Thesis for the Degree of Master of Science

Simultaneous HPLC quantification and comparative anti-inflammatory activity of *Ixeris dentata*, *Ixeris dentata* var. *albiflora* and *Ixeris sonchifolia* in LPS-stimulated RAW 264.7 cells

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(고들빼기, 흰씀바귀, 씀바귀의 HPLC 정량과 LPS로 유도된 RAW 264.7 세포에서의 상대적인 항염증 활성)

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by

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Table of Contents

LIST OF TABLE

Table **Page**

LIST OF FIGURES

Figure **Page**

List of Abbreviations and Symbols

IDA	: *Ixeris dentata* var. *albiflora*
ID	: *Ixeris dentata*
IS	: *Ixeris sonchifolia*
^{1}H NMR	: proton nuclear magnetic resonance
^{13}C NMR	: ^{13}Carbon nuclear magnetic resonance
TLC	: thin layer chromatography
HPLC	: high performance liquid chromatography
IC_{50}	: 50% inhibitory concentration of the test samples
Mp	: melting point
UV	: Ultraviolet
Hz	: hertz (sec^{-1})
RP	: reverse phase
DMSO-d_6	: deuterium dimethyl sulfoxide
Fig	: Figure
J	: coupling constant (Hz)
Δ	: chemical shift

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ABSTRACT

Species of the genus *Ixeris* belonging to Compositae family are used as food garnish and traditional herbal medicines for strengthening of the stomach, sedatives, and diuretic agents in Korea. Extensive phytochemical investigations of the *Ixeris* genus have been carried out, and components including sesquiterpenes lactones, triterpenes, phenylpropanoids, phenols, amino acids, fatty alcohols, and fatty acids have been isolated and shown to exhibit diverse significant bioactivities such as antioxidant, antitumor, hepatoprotective, cardiovascular, and neuroprotective effects. However, *Ixeris* are known to have a high content of flavonoids, which have been established as the main bioactive constituents. Among these plants, *Ixeris dentata* Nakai (ID), *Ixeris dentata* var. *albiflora*

Nakai (IDA) and *Ixeris sonchifolia* Bge Hance (IS) are herbaceous plants used in Korea. Therefore, the aim of the present study was to evaluate the comparative anti-inflammatory activities of *Ixeris dentata* (ID), *Ixeris dentata* var. *albiflora* (IDA) and *Ixeris sonchifolia* (IS) belonging to Compositae family along with simultaneous HPLC quantification of the main compounds present in extracts. Anti-inflammatory activity was evaluated via lipopolysaccharide (LPS)-induced nitric oxide (NO) production in RAW 264.7 murine macrophages. Five main compounds consisting of chlorogenic acid, caffeic acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, and luteolin were used for simultaneous HPLC quantification. The total phenolic content present in ID (30 mg/g GAE), IDA (35.33 mg/g GAE) and IS (43.79 mg/g GAE) is correlated to the corresponding LPS-induced NO production inhibitory effect in RAW 264.7 cells as expressed with IC_{50} values 26.19, 21.43 and 7.59 μg/ml, respectively. Luteolin 7-*O*-glucoside was found as the major compound in ID (8.76 mg/g dry weight) and IDA (10.35 mg/g dry weight) and it was luteolin 7-*O*-glucuronide in case of IS (34.66 mg/g dry weight). The LPS-induced NO production IC_{50} value for luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide was 30 and 4.5 μM, respectively. Furthermore, these two compounds exhibited potent inhibitory activities of IC_{50} 30 and 4.5 μM with regard to LPS-induced NO production in RAW 264.7 cells. Further, luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide suppressed the expression of iNOS and COX-2, and *t*-BHP-induced ROS generation in LPS-stimulated

RAW 264.7 cells. These results clearly showed that the anti-inflammatory potential of ID, IDA and IS extract are primarily due to their higher content of luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide, respectively.

I. Introduction

The genus *Ixeri*s is classified in the Compositae, subfamily Cichorioideae, which is composed of 50 species, mainly found in the East and South East Asia. *Ixeris* species are used as medicines for strengthening of the stomach, sedatives, and also as diuretic agents (Zhong Yao Da Ci Dian, 1985). Many species of the *Ixeris* genus are used as vegetables and traditional herbal medicines. A wide range of phytochemical investigations of the *Ixeris* genus have been carried out, and in total 228 components including sesquiterpenes lactones, triterpenes, phenylpropanoids, phenols, amino acids, fatty alcohols, and fatty acids have been isolated and shown to exhibit significant bioactivities such as antioxidant, antitumor, hepatoprotective, cardiovascular, and neuroprotective effects (Zhang et al., 2013). Plants of the *Ixeris* genus also have a high content of flavonoids, which have been established as the main bioactive constituents. Among these plants, *Ixeris dentata* Nakai (ID), *Ixeris dentata* var. *albiflora* Nakai (IDA) and *Ixeris sonchifolia* Bge Hance (IS) are herbaceous plants used in Korea. ID is also known as Seumbagwi and is a herbal medicine used in Korea to treat indigestion, pneumonia, hepatitis, and tumors (Ahn et al.,

2006). Fermented ID prepared as kimchi is frequently eaten in Korea (Park et al., 2008). ID is known to contain aliphatic compounds, triterpenoids, and sesquiterpene glycosides (Seto et al., 1986) and has been shown to exert various activities such as hypoglycemic, hypolipidemic (Choi et al., 1990), antioxidant (Hong et al., 2010) and anti-inflammatory effects (Kim et al., 2013). IDA known as hinseumbagwi is also used as a traditional medicine in Korea that contains several aliphatics, triterpenoids and sesquiterpenoids (Seto et al., 1986) to treat dyspepsia, diabetes, liver disease, as an appetizer; and for its potent antioxidant effect (Hong et al., 2010). IS is known as Godulbaegi in Korea and has been used traditionally as a folk medicine for its anti-inflammatory and hemostatic effects (Ye et al., 2007). In the southern province of Korea, IS is also used for foodstuffs. One of the preparations of IS, Kudiezi, consists of an injection from the refined extract of whole herb and has been used to treat cerebral infarction, effort angina, and coronary heart disease for several years in clinics (Huang, 2008; Wang, 2008). IS is also known to contain various flavonoids, sesquiterpenes, lactones, and phenolic acids (Shi et al., 2011).

Flavonoids are common secondary metabolites in the plant kingdom which play a vital role in plant metabolism and are considered relatively

non-toxic bioactive substances. Those flavonoids differing by the type and numbers of substitution patterns show anti-inflammatory and free radical scavenging activities (Moroney et al., 1988; Bors and Saran, 1987). Flavonoids have been shown to inhibit cyclooxygenase (COX), lipoxygenase, microsomal mono-oxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADH oxidase, all involved in reactive oxygen species generation (Pietta, 2000; Chang and Hsu, 1992; Hirano et al., 2004). Luteolin a flavone has a C6 - C3 - C6 structure and possesses two benzene rings (A, B), a third, oxygen-containing (C) ring, a 2-3 carbon double bond and hydroxyl groups at carbons 5, 7, 3' and 4' positions. The contributing structure responsible for its biological and biochemical activities is the presence of hydroxyl moieties and 2-3 double bond (Ross and Kasum, 2002; Chan et al., 2003). It is often glycosylated in plants and the glycoside is hydrolyzed to its free form during absorption (Hempel et al., 1999). It is heat stable and losses due to cooking are relatively low (Marchand, 2002). It was reported to have antioxidant, anti-inflammatory activity (Cheng et al., 2013), anti-diabetic and anti-Alzheimer's activity (Liu et al., 2014). Luteolin 7-*O*-glucoside is the glycosylated derivative of luteolin which is known to have anti-diabetic (Azevedo et al., 2010), anti-

oxidant, anti-inflammatory (Hu and Kitts, 2004), hepatic oxidative injury (Quisheng et al., 2004) and anti-atherosclerotic (Kim et al., 2006). Caffeic acid (3, 4-dihydroxycinnamic acid) has been shown to be a α-tocopherol protectant in low-density lipoprotein (LDL) (Laranjinha et al., 1995). Also, its conjugates such as chlorogenic and caftaric acids were demonstrated to be more powerful antioxidants in a number of different systems (Meyer et al., 1998 and Fukumoto and Mazza, 2000). Caffeic acid and its derivatives are good substrates of polyphenol oxidases, and under certain conditions may undergo oxidation in plant tissues or products of plant origin (Kerry and Rice-Evans, 1998). Chlorogenic acid (CGA), formed by esterification of caffeic and quinic acids, is one of the most abundant polyphenol compound found in various agricultural products such as coffee, beans, potatoes, and apples (Zhang et al., 2003). It has been reported to exhibit anti-bacterial (Almeida et al., 2006), anti-oxidant (Kono et al., 1998), anti-inflammatory (Krakauer, 2002) and anti-carcinogenic (Huang et al., 1988) activities.

