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Bioactivity-guided isolation of anti-inflammatory constituents from *Angelica decursiva*

by

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The Graduate School

Pukyong National University

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(자화전호의 활성물질들의 생리활성)

Advisor: Prof. Jae Sue Choi

by

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List of Abbreviations and Symbols

A. decursiva	: *Angelica decursiva*
^{1}H NMR	: Proton nuclear magnetic resonance
^{13}C NMR	: ^{13}Carbon nuclear magnetic resonance
HMBC	: Heteronuclear multiple bond correlation
HMQC	: Heteronuclear multiple quantum coherence
EI-MS	: Electron impact mass spectrometry
TLC	: Thin layer chromatography
IC_{50}	: 50% inhibitory concentration of the test samples
mp	: Melting point
UV	: Ultraviolet
Hz	: Hertz (sec^{-1})
RP	: Reversed phase
DMSO	: Dimethyl sulfoxide
DMSO-d_6	: Deuterated dimethyl sulfoxide
$CDCl_3$	: Deuterated chloroform
J	: Coupling constant (Hz)
δ	: Chemical shift
IC_{50}	: 50% inhibitory concentration

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Abstract

Angelica, a circumboreal genus comprises of more than 60 different species, is by far the largest genus of Apeaceae in the Korean flora, and many species of this genus have long been used in traditional medicine for the treatment of many diseases. Because of the high therapeutic value of the traditionally used *Angelica* species, extensive research has been carried out on different species of this genus which reported wide range of bioactivities including anti-inflammatory, anti-oxidant, anti-diabetic, anticancer, neuroprotective effects. Among them, *Angelica decursiva* has been long used in Korean traditional medicine as an antitussive, analgesic, antipyretic, and cough remedy. In a recent study, the potential antioxidant and anti-inflammatory activities of methanol (MeOH) extract of *A. decursiva* and its different solvent soluble fractions have been reported. Among the fractions, the ethyl acetate (EtOAc) fraction showed strong antioxidant and anti-inflammatory activities, while the dichloromethane (CH_2Cl_2) fraction-derived 90% MeOH soluble fraction showed

the most promising anti-inflammatory activity. Although the 90% MeOH soluble fraction was found as the most active anti-inflammatory fraction, compounds responsible for anti-inflammatory activity were not identified yet. Therefore, the present study was designed to isolate active components from 90% MeOH soluble fraction and to evaluate their anti-inflammatory activity *via* inhibition of nitric oxide (NO) production, tumor necrosis factor-α (TNF-α) production as well as iNOS and COX-2 expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Repeated column chromatography of the 90% MeOH soluble fraction yielded 9 coumarins, including edulisin II (**1**) decursidin (**2**), Pd-C-III (**3**), 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (**4**),Pd-C-I (**5**), Pd-C-II (**6**), (+)-*trans*-decursidinol (**7**), umbelliferone (**8**), and nodakenetin (**9**). Among them, compound **1** was isolated for the first time from *A. decursiva*, while compound **4** was isolated as a new compound from natural sources. Since nodakenetin and umbelliferone showed poor NO production inhibitory activity in previous study, we have selected compounds **1** ~ **7** in the present study. Among the tested compounds, compound **1** exhibited the highest NO production inhibitory activity in a dose-dependent manner in LPS-induced NO production in RAW 264.7 cells with an IC_{50} value of 2.50 ± 0.16 μM. Compound **2** also showed very strong NO production inhibitory effect in LPS-stimulated RAW 264.7 cells with an IC_{50} value of 4.08 ± 0.09 μM, which is nearly similar to that of compound 1. In addition, compounds **3** ~ **5** showed moderate NO production inhibitory activity compared to

compounds **1** and **2** with IC_{50} values of 15.60 ± 0.80, 26.00 ± 1.00, and 31.83 ± 0.36 μM, respectively. On the other hand, compound **6** showed weaker NO production inhibitory activity compared to compounds **1** and **2** with an IC_{50} value of 62.70 ± 1.86 μM. In contrast, compound **7** did not exhibit any suppressive effect on LPS-stimulated RAW 264.7 cells at the concentrations tested. Therefore, considering the NO production inhibitory activity of tested coumarins (compounds **1** ~ **7**), it can be speculated that esterification of –OH at 3' or 4' position of compound **7** with senecioyl or angeloyl or acetyl group is essential for exhibiting NO production inhibitory activity by these coumarins derivatives, and the position and number of senecioyl/angeoloyl/acetyl group on these coumarins largely affects their potency. All six active coumarins (compounds **1** ~ **6**) also inhibited TNF-α production and iNOS protein expression, while compounds **1** ~ **4** inhibited COX-2 protein expression in LPS-stimulated RAW 264.7 cells. These results suggest that coumarins isolated from *A. decursiva* might be used as potential leads for the development of therapeutic remedies for inflammation associated disorders. However, further research is needed to clarify the mechanisms of action behind this anti-inflammatory activity.

I. Introduction

The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs (De Pasquale, 1984). The reasons for this were that pure compounds were easily obtained, structural modifications to produce potentially more active and safer drugs could be easily performed and the economic power of the pharmaceutical companies was increasing. Furthermore, throughout the development of human culture, the use of natural products has had magical-religious significance and different points of view regarding the concepts of health and disease existed within each culture. Despite tremendous advances in modern medicine, plants continue to make important contributions to health care as witnessed by the increasing interest in alternative therapies (Rates, 2001). There have been numerous *in vitro* and *in vivo* studies on herbal medicines which explore the biological mechanisms at which have established the benefits of herbs. Therefore, it suggests that traditional medicine could provide potential source of new drugs (Efferth et al., 2007). Traditional medicine has been used for the treatment of diseases as well as to maintain sound health for

long time and it is still popular in Asian countries, including Korea, China and Japan.

The genus *Angelica* L. belongs to the family Umbelliferae, commonly known as parsley family, and comprises more than 60 species of medicinally important biennial or perennial herbs (Sarkar et al., 2004; GRIN database, 2003). Plants of this genus are distributed widely in Asia, Europe and North America. They grow abundantly in fens, river-banks, damp meadows and woods. *Angelica* species can grow 5 to 8 feet tall, needs rich, moist, well-drained soil in partial shade.

Angelica atropurpurea (American angelica) is indigenous to America and grows wild from Labrador west to Mennesota, south to Maryland, Indiana and Iowa. Other American *Angelica* species, *A. breweri, A. hendersonii*, *A. tomentosa*, etc. grow widely in the Western and Pacific coastal areas (Dobelis et al., 1986). The European version of *Angelica* is *A. archangelica* (Wild parsnip) and the British species is *A. sylvestris* (Wild Angelica). *A. archangelica*, native to Austria, Belgium, Denmark, Germany, Greenland, Hungary, Iceland, Poland and central Russia, has long been naturalized in the UK and other parts of Europe. *A. sylvestris* iscommon throughout the UK and *A. sinensis*, commonly known as ‘Dong quai’, is a

fragrant perennial herb endemic to China and also found widely in Korea and Japan (GIRIN database, 2003; Dobelis et al., 1986; Murray, 1995). The *Angelica* species that originated from Korea include *A. gigas* and *A. koreana*. *A. gigas* is an exquisite ornamental plant, which was introduced in the USA a couple of decades ago. While *A. japonica* (Japanese angelica) is from Japan, *A. glauca* is native to India.

European folkloric reputation of the genus *Angelica* lies in the mythological belief that in the middle ages when Europe was almost destroyed by the plague, apparently an angel came to a monk and offered a herb, which would effect a cure and since then this herb has borne the name *Angelica*. Reports on the traditional medicinal uses of *Angelica* species can be found in various ancient literatures, and the folklore of all North European countries depicts a common belief in their merits as a protection against contagion, for purifying the blood, and for curing every conceivable ailments. Many species of this genus have traditionally been used a s anti-inflammatory, diuretic, expectorant and diaphoretic, and remedy for colds, flu, influenza, hepatitis, arthritis, indigestion, coughs, chronic bronchitis, pleurisy, typhoid, headaches, wind, fever, colic, travel sickness, rheumatism, bacterial and fungal infections and diseases of the urinary organs. In

addition to the medicinal preparations of *Angelica*, fairly large quantities of *Angelica* are used in the confectionery and liquor industries. As a result, the genus *Angelica* has now emerged as one of the commercially important genera. For example, the stems and seeds of *A. archangelica* are used as flavoring in confectionery and liqueurs, and the dried leaves, on account of their aromatic qualities, are used in the preparation of hop bitters.

Species of the genus *Angelica* are not the herbs of the 'past'. Many species of *Angelica* have still been used regularly by the traditional medicine practitioners, especially in the far-east. It is known that the genus *Angelica* still plays an important role in the Chinese traditional medicine systems. In the recent years, with the revival of popularity and renewed interests in traditional herbal remedies, many of these *Angelica*-containing medicine preparations are now commercially available over-the-counter and used regularly not only in China but also in the western countries like France, Germany, UK and USA. *Angelica* has recently become a very popular herb in the United States, and is often recommended by herbalists as a treatment for flatulence and stomach pains, and as a stimulant to invigorate circulation and warm the body. Because of the diverse medicinal properties, several species from this genus have been incorporated and used

in various commercially successful medicine preparations all over the world. Nowadays, products of *Angelica* are available in various forms, either as crude plant parts, e.g. dried roots, stems, seeds etc, or as semi-purified preparations, e.g. crude extracts, essential oils, powder, etc. Modern formulations of *Angelica* include tablets, capsules, etc.

Current medicinal uses of *Angelica* species are not different from traditional uses. In fact, many of the traditional uses are still considered valid. Only difference is that the current *Angelica* products are available as more standardized preparations of higher quality, both in terms of activity and marker constituents, than the traditional preparations. Many of these products have now been recognized and evaluated, and included in various pharmacopoeias, e.g. Swiss, Austrian and German Pharmacopoeias.

The most talked about species of *Angelia*, especially in relation to their traditional medicinal uses and commercial values in modern alternative or herbal medicine, include *A. decursiva*, *A. acutiloba*, *A. archangelica*, *A. atropupurea*, *A. dahurica*, *A. glauca*, *A. gigas*, *A. japonica*, *A. keiskei*, *A. koreana*, *A. pubescens*, *A. sinensis*, and *A. sylvestris*.

A. sinensis ('Dang gui' in Chinese, 'Dang quai' in English, Toki in Japanese, Tanggwi in Korean, and Kinesisk Kvan in Danish) is a fragrant

and perennial herb found commonly in China, Japan, and Korea (Anyones, 2004). The plant typically grows to a height of approximately 2 m, and produces white flowers that bloom umbrella–like clusters in June and July. The dried root is valued for its therapeutic properties. Its flavor is a distinct blend of bitter, sweet, and pungent, and its overall effect is warming in nature (Wei et al., 2009). It is a blood tonic used in Chinese herbal prescriptions and it has been indicated for the treatment of abnormal or painful menstruation, uterine bleeding, premenstrual syndromes and cardiovascular diseases. In traditional Chinese medicine, *A. sinensis* is often referred to as the "female ginseng." Popular for regulating menstruation, 'Dong quai' is believed to promote blood flow to the reproductive organs, and thus to relieve menstrual cramps, regulate uterine contractions, and alleviate some symptoms related to menopause (Duke, 2002; Dobelis, 1986; Murray, 1995; Grieve, 1981). It has also been used to nourish brain, heart, and spleen with increased oxygenated blood supply, boost metabolism, reduce cholesterol, aid digestion, increase immunity, stabilize blood sugar and relieve pain caused by arthritis., and to treat anemia, boils, headache, venous problems and problems of peripheral blood flow. Due to the untold number of constituents, several pharmacological actions might be attributed

to *A. sinensis*. With the development of analysis methods, over 70 chemical components have been identified, including carbohydrates, essential oils, organic acids, phenolic compounds, vitamins, amino acids and other constituents (Chao and Lin, 2011; Wong et al., 2008). Chao et al. demonstrated that the EtOAc extract of *A. sinensis* showed anti-inflammatory activity by inhibiting NF-κB luciferase activity and TNF-α, IL-6, macrophage inflammatory protein-2 (MIP-2) and NO secretions from LPS/IFN-γ-stimulated RAW 264.7 cells, and the effect was attributed to the major compounds ferulic acid and z-lingustilide (Chao et al., 2010). Besides anti-inflammatory activity, several studies also reported wide range of pharmacological activities of *A. sinensis* including antioxidant, hematopoetic, anticancer, antiulcer, anti-diabetic, and neuroprotective (Zhang et al., 2010; Wong et al., 2008; Sun et al., 2009; Li et al., 2007; Liu et al., 2010; Chen et al., 2011).

