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Anti-inflammatory, Gastroprotective, Free-Radical-Scavenging, and Antimicrobial Activities of Hawthorn Berries Ethanol Extract

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Hawthorn [*Crataegus monogyna* Jacq. and *Crataegus oxyacantha* L.; sin. *Crataegus laevigata* (Poiret) DC., Rosaceae] leaves, flowers, and berries are used in traditional medicine in the treatment of chronic heart failure, high blood pressure, arrhythmia, and various digestive ailments, as well as geriatric and antiarteriosclerosis remedies. According to European Pharmacopoeia 6.0, hawthorn berries consist of the dried false fruits of these two species or their mixture. The present study was carried out to test free-radical-scavenging, anti-inflammatory, gastroprotective, and antimicrobial activities of hawthorn berries ethanol extract. Phenolic compounds represented 3.54%, expressed as gallic acid equivalents. Determination of total flavonoid aglycones content yielded 0.18%. The percentage of hyperoside, as the main flavonol component, was 0.14%. With respect to procyanidins content, the obtained value was 0.44%. DPPH radical-scavenging capacity of the extract was concentration-dependent, with EC_{50} value of 52.04 μg/mL (calculation based on the total phenolic compounds content in the extract). Oral administration of investigated extract caused dose-dependent anti-inflammatory effect in a model of carrageenan-induced rat paw edema. The obtained anti-inflammatory effect was 20.8, 23.0, and 36.3% for the extract doses of 50, 100, and 200 mg/kg, respectively. In comparison to indomethacin, given in a dose producing 50% reduction of rat paw edema, the extract given in the highest tested dose (200 mg/kg) showed 72.4% of its activity. Gastroprotective activity of the extract was investigated using an ethanol-induced acute stress ulcer in rats with ranitidine as a reference drug. Hawthorn extract produced dose-dependent gastroprotective activity (3.8 ± 2.1, 1.9 ± 1.7, and 0.7 ± 0.5 for doses of 50, 100, and 200 mg/kg, respectively), with the efficacy comparable to that of the reference drug. Antimicrobial testing of the extract revealed its moderate bactericidal activity, especially against Gram-positive bacteria *Micrococcus flavus*, *Bacillus subtilis*, and *Lysteria monocytogenes*, with no effect on *Candida albicans*. All active components identified in the extract might be responsible for activities observed.

KEYWORDS: ***Crataegus monogyna* Jacq.; *Crataegus oxyacantha* L.; hawthorn fruits; anti-inflammotory effect; antimicrobial activity; free-radical-scavenging activity; gastoprotective activity; phenol compounds; flavonoids**

INTRODUCTION

Crataegus monogyna Jacq. and *Crataegus oxyacantha* L. [sin. *Crataegus laevigata* (Poiret) DC.], Rosaceae, are medicinal plants with a long history of use in European herbal and traditional Chinese medicine (*1*). In Europe, *Crataegus* (hawthorn) leaves, flowers, and both green (unripe) and red (ripe) berries (false fruits) or a combination thereof have traditionally been used as astringent, antispasmodic, cardiotonic, diuretic, hypotensive, and antiatherosclerotic agents. Hawthorn is particularly used in the treatment of various heart problems, including heart failure in cases of declining cardiac performance equivalent to stages I and II of the New York Heart Association (NYHA) classification, angina pectoris, hypertension with myocardail insufficiency, mild alterations of cardiac rhythm, and atherosclerosis (*2, 3*). In traditional Chinese medicine, hawthorn fruits are used as a peptic agent for stimulating digestion and promoting the function of the stomach, improving blood circulation, and removing blood stasis. Also, the fruits

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preparations have a beneficial effect in the treatment of indigestion with epigastric distension, diarrhea, and abdominal pain, as well as amenorrhea, hypertension, and hyperlipidemia (*4*). Hawthorn fruits are consumed not only for medicinal purposes mentioned but also as foodstuff (canned fruit, jam, jelly, drink, and wine).

Hawthorn fruits, leaves, and flowers contain, on the basis of literature data, flavonoids (0.1−2%, including rutin, hyperoside, vitexin, vitexin-2″-*O*-rhamnoside, and acetylvitexin-2″-*O*- rhamnoside), 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 (*1, 3*−*7*). Generally, flavonoids, represented with procyanidins and flavone and flavonol types of flavonoids are considered to be the main groups of active constituents in hawthorn extracts (*4, 8*−*10*), and in many national and international pharmacopoeias, these groups of compounds are used for their standardization and quality control.

Extensive pharmacological and clinical studies have demonstrated that the flavonoids are the active substances largely (but not entirely) responsible for the action of the drug. The main pharmacological activities of hawthorn extracts are primarily cardiovascular ones, including cardiotonic, antiarrithythmic, hypotensive, and hypolipidemic effects (*10*−*19*). Their mechanisms of action are very complex and are thought to be a combination of inhibition of phosphodiesterase activity and increase of cAMP concentration in cardiomyocytes (*20*), inhibition of Na^+/K^+-ATPase in cardiac muscle tissue (*11*) (inotropic action), increase of nitric oxide (NO) production in the vascular endothelium (*8, 9*), inhibition of angiotensin-converting enzyme (*21*) (vasodilating and cardioprotective action), inhibition of LDL cholesterol, enhancing LDL catabolism by way of hepatic receptors (*17, 18*) (hypolipidemic actions), etc.

Over the years the preferred used of leaf and flowers of the drug presumably originated from its traditional use, whereby it is assumed to exhibit a balanced level of procyanidins and other subgroups of flavonoids. Nevertheless, the dried berries are frequently used for herbal preparations. Contrary to the cardiovascular effects mentioned, the data about other pharmacological activities of hawthorn extracts, particularly those prepared from hawthorn berries, are scarce. Because of that, the aim of this study was to estimate free-radical-scavenging, anti-inflammatory, gastroprotective, and antimicrobial activities of hawthorn dry berries ethanol extract, as well as to establish its main groups of active constituents and the correlation between these activities and flavonoid constituents of the extracts, because previous studies have recognized flavonoids as potent biologically active substances (*22, 23*).

