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Potential anti-inflammatory phenolic glycosides from the medicinal plant *Moringa oleifera* fruits

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ABSTRACT

Bioassay-guided isolation and purification of the ethyl acetate extract of *Moringa oleifera* fruits yielded three new phenolic glycosides; 4-[(2′-*O*-acetyl-α-L-rhamnosyloxy) benzyl]isothiocyanate (**1**), 4-[(3′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate (**2**), and *S*-methyl-*N*-{4-[(α-L-rhamnosyloxy)benzyl]}-thiocarbamate (**3**), together with five known phenolic glycosides (**4**–**8**). The structures of the new metabolites were determined on the basis of spectroscopic analyses including 1D- and 2D-NMR and mass spectrometry. The anti-inflammatory activity of isolated compounds was investigated with the lipopolysaccharide (LPS)-induced murine macrophage RAW 264.7 cell line. It was found that 4-[(2′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate (**1**) possessed potent NO–inhibitory activity with an IC_{50} value of 1.67 μM, followed by **2** (IC_{50} = 2.66 μM), **4** (IC_{50} = 2.71 μM), and **5** (IC_{50} = 14.4 μM), respectively. Western blots demonstrated these compounds reduced LPS-mediated iNOS expression. In the concentration range of the IC_{50} values, no significant cytotoxicity was noted. Structure–activity relationships following NO-release indicated: (1) the isothiocyanate group was essential for activity, (2) acetylation of the isothiocyanate derivatives at C-2′ or at C-3′ of rhamnose led to higher activity, (3) un-acetylated isothiocyanate derivatives displayed eight times less activity than the acetylated derivatives, and (4) acetylation of the thiocarbamate derivatives enhanced activity. These data indicate compounds **1**, **2**, **4** and **5** are responsible for the reported NO-inhibitory effect of *Moringa oleifera* fruits, and further studies are warranted.

1. Introduction

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs. This inorganic free radical has been implicated in physiological and pathological processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. Nitric oxide synthase (NOS) isoforms are homodimers that catalyze the oxidation of L-arginine to L-citrulline and nitric oxide in an NADPH- and O_2-dependent process.[1] There are at least three distinct isoforms of NOS. Endothelial isoform (eNOS) and neuronal NOS (nNOS) are constitutively expressed and involved in the regulation of smooth muscle relaxation and neurotransmission, respectively. Unlike eNOS and nNOS, inducible NOS (iNOS) is elevated in macrophages after the exposure of various stimuli, such as the bacterial endotoxin lipopolysaccharide (LPS), IL-1, TNF-α and IFN-γ,[2] and produces NO in normal immune responses. Since iNOS is responsible for the overproduction in inflammation, it has become a new target for drug development in the treatment of chronic inflammatory diseases.[1] Additionally, NO synthesized by iNOS has also been considered as an important mediator of carcinogenesis. NO can react with reactive oxygen species producing reactive nitrogen species that contribute to DNA damage and mutagenesis.[3] In addition, endogenous NO appears to cause a neoplastic transformation of mouse fibroblasts.[4] Overexpressed iNOS has also been detected in several human tumors.[5–7]

Moringa oleifera Lam. (Moringaceae), commonly known as 'Malúñggay' in Hawai'I, is a fast-growing ornamental tree that is widely distributed in tropical areas.[8] The plant is also known to possess high nutritional value, because of the presence of protein and vitamins.[9] The young leaves, flowers, and green pods are used as a vegetable and its roots can be a substitute for horseradish in the Filipino diet. The medicinal value of the seeds and the different parts of the plant has long been recognized in folkloric medicine to treat various ailments related to pain and inflammation.[8,10–14] Several types of bioactive compounds have been isolated from the fruits of *Moringa oleifera*. The fruits, in particular, have been reported to contain antitumor and anti-inflammatory compounds of the glycoside type (i.e., niazirin, niazimicin, niazicin A).[14,15]

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As part of our research program on the isolation of bioactive compounds from Hawaiian plants, the ethyl acetate extract of the fruits of *M. oleifera* was examined for anti-inflammatory effects. We currently describe the isolation and structure elucidation of three new glycosides (**1**–**3**), and report the anti-inflammatory activity observed with LPS-induced RAW 264.7 cells.

