Different spectrophotometric methods for antioxidant for activity assay of four herbs

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Abstract: - The present study investigated the antioxidant activities of some Roumanian plants, using different spectrophotometric methods (FRAP and respectively CUPRAC method). The plants investigated are: hawthorn (Crataegus oxyacantha), bilberry (Vaccinium myrtillus L), rosehip (Rosa canina), chokeberries (Aronia melanocarpa). A good correlation was found between the different methods used for measuring antioxidant activity of some of these herbs. This study focused on finding new natural remedies similar body in cardiovascular therapy.

Key-Words: - Antioxidant activity, Fruits, Herbs, Spectrophotometric methods, Roumanian plants

1 Introduction
Medicinal plants constitute a major source of phyto-pharmaceutical products and natural drugs. The products derived from plants are also becoming more accepted and used in the natural medicines. National flora is remarkable for its richness; many species of plants are known not only for their therapeutic role. However, more research is still needed to explore their utility in modern therapy. Without doubt the herbal drug use is one of the oldest forms of health care.

Existence of plant compound with antioxidant properties and high content of free radicals collectors compounds (derived carotenoids, polyphenols, flavones, anthocyanins, unsaturated fatty acids, vitamins, enzymes and cofactors) has stimulated interest in herbal medicine use and prophylactic cardiovascular healing [1-2].

In the last two decades, the emergence of degenerative processes associated with cardiovascular disease is related to molecular biology with a surplus of harmful free radicals, oxidative processes disastrous promoters of the body.

It is well known that oxidative stress is involved in many disease such as ischemia [2-3], inflammatory [4-7] or neurodegenerative [4,8] that can be combated by using natural products with antioxidant effect.

Antioxidants are a class of compounds of great interest for pharmaceutical, biochemists and other health professionals because they are designed to reduce damage caused by reactive oxygen species (ROS), nitrogen (SNR), or even chlorine (SCR) [9]. The present study try to investigated the use of different spectrophotometric methods (FRAP and CUPRAC) for antioxidant activities determination of some Roumanian plants having medical properties using.

1.1 Plant description
Bilberry fruit contains chemicals known as anthocyanosides, plant pigments that have excellent antioxidant properties. They scavenge damaging particles in the body known as free radicals, helping to prevent or reverse damage to cells. 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. Bilberry also contains vitamin C, which is another antioxidant.
Not many studies have been done to examine bilberry specifically. Even fewer studies have been done in humans. Most of the suggestions about bilberry's effectiveness come from research on similar antioxidants, or from test tube and limited animal studies, and is used in bellow applications: Chronic venous insufficiency, Diabetes, Atherosclerosis, Diarrhea and wounds, vision [10].

Rosahip: (Rosa canina) is very popular in herbal medicine, especially complex because it contains vitamins: vitamin A, B1, B2, C, K, P, E. Rosahip is a reservoir of vitamins for the human body. Rosehip extract treats poisoning, diarrhea, liver disease, fever, intestinal worms (in this case, and rosehip powder is very effective), palpitations. Kidney and bladder disorders can be treated with rosehip seed tea [11].

Rosehip contains dehydroascorbic acid, vitamin A, vitamin B1, vitamin B2, vitamin P, vitamin K, nicotinic acid, terpene compounds, citric and malic acids, water, sugars, proteins, minerals, vitamin E, vitamin PP. The rosehip oil contains essential fatty acids, seeds, lecithin, carotenoids and vitamin F.

Hawthorn: Both fruits and leaves contain many substances that act on the cardiovascular and nervous system, the main effects are: lowers blood pressure by central and peripheral vasoconstriction, has sedative effect on central nervous system. Thanks to the active substances used in treating Hawthorn and improve a range of diseases such as hypertension, ischemic heart disease and other cardiovascular disorders, disorders heart rate or cardiac neurosis [12].

Hawthorn has a significant effect in increasing the flow of oxygenated blood to the heart and brain, is also recommended and tachycardia, atherosclerosis, state of mental instability, emotional pronounced. Also hawthorn fruit and leaves also contain substances that have the ability to stimulate the body's defense system improves general immunity. Hawthorn has cardiotonic action due to vitamin C content and organic acids.

Fruits are a generous reserve of vitamin B.

