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PHENOLIC CONTENT IN THE NEEDLES OF *CRYPTOMERIA JAPONICA* (THUNB. EX L. F.) D. DON, *CUPRESSOCYPARIS* × *LEYLANDII* (A.B. JACKS. & DALLIM.) DALLIM. "CASTLEWELLAN GOLD" AND *SEQUOIADENDRON GIGANTEUM* (LINDL.) J. BUCHHOLZ

Sadržaj fenolnih jedinjenja u iglicama *Cryptomeria japonica* (Thunb. ex L. f.) D. Don, *Cupressocyparis* × *leylandii* (A.B. Jacks. & Dallim.) Dallim. "Castlewellan Gold" i *Sequoiadendron giganteum* (Lindl.) J. Buchholz

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Abstract

Various phenolic compounds can be found in a gymnosperms and have been related to their bioactive properties, esspecially as a allelochemicals. Total phenol, flavonoid (flavone and flavonol) and protoanthocyanindin content was estimated quantitatively by using spectrophotometric method in the needle methanol extracts of mature *Cryptomeria japonica*, *Cupressocyparis* × *leylandii* „Castewellan Gold" and *Sequoiadendron giganteum* individuals. Although there is a strong intraspecific variability on the basis of the studied group of compounds, Duncan's test showed that *C. japonica* is clearly distinguishable from the other two taxa analyzed, and in particular on the basis of total proanthocyanidins and phenolics content. On the other hand, *S. giganteum* and *C.* × *leylandii* only differ on the basis of the flavonoid content calculated in terms of quercetin equivalent. In all three taxa proanthocyanidins had the highest variability. The ratios and relatively high content of analyzed phenolic compounds for all three studied taxa indicate that they may be considerd as a potential both chemotaxonomic characters and valuable sources of antioxidants, which should be confirmed by further researchs.

***Key words**: Cupressaceae, giant sequoia, gymnosperm, Leyland cypress, phenolic compounds, spectrophotometry, sugi*

INTRODUCTION - *Uvod*

Phenolics, heterogeneous group of natural substances, poses at least one aromatic ring with one or more hydroxyl groups attached, and play a major role in the plant defence against herbivores, pathogens, microbial infections and UV radiation, as attractants for pollinators and seed-dispersing animals, in pigmentation, and affect

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microbial decomposers. Since they are structurally diverse with various amounts in plant tissues, and some of them are distributed among a very limited number of plants species, they can be diagnostic in chemotaxonomic studies. Also, they are very interesting for humans since they have a large ecological, agricultural, medical and pharmaceutical importance (LEBRETON 1990, WINK 1999, BÄRLOCHER AND GRAÇA 2005, CROZIER ET AL. 2006, LATTANZIO ET AL. 2006, VERMERRIS AND NICHOLSON 2008, YANG ET AL. 2012, GURAV ET AL. 2013).

Family Cupressaceae, one of the largest and the most widely distributed of all conifer families, have 28 genera with 142 species occurring in diverse habitats on all continents (EARLE 2013). Many of Cupressaceae species produce valuable timber and essential oils, but also many have major importance in the ornamental plantings and environmental forestry (JANJIĆ 1966, 2002; BONNER AND KARRFALT 2008, MIGUEL 2010, JOSHI AND SATI 2012, WINK 2012, EARLE 2013). As the other gymnosperms, Cupressaceae species are adapted to diverse ecological habitats and produce, as a component of their survival and defence strategies, a wide range of secondary metabolites, especially terpenoids and various phenolic compounds (ROMANI ET AL. 2002, BAGAL ET AL. 2012).

