, TFC, antioxidant and antimicrobial activity. All extracts for each sample of leaf, flower, and fruits were prepared in triplicate.

Determination of Total Phenolic Content (TPC): TPC of ethanolic extract of samples was determined spectrophotometrically with Folin-Ciocâlteu reagent [13]. Two milliliters of each sample were added in 10 mL Folin-Ciocâlteu's reagent previously diluted 1/10 with distilled water. After a few minutes, sodium carbonate (8 mL) was added. This solution was stored in dark place for two h and after that the absorbance was measured at 765 nm. A standard curve was prepared using Gallic acid as standard in a concentration range from 100 to 500 μg/mL. Results are expressed as mg of Gallic acid equivalents (mg GAE/g) per g of plant material.

Determination of Total Anthocyanin Content (TAC): TAC was quantified by using a pH differential method [14]. All samples (2 mL) were diluted in two buffer solutions: potassium chloride buffer 0.025 M (pH 1.0) and sodium acetate buffer 0.4 M (pH 4.5), then incubated for 15 minute at room temperature. Absorbance was measured simultaneously at 525 nm and 700 nm using distilled water as blank. The Spectronic GENESYS TM2 UV-Vis spectrophotometer was used for determination. TAC is expressed as mg of cyanidin-3-glucoside equivalents (mg CGE/g) per g of plant material. A molar extinction coefficient (ε) of cyanidin-3-glucoside of 26900 L/mol cm and molar weight (M_W) (449.2 g/mol) was used for calculations of TAC. The anthocyanin content was calculated according to the following equation:

$$TAC = 1000 \times A \times M_w \times D_f / \varepsilon \times l \quad (1)$$

Where:

$$A = (A_{525} - A_{700})\text{pH}_{1.0} - (A_{525} - A_{700})\text{pH}_{4.5} \quad (2)$$

M_W is the molecular weight of cyaniding-3-glucoside, D_f is dilution factor, ε is molar absorbance, and l is the path length.

Determination of Total Flavonoid Content (TFC): The TFC of all selected plant material ethanolic extracts was determined using the aluminium chloride colorimetric method described by Faheem *et al.* [15] Stock solution of each sample extract (0.50 mL) was mixed with 2 mL of distilled water and 0.15 mL of 5% $NaNO_2$ solution. After 6 min of incubation 0.15 mL of 10% $AlCl_3$ solution was added. The mixture was allowed to stand for

additional 6 min. and then 2 mL of 1 mol/L NaOH solution was added. The final volume of the solution was diluted with distilled water to 5 mL. After 15 minutes the absorbance of the mixture was measured at 510 nm. Quercetin (100-500 µg/mL) was used as standard. The total flavonoid content was calculated from a calibration curve, and the results were expressed as mg of quercetin equivalent per g of plant material (mg QE/g).

DPPH Radical Scavenging Activity Assay (RSA): The DPPH radical scavenging activity of flowers, leaves, and fruits extracts was measured according to the standard method described by Baranowska and Bajkacz [16]. According to this method, extracts were mixed with ethanol (96%) and 61 µmol/L solutions of DPPH and incubated at room temperature for 30 min. Trolox (6-hidroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was used as standard, and the absorbance was measured at 517 nm. The comparative analysis of samples was made by calculating DPPH scavenging activity, which stands for the relative decrease of absorbance in the samples analyzed. DPPH scavenging activity converted into percentage was calculated using the following equation:

$$RSA = 100 \times (A_c - A_s)/A_c \quad (3)$$

Where A_c is absorbance of the control at 517 nm and A_s is absorbance of the sample at 517 nm.

Mineral Content Analysis: The fruit samples were dried at 105 °C and ground with mortar and pestle in preparation for chemical analyses. To quantify the mineral content, 1.5±0.0001 g of blackthorn fruits powder from three areas in Bosnia and Herzegovina were digested with 10 mL of 67% HNO_3. The samples were cooled, and 5 mL of H_2O_2 was added to each. The solution was diluted with distilled water before analysis. Flame atomic absorption spectrometry (FAAS) was used to estimate and evaluate the levels of mineral content using a flame atomic absorption spectrophotometer (AA240FS, Varian). For each metal, calibration curves were recorded in the optimal concentration ranges.

Antimicrobial Activity (Bacterial Strains): Standard bacterial strains belonging to Gram-positive bacterial strains (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212), Gram-negative bacterial strains (*Salmonella enterica* ATCC 31194, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027) and fungi *Candida albicans* ATCC 1023 were used in this study. They were obtained from the Faculty of Science (University of Sarajevo, Bosnia, and Herzegovina). The test bacteria strains were cultured on Petri dishes containing Mueller Hinton Agar (MHA) and incubated for 18–24 h.

