JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stilbenoids Isolated from the Seeds of Melinjo (*Gnetum gnemon* L.) and Their Biological Activity

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A 50% EtOH extract of the dried endosperms of melinjo (*Gnetum gnemon* L.) was purified by a combination of column chromatography, including highly porous polymer, octadecylated silica (ODS), hydroxypropylated dextran (LH-20), and silica gel, resulting in the isolation of a new stilbenoid **1**, named gnetin L, along with five previously identified stilbenoids **2**−**6** (gnetin C, gnemonosides A, C, and D, and resveratrol). All of these stilbenoids showed 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity similar to that of ascorbic acid and *dl*-α-tocopherol. With the exception of gnemonoside A (**3**), these stilbenoids showed moderate antimicrobial activity. Inhibition of lipase from porcine pancreas was recognized in four stilbenoids excluding gnemonoside A (**3**) and resveratrol (**6**). Gnetin C (**2**), gnemonoside C (**4**), and gnemonoside D (**5**) inhibited the hydrolysis of starch by α-amylase from porcine pancreas. An EtOH extract containing these stilbenoids also showed DPPH radical scavenging effect, lipase and α-amylase inhibition activity, and antimicrobial activity against food microorganisms and enterobacteria. The present study indicates that melinjo and extracts containing these constituents are useful as health supplements.

KEYWORDS: *Gnetum gnemon*; melinjo; polyphenol; stilbenoid; gnetin; gnemonoside; DPPH radical scavenging effect; antimicrobial activity; lipase inhibition; amylase inhibition

INTRODUCTION

Melinjo (Indonesian name; *Gnetum gnemon* L.), which belongs to the Gnetacea family, is an arboreal dioecious plant that is widely cultivated in Southeast Asia, and its fruits and seeds are used as an ordinary vegetable in Indonesia. The fruits, which change from green to yellow, orange, and red with ripeness, are colorful and attractive (*1*−*4*), and the seed in ripe fruits has a shell. In Indonesia popular dishes include soup cooked from melinjo fruits and leaves and also crackers made by crushing the endosperms obtained through shelling of the heated seeds, drying, and deep-frying. In the present study the slightly bitter taste of melinjo crackers was of interest, and thus the constituents of melinjo seeds and their biological activities were examined.

Wallace and Morris first reported the isolation of *C*-glycosylflavones from melinjo leaf (*5*). Stilbene oligomers were isolated from acetone and methanol extracts of dried melinjo root (*6*−*10*). In addition, the occurrence of various stilbenoids is well-known in other *Gnetum* species (*11*−*23*). Although some Gnetaceae plants are used for the folk treatment of arthritis, bronchitis, and asthma (*24*), and their chemical constituents, stilbene oligomers, lower the level of sugar in the blood (*25*), induce apoptosis in colon cancer (*26*), and show anti-inflammatory activity (*27*) and antibacterial activity (*28*, *29*), there have been no chemical or biological studies on edible melinjo seeds that are readily available in Indonesia.

Resveratrol is a widely available polyphenol contained in red wine, among other sources, and is regarded as having an antiaging effect (*30*). Resveratrol is biosynthesized from coumaroyl CoA and malonyl CoA by resveratrol synthase (*31*). Numerous studies on resveratrol have demonstrated a variety of bioactivities such as antioxidant capacity, cardioprotection, and anticancer activity (reviewed in ref *32*). There are no reports on wine polyphenols relating to digestive enzymes, although there are such reports on tea polyphenols (*33*, *34*).

This paper describes the isolation of a new stilbenoid **1**, named gnetin L, and five known stilbenoids, gnetin C (**2**), gnemonosides A (**3**), C (**4**), D (**5**), and resveratrol (**6**) from the 50% EtOH extract of dried melinjo endosperms as well as their biological activities and the differences between **6** and its dimers **1**−**5** (**Figure 1**).

