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Quantitative HPLC Analysis of Catechin in Wound-Associated Wood and Knots of Beech

Kvantitativna HPLC analiza katehina u ranjenom dijelu i kvrgama bukova drva

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ABSTRACT • *The aim of this research was to examine the content of catechin in normal and traumatic structures of beechwood by high performance liquid chromatography (HPLC). Two discs were sawn from each of six harvested trees. The first disc was taken from the lower, wounded part and the second one from the upper part of each stem. Discs containing the bases of living and dead branches were taken from the crown. Samples of wound-wood, reaction zones, sapwood, as well as living and dead knots, were sampled from discs. Samples were milled and then extracted sequentially by cyclohexane and methanol/water in a Soxhlet apparatus. A method for the separation of catechins from extracts was developed for the present chromatographic investigation. Statistical analysis did not reveal significant differences in total or hydrophilic extractives, nor in the content of catechin among the investigated trees. The content of hydrophilic extractives and catechin were significantly different among the investigated categories of wood tissues. Wound-wood and knot extracts contained the highest amount of this flava-3-ol. Reaction zones contained higher amounts of catechin than discolored wood, but less than sapwood. The extracts of discolored wood showed the lowest amounts of catechin. Sapwood samples that originated from wounded discs exhibited significantly higher contents of catechin than normal sapwood from upper discs. Accumulation of bioactive compound catechin in wound-wood, sapwood and knots is considered to be an important part of the survival strategy of living trees.*

Keywords: *catechin, liquid chromatography, wound-wood, knots, Fagus sylvatica*

SAŽETAK • *Cilj istraživanja bio je ispitati sadržaj katehina u normalnim i traumatskim strukturama bukovine primjenom tekućinske kromatografije (HPLC). Od šest bukovih stabala ispiljena su po dva diska. Prvi je disk uzet iz donjega, ranjenog dijela stable, a drugi od gornjeg dijela svakog stabla. Diskovi koji su sadržavali baze živih i mrtvih grana uzeti su od krošnje. Uzorci ranjenog dijela drva, reakcijskih zona, bjeljike, kao i živih i mrtvih kvrga, izrađeni su od pripremljenih diskova. Ispiljeni su uzorci sekvencijalno ekstrahirani cikloheksanom i smjesom metanola i vode u Soxhlet aparatu. Metoda za odvajanje katehina iz ekstrakata razvijena je za potrebe ovoga kromatografskog istraživanja. Statistička analiza dobivenih podataka nije pokazala značajne razlike između ukupnih i hidrofilnih ekstraktiva, niti u sadržaju katehina među istraživanim stablima. Sadržaj hidrofilnih ekstraktiva i katehina značajno se razlikuje u ispitivanim vrstama drvnog tkiva. Ekstraktivi iz ranjenog drva i kvrga sadržavali*

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su najveću količinu flava-3-ola. Reakcijsko drvo sadržava veće količine katehina nego drvo s diskoloracijom, ali manje nego drvo bijeljike. Ekstraktivi drva s diskoloracijom imali su najmanje količine katehina. Uzorci od drva bjeljike izrađeni od diskova ranjenog drva imali su značajno veći sadržaj katehina nego uzorci normalnog drva bjeljike izrađeni od diskova od gornjeg dijela trupca. Akumulacija bioaktivnih spojeva katehina u ranjenom drvu, drvu bjeljike i kvrgama smatra se važnim dijelom strategije preživljavanja stabala.

Ključne riječi: *katehin, tekućinska kromatografija, ranjeno drvo, kvrge, Fagus sylvatica*

