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# Anti-inflammatory activity of a new cyclic peptide, citrusin XI, isolated from the fruits of *Citrus unshiu*

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**Abstract**

*Ethnopharmacological relevance*: *Citrus unshiu* (Rutaceae) is an easy-peeling citrus fruit, which has been used as a traditional Korean medicine for improving skin elasticity, relieving fatigue and cough, and preventing bronchitis, flu, and various cancers. However, its active components associated with anti-inflammation and underlying mechanisms remain unknown. In this study, we investigated the active constituents from the fruits of *C. unshiu* and evaluated the anti-inflammatory activity in order to support the traditional usage of *C. unshiu*.

*Material and methods:* Repeated column chromatography, together with a semi-preparative HPLC purification was used to separate the bioactive constituent from the EtOAc soluble fraction of the EtOH extract of *C. unshiu* fruits. Anti-inflammatory effects of the isolated compounds on lipopolysaccharide (LPS)-induced production of pro-inflammatory mediators were examined using RAW 264.7 macrophage cells.

*Results:* A new cyclic peptide, citrusin XI (**1**), was isolated and identified from the fruits of *C. unshiu*. The structure of compound **1** was elucidated by spectroscopic analysis, including 1D and 2D nuclear magnetic resonance (NMR) ($^{1}$H, $^{13}$C, COSY, HMQC and HMBC experiments), and high resolution (HR)-mass spectrometry, and its absolute configurations were further confirmed by Marfey's method. Compound **1** decreased NO

production in LPS-stimulated RAW264.7 cells in a dose-dependent manner with an $IC_{50}$ value of 70 μM. Compound **1** suppressed NO production by decreasing iNOS expression but COX-2 expression was slightly associated with the reduction by compound **1** in LPS-induced RAW264.7 cells. Furthermore, compound **1** inhibited NF-κB activation by blocking IκBα degradation and NF-κB phosphorylation in LPS-stimulated RAW264.7 cells.

*Conclusions:* These results indicate that a new cyclic peptide, citrusin XI, from *C. unshiu* fruits has anti-inflammatory properties that inhibit the release of pro-inflammatory mediators. Compound **1** decreases NO production by decreasing iNOS expression and NF-κB activation associated with IκBα degradation and NF-κB phosphorylation in LPS-induced RAW264.7 cells. This is the first study to clarify the underlying mechanism of the anti-inflammatory effect exerted by a pure isolated compound from *C. unshiu* in LPS-stimulated RAW 264.7 macrophage cells. The phytochemical, citrusin XI of *C. unshiu* may serve as lead compound in the design of new agents for preventing and treating inflammatory diseases.

**Keywords**: *Citrus unshiu*; Rutaceae; Peptide; Citrusin XI; Anti-inflammation; RAW264.7 cells

*Abbreviation:*

**NMR,** nuclear magnetic resonance; **NO,** nitric oxide; **LPS,** lipopolysaccharide; **IR,** infrared; **UV,** ultraviolet; **HR,** high resolution; **ESI,** electrospray ionization; **MS,** mass spectrometry; **COSY,** correlation spectroscopy; **HMQC,** heteronuclear multiple quantum coherence; **HMBC,** heteronuclear multiple bond correlation; **HPLC,** preparative high performance liquid chromatography; **RP,** reversed-phase; **TLC,** thin-layer chromatography; **CC,** column chromatography; **iNOS**, inducible nitric oxide synthase; **COX**, cyclooxygenase; **$PGE_2$**, prostaglandin $E_2$; **TNF-α**, tumor necrosis factor-α; **IL**, interleukin; **DMEM,** Dulbecco's modified Eagle medium; **FBS,** fetal bovine serum; **ACTT**, American Type Culture Collection; **BSA**, bovine serum albumin; **PAGE**, polyacrylamide gel electrophoresis; **ECL**, enhanced chemiluminescent; **DPPH**, 2,2-diphenyl-1-picrylhydrazyl; **FDAA**, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; **IκB**, inhibitory κB



**Chemical compounds studied in this article**

D-serine (PubChem CID: 71077); L-proline (PubChem CID: 145742); D-tyrosine (PubChem CID: 71098); L-leucine (PubChem CID: 6106); glycine (PubChem CID: 750)

## 1. Introduction

*Citrus unshiu* Markovich (Rutaceae) is a seedless and easy-peeling citrus fruit, one of the most commonly consumed fruits in Korea. *C. unshiu* is cultivated primarily on Jeju Island of Korea, and in the southern regions of China and Japan. The dried peels from mature fruits of *C. unshiu* have been used as traditional herbal drugs for the treatment of tympanites and nausea since ancient times in East Asia (Oh et al., 2012). The peels of *C. unshiu* also have long been used as traditional Korean medicine, well known as "Jin-pee", for treating conditions ranging from the common cold to cancer, and relieving exhaustion (Lee et al., 2011; Kamei et al., 2000). The whole fruit itself, including the fresh part, also has been used from the ancient times as a natural medicine in Korea (Choi, 2008). It is effective in improving skin elasticity, relieving fatigue and cough, and preventing bronchitis, flu, and various cancers.

Previous studies have demonstrated that *C. unshiu* peels enhance the immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model (Lee et al., 2011). Some studies have shown that *C. unshiu* peels express antioxidant activity (Bocco et al., 1998), and inhibit hydroperoxide production (Higashi-Okai et al., 2002). Additionally, several studies have reported the inhibitory activity of *C. unshiu* peels against the hepatitis-C virus (Suzuki et al., 2005), certain bacteria, and fungi (Jo et al.,

2004). It has also been reported that the extract of *C. unshiu* peels ameliorates hyperglycemia and hepatic steatosis (Park et al., 2013), and gastrointestinal (GI) motility dysfunctions by acting as a prokinetic agent (Lyu and Lee, 2013). In addition, despite relatively low interest of research, compared to that of the dried peels, *C. unshiu* as a whole fruit is known to have inhibitory effects towards tumor growth (Lee et al., 2011). It has also been reported that *C. unshiu* powder has an immunomodulatory effect (Tanabe et al., 2007). Many phytochemicals such as flavonoids and limonoids have been isolated from *C. unshiu* as main biological components (Kuroyanagi et al., 2008; Sawabe et al., 1999; Shin et al., 2012; Bocco et al., 1998; Kim et al., 1999).