MATERIALS AND METHODS

General. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian Unity-600 spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra were recorded on a JMS-700T instrument. Infrared spectra were recorded on a JASCO FT/IR-410 Fourier transform infrared spectrometer. Ultraviolet spectra were recorded on a Hitachi U-3010 spectrophotometer. Optical rotations were

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10.1021/jf803077p CCC: $40.75
Published on Web 02/17/2009

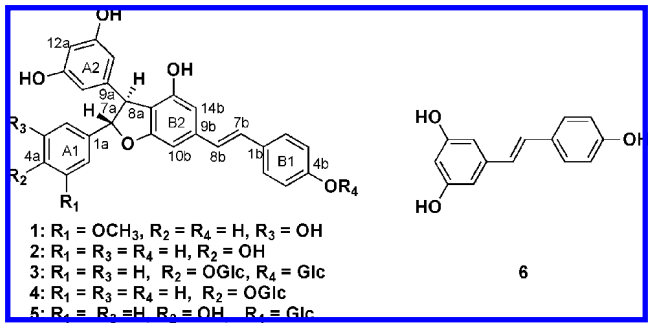

Figure 1. Chemical structures of the isolated compounds.

measured on a JASCO DIP-1000 polarimeter. Fluorescence intensities were measured using a Hitachi F-2000 fluorescence spectrophotometer. Melting points (uncorrected) were measured using a Yanaco MP-J3 instrument. Silica gel column chromatography was performed on Silica Gel 60N (Kanto Chemical, Tokyo, Japan). Reversed-phase ODS column chromatography was carried out on Cosmosil 75C_{18}-OPN (Nacalai Tesque, Kyoto, Japan) and Lichroprep RP-18 (Merck Ltd., Japan, Tokyo). Porous polymer column chromatography was performed on DIAION Sepabeads SP825 (Mitsubishi Chemical, Tokyo, Japan). Hydroxypropylated column chromatography was run on Sephadex LH-20 (Pharmacia, Uppsala, Sweden).

Reagents. All solvents, soluble starch, iodine, potassium iodide, and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Nacalai Tesque. Pancreatic lipase (type IV-S, from porcine pancreas), pancreatic α-amylase (type II, from porcine pancreas), and 4-methylumbelliferyl oleate were obtained from Sigma-Aldrich Japan (Tokyo, Japan). All reagents were of analytical grade. Branched cyclodextrin was obtained from Ensuiko Sugar Refining (Tokyo, Japan). Tryptone−soy peptone−agar and standard method agar were purchased from Eiken Chemical (Tokyo, Japan).

Plant Samples. The melinjo endosperms used in this study were obtained through drying and shelling the seeds, which were purchased in Indonesia in September 2004 and January 2007. Voucher specimens have been deposited in Hosoda SHC Co. Ltd.

Extraction and Isolation. The dried endosperms (40.0 g) were powdered and soaked in 50% EtOH (120 mL) at room temperature for 2 days, and the mixture was filtered. The filtrate was evaporated in vacuo to obtain the melinjo extract (3.73 g, 9.33% yield). This extract was chromatographed on reversed-phase ODS eluted with 25% MeOH, 40% MeOH, and 60% MeOH to produce four fractions. The second fraction (0.63 g, 1.58% yield) was purified by Sephadex LH-20 column chromatography with 50% MeOH to give gnemonoside A (**3**) (0.16 g, 0.40% yield) as a pale brown amorphous powder, $[\alpha]_{546}$ +68.3 (*c* 0.7, MeOH). The third fraction (0.46 g, 1.15% yield) was purified by silica gel column chromatography with $CHCl_3$/MeOH 4:1 (v/v) to give gnemonoside D (**5**) (0.25 g, 0.63% yield) as fine needles. These crystals were recrystallized with 25% MeOH to yield colorless needles, mp 205−206 °C, $[\alpha]_{546}$ −65.6 (*c* 0.6, MeOH). The fourth fraction (0.30 g, 0.75% yield) was purified by silica gel column chromatography with $CHCl_3$/MeOH 9:1 (v/v) to give gnetin C (**2**) (0.25 g, 0.63% yield) as a pale yellow amorphous powder, $[\alpha]_{546}$ −23.2 (*c* 0.5, MeOH).

