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Lipoxygenase Inhibitor from Defatted Nutmeg Seed

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Abstract

Lipoxygenase inhibitory activity of the methanolic extract of 60 different kinds of plant seeds was determined by a spectrophotometric method using a soybean lipoxygenase (SLO) and linolenic acid. Among the extracts examined, the methanolic extract of nutmeg (*Myristica fragrans*) seed showed the most potent SLO inhibitory activity. To isolate SLO inhibitor, hence, the defatted methanol extract was further partitioned with ether, ethyl acetate, and n-butanol, stepwise. The ether souble fraction was successively chromatographed on silica gel, Sephadex LH-20 and preparative TLC. Three phenolic compounds were isolated, and one of them showing a strong SLO inhibition activity was identified as a 2,6-dihydroxy-9-(3',4'-dihydroxyphenyl) nonylphenone (IC_{50}=0.39 μg/ml) by ^{1}H- & ^{13}C-NMR, IR, and MS spectroscopy.

Key words: plant seeds, nutmeg (*Myristica fragrans*), soybean lipoxygenase inhibitor, 2,6-dihydroxy-9-(3',4'-dihydroxyphenyl)nonylphenone

INTRODUCTION

Lipoxygenase (linoleate: oxidoreductase, EC 1.13.11.12) catalyzes the dioxygenation of polyunsaturated fatty acids possessing a 1,4-cis,cis-pentadiene moiety to yield 1,3-cis, trans diene 5-hydroperoxides (1). This enzyme widely exists in plants and mammals (2,3). In plants, soybean lipoxygenase (SLO) has extensively been studied in the production of undesirable off-flavors and off-odors during processing (4). In mammals, a 5-lipoxygenase (5-LO), which is a key enzyme involved in arachidonic acid metabolism, catalyzes the oxygenation of arachidonic acid to 5-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE) and its subsequent dehydration to form several leukotrienes (LTs) (5). LTs play an important roles in the pathology of many inflammatory and allergic diseases (6,7). Therefore, the specific 5-LO inhibitors are expected to be potential therapeutic drugs for the prevention of these diseases. At present, many scientists are widely using SLO as an *in vitro* assay to search for natural 5-LO inhibitors due to structural as well as mechanistic similarities between SLO and human 5-LO (8,9).

Plant seeds have been reported to contain various antioxidative substances, such as tocopherol, γ-oryzanol, sesamol and several flavonoids (10,11). Recently, much attention has been received in the use of a large amount of byproducts obtained from the production of plant seed oils as potentially attractive sources of natural antioxidants. Nutmeg (*Myristica fragrans*) has been widely used as a spice and a valuable remedy for strengthening the stomach and expelling "wind-evil" in Chinese medicine (12). There are many reports on the antioxidative and pharmacological constituents of essential oils from nutmeg (13-15). In particular, eugenol and its derivatives were found to have antibacterial, antiplatelet and antiinflammatory actions (16,17), whereas myristicin, safrole and isoelemecin have psychotropic and carcinogenic actions (18,19). However, few studies on the isolation and identification of phenolic antioxidative and antiinflammatory compounds except essential oils are available. Recently, we have reported that the methanolic extract of nutmeg spice show a significant inhibitory activity against SLO, and especially tetraol is a major component for the inhibition of lipoxygenase (20).

The objective of present study was to isolate and identify novel soybean lipoxygenase inhibitors from the seed kernels of nutmeg.

MATERIALS AND METHODS

Materials and reagents

Most of the dried plant seeds were purchased from local oriental herbal store in Taegu, Korea, and the others were collected, and dried in shade before use. Soybean lipoxygenase (type V), linolenic acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other used for this study were of analytical grade.

Free radical scavenging activity

Free radical scavenging activity of the methanolic extracts of plant seeds was determined by the method of Blois (21).

Methanolic extract of seed (2 ml, final concentration 0.8 mg/ml) decolorized with cartridges (Sep-Pak C_{18}, Waters, USA) were added to a methanol solution (1.0 ml) of 0.2 mM DPPH radical. The mixture was shaken vigorously and left

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to stand for 30 min; the absorbance of the resulting solution was measured at 517 nm with a photodiode array UV-VIS spectrophotometer (S2030, SINCO, Korea).

The percent inhibition of DPPH radical, 100-[(absorbance at 517 nm of sample/absorbance at 517 nm of control)×100] was calculated to express free radical scavenging activity of all seeds. All tests and analysis were run in triplicate.

