Antioxidant Activity of Flavonoids of Different Polarity, Assayed by Modified ABTS Cation Radical Decolorization and EPR Technique

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Modified ABTS cation radical decolorization assay and EPR technique were applied to screen the antioxidant activity of three flavonoids with different polarity: 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine), 4'-methoxy-5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside) and I 3' II 8 biapigenine (amentoflavone), with nonpolar all-trans β-carotene used as standard carotenoid molecule. The ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid] cation radical decolorization assay was modified as follows: (1) measurements extended up to 8 days after preparation, (2) method adapted for flavonoids with different polarity and β-carotene, (3) concentrations in the 0.01-10 μM range of both trolox and antioxidants in order to use the same experimental conditions for both this technique and EPR measurement.

Key words: 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine), 4'-methoxy-5,7-dihydroxyflavone 6-C-β-glucopyranoside (isocytisoside), I 3' II 8 biapigenine (amentoflavone), all-*trans* β-carotene, ABTS cation radical, TEAC, EPR technique, free radicals, oxidation.

INTRODUCTION

Flavonoids are phenylobenzo-γ-pirone derivatives commonly occurring in plants, so they are frequent components of the human diet. Many of the pharmacological effects of flavonoids are linked to their known biological functions as antioxidants, due to free-radical scavenging and metal chelating. Their interactions with enzymes, adenosine receptors and biomembranes are well documented (Bors et al., 1994; Rice-Evans et al., 1996; Cao et al., 1997). Flavonoids possess several biological activities including anti-inflammatory, antiallergic, antiangiogenic and immunomodulatory properties. Flavonoids help prevent age-related, cardiovascular and neurodegenerative diseases and are even thought to reduce the risk of cancer (Steinmetz and Potter, 1996).

In this work we studied the antioxidative activity of selected flavonoids: I 3' II 8 biapigenine (amentoflavone), 4'-methoxy-5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside) and 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine); we used all-*trans* β-carotene as standard carotenoid molecule. In our earlier investigations (Adamska et al., 2003; Jodynis-Liebert et al., 2005) we found that 4'-methoxy-5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside) protected against hepatotoxicity induced by carbon tetrachloride and APAP in rats, and it also inhibited the growth of microorganisms, especially Gram-positive bacteria (Bylka et al., 2004). Amentoflavone possesses anti-inflammatory (Berghöfer and Hölzl, 1989), antiulcerogenic, antioxidant (Conforti et al., 2002) and antiviral activity (Lin et al., 1999; Ma et al., 2001). The immune system is positively influenced by tricine derivatives (Bucar et al., 1998). Liang et al. (2009) reported synergistic interaction of β-carotene with flavonoid (baicalin) against lipid peroxidation and promoting

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ABTS cation radical scavenging. All-*trans* β-carotene (vitamin A precursor) is also a powerful scavenger of singlet oxygen, with a rate constant exceeding 10^{10} M^{-1} sec^{-1}, near that of diffusion control (Foote et al., 1970).

In this work we assessed the antioxidant activity of selected flavonoids and all-*trans* β-carotene in the same solvent (Dimethyl sulfide, DMF) by modified ABTS cation radical decolorization assay and electron paramagnetic resonance (EPR) technique.

MATERIALS AND METHODS

PLANT MATERIAL

Aerial parts of flowering plants of *Axyris amaranthoides* L. and leaves of *Viburnum lantana* L. were collected from the Poznan University of Medical Sciences garden (Department of Medicinal and Cosmetic Natural Products). Leaves with stems of *Aquilegia vulgaris* L. were collected from the Botanical Garden of A. Mickiewicz University. Voucher specimens are deposited at the Department of Pharmacognosy [Nos. KF 0661999, KF 1082000, KF 1261998].

