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THE COMPARISON OF ANTI-OXIDATIVE KINETICS *IN VITRO* OF THE FLUID EXTRACT FROM MAIDENHAIR TREE, MOTHERWORT AND HAWTHORN

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Abstract: The aim of the study was to perform a quantitative analysis of fluid extracts of maidenhair tree (*Ginkgo biloba* L.), motherwort (*Leonurus cardiaca* L.) and hawthorn (*Crataegus monogyna* Jacq.), to evaluate their antioxidant activity and to compare their ability to inactivate free radicals. The antioxidant activity was measured using the $DPPH^{\cdot}$ and the $ABTS^{\cdot+}$ radical scavenging reaction systems. The study showed that the manifestation of the radical scavenging capacity in the $DPPH^{\cdot}$ reaction system was in the following order: the fluid extract of hawthorn (70.37 ± 0.80%) > the fluid extract of maidenhair tree (82.63 ± 0.23%) > the fluid extract of motherwort (84.89 ± 0.18%), while in the $ABTS^{\cdot+}$ reaction system, the manifestation of the radical scavenging capacity was in the following order: the fluid extract of hawthorn (87.09 ± 0.55%) > the fluid extract of motherwort (88.28 ± 1.06%) > the fluid extract of maidenhair tree (88.39 ± 0.72%). The results showed that in the $DPPH^{\cdot}$ reaction system, fluid extract of motherwort manifested higher antioxidant activity, compared to the fluid extracts of maidenhair tree and hawthorn. By contrast, in the $ABTS^{\cdot+}$ reaction system, higher antioxidant activity was found in the fluid extract of maidenhair tree, compared to the fluid extracts of motherwort and hawthorn. This would suggest that preparations manufactured from these herbal raw materials could be used as effective preventive means and valuable additional remedies in the treatment of diseases caused by oxidative stress.

Keywords: maidenhair tree (*Ginkgo biloba* L.), motherwort (*Leonurus cardiaca* L.), hawthorn (*Crataegus monogyna* Jacq.), antioxidant activity

Free radicals – mainly reactive oxygen species – formed in our organisms during normal metabolism, may be among the causes of atherosclerosis, cancer, heart failure, and other diseases (1-3). For this reason, neutralization of free radicals is of great importance. Protection of the organism from the excess of free radicals is provided by protective enzymes of the antioxidant system (superoxide dismutase, catalase, and glutathione peroxidase) and scavengers of the reactive oxygen species (ROS) (tocopherols, ubiquinone, glutathione, L-ascorbic and uric acids, etc.) (2). The most important natural antioxidant is tocopherol (4). The hydrophilic analog of vitamin E is Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), which is a chain-breaking antioxidant that acts as a free radical scavenger *via* the H-donating group in its chromanol nucleus (2, 4).

Flavonoids, phenolic compounds and other bioactive compounds found in medicinal plants also have strong antioxidant properties and scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl, and inhibit oxidative mechanisms that cause degenerative diseases (2, 3, 5). Flavonoids (quercetin, hyperoside, rutin, quercitrin, kaempferol and others) are among the main compounds in *Crataegus monogyna, Leonurus cardiaca, Ginkgo biloba* and other plants that are used for the treatment and prophylaxis of cardiovascular disorders (6-8). The antioxidant activity of *Crataegus monogyna*, a plant that is widely spread in Europe, northwest Africa and western Asia, is based on oligomeric proanthocyanidins (8). In addition, it increases coronary blood flow, enhancing oxygen flow and utilization by the heart. (9). *Leonurus* herb is common throughout Europe; its preparation is used in

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the treatment of cardiac symptoms of neurosis, hypertension, and cardiac failure. However, the antioxidant activity of motherwort has not been sufficiently researched (10, 11). The antioxidant effect of standardized maidenhair tree (EGb 761) extract is related to its ability to scavenge free radicals, reduce nitric-oxide synthase or inhibit xanthine oxidase activity (6). It is postulated that both flavonoid and terpenoid constituents are involved in the antioxidant effects of *Ginkgo biloba* extract due to decreasing tissue levels of ROS and inhibition of membrane lipid peroxidation (6, 8).

