Asian Journal of Chemistry; Vol. 28, No. 1 (2016), 199-202

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2016.19342

Isolation and Identification of Flavonoid Compound from Nutmeg Leaves (*Myristica fragrans* Houtt)

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Received: 12 May 2015; *Accepted*: 25 June 2015; *Published online*: 5 October 2015; AJC-17574

A flavonoid compound *i.e.* 3,5,7,4'-tetrahydroxy-dihydroflavonol or dihydrocaempferol which has contents an antioxidant activity with IC_{50} value about 9.75 ppm were successfully isolated and identified. The structures of the obtained compound are identified using spectral data analysis such as ultra-violet, infrared, 1D NMR (^{1}H NMR, ^{13}C NMR), 2D NMR (COSY, HMQC and HMBC) and mass spectra.

Keywords: Nutmeg, *Myristica fragrans,* Flavonoid, Antioxidant, Spectral data analysis.

INTRODUCTION

The increased discoveries in herbs and spices as sources of natural antioxidants have initiated researchers to look for natural antioxidants with low cytotoxicity [1-3]. Antioxidant are compounds that can inhibit the rate of oxidation or neutralize free radicals [4]. The free radicals [produced in body system in the form of reactive oxygen species (ROS)] which cause oxidative stress and harm to the components of the cell resulting in cellular and metabolic disruptions are believed to play an essential role in human health [2,5].

Myristica fragrans Houtt is one of the high valued spice comodity in trading since Romanian era. The dried and powdered seed (known as nutmeg) is a well-known spice for flavouring many food products and for formulation of medicated balm and perfume. It is also claimed for having anti-inflammatory, aphrodisiac, anthelmintic, hepatoprotective and insecticide properties and a remedy for rheumatism, asthma, atherosclerosis, diarrhea and flatulence. Its aril (known as mace) is the main ingredient in medicated ointment for healing headache, insect bites and rheumatism. The infusion of its leaf is drunk to relieve flatulence intestinal spasm, and hypertension [6,7].

Many chemical and pharmacological studies on flowers, seeds, fruits and nuts of *M. fragrans* have been investigated. The seed kernel of *M. fragrans* contains macelignan compound and shows activity as anticariogenic agents to *S. mutans, Streptococcus sobrinus, Streptococcus salivarius, Streptococcus sanguis, Lactobacillus acidophilus and Lactobacillus casei* [8]. The *n*-hexane extract from a *M. fragrans* seed which acetone-insoluble part of the *n*-hexane extract (AIMF) and trimyristin (TM) shows an anxiogenic activity [9]. The content of total phenolic, antioxidant and antibacterial activity of the different parts of leaf and fruit (pericarp, aril, seedkernel and shell) were compared. The highest content of total phenolic with extracted shell acted as the greatest primary antioxidant are provided by the 80 % extracted methanol of aril, seedkernel and shell [10]. The aril of *M. fragrans* consists of several new tetrahydrofuran lignans [11] and fragransol C, fragransol D, myristicanol-A, myristicanol-B and neolignan [12], the nuts contain (-)-epicatechin and active as antioxidant. The compounds which isolated from nuts, stems and shell of *M. fragrans* usually consist of *meso* dihydroguaiacetic acid, β-phellandren, eugenol, isoeugenol, safrol, cavrol, *etc.* [13]. So far, there are no published reports about chemical compounds from the leaves of a *M. fragrans*. In the course of our research for bioactive metabolites from the plants used in traditional medicine, therefore it's interested in characterizing the chemical constituents. In this paper, the phytochemical extraction of antioxidant compound from leaves of *M. fragrans* is firstly investigated as shown in Fig. 1.

To our best of knowledge, most of the previous scientific studies focused mainly on the seed and aril, neglecting the other edible parts *i.e.* Nutmeg leaf [1,2,8-11,14-16]. The paper presents the investigation of the flavonoid content and antioxidant activity of leaf *of M. fragrans* and reports the first phytochemical finding of antioxidant compound from leaf of *M. fragrans*.

