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Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities

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Abstract

Methanol extracts prepared from five plant materials native to the Mediterranean area, namely olive tree (*Olea europaea*) leaf, St. John's wort (*Hypericum perforatum*), hawthorn (*Crataegus laevigata*), oregano (*Origanum vulgare*) and laurel leaf (*Lauris nobilis*), were examined for their phenolic components. Total phenolic content was determined by the Folin–Ciocalteu method. The content of proanthocyanidins in acid-hydrolysed extracts was determined spectrophotometrically. The contents of free flavones (apigenin and luteolin) and flavonols (kaempferol, myricetin and quercetin) were determined by HPLC analysis. The time of hydrolysis of flavones, flavonols and proanthocyanidins was optimised.

Antioxidant activities of apigenin, luteolin, kaempferol, myricetin, quercetin and of plant extracts were examined. Antioxidative activities were studied in sunflower oil at 98 °C, by measuring peroxide value, and in an aqueous emulsion system of β-carotene and linoleic acid by measuring the absorbance of the sample. Among flavones and flavonols investigated, only myricetin inhibited oxidation of sunflower oil. All other flavones and flavonols showed pro-oxidative activity. Oppositely, in the emulsion system, only apigenin showed pro-oxidative activity while other flavones and flavonols and plant extracts inhibited oxidation of β-carotene.

Keywords: Flavones; Flavonols; Proanthocyanidins; Total phenols; Contents in plant extracts; Antioxidant activity; Oil; Emulsion

1. Introduction

The antioxidant activity of herbs and species, caused mainly by phenolic compounds, has been demonstrated in many studies over recent years. Simple phenols (C_6) seldom occur naturally, so plant phenolics are divided into the following main groups (Bruneton, 1999):

- phenolic acids that are hydroxylated derivatives of benzoic acid (C_6–C_1) and are quite common in the free state as well as combined as esters or glycosides (gallic acid);
- phenolic acids derived from cinnamic acid (C_6–C_3) (coumaric, caffeic, ferulic acid) are widely distributed and occur rarely in the free state and are very often esterified, and
- glycosidic phenylpropanoid esters.

Flavonoids are a group of natural benzo-γ-pyran derivatives and are ubiquitous in photosynthesising cells (Havsteen, 1983). They occur as aglycones, glycosides and methylated derivatives. Depending on the degree of oxidation of the central pyran ring, they can be subdivided into several classes of flavonoids and flavonoid-related compounds: flavones, flavonols, flavanones, isoflavones, flavans, flavanols, and anthocyanins. The pyran ring can be opened (chalcones) and recyclized into a furan ring (aurones) (Bruneton, 1999). The most widespread polyphenolic bioflavonoids (condensed tannins), named proanthocyanidins, are

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polyepicathechins, polyepigallocathechins and polyepiafzelechin (Bruneton, 1999).

For centuries, preparations containing flavonoids as the principal physiologically active constituents have been used to treat human diseases (Havsteen, 1983). There is an increasing interest in the biological effects of these compounds. The intake of plant flavonoids has been shown to be inversely related to the risk of cardiovascular disease. Several flavonoids have been reported to quench active oxygen species and inhibit in vitro oxidation of low-density lipoproteins and therefore reduce thrombotic tendency. Flavonoids may inhibit cycloxygenase and thereby modulate metabolism of arachidonic acid and attenuate inflammation. Many plant flavonoids have also been shown to be anti-carcinogenic in several animal models (Karakaya & Sedef Nehir, 1999; Merken & Beecher, 2000; Miean & Mohamed, 2001). In addition, certain plant flavonoids may interact with carcinogens in the gastrointestinal tract and thereby reduce their adsorption. Natural flavonoids may offer an alternative to protect lipids from oxidation in foods. Some of these flavonoids have been shown to inhibit lipid oxidation in meats, fish oil and lard (Chen, Chan, Ho, Fung, & Wang, 1999).