Inflammation is part of the complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants. Inflammation leads to the up-regulation of several kinds of pro-

inflammatory cytokines (Ferrero-Miliani et al., 2007) which include inducible forms of nitric oxide synthase (NOS) and cyclooxygenase (COX), and are responsible for increasing levels of nitric oxide (NO) (Heiss et al., 2001). Three general isoforms of NOS have been identified, namely, endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) (Garcia-Lafuante et al., 2009). Of these three NOS isoforms, iNOS is highly expressed in lipopolysaccharide (LPS)-activated macrophages, where it contributes to the pathogenesis of septic shock (Petros et al., 1991). LPS is a component of the cell wall of gram-negative bacteria, and triggers the production of pro-inflammatory toxins and cytokines (Ju et al., 2003). Thus, inhibition of NO production by blocking iNOS expression may be a useful strategy for the treatment of various inflammatory diseases. In addition to iNOS, the COX enzyme converts exists as two isoforms (COX-1 and -2). COX-2, the inducible form of COX, is responsible for the production of large amounts of pro-inflammatory cytokines at sites of inflammation (Brock et al., 1991). Enhanced production of NO is responsible for certain forms of acute and chronic inflammation. NO is a short-lived, gaseous radical that diffuses rapidly and directly to intracellular environments, not requiring specific membrane receptors (Schmidt and

Walter, 1994). It is the smallest biosynthetically derived secretory product of mammalian cells (Ialenti et al., 1992) that have anti-inflammatory properties under certain circumstances or pro-inflammatory activities in other cases (Iuvone et al., 1994). NO, produced by the oxidation of L-arginine by a family of isoenzymes (NO synthases) is considered as an important mediator in a variety of physiological and pathophysiological processes. NO produced by the constitutive endothelial NOS (eNOS) is involved in the regulation of blood pressure, organ blood flow distribution and the inhibition of the adhesion and activation of platelets and polymorphonuclear granulocytes. iNOS can be induced by pro-inflammatory agents such as endotoxin, interleukin-1β, tumor necrosis factor-α and interferon-γ in a variety of cells. COX-2 and iNOS are responsible for the production of the mediators like prostaglandins (PG) and NO which in turn are responsible for the progression of inflammation (Schmidt and Walter, 1994; Simon, 1999). Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen. During the normal metabolism of oxygen, ROS is formed as a natural byproduct and exerts important roles in cell signaling and homeostasis. During times of environmental stress (e.g., UV or heat exposure), its levels can increase

dramatically (Devasagayam et al., 2004). This in turn results in tissue damage and leads to various conditions like septicaemia, rheumatoid arthritis and inflammatory bowel disease (Darley-Usmar et al., 1995). Hence, enhanced expression of iNOS and COX-2 proteins are associated with an inflammatory response and it seems vital to reduce their expression in order to reduce inflammation (Chen et al., 2001).

High-performance liquid chromatography with ultraviolet– visible detection (HPLC-UV) has been applied to study IS composition, resulting in the identification of various flavonoids, phenolic acids, and adenosine present in IS (Kong et al., 2006; Yin et al., 2007). HPLC play an important and critical role in the field of pharmaceutical industries and analysis, since it is used to test the products and to detect the raw ingredient used to make them i.e., qualitative and quantitative analysis. The most important benefits gain from the uses of HPLC technique in the analytical field is to help in structure elucidation and quantitative determination of impurities. Quality control analysis of the active components of herbs is very important for their safe and effective use, and quantitative analysis remains the most direct method for this process. Thus, there is a strong demand for simple, rapid, and reliable methods for systematic analysis (Sun et al., 2012). The

chemical constituents of ID, IDA and IS have been studied by a number of researchers. Several components of ID, IDA and IS including flavonoids with potent anti-inflammatory activity have been identified. However, there has been no report on the simultaneous quantification and comparative anti-inflammatory activities of these three plants. Importantly, such simultaneous quantification would enable determination of individual compounds possessing anti-inflammatory activity, thereby providing a suitable means for quality control.

The purpose of this study was to elucidate the comparative anti-inflammatory potential of ID, IDA and IS to establish them as alternate sources of agents to treat inflammation. Further, in order to control the quality and clarify the differentiation of chemical constituents, HPLC method for determining the identities and concentrations of multiple compounds was developed for quality evaluation. We also investigated the anti-inflammatory effects of the active compounds on the inhibition of NO as well as corresponding enzymes, (i.e., iNOS and COX-2) respectively, using mouse macrophage RAW 264.7 cells activated by bacterial LPS. We were particularly interested in the activity of luteolin 7-*O*-glucuronide, which is analyzed here for the first time.

II. Materials and Methods

2-1. General experimental procedures

Uncorrected melting points were measured on a melting point apparatus (Mitamura-Riken Kogyo Inc., Tokyo, Japan). All ^{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). Chemical shifts were referenced to the respective residual solvent peaks (2.50 ppm for ^{1}H NMR and 39.5 ppm for ^{13}C NMR). Thin layer chromatography (TLC) was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 x 20 cm, 0.25 mm) and RP-18 F_{254S} plates (5 x 10 cm, Merck) using 50 % H_2S0_4 as the spray reagent. All solvents for column chromatography were of reagent grade and were acquired from commercial sources.

2-2. Chemicals

LPS from Escherichia coli, Griess reagent, 3-[4,5-dimethylthiazol-2-yl] -2,5-di-phenyl tetrazolium bromide (MTT), *t*-BHP, 2',7'-dichlorodihydrofiuorescein diacetate (DCFH-DA),

ethylenediaminetetraacetic acid (EDTA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 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). Supersignal® West Pico Chemi-luminescent Substrate was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). All other chemicals and solvents were purchased from E. Merck Fluka or Sigma-Aldrich Co. unless stated otherwise.

2-3. Plant materials

ID, IDA and IS leaves were collected from Wonju, Kangwon province and authenticated by Professor H. J. Park (Sangji University, Wonju, Korea). A voucher specimen (No. 20120920) was deposited in the laboratory of Professor Park.

2-4. Methods

2-4-1. Preparation of the 70 % methanol extract

Five grams of dried leaves of ID, IDA and IS were extracted with 70 % (v/v) aqueous methanol (MeOH) under reflux for 3 hours at 95 °C twice, yielding the 70 % MeOH extract (0.69 g, yield: 15.1 %), (0.78 g, yield: 15.6 %), (1.06 g, yield: 21.2 %).

2-4-2. Extraction, fractionation, and isolation

Dried IDA leaves (1.0 kg) were refluxed with MeOH for 3 h (3 × 10 L). The total filtrate was then concentrated to dryness in vacuo at 40 °C to render the MeOH extract (146.2 g). This extract was suspended in distilled water and then successively partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield CH_2Cl_2 (32.1 g), EtOAc (19.0 g), and *n*-BuOH (31.0 g) fractions, respectively, as well as water residue (64.1 g). The EtOAc (19 g) fraction was first chromatographed over a Si gel column (6 × 80 cm) using a mixed solvent of EtOAc and MeOH (EtOAc : MeOH = 10:1 to 1:1, gradient) to yield eight subfractions (F01 ~ F08). Luteolin (340 mg) was separately purified from

fractions F03 (1.8 g) and F05 (2.7 g) with a solvent mixture of EtOAc and MeOH (EtOAc : MeOH = 6:1). Luteolin 7-*O*-glucoside (82 mg) was purified from fractions F05 and F06. Similarly, dried IS leaves (1.0 kg) were refluxed with MeOH for 3 h (3 × 10 L) then concentrated to dryness in vacuo at 40 ℃ to render the MeOH extract. Chlorogenic acid (32 mg), caffeic acid (55 mg) and luteolin 7-*O*-glucuronide (132 mg) were then simultaneously isolated from IS extract. The isolated compounds were identified and characterized by different spectroscopic methods, including ^{1}H- and ^{13}C-NMR, and by comparing with published spectral data and TLC analysis. The structures are shown in Figure 1.