Soybean lipoxygenase (SLO) assay

SLO assay was performed according to a method of Block et al. (9) with a slight modification.

0.1 M Tris buffer (2.0 ml; pH 8.5) and inhibitors in ethanol (20 μl) were added to the cuvette at 18°C, followed by addition of soybean lipoxygenase (type V, 500 units/final concentration) solution in buffer (30 μl). After a 5 min equilibration period the reaction was started by addition of an ethanolic solution of linolenic acid (50 μg; 110 μM/final concentration). The absorbance at 234 nm was recorded as a function of time on a spectrophotometer. The rates were measured from the initial slopes of the linear portions of the curves. A sample containing all of the reagents except the enzyme solution was used as a blank sample. IC_{50} values were determined by regression analysis of the results at three different concentrations of the inhibitor.

Isolation and purification of lipoxygenase inhibitor from nutmeg seed

Nutmeg seeds (600 g) were crushed into small pieces and extracted twice with hot n-hexane (2.0 L) to remove lipids. The residue was extracted twice with MeOH (2.0 L) under reflux. The concentrated methanolic extract was partitioned between n-hexane and 80% aqueous methanol. The 80% aqueous methanolic layer was concentrated to a small volume *in vacuo*, and then partitioned with ether, ethyl acetate, and n-butanol, stepwise. The ether layer (15.8 g), which exhibited the strongest inhibitory activity against SLO, was chromatographed on silica gel (70-230 mesh, Merck, Germany), using mixtures of $CHCl_3$-MeOH (10 : 1) with increasing amounts of MeOH to give five fractions. Among these fractions isolated, the third active fraction was further subjected to column chromatography over Sephadex LH-20 (Pharmacia Biotech, Uppsala, Sweden) with methanol as an eluent and then separated into three fractions (fr.1 with λ_{max} 225 & 270 nm; fr. 2 with λ_{max} 225, 270 & 340 nm; fr. 3 with λ_{max} 225 & 272 nm). The second fraction showing the most strong SLO inhibition, was developed on preparative TLC (Silica gel 60 F_{254}, 0.5 mm, Merck, Germany), using benzene-ethyl acetate-acetic acid (10 : 10 : 1) as solvent, and three bands with Rf 0.65 (compound 1, 18.9 mg), 0.60 (compound 2, 7.2 mg) and 0.51 (compound 3, 11.3 mg) were repeatly isolated. Among them, compound 1 was crystallized from benzene-ethyl acetate (1 : 1) to yield as a light yellow needles.

Instrumental analysis of compound 1

UV absorbance spectrum of the purified active compound 1 was recorded on a spectrophotometer with dilute solution in methanol. The IR spectrum was taken on an IFS 120 HR infrared spectrometer (Bruker, Germany) as KBR discs. ^1H-NMR (300 MHz) and ^{13}C-NMR (75.5 MHz) were measured on a Unity Plus 300 spectrometer (Varian, USA) in CD_3OD containing tetramethylsilane (TMS) as an internal standard. The electron impact mass spectrometry (EI-MS) was measured with a Quattro II mass spectrometer (VG, UK) at an ionization voltage of 70 eV.

Statistical analysis

Statistical analysis was accomplished with Statistical Analysis System (22) software package on replicated test data. Significant differences between means were determined by Duncan's multiple range tests.

RESULTS AND DISCUSSION

Free radical scavenging activity

The radical scavenging activities of the methanolic extracts of 60 different kinds of plant seeds on the DPPH radical are shown in Table 1. Among seed extracts examined, the methanolic extracts of *Vitis vinifera* (91.23%) showed the most strong radical scavenging activity at a concentration 50 mg/ml, followed by *Selosia argentea* (85.90%), *Malva verticillata* (85.32%), *Prunus ishidoyana* (84.91%), *Impatiens balsamina* (84.42%), *Nelumbo nucifera* (82.89%) and *Trigonella foenum-graecum* (82.67%), in a descending order. The next effective seeds were *Phaseolus angularis* (80.95%), *Brassica campestris* (80.58%), *Benincasa hispida* (80.50%) and *Setaria italica* (80%). However, *Allium odorum* (26.15%), *Prunus ansu* (25.26%), and *Sesamum indicum* (28.72%) did not show an appreciable activity. These results support a previous report that plant extracts possessing phenolic antioxidant compounds, such as flavonoids and tannins, exhibited higher radical scavenging activity due to high hydrogen-donating ability (23). Meanwhile, since the *Sesamum indicum* containing antioxidative lignans, such as sesamol and its derivatives showed considerably lower radical scavenging activity (11), an another *in vitro* assay in parallel with this assay for preliminary screening of natural antioxidants is essential.