Dried melinjo endosperms (1430 g) were crushed and soaked in 50% EtOH (4.3 L) at room temperature for 3 days, and the mixture was filtered. The filtrate was evaporated in vacuo to yield melinjo extract (129.8 g). This melinjo extract was separated by porous polymer column chromatography eluted successively with 40% MeOH, 60% MeOH, and 100% MeOH to give three fractions. The second fraction (28.3 g, 1.98%) was again subjected to porous polymer column chromatography with 50% MeOH and 100% MeOH as eluting solvents to give fractions II-1 (9.81 g) and II-2 (13.86 g). The third fraction (25.76 g, 1.80% yield) was subjected to silica gel column chromatography eluted with $CHCl_3$/MeOH in a gradient manner to give fractions III-1 (0.32 g, 0.02% yield), III-2 (13.21 g, 0.92% yield), and III-3 (0.32 g). Fraction III-1 yielded resveratrol (**6**) as fine colorless crystals, mp 275−276 °C (dec). Fraction III-2 contained gnetin C (**2**) (13.21 g, 0.92% yield). Fraction II-2 was chromatographed on silica gel with a mixture of C_6H_6/AcOEt/CH_3COCH_3 in a gradient manner to yield a mixture (8.18 g) of gnemonosides C (**4**) and D (**5**) as a white solid. This mixture was crystallized from 25% MeOH to give gnemonoside D (**5**) (4.61 g, 0.32% yield) as colorless needles. Repeated chromatography of the filtrate on ODS column Cosmosil with 25% MeOH and Lichroprep with MeOH/H_2O/AcOH 54:45:1 (v/v) gave gnemonoside C (**4**) (0.24 g, 0.02% yield) as colorless crystals. These crystals were recrystallized from 25% MeOH to yield colorless fine needles, mp 198−199 °C, $[\alpha]_{546}$ −23.2 (*c* 0.5, MeOH). Fraction III-3 was chromatographed on ODS Lichroprep with MeOH/H_2O/AcOH 54:45:1 (v/v) to yield gnetin L (**1**) (0.118 g, 0.01%).

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of Compounds **1** and **2**

	1		**2**	
no.	δ_C	δ_H	δ_C	δ_H
1a	134.4		134.1	
2a	115.8	6.83 (d, 0.2)	127.8	7.21 (d, 8.5)
3a	147.4		116.2	6.84 (d, 8.5)
4a	119.2	6.82 (d, 0.2)	158.1	
5a	148.4		116.2	6.84 (d, 8.5)
6a	110.2	6.98 (t, 0.2)	127.8	7.21 (d, 8.5)
7a	93.8	5.37 (d, 5.3)	93.6	5.39 (d, 4.9)
8a	56.0	4.42 (d, 5.3)	55.9	4.39 (d, 4.9)
9a	146.1		146.3	
10a	106.7	6.18 (d, 2.3)	106.7	6.17 (d, 2.2)
11a	159.6		159.6	
12a	101.9	6.24 (t, 2.3)	101.9	6.24 (t, 2.2)
13a	159.6		159.6	
14a	106.7	6.18 (d, 2.3)	106.7	6.17 (d, 2.2)
1b	129.9		130.0	
2b	128.7	7.45 (d, 8.5)	128.7	7.44 (d, 8.5)
3b	116.4	6.86 (d, 8.5)	116.2	6.85 (d, 8.5)
4b	158.2		158.2	
5b	116.4	6.86 (d, 8.5)	116.4	6.85 (d, 8.5)
6b	128.7	7.45 (d, 8.5)	128.7	7.44 (d, 8.5)
7b	129.1	7.11 (d, 16.2)	129.2	7.10 (d, 16.1)
8b	126.7	6.98 (d, 16.2)	126.8	6.98 (d, 16.1)
9b	141.1		141.2	
10b	99.1	6.71 (s)	99.2	6.70 (s)
11b	163.1		163.2	
12b	115.1		115.0	
13b	155.4		155.4	
14b	108.0	6.60 (s)	108.0	6.59 (s)
HO-3a		7.70 (s)		
HO-4a				8.38 (s)
HO-11a		8.18 (s)		8.11 (s)
HO-13a		8.18 (s)		8.11 (s)
HO-4b		8.53 (s)		8.44 (s)
HO-13b		8.14 (s)		8.06 (s)
H_3CO-5a	56.3	3.83 (s)		

Gnetin L (1) was obtained as a white amorphous powder, $[\alpha]_{546}$ −27.1 (*c* 0.5, MeOH); Ir (KBr) cm^{-1} 3389, 1605, 1515, 1431, 1341, 1269, 1158, 1004, 836; negative ion HR-FAB-MS *m*/*z* 484.1536 (calcd for $C_{29}H_{24}O_7$, 484.1522); ^{1}H and ^{13}C NMR data, see **Table 1**.

