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Quinic acid derivatives from *Pimpinella brachycarpa* exert anti-neuroinflammatory activity in lipopolysaccharide-induced microglia

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ABSTRACT

Five new quinic acid derivatives (**1**–**5**), together with 10 known quinic acid derivatives (**6–15**), were isolated from the MeOH extract of *Pimpinella brachycarpa* (Umbelliferae). Their structures were established on the basis of spectroscopic analyses including extensive 2D NMR studies (COSY, HMQC and HMBC). Isolated compounds **1–15** were evaluated for their inhibitory activities on nitric oxide (NO) production in an activated murine microglial cell line. Compounds **2**, **3**, **8** and **11** significantly inhibited NO production without high cell toxicity in lipopolysaccharide (LPS)-activated BV-2 cells, a microglia cell line (IC_{50} = 4.66, 12.52, 9.04 and 12.11 μM, respectively).

Microglia, which is a type of neuroglia, is similar to macrophages in the CNS. Activated microglia secrete several inflammatory mediators such as NO and TNF-alpha. Overproduction of toxic mediators by microglia can induce pathogenesis of several neurodegenerative diseases such as ischemia, Alzheimers's disease, Parkinsons's disease and Multiple sclerosis.[1]

Based on the assays in search for anti-inflammatory activities from different plant sources, the MeOH extract from the aerial parts of *Pimpinella brachycarpa* revealed significant anti-neuroinflammatory activities in BV-2 cells.

Pimpinella brachycarpa (Umbelliferae) is widely distributed in Europe, Africa, and Asia.[2] In Korea, *P. brachycarpa*, called as chamna-mul, is well known as a vegetable and a popular edible herb.[3] This species has also been used in Korean folk medicine for the treatment of gastrointestinal disturbances, bronchial asthma, insomnia, and persistent cough.[4] In previous phytochemical studies, terpenes,[4] flavonoids,[5] and essential oil components[6] were isolated from this source. The MeOH extract of *P. brachycarpa* has exhibited antibacterial, antioxidative, anti-proliferative,[3] antifungal,[7] and antithrombotic activities[8] in a previous study. Also, the EtOH extract of *P. brachycarpa* showed positive effects on ethanol-induced chronic hepatotoxicity in rat liver and on lipid metabolism in rats fed with a high cholesterol diet.[4,9] Quinic acid derivatives are a large family of esters formed between quinic acid and one or more of several phenylpropanoic acids such as caffeic acid, ferulic acid, coumaric acid, sinapic acid and cinnamic acid.[10] Quinic acid derivatives have various beneficial effects including antioxidant, anti-inflammatory, anti-HIV, anti-hepatitis B virus, hypoglycaemic, and hepatoprotective activities and inhibition of mutagenesis and carcinogenesis.[11–16]

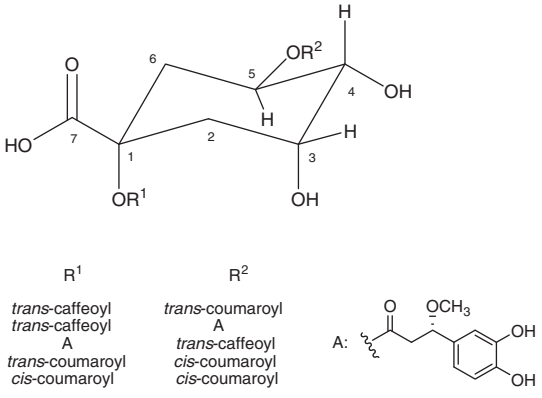

Figure 1. Chemical structures of compounds **1**–**5**.