Soybean lipoxygenase (SLO) inhibitory activity

The SLO inhibitory activities of methanolic extracts of 60 plant seeds are also presented in Table 1. Among the seed extracts tested, nutmeg (*Myristica fragrans*) extract showed the most potent inhibitory activity, causing about 92.65% inhibition of SLO at a concentration of 50 mg/ml, followed by *Sorghum bicolor* (90.52%), *Alpinia katsumadii* (89.35%), *Cuscuta australis* (89.15%), *Torreyta nucifera* (88.01%), *Vitis vinifera* (87.62%) and *Areca catechu* (87.14%) extracts, in a descending order. Other seed extracts, such as *Zizyphus jujuba* (83.33%), *Perilla sikokiana* (82.35%), and *Psoralea corylifolia* (81.28%), also exhibited a considerable SLO inhibitory activity. However, *Brassica juncea* (17.98%), *Malva verticillata* (25.44%) and *Trichosanthes kirilowii* (37.43%), which are

Table 1. Free radical scavenging and soybean lipoxygenase (SLO) inhibitory activities of the methanolic extracts of 60 different plant seeds

No.	Plant seeds	Free radical scavenging activity[1)] (%)	SLO inhibitory activity[2)] (%)
1	*Allium odorum* L.	26.15	35.12
2	*Alpinia katsumadii* Hayata	39.61	89.35
3	*Amomum xanthoides* Wallich	48.12	78.88
4	*Areca catechu* L.	68.20	87.14
5	*Benincasa hispida (Thunb.)* Cogniaux	80.50	24.10
6	*Brassica campestris* L.	80.58	46.04
7	*Brassica juncea* Cosson	77.19	17.98
8	*Capsicum annuum* L.	73.58	34.60
9	*Cassia tora* L.	57.31	40.46
10	*Citrullus vulgaris* Schrad	30.29	47.57
11	*Citrus aurantium* L.	76.89	30.27
12	*Coix ma-yuen* Roman	77.55	30.79
13	*Croton tiglium* L.	77.44	29.10
14	*Cucumis melo* L. var makuwa	43.58	28.58
15	*Cuscuta australis* R. Brown	67.81	89.15
16	*Dianthus sinensis* L.	57.49	46.73
17	*Diospyros kaki* Thunb.	72.33	74.33
18	*Draba nemorosa* L.	78.12	29.62
19	*Euryale ferox* Salisbury	35.83	21.55
20	*Fagopyrum esculentum* Moench	45.25	27.31
21	*Ginkgo biloba* L.	68.27	54.65
22	*Hordeum vulgare* L.	66.51	65.37
23	*Hydnocarpus anthelmintica* Pierre	70.55	74.14
24	*Impatiens balsamina* L.	84.42	46.59
25	*Iris pallasii var. chinensis* Fisch	68.18	56.62
26	*Leonurus sibiricus* L.	74.68	37.20
27	*Luffa cylindrica* Roem	70.82	33.07
28	*Malva verticillata* L.	85.32	25.44
29	*Myristica fragrans* Houttuyn	47.20	92.65
30	*Nelumbo nucifera Gaertner* var. macrorhi zimata Nakai	82.89	68.42
31	*Oenothera odorata* Jacq.	54.74	77.64
32	*Panicum miliaceum* L.	54.21	27.43
33	*Perilla frutescens* var. *japonica* Hara	79.04	68.74
34	*Perilla sikokiana* Nakai	78.24	82.35
35	*Pharbitis nil* Choisy	64.45	37.34
36	*Phaseolus angularis* Wight	80.95	40.80
37	*Piper nigrum* L.	81.30	47.77
38	*Plantago asiatica* Decaisne	74.77	34.71
39	*Prunus ansu* (Maximowics) Komarov	25.26	25.30
40	*Prunus ishidoyana* Nakai	84.91	43.18
41	*Prunus mume* Sieb. et Zucc.	78.12	38.24
42	*Prunus persica* Batsch	77.40	34.15
43	*Psoralea corylifolia* L.	49.66	81.28
44	*Punica granatum* L.	77.90	68.23
45	*Raphanus sativus* L.	67.71	51.45
46	*Rhaseolus radiatus* L.	57.12	22.37
47	*Ricinus communis* L.	35.25	29.04
48	*Selosia argentea* L.	85.90	21.61
49	*Sesamum indicum* L.	28.72	29.00
50	*Setaria italica* BEAUV	81.11	41.79
51	*Sorghum bicolor* Moench	61.83	90.52
52	*Spinacia oleracea* L.	75.31	37.62
53	*Thuja orientalis* L.	69.00	58.64
54	*Torreyta nucifera* Sieb. et Zucc.	74.07	88.01
55	*Trichosanthes kirilowii* Maximowicz	77.62	37.43
56	*Trigonella foenum-graecum* L.	82.67	64.15
57	*Triticum aestivum* L.	45.49	22.32
58	*Vitis vinifera* L.	91.23	87.62
59	*Zea Mays* L.	67.45	75.10
60	*Zizyphus jujuba* Miller	50.07	83.33