MATERIALS AND METHODS

Reagents and Solutions. Sodium bicarbonate (analytical grade), 1,1′-diphenyl-2-picrylhydrazyl (DPPH) (analytical grade), indomethacin and carrageenan (EP grade), and trolox (analytical grade) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Analytical-grade reagents 2,6-di-*tert*-butyl-4-methylphenol (BHT), ether, petrol, dimethyl sulfoxide (DMSO), ethyl acetate, *n*-butanol (BuOH), acetone, hydrochloric acid (HCl), and absolute ethanol (96%, v/v) were purchased from Merck (Darmstadt, Germany). Acetonitrile (MeCN), water, and methanol were of HPLC grade, and they were purchased from Merck (Darmstadt, Germany). Reference HPLC standards of chlorogenic acid, caffeic acid, hyperoside, isoquercitrin, rutin, vitexin, vitexin-2″-*O*-rhamnoside, (−)-epicatechin, and cyanidin chloride were purchased from Carl Roth (Karlsruhe, Germany), and ranitidine (Ranisan ampoules) was purchased from Zdravlje-Actavis Company (Leskovac, Serbia).

Plant Extract. *C. monogyna* and *C. oxycantha* (1:1) berries were collected in Pomoravlje Province, Serbia, 2004. As defined in *European Pharmacopoeia 6.0* (*24*), hawthorn berries consist of the dried false fruits of these two species or their mixture. Assuming that the available hawthorn berries extracts on the world market are usually prepared from *C. monogyna* and *C. oxycantha* mixture, we investigated its mixture with a known ratio of the species. The berries were dried at room temperature, in a shady place. Voucher specimens of the plants (11050904 and 21050904) were deposited at Herbarium of Botanical Garden, Jevremovac, Belgrade, Serbia. Hawthorn dry berries extract was obtained by triple percolation with 70% ethanol, with the drug/eluent ratio of 1:1. The solvent was evaporated, yielding a residue of 10.5% (w/w) in terms of dried starting material. The dried extract was dissolved in DMSO to test the anti-inflammatory, gastroprotective, and antimicrobial action and in methanol for HPLC analysis and DPPH radical-scavenging assays.

Total Phenolics Content. The total phenolics content was determined by the Folin−Ciocalteu method (*25*). A total of 100 μL of MeOH solution of dry extract (62, 124, and 310 μg/mL final quantity) was mixed with 0.75 mL of Folin−Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 °C for 5 min; 0.75 mL of sodium bicarbonate (60 g/L) solution was added to the mixture. After 90 min at 22 °C, absorbance was measured at 725 nm. Results are expressed as gallic acid equivalents.

Procyanidins Content. The percentage content of procyanidins, expressed as cyanidin chloride, was calculated using the method described in *European Pharmacopoeia 6.0* (*24*). Shortly, the investigated extract was hydrolyzed under reflux by an EtOH/HCl mixture. Procyanidins were separated with BuOH from the aqueous layer; the absorbance was measured by a UV−vis spectrophotometer HP 8453 (Agilent Techologies, Santa Clara, CA), at λ_{max} 545 nm, and calculated as cyanidin chloride percentage (%, w/w).

Total Flavonoids Content. *Method 1.* The percentage content of flavonoids expressed as hyperoside was calculated using the method described in DAB 10 (*26*). Briefly, the sample was extracted with acetone/HCl under a reflux condenser; the $AlCl_3$ complex of the flavonoid fraction extracted by ethyl acetate was measured by a UV−vis spectrophotometer HP 8453 at 425 nm. The content of flavonoid, expressed as hyperoside percentage, presented the mean ± standard deviation of three determinations.

Quantification of Total Flavonoid Aglycones Content by HPLC. *Method 2.* The total flavonoid aglycones content after acid hydrolysis using quercetin reference standard was determined applying HPLC. Hydrolysis was performed following the procedure: 1.11 g of investigated extract was treated with 4 mL of 2 M HCl and 3 mL of MeOH at 85 °C and refluxed for 2 h. After adding 3 mL of MeOH and transferring the reaction mixture in a 10 mL volumetric flask, the reaction mixture was subjected to an ultrasonic bath for 10 min on room temperature before being brought to the volume using MeOH. The hydrolyzed mixture was filtered through a 0.2 μm PTFE filter, and the volume of 4 μL was subjected to HPLC analysis for determination of flavonoid content after acid hydrolysis, expressed as the content of quercetin. The determination was performed in triplicate. HPLC method details are described in the next session (see HPLC Procedure).

HPLC Procedure. HPLC fingerprint of the extract and quantification of total flavonoid content after acid hydrolysis was achieved by HPLC (Agilent Technologies 1200). Detection was performed using a diode array detector (DAD), and chromatographs were recorded at $\lambda = 280$ nm [for (−)-epicatechin], 360 nm (for flavonols, flavons, and phenolcarbolic acids), and 520 nm (for cyanidin chloride). HPLC separation of components was achieved using a LiChrospher 100 RP 18e (5 μm), 250 × 4 mm i.d. column, with a flow rate of 1 mL/min and mobile phase, A [500 mL of H_2O plus 9.8 mL of 85% H_3PO_4 (w/w)], B (MeCN), elution, combination of gradient mode: 90−75% A, 0−35 min; 75−60% A, 35−55 min; 60−40% A, 55−60 min). The sample was prepared dissolving 1111.6 mg of the extract (obtained by the procedure previously described) in 10 mL of MeOH, filtered through 0.2 μm PTFE filters. The volume injected was 4 μL. Standard solutions for the determination of flavonoids and polyphenolic acid were prepared at a concentration of 0.2 mg/mL in methanol. The volume injected was 4 μL, the same as the investigated extract. The identification was carried out thanks to retention time and spectra matching. Once spectra

matching succeeded, results were confirmed by spiking with respective standards to achieve a complete identification by means of the so-called peak purity test. Those peaks not fulfilling these requirements were not quantified. Quantification was performed by external calibration with standards.

Determination of the Free-Radical-Scavenging Activity. The α,α-diphenyl-β-picrylhydrazyl (DPPH) scavenging assay was carried out according to the procedure described by Blois, with some modification (*27*). Various concentrations of the samples (100 μL) were mixed with 900 μL of 0.04 mg/mL methanolic solution of DPPH. UV spectra were recorded on a UV−vis spectrophotometer HP 8453. Absorbance at 517 nm was measured after 20 min. The inhibition percentage was calculated using the following equation:

$$I = [(A_c - A_s)/A_c] \times 100$$

where I was the inhibition percentage, A_c was the absorbance of the negative control (containing 100 μL of MeOH instead of the samples), and A_s was the absorbance of the samples. Synthetic antioxidants, trolox and *tert*-butyl hydroxytoluene (BHT), were used as positive controls. The inhibition percentage was plotted against the concentration of the samples, and EC_{50} values, determined by linear regression analysis, presented the mean ± standard deviation of three determinations. The calculation of the EC_{50} value was based on extract total phenolic content.

