Polyphenols of *Rosa* L. Leaves Extracts and their Radical Scavenging Activity

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Antioxidant potential of *Rosa* L. leaves methanolic extracts was evaluated *in vitro* using a spectrophotometric method based on measuring the radical scavenging effect on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals. The contents of ellagic acid, quercetin and kaempferol in the extracts from leaves of seventeen rose species were determined using SPE-RP-HPLC methods. Additionally, total phenolic content was determined spectrophotometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents (GAE). Remarkable high antioxidant activity and high total phenolic content ($5.7\% < \text{GAE} < 15.2\%$), large ellagic acid (EA) content from 9.37 to 19.42 mg/g of dry weight, a quercetin content ranging from 3.68 to 15.81 mg/g of dry weight and kaempferol content from 1.25 to 9.41 mg/g of dry weight were found in rose leaves. Significant correlation between EA ($r^2 = 0.6131$), quercetin ($r^2 = 0.5158$), total phenolic content ($r^2 = 0.8485$) and antioxidant activity was observed.
Basing on the studies conducted one may assume that the extracts of rose leaves are a rich source of natural antioxidants and could be used to prevent free-radical-induced deleterious effects.

Key words: Rosa L., Phenolic Compounds, Antioxidant Activity

Introduction

Plants from the *Rosa* L. genus have been used in medical care, perfumery and in food industry for many years. Their petals are the main source of fragrance compounds. Hips are famous for their high content of vitamins, especially vitamin C. Rose flowers, leaves and roots have been used in Chinese medicine, leaves in treatment of carbuncles, furuncles and burns, roots in treatment of rheumatic arthritis, injuries, prolapse of the anus and uterine prolapse (Fenglin *et al*., 2004).

Recently roses have gained growing attention due to their antioxidant and other beneficial properties (Daels-Rakotoarison *et al.,* 2002; Gao *et al*., 2000; Nowak and Gawlik-Dziki, 2004; Zhang *et al*., 2001).

The antioxidant properties of plant extracts were frequently connected with the content of polyphenols (Sroka, 2005). The importance of antioxidant phenols of plant materials in the maintenance of health and protection from coronary heart disease and cancer is raising interest among scientists, food manufacturers, and consumers as the trend of the future is moving toward functional food with specific health effects. Potential sources of antioxidant compounds have been searched in several types of plant materials such as fruits, leaves, seeds spices, barks and roots (Kähkönen *et al.*, 1999). Flavonoids, phenolic acids and other phenolics are especially common in leaves and flowers. Among phenolic compounds quercetin (Q) and ellagic acid (EA) are known as naturally occurring dietary antimutagens and anticarcinogens with strong antioxidant and anti-inflammatory activities (Falsaperla *et al.*, 2005; Labrecque *et al*., 2005; Narayanan and Re, 2001). The highest levels of ellagic acid were found in fruits *e.g*. raspberries, strawberries, and pomegranates. Extracts from red raspberry leaves or seeds, pomegranates, or other sources are said to contain high levels of ellagic acid, and are available as dietary supplements in capsule, powder, or liquid forms. EA has now been allowed for use as a food additive functioning as an antioxidant, too. Quercetin has a broad range of biological, pharmacological and medical applications (Duthie *et al*., 2000; Harborne and Williams, 2000; Marchand, 2002; Jakubowicz-Gil *et al*., 2005).

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Roses are a rich source of phenolic compounds (Yoshida *et al*., 1993; Hvattum, 2002). However, there are only a few studies concerning the comparison of the polyphenol composition in *Rosa* L. species (Krzaczek and Krzaczek, 1979; Mikanagi *et al*., 1995; Nowak and Tuzimski, 2005), the contents of ellagic acid, as well as flavonols and total polyphenols with respect to antioxidant properties of rose leaves have not been studied earlier.

Experimental

Plant material

The study materials were leaves collected from roses, mostly growing in their natural environment in Lublin Region of Poland, in June 2003 during the florescence of plants.

