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Antioxidant Activity of Extracts from Apple, Chokeberry and Strawberry

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The aim of the study was to determine the protective action of extracts from apple, strawberry and chokeberry with respect to linoleic acid and the biological membrane exposed to oxidation induced by physicochemical factors. The activity of the extracts was determined by measuring inhibition of lipid oxidation in red blood cell membrane, induced with UVC radiation and the AAPH radical. The protective effect of the extracts was essayed fluorimetrically and spectrophotometrically. These results together with the ones obtained earlier explain the mechanism of the interaction between the extracts and the red blood cell membrane. The mechanism consists in the incorporation into the membrane and screening the cell against oxidation. The results indicate that the extracts possess very good antioxidant properties, since at the highest concentrations used (0.1 mg/mL) they protect the biological membranes almost entirely against oxidation. Among the extracts studied the best antioxidant properties were exhibited by the apple fruit, which gave 80% or 100% protection of the membrane at 0.05 mg/mL concentration of dry matter, for UVC and AAPH inductors, respectively.

INTRODUCTION

Phenolic compounds found in plant extracts exhibit many beneficial effects on living organisms, due mostly to their antioxidant properties [Banerjee *et al.*, 2008; Caillet *et al.*, 2007; Naruszewicz *et al.,* 2007; Stratil *et al.*, 2007; Wong *et al.*, 2006; Yeum *et al.*, 2004; Yu *et al.*, 2004]. They are excellent as free radical scavengers including those in the form of the reactive forms of oxygen [Pajk *et al.*, 2006; Skupień *et al.*, 2008]. The radicals are the products of metabolic reactions within the living organism and are also formed in the external medium, *e.g.* by electromagnetic radiation. A high concentration of free radicals within an organism developed as a result of faulty action of natural protective mechanisms leads to oxidative stress that results in many dysfunctions at the molecular and cellular levels, which underlie many serious diseases. An exposed and very important site of attack of free radicals is the biological membrane. Oxidation of membrane lipids by free radicals causes disturbances in the structure and impairs functions of the biological membrane and leads to pathological changes within organism [Bartosz, 2003; Miller *et al.,* 2008].

The toxic effect of free radicals can be reduced or eliminated by consuming adequate amounts of phenolic compounds found in fruits and vegetables. Studies have shown that polyphenolic compounds of plant extracts possess also numerous therapeutic properties aside of the antioxidant ones [Andersen *et al.*, 2004; Awad & de Jager, 2002; Da Silva *et al.*, 2007; Gąsiorowski & Oszmiański, 1997; Ljubuncic *et al.*, 2005; Naruszewicz *et al.*, 2007; Oszmiański *et al.*, 2008; Skupień & Oszmiański, 2004; Zduńczyk *et al.*, 2002]. Attention is also paid to an antihemolytic effect of polyphenols on erythrocyte membranes [Arbos *et al.*, 2008; Chaudhuri *et al.*, 2007].

Numerous studies have shown that the effectiveness of the antioxidant and therapeutic action of polyphenolic substances found in extracts depends both on the quantity and quality of the polyphenols. An effective protection of biological membranes against oxidation may depend on the binding between polyphenols and membranes. Owing to their amphiphilic character, polyphenols are expected to interact mainly with the lipid phase of the biological membrane [Kondo *et al.*, 2007; Lotito & Frei, 2004; Oszmiański & Wojdyło, 2005; Oszmiański *et al.*, 2008; Pajk *et al.*, 2006; Skupień & Oszmiański, 2004; Suwalsky *et al.*, 2008].

Earlier studies [Bonarska-Kujawa *et al.*, 2011a] showed that extracts from apple, chokeberry and strawberry induce changes in biological and model lipid membranes. When incorporated into the membrane, polyphenols alter physical properties of the lipid phase, effecting slightly the fluidity of its hydrophobic region and the packing order of the lipid heads

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in the hydrophilic region. Moreover, in the work by Bonarska-Kujawa *et al.* [2011b] it is shown that apple leaf extract's antioxidative activity is comparable with that of an apple fruit extract with regard to the erythrocyte membrane.