Antimicrobial Activity Detection by Agar Well Diffusion Method: The *in-vitro* antimicrobial and antifungal activity of ethanolic extracts were tested by agar well diffusion method against Gram-positive and Gram-negative bacterial strains. Antibiotic streptomycin (10 µg, Oxoid) and antimycotic nystatin (10 µg, Oxoid) were used as a positive control and 96% ethanol as a negative control. Cavity (wells) was created in the MHA using a sterile Pasteur pipette. A freshly prepared bacterial suspension or spore solution adjusted to 1.5×10^8 CFU/mL was inoculated onto the surface of agar plates using a sterile swab. Wells were filled with 100 µL of each ethanolic extract. The plate was left at +4 ºC for 2 h to facilitate the diffusion of the extracts in the agar [17] and then incubated at 37 °C for 18-24 h. Experiments were performed in triplicate. The diameters of inhibition zones were measured around the well.

Statistical Analyses: All analyses were carried out in triplicate and data was presented with basic descriptive statistical parameters (arithmetic mean - µ, standard deviation - SD).

Analysis of variance (ANOVA) was applied to determine the significance of differences between experimental parameters. The difference in mean values between the analyzed factors was compared with the Newman-Keuls post hoc test for the significance level of $p<0.05$. These analyses were performed in the STATISTICA 10 software (StatSoft. Inc.).

RESULTS AND DISCUSSION:

Total Phenolic, Anthocyanins and Flavonoid Content: TPC, TAC, and TFC, as well as antioxidant activity, expressed as RSA, obtained from different plant parts of *Prunus spinosa* L. are

presented in **Table 1**. The amount of TPC for flower, leaf, and fruit ethanolic extracts expressed as GAE obtained by MAE was found to be 36.81 to 54.45; 17.78 to 27.45 and 4.53 to 6.87 mg/g. Using the Folin-Ciocâlteu assay TPC for extracts prepared by UAM was 19.67 to 24.41; 8.31 to 13.12 and 2.766 to 4.116 mg/g, respectively. Maximum extractable total phenolics were recorded in flower extract from Borije (54.45 mg GAE/g) followed by flower extract from Vareš (44.41). Both extracts were prepared using MAE. Generally, flower and leaf extracts contain higher amounts of TPC in comparison with fruit extracts. All results are expressed as mean ± standard deviation (M±SD).

Presented results for TPC of flower extracts obtained by MAE were in good agreement with the literature results of Lovrić *et al*., [18], which ranges from 45.2 to 63.7 mg GAE/g for blackthorn flower extracts.

TABLE 1: TOTAL PHENOLIC CONTENT, TOTAL ANTHOCYANIN CONTENT, TOTAL FLAVONOID CONTENT AND DPPH RADICAL SCAVENGING ACTIVITY OF FLOWER, LEAF AND FRUIT EXTRACTS FROM THREE DIFFERENT AREAS IN BOSNIA AND HERZEGOVINA

Flowers extracts	Total phenolic content/(mg GAE/g)	Total anthocyanins content/(mg CGE/g)	Total flavonoid content/(mg QE/g)	DPPH radical scavenging activity/%
Borije (MAE)	54.45±0.12^{a}	-	1.551±0.001^{a}	62.698±0.001^{d}
Trnovo (MAE)	36.81±0.06^{c}	0.339±0.063^{b}	1.129±0.001^{c}	62.434±0.002^{d}
Vareš (MAE)	44.41±0.06^{b}	-	1.547±0.001^{b}	68.915±0.001^{b}
Borije (UAM)	24.41±0.03^{d}	-	0.853±0.005^{d}	67.460±0.001^{c}
Trnovo (UAM)	19.67±0.23^{f}	0.718±0.058^{a}	0.501±0.001^{f}	67.725±0.002^{c}
Vareš (UAM)	20.61±0.03^{e}	0.056±0.029^{c}	0.677±0.001^{e}	75.794±0.002^{a}
Leaves extracts				
Borije (MAE)	17.78±0.10^{c}	-	0.386±0.001^{c}	9.656±0.002^{d}
Trnovo (MAE)	27.45±0.12^{a}	1.353±0.060^{b}	0.558±0.001^{a}	7.540±0.001^{e}
Vareš (MAE)	22.81±0.06^{b}	-	0.479±0.001^{b}	7.937±0.002^{e}
Borije (UAM)	8.31±0.03^{f}	4.016±0.036^{a}	0.191±0.003^{f}	20.238±0.001^{c}
Trnovo (UAM)	11.56±0.03^{e}	1.364±0.032^{b}	0.238±0.003^{e}	30.820±0.001^{a}
Vareš (UAM)	13.12±0.03^{d}	0.746±0.067^{c}	0.282±0.001^{d}	27.249±0.001^{b}
Fruit extracts				
Borije (MAE)	6.87±0.01^{a}	2.271±0.203^{a}	0.123±0.001^{b}	71.693±0.001^{b}
Trnovo (MAE)	4.53±0.01^{c}	0.746±0.092^{d}	0.121±0.001^{c}	44.444±0.001^{c}
Vareš (MAE)	6.75±0.01^{b}	0.919±0.121^{e}	0.149±0.001^{a}	76.852±0001^{a}
Borije (UAM)	4.116±0.003^{d}	0.679±0.056^{f}	0.0934±0.0003^{d}	65.476±0.001^{b}
Trnovo (UAM)	2.766±0.003^{f}	1.258±0.029^{b}	0.0664±0.0003^{e}	39.153±0.001^{d}
Vareš (UAM)	2.997±0.003^{e}	1.108±0.013^{d}	0.064±0.001^{f}	71.561±0.002^{b}

