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Anti-Oxidative and Nitric Oxide Production Inhibitory Activities of Phenolic Compounds from the Fruits of *Actinidia arguta*

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Abstract – Phytochemical examination of the fruits of *Actinidia arguta* has led to the isolation three organic acids, one coumarin, and three flavonoids. Structures of these compounds were elucidated as protocatechuic acid (**1**), caffeic acid (**2**), caffeoyl-β-D-glucopyranoside (**3**), esculetin (**4**), quercetin (**5**), quercetin 3-*O*-β-D-galactopyranoside (**6**), and quercetin 3-*O*-α-L-rhamnopyranosyl (1 → 6)-*O*-β-D-glucopyranoside (**7**) by comparisons with previously reported spectral data. To investigate the anti-inflammatory and anti-oxidative effects of these compounds, nitric oxide production inhibitory activity in LPS-stimulated RAW 264.7 cells and DPPH radical scavenging activities were examined. Nitric oxide productions were reduced significantly by the addition of compounds [**1** (IC_{50} = 59.27 μg/ml), **2** (IC_{50} = 27.95 μg/ml), **3** (IC_{50} = 73.09 μg/ml), **4** (IC_{50} = 67.44 μg/ml), **5** (IC_{50} = 17.40 μg/ml), **6** (IC_{50} = 41.99 μg/ml), **7** (IC_{50} = 54.46 μg/ml)], and extracts (IC_{50} = 56.21 μg/ml) compared with positive control, L-NMMA (IC_{50} = 14.48 μg/ml). The phenolic compounds also showed anti-oxidative activities. Especially, Compounds **1** (IC_{50} = 8.87 μg/ml), **4** (IC_{50} = 3.41 μg/ml), and **5** (IC_{50} = 6.06 μg/ml) showed potent anti-oxidative activities similar to L-ascorbic acid (IC_{50} = 5.89 μg/ml).
Keywords – *Actinidia arguta*, organic acid, coumarin, flavonoid, DPPH, nitric oxide

Introduction

The fruits of *Actinidia arguta* have been used in the Korean folk medicine for the treatment of diuresis, fever, icterus, dyspepsia and disease symptomized by thirst (Kim and Xiao, 1995). From the fruits of *A. arguta,* several flavonoids were isolated (Webby and Markham, 1990; Webby, 1991). Anti-infective and anti-tumor effect of the barks (Hou *et al.*, 1995), anti-allergy effect of water-soluble extracts of *Actinidia* species (Park *et al.*, 2004) and phenolic compounds from barks of *Actinidia arguta* Planchon growing in Korea and its anti-oxidative and nitric oxide production inhibitory activities (Lim *et al.*, 2005) were reported.

Nitric oxide (NO) is generated in different cell types by at least three isoforms of NO synthase (NOS). Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed and their enzymatic activity is Ca^{2+}/calmodulin-dependent. The third enzyme is an inducible and Ca^{2+}-independent isoform of NOS (iNOS), virtually expressed in all cell types after stimulation with LPS and/or with different cytokines, such as interferon-γ (IFNγ), interleukin-1β (IL-1β), or tumour necrosis factor-α (TNF-α). Massive amounts of NO produced by iNOS under pathological conditions (e.g., inflammatory diseases) are potentially harmful, especially when time-spatial regulation of iNOS expression becomes compromised. During inflammation associated with different pathogens, NO production increases significantly (Kharitonov *et al.*, 1994) and may become cytotoxic. Moreover, the free radical nature of NO and its high reactivity with oxygen to produce peroxynitrite ($ONOO^-$) makes NO a potent pro-oxidant molecule able to induce oxidative damage, and to be potentially harmful towards cellular targets (Epe *et al.*, 1996). Thus inhibition of NO production in response to inflammatory stimuli might be a useful therapeutic strategy in inflammatory diseases (Hobbs *et al.*, 1999; Sautebin *et al.*, 2000).

Free radicals and other reactive oxygen species are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body. The radicals may cause oxidative damage by oxidizing biomolecules and results in cell death and tissue damage (Kehrer, 1993). Atherosclerosis, cancer, emphysema, cirrhosis, and arthritis have been correlated with oxidative damage (Kehrer, 1993; Jacob, 1994). Therefore, oxidative

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damage plays a significant pathological role in human disease. However, ingestion of anti-oxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human body (Lin and Yen, 1999).