In spite of the intensive research on *C. unshiu* peels for its intriguing pharmacological effects, the fresh part of the fruit has been nearly neglected in this field of research despite its traditional use along with the peels. Moreover, there have been few studies on the anti-inflammatory effects of *C. unshiu* fruit in order to support its traditional usage. Chronic inflammation is an important risk factor for the development of bronchitis and various cancers (Rakoff-Nahoum, 2006; Poulain et al., 2006). A recent study investigated the effects of *C. unshiu* peels on the production of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells (Oh et al., 2012). The research demonstrated that *C. unshiu* peels represses LPS-induced inducible nitric

oxide synthase (iNOS) and cyclooxygenase (COX)-2 gene expression as well as the secretion of nitric oxide (NO), prostaglandin $E_2$ ($PGE_2$), tumor necrosis factor-α (TNF-α), and interleukin (IL)-6 production (Oh et al., 2012). However, the research was conducted based on *C. unshiu* peels rather than *C. unshiu* fruits, and it made no attempt to identify the active components associated with anti-inflammation, which still remains unknown.

As a part of our research program to find new bioactive compounds from the Korean medicinal plants, the fresh parts of *C. unshiu* were chosen for a more detailed investigation to prove the ethnopharmacological use of *C. unshiu* fruits as an anti-inflammatory agent. Repeated chromatography of the EtOAc soluble fraction of its EtOH extract resulted in the isolation and identification of a new cyclic peptide, citrusin XI (**1**) (**Fig. 1**). This paper reports the isolation and structural elucidation of compound **1**, and its effects on LPS-stimulated inflammation and the underlying mechanism of its anti-inflammatory effect in RAW 264.7 macrophage cells.



## 2. Material and methods

### *2.1. General*

Optical rotations were measured on a Jasco P-1020 polarimeter using methanol as a

solvent. Infrared (IR) spectra were recorded on a Bruker IFS-66/S FT-IR spectrometer. Ultraviolet (UV) spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer. High resolution (HR)-electrospray ionization mass spectrometry (ESIMS) and ESIMS spectra were recorded on a Micromass QTOF2-MS. Nuclear magnetic resonance (NMR) spectra, including the spectra in the $^{1}H$-$^{1}H$ correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments, were recorded on a Varian UNITY INOVA 700 NMR spectrometer operating at 700 MHz ($^{1}H$) and 175 MHz ($^{13}C$), with chemical shifts given in ppm ($\delta$). Preparative high performance liquid chromatography (HPLC) purification was carried out on an Agilent 1100 or 1200 Series HPLC system (Agilent Technologies) equipped with a photo diode array detector. LC-MS analysis was performed on an Agilent 1200 Series HPLC system equipped with a diode array detector and a 6130 Series ESI mass spectrometer using an analytical Hydrosphere C18, YMC column (5 μm, 4.6 × 250 mm). Silica gel 60 (Merck, 230-400 mesh) and reversed-phase (RP)-$C_{18}$ silica gel (Merck, 230-400 mesh) were used for column chromatography. Merck precoated silica gel $F_{254}$ plates and RP-18 $F_{254s}$ plates were used for thin-layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with anisaldehyde-sulfuric acid.

*2.2. Plant material*

Fruits of *C. unshiu* were harvested from the National Institutes of Horticultural and Herbal Science on Jeju Island, Korea, in August 2012, and were identified by one of the authors (H. J. Noh). A voucher specimen (SKKU-CU 2012-8) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

*2.3. Extraction and isolation*

Partially fresh whole parts of *C. unshiu* fruits (1.0 kg) were extracted twice with 100% EtOH (2 × 2 days) at room temperature. The resultant extract was then filtered, and concentrated under vacuum pressure to yield crude extracts. The EtOH extracts (89 g) were suspended in distilled water (10 L) and successively partitioned with *n*-hexane, EtOAc, and *n*-BuOH, yielding residues weighing 127 mg, 3.0 g, and 16.0 g, respectively. The EtOAc-soluble fraction (3.0 g) was subjected to fractionation with silica gel column chromatography using a gradient solvent system of *n*-hexane-EtOAc (1:1) and $CHCl_3$-MeOH (50:1, 10:1, 5:1, 2:1, and 1:1) to yield thirty fractions (E1 - E30) according to TLC analysis. Fraction E24 (294 mg) was applied to column chromatography on an RP-$C_{18}$ silica gel column using a gradient of increasing MeOH in $H_2O$ from 40% to 70% to yield 7 subfractions (E24a – E24g). Fraction E24f (18 mg) was further purified by semi-preparative reverse-phase HPLC (250 mm × 10 mm i.d., 10 μm, Phenomenex. Luna

C18(2) column, flow rate; 1.5 mL/min) using a solvent system of 50% MeOH to give compound **1** (7 mg, $t_R$ = 16.8 min).

*2.3.1. Citrusin XI (**1**)*

White powder; $[\alpha]_D^{25}$ -6.09 (*c* 0.07, MeOH); UV (MeOH) $\lambda_{max}$ (log ε): 277 (3.14) nm; IR (KBr) $\nu_{max}$ 3350, 1652 $cm^{-1}$; $^1H$ (700 MHz) and $^{13}C$ (175 MHz) NMR data, see **Table 1**; HR-ESIMS (positive-ion mode) *m/z* 728.3976 $[M + H]^+$ (calcd. for $C_{36}H_{54}N_7O_9$, 728.3983).