Animals. Adult, male Wistar rats weighing 200−300 g were used for estimating hawthorn berries ethanol extract anti-inflammatory (the carrageenan-induced paw edema test) and gastroprotective activities (the absolute ethanol-induced stress ulcer test). Experimental groups consisted of 6−10 animals each. The animals were deprived of food for 18−20 h before the beginning of experiments with free access to tap water.

Carrageenan-Induced Rat Paw Edema. The carrageenan-induced rat paw edema test has been used as an experimental model for screening the anti-inflammatory activity according to the modified method of Oyanagui and Sato (*28*). The extracts were administered p.o. in doses of 50, 100, and 200 mg/kg. Indomethacin, dissolved in DMSO, was used as a reference in a dose of 4 mg/kg p.o., which was a dose producing 50% reduction of rat paw edema. The control animals were given DMSO in a dose of 1 mL/kg p.o. Carrageenan-saline solution (0.5% in a volume of 0.1 mL) was injected into the plantar surface of the right hind paw 1 h after the oral administration of the extracts or indomethacin. A pure saline solution (0.9% NaCl, 0.1 mL) was injected into the left hindpaw, which served as the control one (non-inflamed paw). The animals were killed 3 h after the carrageenan injection, and the paws were cut off for weighing. The difference in weight between the right and left paw, treated versus untreated (control) rats, served as an indicator of the inflammatory response intensity (i.e., anti-inflammatory activity). The percent of anti-inflammatory effect was calculated from the expression

$$\text{anti-inflammatory effect } (\%) = \frac{\Delta k - \Delta e}{\Delta k} \times 100$$

where Δk represent the difference in the paw weight in the control group and Δe is the difference in the paw weight in the tretment group.

Absolute Ethanol-Induced Stress Ulcer in Rats. To study the gastroprotective activity of the ethanol extract of hawthorn berries, an experimental model of acute gastric mucosa damage induced by absolute ethanol (1 mL/rat p.o.) was used. The investigated extract, dissolved in DMSO, were administered p.o. in doses of 50−200 mg/kg, 60 min prior to ethanol. Ranitidine, given in doses of 5−20 mg/kg p.o., was used as a reference drug. The control animals were given the vehicle in a dose of 1 mL/kg p.o., also 60 min before ethanol. The animals were sacrificed 1 h after giving ethanol, and their stomachs were removed and opened along the greater curvature. Lesions were examined under an illuminated magnifier (3×). The intensity of gastric lesions was assessed according to a modified scoring system of Adami et al. (*29*): 0, no lesions; 0.5, slight hyperaemia or ≤5 petechiae; 1, ≤5 erosions ≤5 mm in length; 1.5, ≤5 erosions ≤5 mm in length and many petechiae; 2, 6−10 erosions ≤5 mm in length; 2.5, 1−5 erosions <5 mm in length; 3, >5−10 erosions >5 mm in length; 3.5, >10 erosions >5 mm in length; 4, 1−3 erosions ≤5 mm in length and 0.5−1 mm in width; 4.5, 4−5 erosions ≤5 mm in length and 0.5−1 mm in width; 5, 1−3 erosions >5 mm in length and 0.5−1 mm in width; 6, 4- or 5-grade 5 lesions; 7, ≥6-grade 5 lesions; 8, complete lesion of the mucosa with hemorrhage.

Table 1. Total Phenolics, Total Flavonoids, and Procyanidins Content in Hawthorn Berries Extract (the Mean Value ± SD of Three Measurements)

group of active constituents	amount
total phenolics (mg of GA[a]/g)	35.4 ± 2.48
total flavonoid content, method 1 (%)	0.14 ± 0.02
total flavonoid aglycones content, method 2 (%)	0.18 ± 0.04
procyanidins (%)	0.44 ± 0.09

[a] GA = gallic acid.

