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Chemical Composition and Antioxidant Effect of Free Volatile Aglycones from Nutmeg (*Myristica fragrans* Houtt.) Compared to Its Essential Oil

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The paper reports on the isolation of glycosidically bound volatiles from nutmeg, identification of free aglycones, and determination of the antioxidative power of free aglycones in comparison with nutmeg essential oil. Comparison of the chemical composition of free volatiles with the chemical composition of free volatile compounds found in the essential oil reveals only two common compounds (eugenol, terpinen-4-ol). To measure the antioxidative activities of the essential oil and enzymatically released aglycones from nutmeg, two different assays were performed: the 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and the ferric reducing / antioxidant power assay (FRAP). Both methods showed that the aglycone fraction possesses stronger antioxidant properties than free volatiles from the oil.

Keywords
Myristica fragrans Houtt.
aglycones
glycosides
antioxidant activity

INTRODUCTION

In numerous fruits and other plant organs, many secondary metabolites, including important biologically active compounds, are glycosylated and accumulate as non-volatile glycosides. Glycosidically bound compounds have been extensively studied in grapes and wines,[1–3] fruits[4,5] and aromatic plants.[6,7] To the best of our knowledge, the chemical composition and antioxidant activity of the glycosidically bound volatiles in nutmeg have not been reported to date.

The nutmeg tree (*Myristica fragrans* Houtt.) is a large, leafy evergreen plant originating from Moluccas (the Spice Islands) and now cultivated in the West Indies. It produces two spices – mace and nutmeg. Nutmeg is the seed kernel inside the fruit and mace is the lacy covering (aril) on the kernel. Nutmeg has aromatic, stimulant, narcotic, carminative, astringent, aphrodisiac, hypolipidemic, antithrombotic, anti-platelet aggregation, antifungal, antidysenteric, anti-inflammatory activities.[8] It is used as a remedy for stomach ache, rheumatism and vomiting in pregnancy.[8] The presence of two compounds, myristicin and elemicin, is often related to intoxication and to the hallucinogenic action of nutmeg while safrole has been suspected to be carcinogenic.[9–13] However, the mechanism by which these compounds excrete their activity is still a subject of extensive research.[14]

As a part of the growing consciousness of dietary habits, herbs and spices are becoming an important source of natural antioxidants.[15–17] Since very little is known about the antioxidant activity of glycosidically bound volatile compounds, researches in this field are welcome.

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Owing to recent results in molecular glycobiology, the relations of aglycone and glycoside activity are now more evident; based on these findings, research into the isolation and identification of aglycones is becoming increasingly challenging. It opens up possibilities of developing more powerful glycodrugs.[18]

This study was directed towards gaining a better understanding of the occurrence and the potential antioxidant role of endogenous glycosides in nutmeg. The aim of the study was to reveal the unexploited, high potential of glycosidically bound antioxidant substances by using commercial preparations of nutmeg, such as those which might be bought by people interested in the impacts of dietary supplements on their well-being.

EXPERIMENTAL

Plant Materials

Nutmeg was purchased from Kotanyi spice company (Austria). Commercial supply of the plant material was used throughout. A hundred grams of plant material was subjected to a three-hour hydrodistillation using a Clevenger-type apparatus. The voucher specimen of the essential oil is deposited in the Laboratory of Biochemistry and Food Chemistry, Faculty of Chemical Technology, Split, Croatia.

All chemicals and reagents were of proanalysis purity and were obtained from Fluka Chemie, Buchs, Switzerland.

Isolation of Glycosides Extract

Powdered nutmeg (200 g) was mixed with 1000 mL cold distilled water. After overnight maceration at 4 °C, the mixture was centrifuged for 15 min at 4 °C (7500 g). The supernatant was collected and vacuum-filtered. The glycosidic solution was concentrated to a volume of 100 mL on a vacuum evaporator and applied to an Amberlite XAD-2 (20–60 mesh, obtained from Fluka) column that had been successively washed with 300 mL of distilled water and 200 mL of an pentane/diethyl ether mixture (1:1 *v*/*v*). The glycoside extract was collected by eluting 350 mL of methanol.