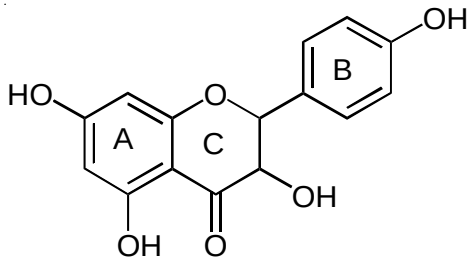

Fig. 1. 3,5,7,4′-tetrahydroxy-dihydroflavonol

EXPERIMENTAL

The leaves of *Myristica fragrans* Houtt (Myristaceae) were collected from Desa Salam Tani, Kecamatan Pancur Batu, Kabupaten Deli Serdang, Indonesia. Authentication of plant material was carried out at the Herbarium Bogoriensis of The Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong 16911, Indonesia, where the herbarium voucher has been kept (Fig. 2).

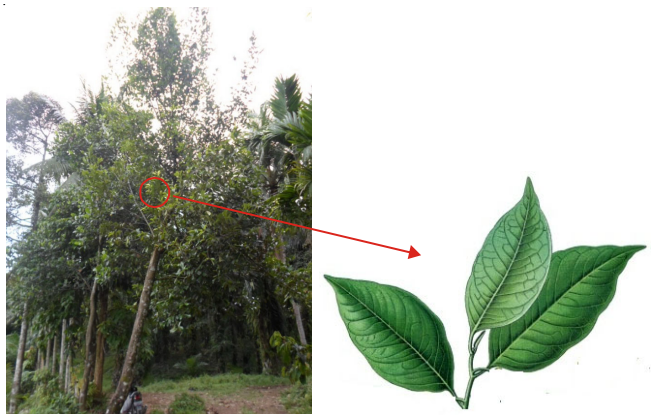

Fig. 2. Plant of Nutmeg (*Myristica fragrans* Houtt)

Preparation of plant extract: The leaves of *M. fragrans* were dried at room temperature, powdered (5 Kg) and macerated with methanol. Methanol extracts (13.92 %) were added with ethyl acetate solvent. Soluble portion of ethyl acetate solvent were collected (5.96 %). Ethyl acetate extracts were partitioned further with mixture of *n*-hexane-methanol to get non-polar compounds free extracts (soluble in *n*-hexane). The methanol extracts were hydrolyzed with 2 N HCl and then filtered, followed with partition using chloroform. The chloroform extracts were considered as total flavonoid (5.50 %) and prepared for isolation, purification and chemical structure further.

Isolation and purification: Purification of total flavonoid was carried out by gradient column chromatography (SiO_2, *n*-hexane-ethyl acetate = 9:1-1:1; and ethyl acetate) with 10 fractions. Based on antioxidant activity test results using free radical scavenging method (DPPH = 1,1-diphenyl-2-picrylhydrazyl), we found out that fraction 4 (a number of little) and 7 provide highest IC_{50} value at 12.19 and 14.38 ppm respectively (Table-1) [17].

Fraction 7 was purified further by gradient column chromatography (SiO_2; chloroform-ethylacetate = 10:1-5:1) and yields 3 fractions *i.e.* fraction 7-1-7.3. Purification for fraction 7-1 by column chromatography (SiO_2; chloroform-ethyl acetate = 4:1) gave 4 fractions (7.1.1-7.1.4). Fraction 7.1.3 was purified by preparative chromatography (chloroform-ethylacetate = 4:1) to give one pure compound as a white, amorf powder (14 mg).

TABLE-1
IC_{50} OF LEAF EXTRACTS NUTMEG AND PURE COMPOUND NUTMEG

No	Extract	% Inhibition at concentration (ppm)			
		25	50	100	IC_{50}
1	Methanol	62.06	88.60	94.00	24.60
2	Ethyl acetate	64.83	77.91	90.40	26.17
3	*n*-Hexane	52.22	53.06	76.83	27.67
4	Total flavonoid	63.48	90.04	97.60	23.57
5	Pure compound (fraction 7.1.3)	61.91	71.24	96.57	9.75
6	Vitamin C	78.15	96.04	96.76	8.84

DPPH radical scavenging activity: The DPPH radical scavenging activity test for the extracts and fractions (1 mg-10 mg) is carried out using method conducted by Gupta, *et al.* [14]. Vitamin C (0.01-0.1 mg) was used as positive control.

