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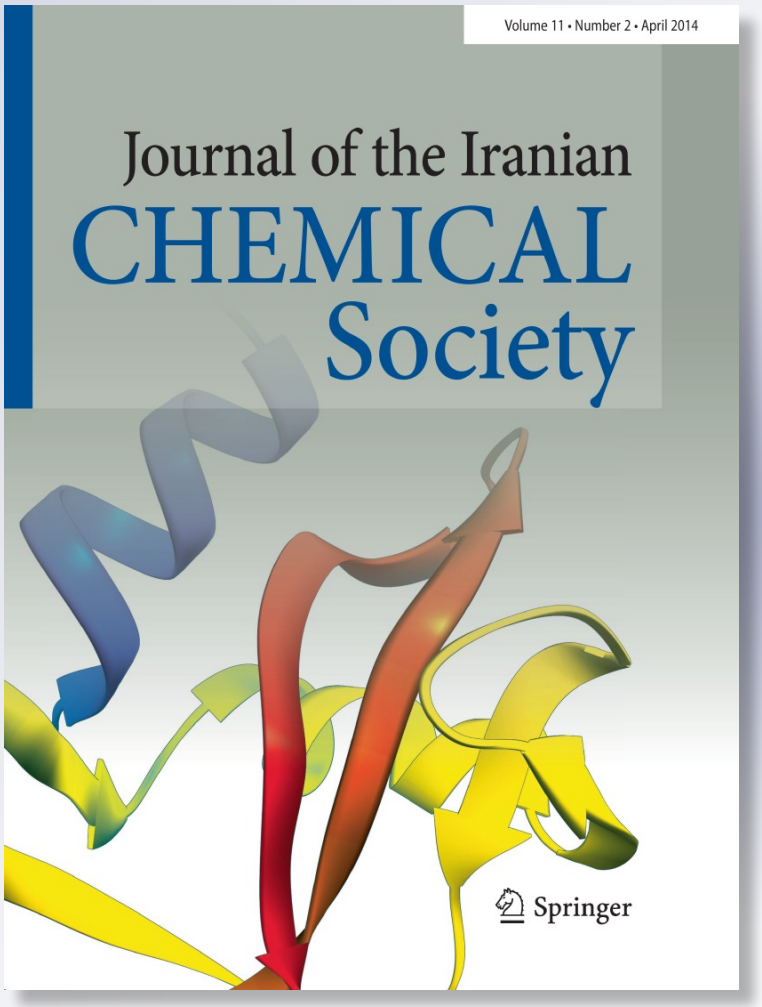

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ORIGINAL PAPER

Different spectrophotometric methods for antioxidant activity assay of four Romanian herbs

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Abstract The present study investigates the antioxidant activities of some Romanian plants, using different spectrophotometric methods (FRAP I, FRAP II, and CUPRAC). The plants investigated are hawthorn (*Crataegus oxyacantha*), bilberry (*Vaccinium myrtillus* L.), rosehip (*Rosa canina*), and chokeberries (*Aronia melanocarpa*). Hawthorn is used to treat a wide variety of inflammatory conditions, but the primary use is generally restricted for treating hypertension, ischemic heart disease, congestive heart failure, and arrhythmia. Investigations have proved the safe and reliable use of plant and plant extracts for treatment of cardiovascular disorders.

Keywords Ischemic heart disease · Antioxidant activity · Spectrophotometric methods · Romanian plants

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Introduction

The medicinal plants represent main products for making natural and fito-pharmaceutical medicines. The derived products from the plants are better accepted and used in the modern medicine. The national plant collection is remarkable owing to its richness: a lot of the plants species are well known for their medical usefulness. Although there are needed much more studies for investigating their importance in the modern therapy, and there is no doubt that the natural medicines are the most used from the oldest times.

In 1987, World Health Organization (WHO) has stated the importance of scientific research on herbal supplements and that there are sufficient pieces of evidence that such products may have beneficial effects.