Enzymatic Hydrolysis

Methanolic extract containing the glycosides was concentrated to dryness under reduced pressure at 30 °C and re-dissolved in 6 mL phosphate-citrate buffer (0.2 mol dm^{-3}, pH 5.0). Remaining volatile compounds were removed by extraction with 5 × 3 mL of pentane. Prior to enzymatic hydrolysis, the absence of volatile compounds was tested by TLC. 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. Volatile compounds were detected using 2 % vanillin in concentrated sulfuric acid. After addition of 1 mL of an almond β-glucosidase solution (Fluka, 5–8 U/mg, concentration of 1 unit/mL buffer), the mixture was incubated overnight at 37 °C. After addition of 1 g of sodium chloride, the released aglycones were extracted with 5 × 5 mL of pentane/diethyl ether mixture (1:1 *v*/*v*). The combined extract was concentrated to 1 mL, and 2 μL were used for GC-MS analysis.

Gas Chromatography-Mass Spectrometry

Analyses of volatile compounds were performed using a Hewlett Packard GC-MS system (GC 5890 series II; MSD 5971A, Hewlett Packard, Vienna, Austria). The fused-silica HP-20 M polyethylene glycol column (50 m × 0.2 mm, 0.2 μm thickness, Hewlett Packard, Vienna, Austria) was directly coupled to the mass spectrometer. The carrier gas was helium (1 mL/min). The program used was 4 min isothermal at 70 °C, then 4 °C/min to 180 °C and 10 min isothermal. 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).

The linear retention indices for all compounds were determined by co-injection of the sample with a solution containing the homologous series of C_8-C_{22} n-alkanes.[19] Individual constituents were identified by their retention indices identical to the compounds known from literature data,[20] and also by comparing their mass spectra with the spectra of either known compounds or those stored in the Wiley mass spectral database (Hewlett Packard, Vienna, Austria).

Free Radical Scavenging Capacity Using the Stable Radical (DPPH)

Antioxidant activity of the nutmeg essential oil and of the released volatile aglycones was measured in terms of hydrogen donating or radical scavenging ability, using the stable radical, DPPH.[21] The 50 mL ethanolic solution of the antioxidant (concentrations of stock solutions were 7, 5, 3.5, 1.5 g/L for aglycones (due to limited quantity of the aglycones, only few concentrations were used), and 50, 30, 20, 16.8, 12.6, 10.5, 8.4, 6.3, 3.5 g/L for the essential oil) was placed in a cuvette, and 1 mL of 6×10^{-5} mol dm^{-3} ethanolic solution of DPPH was added. Absorbance measurements started immediately. The decrease in absorbance at 517 nm was determined using a UV-VIS (double-beam) Perkin-Elmer Lambda EZ 201 spectrophotometer. Ethanol was used to zero the spectrophotometer. Absorbance of the DPPH radical without antioxidant, *i.e.*, the control, was measured daily. All determinations were performed in triplicate.

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

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

Determination of FRAP – Ferric Reducing/Antioxidant Power

Determination of ferric reducing / antioxidant power FRAP is a simple direct test of antioxidant capacity. This method was initially developed to assay plasma antioxidant capacity, but it can be used for plant extracts as well. Total antioxidant potential of the sample was determined using the ferric reducing ability (FRAP) assay[22] as a measure of »an-

tioxidant power«. FRAP assay measures the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from the colorless oxidized Fe^{III} 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 measured at 593 nm (M1 reading). Subsequently, 50 μL of the sample (concentrations of stock solutions were 0.5, 1.5, 3.5, 5 g/L) and 150 μL of deionized water was added to the FRAP reagent. Final dilution of the sample in reaction mixture was 1:34. Absorbance readings were taken every 15 s thereafter during the monitoring period (8 minutes). Since there was little decrease in absorbency between 4 and 8 minutes of incubation, we used its value after 4 minutes for further calculation. 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 was selected for calculation of FRAP values. 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, the antioxidant efficiency of the antioxidant under study was calculated with reference to the reaction signal given by an Fe^{2+} solution of known concentration. The results were corrected for dilution and expressed as $[Fe^{2+}]$ / mmol L^{-1}. All determinations were performed in triplicate.