2. Results and discussion

2.1. Isolation and structural elucidation

The methanolic extract was suspended in H_2O and extracted successively with hexanes and ethyl acetate (EtOAc). The EtOAc layer was subjected repeatedly to column chromatography, and semi-preparative reversed-phase HPLC, to yield three new phenolic glycosides (**1**–**3**) together with five known phenolic compounds. The known compounds were identified as 4-[(4′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate (**4**),[10] 4-[(α-L-rhamnosyloxy)benzyl]isothiocyanate (**5**),[13] niazicin A (**6**),[15] niazinin A (**7**),[16] and niazirin (**8**)[12] (Fig. 1) by comparison of physical and spectroscopic data with published values.

Compound **1** was isolated as a colorless viscous oil and its molecular formula was established as $C_{16}H_{19}NO_6S$ by HRESIMS (*m*/*z* 376.0838 [M+Na]$^+$). The IR absorption bands at 2164 and 2085 cm^{-1} and the ^{13}C NMR resonance at δ_C 131.9 suggested the presence of a N=C=S group.[12] The ^{13}C NMR (Table 1) and DEPT spectroscopic data showed 16 signals of carbons that were identified as two methyls, one methylene, nine methines, three sp^2 quaternary, and one ester carbonyl carbon. The assignment of ^{13}C NMR chemical shifts is based on an HMQC experiment, which also indicated a sugar moiety. The ^{1}H and ^{13}C NMR spectroscopic data (Table 1) indicated the presence of a mono-acetylated sugar moiety from signals at δ_H/δ_C 5.48 (1H, d, *J* = 1.9 Hz, H-1′)/97.1 (C-1′), 5.19 (1H, dd, *J* = 3.4, 1.9 Hz, H-2′)/74.0 (C-2′), 4.02 (1H, dd, *J* = 9.5, 3.4 Hz, H-3′)/70.8 (C-3′), 3.67 (1H, m, H-5′)/70.4 (C-5′), 3.43 (1H, t, *J* = 9.5 Hz, H-4′)/73.7 (C-4′), 2.13 (3H, s, *Me*CO)/20.8, and 1.23 (3H, s, H_3-6′)/18.0 (C-6′). The chemical shifts and coupling constants of the anomeric proton (H-1′) indicated that the sugar is linked to the aglycone with an α-glycosidic linkage. These values are comparable with those of α-L-rhamnose and suggested that the compound is an α-L-rhamonoside.[10,11] The connectivity of the H-1′/H-2′, H-2′/H-3′, H-3′/H-4′, H-4′/H-5′, and H-5′/H-6′ in the COSY spectrum led to the assignment of all of the protons of the sugar moiety. Furthermore, a singlet at δ 4.70 (2H, H_2-7) in the ^{1}H-NMR spectrum connected to a carbon resonance at δ 48.2 in the HMQC experiment, indicating the presence of a benzylic methylene that was adjacent to the N-atom in the structure. Additionally, the ^{1}H NMR spectrum displayed two doublets in the aromatic region at δ 7.30 and 7.10 (each 2H, d, *J* = 8.9 Hz), indicating the presence of a *para*-disubstituted benzene ring. Comparison of ^{1}H and ^{13}C NMR spectral data between compound **1** and 4-[(4′-*O*-acetyl-α-L-rhamnosyloxy)-benzyl]isothiocyanate (**4**)[10] revealed a close structural similarity, except for the rearrangement of an acetyl group. The position of the acetyl group in compound **1** was located at C-2′, whereas those of **4** at C-4′. This was supported by the ^{1}H NMR and HMBC spectra, in which the acetyl methyl of compound **1** resonated at about a 1 ppm lower field than **4** due to the deshielding of the acetate group attached at C-2′. The clear HMBC correlation of H-2′ with the carbonyl carbon of the acetyl group confirmed the assignment. Additionally, HMBC correlations from H-1′ to C-1 and of H_2-7 to C-3, C-4, C-5, and C-8 confirmed the attachments of rhamnose and isothiocyanate moieties at C-1 and C-7, respectively. Thus, compound **1** was assigned as 4-[(2′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate.