FLAVONOID SAMPLE PREPARATION

Air-dried and powdered *Axyris amaranthoides* aerial parts (250 g) were extracted three times with 50% MeOH. Combined extracts were evaporated under reduced pressure and treated with hot water. The precipitate formed in the cooling extract was obtained by filtration. The filtrate was successively partitioned with $CHCl_3$ and *n*-BuOH. The butanol extract was fractionated by column chromatography on cellulose and eluted with EtOAc-MeOH-H_2O (100:16:13) to yield 120 fractions. Fractions 21–44 (~20 ml of each) were subjected to rechromatography on silica gel using EtOAc-MeOH (6:4) as eluent to give 56 fractions. Fractions 25–34 on a Sephadex LH-20 column using MeOH-H_2O (9:1) as eluent yielded crystalline 0.03 g flavonoid compound identified by ^{1}H NMR, ^{13}C NMR, ^{1}H-^{1}H COSY, HMBC and HMQC spectroscopy (Markham and Geiger, 1994) as tricine 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside].

Air-dried and powdered leaves of *Viburnum lantana* (1000 g) were extracted exhaustively three times with boiling MeOH and then MeOH-H_2O (1:1). The combined extracts were evaporated in a vacuum, treated with H_2O and filtered. The H_2O solution was successively extracted with $CHCl_3$, Et_2O and EtOAc. The Et_2O extract was subjected to CC on silica gel (MN-Kieselgel 60) and eluted with $CHCl_3$-MeOH 95:5 (fraction of 10 ml volume). Fractions 44-

66 were rechromatographed on a Sephadex LH-20 column with MeOH as eluent to yield crystalline compound (0.2 g). The structure of the isolated compound was determined by ^{1}H NMR and ^{13}C NMR spectroscopy and identified by comparison with published data as amentoflavone (I 3' II 8 biapigenine) Chari et al. (1977).

The 4'-methoxy-5,7-dihydroxyflavone 6-*C*-β-glucopyranoside was isolated from *Aquilegia vulgaris* and identified by ^{1}H NMR and ^{13}C NMR analysis according to Bylka and Matlawska (1997).

SAMPLE PREPARATION

According to the method of Re et al. (1999), ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Fluka Chemika, Switzerland) was dissolved in water (7.0 mM – stock solution). The ABTS cation radical ($ABTS^{\bullet+}$) was a product of the reaction of the ABTS stock solution with 2.45 mM (final concentration) $Na_2S_2O_8$ sodium peroxo-hexaodisulfate (VI) (Sigma Chemical Co, USA) in water. $ABTS^{\bullet+}$ is a blue/green chromophore with absorption maxima at wavelengths 415 nm, 645 nm, 734 nm and 815 nm (Miller and Rice-Evans, 1996). In this study the 734 nm maximum for detection was used. The molar extinction coefficient (ε) of $ABTS^{\bullet+}$ in water at 734 nm is (1.50 ± 0.05) 10^4 M^{-1} cm^{-1}.

After 20 h of refrigerated storage at 4°C in darkness the $ABTS^{\bullet+}$ solution was diluted with water to get absorbance (0.70 ± 0.01) at 734 nm at room temperature. Next, five different concentrations (10 μM, 1 μM, 0.5 μM, 0.1 μM, 0.01 μM) of trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich, U.S.A.) and the analyzed antioxidants in DMF (dimethylformamide) (POCH, Poland) were prepared. Trolox, a water-soluble vitamin E analogue, has been used as an electron- or H-donating agent which is able to scavenge cation radical ($ABTS^{\bullet+}$) 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) to ABTS.