Chokeberries: Originally considered to be of little medicinal value, new research shows that Aronia melanocarpa has a high concentration of polyphenols and anthocyanins, stimulating circulation, protecting the urinary tract, and strengthening the heart.

Most literature data concerning the chemistry of A. Melanocarpa refers to its berries being a rich source of pharmacologically relevant compounds. Polyphenols, especially anthocyanins and procyanidins, make up the main group of biologically active constituents in black chokeberry fruits. These compounds are responsible for antioxidant properties of the plant. Other phenolics include chlorogenic and neochlorogenic acid as well as a small amount of tannins [13-16].

## 2 Experimental

Absorbance measurements were performed on a UV-VIS spectrophotometer Jasco V 530 apparatus using quartz cell of 1-cm path length. pH measurements were made with special paper pH. The shaker SHKA 2508-ICE (Labo Plus) and centrifuge MPW-350 (LABO-MIX) were used for sample preparation.

All reagents were of analytical grade: 1,10-phenanthroline (Phen, 99%), 2,4,6-tris(2-pyridyl)striazine (TPTZ, 99%) and neocuproine (Neo, 99%) were obtained from Sigma Aldrich, while acetic acid, hydrochloric acid, sodium acetate, iron (III) chloride hexahydrate (FeCl$_3$$\cdot$6H$_2$O), iron (II) sulfate heptahydrate (FeSO$_4$$\cdot$7H$_2$O), methanol (99,8%), acetone (99,5%), copper (II) chloride were obtained from Merck. Redistilled water was used for the preparation of solutions. All the herbal plants used were obtained from Hofigal S.A. Company.

**FRAP I method**: 0,6mL of acetonic or methanolic extracts of an oil sample, 1mL of 0,2% FeCl$_3$ solution in acetone (methanol) and 0,5mL 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 a dark. After 20min, the absorbance of an orange solution was measured at 510nm against a reagent blank (1mL of FeCl$_3$ 0,2% and 0,5 Phen 0,5% made up to 10 mL with acetone or methanol).

**FRAP II method**: The FRAP reagent contained 2,5mL of a 10mmol/L TPTZ solution in 40mmol/L HCl, of 20mmol/L FeCl$_3$ and 25mL of 0,1mol/L acetate buffer (pH 3,6) was prepared freshly and incubated at 37°C for 10min. Then, 0,3mL of acetonic or methanolic extracts of samples and 2mL of FRAP reagent were transferred into a 10 mL volumetric flask and made up to volume with redistilled water. The obtained blue solutions were kept at room temperature for 10 min and centrifuged at 15,000rpm for 10min in a lab centrifuge to remove solids. The absorbance was measured at 593nm against a reagent blank (2mL of FRAP reagent made up to 10mL redistilled water).

**CUPRAC method**: To a test tube were added 1mL of CuCl$_2$ solution (1,0×10$^{-3}$M), 1mL of neocuproine alcoholic solution (7,5×10$^{-3}$M) and 1mL NH$_4$Ac buffer solution and mixed; (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$^{-1}$ cm$^{-1}$ 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 $\varepsilon$ 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 mmol TR/g dry matter.

Solvent extraction of plant materials: The dry plant specimens were crushed in a mill and 5 g samples were taken for each plant species. These samples were soaked in 100 mL EtOH: H$_2$O (solution 1:1), and homogenized in an Ultra-Turrax apparatus by gradually increasing the number of cycles per unit time. The obtained extracts were transferred to centrifuge tubes, centrifuged for 10 min (3000 rpm) and subsequently filtered through a filter paper into 100 mL flasks. The obtained extracts could be analyzed for their antioxidant activities on the next day and stoppered extracts in a refrigerator at +4°C.

Calibration curves were prepared using working solutions of FeSO$_4 \times 7$H$_2$O between 0.010-0.080 and 0.005-0.0040 µmol/mL for FRAP I and FRAP II methods, respectively. Five calibration curves for each method were plotted on the same day. The least/squares method was applied to calculate the lines: $y=13.1x-0.015$ and $y=10.0x-0.014$ for acetonic and methanolic solution of FRAP I method and $y=23.9x+0.035$ for FRAP II method. The correlation coefficients were 0.9988; 0.9981 and 0.9989 for FRAP I and FRAP II methods, respectively.