Compound **2** was obtained as colorless viscous oil. The molecular formula $C_{16}H_{19}NO_6S$ was indicated on the basis of HRESIMS data (*m*/*z* 376.0822 [M+Na]$^+$). The IR absorption bands at 2177 and 2093 cm^{-1} indicated the presence of an N=C=S group**.** The ^{1}H and ^{13}C NMR spectra (Table 1) of **2** were closely related to those of **1**, and differed only in the chemical shifts of H-2′ and H-3′. The oxymethine proton H-3′ of **2** appeared at δ 5.12 (1H, dd, *J* = 9.4, 3.1 Hz), more downfield than that of **1** (δ 4.02, 1H, dd, *J* = 9.5, 3.4 Hz) as a result of the deshielding effect of the OAc group and showed HMBC correlations with the carbons at δ 171.3 (–O*C*OMe), confirming the location of an OAc group at C-3′. Hence, **2** was an isomer of **1**, and identified as 4-[(3′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]isothiocyanate.

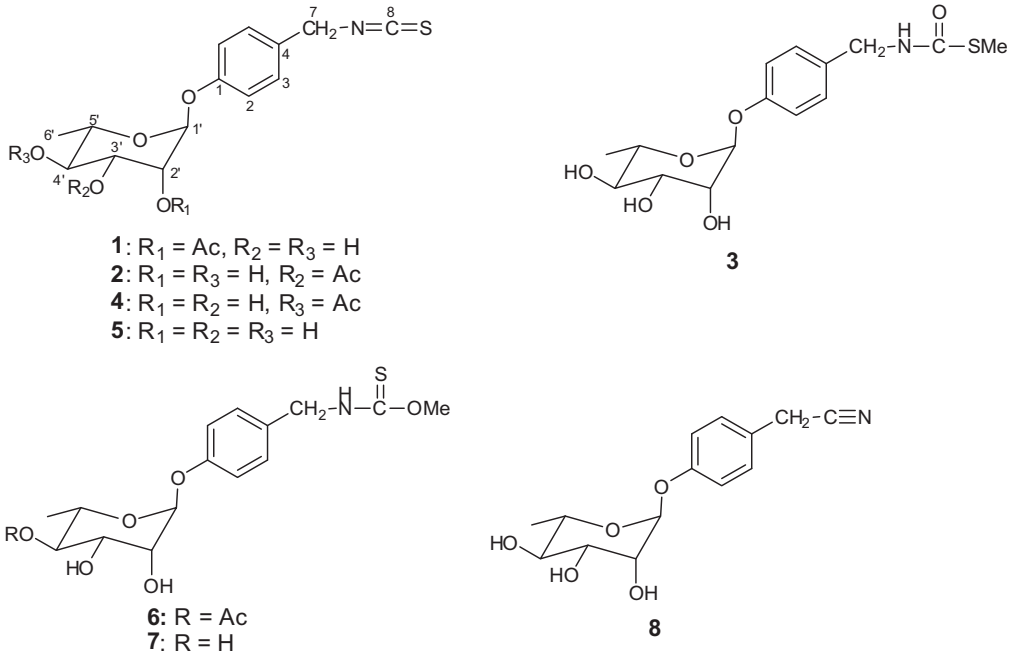

Figure 1. Structures of compounds isolated from the fruits of *M. oleifera*.

