# Anthocyanin Extracts with Antioxidant and Radical Scavenging Effect

Janina Gabrielska[a], Jan Oszmiański[b], Małgorzata Komorowska[c] and Marek Langner[a]

[a] Department of Physics and Biophysics,
[b] Department of Fruit and Vegetable Technology, Agricultural University, Norwida 25, 50–375 Wrocław, Poland
[c] Institute of Physics, Wrocław University of Technology, Wyb. Wyspiańskiego 27, 50–375 Wrocław, Poland

Z. Naturforsch. **54c,** 319–324 (1999); received December 2, 1998/January 22, 1999

Antioxidant, Anthocyanin, Liposome, TBA-Reactive Product

The antioxidative activity of three anthocyanin pigments, extracted from the fruits of chokeberry, honeysuckle and sloe, were studied. Lipid oxidation in the liposome membrane, induced by UV radiation, was evaluated with a thiobarbituric acid-reactive substances assay. The antioxidant efficiency of the studied compounds follows this sequence: chokeberry > sloe > honeysuckle. The extract concentrations at which a 50% reduction of phosphatidylcholine oxidation was observed, were respectively: 48, 54 and 60 mg/l. The end products of lipid membrane oxidation were evaluated using HPLC. It was found that the antioxidative potency of anthocyanin extracts is concentration-dependent. As shown by EPR technique the efficiency of the extracts to eliminate free radicals from the solution follows the order of the antioxidant activity.

## Introduction

The excess production of active oxygen species, such as $\cdot OH$, $O_2^-$, singlet oxygen and other free radicals, causes damage throughout the cell by oxidizing a variety of molecules, including unsaturated lipids. Lipids are a major membrane components and their oxidation leads to significant changes in membrane properties. These changes initiate processes leading to carcinogenesis, mutagenesis, ageing and arteriosclerosis (Cutler, 1992; Pryor, 1986; Stadtman, 1992; Harman, 1992). Cells have limited possibility in eradicating free radicals, hence it is believed that deliverting endogenous antioxidants will enhance its ability to protect vital biological functions (Cutler, 1984; Kohen *et al.,* 1988; Osawa *et al.,* 1990); therefore, there is increasing interest in the application of naturally occurring antioxidants as therapeutic agents. Antioxidants are a common constituent of diets, especialy frequent in dietary plants (Huang and Ferrato, 1992). The anthocyanin pigment is an example of such an antioxidant, as it is a common part of the human diet, present in crops, beans, fruits and vegetables (Harborne and Grayer, 1988). These pigments are believed to play an important role as dietary antioxidants, effective in the prevention of oxidative damage caused by active oxygen radicals. As these compounds originate from natural products, they are expected to have limited toxicity, and thus their extracts may serve as therapeutic agents in preventing or curing diseases induced by oxidative damage.

There are few reports on the biological activity of anthocyanin pigments (Tsuda *et al.,* 1996), including their antioxidative or active oxygen radical scavenging effect. Studies presented in this paper evaluate the inhibition effect of anthocyanin extracts from chokeberry, honeysuckle and sloe fruits on the peroxidation of the phosphatidylcholine membrane and their role as 1,1-diphenyl-2-picrylhydrazyl scavengers.

Reprint request to Janina Gabrielska.
Fax: (+48)-71-205172
E-mail: jaga@ozi.ar.wroc.pl

## Materials and Methods

### *Chemicals*

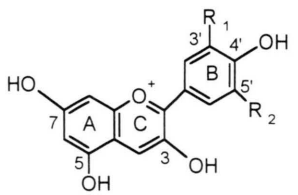
Egg yolk lecithin was prepared in our laboratory, applying the method described by Singleton *et al.* (1965). Anthocyanin extracts were prepared in the Department of Fruit and Vegetable Technology, Agricultural University of Wrocław. Fruits of chokeberry (*Aronia melanocarpa* Elliot), hon-

0939–5075/99/0500–0319 $ 06.00 

eysucle (*Lonicera kamtschatica*) and sloe (*Prunus spinosa*) were obtained from botanical garden of the Department of Biology and Botanics of Medical Academy of Wrocław. 1,1-diphenyl-2-picrylhydrazyl was purchased from Sigma Chemicals (Deisenhofen, Germany), and all other chemicals were of analytical grade.