RESULTS AND DISCUSSION

The paper reports on the isolation of glycosidically bound volatiles from nutmeg, identification of released aglycones, and determination of antioxidative power of the released aglycones in comparison with the nutmeg essential oil. Knowing about the toxicity of oil components, myristicin and elemicin, one of the study aims was to determine the presence and quantity of these two compounds in glycosidic form, as well.

Chemical Composition of Released Volatile Aglycones Compared to the Essential Oil Composition

After evaporation of the solvent, the content of released volatile compounds in dried plant material was 0.11 mg g^{-1}. Percent composition of the volatile aglycones is shown in Table I. The main aglycones identified in nutmeg are phenylpropanoids isoeugenol (46.1 %) and methoxyeugenol (27.7 %). Other aglycones were pulegone (5.6 %), *cis*-isoeugenol (3.7 %), β-thujone (3.4 %), cuminol (3.3 %), isoelimicin (3 %), eugenol (2.8 %), methyl isoeugenol (2.3 %) and terpinen-4-ol (1 %). Some of the volatile compounds, such as eugenol, linalool, geraniol, nerol, benzyl alcohol, 2-phenylethanol and α-terpineol, are ubiquitous in many plants in the form of aglycones.[23] However, only eugenol has been detected in the aglycones fraction of nutmeg.

TABLE I. Percent composition of glycosidically bound volatile compounds in nutmeg isolated by liquid-solid chromatography on Amberlite XAD-2 as adsorbent and hydrolyzed by using β-glucosidase

No.	Component	Rt / min	RI[(a)]	Peak area / %
1	β-Thujone	14.74	1399	3.4
2	Terpinen-4-ol	19.99	1557	1.0
3	Cuminol	34.07	2035	3.3
4	Eugenol	36.06	2094	2.8
5	Methyl isoeugenol	36.79	2121	2.3
6	*Cis*-isougenol	39.31	2187	3.7
7	Isoeugenol[(b)]	43.87	–	46.1
8	Isoelemicin[(b)]	47.18	–	3.0
9	Pulegone[(b)]	50.41	–	5.6
10	Methoxyeugenol[(b)]	58.00	–	27.7
	Total			98.9

[(a)] RI – retention indices relative to C_8-C_{22} n-alkanes on the polar HP-20M column.

[(b)] Retention times of these compounds are outside the retention times of homologous series of C_8-C_{22} *n*-alkanes, which were used for calculation of retention indices. Identification was achieved only by means of their mass spectra.

An amount of 4.92 g of the essential oil was obtained from 100 g of nutmeg. Percent composition of the essential oil from nutmeg is shown in Table II. Major components of nutmeg oil are terpenes, terpene alcohols and phenolic ethers.[24,25] The monoterpene hydrocarbons, β-pinene (23.9 %), α-pinene (17.2 %), and limonene (7.5 %), constituted the main fraction of nutmeg oil. The major phenolic ether is myristicin (16.2 %), accompanied by safrole (3.9 %) and methyl eugenol (1.8 %).

Comparison of the chemical composition of liberated volatiles with the chemical composition of free volatile compounds found in the essential oil reveals only two common compounds (eugenol and terpinen-4-ol). The presence of two major constituents of the aglycone fraction, isoeugenol and methoxyeugenol, have not been detected among the free compounds in the essential oil. Results of other studies[24,25] also show that the percent composition of these two compounds is insignificant compared to other oil constituents. Regarding the substantial interest in the metabolism and action of nutmeg, this paper reveals the presence of compounds whose effect may have been overseen due to the fact that the major amount of these compounds was present in nutmeg in glycosidic form.