[1,2)]Average of triplicate measurements. Standard deviations have been omitted for simplicity. The concentration of samples used was 50 mg/ml of methanolic extracts of plant seeds.

known to have antiinflammatory actions (12,24), did not show appreciable inhibitory activity, and *Rhaseolus radiatus* (22.37 %), *Triticum aestivum* (22.32%) and *Selosia argentea* (21.61 %) showed somewhat inhibitory activity against SLO. Thus, from two above results, we found that grape (*Vitis vinifera*) seed have higher radical scavenging and SLO inhibitory activity than other plant seeds. However, their activity was inclined to decrease slowly due to the autoxidation of several phenolic compounds in them, and the same phenomina were appeared in *Sorghum bicolor* and *Perilla sikokiana* (data not shown). Meanwhile, the extract of *Alpinia katsumadii* and *Myristica fragrans* showed strong SLO inhibitory activity although their radical scavenging activities are low to some extent. Thus, the extract of nutmeg seed may play an important role in inhibition of lipoxygenase-mediated peroxidation of arachidonic acid in cell membrane, thereby can widely used as the potential source of antioxidants or dietary antiinflammatory spice.

SLO inhibitory activity of three compounds isolated from preparative TLC

The SLO inhibitory activity of three compounds isolated from prep. TLC, which is a final purification step, was given in Table 2. Among three compounds isolated, compound **1** showed the strongest inhibitory activity (IC_{50}=0.39 μg/ml) although the activity was weaker than a well known lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA, IC_{50}= 0.23 μg/ml). However, the inhibitory effects of compound **2** and **3** were weaker than that of compound **1**. Thus, compound **1** may be mainly responsible for the potent inhibitory activity of nutmeg seed against SLO.

Structural elucidation of compound 1 from nutmeg seed

The ultraviolet (UV) spectrum (λ_{max} 225, 270 and 340 nm) and IR spectrum (ν_{max} 3421, 2927, 2853, 1680, 1629, 1453, 1203 cm^{-1}) of compound **1** suggested that the presence of an aliphatic hydroxylated phenone moiety. The ^1H-NMR spectrum contained a series of methylene protons at δ_H 1.33 (8H), δ_H 1.40-1.90 (4H) and δ_H 2.57 (2H) & 3.13 (2H), and two dihydroxyphenyl groups at δ_H 6.33 & 7.19 (AB_2 type, A ring), and δ_H 6.47, 6.59, & 6.64 (ABX type, B ring). In a ^{13}C-NMR spectrum, C_2 and C_6 signals and $C_{3'}$ & $C_{4'}$ signals of two dihydroxyphenyl groups were shifted to the lower field, δc 163.44, and δc 146.04 & 144.08, respectively, indicating that two hydroxyl groups of each benzene ring are attached at the C_2 & C_6 and $C_{3'}$ & $C_{4'}$ position, respectively, as compared to the NMR spectral data with the published values (25). In addition, keto group of phenone moiety at δc 209.75, and aliphatic group at δc 25.83-45.78 were also found. Meanwhile, the electron impact-mass spectrometry (EI-MS) gave its molecular ion peak[M^+] at 358 m/z. Furthermore, the prominent fragment ion peak at m/z 248 [358-110 (diphenol core)], 136[248-112 (C_8H_{16})], 108[136-28 (C=O)], 111 (diphenol core) support that the presence of two types of dihydroxylphenyl, and aliphatic and ketone groups. The detailed assignments of compound **1** for UV, IR, NMR and MS spectra are shown in Table 3. On the basis of these spectral data, compound **1** was identified as a 2,6-dihydroxy-9-(3',4'-dihydroxyphenyl)nonylphenone (DDNP, Fig. 1). The isolation and structural elucidation of DDNP is presented here for the first time although its derivative has already been reported in nutmeg seed (26).

