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Phytochemical and antioxidant screening of some extracts of *Juniperus communis* L. and *Juniperus oxycedrus* L.

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Abstract: The content of phytochemicals, total phenolics, total flavonoids and antioxidant potential of extracts of *Juniperus communis* L. and *Juniperus oxycedrus* L. berries were determined. Ethanol, ethyl acetate and chloroform were used for the process of extraction. Phytochemical monitoring was based on already known methods, while *in vitro* antioxidant activities were done by DPPH assay. Phytochemical screening showed a wide spectrum of phytochemicals. Ethanolic extract of *Juniperus communis* L. possesses the strongest antioxidant activity (IC_{50} = 28.55 ± 0.24 µ/ml), as well the higher contents of total phenolics, 189.82 ± 0.27 mg of gallic acid equivalent per g of dried weight extract (mg GAE/g extract DW), and total flavonoids, 42.85 ± 0.13 mg of rutin equivalents per g of dried weight extract (mg RE/g extract DW). The results indicated the potential application of the tested extracts as significant antioxidants.

Keywords: DPPH; extract; *Juniperus* berries; total flavonoids; total phenolics

Since the ancient times, extracts of many plants were used in ethno-pharmacy and have been of vital importance in the treatment of many diseases. The activity of these extracts in the therapeutic sense depended upon the chemical composition of the used plant species. Since at that time it was not much known about the chemical composition of plants, the emphasis was placed on its discovery. Inthe last decades, it has been established that plants have a large number of secondary metabolites as alkaloids, terpenoids, phenolic acids, flavonoids, tannins, lignins, quinones, coumarins and other that are responsible for broad spectrum of biological activities (Liu *et al*. 2016; Shanjani *et al*. 2016; Abbas *et al*. 2017; Venditti *et al*. 2018).

Genus *Juniperus* is one of the most widespread genera, which includes about 67 species throughout the Northern Hemisphere (Rajčevič *et al*. 2015). As an excellent source of antioxidants genus *Juniperus* is very well known, and is widely used in folk medicine. *J. communis* L. is a shrub or small evergreen tree with the largest natural range of any woody plant, and only *Juniperus* species grow in both hemispheres. Also, many previous studies confirm the biological action of *Juniperus communis* L. (*J. communis*) (Banerjee *et al*. 2012; Šojić *et al*. 2017). The berries showed a wide spectrum of pharmacological activity including antioxidant (Höferl *et al*. 2014; Stoilova *et al*. 2014) and antimicrobial (Peruč *et al*. 2018) potential. In folk medicine, they are used as

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antiseptic, diuretic, stomachic, cardiac and rheumatic drugs (Charles 2013; Stoilova *et al*. 2014). *Juniperus oxycedrus* L. (*J. oxycedrus*) appeared as a shrub or small tree in Near East countries (Taviano *et al*. 2011). Leaves, resin, bark and berries extracts possess an anti-cancer (de Marino *et al*. 2014), analgesic (Al-Snafi 2018) and antibacterial (Eryilmaz *et al.* 2016) activities. The berries are used in cosmetics (Loizzo *et al*. 2007), and due to its taste, as a spice and flavor for food. In addition, *J. oxycedrus*is used in folk medicine for parasitic disease, urinary infections and bronchitis (Loizzo *et al*. 2007; Djebaili *et al*. 2013). Also, one of the popular alcoholic drink in Serbia, so-called Klekovača, the type of brandy with unique aroma, was partly made from berries (Lesjak *et al*. 2011).

Based on all mentioned above, and our interests for therapeutic effect of plants, aim of this study was to determine the content of phytochemicals, total phenolics, total flavonoids and evaluate antioxidant activities of the *J. communis* and *J. oxycedrus* berries extracts, prepared with the solvent of different polarity (ethanol, ethyl acetate and chloroform).

MATERIAL AND METHODS

Chemicals. The used solvents were HPLC grade and obtained from Merck (Germany). Galic acid (No. 410860050) and rutin (No. 132390050) were obtained from Acros Organics (Belgium), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (No. D9132-5G) by Sigma-Aldrich (USA), Folin-Ciocalteu's phenol reagent (No. 1090010100), ascorbic acid (No. 1004680100) as well as all other used chemicals from Merck (Germany).

UV-Vis spectroscopy. All absorbance was measured using a UV-1800 Shimadzu (Japan) spectrophotometer.