In the present work the antioxidant activity of apple, chokeberry and strawberry extracts were investigated based on quenching the $ABTS^{\bullet+}$ radical, oxidation of linoleic acid and oxidation induced with AAPH, and also based on the extent of erythrocyte membrane oxidation induced by AAPH and UVC radiation.

MATERIALS AND METHODS

Material

The fruit extracts of chokeberry (*Aronia Melanocarpa* El.), apple (*Malus domestica* B. var. Idared) and strawberry (*Fragaria* × *ananassa* Duch. var. Senga sengana) harvested in 2008, were obtained from the Department of Fruit, Vegetable and Grain Technology of Wrocław University of Environmental and Life Sciences, Wrocław, Poland. The polyphenol content in extract was determined with the HPLC liquid chromatography. The extraction procedure and percent content of polyphenols in individual preparations was presented in a previous publication [Bonarska-Kujawa *et al.,* 2011a]. The total phenolic compounds content in dry matter was 56.59% in an apple extract, 63.58% in a chokeberry extract and 75.04% in a strawberry extract. Erythrocyte ghosts were obtained according to Dodge *et al.* [1963]. The fluorimetric probe DPH-PA (3-(p-(6-phenyl)-1.3.5-hexatrienyl) propionic acid, oxidation inductor AAPH [2.2'-diazobis (2-amidinopropan) dichlorohydrogen], radical ($ABTS^{\bullet+}$) 2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), linoleic acid and Trolox® were purchased from Sigma-Aldrich, Poland.

Oxidation of linoleic acid

Linoleic acid dispersion prepared according to the method of Surrey [1964], was added to UV cuvette containing a phosphate buffer. The oxidation reaction at 37°C was initiated by adding AAPH [Liegeois *et al.*, 2000]. The oxidation was carried out in the presence of different amounts of the fruit extracts. The rate of oxidation was monitored by recording changes in absorbance at λ=234 nm brought about by the formation of conjugated bonds in oxidised linoleic acid. A UV-VIS Evolution 600 (Thermo Scientific) spectrophotometer was used for the measurements. The antioxidant activity index equals the slope of the curve representing inhibition time of linoleic acid oxidation *versus* antioxidant concentration (min/(μg DM/mL)).

Assay of antioxidant capacity of extracts

The assay was based on antioxidant-induced reduction of the 2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) blue-green radical ($ABTS^{\bullet+}$) and converting it into a colourless product. $ABTS^{\bullet+}$ cation radical solution was prepared by mixing 7 mmol/L ABTS and 2.45 mmol/L potassium persulphate solutions at a ratio of 1:1 (v/v). The mixture was incubated in the dark at a room temperature for 16 h. The $ABTS^{\bullet+}$ solution was diluted with water to reach the absorbance of *ca*. 0.9 at 734 nm. To 1960 μL of the $ABTS^{\bullet+}$ solution 40 μL of diluted extracts were added and the decay in absorbance at 734 nm was followed on an Evolution 600 spectrophotometer maintained at 25°C by a Peltier thermostat. The decrease in absorption 5 min after addition of the solution was used for computation. The antioxidant activity of individual extracts was calculated in terms of TEAC (Trolox Equivalent Antioxidant Capacity), *i.e.* μmol Trolox of mg dry matter extract [Re *et al.*, 1999; Van den Berg *et al.*, 1999].