Cryptomeria japonica (Thunb. ex L. f.) D. Don, *Cupressocyparis* × *leylandii* (A.B. Jacks. & Dallim.) Dallim. "Castlewellan Gold" and *Sequoiadendron giganteum* (Lindl.) J. Buchholz, all from Cupressaceae family, are cultivated for landscaping, production of fragrant wood and lumber (VIDAKOVIĆ AND FRANJIĆ 2004, BONNER AND KARRFALT 2008). Also, different parts of these taxa posses various secondary metabolites (essential oils, flavones, flavonoids, lignans, polyprenols, terpenes) with various bioactive properties (CASTRO ET AL. 1996, KOFUJITA ET AL. 2002, ROMANI ET AL. 2002, GUT 2008, SHYUR ET AL. 2008, JOSHI AND SATI 2012, CHENG AND CHANG 2014 and references therein).

The objective of this study was to investigate total phenol, flavonoid (flavone and flavonol) and proanthocyanidin concentrations in the needle's methanol extracts of *Cryptomeria japonica*, *Cupressocyparis* × *leylandii* "Castlewellan Gold" and *Sequoiadendron giganteum.*

MATERIAL AND METHODS - *Materijal i metode*

Collection of plant material and preparation of extracts

The needles were collected in October 2013 from five mature individuals of *C. japonica* and *S. giganteum* at the Arboretum "Slatina" in Sarajevo and of *C.* × *leylandii* "Castlewellan Gold" from Sarajevo's urban area. Voucher specimens have been deposited in the herbarium of the Faculty of Forestry, University of Sarajevo. The plant material was dried at 50-60 °C for 48h and then milled. Forty milligrams of the samples were extracted with 4 mL of 80% methanol, and kept next 24h at +4 °C

(VERMERRIS AND NICHOLSON 2008). Extracts then were centrifuged at 2,000 rpm for 15 min. The obtained supernatants were used for spectrophotometric analysis.

Reagents

All reagents used in the experiment were of the highest grade commercially available by Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Determination of total phenols

Total phenol content in the methanol extracts was determined by the modified Folin-Ciocalteu method (WOLFE ET AL. 2003). An extract aliquot was mixed with 100 μL of Folin-Ciocalteu reagent and 300 μL of fresh prepared 7.5% sodium carbonate. The tubes were vortexed for 15 s and allowed to stand for 30 min at 45 °C for colour development. Absorbance was then measured at 765 nm using the VP1012 Jouan spectrophotometer. Total phenolic content was calculated as mg catechin equivalent/ g of dry weight (mg CE/g) using the following equation based on the calibration curve: $y = 0.0136x$, $R^2 = 0.9922$, where x was the absorbance and y was the catechin equivalent (mg/g).

Determination of total flavonoids using catechin as a standard

Total flavonoid concentrations were estimated by modified ORDOÑEZ ET AL. (2006) method. An extract aliquot was mixed with 25 μL of 10% $AlCl_3$ and 25 μL of 1M sodium acetate water solutions, and vortexed for 15 s. After 20 min at room temperature (24 °C), the absorbance was measured at 415 nm. Total flavonoid content was expressed as mg catechin equivalent/ g of dry weight (mg CE/g) using the following equation based on the calibration curve: $y = 0.0473x$, $R^2 = 0.9711$, where x was the absorbance and y was the catechin equivalent (mg/g).

Determination of total flavonoids using quercetin as a standard

Total flavonoids also were estimated using the modified CHANG ET AL. (2002) and MĂRGHITAŞ ET AL. (2007) methods. To extract aliquot 20 μL of 10% $AlCl_3$ and 20 μL of 1M sodium acetate methanol solutions were added. After homogenization and incubation at room temperature (24 °C) for 30 min, the absorption at 415 nm was read. Total flavonoid content was calculated as mg quercetin equivalent/ g of dry weight (mg QE/g) using the following equation based on the calibration curve: $y = 0.0182x$, $R^2 = 0.9711$, where x was the absorbance and y was the quercetin equivalent (mg/g).

Determination of total proanthocyanidins

Determination of total proanthocyanidins was done by modified vanillin-HCl assay (WETTSTEIN ET AL. 1977, SUN ET AL. 1998). A methanol extract solution was mixed with 750 μL of 4% vanillin-methanol solution and 375 μL of hydrochloric acid. The absorbance was then immediately measured at 500 nm. Total proanthocyanidin contents were expressed as mg catechin equivalent/ g of dry weight (mg CE/g) using

the following equation based on the calibration curve: y = 0.1246x, R^2 = 0.9835, where x was the absorbance and y was the catechin equivalent (mg/g).