The data are presented as mean value±standard deviation of triplicate analyses. Mean with different letters in superscript within a column are statistically different at p≤0.05

TPC for blackthorn leaf and fruit extracts was slightly lower. However, the TPC of all extracts obtained using MAE was significantly higher than TPC of extracts obtained using UAM. Under the influence of microwaves, a polar solvent such as ethanol is exposed to strong rapid heating, which facilitates faster penetration into the cell walls.

That induces tremendous pressure that causes cracks in the cell walls, which increase the extraction of phytoconstituents such as TPC, TAC, TFC *etc*. [19] It can be therefore concluded that the total phenolic content is higher in the extracts obtained by using MAE than those obtained using UAM, which can be seen in **Fig. 2a**, **Fig. 2b,** and **Fig. 2c**.

Anthocyanins are one of the largest and most important groups of water-soluble pigments in the plant kingdom. They are accumulated in cell vacuoles and responsible for diverse pigmentation from orange to red, purple, and blue in flowers and fruits. Anthocyanins in fruits and vegetables are present in glycosylated forms. Depending on the nutritional habits, the daily intake of anthocyanins for individuals has been estimated from several milligrams to hundreds of milligrams per person. Anthocyanins fulfill a protective role against a variety of diseases, particularly cardiovascular disease and some types of cancer [20]. Because of this, it is very important to define the total content of anthocyanins in extracts.

