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CROATICA CHEMICA ACTA
CCACAA **80** (1) 121–126 (2007)

ISSN-0011-1643
CCA-3148
Original Scientific Paper

Chemical Composition and Antioxidant Activity of Free Volatile Aglycones from Laurel (*Laurus nobilis* L.) Compared to Its Essential Oil

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RECEIVED DECEMBER 6, 2005; REVISED JANUARY 30, 2007; ACCEPTED FEBRUARY 2, 2007

The paper examines the chemical composition and antioxidant activity of free volatile aglycones from laurel (*Laurus nobilis* L.) as compared to its essential oil. Comparison of the chemical composition of volatile aglycones with the chemical composition of essential oil showed there was no similarity between the free compounds found in the essential oil and the corresponding volatile aglycones. Only eugenol was found to be identical. To evaluate the said antioxidant activities, two different methods were applied: 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and ferric reducing/antioxidant power assay (FRAP). The results obtained *in vitro* with both methods showed that volatile aglycones from laurel possess lower reducing power and free radical scavenging ability compared to essential oil as well as the well-known synthetic antioxidant butilated hydroxytoluene (BHT).

Keywords
Laurus nobilis L.
volatile aglycones
essential oil
chemical composition
GC-MS
antioxidant activity

INTRODUCTION

Lipids containing polyunsaturated fatty acids and their esters are readily oxidized by molecular oxygen. Such oxidation, called autoxidation, proceeds via a free radical chain mechanism. Autoxidation of lipids has been recognized as a major deterioration process affecting both sensory and nutritional quality of foods. Application of antioxidants is one of the technically simplest ways of reducing lipid oxidation. Widely used artificial antioxidants in food products, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA)[1] are very effective in their role. But, their use in food products is diminishing due to their instability and suspected action as promoters of carcinogenesis.[2–4] These findings, along with consumer interest in natural food additives, have reinforced interest in natural antioxidants.

Many natural compounds extracted from plants have demonstrated biological activities, notably antibacterial, antifungal and antioxidant properties. Among these, volatile oils from aromatic and medicinal plants are particularly interesting, as described by many research works.[5–11] *Laurus nobilis* L. (*Lauraceae)*, laurel, an evergreen tree or shrub, is cultivated in many temperate and warm parts of the word, particularly in the countries bordering on the Mediterranean Basin.[12] Dried leaves, also called »sweet bay« are a highly esteemed flavoring material in culinary preparations (soups, fish, ragouts), especially in Dalmatian and French cuisines. Leaves and essential oil obtained from leaves increase gastric fluid secretion and act against digestive disorders such as flatulent colics.[13] Essential oil, distillated from leaves, has bactericidal and fungicidal properties.[10,14–15] Only few papers have dealt with the antioxidant activity of laurel leaves.[10,16–17]

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Glycosidically bound volatiles can be considered as potential aroma precursors of plants, since enzymatic or acidic hydrolysis of these secondary metabolites releases free volatiles. Occurrence of these compounds in different plant families was established earlier,[18–23] but very little is known about their antioxidant activity. Since, to our knowledge, antioxidant properties of free volatile aglycones from laurel have not been studied to date, the aim of this study was to isolate and identify these compounds as well as to determine their antioxidant power. This is the part of our investigation project dealing with the antioxidant activity of volatile aglycones from aromatic plants.[24,25]

EXPERIMENTAL

Materials

Fresh plant material of laurel leaves was collected in Central Dalmatia (Croatia) in April 2005. The voucher specimen is deposed in the Department of Biochemistry, Faculty of Chemical Technology, University of Split, Croatia. All of the applied chemicals were of *pro analysis* purity and were purchased from Fluka Chemie (Buchs, Switzerland).

Isolation of Essential Oil

100 g of plant material and 500 mL water were placed in a Clevenger type apparatus. The essential oil was isolated by hydrodistillation for three hours, then separated, dried over anhydrous sodium sulphate and, stored under argon in a sealed vial, at –18 °C, before use.

Isolation of Glycosidically Bound Volatile Compounds

Upon addition of internal standard, octyl-β-D-glucopyranoside, 100 g of plant material was extracted with boiling ethyl acetate for 2 hours. After extraction, the extract was filtered and concentrated to dryness in a rotating evaporator, under reduced pressure. The residue was dissolved in boiling water and filtered. The filtrate was subjected to liquid-solid chromatography in a glass column (150 × 20 mm) containing Amberlite XAD-2 as adsorbent.[23] The column was washed with water (500 mL), and the remaining components were subsequently eluted with methanol (100 mL). After evaporation of the solvent, the residue was redissolved in 2 mL citrate-phosphate buffer (pH = 5). The remaining volatile compounds were removed with 4 × 5 mL of n-pentane. Prior to enzymatic hydrolysis, TLC and GC-MS were used to test the absence of free volatile compounds.