Plant material collection. The berries of the two selected plants, *J. communis* and *J. oxycedrus* were collected in September 2017, at region of Šara mountain in south Serbia. The berries of *J. communis* were collected in the village Brezovica (Tršenja, 1300 m asl.) and *J. oxycedrus* in the same village on the opposite side (Piljevac, 1500 m asl.). After 10 days of air-drying in shadow, the plant material was packed in tight-seal dark bottles and kept at dry, dark and cold place (around 15°C) until analysis.

Extraction procedure. Dry, grounded into a fine powdered plant material of 5 g was extracted with 100 ml of appropriate solvent (ethanol, ethyl acetate and chloroform) in a Soxhlet apparatus. The extraction was performed for 5 h at boiling temperature. The obtained extracts were evaporated to dryness under vacuum at 40°C and stored in sterile glass bottles at 4°C until used.

Qualitative phytochemical screening

The ethanol, ethyl acetate and chloroform crude extracts of *J. communis* and *J. oxycedrus* were evaluated for phytochemical. According to previously described methods the presence of alkaloids, tannins, saponins, phenolic compounds, flavonoids, steroids, terpenoids, cardiac glycosides and coumarins in the *J. communis* and *J. oxycedrus* extracts were investigated (Harborne 1984; Kumar *et al*. 2013; Jean *et al*. 2018).

Alkaloids test. About 0.5 g of the crude extract and 5 ml of 1% aqueous HCl was mixed and heated (30°C). After filtration, 2–3 drops of Dragendorff's reagent were added to the filtrate. The presence of alkaloids showed a precipitated orange-red colour.

Tannins test. Crude extract (0.25 g) was dissolved in 10 ml of distilled water and after filtration 1% aqueous $FeCl_3$ was added. The confirmation of presence of tannins was green-purple colour.

Saponins test. In a test tube about 0.5 g of the extract was dissolved with hot distilled water. After 1 min of shaking the formation of 1 cm layer of foam was the preliminary evidence for saponins.

Phenolic test. In 1 ml of extract 2 ml of distilled water was added and after that five drops of 10% $FeCl_3$ solution. Appearance of dark green or blue colour is evidence for phenolic compounds.

Flavonoids test. Into 0.5 ml of extract was added 4 ml of 1% NH_3 and then 1 ml of concentrated H_2SO_4. After a few seconds the yellow colour was indicator for positive result.

Steroids test. Acetic anhydride (2 ml) and 2 ml of H_2SO_4 was added in a test tube with a 0.5 g of the crude extract. The appearance of green or blue colour confirmed steroids in the sample.

Terpenoids (Salkowski's test) test. An extract (5 ml) and 2 ml of $CHCl_3$ were added in a test tube and then 1 ml of conc. H_2SO_4. A positive result for the terpenoids was the formation of reddish-brown coloured layer at the interface.

Cardiac glycosides (Keller Killiani test) test. 5 ml of the extracts and 2 ml of glacial acetic acid mixed in the test tube and after added 1–2 drops of 2% $FeCl_3$. To this solution 1 ml of conc. H_2SO_4 was added carefully. The presence of brown ring and the violet-green ring below, characterized cardiac glycosides.

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Coumarins test. In a test tube which contains 0.1 g of crude extract 1 ml of ethanol was added and then filtered. Afterwards, 1.5 ml of 10% NaOH was added into the filtrate. The yellow colour indicated the presence of coumarins.

Quantitative phytochemical screening

Determination of total phenolic content. The method of Singleton *et al*. (1999) with some modification was used to obtain the total phenolic content in the *Juniperus* berries extracts. The methanolic solution of the extracts (1000 µg/ml) was used in the analysis. Briefly, 0.5 ml of extract was mixed with 2.5 ml of Folin-Ciocalteu reagent (diluted 10-fold). After 1 minute, 2.5 ml of 7.5% Na_2CO_3 was added, and this mixture was incubated at 25°C for 30 minutes. Blank was prepared by mixing of 0.5 ml methanol with 2.5 ml of Folin-Ciocalteu reagent and 2.5 ml of 7.5% Na_2CO_3. Afterwards, the absorbance was recorded at 765 nm. The same method was applied for the standard solution of gallic acid (3.91–250 µg/ml) which was used to construct the calibration curve. The contents of total phenolic compounds were expressed as mg of gallic acid equivalent per g of dried weight extract (mg GAE/g extract DW).