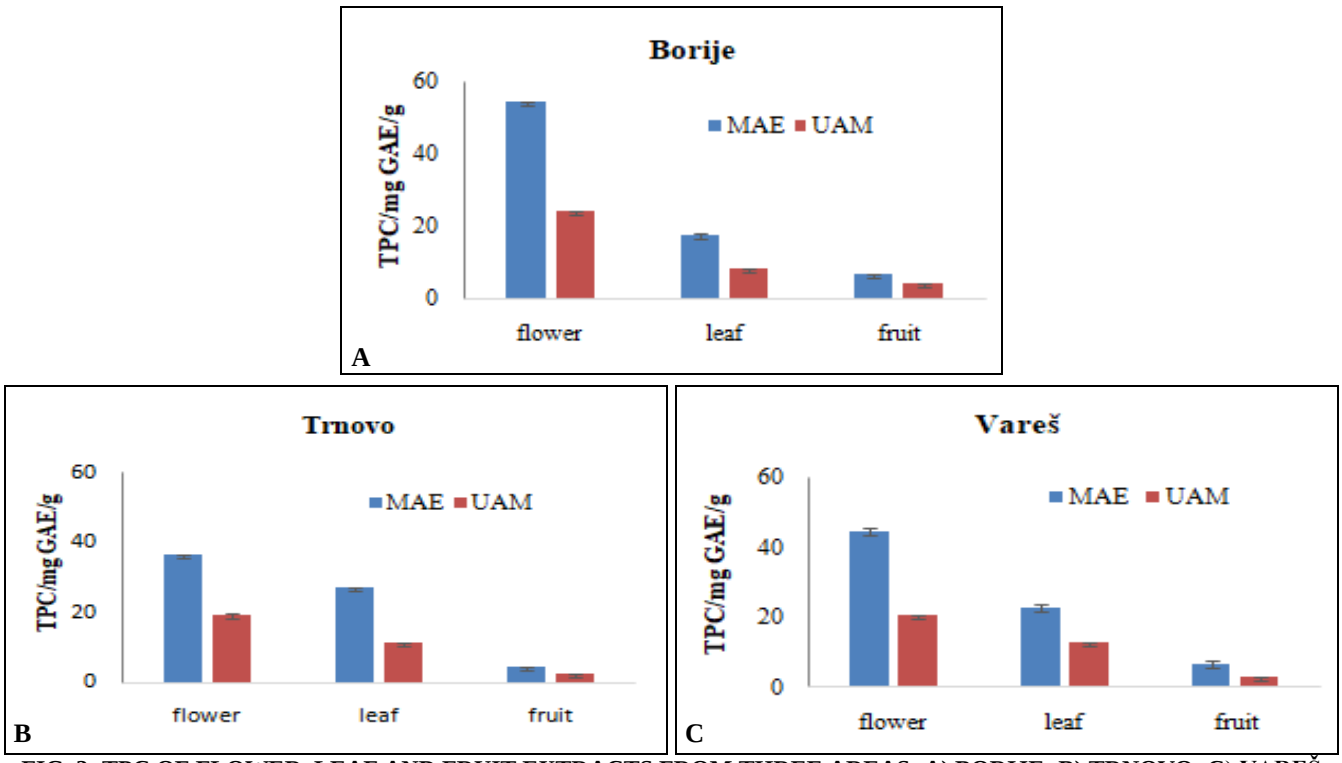

FIG. 2: TPC OF FLOWER, LEAF AND FRUIT EXTRACTS FROM THREE AREAS: A) BORIJE; B) TRNOVO; C) VAREŠ

The total content of anthocyanins was not detected in all samples as was expected. This is due to the type of samples as water extracts were not analyzed but ethanolic. Content of anthocyanins lies between 0.746 to 2.271 mg CGA/g for fruit extract samples obtained using MAE and UAM. Other results for TAC are shown in **Table 1**.

The content of total flavonoids for all extracts is very low and lies between 0.064 to 1.551 mg QE/g of dry flowers, leaves, and fruits **Table 1** as we used dried plant samples. The TFC in different blackthorn extracts obtained from fresh fruit in a study of Veličković *et al*., [8] was higher than in the present study. It ranges from 0.700 to 1.31 expressed as mg QE/g of fresh fruit extracts as is expected to be in fresh fruit samples. However, it is difficult to compare results in this study with results in other studies due to the existence of differences in extraction method; solvents applied as well as differences in plant parts exposed to extraction.

Radical Scavenging Activity: The DPPH radical scavenging assay is a rapid, easy and sensitive method commonly used for the screening and evaluation of the antioxidant activity and free radical scavenging ability of plant extracts. The DPPH assay has been used to test the ability of compounds as free-radical scavengers or hydrogen donors and to evaluate the antioxidant activity of plant extract or foods [21]. The results of RSA for tested extracts are presented in **Table 1**. Usually, high content of phenolic and flavonoid compounds is responsible for antioxidant activity, which does not always have to be directly in correlation with radical scavenging activity since compounds such as terpenoids are also attributed to their antioxidant activity. An important fact in evaluating the antioxidant activity in plant extracts is their interaction with other antioxidants [22].

As it is visible from the results, there was a weak correlation between antioxidant activity and total phenolic, flavonoid, and anthocyanins content of extracts obtained by MAE and UAM. All investigated extracts exhibit good scavenging activity of DPPH radical, which ranges from 7.540 to 76.852%. The highest antioxidant activity showed fruit extract from Vareš obtained using UAM, and the lowest showed leaf extract from Trnovo obtained using MAE. UAM gave better results for an antioxidant activity for flower and leaf extracts than MAE from all three areas. Fruit extracts obtained by MAE showed better results for antioxidant activity than those obtained using UAM **Fig. 3a**, **Fig. 3b,** and **Fig. 3c**.