Enzymatic Hydrolysis and Extraction of Free Volatile Aglycones

In a typical experiment, ß-glucosidase from bitter almonds (10 mg, 5–8 U/mg; Fluka) was added to the glycosidic extract. Enzymatic hydrolysis was run for 48 h at 37 °C. The liberated aglycones were extracted from the aqueous layer with 4 × 5 mL of n-pentane. The combined pentane extract was concentrated to 0.5 mL, and 2 μL were injected for GC-MS analysis.

Gas Chromatography-Mass Spectrometry

Analyses of volatile compounds were run on a Hewlett-Packard GC-MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, Vienna, Austria). Two columns of different polarity were used: a HP-101 column (Methyl silicone fluid, Hewlett Packard; 25 m × 0.2 mm i.d., film thickness 0.2 μm) and a HP-20M column (Carbowax, Hewlett Packard; 50 m × 0.2 mm i.d., film thickness 0.2 μm). Oven temperature was programmed as follows: isothermal at 70 °C for 4 min, then increased to 180 °C, at a rate of 4 °C min^{-1} and subsequently kept isothermal for 15 min (HP-20M column); isothermal at 70 °C for 2 min, then increased to 200 °C, at a rate of 3 °C min^{-1} and kept isothermal for 15 min (HP-101 column). The carrier gas was helium (1 mL/min). The injection port temperature was 250 °C and the detector temperature was 280 °C. Ionization of sample components was performed in the EI mode (70 eV). Injected volume was 1 μL. Linear retention indices for all compounds were determined by co-injection of the samples with a solution containing the homologous series of C_8–C_{22} n-alkanes.[26] Individual constituents were identified by their identical retention indices referring to the compounds known from literature data,[27] and also by comparing their mass spectra with the spectra of known compounds or those stored in the Wiley mass spectral database (Hewlett Packard, Vienna, Austria). Aglycone concentrations were calculated from the GC peak areas related to the 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 a potential aglycone in plant material.

Determination of Antioxidant Activity with 2,2'-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Method

Antioxidant activity of the laurel essential oil and of volatile aglycones was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH.[28] An ethanolic stock solution (50 μL) of the antioxidant (concentrations of stock solutions were 20, 10, 5 and 1 g/L) was placed in a cuvette, and 1 mL of 0.004 % ethanolic solution of DPPH was added. Absorbance measurements commenced immediately. The decrease in absorbance at 517 nm was determined with a UV-Vis Perkin-Elmer Lambda EZ 201 spectrophotometer after 1 h for all samples. Ethanol was used to zero the spectrophotometer. Absorbance of the DPPH radical without antioxidant, *i.e.,* the control, was measured daily. Special care was taken to minimize loss of free radical activity of the DPPH radical stock solution.[29] All determinations were performed in triplicate. Inhibition of the DPPH radical by the samples was calculated according to the formula of Yen & Duh:[30]

$$\text{inhibition / \%} = ((A_{C(0)} - A_{A(t)}) / A_{C(0)}) \times 100$$

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

Determination of Ferric Reducing Antioxidant Power (FRAP Assay)

Determination of ferric reducing/antioxidant power FRAP is a simple direct test for measuring antioxidant capacity. This method was initially developed to assay plasma antioxidant capacity, but can be used for plant extracts as well. Total antioxidant potential of a sample was determined using the ferric reducing ability (FRAP) assay[31] as a measure of »antioxidant power«. This assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{2+}-tripyridyltriazine compound from the colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. The working FRAP reagent was prepared by mixing 10 parts of 300 mmol/L acetate buffer, pH = 3.6, with 1 part of 10 mmol/L TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mmol/L hydrochloride acid and with 1 volume of 20 mmol/L ferric chloride. Freshly prepared FRAP reagent (1.5 mL) was warmed to 37 °C and a reagent blank reading was taken at 593 nm (M1 reading). Subsequently, 50 μL of sample (concentrations of stock solutions were 20, 10, 5 and 1 g/L) and 150 μL of deionized water were added to the FRAP reagent. Final dilution of the sample in the reaction mixture was 1:34. The sample was incubated at 37 °C throughout the monitoring period. The change in absorbance between the final reading (4-min reading) and the M1 reading were selected for calculation of FRAP values. The standard curve was prepared using different concentrations (0.1–5 mmol/L) of $FeCl_2 \cdot 4H_2O$. All solutions were used on the day of preparation. In the FRAP assay, antioxidant efficiency of the antioxidant under test was calculated with reference to the reaction signal given by a Fe^{2+} solution of known concentration. The results were corrected for dilution and expressed in mmol Fe^{2+}/L. All determinations were performed in triplicate.

