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 cardiology 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⁻), peroxyl (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 phytoster 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 vegetable raw material of monogynous hawthorn are flavonoids and their glycosides: hyperoside (quercetin-3-galactoside), quercetin, vitexin, vitexin-O-rhamnoside, isovitexin-O-rhamnoside, acetylvitexin-O-rhamnoside, rutin, quercitrin (quercetin-3-rhamnoside), orientin, kaempferol, saponarin, saponarin, 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 posses 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 (Vy-
tautas 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 perfor-
mated 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 (hypersoside, rutin, quercetin, and
vitexin-O-rhamnoside), and epicatechin derivatives
during this experiment were identical to the amounts
of active substances detected in the studied prepara-
tions. The composition of the flavonoid combination
in the aqueous extract in μg/mL was as follows:
chlorogenic acid 11.04, hypersoside 19.8, rutin 1.89,
quercetin 0.18, vitexin-2O-rhamnoside 1.04, epicate-
chin 172.9, catechin 131.9, procyanidin B3, 3.46. The
composition of the flavonoid combination in the
ethanolic extract in μg/mL was as follows: chloro-
genic acid 34.6, hypersoside 293.2, rutin 26.4, quercetin
12.6, vitexin-2O-rhamnoside 29.6, epicatechin 449.2,
catechin 370.0, procyanidin B3, 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 percent-
age of the DPPH· radical neutralized by the antio-
dioxidant 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 prepara-
tion in a 1-cm cuvette. After 5 minutes following
the mixing, the reduction in the level of absorption of
the studied specimens was measured under a spectro-
photometer 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 per-
centage of the amount of inactivated DPPH· according
to the formula:

DPPH· inactivation percentage =\([\frac{Ab–Aa}{Ab}]\times 100\),
where: Ab – absorption of the inactive specimen (t=0
min), and Aa – 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 per-
sulfate 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
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 peroxyxinitrite solution in 0.1 M sodium hydroxide
solution was drawn and rapidly mixed in the chro-
matograph 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% metlycanide 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-nitrosozyrosine 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 X Terra 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

Ethanolic 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 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⁺ and DPPH⁻, and tyrosine nitration inhibition is applied somewhat less frequently. It has been found that the product – peroxynitrite – can itself cause damage (11). In addition, at physiological pH, peroxynitrite protonates and decomposes to a range of noxious products that are identical with nitronium ion (NO₂⁺), the free radical gas nitrogen dioxide (NO₂), and OH⁻ (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⁺) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH⁻) (6, 7). Miller et al. first developed the ABTS⁺ method, and Brand-Williams et al. established the DPPH⁻ method (13). Reduction of ABTS⁺ or DPPH⁻ by a radical species (R) (ABTS⁻+ or DPPH⁻+R ! ABTS⁻H DPPH⁻R) or by an antioxidant (A) (ABTS⁺ or DPPH⁻+A ! 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⁻, ABTS⁺, 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, µg/mL | Chlorogenic acid | Hyperoside | Rutin | Quercetin | Vitexin-2O-rhamnoside | Epicatechin | Catechin | Procyanidin B₂ |
|-----------------|--------------------------------|----------------|------------|-------|-----------|----------------------|------------|----------|----------------|
| Crataegus extract | 11.04±0.91 | 10.8±1.42 | 1.89±0.70 | 0.18±0.01 | 1.04±0.22 | 172.9±5.83 | 370.0±20.14 | 3.40±0.1 | 7.1±0.6 |
| Ethanol extract | 293.3±3.20 | 126.2±1.22 | 0.5±0.03 | 0.05±0.01 | 84.9±2.34 | 449.0±10.75 | 2.2±0.22 | 0.2±0.01 |
| DPPH⁻ | 34.6±1.21 | 29.3±1.10 | 0.2±0.01 | 0.4±0.01 | 3.0±0.04 | 18.0±0.45 | 0.3±0.02 |
| ABTS⁺ | 34.6±1.21 | 29.3±1.10 | 0.2±0.01 | 0.4±0.01 | 3.0±0.04 | 18.0±0.45 | 0.3±0.02 |
| Tyrosine nitration inhibition | 0.3±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 | 0.1±0.01 |

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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)

a The composition of the flavonoid combination in the aqueous extract in μg/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.

as The composition of the flavonoid combination in the ethanolic extract in μg/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 B, 7.1.

* P<0.05 vs flavonoid combination in the aqueous extract.
experiments), 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 B1 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, astragalain, 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 B1) had weaker free radical-scavenging properties than a combination of these substances did.

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Craetaegus monogyna ekstraktų antiradikalinis aktyvumas

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Raktažodžiai: laisvieji radikalai, antioksidacinis aktyvumas.

Santrauka. Darbo tikslas. Išstirti vandeninio ir etanolinio gudobelio vaisių ekstrakto ir jame esančių flavonoidų bei jų mišinių antiradikalinį aktyvumą.

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Medžiaga ir metodai. Fenolinių junginių ir flavonoidų (chlorogeno rūgšties, hiperozido, rutino, kvercetino, vitexin-O-ramnozido, epikatechnio, katechnio ir procianidino B₃) kiekis nustatytas efektyviosios skyščių 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-azonibis-(3-etilbenztiazolin-6-sulfono rūgšties) (ABTS⁺) radikalo sujungimo testus. Tirozino nitracijos inhibicija nustatyta aukšto slėgio skyščių chromatografijos metodu.

Rezultatai. Etanoliniame gudobelių ekstrakste nustatyta 182±4 mg/100 ml fenolinių junginių, t. y., tris kartus daugiau lyginant su vandeniniu gudobelių ekstraktu, o DPPH⁺- redukcija tarp tiriamoų preparatų skyrėsi 2,3 karto (p<0,05). Tariant ABTS⁺-metodą, etanolinis ekstraktas veikė 2,5 kartą stipriau nei vandeninis (p<0,05). Tirozino nitracijos inhibicijos metodas parodė, kad etanolinis ekstraktas veikė 1,4 kartą stipriau nei vandeninis. Tariant vandeniniame ir etanoliniame ekstraktuose esančių atskirų veiklųjų medžiagų antiradikalinį aktyvumą, nustatyta, kad stipriausiai laisvuosius radikalus suriengė epikatechnas ir katechnas. Procianidinės B₃ anti-radikaliniam aktyvumui turėjo nedidelę įtaką.

Išvada. Ir vandeninis, ir etanolinis gudobelių ekstraktas pasižymi antiradikaliiniu veikimu, tačiau etanolinis ekstraktas laisvuosius radikalus suriengė ir stipriau nei vandeninis. Stipriausiai antioksidacinį aktyvumą tirtuoje preparatuose lemia epikatechnas ir katechnas, tačiau pavienės tirtos medžiagos laisvuosius radikalus suriengė mišyniai savo antiradikalinio veikimo).

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