Table 1
^{1}H and ^{13}C NMR Spectroscopic data (500 MHz) of compounds **1**–**3** in CD_3OD (δ in ppm, multicities, J in Hz)[a]

Position	Compound **1**		Compound **2**[b]		Compound **3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		157.4		156.3		157.0
2	7.10 d (8.9)	117.9	7.13 d (8.9)	116.7	7.01 d (8.5)	117.5
3	7.30 d (8.9)	129.7	7.32 d (8.9)	128.4	7.20 d (8.5)	129.8
4		130.4		128.9		133.8
5	7.30 d (8.9)	129.7	7.32 d (8.9)	128.4	7.20 d (8.5)	129.8
6	7.10 d (8.9)	117.9	7.13 d (8.9)	116.7	7.01 d (8.5)	117.5
7	4.70 s	48.2	4.70 s	48.2	4.32 s	45.1
8		131.9		132.1		170.6
1′	5.48 d (1.9)	97.1	5.45 d (1.5)	98.4	5.39 d (1.4)	99.9
2′	5.19 dd (3.4, 1.9)	74.0	4.16 dd (3.1, 1.5)	69.7	3.97 dd (3.2, 1.4)	72.2
3′	4.02 dd (9.5, 3.4)	70.8	5.12 dd (9.4, 3.1)	74.3	3.82 dd (9.3, 3.2)	72.1
4′	3.43 t (9.5)	73.7	3.66 t (9.4)	69.9	3.44 t (9.3)	73.8
5′	3.67 m	70.4	3.70 m	68.6	3.61 m	70.6
6′	1.23 d (6.5)	18.0	1.25 d (6.1)	17.0	1.21 d (6.5)	18.0
OCOMe		172.3		171.3		
OCO*Me*	2.13 s	20.8	2.14 s	20.0		
-SMe					2.29 s	12.1

[a] Assignments were made using HMQC and HMBC data.
[b] The NMR spectra were measured at 300 MHz.

Compound **3** was isolated as a viscous oil that gave an $[M+H]^+$ ion peak at m/z 344.1162 in the HRESIMS spectrum, consistent with a molecular formula of $C_{15}H_{21}NO_6S$. The IR absorptions (3363 and 1681 cm^{-1}) implied the existence of OH and/or NH, and carbonyl functionalities. The ^{1}H NMR spectral data (Table 1) indicated the presence of one sugar moiety at δ 5.39 (1H, d, J = 1.4 Hz, H-1′), 3.97 (1H, dd, J = 3.2, 1.4 Hz, H-2′), 3.82 (1H, dd, J = 9.3, 3.2 Hz, H-3′), 3.61 (1H, m, H-5′), 3.44 (1H, t, J = 9.3 Hz, H-4′) and 1.20 (3H, d, J = 6.5 Hz, H_3-6′). These values coincided well with those reported for α-L-rhamnose (**7**),[16] and demonstrated that **3** is an α-L-rhamnopyranoside, which was corroborated by the mass fragment at m/z 147.[11] The relative stereochemistry of a sugar moiety in **3** was elucidated to be the same as that of the corresponding moiety in **1** on the basis of the coupling constants and the NOESY data. Moreover, the ^{1}H NMR data displayed characteristics of a *para*-disubstituted benzene ring at δ 7.20, and 7.01 (each 2H, d, J = 8.5 Hz, H-2/H-6, H-3/H-5), and of the benzylic methylenes at δ 4.32 (2H, s, H_2-7). A singlet signal at δ 2.29 (3H) was assigned to a SMe group. In comparison with the known niazinin A (**7**),[16] the ^{13}C NMR signal of C-8 in **3** was shifted upfield from δ 193.1 (C=S) to 170.6 (C=O), suggesting the presence of a thiocarbamate group. HMBC correlations from H_2-7 and *SMe* to C-8 confirmed structure **3** was identified as *S*-methyl-*N*-{4-[(α-L-rhamnosyloxy)benzyl]}thiocarbamate.