Determination of total flavonoid content. The content of total flavonoid was estimated by Ebrahimzadeh *et al*. (2018) with a little modification. Methanolic solution of extracts 0.5 ml (1000 µg/ml) was subsequently mixed with 1.5 ml of methanol, 100 µl of 10% $AlCl_3$, 100 µl of 1M CH_3COOK and 2.8 ml of distilled water, respectively. Blank was prepared in the same way but without the extracts. After 40 min of incubation, the absorbance was read at 415 nm. The standard solution of rutin (3.91–250 µg/ml) was served for the construction of the calibration curve. Contents of total flavonoid was shown as mg of rutin equivalent per g of dried weight extract (mg RE/g extract DW).

Determination of DPPH free radical scavenging activity. Antioxidant activity of extracts was determined by DPPH (2,2-diphenyl-1-picryl-hydrazil) using the previously described method (Braca *et al*. 2001). The crude extracts were dissolved in methanol and an aliquot of 1 ml (1.96–1000 µg/ml) was taken and added 2 ml of 0.004% DPPH methanolic solution. Test tubes were slightly shaken and allowed to stand for 30 min at room temperature. The absorbance was measured at 517 nm against blank. Also, for the standard solution of ascorbic acid (1.96–1000 µg/ml) the same, the above mentioned procedure, was done. The percentage of inhibition by the extracts was determined by using the following Equation 1:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (1)$$

where: $A_{control}$ – absorbance of control solution (2 ml of DPPH radical and 1 ml of methanol); A_{sample} – absorbance of methanolic solution of tested extracts (2 ml DPPH and 1 ml of extracts solution). Using a non-linear regression equation, obtained results were shown as IC_{50} values in µg/ml

Statistical analysis. All analyses were performed in triplicate and the results shown as mean (n = 3) ± s.d. Statistical analyses were performed using a GraphPad Prism ver. 7.00 (Statistical Software Package Inc., USA). The level of probability ($P < 0.05$) was significant.

RESULTS AND DISCUSSION

Extracting yields (%) of powdered plants. The yields of the *J. communis* and *J. oxycedrus* extracts obtained per 100 g of dry plant material in different solvents are given in Table 1. As can be seen, the yields

Table 1. Percentage yields, total phenolics content, total flavonoids content and DPPH free radical scavenging activity of *J. communis* and *J. oxycedrus* extracts

Juniperus species	Extract	Yields (w/w %)	Total phenolics (mg GAE/g DW)	Total flavonoids (mg RE/g DW)	DPPH test IC_{50} (µg/ml)*
Jc	ethanol	59.80	189.82 ± 0.27	42.85 ± 0.13	28.55 ± 0.24
	ethyl acetate	19.80	144.21 ± 0.18	38.40 ± 0.24	106.44 ± 0.27
	chloroform	16.25	132.74 ± 0.13	27.11 ± 0.11	257.66 ± 0.13
Jo	ethanol	49.80	58.73 ± 0.14	21.39 ± 0.33	64.49 ± 0.23
	ethyl acetate	15.60	27.20 ± 0.08	18.43 ± 0.12	130.17 ± 0.15
	chloroform	11.40	15.68 ± 0.14	9.87 ± 0.16	475.36 ± 0.28

Jc – J. communis L.; *Jo – J. oxycedrus* L.; RE – rutin; *ascorbic acid was used as a control (IC_{50} = 3.02 ± 0.12 µg/ml)

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of dried plant extracts are increased with increasing of solvent polarity, and ranging from 11.40% (chloroform extract of *J. oxycedrus*) to 59.80% (ethanol extract of *J. communis*). The extraction with ethanol gives the highest yields (59.80% of *J. communis* and 49.80% of *J. oxycedrus*) while ethyl acetate and chloroform give the lower yields (19.80 and 16.25% for *J. communis*, 15.60 and 11.40% for *J. oxycedrus*, respectively).

