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Full Length Research Paper

Phenolic contents, antioxidant and antimicrobial activity of *Papaver rhoeas* L. extracts from Southeast Serbia

Danijela A. Kostic*, Snezana S. Mitic, Milan N. Mitic, Aleksandra R. Zarubica, Jasmina M. Velickovic, Aleksandra S. Dordevic and Sasa S. Randelovic

Department of Chemistry, Faculty of Natural Sciences and Mathematics, University of Nis, Visegradska 33, 18000 Nis, Serbia.

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Total phenolic and flavonoid contents of different extracts of *Papaver rhoeas* L. were examined. High contents of total phenolic compounds (9.73 - 19.91 mg GAE/g of fresh petals) and total flavonoids (7.904 - 11.45 mg QE/g of fresh petals) were determined. Red pigment present in the flowers of *P. rhoeas* L. originates from anthocyanins, which may act as natural antioxidants. Anthocyanins content in the investigated extracts is very unified and ranged from 4.72 to 5.193 mg of cyanidin-3-O-glukoside/g of fresh petals. The antioxidant activity of different extracts was tested using the spectrophotometric method by means of the ability of extracts to scavenge stable 1,1-Diphenyl-2-picrylhydrazyl (DPPH). All tested extracts exhibited strong scavenging activity against DPPH radicals, that is higher then 80%. Total phenolic and flavonoid contents may be related to the percentages of the scavenging activity of DPPH assay (estimated correlation coefficient are R^2 = 0.965 and 0.752, respectively). The ethanol extract of *P. rhoeas* L. showed antimicrobial activity against the yeast *Candida albicans*, and all tested bacteria except *Bacillus subtilis.* This paper suggests that the investigated extracts of plant *P. rhoes* L. could be potentially applied as an antioxidant and antimicrobial agents.

Key words: *Papaver rhoeas* L., phenolic content, anthocyanins content, flavonoid content, antioxidant activity, antibacterial activity.

INTRODUCTION

Papaver rhoeas L. (Papaveraceae) is an annual herb indigenous to numerous regions in the world. In traditional medicine until synthetic drugs are developed, extracts of this plant have been used for the treatment of a wide range of diseases including inflammation, diarrhea, sleep disorders and, moreover, for cough, analgesia and also the reduction of withdrawal signs of the opioid addiction. It is also claimed that plant *P. rhoeas* exhibits sedative, narcotic, and emollient effects (Zargari, 1994). However, still there is no scientific consensus to clarify the effects of this plant on the withdrawal signs of opioids in animal models. Chemical studies have revealed phytochemical composition data demonstrating major compounds, such as rhoeadine, rhoeadic acid (Kalva and Sanyar, 1989), (Slavik et al., 1989), papaveric acid (Zargari, 1994), rhoeagenine (Rey et al., 1992) and anthocyanins (Matysik and Benesy, 1991). Chronic use of opioids, that is morphine, results in behavioral consequences like physical dependence and withdrawal syndromes (Kreek and Koob, 1998). The mechanisms by which opiates produce these behavioral effects are incompletely understood; however, changes in dopaminergic (Acquas and Di Chiara, 1992), purinergic (Zarrindast et al., 1999; Kaplan and Coyle, 1998), cAMP signaling (Nestler, 2001), Ca^{2+} and GABA alergic (Zarrindast and Mousa-Ahmadi, 1999) impacts, the all appear as relevant ones. The signs of opiate dependence are measured in animals by abrupt drug withdrawal or the administration of a narcotic antagonist (such as naloxone) or both (Kest et al., 2001). Among withdrawal behaviors in rodents, jumping and diarrhea are widely considered as the most sensitive indices of the withdrawal intensity (Kest et al., 2001).

*Corresponding author. E-mail: danijelaakostic@yahoo.com. Tel: + 381 64 2579127. Fax: +38118533014.

