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Research Article

Phenolic Content, and Antioxidant and Antimicrobial Activities of Crataegus Oxyacantha *L (Rosaceae)* Fruit Extract from Southeast Serbia

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Abstract

Purpose: *The aim of this work was to determine the content of total phenols, total flavonoids, anthocyanins, as well as antioxidant and antimicrobial activities of hawthorn (Crataegus oxyacantha L.) alcohol, hydroalcohol and aqueous extracts.*

Methods: *The content of total phenols, flavonoids and anthocyanins of the alcohol, hydroalcohol and aqueous extracts of hawthorn were determined using spectrophotometric methods. Antioxidant assay was based on the measurement of DPPH absorbance at 517 nm caused by the reaction of DPPH with the test sample. Antimicrobial activity was evaluated by measuring the zone of inhibition against selected test microorganisms: Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, and Salmonella abony while antifungal activity was tested against two organisms: Aspergillus niger and Candida albicans.*

Results: *The results of spectrophotometric investigations indicate that the content of total phenol compounds in the investigated extracts varied from 2.12 to 30.63 mg GAE g^{-1} of fresh hawthorn sample. The content of anthocyanins ranged from 0.3207 to 3.168 mg of cyanidin-3-O-glucoside g^{-1} of fresh hawthorn fruit. The fruit extracts showed high antioxidant activity with DPPH radical transformation value as high as 89.9 % in the methanol-water (50/50, v/v%)) extract. The ethanol extract exhibited antimicrobial activity against all test microorganisms except two, Bacillus subtilis and Staphylococcus aureus, and one species of fungi, Aspergillus niger. Flavonoid structure influenced the extract's selectivity towards Gram-*positive *and Gram negative bacteria.*

Conclusion: *Extracts of the fruit of Crataegus oxyacantha L. can be used as natural antioxidant and antimicrobial preparations.*

Keywords: *Crataegus oxyacantha L.; Natural phenolic compounds; Antioxidant and antimicrobial activity, Southeast Serbia.*

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INTRODUCTION

Hawthorn, Crataegus oxyacantha L.(Rosaceae), is a perennial plant, usually a shrub or tree 1 to 6 m in height, and is found in deciduous forests and underbrush in the regions of Southeast Serbia. It grows in moderate, continental climate in the northern hemisphere. Hawthorn is well known in phytotherapy for the treatment of many cardiovascular diseases; it regulates blood pressure, increases the strength of heart muscle, and is used against arteriosclerosis and angina pectoris. Besides, hawthorn has a soothing effect on the nervous system, and is also used as a mild diuretic [1]. The recommended daily dosage of hawthorn is 160 - 900 mg of standardized extract, which corresponds to 3.5 - 19.8 mg of flavonoid. The daily dosage is divided into 2 - 3 individual doses and administered for at least 6 weeks [2].

Apart from phytotherapy, hawthorn is used in the food industry for the production of jam and various beverages including wine, juice, compote and herbal tea[3]. Reference data show that hawthorn leaves and flowers contain flavonoids (0.1 – 2 %, including rutin, hyperoside, vitexin, vitexin-2″-O-rhamnoside, and acetylvitexin-2″-O-rhamnoside), and oligomeric proanthocyanidins (composed of chains of flavan-3-ol units, 1 – 3 %), phenolic acids (including chlorogenic and caffeic acids), triterpene acids (oleanolic and ursolic acids), organic acids and sterols [4].

Generally, flavonoids, represented by procyanidins and flavone and flavonol are considered to be the main groups of active constituents in hawthorn extracts [5,6]. In many national and international pharmacopoeias, these groups of compounds are used for the standardization and quality control of flavonoids [7]. Extensive pharmacological and clinical studies have demonstrated that flavonoids are the substances largely (but not entirely) responsible for the action of the hawthorn extracts [4].

Free DPPH radicals are mostly used in antioxidant tests for the determination of the activity of natural metabolites present in extracts because they can transmit the labile hydrogen atom to free radicals. This represents the simplest and the commonest mechanism of antioxidant protection.

A survey of reference works shows that many authors have studied bioactive components of hawthorn from many aspects. Tadić et al [8] found that extracts of dried hawthorn pyrenes (Crataegus monogyna Jacq. and Crataegus oxyacantha L.. Crataegus laevigata (Poiret) have has significant anti-inflammatory, gastro-protective and antimicrobial propertiest. Tumbas et al have confirmed that the acetone extracts of dried hawthorn pyrenes (Crataegus oxyacantha L.) demonstrated antiradical and anti-proliferative activity [9].