To prepare samples for radical scavenging ability (TEAC) measurements, to 3 ml solution of $ABTS^{\bullet+}$ in water (0.70 absorbance at 734 nm) was added (1) 30 μl of only DMF, (2) 30 μl trolox in DMF or (3) 30 μl the investigated antioxidants (flavonoids or all-*trans* β-carotene) in DMF. The absorbance changes of $ABTS^{\bullet+}$ in samples with antioxidants were measured at 734 nm. Absorbance measurements in the range of 200–1000 nm were taken at room temperature 1 min after sample preparation (as reference sample, DMF in water; 10^{-2} by volume). Next, spectral measurements for extended periods were made: 1 min after sample preparation on the first day, 48 h afterwards on the third day, 120 h afterwards on the sixth day, and 168 h afterwards on the eighth day. Values are averages of two measurements.
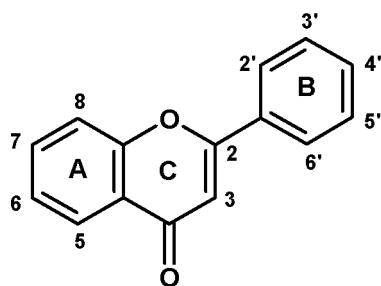
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Fig. 1. Basic structure of flavonoid molecule.

RADICAL SCAVENGING CAPACITY (TEAC) DETERMINATION FOR FLAVONOIDS AND ALL-*TRANS* β-CAROTENE

Trolox equivalent antioxidant capacity (TEAC) was taken as the measure of the radical scavenging ability of flavonoids in comparison to all-*trans* β-carotene as antioxidant standard. Antioxidant activity was expressed as TEAC value (mM concentration of trolox with the same antioxidant activity as a 1 mM concentration of the tested substance) (Lien et al., 1999). The TEAC value of flavonoids and all-*trans* β-carotene was calculated according to the formula:

$$TEAC = (A_{max}-A) / (A_{max}-A_{trolox})$$

where A_{max} is the absorbance of the mixture of 3 ml ABTS$^{\bullet+}$ with 30 µl DMF; A is the absorbance of the mixture of 3 ml ABTS$^{\bullet+}$ with 30 µl flavonoids or all-*trans* β-carotene in DMF; and A_{trolox} is the absorbance of the mixture of 3 ml ABTS$^{\bullet+}$ with 30 µl trolox in DMF.

SPECTRAL MEASUREMENTS

The absorption spectra were measured with a UV/VIS Lambda 20 (Perkin Elmer) spectrophotometer in the 1 cm cell at room temperature. The ^{1}H and ^{13}C NMR spectra of flavonoids were measured with a Varian Unity 300 to check the sample quality. The EPR spectra were measured with a Radiopan SEX-2540 spectrometer. The EPR 335 mT magnetic field parameters were sweep width 10 mT and sweep time 5 min; and the amplitude parameters were modulation frequency 100 kHz, modulation width 0.02 mT, and amplification 3·10^{-3} The parameters for microwave status were frequency 9.38544 ± 0.00001 GHz, power 7.5 mW, phase 72°, digital marker 1 mT and room temperature.

RESULTS AND DISCUSSION

The antioxidant activity of different flavonoids depends on the number and location of hydroxyl groups of the flavonoid ring system (Lien et al., 1999). According to Cos et al. (1998) the o-dihydroxy structure in the B ring, the 2,3-double bond in conjugation with the 4-oxo function in the C ring, and the 3- and 5-OH groups with the 4-oxo function in the A and C rings are very important for effective free radical scavenging activity (Fig. 1). Cao et al. (1997) reported that the relationship between peroxyl radical absorbing activity and the number of hydroxyl groups in flavonoids is a linear function. Another possible contributory mechanism to the antioxidant activity of flavonoids is their ability to stabilize membranes by decreasing their fluidity and partitioning flavonoids into the hydrophobic core of the membrane (Arora et al., 2000; Harborne and Williams, 2000). To assess whether the polarity of flavonoids is an important factor in their antioxidant capacity, we tested three flavonoids having different polarity. From lowest to highest polarity they are I 3' II 8 biapigenine (amentoflavone), 4'-methoxy 5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside) and 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine) (Fig. 2 a–c). The tricine was extracted from *Axyris amaranthoides* L. (Chenopodiaceae), isocytisoside from *Aquilegia vulgaris* L. (Ranunculaceae) (33) and amentoflavone from *Viburnum lantana* L. (Caprifoliaceae).

Our results comprise total antioxidant capacity data for the selected flavonoids in comparison with nonpolar all-*trans* β-carotene as standard carotenoid molecule.