The aim of the present study was to identify the potential components related to the anti-inflammatory activity of the MeOH extract from *P. brachycarpa* on BV-2 microglial cells. Column

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chromatographic separation of the MeOH extract from the aerial parts of *P. brachycarpa* furnished five new quinic acid derivatives (**1**–**5**) (Fig. 1), together with 10 known quinic acid derivatives (**6**–**15**). The structures of these new compounds were elucidated on the basis of 1D and 2D NMR spectroscopic data analyses (^{1}H, ^{13}C NMR, COSY, HMQC and HMBC), as well as chemical means. Also, we evaluated the inhibitory effect of the isolated compounds (**1**–**15**) from *P. brachycarpa* on NO production in LPS-activated BV-2 cells, a microglial cell line.

Compound (**1**) was isolated as a colorless gum. The molecular formula $C_{25}H_{25}O_{11}$ was determined by the positive ion HRFABMS *m/z* 501.1400 $[M+H]^+$ (calcd for 501.1397). The ^{1}H NMR spectrum of **1** (Table 1) showed the presence of three oxygenated methine proton signals at δ_H 5.38 (1H, m, H-5), 4.29 (1H, m, H-3) and 3.78 (1H, m, H-4) and two methylene proton signals at δ_H 2.57, 2.07 (2H, m, H_2-6) and 2.44, 2.43 (2H, m, H_2-2). In the ^{13}C NMR spectrum (Table 2), 7 carbon signals appeared, including oxygenated three methine carbons at δ_C 71.6 (C-4), 70.4 (C-5) and 68.2 (C-3), two methylene carbons at δ_C 35.7 (C-6) and 34.4 (C-2), one quaternary carbon at δ_C 79.8 (C-1) and one carbonyl carbon at δ_C 173.7 (C-7). These spectral data implied that **1** could be a quinic acid derivative.[17,18] In addition, ^{1}H (Table 1) and ^{13}C (Table 2) NMR spectra of **1** showed *trans*-caffeoyl group signals[19] [δ_H 7.57 (1H, d, *J* = 16.0 Hz, H-7′), 7.05 (1H, d, *J* = 2.0 Hz, H-2′), 6.96 (1H, dd, *J* = 8.5, 2.0 Hz, H-6′), 6.78 (1H, d, *J* = 8.5 Hz, H-5′) and 6.29 (1H, d, *J* = 16.0 Hz, H-8′); δ_C 166.8 (C-9′), 148.4 (C-4′), 146.2 (C-7′), 145.9 (C-3′), 126.0 (C-1′), 121.8 (C-6′), 115.3 (C-5′), 114.2 (C-8′) and 114.0 (C-2′)] and *trans-p*-coumaroyl group signals[19] [δ_H 7.65 (d, *J* = 16.0 Hz, H-7″), 7.46 (d, *J* = 8.5 Hz, H-2″, 6″), 6.80 (d, *J* = 8.5 Hz, H-3″, 5″), and 6.33 (d, *J* = 16.0 Hz, H-8″); δ_C 167.4 (C-9″), 160.1 (C-4″), 145.8 (C-7″), 130.0 (C-2″, 6″), 126.6 (C-1″), and 115.6 (C-3″, 5″)]. The position of the *trans-p*-coumaroyl group was confirmed by the HMBC experiment, in which a correlation was observed between the H-5 (δ_H 5.38) of the quinic acid moiety and the C-9″ (δ_C 167.4) of the *trans-p*-coumaroyl unit as shown in Figure 2. The downfield shift of the signals at H-5 ($\Delta\delta_H$ +1.2) and at C-5 ($\Delta\delta_C$ +2.5), compared to the corresponding signals of quinic acid[18] which was isolated from *Centella asiatica*, suggested that the *trans-p*-coumaroyl unit should be placed at C-5 of **1**.[18] The *trans*-caffeoyl unit was placed at C-1, since the carbon chemical shift of the oxygenated quaternary carbon (C-1) was downfield shifted to δ_C 79.8, compared to that of quinic acid containing a free hydroxyl group at C-1 (δ_C 74.5 in **7**; 73.5 in **8**; 74.8 in **9**).[18] The above ^{1}H and ^{13}C NMR spectral data of **1** were very similar to those of **6** which was isolated from *Arnica chamissonis*.[20] The major difference was the acyl moiety at C-5, indicating the presence of the *trans-p*-coumaroyl unit in **1** instead of the *trans*-caffeoyl unit in **6**. Alkaline methanolysis (3% KOH in MeOH) of **1** afforded *trans-p*-coumaric acid methyl ester,[21] *trans*-caffeic acid methyl ester,[22] and quinic acid,[17,18] which were confirmed by comparison of their ^{1}H NMR and optical rotation values with literature values, respectively. Thus, the structure of **1** was determined to be 1-*O*-*trans*-caffeoyl-5-*O*-*trans-p*-coumaroylquinic acid.