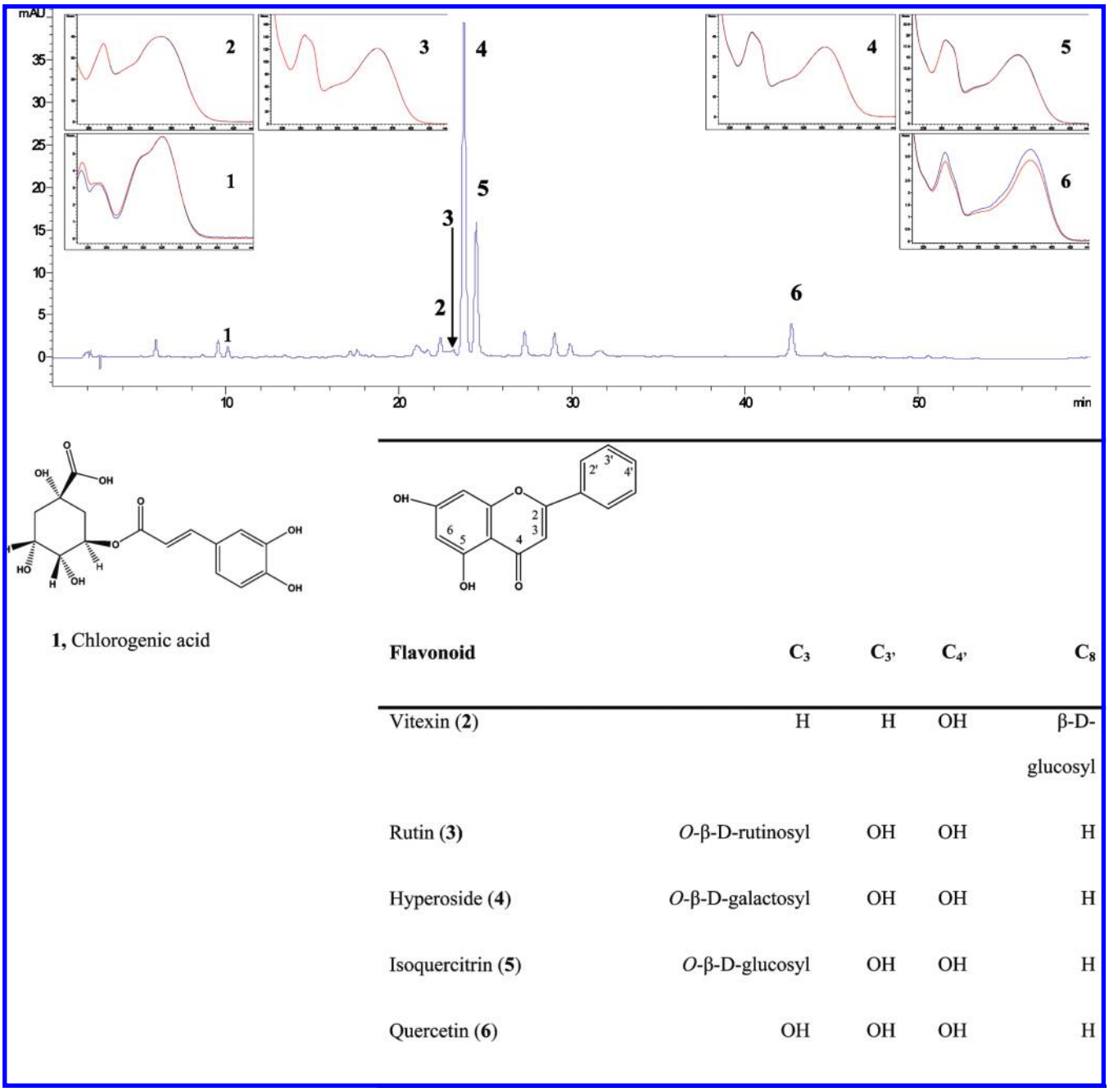
Antimicrobial Activity. The in vitro antimicrobial activities of the extract was tested against a panel of laboratory control strains belonging to the American Type Culture Collection, Rockville, MD: Gram-positive, *Staphylococcus aureus* ATCC 25923, *Streptococcus epidermidis* ATCC 12228, *Bacillus subtilis* ATCC 6051, *Micrococcus luteus* ATCC 10240, *Micrococcus flavus* ATCC 14452, *Lysteria monocytogenes* ATCC 15313, *Enterococcus faecalis* ATCC 19433, *Sarcina lutea* 10054; Gram-negative, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas talaasii* NCTC 387, *Salmonella typhimurium* ATCC 14028, *Salmonella enteritidis* ATCC 13176, *Proteus mirabilis* ATCC 14273; and fungal organism *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231. In vitro antibacterial studies were carried out according to the disk diffusion method by measuring the zones of inhibition against Gram-positive and Gram-negative bacteria and human pathogen fungi. Standars antibiotics streptomicin and nystatin (10 μg/mL) were used to controle sensitivity of the tested bacteria and fungi. The tested fractions were dissolved in DMSO. For each experiment, a control disk with pure solvent was used as a blind control. Muller−Hinton agar was used for growth of bacterial strains, and Sabouraud maltose agar was used for fungi. All agar plates were prepared in 90 mm Petri dishes with 25 mL of agar, giving a final depth of 4 mm.

The 24 h broth cultures of bacteria and fungi were freshly prepared for each assay. The volume (0.3 mL) of each bacterial broth culture (1×10^6 organisms/mL) was added in 3 mL of dissolved (melted) Malt agar and subsequently poured evenly over the surface of the dried agar plates. The plates were placed at 37 °C for approximately 20 min until bacterial overlay had dried on the surface. A 6 mm sterile paper disk was placed onto the dried surface, and 10 μL of test substance was gently placed onto the disk. Plates were subsequently incubated at the appropriate temperature for 24 h. Zones of inhibition were calculated by measuring the diameter in millimeters (including disk). When the inhibitory zone diameter is lower or equal to 6 mm, the sample tested was considered as not active. Two replicates were performed for each analysis. The MIC (minimum inhibitory concentration) values of the fractions in the previous experiment were determined using the broth microdilution method in 96-hole plates. Serial dilutions of the stock solutions of tested fractions in broth medium (Muller−Hinton or Sabouraud broth) were prepared in a microtiter plate (96 walls). The microbial suspensions were added in the microwells at the concentration of 5×10^5 organisms/mL. MICs were determined as the lowest concentrations preventing visible growth. The standard antibiotic streptomicin was used to control the sensitivity of tested bacteria, whereas nystatin was used as control against the fungi (*30*).

Statistical Analysis. Statistical analysis was performed by the Mann−Whitney U test. A value of $p < 0.05$ was considered as significant.

RESULTS

Quantitative analysis of total phenolics, flavonoids, flavonoid aglycones, and procyanidins content, presented in **Table 1**, pointed out a high amount of phenolic compounds in the ethanol extract of hawthorn berries. As analyzed by HPLC, hyperoside (**4**) was the major flavonol component and composed 0.14% of



1, Chlorogenic acid

Flavonoid	C_3	$C_{3'}$	$C_{4'}$	C_8
Vitexin (**2**)	H	H	OH	β-D-glucosyl
Rutin (**3**)	*O*-β-D-rutinosyl	OH	OH	H
Hyperoside (**4**)	*O*-β-D-galactosyl	OH	OH	H
Isoquercitrin (**5**)	*O*-β-D-glucosyl	OH	OH	H
Quercetin (**6**)	OH	OH	OH	H

Figure 1. HPLC chromatogram of the ethanol extract of the dried berries of hawthorn recorded at 360 nm (with the spectrum of identified compounds, compared to UV spectra of reference standards and chemical structures of identified compounds). Numbers refer to the following: **1**, chlorogenic acid; **2**, vitexin; **3**, rutin; **4**, hyperoside; **5**, isoquercitrin; and **6**, quercetin (see **Table 2**).

the extract (**Figure 1**). Other identified components were chlorogenic acid (**1**), vitexin (**2**), rutin (**3**), isoquercitrin (**5**), quercetin (**6**) (**Figure 2** and **Table 2**). Total flavonoids content, applying method 1, revealed the percentage of 0.14%. Determined by the HPLC procedure (method 2), total flavonoid aglycones represented 0.18% of ethanol hawthorn berries extract, expressed as quercetin (**Figure 3**). We applied stronger hydrolysis conditions for the investigated extract then usually proposed but with attention not to degrade the flavonoid aglycones. The successful hydrolysis procedure comprehended the absence of the peaks corresponding to hyperoside and isoquercitrin, as the main components in the investigated extract, and the presence of only one peak corresponding to quercetin in the HPLC chromatogram. Vitexin, whose presence was confirmed in the hydrolisate, because C-glycoside was not hydrolyzed, was what was expected (**Figure 3**). As determined only in the traces, rutin and vitexin (**Table 2**) in original extract and vitexin in the extract after hydrolysis did not significantly contribute to the total flavonoid and total flavonoid aglycones content (**Table 1**). Caffeic acid, vitexin-2″-*O*-rhamnoside, cyanidin chloride, and (−)-epicatechine, although literature data suggested (*1, 3*−*7, 31*), were not identified in the extract. When the HPLC profile of *C. pinnatifida* fruits was compared to the HPLC fingerprint of the investigated extract, we obtained a similar pattern of HPLC chromatogram, with hyperoside and isoquercitrine as main constituents in the flavonol group of compounds (*32*).