The antioxidative (pro-oxidative) effect of natural antioxidants on lipid molecules has been extensively studied in recent years and is influenced by many factors, such as:

- The system (composition of the oil/emulsion (Frankel, Huang, & Aeschbach, 1997; Hopia, Huang, Schwarz, German, & Frankel, 1996; Huang & Frankel, 1997; Huang, Frankel, Schwarz, Aeschbach, & German, 1996), interactions (Medina, Tombo, Satué-Gracia, German, & Frankel, 2002; Meyer, Heinonen, & Frankel, 1998), temperature (Sature, Huang, & Frankel, 1995), pH (Huang, Frankel, Schwarz, & German, 1996), concentration).
- The hydrophobicity/hydrophilicity (Frankel, Huang, Aeschbach, & Prior, 1996; Schwarz et al., 2000).
- The total number and location of hydroxyl groups on aromatic rings (Chen et al., 1999; Das & Pereira et al., 1990; Pekkarinen, Heinonen, & Hopia, 1999; Burda & Oleszek et al., 2001).

In the present work, the contents of phenolic compounds of five plant materials, native to Mediterranean area – olive tree (*Olea europaea*), St. John's wort (*Hypericum perforatum*), hawthorn (*Crataegus laevigata*), oregano (*Origanum vulgare*) and laurel leaf (*Lauris nobilis*) were determined.

Chemical composition of the olive tree leaf is characterized by the presence of several secoiridoids (the main constituent – oleuropein) and unconjugated secoiridoid-type aldehydes (oleacin) (Bruneton, 1999). Triterpenes and flavonoids, namely rutin and the glycosides of apigenin and luteolin, are also found (Bruneton, 1999).

St. John's wort contains essential oil, triterpenes and sterols. It is rich in phenolics: caffeic acids, chlorogenic acid, proanthocyanidins (dimers and oligomers of catechin and epicatechin), prenylated derivatives of phloroglucinol and flavonoids: hyperin, rutin, quercitrin, isoquercitrin, bis-apigenins. A trace of xanthones in the flowering stems is also found (Bruneton, 1999).

The Hawthorn contains pentacyclic triterpenoid acids, aromatic amines, a trace of essential oil, phenolic acids, 1–2% flavonoids (hyperin, 3-galactoside of quercetin, spirein, rutin, glycosides of flavone and apigenin) and 2–3% proanthocyanidins (Bruneton, 1999).

Few literature references are available for phenolic compounds of oregano (Deighton, Glidewell, Deans, & Goodman, 1993; Kikuzaki & Nakatani et al., 1989) wherein the following compounds are reported: protocatechuic acid, caffeic acid, rosmarinic acid, phenyl glycoside and 2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxyphenyl]propionic acid, carvacrol and thymol. Pizzale, Bortolomeazzi, Vichi, Überegger, and Conte (2002) identified three compounds (caffeic acid, rosmarinic acid and carvacrol), which amounted, on average, to 55% of the total phenolic compound contents of oregano samples. The antioxidant activity of extracts of oregano was tested using different methods (Deighton et al., 1993; Kikuzaki et al., 1989; Lindberg Madsen, Nielsen, Bertelsen, & Skibsted, 1996; Pizzale et al., 2002) and it is reported, that the spice extracts have antioxidant activity, which is in good agreement with the total phenolic contents (Lindberg Madsen et al., 1996; Pizzale et al., 2002).

The bay laurel plant is an evergreen tree native to the Mediterranean region and has found its way into all the cuisines of the area. By steam distillation, bay leaves yield about 10–30 ml/kg of an essential oil in which cineole is always the major compound. In addition, the leaf contains sesquiterpenoid lactones and isoquinoline alkaloids (Bruneton, 1999).

In the study, the contents of total phenols, proanthocyanidins, flavones (apigenin and luteolin) and flavonols (kaempferol, myricetin, quercetin) were determined. Chemical structures of flavones and flavonols studied are presented on Fig. 1. Further, the antioxidant effects of pure quercetin, myricetin, kaempferol, apigenin and luteolin, as well as plant extracts were studied. Two methods were used. Sunflower oil oxidation was examined and primary oxidation products – hydroperoxides were determined by peroxide value measurements. In addition, the antioxidant activity was elucidated by heat-induced oxidation in the β-carotene and linoleic acid system. The aim of the study was to elucidate the relationship between the concentrations of the phenolic components in isolated plant extracts and antioxidative activities of these extracts.