Phenolic compounds are often related to the antioxidant activity of plants due to their ability to adsorb and/or neutralize free radicals by means of quenching unpaired oxygen radicals or decomposing peroxides. In the present times, there is an increasing interest in natural antioxidants presented in medicinal and dietary plants, which may contribute to prevent oxidative disorders in humans by replacing use of synthetic antioxidants being also recognized as carcinogenic ones (Ito et al., 1983). Namely, data revealing an oxidative stress as potential cause of Alzheimer' s disease and chronic diseases, such as cancer, arteriosclerosis and rheumatism (Behl and Mosmann, 2002); Halliwell, (1999) speak in favor of how much important prevention of undesirable oxidative processes in humans, is. Now, the *P. rhoeas* genus has received smaller scientific attention, especially indigenous plant species from Southeast Serbia, with an exception comprising studies of the chemical composition (Vardavas et al., 2006), which has also been tested for the protective ability (Conforti et al., 2009).

In the present paper, the evaluation of *P. rhoeas* L. from Southeast Serbia with respect to phenolic content and the antioxidant data of different extracts obtained from plant petals are reported. As it is stated earlier, numerous polyphenols are known to possess excellent antioxidant effects, especially *in vitro,* and the amount of total polyphenols present in a plant has been suggested to correlate with the antioxidant activity. Therefore, this work represents the first report on phenolic content and related antioxidant activity of the extracts of *P. rhoeas* L. from Southeast Serbia. Antimicrobial activity of ethanol extract of *P. rhoeas* was investigated, too.

EXPERIMENTAL

Plant material

The plant was collected at bloom stage in few villages from the South-eastern Cape Province of Serbia in July, 2009. The plant was firstly identified by its vernacular name and later validated by voucher number of the deposited herbarium specimens at the Department of Botany (University of Novi Sad, Serbia).

Preparation of plant extracts

The fresh petals of *P. rhoeas* L. were milled by an appropriate blender. Three samples (each weighed 2.0 g) were separated from the previously homogenized plant material, and extracted with the desired volumes (30, 20 and 20 ml, respectively) of the chosen solvents (methanol, ethanol, methanol-water mixture, ethanol-water mixture and water) three times in the further course. Samples were mixed in an ultrasound bath during the extraction procedure. Such obtained extracts were filtered using the Buchner funnel and Whatman No. 1 filter paper. Solid residues were rinsed for several times in order to gain transparent extracts. Finally, the obtained plant extracts were collected in graduated flask of the same volume of 100 ml.

Chemicals and reagents

1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetin and $AlCl_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent and sodium carbonate were purchased from Merck Chemical Suppliers (Darmstadt, Germany). Sodium chlorate buffer (pH 1.0) and acetate buffer (pH 4.5) were purchased from the same producer. The other used chemicals including solvents were of analytical grade.

Determination of the total phenolics

Total phenolic contents in the extracts were determined by the modified Folin-Ciocalteu method (Singleton and Rossi, 1965). An aliquot of the extracts (1 ml) was mixed with 0.5 ml Folin-Ciocalteu reagent and 1.5 ml of sodium carbonate (20%). Tubes were vortexed for 15 s and allowed to stand at 40°C for 30 min in order to develop colour. Absorbances were then measured at 765 nm using the Hewlett Packard UV-VIS spectrophotometer. Total phenolic content was expressed as mg/g gallic acid equivalent. The result of each one assay was obtained from three parallel determinations.

Determination of the total monomeric anthocyanins

The total monomeric anthocyanin content in the plant extracts was determined using the pH-differential method previously described by Guisti and Wrolstad (2003). Anthocyanins demonstrate maximum of absorbance at 520 nm at pH 1.0. The colored oxonium form predominates at pH 1.0, and the colorless hemiketal form at pH 4.5. The pH-differential method is based on reaction producing oxonium forms and permits accurate and rapid measurement of the total monomeric anthocyanins. The result, considered as the monomeric anthocyanin pigment, was expressed as mg of cyanidin-3-glucoside, by using molar absorptivity (ε) of 26.900 and molecular weight of 449.2. Absorbances of the investigated extracts were calculated by Equation 1:

$$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH4.5} \quad (1)$$

Content of the monomeric antocyanin pigment (MAP) was calculated by Equation 2:

$$MAP(\mu g dm^{-3}) = (AxMWxDFx1000)/(\varepsilon x l) \quad (2)$$

Determination of the total flavonoid content

Total flavonoid contents were determined using the spectrophotmetric method based on formation of flavonoid complex with aluminium (Ordon et al., 2006). A volume of 0.5 ml of 2% $AlCl_3$ methanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420 nm. A yellow color indicated that the extracts contained flavonoids. Total flavonoid content was calculated as quercetin (mg/g) using the equation based on the calibration curve.