2.2. Anti-inflammatory activity

The ethyl acetate extract from *M. oleifera* fruits showed NO-inhibition with an IC_{50} value of 0.136 μg/mL. Further fractionation led to the isolation of eight phenolic glycosides shown in Figure 1. The results indicated 4-[(2′-*O*-acetyl-α-L-rhamnosyloxy)benzyl]-isothiocyanate (**1**) possessed potent NO-inhibitory activity against lipopolysaccharide (LPS)-induced nitric oxide release with an IC_{50} value of 1.67 μM, as well as **2** (IC_{50} = 2.66 μM) and **4** (IC_{50} = 2.71 μM), followed by **5** (IC_{50} = 14.43 μM), whereas other compounds exhibited no activity (Table 2; Figs. 2 and 3). The potency of **1**, **2**, and **4** were comparable to that of L-N^G-monomethyl arginine citrate, a positive control (IC_{50} = 25.5 μM). These compounds did not show appreciable cytotoxicity at their IC_{50} values for NO-inhibitory activity. Further experiments were performed to determine whether these compounds inhibit nitrite production via suppression of iNOS expression in the levels of protein. As shown in Figure 3, in the presence of LPS, RAW 264.7 cells increased the protein expression of iNOS in comparison with control

Table 2
Inhibition of NO production of compounds isolated from *M. oleifera* fruits on LPS-induced NO-release from RAW264.7 cells, (δ) = % inhibition at 45 μM

Compounds	IC_{50} (μM)
4-[(2′-*O*-Acetyl-α-L-rhamnosyloxy) benzyl]Isothiocyanate (**1**)	1.67
4-[(3′-*O*-Acetyl-α-L-rhamnosyloxy)benzyl]Isothiocyanate (**2**)	2.66
S-Methyl-*N*-{4-[(α-L-rhamnosyloxy)benzyl]}thiocarbamate (**3**)	>45 (24.3%)
4-[(4′-*O*-Acetyl-α-L-rhamnosyloxy)benzyl]Isothiocyanate (**4**)	2.71
4-[(α-L-Rhamnosyloxy)benzyl]Isothiocyanate (**5**)	14.43
Niazicin A (**6**)	>45 (17.3%)
Niazinin A (**7**)	>45 (<1%)
Niazirin (**8**)	>45 (<1%)
L-N^G-Monomethyl arginine citrate, positive control	25.49

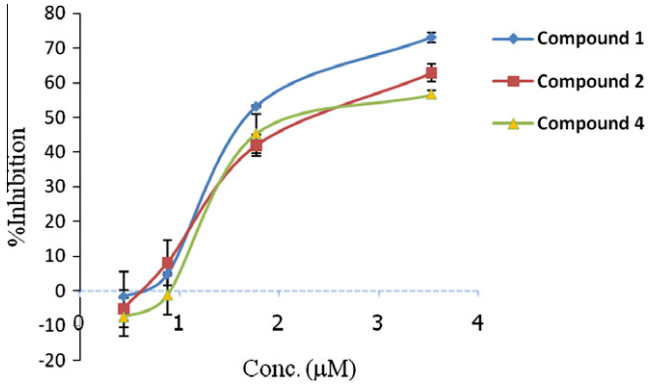

Figure 2. Dose–response curve of compounds **1**, **2** and **4** against lipopolysaccharide (LPS)-induced nitric oxide. Each value represents the mean ± SD of the three determinations.

cells. Compounds **1**, **2**, **4**, and **5** suppressed the expression of iNOS protein levels in a concentration-dependent manner. Compound **1**, which showed the most potent NO-inhibitory activity, down-regulated the expression of iNOS to basal levels. Structure–activity relationships of these compounds against NO-release indicated the following: (1) an isothiocyanate group was essential for activity as observed in **5** (IC_{50} = 14.4 μM) versus **7** (IC_{50} = >45 μM), and **8** (IC_{50} = >45 μM), (2) acetylation of the isothiocyanate derivatives at C-2′ (**1**, IC_{50} = 1.67 μM) gave higher activity than at C-3′ (**2**, IC_{50} = 2.66 μM) and C-4′ (**4**, IC_{50} = 2.71 μM), (3) a non-acetylated