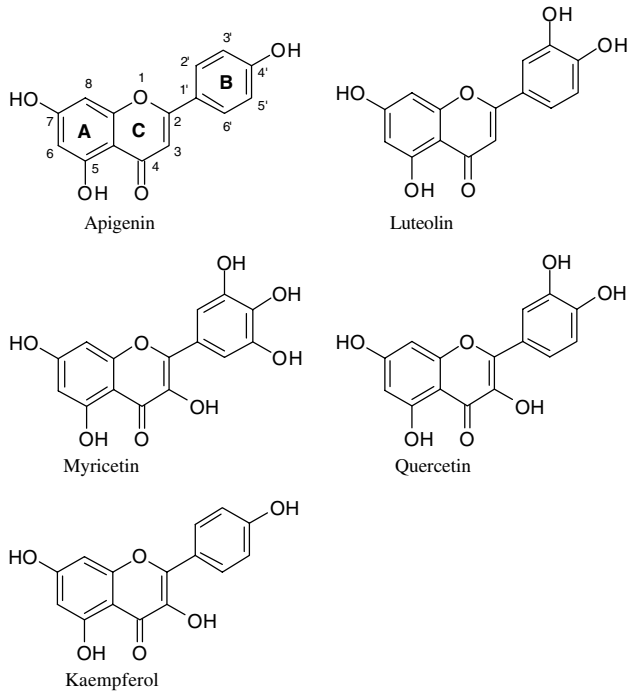

Fig. 1. Chemical structure of flavones (apigenin, luteolin) and flavonols (myricetin, quercetin, kaempferol).

2. Materials and methods

2.1. Materials

The plant materials: leaves of laurel (*Laurus nobilis* L., bay), oregano (*Origanum vulgare* L.), leaves of olive tree (*Olea europaea* L.), hypericum (*Hypericum perforatum* L., St. John's wort) and hawthorn (*Crataegus laevigata* L.) were purchased from Droga (Portorož, Slovenia). All chemicals used for analysis were purchased from Merck (Darmstadt, Germany).

The HPLC-grade flavonoid standard apigenin (Cat. No. 46,074-5) was obtained from Aldrich (Germany) and quercetin (Cat. No. 174070250) from Acros (Belgium). Further, myricetin (Cat. No. 70050), luteolin (Cat. No. 62696) and kaempferol (Cat. No. 60010) were obtained from Fluka (Germany). The standard solutions were prepared by dissolving standards in methanol.

The sunflower oil, without any added antioxidants was purchased from Gea (Slovenska Bistrica, Slovenia).

2.2. Preparation of the extracts

1 g of ground plant material was extracted with 100 ml of pure methanol for 2 h using an ultrasonic bath. The temperature of extraction was 40 °C at atmospheric pressure. The solution was cooled and filtered using a 0.45-μm filter. The solvent was evaporated under vacuum using a rotavapor and the yield of extraction was determined in wt%. The extracts were stored in a dry and cool place.

2.3. Analysis of flavones and flavonols in extract

50 mg of the extract was dissolved in 20 ml of distilled water. 16 ml of this water solution was hydrolysed in a solution of 5 ml 6M HCl and 24 ml pure ethanol. After refluxing at 90 °C for a specific time, the extract was cooled and made up to 50 ml, sonicated and analysed (Wang & Helliwell, 2001). In order to follow the course of hydrolysis and to optimise time, the hydrolysis of particular extract (divided into several portions) was performed by parallel experiments at different refluxing times up to the maximum of 5 h.

The contents of flavones (apigenin, luteolin) and flavonols (quercetin, myricetin, and kaempferol) in hydrolysed extracts were determined by high performance liquid chromatography (HPLC). The HPLC system consisted of a Varian 9012 pump and Varian diode array detector 9065 (Walnutcrek, California). As a stationary phase the Waters symmetry column C-18 250×4.6 mm with 5 μm particle size was used. The mobile phase consisted of two solvents, A: 2% phosphate buffer with pH 3, and B: methanol. The method was isocratic for 5 minutes with 5% B, then changed with a linear gradient from 5% to 100% B over 30 min and for the last 5 min, it remained at 100% B. The flow rate was 0.7 ml/min and the detection was performed at 367 nm. The quantification was made with an external standard.

