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Extracts from apple leaves and fruits as effective antioxidants

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The research aimed at determination of the antioxidant properties of extracts from leaves and fruits of apple tree and of chlorogenic acid and phloridzin, that is compounds contained in the extracts and held responsible for the properties. The very good antioxidant properties of apple fruit extracts have been known since long ago, though it is not yet known what is the antioxidant action of an apple leaf extract. In order to enquire about the antioxidant effect of the extracts with respect to erythrocyte membrane lipids, erythrocyte ghosts were oxidated in the presence of compounds contained in the extracts. The erythrocyte was treated as a model cells and its membrane as an example and model of the biological membrane. The degree of lipid oxidation was determined by using two methods: spectrophotometric and fluorimetric with the DPH-PA probe. Oxidation was induced with two agents: UVC radiation and AAPH radical. The results obtained indicate a strong antioxidant activity of the extracts and phenolic compounds, whose strength follows the sequence: chlorogenic acid > apple leaf extract ≈ apple fruit extract > phloridzin. Thus the present work proves that the apple leaf extract possesses a very high antioxidant activity, which is almost equal to that of the apple fruit extract. In light of these result one can hope that the apple leaf extract may be used as an effective, natural antioxidant in the food, cosmetics and pharmaceutical industries.

Key words: Antioxidant activity, chlorogenic acid, phloridzin, relative fluorescence, vegetable polyphenols.

INTRODUCTION

Natural polyphenolic substances have for long been used in herbal therapy in the form of infusions and extracts, and for pharmacological healing as components of medicines. The compounds, as natural substances, have found application in anticancer therapy and in treating ailments of the vascular, digestive, urinary and respiretory systems; and also in dermatology, obesity treatment and as dietary supplements (Marques and Farah, 2009; McCann et al., 2007; Miller et al., 2008; Peng et al., 2006; Ridgway et al., 1997; Ross and Kasum, 2002). Some life threatening changes in the physicochemical properties of the cell membrane (for example membrane hydrophobicity decrease, membrane depolarization, enzyme inactivation) are the result of peroxidation of the membrane lipids induced by oxidative stress. The stress is caused by exogenous factors, such as ionizing and UV radiation, xenobiotics or endogenous factors both biotic and abiotic (Bartosz, 2003). Based on the research done,

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Abbreviations: AAPH- 2,2' – azobis(2-amidinopropane)dihydrochloride; **DPH-PA-** 3-[p-(6-phenyl)-1,3,5-hexatrienyl] phenylpropionic acid; **HPLC-** high performance liquid chromatography; **MDA-** malonyldialdehyde; **PBS-** phosphate buffer solution, pH 7.4; **TBA-** 2-thiobarbituric acid; **TCA-** trichloracetic acid; **TEAC-** trolox equivalent antioxidant capacity; **TrisEDTA-** triethylenediaminetetraacetic acid; **Trolox®-** 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid**; UVC-** radiation λ = 100 to 280 nm.

it was thought that the main factors that protect against excessive free radicals were vitamin C, E and β-carotene. Further research has, however, shown that vitamin C contained in fruit extracts is only in 15% responsible for the extracts' antioxidant properties. This suggests that the protection against oxidation is due to other compounds, mainly the phenolic ones contained in the extracts (Wang et al., 1996).

Phenolic compounds, depending on their chemical structure, can exhibit an antioxidant activity in the lipid phase, like for instance ascorbic acid, or in the hydrophobic phase where carotenes are located (Rice-Evans et al., 1997). Hydrophilic antioxidants protect the organism mainly via reactions with the reactive forms of oxygen, whereas the hydrophobic antioxidants penetrate the membrane and take part mainly in scavenging of organic free radicals, with an inhibiting effect on lipid peroxidation (Marques and Farah, 2009; McCann et al., 2007; Miller et al., 2008; Rice-Evans et al., 1997; Peng et al., 2006; Ridgway et al., 1997; Ross and Kasum, 2002). In recent years, there have been many works that endeavor to explain the connection between the free-radical scavenging ability and chemical structure of polyphenols. They have shown that the antioxidant activity of the compounds is markedly affected by both the position and number of hydroxyl groups in the phenolic ring (Fukumonto and Mazza, 2000; Kalt et al., 1999; Leja et al., 2003; Lifen et al., 2004; Miller et al., 2008; Rice-Evans et al., 1996; Rice-Evans et al., 1997; Ross and Kasum, 2002; Yang et al., 2001).

