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In vitro antioxidant activity of extracts from leaves and fruits of common dogwood (*Cornus sanguinea* L.)

Activité antioxydante *in vitro* d'extraits de feuilles et de fruits du cornouiller sanguin (*Cornus sanguinea* L.)

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Abstract: This paper deals with first new data for comparative analysis of leaves and fruits from common dogwood (*Cornus sanguinea* L.). Antioxidant activity is expressed as IC_{50} values (μg/ml) that ranged from 1202,85 to 19,84 μg/ml. The total phenolic content ranges from 27,45 to 205,74 mg GA/g. The concentration of flavonoids is between 14,40 and 118,46 mg RU/g. In comparison, methanolic and water extracts from leaves show significant antioxidant activity, IC_{50} values of this extract are better than the values for the *G. biloba* extract. The report of our research of plant parts from *C. sanguinea* can be regarded as promising candidates for natural plant sources of antioxidant with high value.

Keywords: *Cornus sanguinea*; phenolic compounds; antioxidants

Résumé: Cet article traite des premiers résultat d'analyses comparatives des feuilles et des fruits de cornouiller sanguin (*Cornus sanguinea* L.). L'activité antioxydante a été exprimée en valeurs IC_{50} (μg/ml) allant de 1202,85 à 19,84 μg/ml. Le contenu phénolique total se situe entre 27,45 et 205,74 mg GA/g. La concentration des flavonoïdes vaut de 14,40 à 118,46 mg RU/g. Par comparaison, les extraits méthanoliques et aqueuxdes feuilles ont montré une remarquable activité antioxydante, alors que les valeurs IC_{50} de cet extrait ont été meilleures par rapport aux valeurs des extraits de *Ginkgo*. Le rapport de nos recherches sur les feuilles et les fruits de *C. sanguinea* montre des activitiés prometteuses en tant que sources végétales naturelles d'antioxydants à haute valeur.

Mots Clés: Cornus sanguinea; composé phénolique; antioxydants

Introduction

Common dogwood (*Cornus sanguinea* L.) is a species of genus *Cornus* and belongs to the family Cornaceae Link. It is a deciduous shrub with dark greenish-brown branches and twigs that can be up to 5 m high. The leaves are broadly elliptical, 4-10 cm long and 3-6 cm wide. Its creamy flowers are arranged in an umbelliform inflorescence. The fruit is a globose black berry and contains a single seed. The plant inhabits humid, moderately humid and dry forests from southern Scandinavia, Atlantic and Central Europe to the Balkan Peninsula (Ball, 1968; Jovanović, 1974).

The species of the genus *Cornus* are very rich in phenolic compounds. Due to the very strong biological activity they are widely used in both traditional and modern medicine, veterinary medicine, and pharmacy. Their leaves and fruits possess antioxidant, anti-inflammatory, cytoprotective, analgesic, antidiabetic, and anticoagulant activities. Medicinal active substances in species of genus *Cornus* are phenolic compounds, mineral substances, vitamins – especially vitamin C, tannoid substances and anthocyanins (Seeram *et al*., 2002; Viegi *et al*., 2003; Pieroni *et al*., 2004; Serteser *et al*., 2009; Oskay *et al*., 2009; Rop *et al*., 2010).

Free radicals are atoms or groups of atoms that have at least one unpaired electron, which makes them highly reactive. They are produced during normal metabolism or are induced by UV radiation and different pollutants from environment. Their increased concentration in the human body causes many pathological conditions such as inflammatory, neurological, and psychiatric diseases, and carcinogenesis, etc. (Anderson, 1995; Das Sarma *et al*., 2010; Rakesh *et al*., 2010). The negative effects of free radicals

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can be largely prevented by intake of antioxidant substances. Antioxidants act by donating an electron to a free radical and converting it into a nonradical form (Maruthappana & Sakthi, 2010; Guinot *et al*., 2010).

Antioxidants can be of synthetic origin and a great number of secondary metabolites have been isolated from plants, such as various phenolic compounds (Albayrak *et al*., 2008; Fatnassi *et al*., 2010). The most widely used synthetic antioxidants have been suspected to cause or promote negative health influences and genotoxic effects (Ito *et al*., 1986; Chen *et al*., 1992; Kahl and Kappus, 1993). Plants are the source of the most potent free radical scavengers such as different phenolic compounds and vitamins. Therefore, the investigations of biological activity and chemical composition of medicinal plants as a potential source of natural antioxidants are numerous in the recent studies (Albayrak & Aksoy, 2010; Salas *et al*., 2010).