Extent of membrane lipid oxidation by fluorimetric method

The antioxidant activity of the extracts was determined by using the fluorimetric method, based on the relation between fluorescence intensity of the DPH-PA probe and concentration of free radicals in the solution. Fluorescence intensity decreases with an increasing concentration of free radicals [Arora & Strasburg, 1997]. To a phosphate buffer (131 mmol/L NaCl, 1.79 mmol/L KCl, 0.86 mmol/L $MgCl_2$, 11.79 mmol/L $Na_2HPO_4 \times 2H_2O$, 1.80 mmol/L $Na_2H_2PO_4 \times H_2O$, pH 7.4) containing erythrocyte ghosts and the fluorescence probe were added proper amounts (0.001, 0.005, 0.0075, 0.01, 0.05, 0.01 mg/mL) of the extracts. The samples were then subjected to UVC irradiation of 3.5 mW/cm^2 intensity from a bactericidal lamp for 30 min to induce oxidation, and fluorescence of the DPH-PA probe was recorded every 5 min. The measurements were conducted at a room temperature using a Carry Eclipsce (Varian) spectrofluorimeter. The excitation and emission wavelengths were: λ_{ex}=364 nm, λ_{em}=430 nm. The relative change in fluorescence intensity F/F_0 (F_0 – initial fluorescence, F – current fluorescence) was assumed as a measure of the extent of membrane oxidation. Percentage of oxidation inhibition was calculated according to the formula:

$$inhibition\% = \frac{(F_x - F_u)}{(F_k - F_u)} \times 100\%,$$

where: F_x – is relative fluorescence for oxidised sample (*via* UV or AAPH) in the presence of an extract after 30 min; F_U – is relative fluorescence for oxidised sample (*via* UV or AAPH) without an extract after 30 min; and F_K – is relative fluorescence for control sample, not oxidised and without extract after 30 min.

Statistical analysis

The results are presented in the form: mean value ± standard deviation calculated at a confidence level of α=0.05 ($p<0.05$) from at least 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's test. The calculations were performed using StartSoft STATISTICA 9.

RESULTS AND DISCUSSION

In $ABTS^{\bullet+}$ assay radicals are deactivated by single electron transfer. This method is most common and simple assay for the fast assessment of antioxidant activity of various plant extracts [Prior *et al.,* 2005]. The apple extract had the highest total antioxidant activity (5.65 μmol TEAC/mg DM).

TABLE 1. Antioxidant activity and total phenolic content of extracts (mean±SD, n=4).

Extract	Antioxidant activity (μmol TEAC/mg DM)	Antioxidant activity index (min/(μg DM/mL))
Chokeberry	4.15±0.34	44.4±3.7
Apple	5.65±0.45	47.1±3.4
Strawberry	4.80±0.40	43.1±2.8

The efficacies of the other extracts were over 20% lower (Table 1). It is difficult to compare the results obtained with published data, because different units are used in $ABTS^{\bullet+}$ assay, but investigated extracts had very high antioxidant activity [Raudoniute *et al.,* 2011; Vasco *et al.,* 2008; Zulueta *et al*., 2009].

In the absence of a radical initiator the rate of linoleic acid oxidation is negligible. AAPH induces oxidation that proceeds at a constant rate and results in conjugated double bonds formation. Addition of an extract inhibits the process for a certain time. Therefore, for each of the antioxidant concentrations the reaction inhibition time T_{inh} was determined. The measured inhibition time is directly proportional to the concentration of the extract. The slope of the curve T_{inh} *versus* the antioxidant concentration can be used as an antioxidant activity index [Liegeois *et al.*, 2000]. The results obtained for the extracts, presented in Table 1, confirm the highest antioxidative activities of the apple extract. Two other extracts had a slightly lower antioxidant activity index. The investigated extracts protect lionleic acid micelles against oxidation 15–25% better than Trolox®.

Antioxidative activities of apple, chokeberry and strawberry extracts were studied with the fluorometric technique coupled with the DPH-PA probe. AAPH free radicals, or those induced by UVC radiation, cause the quenching of DPH-PA fluorescence probe, evidenced by a decrease in fluorescence intensity in time. As a measure of membrane lipid oxidation there was assumed relative fluorescence, *i.e.* the ratio of fluorescence of an oxidised sample to the initial fluorescence of the sample. As a control in this method was assumed the relative fluorescence of oxidised sample referred to relative fluorescence of unoxidised sample (blank). Figure 1 shows a representative kinetics of UVC-induced oxidation of erythrocyte membrane lipids for chokeberry extract, expressed by relative fluorescence as a function of time and extract concentration. As seen in Figure 1, relative fluorescence is decreasing in time of oxidation, which indicates that the concentration of free radicals in the samples decreases.