The intensive research conducted throughout the world on the composition and antioxidant properties of extracts from fruits, vegetables, herbs and plant leaves allowed to determine the antioxidant activity of the substances. It was found for instance that phenolic compounds contained in fruits exhibit better antioxidant properties than compounds contained in vegetables. It was also shown that mixtures of various phenols have better antioxidant properties than vitamins and synthetic phenolic compounds (Nagai et al., 2001; Tzanakis et al., 2000; Vinson et al., 2001; Wang et al., 1996). Since ancient times the apple is the most often consumed fruit all over the world. For instance, it was shown that the antioxidant properties of 100g of fresh apples are equivalent to 1500 mg of vitamin C. However, even 500 mg of vitamin C is detrimental to health because it can cause oxidative effects (Eberhardt et al., 2000). Hence, it seems justifiable to say, with the English, that an apple a day keeps the doctor away. Among the phenolic com-pounds occurring in apple leaves and fruits a substantial part is constituted by chlorogenic acid (CGA) that exhibits a strong antioxidant activity, which is 2-3 times stronger than that of vitamin C and E (Lu and Foo, 2000).

Chlorogenic acid (CGA), belonging to derivatives of hydroxycinnamic acid, is the ester of caffeic and quinic acids. It occurs in large quantities, among others, in coffee grains, beans, potatoes and apples. CGA is an antioxidant created by plants in response to environmental stress, and also in response to infections caused by microorganisms, mechanical damage or excessive irradiation within the wavelengths 220 to 1100 nm. Its antioxidant properties are thought to be connected with its chemical structure. Owing to its ring structure and functional groups, this acid can play the role of a center for binding free radicals. It is known as a very good scavenger of reactive forms of oxygen, nitrogen, and the hydroxyl radical $^{\cdot}OH$ (Pchelkin, 2003; Zang et al., 2003). The acid is also able to chelate transitional metals, for example iron or copper, and protects vitamin A against oxidation (Marques and Farah, 2009). The studies performed have shown that the presence of CGA in a diet has a positive influence on the human organism owing to the acid's antioxidant, antidiabetic and antibacterial properties, and protective properties towards liver and other organs (Marques and Farah, 2009). It was also found that the acid protects erythrocytes against oxidation, which due to their function are especially exposed to the reactive forms of oxygen (Lekse et al., 2004; Mikstacka et al., 2010). Another component that occurs in all parts of fruits and in young shoots, roots and bark is phloridzin. This compound is a dihydroderivative of glycozilated chalcones and occurs in large quantities in apple seeds.

Phloridzin and its derivatives are biologically active, in particular they exhibit antioxidant properties towards membrane lipids (Rezik et al., 2002; Ridgway et al., 1996; Ridgway et al., 1997). Compared with other phenolic compounds, phloridzin has a lower antioxidant activity, which is probably due to only one OH group in its phenol ring (Dziedzic and Hudson, 1983; Dziedzic et al., 1985; Lu and Foo, 2000). In many works were determined the content and antioxidant properties of polyphenols present in all parts of the apple fruit (skin, pulp and seeds) for various apple cultivars (Hossain et al., 2009; Kondo et al., 2002; Lu and Foo, 1997,1998, 2000; Miller and Rice-Evans, 1997; Oszmiański et al., 2008; Shoji et al., 2004). Studies were also conducted on the influence of phenolic compounds on the taste and color of food products and apple fruit preserves. It was documented that the lack of mutagenicity and genotoxicity of the preparation "applephenon" that contains apple fruit extract and has been broadly used in Japan for many years as an additive to food and dietary supplement (Shoji et al., 2004). It was proved in many studies that plant extracts containing phenolic compounds are a group of good antioxidants. It is the aim of much contemporary research, also in our laboratory, to explore the connection between the high biological activity of phenolic compounds (evident in their influence on the organism, on the cell and at molecular level) and their ability to incorporate into the biological membrane (Bonarska-Kujawa et al., 2010). The aim of the work was to investigate the antioxidant properties of apple fruit and leaf extracts and their phenolic components, that is

Table 1. Contents of phenolic compounds (%) in apple leaf and fruit extracts.

