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Research Article

# Identification of a novel triterpene saponin from *Panax ginseng* seeds, pseudoginsenoside RT$_8$, and its antiinflammatory activity

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## ARTICLE INFO

## ABSTRACT

*Background:* *Panax ginseng* Meyer (Araliaceae) is a highly valued medicinal plant in Asian regions, especially in Korea, China, and Japan. Chemical and biological studies on *P. ginseng* have focused primarily on its roots, whereas the seeds remain poorly understood. This study explores the phytochemical and biological properties of compounds from *P. ginseng* seeds.

*Methods:* *P. ginseng* seeds were extracted with methanol, and 16 compounds were isolated using various chromatographic methods. The chemical structures of the isolates were determined by spectroscopic data. Antiinflammatory activities were evaluated for triterpene and steroidal saponins using lipopolysaccharide-stimulated RAW264.7 macrophages and THP-1 monocyte leukemia cells.

*Results:* Phytochemical investigation of *P. ginseng* seeds led to the isolation of a novel triterpene saponin, pseudoginsenoside RT$_8$, along with 15 known compounds. Pseudoginsenoside RT$_8$ exhibited more potent antiinflammatory activity than the other saponins, attenuating lipopolysaccharide-mediated induction of proinflammatory genes such as interleukin-1β, interleukin-6, inducible nitric oxide synthase, cyclooxygenase-2, and matrix metalloproteinase-9, and suppressed reactive oxygen species and nitric oxide generation in a dose-dependent manner.

*Conclusion:* These findings indicate that pseudoginsenoside RT$_8$ has a pharmaceutical potential as an antiinflammatory agent and that *P. ginseng* seeds are a good natural source for discovering novel bioactive molecules.

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## 1. Introduction

*Panax ginseng* Meyer (Araliaceae) is a representative medicinal plant that is highly valued in several Asian countries, including Korea, China, and Japan, and has been traditionally used to prevent or treat a wide range of ailments and diseases [1]. A number of studies have shown that *P. ginseng* has various beneficial biological effects on human health and relieves many chronic diseases, including Alzheimer's disease, atherosclerosis, cancer, fatty liver, hyperlipidemia, inflammatory disease, insulin resistance, obesity, Parkinson's disease, and pulmonary disease [2—12]. It is thought that the biological effects of *P. ginseng* derive from a combination of diverse compounds, such as saponins, polysaccharides, peptides, phytosterols, polyacetylenes, and fatty acids [1,13]. Ginseng saponins, called ginsenosides, are characteristic constituents of *P. ginseng* which are responsible for many of its pharmacological effects [14]. More than 100 ginsenosides have been identified in *P. ginseng*, and vigorous research efforts are still underway to discover novel ginsenosides with unique pharmacological activities.

Inflammation is a complex immune response to defend living organism against harmful stimuli such as mechanical injury, pathogens, or irritants [15]. Once such inducers of inflammation initiate inflammatory process, stimulated inflammatory cells generate higher level of proinflammatory cytokines [e.g., interleukin (IL)-1β, IL-6, and tumor necrosis factor-α] and inflammatory mediators including nitric oxide (NO), inducible nitric oxide synthase (iNOS), and cyclooxygenase (COX)-2 to modulate the functionality of tissues and organs. However, abnormal regulation of the

inflammatory response can be triggered by nonpathogenic means [15,16]. For example, it has been proposed that free fatty acids can bind to toll-like receptor 4 to trigger a proinflammatory response [17]. Low-grade, chronic inflammation is closely related to the development of various metabolic disorders, including atherosclerosis, cancer, fatty liver disease, insulin resistance, rheumatoid arthritis, type-2 diabetes, and vascular diseases [15,16]. Therefore, it is important to maintain a balanced inflammatory status to remain healthy.

Most of the phytochemical research on *P. ginseng* has focused on roots, whereas other parts of the plant remain relatively poorly understood. Recently, the fruits of *P. ginseng*, called ginseng berries, have been used as dietary supplements because they also exert multiple beneficial effects on metabolic disorders, such as hyperglycemia, hyperlipidemia, diabetes, and insulin resistance [18−20]. It is feasible that other parts of *P. ginseng*, e.g., the stems, flowers, and seeds, may also contain functional components.

In this study, phytochemical evaluation of ginseng seeds led to the determination of a novel ginsenoside and six known ginsenosides, three steroidal saponins, three phenolics, and three primary metabolites. In addition, the antiinflammatory activities of triterpene and steroidal saponins were evaluated by estimating proinflammatory genes expression [IL-1β, IL-6, iNOS, COX-2, and matrix metalloproteinase (MMP)-9] in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages and the production of reactive oxygen species (ROS) and nitric oxide (NO).

## 2. Material and methods

### 2.1. Instrumentation

Semipreparative-scale high-performance countercurrent chromatography (HPCCC) was conducted with a Spectrum HPCCC (Dynamic Extractions, Berkshire, UK) consisting of a 1525 binary high-performance liquid chromatography (HPLC) pump (Waters, Milford, MA, USA), Sedex 75 ELSD (Sedere, Olivet, France) and a Foxy R2 fraction collector (Teledyne Isco, Lincoln, NE, USA). A CCA-1111 circulatory temperature regulator (Eyela, Tokyo, Japan) was used to control the inner temperature of the HPCCC at 30°C. Preparative HPLC was conducted with a Gilson HPLC system (Middleton, WI, USA) composed of a liquid handler, ultraviolet−visible (UV/VIS) detector, binary pumps, and a Luna C18 column (21.2 × 250 mm I.D., 5 μm; Phenomenex, Torrance, CA, USA). Chemical structures of the isolated compounds were identified using one-dimensional and two-dimensional nuclear magnetic resonance (NMR) data collected using an AVANCE 500 spectrometer (Bruker, Karlsruhe, Germany). Optical rotation was recorded on a Jasco P-2000 polarimeter (Tokyo, Japan). Electrospray ionization−quadrupole−time-of-flight−mass spectrometry (ESI-Q-TOF-MS) spectra were measured on a 6460 QTOF-MS spectrometer (Agilent Technologies, Santa Clara, CA, USA).