*Preparation of anthocyanin extracts*

Fruits of chokeberry, honeysuckle and sloe may contain the following amounts of anthocyanins: 300–600, 300–1200 and 80–170, mg/100 g of fruit, respectively (Oszmiański and Sapis, 1988). Fruits of chokeberry, honeysuckle and sloe were homogenized using Ultra Turrax aparatus (Heidolph, Germany) durring 2 min, and anthocyanins were extracted using a water solution containing 200 ppm of $SO_2$. The solution was run through Duolite S761 resin column (Saquet-Barel *et al.*, 1982). Impurities were washed off with distilled water and pigments were eluted with 80% ethanol. The resulting ethanol solution of anthocyanin was concentrated under vacuum and freeze dried. Obtained anthocyanins (chemical structures are presented in Fig. 1) were analyzed using high pressure liquid chromatography (HPLC). The composition of anthocyanin monomers was found to be: chokerberry extract contained 64.5% of cyanidin-3-galactoside, 28.9% of cyanidin-3-arabinoside, 4.2% cyanidin-3-xyloside and 2.4% of cyanidin-3-glycoside (Oszmiański and Sapis, 1988); extract of plum contained 48% of cyanidin-3-rutinoside, 37.8% of cyanidin-3-glycoside, 11.2% of peonidin-3-rutinoside and 2.8% of peonidin-3-glycoside (Werner *et al.*, 1989); honeysuckle extract contained 90.9% of cyanidin-3-galactoside, 4.1% of cyanidin-3-rutinoside, 2.5% of cyanidin-3-diglycoside and 2.5% of malwidin-3-glycoside (Oszmiański *et al.*, 1995). Mean molecular weight of the anthocyanin extracts was estimated to be 491.6 g.




| $R_1$ | $R_2$ | |
|---|---|---|
| OH | H | cyanidin |
| $OCH_3$ | H | peonidin |
| $OCH_3$ | $OCH_3$ | malwidin |

Fig 1. Chemical formulas of the anthocyanin extracts studied.

*Induction of lipid peroxidation*

Egg lecithin in chloroform was dried under vacuum in a nitrogen atmosphere (Gabrielska *et al.*, 1997). A 0.05 mM Tris:HCl [(hydroxymethyl) aminomethane] buffer of pH 7.4 was added and the sample was shaken for 15 min (control sample), and after adding the appropriate anthocyanin extracts for another fifteen min. Anthocyanin extracts were added from the ethanol stock solution (the amount of ethanol never exceeded 2% of the final volume of the sample). The same amount of ethanol alone was added to the control sample. The final lipid concentration in each sample was 1.5 mg phosphatidylcholine (PC) per ml.

Lipid peroxidation was induced with an ultraviolet radiation of intensity 3.0 mW/cm$^2$. The concentration of accumulated phospholipid peroxidation products was determined by measuring 2-thiobarbituric acid (TBA)-reactive products (Buege and Aust, 1978). The amount of reaction products by the reaction between peroxides and TBA was determined measuring the absorbance at 535 nm (Specol 11, Zeiss Jena).

*HPLC analysis of 2-hydroxypyrimidine (reaction of malonaldehyde and urea)*

Egg lecithin was mixed with an appropriate amount of anthocyanin extract in chloroform, dried under vacuum in a nitrogen atmosphere (Gabrielska *et al.*, 1997), and hydrated with a 0.05 mM Tris:HCl [(hydroxymethyl)aminomethane] buffer, pH 7.4. The sample was than shaken for 15 min. The final lipid concentration of each sample was 6 mg PC/ml.

The oxidation of phospholipids was induced by UV-radiation of intensity 3.0 mW/cm$^2$. Samples were irradiated for 2 hours. The resulting sample was treated according to the method described by Osawa and Shibamoto (1992), with minor modifications: The oxidized sample was heated to 100 °C and kept at that temperature for an hour with 0.3 ml urea (120 μmol/ml) and 0.3 ml 1.2 N HCl. The reaction solution was passed through Sep Pak a C-18 cartridge (Waters Millipore Milford, MA, USA) and washed with 1.5 ml distilled water prior to HPLC application. The final volume of each sample was adjusted to 3 ml with distilled water. 20 μl of such a preparation was than injected into an HPLC instrument (WATERS) using a variable

wavelength absorbance detector (Waters 486), which was adjusted for detecting 2-hydroxypyrimidine. The column (Lichrospher 100 RP-18, 5 μm, MERCK) was eluted with water (HPLC).