MONITORING OF ANTIOXIDANT ACTIVITY OF SELECTED FLAVONOIDS AND ALL-*TRANS* β-CAROTENE BY MODIFIED ABTS RADICAL CATION DECOLORIZATION ASSAY

Figure 3 shows the decreasing of absorbance (at 734 nm) of the cation radical ABTS$^{\bullet+}$ during testing time. The kinetic curve of ABTS$^{\bullet+}$ bleaching was similar to that of ABTS$^{\bullet+}$ with 0.01 and 0.1 µM trolox, so we used trolox concentrations higher than 0.5 µM (1 µM and 10 µM trolox) for TEAC calculation. Figure 4 a–d presents the antioxidant activity curves of the flavonoids and all-*trans* β-carotene for concentrations of 0.5 µM (s), 1 µM (r) and 10 µM(○).

The all-*trans* β-carotene scavenging time of the cation radical ABTS$^{\bullet+}$ is much longer than that of flavonoids; the slope of the all-*trans* β-carotene curve is smaller than for flavonoids. The slope is highest for the flavonoid of the highest polarity, tricine, so the cation radical ABTS$^{\bullet+}$ scavenging time is the shortest among the investigated samples.

The concentration range is an important factor for antioxidant action in the case of flavonoids, but not so much for all-*trans* β-carotene. The TEAC values of the flavonoids and all-*trans* β-carotene are summarized in Table 1.
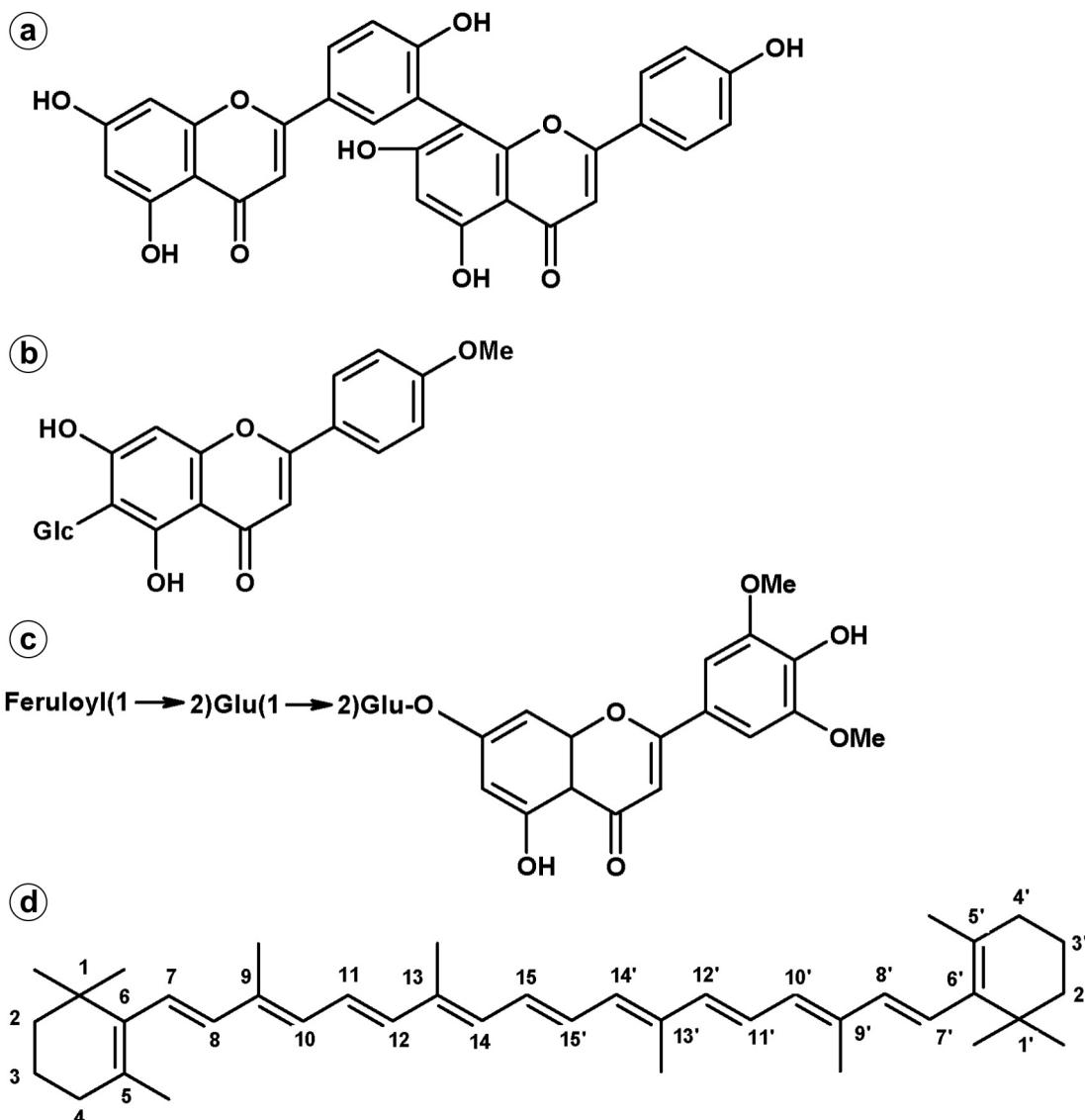
Fig. 2. Molecular structure of the applied flavonoids: **(a)** I 3' II 8 biapigenine (amentoflavone), **(b)** 4'-methoxy 5,7-dihydroxyflavone 6-*C*-*β*-glucopyranoside (isocytisoside), **(c)** 7-*O*-*β*-[2-*O*-feruloyl-*β*-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine), **(d)** all-*trans* β-carotene.