### 2.2. Chemicals and plant materials

Deionized water was produced using a Milli-Q water purification system (Millipore, Billerica, MA, USA). Solvent for HPCCC, HPLC, and medium pressure liquid chromatography (MPLC) were provided by Daejung Chemical and Metals Co. Ltd. (Kyeonggi-Do, Korea). LPS, N-acetyl-L-cysteine (NAC), and Nω-nitro-L-arginine methyl ester were obtained from Sigma-Aldrich (St. Louis, MO, USA). H2-2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) was purchased from Invitrogen (Carlsbad, CA, USA). Seeds of *P. ginseng* were provided by Amorepacific Co. (Yongin, Korea), and the voucher specimen (CU-PGS 20160824) was deposited in the herbarium of the College of Pharmacy at the Catholic University of Korea.

### 2.3. Cell culture

RAW264.7 mouse macrophage and THP-1 human monocyte leukemia cell lines were provided from the American Type Culture Collection (Manassas, VA, USA). RAW264.7 cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (HyClone, Logan UT, USA). THP-1 cells were maintained with Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum. All media included 100 units/mL of penicillin and 100 mg/mL of streptomycin (Sigma-Aldrich). Cells were grown at 37°C in a humidified atmosphere containing 5% $CO_2$.

### 2.4. Preparation of cDNA and quantitative real-time polymerase chain reaction

RNA was isolated using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Each RNA sample (1 μg) was subjected to cDNA synthesis with a RevertAid 1st Strand cDNA Synthesis kit (Thermo Fisher Scientific). Relative mRNA levels were evaluated by quantitative real-time polymerase chain reaction (Bio-Rad CFX96; Bio-Rad, Hercules, CA, USA) with a QuantiSpeed SyBR One-step kit (PhileKorea, Seoul, South Korea) and appropriate quantitative real-time polymerase chain reaction primers (Bioneer, Daejeon, South Korea).

### 2.5. Measurement of ROS and NO concentrations

ROS was measured using DCF-DA reagent. Briefly, after being treated with saponins and LPS, cells were washed with phosphate-buffered saline (Sigma-Aldrich) and incubated in 5 μM H2DCF-DA for 30 minutes at 37°C in the dark condition. After two additional washes with phosphate-buffered saline, the fluorescence intensity was measured with a Tecan Infinite 200 Pro multi-plate reader (Tecan trading AG, Männedorf, Switzerland; excitation wavelength: 492 nm, emission wavelength: 530 nm).

NO production was analyzed using Griess reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol. In brief, 50 μL of sulfanilamide solution was added to 50 μL of each sample, followed by 10 minutes of incubation at room temperature (RT), protected from light. Then, a solution of N-1-napthylethylenediamine (50 μL) was dispensed into the mixture and incubated for an additional 10 minutes at RT without light. The colorimetric intensity of NO was estimated with a Tecan Infinite 200 Pro multi-plate reader at a wavelength of 540 nm. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

### 2.6. Statistical analysis

All data points represent the average of at least triplicate samples, and standard deviation was represented as error bar. The *p* values were calculated by a one-way analysis of variance and Dunnett's test. *p* values less than $<0.05$ were regarded to be statistically significant.