As continuous studies on anti-oxidative and anti-inflammatory agents from natural sources (Lim *et al.*, 2005), we tried separation of the main compounds from the fruits of *Actinidia arguta* and investigated the free radical scavenging and nitric oxide (NO) production inhibitory activities on the isolated compounds.

Experimental

General experimental procedures – ^{1}H-(500 MHz) and ^{13}C-(125 MHz) NMR spectra were obtained on a Varian Unity INOVA 500 spectrometer (Varian, Inc., U.S.A.). Chemical shifts were expressed in parts per million (ppm) relative to TMS as an internal standard, and coupling constants (*J*) were given in Hz. MS were obtained on a Varian Saturn 4D mass spectrometer (Varian, Inc., U.S.A.) and JEOL JMS HX-110/110A tandem mass spectrometer (JEOL Ltd., Japan). HPLC was performed on a Waters LC 600E pump using a Waters PorasilTM column (5 μm, 300 × 7.8 mm i.d.) and Supelco Silica column (3 μm, 150 × 4.6 mm i.d.). TLC was carried out on Merck silica gel F_{254}-precoated glass plates and RP-18 F_{254s} plates.

Reagents - 1,1-diphenyl-2-picrylhydrzyl (DPPH), L-NMMA, L-ascorbic acid, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide], RPMI 1640 medium, Lipopholysaccharide (LPS), Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution) and DMSO were obtained from Sigma (St. Louis, MO, USA). Penicillin (100 units/ml)-streptomycin (100 μg/ml) and 10% fetal bovine serum (FBS) were obtained from Gibco Ltd. (USA). Liquid nitrogen and CO_2 gas were obtained from Dong-A Co. (Korea), while first grade dichloromethane, methanol, diethyl ether, ethyl acetate and n-butanol were obtained from Sigma (St. Louis, MO, USA).

Plant material – The fruits of *Actinidia arguta* were purchased from WanSan-Dong, YungChun-City, KyungBuk, South Korea in June of 2004. A voucher specimen has been deposited at the herbarium, College of Pharmacy, Chung-Ang University.

Extraction and isolation – The fruits of *Actinidia arguta* (2.5 kg) were extracted with 80% aqueous acetone (3 × 10 L) for 3 days. After removal of Me_2CO in vacuo, the aqueous solution was filtered. The filtrate was concentrated and then applied to a column of Sephadex LH-20 (450 g, 10 × 70 cm). Elution with H_2O containing increasing proportion of MeOH afforded 9 fractions, I (300 g), II (50 g), III (4 g), IV (5 g), V (6 g), VI (14 g), VII (15 g), VIII (3 g) and IX (2 g).

Repeated column chromatography of fraction V on MCI gel with a H_2O : MeOH gradient, followed by Sephadex LH-20 column chromatography with 20% H_2O : MeOH gradient yielded **1** (150 mg). After the residue on YMC ODS gel (400/230 mesh) low pressure liquid column chromatography (20% H_2O : 80% MeOH, gradient system) and Sephadex LH-20 column chromatography (60% MeOH) yielded **2** (95 mg). Column chromatography of fraction VI over MIC gel with H_2O : MeOH gradient and YMC ODS gel with 60% MeOH yielded two subfraction (VI-I and VI-II). Repeated column chromatography of fr. VI-I on YMC ODS gel (400/230 mesh) low pressure liquid column (20% H_2O : 80% MeOH, gradient system) and Sephadex LH-20 column chromatography (60% MeOH) yielded **7** (50 mg). Column chromatography of fr. VI-II on YMC ODS gel (400/230 mesh) low pressure liquid column (40% H_2O : MeOH, gradient system) and Sephadex LH-20 column (60% MeOH) yielded **4** (80 mg). Column chromatography of fraction VII over YMC ODS gel with 20% H_2O : MeOH gradient and Sephadex LH-20 column (40% H_2O : MeOH, gradient system) yielded two subfraction (VII-I and VII-II). Repeated column chromatography of fr. VII-I on Sephadex LH-20 column (60% MeOH) yielded **6** (250 mg). Column chromatography of fr. VII-II on YMC ODS gel (400/230 mesh) low pressure liquid column (40% H_2O : MeOH, gradient system) yielded **3** (80 mg). Column chromatography of fraction IX over Sephadex LH-20 column (40% H_2O : MeOH, gradient system) yielded **5** (75 mg).