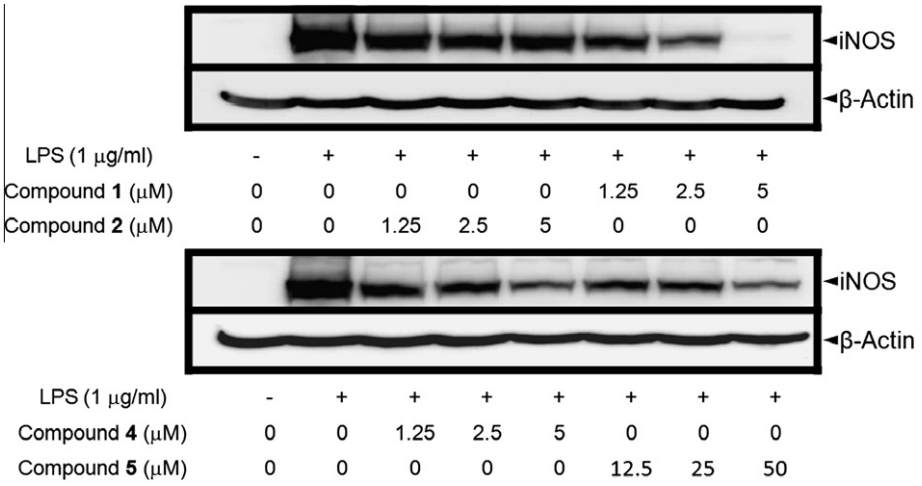

Figure 3. Inhibitory effect of compound **1**, **2**, **4**, and **5** on iNOS protein expression. RAW 264.7 cells (5×10^5 cells) were incubated in 12 well culture plate for 24 h, and then treated with compounds and LPS (1 μg/ml) for 18 h. After incubation, cells were lysed, and protein (15 μg) was applied on SDS-polyacrylamide gel. The level of iNOS protein expression was examined by Western blot analysis.

isothiocyanate derivative (**5**, IC_{50} = 14.4 μM) displayed eight times less activity than an acetylated derivative (**1**, IC_{50} = 1.67 μM), (4) acetylation of thiocarbamate derivatives enhanced activity, as observed in **6** (17.3% inhibition) versus **7** (<1% inhibition).

In conclusion, the isothiocyanate group was essential for activity, and acetylation of the isothiocyanate derivatives at C-2′ (**1**), at C-3′ (**2**), and at C-4′ (**4**) of rhamnose led to higher activity versus the non-acetylated isothiocyanate (**5**).

3. Conclusions

Recently, Sashidhara et al. reported potential anti-inflammatory agents, aurantiamide acetate and 1,3-dibenzyl urea, from the roots of *M. oleifera*.[17] 4-[(α-L-rhamnosyloxy)benzyl]Isothiocyanate (**5**) and niazirin (**8**) have been reported having antitumor promoting activity against EBV-EA activation[13] and antibiotic activities[13] whereas niazicin A (**6**) significantly stimulated insulin-release and inhibited peroxidation reactions. We currently report compounds **1**, **2**, **4** and **5**, isolated from *Moringa oleifera* fruits, are responsible for NO-inhibitory effects. The structure–activity relationships of these compounds require the isothiocyanate group and acetylation of the rhamnopyranoside ring. These data suggest the most potent of these compounds might be investigated as anti-inflammatory agents and might help in understanding the mechanism of action of this traditional plant on inflammation.