DPPH Radical Scavenging Assay. The DPPH radical scavenging effect was evaluated in accordance with the method reported by Yoshikawa et al. (*35*). Two milliliters of DPPH ethanol solution (250 μM, final concentration = 50 μM) was added to a mixture of 4 mL of a 50% EtOH solution of various sample concentrations and 4 mL of 0.1 M acetate buffer (pH 5.5) and mixed. After mixing, the solutions were left at room temperature. The optical density of each solution was measured at 517 nm. Ascorbic acid and *dl*-α-tocopherol were tested as positive controls and 50% EtOH was used instead of the sample solution as a negative control. The ED_{50} (concentration scavenging half of 50 μM DPPH, that is, the concentration producing half of OD_1) of the test sample was calculated by the least-squares method from the plots of the sample concentration (μM or μg/mL) versus the following equation: $1 - OD_2/OD_1$ (where OD_1 is the optical density of the negative control without the sample and OD_2 is the optical density of the positive control or sample solution).

Pancreatic Lipase Inhibiting Assay. The inhibitory action against pancreatic lipase hydrolysis of 4-methylumbelliferyl oleate (MUO) was

evaluated according to a method modified for utilization of a spectrophotometer on the basis of the method reported by Nakai et al. (*33*). One milliliter of MUO solution (0.1 mM) in 10% 2-methoxyethanol was added to a mixture of 1 mL of a 10% MeOH solution of various sample concentrations and 1 mL of the lipase solution (50 U/mL) in a buffer consisting of 0.1 M Tris-HCl, 0.3 M NaCl, and 2.6 mM $CaCl_2$ (pH 7.4). After incubation at 25 °C for 30 min, 7 mL of citric acid solution (25 mM) in 20% 2-methoxyethanol was added to the reaction mixture to stop the reaction. This mixture was diluted 10 times with H_2O. The amount of 4-methylumbelliferone (MU) released by lipase in the diluted solution was measured at an excitation wavelength of 319 nm and an emission wavelength of 449 nm. The IC_{50} of the test sample was determined by the least-squares method from the plots of the sample concentration (μM or μg/mL) versus the lipase activity (%).

Pancreatic α-Amylase Inhibiting Assay. The inhibition of pancreatic α-amylase activity was evaluated using soluble starch as a substrate by iodine−starch coloration after enzyme reactions under the conditions reported by Hara et al. (*34*). One milliliter of a 30% MeOH solution of various sample concentrations was mixed with 0.1 mL of the α-amylase solution (5 units/mL) in a buffer consisting of 80 mM 3,3-dimethylglutaric acid/NaOH, 40 mM NaCl, and 5 mM $CaCl_2$ (pH 6.9), and then 0.9 mL of 0.56% soluble starch in the same buffer was added. After incubation at 37 °C for 10 min, 1 mL of 0.5 M HCl was added to the reaction mixture to stop the reaction, and then 7 mL of 0.2 mM KI_3 as color-producing reagent and 10 mL of 3% MeOH were added. The optical density of each dilute solution was measured at 650 nm. Reference and control samples were tested using the buffer and 30% MeOH instead of the α-amylase solution and the sample solution, respectively. The IC_{50} of the test sample was calculated according to the least-squares method from the plots of the sample concentration (μM or μg/mL) versus the following ratio: $(OD_3 - OD_2)/(OD_1 - OD_2)$ (where OD_1 is the optical density of the reference solution, OD_2 is the optical density of the control solution, and OD_3 is the optical density of the sample solution).

