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Comparison of Chemical Composition and Free Radical Scavenging Ability of Glycosidically Bound and Free Volatiles from Bosnian Pine (*Pinus heldreichii* Christ. var. *leucodermis*)

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Abstract: The results obtained show that Bosnian pine is rich in glycosidically bound volatile compounds with strong free radical scavenging properties. Since volatiles can be released from nonvolatile glycoside precursors, these compounds can be considered as a hidden potential source of antioxidant substances and may contribute to the total free radical scavenging ability of Bosnian pine.

Keywords: *Pinus heldreichii* Christ., antioxidants, DPPH, glycosides, essential oil

Introduction

Bosnian pine (*Pinus heldreichii* Christ. var. *leucodermis*) is a plant endemic to the Balkan peninsula and Southern Italy. The free volatile constituents in essential oil of this plant have been extensively studied in the context of biosystematic investigations related to the environment [1]. Essential oils are dominated by monoterpene hydrocarbons such as limonene , pinenes and terpinenes, but also by sesquiterpenes germacrene, γ-cadinene, γ-muurolene, caryophyllene. In numerous fruits

and other plant organs, many secondary volatile metabolites are glycosylated and accumulate as non-volatile glycosides. Glycosidically bound compounds in grapes and wines, fruits and aromatic plants have been extensively studied [2]. Owing to recent results in molecular glycobiology, the relations of aglycone and glycoside activity are now more evident [3], and based on these findings, interest in the isolation and identification of aglycones has been increasing. As far as we know, the chemical composition of glycosidically bound volatile compounds of Bosnian pine has not been investigated yet. The aim of this study was to isolate and identify these compounds as well as to determine the free radical scavenging properties of both free and glycosidically bound volatiles in essential oil.

Results and Discussion

Chemical composition of free volatile aglycones compared to essential oil composition

The content of free volatile aglycones in dried plant material was 0.05 mg/g. As shown in Table 1, the GC–MS analysis of the aglycones revealed nine compounds, representing 76.8% of the total aglycone fraction. The main aglycones were vanillin (24.8%), phenylethyl alcohol (15.9%), zingerone (7.3%) and an unidentified compound (16.7%). Other aglycones were presents in relatively small percentages. The obtained results show a qualitative similarity to the chemical composition of aglycones of several other plants. This is in agreement with the hypothesis that the aglycones such as, phenylethyl alcohol, vanillin, eugenol, linalool, geraniol, nerol, α-terpineol, and aliphatic alcohols, can be considered to be more or less common in most plants [2b].

Table 1. Chemical composition of free volatile aglycones isolated from Bosnian pine.

No.	**Identified compound**	**RI HP-20M**	**Peak area (%) X ± SD**
1.	Borneol	1647	3.6 ± 0.1
2.	Exo-2-hydroxycineole	1797	0.6 ± 0.0
3.	Phenylethyl alcohol	1845	15.9 ± 1.0
4.	Dihydro-β-ionol	1907	0.5 ± 0.0
5.	Eugenol	2085	0.7 ± 0.1
6.	Zingerone	-	10.3 ± 0.1
7.	1-Phenylethanolamine	-	3.7 ± 0.3
8.	Vanillin	-	24.8 ± 5.1
9.	Unidentified compound	-	16.7 ± 3.1
	Total		76.8 ± 9.8

The total content of the essential oil (yield = 4.5 mg/g), determined by the gravimetric method, is 90 times higher than that of the aglycones. Among 20 compounds identified in Bosnian pine essential oil, representing 97.3% of the total oil (Table 2), the major constituents were limonene (52.8%), germacrene D (15.8%), α-pinene (10.2%), caryophyllene (7.7%) and β-pinene (3.0%). Other important components were myrcene, Δ-cadinene and α-humulene. The chemical composition of the essential oil was similar to that reported for *Pinus heldreichii* Christ., found in Bulgaria [1]. A comparison between the chemical compositions of the essential oil and the aglycones showed no evidence of common compounds.