Using thin layer chromatography method, Mihajlović et al identified flavonoids, vitexin and hyperoside in the extracts of hawthorn leaves and flowers [10]. Gudžić et al analyzed the extracts of white hawthorn (Crataegus monogyna Jacq.) flowers prepared with 0.1M HCl, and found that they contained quercetin [11].

Thus, there is growing interest in natural antioxidants and their application in nutrition and medical treatments since they contribute to the prevention of oxidative stress [12]. Based on However, to the best of our knowledge, there are no studies on the content of total phenol compounds, total flavonoids, anthocyanins, or their antioxidant and antimicrobial activity of hawthorn (Crataegus oxyacantha L.) fruit.

EXPERIMENTAL

Preparation of the material

Fully ripe fruits, collected throughout the month of October 2009, were used for the investigation. This region of Serbia is said to be free of negative environmental influences,

as it is largely devoid of industries and major highways. A voucher specimen was deposited in the herbarium of the Institute of Botany and Botanical Garden “Jevremovac", Faculty of Biology, University of Belgrade, under accession number 16476, BEOU [13]. The plant species were identified by Mirjana Milenkovic, Faculty of Biology, University of Belgrade.

Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetin and aluminium chloride ($AlCl_3$) were purchased from Sigma Chemical Co, St. Louis, MO, USA. Folin-Ciocalteu’s phenol reagent and sodium carbonate were obtained from Merck Chemical Suppliers (Darmstadt, Germany). Sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were also purchased from Merck. All other chemicals used, including solvents, were of analytical grade.

Preparation of herbal extracts

The fresh fruits of hawthorn (Crataegus oxyacantha L.) were ground in a blender, and 2 g was extracted by either of the following solvents: ethanol, ethanol-water (50/50, v/v%), methanol, methanol-water (50/50, v/v%), and water. Extraction was carried out in an ultrasonic bath for 15 min three times in succession with 30, 20, and 20 ml of the solvent, respectively. The extract was filtered through a Buchner funnel and filter paper (blue collar) (CHMLAB, Spain), transferred into a 100 ml flask and made up to mark with the same solvent.

Determination of the total phenolics

Total phenol contents of the extracts were determined by the modified Folin-Ciocalteu method [14]. An aliquot of extract (1 ml) was mixed with 0.5 ml of Folin-Ciocalteu reagent and 1.5 ml of sodium carbonate (20 %) in a tube. The tubes were vortexed for 15 sec and allowed to stand at 40 ^{o}C for 30 min to develop color. Absorbance was then measured at 765 nm using a Hewlett Packard UV-VIS spectrophotometer. Gallic acid was used as the standard and a calibration curve in the linear range of 0 - 2 mgml. Total phenol content was expressed as mg g^{-1} gallic acid equivalent (GAE). The assay was carried out in triplicate and the mean result taken.

Determination of the total monomeric anthocyanins

The total monomeric anthocyanin content of the plant extracts was determined using the pH-differential method previously described [15]. Anthocyans demonstrate maximum absorbance at 520 nm at pH 1.0. The colored oxonium form predominates at pH 1.0, and the colorless hemiketal form at pH 4.5. The pH-differential method is based on a reaction that produces oxonium forms and it permits accurate and rapid measurement of total monomeric anthocyanins. The extract (1 ml) was transferred 10 ml flask and made to volume with pH 1.0 buffer. Fifteen minutes later, the absorbance of the solution was measured at 520 and 700 nm. The procedure was using pH4.5 buffer instead of pH 1.0 buffer. The absorbance of the extracts was calculated as in Eq 1.

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5} \quad \ldots\ldots (1)$$

The content of monomeric anthocyanin pigment (MAP) was calculated using Eq 2.

$$MAP\ (mgdm^{-3}) = (A \times MW \times DF \times 1000)/(\varepsilon \times l) \quad (2)$$

where (ε) is the molar absorptivity (26.900), MW is the molecular weight (449.2), and DF is the dilution factor, l is the path length. The result, taken as the monomeric anthocyanin pigment (MAP), was expressed as mg of cyanidin-3-O-glucoside per dm^3.