Preliminary qualitative phytochemical evaluation. The results of the preliminary phytochemical screening revealed that ethanol extracts of *J. communis* and *J. oxycedrus* have the wide range of secondary metabolites (Table 2). Both extracts demonstrated the positive tests of screened phytochemicals, with exception of *J. oxycedrus* extract where the cardiac glycosides were absent. Ethyl acetate and chloroform extracted a similar phytoconstituents but in lower range. Both ethyl acetate extracts showed negative tests for saponins and cardiac glycosides. Chloroform extracts of *J. communis* and *J. oxycedrus* were absent in tannins, saponins and cardiac glycosides, while *J. oxycedrus* extract also showed negative test for coumarins. The obtained results for ethyl acetate and chloroform extract of *J. communis* are in agreement with previously reported (Fernandez *et al*. 2016) and the only difference is the absence of saponins in our case. There was no literature data of phytochemical evaluation of ethanol, ethyl acetate and chloroform extracts of *J. oxycedrus* berries. Alkaloids, steroids, terpenoids, phenolics, and flavonoids were present in all tested extracts.

Quantitative phytochemical analysis

Total phenolic content. Phenolic components represent the largest class of secondary metabolites which are known as exceptional antioxidants and their antioxidant activity is based on their redox potential (Marimuthu *et al*. 2008). They include phenolic acids, flavonoids, tannins, stilbenes, curcuminoids, coumarins, lignans, quinones and others. Various bioactivities of phenolic compounds are responsible for their biological properties like antimicrobial, anticarcinogenic, anti-inflammatory and therapeutic effects. The content of total phenolics is ranged from 15.68 ± 0.14 to 189.82 ± 0.27 mg GAE/g extract DW (Table 1). The highest concentrations were in ethanolic extracts of *J. communis* (189.82 ± 0.27 mg GAE/g extract DW) and *J. oxycedrus* (58.73 ± 0.14 mg GAE/g extract DW). Also, it is evident that the extracts of *J. communis* have a substantially higher amount of phenolics than *J. oxycedrus* extracts. Ethyl acetate and chloroform extracts contain a significantly lower total phenolic contents compared to ethanol extracts. The total phenolic content depends on polarity of used solvent (Quy-Diem *et al*. 2014; Hafiza *et al*. 2017). The obtained results showed that the content of total phenolic decrease in order: ethanol > ethyl acetate > chloroform extract. In many previous reported studies the total phenolics content of *J. communis* and *J. oxycedrus* berries in ethanol, methanol, hexane, acetone and aqueous extracts has been determined (Miceli *et al*. 2009; Orhan *et al*. 2011; Öztürk *et al*. 2011; Taviano *et al*. 2013). The obtained results results very well corresponded with the literature data for *J. communis* and *J. oxycedrus* ethanolic extracts (Orhan *et al*. 2011), while the total content of phenols in ethyl acetate and chloroform extracts could not be compared with the previously mentioned studies, mainly due to various solvents which were used during the extraction process.

Total flavonoid content. Flavonoids are phenolic compounds with numerous biological properties such as hepatoprotective, antithrombotic, antiin-

Table 2. Qualitative phytochemical screening of *J. communis* and *J. oxycedrus* extracts

Juniperus species	Extract	Phytochemicals								
		alkaloids	tannins	saponins	phenolics	flavonoids	steroids	terpenoids	cardiac glycosides	coumarins
Jc	ethanol	+	+	+	+	+	+	+	+	+
	ethyl acetate	+	+	–	+	+	+	+	–	+
	chloroform	+	–	–	+	+	+	+	–	+
Jo	ethanol	+	+	+	+	+	+	+	–	+
	ethyl acetate	+	+	–	+	+	+	+	–	+
	chloroform	+	–	–	+	+	+	+	–	–

Jc – J. communis L.; *Jo – J. oxycedrus* L.; $^{+}$present; $^{-}$absent

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flammatory and antiviral activity which associated to their antioxidant capability (Akhtar *et al.* 2015; Spagnuolo *et al.* 2018). They are widely distributed in the plant kingdom and are present in considerable amounts in fruits, vegetables and spices, have been used to prevent many human diseases, such as diabetes, cancers and coronary heart diseases. The concentration of flavonoids in tested extracts ranged from 9.87 ± 0.16 to 42.85 ± 0.13 mg RE/g extract DW (Table 1). Ethanolic extracts of *J. communis* and *J. oxycedrus* contain the highest flavonoids content (42.85 ± 0.13 and 21.39 ± 0.33 mg RE/g extract DW, respectively). The lowest flavonoids concentration was observed in ethyl acetate and chloroform extract. The flavonoids content has been determined in many literature data (Miceli *et al.* 2009; Öztürk *et al.* 2011; Orhan *et al.* 2011) and our results obtained for *J. communis* and *J. oxycedrus* ethanolic extracts are comparable with those reported (Orhan *et al.* 2011), wherein a greater amount of total flavonoids was recorded than other above mentioned literature data. Results obtained for the total content of flavonoids for the remaining tested extracts (ethyl acetate and chloroform) could not be compared with the previous studies because of different extracts. The content of flavonoids is less than phenolic compounds in all tested extracts.