Protocatechuic acid (**1**) – white amorpous powder, IR ν_{max} cm^{-1}: 3322 (OH), 1617, 1523 aromatic (C = C), Negative-FAB MS *m/z* : 153 ([M - H]$^-$), ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.31 (1H, d, *J* = 2.1 Hz, H-2), 7.31 (1H, dd, *J* = 8.1, 1.8 Hz, H-6), 6.80 (1H, d, *J* = 8.1 Hz, H-5). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 115.3 (C-5), 116.7 (C-2), 121.9 (C-1), 122.0 (C-6), 145.1 (C-3), 150.2 (C-4) 167.6 (COOH).

Caffeic acid (**2**) – white amorpous powder, IR ν_{max} cm^{-1}: 3438 (OH), 1648, 1524 aromatic (C = C), Negative-FAB MS *m/z* : 179 ([M - H]$^-$), ^{1}H-NMR (300 MHz, DMSO-d_6 + D_2O): δ 7.43 (1H, d, *J* = 15.9 Hz, H-7), 7.04 (1H, d, *J* = 1.8 Hz, H-2), 6.97 (1H, dd, *J* = 8.1, 2.1 Hz, H-6), 6.77 (1H, d, *J* = 8.1 Hz, H-5), 6.15 (1H, d, *J* = 15.9 Hz, H-8). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 114.8 (C-2), 115.2 (C-8), 115.9 (C-5), 121.3 (C-6), 125.8 (C-1), 144.8 (C-7), 145.7 (C-3), 148.3 (C-4), 168.1 (COOH).

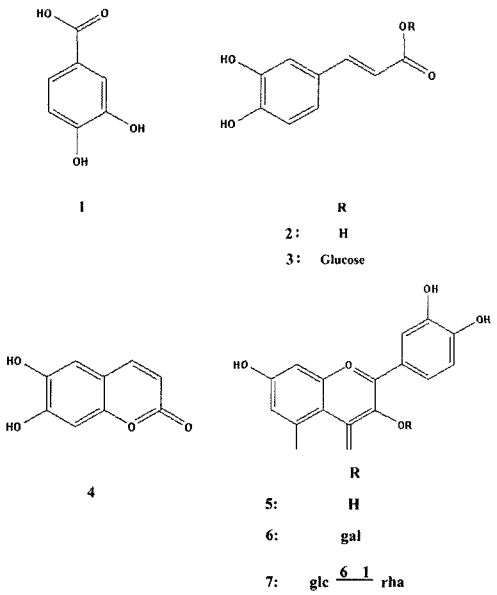

Chart. 1. Stuctures of the compounds **1-7**.

Caffeoyl-β-D-glucopyranoside (**3**) – white amorpous powder, IR ν_{max} cm^{-1}: 3361 (OH), 1687, 1529 aromatic (C = C), Negative-FAB MS *m/z* : 341 ($[M - H]^-$), ^{1}H-NMR (300 MHz, DMSO-d_6): δ 7.48 (1H, d, *J* = 15.9 Hz, H-7), 7.23 (1H, d, *J* = 2.1 Hz, H-2), 7.20 (1H, dd, *J* = 8.1, 2.1 Hz, H-6), 6.84 (1H, d, *J* = 8.1 Hz, H-5), 6.32 (1H, d, *J* = 15.9 Hz, H-8), 4.81 (1H, d, *J* = 7.2 Hz, glc H-1). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 60.9 (glc-6), 70.0 (glc-4), 73.4 (glc-2), 76.0 (glc-5), 77.3 (glc-3), 101.9 (glc-1), 115.8 (C-2), 116.1 (C-8), 116.2 (C-5), 124.3 (C-6), 126.1 (C-1), 144.5 (C-7), 145.7 (C-3), 149.2 (C-4), 168.2 (COOH).