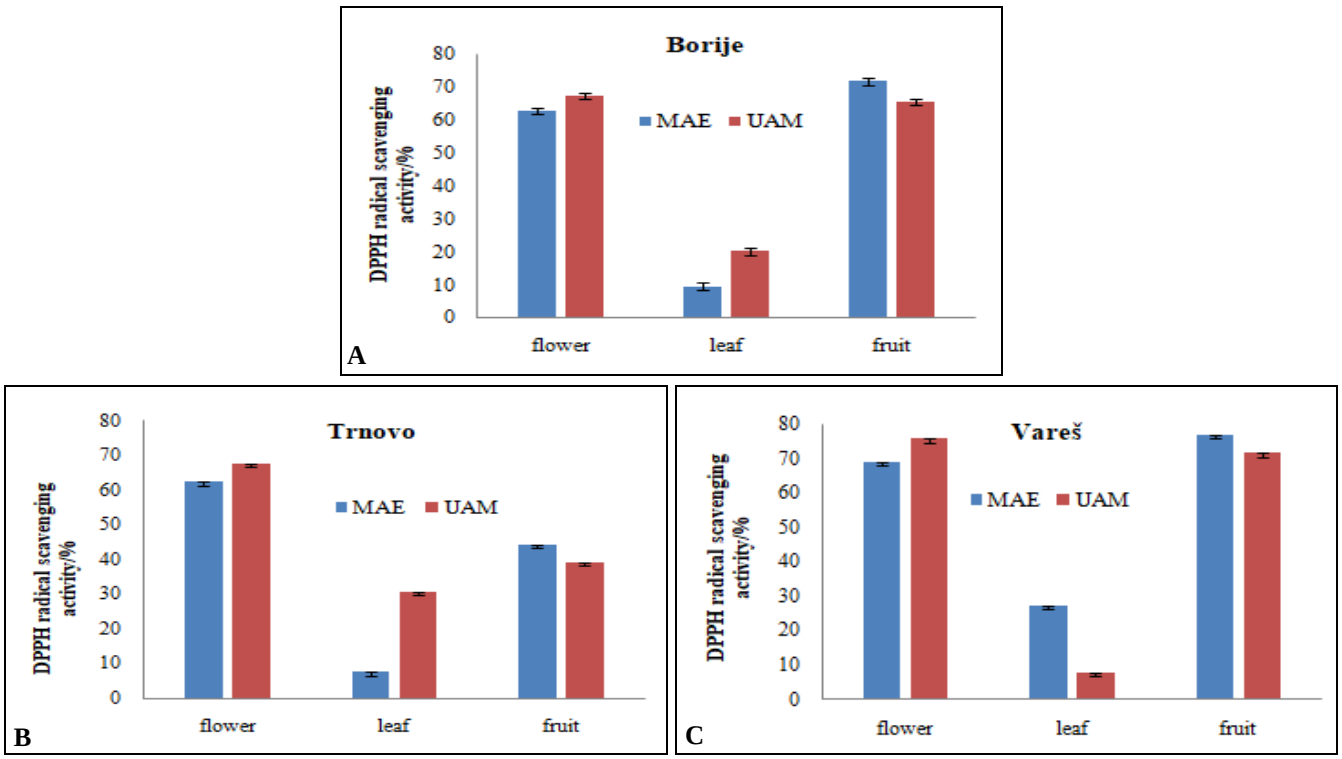

FIG. 3: RSA OF FLOWER, LEAF AND FRUIT EXTRACTS FROM THREE AREAS: A) BORIJE; B) TRNOVO; C) VAREŠ

The difference in the results for the total content of phenol, flavonoid, anthocyanin, and antioxidant activity can be explained by the type of extraction method used for the preparation of ethanolic extracts (MAE and UAE). Also, the antioxidant activity of blackthorn extracts depends on the area from which samples were collected. The content of polyphenols extracted from the plant matrix depends on the plant material, conditions of growth, plant maturity, and plant storage [23].

Mineral Profile of Blackthorn Fruit Samples: Mineral content of blackthorn fruits growing in Bosnia and Herzegovina were determined by FAAS and reported in **Table 2**. Potassium, calcium, magnesium, sodium, and iron were established as major minerals of the fruits. Calcium is the most abundant mineral in the body. It is essential for a number of vital functions, healthy bones, and teeth. Magnesium is an essential mineral present in all human tissues, especially in bones, and has important interrelationships with calcium, potassium, and sodium. Potassium and sodium balance fluids in the body, helps make muscles contract, and may benefit bones and blood pressure [24]. Others were determined at minor levels. All fruit extracts contained high amounts of K (1035.826-1245.379 µg/g), Ca (19.859-34.234 µg/g), Mg (8.574-11.827 µg/g), Na (2.560-12.223 µg/g) and Fe (3.399-9.167 µg/g). Some other essential minerals found in fruits samples of blackthorn were: Mn (0.796-2.379 µg/g), Cu (0.928-2.454 µg/g), Zn (0.350-1.803 µg/g), and Ni (0.154-0.212 µg/g).