The decrease is the biggest for the sample without the extract. For samples containing the chokeberry extract the relative fluorescence in time with an increasing extract concentration is smaller and smaller, indicating that the concentration of free radicals is decreasing as they are being scavenged by the polyphenolic substances present in the extract. Figure 2 shows an example of membrane lipid oxidation kinetics induced by the AAPH radical in the presence of the strawberry extract. In this case, a significant decrease in relative fluorescence is observed when there is no extract or when its concentration is the lowest, and that of free radicals is high. At extract concentration of 0.01 mg/mL and higher, the concentration of free radicals in the samples decreases markedly, resulting in a slow decrease in relative fluorescence (Figure 2). As seen, the kinetics of lipid oxidation in the erythrocyte membrane is of different character in the case of UVC radiation and AAPH radical.

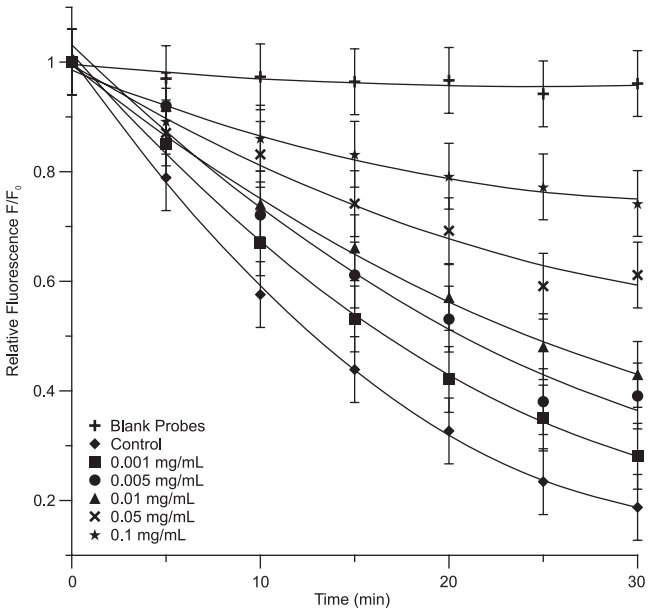

FIGURE 1. Relative fluorescence *versus* time of UVC irradiation for various concentrations of a chokeberry extract.

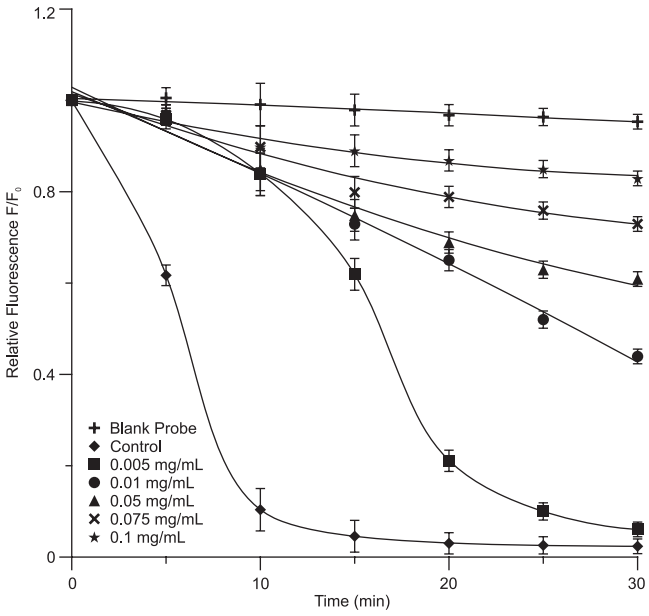

FIGURE 2. Relative fluorescence *versus* time of AAPH induced oxidation for various concentrations of a strawberry extract.