### 2.7. Extraction and isolation of compounds *1−10*

Seeds of *P. ginseng* (5.5 Kg) were ground to make a fine powder. The powder was extracted with methanol to yield a methanol extract (481 g). The methanol extract was suspended in $H_2O$ and partitioned sequentially with *n*-hexane and *n*-butanol to give *n*-hexane−soluble (322 g), *n*-butanol−soluble (114 g), and water-soluble (45 g) extracts. Further isolation process for compounds *1−16* were described in Supplementary Information S1.
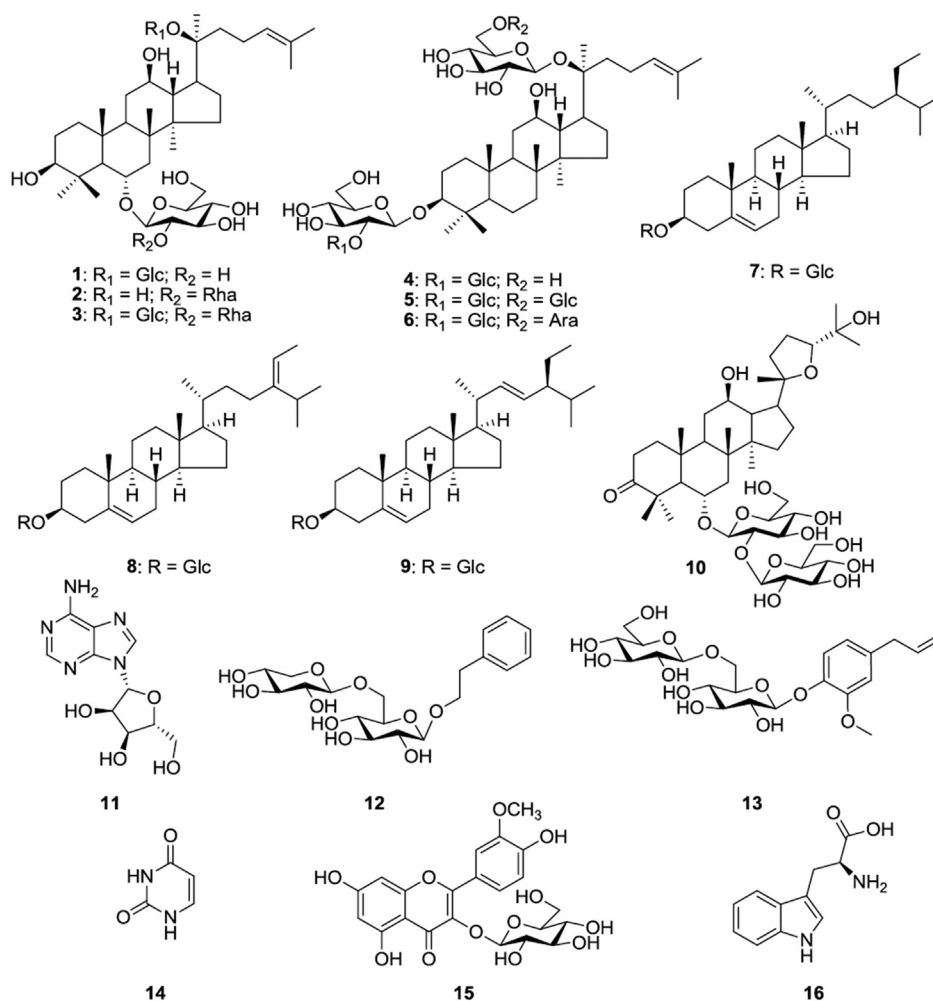
**Fig. 1.** Chemical structures of compounds **1**−**16** from *P. ginseng* seeds.

## 2.8. HPLC-ESI-Q-TOF-MS analysis of P. ginseng extracts

HPLC-ESI-Q-TOF-MS analysis was conducted on compounds **1**−**9** from methanol extract and compound 10 from *n*-butanol−soluble extracts of *P. ginseng* seeds. The isolated compounds **1**−**10** were used as authentic samples. For mobile phases of HPLC, the gradient elution of methanol (0.1% formic acid) (solvent A) and water (0.1% formic acid) (solvent B) mixture was used (50% A in 0−5 min, 50−100% A in 5−30 min, and 100% A in 30−50 min). The flow rate was 1.0 mL/min, and the injection volume was 5 μL. The effluent of HPLC was divided by a split valve to flow one-tenth of the effluent to ESI-Q-TOF-MS. HPLC column Eclipse XDB-C18 (Agilent Technologies, Santa Clara, CA, USA). Positive ion mode ESI-Q-TOF-MS parameters are as follows: flow rate and temperature of drying gas: 13.0 mL/min and 350°C, respectively, nebulizer gas pressure: 35 psig, capillary voltage: 3500 V, nozzle voltage: 1000 V, OCT RFV voltage: 750 V, fragmentor voltage: 50 V, and skimmer voltage: 65 V. The data acquisition was performed using a MassHunter Workstation Software with a version of B.05.00.

## 3. Results and discussion

### 3.1. Structure elucidation of compounds 1−16

Phytochemical work of ginseng seeds led to the determination of a new saponin (**10**), three known protopanaxatriol saponins

(**1**−**3**) [21,22], three protopanaxadiol saponins (**4**−**6**) [23], three sterol glycosides (**7**−**9**) [24,25], two phenolic glycosides (**12**−**13**) [26,27], a flavonoid (**15**) [28], and three primary metabolites (**11**, **14**, and **16**) [29−31]. Fifteen known molecules were identified as ginsenoside (G)-Rg1 (**1**), G-Rg2 (**2**), G-Re (**3**), G-Rd (**4**), G-Rb1 (**5**), G-Rb2 (**6**), β-sitosterol 3-*O*-β-D-glucopyranoside (**7**), daucosterol (**8**), stigmasterol-3-*O*-β-D-glucopyranoside (**9**), adenosine (**11**), phenethyl alcohol β-D-xylopyranosyl(1→6)-β-D-glucopyranoside (**12**), eugenyl β-gentiobioside (**13**), uracil (**14**), isorhamnetin 3-*O*-β-D-glucopyranoside (**15**), and L-tryptophan (**16**). Spectroscopic evidence and structures for **1**−**9** and **11**−**16** are provided in the Fig. 1 and Supplementary Information S2.