The following taxons were examined (Popek, 1996; Zieliński, 1987; Henker, 2000): *R. canina* L. var. *canina*, *R. canina* L. var. *corymbifera* (Borkh.) Boulenger, *R. canina* L. var. *puberula* R. Keller, *R. canina* L. var *dumalis* Baker, *R. vosagiaca* Desportes, *R. subcanina* (H. Christ) Dalla Torre et Sarnath, *R. coriifolia* Fri., *R. caryophyllacea* Besser pro parte, *R. subcollina* (H. Christ) Dalla Torre et Sarnath, *R. tomentosa* Sm., *R. rubiginosa* L., *R. villosa* L., *R. inodora* Fri., *R. agrestis* Savi, *R. jundzilli* Besser, *R. pendulina* L., *R. rugosa* Thunb., *R. gallica* L.

The plants were authenticated by Prof. Dr. T. Krzaczek, Department of Pharmaceutical Botany, Medical University of Lublin, and voucher specimens were deposited in the herbarium of the Department of Pharmaceutical Botany, Medical University, Lublin (Poland).

Chemicals

Standards of quercetin, kaempferol, ellagic and gallic acids were purchased from Roth (Karlsruhe, Germany). Compounds were dissolved in methanol to obtain a stock solution (0.5 mg ml^{-1}). All solvents used were of analytical or HPLC grade (Merck, Darmstadt, Germany).

HPLC analysis

All chromatographic separations were performed using a HPLC system (Knauer, Berlin, Germany) consisting of a HPLC Pump K-1001, Solvent Organizer K-1500, UV-VIS Detector Fast Scanning SpectroPhotometer K-2600, Degasser K-5004, Column Thermostat and 20 μl sample injector (Rheodyne, Cotati, CA, USA). Chromatographic data were collected and recorded using the computer program Eurochrom 2000.

The analytical column was a Zorbax SB C18 (200 × 4.6 mm I. D., 5 μm; Agilent Technologies, Germany) with a guard column Zorbax SB C18 (5 μm, 125 mm × 4.6 mm I. D.). A 10 μl volume of each sample was injected into the chromatographic system.

After preparation, the mobile phases were filtered through a 0.45 μm filter (J. T. Baker, Philipsburg, NY, USA). The sample solutions were also filtered through a 0.45 μm filter before HPLC. A 10 μl volume of each sample was injected into the chromatographic system.

The identification of compounds was accomplished by comparison of their retention times and UV spectra with those of appropriate standard compounds. UV spectra were recorded between 200 and 400 nm.

The quantitative determination of compounds was carried out with external standard methods calculating the peak areas. The calibration curves were obtained with six samples in the concentration range 0.1–100 μg/ml using linear regression analysis. Each sample was measured three times.

For preliminary purification of samples by SPE extractions a vacuum manifold processor (system Baker SPE-12G, J. T. Baker) was used.

Preparation of extracts

10 g crushed rose leaves were twice extracted using 60 ml and 40 ml of 70% aqueous methanol in a hot water bath for 1 h. The combined extracts were filtered and placed in 100 ml volumetric flasks and filled up to the mark.

Determination of total phenol contents

Total phenol content was determined by the colorimetric method adapted from Singleton and Rossi (1965). 1 ml of each aqueous sample solution was added to 4 ml distilled water and 1 ml of Folin-Ciocalteu reagent. After 3 min, 4 ml of Na_2CO_3 (70 g/l) were added. The solutions were mixed and allowed to stand for 30 min. The absorption was measured at 660 nm (UV-VIS spectrophotometer; Carl Zeiss, Jena). The concentration of phenols in the sample was estimated from a standard curve of gallic acid in the concentration range of 10–120 μg/ml. Results are expressed as mg gallic acid equivalents (GAE) per 1 g of dry

material and in percentage (% = g GA per 100 g of dry leaves). All samples were analyzed in three replications.