The present study is prompted by the fact that no data on antioxidant activity, phenol concentration and flavonoid content of *C. sanguinea* has been provided so far. In addition, there is no data concerning the comparative analysis of the antioxidant activity of the different plant extracts of *C. sanguinea* leaves and fruits. The basic aim of our research is to determine the total phenolic contents and concentrations of flavonoids in various extracts of the species *C. sanguinea* using spectrophotometric methods, as well as to examine the antioxidant activity of plant extracts *in vitro* using model system.

Material and methods

Chemicals

Acetone, methanol, petroleum ether, ethyl acetate and sodium hydrogen carbonate ($NaHCO_3$) were purchased from "Zorka pharma" Šabac, Serbia. Standards of phenolic acids (gallic acid) and flavonoids (rutin hydrate), chlorogenic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma Chemicals Co., St Louis, MO, USA. The Folin-Ciocalteu's phenol reagent, 3-tert-butyl-4-hydroxyanisole (BHA) and aluminium chloride ($AlCl_3$) were from Fluka Chemie AG, Buchs, Switzerland. A standardized extract of *Ginkgo biloba* was obtained from Pharmaceutical Company „Ivančić i Sinovi", Belgrade, Serbia. All other solvents and chemicals were of analytical grade.

Plant material

Leaves of *C. sanguinea* were collected in July and fruits in September 2010 from the region of Šumarice, Kragujevac in central Serbia. The voucher specimen of *C. sanguinea* was confirmed and deposited in Herbarium at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac. The collected leaves were air-dried in darkness at room temperature (20 °C). Harvested fresh fruits are immediately used to prepare extracts.

Preparation of plant extracts

The prepared plant material (10 g) was coarsely crushed in small pieces of 2-6 mm by using the cylindrical crusher and extracted with water, methanol, acetone, ethyl acetate and petroleum ether. The extract was filtered through a paper filter (Whatman, No. 1) and evaporated under reduced pressure by the rotary evaporator. The obtained extracts were stored in dark glass bottles for further processing.

Determination of total phenolics in the plant extracts

Total soluble phenolic compound in the different extracts of *C. sanguinea* were determined with Folin-Ciocalteu reagent (Singleton *et al*., 1999) using gallic acid as a standard. Methanolic extract was diluted to the concentration of 1 mg/ml and 0.5 ml of the soluted extract was mixed with 2,5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7,5%). After 15 min of staying at the 45 °C, the absorbance was measured at 765 nm versus blank sample on spectrophotometer (Iskra, MA9523-Spekol 211). Content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg GA/g extract). Values were uniformly expressed as the corresponding dry weight of plant extract (1 g). All measures were repeated three times.

Determination of total flavonoids in the plant extracts

The total flavonoid contents were determined spectrophotometrically (Quettier *et al*., 2000). Briefly, 0.5 ml of 2% solution of $AlCl_3$ in methanol was mixed with the same volume of extract (1 mg/ml). Absorption readings at 415 nm were taken after 1 h against a blank (methanol). The total flavonoid content was determined using a standard curve with rutin (0-50 mg/l). Values were uniformly expressed as the corresponding dry weight of plant extract (1 g). All measures were repeated three times.

Evaluation of antioxidant activity

The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method (Tekao *et al*., 1994), adopted with suitable modifications (Kumarasamy *et al*., 2007). DPPH (20 mg) was dissolved in methanol (250 ml) to obtain a concentration of 80 μg/ml. The stock solution of plant extract was prepared in methanol to achieve the concentration of 1 mg/ml. Dilutions were made to obtain concentrations of 500; 250; 125; 62,5; 31,25; 15,62; 7,81; 3,90; 1,99; 0.97 μg/ml. Diluted solutions (1 ml each) were mixed with 1 ml oh DPPH solution. After 30 min in darkness at room temperature (23 °C), the absorbance was recorded at 517 nm. Control samples contained all the reagents except the extract. Percentage of inhibition was calculated using eq. (1), whilst IC_{50} values were estimated from the % inhibition versus concentration plot, using a non-linear

regression algorithm. The data were presented as mean values ± standard deviation (n = 3).

$$\%\,inhibition = \left(\frac{A\text{ of control} - A\text{ of sample}}{A\text{ of control}}\right)x100 \quad (1)$$

Results and discussion

Ten leaves and fruits extracts are prepared using water, methanol, ethyl acetate, acetone and petroleum ether in order to examine the total soluble phenolic compound, flavonoid concentrations and *in vitro* antioxidant activity. Various solvents are used to achieve the extraction of active substances with diversity in their polarity. The choice of solvents proved to be very effective in earlier studies (Stanković *et al*., 2010).