Lithuanian pharmaceutical industry uses these medicinal raw materials in the production of "Hawthorn extract", "Motherwort extract", and "Maidenhair tree extract". We did not find any literature data about the antioxidative kinetics of these tinctures *in vitro*. Thus, the aim of the study was to perform a quantitative analysis of fluid extracts of maidenhair tree (GE), motherwort (LE) and hawthorn (CE), to evaluate their antioxidant activity, and to compare their ability to inactivate free radicals. Two techniques were selected for the study – 2.2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2.2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) free radicals scavenging reactions.

EXPERIMENTAL

Materials

The materials used in the study were fluid extracts from *Ginkgo biloba* L.*, Leonurus cardiaca* L., and *Crataegus monogyna* Jacq. raw materials (JSC Acorus Calamus, Lithuania). The freshly cut plants were sorted out and dried in the drying room with active ventilation at ambient temperature. Dried raw materials with no signs of external damage were used for analysis.

A 70% (v/v) ethanol (JSC Stumbras, Lithuania) and Trolox (6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid) (Sigma Aldrich, Germany) were used for the production of experimental fluid extracts and for the determination of the antioxidant activity.

Standards of chlorogenic acid, vitexin, isovitexin, rutin, hyperoside, astragalin, quercetrin, luteolin, quercetin, kaempferol, procyanidin B_2, epicatechin and ursolic acid were purchased from Carl Roth (Germany).

The antioxidant activity was evaluated using the following substances: 2.2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical, 2.2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) radical, methanol, (Sigma Aldrich, Germany), sodium chloride, potassium chloride, potassium dihydrogen phosphate, disodium phosphate dihydrate (Carl Roth, Germany) and potassium persulfate (Merck, Germany).

Methods

Extraction procedure

The experimental fluid extract was produced from *Ginkgo biloba* L., *Leonurus cardiaca* L., and *Crataegus monogyna* Jacq. raw materials by percolation, at the ratio of 1:5. Ethanol at the concentration of 70% (v/v) was used for the extraction. The plant material was moistened with ethanol for 1 h, and then extracted in a percolator for 24 h with a flow of the solvent (3 drops per min). The produced fluid extracts (GE, LE, and CE) were kept at 8°C for 72 h, filtered through a paper filter and then used for analysis.

Analysis of active compounds

Quantitative determination of flavonoids using the HPLC technique

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

Determination of the amount of phenolic compounds using colorimetry

Suitably diluted fluid extracts were oxidized using 0.2 M Folin-Ciocalteu reagent, and were later neutralized using sodium carbonate (75 g/L) solution. After 2 h, the suspension was centrifuged (at 5000 rpm, for 10 min), and the absorption level was measured at 760 nm wavelength. The amount of phenolic compounds was determined using the calibration curve for gallic acid. The results were expressed as gallic acid equivalent (GAE) for 100 mL of the fluid extract.

Antioxidant activity

The antioxidant activity was evaluated using DPPH and ABTS techniques. The antioxidant activity of the experimental fluid extracts (GE, LE, and

CE), Trolox and ethanol was assessed by measuring 2.2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) and 2.2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt ($ABTS^{\bullet+}$) free radicals.

Preparation of the Trolox solution

The concentration of the Trolox solution was selected on the basis of the extract technology. Trolox was dissolved in 70% ethanol at the ratio of 1:5 immediately before use.

The DPPH method

The $DPPH^{\bullet}$ radical was prepared by dissolving 2.36 mg of DPPH in 100 mL of methanol. Fresh DPPH stock solution was prepared on each day of analysis.