The WHO estimates that about 80 % of the world population still relies on botanic medication [1]. Some used products have become to be widely used in standard allopathic medicine. Their use is regulated by the Food and Drug Administration (FDA) or European Medicines Agency (EMA) standards.

It is well known that oxidative stress is involved in many diseases such as ischemia [2–4], inflammatory [5] or neurodegenerative [6], and all these can be combated using natural products with antioxidant effect.

Antioxidants are a class of compounds of great interest for pharmaceuticals, biochemists and other health professionals because they are designed to reduce the damage caused by reactive oxygen species (ROS), reactive nitrogen species (RNS), or even reactive chlorine species (SCR) [7]. There is also a great interest in trying to replace partially or totally the synthetic antioxidants with natural ones, because of concerns about possible side effects—cancer, of some synthetic antioxidants in food. Antioxidants have been shown to help prevent a number of long-term illnesses such

as heart disease, cancer, and an eye disorder called macular degeneration. They destroy damaging particles in the body known as free radicals, helping to prevent or reverse damage to cells.

The importance of the mechanism of oxidation in the body and food was widely accepted. Oxidative metabolism is an essential process for cell survival. A side effect of this metabolism is the excess production of free radicals and other ROS that can cause undesirable oxidative changes. There is an increasing evidence for the involvement of high reactivity of these species in a variety of diseases.

There are numerous studies related to the evaluation of antioxidant capacity of plant materials. Methods that measure the antioxidant's radical scavenging ability include total peroxyl radical trapping parameter (TRAP) [8], oxygen radical absorbance capacity (ORAC) [9], Trolox equivalent antioxidant capacity (TEAC) [10], and 2,2-diphenyl-l-picrylhydrazyl radical (DPPH) [11] assays. The reducing ability of plasma in the presence of antioxidant was determined also by the ferric ion-reducing antioxidant parameter (FRAP) assay [12]. Other commonly used antioxidant activity methods include cupric-reducing antioxidant capacity (CUPRAC) [13], cerium (IV) reduction [14], phosphomolybdenum complex [15] and inhibited oxygen uptake (IOU) [16]. There are also methods that use FTIR measurements in such determinations [17].

In the present study, we investigated the use of three different spectrophotometric assays (FRAP I, FRAP II, and CUPRAC) for antioxidant activities determination of some Romanian plants that have medical properties.

Experimental

Plant description

Hawthorn (Crataegus monogyna)

Hawthorn extract is commonly used by herbalists for treatment of angina, congestive heart failure, bradyarrhythmia, and cerebral insufficiency. Hawthorn has positive isotropic and vasodilatory effects and is thought to increase myocardial perfusion and reduce after load. As an adjuvant treatment for congestive heart failure, hawthorn has been reported to have beneficial effects on symptom control and physiologic outcomes [18], but the efficiency and safety of its supposed isotropic activity and effect on morbidity and mortality have not been systematically followed. Hawthorn suggests that the activity of digitalis and its concomitant use should be monitored carefully for potential toxic effects. Hawthorn also inhibits the biosynthesis of thromboxane A2, and it could potentially increase the risk of bleeding in patients taking antiplatelet or anticoagulant agents. Without additional data on safety and efficacy, clinicians should discourage unsupervised use of hawthorn in patients with congestive heart failure who are taking heart failure medications.

Usually associated with flavonoid content structures, linked by glycosidic (e.g., Vitexin-2-O-rhamnoside or its acetate, luteolin seven glucoside, hyperoside and routine), several beneficial properties on the cardiovascular system have been raised from flowers in the recent year. Essential and proven by pharmacological determinations these are increasing myocardial flow, a positive inotropic effect and positive chromotropic associated with hypotensive effect and an antioxidant effect antisclerotic. The polyphenols and flavonoids contained in hawthorn, rosehip, and bilberry form a group that is evidenced by its important antioxidant properties. Plant extracts containing low molecular mass compounds have been successively used in phytotherapy since ancient times, as ROS are involved in several diseases in this study, the research on the antioxidant potential of medicinal plants was considered seriously [19]. Many plants have been also found to possess free radical scavenging activity (polyphenols, flavons, and anthocians). Low levels of one or more of the essential antioxidants have been shown to be associated with many disorders including cancer, inflammation, atherosclerosis, coronary heart disease, and diabetes. Thus, in such cases, the administration of exogenous antioxidants seems to be salutary.