*2.4. Determination of the absolute configurations of the amino acid units in **1***

A 0.5 mg quantity of **1** was hydrolyzed in 0.5 mL of 6 N HCl at 110°C for 5 h. The reaction solvent was evaporated *in vacuo*, and residual HCl was completely removed by adding 0.5 mL of water and removing the solvent three times. The hydrolysate was purified by column chromatography using a C18 Sepak column (0.5 g). The free amino acids were eluted with 10 % $CH_3CN$ in water. A solution of Marfey's reagent (20 μL, 10 mg/mL in acetone) was added to the purified hydrolysate containing the free amino acids, followed by 1 N aqueous $NaHCO_3$ (100 μL). The reaction was heated to 80 °C for 10 min, cooled to room temperature, and acidified with 2 N HCl (50 μL). The reaction mixture was filtered and analyzed by LC-MS with a gradient solvent system from 20 % to 70 % $CH_3CN$ containing 0.1% formic acid over 50 min (Agilent 1200

Series HPLC / 6130 Series mass spectrometer, Hydrosphere C18, YMC column 5 μm, 4.6 mm × 250 mm, 0.7 mL/min flow rate). Standards were prepared from the appropriate authentic D- or L-amino acids (0.2 mg) by derivatizing them with Marfey's reagent using the above procedure. The retention times for Marfey's derivatives were as follows: serine (L-Ser, 15.62 min and D-Ser, 15.98 min), proline (L-Pro, 21.87 min and D-Pro, 22.24 min), tyrosine (L-Tyr, 24.71 min and D-Tyr, 25.95 min), and leucine (L-Leu, 32.04 min and D-Leu, 33.82 min). Sample prepared from **1** was also co-injected with standards to confirm its assignment which proved that compound **1** contains D-serine (15.99 min), L-proline (21.85 min), D-tyrosine (25.90 min), and L-leucine (32.07 min).

*2.5. Cell culture*

RAW264.7 mouse macrophage cells, A549 (human lung adenocarcinoma cells), SK-OV-3 (human ovarian adenocarcinoma cells), MCF-7 (human breast adenocarcinoma cells), HepG2 (human liver adenocarcinoma), and JEG-3 (human placental choriocarcinoma cells) were obtained from American Type Culture Collection (ACTT) (Manassas, VA, USA). RAW264.7, MCF-7 and JEG-3 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and A549, SK-OV-3, and HepG2 cells were grown in RPMI 164 medium. All media are supplemented with 10% fetal bovine serum (FBS),

penicillin and streptomycin and grown in a humidified incubator at 37 °C and 5% $CO_2$/95% air.

*2.6. Cell viability assay*

Cell viability was assessed using water-soluble tetrazolium salt (WST-1) assay. RAW264.7 cells were seeded in 96-well plates at 1 x $10^5$ cells/ well. After 24 h incubation, cells were treated with compound **1** (0 - 200 μM) and LPS (1 μg/mL) for 24 h. The medium was removed and 100 μL of DMEM was added to each well. 10 μL of WST-1® (Daeil Lab service, Seoul, Korea) was added and incubated for an additional 3 h. The absorbance at 460 nm was measured with ELISA reader.

*2.7. Nitric oxide (NO) assay*

RAW264.7 cells ($5 \times 10^5$ cells/ well) were cultured in 24-well plates for 24 h. The cells were pretreated with compound **1** (0 - 200 μM) for 2 h, and treated with LPS (1 μg/mL) for 24 h. A series of known concentration of sodium nitrate was used as a standard and dexamethasone (100 nM) was used as a positive control. NO in the culture medium was examined as an indicator of NO production by Griess reagent (Sigma-Aldrich, St. Louis, MO, USA). The Griess reagent (1% sulfanilamide, 0.1% N-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid) was mixed with culture medium at 1:1 ratio and incubated at room temperature for 15 min. The absorbance at

540 nm was measured with ELISA reader (Choi et al., 2012).

*2.8. Western blot analysis*

RAW264.7 cells were treated and harvested by cell scraper. The harvested cells were collected by centrifugation, lysed with ice-cold lysis buffer containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% Deoxycholate, 0.1% SDS, and a cocktail of proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, and prostatin A) (Intron biotechnology, Gyeonggi, Korea). After incubation for 30 min in ice, cell debris was removed by centrifugation at 14,000 rpm for 20 min at 4°C. The protein concentration was examined using a Protein Quantification Kit (CBB solution®) (Dojindo Molecular Technologies, Rockvile, MD, USA) with bovine serum albumin (BSA) as the standard. An aliquot from each sample was boiled with sample buffer for 5 min, and then resolved by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrotransferred to a nitrocellulose membrane (PALL Life Sciences, Pensacola, MI, USA) and blocked with 5% skim milk in PBST buffer (4.3 mM $NaPO_4$, 1.4 mM $KH_2PO_4$, 135 μM NaCl, 2.7 mM KCl, and 0.5% Tween-20). After blocking, the membrane was probed with primary antibodies (Cell Signaling Technology Inc., Beverly, MA, USA) and washed three times with PBST buffer. The membrane was followed by incubation for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG

or anti-mouse IgG secondary antibodies (Cell Signaling Technology Inc.). The blots were then washed with PBST buffer and visualized by an enhanced chemiluminescent (ECL) detection solution (AbFontier, Gyeonggi, Korea).

*2.9. Antiproliferative and antioxidant activities*

The antiproliferative activity was assessed using WST-1 method. A549, SK-OV-3, MCF-7, HepG2, and JEG-3 cells (1,000 cells/well) were seeded into 96-well plates and attached for 24 hrs. The seeding medium was replaced with the experimental medium (5% CDFBS supplemented with phenol red-free DMEM) and compound **1** was added to this medium at concentration from 0.1 μg/mL to 50 μg/mL. After 72 hrs incubation, according to manufacturer's instructions, experimental medium was replaced with serum-free medium containing cell proliferation Reagent WST-1 and incubated the plates in 5% $CO_2$ at 37 °C for 1 hr. Cell viability was quantified by using the absorbance at 440 nm using a spectrophotometer (VERSAmax, Molecular Devices, Sunnyvale, CA, USA). The DPPH radical-scavenging effect of compound **1** were assessed by the decoloration of a methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Kumarasamy et al., 2002). A methanol solution (100 μL) of **1** at various concentrations was added to DPPH-MeOH (78 μg/mL) solution, and the absorbance of the remaining DPPH was measured on a spectrophotometer at 517 nm after 10 min incubation in a

dark area. The radical-scavenging activity was determined by comparing the absorbance with that of blank (100%) containing DPPH and solvent.

