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Free and Glycosidically Bound Volatile Compounds from Cypress Cones *(Cupressus Sempervirens* L.*)*

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Isolations of glycosides from fresh cypres cones, *Cupressus sempervirens* L. (*Cupressaceae*), were performed by cold and hot ethyl acetate extraction. After enzymatic hydrolysis by means of β-glucosidase, 18 aglycones were released. The glycosidically bound volatile compounds amounted to 7–8 mg kg^{-1}. The main aglycones were 3-hydroxybenzoic acid methyl ester (15.5%) and thymoquinone (5-isopropyl-2-methyl-1,4-benzoquinone: 3.7–9.7%). Other important aglycones were perilla alcohol (3.6–8.2%), *p*-cymen-8-ol (5.3–6.4%), 2-phenylethanol (2.7–6.9%) and carvacrol (2.5–6.3%). There was no similarity between the glycosidically bound aglycones and the corresponding free compounds found in the essential oil.

INTRODUCTION

Cupressus sempervirens L. (*Cupressaceae*) is a tree widely distributed in the Mediterranean basin.[1] Its leaves and cones play an important role in traditional medicine. For many years this plant has been used as an antiseptic, antirheumatic, antihemorrhoidal, antidiarrhoeic and vasoconstrictive agent.[2] Thus, the study of the free and glycosidically bound volatile compounds may be of pharmacological interest, but also interesting for the food and perfume industries.

A thorough investigation of the essential oil of Egyptian *C. sempervirens* cones led to identification of 49 components with α-pinene (48.2%) and 3-carene (19.1%) as the main components.[3]

Along with the essential oil, there is a growing interest in the study of glycosidically bound volatile compounds.[4] Many publications have dealt with the chemistry of glycosidically bound volatiles and their distribution in

the vegetable kingdom. The glycosidically bound volatile compounds in *C. sempervirens* cones have not been studied to date. As potential precursors of the volatile compounds from *C. sempervirens* leaves, they were investigated in our previously published work.[5] The main aglycone was thymoquinone. Other important aglycones were guaiacol, carvacrol, 3-phenyl-2-propen-1-ol, 2-phenylethanol, benzyl alcohol and myrtenol.

The aim of this study was to determine the composition of free and glycosidically bound volatile compounds from *C. sempervirens* cones and to show their possible similarity with the corresponding compounds found in *C. sempervirens* leaves.

EXPERIMENTAL

Plant Material

The *C. Sempervirens* cones were collected in the region of central Dalmatia in September 1996. The voucher specimen was deposited in the Laboratory of Organic Chemistry, Faculty of Chemical Technology, Split, Croatia.

Isolation of Free Volatile Compounds

Partially crushed (100 g), fresh cones were subjected to hydrodistillation using 500 mL of distilled water. Hydrodistillation was performed in a Clevenger type apparatus for three hours.

Isolation of Glycosidically Bound Volatile Compounds

Isolation of glycosidically bound volatile compounds was effected by simple, cold and hot, ethyl acetate extraction.[6]

Cold extraction. – Internal standard, octyl-β-D-glucopyranoside (0.5 mg), was added to ground fresh *C. Sempervirens* cones (100 g). Extraction was achieved by maceration with ethyl acetate (300 mL) at room temperature for 24 h. After filtration, the extract was concentrated to dryness and the residue was dissolved in 4 mL citrate-phosphate buffer (pH 5). The remaining volatiles were removed by extraction with *n*-hexane and *n*-pentane in several steps (10 × 2 mL). Prior to enzymatic hydrolysis, the negative test on the presence of any volatiles was carried out by TLC and GC-MS.

Hot extraction. – Upon the addition of octyl-β-D-glucopyranoside (0.5 mg), the ground fresh cypress cones were subjected to boiling with reflux using 300 mL of ethyl acetate. After one hour, the ethyl acetate extract was cooled and filtered. The extract was concentrated to dryness and the residue was dissolved in 4 mL citrate-phosphate buffer (pH 5). The remaining volatiles were removed as described above.

Enzymatic Hydrolysis

β-Glucosidase from bitter almonds (10 mg, 5–8 U/mg; Fluka) was added to the glycosidic extract and enzyme catalysis was carried out for 48 h at 37° C. The relea-

sed aglycones were extracted with *n*-pentane (10 × 2 mL). The collected pentane extracts were concentrated at 0.5 mL, and 2 μL were used for GC-MS analysis.