Antioxidant Activity of Released Volatile Aglycones Compared to that of Essential Oil

To measure the antioxidative capacities of the essential oil and enzymatically released aglycones from nutmeg,

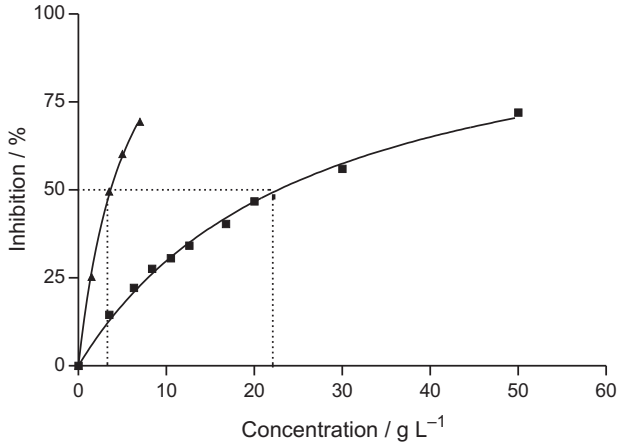

Figure 1. Antioxidant activity of nutmeg essential oil (–■–) and its volatile aglycones (–▲–), measured by the DPPH method. Concentrations of the essential oil and the aglycones are given as the concentrations of stock solutions.

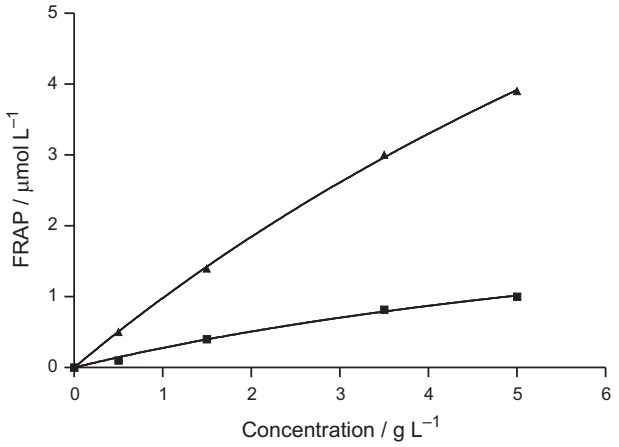

Figure 2. Dose-response curves of the antioxidant capacity of nutmeg essential oil (–■–) and its volatile aglycons (–▲–), measured by the FRAP assay.

two different assays were performed: the 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) and the ferric reducing / antioxidant power assay (FRAP). Owing to the advantage of being very simple to carry out, procedures in which the consumption of a stable free radical (DPPH$^{\cdot}$, ABTS$^{\cdot}$+) is measured following addition of the tested compound are widely used in the assessment of radical scavenging activity.[26] The method using a stable organic DPPH radical is based on the reduction of alcoholic DPPH solution at 517 nm in the presence of a hydrogen donating antioxidant as a result of the formation of the non-radical form DPPH-H. The remaining DPPH, measured after a certain time, corresponds inversely to the radical scavenging activity of the antioxidant.[27] The method was applied to evaluation of antioxidant properties of the nutmeg essential oil and the corresponding released aglycones in comparison with three known antioxidants (BHT, α-tocopherol, and ascorbic acid). Therefore, compared to compounds with a strong antioxidant activity, such as the BHT, ascorbic acid and α-tocopherol, the essential oil and the aglycones fraction show significant scavenging activity.[28] Although known as an essential oil with good antioxidative properties, comparison between the essential oil of nutmeg and its released aglycones shows (Figure 1) that the aglycone fraction possesses an even better scavenging activity with IC_{50}-values for the aglycones and the nutmeg oil being 3.5 g/L and 22 g/L, respectively.

TABLE II. Percent composition of the essential oil isolated from nutmeg

No.	Component	Rt / min	RI[(a)]	Peak area / %
1	α-pinene	5.38	1038	17.2
2	β-pinene	6.68	1102	23.9
3	β-myrcene	7.48	1149	2.2
4	α-terpinene	7.82	1161	3.8
5	Limonene	8.31	1183	7.5
6	γ-terpinene	9.43	1231	6.8
7	*p*-cymene	9.96	1247	0.9
8	Terpinolene	10.33	1262	1.8
9	α-copaene	16.99	1466	0.7
10	Terpinen-4-ol	20.04	1557	7.9
11	Terpineol	23.00	1624	0.8
12	Safrole	28.04	1824	3.9
13	Methyl eugenol	31.81	1958	1.8
14	Eugenol	36.12	2094	0.3
15	Methyl isoeugenol	36.81	2121	0.4
16	Elemicin	39.00	2179	1.1
17	Myristicin[(b)]	40.36	–	16.2
	Total			97.2

[(a)] RI – retention indices relative to C_8-C_{22} n-alkanes on the polar HP-20M column.