The respective spectral data are as follows:

Luteolin: yellow powder; mp: 328-330 ℃; IR λmax: 3423, 2920, 1654, 1611,1500, 1367, 1267, 1164, 1032, 839 cm-1; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 12.97 (5-OH), 10.82, 9.92, 9.41 (aromatic OH), 7.41 (1H, dd, J = 9.4, 2.2 Hz, H-6′), 7.39 (1H, d, J = 2.2 Hz, H-2′), 6.88 (1H, d, J = 9.4 Hz, H-5′), 6.66 (1H, s, H-3), 6.44 (1H, d, J = 2.2 Hz, H-8), 6.18 (1H, d, J = 2.2 Hz, H-6); ^{13}C-NMR (100 MHz, DMSO-d_6): δ 181.6 (C-4), 164.1 (C-2), 163.9 (C-7), 161.5 (C-5), 157.3 (C-9), 149.7 (C-4′), 145.7 (C-3′), 121.5 (C-6′), 119.0 (C-1′), 116.0 (C-5′), 113.4 (C-2′), 103.7 (C-10), 102.9 (C-3), 98.8 (C-6), 93.8 (C-8).

Luteolin 7-*O*-β-D-glucopyranoside: pale yellow powder; mp: 254-255 °C; ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.0 (5-OH), 7.41 (1H, dd, J = 8.1, 2.1 Hz, H-6′), 7.41 (1H, d, J = 2.1 Hz, H-2′), 6.89 (1H, d, J = 8.1 Hz, H-5′), 6.78 (1H, d, J = 2.1 Hz, H-8), 6.74 (1H, s, H-3), 6.43 (1H, d, J = 2.1 Hz, H-6), 5.06 (1H, d, J = 6.9 Hz, H-1″); ^{13}C-NMR (100 MHz, DMSO-d6): δ 182.1 (C-4), 164.7 (C-2), 163.2 (C-7), 161.3 (C-5), 157.2 (C-9), 150.2 (C-4′), 146.0 (C-3′), 121.6 (C-6′), 119.4 (C-1′), 116.2 (C-5′), 113.8 (C-2′), 105.6 (C-10), 103.4 (C-3), 100.1 (C-1″), 99.8 (C-6), 95.0 (C-8), 77.4 (C-3″),76.6 (C-5″), 73.3 (C-2″), 69.8 (C-4″), 60.8 (C-6″).

Luteolin 7-*O*-glucuronide: pale yellow powder; ^{1}H-NMR (300 MHz, DMSO-d6): δ 7.35 (dd, J = 2.0, 8.0 Hz, H-6′), 7.34 (d, J = 2.0 Hz, H-2′), 6.76 (d, J = 2.2 Hz, H-8′), 6.74 (d, J = 8.0 Hz, H-5′), 6.66 (s, H-3), 6.41 (d, J = 2.0 Hz, H-6), 5.07 (d, J = 7.2 Hz, H-1″), 3.60 (d, J = 9.8 Hz, H-5″), 3.17-3.41 (m, sugar-H); ^{13}C-NMR (300 MHz, DMSO-d6): δ 181.97 (C-4), 172.21 (C-6″) 165.45 (C-2), 163.2 (C-7), 161.3 (C-5), 157.2 (C-9), 154.0 (C-4′), 147.38 (C-3′), 119.89 (C-6′), 118.36 (C-1′), 116.2 (C-5′), 112.68 (C-2′), 105.6 (C-10), 103.4 (C-3), 100.1 (C-1″), 99.8 (C-6), 95.0 (C-8), 77.4 (C-3″),76.6 (C-5″), 73.3 (C-2″), 72.31 (C-4″), 60.8 (C-6″).

Chlorogenic acid: White greyish powder ^{1}H-NMR (300 MHz, DMSO- d_6) δ ppm; 9.57 (1H, s, 4'-OH), 9.15 (1H, s, 3'-OH), 7.43 (1H, d, J = 16.11 Hz, H-7'), 7.04 (1H, d, J = 1.17 Hz, H-2'), 6.99 (1H, dd, J = 1.17 & 8.20 Hz, H-6'), 6.77 (1H, d, J = 8.20 Hz, H-5'), 6.16 (1H, d, J = 16.11 Hz, H-8'), 5.07 (1H, m, H-2 & H-6), 1H-NMR (300 MHz DMSO-d6 + D2O) δ ppm; 7.46 (1H, d, J=15.4 Hz, H-7'),7.09 (1H, s, H-2'), 7.06 (1H, d, J=8.1 Hz, H-6'), 6.83 (1H, d, J= 8.1 Hz, H-5'), 6.23 (1H, d, J=15.4 Hz), 5.11 (1H, m H-3), 3.94 (1H, m, H-5), 3.63 (1H, m, H-4),1.81 ~ 2.09 (4H, m, H-2 & H-6), ^{13}C-NMR (75.5 MHz, DMSO-d_6+D_2O) δ ppm; 174.85 (COOH), 165.71 (COO), 148.30 (C-4'), 145.53 (C-3'), 144.89 (C-7') 125.60 (C-1'), 121.31(C-

6'), 115.74 (C-2') 114.73 (C-5'), 114.31 (C-8'), 73.50 (C-1),70.44 (C-5), 68.40 (C-4), 37.18 (C-6), 36.45 (02).

Caffeic acid: yellow amorphous solid: ^{1}H NMR (CD_3OD, 500 MHz) δ: 7.55 (1 H, d, J = 15.9 Hz, H-7), 7.07 (1 H, d, J = 2.0 Hz, H-2), 6.95 (1 H, dd, J = 8.2, 2.0 Hz, H-6), 6.81 (1 H, d, J = 8.2, H-5), 6.24 (1 H, d, J = 15.9 Hz, H-8); ^{13}C-NMR (CD_3OD, 125 MHz) δ 171.6 (C-9), 149.8 (C-4), 147.6 (C-7), 147.2 (C-3), 128.3 (C-1), 123.4 (C-6), 117.0 (C-5), 116.0 (C-8), 115.7 (C-2).

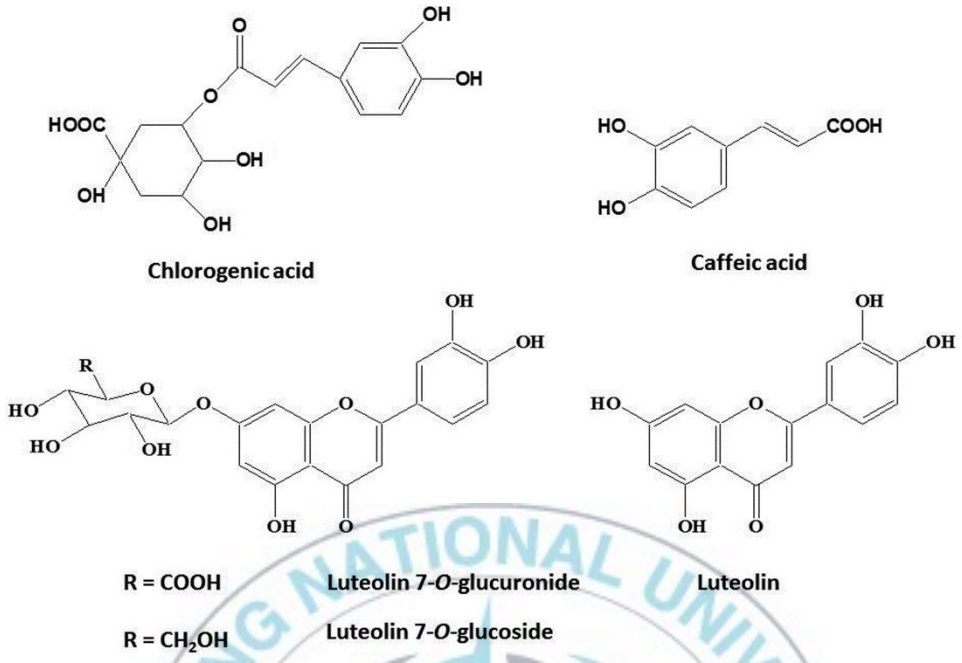

Figure 1. Structure of chlorogenic acid, caffeic acid, luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide.