Compound **10** was isolated as a white amorphous powder showing a molecular formula of $C_{42}H_{70}O_{15}$, based on a sodiated pseudomolecular ion peak at *m/z* 837.4617 [(M+Na)$^+$, calcd. 837.4612] in its positive ion ESI-Q-TOF-MS spectrum. The $^1$H NMR spectrum of **10** contained eight methyl resonances at [$\delta_H$ 1.86 (3H, s, H-28), 1.69 (3H, s, H-29), 1.47 (3H, s, H-27), 1.25 (6H, s, H-21, 26), 1.10 (3H, s, H-18), 0.81 (3H, s, H-30), 0.75 (3H, s, H-19)]. In addition, two pairs of signals corresponding to anomeric proton and carbon atoms in two sugar moieties were detected at $\delta_H$ 6.02 (1H, d, *J* = 7.8, H-2″)/$\delta_C$ 104.08 (C-1′) and $\delta_H$ 4.91 (1H, d, *J* = 7.7, H-1′)/$\delta_C$ 104.32 (C-1″). $^{13}$C NMR and heteronuclear single quantum correlation spectra revealed 42 carbon signals. Apart from the two sugar moieties, the aglycone of **10** possessed eight methylenes, four methines, three oxygen-bearing methines [$\delta_C$ 79.79 (C-6), 71.40 (C-12), and 86.09
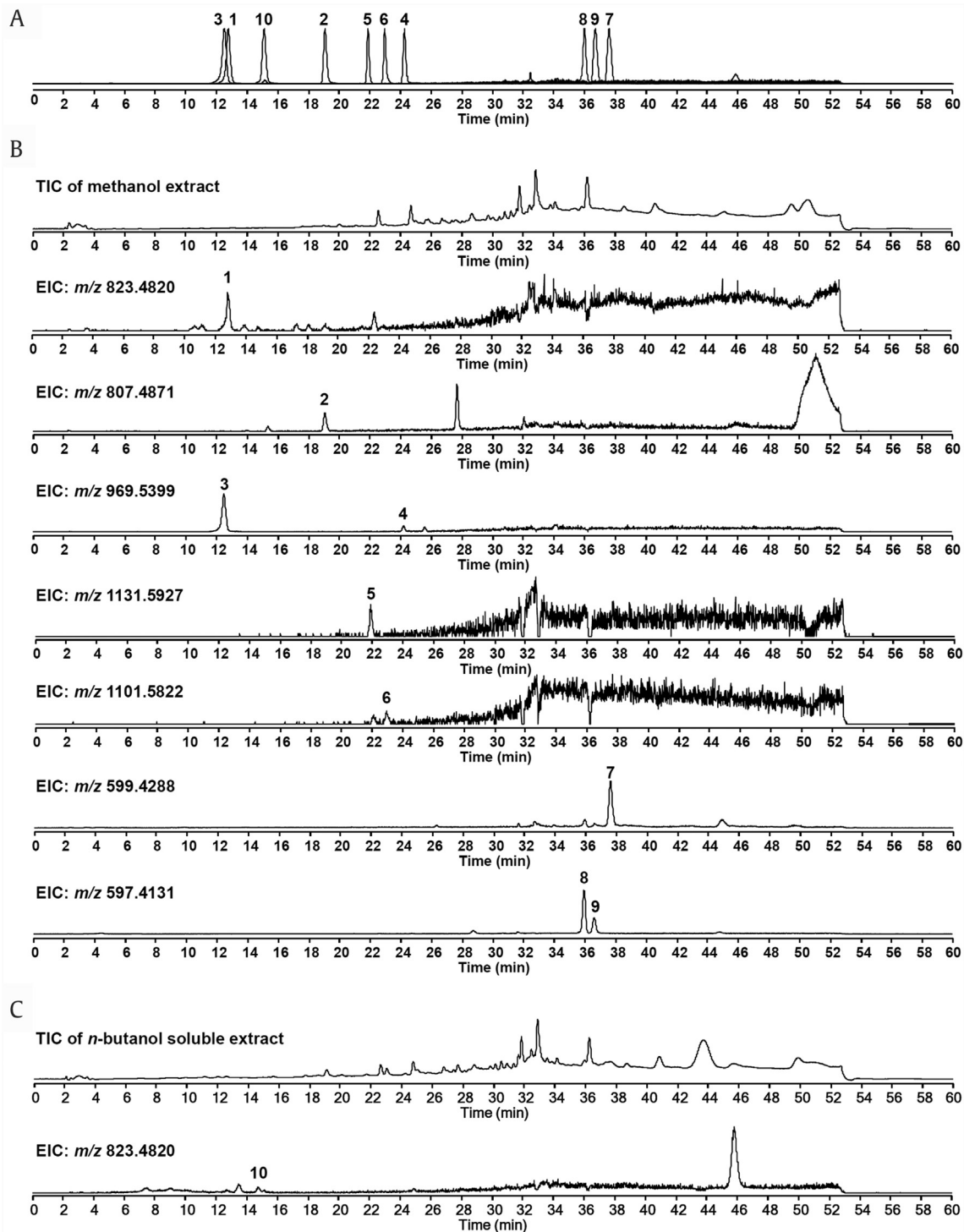
**Fig. 2.** HPLC-ESI-Q-TOF-MS analysis of *P. ginseng* seeds extracts. (A) EIC chromatogram of authentic samples of compounds **1−10**. (B) TIC and EIC chromatograms of methanol extract of *P. ginseng* seeds. (C) TIC and EIC chromatograms of *n*-butanol−soluble extract of *P. ginseng* seeds. Peaks: G-Rg1 (**1**), G-Rg2 (**2**), G-Re (**3**), G-Rd (**4**), G-Rb1 (**5**), G-Rb2 (**6**), β-sitosterol 3-*O*-β-D-glucopyranoside (**7**), daucosterol (**8**), stigmasterol-3-*O*-β-D-glucopyranoside (**9**), and pseudoginsenoside RT$_8$ (**10**).
EIC, extracted ion chromatogram; HPLC-ESI-Q-TOF-MS, high-performance liquid chromatography−electrospray ionization−quadrupole−time-of-flight−mass spectrometry; TIC, total ion chromatogram.