Determination of quercetin and kaempferol

Samples of 10 ml of rose leaves extract were heated for 1 h with 4 ml of 4 M HCl solution and TBHQ (0.05 g) in a water bath by reflux. The obtained extracts were filtered through cotton-wool into volumetric flasks and filled up with 50% methanol in water to 25 ml.

A 5 ml portion of each hydrolyzed extract was diluted with 5 ml water and passed through a octadecyl SPE microcolumn (500 mg; J. T. Baker Inc.) previously conditioned with methanol (10 ml), then with water (10 ml) and methanol/water 25:75 in 0.1% HCl. After the application of samples, the individual microcolumn was washed with 2 ml of water. The flavonoid aglycones adsorbed on the packing were desorbed by means of 5 ml of pure methanol. Collected methanol eluates containing flavonol aglycones were directly analyzed by HPLC.

The following mobile phases and gradient elution were used: D, acetonitrile/0.1% trifluoroacetic acid in water (18:82, v/v); E, acetonitrile/0.1% trifluoroacetic acid in water (35:65, v/v); F, acetonitrile/0.1% trifluoroacetic acid in water (80:20, v/v). The gradient elution profile was: 0–15 min: 100% D to 100% E; 15–25 min: 100% E (isocratic); 25–30 min: 100% E to 100% F; 30–35 min: 100% F (isocratic); 35–40 min: 100% F to 100% D; and post-time 5 min before the next injection. The flow rate was 1 ml/min, column temperature 25 °C, injection volume 10 μl. Detection was effected at 254 nm and 360 nm.

The contents of quercetin and kaempferol in the examined extracts were evaluated according to line dependence of peak area of analyzed substances and standard peaks from their concentrations. Total flavonol aglycone content was estimated as the sum of quercetin and kaempferol amounts.

Estimation of free ellagic acid content

The modified method of Amakura *et al*. (2000) was used. 10 ml of each rose leaves extract were placed in volumetric flasks and filled up with 0.1% HCl in water to 25 ml. A 10 ml portion of diluted extract was passed through octadecyl SPE microcolumns (500 mg; J. T. Baker Inc.) previously conditioned with methanol (10 ml), then with water (10 ml) and methanol/water 30:70 in 0.1% HCl. After the application of samples the individual microcolumn was washed with 2 ml of water. The ellagic acid adsorbed on the packing was eluted with 10 ml of methanol. Solution was evaporated in vacuum to 1 ml and analyzed by HPLC.

Estimation of total ellagic acid content

The extraction method applied for ellagic acid determinations was based on the method of Häkkinen *et al*. (2000) with some modifications. 1 g of each sample was hydrolyzed with 2 M HCl in 50% methanol (25 ml) and TBHQ (0.01 g). The mixture was refluxed for 5 h at 90 °C. The cold extract was filtered and diluted to 50 ml with water.

10 ml of the extraction solution were filtered through a C_{18} SPE cartridge (octadecyl, 500 mg; J. T. Baker) previously conditioned with water, methanol and 25% methanol in 0.1% HCl, respectively. The microcolumn was washed with 2 ml water and then ellagic acid was eluted with 10 ml of methanol. After evaporation to 1 ml EA was analyzed using HPLC.

The HPLC chromatographic conditions were as follows: isocratic mobile phase A, methanol/water/phosphoric acid (49.5:49.5:1, v/v/v), flow rate 1.0 ml/min, column temperature 20 °C, UV detection at λ = 254 and 360 nm; gradient elution phases B, methanol/water/phosphoric acid (199.5:799.5:1, v/v/v), and C, methanol/water/phosphoric acid (599.5:399.5:1, v/v/v), elution profile: 0–5min: 100% B to 50% B/50% C; 5–10 min: 50% B/50% C to 20% B/80% C; 10–15 min: 20% B/80% C to 100% C; 15–25 min: 100% C (isocratic); 25–30 min: 100% C to 100% B; post-time 5 min before the next injection. Quantitative analysis of ellagic acid was performed using system elution A.