Flavonoid TEAC values range from 0.79 to 2.10 and depend on the number and position of OH and other substituents of the molecule (Lien et al., 1999). Re et al. (1999) gave flavonoid TEAC values of 1.29 at 1 min and 2.06 for 6 min after sample preparation; the value for all-*trans* β-carotene is 2.47, and the reaction between carotenoids and ABTS$^{\bullet+}$ is essentially completed after 1 min although a further inefficient reaction takes place thereafter.

In our study (Tab. 1) the TEAC value of all-*trans* β-carotene was 0.12 for 1 min and 0.33 at 8 days after sample preparation. For the flavones the TEAC values 1 min after sample preparation were 0.68 for amentoflavone, 0.32 for isocytisoside and 0.73 for tricine, but after 8 days the TEAC value was 1 for all flavonoid samples.

Our data and literature data (Re et al., 1999) indicate that the solvent, the antioxidant concentration and the test time are very important factors in TEAC measurements. In our method the same solvent (DMF) was used for the different antioxidants: (flavonoids) and nonpolar all-*trans* β-carotene. The use of DMF in our study eliminated the possible effects of differences in polarity between solvents.

TABLE 1. Antioxidant activity (TEAC) of flavonoids and all-*trans* β-carotene at test times

| Day | Trolox equivalent antioxidant capacity (TEAC) [mM trolox] ± 0.02* | | | |
	I 3' II 8 biapigenine (amentoflavone)	4'-methoxy 5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside)	7-*O*-*β*-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine)	all-*trans* β-carotene
1[a]	0.68	0.32	0.73	0.12
3[b]	0.91	0.81	1.00	0.16
6[c]	1.00	0.90	1.00	0.27
8[d]	1.00	1.00	1.00	0.33

[a] 1 min after sample preparation; [b] 48 h after sample preparation; [c] 120 h after sample preparation; [d] 168 h after sample preparation
* maximal error (differential calculus)

TABLE 2. Amplitude of EPR signals of ABTS$^{\bullet+}$ with admixture of analyzed compounds