Compound (**2**) was isolated as a colorless gum. The molecular formula $C_{26}H_{28}NaO_{13}$ was inferred from the positive ion HRESIMS *m/z* 571.1431 $[M+Na]^+$ (calcd for 571.1428). The ^{1}H (Table 1) and ^{13}C (Table 2) NMR data were similar to those of **1**, except for the presence of 7,8-dihydro-7-methoxycaffeoyl signals[23] [δ_H 6.74 (1H, d, *J* = 2.0 Hz, H-2″), 6.73 (1H, d, *J* = 8.5 Hz, H-5″), 6.66 (1H, dd, *J* = 8.5, 2.0 Hz, H-6″), 4.48 (1H, m, H-7″), 3.16 (3H, s, 7″-OMe)

Table 2
^{13}C NMR data of compounds **1**–**5** (in CD_3OD, 125 MHz, δ in ppm)

Position	1	2	3	4	5
1	79.8	80.1	79.7	79.8	79.8
2	34.4	34.5	34.2	34.2	34.0
3	68.2	68.3	68.2	68.3	68.2
4	71.6	71.6	71.5	71.7	71.5
5	70.4	70.7	70.4	69.8	69.9
6	35.7	35.8	35.2	35.8	35.9
7	173.7	173.8	173.6	173.7	173.7
1′	126.0	126.7	132.1	125.8	126.2
2′	114.0	113.9	113.4	129.8	132.3
3′	145.9	145.6	145.4	115.3	114.4
4′	148.4	148.4	145.1	159.8	158.7
5′	115.3	115.3	115.1	115.3	114.4
6′	121.8	121.9	118.3	129.8	132.3
7′	146.2	146.2	79.8	145.6	143.9
8′	114.2	114.2	43.4	114.0	115.4
9′	166.8	166.8	170.4	166.6	166.1
7′-OMe			55.5		
1″	126.6	131.9	126.7	126.0	126.1
2″	130.0	113.4	113.9	132.3	132.3
3″	115.6	145.4	145.6	114.4	114.3
4″	160.1	145.1	148.4	158.7	158.6
5″	115.6	115.0	115.3	114.4	114.3
6″	130.0	118.4	121.8	132.3	132.3
7″	145.8	79.9	146.1	143.8	143.8
8″	114.1	43.3	114.1	115.2	115.3
9″	167.4	171.0	167.3	166.2	165.7
7″-OMe		55.5			

Table 1
^{1}H NMR data of compounds **1**–**5** (in CD_3OD, 500 MHz, δ in ppm, *J* in Hz)