A Korean traditional herbal medicine, Cham-Dang-Gui (Korean Angelica, the dried root of *A. gigas* Nakai), has been widely used in the treatment of dysmenorrhea, amenorrhea, menopausal syndromes, anemia, abdominal pain, injuries, migraine headaches and arthritis (Lee et al., 2003). It is also known that this herbal medicine ensures healthy pregnancies and

easy deliveries, and that coumarins such as decursin and decursinol angelate are the major constituents of this plant (Konoshima et al., 1968; Kang et al., 2003; Pachaly et al., 1996). Recently, it has been reported that decursinol and decursin exhibited significant neuroprotecitve activity against glutamate induced neurotoxicity in primary cultures of rat cortical cells and improved scopolamine-induced amnesia *in vivo* with another coumarin constituent, nodakenin (Kang et al., 2005; Kang et al., 2003; Kim et al., 2007). Anticancer activities of decursin have been also reported against human prostate carcinoma cells, human K562 erythroleukemia and U937 myeloleukemia cells (Jiang et al., 2005; Kim et al., 2005). Meanwhile, Dang-Gui, one of the most important traditional herbal medicines in Asia, is also marketed as a functional food product for women's health care in Europe and America. However, three different *Angelica* species are respectively recorded in Chinese, Korean and Japanese pharmacopoeia. Korean Angelica (the root of *A. gigas*) is quite distinct in having deep purple flowers while Chinese Angelica (the root of *A. sinensis* (Oliv.) Diels) and Japanese Angelica (the root of *A. acutiloba* Kitagawa or *A. acutiloba* Kitagawa var. *sugiyama* Hikino) have white ones. Chinese Angelica and Japanese Angelica are sold in Korean markets with Korean angelica as just

'Dang-Gui'. Although the three roots are known to have similar pharmacological efficacy, they show variation in their chemical compositions and pharmacological properties. Instead of coumarins in Korean Angelica, phthalides are the principal components in the former two herbal drugs (Lu et al., 2005; Lu et al., 2004; Zhao et al., 2003). Coumarins have not been found in those herbal medicines. Therefore, coumarins could be standard compounds to differentiate between Korean Angelica and Chinese or Japanese Angelica and to characterize the Korean Angelica's own biological activity.

Angelica radix is one of the most popular herbal medicines used by Japanese people; traditionally, it has been primarily used for health conditions in women particularly in gynecological disorders such as painful menstruation (The Japan Society for Oriental Medicine, 2005a). *Angelica acutiloba* Kitagawa radix (Yamato-toki) produced in the Yamato and neighboring areas in Japan was recognized as a good quality product. This recognition was described in two Japanese historical manuscripts written in the 18th century (Kaibara, 1709; Terashima, 1713). The recognition continues today, and is reflected in the market price of the radix. In present-day Japan, two varieties of *Angelica radix*, Yamato-toki *and Angelica*

acutiloba Kitagawa var. sugiyama Hikino radix (Hokkai-toki) can be used medicinally by rule on the Japanese Pharmacopoeia. Dried roots of *Angelica acutiloba*, or Yamato-toki in Japanese, possesses several beneficial therapeutic properties, and can be used in the treatment of gynecological disorders and as an aid for modern conventional methods in treating cancer (e.g., chemotherapy and radiotherapy) (Liu et al., 2001; Yang et al., 2007a; Yang et al., 2007b; Han et al., 2006). Thus, Yamato-toki is an important and popular herbal product that is readily accessible in global markets. Several pharmacological effects of ligustilide, a major component of Angelica radix extract, have been reported. Anti-cholinergic action, such as inhibition of acetylcholine (ACh)-induced contraction in rat intestine (Mitsuhashi et al., 1960) and rat uterus (Du et al., 2006), has been demonstrated. On the other hand, cholinergic action of the radix extract has also been reported, such as increasing the activity of uterus contraction in rabbit (Harada et al., 1984). Even though there is no clear explanation for these contradictory reports, it has been suggested that choline-related activity is one of the noticeable pharmacological effects of the radix. Assay of inhibition of ACh-induced contraction in guinea pig ileum is a method of estimating anti-cholinergic actions of a test compound, while assay of ileum contraction is a method of

estimating cholinergic actions. In this study, both assays were performed to compare the choline-related activities between excellent- and lowgrade Yamato-toki, which were, respectively, produced in Nara Prefecture, Japan, and in China. Then, ligustilide content in the radixes was quantitatively measured and compared between the excellent grades and the other grades. Differences in the choline related activity and the ligustilide content of the radix grades were observed.

According to TCM, *A. decursiva* belongs to Plantae, Angiospermea Phylum, Dicotyledoneae Class. Umbelliflorae Order, Apiaceae Family, *Angelica* Genus (Catalogue of life, China, 2009). It grows throughout Japan, China and Korea, is mainly distributed in hillside, grass land or sparse forest (Chi et al., 2001). It is called 'Jahwajeonho' in Korean and 'Zi hua qian hu' in Chinese, is widely employed in traditional medicine to cure diseases such as cough from pathogenic wind heat, and the accumulation of phlegm and heat in the lungs (Kong et al., 1996). *A. decursiva* is also used in Korea as salad, it has been reported has no toxicity (Yi et al., 2008). The usage of the roots of *A. decursiva* has long history in China to clean heat, resolve summer heat and stop bleeding. It is also officially listed in the Chinese pharmacopoeia. This plant is a rich source of different types of coumarin

derivatives which include decursin, decursidin, nodakenetin, umbelliferone, andelin, nodakenin, decursinol, methyl decursinol, bergapten, isoimperatorin, imperatorin and hydroxypeucedanin (Hata and Sano, 1969; Avramenko et al., 1970; Yook, 1975). In addition, different type of compounds such as furanocoumarin, psoralen, dihydropsoralen, angelicin, dihydroangelicin, pyranocoumarin, dihydroxanthyletin, dihydroseselin also reported (Chen et al., 2008). Recently, several coumarin derivatives, such as, nodakenin, nodakenetin, and umbelliferone, isolated from this plant, have been reported numerous pharmacological effects which include anti-tumor (Ahn et al., 1997; Kim et al., 2005; Jiang et al., 2006), anti-platelet aggregation (Lee et al., 2003), anti-*Helicobacter pylori* (Bae et al., 1998) and neuroprotective (Lee et al., 2003a; Kang et al., 2005) effects, as well as memory-enhancing (Kang et al., 2001; Kang et al., 2003), inhibit acetyl cholinesterase, anti-amnesic (Kim et al., 2007) activities.

In recent years, pharmacological studies have shown that the alcohol extract of *A. decursiva* has antispasmodic, expectorant, anti-platelet aggregation, anti-inflammatory activities, and inhibit cancer cell growth and metabolism. Research shows that nodakenin is the effective ingredient in the extract of *A. decursiva*, the higher content of nodakenin the better its

efficacy. So the content of nodakenin can be used as the evaluation indicators of extraction technology of *A. decursiva* (http://baike.baidu.com/view/441874.htm). Another classical composition of *A. decursiva* is umbelliferone, which minimize the risk of diabetic complications (Ramesh et al., 2007), has antioxidantive and antihyperlipidemic (Ramesh and Pugalendi, 2005a; Ramesh and Pugalendi, 2005b; Ramesh and Pugalendi, 2006; Singh et al., 2010); immunomodulatory (Park et al., 2004; Qin and Sun, 2005) and potential anti-inflammatory (Hoult and Payá, 1996; Juliana et al., 2009; Toyama et al., 2009) activities.

Inflammation is a complex normal response of the mammalian body to a variety of hostile agents such as parasites, pathogenic microorganisms, toxic chemical substances, and physical damage of the body which disturbed homeostasis. It is known to contribute to physiological and pathological processes by the activation of the immune system, local vascular system, and various cells within the damaged tissue (Coussens and Werb, 2002). Prolonged inflammation, known as chronic inflammation, is caused by a variety of factors, including microbial pathogen infection, physical, chemical, and surgical irritation, and/or wounding. The classical

characteristics of inflammation are pain, swelling, edema, redness and heat (Mantovani, 2010). The main symptoms of the body against an inflammatory stimulation are increased body temperature and pain. In general, controlled inflammation is a beneficial response that can defend and protect the body from harmful factors, but if the body's regulation of inflammation is dysfunctional, then inflammation will have an adverse effect on the body, such as the emergence of chronic inflammation and a series of chain reactions. A large number of inflammatory mediators lead to harmful effects on the body (Nathan, 2002; Medzhitov, 2008), including excessive degeneration, exudation, necrosis, or the formation of abnormal granulation formation, that result in different degrees of injury to the body (Serhan and John, 2005; Karin et al., 2006). Epidemiological studies have demonstrated that chronic inflammation is associated with a wide variety of diseases, including cardiovascular diseases, cancers, diabetes, arthritis, Alzheimer's disease, pulmonary diseases, and autoimmune diseases (Lin and Tang, 2008). From ancient times many plants have been used as a remedy form inflammation-associated disorders, and thus they may serve as the alternative sources for the development of new anti-inflammatory agents due to their biological activities. Several phytoconstituents such as alkaloids,

coumarins, flavonoids, polyphenolics, glycosides, steroids, saponins, and essential oils derived from plants have shown anti-inflammatory activity in both *in vitro* and *in vivo* experimental models (Minky and Ankush, 2013). In a recent study, the potential antioxidant and anti-inflammatory activity of methanol extract of *A. decursiva* and its different solvent soluble fractions have been reported. Among the fractions, the ethyl acetate (EtOAc) fractions showed strong anti-oxidant and anti-inflammatory activities, while the dichloromethane (CH_2Cl_2) fraction-derived 90% MeOH soluble fraction showed the most promising anti-inflammatory activity (Dafang et al., 2012). Although the 90% MeOH soluble fraction of the CH_2Cl_2 fraction was found as the most active anti-inflammatory fraction, compounds responsible anti-inflammatory activity were not identified yet. Therefore, the present study was focused on the bioactivity-guided isolation of anti-inflammatory components from the active 90% MeOH soluble fraction.

II. Materials and Methods

2-1. Plant materials

Whole plants of *A. decursiva* were purchased from the Korean Plant Extract Bank under the Korea Research Institute of Bioscience and Biotechnology. A voucher specimen as a whole plant registered and deposited in the Department of Food Science and Nutrition, Pukyong National University, Busan, South Korea for future reference.

2-2. Reagents and equipments

2-2-1. Reagents

LPS from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), fetal bovine serum (FBS), and antibiotics were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and Dulbecco's Modified Eagle's Medium (DMEM) from Hyclone (Logan, Utah, USA). Various primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling

Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO). Column chromatography was conducted using silica (Si) gel 60 (70–230 mesh, Merck, Germany). TLC was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm) using 50 % H_2SO_4 as a spray reagent. All the solvents used for column chromatography were of reagent grade, and were purchased from commercial sources.

2-2-2. Equipments

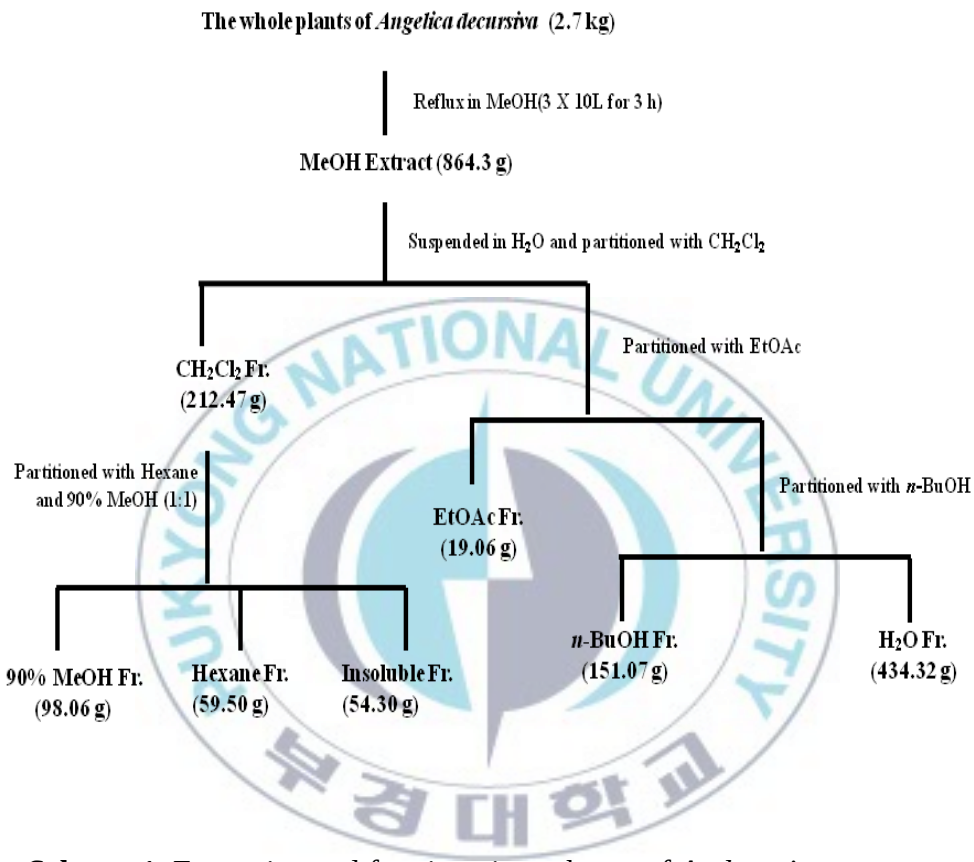
The EI-MS spectra were determined using a Hewlett-Packard 5989B spectrometer (Agilent Technologies) and a JEOL JMS-700 spectrometer. The ^{1}H- and ^{13}C-NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR in deuterated dimethylsulfoxide (DMSO-$d_{6)}$ and chlroform-*d* ($CDCl_3$). Chemical shifts were referenced to the respective residual solvent peaks (2.50 ppm for ^{1}H and 39.5 ppm for ^{13}C NMR for DMSO-d_6; 7.24 for ^{1}H and

77.0 for ^{13}C for $CDCl_3$). Column chromatography was performed using silica gel (Si-gel) 60 (70~230 mesh, Merck, Darmstadt, Germany). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm) and RP-18 F_{254s} plates (5 × 10 cm, Merck), using 50% H_2SO_4 as a spray reagent.

