

Free radical-scavenging activities of *Crataegus monogyna* extracts

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Key words: free radicals; antioxidant activity.

Summary. The aim of this study was to investigate antiradical activity of aqueous and ethanolic hawthorn fruit extracts, their flavonoids, and flavonoid combinations.

Material and methods. Total amount of phenolic compounds and the constituents of flavonoids were determined using a high-performance liquid chromatography. The antioxidant activity of *Crataegus monogyna* extracts and flavonoids (chlorogenic acid, hyperoside, rutin, quercetin, vitexin-2O-rhamnoside, epicatechin, catechin, and procyanidin B₂) quantitatively was determined using the method of spectrophotometry (diphenyl-1-picrylhydrazyl (DPPH·) radical scavenging assay and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)(ABTS·+) radical cation decolorization assay). The level of tyrosine nitration inhibition was determined using a high-performance liquid chromatography.

Results. Ethanolic hawthorn fruit extract contained 182±4 mg/100 mL phenolic compounds, i.e. threefold more, as compared to aqueous extract. The antioxidant activity according to DPPH· reduction in the ethanolic extracts was higher 2.3 times ($P<0.05$). The ABTS·+ technique showed that the effect of ethanolic extracts was by 2.5 times stronger than that of aqueous extracts. Tyrosine nitration inhibition test showed that the effect of ethanolic extracts was by 1.4 times stronger than that of aqueous extracts. The investigation of the antiradical activity of the active constituents in aqueous and ethanolic extracts revealed that epicatechin and catechin contribute to radical-scavenging properties more than other components. Procyanidin B₂ only insignificantly influenced the antiradical activity of the extracts.

Conclusion. Both aqueous and ethanolic hawthorn extracts had antiradical activity, but ethanolic extract had stronger free radical-scavenging properties, compared to the aqueous extract. The antioxidant activity of the studied preparations was mostly conditioned by epicatechin and catechin. The individual constituents of both extracts had weaker free radical-scavenging properties than the combination of these substances did.

Introduction

Despite the possibilities of modern scientifically oriented cardiologic diagnostics and treatment, mortality from cardiovascular diseases remains high, and cardiovascular diseases rank first among the causes of death (1). It is stated that one of the causes of heart failure is free radicals formed during metabolic processes in the body – organic substances with one or more unpaired electrons, such as hydroxyl (OH·), peroxy (ROO·), alkoxy (RO·), and peroxonitrite (ONOO·) (2). For this reason, it is important to neutralize these free radicals. Neutralization of free radicals may be affected not only by the enzymes of the antioxidant protection system (catalase, superoxide dismutase, etc.), but also possessing antiradical activity

in phyto preparations.

Preparations from hawthorn fruits have a cardio-stimulating effect and are used for teas, dry and liquid extracts, tinctures, and juice. The main biologically active substances detected in the medicinal vegetal raw material of monogynous hawthorn are flavonoids and their glycosides: hyperoside (quercetin-3-galactoside), quercetin, vitexin, vitexin-O-rhamnoside, isovitexin-O-rhamnoside, acetyl vitexin-O-rhamnoside, rutin, quercitrin (quercetin-3-rhamnoside), orientin, kaempferol, spiroside, saponaretin, oligomeric procyanidins, catechins, and phenolic acids (chlorogenic acid, caffeic acid, triterpene saponins, etc.) (3, 4).

Literature provides abundant data proving that various biologically active compounds possess differ-

ent free radicals scavenging ability (5). However, we did not find any data on how active substances and preparations of hawthorn fruit scavenge free radicals, and therefore, we have selected aqueous and ethanolic hawthorn extracts for experimental studies.

The aim of this study was to investigate antiradical activity of aqueous and ethanolic hawthorn fruit extracts, their flavonoids, and flavonoid combinations.

2. Material and methods

2.1. Plant material

The fruit of hawthorn (*Crataegus monogyna* (L.) (*Rosaceae*)) were harvested from the collection of medicinal plants at Kaunas Botanical Garden (Vytautas Magnus University, Lithuania). The fresh fruit were sorted out and dried in the drying room with active ventilation at ambient temperature.