Statistical analysis

The obtained results were expressed as mean ± standard deviation (SD) of three replicates. The data were subjected to one-way analysis of variance (ANOVA), and differences between samples were determined by Duncan's multiple range test using the SPSS program (ver. 15.0). p values <0.05 were regarded as significant and p values <0.01 as very significant.

RESULTS AND DISCUSSION - *Rezultati i diskusija*

The presence of phenolic compounds in conifers' needles is relatively well documented (RIFFER AND ANDERSON 1967, HUTZLER ET AL. 1998, SINGH ET AL. 1999, CARNACHAN AND HARRIS 2000, ROMANI ET AL. 2002, IVANESCU AND GOSTIN 2008, VERMERRIS AND NICHOLSON 2008), but much more attention is paid to the terpenoids and essential oils, and their applications (ALONSO ET AL. 2002, MIGUEL 2010, JOSHI AND SATI 2012). The family Cupressaceae is no exception.

The total phenol, flavonoid (using catechin/quercetin as a standards) and proanthocyanidin contents in the needle's methanol extracts of analyzed taxa were determined in this study (Table 1). The mean concentrations of total phenols and proanthocyanidins were almost two fold higher in *C. japonica* (14.93/15.22 mg CE/g) as for *S. giganteum* and *C.* × *leylandii* (8.18/7.31 and 8.47/6.37 mg CE/g respectively), while *C.* × *leylandii* had two fold lower mean content of total flavonoids calculated in terms of quercetin equivalent (0.24 mg QE/g) than *C. japonica* and *S. giganteum* (0.47 and 0.55 mg QE/g respectively). Mean total flavonoid content, calculated in terms of catechin equivalent, for *C. japonica* (0.52 mg CE/g) was slightly lower than for *C.* × *leylandii* and *S. giganteum* (0.70 and 0.73 mg CE/g respectively).

Analysis of variance indicated the presence of significant differences between the taxa analyzed (p<0.01). The presence of intraspecific variability, especially of total proanthocyanidin contents, in all three analyzed taxa was confirmed by Duncan's test. Duncan's test also confirmed both that *C. japonica* differ from *S. giganteum* and *C.* × *leylandii* based on the concentrations of total proanthocyanidins, phenols and flavonoids calculated in terms of catechin equivalent, and that *C.* × *leylandii* differ from *S. giganteum* and *C. japonica* based on total flavonoid contents calculated in terms of quercetin equivalent. Canonical discriminant analysis showed that there is a clear differentiation of taxa analyzed (Figure 1). The first canonical function took over 74.82%, and significant discriminatory characters are total concentrations of proanthocyanidins, phenols and flavonoids calculated in terms of catechin equivalent, which implies that analyzed parameters may be used as chemotaxonomic characters, but applied to a larger number of individuals and populations for better accuracy.

Table 1. Basical descriptive parameters of phenolics for species analyzed (SD – standard deviation, CV – coefficient variation)

Tabela 1. Osnovni deskriptivni parametri fenolnih jedinjenja za istraživane vrste (SD – standardna devijacija, CV – koeficijent varijacije)

Species		Phenols (mg CE/g)*	Flavonoids (mg CE/g)	Flavonoids (mg QE/g)**	Proanthocyanidins (mg CE/g)
Cryptomeria japonica	Min	7.78	0.33	0.29	11.36
	Max	22.75	0.86	0.97	19.66
	X	14.93	0.52	0.47	15.22
	S. D.	4.49	0.16	0.17	2.45
	C.V. (%)	30.05	30.84	36.74	16.10
Cupressocyparis × leylandii	Min	4.32	0.38	0.10	1.64
	Max	18.91	1.18	0.40	12.11
	X	8.47	0.70	0.24	6.37
	S. D.	4.50	0.24	0.09	3.28
	C.V. (%)	53.10	34.23	37.29	51.60
Sequoiadendron giganteum	Min	2.69	0.41	0.30	4.26
	Max	15.46	1.15	1.10	14.80
	X	8.18	0.73	0.55	7.31
	S. D.	3.73	0.25	0.21	3.71
	C.V. (%)	45.55	34.14	37.90	50.71