2-3. Methods

2-3-1. Extraction and fractionation

The whole plants of *A. decursiva* used in this study was extracted and fractionated according to the procedure described by Dafang et al. (2012). The CH_2Cl_2 fraction was further partitioned with hexane and 90% MeOH at a ratio of 1:1 to yield 90% MeOH soluble fraction (98.06 g) and hexane soluble fraction (59.50 g) along with an insoluble intermediate (54.30 g) as shown in **Scheme 1.**

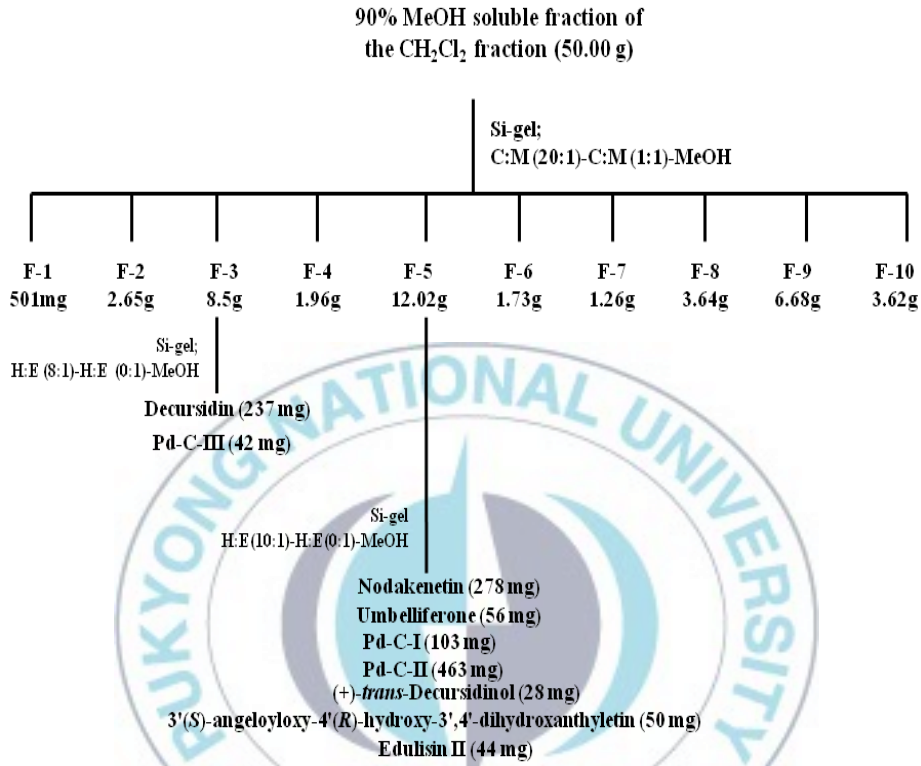


Scheme 1. Extraction and fractionation scheme of *A. decurisva*

2-3-2. Isolation of active compounds from the active CH_2Cl_2 fraction

A part of the 90% MeOH fraction (50.00 g) was subjected to column chromatography over a Si-gel column using CH_2Cl_2-MeOH (20:1→0:1) in order to get 10 subfractions (F-1 to F-10). Among them, F-3 was chromatographed over Si-gel column using hexane-EtOAc (8:1→0:1) to obtain compound **2** (237 mg) and compound **3** (42 mg). F-5 was chromatographed over Si-gel column using hexane-EtOAc (10:1→0:1) to afford 12 subfractions (F-5-1 to F-5-12). F-5-5 was chromatographed over Si-gel column using hexane-EtOAc (3:1→0:1) which yielded compound **1** (44 mg). F-5-7 was chromatographed repeatedly using hexane-EtOAc (5:1→0:1) to get compound **5** (463 mg). Repeated chromatography of F-5-8 using CH_2Cl_2-MeOH (100:1→0:1) yielded three compounds, compound **8** (56.8 mg), compound **7** (28.1 mg), and compound **4** (50 mg). F-5-9 was chromatographed using CH_2Cl_2-MeOH (100:1→0:1) to get Pd-C-I (103 mg), while F-5-10 was filtered to get compound **9** (278 mg) as a precipitate. Compounds **1** ~ **9** isolated from the 90% MeOH fraction were characterized and identified as edulisin II (Kawasaki et al., 1984), decursidin (Liu et al., 2005), Pd-C-III (Sakakibara et al., 1982), 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin, Pd-C-I, Pd-C-II (Sakakibara et al., 1982), (+)-

trans-decursidinol (Yao and Kong, 1994), umbelliferone (Singh et al., 2010), and nodakenetin (Lee et al., 2002), respectively based on spectroscopic methods, including ^{1}H- and ^{13}C-NMR, as well as through comparison with published spectral data. Summary of the isolation procedure of active compounds from the active CH_2Cl_2 fraction is given in **Scheme 2**. The structure of isolated compounds is shown in **Figure 1**.



Scheme 2. Isolation of active compounds from the active 90% MeOH soluble fraction of the CH_2Cl_2 fraction of *A. decursiva*

The respective spectral data of isolated compounds are as follows:

Edulisin II (1)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.60 (1H, d, *J* = 9.4 Hz, H-4), 7.40 (1H, d, *J* = 8.5 Hz, H-5), 7.05 (1H, s, *J* = 7.2 Hz, H-3'), 6.85 (1H, d, *J* = 8.5 Hz, H-6), 6.22 (1H, d, *J* = 9.4 Hz, H-3), 5.60 (1H, t, *J* = 6.1 Hz, H-4'), 5.56 (1H, t, *J* = 6.1 Hz, H-3'), 5.20 (1H, d, *J* = 7.2 Hz, H-2'), 2.21 (3H, s, H-5'''), 2.14 (3H, m, H-5''), 1.87 (3H, m, H-4'''), 1.85 (3H, m, H-4''), 1.73 (3H, s, gem-CH_3), 1.64 (3H, s, gem-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 165.8 (C-1''), 163.9 (C-1'''), 163.7 (C-7), 160.0 (C-2), 159.2 (C-3'''), 156.3 (C-3''), 151.8 (C-9), 143.4 (C-4), 131.1 (C-5), 117.0 (C-2''), 115.0 (C-2'''), 113.3 (C-10), 113.1 (C-8), 113.0 (C-2), 107.6 (C-6), 88.9 (C-2'), 80.5 (C-4'), 67.2 (C-3'), 27.5 (C-4''), 27.3 (C-4'''), 25.1 (gem-CH_3), 22.2 (gem-CH_3), 20.4 (C-5''), 20.2 (C-5''').

Decursidin (2)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.60 (1H, d, *J* = 9.6 Hz, H-4), 7.39 (1H, s, H-5), 6.81 (1H, s, H-8), 6.24 (1H, d, *J* = 9.3 Hz, H-3), 6.05 (1H, d, *J* = 5.5 Hz, H-4'), 5.69 (1H, t, 5.69, H-2'''), 5.68 (1H, t, H-2''), 5.27 (1H, d, *J* = 5.5

Hz, H-3'), 2.22 (3H, d, *J* = 1.0 Hz, H-4'''), 2.16 (3H, d, *J* = 1.0 Hz, H-5'''), 1.93 (3H, d, *J* = 1.5 Hz, H-5(3H, d, *J* = 1.5 Hz, H-4'''), 1.90 (3H, d, *J* = 1.5 Hz, H-4''), 1.46 (3H, s, H-2'-CH_3), 1.39 (3H, s, H-2'-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 165.9 (C-1'''), 164.9 (C-1''), 160.9 (C-2), 159.3 (C-3'''), 159.2 (C-3''), 156.2 (C-7), 155.2 (C-9), 143.4 (C-4), 129.3 (C-5), 117.2 (C-6), 115.1 (C-2'''), 114.9 (C-2''), 113.6 (C-3), 113.2 (C-10), 104.8 (C-8), 77.9 (C-2'), 71.2 (C-3'), 66.1 (C-4'), 27.5 (C-5'''), 27.4 (C-5''), 24.9 (C-2'-CH_3), 22.5(C-2'-CH_3), 20.5 (C-4'''), 20.4 (C-4'').

Pd-C-III (3)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.60 (1H, d, *J* = 9.6 Hz, H-4), 7.36 (1H, s, H-5), 6.81 (1H, s, H-8), 6.26 (1H, d, *J* = 9.6 Hz, H-3), 6.16 (1H, m, H-3''), 6.08 (1H, m, H-4'), 5.32 (1H, d, *J* = 6.1 Hz, H-3'), 2.16 (3H, s, H-2''), 1.96 (3H, m, H-4'''), 1.88 (3H, m, H-5'''), 1.47 (3H, s, gem-CH_3), 1.40 (3H, s, gem-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 170.7 (C-1''), 166.2 (C-1'''), 160.7 (C-2), 156.1 (C-7), 155.4 (C-9), 143.0 (C-4), 140.1 (C-3'''), 128.8 (C-5), 126.8 (C-2'''), 116.7 (C-6), 113.9 (C-3), 113.3 (C-10), 104.9 (C-8), 77.9 (C-2'), 71.9 (C-3'), 67.4 (C-4'), 25.1 (gem-CH_3), 22.2 (gem-CH_3), 21.0 (C-2''), 20.4 (C-5'''), 15.8 (C-4''').

3' (*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (4)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.63 (1H, d, *J* = 9.5 Hz, H-4), 7.63 (1H, s, H-5), 6.76 (1H, s, H-8), 6.24 (1H, d, *J* = 9.5 Hz, H-3), 6.21 (1H, dd, *J* = 7.0 & 1.3 Hz, H-3"), 5.11 (1H, d, *J* = 7.8 Hz, H-3'), 4.77 (1H, d, *J* = 7.8 Hz, H-4'), 2.01 (3H, d, *J* = 1.2 Hz, H-4"), 1.93 (3H, d, *J* = 1.3 Hz, H-5"), 1.46 (3H, s, gem-CH_3), 1.35 (3H, s, gem-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 167.8 (C-1"), 161.1 (C-2), 155.3 (C-7), 155.0 (C-9), 143.3 (C-4), 140.6 (C-3"), 128.1 (C-5), 126.8 (C-2"), 121.0 (C-6), 113.5 (C-3), 113.3 (C-10), 104.4 (C-8), 78.3 (C-2'), 76.3 (C-3'), 67.4 (C-4'), 26.0 (gem-CH_3), 21.1 (gem-CH_3), 20.5 (C-5"), 16.0 (C-4").

Pd-C-I (5)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.64 (1H, d, *J* = 9.6 Hz, H-4), 7.62 (1H, s, H-5), 6.77 (1H, s, H-8), 6.25 (1H, d, *J* = 9.6 Hz, H-3), 5.77 (1H, t, *J* =1.0 Hz, H-2"), 5.02 (1H, d, *J* = 7.8 Hz, H-3'), 4.75 (1H, d, *J* = 6.0 Hz, H-4'), 3.00 (1H, d, *J* = 6.0 Hz, H-4'-OH), 2.20 (3H, d, *J* = 1.0 Hz, -CH_3), 1.95 (3H, d, *J* = 1.0 Hz, -CH_3), 1.44 (3H, s, gem-CH_3), 1.33 (3H, s, gem-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 166.6 (C-1"), 161.0 (C-2), 160.1 (C-3"), 155.4 (C-7), 155.1 (C-9), 143.3 (C-4), 128.1 (C-5), 120.9 (C-6), 114.9 (C-2"),

113.6 (C-3), 113.3 (C-10), 104.4 (C-8), 78.4 (C-2'), 75.8 (C-3'), 67.5 (C-4'), 27.6 (3"-CH_3), 26.0 (gem-CH_3), 20.9 (gem-CH_3), 20.5 (3"-CH_3).