Chokeberries (Arona melanocarpa)

The broadly defined cardioprotective action of Aronia extracts was also confirmed in several studies. It may be suggested that this activity is somehow related to antioxidative properties of Aronia, as some oxidative stress symptoms have been reported during viral infections, such as the common cold and influenza. So far no research has been undertaken to support this hypothesis, although some antiviral activity of chokeberry extracts has been reported. The use of Aronia fruits in hemorrhoid treatment, although not clinically supported, may be attributed to the hemostatic properties of tannins, as well as the improvement of microcirculation by polyphenolic compounds. Many of the pharmacological activities of the black chokeberry, such as antimutagenic, hepatoprotective, and cardioprotective, are directly or indirectly related to its antioxidative properties resulting from the high polyphenol content. Because of their health-promoting effects, *A. melanocarpa* extracts may constitute a valuable dietary supplement for people with risk factors of cardiovascular diseases or metabolic syndrome. Moreover, regular consumption of black chokeberry products, considering their high antioxidant and antimutagenic potential, may show some long-term effects, such as cancer prevention [20–22].

Rosehip (Rosa canina)

Rosehip contains different pharmaceutical compounds, such as ascorbic acid (vitamin C) and dehydroascorbic acid, vitamin A, vitamins B1 and B2, vitamin P, nicotinic acid, vitamin K, changed sugar radicals, tannins, citric and malic acid, peptine, volatile oils, fat oil, flavonoids, carotenoids, mineral substances, traces of vanillin, alpha and beta tocopherol (vitamin E), lecithin, sugars, etc.

Because the ascorbic acid (vitamin C) and the dehydroascorbic acid form together a "redox" system, it has an important influence in the biological oxidoreductases and the cellular breath. The vitamins (especially vitamin P) have the property of reducing the permeability and the fragility of the capillaries creating a normal blood pressure. They offer a quantity of natural substances necessary for the organism and have a digestive power. It is a good coagulator and tonic, rich in vitamins and it delates peripheral blood vessels.

Rosehip extract treats poisoning, diarrhea, liver disease, fever, intestinal worms (in this case, rosehip powder is very effective) and palpitations. Kidney and bladder disorders can be treated with rosehip seed tea [18].

Bilberry fruit (Vaccinium myrtillus L.)

The bilberry fruit contains anthocyanins, flavonoids, tannins, and vitamin C, all of them having excellent antioxidative properties. The antioxidants are substances that stop the oxidation processes, preventing this way some diseases, such as cardiovascular diseases, cancer, the muscles' degeneration of the eye and rheumatoid illnesses. The study for determining a medication prophylaxis using the almonds' fruit is evolving continually. Every year new natural treatments for different illnesses are discovered using the almond fruits or the whole plant.

Reagents and apparatus

All reagents were of analytical grade: 1,10-phenantroline (Phen, 99 %), TPTZ [2,4,6-tri (2-pyridyl)-*S*-triazine 99 %], and neocuproine (Neo, 99 %) were obtained from Sigma-Aldrich—Germany, while acetic acid, hydrochloric acid, sodium acetate, iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), methanol (99.8 %), acetone (99.5 %), and copper (II) chloride were obtained from Merck, Germany. All the herbal plants used were obtained from Hofigal S.A. Company.

Absorbance measurements were performed on an UV spectrometer LAMBDA 45 Perkin Elmer apparatus using quartz cell of 1-cm path length. The pH measurements were made with special paper pH. The shaker SHKA 2508-ICE (Labo Plus, Poland) and centrifuge MPW-350 (LABO-MIX, Poland) were used for sample preparation.