Phenolic compound (%)	Apple fruit	Apple leaf
Cyanidin-3-galactoside	0.12	0
Chlorogenic acid	16.67	0.92
Derivative of caffeic acid	0	0.12
Derivative of p–coumaric acid	0	0.29
p-coumaroyl-glucoside	1.56	0.16
(+)-catechin	0.7	0
(-)-epicatechin	4.32	0
Procyanidins B2	2.43	0
Procyanidins C1	1.63	0
Polimeric procyanidins	17.00	0
Phloretin-2'-O-xyloglucoside	0.72	5.38
Phloridzin	9.13	2.98
Quercetin-3-O-galactoside	0.84	3.40
Quercetin-3-O-glucoside	0.12	1.40
Quercetin-3-O-arabinoside	0.41	1.39
Quercetin-3-O-xyloside	0.94	2.44
Quercetin-3-O- rhamnoside	0	8.54
Total	56.59	27.39

chlorogenic acid and phloridzin, with respect to erythrocyte membrane lipids. The studies performed have shown that the extracts, owing to their lipophilic character, easily interact with biological membranes and even at small concentrations exhibit antioxidant properties without causing negative effects in the membrane structure.

MATERIALS AND METHODS

The plant extracts studied were obtained from the Department of Fruit, Vegetable and Grain Technology, Wroclaw University of Environmental and Life Sciences. Polyphenol content was determined by HPLC chromatography (Oszmiański and Wojdyło, 2005; Skupień and Oszmiański, 2004), using the method described by Oszmiański et al. (2007). Polyphenols were isolated from apple leaves and fruits by extraction with water containing 200 ppm of SO_2, the ratio of solvent to fruits or leaf being 3:1. The extract was adsorbed on Purolite AP 400 resin (UK) for further purification. The polyphenols were then eluted out with 80 % ethanol, concentrated and freeze-dried. By means of the above method a mixture of polyphenols was obtained (Gąsiorowski et al., 1997). The percent content of polyphenols in individual preparations was determined by means of the liquid chromatography HPLC (Oszmiański and Wojdyło, 2005; Oszmiański et al., 2008; Skupień and Oszmiański, 2004) (Table 1).

The research was carried out on isolated pig erythrocyte membranes (ghosts). Erythrocyte ghosts were obtained according to Dodge et al. (1963). The pig red blood membrane is known to be the closest to the human erythrocyte membrane as far as lipid composition is concerned. The fluorescence probe DPPH-PA was purchased from "molecular probes", Eugene, Oregon USA. The oxidation inductor AAPH was purchased from Aldrich-Sigma. Chlorogenic acid and Phloridzin were purchased from Extrasynthese® France (Figure 1).

Erythrocyte ghosts were suspended in isotonic phosphate buffer solution of pH 7.4. Membrane protein concentration was assayed using Bradford's method (Bradford, 1976) and it was 1 mg/l. The control sample contained erythrocyte ghosts only, whereas to the remaining samples were added appropriate amounts of extracts. The ghosts suspension was oxidized with UVC radiation of 3.5 mW/cm^2 intensity, emitted from a bactericidal lamp. The measure of lipid oxidation was the concentration of malondialdehyde (MDA) released during lipid peroxidation. Its concentration was determined spectrophotometrically owing to the color reaction between MDA and thiobarbituric acid (TBA). Absorption was measured at $\lambda = 535$ nm after different time of radiation of the samples. In this method the measure of membrane lipid peroxidation is the concentration of MDA released during the oxidation. The color MDA-TBA reaction allows determining the concentration of MDA from its light absorption at 535 nm. The extent of lipid oxidation inhibition was expressed in percentage calculated from the formula:

$$\text{Inhibition (\%)} = \{ (A_0 - A)/ A_0 \} \times 100$$

Where:

A_0 – absorption of control sample,
A – absorption of sample with extract added.