Antimicrobial Assay. The suppression of multiplication of food microorganisms was tested by the agar dilution method. A 50% EtOH solution of various sample concentrations was mixed with warm tryptone−soypeptone−agar and cooled to perform a plate culture in a Petri dish (final concentrations = 5, 10, 20, 50, 100, 250, 500, 1000, 2000, and 3000 μg/mL). Thirty microliters of each microorganism solution (about 10^4 cfu/mL) was inoculated onto the plate and cultivated at 30 °C for 72 h. *Bacillus subtilis* Marburg 168 (*B. subtilis*), *Luconostoc mesenteroides* 9a4 (*L. mesenteroides*), *Lactobacillus plantarum* NRIC1067 (*L. plantarum*), *Escherichia coli* IFO3301 (*E. coli*), *Saccharomyces cerevisiae* IFO2347 (*S. cerevisiae*), and *Penicillium expansum* IFO6096 (*P. expansum*) were used as the test microorganisms. *Clostridium perfringens* NCT8238 (*C. perfringens*) and *Bifidobacterium bifidum* NRBC100015 (*B. bifidum*) were cultivated at 37 °C with GAM agar and exclusive medium (1% casein peptone, 0.5% meat extract, 0.5% yeast extract, 1% glucose, 0.3% K_2HPO_4, 0.1% Tween 80, 0.1% Cys.HCl, 0.05% AcONa, 1.5% agar), respectively. The minimum growth inhibitory concentration (MIC, μg/mL) of the test sample was judged by the lack of emergence of colonies of the microorganism.

Storage Test of Potato Salad. Mayonnaise (120 g) was mixed together with crushed boiled potato (600 g) to obtain potato salad. To 200 g of salad was added 0.4 or 0.8 g of powdered melinjo extract, which was prepared by evaporation of a mixture of the melinjo extract and branched cyclodextrin (1:1) in aqueous solution. Each mixture was fully compounded, divided into four parts, and then put into polyethylene bags. These bags were sealed and kept in a thermostat-controlled room at 20 °C. Test solutions were obtained by mixing each salad sample (20 g) with saline (180 mL), and tests were performed by using the standard method agar medium to measure the number of living microbial cells.

RESULTS AND DISCUSSION

Chemistry. Activity-guided fractionation of the EtOH extract of melinjo endosperms using DPPH radical scavenging and antimicrobial activities resulted in the isolation of a new

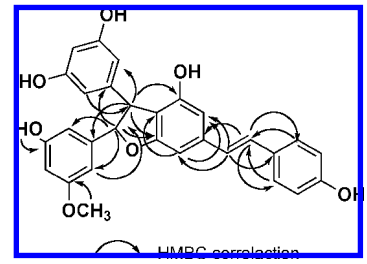

Figure 2. Representative HMBC correlations of **1**.

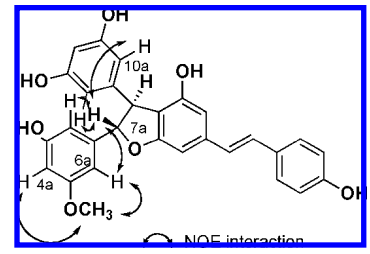

Figure 3. Representative NOE correlations of **1**.

stilbenoid, **1**, gnetin L, along with the previously identified stilbenoids gnetin C (**2**), gnemonoside A (**3**), gnemonoside C (**4**), gnemonoside D (**5**), and resveratrol (**6**) (*11*, *19*) as active components. The known compounds **2**−**6** were identified by analyses of NMR data and comparison of spectroscopic data with those recorded in the literature.