Position	1	2	3	4	5
2	2.43 m, 2.44 m	2.35 m, 2.48 m	2.38 m, 2.40 m	2.33 m, 2.45 m	2.35 m, 2.48 m
3	4.29 m	4.23 m	4.26 m	4.23 m	4.23 m
4	3.78 m	3.68 m	3.75 m	3.70 m	3.69 m
5	5.38 m	5.29 m	5.33 m	5.37 m	5.31 m
6	2.07 m, 2.57 m	1.98 m, 2.48 m	2.04 m, 2.50 m	1.98 m, 2.54 m	1.98 m, 2.52 m
2′	7.05 d (2.0)	7.05 d (2.0)	6.74 d (2.0)	7.46 d (8.5)	7.65 d (8.5)
3′				6.80 d (8.5)	6.74 d (8.5)
5′	6.78 d(8.5)	6.76 d (8.0)	6.73 d (8.5)	6.80 d (8.5)	6.74 d (8.5)
6′	6.96 dd (8.5, 2.0)	6.95 dd (8.0, 2.0)	6.66 dd (8.5, 2.0)	7.46 d (8.5)	7.65 d (8.5)
7′	7.57 d (16.0)	7.57 d (16.0)	4.51 m	7.65 d (16.0)	6.86 d (13.0)
8′	6.29 d (16.0)	6.28 d (16.0)	2.57 m, 2.74 m	6.36 d (16.0)	5.82 d (13.0)
7′-OMe			3.18 s		
2″	7.46 d (8.5)	6.74 d (2.0)	7.05 d (2.0)	7.65 d (8.5)	7.64 d (8.5)
3″	6.80 d (8.5)			6.75 d (8.5)	6.73 d (8.5)
5″	6.80 d (8.5)	6.73 d (8.5)	6.76 d (8.5)	6.75 d (8.5)	6.73 d (8.5)
6″	7.46 d (8.5)	6.66 dd (8.5, 2.0)	6.96 dd (8.5, 2.0)	7.65 d (8.5)	7.64 d (8.5)
7″	7.65 d (16.0)	4.48 m	7.57 d (16.0)	6.86 d (13.0)	6.86 d (13.0)
8″	6.33 d (16.0)	2.59 m, 2.74 m	6.25 d (16.0)	5.79 d (13.0)	5.78 d (13.0)
7″-OMe		3.16 s			

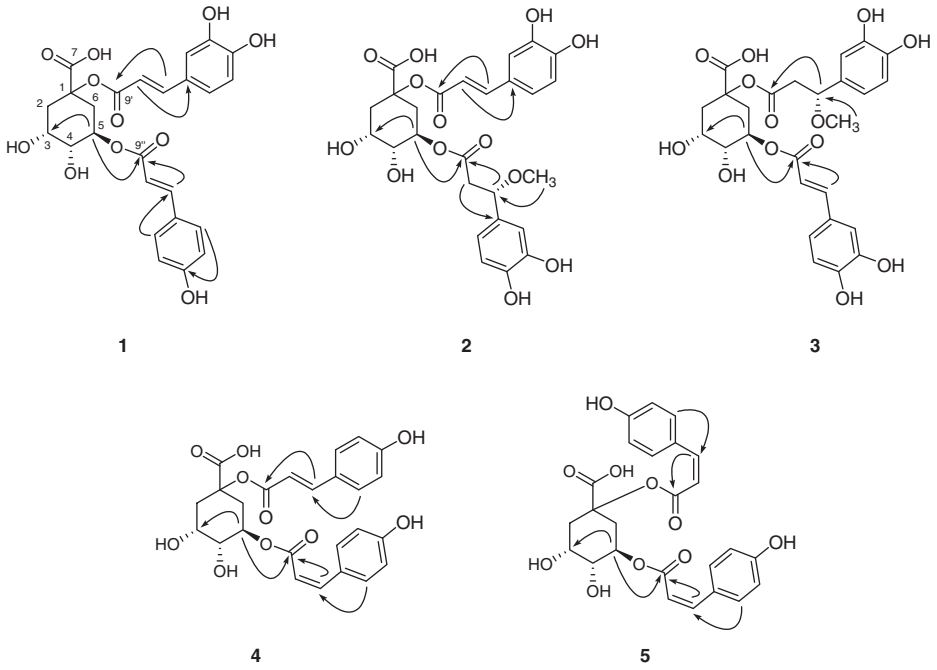

Figure 2. Key HMBC (→) correlations of **1**–**5**.