TABLE 2: MINERAL CONTENT OF BLACKTHORN FRUITS

Minerals	*w*(minerals)/(µg/g)		
	Vareš	Borije	Trnovo
Cr	0.292±0.004	N.D*	N.D*
Cu	2.454±0.005	0.928±0.006	2.034±0.008
Mn	1.868±0.002	2.379±0.001	0.796±0.001
Fe	7.821±0.021	9,167±0,051	3.399±0.023
Ni	0.212±0.002	N.D*	0.154±0.005
Zn	1.803±0.006	0.350±0.006	0.551±0.008
Cd	0.150±0.005	N.D*	N.D*
Pb	N.D*	N.D*	N.D*
K	1035.826±0.029	1245.379±0.055	1202.822±0.002
Ca	34.234±0.015	22.356±0.006	19.859±0.030
Mg	11.827±0.02	10.228±0.002	8.574±0.012
Na	12.223±0.144	6.248±0.026	2.560±0.032

The data are presented as mean value±standard deviation of triplicate analyses. *N.D.= not detected, below limit of detection. Limit of detection of instrument in ppm.

Cadmium and lead are toxic elements. Their toxicity depends on several factors, including chemical species, the dose, route of exposure, as well as the age, gender, and nutritional status of exposed individuals [25]. In our study, lead was not detected in any fruit sample, but cadmium was detected in one sample (0.150 µg/g) from Vareš.

Chromium is a nutrient that is present in tissues in small quantities. It is required for some biochemical functions, but in huge quantities, it is toxic. Chromium was detected in the fruits sample from Vareš (0.292 μg/g).

Antimicrobial Activity of Blackthorn Fruit Extracts: Evaluation of the antimicrobial activity of ethanolic extracts of blackthorn flowers, leaves and fruits was screened against 7 microorganisms, including 6 bacteria and 1fungus, and their potency was determined by the diameters of the inhibition zones. These bacterial strains are Gram-positive and Gram-negative species frequently encountered in infectious diseases.

The observed antimicrobial activities were classified as follows: sensitive-inhibition zone, >18 mm; intermediate-inhibition zone, 13–17 mm; and resistance-inhibition zone, <13 mm [26]. They were then compared to the growth inhibition results obtained for the controls (Streptomycin for bacteria and Nystatin for fungi). **Tables 3, 4, 5** show inhibition zones for all tested extracts. The obtained results depend on the extraction method and tested microorganisms.

TABLE 3: ANTIMICROBIAL ACTIVITY OF BLACKTHORN FLOWER EXTRACTS FROM THREE AREAS

Strains	Inhibition zone/mm					
	MAE Borije	MAE Trnovo	MAE Vareš	UAM Borije	UAM Trnovo	UAM Vareš
Gram-positive strains						
Bacillus subtilis	17.00±3.46^{a}	13.67±0.58^{b}	13.33±0.58^{b}	8.34±1.15^{cd}	9.10±0.76^{cd}	8.92±2.52^{cd}
Staphylococcus aureus	12.00±0.00^{a}	12.00±1.00^{a}	11.17±0.29^{ab}	6.34±0.58^{c}	6.00±0.00^{c}	5.92±0.29^{cd}
Enterococcus faecalis	1.83±0.29^{ab}	14.00±1.00^{ab}	13.33±0.58^{b}	7.50±1.00^{c}	6.17±1.15^{d}	8.50±1.73^{c}
Gram-negative strains						
Escherichia coli	10.67±0.58^{a}	-	-	-	6.00±1.00^{b}	-
Salmonella enterica	-	-	-	-	-	-
Pseudomonas aeruginosa	17.67±0.58^{a}	15.33±0.58^{b}	16.67±0.58^{a}	8,17±1.53^{d}	8.17±0.58^{cd}	8.34±1.15^{cd}
Fungi						
Candida albicans	11.67±0.58^{a}	11.67±0.29^{a}	11.17±0.29^{a}	-	5.92±0.29^{b}	5.84±0.58^{b}

The data are presented as mean value ± standard deviation of triplicate analyses. Mean with different letters in superscript within a row are statistically different at p≤0.05

It can be noted that all plant extracts exhibited varying degrees of antimicrobial activity against bacterial strains tested. The best antimicrobial activity had leaf extract obtained using MAE from Trnovo with inhibition zone 21.67 mm against gram-positive bacterial strains *B. subtilis* **Table 4**. This result was significantly different from the others for the same bacterial strain (p<0.05). The MAE extracts of blackthorn flowers, leaves and fruits from Borije, Trnovo and Vareš present an intermediate-inhibition zone against Gram-negative bacterial strain *P. aeruginosa* with a diameter from 15.33 mm to 17.67 mm. The highest zone of inhibition against this bacterial strain (17.67 mm) shows flower ethanolic extract from Borije.