Gnetin L (**1**), a white amorphous powder, had a molecular formula of $C_{29}H_{24}O_7$, which was determined by negative ion high-resolution fast-atom bombardment mass spectroscopy (HR-FAB-MS) (*m*/*z* 484.1536). The NMR spectroscopic data (**Table 1**) of **1** were found to be similar to those of **2** except for the presence of a methoxy group at δ_H 3.83 (3H, s) and δ_C 56.3 and different signals due to ring A1, which showed the presence of a 1,3,5-trisubstituted benzene ring at δ_H 6.82 (1H, d, J = 0.2 Hz, H-2a), 6.83 (1H, d, J = 0.2 Hz, H-4a), and 6.98 (1H, t, J = 0.2 Hz, H-6a) and δ_C 134.4 (C-1a), 115.8 (C-2a), 147.4 (C-3a), 119.2 (C-4a), 148.4 (C-5a), and 110.2 (C-6a) (**Table 1**). A hydroxy proton signal resonated at δ_H 7.70 (1H, s), and the methoxy signal showed heteronuclear multiple-bond correlations (HMBC) with C-3a and C-5a (**Figure 2**). In addition, the methoxy signal showed nuclear Overhauser effect (NOE) interactions with H-4a and H-6a (**Figure 3**). These spectroscopic data indicated that the *p*-hydroxy A1 ring in **2** was modified to a methoxybenene ring in **1**. Other HMBC data (**Figure 2**) were consistent with the structure of **2** except for ring A1. The relative configuration between H-7a and H-8a was assigned as *trans* from NOE correlations between H-7a and H-10a (14a) on the A2 benzene ring. A *trans* geometry of the $\Delta^{7b,8b}$ double bond was evident from a large vicinal J value (16.2 Hz).

On the basis of the aforementioned spectroscopic data, the structure of gnetin L was elucidated as **1**.

DPPH Radical Scavenging Effect. The EtOH melinjo extract gradually scavenged DPPH radicals and reached a maximum effect after 5 h (**Figure 4**). This manner of scavenging activity was found to be quite different from that of *dl*-α-tocopherol, which showed the highest activity within 1 h. The ED_{50} for the EtOH melinjo extract was calculated as 23 μg/mL at 5 h. Next, the DPPH radical scavenging effect of compounds **1**−**6** was examined. All of the compounds could maintain DPPH radical scavenging effects even after 5 h, and their ED_{50} values decreased with time (**Table 2**). On the other hand, the ED_{50} values of ascorbic acid and *dl*-α-tocopherol showed their highest potencies of 14.7 and 15.1 μM, respectively, at 30 min. The delayed oxidative activity of compounds **1**−**6** could be rational-

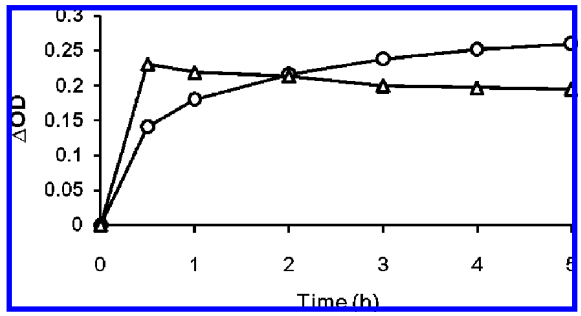

Figure 4. DPPH radical (50 μM) scavenging activity of the melinjo extract with time: (○) melinjo extract 20 μg/mL; (△) *dl*-α-tocopherol 5.4 μg/mL; (ΔOD) $OD_1 - OD_2$; (OD_1) optical density of the negative control (only DPPH) solution without sample; (OD_2) optical density of the sample solution. Each point represents the mean of two determinations.

Table 2. Concentration Scavenged for Half of DPPH Radicals (ED_{50}, μM) with Time[a]

sample	0.5 h	1 h	3 h	5 h
1	17.0 ± 0.8	14.7 ± 1.4	11.8 ± 1.5	11.1 ± 1.6
2	19.7 ± 0.8	14.5 ± 0.7	12.0 ± 0.5	10.7 ± 0.2
3	14.9 ± 1.5	12.5 ± 1.4	9.5 ± 0.9	8.3 ± 0.7
4	21.4 ± 0.5	18.5 ± 0.2	13.2 ± 0.2	11.3 ± 0.1
5	18.9 ± 0.4	15.3 ± 0.6	11.1 ± 1.3	9.4 ± 0.8
6	16.7 ± 1.2	15.3 ± 0.8	13.9 ± 0.7	13.2 ± 0.8
ascorbic acid	14.7 ± 0.4	14.9 ± 0.9	15.0 ± 0.4	14.1 ± 0.3
dl-α-tocopherol	15.1 ± 0.9	16.1 ± 0.9	16.8 ± 1.2	17.1 ± 1.7

[a] DPPH radical concentration = 50 μM. Each value is the mean ± SD of three independent experiments.