The fluid extracts (GE, LE and CE), ethanol or Trolox solution (50 µL) were mixed with $DPPH^{\bullet}$ methanolic solution (2 mL, 6×10^{-5} M) in 1 cm path length measuring cuvette. The decrease in the absorbance of the $DPPH^{\bullet}$ radical was measured at 517 nm during 10 min. Methanol was used as the reference (12).

The ABTS method

The $ABTS^{\bullet+}$ radical cation was generated by reacting 2 mM of ABTS solution (phosphate buffered saline, pH 7.4) with 70 mM of potassium persulfate, and allowing the mixture to stand in the dark at room temperature for 16 h. The fluid extracts (GE, LE and CE), ethanol or Trolox solution (30 µL) were mixed with $ABTS^{\bullet+}$ radical cation solution (3 mL) in 1 cm path length measuring cuvette. The decrease in the absorbance of the $ABTS^{\bullet+}$ radical was measured at 734 nm during 10 min. The reference solution was phosphorus and salt buffer solution (13).

The absorbance of the $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical was measured by using UNICAM Helios α UV spectrophotometer (Unicam, Cambridge, UK).

All samples were assayed in triplicate.

The percent inhibition of the $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ radical was calculated according to the following formula:

$$DPPH^{\bullet} \text{ or } ABTS^{\bullet+} \% \text{ inhibition} = [(A_{C(O)} - A_{C(t)})/A_{C(O)}] \times 100,$$

where A is the absorbance of the control at t = 0 min, and A is the absorbance of the reaction solution at t = 10 min.

Statistical analysis

Data are presented as the means ± S.E.M. Non-parametric methods were applied for making inferences about data. Differences between mean values in dependent groups were tested using Wilcoxon matched pairs test. Differences between mean values in independent groups were tested using non-parametric Kruskal-Wallis test with Dunn's *post-hoc* evaluation. $p < 0.05$ was taken as the level of significance. Statistical analysis was performed by using the software Statistica 1999, 5.5 StatSoft Inc., USA.

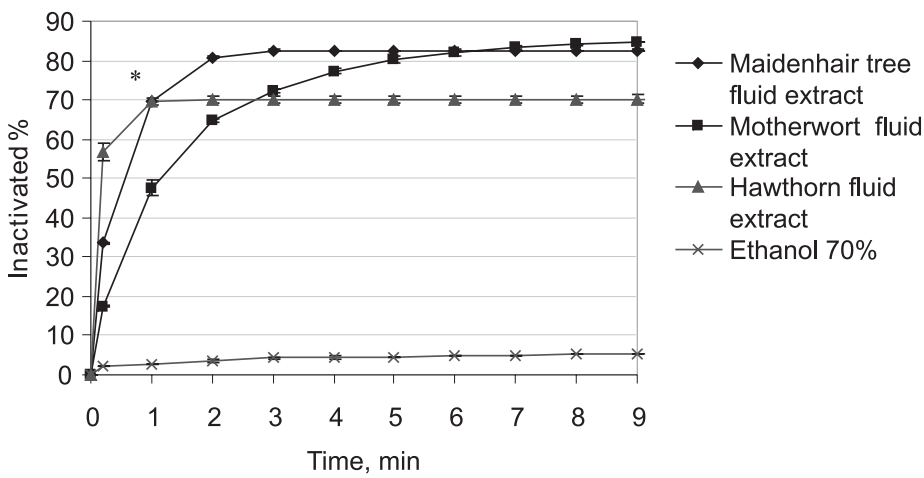

Figure 1. Reaction kinetics of the fluid extracts of maidenhair tree, motherwort, hawthorn, and ethanol (control) using the DPPH method. * $p < 0.05$ *vs.* motherwort fluid extract, n = 3-4