Determination of total flavonoid content

Total flavonoid content was determined using a spectrophotmetric method based on formation of flavonoid complex with aluminum *[16]. A volume of 0.5 ml of 2 % $AlCl_3$*

methanol solution was added to 0.5 ml of sample solution at room temperature. One hour later, its absorbance was measured at 420 nm. Yellow color indicated that the extracts contained flavonoids. Quercetin was used as standard for calibration in the linear range 0 - 30 mgdm$^{-3.}$ Total flavonoid content was calculated as quercetin equivalent (QE)).

Evaluation of free radical scavenging activity

The free radical scavenging activity of the plant extracts was analyzed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [17,18]. The antioxidant assay is based on the spectrophotometric measurement of the loss of color of DPPH solution, caused by the reaction of DPPH with the test sample, by measuring its absorbance at 517 nm. In the assay, the extract (0.2 ml) and 1.8 ml of freshly prepared DPPH in methanol (20 mg dm^{-3}) were allowed to react in a cuvette at room temperature for 20 min at room temperature, and the absorbance read against a blank at 517 nm. .The determinations were performed in triplicate. Inhibition of DPPH (RSC %) by the extract was calculated from Eq 3.

$$RSC\ (\%) = (1 - A_{sample}/A_{blank}) \times 100 \ldots\ldots \quad (3)$$

where A_{blank} is the absorbance of control ($1 \cdot 10^{-4}$ mol dm^{-3} DPPH methanol solution), and A_{sample} is the absorbance of the test sample.

Assessment of antimicrobial activity

The in vitro antimicrobial activity of the ethanol extract of Crataegus oxyacantha L. was tested against a selection of laboratory control strains from American Type Culture Collection (ATCC), Maryland, USA and National Collection of Type Cultures (NCTC). Antibacterial activity was evaluated against two Gram-positive and three Gram-negative bacteria. The Gram-positive bacteria used were Bacillus subtilis ATCC 6633 and Staphylococcus aureus ATCC 6538 while the Gram-negative bacteria were: Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027 and Salmonella abony NCTC 6017. Antifungal activity was tested against two organisms: Aspergillus niger ATCC 16404 and Candida albicans ATCC 10231.

The disc-diffusion method was employed for the determination of the antimicrobial activity of the extracts [19]. The inocula of the bacterial and fungal strains were prepared from overnight broth cultures and the suspensions were adjusted to 0.5 McFarland standard turbidity. An aliquot (100 µl) of the suspension containing 1.0 x 108 CFU ml^{-1} of bacteria or 1.0 × 104 CFU ml^{-1} of fungal spores was spread on Mueller-Hinton agar (MHA, Torlak) or Sabouraud dextrose agar (SDA, Torlak), respectively, in sterilized Petri dishes (90 mm in diameter). Discs (9 mm diameter, Macherey-Nagel, Düren, Germany) were impregnated with 20 µl and 50 µl of the extract (30 mg ml^{-1}) and placed on the inoculated agar. Negative controls were prepared using the same solvent (ethanol). Tetracycline (30 µg, Torlak) and nystatin (30 µg, Torlak) were used as positive reference standards for the antibacterial and antifungal tests, respectively. The inoculated plates were kept at 4 ^{o}C for 2 h and incubated at 37 ^{o}C (24 h) for bacterial strains, and at 28 ^{o}C (48 h) for fungal strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Each assay was performed in triplicate.

Statistical analysis

The results obtained were expressed as mean ± standard error of mean (SEM) of three determinations. Where applicable, the data were subjected to a one-way analysis of variance (ANOVA test), and differences among among data were determined by Duncan's Multiple Range test using Statistical Analysis System software (SAS, 1999) [20].

RESULTS

Total phenols, anthocyanin and flavonoid contents, and antioxidant activity of Crataegus oxyacantha L fruit extracts are given in Table 1.

Table 1: Total phenols, anthocyanin and flavonoid contents, and antioxidant activity of Crataegus oxyacantha L. extracts

Extract	Total phenols content[a]	Anthocyan content[b]	Flavonoid content[c]	RSC(%)	RSC[d]
Ethanol extract	2.12±0.12	0.749±0.006	0.560±0.000	60.61±0.00	3.53±0.74
Ethanol-water extract (1:1)	19.32±0.49	1.118±0.006	0.556±0.023	88.60±0.58	5.22±0.55
Methanol extract	18.21±0.51	3.168±0.033	0.990±0.038	89.56±0.16	5.28±0.3
Methanol-water extract (1:1)	30.63 ±2.56	1.978±0.030	0.855±0.033	89.89±0.22	5.30±0.81
Water extract	24.89±0.67	0.320±0.064	0.766±0.047	75.95±0.85	4.46±0.64