1 INTRODUCTION
1. UVOD

Utilization of hardwood has been one of the main industrial and research challenges of the European wood sector in recent years. European beech (*Fagus sylvatica* L.) is an economically important tree species in the Dinaric region of south-eastern Europe. Beech wood has a favorable density and relatively homogenous structure, satisfactory workability and exceptionally good steam bending properties, which makes beechwood an all-purpose wood with a wide spectrum of applications, ranging from furniture, especially curved and turned parts of chairs, parquet, boats, toys, textile weaving shuttles, tool handles, piano parts, railway sleepers, veneer, plywood, particle and fiber boards, pulp and paper to food containers, because it does not impart taste or odor (Torelli, 1994). One of the main deficiencies of this tree species is its tendency to develop discolored wood in the central part of the tree, often called red heart, red heartwood, facultatively colored heartwood, false heartwood or red core (Bosshard, 1974; Torelli, 1984; Shigo, 1986; Sachsse, 1991; Wernsdörfer *et al.*, 2005). As opposed to normal wood, discolored beech wood is characterized by its unfavorable technological properties, including hard impregnation, problems in drying processes and veneer production and esthetic insufficiency (Koch *et al.*, 2000; Pöhler *et al.*, 2006). In addition, wood of trees from unevenly managed forests is regularly subjected to mechanical wounding, which results in a relatively predictable response of the tree. This includes the development of discoloration and eventual decay, accompanied by the formation of reaction zones, which separate compromised and sound sapwood, and the formation of wound-wood, which attempts to overgrow the wound. These compartmentalization barriers that try to block spreading of negative consequences of wounding towards intact and vital tissues, are generally described by anatomical alterations and cell necrosis accompanied with the accumulation of antimicrobial compounds and formation of polyphenol deposits (Shortle, 1979; Bauch, 1984; Shigo, 1986; Torelli *et al.*, 1994; Schwarze and Baum, 2000; Dujesiefken *et al.*, 2005; Dujesiefken and Liese, 2006; Oven *et al.*, 2008; Vek *et al.*, 2013a).

It has been recently showed by spectrophotometric analysis that the content of total phenols, flavonoids and proanthocyanidins is markedly lower in red heart than in reaction zones and wound-wood extracts (Vek *et al.*, 2013a; 2013b). It was reported that differences in the content of total phenols in reaction zones indicate differences in their formation process, differences in alterations to surrounding tissues and in the characteristics of individual trees (Vek *et al.*, 2013b). It was demonstrated that fungicidal properties on wood decaying fungi can be ascribed to extracts of wound-wood, as well as to that of healthy sapwood of beech (Vek *et al.*, 2013a). Recent chromatographic research on hydrophilic extractives of discolored and normal wood demonstrated that catechin is the dominant phenolic compound in beechwood (Baum and Schwarze, 2002; Koch *et al.*, 2003; Zule and Može, 2003; Hofmann *et al.*, 2004; 2008; Jamalirad *et al.*, 2011; 2012), but there is no information on the occurrence of catechin in wound-associated beechwood. As catechin has proven antimicrobial and fungistatic properties and it is also considered as relevant antioxidant, this flavonoid can potentially be applied in the field of wood preservatives or as technical antioxidant (Malterud *et al.*, 1985; Feucht *et al.*, 1994; Choi *et al.*, 2001; Mantani *et al.*, 2001; Baum and Schwarze, 2002; Hsu *et al.*, 2007; Yen and Chang, 2008; Rosales-Castro *et al.*, 2012).

The aim of our research was to examine the content of catechin in normal and traumatic structures of beechwood, using high performance liquid chromatography (HPLC). A chromatographic method was developed for this purpose and samples of wound-wood, reaction zones, sapwood, as well as living and dead knots, were removed from mechanically wounded trees. Knots were included because there are parallels in the function of these tissues after branch breakage (Willför *et al.*, 2003a) and wound-associated tissues.

2 MATERIAL AND METHODS
2. MATERIJAL I METODE

2.1 Chemicals
2.1. Kemikalije

Methanol (HPLC grade) was provided by J.T. Baker (Deventer, Netherlands), (+)-catechin (analytical standard) and formic acid (Puriss. p.a., ~ 98 %) were purchased from Sigma-Aldrich (Steinheim, Germany), while cyclohexane (99 %) was supplied by Carlo Erba Reagents (Val de Reuil, France). Ultrapure water was produced with a Millipore water purification system A10 (Billerica, USA), which was kindly provided by the Department of Forest Ecology of the Slovenian Forestry Institute.