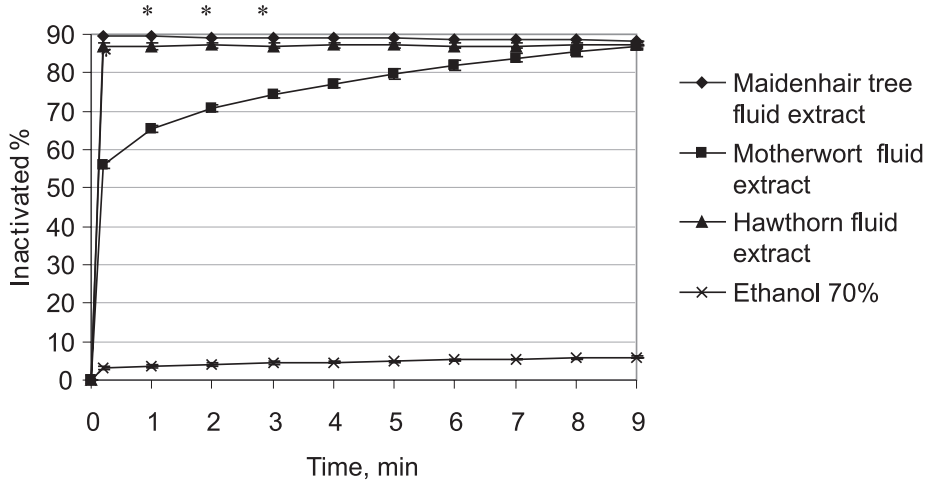

Figure 2. Reaction kinetics of the fluid extracts of maidenhair tree, motherwort, hawthorn, and ethanol (control) using the ABTS method. * $p < 0.05$ *vs.* motherwort fluid extract, n = 3

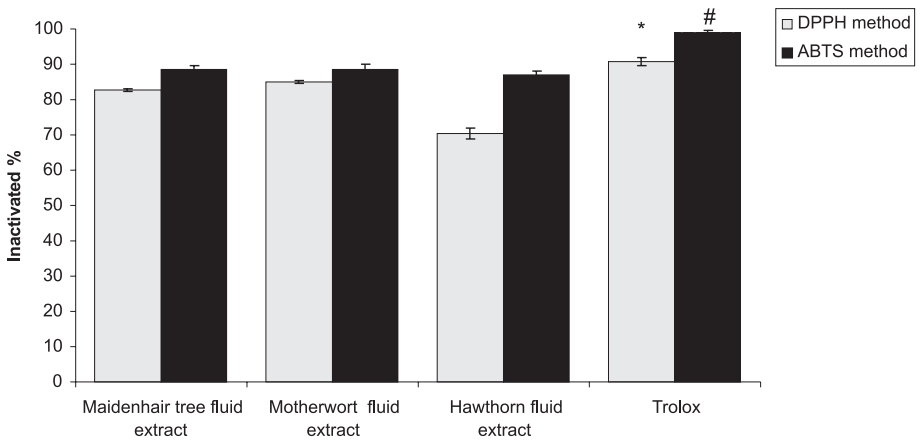

Figure 3. Antioxidant activity (after 10 min) of the fluid extracts of maidenhair tree, motherwort and hawthorn evaluated using the DPPH (DPPH inactivated %) and ATBS (ABTS inactivated %) methods, n = 3. * $p < 0.05$ *vs.* hawthorn fluid extract, # $p < 0.05$ *vs.* maidenhair tree, motherwort, and hawthorn fluids extracts

RESULTS AND DISCUSSION

The determination of the total amount of phenolic compounds showed that the extract of maidenhair tree (Ginkgo) contained the highest amount of these compounds (340 ± 4 mg GAE/100 mL), compared to other investigated extracts. The extract of motherwort contained 227 ± 6 mg GAE/100 mL, and the extract of hawthorn fruit – 139 ± 7 mg GAE/100 mL of phenolic compounds. The literature sources indicate that phenolic compounds have major influence on the antioxidant activity of the preparation (14, 15). Some papers state that the amount of phenolic compounds is in a direct proportion to antioxidant activity of the preparation (16, 17). Our study showed that although the extract of maidenhair tree contained by 1.5- and 2.4-fold higher amounts ($p < 0.05$) of phenolic compounds, compared to the extract of hawthorn fruit and the extract of motherwort, the investigation of the antioxidant activity yielded no statistically significant differences in free radical scavenging between the extract of maidenhair tree and the extract of motherwort (Fig. 1 and Fig. 2). Other literature sources also state that there is no direct correlation between the amount of phenolic compounds in the preparation and its antioxidant activity. For instance, R. Masteikova et al. found that despite statistically sig-