Acidic Hydrolysis

After enzymatic hydrolysis, the glycosidic extract was acidified to pH 1 by addition of HCl. Hydrolyzed aglycones were simultaneously removed by liquid-liquid extraction with pentane within 24 h at 37 °C. The concentrated extract was subjected to GC-MS analysis.

Gas Chromatography-Mass Spectrometry

Analyses of the volatiles were run on a Hewlett-Packard GC-MS system (GC: 5890 series II; MSD 5971A) equipped with two fused-silica capillary columns HP-20M (polyethylene glycol; 50 m × 0.2 mm i.d.; film thickness 0.2 μm) and HP-101 (dimethylpolysiloxan fluid; 25 m × 0.2 mm i.d.; film thickness 0.2 μm). The columns were directly coupled to the MS. The carrier gas was helium, flow rate 1 mL/min. For HP-20M, the oven temperature was programmed: 70 °C for 4 min, then 70–180 °C at 4 °C/min and then it was held isothermal for 10 min. The programme for HP-101 column was as follows: 70 °C for 2 min, then 70–200 °C at 3 °C/min, held isothermal for 10 min. Injection port 250 °C; detector, 280 °C; split ratio 1:50; and ionization of the sample components was performed in the *EI* mode (70 eV) for both columns.

Identification and Quantitative Determination of Components

Retention indices for all compounds were determined by coinjection of the sample with a solution containing a homologous series of *n*-hydrocarbons (C_8–C_{22}) according to the Kováts method.[7] Individual constituents were identified by comparison of their retention indices with those obtained from known plant sources and by comparison of their spectra with the Wiley MS library. Aglycone concentrations were calculated from the GC peak areas related to the GC peak area of 1-octanol (from internal standard; octyl-β-D-glucopyranoside). Preliminary GC-MS analysis indicated the absence of 1-octanol as potential aglycone.

RESULTS AND DISCUSSION

Free Volatile Compounds of C. Sempervirens *Cones*

From 100 g of fresh *C. sempervirens* cones, 0.45 g of essential oil was obtained, which was used for GC-MS identification of the free volatile compounds without preliminary separation. A total of 16 compounds, representing 98.2% of the total oil, were identified by GC-MS analysis (Table I). The major compound was α-pinene (69.9%), which was also found as a major constituent in *C. sempervirens* leaves oil[5] and in the Egyptian *C. sempervirens* cones oil.[3] The Δ-3-carene was the second most important compound with a peak area of 11.7%. Other important compounds were α-terpinolene (3.6%) and β-cubebene (1.9%)

TABLE I

Percentage composition of the essential oil of *Cupressus sempervirens* L. cones

Component	RIA[a]	RIP[b]	Peak area (%)
α-pinene	937	1023	69.9
β-pinene + sabinene	973	1092	2.3
Δ-3-carene	1007	1131	11.7
β-myrcene	987	1148	2.5
limonene	1025	1179	1.4
α-terpinolene	1081	1260	3.6
α-cubebene	1342	1433	0.6
bornyl acetate	1268	1550	0.5
terpinene-4-ol	1168	1561	0.7
caryophyllene	–	1585	0.4
α-humulene	1438	1638	0.3
α-terpineol	1184	1624	0.6
β-cubebene	1464	1694	1.9
Δ-cadinene	1504	1716	0.3
α-cedrol	1580	2063	1.5
Total			98.2

[a]RIA – Retention indices on apolar HP-101 column
[b]RIP – Retention indices on polar HP-20 M column

Glycosidically Bound Volatile Compounds of C. Sempervirens *Cones*

Glycoside isolations were performed by simple cold and hot ethyl acetate extraction. Enzymatic hydrolysis by means of β-glucosidase released approximately 95% of total aglycones, then acidic hydrolysis released another 5%. As previously described,[8, 9] purified β-glucosidase is specific for the hydrolysis of β-glucosides, suggesting that in our case the majority of aglycones were bound to glucose. Acidic hydrolysis released all the remaining glycosides without selectivity, but also produced their rearrangement.[10] The total amount of released aglycones during acidic hydrolysis shows that less than 5% of aglycones were bound in other forms of glycosides. For detailed research of these glycosides, hydrolysis by some other enzymes (maltase, β-D-apiosidase, α-L-rhamnosidase, *etc*.) should be performed. Because of the formation of different artifacts during acidic hydrolysis, our attention was directed only to the study of the chemical composition of aglycones released during enzymatic hydrolysis.