and 2.74, 2.59 (2H, m, H-8″); δ_C 171.0 (C-9″), 145.4 (C-3″), 145.1 (C-4″), 131.9 (C-1″), 118.4 (C-6″), 115.0 (C-5″), 113.4 (C-2″), 79.9 (C-7″), 55.5 (7″-OMe) and 43.3 (C-8″)] in **2** instead of the *trans*-*p*-coumaroyl group in **1**. The position of the 7,8-dihydro-7-methoxycaffeoyl group was confirmed by the HMBC experiment, in which a correlation was observed between the H-5 (δ_H 5.29) of the quinic acid moiety and the C-9″ (δ_C 171.0) of the 7,8-dihydro-7-methoxycaffeoyl unit. In addition, this HMBC correlation between the methoxy signals at δ_H 3.16 and C-7″ (δ_C 79.9) implied that the methoxy group was located at C-7″ (Fig. 2). Alkaline hydrolysis of **2** afforded *trans*-caffeic acid methyl ester,[22] quinic acid[17,18] and 7,8-dihydro-7-methoxycaffeic acid methyl ester (**2a**). The ^{1}H, ^{13}C NMR, optical rotation value and HRFABMS data of **2a** were similar to those of pisnoninol I isolated from *Pisonia unbellifera*,[24] except for the presence of an additional methoxy signal (δ_H 3.15; δ_C 55.9). **2a** had the same planar structure as taraxafolin isolated from *Taraxacum formosanum*,[23] except for the optical rotation value. The optical rotation of 7,8-dihydro-7-methoxycaffeic acid methyl ester ([α] +30.5) (Ref. 24 [α] +37.8) was almost of the same value but of opposite sign to that of taraxafolin C ([α] −32.0), which suggested that 7,8-dihydro-7-methoxycaffeic acid methyl ester could be a stereoisomer of taraxafolin. The above data indicated that the methoxyl group at C-7 has α-orientation.[25] Therefore, the structure of **2** was determined to be 1-*O*-*trans*-caffeoyl-5-*O*-7,8-dihydro-7α-methoxycaffeoylquinic acid.

Compound (**3**) was obtained as a colorless gum, and its molecular formula $C_{26}H_{28}NaO_{13}$ was inferred from the positive ion HRESIMS *m/z* 571.1432 [M+Na]$^+$ (calcd for 571.1428). The ^{1}H (Table 1) and ^{13}C (Table 2) NMR data were very similar to those of **2**. The difference was the position of two acyl groups. The location of the *trans*-caffeoyl unit was determined by HMBC correlation between H-5 (δ_C 5.33) of the quinic acid moiety and C-9″ (δ_C 167.3) of the *trans*-caffeoyl unit. In addition, a comparison of the carbon signal (δ_C 79.8, C-1) of **1** with the corresponding signal (δ_C 79.7) of **3** revealed that another acyl group, 7,8-dihydro-7α-methoxycaffeoyl unit, should be placed at C-1 in **3**. Alkaline hydrolysis of **3** with 3% KOH in MeOH at room temperature afforded *trans*-caffeic acid methyl ester,[22] quinic acid[17,18] and 7,8-dihydro-7α-methoxycaffeic acid methyl ester.[23,25] Therefore, the structure of **3** was determined to be 1-*O*-7,8-dihydro-7α-methoxycaffeoyl-5-*O*-*trans*-caffeoylquinic acid.