2.2 Material
2.2. Materijal

Samples included in the present investigation were obtained from six adult, mechanically wounded beech trees. They were selected and felled in the forest area of Kočevski Rog in southern Slovenia. Two sample discs, approximately 10 cm thick, were sawn from each harvested tree. The first disc was taken from the lower, wounded part and the second one from the upper part of each stem. Discs containing both wound-associated tissues and abiotic discoloration were thus obtained. Furthermore, discs containing the bases of living and dead branches were taken from the crown region of individual trees. These samples will be referred to as knots (Torelli, 1990). Wood blocks of wound-wood (W), intact sapwood (S), reaction zone (RZ), discolored wood (DW) and living (LK) and dead knots (DK) were sawn from the sample discs and ground by a Retsch ZM 200 centrifugal mill (Haan, Germany), producing particles that passed through a 0.5 mm sieve (35 mesh screen). Heating of samples was prevented by applying dry ice during the milling process. The obtained wood meals were stored in a cool dark place until further processing. Sixty samples, ten per trees, were thus prepared for subsequent extraction and chromatographic analysis.

2.3 Extraction
2.3. Ekstrakcija

Wood and knot samples were extracted in two steps in a Soxhlet apparatus. Lipophilic compounds, which can have a deleterious effects on chromatographic instrumentation due to column clogging (Slanina and Glatz, 2004), were removed from 2.5 g of wood sample using 250 ml of cyclohexane for 4 hours. Hydrophilic extractives and catechin were subsequently extracted for 6 hours with 250 ml of methanol/water mixture (95:5, v/v). The extraction procedure was defined in a preliminary experiment. Total extractives and the content of hydrophilic extractives were determined gravimetrically, whereby the results were expressed in percentage of dry matter (%) and in milligrams per gram of dry wood ($mg \cdot g^{-1}$), respectively.

2.4 HPLC analysis
2.4. HPLC analiza

The chromatographic separation of catechin was performed on a Thermo Fischer Scientific Accela HPLC modular system (Waltham, USA), equipped with an Accela 600 quarter pump and Accela photodiode array detector (PDA). Methanolic extracts were filtered through Chromafil 0.45 μm polyamide filter (Macherey-Nagel, Düren, Germany) and 3 μl of each sample was directly injected into the column. A method for the separation of catechins from the extracts of wound-associated beechwood was developed for the present chromatographic investigation. Extractives were separated in an Accucore PFP column (Thermo Fischer Scientific) with particle size of 2.6 μm and dimensions of 2.1 mm × 150 mm. The column was thermostatted at 30 °C. The mobile phase consisted of water with 0.1 % of formic acid (v/v) as solvent A and methanol containing 0.1 % of formic acid as solvent B. The 10 minutes gradient from 5 to 65 % of solvent B was applied for elution of catechin, whereby the mobile phase flow rate was defined at 400 $\mu l \cdot min^{-1}$. The detection wavelength was adjusted to 275 nm and UV spectra from 200 to 400 nm were recorded for peak identification. Quantitative analysis was based on a three point calibration curve, consisting of standard solutions with mass concentration of 0.5, 5 and 50 $\mu g \cdot ml^{-1}$. Chromatograms were evaluated by ChromQuest 5.0 software. Peak identification was achieved by comparison of retention times and UV spectra of separated compounds with analytical standards. The content of catechin was expressed in mg of catechin per gram of dry sample ($mg \cdot g^{-1}$)

2.5 Statistics
2.5. Statistika

A comparison of total extractives, contents of hydrophilic extractives and content of catechin in tissues of wounded beech trees was performed by Statgraphics software. Values of measurements were first checked for normal distribution. Significant differences were then investigated by means of ANOVA at a 0.95 confidence interval. The contents of phenolic extractives in different trees and in different categories of wound-associated wood and knots were further compared by means of the multiple range test (LSD procedure).

3 RESULTS AND DISCUSSION
3. REZULTATI I RASPRAVA

3.1 Total extractives and content of hydrophilic extractives
3.1. Ukupni ekstraktivi i sadržaj hidrofilnih ekstraktiva

The average values for total extractives (%) and content of hydrophilic extractives (mg/g) among the investigated beech trees are presented in Table 1. Statistical analysis (ANOVA) did not reveal significant differences, either in average content of total extractives or in average content of hydrophilic extractives among the investigated beech trees. However, hydrophilic extractives were significantly different between tree No. 2 and tree No. 4 (LSD procedure) (Table 1).