Note: * - mg catechin equivalent/g dry weight; ** - mg quercetin equivalent/g dry weight

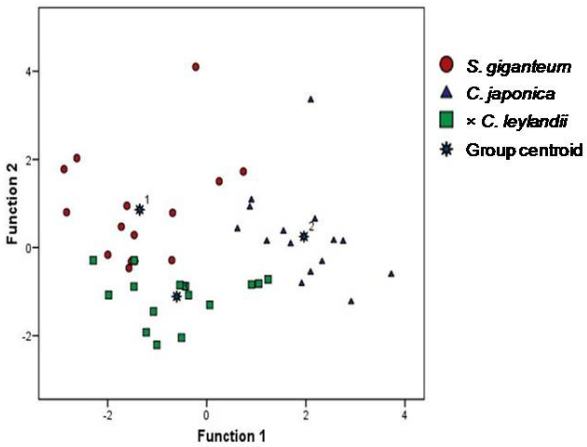

Figure 1. Canonical discriminant analysis

Slika 1. Kanonička diskriminaciona analiza

According to the available literature, in the needles of *C. japonica* is detected the presence of diterpenes, essential oils, flavones, flavonoids, lignans, monoterpenes, polyprenols, and sesquiterpenes with different antimicrobial and antifungal activity (APPLETON ET AL. 1970, IBATA ET AL. 1984, PERRY AND WEAVERS 1985, NAGAHAMA ET AL. 1993, SU ET AL. 1993, SU ET AL. 1995a, SU ET AL. 1995b, CASTRO ET AL. 1996, CASTILO ET AL. 2012). In the bark KOFUJITA ET AL. (2002) are determined various terpenoids with antifungal and cytostatic activities, while sugi's wood is very rich with lignans and norlignans, which give it specific colour and have a role in the defence against microbial invasions (CASTRO ET AL. 1996, YOSHIDA ET AL. 2004, KAZUMASA ET AL. 2007, SHYUR ET AL. 2008). In the needles of intergeneric taxon *C. × leylandii* are detected monoterpenes, sesquiterpenes and biflavones (SCHEFFER ET AL. 1980, HANSON 2007). In *S. giganteum* needles are found volatile oils and lignans (LEVINSON ET AL. 1971, CASTRO ET AL. 1996), in wood norlignans (HENLEY-SMITH AND WHITING 1976, CASTRO ET AL. 1996), and in cones phenol phloroglucinol (GUT 2008).

According to HATCHER (1990, and references therein) and GIERTYCH ET AL. (1999), concentrations of phenolics and tannins are usually slightly higher in the elderly than in younger conifers' leaves (*Pinus sylvestris*, *P. nigra*, *P. leucodermis*, *Picea abies*, *Tsuga heterophylla*), similarly to the deciduous species. However, there is evidence that in some conifers (eg. *Pseudotsuga menziesii*) phenol concentrations are higher in the buds than in developed needles. As the causes of this variation are listed: cell's compartmentalization and age (especially autotoxicity), impact of various environmental stresses (drought, air pollution, soil contamination, fire, etc.), canopy damage, mineral composition of the soil (especially N, Mg, Fe, P and S), and the application of different both methods for determination and standards for phenolics analysis (HATCHER 1990, GIERTYCH ET AL. 1999, ALONSO 2002). Additionally, because of the influence of different microbial organisms, pathogens, herbivores and other plant species, many conifers express the allelopathic effect, by increasing the production of certain, usually specific to them, phenolic compounds (SINGH ET AL. 1999).