*EPR measurements*

The effect of anthocyanin extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured using EPR as described previously by Ushida *et al.* (1987); in short: 100 μl of a 560 μM DPPH solution (in ethanol) was mixed with an appropriate amount of anthocyanins and immediately placed in a special flat cell (volume 160 μl). After 2 min, the first spectrum of DPPH radicals was recorded using an EPR spectrometer an (X-band spectrometer made at the Technical University in Wrocław) and subsequently, further spectra were taken in following appropriate intervals of time. The initial concentration of DPPH free radicals in each samples was constant and equal 280 μM.

## Results

Peroxidative damage inflicted by UV radiation on phosphatidylcholine in vesicle membranes as a function of time is shown in Fig. 2. Absorbance at 535 nm ($\Delta A_{535}$) reflects the changes in concentration of TBA-reactive products. The addition of anthocyanin extract to the sample (24.5, 49, 73.5 and 98 mg/l) reduced the amount of oxidation products in a concentration-dependent manner, which shows itself as a decreased absorbance level. Lipid oxidation inhibition by anthocyanins for selected antioxidant concentrations (24.5 and 98 mg/l) is presented in Fig. 3. This inhibition was calculated according to $[1\text{-}\Delta A'_{535}/\Delta A_{535}]$, expressed as percentage change. $\Delta A'_{535}$ and $\Delta A_{535}$ represent changes in absorbance in the presence and absence of anthocyanin extract, respectively. The inhibition level changed proportionally to time to UV irradiation time for small extract concentrations (24.5 mg/l), and for higher concentrations of extracts (98 mg/l) saturates around 80% oxidation inhibition after a short period of time.

The amount of oxidation products can be directly determined using the HPLC technique. Fig. 4 shows examples of HPLC chromatograms, from which the amount of oxidation products induced by irradiation were determined. These chromatograms show that

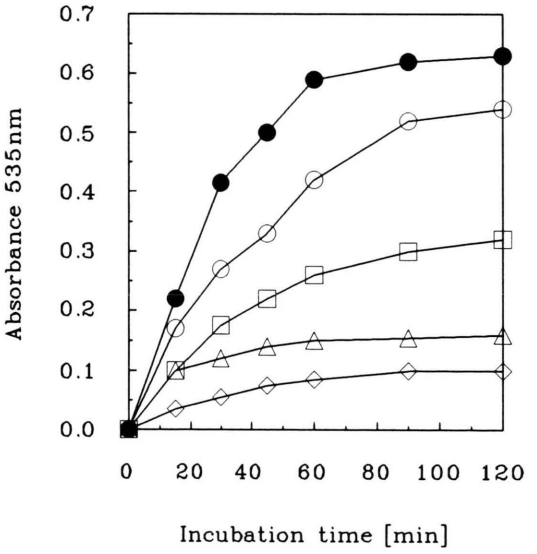



Fig 2. The effect of chokeberry extract on UV-induced oxidation within phosphatidylcholine liposomes as a function of time. The concentration of chokeberry extract was zero (closed circles), 24.5 mg/l (open circles), 49 mg/l (squares), 73.5 mg/l (triangles) and 98 mg/l (rhombuses).

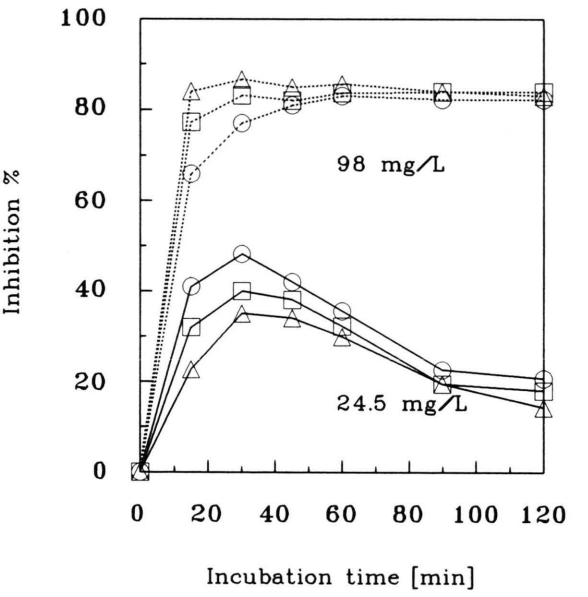



Fig 3. The dependence of inhibition of phosphatidylcholine (estimated on the basis of results presented in Fig. 2 – details in text) on exposure time to UV-irradiation when the anthocyanin pigment is present in the sample at two concentration: 24.5 mg/l and 98 mg/l (circles – honeysucle; squares – sloe and triangles – chokeberry).