2.4. Analysis of proanthocyanidins in extracts

The proanthocyanidins were determined by UV spectrophotometry method (Varian-UV–VIS Spectrophotometer) based on acid hydrolysis and colour formation (Porter, Hrstich, & Chan, 1986). 50 mg of the extract was dissolved in 25 ml distilled water. To 2 ml of this solution, 20 ml of Fe sulphate solution (77 mg of $FeSO_4 \cdot 7H_2O$ in 500 ml of HCl: *n*-buthanol = 2:3) was added. For a control sample, 2 ml of distilled water was used. The sample was incubated for a specific time at 95 °C. In order to follow the course of hydrolysis and to optimise time, the hydrolysis of a particular extract (divided into several portions) was done by parallel experiments at different incubating times up to the maximum of 15 min. After incubation, the sample was cooled and analysed by measuring absorbance at 540 nm. The result was presented as the percentage of proanthocyanidins in the sample.

2.5. Analysis of total phenols in extracts

The concentration of total phenols in extracts was measured by UV spectrophotometry, based on a colorimetric oxidation/reduction reaction. The oxidizing agent used was Folin–Ciocalteu reagent (AOCS, 1990).

To 0.5 ml of diluted extract, 2.5 ml of Folin–Ciocalteu reagent (Merck, diluted 10 times with water) was

added and, after that (within time interval from 0.5 to 8 min), 2 ml of Na_2CO_3 (75 g/l) was added. The sample was incubated for 5 min at 50 °C and then cooled. For a control sample, 0.5 ml of distilled water was used. The absorbance was measured at 760 nm. The results were expressed in gram of gallic acid per kilogram of extract (g GA/kg extract).

2.6. Antioxidant activity determination

In order to determine the antioxidative activity of pure flavones and flavonols and that of plant extracts two methods were applied: measuring the peroxide value of sunflower oil, and measuring the oxidation of an aqueous emulsion system of β-carotene and linoleic acid.

2.6.1. Determination of peroxide value

Peroxide values (*PV*) of sunflower oil were measured to determine the antioxidative activity of pure flavones and flavonols. Substances were dissolved in oil and the solution was stored at 98 °C. Peroxide values were determined periodically, using AOCS (1990).

About 1 g sample was weighed into 250 ml glass Erlenmeyer each hour. 30 ml of a mixture of acetic acid and chloroform was added and swirled by hand to dissolve. 0.5 ml of saturated KJ solution was added and it was shaken for 1 min. Finally, 30 ml of water was added. The liberated iodine was titrated with the 0.01 N sodium thiosulphate solution with vigorous shaking until the yellow colour almost disappeared. 1 ml of 1% starch solution was added and the solution was titrated until the blue colour disappeared. The peroxide value (*PV*), expressed in mmol of active oxygen per kg of sample, is given by the formula:

$$PV = \frac{VT}{m}1000, \tag{1}$$

where V is the volume, in ml, of the standardized sodium thiosulphate solution, used for the test, corrected to take into account the blank test, T is the exact normality of the sodium thiosulphate solution used, and m is the mass, in g, of the test sample.

PV for each sample was analysed three times and the standard deviation value was 0.01.

Antioxidant activity of pure flavones and flavonols in the sunflower oil was calculated as percent variation of *PV* of the sample (s) versus that of control sample (c):

$$\%\ \text{antioxidant activity}_{\text{sunflower oil}} = 100 \times \left[1 - \frac{PV_s}{PV_c}\right]. \tag{2}$$

2.6.2. Determination of the oxidation of an aqueous emulsion system of β-carotene and linoleic acid

The antioxidant activity was elucidated on heat-induced oxidation of aqueous emulsion system of β-carotene and linoleic acid (Burda et al., 2001). 1 ml of β-carotene chloroformic solution (0.2 mg/ml) was added to an Erlenmeyer flask containing linoleic acid (0.02 ml), Tween 20 (0.2 ml) and the corresponding flavonoid in methanol solution at a concentration of 10^{-3}M. 50 ml of distilled water was saturated with oxygen for 15 min and added to the flask. The mixture was shaken and stored for 2 h at 50 °C. The absorbance of the samples was measured on the Varian UV–VIS spectrophotometer at 470 nm at the beginning ($t = 0$ min) and after the experiment ($t = 120$ min). Antioxidant activity of the sample (s) was calculated as percent inhibition of oxidation versus control sample (c), using the equation

$$\%\ \text{antioxidant activity}_{\text{emulsion system}} = 100 \times \left[1 - \frac{(A_s^0 - A_s^{120})}{(A_c^0 - A_c^{120})}\right], \tag{3}$$

where A_s is the absorbance of sample at $t = 0$ min and $t = 120$ min, and A_c is the absorbance of control sample at $t = 0$ min and $t = 120$ min.