Determination of antioxidant activities

Radical scavenging activity of rose extracts against stable DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals was determined according to the method of Brand-Williams *et al.* (1995) which has been widely used to evaluate the activity of natural antioxidants.

The changes in color from deep-violet to light-yellow were measured at 515 nm on a UV-VIS light spectrophotometer (Lambda 40; Perkin-Elmer).

The solution of DPPH in methanol (6×10^{-5} M) was prepared. 1.96 ml of this solution were mixed with 40 μl methanolic extract. The decrease in absorbance at 515 nm was determined continuously with data capturing at 60 s intervals. Blank sample contained 40 μl of methanol instead of the extract. All determinations were performed in duplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ inhibition} = [(A_B - A_A)/A_B] \times 100,$$

where A_B is the absorption of blank sample and A_A the absorption of tested solution.

Statistical analysis

Statistical analysis was done by one-way analysis of variance (Statistica 6.0). Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

In this work evaluating of rose leaves extracts of Polish origin with respect to their phenolic content and antioxidant activity in order to find a new potential source of natural antioxidants was performed. All investigated rose leaves extracts exhibited high total phenolic contents. The amount of total phenolics varied in the extracts and ranged from 5.7% to 15.2% GAE in dry material (Table I). Among the extracts, low levels were found in *R. rugosa* leaves (5.7%) and *R. vosagiaca* (7.7%), whereas *R. canina* var. *dumalis* and other *R. canina* varieties (except for *R. canina* var. *canina*) contained the highest amounts of phenolics.

It is obvious that the total phenolic content measured by the Folin-Ciocalteu procedure does not give a full picture of the quantity or quality of the phenolic constituents in the extracts. It is known, that there may be some interference in used method rising of phenolics from other chemical components present in the extract, such as sugars or ascorbic acid (Singleton and Rossi, 1965). Thus, further investigation of the composition of the phenolic fraction was necessary.

Using the HPLC method, ellagic acid was detected and quantified in the extracts of rose leaves. The elaborated simple and rapid method for this purpose based on the gradient elution with UV detection using a solid-phase extraction of EA. Good chromatographic resolution of the analyzed compound was achieved. Recently, ellagic acid has drawn more attention because of its biological properties, especially antioxidant and anticarcinogenic ones. For this reason, new sources of EA are searched.

Free ellagic acid content of leaves ranged from 3.32 to 7.41 mg/g of dry weight (mean 5.11) for 17

Table I. Antioxidant activity, total phenolic content, quercetin, kaempferol and ellagic acid contents in the investigated leaves of some rose species.