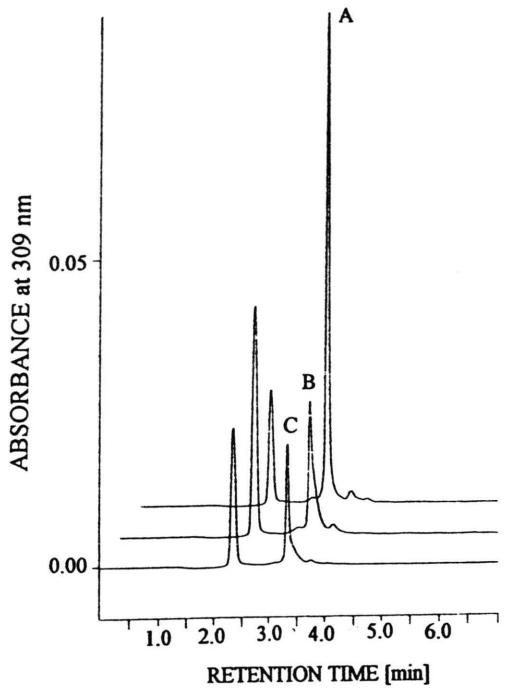



Fig 4. Examples of phosphatidylcholine liposome suspension HPLC chromatograms exposed to UV-radiation in the absence (A) and in the presence of anthocyanin extract at a concentration of 24.5 mg/l (B). Peaks labeled with A,B and C are 2-hydroxypyrimidine, resulting from the reaction of the membrane oxidation product, malonaldehyde, and urea.

the presence of anthocyanins in the sample reduces the amount of oxidative products (B – 24.5 mg/l anthocyanins from chokeberry and C – 24.5 mg/l of anthocyanins from honeysuckle), when compared to the chromatogram obtained with no anthocyanin was present (A). Quantitative results derived form HPLC chromatograms are compiled in Table I.

Table I. The effect of the anthocyanin extract on the level of phosphatidylcholine oxidation. Oxidation products were estimated based on HPLC chromatograms. It was assumed that full oxidation was obtained in the liposome suspension.

| | Percentage of oxidation inhibition of liposome membranes as a function of anthocyanin extract concentration [mg/l] | | | |
|---|---|---|---|---|
| Concentration | 9.8 | 19.8 | 39.6 | 58.8 |
| Chokeberry | 37 | 51 | 58 | 61 |
| Honeysuckle | 40 | 51 | 62 | 70 |
| Sloe | 35 | 48 | 65 | 80 |

DPPH is a free radical commonly used in EPR studies. Here, the concentration of DPPH was used to measure the ability of anthocyanins to eliminate highly reactive species from the lipid bilayer. The concentration of DPPH in the sample was estimated from the amplitude of the EPR signal. The dependence of the amount of DPPH on the concentration of added anthocyanins is summarized in Fig. 5.

The antioxidative potency of extracts can be expressed as concentrations required to eliminate half of the free radicals present in the sample are ($IC_{50}$). A similar parameter can be used to express the pigments' protective potency against damage inflicted on the lipid bilayer by UV radiation, applied for an arbitrarily chosen period of time (90 min). Such data presentations enable us to qualitatively compare results obtained using both methods mentioned in this paper (Table II).

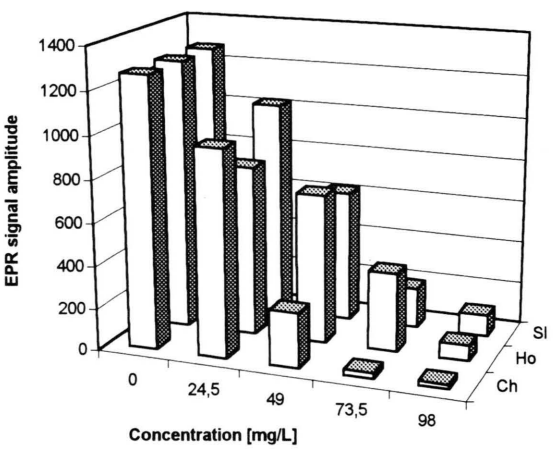



Fig 5. The dependence of the amplitude of EPR signals obtained from DPPH free radicals on the amount of anthocyanin present in the medium. The EPR spectrum was taken after 10 minutes of sample incubation. Sl – sloe; Ho – honeysuckle; Ch – chokeberry.