(C-24)], five quaternary carbon atoms, two oxygenated quaternary carbon atoms [δ$_C$ 87.15 (C-20) and 70.78 (C-25)], eight methyl groups, and a carbonyl carbon [δ$_C$ 218.85 (C-3)]. Exhaustive interpretation of $^1$H and $^{13}$C NMR data revealed that the aglycone of **10** was superimposed on pseudoginsengenin R1 [(20*S*,24*R*)-dammar-

3-one-20,24-epoxy-6α,12β,25-triol] [32−34]. The absolute configuration of C-20 in **10** was deduced to be *S* from the chemical shift of C-21 (δ$_C$ 27.67), and the 24*R* configuration was determined by the chemical shift of C-24 (δ$_C$ 86.09), in accordance with previously published evidence [32−34]. The identities of the two sugar units

were elucidated as β-D-glucopyranosyl moieties from their coupling constants of anomeric protons in the $^1$H NMR spectrum and 12 carbon resonances, along with acid hydrolysis data and gas chromatography analysis results. The glycosidic linkages were determined by heteronuclear multiple bond correlation, which showed cross peaks at $\delta_H$ 6.02 (H-1″)/$\delta_C$ 79.49 (C-2′) and $\delta_H$ 4.91 (H-1′)/$\delta_C$ 79.79 (C-6), demonstrating that the 2-O-(β-D-glucopyranosyl)-β-D-glucopyranosyl moiety was linked to C-6 of the aglycone in pseudoginsengenin R1 (Supplementary Information S3–S9). Therefore, the chemical structure of **10** was determined as (20S,24R)-6-O-[2-O-(β-D-glycophyrnosyl)-β-D-glucopyranosyl]-dammar-3-one-20,24-epoxy-6α,12β,25-triol, namely pseudoginsenoside RT₈ (PG-RT₈). The spectroscopic data of compound **10** are as follows:

Compound **10**: $C_{42}H_{70}O_{15}$; ESI-Q-TOF-MS: *m/z* 837.4617 [M+Na]$^+$; [α]$_D^{24}$ −4.82 (*c* 0.30, MeOH); $^1$H-NMR (500 MHz, pyridine-$d_5$): δ 6.02 (1H, d, *J* = 7.8 Hz, H-1″), 4.91 (1H, d, *J* = 7.7 Hz, H-1′), 4.54 (1H, H-6′a)*, 4.54 (1H, H-6″a)*, 4.48 (1H, H-2′)*, 4.38 (1H, H-3′)*, 4.32 (1H, H-6′b)*, 4.32 (1H, H-6″b)*, 4.27 (1H, H-5″)*, 4.18 (1H, H-2″)*, 4.16 (1H, H-4′)*, 4.15 (1H, H-5′)*, 4.15 (1H, H-6)*, 4.12 (1H, H-4″)*, 3.99 (1H, H-3″)*, 3.94 (1H, t, *J* = 7.5 Hz, H-24), 3.68 (1H, td, *J* = 10.6, 4.5 Hz, H-12), 2.57 (1H, H-7a)*, 2.23(1H, H-2a)*, 2.22 (1H, H-11a)*, 2.21 (1H, H-17)*, 2.17 (1H, H-16a)*, 2.06 (1H, d, *J* = 10.6 Hz, H-5), 1.87 (1H, H-16b)*, 1.86 (3H, s, H-28), 1.82 (1H, H-7b)*, 1.82 (1H, H-23a)*, 1.81 (1H, H-13)*, 1.78 (1H, H-2b)*, 1.69 (3H, s, H-29), 1.67 (1H, H-1a)*, 1.64 (1H, H-15a)*, 1.60 (1H, H-9)*, 1.60 (1H, H-22a)*, 1.49 (1H, H-1b)*, 1.45 (3H, s, H-27), 1.37 (1H, H-22b)*, 1.32 (1H, H-11b)*, 1.26 (1H, H-15b)*, 1.25 (3H, s, H-26), 1.25 (3H, s, H-21), 1.25 (1H, H-23b)*, 1.10 (3H, s, H-18), 0.81 (3H, s, H-30), 0.75 (3H, s, H-19) (*peak overlapped); $^{13}$C-NMR (125 MHz, pyridine-$d_5$): δ 218.85 (C-3), 104.32 (C-1″), 104.08 (C-1′), 87.15 (C-20), 86.09 (C-24), 80.55 (C-3′), 79.94 (C-5′), 79.79 (C-6), 79.49 (C-2′), 79.11 (C-5″), 78.64 (C-3″), 76.34 (C-2″), 73.05 (C-4′), 72.34 (C-4″), 71.40 (C-12), 70.78 (C-25), 63.93 (C-6″), 63.53 (C-6′), 58.35 (C-5), 52.76 (C-14), 49.97 (C-13), 49.47 (C-9), 48.75 (C-17), 48.58 (C-4), 43.47 (C-7), 40.63 (C-1), 40.47 (C-8), 38.82 (C-10), 33.61 (C-2), 33.47 (C-11), 33.19 (C-15), 32.95 (C-28), 32.09 (C-22), 29.25 (C-23), 28.18 (C-27), 27.67 (C-21), 27.43 (C-26), 25.94 (C-16), 20.42 (C-29), 18.52 (C-30), 18.48 (C-19), 16.13 (C-18)