The CUPRAC method is expressed in grams TR/LOX/g sample.

$$A.O.\text{activity} = \frac{\text{Conc} \times 4.1 \times 50 \times 30 \times 10^6}{X \times m_p} \quad (1)$$

where:
- Conc is the concentration determined after reading on the calibration curve (micrograms TR/LOX)
- 4.1 is the final volume of the sample to be analysed,
- 50 is the sample solution volume after extraction (ml)
- 30 is the reaction time (min)
- $m_p$ is the analysed sample mass (g)
- $X$ is the analysed sample solution volume

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

- For the CUPRAC method:

$$A.O.\text{activity} = \frac{\text{Conc} \times 10}{0.6 \times m_p} \quad (2)$$

where:
- Conc is the concentration determined after reading on the calibration curve (micrograms Fe)
- 10 is the sample solution volume after extraction (ml)
- $m_p$ is the analysed sample mass (g)
- 0.6 is the analysed sample solution volume

- For the FRAP II method:

$$A.O.\text{activity} = \frac{\text{Conc} \times 10}{0.3 \times m_p} \quad (3)$$
3 Results and Discussion

Figures (4-5) presents the chemical reactions involved in the antioxidant activity determination using Cuprac and respectively Frap methods.

The results referring to the antioxidant activity (A.O.) for the analysed plants are presented in figure 6.

The best results were obtained for the hawthorn, as it can be seen in the figure 6.

Experiments were performed in the concentration range 0.2 to 1µg/ml, to highlight the antioxidant activity, with Cuprac method. The highest value obtained for bilberry antioxidant activity was 5.97 g_trolox/g_sample (fig.7)

The highest value obtained for antioxidant activity for chokeberries was 6.38 g_trolox/g_sample, with Cuprac method (fig. 8).

Modern pharmacological research presents chokeberries as a plant with numerous health-promoting activities. Biological activities of anthocyanin-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 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 healthpromoting 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 exert some long-term effects such as cancer prevention.

The highest value obtained for antioxidant activity for rosahip was 6.36 g_trolox/g_sample with Cuprac method (fig.9).

Besides vitamin C, rosahip also contains vitamins B1, B2, K and PP, protein, acids, cellulose, minerals, provitamin A and sugars. Protects the heart. Heart disease can be prevented with rosahip extract. Along with hawthorn fruit, rosahip are true champions in the prevention of angina pectoris and myocardial crisis.

The highest value obtained for antioxidant activity for hawthorn was 7.73 g_trolox/g_sample with Cuprac method (fig.10).

The Frap I method consisted in the reduction of Fe^{3+} to Fe^{2+}, and the best results were obtained for the rosahip due to its high content in polyphenols than other samples analysed (fig. 11).

The Frap II method versus volume for all the herbs, with FRAP II method

Fig.9 Antioxidant activity versus concentration for rosahip at different concentration

Fig.10 Antioxidant activity versus concentration for hawthorn at different concentration

Fig.11 Antioxidant activity versus volume for all the herbs, with FRAP I method

Fig.12 Antioxidant activity versus volume for all the herbs, with FRAP II method
With the Frap II method, hawthorn presents the best results due to its high content in flavonoid compounds and polyphenols. The best results obtained for the antioxidant activity were 3238.57 µFe/g sample (fig.12). The hawthorn has the following composition: essential oils, tannins, flavonoids, triterpene acids, crategic acid, ursolic acid, pectin, phytosterols, vitamin C, B complex.

4 Conclusions
The results obtained following the determinations of antioxidant activity via the CUPRAC FRAP II and FRAP II methods demonstrate that hawthorn, when compared to the other analysed herbs, has the highest level of antioxidant activity (followed by rosehip, chokeberries and bilberry). This activity correlates well with its content of polyphenolic structured compounds.

Flowers and fruits of hawthorn are used to treat cardiovascular diseases. Usually associated with flavonoid content structures, linked by glycosidic linked sugars (eg. Vitexin-2-0-rhamnozida or its acetate, luteolin 7 glucoside, hiperoside and routine), several beneficial properties on the cardiovascular system have been raised for flowers hawthorn in recent year.
Essential and proven by pharmacological determinations are increasing myocardial flow, a positive inotropic effect and positive cromotrop 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. These data are used to develop new cardiovascular phytotherapy natural remedies.

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