2-4-3. HPLC quantitative analysis

Reverse-phase HPLC was performed on a JASCO HPLC system (Tokyo, Japan) consisting of a PU-1580 Intelligent HPLC pump, LG-1580-04 quaternary gradient unit, UV-1575 intelligent UV/vis detector, PG-1580-54 four-line degasser, and CO-1560 intelligent column thermostat. The BORWIN chromatographic system (Le Fontanil, France) was used for HPLC data analysis. Chromatographic separation was accomplished on a Phenomenex C18 reverse-phase column (Phenomenex, 4.6 x 250 cm, 5 μm) at 25 ^{o}C and monitored at 340 nm. The linear gradient solvent system consisted of 0.5 % phosphoric acid in water (solvent A) and 100 % methanol (solvent B), and was adjusted from 75 % (solvent A): 25 % (solvent B) to 0 % (solvent A) and 100 % (solvent B) over 60 min at a flow rate of 0.5 ml/min. For preparation of stock solutions, plant extracts were dissolved in 100 % MeOH at concentrations of 1 mg/ml, respectively. After filtration through a centrifugal filter device (0.45 μm, Millipore Co., Bedford, MA, USA), 10 μL of each sample was injected into the HPLC system. The retention times of respective compounds were determined using the resulting chromatograms. A calibration curve was drawn using the five standards at concentration ranging from 10 to 500 μg/ml, and the regression

equation was calculated in the form of y = ax - b, where y and x correspond to the peak area and concentration, respectively. The regression equations and correlation coefficients of **1**, **2**, **3**, **4** and **5** were as follows: y = 99265x – 429391, r^2 = 0.9989; y = 151095x – 617857, r^2 = 0.9994; y = 33194x – 292955, r^2 = 0.9995; y = 16826x – 10216, r^2 = 0.9992; and y = 73601x – 226274, r^2 = 0.9997, respectively. The relative quantities of compounds in each extract (mg/g of the extract) were calculated from these equations.

2-4-4. Determination of total phenolic content

The total phenolic content of the extract was determined using Folin-Ciocalteu reagent following a slightly modified method of (Ainsworth and Gillespie, 2007). Briefly, a volume of 0.5 mL of the 70 % MeOH extract (l00 μg/ml) was mixed with 2 mL of Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and neutralized with 4 mL of sodium carbonate solution (7.5 %, w/v). The reaction mixture was then incubated at room temperature for 30 min with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765 nm using a double beam UV-VIS spectrophotometer (UV Analyst-CT 8200). The total phenolic content was determined from the linear equation of a standard

curve prepared with gallic acid as follows: A = 0.0742 x, R = 0.9658, where A is absorbance and x is the amount of gallic acid.

2-4-5. Cell culture

RAW 264.7 murine macrophages were obtained from the American Type Culture Collection (ATCC Rockville, MD, USA). RAW 264.7 cells were cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 ^{o}C with humidified air containing 5 % CO_2.

2-4-6. Cell viability assay

Cell viability was assessed using the MTT assay as described previously (Mossman, 1983) Briefly, RAW 264.7 cells were seeded into 96-well plates at a density of 1.0 x 10^4 cells per well and incubated at 37 ^{o}C for 24 h. The cells were then treated with various concentrations of the samples. After incubation for an additional 24-h at 37 ^{o}C, 100 μL of MTT (0.5 mg/mL in PBS) was added to each well and the incubation continued for another 2 h. The resulting color was assayed at 540 nm using a microplate spectrophotometer (Molecular Devices, CA, USA).

2-4-7. Assay for inhibition of cellular NO production

The nitrite concentration in the medium was measured using Griess reagent as an indicator of NO production as previously described (Shin et al., 2008). Briefly, RAW 264.7 cells (1.0 x 10^5cells/well in a 24-well plate with 500 μL of culture medium) were pretreated with samples for 2 h and incubated for 18 h with LPS (1.0 μg/mL). After incubation, the nitrite concentration of the supernatants (100 μL/well) was measured by adding 100 μL of Griess reagent. To quantify the nitrite concentration, standard nitrite solutions were prepared. The absorbance values of mixtures were determined using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 540 nm. The iNOS inhibitor AMT was used as a positive control.

2-4-8. Analysis for inhibition of iNOS and COX-2 protein expression

Western blotting was used to measure the protein levels of iNOS and COX-2. First, RAW 264.7 cells were cultured in 100-mm culture dishes in the presence or absence of LPS (1.0 μg/ml) and with or without test samples for 18 h. Afterwards, the cells were washed twice with ice-cold PBS and lysed with cell lysis buffer (Cell Signaling Technology, Inc., #9803) on ice

for 30 min. Cell extracts were obtained by centrifugation at 14,000 g at 4 ^{o}C for 20 min. Cytosolic proteins were electrophoretically separated on SDS-PAGE and transferred to PVDF membranes. 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 then washed three times (10 mm) in TBST buffer and incubated with primary antibody, diluted 1:1000 in 5 % (w/v) non-fat dry milk in TBST buffer, at 4 ^{o}C overnight. After three washings in TBST buffer (10 min), the membranes were incubated with a 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 labeling was visualized with Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and was exposed to X-ray film (GE Healthcare Ltd., Amersham, UK). Pre-stained blue protein markers were used for molecular weight determination.

2-4-9. Assay for inhibition of intracellular reactive oxygen species generation

Reactive oxygen species (ROS) generation was assessed using the ROS-sensitive fluorescence indicator DCFH-DA (Lebel and Bondy, 1990). When DCFH-DA is introduced to viable cells, it can penetrate the cell and become deacetylated by intracellular esterases to form 2',7'-dichlorodihydrofluorescein (DCFH), which can react quantitatively with ROS within the cell and be converted to 2',7'-dichlorofluorescein (DCF), which in turn are detected using a fluorescence spectrophotometer. To determine intracellular ROS scavenging activity, RAW 264.7 cells (2.0 x 10^4 cells/well) were first seeded in black 96-well plate. After 24 h, cells were treated with samples for 2 h, followed by *t*-BHP (200 μM) to induce ROS generation. After the cells were incubated with DCFH-DA (20 μM) for 30 min, the fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a fluorescence microplate reader FL x 800 (Bio-Tek Instruments Inc., Winooski, UT, USA).

2-4-10. Statistics

Data are presented as the mean ± SD of at least four independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t* test.

III. Results

3-1. Effect of the 70 % MeOH extracts of ID, IDA and IS on cell viability and NO production in LPS-induced RAW 264.7 cells

We first evaluated the cytotoxicity of the ID, IDA and IS using an MTT assay. As shown in Fig. 2; ID, IDA and IS did not exhibit pronounced toxicity even at a concentration of 100 μg/ml. Increased NO production is a typical phenomenon that occurs in LPS-stimulated macrophages and serves as an indicator of a typical inflammatory response. Thus, the inhibitory effect of ID, IDA and IS extract on LPS-induced NO production in RAW 264.7 cells was investigated (Fig. 2). Nitrite, a stable metabolite of NO, was used as an indicator of NO production in the medium. During the 18-h incubation with LPS (1.0 μg/mL), NO production in RAW 264.7 cells increased dramatically. As shown in Fig. 2, pretreatment with the ID, IDA and IS 70 % MeOH extract resulted in a relatively high inhibition of cellular NO production. Specifically, the IC_{50} of ID was found to be 26.19 μg/ml while that of IDA and IS was found to be 21.43 μg/ml and 7.59 μg/ml. With respect to the cellular NO production assay, ID at the concentration of 100 μg/ml resulted in an 87 % inhibition while IDA and IS at the same

concentration resulted in a 89 % and 92 % inhibition compared with the positive control AMT (90 % at 0.5 μg/ml). Thus, the NO inhibitory potency of IS was greater than that of ID and IDA.