Based on the results of lipid oxidation kinetics in erythrocyte membranes with the fluorimetric method in the presence of apple, chokeberry and strawberry extracts, bar graphs were plotted that show the inhibition percentage after 30 min of oxidation at different extract concentrations (Figures 3, 4 and 5).

As seen from the bar graphs, in the case of UVC radiation inducing membrane lipid oxidation, for all the extracts a smaller effect of concentration on the inhibition of lipid

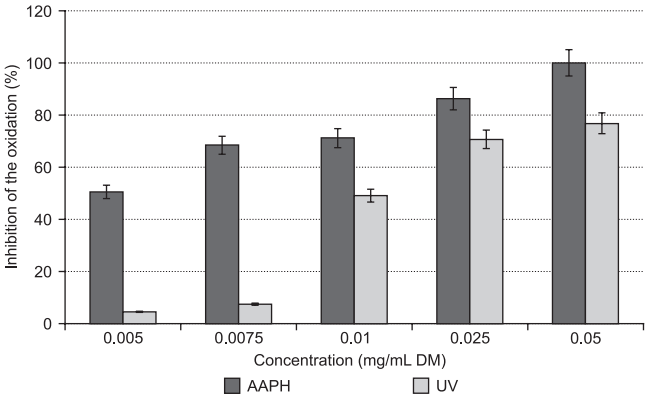

FIGURE 3. Apple extract-induced oxidation inhibition for erythrocyte ghosts after 30 min of UVC irradiation and AAPH oxidation.

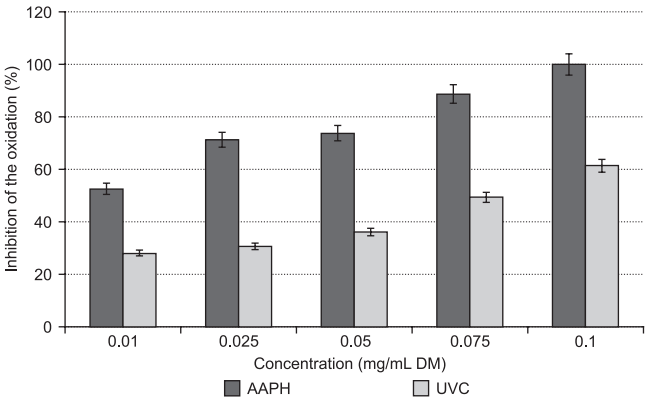

FIGURE 4. Chokeberry extract-induced oxidation inhibition for erythrocyte ghosts after 30 min of UVC irradiation and AAPH oxidation.

oxidation is observed than in the case of the AAPH inductor. At the highest concentrations of the extracts, the inhibition of oxidation does not exceed 80%. Moreover, the rate of increase in the inhibition with extract concentration differs. For the apple there is a dynamic increase in the inhibition with concentration (Figure 3), and a slower increase of inhibition for chokeberry and strawberry extracts (Figures 4 and 5). For the AAPH radical, at the highest concentrations of the extracts lipid oxidation in the erythrocyte membrane was completely inhibited. Due to the differentiated activity of the extracts, various concentration ranges were applied, as in the case of the strawberry extract (Figure 5). With the results obtained, and based on earlier results [Bonarska-Kujawa *et al.*, 2011a], one can expect that an effective protection of the biological membrane against oxidation is possible when the polyphenolic compounds of the extracts bind to the membrane surface. The percentage of oxidation inhibition will thus depend on the concentration of polyphenolic substances bound to the membrane surface. As indicated by the research done, for UVC radiation the concentrations of the extracts ought to be higher than with the AAPH inductor in order to obtain similar inhibition effect. This may mean that UVC radiation induces more free radicals.