Table 1. Compounds identified in the extracts of maidenhair tree, motherwort, and hawthorn using the HPLC technique, n = 4

Active compounds µg/mL	The fluid extracts of maidenhair tree (GE)	The fluid extracts of motherwort (LE)	The fluid extracts of hawthorn (CE)
Chlorogenic acid	86.9 ± 8.0	46.0 ± 4.0	10.0 ± 0.9
Vitexin	23.1 ± 5.0	17.0 ± 0.3	4.0 ± 0.1
Isovitexin	145.8 ± 9.6	14.0 ± 0.7	4.0 ± 0.01
Rutin	227.5 ± 12.0	52.0 ± 7.3	5.0 ± 1.0
Hyperoside	41.2 ± 5.1	14.0 ± 2.0	20.0 ± 5.0
Astragalin	50.2 ± 6.0	1.0 ± 0.02	4.0 ± 0.1
Quercitrin	93.1 ± 4.0	1.0 ± 0.02	2.0 ± 0.02
Luteolin	33.2 ± 2.1	1.0 ± 0.01	2.0 ± 0.02
Quercetin	8.8 ± 0.2	8.0 ± 0.1	2.0 ± 0.04
Kaempferol	90.3 ± 2.4	2.0 ± 0.03	-
Procyanidin B	-	-	1.0 ± 0.01
Epicatechin	-	-	40.0 ± 2.3
Ursolic acid	-	250.0 ± 8.1	-

nificantly higher content of phenolic compounds, the extract of maidenhair tree exhibited lower antioxidant activity, compared to the extract of *Echinacea* or the extract of ginseng (18).

The analysis of the studied extracts using the HPLC technique allowed for the determination of their flavonoid (quercetin and epicatechin derivatives) content. The analysis of the extract of maidenhair tree showed that the bulk of the identified flavonoids consisted of quercetin glycosides: rutin (quercetin-3-rutinoside), quercitrin (quercetin-3-rhamnoside), and hyperoside (quercetin-3-galactoside). Rutin (227.5 ± 12.0 µg/mL) predominated in the extract of maidenhair tree. The extract of hawthorn fruit mostly contained epicatechin and hyperoside, while the extract of motherwort – ursolic acid. Other compounds identified in the studied preparations are presented in Table 1. The results of the HPLC analysis showed that the chemical compounds and their amounts detected in the extract of maidenhair tree and the extract of hawthorn fruit are similar to those mentioned in other studies on the chemical composition of these extracts (19, 20). Data on the chemical composition and pharmacological effect of motherwort are scarce. Although several studies do provide the composition of essential oils of motherwort, we found no studies in the chemical composition of ethanolic extracts of this herb.

In the next series of our experiments, the antioxidant activity of the samples was measured. Various methods were used for the evaluation of the antioxidant activity of the vegetal preparations, including oxygen radical absorbance capacity (ORAC) (21), ferric reducing antioxidant power (FRAP) (22), using the computerized spectrometer system (23), $DPPH^{\bullet}$, $ABTS^{\bullet+}$ (2, 14, 15, 18). Awika et al. observed high correlation between ABTS, DPPH, among sorghum and its products (24). On the basis of the literature data, we selected $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ techniques for the evaluation of the antioxidant activity of the studied preparations.