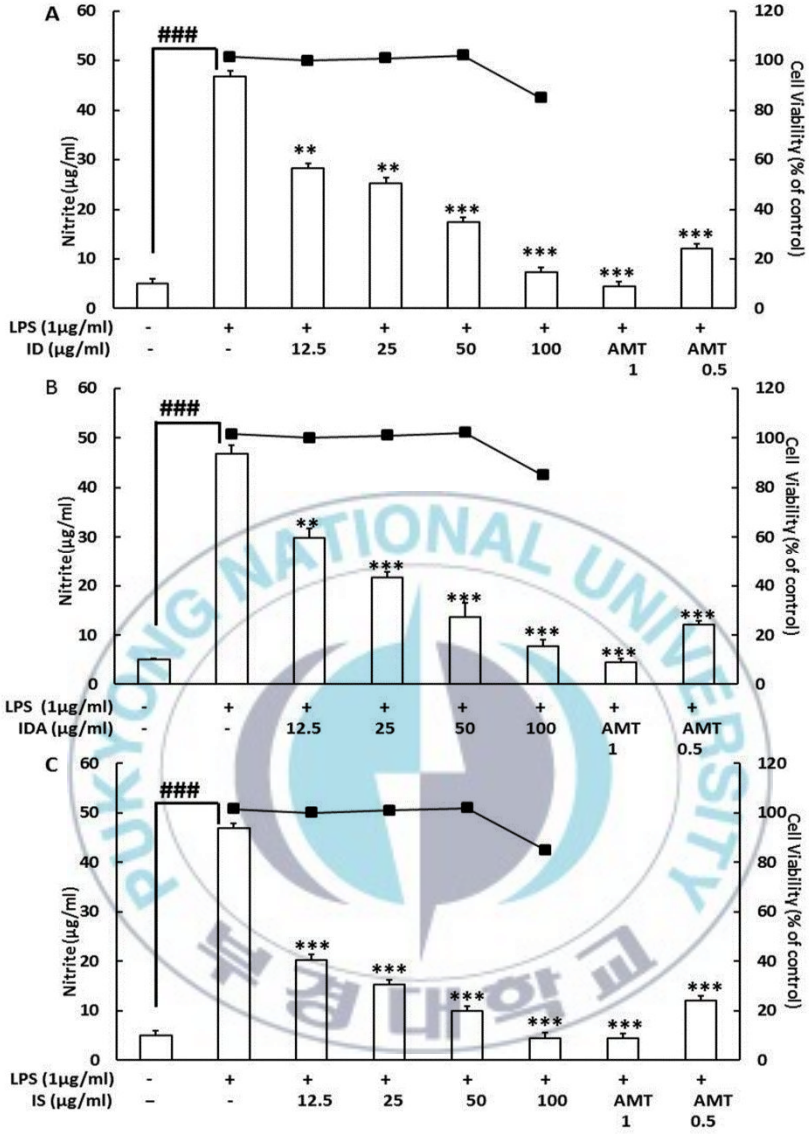

Figure 2. Effect of 70% MeOH extracts of ID (A), IDA (B) and IS (C) on LPS-induced NO production and viability in RAW 264.7 cells. RAW 264.7

cells were pretreated with the indicated concentrations for 2 h followed by LPS (1.0 μg/mL). After incubation for 18 h, the amount of NO in the culture supernatant was measured by Griess reaction assay. Cell viability was determined by the MTT method. Data represent the mean ± SD of triplicate experiments and were analyzed one-way ANOVA ###P < 0.001 versus an untreated control, **P < 0.01, ***P < 0.001 versus each treated group.

3-2. Quantitative analysis of active compounds from ID, IDA and IS

The total phenolic contents (in GAE) of 70 % MeOH extracts of ID, IDA and IS were 30 mg/g, 35.33 mg/g and 43.79 mg/g. Next, simultaneous HPLC quantitative analysis was conducted to determine the relative presence of compounds **l**, **2**, **3**, **4** and **5** in ID, IDA and IS (Fig. 3). The retention times for standard flavonoid compounds 1-5 were 16.25, 21.94, 29.65, 30.40 and 42.77 min, respectively (Fig. 3). The 70% MeOH extracts of the whole ID, IDA and IS plants exhibited major peaks with retention times of 16.25, 21.94, 29.65, 30.40 and 42.77 min, corresponding to chlorogenic acid (**1**), caffeic acid (**2**), luteolin 7-*O*-glucoside (**3**), luteolin 7-*O*-glucuronide (**4**), and luteolin (**5**) respectively. The relative amounts of respective compounds (**1**-**5**) from the 70 % MeOH extract of ID is given in table 1. The quantitative amount of compounds **1**, **2**, **3**, **4** and **5** in ID, IDA and IS as determined by HPLC was found to be in the order of 9.26, 5.10, 56.23, 2.88, 8.39 mg/g, 12.36, 6.67, 76.12, 2.32, 12.53 mg/g and 5.93, 5.3, 38.16, 175.34, 7.67 mg/g, respectively. IS contained the highest content of compound **4** whereas in the case of ID and IDA, compound **3** constituted the major portion.

Table 1: Relative amount of compounds from 70 % methanol extract

Compounds	*Ixeris dentata* (%)	*Ixeris dentata* var. *albiflora* (%)	*Ixeris sonchifolia* (%)
Chlorogenic acid	6.5	8.25	2.15
Caffeic acid	2.03	3.23	1.45
Luteolin 7-*O*-glucoside	20.87	24.31	3.68
Luteolin 7-*O*-glucuronide	0.45	1.23	49.6
Luteolin	5.18	7.34	4.89