Table 1 Total extractives and content of hydrophilic extractives in beech trees; average values are accompanied by standard deviations
Tablica 1. Ukupni ekstraktivi i sadržaj hidrofilnih ekstraktiva u bukovini; prosječne vrijednosti dane su sa standardnom devijacijom

Tree No. *Broj stabla*	**Total extractives,** % *Ukupni ekstraktivi, %*	**Hydrophilic extractives,** $mg \cdot g^{-1}$ *Hidrofilni ekstraktivi,* $mg \cdot g^{-1}$
1	1.82 ± 0.43[a]	47.47 ± 14.46[a, b]
2	2.03 ± 0.54[a]	41.33 ± 7.11[a]
3	1.97 ± 0.52[a]	44.27 ± 13.93[a, b]
4	1.77 ± 0.48[a]	55.53 ± 19.73[b]
5	1.92 ± 0.54[a]	47.04 ± 12.54[a, b]
6	1.81 ± 0.64[a]	49.64 ± 15.62[a, b]

[a - b] Different letters within the same column indicate statistically significant differences at a 95.0% confidence level (Fisher's least significant difference (LSD) procedure). / *Različita slova u istom stupcu označavaju statistički značajne razlike s razinom pouzdanosti od 95 % (Fisherov postupak najmanje značajne razlike, LSD)*

Average values for total extractives and average content of hydrophilic extractives obtained from wound-associated tissues and knots are given in Table 2. Total extractives showed no significant differences among categories of beechwood (ANOVA, $p = 0.0585$). However, the highest percentage of extractible compounds was distinctive for wound-wood and knot extracts, while the lowest amounts of these compounds described discolored wood. Wound-wood contained a significantly higher amount of total extractives than intact sapwood (LSD procedure). In contrast, extraction of discolored wood gave significantly less extractives than wound-wood and living knots (Table 2).

Table 2 Total extractives and content of hydrophilic extractives in wound-associated wood and knots of beech. Average values are accompanied by standard deviations.
Tablica 2. Ukupni ekstraktivi i sadržaj hidrofilnih ekstraktiva u ranjenom drvu i kvrgama bukova drva; prosječne vrijednosti dane su sa standardnom devijacijom

Category of wood *Kategorija drva*	**Total extractives, %** *Ukupni ekstraktivi, %*	**Hydrophilic extractives, mg·g^{-1}** *Hidrofilni ekstraktivi, mg·g^{-1}*
Wound-wood *ranjeno drvo*	2.24 ± 0.36^{a}	55.05 ± 20.29$^{a, d}$
Intact sapwood *zdrava bjeljika*	1.75 ± 0.44$^{b, c}$	47.22 ± 13.54$^{a, b}$
Reaction zone *reakcijsko drvo*	1.97 ± 0.44$^{a, b, c}$	42.61 ± 8.76$^{b, c}$
Discolored wood *drvo s diskoloracijom*	1.61 ± 0.67^{b}	37.94 ± 9.44^{c}
Living knot *živa kvrga*	2.14 ± 0.40$^{a, c}$	65.75 ± 5.92^{d}
Dead knot *mrtva kvrga*	2.11 ± 0.42$^{a, b, c}$	49.53 ± 17.14$^{a, b, c}$

$^{a-d}$ Different letters within the same column indicate statistically significant differences at a 95.0% confidence level (Fisher's least significant difference (LSD) procedure). / *Različita slova u istom stupcu označavaju statistički značajne razlike s razinom pouzdanosti od 95 % (Fisherov postupak najmanje značajne razlike, LSD)*

In contrast to the distribution of total extractives, a statistically significant difference is evident among different categories of the investigated wood tissues for hydrophilic extractives (ANOVA, $p = 0.0005$), ranging from 28.50 to 75.34 mg·g^{-1} (Table 2). The highest average content of hydrophilic extractives was determined for methanolic extracts of living knots. Wound-wood had significantly more hydrophilic extractives than the reaction zone. Discolored wood was the poorest in hydrophilics, whereby its content was significantly different from wound-wood, healthy sapwood and living knots. Average values for the content of hydrophilic extractives were significantly different between living and dead knots (Table 2).