The radical scavenging capacity in $DPPH^{\bullet}$ reaction systems of the fluid extracts of maidenhair tree (GE), motherwort (LE) and hawthorn (CE), and ethanol (control) is presented in Figure 1. The results showed that the free radical scavenging ability of GE, LE and CE was relatively high. The samples reacted with $DPPH^{\bullet}$ radical immediately (Fig. 1). The investigation showed that the antioxidant activity of the tested samples depended on the extract. In the $DPPH^{\bullet}$ model system, after 10 seconds, the highest amount of $DPPH^{\bullet}$ radicals was scavenged by CE – 56.69 ± 2.15%. GE and LE scavenged, accordingly, 33.55 ± 0.13% and 17.47 ± 0.40% of radicals. The obtained findings about the antioxidant activity after 1 min showed a statistically significant difference of the activity of GE (69.70 ± 0.09%) and CE (69.72 ± 0.99%), compared to that of LE (47.64 ± 2.08%). However, no statistically significant difference between GE, CE, and LE was found after 3 min (82.63 ± 0.13%, 69.98 ± 0.92%, and 72.27 ± 0.40%, respectively). After 6 minutes, GE, CE, and LE equally inactivated free radicals

(82.54 ± 0.05%, 70.15 ± 0.77%, and 82.04 ± 0.72%, respectively). The highest ability to scavenge DPPH$^{\bullet}$ free radicals after 10 min was seen in the fluid extract from motherwort raw material (LE). The lowest ability to scavenge DPPH$^{\bullet}$ free radicals was observed in the fluid extract prepared from hawthorn raw material (CE). The study showed that the radical scavenging capacity in DPPH$^{\bullet}$ reaction system (after 10 min) was in the following order: CE (70.37 ± 0.80%) > GE (82.63 ± 0.23%) > LE (84.89 ± 0.18%) (Fig. 3).

We also studied the radical scavenging capacity of GE, LE and CE in the ABTS$^{\bullet+}$ reaction system. The results are presented in Figure 2. The free radical scavenging ability of the samples in this case was very high as well. In the ABTS$^{\bullet+}$ model system, GE and CE scavenged the highest amount of free radicals after 10 s (89.42 ± 0.59% and 86.84 ± 0.78%, respectively); after 9 min, the amount of scavenged free radicals remained nearly unchanged (88.43 ± 0.69% and 87.09 ± 0.55%, respectively). Meanwhile, LE after 10 s scavenged only 55.97 ± 1.05% of free radicals, while the data of antioxidant activity after 3 min (74.38 ± 0.99%) and 6 min (81.96 ± 1.26%) showed a different picture. The results showed that the highest ability to scavenge ABTS$^{\bullet+}$ free radicals after 9 min was exhibited by the fluid extract from maidenhair tree raw material (GE). The lowest ability to scavenge DPPH$^{\bullet}$ free radicals was seen in the fluid extract prepared from hawthorn raw material (CE). The study showed that the radical scavenging capacity in the ABTS$^{\bullet+}$ reaction system (after 10 min) was in the following order: CE (87.09 ± 0.55%) > LE (88.28 ± 1.06%) > GE (88.39 ± 0.72%) (Fig. 3).

The antioxidant activity (after 10 min) of the samples of GE, LE and CE for the DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ free radicals was compared between these samples and with a natural antioxidant Trolox (Fig. 3). The results showed relatively high antioxidant activity of the samples for the ABTS$^{\bullet+}$ radical compared to the antioxidant activity for the DPPH$^{\bullet}$ free radical. The antioxidant activity of GE, LE, and CE for the ABTS$^{\bullet+}$ free radical was 88.39 ± 0.72%, 88.28 ± 1.06%, and 87.09 ± 0.55%, respectively, while the antioxidant activity of GE, LE, and CE for the DPPH$^{\bullet}$ free radical was 82.63 ± 0.23%, 84.89 ± 0.18%, and 70.37 ± 0.80%, respectively. The investigation showed that the lowest antioxidant activity in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ reaction systems was found in the fluid extract of CE, compared to the fluid extracts of GE and LE.