Esculetin (**4**) – yellow amorpous powder, IR ν_{max} cm^{-1}: 3302 (OH), 16717, 1526 aromatic (C = C), Negative-FAB MS *m/z* : 177 ($[M - H]^-$), ^{1}H-NMR (500 MHz, DMSO-d_6 + D_2O): δ 7.88 (1H, d, *J* = 9.5 Hz, H-4), 6.97 (1H, s, H-8), 6.74 (1H, s, H-5), 6.10 (1H, d, *J* = 9.5 Hz, H-3). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 103.0 (C-8), 111.1 (C-3), 111.8 (C-10), 112.7 (C-5), 143.2 (C-6), 144.8 (C-4), 148.8 (C-9), 150.7 (C-7), 161.3 (C-2).

Quercetin (**5**) – yellow amorpous powder, IR ν_{max} cm^{-1}: 3407 (OH), 1660, 1562 aromatic (C = C), Negative-FAB MS *m/z* : 301 ($[M - H]^-$), ^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.51 (1H, s, 5-OH), 7.70 (1H, d, *J* = 1.8 Hz, H-2'), 7.60 (1H, dd, *J* = 8.1, 1.8 Hz, H-6'), 6.91 (1H, d, *J* = 8.1 Hz, H-5'), 6.42 (1H, d, *J* = 2.1 Hz, H-8), 6.22 (1H, d, *J* = 2.1 Hz, H-6). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 93.5 (C-8), 98.3 (C-6), 103.1 (C-10), 115.2 (C-2'), 115.8 (C-5'), 120.1 (C-6'), 122.1 (C-1'), 135.9 (C-3), 145.2 (C-3'), 147.0 (C-2), 147.9 (C-4'), 156.3 (C-9), 160.9 (C-5), 164.1 (C-7), 176.1 (C-4).

Quercetin-3-*O*-β-D-galactopyranoside (**6**) – yellow amorpous powder, IR ν_{max} cm^{-1}: 3399 (OH), 1667, 1543 aromatic (C = C), Negative-FAB MS *m/z* : 463 ($[M - H]^-$), ^{1}H-NMR (300 MHz, DMSO-d_6): δ 12.65 (1H, s, 5-OH), 7.69 (1H, dd, *J* = 7.8, 2.1 Hz, H-6'), 7.55 (1H, d, *J* = 2.1 Hz, H-2'), 6.83 (1H, d, *J* = 8.7 Hz, H-5'), 6.42 (1H, d, *J* = 2.1 Hz, H-8), 6.22 (1H, d, *J* = 2.1 Hz, H-6), 5.50 (1H, d, *J* = 7.8 Hz, gal H-1). ^{13}C-NMR (75 MHz, DMSO-d_6): δ 60.2 (gal-6), 68.0 (gal-4), 71.2 (gal-2), 73.2 (gal-3), 75.9 (gal-5), 93.6 (C-8), 98.8 (C-6), 101.8 (C-1), 104.0 (C-10), 115.3 (C-2'), 116.0 (C-5'), 121.2 (C-1'), 122.2 (C-6'), 133.6 (C-3), 145.0 (C-3'), 148.6 (C-4'), 156.4 (C-9), 156.5 (C-2), 161.4 (C-5), 164.3 (C-7), 177.7 (C-4).

Quercetin 3-*O*-α-L-rhamnopyranosyl(1 → 6)-*O*-β-D-glucopyranoside (**7**) – yellow amorpous powder, IR ν_{max} cm^{-1}: 3421 (OH), 1658, 1554 aromatic (C = C), Negative-FAB MS *m/z* : 609, 301 ($[M - H]^-$), ^{1}H-NMR (300 MHz, DMSO-d_6 + D_2O): δ 7.56 (1H, dd, *J* = 8.3, 1.8 Hz, H-6'), 7.53 (1H, d, *J* = 1.8 Hz, H-2'), 6.84 (1H, d, *J* = 8.3 Hz, H-5'), 6.37 (1H, d, *J* = 1.8 Hz, H-8), 6.18 (1H, d, *J* = 1.8 Hz, H-6), 5.34 (1H, d, *J* = 7.2 Hz, glc H-1), 4.39 (1H, s, rha H-1), 0.99 (3H, s, rha-CH_3). ^{13}C-NMR (125 MHz, DMSO-d_6): δ 18.0 (rha-6), 67.4 (glc-6), 68.6 (rha-5), 70.3 (glc-4), 70.6 (rha-2), 70.8 (rha-3), 72.2 (rha-4), 74.3 (glc-2), 76.1 (glc-5), 76.6 (glc-3), 94.2 (C-8), 99.1 (C-6), 101.1 (rha-1), 101.5 (glc-1), 104.3 (C-10), 115.6 (C-2'), 116.6 (C-5'), 121.5 (C-1'), 122.1 (C-6'), 133.7 (C-3), 144.9 (C-3'), 148.6 (C-4'), 156.6 (C-9), 157.1 (C-2), 161.2 (C-5), 164.5 (C-7), 177.7 (C-4).