2.2. Preparation of ethanolic and aqueous extracts

Water extraction: 20.0 g of fruit chopped into pieces of up to 0.5–1 mm in size was extracted with 100 mL ultra-filtered water (absorption coefficient, 2.8) at 80°C for 20 min in a water bath shaker (Shaking Bath 5B-16) (Techne, Ltd., UK). After cooling, the extract was centrifuged at 5000 rpm for 10 min and filtered by a Millipore filter with a nylon membrane under vacuum at 25°C. The filtrate was stored at 4°C until use within 24 h. Ethanol extraction was performed as follows: a 20.0-g sample was extracted with 100 mL 70% ethanol via percolation.

2.3. Evaluation of antioxidant activity

We studied the influence of chlorogenic acid, quercetin derivatives (hyperoside, rutin, quercetin, and vitexin-O-rhamnoside), and epicatechin derivatives (epicatechin, catechin, and procyanidin B₂) on the antiradical activity. Flavonoid concentrations used during this experiment were identical to the amounts of active substances detected in the studied preparations. The composition of the flavonoid combination in the aqueous extract in µg/mL was as follows: chlorogenic acid 11.04, hyperoside 19.8, rutin 1.89, quercetin 0.18, vitexin-2O-rhamnoside 1.04, epicatechin 172.9, catechin 131.9, procyanidin B₂ 3.46. The composition of the flavonoid combination in the ethanolic extract in µg/mL was as follows: chlorogenic acid 34.6, hyperoside 293.2, rutin 26.4, quercetin 12.6, vitexin-2O-rhamnoside 29.6, epicatechin 449.2, catechin 370.0, procyanidin B₂ 7.1 (Sigma, USA).

2.3.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH·) radical scavenging assay

The antioxidant activity of vegetal tinctures and

flavonoids was determined by measuring the percentage of the DPPH· radical neutralized by the antioxidant compounds in the studied samples as described elsewhere (6). In brief, 1 mL of 0.1 mM methanol DPPH· solution was mixed with the studied preparation in a 1-cm³ cuvette. After 5 minutes following the mixing, the reduction in the level of absorption of the studied specimens was measured under a spectrophotometer at the wavelength of 517 nm. In parallel, the level of absorption was measured in the control specimen (1 mL of 0.1 mM methanol DPPH· solution without studied preparations).

The antioxidant activity was calculated as the percentage of the amount of inactivated DPPH· according to the formula:

$$\text{DPPH}\cdot \text{ inactivation percentage} = [(A_b - A_a)/A_b] \times 100,$$

where: A_b – absorption of the inactive specimen ($t=0$ min), and A_a – absorption of the specimen with the studied preparation (6).

2.3.2. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS·+) radical cation decolorization assay

We used standardized ABTS·+ method (7). Namely, ABTS·+ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature (23°C) in the dark for 16 h. The ABTS·+ solution was diluted with 80% ethanol. The filtered sample was diluted with ultra-filtered water or 80% ethanol to give a 20–80% inhibition of the blank absorbance with 0.1 mL of sample. ABTS·+ solution was added to 0.1 mL of the samples tested and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 10 min, and the absorbance was immediately recorded at 734 nm. The antioxidant activity was calculated as ABTS·+ inactivation percentage using the similar formula as in case of the DPPH· technique (7).

2.3.3. Determination of the level of tyrosine nitration inhibition using the HPLC technique

The study was performed using high-performance liquid chromatography (HPLC) system HP 1100 (Agilent Technologies). UV spectra were registered using HP 8453 (Agilent Technologies) detector. We used standardized method (6, 7). Namely, 8 µL of 10 mM peroxyxynitrite solution in 0.1 M sodium hydroxide solution was drawn and rapidly mixed in the chromatograph injector with 42 µL of vegetal extract or standard ethylene glycol monomethyl ether mixture (diluted with water at 1:1 ratio), containing 1.0 mM of tyrosine solution in 0.15 M potassium dihydrogen phosphate and sodium hydrogen buffer (pH 6.0). The

reaction mixture was directly injected into ESC cartridge *Supelcosil ABZ Plus* 250×4.6 mm, 5 μm. The mobile phase was composed of 90% 40 mM formic acid and 10% metylecyanide at the flow rate of 1 mL/min. Chromatograms were recorded at the wavelength of 276 nm. The activity of the studied substances was evaluated by comparing the peak of 3-nitrozotyrosine with that of the control sample.