ized as the donation of multiple electrons to the DPPH single radical. Namely, stilbenoids **1**−**5**, other than the similar resveratrol (**6**) to ascorbic acid, which is known as a double-electron reductant, give one electron within 30 min, a second electron in 1 h, and a third electron in 5 h to the DPPH radicals of a single electron oxidant. Thus, stilbenoids **1**−**5** scavenged three times as many as DPPH radicals over 5 h. This fact suggests that the resorcin structure of ring A2 in **1**−**5**, which are dimers of **6**, plays a crucial role in scavenging radicals because it acts as an electron donor, gradually donating two electrons (one by one). In dimers, ring B2 donates an additional electron regardless of rings A1 and B1, but a hydroxyphenyl moiety has no influence on ED_{50} even if it is substituted by glucosyl or methyl groups. Although no products derived by oxidation of DPPH radicals have been identified, in analogy with the oxidative coupling reaction of (*E*)-isorhapontigenin using ferric chloride (*36*), dimers **1**−**5** may be formed by single-electron oxidation of the resorcin moiety in resveratrol and its glycoside, which are generated by biosynthesis from coumaroyl CoA and malonyl CoA.

Pancreatic Lipase Inhibiting Effect. The inhibitory activities of melinjo extract and its constituents against pancreatic lipase are shown in **Table 3**, although these activities were lower than those of oolong tea polyphenols (*33*). Gnetin L (**1**), with an IC_{50} of 7.2 μM, showed the strongest activity among **1**−**6**. The effect of gnetin C (**2**) was higher than that of its monoglucosides **4** and **5**. Whereas diglucoside **3** showed weak inhibitory activity, resveratrol (**6**) showed no activity. These results show that the inhibitory activities of dimer stilbenoids were higher than that of monomer and glycoside-linked constituents. This suggests that a hydrophobic benzofuran skeleton formed by the dimerization of **6** elevates lipophilicity and increases affinity for the hydrophobic regions of lipase, leading to competitive inhibition with the esters of fatty acids, which are the substrates of lipase.

Table 3. Inhibitory Activities against Pancreatic Digestive Enzymes[a]

sample	lipase	amylase
melinjo extract	11.6 ± 9.4	352 ± 84
1	7.2 ± 1.8	>1000
2	12.2 ± 2.2	203 ± 57
3	125.7 ± 6.4	>1000
4	19.6 ± 3.0	840 ± 74
5	41.4 ± 2.5	277 ± 54
6	>200	>1000

[a] Concentration of melinjo extract = μg/mL; concentration of compounds **1**−**6** = μM. Each value is the mean ± SD of three independent experiments.

Table 4. Antimicrobial Action (pH 7; MIC, μg/mL)[a]

sample	*B. subtilis*	*E. coli*	*S. cerevisiae*	*P. expansum*
melinjo extract	250	2500	3000	3000
1	10	>3000	2000	3000
2	20	1000	500	500
3	>3000	>3000	>3000	>3000
4	500	>3000	>3000	>3000
5	250	>3000	>3000	>3000
6	500	>3000	2000	>3000
sorbic acid (pH 6.5)	>3000	>3000	>3000	3000

[a] Cultivated at 30 °C for 72 h. Each value is mean of three independent experiments. The sample solution of sorbic acid was neutralized with 0.5 M NaOH.

The IC_{50} of the melinjo extract was 11 μg/mL. The IC_{50} values of gnetin C (**2**), gnemonoside D (**5**), and gnemonoside A (**3**), if their concentrations in the solution were calculated on the basis of their crude yields, were about 2, 0.7, and 0.1 μM, respectively.

Pancreatic α-Amylase Inhibiting Effect. The inhibitory activity of the melinjo extract and its constituents against pancreatic α-amylase was one order of magnitude lower than that against lipase (**Table 3**). Although gnetin C (**2**) and gnemonoside D (**5**) showed weaker inhibitory effects than tea polyphenols (*34*) with IC_{50} values of 203 and 277 μM, respectively, gnetin L (**1**), which showed lipase inhibitory activity, did not inhibit pancreatic α-amylase. Furthermore, resveratrol (**6**) did not show activity, as seen for lipase inhibition. Considering this result, the hydrophobic benzofuran moiety may lead to lower affinity for the hydrophilic part of the enzyme that recognizes the glucose linkage. In the melinjo extract, the same discrepancy in the IC_{50} values and the estimates as in lipase inhibition was recognized.