It is well-known that the aliphatically dihydroxylated phenolic compounds and flavonoids with a catechol structure,

Table 2. Inhibitory effects of three compounds isolated from preparative TLC on soybean lipoxygenase (SLO)

Compounds	SLO inhibitory activity (IC_{50}, μg/ml)
Compound 1	0.39±0.02
Compound 2	2.87±0.03
Compound 3	11.53±0.06
NDGA[1)]	0.23±0.01

[1)]Nordihydroguaiaretic acid, a positive reference.
Values within a column are significantly different ($p<0.05$).

Table 3. UV, IR, NMR and EI-MS spectral data of compound **1** (2,6-dihydroxy-9-(3',4'-dihydroxyphenyl)nonylphenone (DDNP) isolated from nutmeg seed

Instrumental analysis	DDNP
UV λ_{max} nm (log ε)	225 (4.68), 270 (4.34), 340 (s)
IR ν_{max} (cm^{-1})	3421 (OH), 2927, 2853, 1680 (C=O), 1629, 1453, 1203
^1H-NMR	
-$COCH_2CH_2(CH_2)_4CH_2$-	
-$COCH_2CH_2$-,	1.33 (8H, brs)
Ph-$CH_2^*CH_2$-	1.5-1.8 (4H, m)
Ph-$CH_2CH_2^*$-	2.44 (2H, t, J=7.2 Hz)
	3.11 (2H, t, J=7.2 Hz)
	6.33 (2H, d, J=8.1Hz, H-3 & H-5),
	6.47 (1H, dd, J=8.1 & 2.1Hz, H-6'),
	6.59 (1H, d, J=2.1Hz, H-2'),
	6.64 (1H, d, J=8.1Hz, H-5'),
	7.19 (1H, t, J=8.1Hz, H-4)
^{13}C-NMR	
C=O	209.75
	163.44 (C_2 & C_6),
	146.04 ($C_{4'}$),
	144.08 ($C_{3'}$),
	136.86 (C_4),
	135.79 ($C_{1'}$),
	120.66 ($C_{6'}$),
	116.55 ($C_{5'}$),
	116.22 ($C_{2'}$),
	111.89 (C_1)
	108.38 (C_3 & C_5)
-$CH_2CH_2(CH_2)_4CH_2CH_2$-	45.78, 36.33, 32.68, 30.65, 30.58, 30.33, 25.83
EI-MS (m/z)	358 [M^+], 248, 139, 138, 123, 111

Coupling constants (J in Hz) in parentheses.
Brs, broad singlet; d, double; dd, double doublet; t, triple; m, multiple

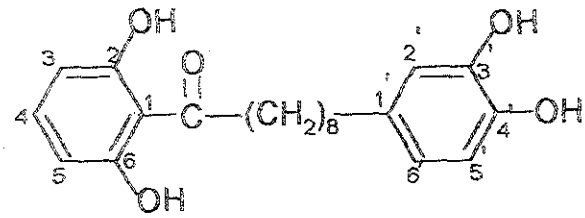

Fig. 1. Chemical structure of 2,6 dihydroxy-9-(3',4'-dihydroxy-phenyl)nonylphenone isolated from nutmeg (*Myristica fragrans*) seed.

such as NDGA, cucurmin, rosmarinic acid, esculetin, and luteolin have potent lipoxygenase inhibitors (11,27-29). DDNP have the similar structural backbone to NDGA and curcumin. This finding suggest that the DDNP from nutmeg seed can be used as potentially dietary antiinflammatory compounds.

Meanwhile, DDNP and tetraol isolated from nutmeg drug and spice, respectively, have not as yet been isolated and elucidated from several nutmeg species. This fact suggest that eugenol and its derivatives or several cyclic and acyclic phenylpropanoids in nutmeg (*Myristica fragrans*) may be converted into tetraol or DDNP by alkaline treatment in the manufacturing process of nutmeg spice and drug (26,30). Futher investigations on the structural elucidation of compounds 2 and 3, and their antiinflammatory actions in an *in vivo* system are needed.

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