2.3.4. Evaluation of total phenolics

Evaluation was performed using the Folin-Ciocalteu colorimetric method described previously with a little modification (8). Briefly, the appropriate dilutions of the filtered extracts were oxidized with 0.2 N Folin-Ciocalteu reagent, and then the reaction was neutralized with saturated sodium carbonate (75 g/L). The absorbance intensity of the resulting blue color was measured at 760 nm with a spectrophotometer after incubation for 2 h at 23°C. Quantification was performed based on the standard curve of gallic acid. Results were expressed as grams of gallic acid equivalent (GAE) per weight of 100 mL of vegetal tincture.

2.3.5. Determination of the amount of flavonoids using high-performance liquid chromatography (HPLC)

For the study, we used the chromatographic system *Waters 2690* with UV/Vis detector *Waters 2487* (Waters, Milford, USA), column *XTerra RP18* 150×3.9 mm, 3.5 μm. The mobile phase A was 0.1% aqueous trifluoroacetic acid (TFA) solution, and the mobile phase B – 0.1% TFA solution in acetonitrile. The change in the concentration of the solvents of the phase was a direct gradient from A 5% B to 45% B per 45 min, and the flow rate was – 0.4 mL/min. Chromatograms were recorded at 360 and 275 nm wavelengths. The amount of flavonoids was calculated according to the peak areas, using flavonoid standard calibration curves.

Data analysis

Statistical analysis was performed using statistical software package *Statistica 5.5*. The data were presented as means ± S.E.M. Statistical analysis was performed using Student's *t* test, and $P < 0.05$ was used as the level of significance.

Results

Ethanollic hawthorn fruit extract (ECE) contained 182 mg/100 mL phenolic compounds, i.e. threefold more, as compared to aqueous extract (ACE) (61 mg/100 mL). Meanwhile, when applying the HPLC technique, the amount of the derivative of phenolic

acids – chlorogenic acid – in aqueous extract was 11 μg/mL, i.e. 3.13 times less than in ECE (Table). In ACE, hyperoside was the most abundant among the studied quercetin derivatives, but its amount was by 7 times lesser than that in ECE. Of the studied epicatechin derivatives, the most abundant ones were epicatechin (by 2.6 times less than in ECE) and catechin (by 2.8 times less than in ECE) (Table).

Although the total amount of phenolic compounds in ECE was three-fold greater than in ACE, DPPH· reduction in the studied preparations differed by 2.3 times ($P < 0.05$) (53.4% – in ECE, and 23.1% – in ACE) (Fig. 1). The examination of the antioxidant activity using the ABTS·+ technique showed that the effect of ECE was by 2.5 times stronger than that of ACE (ECE – 45%, ACE – 18%) ($P < 0.05$). Meanwhile, the TNI test showed that the effect of ECE was by 1.4 times stronger than that of ACE (ECE – 34%, ACE – 24%) (Fig. 1).

In further studies, we tried to clarify how actively substances neutralized free radicals in the studied preparations (Table). The investigation of the antiradical activity of the active substances in ACE using all three techniques was applied. The strongest free radical-scavenging properties were observed in epicatechin (3.4%, 3.0%, and 2.3%, respectively for DPPH·, ABTS·+, and TNI) and catechin (2.2%, 1.8%, and 1.3%, respectively using DPPH·, ABTS·+, and TNI). Procyanidin B₂ only insignificantly influenced the antiradical activity (0.2%, 0.3%, and 0.2%, respectively) (Table). For the group of quercetin derivatives, the strongest free radical-scavenging properties were observed in hyperoside (0.3% and 0.4%, respectively using DPPH· and TNI), whereas its examination using the ABTS·+ technique showed that its antiradical activity equaled to that of chlorogenic acid – 0.2% (Table).