Antimicrobial Effect. The antimicrobial activity, measured as minimum inhibitory concentration (MIC), of the melinjo extract and its constituents against food microorganisms and enterobacteria is shown in **Table 4**. Compared with *B. subtilis*, their activities for other microbes were low. The MICs of the melinjo extract were 250 μg/mL against *B. subtilis*, *C. perfringens* (enterobacteria), and *L. mesenteroides* (globular lactic acid bacteria), 500 μg/mL against *L. plantarum* (cylindrical lactic acid bacteria), 2500 μg/mL against *E. coli* and *B. bifidum* (enterobacteria), and 3000 μg/mL against *S. cerevisiae* and *P. expansum*. Gnetin C (**2**) showed antimicrobial activity against four species and had a moderate effect, with an MIC of 20 μg/mL against *B. subtilis*. This value agrees with the concentration of gnetin C on the basis of the calculated concentration of the crude yield (8%). Gnetin L (**1**) showed the strongest activity among the isolated constituents, with an MIC of 10 μg/mL against *B. subtilis*, but was not effective against other species. Resveratrol (**6**) and gnemonosides C (**4**) and D (**5**) showed weak activities against only *B. subtilis*, and gnemonoside A (**3**) had no antimicrobial activity.

Table 5. Live Microbial Cells in Potato Salad Kept at 20 °C

salad	amount (%)	0 days	1 day	2 days	3 days
control	0	<300	<300	500	2.0×10^5
melinjo extract	0.1	<300	<300	500	3.4×10^4
	0.2	<300	<300	300	7.9×10^3

The above results indicated that the activity was reduced with the increase in glycosidic linkages, suggesting the involvement of the solubility of the dimer in water, in the order practically insoluble **1**, slightly soluble **2**, soluble **4** and **5**, and freely soluble **3**.

Storage of Food. Because the melinjo extract possessed antimicrobial activity against food microorganisms, potato salad mixed with the mixture of the extract and branched cyclodextrin was examined to determine the possibility of using melinjo extract as a preservative. The salad was kept at 20 °C, and the number of live microbial cells was measured over the course of the incubation (**Table 5**). The number of cells in the salad (control) without extract exceeded 1×10^5 after 3 days, whereas the salads containing extract at 0.1 and 0.2% contained 1×10^4 and 1×10^3 bacterial cells, respectively. This result indicated that the melinjo extract depressed the multiplication of bacteria depending upon the amount of melinjo extract and may be useable to extend the shelf life of salad.

Conclusion. This investigation was carried out to clarify the biological activity and identify the chemical constituents of the EtOH extract of melinjo seeds, which is frequently consumed as a foodstuff in Indonesia. The melinjo extract contains stilbenoids, such as gnemonoside A (**3**), gnemonoside D (**5**), and gnetin C (**2**), as major constituents, and resveratrol (**6**), gnemonoside C (**4**), and gnetin L (**1**) as minor constituents. It should be noted that a new stilbenoid, gnetin L (**1**), was found and its structure elucidated. All of these stilbenoids could scavenge DPPH radicals. Among the constituents, gnetins L (**1**) and C (**2**) possessed moderate inhibitory effect against porcine pancreatic lipase, and **2** showed relatively weak inhibition against porcine pancreatic α-amylase as well as moderate antimicrobial activity against food microorganisms. From these results, except for **6**, melinjo extract has the potential to scavenge active oxygen as an antioxidant, suppress fat absorption, control the level of sugar in the blood, improve intestinal bacterial flora by depressing the multiplication of harmful enterobacteria such as *C. perfringens*, and also contribute to the extension of the shelf life of foods.

ACKNOWLEDGMENT

We are grateful to Dr. Nobuyuki Hamanaka for analysis of the NMR spectra and thank Prof. Yoshiyasu Fukuyama of Tokushima Bunri University for helpful discussions.

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Received for review October 2, 2008. Revised manuscript received January 10, 2009. Accepted January 21, 2009.

JF803077P