RESULTS AND DISCUSSION

Chemical Composition of Free Volatile Aglycones Compared to Essential Oil Composition

The content of free volatile aglycones in fresh plant material was 0.14 mg/g. Twenty-three aglycones were identified, representing 92 % of the total aglycone fraction. The mixture of liberated aglycones is composed of phenylpropane derivatives, acids, alcohols, esters and terpene compounds. Results are shown in Table I. The major compound was an aromatic compound benzyl alcohol (63.4 %), followed by 2-butenoic acid (8.1 %) and vanillin (8.0 %). Butanoic acid (1.9 %), benzoic acid (1.7 %), 4-hydroxy-3,5-dimethoxybenzaldehyde (1.4 %) and chavicol (1.1 %) were identified in small quantities. This is the second report on the chemical composition of laurel volatile aglycones. The obtained results showed partial similarity with those reported by Kilic *et al.*,[32] published this year. They compared amounts of aglycons obtained from different parts (leaves and buds) of *Laurus nobilis* L. (collected from Black Sea, Turkey) and they found benzyl alcohol, some linalool-diols, 2-hydroxy-1,8-cineole and its derivatives as major aglycons. The aglycones such as benzyl alcohol, 2-phenylethanol and eugenol are considered to be ubiquitous in aglycone fractions in different plants.[33] According to Siegel,[34] eugenol and other *p*-hydroxyphenylpropanes can be connected with lignin biosynthesis via the peroxidase-hydrogen peroxide system.

TABLE I. Chemical composition of free volatile aglycones isolated from laurel

No.	Identified compound	Peak area %	RI[(a)] HP-20M	RI[(b)] HP-101
1	Acetic acid	0.4	1402	–[(c)]
2	Benzaldehyde	0.2	1480	–
3	Butanoic acid	1.9	1576	879
4	2-Butenoic acid[(d)]	0.3	1651	–
5	Benzyl acetate	0.5	1682	1141
6	2-Butenoic acid[(d)]	8.1	1708	986
7	Benzyl alcohol	63.4	1836	1102
8	2-Phenylethanol	0.3	1858	1144
9	*cis*-1,3,3-Trimethyl-2-oxabicyclo[2.2.2]octan-5-ol	0.9	1864	1234
10	2-Hexenyl butanoate[(d)]	0.3	1896	–
11	4-Methylbenzyl alcohol	0.1	1910	–
12	Phenylpropanol	0.2	1980	–
13	4-Ethylbenzyl alcohol	0.3	2145	–
14	2-Hexenyl butanoate[(d)]	0.3	2067	–
15	3,7-Dimethyl-1,5-octadiene-3,7-diol[(d)]	0.7	–	1219
16	Eugenol	0.3	2103	1363
17	Cinnamyl alcohol	0.3	[(e)]	1384
18	Benzoic acid	1.7	[(e)]	1340
19	Chavicol	1.1	[(e)]	1373
20	Vanillin	8.0	[(e)]	1496
21	*exo*-2-Hydroxycineole	0.5	–	1212
22	1-Cyclohexene-1-methanol	0.8	–	1620
23	4-Hydroxy-3,5-dimethoxybenzaldehyde	1.4	–	1746
	Total:	92.0		

[(a)] Retention indices relative to C_8–C_{22} n-alkanes on polar HP-20M column.
[(b)] Retention indices relative to C_8–C_{22} n-alkanes on apolar HP-101 column.
[(c)] –, not identified on this column.
[(d)] Correct isomer not identified.
[(e)] Retention time is outside retention times of homologous series of C_8–C_{22} n-alkanes.

Total content of the essential oil (yield = 6.20 mg/g), determined by the gravimetric method, is 43 times higher than that of the free aglycones. Twenty-two compounds were identified, representing 98.5 % of total oil. Monoterpene, sesquiterpene and phenylpropane compounds were identified. The major oil component was an oxygenated monoterpene: 1.8-cineole (45.5 %). The other important compounds were methyl eugenol (10.0 %), α-terpinyl acetate (9.1 %), linalool (8.5 %) and sabinene (5.7 %). The essential oil also contained smaller amounts of eugenol (2.5 %), α-pinene (2.1 %), terpinen-4-ol (2.1 %), *trans*-caryophyllene (2.1 %), camphor (2.1 %), carryophyllene oxide (1.7 %), α-terpineol (1.5 %) as well as other compounds. Similar results were obtained by other researchers.[35–44] Comparison of the chemical composition of the essential oil and volatile aglycones showed there was no similarity between the free compounds found in the essential oil and the corresponding glycosidically bound aglycones. Only one compound, eugenol, was established to be identical. These results support the Baerheim Svendsen hypothesis[45–46] that glycosidically bound volatile compounds seem to occur all over the plant kingdom, independently of essential oil occurrence.