[a]Expressed as mg of gallic acid g^{-1} of fresh fruit; [b]Expressed as mg of cyanidin-3-O-glukoside g^{-1} of fresh fruit; [c, d] Expressed as mg of quercetin g^{-1} of fresh fruit

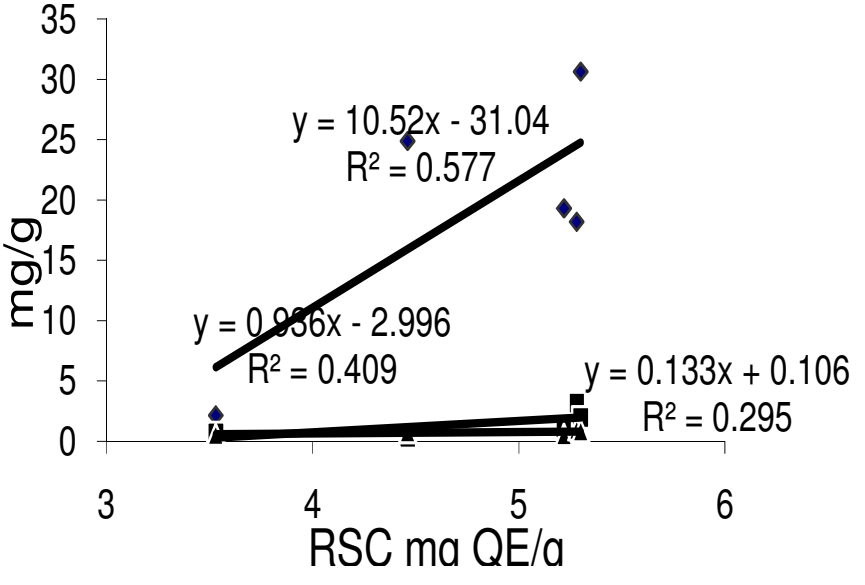

Fig 1: Correlation of total phenol (♦)flavonoid (■) and anthocyanin (▲) contents with antioxidant activity of hawthorn (*Crataegus oxyacantha* L.) fruit

The results show that the content of total phenols in the investigated extracts was high, ranging from 2.13 mg GAE g^{-1} for the ethanol extract to 30.63 mg GAE g^{-1} for the methanol-water (50/50 v/v%) extract. Extracts prepared using methanol-water (50/50 v/v%) showed the highest content of phenol compounds. Anthocyan content was between 0.320 mg cyanidin 3-glucoside g^{-1} for water extract, and 3.168 mg cyanidin 3-O-glucoside g^{-1} for methanol extract (Table 1). The total flavonoid contents of the extracts was low, ranging from 0.556 mg (ethanol-water, 50/50 v/v% extract) to 0.99 mg QE g^{-1} (methanol extract). All the extracts exhibited strong scavenging activity against DPPH radicals, rangeing from 60.6 to 89.9 %.

Fig 1 indicates that total phenol content showed little correlation with antioxidant activity of the extracts (R^2 = 0.5778).

As Fig 1 shows, the correlation coefficient of monomer anthocyanin contents and antioxidant activity was R^2 = 0.4096, indicating poor correlation. The correlation of total flavonoids to antioxidant activity was not significant (R^2 = 0.2955).

Antimicrobial activity of extract

The antimicrobial activity data for the ethanol extract of Crataegus oxyacantha L. is given in Table 2.

DISCUSSION

Some studies suggest that the polyphenolic content of plant fruits is influenced by genotype, habitat conditions and ripeness of the fruits [21]. Other factors, such as altitude, light, temperature, and content of nutritive matter in the soil, can influence phenylpropanoid metabolism [22].
Phenol content often determines the pharmacological properties of plants. In medicinal herbs, phenol concentration is between 0.23 and 2.85 mg GAE g^{-1}, while phenol concentration in nutritive plant species is between 0.26 and 17.51 mg GAE g^{-1} [23]. According to literature, the highest content of phenol compounds is found in spice herbs from the genus Origanum (20 mg GAE g^{-1}).

Based on the foregoing, it can be said that Crataegus oxyacantha L.fruit ranks high among the medicinal plants in terms of phenolic content with a avlue as high as 30.63 mg GAE g^{-1}. fact that polyphenol content in hawthorn (Crataegus oxyacantha L.) fruit is 306 ± 12.65 The content of total polyphenolic compounds determined by Folin-Ciocalteu method did not give a complete representation of the quantity and quality of polyphenolic compounds in the extracts due to possible presence of interfering compounds (sugars, aromatic amines, sulfur dioxide, vitamin C, organic acids, iron (II) and other substances of non-polyphenolic origin), which can produce misleadingly high results [24].