## 3. Results and discussion

### *3.1. Structure elucidation*

Compound **1** was isolated as a white powder, and the molecular formula was established as $C_{36}H_{53}N_7O_9$ by an analysis of HR-ESIMS data (*m/z* 728.3976 $[M + H]^+$, calcd for $C_{36}H_{54}N_7O_9$, 728.3983) coupled with $^1H$ and $^{13}C$ NMR spectra. The $^1H$ NMR spectrum showed typical features of a peptide-derived compound, including 7α proton signals ($\delta_H$ 4.67, 4.60, 4.59, 4.40, 4.22, 4.18, and 4.04). The $^{13}C$ NMR and HMQC spectra were also consistent with a peptide-derived compound and displayed 7 amide or ester carbonyl carbons at $\delta_C$ 174.5-170.9.

Interpretation of the COSY, HSQC, and HMBC NMR spectra enabled the straightforward identification of 7 standard amino acids. These include two prolines ($Pro^1$ and $Pro^2$), two leucines ($Leu^3$ and $Leu^4$), a tyrosine ($Try^5$), a glycine ($Gly^6$), and a serine ($Ser^7$) (Figure 2). In particular, the UV maximum at 277 nm of **1** supported the presence of the Try residue. The sequence of the 7 identified amino acid units was established by extensive analyses of the HMBC data (**Fig. 2**). The connectivity of the

amino acids was elucidated by HMBC correlations for each α proton to the amide carbonyl carbon.

First, the α proton ($\delta_H$ 4.60) of $Pro^1$ correlated with the carbonyl carbon ($\delta_C$ 173.7; C-6) of $Pro^2$ in the HMBC spectrum, establishing the linkage between $Pro^1$ and $Pro^2$. The α proton ($\delta_H$ 4.59) of $Pro^2$ clearly showed HMBC correlation to C-11 ($\delta_C$ 170.9), the carbonyl carbon of $Leu^3$, thus establishing the connectivity between $Pro^2$ and $Leu^3$. The next amino acid was deduced as $Leu^4$ on the basis of the HMBC correlation from the α proton ($\delta_H$ 4.67) of $Leu^3$ to C-17 ($\delta_C$ 174.4; the carbonyl carbon of $Leu^4$). Further analysis of the HMBC spectrum enabled us to establish the connectivity of $Leu^4$-$Try^5$-$Gly^6$-$Ser^7$. Finally, the last amino acid, $Ser^7$ was then connected to $Pro^1$ by the HMBC correlation between the α proton ($\delta_H$ 4.40) of $Ser^7$ to C-1 ($\delta_C$ 174.1; the carbonyl carbon of $Pro^1$), which completed the planar structure of **1** as a cyclic peptide composed of 7 amino acid residues.

The Marfey's method using L-FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide; Marfey's reagent) was applied to determine the absolute configurations of the amino acid α-carbons (Marfey, 1984). A small sample of **1** (0.5 mg) was hydrolyzed with 6N HCl (0.5 mL) at 110 °C for 5 h. Although conventional acid hydrolysis is usually performed as an overnight reaction, we performed the reaction for 5 h to preserve each

of the amino acid residues. The hydrolysate was derivatized with L-FDAA, and the derivatives were analyzed by LC/MS to determine the absolute configurations of the α-carbon of each of the amino acid residues. A LC/MS comparison of the Marfey's derivatives derived from **1** allowed us to determined that compound **1** contains D-serine, L-proline, D-tyrosine, and L-leucine, which indicated the absolute configuration of α-position of amino acid residue as 2*S*, 7*S*, 12*S*, 18*S*, 24*R*, and 35*R*. Thus, the structure of **1** was established as a cyclic peptide, cyclo(L-Pro-L-Pro-L-Leu-L-Leu-D-Tyr-Gly-D-Ser), namely citrusin XI. To our knowledge, a stereoisomer of **1**, cyclonatsudamine A, was previously reported from the peels of *C. natsudaidai* (Morita et al., 2007). Cyclonatsudamine A was structurally composed of all L-amino acids, and relaxed norepinephrine-induced contractions in rat aorta.

As mentioned above, *C. unshiu* fruits have been nearly ignored in the phytochemical investigation, compared to that of its dried peels, and there are few compounds reported from *C. unshiu* fruits. Literature survey revealed that some flavonoid glycosides such as hesperidin, didymin, and narirutin were isolated as the main constituents of *C. unshiu* fruits (Shalashvili et al., 1986; Matsuda et al., 1991). On the other hand, various constituents including cyclic peptides, flavonoids, and limonoids were mainly isolated

from *C. unshiu* peels (Matsubara et al., 1991; Sawabe et al., 1999; Shin et al., 2012; Bocco et al., 1998; Kim et al., 1999).

### *3.2. Anti-inflammatory activity*

#### *3.2.1. Effect of compound **1** on the viability of RAW264.7 cells*

Cell viability assay was used to examine the anti-inflammatory effect of compound **1** on RAW264.7 cells. Figure 3 shows that compound **1** did not affect cell toxicity when concentrations less than 200 μM were used to treat the cells.