Antioxidant Activity of Glycosidically Bound Volatile Aglycones Compared to Essential Oil

The aim of this work was to evaluate the antioxidant activity of laurel volatile aglycones and to compare it to the antioxidant activity of the laurel essential oil as well as to known synthetic antioxidant butylated hydroxytoluene (BHT). To evaluate antioxidant properties of the essential oil and enzymatically released volatile aglycones from laurel, two different assays were used: 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and ferric reducing/antioxidant power assay (FRAP). Relatively stable organic radical DPPH has been widely used in determination of antioxidant activity of single compounds as well as different plant extracts.[47–50] The DPPH radical scavenging activities are shown in Figure 1. The data showed that the decrease in concentration of volatile aglycones caused a reduction of antioxidant activity. The sample of stock solution concentration of 20 g/L showed the highest activity (AI = 49.1 % inhibition), while the sample of 1 g/L showed the lowest activity (AI = 8.0 %). The same phenomenon was observed for the essential oil as well as BHT, but for all the applied concentrations, all samples showed higher activity compared to volatile aglycones. The best activity was recorded for samples of the essential oil and BHT at the concentration of 20 g/L (AI = 89.6 % and 91.4 %).

The FRAP assay is quick and simple to perform, and the reaction is reproducible and linearly related to the amount concentration of the antioxidant present.[51] The antioxidant capacities of the liberated volatile aglycones and the essential oil are shown in Figure 2. Like in the

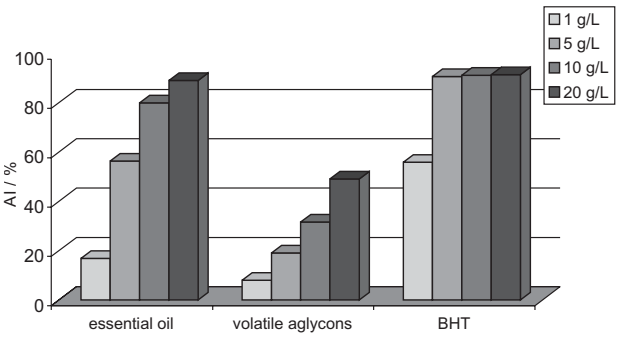

Figure 1. Antioxidant activity of laurel essential oil and the corresponding volatile aglycones plus BHT by the DPPH method.

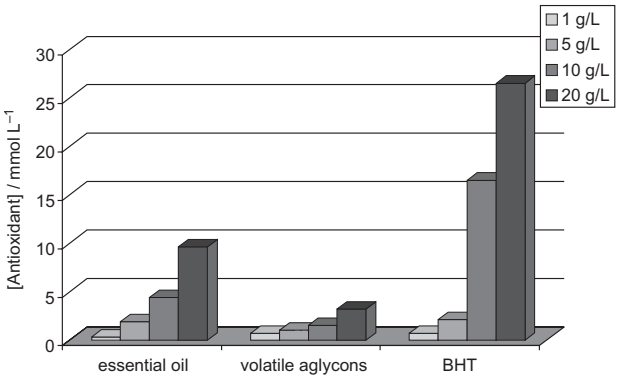

Figure 2. Antioxidant activity of laurel essential oil and the corresponding volatile aglycones plus BHT by the FRAP method.

case of the DPPH method, the decrease in concentration caused a reduction in the activity of volatile aglycones. For applied concentrations of stock solutions, activities ranged from 0.7 mmol/L for 1 g/L to sample 3.2 mmol/L for 20 g/L. Compared to the compound with strong antioxidant activity, BHT, the volatile aglycone fraction and the essential oil showed lower activity. The reducing capacity decreased in the following order BHT > essential oil > volatile aglycones. The best capacity was shown by BHT at concentrations of 20 g/L and 10 g/L (26.5 mmol/L and 16.5 mmol/L) and the essential oil of a concentration of 20 g/L (9.6 mmol/L).