Procedures

Solvent extraction of plant materials

The dry plant specimens were crushed in a mill and 1 g samples were taken for each plant species. These samples were soaked in 100-mL EtOH: H_2O (solution 1:1), and homogenized in an Ultra-Turrax apparatus by gradually increasing the number of cycles per unit of time. The obtained extracts were transferred to centrifuge tubes, centrifuged for 10 min (3,000 rpm) and subsequently filtered through a filter paper into 100-mL flasks. The obtained extracts could be analysed for their antioxidant activities the next day. They were kept in a refrigerator at +4 °C.

CUPRAC assay

The CUPRAC method was applied as for interrelated procedures, i.e., normal (N), incubated (I), hydrolyzed & incubated (H&I) versions of the assay, depending on the nature of the sample. The standard procedure that has to be applied for completing all procedures during the development of final color is summarized below. In a test tube, 1-mL $CuCl_2$ solution (1.0×10^{-2} M), 1-mL neocuproine alcoholic solution (7.5×10^{-3} M), and 1-mL NH_4Ac buffer solution at pH 7.0 were added and mixed. Then (**X**) mL of herbal extract followed by (1.1-**X**) mL of water were added (total volume = 4.1 mL) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min. Since the molar absorptivity of Trolox in the CUPRAC method is $\varepsilon = 1.67 \times 10^4$ L/mol cm and the calibration curve for Trolox is a line passing through the origin, the Trolox equivalent molar concentration of the herbal extract sample in the final solution may be found by dividing the observed absorbance to the ε for Trolox. The Trolox equivalent antioxidant activity may be traced back to the original extract considering all dilutions and proportionated to the initial mass of herbal material taken to find a capacity in the units of μgTR/g dry matter.

FRAP I (1,10-o-phenanthroline) assay

0.6 mL of acetonic or methanolic extracts of the sample, 1 mL of 0.2 % $FeCl_3$ solution in acetone (methanol), and 0.5 mL of 0.5 % 1,10-phenanthroline solution in acetone (methanol) were placed into a 10-mL volumetric flask and made up to volume with acetone or methanol. The obtained solution was mixed and left at room temperature in dark. After 20 min, the absorbance was measured at 510 nm

against a reagent blank (1 mL of $FeCl_3$ 0.2 % solution and 0.5-ml Phen 0.5 % solution made up to 10 mL with acetone or methanol).

FRAP II (TPTZ) assay

A modified method of Benzie and Strain [12] was adopted for the FRAP assay. The stock solutions included 300-mM acetate buffer, pH 3.6, 10-mM TPTZ (2,4,6-tripyridyl-*S*-triazine) solution in 40-mM HCl, and 20-mM $FeCl_3{\cdot}6H_2O$. The fresh working solution was prepared by mixing 25-mL acetate buffer, 2.5-mL TPTZ and $FeCl_3{\cdot}6H_2O$. The temperature of the solution was raised to 37 °C before using. Plant extracts (0.15 mL) were allowed to react with 2.85 mL of FRAP II solution for 30 min in the dark condition. Readings of the colored product (Ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 20- and 100-μM $FeSO_4$. Results are expressed in μM Fe(II)/g dry mass and compared with that of ascorbic acid.

Calculation of the total antioxidant capacity of herbal material

The molar absorptivities of trolox in the above reference methods were as follows: $\varepsilon = 1.67 \times 10^4$ L/mol cm (CUPRAC method), $\varepsilon = 2.6 \times 10^4$ L/mol cm (ABTS method). If a herbal infusion (initial volume $= V_{cup}$) prepared from (m_p) grams of dried plant was diluted (r) times prior to analysis, and a sample volume of V_s was taken for analysis from the diluted extract, and color development (after addition of reagents) was made in final volume of (V_f) to yield an absorbance of (A_f), then the trolox equivalent antioxidant capacity of the herb (in μgTR per gram of dried plant, or simply in μgTR/g) was found using the equation:

$$\text{Capacity}(\text{in } \mu\text{gTR/g sample}) = \left(\frac{A_f}{\varepsilon_{TR}}\right)\left(\frac{V_f}{V_s}\right) r \left(\frac{V_{cup}}{m_p}\right)$$