Because of the interesting outcomes, it will be required in the future investigations to increase the number of studied individuals, populations and species within the family Cupressaceae; to apply methods for the quantification of isolated phenolic compounds; to verify their bioactive properties; and to monitor the temporal dynamics as well as changes in the concentration of phenolic compounds in young and old leaves.

CONCLUSIONS - *Zaključci*

Gymnosperms, very valuable forestry and ornamental trees, also have a medicinal value due to the presence of different phytoconstituents. Our results show that *Cryptomeria japonica*, *Cupressocyparis* × *leylandii* "Castlewellan Gold" and *Sequoiadendron giganteum* possess relatively high values of total phenols and proanthocyanidins. The ratios and relatively high content of analyzed phenolic compounds for studied taxa indicate that they may be considerd as a potential both chemotaxonomic characters and valuable sources of antioxidants.

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SAŽETAK

Prisustvo različitih fenolnih jedinjenja kod četinara je dosta dobro dokumentirano, ali se mnogo više pažnje posvećivalo izučavanju terpenoida i esencijalnih ulja te njihovoj primjeni. Porodica Cupressaceae nije izuzetak. Ukupni sadržaj fenola, flavonoida (flavona i flavonola) izražen preko katehina/kvercetina kao standarda, te proantocijanidina u metanolnim ekstraktima iglica zrelih stabala *Cryptomeria japonica*, *Cupressocyparis* × *leylandii* „Castewellan Gold“ i *Sequoiadendron giganteum* je kvantitativno određen spektrofotometrijskim putem. Srednje vrijednosti očitanih koncentracija ukupnih fenola iznosile su 8.18 i 8.47 mg CE/g DW za *S. giganteum* i *C.* × *leylandii*, dok su za *C. japonica* bile skoro dvostruko veće (14.93 mg CE/g DW). Srednje vrijednosti koncentracija ukupnih flavonoida izraženih preko katehina, kao standarda, kod *S. giganteum* (0.73 mg CE/g DW) i *C.* × *leylandii* (0.70 mg CE/g DW) su isto bile dosta ujednačene ali i nešto veće nego kod *C. japonica* (0.52 mg CE/g DW). Srednje koncentracije ukupnih flavonoida izraženih preko kvercetina, kao standarda, su bile dosta ujednačene za *S. giganteum* (0.55 mg QE/g DW) i *C. japonica* (0.47 mg QE/g DW), dok su kod *C.* × *leylandii* bile upola manje (0.24 mg QE/g DW). Srednje vrijednosti ukupnih proantocijanidina za *S. giganteum* i *C.* × *leylandii* (7.31 i 6.37 mg CE/g DW) su bile dosta bliske ali i dvostruko manje od očitanih vrijednosti za *C. japonica* (15.22 mg CE/g DW). Iako postoji izražena intraspecijska varijabilnost na osnovu istraživanih grupa jedinjenja, Duncanov test je pokazao da se *C. japonica* jasno razlikuje od druge dvije analizirane svojte, i to naročito na osnovu ukupnih proantocijanidina i fenola. S druge strane, *S. giganteum* i *C.* × *leylandii* su se jedino međusobno razlikovale na osnovu ukupnih koncentracija flavonoida izraženih preko kvercetina kao standarda. Kod sve tri ispitivane svojte proantocijanidini su imali najveću varijabilnost. Odnos i relativno visok sadržaj analiziranih fenolnih jedinjenja kod istraživanih svojti ukazuje na to da se oni mogu smatrati potencijalnim hemotaksonomskim karakterima i vrijednim izvorima antioksidanata, što bi se trebalo potvrditi daljnjim istraživanjem. Dobiveni podaci ukazuju da bi u budućim istraživanjima bilo potrebno povećati broj individua, populacija i vrsta unutar porodice Cupressaceae; primjeniti metode za kvantifikaciju izoliranih fenolnih jedinjenja; procijeniti njihova bioaktivna svojstva; te pratiti temporalnu dinamiku i promjene koncentracija fenolnih jedinjenja u mladim i starim listovima.