Biological Assay

Cell culture – Raw 264.7 cells were purchased from the Korean Cell Line Bank. The cells were grown at 37°C in a humidified atmosphere (5% CO_2) in a RPMI 1640 medium containing 10% fetal bovine serum, penicillin G (100 IU/ml) and streptomycin (100 mg/ml) (Mosmann, 1983).

MTT assay – The cytotoxicity was measured by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] to formazan (Mosmann, 1983). The cells were seeded at a density 1 × 10^6 cells/ml in 96 well-plates. After incubating for 2 h, the cells were treated with the samples (12.5, 25. 50 and 100 μg/ml). The cells were incubated for an additional 24 h, and the medium was replaced with fresh medium. The medium contained MTT (final concentration : 0.5 mg/ml), and the incubation continued for a further 1 h at 37 °C The medium was then removed and the MTT-formazan produced was dissolved in 200 μl DMSO. The

extent of the reduction of MTT to formazan within the cells was quantified by measuring the absorbance at 540 nm using an ELISA reader (TECAN, Salzburg, Austria) (Mosmann, 1983; Feelisch and Stamler, 1996; Park *et al.*, 2005).

Nitrite assay – Raw 264.7 macrophage cells were cultured in a 96-well plate and preincubated for 2 h at 37 °C in a humidified atmosphere (5% CO_2). The cells were then incubated in a medium containing LPS (1 μg/ml) and the test samples (12.5, 25. 50 and 100 μg/ml). After incubating for an additional 24 h, the media were removed and analyzed for the level of nitrite accumulation, as an indicator of NO, using the supernatant by a Griess assay. The Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H_3PO_4 solution, 100 μl) was added to 100 μl of each of the supernatants from the cells treated with the samples (12.5, 25. 50 and 100 μg/ml). L-NMMA were used as positive control. The samples were then read at 540 nm against a standard sodium nitrite curve (Feelisch and stamler, 1996; Park *et al.*, 2005). The amount of nitrite in the samples was calculated from a sodium nitrite standard curve.

DPPH radical scavenging activity – The antioxidant activity was determined on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrzyl (DPPH) free radical by a previously described method with a slight modification (Hatano *et al.*, 1989). 20 μl of the compound in EtOH was added to 180 μl of a DPPH solution (7.887 mg DPPH in 200 ml EtOH). After mixing gently and standing at for 30 min, the optical density was measured at 492nm using an ELISA reader (TECAN, Salzburg, Austria). The free radical scavenging activity was expressed as follow:

DPPH scavenging activity (%) = {(Ac-As)/(Ac-Ab)} × 100

Where Ac was the absorbance of the control, As was the absorbance of the sample and Ab was the absorbance of the blank. The IC_{50} values were defined as the concentration that could scavenge 50% DPPH free radical. L-ascorbic acid were used as positive control.

Results and Discussion

Chromatographic isolation of 80% aqueous Me_2CO extract of the fruits of *Actinidia arguta* afforded seven phenolic compounds. Structures of compounds **1-7** were identified as procatechuic acid (**1**, Sroka and Cisowski, 2003), caffeic acid (**2**, Kim *et al.*, 2005), caffeoyl-β-D-glucopyranoside (**3**, Berregi *et al.*, 2003; Kim *et al.*, 2005), esculetin (**4**, Liu *et al.*, 2005), quercetin (**5**, Harborne and Mabry, 1982; Agrawal, 1989; Lee *et al.*, 2004), Quercetin-3-*O*-β-D-galactopyranoside (**6**, Harborne and Mabry, 1982; Agrawal, 1989; Lim *et al.*, 2005) and quercetin 3-*O*-α-L-rhamnopyranosyl(1 → 6)-*O*-β-D-glucopyranoside (**7**, Harborne and Mabry, 1982; Agrawal, 1989; Kim *et al.*, 2004), respectively, by comparing the spectral (IR, MS, NMR) data with the values reported in the literatures. Compounds **1**, **2**, **3**, **4**, **6** and **7** were isolated first from the fruits of *A. arguta*.