#### *3.2.2. Inhibition of NO production and expression of iNOS and COX-2*

To determine whether compound **1** can regulate inflammatory mediators in LPS-induced RAW264.7 cells, NO production was measured in the culture medium using Griess reagent (Choi et al., 2012). Inflammatory stimuli implicate high levels of NO and it causes induction of pro-inflammatory mediators (Korhone et al., 2005). Figure 4 (A) shows that compound **1** decreased the NO production in LPS-stimulated RAW264.7 cells in a dose-dependent manner with an $IC_{50}$ value of 70 μM. Dexamethasone was used as a positive control. To investigate suppression of NO production by compound **1**, iNOS expression was mediated by western blot analysis. NO is regulated by NO synthases (NOSs), which oxidize L-arginine to L-citrulline and produce NO at the same

time. NOS encodes three genes including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). iNOS is involved in various pathological conditions by producing high concentrations of NO whereas other NOSs produce low concentrations that are essential for maintaining tissue homeostasis (MacMicking et al., 1997). Figure 4 (B) shows that compound **1** significantly decreased the expression of iNOS in a dose-dependent manner. Cyclooxygenase-2 (COX-2) is only expressed by stimuli in mammalian tissues, and is commonly the target of anti-inflammatory treatment (Murakami et al, 2007). Compound **1** slightly inhibited COX-2 expression in LPS-stimulated RAW264.7 cells. These results suggest that compound **1** decreased NO production by decreasing iNOS expression, but COX-2 expression was only slightly associated with the reduction by compound **1** in LPS-induced RAW264.7 cells.

*3.2.3. Inhibitory effect of NF-κB pathway in LPS-induced RAW264.7 cells*

NF-κB is a transcription factor which encodes several pro-inflammatory genes such as cytokines, chemokines, and inducible enzymes including iNOS and COX-2, and is inactivated by inhibitory κB (IκB) in the absence of stimuli (Lawrence et al., 2002). Upon stimulation of pro-inflammatory signals such as LPS or cytokines, NF-κB is activated by phosphorylation of IκBα and NF-κB. Phosphorylation of IκBα leads to ubiqutination which degrades IκBα, and translocates NF-κB into the nuclear (Pereira et

al., 2008; Kumar et al., 2004). We studied whether compound **1** inhibited degradation of IκBα and phosphorylation of NF-κB in LPS-induced RAW264.7 cells by western blot analysis. As shown in figure 5, compound **1** significantly decreased the degradation of IκBα and phosphorylation of NF-κB in a dose-dependent manner. These results suggest that compound **1** inhibited NF-κB activation by blocking IκBα degradation and NF-κB phosphorylation in RAW264.7 cells. As mentioned above, iNOS gene transcription is controlled by a ~1 kb promoter, which includes several transcription factors such as NF-κB. When NF-κB is regulated, iNOS expression and NO production are also controlled (Korhonen et al. 2005). Thus, these results suggest that compound **1** reduces NO production and iNOS expression through NF-κB pathways.

*3.3. Antiproliferative and antioxidant activities*

The antiproliferative activity of compound **1** was additionally tested at various concentrations from 0.14 μM (0.1 μg/mL) to 68.64 μM (50 μg/mL) against A549, SK-OV-3, MCF-7, HepG2, and JEG-3 cells. However, after being incubated for 72-hours with compound **1**, there was no significant effect on the growth of each cell line. Furthermore, compound **1** was tested for radical-scavenging activity against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals (Kumarasamy et al., 2002). Compound **1**

showed no free-radical scavenging activity (antioxidant activity) in DPPH assay [$IC_{50}$ > 274.56 μM (200 μg/mL)].

## 4. Conclusions

In search for new bioactive compounds, this study determined the structure of a new cyclic peptide, citrusin XI (1), that is present in EtOH extract of *C. unshiu* fruits, using spectroscopic data analysis and chemical methods. The present findings suggests for the first time that citrusin XI (**1**) is potential inhibitor of LPS-induced pro-inflammatory mediator, NO, in RAW264.7 cells through the inhibition of iNOS expression and NF-κB activation associated with IκBα degradation and NF-κB phosphorylation. Thus, citrusin XI (**1**) may have therapeutic potential for the modulation and regulation of macrophage activation in the treatment of inflammatory diseases. Taking into account that compound **1** was significantly involved in reducing inflammation, it could be concluded that citrusin XI (**1**) contributes to health benefits and ethnopharmacological usage of *C. unshiu* as an anti-inflammatory agent.

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**References**

Bocco, A., Cuvelier, M.E., Richard, H., Berset, C., 1998. Antioxidant activity and phenolic composition of citrus peel and seed extracts. Journal of Agricultural and Food Chemistry 46, 2123–2129.

Choi, K.J., 2008. Functional Foods and Nutraceuticals, first ed. Dong Myeong Publishers, Seoul. P. 147.

Choi, W.S., Shin, P.G., Lee, J.H., Kim, G.D., 2012. The regulatory effect of veratric acid on NO production in LPS-stimulated RAW264.7 macrophage cells. Cellular Immunology 280, 164-170.

Higashi-Okai, K., Kamimoto, K., Yoshioka, A., Okai, Y., 2002. Potent suppressive activity of fresh and dried peels from Satsuma mandarin *Citrus unshiu* (Marcorv.) on hydroperoxide generation from oxidized linoleic acid. Phytotherapy Research 16, 781–784.

Jo, C.R., Park, B.J., Chung, S.H., Kim, C.B., Cha, B.S., Byun, M.W., 2004. Antibacterial and anti-fungal activity of citrus (*Citrus unshiu*) essential oil extracted from peel by-products. Food Science and Biotechnology 13, 384–386.

Kamei, T., Kumano, H., Iwata, K., Nariai, Y., Matsumoto, T., 2000. The effect of a traditional Chinese prescription for a case of lung carcinoma. Journal of Alternative and

Complementary Medicine 6, 557-559.

Kim, D.K., Lee, K.T., Eun, J.S., Zee, O.P., Lim, J.P., Eum, S.S., Kim, S.H., Shin, T.Y., 1999. Anti-allergic components from peels of *Citrus unshiu*. Archives of Pharmacal Research 22, 642–645.