4. Experimental section

4.1. General experimental procedures

The optical rotation $[\alpha]_D$ values were determined with an Autopol® IV Automatic polarimeter. UV and IR spectra were measured on a Shimadzu PharmaSpec-1700 UV–visible and a Shimadzu-8400S FT-IR spectrophotometer, respectively. Mass spectra and high-resolution MS spectra were taken with a BioTOF II ESI mass spectrometer. 1D- and 2D-NMR spectra were recorded in methanol-d_4 on an INOVA Unity (500 MHz) Varian and a Bruker FT NMR Ultra Shield™ (300 MHz) spectrometers. Reversed-phase HPLC was carried out on a Beckman Coulter Gold-168 system equipped with a photodiode array detector using an Alltech semi-preparative Econosil C_{18} column (10 μm, 10 × 250 mm) run with a flow rate of 2.0 mL/min. Flash column chromatography and column chromatography (CC) were carried out with Merck Si Gel 60 F_{254} and Merck Si Gel 100, respectively. TLC was performed on Merck precoated aluminum Si Gel 60 F_{254} and RP-18 F_{254} sheets.

4.2. Plant material

Fruits of *M. oleifera* were collected at Waialua, Hawai'i in February 2009 and identified by Liloa Dunn (Botanist, Ethonobotanist) University of Hawai'i Manoa. A voucher specimen (collection C. Barit 1, BISH) was deposited at Bishop Museum, Honolulu, Hawaii.

4.3. Extraction and isolation

The chopped-fresh fruits (4.9 kg) of *M. oleifera* were extracted with MeOH (3 × 6 L, overnight) at room temperature and the solvent was evaporated under reduced pressure to afford the MeOH extract. The extract was suspended in H_2O (1:1) then partitioned successively with hexanes and EtOAc (3 × 250 mL each). The EtOAc extract (20.3 g) was subjected to QCC on silica gel (400 g) eluting with hexanes/EtOAc/MeOH (100:0:0, 4:1:0, 7:3:0, 3:2:0, 1:1:0, 3:7:0, 1:4:0, 1:9:0, 0:100:0, 0:9:1, 0:4:1, 0:1:1, and 0:0:100, each 500 mL) to yield eight fractions (F1–F10). Fraction F5 (1.46 g) was further separated by CC using MeOH/$CHCl_3$ (1:19, 2.0 mL) over silica gel (90 g) to give eight subfractions (F5a–F5h). Subfraction F5c was recrystallized with MeOH/$CHCl_3$ (1:1) to afford compound **4** (51.0 mg). Subfraction F5d (77.3 mg) was purified by RP-HPLC using MeOH/H_2O/TFA (65:35:0.05) to give compounds **6** (13.4 mg, t_R 15.2 min), **3** (12.9 mg, t_R 16.6 min), **1** (7.0 mg, t_R 20.1 min), and **4** (38.4 mg, t_R 23.3 min). Fraction F6 (13.0 g) was subjected to QCC over Silica Gel 60 (350 g) using $CHCl_3$/MeOH (100:0, 1:49, 1:19, 1:9, 3:17, 1:4, 3:7, 1:1, 0:100, each 300 mL) to give five subfractions (F6a–F6e). A part of subfraction F6e (1.67 g) was separated by reversed-phase CC eluting with MeOH/H_2O (1:1, 2.5 L) to give four subfractions (F6e-1–F6e-4). A part of subfractions F6e-1 (80.2 mg) was further purified by RP-HPLC eluting with CH_3CN/H_2O/TFA (25:75:0.05) to give compounds **8** (39.8 mg, t_R 14.2 min), **3** (1.5 mg, t_R 18.1 min), and **7** (10.3 mg, t_R 35.1 min). Subfraction F6e-4 (100.3 mg) was isolated by RP-HPLC using MeOH/H_2O/TFA (45:55:0.05) to give compound **5** (68.2 mg, t_R 35.1 min).