DPPH free radical scavenging activity. Antioxidant activity, particularly radical scavenging activities, is very important due to the deleterious role of free radicals in foods and in biological systems. Plants rich in secondary metabolites, including phenolics and flavonoids, have antioxidant activity because of their redox properties and chemical structures. The DPPH screening is one of the inevitable tests when determining the antioxidant activity of the extracts. The results showed a remarkable antioxidant effect of extracts of berries of both plants. Is evident that the extracts of more polar solvents possess higher antioxidant activity, as well as that the activity of *J. communis* extracts are significantly higher than the corresponding *J. oxycedrus* extracts. The DPPH radical scavenging activity of various extracts of *J. communis* and *J. oxycedrus* are shown in Figure 1. Due to the great activity of tested extracts, the IC_{50} values were calculated (Table 1). The greatest ability to neutralize DPPH radicals was found for ethanolic extracts for both plants. Their IC_{50} values are 28.55 ± 0.24 and 64.49 ± 0.23 µg/ml, for *J. communis* and *J. oxycedrus*, respectively. Ethyl acetate extracts of *J. communis* (106.44 ± 0.27 µg/ml) and *J. oxycedrus*

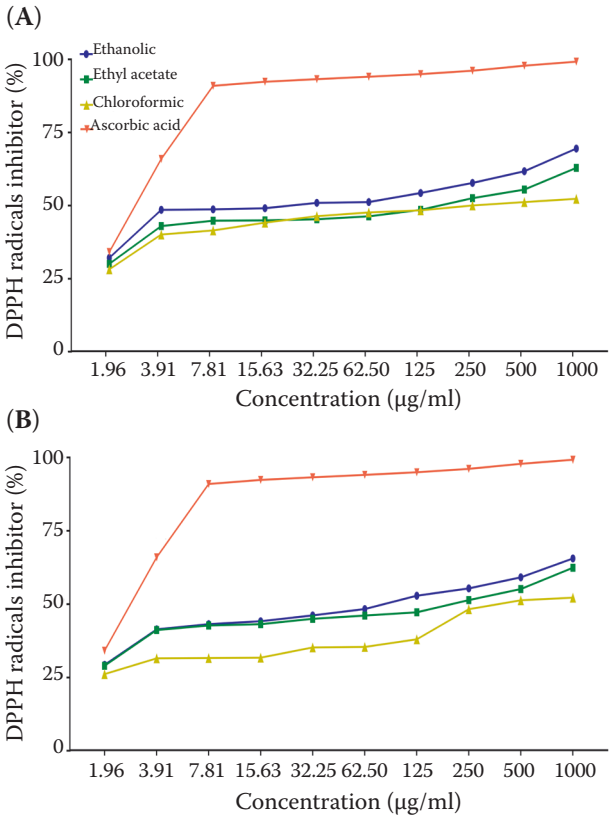

Figure 1. DPPH radical scavenging activity of ethanol, ethyl acetate and chloroform extracts: (**A**) *J. communis* and (**B**) *J. oxycedrus*

Ascorbic acid was used as positive control

(130.17 ± 0.15 µg/ml) also possess significant capacity, while the scavenging activity of chloroform extracts is moderate with IC_{50} values of 257.66 ± 0.13 µg/ml (*J. communis*) and 475.36 ± 0.28 µg/ml (*J. oxycedrus*). It can be noticed that the *J. communis* possess significantly higher antioxidant activity than *J. oxycedrus*. Also IC_{50} value for ascorbic acid was performed in order to obtain a better picture of effectivity of the tested extracts. There have been diverse reports on antioxidant activity of a number of *Juniperus* species (Miceli *et al.* 2009; Orhan *et al.* 2011; Öztürk *et al.* 2011; Taviano *et al.* 2013). Obtained results for tested ethanolic extracts of *J. communis* and *J. oxycedrus* displayed similar DPPH radical scavenging activity as previously reported (Orhan *et al.* 2011), and higher antioxidant activities than other above reported studies. This could be due to the differences in extraction procedures and used solvents.