## 3.2. HPLC-ESI-Q-TOF-MS analysis of P. ginseng extracts

HPLC-ESI-Q-TOF-MS analysis was performed to confirm the presence of compounds **1–10** from the extracts of *P. ginseng* seeds. The identification of compounds **1–10** in ginseng extracts was achieved by comparison with retention times and extracted ion chromatograms (EICs) established by theoretical *m/z* values of sodiated pseudomolecular ion [M+Na]$^+$ of authentic samples [*m/z* 823.4820 (G-Rg1); *m/z* 807.4871 (G-Rg2); *m/z* 969.5399 (G-Re and G-Rd); *m/z* 1131.5927 (G-Rb1); *m/z* 1101.5822 (G-Rb2); *m/z* 599.4288 (β-sitosterol 3-O-β-D-glucopyranoside); *m/z* 597.4131 (daucosterol and stigmasterol-3-O-β-D-glucopyranoside); 823.4820 (PG-RT₈)]. As shown in Figs. 2A, 2B, all saponins were well detected in methanol extract of *P. ginseng* except for compound **10**, which indicated that the content of compound 10 was relatively lower than that of the other ginseng saponins. However, compound **10** was observed in *n*-butanol–soluble extract because saponins are well concentrated in *n*-butanol (Fig. 2C).

The results of the present phytochemical work are comparable with those of a previously published study by Sugimoto et al [26], which identified 11 compounds from *P. ginseng* seeds: a dammarane-type triterpene ketone, two dammarane- and lupine-type triterpenes, an aromatic oligo-glycoside, three sterol glycosides, G-Rd, G-Re, and G-Rg2. The previous study revealed that the content of G-Re was the highest among the ginsenosides. In the present phytochemical work, the isolation yield of G-Re was the highest among the saponins. Therefore, we carefully assume that G-Re is a characteristic ginsenoside in *P. ginseng* seeds.

## 3.3. Inhibition of proinflammatory genes expression of compounds **1–10**

Many studies have revealed that ginsenosides exert antiinflammatory effects of *P. ginseng* [12,35–38]. In this study, seven triterpene saponins (**1–6** and **10**) and three steroidal saponins (**7–9**) were tested to evaluate and compare their inhibition level of
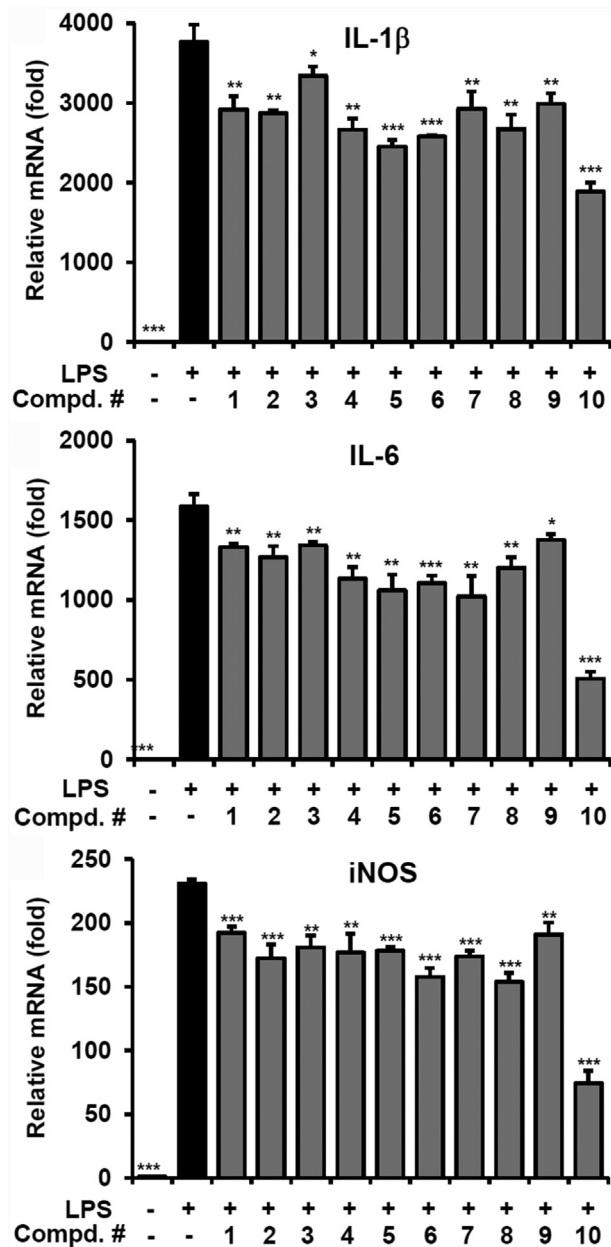


**Fig. 3.** *P. ginseng* seed–derived saponins exhibit antiinflammatory properties. RAW264.7 macrophages were treated for 2 h with 10 μM of each ginseng seed–derived triterpene and steroidal saponins (**1–10**) and then with lipopolysaccharide (LPS) (10 ng/mL) for an additional 6 h. mRNA expression of inflammatory genes was measured by quantitative polymerase chain reaction analyses and normalized by cyclophilin. *P < 0.05 vs. LPS, **P < 0.01 vs. LPS. ***P < 0.001 vs. LPS.
IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase.

proinflammatory genes expression (IL-1β, IL-6, and iNOS) using LPS-stimulated RAW264.7 macrophages. The primary metabolites and phenolic compounds were excluded from the experiments.

Cytokines, IL-1β, and IL-6 are produced from diverse cell types, most importantly macrophages and mast cells. Both cytokines possess important homeostatic functions in normal condition. In most inflammatory states, they are overproduced, giving rise to pathophysiological changes in the human body and inducing many inflammatory diseases [39,40]. iNOS is one of the nitric oxide synthase isoforms, which generates high levels of NO in inflammatory events, causing cellular death and tissue destruction [41,42].