The fluorimetric experiment was carried out with the fluorescence probe DPH-PA. Erythrocyte ghosts, with and without (control) addition of extracts, were suspended in a phosphate buffer of pH 7.4 and UVC irradiated or treated with the chemical oxidation inductor AAPH for 30 min. A Carry Eclipsce spectrofluorimeter (Varian) was used to measure free radicals concentrations in the samples. Excitation and emission wavelengths were $\lambda_{ex} = 364$ nm and $\lambda_{em} = 430$ nm. The measure of lipid oxidation was the relative change in fluorescence intensity (F/F_0), where F_0 is the starting fluorescence and F the one measured during an oxidation procedure (Arora and Strasburg, 1997). Percentage of lipid oxidation inhibition was calculated from the following formula:

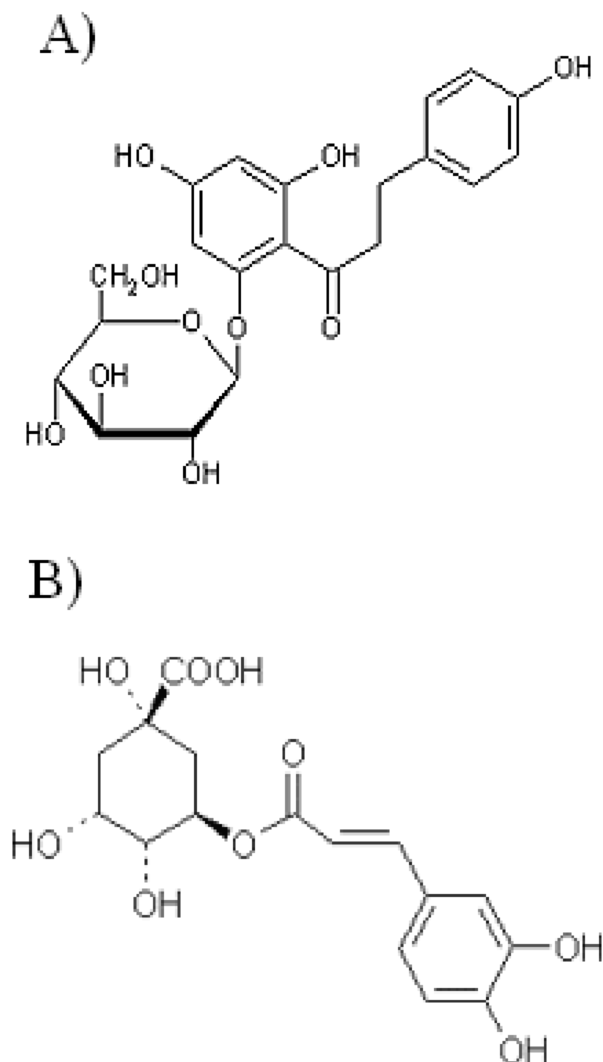

Figure 1. Chemical structures of phenolic compounds: A) Phloridzin, B) Chlorogenic acid.

Inhibition (%) = $\{(F_x - F_u)/(F_k - F_u)\} \times 100$

F_X – relative fluorescence of UVC irradiated sample, or oxidized by AAPH, for 30 min in the presence of an extract and compound, F_U - relative fluorescence of control sample, oxidized by AAPH or UVC irradiation, without extract or compound, measured after 30 min, F_K - relative fluorescence of blank sample, not subjected to oxidation procedures, measured after 30 min.

The results of the assay were expressed relative to Trolox, in terms of TEAC (Trolox equivalent antioxidant capacity). The results are presented in the form: mean value ± standard deviation calculated at confidence level α = 0.05 ($p<0.05$) from 5 independent measurements. Two-factor analysis of variance was carried out with ANOVA. The differences between control and extract containing samples were found significant with the Dunnett test. The calculations were performed using StartSoft statistica 9.

RESULTS AND DISCUSSION

Based on the results of spectrophotometric measurements, the kinetics of the process of membrane lipid oxidation in the presence of the substances studied was determined. With increasing time of irradiation the absorption rises, indicating increasing degree of lipid oxidation. Representative curves are shown in Figures 2 and 3, for each of the compounds at one chosen concentration. Figure 2 shows the dependence of absorption on irradiation time of erythrocyte suspension for control and samples with addition of two studied compounds: chlorogenc acid at 0.005 mg/ml and phloridzin at concentration ten times higher (0.05 mg/ml). The plots clearly show that lipid oxidation is markedly inhibited by both the compounds, though the chlorogenic acid is more effective than phloridzin. The dependence of absorption on irradiation time for control and two samples containing equal concentrations of apple leaf and fruit extracts (0.025 mg/ml) are shown in Figure 3. The absorption in the presence of extracts is markedly lower, relative to control; which testifies that both the extracts inhibit lipid oxidation well. However, the apple leaf extract's antioxidant activity is a bit stronger compared with fruit extract.