The studies of ECE revealed that of the flavonoids of the epicatechin group, the strongest free radical-scavenging properties were displayed by the same active compounds as in ACE, i.e. epicatechin (8.4, 7.2 and 4.1%, respectively using DPPH·, ABTS·+, and TNI), and of the quercetin group derivatives – hyperoside (6.3, 4.3 and 1.3%, respectively using DPPH·, ABTS·+, and TNI).

The total amount of the activity of individual constituents was obtained by summation, and it is equal to: 6.6, 5.9, and 4.7%, respectively using DPPH·, ABTS·+, and TNI in ACE, and – by 24.5, 20.1, and 11.3%, respectively using DPPH·, ABTS·+, and TNI in ECE. Further, we investigated how the combination of individual active substances influenced the antiradical activity of the preparation. The application of

all techniques showed that a combination of flavonoids in both ACE and ECE more strongly scavenged free radicals than individual studied substances, compared to the data presented in Table, but less strongly than ECE or ACE (Fig. 2).

Discussion

The extraction of the active compounds from vegetal crude material may be performed using various solvents as extractants. Cai et al. studied 112 Chinese medicinal plants and used methanol and water as extractants (9). Miliauskas et al. extracted with three solvents: acetone, ethyl acetate, and methanol (10). We chose ethanol and water as extractants, since they are frequently used in the manufacturing of liquid hawthorn fruit preparations – infusions, decoctions, or extracts.

The determination of the antiradical activity of the preparations *in vivo* and *in vitro* is performed by applying a number of techniques; the most commonly applied techniques are ABTS $\cdot^+$ and DPPH $\cdot$, and tyrosine nitration inhibition is applied somewhat less frequently. It has been found that the product – peroxy-nitrite – can itself cause damage (11). In addition, at physiological pH, peroxy-nitrite protonates and decomposes to a range of noxious products that are identical with nitronium ion (NO $_2^+$), the free radical gas nitrogen dioxide (NO $_2$), and OH $\cdot$ (12). The most common and reliable method involves the determination of the disappearance of free radicals using a spectrophotometer, such as 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic) acid radical (ABTS $\cdot^+$) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH $\cdot$) (6, 7). Miller et al. first developed the ABTS $\cdot^+$ method, and Brand-Williams et al. established the DPPH $\cdot$ method (13). Reduction of ABTS $\cdot^+$ or DPPH $\cdot$ by a radical species (R) (ABTS $\cdot^+$ + R $\rightarrow$ ABTS-H + DPPH-R) or by an antioxidant (A) (ABTS $\cdot^+$ + A $\rightarrow$ ABTS-H or DPPH-H + A) causes a loss of absorbance at 734 nm or 515 nm, respectively. We successfully applied these three techniques for assaying total antioxidant activity of hawthorn fruit extracts on a large scale. Thus, three antioxidant study techniques showed that ECE had statistically reliably stronger activity in free radical scavenging. Differing indices of the antioxidant activity of the same product obtained when applying different techniques allow assuming that there are various chemical substances that participate in the inactivation of free radicals in DPPH $\cdot$, ABTS $\cdot^+$, or TNI (Fig. 1).

Zheng and Wang determined that phenolic compounds had a major contribution to antioxidant activity (14). Of our studied preparations (ACE and ECE