The results demonstrated that the extract tested possessed DPPH free-radical-scavenging activity. When the extract was applied in the concentration range of 6130−1240 μg/mL, its DPPH free-radical-scavenging activity varied approximately from 90 to 40%, respectively, with EC_{50} value of 1470 μg/mL.

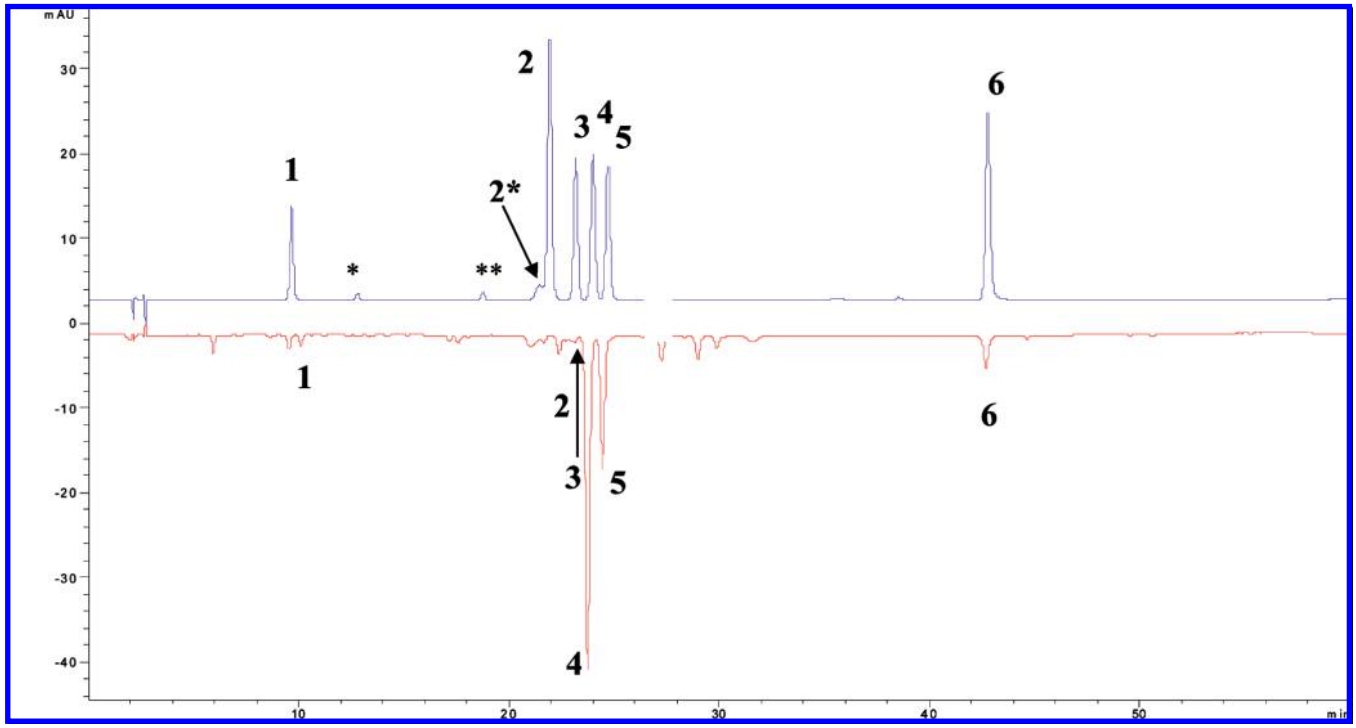


Figure 2. HPLC chromatogram of the ethanol extract of the dried berries of hawthorn recorded at 360 nm compared to the standard mix of identified flavonoids. Numbers refer to the following: **1**, chlorogenic acid; **2**, vitexin; **2***, vitexin-2″-*O*-rhamnoside; **3**, rutin; **4**, hyperoside; **5**, isoquercitrin; and **6**, quercetin. (∗) Impurity from chlorogenic acid standard. (∗∗) Impurity from vitexin standard.

Table 2. Quantitative Determination of Flavonoids and Phenolcarbonic Acids in Ethanol Hawthorn Berries Extract

compound name[a]	Rt[b]	Rt[c]	λ_{max} of investigated compounds	percentage (%)
chlorogenic acid (**1**)	10.103	9.969	218, 238, 296, 324	0.02
caffeic acid		12.458	218, 238, 296, 324	not found
(−)-epicatechin		13.394	278	not found
vitexin-2″-*O*-rhamnoside (**2***)		21.340	268, 338	not found
cyanidin chloride		21.940	276	not found
vitexin (**2**)	22.379	21.999	268, 338	trace
hyperoside (**3**)	23.747	23.998	256, 266, 300, 354	0.14
rutin (**4**)	23.099	23.174	256, 266, 298, 354	trace
isoquercitrin (**5**)	24.459	24.707	256, 266, 298, 354	0.03
quercetin (**6**)	42.679	42.773	254, 370	0.01
quercetin[d] (**6**)	43.140	42.773	254, 370	0.18

[a] The numbers refer to compounds signed on the HPLC spectrum (**Figures 1**−**3**). [b] Retention times of the compounds identified in the extract. [c] Retention times of the standards in the HPLC chromatogram of standards mix. [d] Percentage determined after acid hydrolysis.

It was much more compared to those of trolox and BHT (EC_{50} = 5.9 and 6.0 μg/mL, respectively) (**Table 3**). However, the trolox and BHT EC_{50} values are for pure compounds, whereas the value for the extract is expressed in the amount of dry weight biomass. If one assumes that phenolics contained in the extract are the carriers of its free-radical-scavenging activity, then, according to their content in the extract (**Table 1**), the EC_{50} value is calculated to be 52.04 μg/mL.