The similar character of the plots in Figures 2 and 3 (parallel shift) suggests that the mechanism of membrane lipid inhibition is similar for the compounds and extracts. For comparison of the activity of the extracts, on the basis of plots for 5 different concentrations and 60 min irradiation time, were determined concentrations (IC_{50}) causing 50% inhibition of lipid oxidation for the substances studied and Trolox®, which is regarded as a standard antioxidant substance (Table 2). From Table 2 it follows that all the compounds studied exhibit different antioxidant properties, and at concentrations not exceeding 0.04 mg/ml they cause 50% inhibition of membrane lipid oxidation. The best antioxidant is the chlorogenic acid which at concentration even smaller than Trolox® (0.0074 mg/ml) causes 50% inhibition of lipid oxidation. The remaining substances used induce the same effect at higher concentrations, and their activity, together with Trolox®, following the sequence: chlorogenic acid > Trolox® > apple leaf extract ≥ apple fruit extract > phloridzin. The antioxidant activity of the substances used was also investigated with the fluorimetric method. Free radicals produced during irradiation of membrane lipids cause quenching of DPH–PA probe fluorescence. As a measure of lipid oxidation was assumed the relative fluorescence that is the ratio of the fluorescence emitted by a sample oxidized with UVC radiation to initial fluorescence of the sample. As a control was assumed the relative fluorescence of sample containing a suspension of erythrocyte ghosts and DPH-PA probe, oxidized with UVC or AAPH radical; the blank being the relative fluorescence of sample of the same composition but not oxidized.

Figure 4 shows the relation between relative

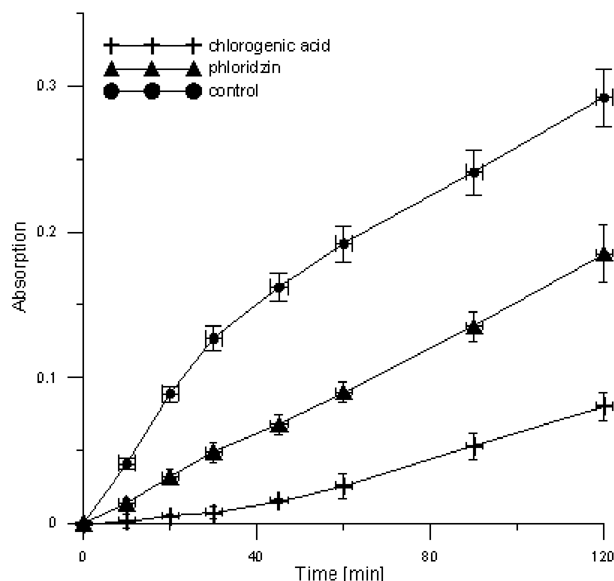

Figure 2. Light absorption vs. time of UVC irradiation of erythrocyte ghosts, for control and samples containing chlorogenic acid at 0.005 mg/ml and phloridzin at 0.05 mg/ml. Light absorption upon the color TBA-MDA reaction was measured for the irradiation times 0, 10, 20, 30, 45, 60, 90 and120 min.