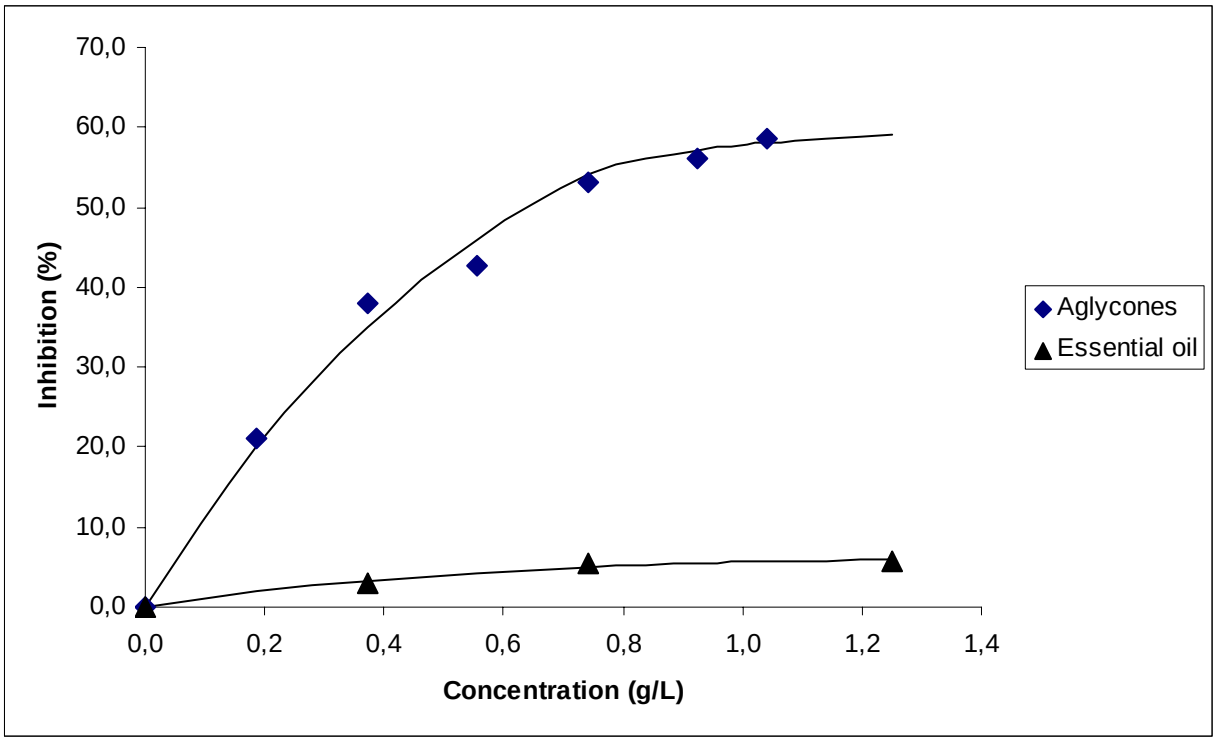
Table 2. Chemical composition of the essential oil isolated from Bosnian pine.

No.	Identified compound	RI HP-20M	Peak area (%)
1.	α- Pinene	1036	10.2 ± 0.9
2.	β- Pinene	1105	3.0 ± 0.3
3.	Myrcene	1149	1.5 ± 0.3
4.	1- Limonene	1208	52.8 ± 1.9
5.	γ- Terpinene	1230	0.1 ± 0.1
6.	α- Terpinolene	1262	0.3 ± 0.1
7.	α- Cubebene	1440	0.2 ± 0.1
8.	α- Copaene	1473	0.3 ± 0.2
9.	Bornyl acetate	1546	0.3 ± 0.1
10	β- ocimene	1235	0.6 ± 0.4
11.	trans-Caryophyllene	1583	7.7 ± 0.0
12.	α- amorphene	1612	1.1 ± 1.1
13.	Citronellyl propionate	1625	0.1 ± 0.1
14.	α- humulene	1641	1.5 ± 1.1
15.	Germacrene -D	1681	15.8 ± 1.8
16.	Ledene	1688	0.3 ± 0.0
17.	Δ- Cadinene	1722	1.4 ± 0.4
18.	Cadina-1,4-diene	1742	0.1 ± 0.1
19.	Δ- Cadinol	-	0.1 ± 0.1
20.	Cembrene	-	0.1 ± 0.1
	Total		**97.3 ± 9.2**

Free radical scavenging ability of volatile aglycones compared to essential oil

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method was used to evaluate the free radical scavenging ability of the volatile aglycones as well as the essential oil. The decrease in absorbance was measured at room temperature every 15 min until the reaction reached steady state or until absorbance declined below 10%. The percent inhibition of the DPPH radical as a function of the antioxidant concentrations is shown in Figure 1. The free radical scavenging ability for series of concentrations of volatile aglycones and essential oils were used to calculate the effective relative concentration EC_{50}. The term EC_{50} corresponds to the concentration of a compound where 50% of its maximal effect on reduction of DPPH radical is observed. EC_{50} values were determined graphically from the graph in Figure 1. The EC_{50} values for aglycones was 0.7 g/L while EC_{50}s for essential oil was undetermined because at its maximum concentration only 6% of DPPH inhibition was achieved. These measurements show that the aglycones fraction exhibits much stronger free radical scavenging potential than the essential oil.

Figure 1. Antioxidant activity of the volatile aglycones and the essential oil from Bosnian pine measured by the DPPH method.



Conclusions

The obtained results show that Bosnian pine is rich in glycosidically bound volatile compounds with strong free radical scavenging properties. Since volatiles can be released from nonvolatile glycoside precursors, these compounds can be considered as a hidden potential of antioxidant substances and may contribute to the total free radical scavenging ability of Bosnian pine. The free radical scavenging properties as well as other biological activity of glycosidically bound volatiles merit further study.