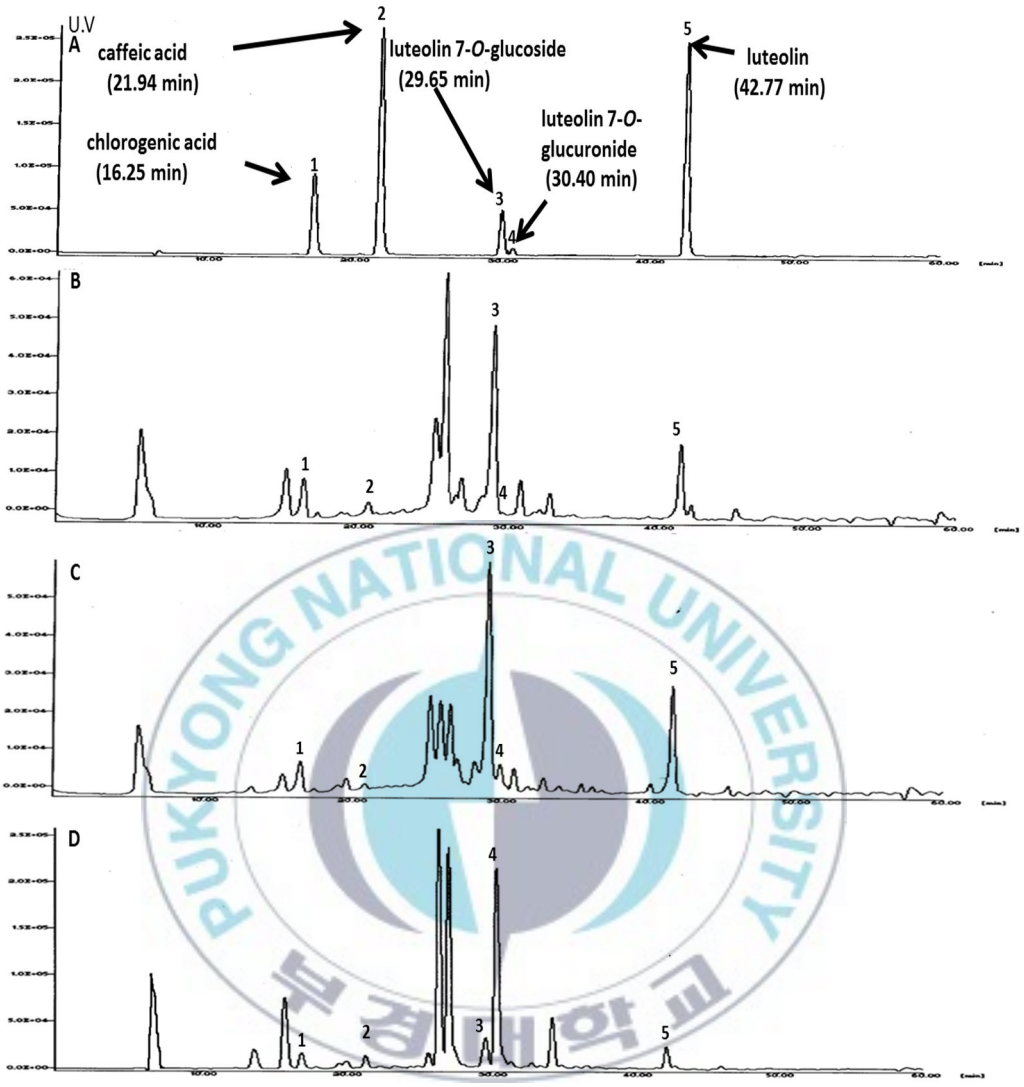

Figure 3. HPLC chromatograms of standards (A), ID (B), IDA (C) and (IS) (D). Peaks: 1. Chlorogenic acid, 2. Caffeic acid, 3. Luteolin 7-*O*-glucoside, 4. Luteolin 7-*O*-glucuronide 5. Luteolin

3-3. Effect of luteolin, luteolin 7-*O*-glucoside, chlorogenic acid, caffeic acid and luteolin 7-*O*-glucuronide on cell viability, LPS-induced NO production.

MTT assays were performed in order to evaluate the cytotoxicity of luteolin, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, chlorogenic acid, and caffeic acid obtained from ID, IDA and IS. As shown in Fig. (4A~4E), luteolin (Fig. A), luteolin 7-*O*-glucoside (Fig. B), caffeic acid (Fig. C), chlorogenic acid (Fig. D) and luteolin 7-*O*-glucuronide (Fig. E) were not toxic upto 30 μM concentration. We next investigated the inhibitory effect of the respective compounds on LPS-induced NO production in RAW 264.7 cells (Fig. A~E). The amount of nitrite, a stable metabolite of NO, was used as an indicator of NO production in the medium. During the 18-h incubation with LPS (1μg/ml), NO production in RAW 264.7 cells increased dramatically. As shown in Fig. 4A, pretreatment with compounds at concentrations of 5, 15 and 30 μM inhibited cellular NO production in a dose-dependent manner. Luteolin exerted an almost 97 % inhibitory activity at a concentration of 30 μM with an IC_{50} of 12.23 μM. Luteolin 7-*O*-glucoside exerted 50 % inhibitory activity at 30 μM with an IC_{50} of 30 μM (Fig. 4B). Likewise, caffeic acid exerted 62 % inhibitory activity with an

IC_{50} of 5.23 μM (Fig. 4C). Similarly, chlorogenic acid exerted a dose dependent inhibitory effect, with a 57 % inhibitory effect at 30 μg/ml with an IC_{50} value of 14 μM (Fig. 4D). Luteolin 7-*O*-glucuronide exerted 71 % inhibitory activity at 30 μM with an IC_{50} of 4.5 μM (Fig. 4E). Thus, all of the individual compounds appeared to have potent inhibitory effects on NO production.

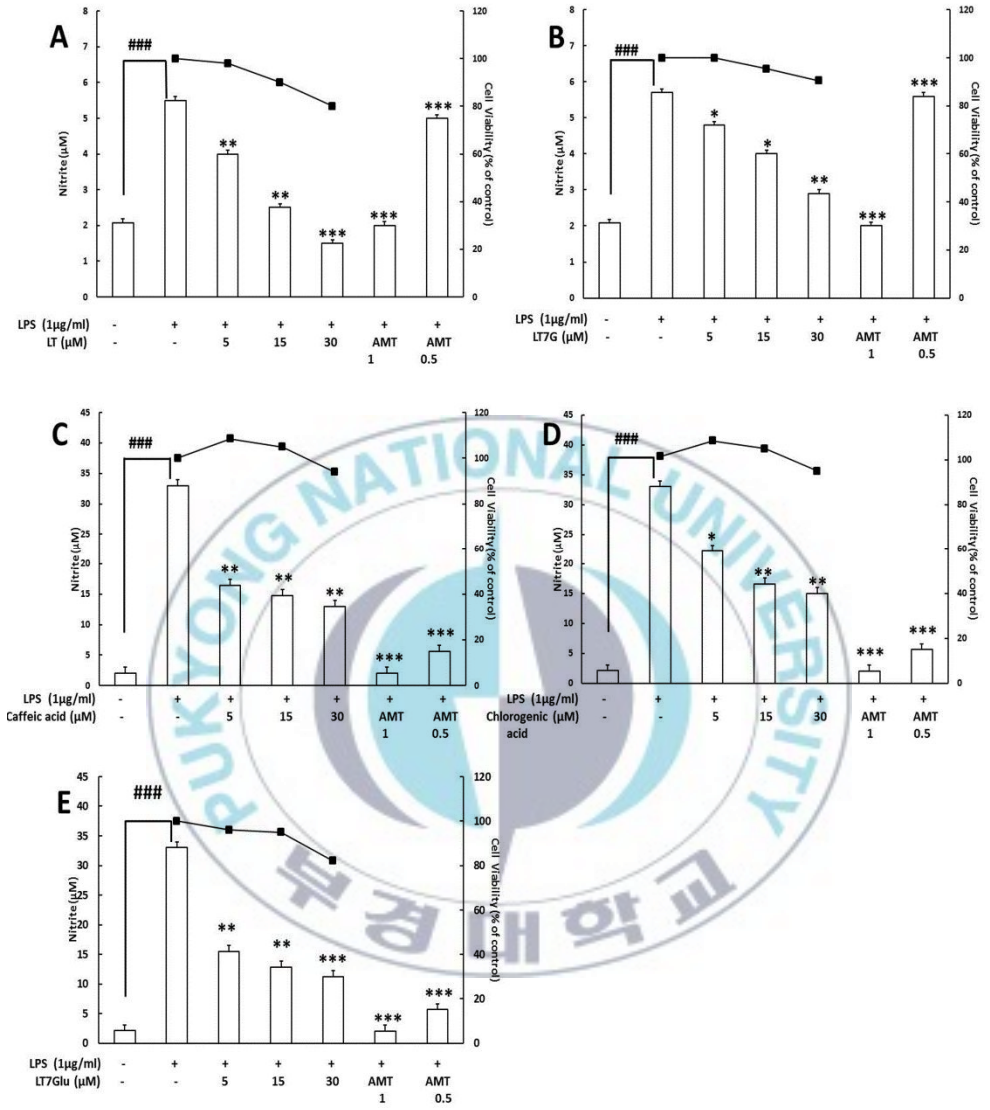

Figure 4. Effects of luteolin (LT) (A), luteoin 7-O-glucoside (LT7G) (B),

caffeic acid (C) chlorogenic acid (D) and luteolin 7-O-glucuronide (LT7Glu) (E) on cell viability and LPS-induced NO production in RAW 264.7 cells. Cell viability was determined by MTT assay. Cells were seeded in 96-well plates at a density of 1.0 × 104 cells/well and incubated at 37 ^{o}C for 24 h. The cells were then treated with various concentrations of sample (5-30 μM). After an additional 24 h of incubation at 37^{o}C, 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells and mixed well. Cells were pretreated with the indicated concentrations (5, 15, and 30 μM) for 2 h, after which LPS (1.0 μg/mL) was added. After 18 h of incubation, the NO in the culture supernatants was measured using Griess reagent. 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, ***P < 0.001 indicate significant differences from the LPS-stimulated control group.