Table 2: Antimicrobial activity (expressed as zone of inhibition) of ethanol extract of Crataegus oxyacantha L. against selected microbial strains (mean ± SEM, n = 3)

Test microorganism	ZONE OF INHIBITION (mm)				
	Extract (10 mg ml^{-1})		Reference antibiotic	Reference antimycotic	Negative control
	20µl	***50µl***	***Tetracycline (30 µg)***	***Nystatin (30µg)***	***Ethanol (96%)***
Salmonella abony NCTC 6017	12±0.2	21±0.15	28±0.25	-	-
Escherichia coli ATCC 25922	12±0.2	22±0.15	30±0.25	-	-
Pseudomonas aeruginosa ATCC 9027	14±0.2	27±0.2	19±0.2	-	-
Bacillus subtilis ATCC 6633	-	-	36±0.3	-	-
Staphylococcus aureus ATCC 6538	-	-	34±0.3	-	-
Candida albicans ATCC 10231	13±0.1	20±0.25	-	20±0.2	-
Aspergillus niger ATCC 16404	-	-	-	19±0.2	-

As expected, blank (ethanol) which served as negative control, had no inhibitory effect on any of the test microorganisms. The ethanol extract exhibited antimicrobial activity against all microorganisms except two strains of bacteria, Bacillus subtilis *and* Staphylococcus aureus*, and one strain of fungi,* Aspergillus niger*.*

It has been reported that the content of total phenols in the ethanol extract of dried hawthorn (Crataegus oxyacantha L.) fruit is 35.4 ± 2.48 mg of gallic acid equivalent g^{-1} of dried mass [7]. The results of this study agree with those of our work with respect to total phenols. However, the flavonoids content found in an earlier study (165.16 ± 7.28 mg of rutin equivalent g^{-1} (which used 80 % acetone and 0.5 % acid as extracting solvents, and standard rutin) [8], is significantly higher ($p = 0.05$) than our own finding. This is probably because less flavonoids are extracted by polar solvents, such as ethanol, than by less polar solvents . The differences in total phenol compounds content depended on the extraction medium used are a consequence of different polarity of the organic solvents used and their mixtures, which selectively extract individual phenol compounds.Another study reported total flavonoids ranging from 0.14 ± 0.02 to 0.18 ± 0.04 mg of rutin equivalent per 1 g of dry residue for ethanol extract [8]. These values are significantly lower than the total flavonoid values found in our study.

The ethanol extract exhibited antimicrobial activity against the test microorganism except two species of bacteria, Bacillus subtilis and Staphylococcus aureus, and one species of fungi, Aspergillus niger. The antibacterial activity of flavonoids against both Gram-positive and Gram-negative bacteria has been reported. [25]. Activity against Gram-positive bacteria (S aureus) was demonstrated mainly by compounds that contained hydroxyl groups in ring B. The most active were the 3',4',5'-trihydroxyflavonoids, eg, myricetin-3,5,7, 3',4',5'-hexahydroxyflavone. Flavanone aglycones (naringenin, pinocembrin) and their C-6 or C-8 prenylated derivatives also turned out to be active. Only those 3-methoxy flavones having additional hydroxyl groups at C-5 and C-7 showed activity against B cereus. Flavones such as apigenin and its C- and O- glycosides were not active.

Activity against Gram-negative bacilli (E coli, Ps aeruginosa, P vulgaris, and K pneumoniae) was demonstratedby the flavones apigenin, vitexin, and saponarin, while flavonoid compounds having two or three hydroxyl groups in rings A or B were active against Gram-positive bacteria. Glycosides in general were less active, but acetyl derivatives of kaempferol-3-glucoside showed some activity against S aureus, and B cereus. Their activity, however, was directly related to the presence of an acyl substitution in the cis form.

CONCLUSION

From the medicinal point of view, the fruit extracts of hawthorn (Crataegus oxyacantha L.) contain a high proportion of polyphenolic compounds and exhibited good antioxidant and antimicrobial activities. Phenolic compounds accounted mainly for the antioxidant activity of the fruit extracts. Thus, the fruit of hawthom is of significant biological importance for its antioxidant and microbiological properties.

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