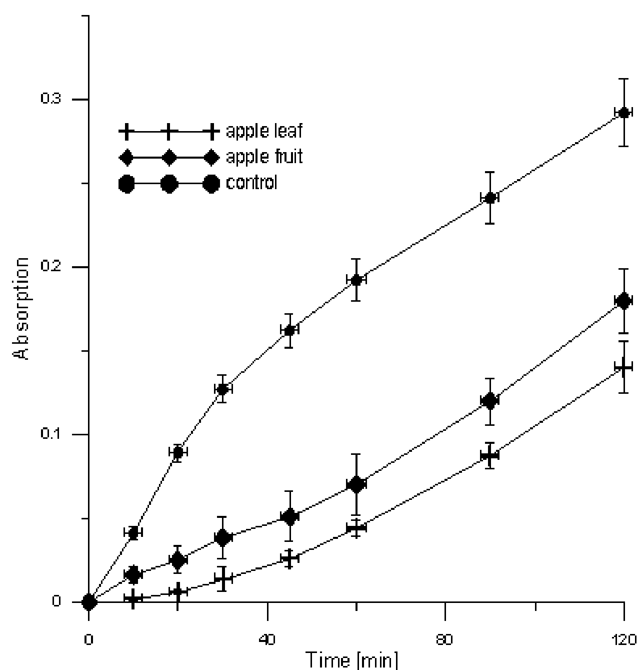

Figure 3. Light absorption vs. time of UVC irradiation of erythrocyte ghosts, for control and samples containing apple leaf or fruit extract at 0.025 mg/ml. Light absorption upon the color TBA-MDA reaction was measured for the irradiation times: 0, 10, 20, 30, 45, 60, 90 and 120 min at λ= 535 nm.

fluorescence and time of erythrocyte ghosts oxidation with UVC, for samples containing apple fruit extract at

Table 2. Concentration values IC_{50} for apple leaf and fruit extracts, chlorogenic acid, phloridzin and Trolox®, for UVC induced oxidation, from spectroscopic measurements.

Compound	Concentration IC_{50} (mg/ml)
Chlorogenic acid	0.0074 ± 0.0005
Phloridzin	0.0320 ± 0.0046
Apple leaf	0.0218 ± 0.0026
Apple fruit	0.0230 ± 0.0023
Trolox®	0.0090 ± 0.0006

concentrations from 0.005 to 0.05 mg/ml. As can be seen from Figure 4, relative fluorescence decreases with increasing time of oxidation and concentration of the extracts; the decrease being less pronounced with increasing concentration, until 80% inhibition of membrane lipid oxidation that occurs at 0.05 mg/ml. In order to compare the antioxidant activities of the substances, like in the spectrophotometric method, the concentrations (IC_{50}) of the compounds causing 50 % inhibition of membrane lipids oxidation were determined; which, together with the results for Trolox® are compiled in Table 3. This method confirmed the antioxidant properties of the compounds. Like in the spectophotometric method, the chlorogenic acid protects lipid membranes against oxidation best. It was also found that, of the substances studied, the weakest protection is rendered by phloridzin. In this method it is also evident that apple leaf and fruit extracts have a lower antioxidant activity than Trolox®.

DPH-PA probe fluorescence quenching in the presence of the compounds at various concentrations, that is their ability to scavenge free radicals, was also investigated when erythrocytes oxidation was induced with the free radical AAPH used at 60 µM concentration. Figure 5 shows representative plots of the relation between relative fluorescence and time of irradiation for apple leaf extract applied at concentrations from 0.005 to 0.008 mg/ml.

The results obtained shows that the extract clearly inhibits erythrocyte membrane lipids oxidation for all the concentrations used. Previously, concentrations were determined for the compounds and Trolox® that cause 50% inhibition of membrane lipids. Results are shown in Table 4. Like in the case of UVC radiation, the best antioxidant properties were exhibited by chlorogenic acid, the weakest antioxidant was phloridzin. Apple leaf and fruit extracts exhibited a lower activity than Trolox®.

Conclusions

The results obtained using two different research techniques and two oxidation indicators have shown that chlorogenic acid has the best antioxidant properties,