3 ml ABTS$^{\square+}$ + 30 µl : one of the following compounds	Amplitude (A) of EPR signal (a. u.) ΔA = ± 0.1*
DMF (control)	7.8
trolox	6.4
I 3' II 8 biapigenine (amentoflavone)	7.3
4'-methoxy-5,7-dihydroxyflavone 6-*C*-*β*-glucopyranoside (isocytisoside)	7.8
7-*O*-*β*-[2-*O*-feruloyl-*β*-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine)	6.3
all-*trans* β-carotene	6.4

* equipment error; ** measurement immediately after sample preparation; *** line width ΔB = (1.0 ± 0.1) mT, g = 2.0051 ± 0.0001
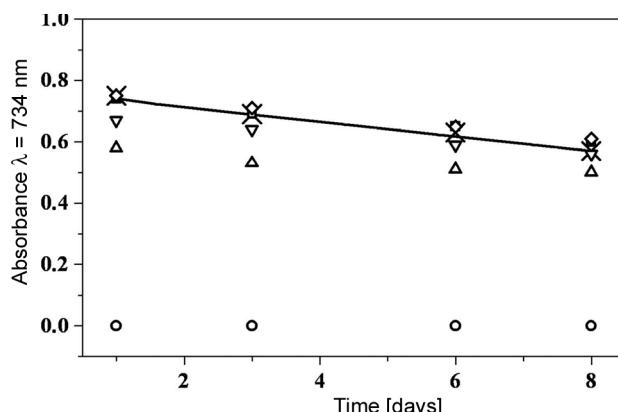

Fig. 3. Time effect on the suppression of the absorbance (at 734 nm) of the ABTS$^{\bullet+}$. Control ABTS$^{\bullet+}$ (✕). Trolox: 10 µM (○), 1 µM (△), 0.5 µM (▽), 0.1 µM (☐) [overlapped with (◇) and (✕)] and 0.01 µM (◇).

The relation between free radical scavenging and the concentrations of flavonoids and trolox was linear (Fig. 5). In the case of all-*trans* β-carotene, however, even at its highest concentration (10 µM) there was no such linear relationship (Fig. 6); the absorbance changes at 734 nm were not linear with sample concentration.

A high all-*trans* β-carotene concentration generates crystals; the so-called Shibata peak is at 540 nm (Shibata, 1956) and it aggregates at ~500 nm, modifying the physical properties of the membrane (Gruszecki, 1999). Aggregated forms of carotene are less effective antioxidants than monomeric forms (Cantrell et al., 2003). Such peaks were not observed in our electronic absorption steady-state study, but recent time-resolved (fs) stimulated emission data from Bialek-Bylka et al. (2008) indicate the presence of aggregated forms of β-carotene, so our conclusion that all-*trans* β-carotene is a less efficient antioxidant than the investigated flavonoids is open to question.