3-4. Effect of luteolin, luteolin 7-*O*-glucoside, and luteolin 7-*O*-glucuronide on *t*-BHP-induced ROS generation in RAW 264.7 cells

As shown in Fig. (5), ROS generation in RAW 264.7 cells increased gradually during the 30 min incubation with *t*-BHP (200 μM). Pretreatment with luteolin (1.25 to 5 μM) or its 7-*O*-glucoside (0.31 to 1.25 μM) and luteolin 7-*O*-glucuronide (5 to 30 μM) for 1 h resulted in dose-dependent inhibitory effects. In particular, luteolin 7-*O*-glucoside was more active in inhibiting ROS generation with an inhibitory activity of 60 % at 1.25 μM; luteolin at the same concentration had 14.28 % inhibition. Luteolin and luteolin 7-*O*-glucoside had IC_{50} values of 2.21 μM and 0.91 μM, respectively. Similarly, the respective inhibitory activities of luteolin 7-*O*-glucuronide at 5, 15, and 30 μM were 44 %, 63 %, and 72 %, with an IC_{50} of 8.16 μM. This result was compared with trolox (positive control) which had an inhibitory activity of 75 % at 10 μM. Thus, luteolin 7-*O*-glucoside was found to be active than luteolin or luteolin 7-*O*-glucuronide.

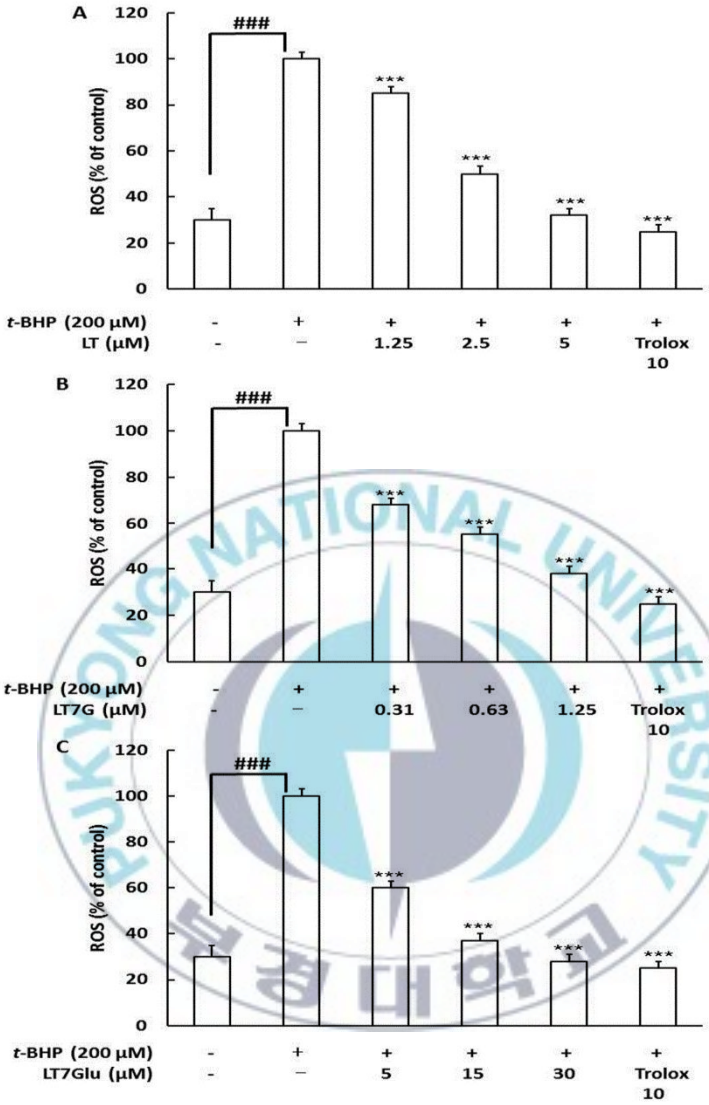

Figure 5. Effects of luteolin (LT) (A), luteolin 7-*O*-glucoside (LT7G) (B) and luteolin 7-*O*-glucuronide (LT7Glu) (C) on *t*-BHP-induced ROS

generation in RAW 264.7 cells. Cells were pre-treated with different concentrations of LT (1.25-5 μM), LT7G (0.31-1.25 μM) and LT7Glu (5-30 μM) and *t*-BHP (200 μM) and incubated for 1 hr. Control cells were treated with *t*-BHP (200 μM) in the absence of either flavonoid. The values are expressed as the mean ± SD of triplicate experiments and were analyzed by one-way ANOVA ###P < 0.001 versus an untreated control, ***P < 0.001 versus *t*-BHP treated group.

3-5. Effect of luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide isolated from ID, IDA and IS on iNOS and COX-2 expression in RAW 264.7 cells

We next used Western blot analysis to assess the activity of luteolin, its 7-*O*-glucoside and luteolin 7-*O*-glucuronide on the expression of iNOS and COX-2, which are well-characterized markers of NF-κB-responsive inflammation (Sharif et al., 2007). As shown in Fig. 6, iNOS and COX-2 protein expression in unstimulated RAW 264.7 cells was almost undetectable, but was significantly stimulated upon treatment with 1 μg/ml of LPS. Pretreatment with luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide at concentrations of 5, 15, and 30 μM significantly down-regulated the expression of iNOS and COX-2 protein in a dose dependent manner. Luteolin inhibited both the expression of COX-2 proteinn and iNOS protein also its 7-*O*-glucoside inhibited both protein expression. The effect of luteolin 7-*O*-glucuronide against COX-2 protein levels were less pronounced although dose dependent as shown by densitogram, and only at 30 μM did there appear to be a potential decrease in COX-2. However, it showed the inhibitory potential for iNOS protein expression.

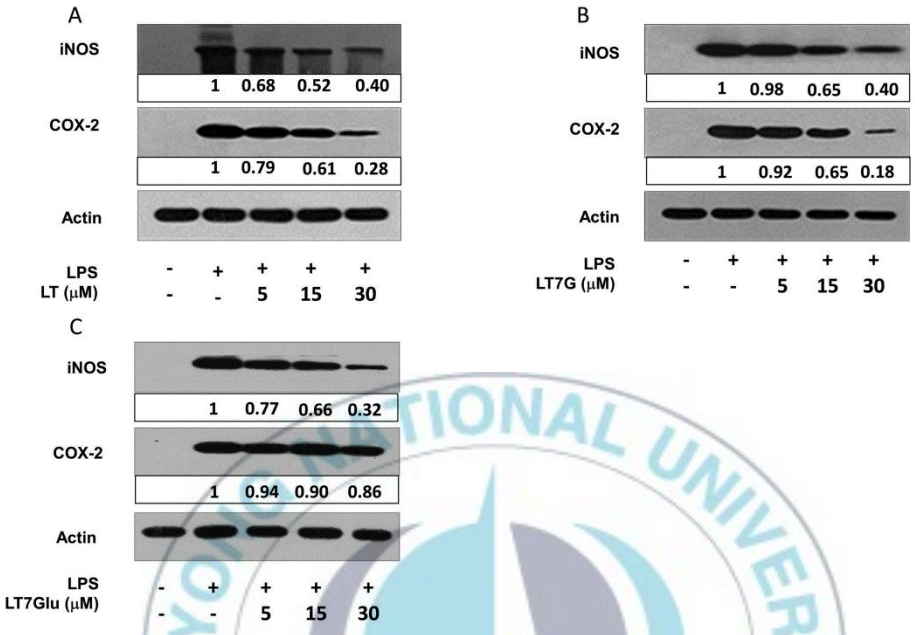

Figure 6. Effects of luteolin (LT) (A), luteolin 7-*O*-glucoside (LT7G) (B) and luteoin 7-*O*-glucuronide (LT7Glu) (C) on the expression of iNOS and COX-2 protein in RAW 264.7 cells. Cytosolic lysates from RAW 264.7 cells stimulated for 16 h were separated by SDS-PAGE and the protein levels of iNOS, COX-2, and *β*-actin were detected by Western blot.