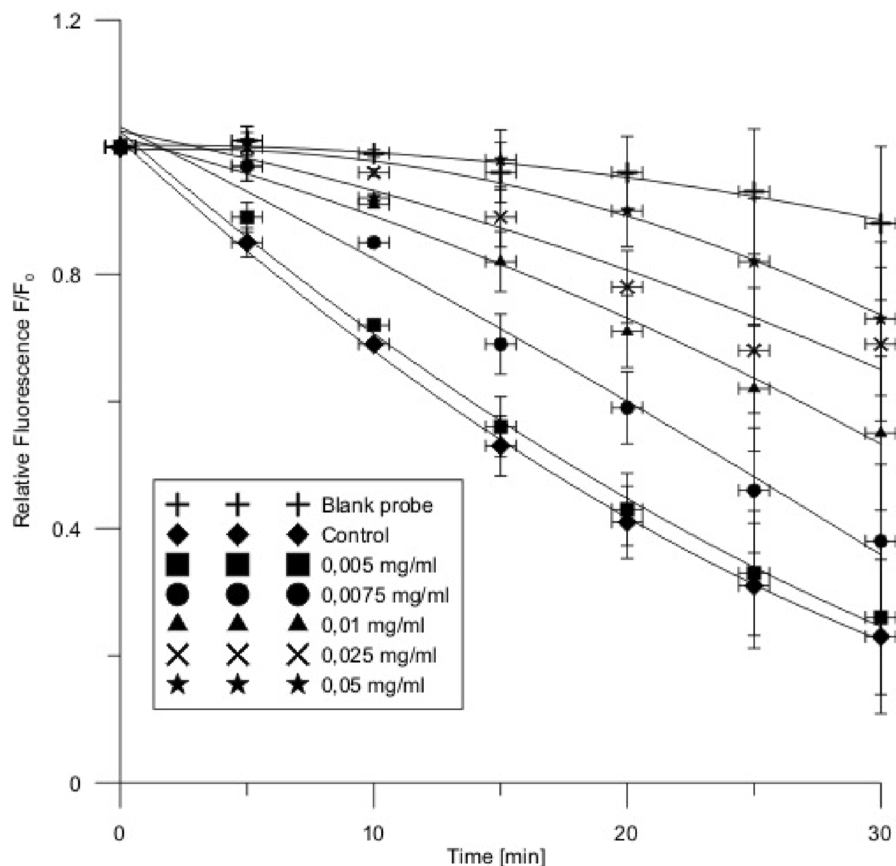

Figure 4. Relative fluorescence vs. time of irradiation of erythrocyte ghosts labeled with DPH-PA probe with UVC for control and samples containing different concentrations of apple fruit extracts (0.005 to 0.05 mg/ml). Relative fluorescence of DPH-PA probe was measured for the irradiation times: 0, 5, 10, 15, 20, 25 and 30 min.

Table 3. Concentration values IC_{50} for apple leaf and fruit extracts, chlorogenic acid, phloridzin and Trolox®, for UVC induced oxidation, from fluorimetric measurements.

Compound	Concentration IC_{50} (mg/ml)
Chlorogenic acid	0.0078 ± 0.0007
Phloridzin	0.0720 ± 0.0046
Apple leaf	0.0260 ± 0.0029
Apple fruit	0.0286 ± 0.0026
Trolox®	0.0146 ± 0.0013

while phloridzin the weakest. Both the leaf and fruit extracts, being a mixture of polyphenols and containing both the compounds in different amounts, exhibit a good, similar antioxidant properties; which are weaker than those of chlorogenic acid and Trolox® but better than phloridzin (Table 5). Both the extracts studied contain different amounts of polyphenolic compounds. In apple fruit extract they constitute 60% of all components, in the leaf extract 30%. In spite of the differences, the present studies have shown that the extracts' antioxidant activities are at similar level, which allows to postulate that their antioxidant properties are determined both by the amount of polyphenolic compounds they contain and the kind of the compounds. It has also been shown that phenolic compounds present in apple leaf and fruit extracts have strong antioxidant properties, but weaker than those of chlorogenic acid and stronger than phloridzin's. Since the chlorogenic acid is the dominant phenolic compound in apple fruit extract and constitutes 30% of all the polyphenols there, one can expect that the