3.2 Quantitative HPLC analysis of catechin
3.2. Kvantitativna HPLC analiza katehina

The average content of catechin was not significantly different among the examined beech trees (ANOVA, $p = 0.5455$). The content of catechin in the investigated trees ranged between 0.052 and 2.080 mg·g^{-1}, with the highest average value being measured in tree No. 4 (Figure 1). The high variability in the content of catechin within an individual tree (Figure 1) reflects differences among categories of wood tissues.

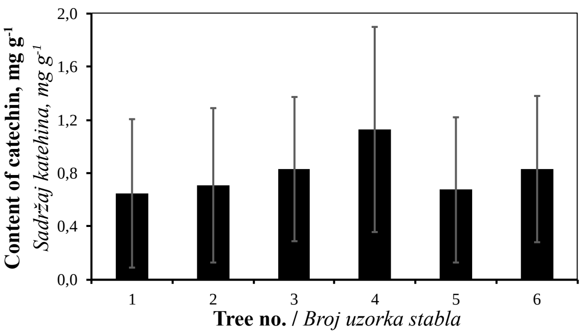

Figure 1 Average content of catechin in investigated beech trees. Error bars represent standard deviation.
Slika 1. Prosječni sadržaj katehina u istraživanim stablima bukve; na stupcima su označene standardne devijacije

The distribution of catechin among wound-associated tissues and knots of beech are given in Figure 2. Chromatograms of wound-wood, reaction zone and living knot are shown in Figure 3 to illustrate the occurrence of catechin. The average content of catechin was significantly different among different categories of wood tissues (ANOVA, $p = 0.0000$). Wound-wood and knot extracts contained the highest amount of this flavanol, while extracts of discolored wood showed the lowest amounts of catechin. Knot extracts contained significantly higher contents than sapwood, reaction zone and discolored wood. In comparison to wound-wood, extraction of dead knots resulted in a higher content of catechin. The difference between dead and living knots was not statistically significant. The amount of catechin in wound-wood was significantly higher than in sapwood, reaction zone and discolored wood. Sapwood samples contained significantly higher contents than reaction zone. The content of catechin in the reaction zone is highly variable and not significantly different from that in discolored wood (Figure 2).

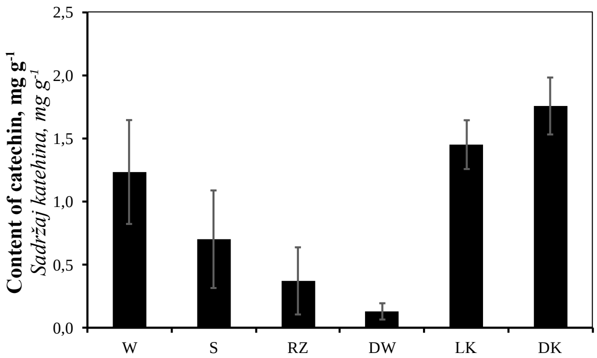

Figure 2 Content of catechin in investigated categories of beechwood. W = wound-wood, S = intact sapwood, RZ = reaction zone, DW = discolored wood, LK = living knot and DK = dead knot. Error bars represent standard deviation.
Slika 2. Sadržaj katehina u istraživanim kategorijama drva; W – ranjeno drvo, S – zdrava bjeljika, RZ – reakcijsko drvo, DW – drvo s diskoloracijom, LK – živa kvrga, DK – mrtva kvrga; na stupcima su označene standardne devijacije