Free radical scavenging activity

The free radical scavenging activity of the plant extracts was analyzed by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Fuhrman et al., 2001; Lachman et al., 2007; Sanchez-Moreno et al., 1999; Turkoglu et al., 2007; Villano et al., 2006). The antioxidant assay is based on the measurement of the loss of colour of DPPH solution by the change of absorbance at 517 nm caused by the reaction of DPPH with the tested sample. The reaction was monitored using UV-VIS spectrophotometer. Plant extracts 0.2 and 1.8 ml of freshly prepared DPPH in methanol (20 $mgdm^{-3}$) were put into a cuvette at room temperature. After 20 min of incubation

Table 1. The total phenolic, anthocyanin and flavonoid content and antioxidant activity of *P. rhoeas* L. extracts.

Sample	Total phenols content[a]	Anthocyanin content[b]	Flavonoid content[c]	RSC (%)	RSC[d]
Ethanol extract	13.127 ± 1.553	5.193 ± 0.082	9.530 ± 0.045	84.91 ± 0.94	2.538 ± 0.74
Ethanol-water extract (1:1)	14.307 ± 0.255	4.729 ± 0.194	9.075 ± 0.073	86.1 ± 1.58	22.867 ± 0.55
Methanol extract	12.404 ± 0.911	4.909 ± 0.259	9.723 ± 0.067	83.11 ± 1.16	2.041 ± 0.37
Methanol-water extract (1:1)	9.734 ± 0.425	4.717 ± 0.177	7.904 ± 0.01	81.47 ± 1.22	1.588 ± 0.81
Water extract	19.912 ± 0.442	4.955 ± 0.171	11.45 ± 0.023	89.71 ± 0.85	23.864 ± 0.64

[a]Expressed as mg of gallic acid/g of fresh petals, [b]Expressed as mg of cyanidin-3-O-glukoside/g of fresh petals, [c, d] Expressed as mg of quercetin/g of fresh petals.

period at room temperature, the absorbance was read against a blank at 517 nm. The determinations were performed in triplicate. An inhibition of DPPH in percents (RSC %) of each plant extract sample was calculated from the decrease of absorbance according to the relationship:

$$RSC\ (\%) = (1-A_{sample}/A_{blank}) \times 100(\%) \quad (3)$$

where A_{blank} is the absorbance of control reaction, and A_{sample} is the absorbance of the tested sample.

Antimicrobial activity

The *in vitro* antimicrobial activity of ethanol extract of *P. rhoeas* L. was tested against a panel of laboratory control strains belonging to the American Type Culture Collection Maryland, USA (except one, belonging to National Collection of Type Cultures, see below). Antibacterial activity was evaluated against two gram-positive and three gram-negative bacteria. The gram-positive bacteria used were: *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538. The gram-negative bacteria utilized in the assay were: *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NCTC 6017. The antifungal activity was tested against two organisms *Aspergillus niger* ATCC 16404 and *Candida albicans* ATCC 10231.

A disc-diffusion method was employed for the determination of the antimicrobial activity of the extracts, according to NCCLS (1997). The inoculate of the bacterial and fungal strains were prepared from overnight broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. 100 µl of suspension containing 1.0 x 10^8 CFU/ml of bacteria and 1.0 x 10^4 CFU/ml of fungal spores spread on Mueller-Hinton agar (MHA, Torlak) and sabouraud dextrose agar (SDA, Torlak) respectively, in sterilized Petri dishes (90 mm in diameter). The discs (9 mm in diameter, Macherey-Nagel, Düren) were impregnated with 20 and 50 µl of the extracts (conc. 30 mg/ml) and placed on the inoculated agar. Negative controls were prepared using the same solvent (ethanol). Tetracycline (30 µg, Torlak) and Nystatin (30 µg, Torlak) were used as positive reference standards to determine the sensitivity of a strain of each tested microbial species. The inoculated plates were kept at 4°C for 2 h and incubated at 37°C (24 h) for bacterial strains and at 28°C (48 h) for fungal strains. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test microorganisms. Each assay in this experiment was repeated triplicate.