Chemical structure elucidation: The structure of the isolated compound was determined by its NMR data [^{1}H NMR and ^{13}C NMR on JEOL 500 spectrometer; 500 MHz for ^{1}H NMR; 125 MHz for ^{13}C NMR)]; UV spectra (Shimadzu Varian Cary 100 Conc); IR spectra [Perkin Elmer FTS FT-IR spectrometer] and EI-MS mass spectra by LC-MS/MS (HPLC Alliance 2695, Waters Detector Photodiode Array 2996).

RESULTS AND DISCUSSION

Phytochemical test: Phytochemical screening results show that leaves of *M. fragrans* containing chemical compound groups alkaloids, flavonoids, terpenoids, saponins and tanins.

Antioxidant activity: Several extracts with antioxidant activity obtained from leaf of *M. fragrans* were tested with radical DPPH (its stability is measured). When radical DPPH reagent mixed with antioxidant compound that donor hydrogen atoms, the reaction formed non-radical compound diphenypicryl-hydrazine and inducted colour change in the mixture from purple to yellow and decreased absorbance value at wavelength 517 nm. Antioxidant activity of several extracts and pure compounds were compared to standard vitamin C (Table-1, Fig. 3, Table-2, Fig. 4).

TABLE-2
IC_{50} OF OF TOTAL FLAVONOID FRACTIONS

No	Fraction	% Inhibition at concentration (ppm)			
		25	50	100	IC_{50}
1	Fraction 1	13.65	21.68	58.29	204.33
2	Fraction 2	46.17	71.17	89.80	23.81
3	Fraction 3	52.63	62.76	86.48	29.51
4	Fraction 4	53.95	74.62	95.28	12.19
5	Fraction 5	48.72	77.30	97.07	28.56
6	Fraction 6	51.66	68.75	92.98	33.84
7	Fraction 7	50.64	77.17	94.13	14.38
8	Fraction 8	39.29	65.82	89.41	35.16
9	Fraction 9	40.94	68.49	90.56	31.88
10	Fraction 10	44.13	67.35	90.18	29.22

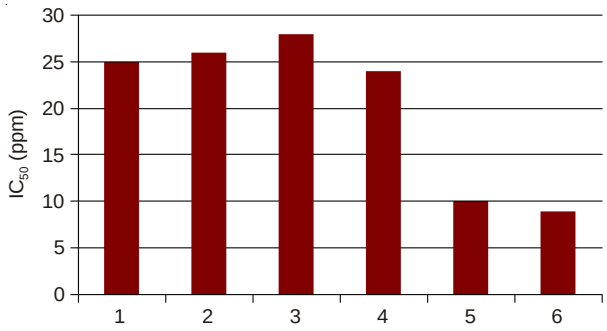

Fig. 3. IC_{50} of leaf extracts Nutmeg and pure compound nutmeg; 1 = methanol extract, 2 = ethyl acetate extract, 3 = *n*-hexane extract, 4 = total flavonoid of nutmeg, 5 = pure compound (fraction 7.1.3) and 6 = standard vitamin C

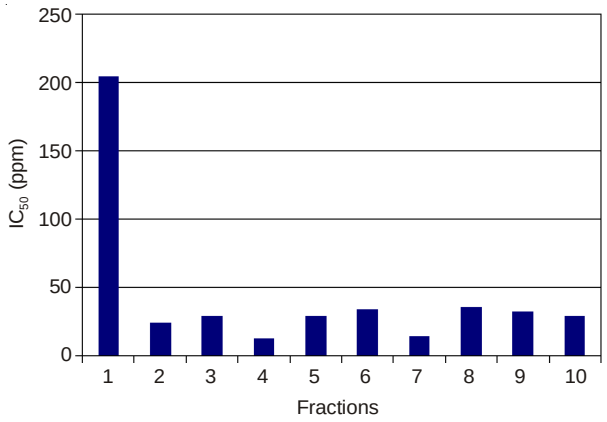

Fig. 4. IC_{50} of total flavonoid fractions

As shown in Tables 1 and 2, all extracts provide the values of IC_{50} less than 1000 ppm which are around 23 to 28 ppm and around 12 to 205 ppm for Tables 1 and 2, respectively. The more excited result which is only slighly higher than value from vitamin C is achieved from pure compound (fraction 7.1.3) about 9.75 ppm. The cathechol structure with phenolic hydrogen donor or electron to radical acceptor characteristics is obtained. The same characteristics with similar compound has also been reported by Shan *et al.* [18].