Species	Antioxidant activity (% inhibition)	Total phenols (% of dry weight)	Content of flavonols (mg/g of dry weight)			Content of ellagic acid (mg/g of dry weight)	
			Total	Quercetin	Kaempferol	Free	Total
1. *R. canina* var. *canina*	87.7 ± 0.05	9.9 ± 0.1	8.53 ± 0.03	6.84 ± 0.04	1.69 ± 0.02	4.42 ± 0.02	14.97 ± 0.03
2. *R. canina* var. *corymbifera*	95.7 ± 0.1	13.9 ± 0.24	11.78 ± 0.04	8.03 ± 0.04	3.75 ± 0.03	6.4 ± 0.03	19.42 ± 0.09
3. *R. canina* var. *puberula*	91.7 ± 0.08	12.2 ± 0.32	9.46 ± 0.05	7.12 ± 0.07	2.34 ± 0.02	4.58 ± 0.02	15.15 ± 0.1
4. *R. canina* var. *dumalis*	93.6 ± 0.07	15.2 ± 0.21	8.84 ± 0.04	8.11 ± 0.07	0.73 ± 0.01	5.27 ± 0.05	18.04 ± 0.17
5. *R. vosagiaca*	84.2 ± 0.1	7.7 ± 0.08	5.55 ± 0.03	4.22 ± 0.03	1.33 ± 0.02	5.0 ± 0.03	10.87 ± 0.02
6. *R. subcanina*	91.3 ± 0.1	10.2 ± 0.12	6.41 ± 0.03	5.16 ± 0.04	1.25 ± 0.01	4.49 ± 0.02	13.87 ± 0.15
7. *R. coriifolia*	94.2 ± 0.2	13.2 ± 0.27	12.77 ± 0.06	8.95 ± 0.1	3.82 ± 0.01	7.41 ± 0.05	14.37 ± 0.17
8. *R. caryophyllaceae*	93.5 ± 0.05	11.1 ± 0.14	17.20 ± 0.05	7.79 ± 0.08	9.41 ± 0.02	4.36 ± 0.04	10.42 ± 0.15
9. *R. subcollina*	88.2 ± 0.5	9.3 ± 0.08	11.27 ± 0.04	6.15 ± 0.05	5.12 ± 0.02	6.08 ± 0.05	12.01 ± 0.02
10. *R. tomentosa*	94.5 ± 0.1	11.1 ± 0.2	11.67 ± 0.03	7.48 ± 0.1	4.19 ± 0.05	3.94 ± 0.04	16.31 ± 0.15
11. *R. rubiginosa*	92.1 ± 0.25	10.8 ± 0.3	18.27 ± 0.03	10.68 ± 0.1	7.59 ± 0.05	4.24 ± 0.02	9.37 ± 0.12
12. *R. villosa*	89.9 ± 0.1	10.5 ± 0.2	12.39 ± 0.05	6.86 ± 0.07	5.53 ± 0.03	3.32 ± 0.01	13.80 ± 0.23
13. *R. inodora*	90.5 ± 0.05	13.2 ± 0.09	8.88 ± 0.05	7.22 ± 0.08	1.66 ± 0.01	3.98 ± 0.02	13.00 ± 0.28
14. *R. agrestis*	88.9 ± 0.14	9.7 ± 0.18	8.79 ± 0.04	3.68 ± 0.05	5.11 ± 0.02	4.43 ± 0.02	11.20 ± 0.08
15. *R. jundzilli*	87.9 ± 0.08	10.0 ± 0.11	10.78 ± 0.03	6.66 ± 0.04	4.12 ± 0.01	7.14 ± 0.05	11.53 ± 0.03
16. *R. rugosa*	83.4 ± 0.05	5.7 ± 0.08	6.57 ± 0.03	5.03 ± 0.05	1.54 ± 0.01	4.48 ± 0.02	9.59 ± 0.05
17. *R. gallica*	92.2 ± 0.12	11.5 ± 0.24	19.01 ± 0.1	15.81 ± 0.2	3.20 ± 0.02	6.73 ± 0.04	13.20 ± 0.22

rose species examined (Table I). Total ellagic acid content in the plant material was estimated after hydrolysis and ranged from 9.37 to 19.42 mg/g of dry weight (mean 13.26). The level of ellagic acid in rose leaves is comparable with the amount of this compound in strawberry leaves, which are considered to be one of the most potent sources of EA (Maas *et al*., 1991). Large differences in ellagic acid content were found among the species examined. The highest amount was found in all varietas of *R. canina* and in *R. tomentosa.* The lowest content of EA was estimated in *R. rubiginosa* and *R. rugosa*.

The RP-HPLC technique with spe preliminary extraction was used to describe the qualitative and quantitative composition of flavonol compounds in the analyzed plant samples. The reported method is very suitable for the analysis of flavonoid aglycones comparing the spectrophotometric and colorimetric methods described in several pharmacopoeias, which are not very specific and permit only a limited statement regarding the total flavonoids in plants. Quercetin content ranged from 3.68 in *R. agrestis* to 15.81 mg/g of dry weight in *R. gallica* (mean 7.43) and kaempferol level ranged from 1.25 in *R. subcanina* to 9.41 mg/g of dry weight in *R. caryophyllacea* (mean 3.79). These results clearly showed, that quercetin glycosides are dominant flavonols in most rose leaves and have greater influence on the antioxidant activity of rose leaves than derivatives of kaempferol. Among the investigated leaves, *R. gallica* had a clearly higher quercetin content than the other species. As noted previously, quercetin is the most common antioxidant flavonoid. Over the past several years there has been considerable interest in the antiproliferative, anticarcinogenic and antioxidant activity of this flavonoid.