Pd-C-II (6)

^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.59 (1H, d, *J* = 9.6 Hz, H-4), 7.31 (1H, s, H-5), 6.78 (1H, s, H-8), 6.24 (1H, d, *J* = 9.5 Hz, H-3), 5.92 (1H, dd, *J* = 7.55 & 0.7 Hz, H-4'), 5.80 (1H, t, *J* = 1.4 Hz, H-2"), 3.86 (1H, d, *J* = 7.9 Hz, H-3'), 3.18 (1H, d, *J* = 6.0 Hz, H-4'-OH), 2.26 (3H, d, *J* = 1.0 Hz, 3"-CH_3), 1.96 (3H, d, *J* = 1.0 Hz, 3"-CH_3), 1.52 (3H, s, gem-CH_3), 1.35 (3H, s, gem-CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 167.9 (C-1"), 160.9 (C-2), 160.5 (C-3"), 156.4 (C-7), 155.3 (C-9), 143.1 (C-4), 128.1 (C-5), 117(C-6), 114.9 (C-2"), 113.7 (C-3), 113.1 (C-10), 104.7 (C-8), 79.9 (C-2'), 73.9 (C-3'), 70.7 (C-4'), 27.7 (3"-CH_3), 25.9 (gem-CH_3), 20.6 (3"-CH_3), 19.7 (gem-CH_3).

(+)-*trans*-Decursidinol (7)

^{1}H NMR (400 MHz, CD_3OD): δH: 7.87 (1H, dd, *J* = 9.2 Hz, H-4), 7.69 (1H, s, H-5), 6.67 (1H, s, H-8), 6.22 (1H, d, *J* = 9.6 Hz, H-3), 4.53 (1H, d, *J* = 8.5 Hz, H-4'), 3.54 (1H, d, *J* = 8.5 Hz, H-3'), 1.47 (3H, s, H-2'-CH_3), 1.23 (3H, s, H-2'-CH_3). ^{13}C NMR (100 MHz, CD3OD): δC: 163.2 (C-2), 157.6

(C-), 156.1 (C-), 145.8 (C-4), 129.5 (C-5), 124.3 (C-6), 114.3 (C-10), 113.5 (C-3), 104.5 (C-8), 81.5 (C-2'), 76.3 (C-3'), 69.4 (C-4'), 27.1 (C-2'-CH_3), 19.8 (C-2'-CH_3).

Umbelliferone (8)

White prisms; ^{1}H NMR (400 MHz, DMSO-d_6): $\delta_{H:}$ 10.56 (1H, brs, 7-OH), 7.93 (1H, d, J = 9.2 Hz, H-4), 7.51 (1H, d, J = 8.2 Hz, H-5), 6.77 (1H, dd, J = 8.5, 2.4 Hz, H-6), 6.70 (1H, d, J = 2.1 Hz, H-8), 6.19 (1H, d, J = 9.6 Hz, H-3); ^{13}C NMR (100 MHz, DMSO-d_6): δ_C: 161.3 (C-2), 160.4 (C-7), 155.5 (C-9), 144.5 (C-4), 129.7 (C-5), 113.1 (C-6), 111.4 (C-3), 111.2 (C-10), 102.1 (C-8).

Nodakenetin (9)

Colorless needles $[\alpha]_D^{20}$ = -0.028^0 (Conc. 11.4 mg/mL, EtOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 7.58 (1H, d., J = 9.2 Hz, H-4), 7.21 (1H, s, H-5), 6.71 (1H, s, H-8), 6.19 (1H, d, J = 9.6, H-3), 4.74 (1H, t, H-2'), 3.21 (2H, m, H-3'), 1.37 (3H, s, 4'-CH_3), and 1.24 (3H, s, 4'-CH_3). ^{13}C-NMR (100 MHz, DMSO-d_6): δ_C: 163.2 (C-7), 161.4 (C-2), 155.6 (C-9), 143.7 (C-4), 125.1 (C-6), 123.4 (C-5), 112.7 (C-10), 111.2 (C-3), 97.8 (C-8), 91.1 (C-2), 71.6

(C-4), 29.4 (C-3'), 26.0 (C-4'), 24.3 (C-4').

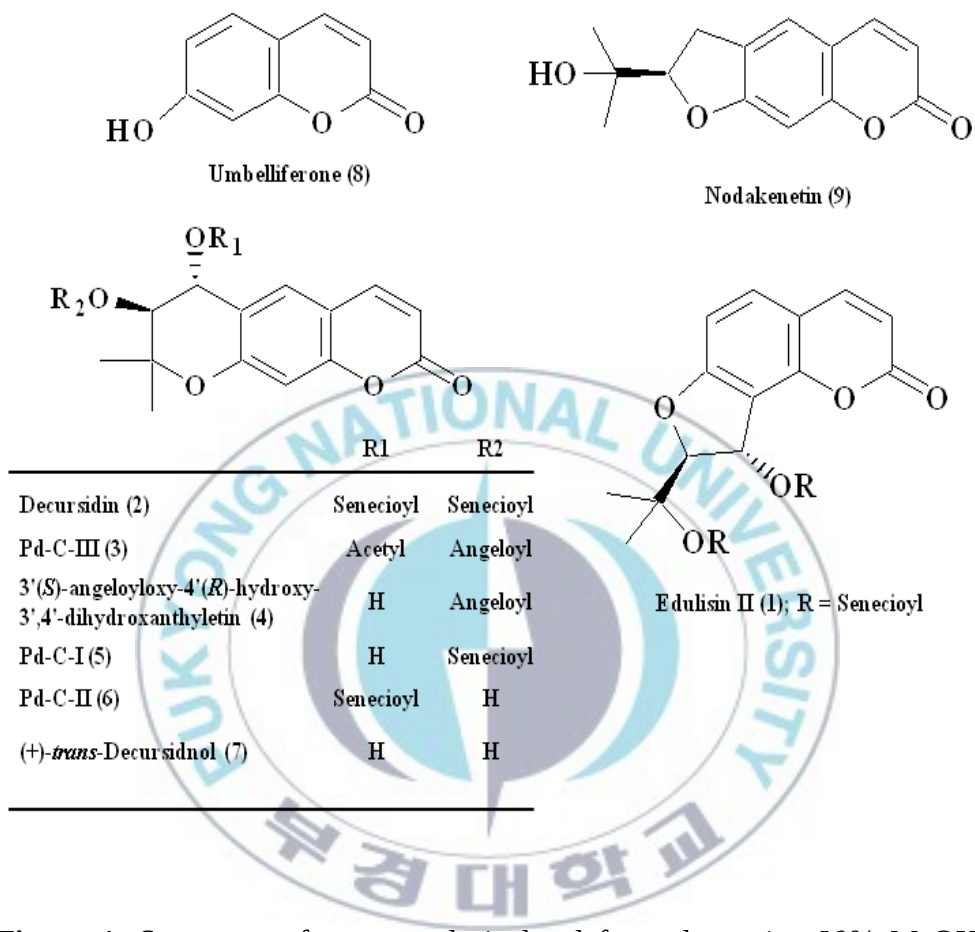


Figure 1. Structures of compounds isolated from the active 90% MeOH soluble fraction of the CH_2Cl_2 fraction of *A. decursiva*

2-3-3. Cell viability in RAW 264.7 cells

The macrophage RAW 264.7 cell line obtained from American Type culture Collection (ATCC) was incubated in Dulbecco's modified Eagle's medium (DMEM) at 37 ℃ under 5% CO_2 humidified air. The cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated at 37 ℃ for 24 h. The cells were then treated with various concentrations of samples (both extract and compounds). After additional 24 h incubation at 37 ℃, 100 µl of MTT (0.5 mg/mL in PBS) was added to the wells and mixed well. The resulting color was assayed at 540 nm using microplate reader (VERSA max Molecular Devices, Sunnyvale, CA, U.S.A.).

2-3-4. Estimation of the inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells

RAW 264.7 cells were cultured with DMEM supplemented with 10% FBS and 1% antibiotics in a 5% CO_2 atmosphere at 37 ^{0}C. The cells were activated with LPS as previously described (Chi et al., 2001). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2h, test samples (both extract and compounds) and LPS (1.0 µg/ml) were

added and incubated for 18h unless otherwise specified. Test samples (both subfractions and compounds) dissolved in DMSO were diluted to appropriate concentrations with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1%. To assess NO production, the stable conversion product of NO was measured using Griess reagent and the optical density was determined at 540 nm.

2-3-5 Analysis for inhibition of iNOS and COX-2 protein expression

Western blotting was used to measure the protein levels of iNOS and COX-2. RAW 264.7 cells were cultured in 100 mm culture dishes in the presence or absence of LPS (1.0 μg/ml) and with/without test compounds for 18 h. Afterwards, the cells were washed twice with ice-cold PBS and lysed with buffer on ice for 30 min. Cell extracts were obtained by centrifugation at 14,000 × g and 4℃ for 20 min. Cytosolic proteins were electrophoretically separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were immediately blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (pH 7.4) (TBST) buffer at room temperature for 1 h. The membranes were washed three times (10 min) in TBST buffer and incubated with primary antibody,

diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST buffer, at 4°C overnight. After three washings in TBST buffer (10 min), the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody, diluted 1:2000 in 5% (w/v) non-fat dry milk in TBST buffer, at room temperature for 1 h. After three washings in TBST buffer (10 min), the antibody was visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), according to the manufacturer's instructions and exposed to an X-ray film (GE Healthcare Ltd., Amersham, UK). Pre-stained blue protein markers were used for molecular weight determination.

2-3-6. Measurement of TNF-α production in the medium

The TNF-α production in the culture medium was determined using a commercially available TNF-α ELISA kit (eBioscience, Inc., CA) according to the manufacturer's instructions.

2-4. Statistics

All results were expressed as mean ± standard deviation (SD) of triplicate experiments. The statistical significance was determined by one-

way ANOVA, followed by student's *t*-test using a computerized statistical program. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

III. Results

3-1. Effect of 90% MeOH fraction-derived subfractions on the cell viability in RAW 264.7 cells

Prior to our study on the anti-inflammatory activity, the MTT assay was performed to determine the potential toxicity of the crude extract and its different fractions. Chromatography of 90% MeOH soluble fraction yielded 10 subfractions (F-1 to F-10), which were evaluated for their cytotoxicity at a concentration of 5 μg/ml. All these fractions were found to be nontoxic up to 5 μg/ml, and therefore they were used at this concentration in subsequent experiments (**Table 1**).

3-2. Effect of 90% MeOH fraction-derived subfractions on NO production in LPS-stimulated RAW 264.7 cells

The inhibitory effect of different subfractions derived from the 90% MeOH fraction on NO production was evaluated in LPS-stimulated RAW 264.7 cells and results are summarized in **Table 1**. It is evident from **Table 1** that F-5 exhibited the most potent NO production inhibitory activity (% inhibition: 78.06 ± 3.50) followed by F-4 (% inhibition: 72.26 ± 2.54)

compared to the positive control AMT (% inhibition: 99.27 ± 3.63 at 1.6 μg/ml) in LPS-stimulated RAW 264.7 cells at a concentration of 5 μg/ml without affecting cell viability. Besides these two subfractions, F-3 was also found as the potent inhibitor of NO production in LPS-stimulated RAW 264.7 cells. On the other hand, F-1 and F-2 showed weak NO production inhibitory activity in LPS-stimulated RAW 264.7 cells, however, F-7 to F-10 were found to be inactive at the tested concentration.

Table 1. Effect of different subfractions derived from the 90% MeOH fraction on LPS-induced NO production and cell viability in RAW 264.7 cells.

Sample	Yield (g)	5 μg/ml		
		NO production (μM)	Inhibition (%)	Cell viability (%)
		Mean ± SD	Mean ± SD	Mean ± SD
Control	-	3.77 ± 0.13	-	100.00 ± 1.68
LPS	-	34.06 ± 0.84$^{###}$	-	-
F-1	0.51	25.17 ± 0.29*	29.37 ± 3.12	93.44 ± 3.18
F-2	2.65	26.34 ± 0.21*	25.48 ± 2.13	99.00 ± 2.18
F-3	8.50	17.54 ± 0.45***	54.56 ± 2.61	98.14 ± 3.85
F-4	1.96	12.17 ± 0.28***	72.26 ± 2.54	99.25 ± 1.07
F-5	12.02	10.42 ± 0.19***	78.06 ± 3.50	100.89 ± 0.02
F-6	1.73	22.54 ± 0.07**	38.04 ± 2.11	98.57 ± 0.05
F-7	1.25	29.18 ± 1.12	16.11 ± 1.69	99.04 ± 3.50
F-8	3.64	29.49 ± 1.22	15.09 ± 1.02	109.42 ± 3.92
F-9	6.68	30.64 ± 2.14	11.31 ± 2.11	104.66 ± 4.23
F-10	3.62	31.01 ± 0.87	10.06 ± 0.68	105.02 ± 2.38
AMT[a]		3.99 ± 0.08	99.27 ± 3.63	-

[a]AMT (1.6 μg/ml) was used as a positive control in the experiment. ###p < 0.001 indicates a significant difference from the unstimulated control group, *p < 0.05 **p < 0.01, and ***p < 0.001 indicate a significant difference from the LPS-stimulated control group.

3-3. Isolation of active compounds from the 90% MeOH fraction

The TLC profile of F-3 and F-4 is quite similar. Therefore, based on the NO production inhibitory activity of the different subfractions derived from the 90% MeOH fraction, particular emphasis was given to F-3 and F-5. Repeated column chromatography of the F-3 yielded two coumarins, including compounds **2** and **3**, while repeated chromatography of the F-5 yielded seven coumarins, including compounds **1**, **4**, **5**, **6**, **7**, **8**, and **9**. These compounds **1** ~ **9** were identified as edulisin II, decursidin, Pd-C-III, 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin, Pd-C-I, Pd-C-II, (+)-*trans*-decursidinol, umbelliferone, and nodakenetin, respectively based on spectroscopic methods, including ^{1}H- and ^{13}C-NMR, as well as through comparison with published spectral data.