Compound (**4**) was obtained as a colorless gum, and its molecular formula $C_{25}H_{25}O_{10}$ was inferred from the positive ion HRFABMS *m/z* 485.1446 [M+H]$^+$ (calcd for 485.1448). The ^{1}H and ^{13}C NMR data were very similar to those of **15**, except for the presence of additional *trans*-*p*-coumaroyl group signals[19] [δ_H 7.65 (1H, d, *J* = 16.0 Hz, H-7′), 7.46 (2H, d, *J* = 8.5 Hz, H-2′,6′), 6.80 (2H, d, *J* = 8.5 Hz, H-3′,5′) and 6.36 (1H, d, *J* = 16.0 Hz, H-8′); δ_C 166.6 (C-9′), 159.8 (C-4′), 145.6 (C-7′), 129.8 (C-2′,6′), 125.8 (C-1′), 115.3 (C-3′,5′) and 114.0 (C-8′)]. The location of another *trans*-*p*-coumaroyl group should be placed at C-1 (δ_C 79.8) in **4** due to the downfield shift of the signal at C-1 ($\Delta\delta_C$ +3.0), compared to the corresponding signal of **15**. Alkaline methanolysis (3% KOH in MeOH) of **4** yielded *trans*-*p*-coumaric acid methyl ester, *cis*-*p*-coumaric acid methyl ester[21] and quinic acid.[17,18] Therefore, the structure of **4** was determined to be 1-*O*-*trans*-*p*-coumaroyl-5-*O*-*cis*-*p*-coumaroylquinic acid.

Compound (**5**) was obtained as a colorless gum, and its molecular formula $C_{25}H_{25}O_{10}$ was inferred from the positive ion HRFABMS *m/z* 485.1450 [M+H]$^+$ (calcd for 485.1448). The ^{1}H and ^{13}C NMR data were very similar to those of **4** except for the presence of *cis*-*p*-coumaroyl group signals[26] [δ_H 7.65 (2H, d, *J* = 8.5 Hz, H-2′,6′), 6.86 (1H, d, *J* = 13.0 Hz, H-7′), 6.74 (2H, d, *J* = 8.5 Hz, H-3′,5′) and 5.82 (1H, d, *J* = 13.0 Hz, H-8′); δ_C 166.1 (C-9′), 158.7 (C-4′), 143.9 (C-7′), 132.3 (C-2′,6′), 126.2 (C-1′), 115.4 (C-8′) and 114.4 (C-3′,5′)] in **5**, instead of the *trans*-*p*-coumaroyl group in **4**. The coupling constant values (13.0 Hz) at H-7′ (δ_H 6.86) and H-8′ (δ_H 5.82) in the *p*-coumaroyl unit proton signals indicated the *cis* form.[26] Alkaline hydrolysis (3% KOH in MeOH) of compound **5** gave quinic acid,[17,18] and *cis*-*p*-coumaric acid methyl ester.[21] Thus, the structure of **5** was determined to be 1,5-di-*O*-*cis*-*p*-coumaroylquinic acid.

The structures of the known compounds (**6–15**) were identified as 1,5-*O*-*trans*-dicaffeoylquinic acid (**6**),[20] 3,4-*O*-*trans*-caffeoylquinic acid methyl ester (**7**),[27] 3,5-*O*-*trans*-dicaffeoylquinic acid methyl ester (**8**),[15] 4,5-*O*-*trans*-dicaffeoylquinic acid methyl ester

Table 3
Inhibitory effect on NO production of compounds **1**–**15** in LPS-activated BV-2 cells

Sample	IC_{50}[a] (μM)	Cell viability[b] (%)
1	23.53	94.3 ± 1.9
2	4.66	93.2 ± 1.0
3	12.52	90.9 ± 2.3
4	59.82	98.1 ± 2.1
5	52.79	96.0 ± 3.7
6	22.63	93.7 ± 3.8
7	20.15	96.8 ± 2.9
8	9.04	91.1 ± 6.8
9	24.58	92.2 ± 3.8
10	16.57	92.6 ± 1.2
11	12.11	95.3 ± 3.0
12	17.04	90.6 ± 0.9
13	44.99	108.1 ± 7.5
14	81.97	108.8 ± 0.9
15	86.41	108.7 ± 5.5
NMMA[c]	16.32	103.2 ± 4.3

[a] IC_{50} value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells.
[b] Cell viability after treatment with 20 μM of each extract was expressed as a percentage (%) of the LPS only treatment group. The results are averages of three independent experiments, and the data are expressed as mean ± SD.
[c] NMMA as a positive control.