The total amount of flavonol aglyones in rose leaves after hydrolysis was calculated as the sum of levels of quercetin and kaempferol and ranged from 5.55 to 19.01 mg/g of dry weight (mean 11.23).

The method employed to determine the radical scavenging activity of rose leaves extracts is based on the reduction of DPPH solution to the non-radical form (Brand-Williams *et al*., 1995). The remaining DPPH, measured at 515 nm after a certain time, corresponds inversely to the radical scavenging activity of the sample. This method is simple, rapid, sensitive and generally accepted for screening the antioxidant capacity of plant extracts (Fenglin *et al.*, 2004).

All of the examined rose leaves extracts showed high antioxidant activity in the DPPH assay (Table I). The inhibition values ranged from 83.4% (*R. rugosa*) to 95.7% (*R. canina* var. *corymbifera)*. Most of the extracts showed a very strong antioxidant response (over 90%). Their activity decreased in the following order: *R. canina* var. *corymbifera* > *R. tomentosa* > *R. coriifolia* > *R. canina* var. *dumalis* > *R. caryophyllacea* > *R. gallica* > *R. rubiginosa* > *R. canina* var. *puberula* > *R. subcanina* > *R. inodora.*

The correlation coefficient between the DPPH radical scavenging activity and phenolic content was determined (Table II). The values of the DPPH radical scavenging activity showed positive correlation with those of total phenolics: the correlation coefficient, r^2, was 0.8485 (Fig. 1). This result is in agreement with other studies. There are some literature data revealing a strong correlation between the total number and content of phenolics and the antioxidant activity of food, medicinal

Table II. Correlation coefficients (r^2) between the DPPH radical scavenging activity and content of phenolic compounds.

Compound	r^2
Total phenols	0.8485
Free EA	0.1333
Total EA	0.6131
Total flavonols	0.5227
Quercetin	0.5158
Kaempferol	0.2714

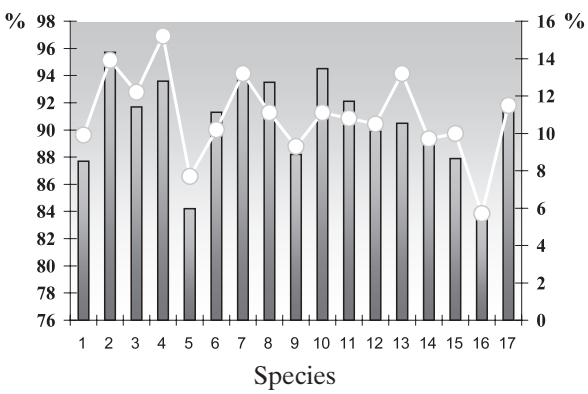

Fig. 1. Relationship between total polyphenols contents (% of dry weight leaves – points) and antioxidant activity (% of inhibition after 30 min – columns).

plants, fruits or vegetables (Vinson *et al*., 1998; Velioglu *et al*., 1998; Pellati *et al*., 2004). However, Kähkönen *et al.* (1999) showed no significant correlations between the total phenolic content and antioxidant activity of the plant extracts in the studied plant materials subgroups. As it can be observed, antioxidant activity does not necessarily correlate with high amounts of phenolics.

A possible explanation for these different results is that the antioxidant response of phenolic compounds depends on their chemical structure and a detailed description of the chemical composition of the analyzed extracts is needed.