Table II. Compared antioxidant activity of various anthocyanin extracts. The inhibition of phosphatidylcholine oxidation and ability to eliminate free radicals from the aqueous phase are presented. The inhibition potency was determined as a concentration at which phospholipid oxidation was reduced by 50% ($IC_{50}$) or 50% of all free radical were eliminated from the aqueous phase.

| | $IC_{50}$ [mg/l] | |
|---|---|---|
| | EPR | $\Delta A_{535}$ |
| Chokeberry | 36 | 48 |
| Sloe | 49 | 54 |
| Honeysuckle | 55 | 60 |

## Discussion

Data presented in this paper shows that anthocyanins are potent antioxidants, readily available from products used in common diets. This fact opens the possibility to obtain a natural drug for preventing oxidative damage caused by active oxygen radicals in living systems. Free radicals are the major factor that cause oxidative damage to membrane lipids. Pigments studied in this paper are likely to be located within the lipid bilayer, where major oxidative damage is inflicted by free radicals entering the lipid matrix from the aqueous phase or those produced within the membrane itself (by UV radiation). We have found that the extracts exhibited a marked stability in their protective effect on the liposome membrane when applied at high concentrations, *i.e.* 98 mg/l; whereas, at a concentration of 24.5 mg/l their potency to inhibit lipid oxidation rises at first and then after 60 min steadily declines (Fig. 3). This may indicate that at low concentrations the pool of active antioxidant molecules needed to react with free radicals continually being produced is depleted.

Limited differences between the three studied anthocyanins are most likely a result of variations in their chemical structures. The chokeberry extract had the highest antioxidant potency, whereas sloe and honeysuckle having potency is roughly 40% lower (Table II). The higher activity of chokeberry anthocyanins may result from, present in the extract, cyanidin derivatives, which posses two hydroxyl groups in the ortho position on the B ring. Sloe is less active, because it contains peonidin derivatives, in which one of the two hydroxyl groups is methylated, which presumably causes lower polyphenol antioxidant activity. The same is evident for phenolic acid antioxidant activity, which shows that caffeic acid (with two *ortho*-hydroxyl groups) exhibits higher antioxidant activity than ferulic acid (with one methylated group) (Cuvelier *et al.,* 1992). Among the anthocyanins of honeysuckle, there are malvidin derivatives, whose ring B hydroxyl groups are methylated (Oszmiański *et al.,* 1995). This reduces the antioxidant activity of that particular preparation. The higher antioxidative activity of chokeberry anthocyanins can also be ascribed to the fact that it contains only monoglycoside derivatives, whereas the other anthocyanins contain diglycosides, presumably causing lower activity. Such lowering of antioxidation activity of rutin, which has a diglycoside substitute, was observed in the case of quercetin (Rice-Evans *et al.,* 1997). All studied anthocyanin extracts proved to be effective radical scavengers when tested against the diphenylpicrylhydrazyl radical (Fig. 5), frequently used as a model compound for free radicals in lipids (Hatano *et al.,* 1997; Malterud *et al.,* 1996). This indicates that these compounds may act as preventives or chain-breaking antioxidants of radicals, such as hydroxyl, alkoxyl or peroxyl, which can initiating and promoting the peroxidation process in the phosphatidylcholine membrane. Experiments indicate that extracts may have similar activities towards free radicals formed by UV radiation.

*Acknowledgments*

Work was sponsored by Agricultural University grant no. 332/GW/98 and partly by KBN under Wrocław University of Technology research project no. 33153-3.

Cutler R. G. (1984), Antioxidants, aging, and longevity. Free Radicals in Biology (Pryor W. A., ed.) Vol. VI. Academic Press, Orlando, FL, pp. 371–423.

Cutler R. G. (1992), Genetic stability and oxidative stress. In: Free Radicals and Aging (Emerit I. and Chance B., eds). Birkhäuser Verlag, Basel, Switzerland. pp. 31–46.