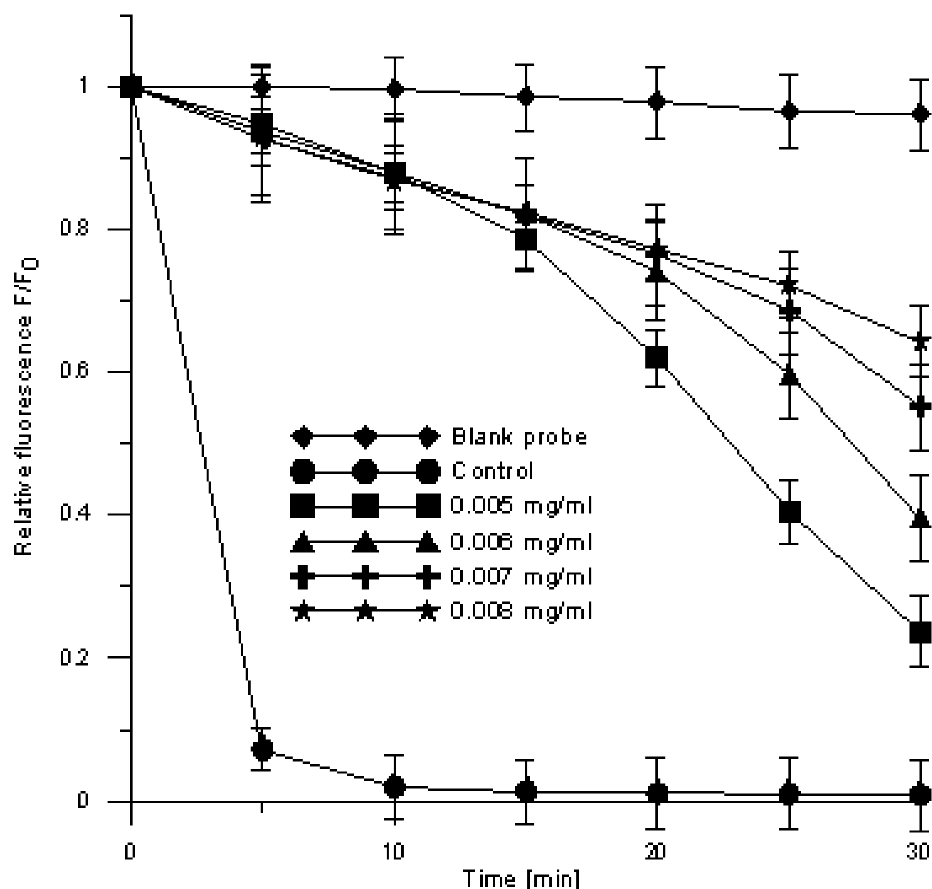

Figure 5. Relative fluorescence vs. time of oxidation of erythrocyte ghosts labeled with DPH-PA probe and containing the AAPH radical at 20 mM; the apple leaf extract concentration was 0.005 to 0.008 mg/ml. Measurements of DPH-PA relative fluorescence were performed for the irradiation times: 0, 5, 10, 15, 20, 25 and 30 min.

Table 4. Concentrations IC_{50} for apple leaf and fruit extracts, chlorogenic acid, phloridzin and Trolox®, from the fluorimetric method with AAPH as oxidation inductor.

Compound	Concentration IC_{50} (mg/ml)
Chlorogenic acid	0.0009 ± 0.00008
Phloridzin	0.22 ± 0.01
Apple leaf	0.0068 ± 0.0005
Apple fruit	0.0079 ± 0.0007
Trolox®	0.0039 ± 0.0003

good antioxidant properties of the extract are due to chlorogenic acid, which has been shown here to be a very good antioxidant, better than Trolox®.

The antioxidant activity of apple leaf extract is surely affected by chlorogenic acid, but to a lesser degree since it constitutes only 3% of all the polyphenols in the extract. Phloridzin, however, as documented in the present work, is a markedly weaker antioxidant than the extracts studied, and thus it should not markedly affect the antioxidant properties of the extracts. The similar antioxidant properties of apple leaf and fruit extracts are evidently resulting from the antioxidant potential of a mixture of various polyphenols in the extracts. The results of the present work indicate unequivocally that apple leaf extract is a very good scavenger of free radicals and should find application as a natural antioxidant in the pharmaceutical, cosmetics or food industry. The authors, by confirming the excellent antioxidant properties (with

Table 5. TEAC values for apple leaf and fruit extracts, chlorogenic acid and phloridzin.

Compound	TEAC		
	AAPH	UVC/60	UVC/30
Chlorogenic acid	0.236 ± 0.012	0.822 ± 0.041	0.534 ± 0.014
Phloridzin	56.410 ± 2.847	3.555 ± 0.255	4.931 ± 0.260
Apple leaf	1.744 ± 0.085	2.422 ± 0.128	1.780 ± 0.11
Apple fruit	2.026 ± 0.103	2.556 ± 0.278	1.959 ± 0.098

respect to membrane lipids) of the polyphenolic compounds contained in apples, hope that the equally active antioxidant in the form of apple leaf extract will also find application. It can protect living organisms against exogenous oxidation as a component, for instance, of protective creams.

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