(**9**),[27] 4-*O*-*trans*-caffeoylquinic acid methyl ester (**10**),[28] 5-*O*-*trans*-caffeoylquinic acid methyl ester (**11**),[28] 5-*O*-*cis*-caffeoylquinic acid methyl ester (**12**),[29] 4-*O*-*trans*-coumaroylquinic acid methyl ester (**13**),[30] 5-*O*-*trans*-coumaroylquinic acid methyl ester (**14**),[31] and 5-*O*-*cis*-coumaroylquinic acid methyl ester (**15**)[26] by comparison of their spectroscopic data with those reported in the literature.

Lipopolysaccharide (LPS) is an important activator of microglia. Overactivated microglia cells secrete excessive inflammatory and cytotoxic mediators which are deleterious to neuronal cells. Among those factors, NO is a primary released factor in activated microglia. The IC_{50} values of **2**, **3**, **8** and **11** were 4.66, 12.52, 9.04, and 12.11 μM, respectively. L-NMMA, a NOS inhibitor (a positive control), showed a low IC_{50} value of 16.32 μM (Table 3). Compounds **2**, **3**, **8** and **11** were more active than the L-NMMA. In particular, compounds **2** and **8** exhibited very strong anti-neuroinflammatory properties. Moreover, both compounds **2** and **8** did not affect cell viability at a concentration of 20 μM. To confirm effects of these compounds on NO production in different cell line, NO levels in the medium of murine macrophage RAW264.7 activated by LPS were also measured. The IC_{50} values of **2** and **8** were 174.89 and 42.87 μM. And both compounds had no influence on cell viability at a concentration of 20 μM in RAW264.7 cells (data not shown). These data confirmed that the inhibitory effects of compounds **2** and **8** on NO production were more potent in BV2 than RAW264.7.

In microglia, NO production is primarily regulated by the inducible nitric oxide synthase (iNOS).[32] Based on western blot analysis and iNOS activity assay, we determined whether the NO inhibitory effects of compounds **2** and **8** are related to the regulation of the protein expression and/or enzymatic activity of iNOS. As shown in Figure 3A, pretreatment with 10 μM of compound **2** led to a decrease in iNOS protein level. However, compound **8** did not showed an inhibitory effect on LPS-induced iNOS expression in concentration range of 1–10 μM. As shown in Figure 3B, both compounds **2** and **8** reduced iNOS activity in LPS-pretreated BV-2. One and 10 μM of compound **2**, and 1 and 5 μM of compound **8** inhibited iNOS activity, significantly. To investigate the precise mechanisms of compounds **2** and **8** on NO regulation, NO radical scavenging assay was also performed. As shown in Figure 3C, 5 and 10 μM of compound **2** reduced the accumulation of nitrite upon decomposition of NO donor, sodium nitroprusside (SNP). Moreover, the effect of compound **8** is similar to that of compound **2** in 1–10 μM. These results indicate that quinic acid derivatives isolated from *P. brachycarpa*