The total phenolic content in the examined plant extracts using the Folin-Ciocalteu reagent is expressed in terms of gallic acid equivalent, GAE (the standard curve equation: y = 7,026x – 0.0191, r^2 = 0.999) as mg of GA/g of extract (Table 1). The total phenolic content in the examined extracts ranges from 27,45 to 205,74 mg GA/g. The high content of phenols is measured in methanolic (205,74 mg GA/g), also in water extract (98,21 mg GA/g) from leaves. In all other extracts (acetone, ethyl acetate and petroleum ether extracts from leaves and all fruits extracts) total phenolic contents are in the range from 27,45 to 59,59 mg/g.

In the comparison of these two groups of extracts, it appears that the leaves of *C. sanguinea* contain higher concentrations of phenolic compounds in relation to the fruits. Analyzing the concentration of total soluble phenolic compounds in all leaf extracts it is noticed that the highest concentration of phenolic compounds are in the extracts obtained using solvents of high polarity. It has been reported that methanolic extract manifest greater power of extraction of phenolic compounds from the leaves of *C. sanguinea*. The values obtained for the concentration of phenolic compounds in the extracts from fruits are small in comparison with the ones from leaves (Table 1).

In previous studies (Zhang *et al*., 2007; Barrira *et al*., 2008), the authors who have examined the difference between the phytochemical characteristics of different plant parts (leaves, flower, fruits, etc.) of some plants confirm the higher concentration of phenolic compounds in leaf extracts if compared to the fruit extracts.

Table 1. Total phenolic content in the plant extracts expressed in terms of gallic acid equivalent (mg GA/g extr). Each value in the table is obtained by calculating the average of three analysis ± standard deviation.
Tableau 1. Contenu phénolique total des extraits végétaux exprimé en termes d'équivalent d'acide gallique (mg GA/g extr). Chaque valeur du tableau est obtenu en calculant la moyenne des trois analyses ± déviation standard.

type of extract	leaves	fruits
Methanol	205,74 ± 0.49	34,19 ± 0.25
Water	98,21 ± 0.38	27,45 ± 0.15
Ethyl acetate	39,73 ± 0.11	45,34 ± 0.19
Acetone	59,59 ± 0.21	43,51 ± 0.27
Petroleum ether	55,04 ± 0.13	38,31 ± 0.31

Table 2. Total flavonoid contents in the plant extracts expressed in terms of rutin equivalent (mg Ru/g extr). Each value in the table is obtained by calculating the average of three analysis ± standard deviation.
Tableau 2. Contenus totaux des flavonoïdes dans des extraits végétaux exprimés en termes d'équivalent de rutine (mg Ru/g extr). Chaque valeur du tableau est obtenu en calculant la moyenne des trois analyses ± déviation standard.

type of extract	leaves	fruits
Methanol	23,24 ± 0.16	14,40 ± 0.09
Water	91,00 ± 0.23	24,22 ± 0.12
Ethyl acetate	118,46 ± 0.98	32,38 ± 0.20
Acetone	112,22 ± 0.76	31,54 ± 0.29
Petroleum ether	57,12 ± 0.32	28,60 ± 0.19

The concentration of flavonoids in plant extracts from leaves and fruits of *C. sanguinea* is determined using spectrophotometric method with $AlCl_3$. The concentrations of flavonoids is expressed in terms of rutin equivalent, RuE (the standard curve equation: y = 17,231x – 0.0591, r^2 = 0.999), as mg of Ru/g of extract. The summary of quantities of flavonoids identified in the tested extracts is shown in Table 2. The concentrations of flavonoids in plant extracts range from 14,40 to 118,46 mg Ru/g.

The values of concentration of flavonoids in the extracts from leaves vary in a wide range. The lowest values are measured in methanolic extract (23,24 mg Ru/g) and the highest are in ethyl acetate (118,46 mg Ru/g) and acetone (112,22 mg Ru/g) extracts. The concentration of flavonoids in the extracts from fruit is smaller in comparison with extracts from leaves, but there is not much variation in the values. Similar to the extracts from leaves, the lowest concentration of flavonoids is in the methanol extract from fruit (14,40 mg Ru/g) and the highest in the ethyl acetate (32,38 mg Ru/g) extract from fruit. Based on the obtained values of concentration of flavonoids in the examined extracts of *C. sanguinea*, it is found that the highest concentration of these compounds is in the extracts obtained using solvents of moderate polarity.

The concentration of total phenolics and contents of flavonoids in the extracts of *C. sanguinea* depend on the polarity of solvents and the of type of plant material used for the extractions. Generally, higher concentrations of total phenolic compounds and flavonoids are recorded in the leaf extracts.