3-4. Effect of isolated coumarins on NO production in LPS-stimulated RAW 264.7 cells

Since compounds **8** and **9** showed poor NO production inhibitory activity in a previous study (Dafang et al., 2012), we have selected compounds **1** ~ **7** in order to evaluate their anti-inflammatory potential. As shown in Figure 2, the cytotoxic effect of the selected coumarins derivatives

isolated from the active 90% MeOH fraction was measured prior to evaluate their effect on NO production in LPS-stimulated RAW 264.7 cells. All the selected coumarins derivatives showed different degree of cytotoxicity in RAW 264.7 cells, such as compound **1** showed no cytotoxicity up to the concentration 4 μM (**Figure 2**), compound **2** showed no cytotoxicity up to 12 μM (**Figure 3**), compound **3** showed no cytotoxicity up to 60 μM (**Figure 4**), compound **4** and **5** showed no cytotoxicity up to 40 μM (**Figures 5 & 6**), compound **6** showed no cytotoxicity up to 80 μM (**Figure 7**), and compound **7** showed no cytotoxicity up to 200 μM (not shown). Therefore, all the selected coumarins derivatives isolated from the active 90% MeOH fraction were treated at a range of respective noncytotoxic concentrations in the subsequent experiments. Intracellular anti-inflammatory effects of the selected compounds were evaluated *via* their inhibitory activities against LPS-induced NO production in RAW 264.7 cells. The inhibitory activity of selected coumarin derivatives on LPS-stimulated cellular NO production is summarized in **Figures 2 ~ 8** and **Table 2**. Among the tested coumarin derivatives, compound **1** exhibited the highest NO production inhibitory activity in a dose-dependent manner in LPS-induced RAW 264.7 cells with an IC_{50} value of 2.50 ± 0.16 μM. In

particular, compound **1** showed 59.66% inhibition at 4 μM compared to 99% inhibition exerted by AMT at 10 μM, a positive control used in the assay (**Figure 2**). Compound **2** also showed very strong NO production inhibitory effect in LPS-stimulated RAW 264.7 cells with an IC_{50} value of 4.08 ± 0.09 μM, which is nearly similar to that of compound **1** (**Figure 3**). In addition, compounds **3**, **4**, and **5** showed moderate NO production inhibitory activity compared to compounds **1** and **2** with IC_{50} values of 26.00 ± 1.00, 15.6 ± 0.80, and 31.83 ± 0.36 μM, respectively (**Figure 4, 5** & **6**). On the other hand, compound **6** showed weaker NO production inhibitory activity compared to compounds **1** and **2** with IC_{50} value of 62.7 ± 1.86 μM (**Figure 7**). In contrast, compound **7** did not exhibit any suppressive effect on LPS-stimulated RAW 264.7 cells at the concentration tested (**Figure 8**).

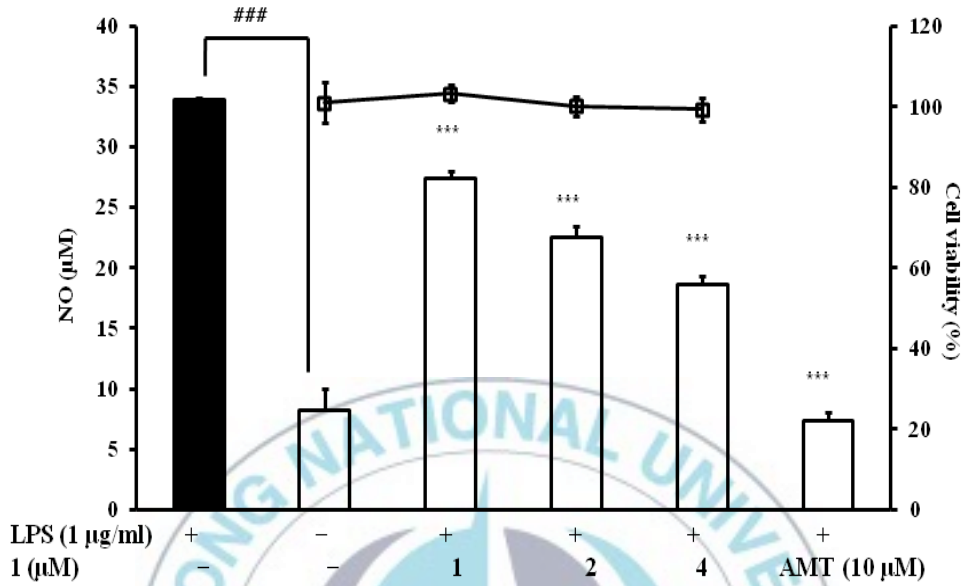


Figure 2. Effect of edulisin II (**1**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

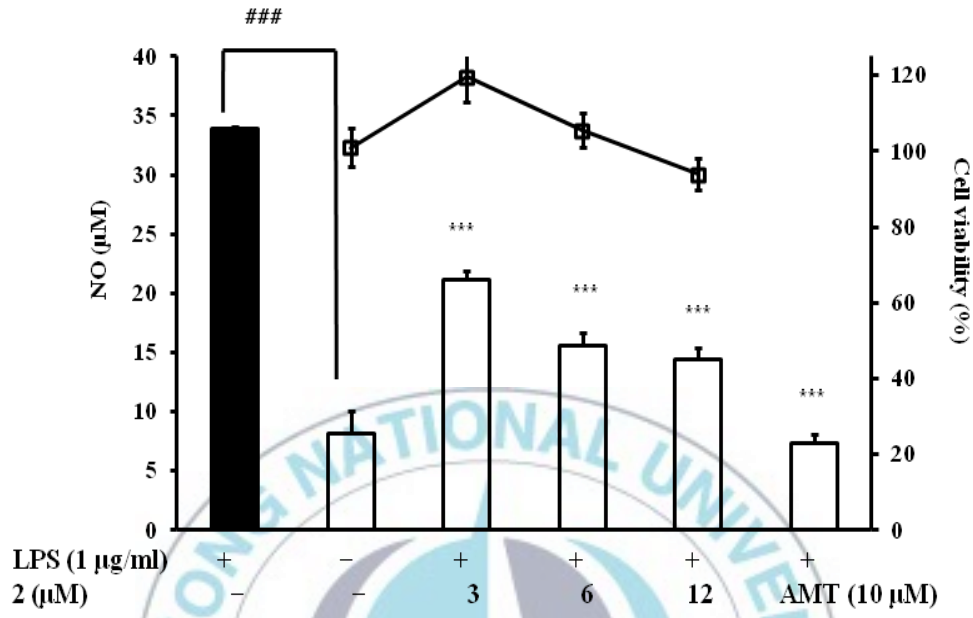


Figure 3. Effect of decursidin (**2**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

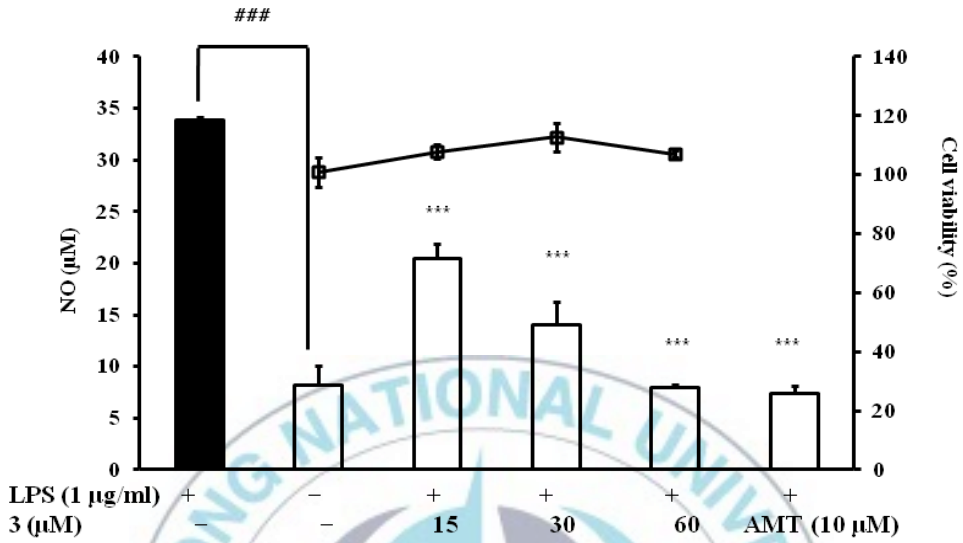


Figure 4. Effect of Pd-C-II (**3**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

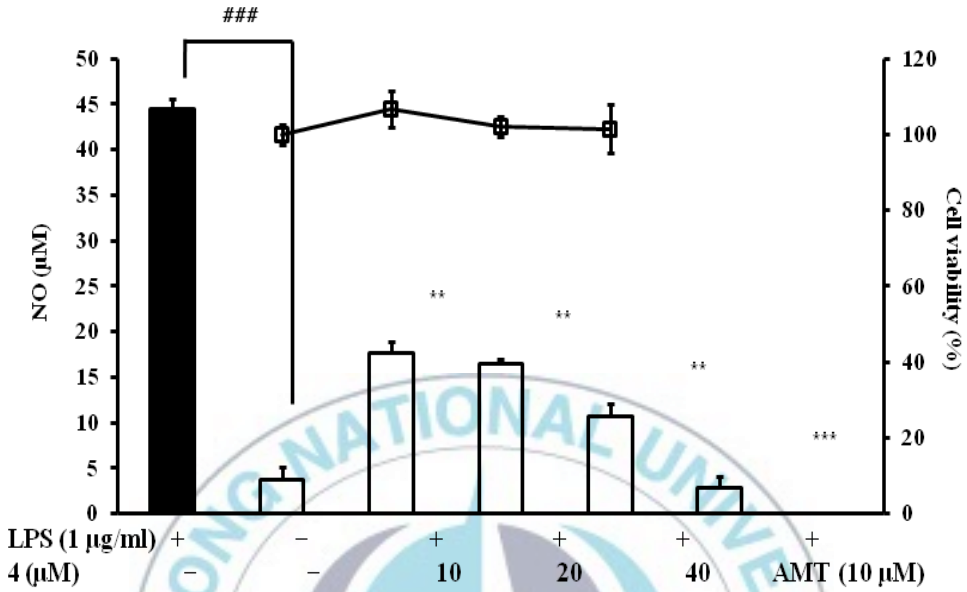


Figure 5. Effect of 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (**4**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###p < 0.001 indicates a significant difference from the unstimulated control group, *p < 0.05 **p < 0.01, and ***p < 0.001 indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

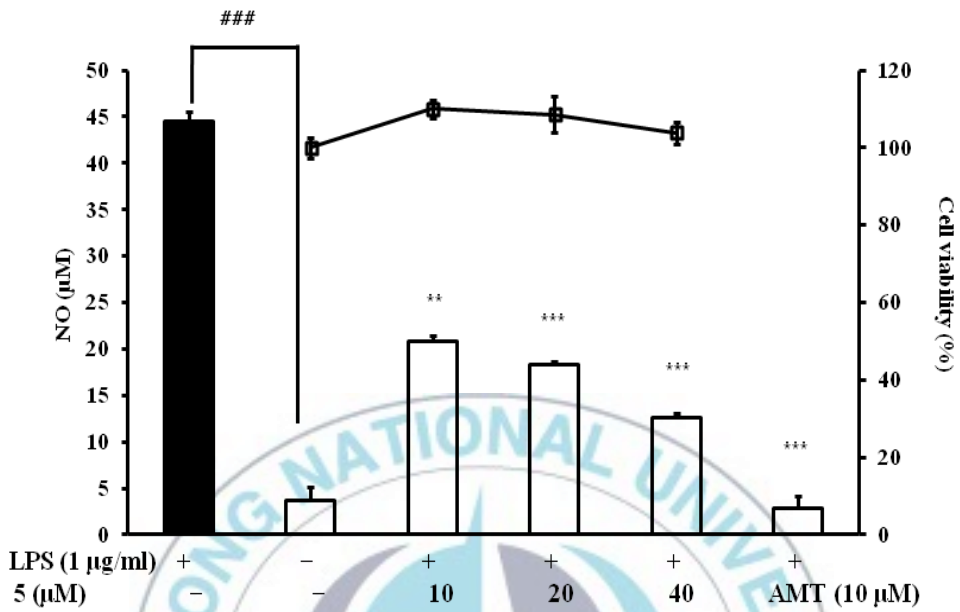


Figure 6. Effect of Pd-C-I (**5**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