3. Results and discussion

3.1. Hydrolysis and contents of phenolic compounds in plant extracts

In the literature (Porter et al., 1986) it is reported that 2 h of hydrolysis are not enough to produce all free flavonoid aglycones for quantification. In order to optimise the time of hydrolysis in this study, the contents of apigenin, quercetin, myricetin, luteolin and kaempferol in plant extracts were measured after hydrolysis of parallel samples was performed at various times. In Fig. 2 the contents of flavones and flavonols of hydrolysed Olive tree and Laurel extracts are presented. The results show that, after 2 h of hydrolysis, the concentrations of myricetin, apigenin and quercetin remain constant with further hydrolysis. On the other hand, the content of luteolin still rises and, after 4 h, finally reaches the end value and stays constant with further hydrolysis. Similar conditions are necessary in the case of kaempferol; 4 h of hydrolysis are needed for obtaining free kaempferol (Fig. 2(b)). The results obtained are in agreement with findings of Wang and Helliwell (2001), who investigated the optimal conditions for hydrolysis of flavonols myricetin, quercetin and kaempferol in tea leaves and tea infusion. They reported that, when 6 M HCl is used for hydrolysis, the highest yield, for both myricetin and quercetin, is obtained within 2 h, and for kaempferol within 3 h.

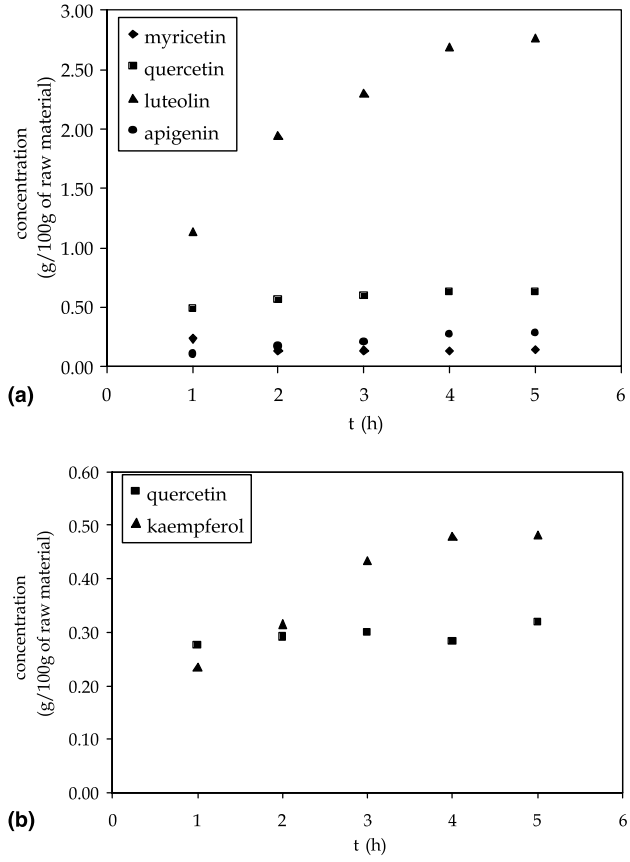

Fig. 2. Optimisation of hydrolysis of flavones and flavonols in methanol extracts from: (a) olive tree; (b) laurel.

Similarly, the optimisation of time for hydrolysis of proanthocyanidins was determined. According to literature data (Porter et al., 1986) the sample has to be incubated for 15 min at 95 °C before analysing. The time of incubation in our experiments was extended up to 5 h. When the incubation of parallel samples was performed

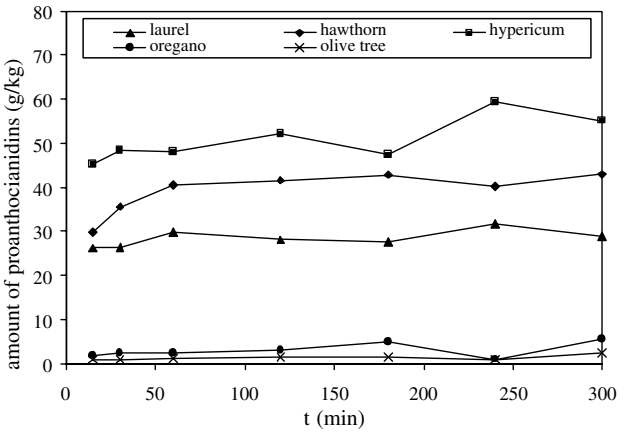

Fig. 3. Optimisation of hydrolysis of proanthocyanidins in methanol plant extracts.

for various times, the contents of proanthocyanidins were measured. It was found that 1 h of incubation is necessary to obtain the maximum concentration of proanthocyanidins. After that time, the concentration of proanthocyanidins stays approximately constant (Fig. 3) for all five plant materials investigated.