Calibration curves were prepared using working solutions of $FeSO_4{\cdot}7H_2O$ and trolox between 0.010–0.080 and 0.005–0.0040 μmol/mL for FRAP I, FRAP II, and CUPRAC methods, respectively. The calibration curves for each method were created for a week. The correlation coefficients were 0.9987, 0.9994, and 0.9988 for CUPRAC (Fig. 1), FRAP I (Fig. 2), FRAP II (Fig. 3) methods, respectively.

Fe(II) ion is known as a pro-oxidant; therefore, not every substance that reduces Fe(III) is an antioxidant and can, thus, negatively influence the outcome of the analysis. Furthermore, an antioxidant, such as glutathione (Fig. 1) which also can be a pro-oxidant, cannot be determined by this method (refer to Fig. 3). Accordingly, problems can occur when there are other chemical species containing Fe(III), which can react with antioxidants (is a parallel between Figs. 2, 3).

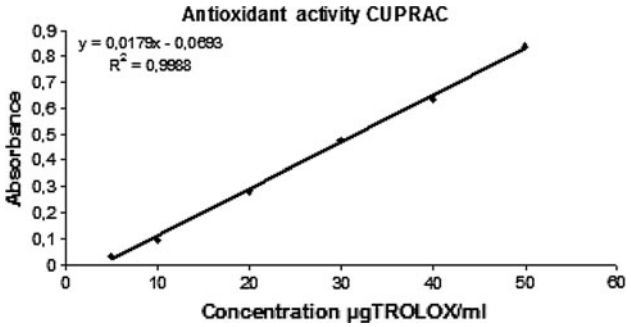

Fig. 1 Absorbance versus concentration calibration graph (CUPRAC in hydro-alcoholic media)

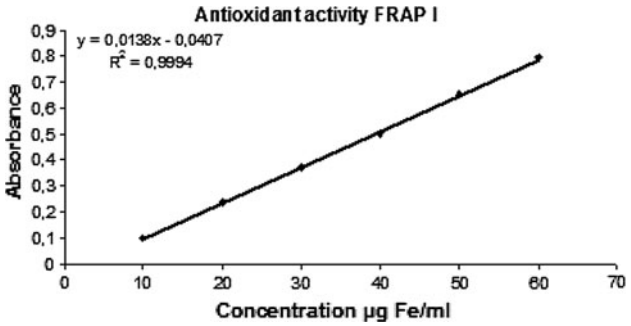

Fig. 2 Absorbance versus concentration calibration graph (FRAP I in acetonic media)

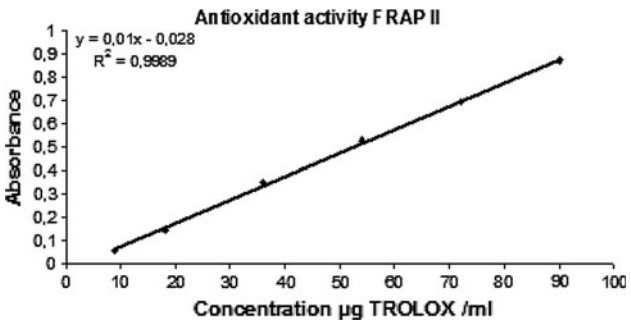

Fig. 3 Absorbance versus concentration calibration graph (FRAP II in methanolic media)

For the determination of the antioxidant activity of the studied products, the following calculation formulas were used:

- For the CUPRAC method, the results are expressed in μg Trolox/g sample.

$$\text{Capacity}(\text{in } \mu\text{gTR/g sample}) = \left(\frac{A_f}{\varepsilon_{TR}}\right)\left(\frac{V_f}{V_s}\right) r \left(\frac{V_{cup}}{m_p}\right) \quad (1)$$

where A_f = Conc. is the concentration determined after reading on the calibration curve (micrograms Trolox), $V_{cup} = 4.1$ mL is the final volume of the sample to be tested, V_f is the sample solution volume after extraction (50 mL); r is the factor of the dilution for each sample; m_p is the tested sample mass (g); V_s is the tested sample solution volume (4.1-x mL).