Correlation between antioxidant activity and total phenolic and total flavonoid content. A significant correlation between the antioxidant activity

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and the total phenolic, as well the total flavonoid contents was found with a correlation coefficient $r = -0.879$ in *J. communis*, $r = -0.809$ in *J. oxycedrus*, $r = -0.997$ in *J. communis* and $r = -0.996$ in *J. oxycedrus*, respectively (Figure 2).

The correlation coefficient of *J. communis* extracts for total phenol contents with its antioxidant activity was moderate high, while the phenol contents of *J. oxycedrus* extracts was slightly lower which proved that the phenolic contents of *J. communis* and *J. oxycedrus* berries extract attributed a lot to their antioxidant activities. Regarding the correlation coefficients of *J. communis* and *J. oxycedrus* extracts when we compare antioxidant activity and total flavonoids, they were significantly high and showed an excellent correlation with antioxidant activities. A large number of studies showed an excellent correlation between the total content of phenolic compounds and antioxidant activities in different plant extracts (Guedes Mayara *et al*. 2017).

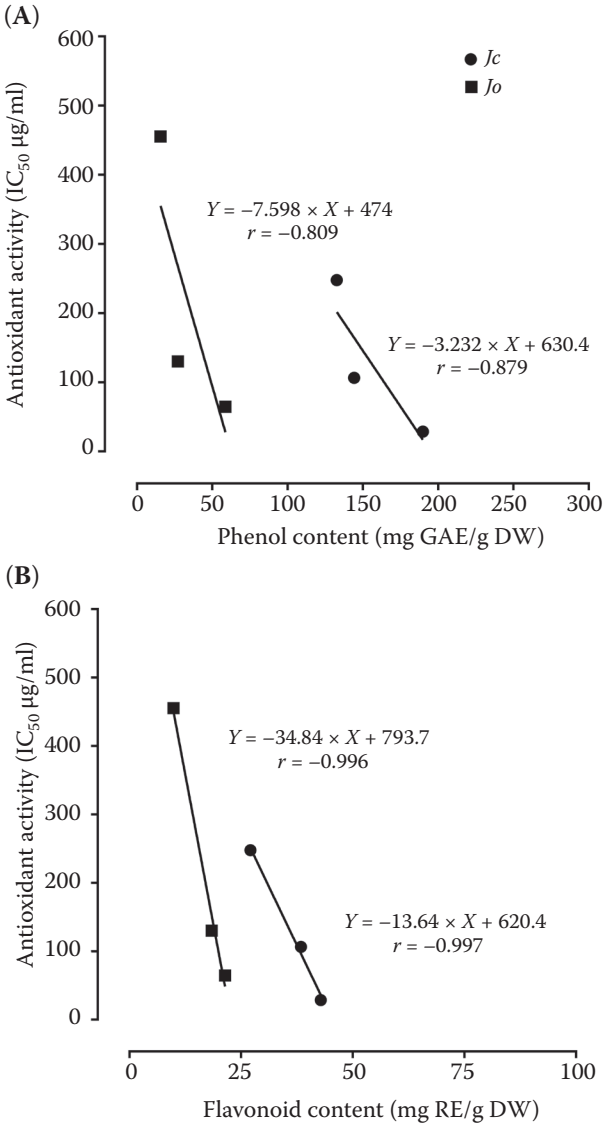

Figure 2. Linear correlation between antioxidant activity and total phenolic (**A**) or flavonoid contents (**B**)
Jc – J. communis; *Jo – J. oxycedrus*; the correlation analyses were described as correlation coefficient (*r*)

CONCLUSION

The obtained results are very important for further research of *J. communis* and *J. oxycedrus* berries and for even wider therapeutical use. Ethanol and ethyl acetate extracts possess strong antioxidant activity for both *Juniperus* species, where they showed a high potential as natural sources of antioxidant activity which may be used in the food industry as preservative agents or to extend the shelf-life of raw and processed foods. Also, there was a significant correlation between phenolic and flavonoid content on the one, and antioxidant activity on the other side, for all tested extracts. The obtained results in this study contribute to the ethnopharmacological use of these *Juniperus* species and also could be suitable for applications in the food industry.

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