Plant parts from all three areas prepared using UAM have shown a resistance-inhibition zone against all bacterial strains. The lowest anti-microbial activity had leaf extract from Trnovo against *S. enterica* (5.34 mm). Results for black-thorn leaves are shown in **Table 4**.

TABLE 4: ANTIMICROBIAL ACTIVITY OF BLACKTHORN LEAF EXTRACTS FROM THREE AREAS

Strains	Inhibition zone/mm					
	MAE Borije	MAE Trnovo	MAE Vareš	UAM Borije	UAM Trnovo	UAM Vareš
Gram-positive strains						
Bacillus subtilis	16.00±1.00^{c}	21.67±1.04^{a}	17.67±0.58^{b}	8.84±2.08^{e}	11.00±1.73^{d}	7.75±0.50^{e}
Staphylococcus aureus	12.67±2.89^{a}	11.33±0.58^{a}	13.67±3.79^{a}	5.84±0.58^{b}	6.50±1.00^{b}	6.17±0.58^{b}
Enterococcus faecalis	15.67±1.15^{a}	14.67±2.89^{a}	15.67±0.58^{a}	9.00±3.61^{b}	8.17±0.58^{b}	8.50±1.73^{b}
Gram-negative strains						
Escherichia coli	11.00±1.00^{a}	-	11.33±0.58^{a}	5.67±0.58^{b}	-	-
Salmonella enterica	10.67±0.58^{a}	-	0.67±0.58^{a}	-	5.34±0.58^{b}	-
Pseudomonas aeruginosa	16.33±0.58^{b}	16.00±0.00^{b}	17.00±0.00^{a}	8.17±0.58^{d}	9.00±0.00^{c}	9.25±0.50^{c}
Fungi						
Candida albicans	11.83±0.76^{b}	12.33±0.58^{b}	16.83±1.04^{a}	5.75±0.50^{c}	7.34±3.06^{c}	6.17±1.26^{c}

The data are presented as mean value ± standard deviation of triplicate analyses. Mean with different letters in superscript within a row are statistically different at p≤0.05

UAM blackthorn flower extracts showed no activity against *S. enterica*. These plants parts extract samples also showed a low activity, particularly against *E. coli*, *S. aureus* and *E. faecalis*.

Results for antimicrobial activity of blackthorn fruit extracts against gram-positive (*B. subtilis*, *S. aureus*, *E. faecalis*) and gram-negative bacterial strains (*P. aeruginosa*) obtained using MAE were significantly different ($p<0.05$) than the results obtained by UAM.

Fruit extract from Trnovo had the best result compared to other fruit extracts against all bacterial strains. The inhibition zone for this plant extract was 16.33 mm **Table 5**.

TABLE 5: ANTIMICROBIAL ACTIVITY OF BLACKTHORN FRUIT EXTRACTS FROM THREE AREAS

Strains	Inhibition zone/mm					
	MAE Borije	MAE Trnovo	MAE Vareš	UAM Borije	UAM Trnovo	UAM Vareš
Gram-positive strains						
Bacillus subtilis	13.67±0.76^{a}	13.00±0.00^{a}	13.00±1.73^{a}	7.00±1.00^{b}	6.92±1.26^{b}	7.93±0.76^{b}
Staphylococcus aureus	11.17±0.29^{a}	11.33±0.58^{a}	11.00±0.00^{a}	5.67±0.58^{c}	5.42±0.29^{c}	7.50±0.50^{b}
Enterococcus faecalis	14.33±1.15^{a}	13.00±1.00^{a}	12.33±0.58^{a}	7.75±3.12^{b}	-	7.67±2.84^{b}
Gram-negative strains						
Escherichia coli	-	-	10.00±0.00^{a}	5.00±0.00^{b}	5.42±0.29^{b}	-
Salmonella enterica	-	-	10.33±0.58^{a}	5.25±0.50^{b}	-	5.50±0.00^{b}
Pseudomonas aeruginosa	15.67±0.58^{a}	16.33±0.58^{a}	15.50±0.87^{a}	7.67±0.58^{b}	6.00±0.00^{c}	8.17±0.58^{b}
Fungi						
Candida albicans	11.00±0.00^{b}	12.33±1.15^{b}	15.50±1.80^{a}	5.50±0.00^{c}	5.84±0.58^{c}	5.92±0.29^{c}

The data are presented as mean value ± standard deviation of triplicate analyses. Mean with different letters in superscript within a row are statistically different at $p\leq0.05$

From the results presented in **Tables 3, 4, 5** it can be seen that extracts obtained by both methods exhibited antifungal activity against *C. albicans* (except UAM flower extract from Borije).