Further, the standard analysis of total phenolic contents (AOCS, 1990) takes 5 min of incubation of sample with Folin–Ciocalteu reagent at 50 °C. In the present work, it was verified that the highest amount of GA is determined after 5 min and further incubation causes decrease of GA, probably due to the decomposition of phenols.

The extraction yields of various plant materials with methanol and the composition of extracts are presented in Table 1. The contents of quercetin, myricetin, kaempferol, apigenin and luteolin in plant extracts, determined after optimal hydrolysis of methanol extracts are presented in Table 2.

Table 1
Extraction of plant materials with methanol: yields and composition of extracts

	Extraction yield (%)	Total phenols (g GA/kg)	Proanthocyanidins (g/kg)	Flavonoids[a] (mg/kg)
Laurel	24.6	99.7	29.9	80.1
Oregano	26.2	186	2.53	257
Olive tree	34.7	144	1.22	382
Hypericum	32.9	191	48.2	1479
Hawthorn	28.0	160	40.6	245

[a] Quercetin, luteolin, apigenin, kaempferol and myricetin.

Table 2
Contents of flavones and flavonols in hydrolysed methanol extracts from plants

	Quercetin (mg/kg)	Luteolin (mg/kg)	Apigenin (mg/kg)	Kaempferol (mg/kg)	Myricetin (mg/kg)
Laurel	31.90	–	–	48.22	–
Oregano	219	–	17.0	–	21.0
Olive tree	62.4	277	28.4	–	14.30
Hypericum	1451	–	–	12.72	15.8
Hawthorn	241	–	3.95	–	–

3.2. Antioxidant activity

The antioxidative effect of pure flavones and flavonols in sunflower oil at 98 °C at various concentrations can be seen in Figs. 4(a) and (b). Among flavones and flavonols investigated, only myricetin showed protection for sunflower oil against oxidation while all other flavones and flavonols showed pro-oxidative activity.

The influence of the amount of flavones and flavonols added to the sunflower oil on peroxide value change can also be seen in Fig. 4. Generally, a higher concentration of flavonoid causes a higher oxidative or pro-oxidative effect.

The results show that, in sunflower oil, the antioxidant activity of the five flavones and flavonols investigated depends on the number and location of hydroxyl groups on the aromatic ring. All five tested flavones and flavonols have hydroxyl groups in positions R5, R7 and 4′ and differ in substituents located in positions R3, 3′ and 5′. Among flavones and flavonols investigated, only myricetin showed protection for sunflower oil against oxidation, and this is the only molecule containing six hydroxyl groups. It differs from the quercetin molecule in the substituent at position 5′; the hydroxyl group in this position probably increased the antioxidative effectiveness of myricetin. All other flavones and flavonols showed pro-oxidative activity in sunflower oil in the following order: quercetin < luteolin < kaempferol < apigenin. The molecule of quercetin contains five hydroxyl groups while luteolin and kaempferol have four hydroxyl groups located in different positions. The hydroxyl group in position 3′ (luteolin) causes a lower pro-oxidative effect than the hydroxyl group in position R3 (kaempferol). Apigenin, which showed the highest pro-oxidative effect in sunflower oil, contains only three hydroxyl groups.

The problem observed in performing experiments with sunflower oil was the low solubility of flavones, flavonols, and especially plant extracts in the oil. Further, it is generally believed, that the lipid oxidation begins and is most intense in the membrane or interphases of an emulsion (Chen et al., 1999). Therefore, the antioxidant activities of pure flavones and flavonols and extracts were also tested in emulsion. The results, summarised in Fig. 5, show that only apigenin enhances the oxidation process of β-carotene in the water emulsion system. In contrast, quercetin, myricetin and kaempferol inhibit β-carotene oxidation. The results of antioxidant activity obtained for pure flavones and flavonols are comparable with those presented in the literature (Burda et al., 2001) and indicate that flavonoids with a free hydroxyl group in position R3 have antioxidant potential in the emulsion system. The antioxidant activities of pure flavones and flavonols obtained in water-containing systems differ from that obtained for sunflower oil. The reason is certainly the variation of solubility of individual flavones and flavonols in the oil and their hydrophobicity/hydrophilicity.