Nitric oxide production inhibitory activities in LPS-stimulated RAW 264.7 cells and DPPH radical scavenging activities were examined on the isolated compounds. The NO levels were moderately reduced as a result of the addition of the compounds **1-7** and extract to the RAW 264.7 cells stimulated by LPS. All compounds and extract were found to inhibit NO production in a dose dependent manner with an IC_{50} of **1** (IC_{50} = 59.27 μg/ml), **2** (IC_{50} = 27.95 μg/ml), **3** (IC_{50} = 73.09 μg/ml), **4** (IC_{50} = 67.44 μg/ml), **5** (IC_{50} = 17.40 μg/ml), **6** (IC_{50} = 41.99 μg/ml), **7** (IC_{50} = 54.46 μg/ml) and extracts (IC_{50} = 56.21 μg/ml), respectively compared with positive control, L-NMMA with an IC_{50} value of 14.48 μg/ml (Table 1). The MTT assay showed that all compounds **1-7** and extract did not cause cell cytotoxicity in the treatment ranges of the compounds, 0 –100 μg/ml (Fig. 1).

The anti-oxidative activities were tested on the isolated compounds **1-7** and extract by DPPH free radical scavenging method. Among them, **1**, **4** and **5** exhibited a potent free radical scavenging effect with an IC_{50} value of 8.87 μg/ml, 3.41 μg/ml and 6.06 μg/ml, respectively, compared with positive control, L-ascorbic acid (IC_{50} = 5.89 μg/ml). Compounds **2** (IC_{50} = 11.02 μg/ml), **3** (IC_{50} = 33.46 μg/ml), **6** IC_{50} = 10.77 μg/ml), **7** (IC_{50} = 11.18 μg/ml) and extract (IC_{50} = 90.10 μg/ml) also showed moderate DPPH radical scavenging effects (Table 1).

Table 1. Effects of the compounds **1-7** on DPPH radical scavenging and LPS-induced NO production inhibition in the Raw 264.7 macrophages

compounds	DPPH	NO
	IC_{50} (μl/ml)	IC_{50} (μl/ml)
1	**8.87**	59.27
2	11.02	**27.95**
3	33.46	73.09
4	**3.41**	67.44
5	**6.06**	**17.40**
6	10.77	41.99
7	11.18	54.46
extract	90.10	56.21
L-ascorbic acid	**5.89**	–
L-NMMA	–	**14.84**

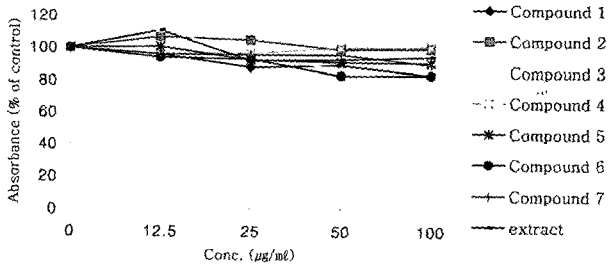

Fig. 1. Effect of Compound **1-7** and extract on the viability of RAW 264.7 cells. The viability of the cells was measured by MTT assay. Results were expressed as % of control absorbance.

The structural activity relationship among these phenolic compounds, phenolic acids (**1**, **2** and **3**), coumarin (**4**), flavonoids (**5**, **6**, and **7**) showed that the presence of the ortho-dihydroxyl in the aromatic ring, a number of hydroxyl groups and combination of glycoside were important for the inhibitory activities on NO production (Kim *et al.*, 2004), and DPPH radical scavenging activity (Hou *et al.*, 2004).

These results suggest that the phenolic compounds which were isolated from the fruits of *A. arguta* might be used as a potent anti-inflammatory and anti-oxidative agents.

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