Statistical analysis

The experimental results were expressed as mean value ± standard error of mean value of three replicates. In order to estimate statistically any significant differences among mean values, where it was applicable, the data were subjected to a one-way analysis of variance (ANOVA test), and differences among samples were determined by Duncan's Multiple Range test using the Statistical Analysis System (SAS, 1999) software.

RESULTS

The phenolic content of the examined extracts of *P. rhoeas* L., obtained using the Folin-Ciocalteu reagent, is presented in Table 1. These contents, shown in Table 1, are expressed as Gallic Acid Equivalents (GAE) per gram of fresh plant petals. Total phenolic content of the investigated plant extracts ranged from 9.73 mg of GAE/g of fresh plant petals for the (methanol-water: 50/50, v/v(%)) to 19.91 mg GAE/g of fresh plant parts in the case of water extract. In other extracts the total phenolic contents values are very similar. The observable differences concerning total phenolic content, Table 1, may be related to different polarity of the used organic solvents, which have extracted exclusively selective compounds.

The antioxidant activity and total phenolic contents may vary considerably among herbs. This activity of the herbal extracts here investigated using the reduction of DPPH· by natural antioxidants, expressed as the percentage of radical scavenging activity or mg of quercetin equivalent (QE) per gram of fresh petals, is also shown in Table 1. The total phenolic content was correlated to the antioxidant activity of the extracts (R^2 = 0.9654), as can be seen in Figure 1.

The antioxidant properties of herbs may be also connected with the plant pigments originated from the main components of each herbal extract. The red pigment exhibits in the flowers of *P. rhoeas* L. comes of anthocyanins, which may act as natural antioxidants. Total monomeric anthocyanin content in the all investigated extracts is very unified and ranged from 4.72 to 5.3 mg of cianidin-3-O-glukoside/g of fresh petals, Table 1. The total monomer antocyanins content values do not significantly differ in aqueous, ethanolic and methanolic extracts. Overall correlation coefficient in the plant petals

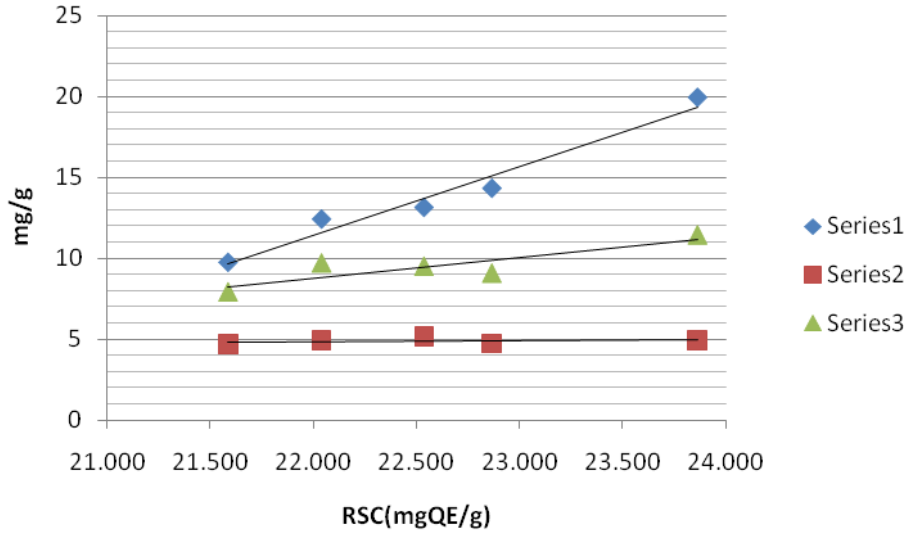

Figure 1. Corellation of total phenolic compounds (series 1), flavonoid (series 2) and anthocyanins (series 3) contents as well as the antioxidant activity of plant extracts of *P. rhoeas* L.