The hawthorn ethanol extract reduced the carrageenan rat paw edema in a dose-dependent manner. Doses of 50, 100, and 200 mg/kg led to 20.8, 23.0, and 36.3% reduction of the edema, respectively. The reduction obtained by the highest dose used was statistically significant, amounting 72.4% of the activity achieved by indomethacin that was given in a dose producing 50% reduction of the rat paw edema (**Figure 4**).

The hawthorn berries ethanol extract exhibited significant dose-dependent gastroprotective activity comparable to that of the reference drug ranitidine. Moreover, in the highest tested dose (200 mg/kg p.o.), the extract produced an even stronger protective effect than ranitidine given in a dose of 20 mg/kg but this difference was not statistically significant (**Table 4**).

The results of antimicrobial activity of the ethanolic extract of hawthorn berries, tested in a disk diffusion assay, are presented in **Table 5**. In comparison to the standard antibiotic streptomycin, the extract produced moderate bactericidal effect, especially against Gram-positive bacteria *M. flavus*, *B. subtilis*, and *L. monocytogenes* (80.0, 62.2, and 60.7%, respectively). On the other hand, the extract failed to produce an effect against *C. albicans*.

DISCUSSION

Recent therapeutic uses of hawthorn concern cardiovascular effects, especially with regard to atherosclerosis, hypertension, and congestive heart failure. Its efficacy in these indications is supported by both experimental and clinical data. The current study, however, provides evidence on some other pharmacological effects of the plant. It is shown that the ethanolic extract of hawthorn berries, containing 3.54% of phenolic compounds (expressed as gallic acid equivalents) with 12.4% of procyanidins, 5.1% of flavonols (4.0, 0.8, and 0.3% of hyperoside, isoquercitrin, and quercetin, respectively), and 0.6% of chlorogenic acid of the extract total phenolics content, produces free-radical-scavenging, anti-inflammatory, gastroprotective, and antimicrobial activities.

Reactive oxygen species (ROS) are generated by many redox processes that normally occur in the metabolism of aerobic cells. These species are highly reactive and harmful to the cells. If not eliminated, ROS can damage important molecules, such as proteins, DNA, and lipids. Their excess has been implicated in the development of a number of chronic diseases, such as cancer, aterosclerosis, heart diseases, and rheumatism, as well as in the development of neurodegenerative disorders, such as Alzhe-

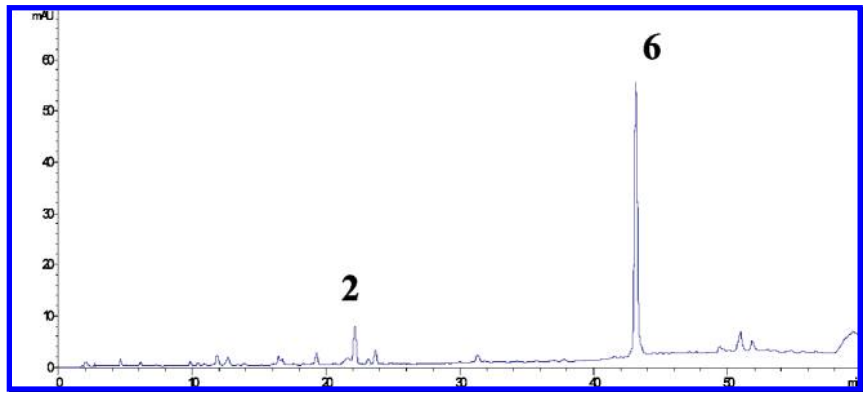


Figure 3. HPLC chromatogram of the hydrolyzed ethanol extract of the dried berries of hawthorn recorded at 360 nm. Numbers refer to the following: **2**, vitexin; **6**, quercetin.

Table 3. DPPH Free-Radical-Scavenging Activity of the Hawthorn Berries Ethanol Extract (HBE), Expressed According to the Total Phenolic Content

sample	EC_{50}[a] (μg/mL)
HBE	52.04 ± 0.06
trolox	5.9 ± 0.8
BHT	6.0 ± 0.2

[a] The mean value ± SD of three measurements.

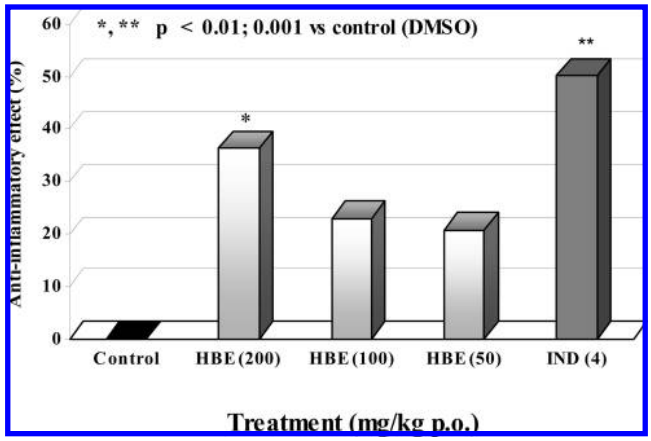


Figure 4. Effect of hawthorn berries extract (HBE) and indomethacin (IND) on carrageen-induced rat paw edema.

imer's and Parkinson's diseases (*33*−*36*). Because of this, there is an increasing interest in natural antioxidants, namely, phenolics, present in medicinal and dietary plants, that may help prevent oxidative damage (*33*−*38*). In cases of increased free-radical production, the reinforcement of endogenous antioxidants via intake of dietary antioxidants may be of great importance in attenuating the cumulative effects of oxidatively damaged molecules.

Flavonoids are the most common and widely distributed group of plant phenolics. Many of them act as potent metal chelators and free-radical scavengers, being suggested as powerful chain-breaking antioxidants (*22*). The major flavonoids in hawthorn are hyperoside, isoquercitrin, vitexin, quercetin, rutin, and vitexin-2-rhamnoside. Other important flavonoids in this plant are (−)-epicatechin and procyanidins B2, B5, and C1 and oligomeric proanthocyanidins with varying degrees of polymerization, catechin-1-epicatechin, cyanidin, and antocyanidin (*1, 3*−*7*).