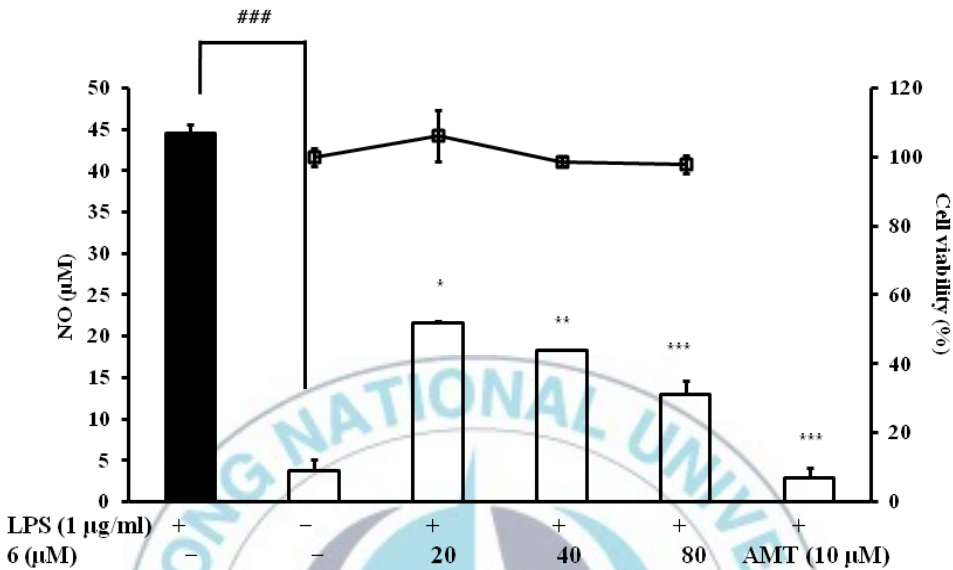


Figure 7. Effect of Pd-C-II (**6**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

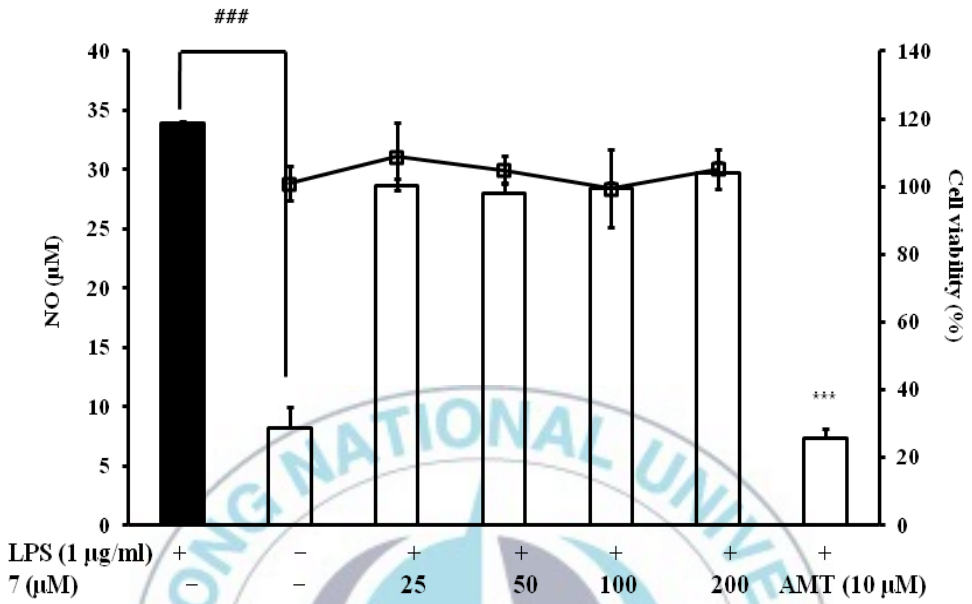


Figure 8. Effect of (+)-*trans*-decursidinol (**7**) on LPS-induced NO production and cell viability in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compound for 2 h and LPS (1.0 μg/ml). After 18 h of incubation, the amount of NO in the culture supernatants was measured by the Griess reaction assay. Cell viability was determined using the MTT method. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$ **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group. AMT was used as a positive control.

Table 2. Summary of anti-inflammatory potential of coumarin derivatives obtained from the inhibition of NO production in LPS stimulated RAW 264.7 cells.

Compounds name	IC_{50} values (μM) ± SD
Edulisin II (**1**)	2.50 ± 0.16
Decursidin (**2**)	4.08 ± 0.09
Pd-C-III (**3**)	15.60 ± 0.80
3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (**4**)	26.00 ± 1.00
Pd-C-I (**5**)	31.83 ± 0.36
Pd-C-II (**6**)	62.70 ± 1.86
(+)-*trans*-Decursidinol (**7**)	NA

NA: No activity at tested concentrations.

3-5. Effect of isolated coumarins on iNOS protein expression in LPS-stimulated RAW 264.7 cells

To investigate whether the inhibitory effects of the isolated compounds on NO production were mediated by inhibiting gene expression, we examined the effect of compounds **1** ~ **6** on the expression of iNOS protein in LPS-stimulated RAW 264.7 cells by Western blot analysis. Western blot analyses showed that iNOS protein expression in unstimulated RAW 264.7 cells was almost undetectable; however, treatment with LPS significantly increased iNOS protein expression in RAW 264.7 cells. Interestingly, pretreatment with compounds **1** ~ **6** at nontoxic concentrations dose-dependently inhibited the expression of iNOS protein in LPS-stimulated RAW 264.7 cells (**Figure 9(a)** ~ **(f)**). In particular, pretreatment with compound **2** at 12 μM and compound **4** at 40 μM strongly inhibited the expression of iNOS protein which was barely detectable (**Figure 9(b)** & **(d)**).

3-6. Effect of isolated coumarins on COX-2 protein expression in LPS-stimulated RAW 264.7 cells

The inhibitory effects of the isolated compounds on the expression of

COX-2 protein in LPS-stimulated RAW 264.7 cells were also evaluated by Western blot analysis. As shown in the **Figure 9**, LPS treatment markedly elevated COX-2 protein levels as compared to the untreated RAW 264.7 cells. However, pretreatment with compound **1**, **2**, **3**, and **4** significantly attenuated the expression of COX-2 protein expression in a concentration-dependent manner (**Figure 9(a)** ~ **(d)**). On the other hand, compounds **5** and **6** did not inhibit COX-2 protein expression at the tested concentrations (**Figure 9(e)** & **(f)**).

Figure 9. Effect of edulisin II (a), decursidin (b), Pd-C-III (c), 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (d), Pd-C-I (e), and Pd-C-II (f) on LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of compounds for 2 h and LPS (1.0 μg/ml). Cytosolic lysates were separated on SDS-PAGE. iNOS, COX-2 and β-actin were detected by Western blot analysis.

3-7. Effect of isolated coumarins on TNF-α production in LPS-stimulated RAW 264.7 cells

Since TNF-α is a well known proinflammatory mediators in many acute and chronic inflammatory diseases as well as in normal defense reactions, we also examined whether the isolated compounds modulate TNF-α production in LPS-stimulated RAW 264.7 cells. As shown in the Figure **10** ~ **15**, LPS treatment significantly increased TNF-α production in cell supernatant compared to the untreated cells. But a significant inhibition of the TNF-α secretion was observed while treatment with compounds **1** ~ **6** in LPS-stimulated RAW 264.7 cells (**Figures 10** ~ **15**).

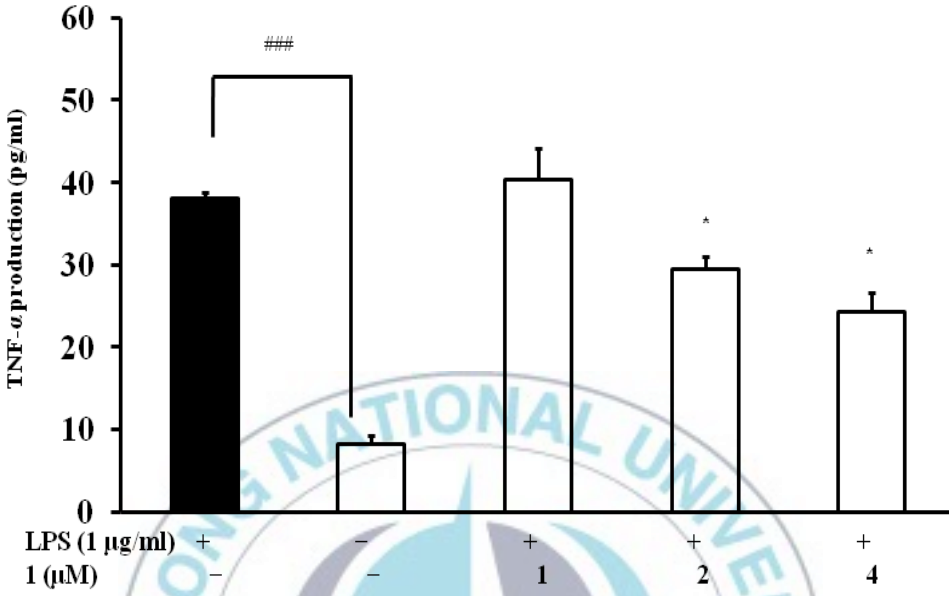


Figure 10. Effect of edulisin II (**1**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

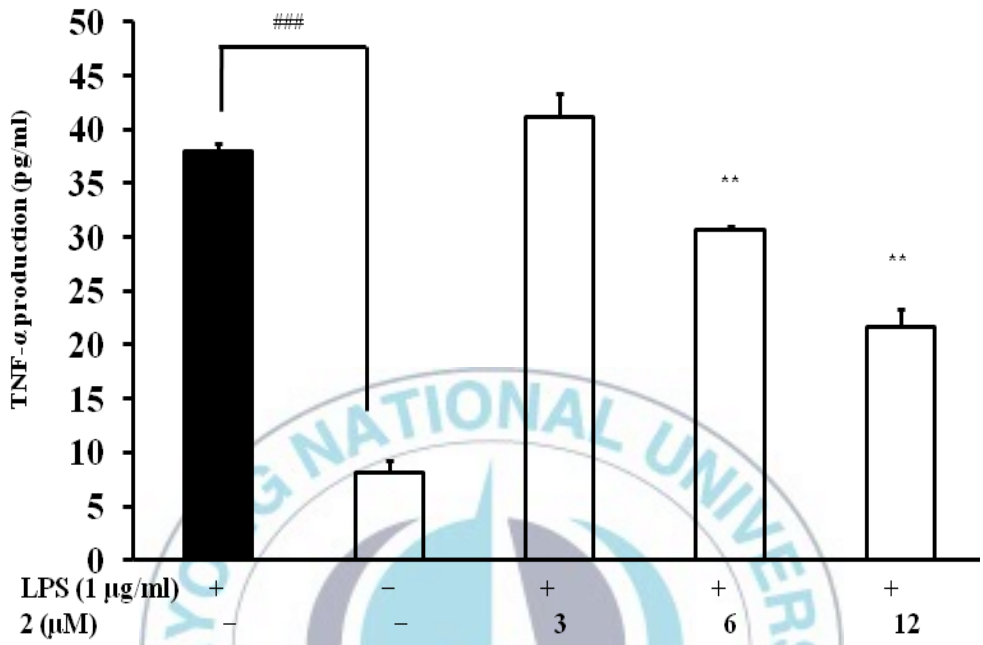


Figure 11. Effect of decursidin (**2**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

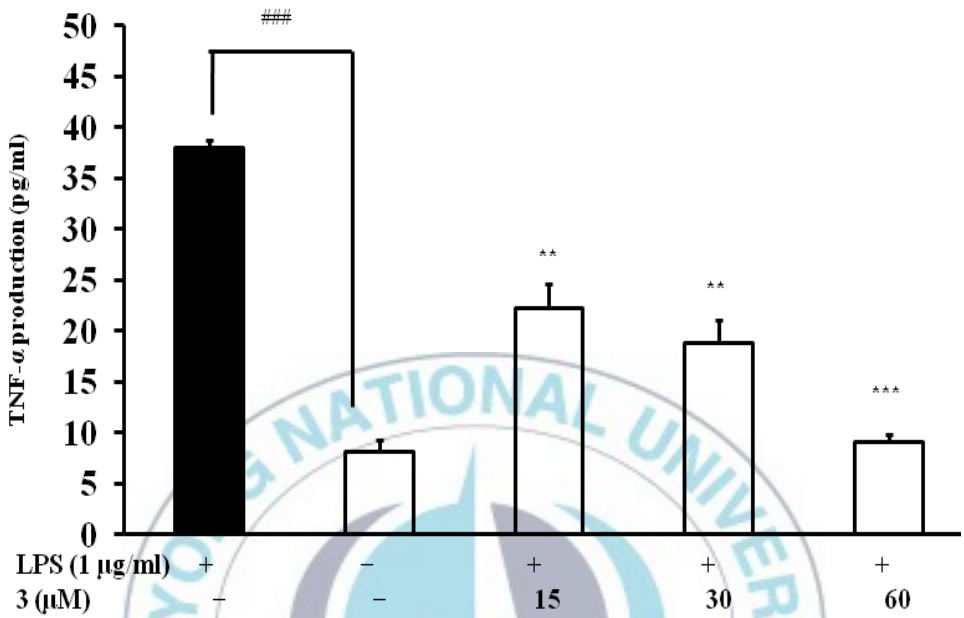


Figure 12. Effect of Pd-C-III (**3**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