As shown in Fig. 3, nine of the tested saponins (1−9) showed moderate inhibition of LPS-induced proinflammatory genes expression at a concentration of 10 μM. Remarkably, compound 10, PG-RT$_8$, showed the most potent inhibitory activities against IL-1β, IL-6, and iNOS genes expression compared with those of compounds 1−9 at the same concentration. To rule out the possibility that the saponins may be affecting inflammatory gene expression through cytotoxic activity, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were conducted to assess cell growth in the presence of 10 saponins (Supplementary Information S10). None of the tested saponins showed any cytotoxicity up to 50

μM. These results indicate that all saponins negatively regulate the expression of proinflammatory genes without harmful effects on cell viability.

The antiinflammatory properties of G-Rg1 (1), G-Re (3), G-Rd (4), G-Rb1 (5), and G-Rb2 (6) have been reviewed previously [12], which described that such ginsenosides exert antiinflammatory activities of Korean White Ginseng and Korean Red Ginseng by suppressing the generation of proinflammatory cytokines and regulating inflammatory signaling pathways. It is notable that a novel saponin, PG-RT$_8$, showed most potent antiinflammatory properties than well-known ginsenosides and steroidal saponins. Thus, detailed antiinflammatory experiments were performed on PG-RT$_8$.

### 3.4. Pseudoginsenoside RT$_8$ relieved proinflammatory responses of LPS-stimulated RAW264.7 macrophages and THP-1 monocytes leukemia cells in a dose-dependent manner

As described previously, PG-RT$_8$ decreased the mRNA expression of IL-1β, IL-6, and iNOS at a concentration of 10 μM. To confirm the inhibitory effects of PG-RT$_8$ against antiinflammatory genes expression, further experiments were performed to measure the
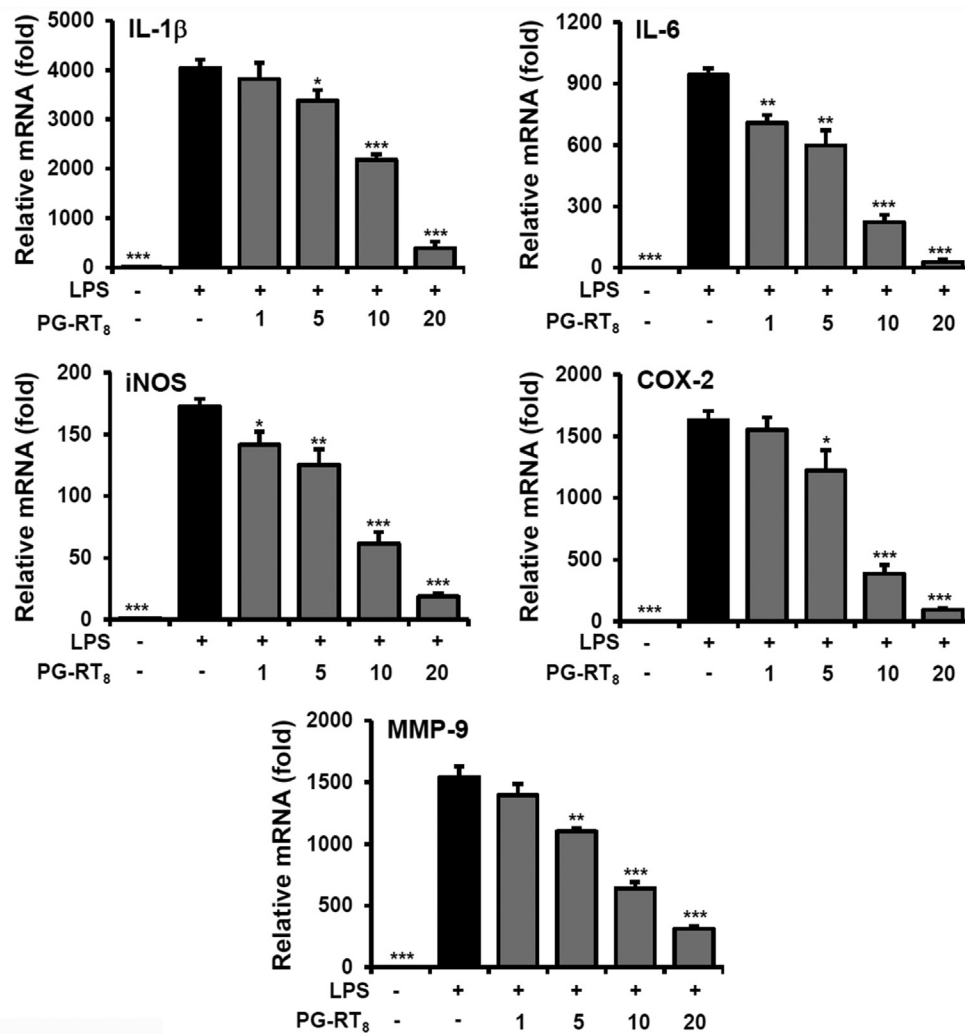


Fig. 4. PG-RT$_8$ suppresses the expression of proinflammatory genes in a dose-dependent manner. RAW264.7 macrophages were treated with PG-RT$_8$ (1, 5, 10, and 20 μM) for 2 h and then with LPS (10 ng/mL) for an additional 6 h. mRNA expression of inflammatory genes was measured by quantitative polymerase chain reaction analyses and normalized by cyclophilin. *P < 0.05 vs. LPS, **P < 0.01 vs. LPS. ***P < 0.001 vs. LPS.
COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase, LPS, lipopolysaccharide; MMP-9, metalloproteinase-9; PG-RT$_8$, pseudoginsenoside RT$_8$.

relative amounts of transcripts of COX-2 and MMP-9, as well as IL-1β, IL-6, and iNOS, in four concentration levels (1, 5, 10, and 20 μM) of PG-RT$_8$.