FRAP assay is based on comparison of the total amount of antioxidants with the reducing capacity of the sample. Absorbance changes are linear over a wide range with the antioxidant mixture. The advantage of the FRAP assay is in being fast, easy to handle, with highly reproducible results.[26] Antioxidant capacities of the essential oil and the released volatile aglycones, as determined by the FRAP assay, are shown in Figure 2. From the results shown, a linear dose-response concentration line can be seen for the released aglycones and the essential oil measured by the FRAP assay. The results obtained from antioxidative measurements by both assays show that the aglycones fraction exhibits much stronger antioxidative capacity than the essential oil.

According to Dorman's[29] assessment of antioxidant activity of 33 phytoconstitutents from nutmeg, black pepper, clove, geranium and melissa, antioxidant activity of eugenol, carvacrol and thymol is much higher compared

to other compounds such as α-pinene, *p*-cymene, limonene, terpinen-4-ol, and terpinolene. When the composition of the nutmeg oil and its released volatile aglycones is considered, the difference in the antioxidant capacity of the aglycones is probably due to differing amounts of eugenol and isoeugenol. As shown in Tables I and II, the percent composition of eugenol forms in the essential oil is very low, while isoeugenol and methoxyeugenol constitute the major portion of released aglycones. However, in the case of complex mixtures such as nutmeg essential oil, the antioxidant activity of the oil is probably due to synergy among the oil components. Thus, although scarce in a powerful single antioxidant constituent, the essential oil still exhibits a relatively significant antioxidant activity.

The hydroxyl group in the phenolic ring of eugenol/isoeugenol is responsible for the antioxidative activity of both compounds while lack of the free hydroxyl group can explain the low activity of *p*-cymene.[30] Based on interest[31,32] in studying the activity of compounds isolated from nutmeg, it is interesting how distribution of these compounds differs between the aglycones and the essential oil fraction. Myristicin and safrole occur in the essential oil while a significant amount of methoxyeugenol is restricted to the aglycone fraction. This can serve as important evidence relating to the consumption and metabolism of the volatile compounds in nutmeg.

Conversant with the glycolitic cleavage of glycoconjugates in the stomach and in the intestine, this area of research provides predictions of glycosides activities in the organism. Hence the released aglycones are interesting as a hidden antioxidant potential in nutmeg. Due to the fact that attachment of glycosidic moiety to a molecule enhances its hydrophilicity, which effects pharmacokinetics of that particular compound, this area of research offers a vast potential of showing the effects and applications of the compounds carrying glycosidic moiety compared to the respective aglycones.

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SAŽETAK

Kemijski sastav i antioksidacijski učinak oslobođenih hlapljivih aglikona iz muškatnoga oraščića (*Myristica fragrans* Houtt.) u usporedbi s njegovim eteričnim uljem

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Rad izvještava o izolaciji glikozidno vezanih hlapljivih spojeva iz muškatnoga oraščića, identifikaciji oslobođenih aglikona i određivanju antioksidacijskih svojstava oslobođenih aglikona u usporedbi s eteričnim uljem istoga. Usporedba kemijskoga sastava oslobođenih aglikona s kemijskim sastavom slobodnih hlapljivih spojeva iz eteričnoga ulja otkriva samo dva zajednička spoja (eugenol i terpinen-4-ol). Antioksidacijski kapacitet eteričnoga ulja i enzimatski oslobođenih aglikona iz muškatnoga oraščića ispitan je pomoću dvije različite metode: 2,2'-diphenyl-1-picrylhydrazyl radical scavenging method (DPPH) i ferric reducing / antioxidant power assay (FRAP). Obje metode su pokazale da aglikonska frakcija posjeduje jača antioksidacijska svojstva nego slobodni hlapljivi spojevi iz ulja.