Table. The influence of chlorogenic acid and flavonoids on antiradical activity

Active substance	Determined concentration, $\mu\text{g/mL}$		Amount of inactivated radical, %					
			DPPH $\cdot$		ABTS $\cdot^+$		Tyrosine nitration inhibition	
	Aqueous Crataegus extract	Ethanol Crataegus extract	Aqueous Crataegus extract	Ethanol Crataegus extract	Aqueous Crataegus extract	Ethanol Crataegus extract	Aqueous Crataegus extract	Ethanol Crataegus extract
Chlorogenic acid	11.04 $\pm$ 0.91	34.6 $\pm$ 1.21	0.1 $\pm$ 0.01	0.6 $\pm$ 0.02	0.2 $\pm$ 0.02	0.4 $\pm$ 0.07	0.1 $\pm$ 0.00	0.3 $\pm$ 0.01
Hyperoside	19.8 $\pm$ 1.42	293.2 $\pm$ 3.20	0.3 $\pm$ 0.01	6.3 $\pm$ 0.10	0.2 $\pm$ 0.03	4.3 $\pm$ 0.87	0.4 $\pm$ 0.01	1.3 $\pm$ 0.23
Rutin	1.89 $\pm$ 0.70	26.4 $\pm$ 1.22	0.1 $\pm$ 0.02	0.4 $\pm$ 0.04	0.1 $\pm$ 0.02	0.3 $\pm$ 0.00	0.1 $\pm$ 0.00	0.3 $\pm$ 0.01
Quercetin	0.18 $\pm$ 0.01	12.6 $\pm$ 1.10	0.1 $\pm$ 0.02	0.5 $\pm$ 0.03	0.1 $\pm$ 0.01	0.4 $\pm$ 0.02	0.1 $\pm$ 0.01	0.3 $\pm$ 0.02
Vitexin-2O-rhamnoside	1.04 $\pm$ 0.22	29.6 $\pm$ 2.34	0.1 $\pm$ 0.01	0.5 $\pm$ 0.02	0.1 $\pm$ 0.03	0.3 $\pm$ 0.05	0.1 $\pm$ 0.01	0.3 $\pm$ 0.01
Epicatechin	172.9 $\pm$ 5.83	449.2 $\pm$ 10.75	3.4 $\pm$ 0.14	8.4 $\pm$ 0.32	3.0 $\pm$ 0.54	7.2 $\pm$ 0.47	2.3 $\pm$ 0.11	4.1 $\pm$ 0.71
Catechin	131.9 $\pm$ 11.20	370.0 $\pm$ 20.14	2.2 $\pm$ 0.22	8.4 $\pm$ 0.54	1.8 $\pm$ 0.45	6.5 $\pm$ 0.78	1.3 $\pm$ 0.08	4.3 $\pm$ 0.15
Procyanidin B $_2$	3.46 $\pm$ 0.1	7.1 $\pm$ 0.6	0.2 $\pm$ 0.01	0.4 $\pm$ 0.01	0.3 $\pm$ 0.02	0.7 $\pm$ 0.03	0.2 $\pm$ 0.01	0.4 $\pm$ 0.01

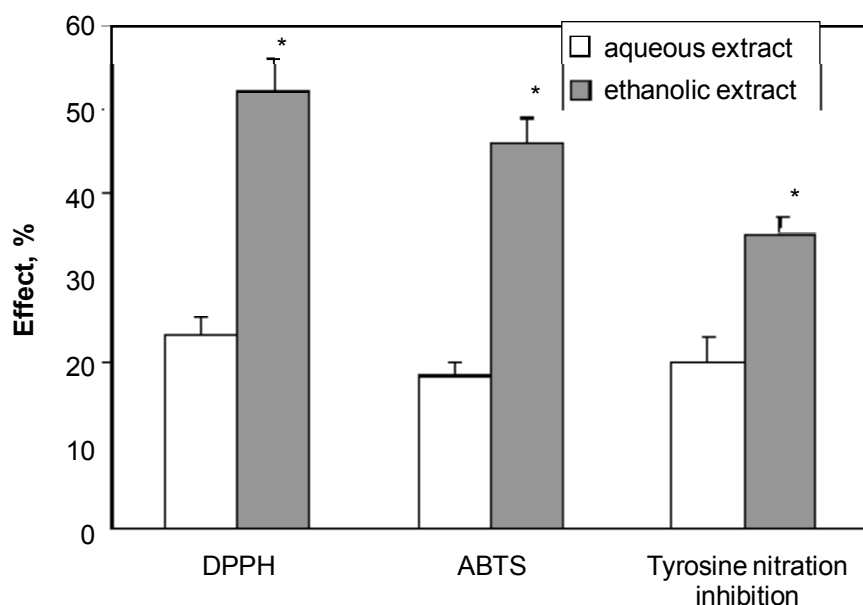


Fig. 1. Antiradical activity of the hawthorn fruit extracts determined using DPPH, ABTS+, and tyrosine nitration inhibition techniques

* $P < 0.05$ vs aqueous extract.