MONITORING OF ANTIOXIDANT ACTIVITY
OF SELECTED FLAVONOIDS AND ALL-*TRANS*
β-CAROTENE BY EPR TECHNIQUE

According to Subramanian et al. (2003), the antioxidant activity of compounds can be evaluated by monitoring free radical generation in the presence of antioxidants using the EPR technique. The free radical concentration is proportional to the second integral of the EPR spectrum. In our case, the addition of the antioxidants tricine, isocytisoside, amento-flavone and all-*trans* β-carotene to the
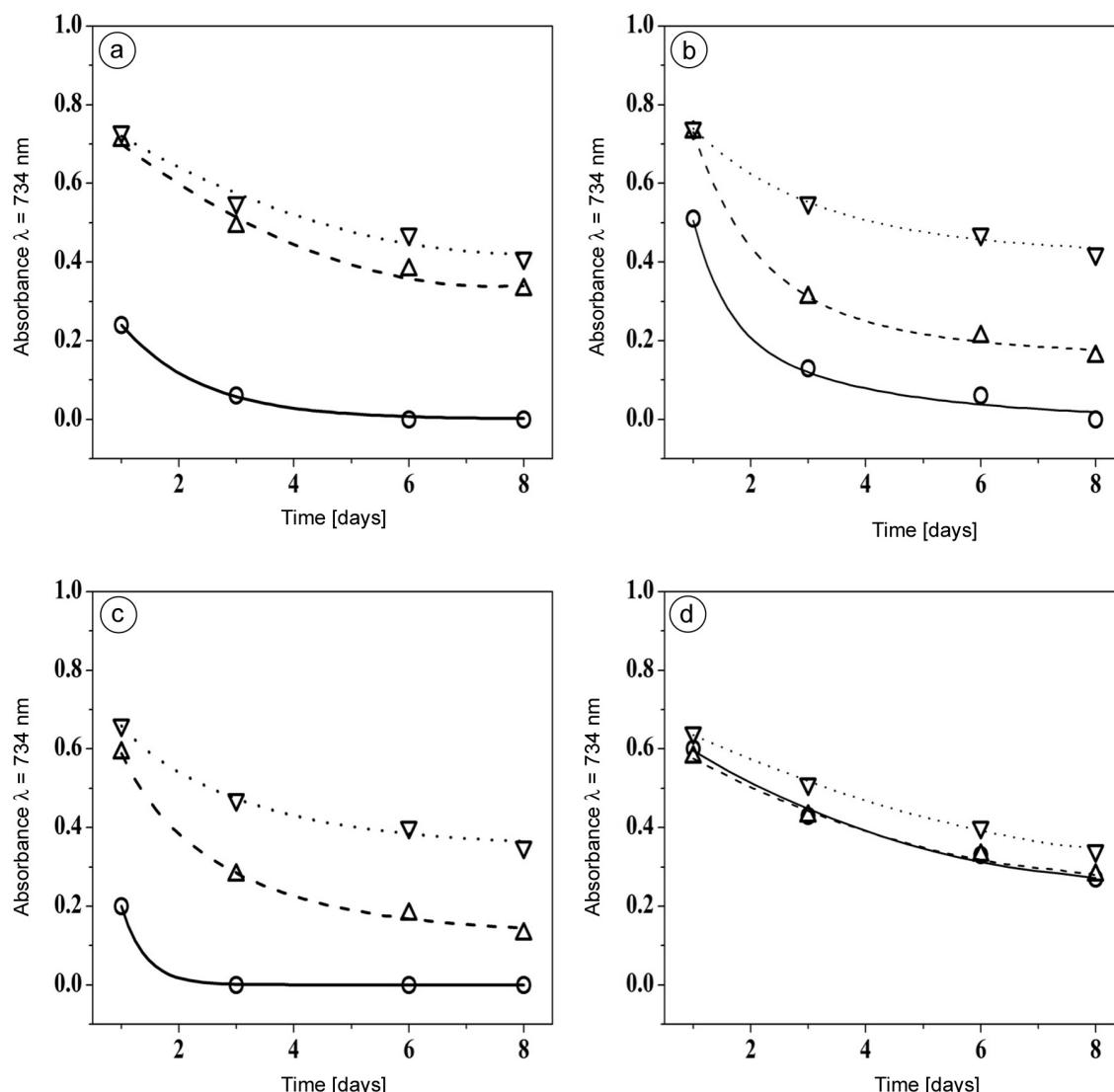
Fig. 4. Time effect on suppression of ABTS$^{\bullet+}$ absorbance at 734 nm (averages of 2 measurements). **(a)** I 3' II 8 biapigenine (amentoflavone), **(b)** 4'-methoxy 5,7-dihydroxyflavone 6-C-β-glucopyranoside (isocytisoside), **(c)** 7-O-β-[2-O-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine), **(d)** all-*trans* β-carotene; concentrations 10 μM (–O–), 1 μM (--$\triangle$--) and 0.5 μM ($\cdots\triangledown\cdots$).

ABTS$^{\bullet+}$ mixture did not change the EPR spectra in terms of line width and line shape (line width ΔB = 1.0 ± 0.1 mT and g = 2.0051 ± 0.0001 for samples with and without antioxidants). In all the samples investigated with EPR only the signal of the ABTS cation radical is detected. The free radical concentrations (proportional to the EPR amplitude signals) of ABTS$^{\bullet+}$ are different because of the scavenge effect of antioxidants present in the samples. The EPR data are given in Table 2.