Our results suggest that hyperoside and isoquercitrin are the main flavonols in the ethanolic extract of hawthorn berries tested in this study. Hyperoside was also established as a major flavonoid in dried fruits of another species of *Cratageus*, i.e., *C. pinnatifida* (*39*). It was shown that its flavonoids exhibited

Table 4. Effect of Hawthorn Berries Ethanol Extract (HBE) against Gastric Lesions Induced by Ethanol in Rats

treatment	dose (mg/kg p.o.)	gastric damage score[a]
control (DMSO, 1 mL/kg p.o.)		5.9 ± 1.1
	20	1.8 ± 1.1[b]
ranitidine	10	2.83 ± 1.03[c]
	5	3.08 ± 0.8[c]
	200	0.7 ± 0.8[d]
HBE	100	1.9 ± 1.7[c]
	50	3.8 ± 2.1

[a] 0, no lesions; 0.5, slight hyperaemia or ≤5 petechiae; 1, ≤5 erosions ≤5 mm in length; 1.5, ≤5 erosions ≤5 mm in length and many petechiae; 2, 6−10 erosions ≤5 mm in length; 2.5, 1−5 erosions <5 mm in length; 3, >5−10 erosions >5 mm in length; 3.5, >10 erosions >5 mm in length; 4, 1−3 erosions ≤5 mm in length and 0.5−1 mm in width; 4.5, 4−5 erosions ≤5 mm in length and 0.5−1 mm in width; 5, 1−3 erosions >5 mm in length and 0.5−1 mm in width; 6, 4- or 5-grade 5 lesions; 7, ≥6-grade 5 lesions; 8, complete lesion of the mucosa with hemorrhage. [b] $p < 0.01$ versus the control. [c] $p < 0.05$ versus the control. [d] $p < 0.001$ versus the control.

Table 5. Antimicrobial Activity of the Hawthorn Berries Extract (Mean Values of the Growth Inhibition Zones in Millimeters, Including the Disk Diameter of 12.7 mm)

microorganism	hawthorn berries ethanol extract (at concentration of 10 mg/ml DMSO, 50 μL per disk)	MIC (μg/mL)	MBC (μg/mL)	streptomycin sulfas (10 mm)	nystatin
Escherichia coli	11	119	187	22	nt[a]
Salmonella typhimurium	na[b]	nt	nt	25	nt
Staphylococcus aureus	8	151	187	30	nt
Enterococcus faecalis	na	nt	nt	20	nt
Streptococcus epidermidis	12	151	187	23	nt
Bacillus subtilis	23	119	187	37	nt
Micrococcus luteus	15	119	187	28	nt
Micrococcus flavus	20	151	187	25	nt
Pseudomonas aeruginosa	13	151	187	22	nt
Pseudomonas talaasii	na	nt	nt	15	nt
Proteus mirabilis	na	nt	nt	22	nt
Sarcina lutea	na	nt	nt	8	nt
Lysteria monocytogenes	17	151	187	28	nt
Saccharomyces cerevisiae	na	nt	nt	20	nt
Candida albicans	11	119	187	nd	20

[a] nt = not tested. [b] na = no activity.

a free-radical-scavenging capacity, implying their antioxidative potential (*40*), as well as a strong anti-inflammatory activity (*39*). In our study, the free-radical-scavenging activity of the hawthorn berries ethanol extract was examined by the DPPH method. DPPH is a useful reagent for investigating free-radical-scavenging activities of phenolic compounds (*41*). The reduction

of DPPH absorption is indicative of the capacity of the extracts to scavenge free radicals, independently of any enzymatic activity. The results of our study demonstrated that ethanol extract of hawthorn berries produced concentration-dependent free-radical-scavenging activity that was lower than those of trolox and BHT used as positive controls (EC_{50} values of 52.04, 5.9, and 6.0 μg/mL, respectively). It has been shown that substances with EC_{50} values $\leq$ 50 μg/mL in the DPPH assay are considered as active antioxidants (*42, 43*), and accordingly, the extract tested might be put in this category. Other authors also studied free-radical scavenging, i.e., antioxidative activity of hawthorn extracts. For example, a comparative study of antioxidant activities of flavonoid fractions from several medicinal plants on lecithin liposomes showed that concentrations required to inhibit oxidation by 50% were somewhat higher for hawthorn fruits (60 μM) compared to 55 μM for grape seeds, 38 μM for Japanese quince, and 32 μM for rose hips (*44*). Another group tested the total phenolic content of the hawthorn flower (*C. monogyna*) solvent extract for antioxidant activity against ROS hydrogen peroxide and hypochlorous acid, and the EC_{50} values obtained were 12.88 $\pm$ 0.47 and 35.18 $\pm$ 0.34 mg/mL, respectively. The most active antioxidant constituents of the hawthorn flower extract were shown from the flavonoid fraction and proanthocyanidin B_2 (*45*). In tests of a standardized extract of hawthorn, known as WS 1442, the flavonoid-free, oligomeric procyanidin-rich (OPC) fraction (21.3% of WS 1442) produced potent *in vitro* radical-scavenging activity (lipid peroxidation inhibition $EC_{50} = 0.3$ μg/mL, and human neutrophil elastase inhibition $EC_{50} = 0.84$ μg/mL). The extract itself was also active, whereas the flavonoid-rich fraction (14.9% of WS 1442) showed half of the activity (*9*). The OPC fraction (20 mg kg^{-1} day^{-1}) orally administered in rats showed a similar degree of protection from ischemia-reperfusion-induced pathologies as those of the administered extract (100 mg kg^{-1} day^{-1}) (*14*). This study emphasized procyanidins as another group of hawthorn constituents with strong antioxidative potential. Because the extract investigated in our study, besides flavon- and flavonol-types of flavonoids, also contains procyanidins, it could be concluded that its free-radical-scavenging activity might be, at least in part, due to the presence of this group of polyphenolic compounds.

The high total phenolic content and capability of the extract tested for scavenging free radicals might partly be responsible for both its anti-inflammatory and gastroprotective activities as demonstrated in carrageenan-induced paw edema test and ethanol-induced acute gastric damage, respectively. Namely, carrageenan injected in rat paw produces an acute local inflammatory response, which consists of two phases. It is thought that in the early phase (within the first hour after injecting carrageenan) many vasoactive substances (e.g., histamin, 5-hydroxytryptamin, bradykinins, and prostaglandins) are released. On the contrary, the second phase is related to neutrophil infiltration, as well as to the continuing of the production of arachidonic acid metabolites (*46*−*48*). It is known that, in the second phase of acute inflammation induced by carrageenan and characterized by neutrophil infiltration, activated polymorphonuclear cells produce a great amount of free-radical species, which may additionally damage the tissue caught by inflammation (*49*). Numerous investigations have shown that many active constituents from medicinal plants, mainly flavonoids and phenolcarbonic acids, preventing neutrophil infiltration in the inflammed area and neutralizing free-radical species, act as anti-inflammatory agents (*49, 50*). Regarding the DPPH-scavenging capacity of the hawthorn berries ethanol extract tested and high total phenol content, it could be hypothesized that its anti-inflammatory effect in the model of carrageenan-induced acute inflammation is a consequence, at least partly, of its flavonols, procyanidins, and phenolcarbonic acid content.