Table 2. Antimicrobial activity of *P. rhoes* L. ethanol extract (zone size, mm).

Microorganism	Tested sample		Positive control (referent standard)		Negative control
	Ethanol extract (10 mg/ml)		Tetracycline (30 µg)	Nystatin (30 µg)	Ethanol
	20 µl	50 µl			
B. subtilis	na	na	36 ± 0.3	nt	na
S. aureus	12 ± 0.15	18 ± 0.1	34 ± 0.3	nt	na
E. coli	17 ± 0.2	24 ± 0.15	30 ± 0.25	nt	na
P. aeruginosa	11 ± 0.2	20 ± 0.2	19 ± 0.2	nt	na
S. abony				nt	na
C. albicans	13 ± 0.1	26 ± 0.25	nt	20 ± 0.2	na
A. niger	na	na	nt	19 ± 0.2	na

Nt: not tested, na: not active, Data are expressed as the mean of three replicates ± standard deviation.

between monomer anthocyanins content and antioxidant activity is not significant (only R^2 = 0.076) due to unified anthocyanins amount in the examined plant extracts (Figure 1).

Total flavonoid contents ranged from 7.904 mg QE/g of fresh petals to 11.45 mg QE/g of fresh petals, Table 1. There is significant correlation of flavonoid content and antioxidant activity; namely, correlation coefficient is R^2 = 0.7524 (Figure 1). The obtained and discussed results may impose that the phenolic compounds have a major contribution to the antioxidant capacity of plants. The antimicrobial activities of the ethanol extracts of *P. rhoeas* L. are given in Table 2. As expected, the control treatment (ethanol) had no inhibitory effect on any of the tested microorganisms. The antimicrobial activity was assessed qualitatively and quantitatively by the presence or absence of inhibition zones. The ethanolic extract from *P. rhoes* L. showed antimicrobial activity against all tested microorganisms, with the exception both of bacteria *B. subtilis* and mould *A. niger.*

DISCUSSION

Polyphenols are the major plant compounds that are characterized by antioxidant activity. This activity is believed to be mainly due to their redox properties (Zheng and Wang, 2001), polyphenols in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. The results from this study, presented in Table 1, strongly suggest that phenolic compounds are important components of this plant, and some of their pharmacological effects could be attributed to the presence of these valuable constituents.

The phenolic concentration of the medicinal herbs ranged from 0.23 to 2.85 mg GAE/g of fresh weight, while the phenolic content of the culinary herbs ranged from 0.26 to 17.51 mg GAE/g of fresh weight (Zheng and Wang, 2001). It is obvious from a survey of the literature that certain culinary plants species, especially of the genus *Origanum*, have extremely high total phenolic content of about 20 mg GAE/g of fresh weight. Based on this finding, the authors claim that here investigated plant possesses high amount of total phenolic content comparable to one of several culinary herbs extensively studied as effective antioxidants in the lipid system (Lagouri and Boskou, 1996).

Among the studied plant extracts, the water extract showed the highest phenolic content and the highest resulted antioxidant activity. Thus, the antioxidant activity of an extract could be predicted from its total phenolic content. Statistically significant relationships were also observed between the total phenolics and the antioxidant activity in the case of virgin olive oils (R^2 = 0.991), flaxseed (R^2 = 0.963) and cereal products (R^2 = 0.905) (Velioglu et al., 1998).

The total monomer antocyanins content values do not significantly differ in aqueous, ethanolic and methanolic extracts. Overall correlation coefficient in the plant petals between monomer anthocyanins content and antioxidant activity is not significant (only R^2 = 0.076) due to much unified anthocyanins amount in the all examined plant extracts. Significant correlation of the flavonoid content and antioxidant activity was obtained and discussed results may impose that the phenolic compounds have a major contribution to the antioxidant capacity of plants.

P. rhoeas L. ethanolic extract showed antimicrobial activity against all tested microorganisms, except *B. subtilis* and *A. niger.* From the medicinal point of view, watery, ethanolic and ethanol-watery extracts are very important due to their high total content of phenolic compounds, and thus the appropriate antioxidant activity. Among them, watery extract exhibited the highest total content of phenolic compounds and resulted antioxidant activity.

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