Determination of chemical structure: Identification of chemical structure of flavonoid compound was found in fraction 7.1.3 as creamy white amorphous crystals. Infrared spectroscopy analysis showed sharp absorbance bands at 3082, 2920, 1722, 1595, 1495 and 1170 cm^{-1}. It contains functional groups OH, C-H streching; C=O (carbonyl); C=C aromatic; C-O-C ether respectively that characterizes the flavonoid compound. Ultraviolet spectroscopy data analysis showed band peak I at λ_{max} 365 nm and band peak II at λ_{max} 265 nm which is the characteristic of flavonoid type flavonol. The ^1H NMR and ^{13}C NMR spectra of flavonoid compound were measured in $CDCl_3$ (Table-3).

Table-3 showed one methine proton in splitting doublet (d) pattern at δ_H 4.61 ppm (H-3) (CH) and one proton in splitting doublet (d) pattern at δ_H 5.05 ppm (H-2). One methine proton (=CH) at δ_H 5.92 (H-7) and 5.96 ppm (H-8) each in splitting doublet (d) pattern showed characteristic of A ring of flavonoid skeleton. Four methin protons (=CH) also found at δ_H 6.8 (H-3′ and H-5′) and δ_H 7.2 ppm (H-2′ and H-6′), each in splitting doublet (d) pattern that showed characteristic of B

TABLE-3
^1H NMR AND ^{13}C NMR SPECTRA OF
FLAVONOID COMPOUND (FRACTION 7.1.3)

Carbon No.	(500 MHz; $CDCl_3$; ppm; *J* in Hz)	
	^1H NMR, δ	^{13}C NMR, δ
1	-	-
2	5.05 (d, *J* = 11.7)	83.45 (d)
3	4.61 (d, *J* = 11.7)	72.23 (d)
4	-	198.87 (s)
5	-	164.05 (s)
6	5.96 (d, *J* = 2.0)	96.50 (d)
7	-	168.47 (s)
8	5.92 (d, *J* = 2.0)	95.53 (d)
9	-	163.31 (s)
10	-	100.19 (s)
1’	-	128.41 (s)
2’	7.42 (d, *J* = 8.4)	129.44 (d)
3’	6.80 (d, *J* = 8.4)	115.05 (d)
4’	-	157.99 (s)
5’	6.80 (d, *J* = 8.4)	115.05 (d)
6’	7.42 (d, *J* = 8.4)	129.44 (d)

ring of flavonoid skeleton. DEPT data analysis showed that fraction 7.1.3 has 15 C atoms. At δ_C 72.23 ppm (C-3), δ_C 83.45 ppm (C-2), δ_C 95.53 ppm (C-6), δ_C 96.50 ppm (C-8), each of them has one methine carbon (CH) in splitting doublet (d) pattern. At δ_C 115.05 ppm (C-3′ and C-5′) and δ_C 129.44 ppm (C-2′ and C-6′) each of them has also had two methine carbons in splitting doublet (d) pattern. Quartenary carbons were appeared at δ_C 100.19 ppm (C-10), δ_C 128.41 ppm (C-1′), δ_C 157.99 ppm (C-4′), δ_C 1163.31 ppm (C-9), δ_C 164.00 δ_C ppm (C-5), δ_C 168.47 ppm (C-7) and δ_C 198.87 ppm (C-4).