The best-registered result of MAE fruit extract was the one from Vareš with a diameter zone of 15.50 mm and the worst from Borije obtained by UAM (5.50).

Correlation between Antioxidant Activity and TPC, TFC, TAC and Mineral Content: The trace minerals such as Zn, Se, Cu, and Mn are components that support metabolism, growth, production, reproduction and there are included in human cell defence mechanisms against reactive oxygen species (ROS). These minerals act as cofactors of several antioxidant enzymes such as superoxide dismutase and glutathione peroxidise [27]. The correlation coefficient (r) was obtained from bivariate correlation analysis and used to describe the correlation between the antioxidant activity and the content of antioxidant components (TPC, TFC, TAC, Cu, Fe, Mn, and Zn). Three correlation levels were defined: strong (r=±0.600–1.000), moderate (r=±0.400–0.599), and weak (r=±0.000–0.399) [28]. Positive weak correlations of antioxidant activity with TPC, TFC, and TAC were found in flower, leaf and fruit extracts of blackthorn. Correlation between antioxidant activity and TPC in flower, leaf and fruit extracts were r=0.3705, r=0.0911, r=0.3247, respectively. Results of the correlation coefficient for antioxidant activity and TFC were also weak and found to be r=0.2394 for flower extracts, r=0.1017 for leaf, and the least was for fruit extract (r=0.1369). Correlation values for TAC are r= 0.0237 (flower extract), r=0.0187 (leaf extract) and r=0.0475 (fruit extract). TPC, TFC, and TAC may not contribute to the antioxidant activity since r values were weak positive. For these reasons, the antioxidant activity of samples was considered to be caused by the combined effects of TPC, TFC, and TAC and other compounds that are present in extracts. These findings suggest the necessity of choosing different methods for the determination of *in-vitro* antioxidant activity and determination of compounds of interest to get the right antioxidant profile of plant extracts. However, a strong correlation was found between antioxidant activity in fruit extracts and the content of Fe and Mn (r=0.8668 for Fe and r=0.7945 for Mn). The results obtained indicate that the content of Fe and Mn

contribute to the antioxidant activity. Furthermore, Manganese and Copper participate in reduction reactions as components of metallo-enzymes. These essential minerals are involved in multiple physiological processes including respiration, carbohydrate and lipid metabolism, antioxidant activities, and collagen formation [29]. Cu and Zn have weak correlation with antioxidant activity because correlation coefficient was r=0.011 and r=0.2681 respectively. Flower, leaf and fruits extracts of blackthorn have different content of phytoconstituents. Due to this, the correlation study suggested that the antioxidant activity of analysed extracts seem to be the combination of activities of these compounds.

CONCLUSION: In the present study, antioxidant and antimicrobial activity of blackthorn flower, leaf and fruit extracts obtained by MAE and UAM were observed.

The results of current comprehensive analysis demonstrated that blackthorn flowers, leaves and fruits possess high phenolic, flavonoid and antho-cyanin contents and high antioxidant activity. The potent antioxidative activity might result from its high contents of polyphenolic compounds. Due to this, it could be exploited for functional food, nutraceuticals and health applications especially in the prevention of diseases in which free radicals are implicated.

Fruit extracts from three different areas from Bosnia and Herzegovina were found to have the highest antioxidant activity, as determined through DPPH free radical activity irrespective of the extraction method used, while leaf extracts were found to have the highest antimicrobial activity against Gram-positive / Gram-negative bacterial strains.

Significant amount of anthocyanins were obtained in fruit extracts. Better results for TPC, TAC, and TFC of all plant parts were obtained using MAE than UAM. This can be explained due to high power level of microwaves and the possibility of fast extraction of compounds of interest. At the same time, high speed of extraction offers protection to thermo-labile constituents present in plant material.

The flower, leaf, and fruit extracts displayed effective antimicrobial activity against *B. subtilis*, *S. aureus*, *E. faecalis*, and *P. aeruginosa* as well as antifungal activity.

In summary, it can be concluded that the extraction of different parts of plant material originates from different areas from Bosnia and Herzegovina, as well as extraction methods, are the cause of the variety of results obtained for TPC as well as for TFC, TAC, antioxidant and antimicrobial activities. At the same time, it confirmed the validity of their traditional usage.

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