Korhone, R., Lahti, A., Kankaanranta, H., Moilanen, E., 2005. Nitric oxide production and signaling in inflammation. Current Drug Targets - Inflammation & Allergy 4, 471-479.

Kumar, A., Takada, Y., Boriek, A.M., Aggarwal, B.B., 2004. Nuclear factor- κB: its role in health and disease. Journal of Molecular Medicine 82, 434-448.

Kumarasamy, Y., Fergusson, M.E., Nahar, L., Sarker, S.D., 2002. Bioactivity of moschamindole from *Centaurea moschata*. Pharmaceutical Biology 40, 307-310.

Kuroyanagi, M., Ishii, H., Kawahara, N., Sugimoto, H., Yamada, H., Okihara, K., Shirota, O., 2008. Flavonoid glycosides and limonoids from *Citrus* molasses. Journal of Natural Medicines 62, 107-111.

Lawrence, T., Willoughby, D.A., Gilroy, D.W., 2002. Anti-inflammatory lipid mediators and insights into the resolution of inflammation. Immunology 2, 787-795.

Lee, S., Ra, J., Song, J.Y., Gwak, C., Kwon, H.J., Yim, S.V., Hong, S.P., Kim, J., Lee, K.H., Cho, J.J., Park, Y.S., Park, C.S., Ahn, H.J., 2011. Extracts from *Citrus unshiu*

promote immune-mediated inhibition of tumor growth in a murine renal cell carcinoma model. Journal of Ethnopharmacology 133, 973-979.

Lyu, J.H., Lee, H.T., 2013. Effects of dried *Citrus unshiu* peels on gastrointestinal motility in rodents. Archives of Pharmacal Research 36, 641-648.

MacMicking, J., Xie, Q., Nathan, C., 1997. Nitric oxide and macrophage function. Annual Review of Immunology 15, 323-350.

Marfey, P., 1984. Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsberg Research Communications 49, 591-596.

Matsubara, Y., Yusa, T., Sawabe, A., Iizuka, Y., Takekuma, S., Yoshida, Y., 1991. Structures of new cyclic peptides in young unshiu (*Citrus unshiu* Marcov.), orange (*Citrus sinensis* Osbeck.) and amanatsu (*Citrus natsudaidai*) peelings. Agricultural and Biological Chemistry 55, 2923-2929.

Matsuda, H., Yano, M., Kubo, M., Iinuma, M., Oyama, M., Mizuno, M., 1991. Pharmacological study on citrus fruits. II. Antiallergic effect of fruit of *Citrus unshiu* Markovich (2). On flavonoid components. Yakugaku Zasshi 111, 193-198.

Morita, H., Enomoto, M., Hirasawa, Y., Iizuka, T., Ogawa, K., Kawahara, N., Goda, Y., Matsumoto, T., Takeya, K., 2007. Cyclonatsudamine A, a new vasodilator cyclic peptide from *Citrus natsudaidai*. Bioorganic & Medicinal Chemistry Letters 17, 5410-5413.

Murakami, A., Ohigashi, H., 2007. Targeting NOX, INOS, and COX-2 in inflammatory cells: Chemoprevention using food phytochemicals. International Journal of Cancer 121, 2357-2363.

Oh, Y.C., Cho, W.K., Jeong, Y.H., Im, G.Y., Yang, M.C., Hwang, Y.H., Ma, J.Y., 2012. Anti-inflammatory effect of *Citrus Unshiu* peel in LPS-stimulated RAW 264.7 macrophage cells. The American Journal of Chinese Medicine 40, 611-629.

Park, H.J., Jung, U.J., Cho, S.J., Jung, H.K., Shim, S., Choi, M.S., 2013. *Citrus unshiu* peel extract ameliorates hyperglycemia and hepatic steatosis by altering inflammation and hepatic glucose- and lipid-regulating enzymes in db/db mice. The Journal of Nutritional Biochemistry 24, 419-427.

Pereira, S.G., Oakley, F., 2008. Nuclear factor-κB: Regulation and function. International Journal of Biochemistry & Cell Biology 40, 1425-1430.

Poulain, M., Doucet, M., Major, G.C., Drapeau, V., Sériès, F., Boulet, L.P., Tremblay, A., Maltais, F., 2006. The effect of obesity on chronic respiratory diseases: pathophysiology and therapeutic strategies. Canadian Medical Association Journal 174, 1293-1299.

Rakoff-Nahoum, S., 2006. Why cancer and inflammation?. Yale Journal of Biology and Medicine 79, 123-130.

Sawabe, A., Morita, M., Kiso, T., Kishine, H., Ohtsubo, Y., Minematsu, T., Matsubara,

Y., Okamoto, T., 1999. Isolation and characterization of new limonoid glycosides from *Citrus unshiu* peels. Carbohydrate Research 315, 142-147.

Shalashvili, A.G., Durmishidze, S.V., Targamadze, I.L., 1986. Flavanones of the *Citrus unshiu* Marc. Fruits. Prikladnaya Biokhimiya i Mikrobiologiya 22, 423-427.

Shin, H.J, Nam, J.W., Yoon, U.J., Han A.R., Seo, E.K., 2012. Identification of three new flavonoids from the peels of *Citrus unshiu*. Helvetica Chimica Acta 95, 240–245.

Suzuki, M., Sasaki, K., Yoshizaki, F., Oguchi, K., Fujisawa, M., Cyong, J.C., 2005. Anti-hepatitis C virus effect of *Citrus unshiu* peel and its active ingredient nobiletin. The American Journal of Chinese Medicine 33, 87–94.

Tanabe, S., Kinuta, Y., Yasumatsu, H., Takayanagi, M., Kobayashi, S., Takido, N., Sugiyama, M., 2007. Effects of *Citrus unshiu* powder on the cytokine balance in peripheral blood mononuclear cells of patients with seasonal allergic rhinitis to pollen. Bioscience, Biotechnology, and Biochemistry 71, 2852-2855.