Among the identified compounds in the volatile aglycone fraction and the essential oil from laurel, eugenol and methyl eugenol may be considered the main contributors to the antioxidant activity. The antioxidant activity of eugenol has been reported several times.[5,52–54] The most abundant component of the volatile aglycone fraction, benzyl alcohol, was also investigated for antioxidant activity.[5,55] The results showed antioxidant activity, but lower than that of eugenol. For this reason, antioxidant activity of laurel volatile aglycones was lower than the essential oil activity.

The results obtained from antioxidative measurements *in vitro* by both methods showed that the best antioxi-

TABLE II. Chemical composition of the laurel essential oil

No.	Identified compound	Peak area %	RI[(a)] HP-20M	RI(b) HP-101
1	α-Pinene	2.1	1030	938
2	Sabinene	5.7	1097	968
3	δ-3-Carene	0.4	1136	1018
4	α-Terpinene	0.3	1166	–[(c)]
5	1.8-Cineole	45.5	1183	1035
6	Limonene	0.9	1189	–
7	γ-Terpinene	0.7	1214	1065
8	*trans*-β-Ocimene	0.2	–	1044
9	*p*-Cymene	0.2	1234	1029
10	Terpinolene	0.3	1257	1080
11	Camphor	2.1	1475	1126
12	Linalool	8.5	1502	1100
13	Bornyl acetate	0.3	1534	1278
14	Terpinen-4-ol	2.1	1542	1171
15	*trans*-Caryophyllene	2.1	1561	1403
16	α-Terpineol	1.5	1620	1190
17	α-Terpinyl acetate	9.1	1648	1341
18	α-Humulene	0.2	1632	1442
19	δ-Cadinene	0.2	1715	1505
20	α-Farnesene	0.6	1725	1521
21	Caryophyllene oxide	1.7	1917	1576
22	Methyleugenol	10.0	1943	1402
23	Eugenol	2.5	2081	1397
24	*cis*-Isoelemicin	0.6	–	1561
25	Aromadendrene	0.7	–	1654
	Total:	98.5		

[(a)] Retention indices relative to C_8–C_{22} n-alkanes on polar HP-20M column.
[(b)] Retention indices relative to C_8–C_{22} n-alkanes on apolar HP-101 column.
[(c)] –, not identified on this column.

dant activity possessed the known synthetic antioxidant butylated hydroxytoluene (BHT), while potential natural antioxidants isolated from laurel showed lower or comparable (higher concentration of essential oil activity tested by the DPPH method) activity. These, not significant, differences can be explained by different solvent polarity in the two assays. Bound volatile aglycones from laurel possessed lower reducing power and free radical scavenging ability compared to the essential oil, tested by both methods.

In spite of the lower antioxidant ability of laurel volatile aglycones, this was a valuable antioxidant potential of this plant. During maturation, storage, industrial pretreatment or processing, volatile compounds can be released from these nonvolatile precursors by enzymatic or chemical reactions. For this reason, glycosidically bound volatile compounds can be considered a hidden antioxidant potential of plant materials and may contribute to total antioxidant activity of plants. This confirms the potential use of herbs and spices in the food and pharmaceutical industries to increase the shelf life of foodstuffs, as well as to prevent cellular damage, a cause of human diseases and ageing. The antioxidant properties of glycosidically bound volatile compounds from other plants deserve to be the objective of future research.

Acknowledgments. – This work was supported by the Ministry of Science, Education and Sports of the Republic of Croatia, Project 0011–003.

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SAŽETAK

Kemijski sastav i antioksidacijska aktivnost oslobođenih hlapljivih aglikona iz lovora (*Laurus nobilis* L.) u usporedbi s njegovim eteričnim uljem

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Rezultati istraživanja kemijskog sastava i antioksidacijske aktivnosti oslobođenih hlapljivih aglikona iz lovora (*Laurus nobilis* L.) uspoređeni su s kemijskim sastavom i antioksidacijskom aktivnošću eteričnog ulja lovora. Usporedba kemijskog sastava hlapljivih aglikona s kemijskim sastavom eteričnog ulja pokazuje da nema sličnosti između spojeva utvrđenih u eteričnom ulju i odgovarajućih aglikona. Jedino je eugenol identificiran kao zajednički. Za ispitivanje antioksidacijske aktivnosti korištene su dvije različite metode: metoda vezivanja slobodnih radikala (DPPH metoda) i metoda određivanja sposobnosti redukcije željeza (FRAP metoda). Rezultati dobiveni *in vitr*o objema metodama pokazuju da hlapljivi aglikoni iz lovora imaju slabiju reduktivnu sposobnost, kao i sposobnost vezivanja slobodnih radikala, u usporedbi s eteričnim uljem i poznatim sintetskim antioksidantom butiliranim hidroksitoluenom (BHT).