Analysis of HMQC (homonuclear quantum coherence) of fraction 7.1.3 (Fig. 5) consists of two bonds of C-H at the ring of **C** that obtained from chemical movement of δ_H 4.61 ppm (H-3) and δ_H 5.05 ppm (H-2), respectively which are correlated with δ_C 72.23 ppm (C-3) and δ_C 83.45 ppm (C-2), respectively. Two bonds of C-H at the ring **A** were formed from chemical movement of δ_H 5.95 ppm (H-6) and δ_H 5.96 ppm (H-8), respectively which are correlated with δ_C 95.53 ppm (C-6) and δ_C 96.50 ppm (C-8), respectively. The four bonds of C-H at ring **B** were formed from chemical movement of δ_H 5.92 ppm (H-3′ and H-5′) which are correlated with δ_C 95.53 ppm (C-3′ and C-5′) (formed two overlapped bonds of C-H) and δ_H 5.96 ppm (H-2′ and H-6′) which are correlated with δ_C 96.50 ppm (C-3′ and C-5′) (formed two overlapped C-H bonds).

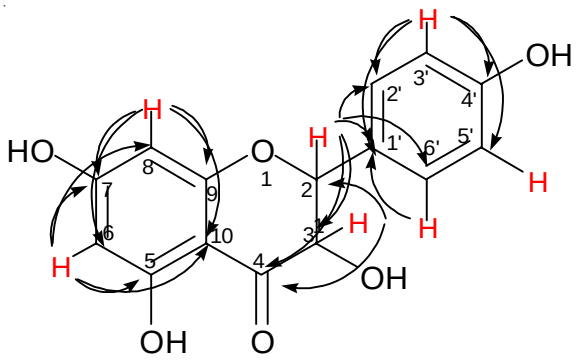

Fig. 5. HMBC analysis for chemical structure of fraction 7.1.3

Homonuclear correlation spectra (^{1}H-^{1}H COSY) is analyzed to determine correlation between proton and proton of ^{1}H NMR spectra of fraction 7.1.3. It is shown that methine proton (=CH) at δ_H 4.61 (H-6) is correlated with methine proton at δ_H 5.05 (H-8). Two methine protons at δ_H 6.8 (H-3′) and (H-5′) were correlated with two methine protons at δ_H 7.42 (H-2′) and (H-6′).

Hetero multiple bond connection (HMBC) spectra analysis (Fig. 5) showed correlation between proton and carbon for more than one bond. Ordinate axis in HMBC spectra can be plotted with chemical shift of proton nuclear ^{1}H HMBC spectra analysis for fraction 7.1.3 showed proton signal at δ_H 5.92 ppm (H-6) and vertical line stretched from that signal gave 4 bonds as results of long distance correlation with carbon atom. Those bonds were belong to carbons at C-5 (δ_C 100.19); C-7 (δ_C 163.31); C-8 (δ_C 95.50); and C-2' (δ_C 168.46). Vertical line stretched from δ_H 5.96 ppm (H-6) gave 3 bonds at C-6 (δ_C 95.53); C-10 (δ_C 100.19); and C-5 (δ_C 163.31). Vertical line stretched from δ_H 6.80 ppm (H-3 dan H-6) provide 2 bonds at C-2′ and C-5′ (δ_C 129.44); and C-4′ (δ_C 157.99).

EI-MS mass spectra characterization by LC-MS/MS showed that this compound has similarity with dihydrocaempferol. Fraction 7.1.3 spectra showed molecule ion at *m/z* 288 (M^+) *i.e*. [M+H_2] that indicated molecular weight of 286) with molecular formula $C_{15}H_{12}O_6$. This might cause by H_2 molecules movement resulted in addition of 2 electrons. Those two molecules were fragmented which causes molecular ion peaks and ion fragments at *m/z* = 256 by releasing CH_3OH molecule, at *m/z* = 230 by releasing $C_2H_2O_2$ molecules and at *m/z* = 106 by releasing $C_9H_8O_4$ molecule.

Conclusion

Based on infrared, ultraviolet, ^{1}H NMR, ^{13}C NMR spectral data analysis and supported by HMQC, ^{1}H-^{1}H-COSY, HMBC and MS spectral data, pure compound isolated from leaf of *M. fragrans* (fraction 7.1.3) was determined as flavonoid compound 3,5,7,4′-tetrahydroxy-dihydroflavonol. Both extracted and pure compound had high antioxidant activity which can be used for preventing or treating oxidative diseases.

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