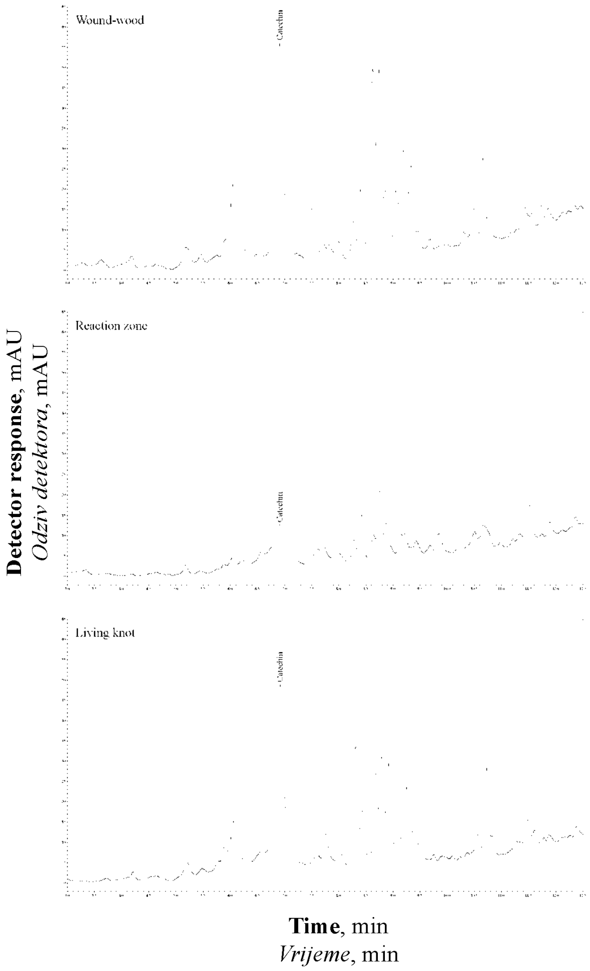

Figure 3 High performance liquid chromatograms of wound-wood, reaction zone and living knot showing the occurrence of catechin in the tissues
Slika 3. Tekućinska kromatografija ranjenog drva, reakcijskog drva i živih kvrga iz koje se vidi pojava katehina u drvnom tkivu

Variance component analysis revealed that 77.46 % of the variability in the content of catechin could be explained by the category of wood tissue, whereby 22.54 % of variability remained unexplained. As shown in Figure 4, a significant correlation between the content of catechin and hydrophilic extractives was found (R^2 = 64. 67 %, p = 0.0000).

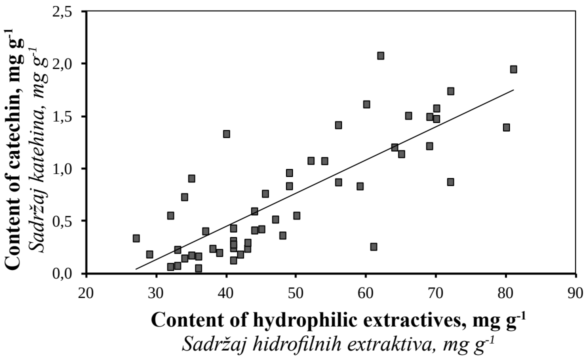

Figure 4 Correlation between the content of catechin and content of hydrophilic extractives in beech
Slika 4. Korelacija između sadržaja katehina i sadržaja hidrofilnih ekstraktiva u bukovu drvu

The content of catechin in samples taken from lower, directly wounded discs and from upper discs with discolored red heart is shown in Figure 5. Sapwood samples that originated from wounded discs had a significantly higher content of catechin than sapwood from the upper disc (ANOVA, p= 0.0005). As shown in Figure 5, differences in the content of catechin of reaction zones and discolored wood from two heights along the stem were not statistically significant.

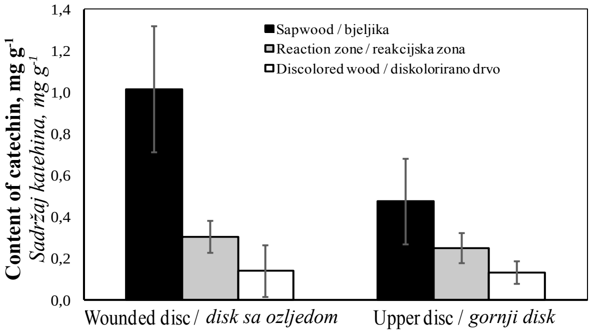

Figure 5 Content of catechin in wound-associated wood samples taken from a wounded disc and from a disc containing abiotic discoloration. Error bars represent standard deviation.
Slika 5. Sadržaj katehina u uzorcima ranjenog drva uzetima iz diska ranjenog drva i diska koji ima abiotsku diskoloraciju; na stupcima su označene standardne devijacije