To compare the antioxidative activity of the extracts, kinetics plots were used to find extract concentrations which cause 50% inhibition (I_{50}) of membrane lipid oxidation in 30 min of the oxidation process.

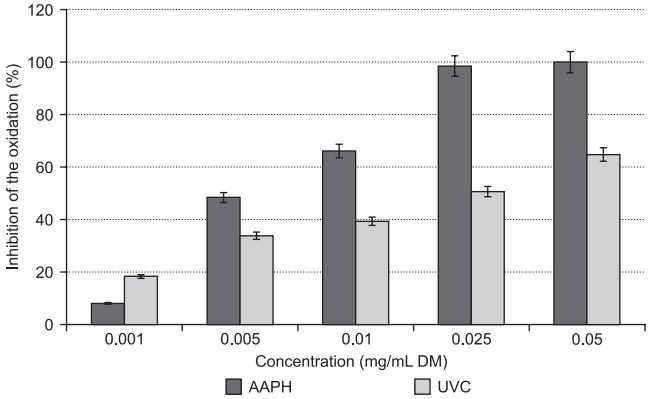

FIGURE 5. Strawberry extract-induced oxidation inhibition for erythrocyte ghosts after 30 min of UVC irradiation and AAPH oxidation.

TABLE 2. I_{50} values obtained from experiments with erythrocyte membranes induced by AAPH and UVC radiation (mean±SD, n=5).

	I_{50} (mg/mL DM)	
Extract	UV / 30 min	AAPH /30 min
Chokeberry	0.0520±0.0047	0.00955±0.00086
Apple	0.0286±0.0026	0.00794±0.00071
Strawberry	0.0529±0.0048	0.02423±0.00218
Trolox	0.0146±0.0013	0.00390±0.00035

The values of I_{50} of the extracts and Trolox®, which is a standard antioxidant, are presented in Table 2.

CONCLUSIONS

The results presented indicate that all the extracts studied possess a high antioxidant activity, which is confirmed by the investigation of quenching the $ABTS^{\bullet+}$ radical, oxidation of linoleic acid and biological membranes. As regards erythrocyte membranes, the process of oxidation inhibition showed differentiated kinetics, both for the extracts and different oxidation inductors. The high but differentiated antioxidative activity of the extracts is due to different contents of polyphenolic compounds. The percent content of polyphenols in the extracts was determined with the liquid chromatography (HPLC) method described in a study by Bonarska-Kujawa *et al.* [2011a].

Earlier research on the interaction between the extracts and biological and lipid membranes have shown that apple, chokeberry and strawberry extracts induce changes in biological membranes and model lipid membranes [Bonarska-Kujawa *et al.*, 2011a]. Compounds contained in the extracts interact with the erythrocyte and lipid membranes, incorporating into and binding to the outer hydrophilic part of the membrane, and thereby changing its properties, while not effecting fluidity of the hydrophobic region, *i.e.* where the hydrocarbon chains are. Taking into account the above results on the antioxidative activity of the extracts, and those of the present investigation, one can propose the mechanism of their antioxidative action at a cell membrane level. The polyphenolic compounds of the extracts bind to the outer part of membrane and thus constitute a kind of screen (barrier) that prevents free radicals from penetrating the membrane. This applies both to ambient free radi-

cals and those induced by UVC radiation. It seems that the differentiated antioxidant activity of the extracts follows from the extent an extract's polyphenols bind to the membrane, this in turn depending on the kind and content of the polyphenols in the extract. The high activity of the apple extract can be ascribed to the presence of chlorogenic acid which easily binds to the membrane [Bonarska-Kujawa *et al.*, 2011a].

Thus the present research documents that the extracts studied are effective antioxidants that can protect erythrocyte membranes against oxidation. Moreover, their presence at the membrane surface does not provoke alterations in structure nor properties [Bonarska-Kujawa *et al.*, 2011a] and protects the membrane structure by keeping free radicals at a safe level.

The protective effect of the extracts on membrane structures is expected to extend to the whole organism.

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