Experimental Section

Materials

Bosnian pine plant material (pine needles) was collected in central Herzegovina near Mostar (Bosnia and Herzegovina) in August 2006. All of the chemicals used were of pro analysis purity and were purchased from Fluka Chemie (Buchs, Switzerland).

Isolation of essential oil

Fresh plant material (100 g) was subjected to three-hours of hydrodistillation in a Clevenger type apparatus. The obtained essential oil was dried over anhydrous sodium sulphate and stored under argon in a sealed vial, at -18 ^{o}C before use.

Isolation of glycosidically bound volatile compounds

After addition of the internal standard (octyl-β-D-glucopyranoside) plant material (200 g) was extracted with boiling ethyl acetate under reflux for 2 h. After percolation, the extract was concentrated to dryness under reduced pressure in a rotating evaporator. The residue was dissolved in boiling water and after cooling the sediment was removed by filtration. The filtrate was applied to Amberlite XAD-2 column at rate of 2 mL/min [2a]. Sugars, amino acids and proteins were removed by washing with distilled water (500 mL). The glycosides extract was collected by eluting with methanol (100 mL). The methanolic extract containing the glycosides was concentrated to dryness under reduced pressure and redissolved in citrate-phosphate buffer (2 mL, 0.2 M, pH 5.0). The remaining volatile compounds were removed by liquid–liquid extraction with *n*-pentane (4 x 5 mL) over 24 h. Prior to enzymatic hydrolysis the absence of volatile compounds was tested by TLC and GC–MS. Thin layer chromatography was performed on 0.2 mm precoated silica plates (Kiesegel 60, Merck) with hexane/ethyl acetate (85:15, v/v) as eluent. The volatile compounds were detected using 2% vanillin in concentrated sulfuric acid. Isolation was performed in triplicate.

Enzymatic hydrolysis and extraction of free volatile aglycones

After addition of an almond β-glucosidase solution (20 mg, 5–8 U/mg; Fluka) the mixture was incubated during 48 h at 37 ^{o}C with occasional shaking. After hydrolysis, the liberated volatile aglycones were extracted from aqueous layer with *n*-pentane (4 x 5 mL). The combined pentane extract was concentrated to 0.5 mL and 2 μL was used for GC–MS analysis.

Gas chromatography–mass spectrometry

The analyses of the volatile compounds were run on a Hewlett–Packard GC–MS system (GC 5890 series II; MSD 5971A, Hewlett–Packard, Vienna, Austria). The HP-20M polar column was used (Carbowax, Hewlett–Packard; 50 m x 0.2 mm i.d., film thickness 0.2 μm). Oven temperature was programmed as follows: isothermal at 70 ^{o}C for 4 min, then increased to 180 ^{o}C, at a rate of 4 ^{o}C/min and subsequently held isothermal for 15 min. The carrier gas was helium (1 mL/min). The injection port temperature was 250 ^{o}C and the detector temperature was 280 ^{o}C. Ionization of the sample components was performed in the EI mode (70 eV). Injected volume was 1 μL. The linear Kováts retention indices (RI) for all the compounds were determined by co-injection of the samples with a solution containing the homologous series of C_8–C_{22} *n*-alkanes [6]. The individual constituents were identified by their identical retention indices referring to the compounds known from the literature data, and also by comparing their mass spectra with spectra of either the known compounds or with those stored in the Wiley mass spectral database (Hewlett–Packard, Vienna, Austria). The aglycone concentrations were calculated from the GC peak areas related to GC peak area of 1-octanol (from the internal standard octyl-β-D-glucopyranoside). Preliminary GC–MS analysis showed the absence of 1-octanol as potential aglycone in plant material. GC-MS analyses were performed in triplicate.

Determination of free radical scavenging ability by the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) method

The free radical scavenging ability of Bosnian pine essential oil and of its volatile aglycones was measured using the stable DPPH radical [4]. The ethanolic stock solution of antioxidants (50 μL, concentrations of stock solutions were 20.8, 18.6, 14.9, 11.5, 7.4 and 3.7 g/L for volatile aglycones and 20, 15, 10 g/L for essential oil) were placed in a cuvette, and 0.004% ethanolic solution of DPPH (1 mL) was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined using UV–VIS Perkin–Elmer Lambda EZ 201 spectrophotometer after 1 h for all samples. Pure ethanol was used to zero the spectrophotometer. The absorbance of the DPPH radical without the antioxidant, i.e. the control, was measured daily. All determinations were performed in triplicate. The percent inhibition of the DPPH radical by the samples was calculated according to the formula [5]:

$$\% \text{ inhibition} = ((A_{C(o)} - A_{A(t)}) / A_{C(o)}) \times 100$$

were $A_{C(o)}$ is the absorbance of the control at t=0 min and $A_{A(t)}$ is the absorbance of the antioxidant at t=1h.

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Sample availability: Available from the authors.