Investigation of the content of both total and hydrophilic extractives in wood of selected trees confirmed the finding that *Fagus sylvatica* is a species with a relatively low amount of extractives (Rowe and Conner, 1979). Comparison of the extractive content of the examined trees revealed relatively little variation between trees from the same growing site. However, high variability in the content of hydrophilic extractives and catechin among different categories of wound associated wood tissues and knots revealed that different tissues could have different physiological functions in a living tree. It has previously been reported that sapwood of American beech (*Fagus grandifolia* Ehrh.) contains up to 4 % of extractives and discolored wood (heartwood in the original reference) contains less than 2 % of extractives (Rowe and Conner, 1979). Our results on the content of extractives in various categories of wood should also be elucidated from the methodological aspect, because the sampling procedure could have a crucial effect on the content of extractives measured in wood, irrespective of the extraction procedure and solvents used. For example, 5 to 9 times less hydrophilic extractives were measured in beech wood chips that were sequentially extracted with hexane and acetone by Soxhlet extraction (Zule and Može, 2003) than in our study.

Detailed chromatographic analysis of more polar extractives revealed that they consisted mainly of typical wood monosaccharides, whereas catechin was the predominant phenolic compound (Kubel *et al.*, 1988; Zule and Može, 2003). The distribution of catechin in different categories of wood tissues was in strong correlation with the content of hydrophilic extractives (Figure 4). This relationship could have practical consequences as a means of relatively simple determination of catechin in wood of living trees. A relationship between the content of an individual compound and assessment of the content of a particular group of compounds has also been found for wood in other species. For instance, a strong relationship was confirmed for pinosylvin and the content of total phenols in wood extracts of pines (Venäläinen *et al.*, 2003; 2004). Our results on the distribution of catechin in sapwood, reaction zone and discolored wood of beech showed that reaction zones, which have been postulated as compartmentalization tissues, contained a higher amount of catechin than the adjacent discolored wood, but lower than sound sapwood. Our results are in accordance with the observations of Baum and Schwarze (2002). On the other hand, Hofmann *et al.* (2004) reported an abrupt change in the content of catechin, i.e., a high amount of catechin in inner sapwood and a sharp decrease beyond the color boundary. Nevertheless, our results support the suggestion of Hofmann *et al.* (2004; 2008) that catechin participates in the molecular process of red heart formation and in the formation of the chromophores of discolored wood. The relatively low amounts of catechin in discolored wood could be explained by the participation of this flavonoid in the formation of proanthocyanidins, as previously reported by some research groups (Schwarze and Baum, 2000; Baum and Schwarze, 2002). Wound-wood and especially knots exhibited a higher content of catechin than other tissues. A higher content of phenolic extractives has been found in knots of various tree species (Willfor *et al.*, 2003b; 2005; 2007). Wound-wood, which is ontogenetically young tissue, contained a higher content of catechin than sapwood in our experiment. It should be noted here that young sapwood has been shown to contain less catechin than old, inner sapwood (Hofmann *et al.*, 2004). Comparison of the catechin content in sapwood from different tree heights in our study, which also corresponds to different ages, showed that sapwood of older samples contained a higher amount of catechin. It appears that a high content of catechin in the tissue could have an important function in restricting the growth of wood decaying fungi. It has been recently demonstrated that methanolic extracts of wound-wood and sapwood inhibited the growth of wood decaying fungi, whereas extracts of reaction zones did not have an inhibitory effect (Vek *et al.*, 2013a). Extract of knots were not included in our antifungal experiment. However, the high content of catechin in both living and dead knots in beech could have an important protective function, due to its occurrence at the position in which trees, long living organisms, are exceptionally vulnerable to the ingress of pathogenic organisms after branch breakage.

4 CONCLUSIONS
4. ZAKLJUČAK

The content of catechin in wood of the investigated beech trees revealed high within-tree variation, as a result of different categories of wood tissues involved in the investigation. Our findings revealed that a high content of catechin is present in knots, wound-wood and sapwood. Wound-wood, which can be considered to be physiologically and ontogenetically young tissue, contained a higher amount of catechin than the remaining stem samples. Knots are actually the bases of branches, which are attached to stem wood in a complex way, and represent an extremely vulnerable point in the tree in case of branch breakage. The accumulation of a bioactive compound such as catechin in wound-wood, sapwood and knots is considered to be an important part of the survival strategy of trees.

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