Table legends

**Table 1.** $^{1}H$ (700 MHz) and $^{13}C$ NMR (175 MHz) data of compound **1** in $CD_3OD$ (δ in ppm, *J* values in parentheses)[a]

Figure captions

**Fig 1.** Chemical structure of compound **1**.

**Fig. 2.** Key $^{1}H$-$^{1}H$ COSY (—) and HMBC (⟶) correlations of **1**.

**Fig. 3.** Effects of compound **1** on the cell viability of RAW264.7 cells. Cells were treated with compound **1** (0 - 200 μM) and LPS (1 μg/mL) for 24 h. Cell viability was measured by MTT assay using WST-1 solution. The data were represented the mean ± SD of three independent experiments.

**Fig. 4.** Effects of compound **1** on (A) NO production and (B) iNOS and COX-2 expression in LPS-induced RAW264.7 cells. Cells were pretreated with the indicated concentrations of compound **1** for 2 h and stimulated with LPS (1 μg/mL) for (A) 24 h and (B) 18 h. (A) was measured with a microplate reader to determinate NO production (540 nm) and 100 nM of dexamethasone (Dex) was used as a positive control, and (B) was examined by western blot analysis. The data were represented the mean ± SD of three independent experiments. ****P<0.0001 significantly different from LPS-induced group.

**Fig. 5.** Effects of compound **1** on NF-κB pathway in LPS-stimulated RAW264.7 cells. Cells were pre-treated with compound 1 (100 and 200 μM) for 2 h and treated with LPS (1 μg/mL) for 18 h and examined by western blot analysis.

**Table 1.** $^{1}H$ (700 MHz) and $^{13}C$ NMR (175 MHz) data of compound **1** in $CD_3OD$ (δ in ppm, *J* values in parentheses)[a]

| Position | | Citrusin XI | |
|---|---|---|---|
| | | $\delta_H$ | $\delta_C$ |
| Pro[1] | 1 | | 174.1 |
| | 2 | 4.60 br d (8.0) | 62.6 |
| | 3 | 2.43 m | 32.6 |
| | | 2.20 m | |
| | 4 | 1.97 m | 23.0 |
| | | 1.81 m | |
| | 5 | 3.67 m | 48.1 |
| | | 3.55 overlap | |
| Pro[2] | 6 | | 173.7 |
| | 7 | 4.59 dd (5.5, 8.5) | 61.2 |
| | 8 | 2.29 m | 29.5 |
| | | 1.84 m | |
| | 9 | 2.09 m | 26.3 |
| | | 2.02 m | |
| | 10 | 3.63 m | 48.7 |
| | | 3.56 overlap | |
| Leu[3] | 11 | | 170.9 |
| | 12 | 4.67 dd (6.5, 7.5) | 50.8 |
| | 13 | 1.68 m | 41.9 |
| | | 1.34 m | |
| | 14 | 1.56 m | 25.8 |
| | 15 | 0.95 d (7.0) | 23.0 |
| | 16 | 0.94 d (7.0) | 23.4 |
| Leu[4] | 17 | | 174.4 |
| | 18 | 4.18 dd (4.5, 11.0) | 54.4 |
| | 19 | 1.67 m | 40.7 |
| | | 1.56 m | |
| | 20 | 1.34 m | 25.7 |
| | 21 | 0.86 d (6.5) | 21.4 |
| | 22 | 0.93 d (6.5) | 23.7 |
| Try[5] | 23 | | 174.5 |
| | 24 | 4.22 dd (7.0, 7.5) | 59.3 |
| | 25 | 2.98 dd (7.5, 14.0) | 37.2 |
| | | 2.94 dd (7.0, 14.0) | |
| | 26 | | 127.9 |
| | 27 | 7.07 d (8.0) | 131.3 |
| | 28 | 6.73 d (8.0) | 116.5 |
| | 29 | | 157.8 |
| | 30 | 6.73 d (8.0) | 116.5 |
| | 31 | 7.07 d (8.0) | 131.3 |
| Gly[6] | 32 | | 172.2 |
| | 33 | 4.04 d (1.0, 17.0) | 43.1 |
| | | 3.93 overlap | |
| Ser[7] | 34 | | 172.3 |
| | 35 | 4.40 dd (4.5, 7.0) | 59.5 |
| | 36 | 3.98 dd (7.0, 13.0) | 62.6 |
| | | 3.96 dd (4.5, 13.0) | |

[a] The assignments were based on $^{1}H,^{1}H$-COSY, HMQC and HMBC experiments.