- For the FRAP I method, the results are expressed in μg Fe/g sample:

$$\text{A.O.Ac} = \frac{\text{Conc.}}{V_s \times m_p} \quad (2)$$

where A.O.Ac is antioxidant activity for the FRAP I method, Conc. is the concentration determined after reading on the calibration curve (micrograms Fe), V_f is the sample solution volume after extraction (10 mL), m_p is the tested sample mass (g), V_s is the tested sample solution volume (0.6 mL).

- For the FRAP II method, the results are expressed in μg Fe/g sample:

$$\text{A.O.Ac} = \frac{\text{Conc.}}{V_s \times m_p} \quad (3)$$

where Conc. is the concentration determined after reading on the calibration curve (micrograms Fe), V_f is the sample solution volume after extraction (10 mL), m_p is the tested sample mass (g), V_s is the tested sample solution volume (0.15–0.3 mL).

Results and discussion

Figures 4, 5, and 6 present the chemical reactions involved in the antioxidant activity determination using CUPRAC and, respectively, FRAP methods.

The FRAP II assay also has an advantage of electron-transfer reactions. In this study, the ferric salt Fe(III) $(TPTZ)_2Cl_3$ (TPTZ = 2,4,6-tripyridyl-*S*-triazine) is used as an oxidant.

The results referring to the antioxidant activity (A.O.Ac.) for the analysed plants are presented in Fig. 7.

These results' differences are due to the extraction media. In hydro-alcoholic media, the principal active substances are extracted well than in methanol or acetone media. An increase in absorbance as a function of extracts

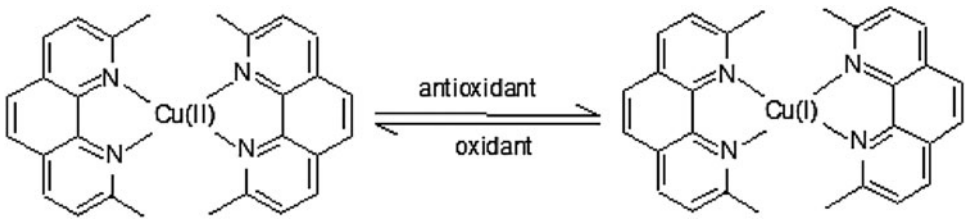

Fig. 4 The reduction of the Cu(II)–Phen chelate to Cu(I)–Phen chelate via the CUPRAC method

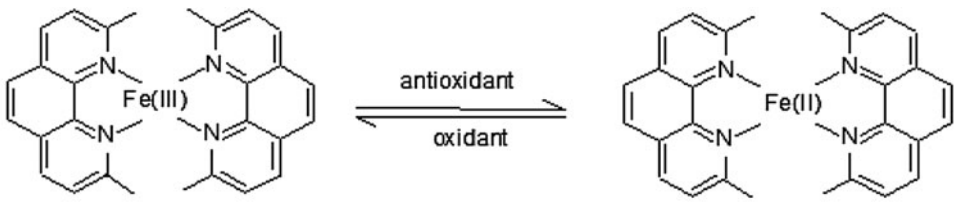

Fig. 5 The reduction of the Fe(III)–Phen chelate ion to Fe(II)–Phen chelate via the FRAP I method