IV. Discussion

A number of species of the *Ixeris* genus are used in Korea as traditional medicines. ID and IDA are traditional herbal medicine used to treat indigestion, pneumonia, hepatitis, and tumors, while IS is a well-known folk medicine, with parts of the entire plant used to invigorate blood circulation, normalize menstruation, and eliminate blood stasis to relieve pain. Thus, we were interested in evaluating and comparing the potential anti-inflammatory effects of ID, IDA and IS as well as to clarify the individual compounds present in these plants. We also sought to characterize the potential collective role of the components of ID, IDA and IS contributing to their anti-inflammatory effect and to develop a simple method for quality control. To this end, we systematically evaluated the potential anti-inflammatory activity of the plant extract. In addition, we determined the individual compounds present in the extract using HPLC and investigated the anti-inflammatory activity of individual compounds in RAW 264.7 cells to correlate their potential activity on LPS-induced NO production, iNOS and COX-2 protein expression, and *t*-BHP-induced ROS generation.

ID was previously reported to have no cytotoxic effects on human salivary gland cells up to a concentration of 80 μg/ml (Lee et al., 2013). Although we utilized a different cell line, our results were similar for ID, IDA and IS even at 100 μg/ml with respect to cytotoxicity. Specifically, because IDA and IS both contain similar compounds as ID, their similar cytotoxicity profiles are reasonable. Both IS and IDA exhibited potent dose-dependent anti-inflammatory effects like ID. Previously, Chung et al. (2002) and Lee. (2011) reported that ID does not have anti-inflammatory activity, but does inhibit anti-inflammatory mediators in LPS stimulated rat and RAW 264.7 cells. In addition, the presence of the active flavonoids chlorogenic acid, caffeic acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, and luteolin support our findings. IS was relatively more potent than ID and IDA at inhibiting NO, which may correlated with the fact that IS had a higher presence of phenolic compounds. In particular, IS had a significant level of individual phenolic compounds, particularly luteolin 7-*O*-glucuronide, as determined by HPLC analysis in IS.

Consistent with previous results for *Sonchus brachyotus* (Nugroho et al., 2012), the HPLC fingerprints showed the presence of the compounds chlorogenic acid, caffeic acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-

glucuronide, and luteolin. Conversely, Yin et al. (2007) stated that the earlier retention time of luteolin 7-*O*-glucuronide compared with luteolin 7-*O*-glucoside was due to the difference in the solvents system used. Luteolin 7-*O*-glucoside constituted the major portion (56.23 mg/g) of the ID and IDA (76.12 mg/g) of the extract, whereas in case of IS, luteolin 7-*O*-glucuronide (175.34 mg/g) constituted the major portion along with luteolin, chlorogenic acid and caffeic acid. The chromatograms of ID, IDA and IS helped to compare the compounds present in each. Thus, we developed a suitable analytical method for the quality control of the individual compounds of ID, IDA and IS.

Polyphenol compounds are important plant constituents because of their ability to scavenge free radicals, which is facilitated by their hydroxyl groups (Husain et al., 1989; Rice-Evans et al., 1997). IS was found to contain the highest abundance of phenolic compounds at 43.79 mg/g GAE, while ID and IDA was found to contain 30 mg/g and 35.33 mg/g GAE. Thus, the rich phenol content of both extracts supports their antioxidant activity. More specifically, the high relative contents of luteolin 7-*O*-glucoside in ID and IDA and luteolin 7-*O*-glucuronide in IS combined with their overall high anti-inflammatory activity suggests their role in

contributing anti-inflammatory activity.

Inflammation is known to cause oxidative stress with accompanying up-regulation of iNOS and increased production of NO (Valko, 2007). The ability to screen the anti-inflammatory activity of individual compounds can be used to determine the potential expected activity of totals extracts. Thus, in the present study, the respective anti-inflammatory activity of individual compounds was evaluated. All of the evaluated compounds exhibited pronounced NO inhibitory activity without cytotoxic effects in LPS-induced RAW 264.7 cells, which was consistent with previous results for chlorogenic acid (Shan et al., 2009; Fransisco et al., 2013; Kwon et al., 2010), caffeic acid (Park et al., 2009; Huang et al., 2013), luteolin (Jung et al., 2012; Nishitani et al., 2013), and luteolin 7-*O*-glucoside (Park and Song, 2013). However, luteolin showed tendency to exhibit cytotoxicity above 30 μM concentration, which was consistent with previous findings. (Hu and Kitts, 2004)

Interestingly, we found that luteolin 7-*O*-glucuronide exhibited potent anti-inflammatory activity with an IC_{50} of 4.5 μM. Thus, the activity of the extract can be correlated with the potent activities of individual compounds as suggested above. We further evaluated luteolin, luteolin 7-*O*-

glucoside and luteolin 7-*O*-glucuronide for its inhibitory effects on iNOS and COX-2 protein expression. Luteolin and luteolin 7-*O*-glucoside showed the inhibitory activity against iNOS and COX-2 protein consistent with earlier reported data (Hu and kitts, 2004). However, luteolin 7-*O*-glucuronide inhibited iNOS expression in a dose-dependent manner and had a weak effect on COX-2 expression, suggesting that it may be a potential candidate against inflammation. Nevertheless, the inhibition of both NO production and ROS generation in a dose-dependent manner in LPS- or *t*-BHP-induced macrophages was attributed to the ability of luteolin 7-*O*-glucuronide to downregulate the protein expression of iNOS and COX-2. Specifically, the oxidative stress caused by *t*-BHP generates butoxyl radicals, which in turn mediate depletion of the antioxidant system via reduction of intracellular thiols and gluthathione reserves (Quillet-Mary et al., 1997). Thus, the inhibitory activity of luteolin 7-*O*-glucuronide against *t*-BHP-induced generation of intracellular ROS in cultured RAW 264.7 cells might be attributed to its ability to scavenge free radicals.

The potent activity of luteolin and its substituted glucoside and glucuronide emphasizes the role of flavonoids attached to carbohydrates. Over the past fifteen years, a considerable number of *in vitro* studies have

sought to arrive at a common hierarchy of flavonoids in terms of substitutions of glucose moiety. These comparisons of luteolin with its glucoside and glucuronide enables a better understanding of the anti-inflammatory effects of flavonoids, and offer reasonable predictions of the influence of structural modifications conferred by glucose substitution. Most dietary flavonoids occur in food as glycosides and the incremental increase in bioavailability conferred by glucose attachment (Heim et al., 2002), highlights the importance of glucose substitution. Thus, the potent activity of luteolin glucoside and glucuronide may serve to contribute as potential alternative sources of anti-inflammatory agents. There are numerous reports regarding the potent anti-inflammatory activity of luteolin (Hu and Kitts, 2004) and luteolin 7-*O*-glucoside (Park and Song, 2013) in RAW cells. Meanwhile, luteolin 7-*O*-glucuronide is known to contribute to the potent peroxynitrite inhibition in *Sonhus brachyotus* when present in sufficiently high amounts (Nugroho et al., 2012). Thus, our identification of luteolin-7-*O*-glucuronide, which is the first report of its presence in IS extracts, may suggest that it contributes to the overall potent anti-inflammatory effect of IS. Taken together, our results elucidated the identity of contributing compounds responsible for the activity of ID, IDA and IS, and add further

support to the importance of glucuronide and glucoside attached flavonoids as anti-inflammatory agents

V. Conclusions

Chlorogenic acid, caffeic acid, luteolin 7-*O*-glucoside, luteolin 7-*O*-glucuronide, and luteolin isolated from ID, IDA and IS possess potent anti-inflammatory activity as illustrated by their inhibition of NO production and iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells. Luteolin, luteolin 7-*O*-glucoside and luteolin 7-*O*-glucuronide also inhibited iNOS and COX-2 protein and *t*-BHP-induced ROS generation in RAW 264.7 cells in a dose-dependent manner, which indicates their potential anti-inflammatory and antioxidant activity. Further, the HPLC methods used in our study can be used as a guideline to standardize individual *Ixeris* species based on luteolin 7-*O*-glucoside, the major component in ID, IDA, and luteolin 7-*O*-glucuronide, the major component of IS. Thus, we established a correlation between the anti-inflammatory activity of ID, IDA and IS, and their major components via HPLC profile and predicted both as an alternative source for anti-inflammatory compounds. However, additional unidentified phenolic compounds of ID, IDA and IS should be identified, and their respective anti-inflammatory activities and pharmacologic profiles should be determined *in vivo*.

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