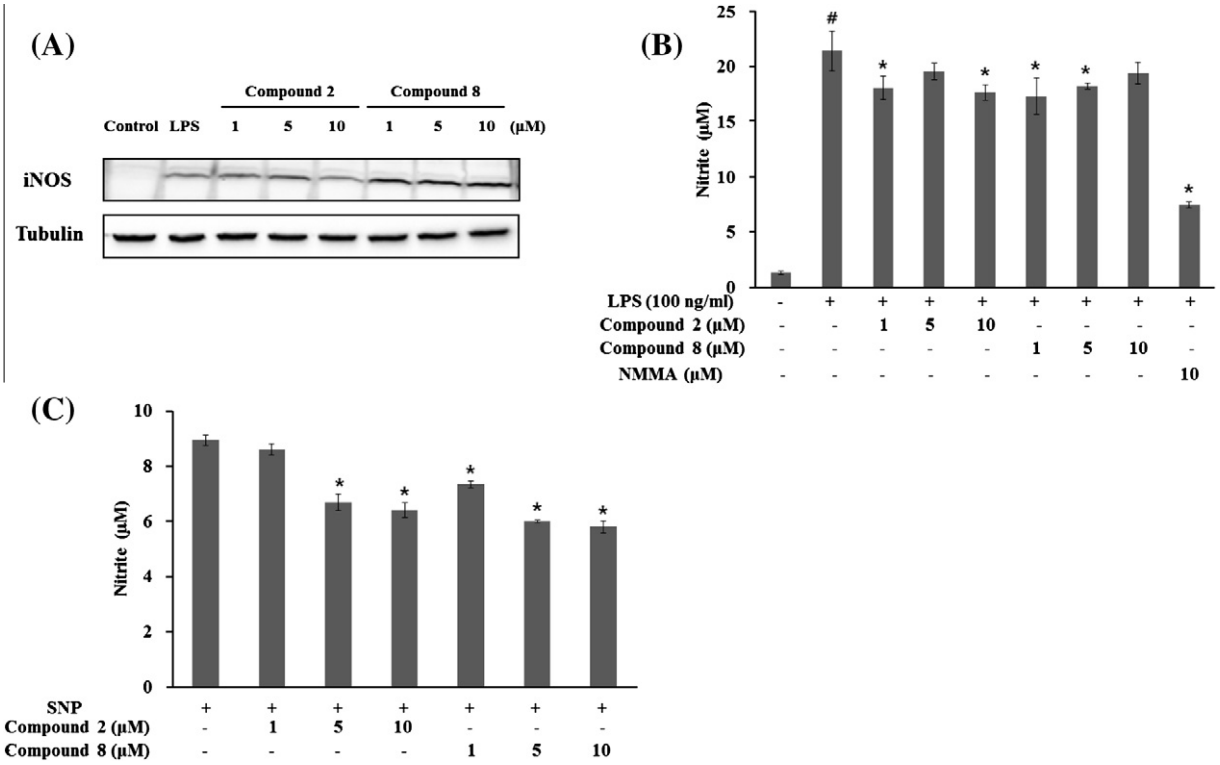

Figure 3. Effects of compounds **2** and **8** on NO regulation. (A) Effects of compounds **2** and **8** on LPS-induced iNOS expression in BV-2 cells. (B) Effects of compounds **2** and **8** on iNOS activity in BV-2 cells. (C) Effects of compounds **2** and **8** on NO scavenging. Sodium nitroprusside (SNP) is a NO generator. $^{\#}p$ <0.05 indicates statistically significant difference between the control and LPS alone-treated groups. $^{*}p$ <0.05 indicate statistically significant differences compared to treatment with LPS alone.

exert attenuate LPS-induced pro-inflammatory responses in microglial BV2 cells through down regulation of NO. NO regulation of compounds **2** and **8** might be attributed to the inhibition of iNOS expression, enzymatic activity, and NO scavenging property in all.

In Korea, *P. brachycarpa*, called as cham-na-mul, is well known as a vegetable and a popular edible herb. In the course of our continuing search for biologically active constituents from Korean medicinal plants, we investigated the MeOH extract from the aerial parts of *P. brachycarpa* and isolated five new quinic acid derivatives (**1**–**5**), together with 10 known quinic acid derivatives (**6**–**15**). The chemical structures of the new compounds (**1**–**5**) were determined by the mean of ^{1}H, ^{13}C NMR, COSY, HMQC, HMBC and HRMS. Compounds **2** and **8** isolated from *P. brachycarpa* may have neuroprotective properties by inhibiting inflammatory activation of microglia. This study supports that *P. brachycarpa,* a well known vegetable, may be an invaluable functional food source for the regulation of neurodegenerative diseases related with neuroinflammation.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.01.115.

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