On the other hand, considering that the production of arachidonic acid metabolites is the main factor involved in both phases of carrageenan-induced inflammation, it might be proposed that the anti-inflammatory effect of the hawthorn extract may be related to the inhibition of synthesis or release of these metabolites. At the moment, we have no data about such an effect of the ethanolic extract of hawthorn (*C. monogyna* and *C. oxycantha*) berries that we tested in this study. However, it was recently shown that the flavonoid fraction of the aqueous extract of dried fruits of *C. pinnatifida* produced a strong anti-inflammatory effect reducing lipopolysaccharide (LPS, an endotoxin) induced production of nitric oxide (NO) and prostaglandins as a result of inhibition of iNOS and COX-2 expression. Futhermore, it was established that hyperoside is the major flavonoid in *C. pinnatifida* dried fruits and that it dose-dependently inhibited both COX-2 and iNOS (*39*). Hyperoside is also found to be the major flavonoid in the hawthorn extract tested in our study. Beacause of that, it seems reasonable to assume that the above-described effect of hyperoside on prostaglandin production in inflammation settings might be involved in the whole anti-inflammatory effect of this extract. On the other hand, it is well-known that, in macrophages and in other cell types, cytokines (such as interferon γ) and lypopolysaccharide induce co-expression of iNOS and COX-2. Both iNOS and COX-2 are responsible for the production of large pro-inflammatory mediators, nitric oxide and prostaglandins, at the inflammatory site. Raso et al. suggested that quercetin was responsible for inhibition of iNOS and COX-2 enzyme expression in stimulated macrophages (*51*−*53*). Besides, recently, it was confirmed that quercetin and isoquercitrin are effective inflammation suppressors (*54*). The presence of quercetin and isoquercitrin in investigated extract was confirmed, as shown in **Table 2**. On the basis of the mentioned investigations, our assumption addressed these flavonols among other substances as the potential carriers of anti-inflammatory activity of ethanol hawthorn berries extract.

The results of the present study demonstrated that the hawthorn berries ethanol extract offered significant protection against the ulcerogenic effect of absolute ethanol in rats and that this effect was very close to that achieved by the known anti-ulcer drug ranitidine. It is known that absolute ethanol is noxious for the stomach and that it affects the gastric mucosa topically by distrupting its barrier and thus causing hydrogen back diffusion that leads to necrosis. As a result of the disturbed barrier function of gastric mucosa, significant microvascular changes (rapid and strong vasoconstriction accompanied by rapid and vigorous arterial dilation) occur. As a consequence, oxyradical-mediated injury of gastric mucosa results from ischemia followed by reperfusion (*55*−*57*). Salim (*58*) concluded that oxygen-derived radicals were directly implicated in that mechanism and that their removal stimulated healing of ethanol-induced gastric lesions.

Many studies have demonstrated that substances with antioxidant properties (e.g., polyphenolic compounds, such as flavonoids and phenolic acids) may protect against gastric-damaging effects of absolute ethanol (*59*−*63*). Because the ethanolic extract of hawthorn berries tested in this study contained the flavonoids component (**Tables 1** and **2**) and showed antioxidant activity, it could be suggested that a

significant gastroprotective effect of the extract, similarly to its anti-inflammatory effect, might at least in part be the consequence of the presence of flavonoids and this activity. Phenolic compounds in this extract might be marked as responsible for producing such effects. Furthermore, for flavonoids, several other mechanisms have also been proposed to explain their gastroprotective effect, including an increase of mucosal prostaglandin content, a decrease of histamin secretion from mast cells, an inhibition of acid secretion, and an inhibition of *Helicobacter pylori* growth (*64*).

In the late 1980s and early 1990s, NO emerged as an important mediator of gastric mucosal defense. Application to the stomach of a solution of NO or a NO donor significantly protected mucosa from injury (*65, 66*). The mechanisms through which NO protects gastric mucosa and promotes ulcer healing are not entirely clear. It is thought that NO exerts beneficial effects on the mucosa by maintaining gastrointestinal blood flow and inhibiting adherence and activation (i.e., release of reactive oxygen metabolites and proteases) of white blood cells within gastrointestinal microcirculation. As a consequence, a number of gastrointestinal-sparing nonsteroidal anti-inflammatory drugs (NSAIDs) with NO-releasing moiety (so-called NO−NSAIDs) have been synthetized to diminished their ulcerogenic potential (*67*). It has been shown that the hydroalcoholic extract from hawthorn leaves with flowers, standardized to contain 18.75% oligomeric procyanidins, may enhance endothelial NO synthesis causing vasorelaxation and cardioprotection (*9, 68*). The hawthorn extract used in our study had a low content of procyanidins (0.44%), but the possibility for their involvement in the stimulation of NO production and consequently its influence in gastric mucosal defense could not be excluded. Besides, recent investigations of chlorogenic acid biological activity provided evidence that this phenolcarbonic acid formed *o*-semiquinone radicals and nitrated polyphenols, suggesting the scavenging of $*NO_2$ by *o*-semiquinones and thereby promoted NO bioavailability at the gastric level (*69*). The presence of chlorogenic acid, confirmed in tested hawthorn berries extract (**Table 2**), might contribute to the shown beneficial effect on the gastric mucosa.

Finally, in a separate part of the experiment, we tested antimicrobial activity of the hawthorn berries ethanol extract. It was shown that, in comparison to the standard antibiotic streptomycin, the extract produced moderate bactericidal effect, especially against Gram-positive bacteria *M. flavus*, *B. subtilis*, and *L. monocytogenes* (80.0, 62.2, and 60.7%, respectively). On the other hand, the extract failed to produce an effect against *C. albicans*.

The overall aim of this study was to contribute to the global search for bioactive natural products. As documented, our investigated extract showed antioxidant, anti-inflammatory, and gastroprotective effects and antimicrobial activity. Anti-inflammatory and gastroprotective activities might be of particular therapeutic importance because most of the anti-inflammatory drugs used in modern medicine are also ulcerogenic. The results raise an interesting possibility of applying the traditional cardiotonic drug in preventing the inflammatory processes.

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