The released aglycones were extracted with *n*-pentane and analyzed by GC-MS. Approximately 7–8 mg/kg of the glycosidically bound volatile com-

TABLE II

Percentage composition of glycosidically bound volatile compounds in fresh cones of *Cupressus sempervirens* L., isolated by cold and hot ethyl acetate extraction and hydrolyzed by means of β-glucosidase

Component	RIA[a]	RIP[b]	Peak area (%) Ethyl acetate extraction	
			Cold	Hot
terpinen-4-ol	1163	1557	3.5	2.2
thymoquinone	1220	1686	9.7	3.7
myrtenol	1193	1735	5.4	2.1
trans-carveol	1093	1776	3.6	2.8
benzyl alcohol	1102	1807	5.4	2.3
2-phenylethanol	1150	1844	6.9	2.7
guaiacol, 4-methyl	–	1889	2.4	0.7
unknown	1220	1912	5.5	–
2-methylphenol	–	1933	2.2	0.8
perilla alcohol	1301	1939	8.2	3.6
3-hydroxybenzoic acid methyl ester	1365	2012	–	15.5
unknown	1415	2027	–	6.3
p-cymen-8-ol	1315	2037	6.4	5.3
4-hydroxy-3-methoxy benzaldehyde	1497	2065	–	2.6
eugenol	1371	2099	4.2	1.4
thymol	1364	2118	5.4	1.8
carvacrol	1371	2140	6.3	2.5
trans-2-caren-4-ol	1523	–	1.8	2.2
Total			76.9	58.5

[a]RIA – Retention indices on apolar HP-101 column
[b]RIP – Retention indices on polar HP-20 M column

pounds were released by β-glucosidase activity during 24 h of incubation, depending on the method of isolation.

As shown in Table II, 18 volatile compounds were identified, representing 76.9% and 58.5% of the total aglycones released in cold or hot extraction, respectively. Global results, concerning the composition of aglycones, obtained by the two methods of isolation show a relatively good similarity. The major aglycone, 3-hydroxybenzoic acid methyl ester (15.5%), which was isolated by hot extraction, was not identified after cold extraction. This fact suggests that 3-hydroxybenzoic acid methyl ester was probably a product of unintentional derivatization, produced by heating of the sample. Other im-

portant aglycones were thymoquinone (3.7–9.7%), perilla alcohol (3.6–8.2%), *p*-cymen-8-ol (5.3–6.4%), 2-phenylethanol (2.7–6.9%) and carvacrol (2.5––6.3%).

Comparison of bound and free volatiles shows that there is no similarity between the glycosidically bound aglycones (Table II) and the corresponding free compounds found in the essential oil (Table I). This is in good correlation with Svendsen's hypothesis that glycosidically bound volatile compounds seem to appear independently of essential oil all over the plant kingdom.[11,12]

Comparison of the chemical and percentage composition of released aglycones from *C. sempervirens* cones and from *C. sempervirens* leaves[5] shows that the quantity of certain compounds varies but the general similarity is very good. In comparison with other species from the family *Cupressaceae*[11,12] *C. sempervirens* shows a noticeable difference in the composition of the glycosidically bound volatile compounds. Partial similarity is observed in aromatic aglycones of *Pinus silvestris* from the familly *Pinaceae*.[13]

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SAŽETAK

Slobodni i glikozidno vezani hlapljivi spojevi češera čempresa (*Cupressus Sempervirens* L.)

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Ekstrakcija glikozida iz svježih češera čempresa, *Cupressus sempervirens* L. (*Cupressaceae*), izvršena je hladnim i vrućim etil-acetatom. Nakon provedene enzimske hidrolize oslobođeno je 18 aglikona. Glavni aglikoni jesu metilni ester 3-hidroksi-benzojeve kiseline (15,5%) i timokinon (5-izopropil-2-metil-1,4-benzokinon: 3,7–9,7%). Ostali aglikoni, prisutni u većim količinama, jesu perila-alkohol (3,6–8,2%), *p*-cimen-8-ol (5,3–6,4%), 2-feniletanol (2,7–6,9%) i karvakrol (2,5–6,3%). Nema sličnosti između glikozidno vezanih hlapljivih spojeva i slobodnih hlapljivih spojeva prisutnih u eteričnom ulju.