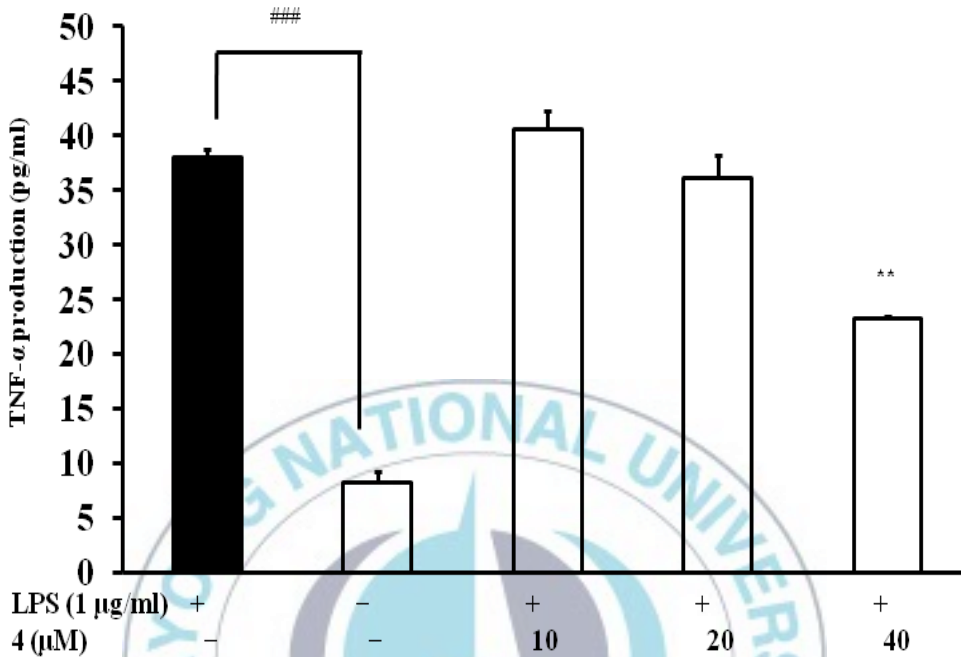


Figure 13. Effect of 3'(*S*)-angeloyloxy-4'(*R*)-hydroxy-3',4'-dihydroxanthyletin (**4**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

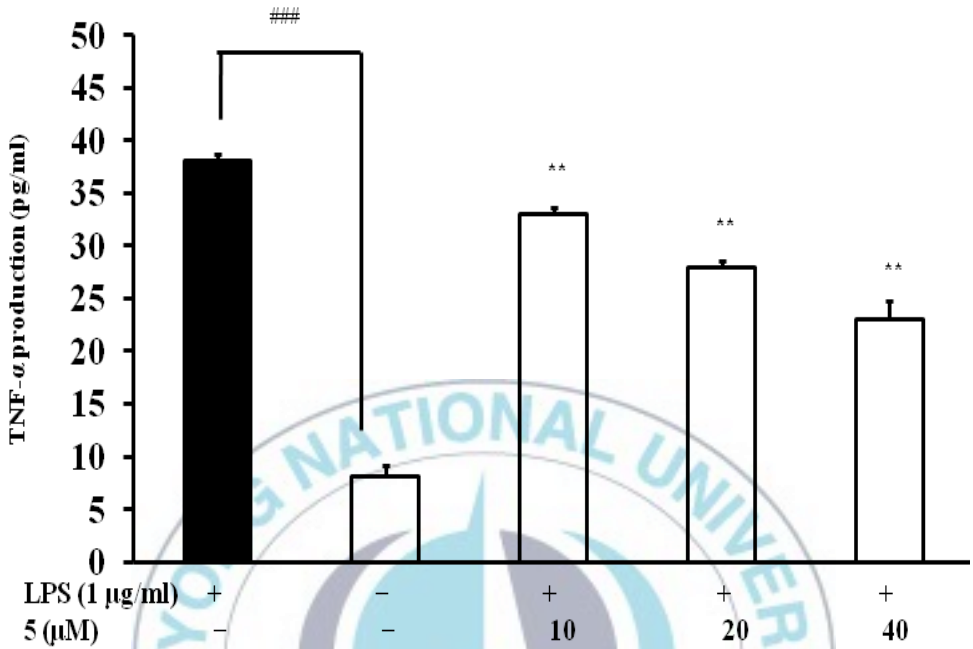


Figure 14. Effect of Pd-C-I (**5**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

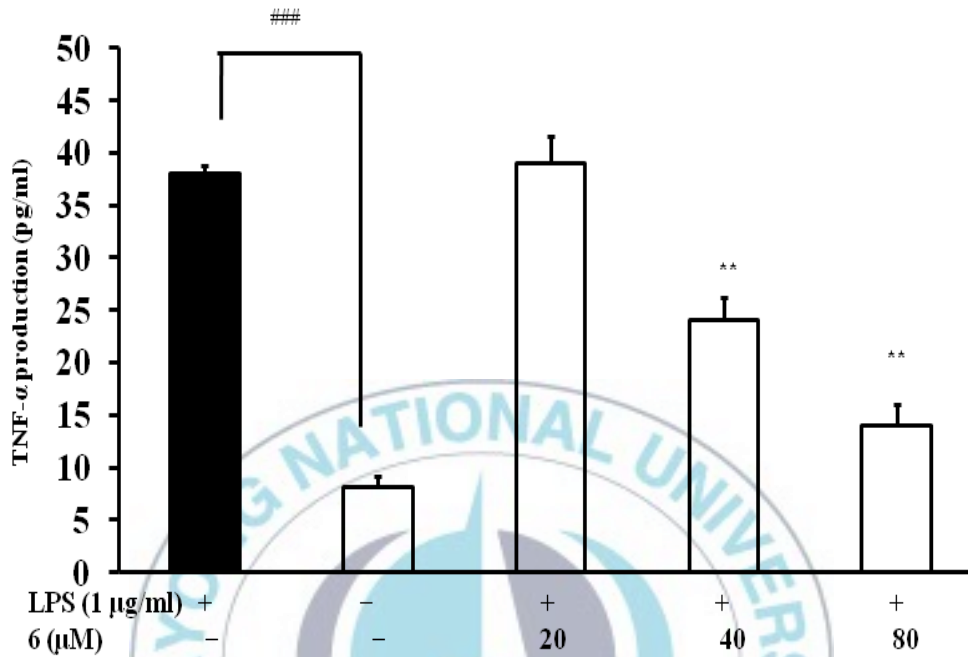


Figure 15. Effect of Pd-C-II (**6**) on LPS-induced TNF-α production in RAW 264.7 cells. TNF-α in the culture medium was determined using a commercially available TNF-α ELISA kit. The values are expressed as the mean ± SD of triplicate experiments. ###$p < 0.001$ indicates a significant difference from the unstimulated control group, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ indicate a significant difference from the LPS-stimulated control group.

IV. Discussion

Inflammation is a normal biological process involving the innate and adaptive immune systems in response to tissue injury, microbial pathogen infection and chemical irritation that helps to inactivate or destroy invading organisms, remove irritants, and set the stage for tissue repair. At a damaged site, inflammation is initiated by migration of immune cells from blood vessels and release of mediators, followed by recruitment of inflammatory cells and release of reactive oxygen species (ROS), reactive nitrogen species (RNS) and proinflammatory cytokines to eliminate foreign pathogens, resolving infection and repairing injured tissues (Pan et al., 2009; Medzhitov, 2008). A typical inflammatory response consists of four components: inflammatory inducers, the sensors that detect them, the inflammatory mediators induced by the sensors, and the target tissues that are affected by the inflammatory mediators. Each component comes in multiple forms and their combinations function in distinct inflammatory pathways. The type of pathway induced under given conditions depends on the nature of the inflammatory trigger. Thus, bacterial pathogens are detected by receptors of the innate immune system, such as Toll-like

receptors (TLRs) which are expressed on tissue-resident macrophages and induce the production of inflammatory cytokines (e.g., TNF, IL-1, IL-6) and chemokines (e.g., CCL2 and CXCL8), as well as prostaglandins. These inflammatory mediators then act on target tissues, including local blood vessels, to induce vasodilation, extravasation of neutrophils, and leakage of plasma into the infected tissue. Neutrophils recruited from the circulation, tissue-resident macrophages, and mast cells seek and destroy invading pathogens. This process is aided by plasma components, including antibodies and complement. In addition, IL-1, TNF, and IL-6 can have systemic effects when secreted in sufficient amounts. They induce liver cells (hepatocytes) to produce acute phase proteins such as C-reactive protein and coagulation factors, and they activate brain endothelium to produce prostaglandins, including the major proinflammatory prostaglandin, PGE2. Locally produced PGE2, in turn, induces specific populations of neurons in the central nervous system to promote so-called sickness behavior: fever, anorexia, fatigue, sleepiness, and social withdrawal (Pecchi et al., 2009). A successful acute inflammatory response results in the elimination of the infectious agents followed by a resolution and repair phase, which is mediated mainly by tissue-resident and recruited macrophages (Serhan and

Savill, 2005). Thus, the acute inflammatory responses are beneficial for host defense, however, the chronic or persistent inflammation is considered to be the hallmark of a multitude of severe and prevalent diseases, such as atherosclerosis, rheumatoid arthritis, multiple sclerosis, psoriasis, Crohn's disease, and asthma (Barreiro et al., 2010). Because inflammation involves many inflammatory mediators and path- ways that lead to a wide range of changes in pathology, it is difficult to target the desired area when treating inflammation. The current treatment of inflammatory disorders in Western medicine often involves the extensive use of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids. Recently, more attention has been focused on both plants and plants-derived components due to their anti-inflammatory efficacy, which results from their multi-component features including the ability to affect multiple targets and levels signaling pathways and their multiple mechanisms of mitigating inflammation (Drayton et al., 2006).

Angelica, a circumboreal genus, is by far the largest genus of Apeaceae in the Korean flora. The genus *Angelica* comprises of more than 60 different species, among them about 20 species are found in Korea (Woo et al., 1980). Many species of this genus have traditionally been used as anti-

inflammatory, diuretic, expectorant and diaphoretic, and remedy for colds, flu, influenza, hepatitis, arthritis, indigestion, coughs, chronic bronchitis, pleurisy, typhoid, headaches, wind, fever, colic, travel sickness, rheumatism, bacterial and fungal infections and diseases of the urinary organs (Sarkar and Nahar, 2004). Because of the high therapeutic value of the traditionally used Angelica species, extensive research has been carried out on this genus. In particular, many species have been reported to have promising therapeutic potential in treating inflammation-associated disorders. Lee et al. reported that the ethanolic extract of *A. dahurica* significantly reduced airway eosinophilia, cytokine levels, including interleukin (IL)-4, IL-5, and tumor necrosis factor (TNF)-alpha levels, mucus production and immunoglobulin (Ig)E *via* induction of hemeoxygenase (HO)-1 in an ovalbumin-induced airway inflammation model in mice, and thereby mitigating airway inflammation (Lee et al., 2011). In addition, the EtOAC fraction of *A, dahurica* also inhibited LPS-induced NO, PGE2 and TNF-α production as well as expression of iNOS and COX-2 in RAW 264.7 cells through blockade of the phosphorylation of MAPKs, following I-κBα degradation and NF-κB activation (Kang et al., 2007). The ethanolic extract of *A. gigas* inhibited local and systemic inflammatory and allergic reactions

through nuclear factor-κB and extracelullar signal-regulated protein kinase-dependent inflammatory pathways (Joo et al., 2010). Low dose of *A. sinensis* EtOAc extract that inhibits the production of inflammatory mediators such as TNF-α, IL-6, macrophage inflammatory protein-2 (MIP-2) and NO secretions from LPS/IFN-γ-stimulated RAW 264.7 cells, alleviates acute inflammatory hazards and protect mice from endotoxic shock. Two compounds, ferulic acid and z-ligustilide were identified as the major contributor to the anti-inflammatory activity of *A. sinensis* (Chao et al., 2010).

Among them, *A. decursiva* has been long used in Korean traditional medicine as an antitussive, analgesic, antipyretic and cough remedy (Lee et al., 2009). In a recent study, the potential antioxidant and anti-inflammatory activity of the MeOH extract of *A. decursiva* and its different solvent soluble fractions have been reported. Among the fractions, the EtOAc fractions showed strong antioxidant and anti-inflammatory activities, while the CH_2Cl_2 fraction-derived 90% MeOH soluble fraction showed the most promising anti-inflammatory activity. Five compounds including nodakenin, nodakenetin, umbelliferone, vanillic acid and umbelliferone 6-carboxylic acid were isolated from the active EtOAc fraction, among which vanillic

acid and umbelliferone 6-carboxylic acid were isolated first time from this plant. Vanillic acid showed promising antioxidant activity but no anti-inflammatory activity, however, umbelliferone 6-carboxylic acid showed strong antioxidant and anti-inflammatory activities (Dafang et al., 2012). Another *in vitro* and *in vivo* study revealed that umbelliferone 6-carboxylic acid could be a promising anti-inflammatory agent, and its anti-inflammatory effect was attributed to the inhibition of inflammatory mediators such as NO, prostaglandin E2 (PGE2), TNF-α, iNOS, and COX-2 *via* the inhibition of nuclear factor κB (NF-κB) activation pathway (Islam et al., 2012). Although the 90% MeOH soluble fraction of the CH_2Cl_2 fraction was found as the most active anti-inflammatory fraction, compounds responsible for anti-inflammatory activity were not identified yet. Therefore, the present study was focused on the bioactivity-guided isolation of anti-inflammatory constituents from the most active 90% MeOH fraction of *A. decursiva*.