We applied a modified ABTS cation radical decolorization assay method to screen the antioxidant activity of selected flavonoids of different polarity and also nonpolar all-*trans* β-carotene as a standard representative of carotenoid molecules, in the same solvent (DMF). Antioxidant capacity was highest for the flavonoid with the highest polarity, tricine. At the 10 μM flavonoid concentration, total bleaching of ABTS$^{\bullet+}$ occurred after 48 h. This flavonoid possesses an -OH group at position 4' in the B ring, which is very important for high antioxidant capacity, and it also has an -OMe group at positions 3' and 5' in the B ring, responsible for increasing antioxidant capacity (Dugas et al., 2000).

Similarly to tricine, amentoflavone has an -OH group at position 4' in the B ring but it has no -OMe group; that is why its antioxidant capacity is lower
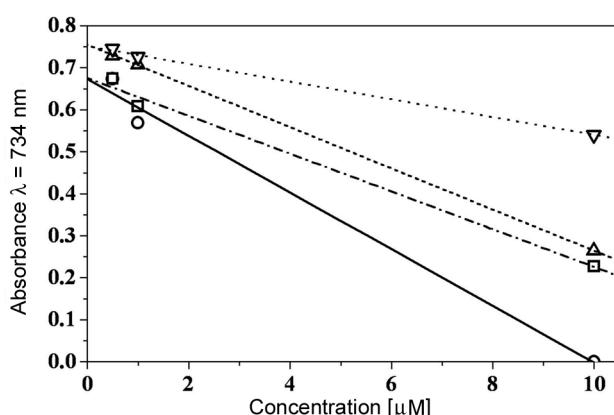
Fig. 5. Effect of flavonoid and trolox concentration on suppression of ABTS$^{\bullet+}$ absorbance at 734 nm. Trolox (O), I 3' II 8 biapigenine (amentoflavone) (△), 4'-methoxy 5,7-dihydroxyflavone 6-*C*-β-glucopyranoside (isocytisoside) (▽), 7-*O*-β-[2-*O*-feruloyl-β-glucuronopyranosyl (1→2) glucuronopyranoside] (tricine) (▢).
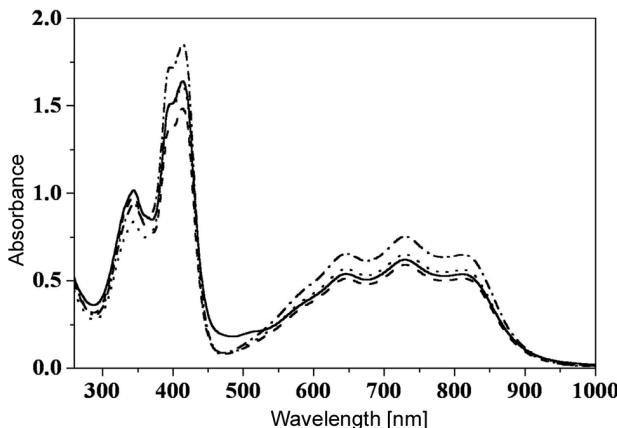

Fig. 6. Effect of 10 μM (—), 1 μM (– –) and : and 0.5 μM (⋯) β-carotene concentrations on suppression of the ABTS$^{\bullet+}$ control signal (–·–).

than that of tricine derivatives. For antioxidant capacity, the angle between the B ring and the plane of the A and C rings can also play a role (Harborne and Williams, 2000). In amentoflavone one of the B rings is less mobile than the other.

For efficient free radical scavenging the flavonoid concentration is an important factor: 10 μM (the highest investigated concentration) had the most pronounced effect.

In the experimental conditions of both the spectroscopic methods we used, the selected flavonoids showed better antioxidant activity and scavenged free radicals faster than all-*trans* β-carotene.

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