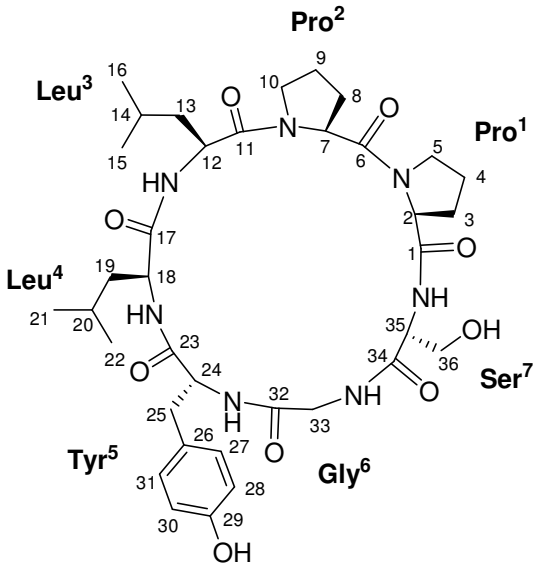



**Fig 1.** Chemical structure of compound **1**.

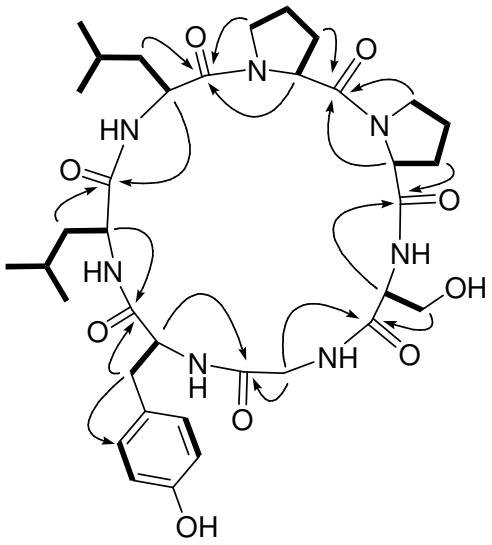



**Fig. 2.** Key $^{1}$H-$^{1}$H COSY (—) and HMBC (⟶) correlations of **1**.

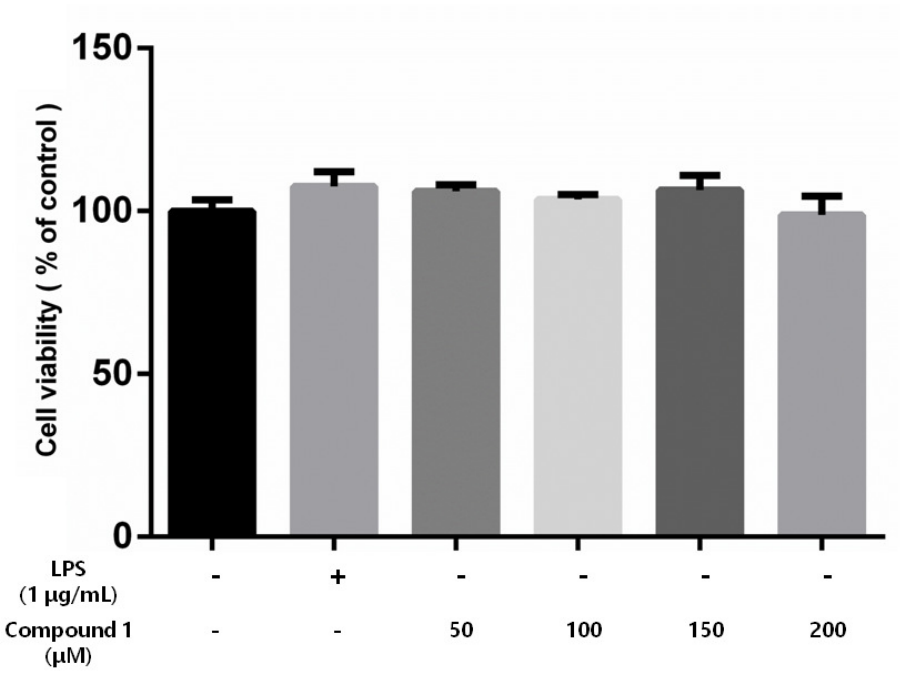
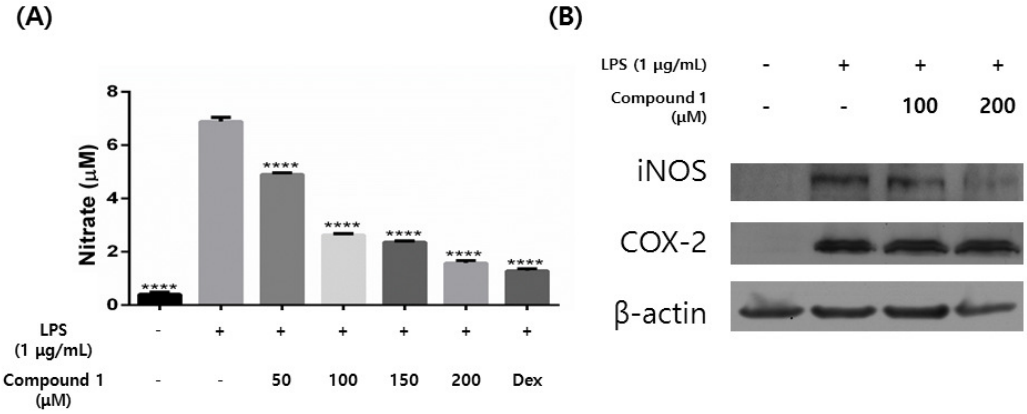



**Fig. 3.** Effects of compound **1** on the cell viability of RAW264.7 cells. Cells were treated with compound **1** (0 - 200 μM) and LPS (1 μg/mL) for 24 h. Cell viability was measured by MTT assay using WST-1 solution. The data were represented the mean ± SD of three independent experiments.

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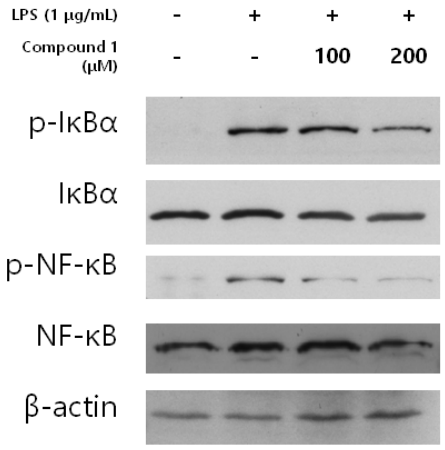


**Fig. 5.** Effects of compound **1** on NF-κB pathway in LPS-stimulated RAW264.7 cells. Cells were pre-treated with compound 1 (100 and 200 μM) for 2 h and treated with LPS (1 μg/mL) for 18 h and examined by western blot analysis.

# Anti-inflammatory activity of a new cyclic peptide, citrusin XI, isolated from the fruits of *Citrus unshiu*

Hyung Jun Noh, Dukhyun Hwang, Eun Suk Lee, Jae Wook Hyun, Pyoung Ho Yi, Geum Soog Kim, Seung Eun Lee, Changhyun Pang, Yong Joo Park, Kyu Hyuck Chung, Gun Do Kim, Ki Hyun Kim