NO is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the human body including circulation and blood pressure, platelet function, host defense, and neurotransmission in central nervous system and in peripheral nerves. To understand the role of

NO in regulating host defense and immunoresponses has become a target of intensive research and drug development since its discovery (Riku et al., 2005). NO is synthesized and released into the endothelial cells from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes. Active NOS is a tetramer formed by two NOS proteins and two calmodulin molecules which convert L-arginine into L-citrulline producing NO in the process, and it requires NADPH and O_2 as cosubstrates while (6R)-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haem) as co-factors in such conversion (Knowles and Monacada, 1994; Marletta, 1994; Alderton et al., 2001). Three quite distinct isoforms of NOS have been identified, products of different genes, with different localization, regulation, catalytic properties and inhibitor sensitivity, and with 51-57% homology between the human isoforms. These isoforms are referred to by the most common nomenclature: nNOS, being the isoform first found in neuronal tissue, iNOS being the isoform which is inducible in a wide range of cells and tissues, and eNOS, being the isoform first found in vascular endothelial cells. These isoforms have in the past been also differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression, and their calcium-dependence (eNOS and

nNOS) or –independence (iNOS), but such things now appear to be by no means that straightforward (Wendy et al., 2001). The molecular mechanisms that mediate the biological activities of NO can be divided into three categories: Firstly, NO reacts readily with transition metals, such as iron, copper and zinc present in prosthetic groups of enzymes and other proteins, and regulates the activity of various enzymes; Secondly, NO is able to induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation, and modifies the activity of several proteins involved in cellular regulatory mechanisms (Stamler et al., 2001); Thirdly, NO reacts very quickly with superoxide anion (O_2^-), resulting in the formation of peroxynitrite (ONOO-), a powerful oxidant that is able to modify proteins, lipids and nucleic acids. The reaction between NO and superoxide anion is very rapid, the rate constant of the reaction being about three times greater than the rate of superoxide decomposition by superoxide dismutase (SOD). The rate of ONOO- production is strongly dependent on the presence of NO and superoxide, and ONOO- formation is favored in an environment containing equivalent amounts of NO and superoxide. Sources of superoxide production are mainly considered to be mitochondria and immune cells (macrophages and granulocytes), and the synthesis of both

NO and superoxide is increased in inflammation (Davis et al., 2001). Excessive peroxynitrite formation leads to nitrated proteins, inhibition of mitochondrial respiration, depletion of cellular energetics, DNA damage, apoptosis and necrotic cell death, resulting in cellular/tissue injury (Stamler et al., 2001; Davis et al., 2001). Endothelial cells normally produce small amounts of NO in response to specific stimuli, but when macrophages exposed to certain cytokines, interferon-γ or to endotoxin, a bacterial cell wall product, they can produce much larger, potentially cytotoxic amounts of NO; such levels of NO play a key role in macrophage-mediated killing of tumor cells and microbial pathogens (Hibbs et al., 1987; Kilbourn and Belloni, 1990). Several studies have revealed that the disruption of this signaling pathway due to massive and sustained production of NO may lead to various inflammatory diseases such as vasculitis (Mulligan et al., 1992), arthritis (Farrell et al., 1992), asthma (Hamid et al., 1993).

In the present study, the 90% MeOH fraction was selected for chromatographic separation based on its potent NO production inhibitory activity as well as relatively high yield. Chromatography of the 90% MeOH fraction yielded 10 subfractions (F-1 ~ F-10), among which F-3 ~ F-5 were found to be potent inhibitors of LPS-stimulated NO production in RAW

264.7 cells. It is to be noted that the TLC profile of F-3 and F-4 is quite similar, therefore, F-3 and F-5 were selected for extensive chromatography in order to isolate active anti-inflammatory components. Repeated column chromatography of F-3 yielded two coumarins, including compounds **2** and **3**, while repeated chromatography of F-5 yielded seven coumarins, including compounds **1**, **4**, **5**, **6**, **7**, **8**, and **9**. Compounds **2** ~ **3** and **5** ~ **9** were previously reported from *A. decursiva*, however, this is the first report of compounds **1** and **4** in *A. decursiva*. Compound **1** was previously isolated from *A. edulis* (Kawasaki et al., 1984), while compound **4** was isolated for the first time from natural sources. Compound **4**, [α] +138.5 ($CHCl_3$), isolated as an amorphous powder with a molecular formula of $C_{19}H_{20}O_6$ on the basis of a pseudo-molecular ion peak at m/z 367.3585 [M+Na] in its HRFAB mass spectrum. Its IR (νmax 1720^{-1}cm, of α-pyrone and ester) and UV (λmax 324.8 and 257.2 nm, α, β-unsaturated C=O) spectra were almost identical to that of compound **5**, indicating their similar nature. The ^{1}HNMR spectrum of compound 4 was very similar to that of compound 5, except for the replacement of the senecioyl group at C-3' position in compound 5 by an angeloyl group [δ 6.21 (1H, dd, *J* = 1.3 & 7.0 Hz, H-3"), δ 1.93 (3H, d, *J* = 1.2 Hz, H-5"), δ 2.01 (3H, d, *J* = 1.2 & 7.0 Hz, H-4")]. The angeloyl group

could be located at C-3′ by a HMBC correlation between carbonyl ester carbon at δ 167.8 and C-3'at δ 76.3. The coupling constants of methine signals at δ 5.11 (H-3') and 4.77 (H-4') are 7.8 Hz, respectively, thus showing the 3'4'-*trans* configuration in compound 4. Basic hydrolysis (0.5 N KOH in dioxane) of compound **4** afforded (-)-*cis*-decursidinol and (+)-*trans*-decursidinol (**7**), identified on TLC analysis by comparison with authentic samples, indicating the absolute configurations of C-3' and C-4' are *S* and *R*, respectively (Sano et al., 1975). Detailed analysis of the NMR data, aided by ^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC spectra and comparison with compound **5**, allowed establishment of the structure of compound **4**. Thus the structure of compound **4** was elucidated as 3'(*S*)-angeloyloxy-4'-(*R*)-hydroxy-3,4,-dihydroxanthyletin, and was given a trivial name, angedecursin. An structural isomer having an angeloyl group in an alternative position at C-4' [3'(*S*)-hydroxy-4'-(*R*)-angeloyloxy-3,4,-dihydroxanthyletin (decursitin D)] from the same plant *Peucedanum decursivum* (Yao and Kong, 2001) and a diasteriomeric angeloyl derivative of which C-4' configuration was the same as that of **4** but C-3' differ [3'(*R*)-angeloyloxy-4'(*S*)-hydroxy-3,4,-dihydroxanthyletin (L1-2)] from *Libanotis laticalycina* (Baba et al., 1991) were reported.

Among the isolated coumarins, compounds **8** and **9** were previously reported to possess poor NO production inhibitory activity, therefore, compounds **1** ~ **7** were selected in this study. Among the tested coumarin derivatives, compound **1** was found as the most potent inhibitor of NO production in LPS-stimulated RAW 264.7 cells followed by compound **2**. In addition, compounds **3**, **4**, and **5** showed moderate NO production inhibitory activity, while compound **6** showed weaker NO production inhibitory activity compared to compound **1** and **2**. In contrast, compound **7** did not exhibit any suppressive effect on NO production in LPS-stimulated RAW 264.7 cells at the concentration tested. Therefore, considering the NO production inhibitory activity of compounds **2** ~ **5**, it can be speculated that esterification of –OH at 3' or 4' position of compound **7** with senecioyl or angeloyl or acetyl group is essential for exhibiting NO production inhibitory activity by these coumarins derivatives, and the position and number of senecioyl or angeoloyl group on these coumarins largely affects their potency. Moreover, compound **5** is more active than compound **6** implying that esterification with senecioyl group at 3' position has more influence on NO production inhibitory activity than esterification at 4' position for the senecioyl substituted dihydroxanthyletin type coumarins. In addition,

esterification with angeloyl group is more active than esterification with senecioyl group which is evidenced by comparing NO production inhibitory activity of compound **4** and **5**. Interestingly, free OH group at either 3' or 4' positions decreased the NO production inhibitory potential of these coumarins, while the presence of free OH group at both 3' and 4' positions completely diminished their inhibitory potential as observed in case of compound **7**.

To investigate whether the inhibitory effects of the compounds on NO production *via* the inhibition of the corresponding gene expressions, the protein iNOS was evaluated by Western blot analysis. Consistent with our previous results, all active coumarins (**1** ~ **6**) that showed potential NO production inhibitory potential also inhibited iNOS protein levels in a dose-dependent manner (**Figure 9(a)** ~ **(f)**). Therefore, it can be concluded that the potent NO production inhibitory activity of these coumarin derivatives isolated from *A. decursiva* was attributed to the inhibition of iNOS protein expression.

COX is the key enzyme responsible for the biosynthesis of prostaglandin from arachidonic acid. Prostaglandins synthesis involves phospholipase catalyzed release of arachidonic acid from membranes

phospholipids and its conversion by the COX enzyme to prostaglandins. There are mainly two isoforms of COX: COX-1 exhibits the futures of housekeeping enzyme, and is constitutively expressed virtually in all tissues and plays housekeeping role, while COX-2 is an inducible enzyme produced in response to a variety of proinflammatory stimuli (Smith et al., 1996; Griswold and Adams, 1996). Therefore, selective inhibition of COX-2, sparing COX-1, emerged as the new way of treating inflammatory disorders with greater safety. In the present study we have also investigated the effect of isolated compounds from the 90% MeOH fraction on LPS-stimulated COX-2 expression in RAW 264.7 cells. Among the coumarin derivatives, compound **1** ~ **4** showed promising COX-2 inhibitory potential in a dose-dependent manner in LPS-stimulated RAW 264.7 cells (**Figure 9(a)** ~ **(d)**). Although compound **5** and **6** inhibited iNOS protein expression, they did not inhibit COX-2 protein expression at the tested concentrations (**Figure 9(e)** & (**f)).**

TNF-α, a macrophage-derived pro-inflammatory cytokine, can have direct effects on vascular endothelial cells to induce chemotactic factors, other cytokines, cell adhesion molecules, and to facilitate the infiltration of leukocytes, all of which contribute to the inflammatory process (Chai et al.,

2008; Zhou et al., 2005). Therefore, identification of novel agents which can reduce the pro-inflammatory effects of TNF-α protein is expected to be beneficial for the treatment of inflammation-associated disorders (Reale et al., 2009). Inhibitory effect of the isolated compounds from the 90% MeOH fraction on TNF-α production in LPS-stimulated RAW 264.7 cells is presented in **Figures 10 ~ 15**. As shown in Figure 4, all tested coumarins (**1 ~ 6**) 1derivatives showed different degree of inhibitory potential against LPS-stimulated TNF-α production in RAW 264.7 cells (**Figures 10 ~ 15**).

V. Conclusion

By virtue of the development of biomedical science, it is well known that chronic inflammation is clearly involved in the pathogenesis of many diseases such as Alzheimers disease, heart disease, cancer, cardiovascular diseases, and diabetes mellitus. In this study, bioactivity-guided isolation of active principles from the most active 90% MeOH fraction yielded 8 known coumarin derivatives along with a new coumarin, 3'(*S*)-angeloyloxy-4'-(*R*)-hydroxy-3,4,-dihydroxanthyletin (**4**). Among them, compounds **1** ~ **6** strongly inhibited NO production in LPS-stimulated RAW 264.7 cells, which might be attributed to their ability to down-regulate LPS-stimulated iNOS protein expression. In addition, they also significantly inhibited TNF-α production in LPS-stimulated RAW 264.7 cells. Moreover, compounds **1** ~ **4** strongly down-regulated the expression of COX-2 protein in LPS-stimulated RAW 264.7 cells. Although these coumarins except compounds **1** and **4** have been previously reported from *A. decursiva*, the potential anti-inflammatory activity of these coumarin derivatives are reported herein for the first time. Considering all results obtained from this study, the potential

anti-inflammatory activity of the 90% MeOH fraction of *A. decursiva* might be attributed, at least in a part, to the presence of these coumarin derivatives. Therefore, *A. decursiva* might be a potential source of anti-inflammatory constituents which could be explored further in order to develop therapeutic remedy for the treatment of inflammation associated disorders. However, further research should be carried out to clarify the mechanism of actions of these coumarin derivatives.

VII. References

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