Buege J. A. and Aust S. D. (1978), Microsomal lipid peroxidation. Methods Enzymol. **52c**, 302–310.

Cuvelier M.-E., Richard H. and Berset C. (1992), Comparison of the antioxidative activity of some acid-phenols: structure-activity relationship. Biosc. Biotech. Biochem. **56**, 324–325.

Gabrielska J., Oszmiański J., Żyłka R. and Komorowska M. (1997), Antioxidant activity of flavones from *Scutellaria baicalensis* in lecithin liposomes. Z. Naturforsch. **52c**, 307–317.

Harborne J. B. and Grayer R. J. (1988), The anthocyanins. In: The Flavonoids (Harborne J. B., ed.). Chapman & Hall, London, pp.1–20.

Harman D. (1992), Free radical theory of aging. Mutat. Res. **275**, 257–266.

Hatano T., Takagi M., Ito H. and Yoshida T. (1997), Phenolic constituents of liquorice. VII. A new chalcone with a potent radical scavenging activity and accompanying phenolics from liquorice. Chem. Pharm. Bull. **45**, 1485–1492.

Huang M. T. and Ferraro T. (1992), Phenolic Compounds in Food and Their Effects on Health II (Huang M. T., Ho C. T., and Lee C. Y., eds.). Amer. Chem. Soc., Washington, DC, pp. 8-34.

Kohen R., Yamamoto Y., Cundy K. C. and Ames B. N. (1988), Antioxidant activity of carnosine, homocarnosine, and anserine present in muscle and brain. Proc. Natl. Acad. Sci. USA **85**, 3175–3179.

Malterud K. E., Diep O. H. and Sund R. B. (1996), C-Methylated dihydrohalcones from *Mirica gale* L.: Effect as antioxidants and as scavengers of 1,1-diphenyl-2-picrylhydrazyl. Pharmacol. & Toxicol. **78**, 111–116.

Osawa T., Namiki M. and Kawakishi S. (1990), Role of dietary antioxidants protection against oxidative damage. In:Animutagenesis and Anticarcinogenesis Mechanism II (Kuroda Y., Shankel D. M. and Waters D., eds.). Plenum Press, New York, pp. 139–153.

Osawa T. and Shibamoto T. (1992), Analysis of free malonaldehyde formed in lipid peroxidation systems *via* a pyrimidine derivative. JAOCS **69**, 466–468.

Oszmiański J. and Sapis J. C. (1988), Anthocyanins in fruits of *Aronia melanocarpa* (chokeberry). J. Food Science **53 (4),** 1241–1242.

Oszmiański J., Souquet J. M. and Moutounet M. (1995), Honeysucle fruit anthocyanins. Zesz. Nauk. AR Wrocław, Food Technol. VIII, **273**, 67–72.

Pryor W. A. (1986), Cancer and free radicals. In: Antimutagenesis and Anicarciogenesis Mechanism (Shankel D. M., Hartman P. E., Kada T. and Hollaender A., eds.). Plenum Press, New York, pp. 45–59.

Rice-Evans C. A., Miller N. J., and Papanga G. (1997), Antioxidant properties of phenolic compounds. Trends Plant Science **2,** 152–159.

Saquet-Barel H., Comte F., Kiepferie H. and Crouzet J. (1982), Purification et concentration des anthocyanes par adsorption sur polymeres poreux. Lebensmwiss. Technol. **15**, 199- 202.

Singleton W. S., Gray M. S., Brown L. M. and White J. L. (1965), Chromatographically homogeneous lecithin from egg phospholipid. J. Am. Oil Chem. Soc. **42**, 53–56

Stadtman E. R. (1992), Protein oxidation and aging. Science **257**, 1220–1224.

Tsuda T., Shiga K., Oshima K., Kawashiki S. and Osawa T. (1996), Inhibition of lipid peroxidation and the active oxygen radical scavenging effect of anthocyanin pigments isolated from *Phaseolus vulgaris* L. Biochem. Pharmacol. **52**, 1033–1039.

Uchida S., Edamatsu R., Hiramatsu M., Mori A., Nonaka G., Nishioka I., Niwa M. and Ozari M. (1987), Condensed tannins scavenge active oxygen free radicals. Med. Sci.Res. **15**, 831–832.

Werner D., Maness E., Ballinger W. E. (1989), Fruit anthocyanins in tree sections of *Prunus* (Rosaceae). Hort. Science **24**, 488.