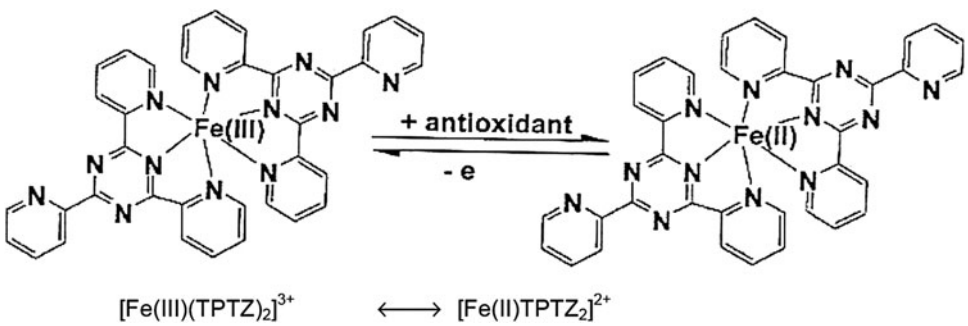

Fig. 6 The reduction of the Fe(III)–TPTZ chelate ion to Fe(II)–TPTZ chelate via the FRAP II (TPTZ) method

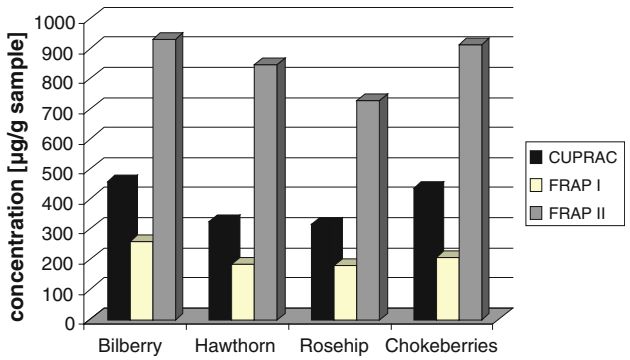

Fig. 7 Antioxidant activity for the analysed products via the methods used

concentration indicated a high antioxidant activity of the extracts. Modern pharmacological research presents chokeberry as a plant with numerous health-promoting activities. Biological activities of anthocyanins—rich chokeberry fruit extracts include antioxidative, antimutagenic, cardioprotective, and antihyperglycemic, among others. Interestingly, this application of black chokeberry was not reported in Western countries.

The FRAP I method consisted in the reduction of Fe^{3+} to Fe^{2+}, and the best results were obtained for the rosehip due to its high content in phenols than other samples analysed (Fig. 7). The FRAP assay measures directly the antioxidant activity of the substances, which is an important parameter for a component to be a good antioxidant. Besides vitamin C, rosehip also contains vitamins B_1, B_2, K, and PP, protein, acids, cellulose, minerals, provitamin A, and sugars. The heart disease can be prevented with rosehip extract. Along with hawthorn fruit, rosehip is a true champion in the prevention of angina pectoris and myocardial crisis.

With the FRAP II method, hawthorn presents the good results due to its high content in flavones and phenols compounds. The best results obtained for the antioxidant activity were 847.55 µg Fe/g sample (Fig. 7). The hawthorn has the following composition: essential oils, tannins, flavones, triterpene acids, crataegic acid, ursolic acid, pectin, phytosterols, vitamin C, B complex.

In this assay, antioxidants in the fruit extracts may disrupt the Fe^{3+} to Fe^{2+} transformation by competing with O_2^- and thereby causing a decrease in the formation of hydroxyl radicals.

The antioxidant activity properties are generally associated with the presence of reduction agents and their action is based on the breaking of the free radical chain by donating a hydrogen atom. Generally, the radical scavenging and reducing power of the extracts may decrease as a function of storage time at room temperature.