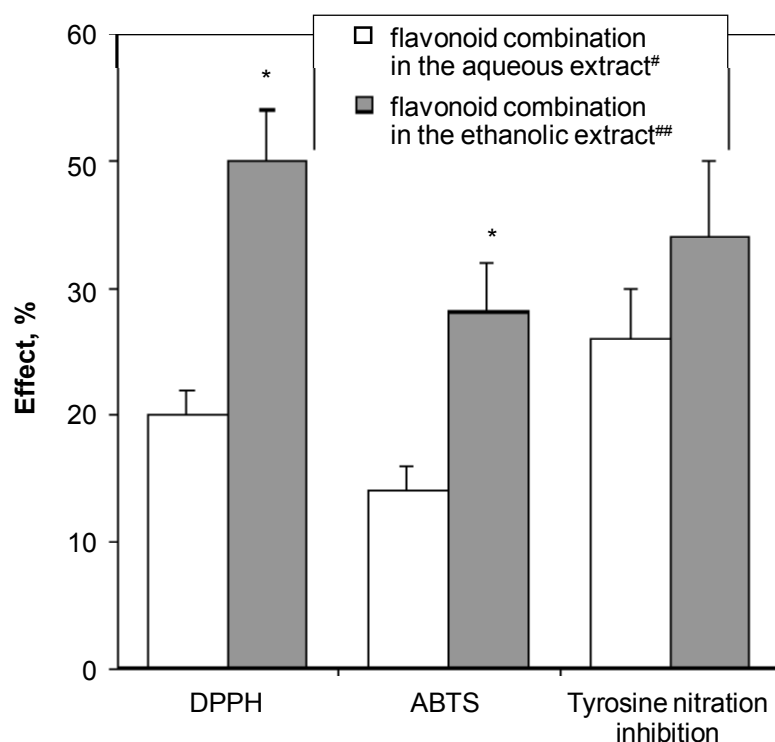


Fig. 2. The antiradical activity of standard flavonoids combinations (mimic composition of hawthorn fruit extracts)

The composition of the flavonoid combination in the aqueous extract in $\mu\text{g/mL}$: chlorogenic acid 11.04, hyperoside 19.8, rutin 1.89, quercetin 0.18, vitexin-2O-rhamnoside 1.04, epicatechin 172.9, catechin 131.9, procyanidin B₂ 3.46.

The composition of the flavonoid combination in the ethanolic extract in $\mu\text{g/mL}$: chlorogenic acid 34.6, hyperoside 293.2, rutin 26.4, quercetin 12.6, vitexin-2O-rhamnoside 29.6, epicatechin 449.2, catechin 370.0, procyanidin B₂ 7.1.

* $P < 0.05$ vs flavonoid combination in the aqueous extract.

extracts), ECE extract was found to have more phenolic compounds, and the application of all three techniques showed that this extract had stronger free radical-scavenging activity than ACE. Meeker et al. found that the activity of phenolic compounds is conditioned by their chemical composition, and for this reason in our study, we determined the antiradical activity of individual flavonoids in the studied extracts (15). Epicatechin and catechin had the strongest influence on the antiradical activity of both hawthorn fruit preparations, but the studied preparations contained the largest amounts of these substances. It has been found that flavonoids of the epicatechin group scavenge free radicals like a known antioxidant – vitamin E – does (16). Some scientists determined that tea catechins and polyphenols are effective scavengers of reactive oxygen species in vitro and may function indirectly as antioxidants through their effects on transcription factors and enzyme activities (17). Thus, our findings confirmed the reports of other authors stating that epicatechin has strong antiradical activity. It has been found that procyanidin also had the highest antioxidant activity in both the microsomal lipid peroxidation and the hydroxyl radical scavenging assay (18). However, in all our studied preparations, the antiradical activity of procyanidin B₂ was not strong. This may be related to low concentrations of procyanidin in the studied preparations.