The antioxidant activity of Trolox solution investigated using the DPPH$^{\bullet}$ technique was comparable to that of maidenhair tree or motherwort extracts, while the antioxidant activity of hawthorn extract was by 20% lower ($p < 0.05$). The antioxidant activity of the studied preparations determined using the ABTS$^{\bullet+}$ technique was by 9-11% lower than that of the natural antioxidant Trolox. Our results confirmed the findings obtained by other scientists, indicating that the various methods used to measure the antioxidant activity can give varying results depending on the specific free radical being used as a reactant (12, 14). Bizimenyera et al. found that the antioxidant activity of *Ginkgo biloba* extract (EGb 761) was higher, compared to that of L-ascorbic acid and Trolox (25). The concentration of the active substances in GE used in our study was lower than that in EGB 761, and thus the comparison of the results is complicated. Matkowski et al. found that the antioxidant activity of the methanolic extract of *Leonurus cardiaca* measured using the DPPH technique was 70% (26). Our results showed that anti-radical activity of LE was higher, but motherwort extract was prepared using ethanol rather than methanol in this case. Theeshan et al. studied the antioxidant activity of *Crataegus monogyna* on the Trolox equivalent antioxidant capacity (TEAC) as ferric reducing antioxidant power (FRAP) and found that *Crataegus monogyna* cell culture represents an important alternative source for natural antioxidants (27). Our results also confirm the antioxidant activity of CE, yet this activity is lower than that exhibited by the natural antioxidant Trolox (using the DPPH and ABTS techniques), GE, and LE (Fig. 3)

The experimental fluid extracts and Trolox solution were prepared using 70% (v/v) ethanol. Therefore, for comparable evaluation, 70% (v/v) ethanol was used for the analysis in order to evaluate their antioxidant activity and to compare the ability of ethanol to inactivate free radicals with that of the fluid extracts. The findings demonstrated that the samples of GE, LE, and CE exhibited higher antioxidant activity, compared to ethanol (using the DPPH and ABTS methods, Figs. 1 and 2). The results showed that 70% (v/v) ethanol had very low ability to scavenge DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ free radicals. The antioxidant activity of 70% (v/v) ethanol for DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ free radicals (after 10 min) was 5.59 ± 0.13% and 6.02 ± 0.22%, respectively. It may be concluded that the influence of ethanol on the free radical scavenging ability is insignificant, yet the scavenging ability of ethanol was subtracted from results given in the paper.

Therefore, further studies are needed to examine the antioxidant activity of GE, LE, CE using the

ORAC and FRAP techniques, and to compare this antioxidant activity to that of such compounds as ascorbic acid, Trolox, rutin, and carotenoids.

CONCLUSIONS

1. The antioxidant activity of the fluid extracts of maidenhair tree (*Ginkgo biloba* L.), motherwort (*Leonurus cardiaca* L.), and hawthorn (*Crataegus monogyna* Jacq.) were investigated using DPPH and ABTS methods. The investigation showed that antioxidant activity of the tested samples was relatively high. For this reason, preparations produced from this herbal raw material may be used as effective prevention and as valuable additional treatment for diseases caused by oxidative stress.

2. The antioxidant activity of the samples was different in $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging reaction systems. The results showed that in the $DPPH^{\bullet}$ reaction system, the fluid extract of motherwort exhibited higher antioxidant activity, compared to fluid extracts of maidenhair tree and hawthorn fruit. By contrast, in the $ABTS^{\bullet+}$ reaction system, higher antioxidant activity was found in the fluid extract of maidenhair tree, compared to the fluid extracts of motherwort and hawthorn fruit.

3. The study showed that fluid extracts of maidenhair tree, motherwort and hawthorn fruit exhibited higher antioxidant activity for the $ABTS^{\bullet+}$ radical, compared to the $DPPH^{\bullet}$ free radical.

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