Due to the very low solubility of plant extracts observed in sunflower oil, the antioxidative activity of

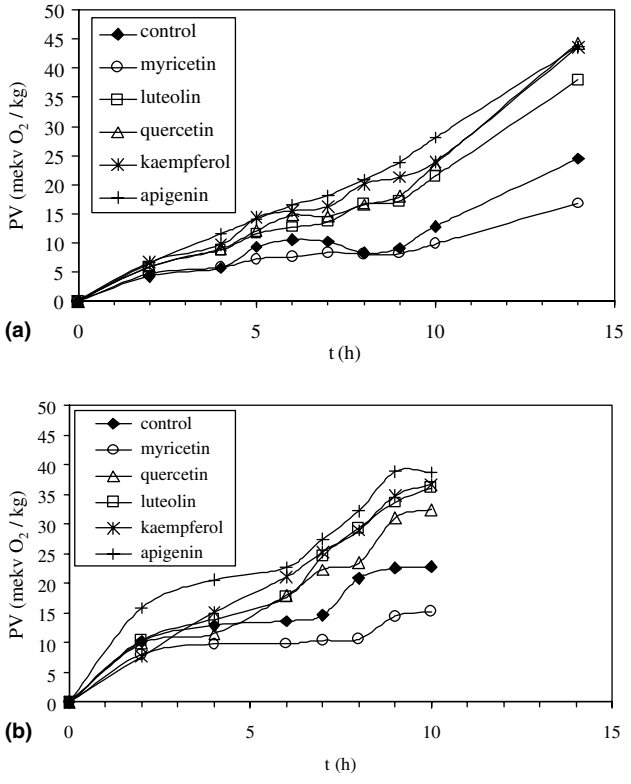

Fig. 4. Antioxidative effect of pure flavones and flavonols in sunflower oil at 98 °C: peroxide value (*PV*) at concentrations: (a) 200 ppm and (b) 500 ppm.

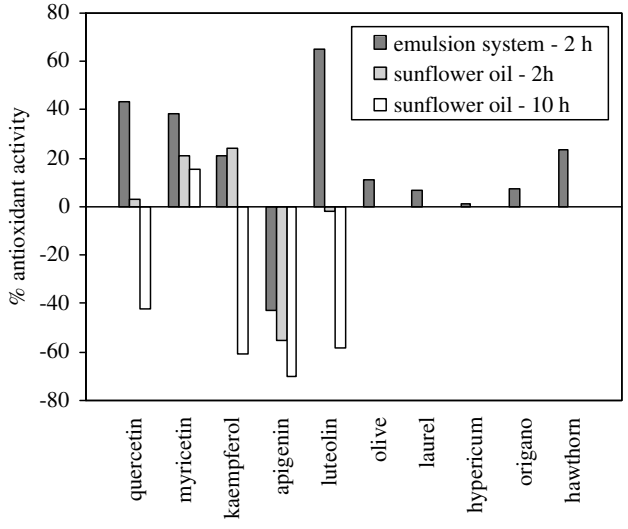

Fig. 5. Antioxidant activity of flavones, flavonols and nonhydrolysed methanol plant extracts measured in emulsion system and sunflower oil.

unhydrolysed plant extracts was tested only in the emulsion system. The results show that all plant extracts examined have antioxidative activity. However, this activity is not simply related to the total phenolic components determined in extracts. In addition to various antioxidative activities of different extract constituents contributing to total extract activity, the following facts should also be considered for the evaluation of results: the phenolics content was determined by hydrolysis of plant extracts and therefore free aglycones were measured. According to the observations made before, namely that the flavonoid aglycones are more potent in their antiperoxidative action than their corresponding glycosides (Das et al., 1990), besides the total phenolics in the extract the ratio of free and esterified phenolics/glycosides should also be known. Further, interactions may occur between the extract components. The synergism of flavonoids with tocopherols, ascorbyl palmitate and citric acid has already been reported (Hudson & Lewis, 1983; Nieto et al., 1993; Pekkarinen et al., 1999; Rižner Hraš, 2000).

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