Other studied flavonoids did not significantly influence the antioxidant activity of the preparations – this may also be related to their low concentrations in the studied preparations. Antioxidant activity of phenolic depends on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules. Jung et al. determined that flavonol aglycones, such as quercetin and kaempferol, had multiple hydroxyl groups, and their antioxidant

activity is stronger than that of their glycosides such as rutin, astragalin, etc. (6). Our findings confirmed this opinion, since quercetin was statistically significantly less abundant in ACE than rutin was, but the antioxidant activity of both substances was equal. Similar results were obtained when studying the ethanolic hawthorn extract as well. Scientists have determined that quercetin is found to be the most active of the flavonoids, and many medicinal plants owe much of their activity due to their high quercetin content. Quercetin has demonstrated significant anti-inflammatory activity because of direct inhibition of several initial processes of inflammation. In addition, it exerts potent antioxidant activity and vitamin C-sparing action.

Individually studied substances only insignificantly influenced the antiradical activity, but the activity of their combination was stronger. We could not compare our findings with those of other authors, because we failed to find any reports on the studies of a similar combination of flavonoids.

Conclusions

Both aqueous and ethanolic hawthorn extracts had antiradical activity, but ethanolic extract had stronger free radical-scavenging properties, compared to the aqueous extract. The antioxidant activity of the studied preparations was mostly conditioned by epicatechin and catechin, but individual studied substances (chlorogenic acid, hyperoside, rutin, quercetin, vitexin-O-rhamnoside, epicatechin, catechin, and procyanidin B₂) had weaker free radical-scavenging properties than a combination of these substances did.

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Crataegus monogyna ekstraktų antiradikalinis aktyvumas

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Raktažodžiai: laisvieji radikalai, antioksidacinis aktyvumas.

Santrauka. Darbo tikslas. Ištirti vandeninio ir etanolinio gudobelių vaisių ekstrakto ir jame esančių flavonoidų bei jų mišinio antiradikalinį aktyvumą.

Medžiaga ir metodai. Fenolinių junginių ir flavonoidų (chlorogeno rūgšties, hiperozido, rutino, kvercetino, viteksin-O-ramnozido, epikatechino, katechino ir procianidino B₂) kiekis nustatytas efektyviosios skysčių chromatografijos metodu. *Crataegus monogyna* vaisių ekstraktų ir juose esančių flavonoidų antiradikalinis aktyvumas tirtas spektrofotometrijos metodu naudojant 2,2-difenil-1-pikrilhidrazilo (DPPH·) ir 2,2-azino-bis-(3-etilbenzotiazolin-6-sulfono rūgšties) (ABTS·+) radikalo sujungimo testus. Tirozino nitracijos inhibicija nustatyta aukšto slėgio skysčių chromatografijos metodu.

Rezultatai. Etanoliniame gudobelių ekstrakte nustatyta 182±4 mg/100 ml fenolinių junginių, t. y., tris kartus daugiau lyginant su vandeniniu gudobelių ekstraktu, o DPPH· redukcija tarp tiriamųjų preparatų skyrėsi 2,3 karto ($p < 0,05$). Tiriant ABTS·+ metodu, etanolinis ekstraktas veikė 2,5 karto stipriau nei vandeninis ($p < 0,05$). Tirozino nitracijos inhibicijos metodas parodė, kad etanolinis ekstraktas veikė 1,4 karto stipriau nei vandeninis. Tiriant vandeniniame ir etanoliniame ekstraktuose esančių atskirų veikliųjų medžiagų antiradikalinį aktyvumą, nustatyta, kad stipriausiai laisvuosius radikalus sujungė epikatechinas ir katechinas. Procianidinas B₂ antiradikaliniam aktyvumui turėjo nedidelę įtaką.

Išvada. Ir vandeninis, ir etanolinis gudobelių ekstraktas pasižymi antiradikaliniu veikimu, tačiau etanolinis ekstraktas laisvuosius radikalus suriša stipriau nei vandeninis. Stipriausiai antioksidacinį aktyvumą tirtuose preparatuose lemia epikatechinas ir katechinas, tačiau pavienės tirtos medžiagos laisvuosius radikalus suriša silpniau nei šių medžiagų mišinys.

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