The antioxidant activity of different plant extracts of *C. sanguinea* is determined using methanol solution of DPPH reagent. DPPH method has also been used to quantify antioxidants in complex biological systems in recent years and is based on the reduction of methanolic solution of colored free radical DPPH by free radical scavenger. The scavenging activity is measured as the decrease in absorbance of the samples versus DPPH standard solution (Brand-Williams *et al*., 1995).

Table 3. DPPH scavenging activity of investigated plant extracts presented as IC_{50} values (μg/ml). Each value in the table is obtained by calculating the average of three analysis ± standard deviation.
Tableau 3. Activité antioxydante des extraits végétaux observés présentée en valeurs IC_{50} (μg/ml). Chaque valeur du tableau est obtenu en calculant la moyenne des trois analyses ± déviation standard.

type of extract	leaves	fruits
Methanol	19,84 ± 0.11	358,59 ± 1,14
Water	22,37 ± 0.17	384,45 ± 2,01
Ethyl acetate	738,20 ± 2,43	537,83 ± 1,98
Acetone	225,72 ± 1,56	247,83 ± 2,l24
Petroleum ether	515,37 ± 1,92	1202,85 ± 3,01

The antioxidant activity of ten different extracts from leaves and fruits of *C. sanguinea* is expressed in terms of IC_{50} (μg/ml) values (Table 3). In parallel with the examination of the antioxidant activity of the plant extracts, the values for three standard compounds (Table 4) and *G. biloba* standardized extract are obtained and compared to the values of the antioxidant activity of *C. sanquinea*. The standard substances used are rutin, chlorogenic acid and BHA.

The obtained values of antioxidant activity examined by DPPH radical scavenging activity range from 1202,85 to 19,84 μg/ml. The largest capacity to neutralize DPPH radicals is measured in methanol and water extracts from leaves of *C. sanguinea*, which neutralized 50% of DPPH free radicals in small concentration, of 19,84 and 22,37 μg/ml respectively. In the group of fruit extracts, the highest antioxidant activity is measured in the acetone extract. The leaf extracts appear to have higher antioxidant activity when compared to extracts obtained from fruit.

In comparison to IC_{50} values of BHA, rutin and chlorogenic acid (Table 4), methanolic and water leaf extracts of *C. sanguinea* manifest the strongest capacity to neutralize DPPH radicals. Methanolic and water extracts from leaves of *C. sanguinea* are found to be more active than *G. biloba* extract.

Following a comparative review of two most active extracts of *C. sanguinea* with values related to standard substances and *G. biloba* extract, the range of antioxidant power is as follows: BHA > rutin > chlorogenic acid > methanolic extract from leaves > water extract from leaves > *G. biloba* extract.

Table 4. Values for antioxidant (DPPH scavenging) activity of standard substances obtained for comparison with *C. sanguinea*. Each value in the table is obtained by calculating the average of three analyses ± standard deviation.
Tableau 4. Les valeurs[1] pour l'activité antioxydante (piègeage DPPH) des substances standard obtenues pour la comparaison avec *C. sanguinea*. Chaque valeur du tableau est obtenu en calculant la moyenne des trois analyses ± déviation standard.

substances	IC_{50} μg/ml
BHA	5,39 ± 0.31
Rutin	9,28 ± 0.27
Chlorogenic acid	11,65 ± 0.52
Ginkgo biloba	33,91 ± 1,16

Comparing the concentration of phenolic compounds and antioxidant activity we find that extracts with the highest concentrations of phenolic compounds also have strong antioxidant activity. Based on this results, each extracts of *C. sanguinea* exhibit phenol concentration-dependent scavenging effects.

In the literature there is no data for comparative analysis of antioxidant activity, total phenolic contents and flavonoid concentrations of different extracts from plant parts of *C. sanguinea*. The authors who have comparatively analyzed the antioxidant activity of different parts of other plants have obtained similar results. Our data confirms the results of Barreira *et al*. (2008) in their comparative study of chestnut: leaf extracts have higher concentrations of phenolic compounds and greater antioxidant activity than other plant parts.

This data suggests that methods used in the analysis are very effective with leaves, but when examining fruits it is necessary to use more effective methods for preparing extracts and even a variety of methods for the examination of antioxidant activity. In addition, the phenolic contents of extracts depend on the plant part used in the experiment, the extraction method employed, and solvents used for extraction, and not only the concentration of phenolic contents but also properties of these compounds contribute to the activites of different extracts.

The results of our study suggest the great value of the species *C. sanguinea* for use in pharmacy and phytotherapy. Therefore, the plant parts of this plant are natural sources of antioxidant substances of high importance. Further studies of this plant species should be directed to a detailed qualitative analysis of all its parts and to carry out *in vivo* studies of its medicinally active components in order to prepare pharmaceutical natural products of high value.

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