4.3.1. 4-[(2′-*O*-Acetyl-α-L-rhamnosyloxy)benzyl]Isothiocyanate (1)

Colorless viscous oil; $[\alpha]_D^{21}$ −107.4 (*c* 0.027, MeOH); IR (film) ν_{max} 3429, 2926, 2164, 2085, 1706, 1237, 1129 cm^{-1}; UV (MeOH)

λ_{max} (log ϵ) 203 (4.10), 222 (4.00) nm; ^{1}H and ^{13}C NMR (CD_3OD, 500 MHz) data see Table 1; HRESIMS *m*/*z* 376.0838 $[M+Na]^+$ (calcd for $C_{16}H_{19}NO_6SNa$, 376.0831).

4.3.2. 4-[(3′-*O*-Acetyl-α-L-rhamnosyloxy)benzyl]Isothiocyanate (2)

Colorless, viscous oil; $[\alpha]_D^{21}$ −118.9 (*c* 0.037, MeOH); IR (film) ν_{max} 3417, 2913, 2177, 2093, 1735, 1577, 1211 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 204 (3.91), 224 (3.67) nm; ^{1}H and ^{13}C NMR (CD_3OD, 300 MHz) data see Table 1; HRESIMS *m*/*z* 376.0822 $[M+Na]^+$ (calcd for $C_{16}H_{19}NO_6SNa$, 376.0831).

4.3.3. *S*-Methyl-*N*-{4-[(α-L-rhamnosyloxy)benzyl]} thiocarbamate (3)

Colorless, viscous oil; $[\alpha]_D^{21}$ −97.8 (*c* 0.080, MeOH); IR (film) ν_{max} 3363, 2984, 1681, 1373, 1178 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 222 (4.12), 266 (2.91) nm; ^{1}H and ^{13}C NMR (CD_3OD, 500 MHz) data see Table 1; HRESIMS *m*/*z* 344.1162 $[M+H]^+$ (calcd for $C_{15}H_{22}NO_6S$, 344.1168).

4.4. Assay for NO-inhibitory effect using RAW 264.7 cells

The inhibitory effects of samples on NO production were evaluated in lipopolysaccharide (LPS)-activated murine macrophage RAW 264.7 cells, using a method modified from that previously reported.[18] Briefly, RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin G sodium (100 units/mL), streptomycin sulfate (100 μg/mL), amphotericin B (0.25 μg/mL), and 10% fetal bovine serum (FBS). The cells were seeded in 96-well culture plates with 1×10^5 cells/well and allowed to adhere for 24 h at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were treated with samples dissolved in phenol red-free DMEM for 30 min followed by 1 μg/mL of LPS treatment for 20 h. The concentration of NO in the cultured medium was measured with Griess reagent [90 μL 1% sulfanilamide and 90 μL 0.1% *N*-(1-naphthyl)ethylenediamine in 2.5% H_3PO_4 in each well]. A standard curve was created by the use of known concentrations of sodium nitrite, and absorbance was measured at 540 nm. To evaluate the cytotoxic effect of samples in RAW 264.7 cells in the assay condition, sulforhodamine B (SRB) assay was performed. Briefly, after the fixation with 10% trichloroacetic acid (TCA), cells were stained with 0.4% SRB solution in 1% acetic acid followed by dissolving bound SRB in 10 mM Tris-buffer. The optical density was determined at 515 nm.

4.5. Western immunoblot analysis

RAW 264.7 cells were pretreated with various concentrations of samples for 15 min before treatment with 1 μg/ml LPS for 18 h and examining the expression of iNOS protein. Cells were lysed with lysis buffer. 15 μg of total protein in each cell lysate was resolved using 9% SDS–PAGE, and electrotransferred to PVDF membranes. The membranes were incubated with 5% skimmed milk in 0.1% Tween 20 containing TBS (TBST) for 1 h at room temperature to block non-specific protein binding. Then, membranes were incubated overnight at 4 °C with corresponding primary antibodies in 3% skimmed milk in TBS followed by the incubation with horse radish peroxidase (HRP)-conjugated secondary antibodies, and visualization using enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.03.057.

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