In this work, significant positive correlations between total EA (r^2 = 0.6131), total flavonol (r^2 = 0.5227), quercetin content (r^2 = 0.5158) and antioxidant activity of rose leaves extracts were found (Table II, Figs. 2 and 3). However, these correlations are surprisingly lower than it should be expected. The similarity of antioxidant activity between rose species with different contents of EA, quercetin and phenolics may result partly from the presence of other compounds, *e. g.* other phenolic acids or ascorbic acid (Gardner *et al*., 2000). To explain this inconsistency, it would be necessary to study the composition of these extracts to a fuller extent. The HPLC analysis showed that methanolic extracts of rose leaves are a wide mixture of phenolic compounds, not identified in this study. It is possible that these constituents may interact to produce synergistic or antagonistic antioxidant effects with each other and with other compounds. Very little is known about such effects. Meyer *et al*. (1998) have been investigating potential synergistic or antagonistic effects between some phenolic compounds. All antioxidant effects of the analyzed substances were additive except for combinations with ellagic acid and catechin, where ellagic acid exerted a significant antagonistic effect.

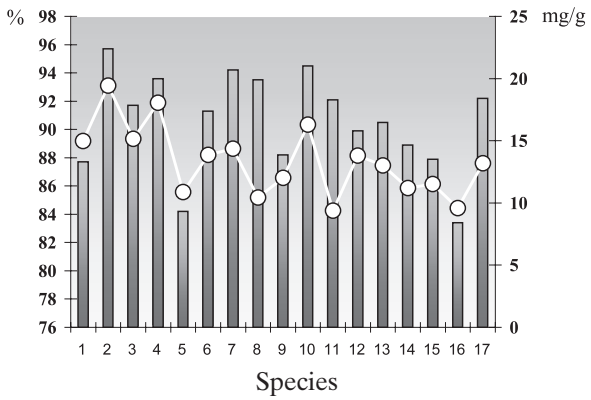

Fig. 2. Relationship between total ellagic acid content (mg/g of dry leaves – points) and antioxidant activity (% of inhibition after 30 min – columns)

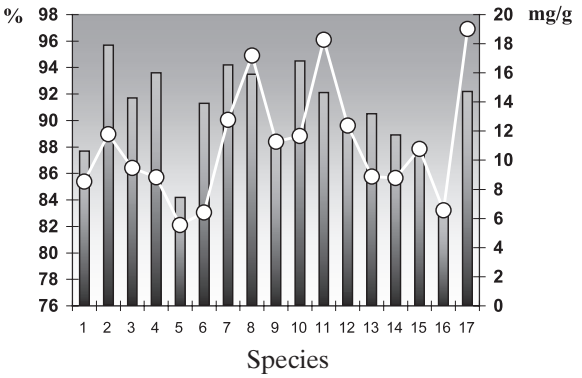

Fig. 3. Relationship between total flavonol content (mg/g of dry leaves – points) and antioxidant activity (% of inhibition after 30 min – columns).

Other authors revealed another kind of interaction of ellagic acid with quercetin. For example, EA significantly potentiated the effects of quercetin in the reduction of proliferation and viability and the induction of apoptosis (Mertens-Talcott *et al*., 2003). The interaction of ellagic acid and quercetin was demonstrated by an enhanced anticarcinogenic potential of polyphenol combinations, which was not based solely on the additive effect of individual compounds, but rather on synergistic biochemical interactions. Thus, plant extracts, like from rose leaves, with large amounts of both these compounds seem to be especially interesting. Unfortunately, little is known about the bioavailability of phenol compounds. The nutritional relevance of some phenolics is uncertain as they may be poorly absorbed and thus have limited antioxidant ability *in vivo*. However, further studies in this subjects are needed.

The results of this work show, that rose leaves accumulate large amounts of phenolic compounds and are very promising sources of natural phenolic antioxidants, with an especially high content of ellagic acid. Interest in sources of plant phenolics has increased greatly because of the antioxidant and free radical scavenging abilities associated with some compounds and their potential effects on human health. Considering the antioxidative activity on DPPH assay, the extracts of roses showed a possibility to be used as a new material for natural antioxidant and functional food.

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