Conclusions

Plants generally play a key role in the maintenance of the human health and enhancing the quality of human life. Plant biochemicals are a gift from nature. Phenolic compounds, such as flavonoids are typical representatives of these botanical gifts. The results obtained following the determinations of antioxidant activity via the CUPRAC, FRAP I, and FRAP II methods, respectively, demonstrate that hawthorn, when compared to the other analysed herbs, has the highest values of antioxidant activity (followed by rosehip, chokeberries, and bilberry). This activity correlates well with its content of phenolic-structured compounds. Flowers and fruits of hawthorn are used to treat cardiovascular diseases. Nowadays, a great deal of effort being expended to find effective antioxidants for the treatment or prevention of free radical-mediated deleterious effects. These data are used to develop new cardiovascular phytotherapy natural remedies.

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Conflict of interest The authors confirm no conflict of interests.

References

1. A. DerMarderosian, *Guide to popular natural products* (Facts and Comparisons, St. Louis, 1999), p. vii
2. X.Y. Ji, B.K.-H. Tan, Y.Z. Zhu, Acta Pharmacol. Sin. **21**, 1089–1094 (2000)
3. J. Sun, B.K. Tan, S.H. Huang, M. Whiteman, Y.Z. Zhu, Acta Pharmacol. Sin. **23**, 1142–1151 (2002)
4. Z.Q. Wang, F. Porreca, S. Cuzzocrea, K. Galen, R. Lightfoot, E. Masini, C. Muscoli, V. Mollace, M. Ndengele, H. Ischiropoulos, D. Salvemini, J. Pharmacol. Exp. Ther. **309**, 869–878 (2004)
5. J. Emerit, M. Edeas, F. Bricaire, Biomed. Pharmacother. **58**, 39–46 (2004)
6. R. Olinski, D. Gackowski, M. Foksinski, R. Rozalski, K. Roszkowski, P. Jaruga, Free Radical Biol. Med. **33**, 192–200 (2002)
7. M. Sato, G. Maulik, D. Bagchi, D.K. Das, Free Radical Res. **32**, 135–144 (2000)
8. D.D. Wayner, G.W. Burton, K.U. Ingold, S. Locke, FEBS Lett. **187**, 33–37 (1985)
9. G. Cao, C.P. Verdon, A.H.B. Wu, H. Wang, R.L. Prior, Clin. Chem. **41**, 1738–1744 (1995)
10. N.J. Miller, C.A. Rice-Evans, M.J. Davies, V. Gopinathan, A.A. Milner, Clin. Sci. **84**, 407–412 (1993)
11. W. Brand-Williams, M.E. Cuvelier, C. Berset, LWT-Food Sci. Technol. **28**, 25–30 (1995)
12. I.F.F. Benzie, J.J. Strain, Anal. Biochem. **239**, 70–76 (1996)
13. R. Apak, K.C. Guclu, M. Ozyurek, S.E. Karademir, J. Agr. Food Chem. **52**, 7970–7981 (2004)
14. D. Ozyurt, B. Demirata, R. Apak, Talanta **71**, 1155–1165 (2007)
15. P. Prieto, M. Pineda, M. Aguilar, Anal. Biochem. **269**, 337–341 (1999)
16. K.L. Empson, T.P. Labuza, E. Graf, J. Food Sci. **56**, 560–563 (1991)

17. A.A. Bunaciu, H.Y. Aboul-Enein, S. Fleschin, Appl. Spectrosc. Rev. **47**, 245–255 (2012)
18. J.H. Kramer, Surv. Ophthalmol. **49**, 618 (2004)
19. M. Blumenthal, A. Goldberg, J. Brinckmann, "Herbal Medicine Expanded Commission" E Monographs Newton, Mass: "Integrative Medicine Communications" 18–19 (2000)
20. J.K. Willcox, S.L. Ash, G.L. Catignani, Crit. Rev. Food Sci. Nutr. **44**(4), 275–295 (2004)
21. A. Contestabile, Curr. Top. Med. Chem. **1**, 553–568 (2001)
22. M.M. Silva, Santos, C. Goncalo, R. Rocha, J. Goncalo, L. Mira, Free Radic. Res **36**, 1219–1227 (2002)