COX-2 is an inducible form of prostaglandin synthetase enzyme that is involved in prostaglandin production pathway and closely related to generating prostaglandin E$_2$ (PGE$_2$) from arachidonic acid. The expression of COX-2 is elevated in inflammatory and malignant states by proinflammatory cytokines such as tumor necrosis factor-α, IL-1β, and epidermal growth factor (EGF) [43,44].

MMP-9 is one of the MMPs of gelatinase family which cleave extracellular matrix and mediate immune cells migration into tissues. MMP-9 is mainly found in monocytes, macrophages, and neutrophils and highly induced by chemokines and cytokines during inflammatory events. The enhanced level of MMP-9 from immune cells is related to diverse inflammatory pathologies, including autoimmunity, arthritis, and transplant rejection [45,46].

As demonstrated in Fig. 4, PG-RT$_8$ strongly attenuated the LPS-mediated induction of proinflammatory genes expression (IL-1β, IL-6, iNOS, COX-2, and MMP-9) in LPS-stimulated macrophages at 20 μM (P < 0.001). Moreover, transcripts of IL-1β, IL-6, and MMP-9 were also downregulated by PG-RT$_8$ in LPS-stimulated THP-1 monocyte leukemia cells in a dose-dependent manner (Fig. 5). These results demonstrate that PG-RT$_8$ possesses potent antiinflammatory activity. Because MMP-9 is also known to play an important role in cancer progression and metastasis, our results imply a therapeutic value of PG-RT$_8$ in treating cancers and immune-related diseases.
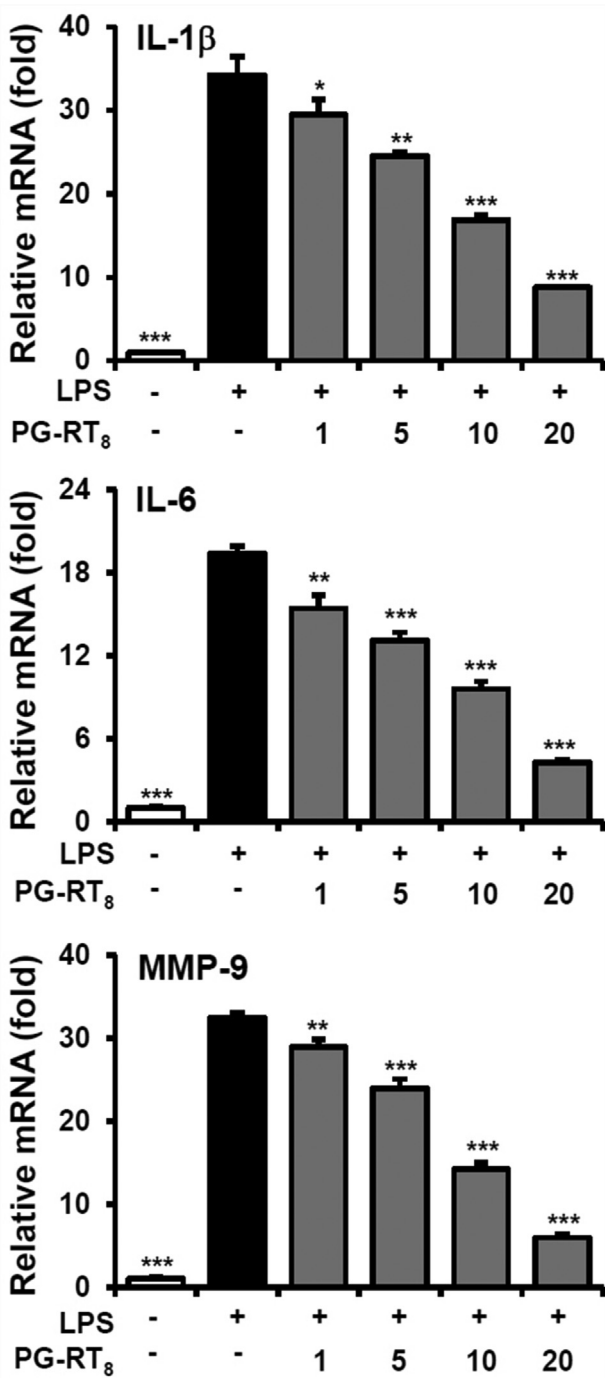


**Fig. 5.** PG-RT$_8$ suppresses the LPS-stimulated induction of proinflammatory genes in THP-1 monocytes leukemia cells. THP-1 monocytes leukemia cells were treated with PG-RT$_8$ (1, 5, 10, and 20 μM) for 2 h and then with LPS (10 ng/mL) for an additional 6 h. mRNA expression of inflammatory genes was measured by quantitative polymerase chain reaction and normalized by cyclophilin. *P < 0.05 vs. LPS, **P < 0.01 vs. LPS. ***P < 0.001 vs. LPS.
IL-1β, interleukin-1β; IL-6, interleukin-6; LPS, lipopolysaccharide; MMP-9, metalloproteinase-9; PG-RT$_8$, pseudoginsenoside RT$_8$.
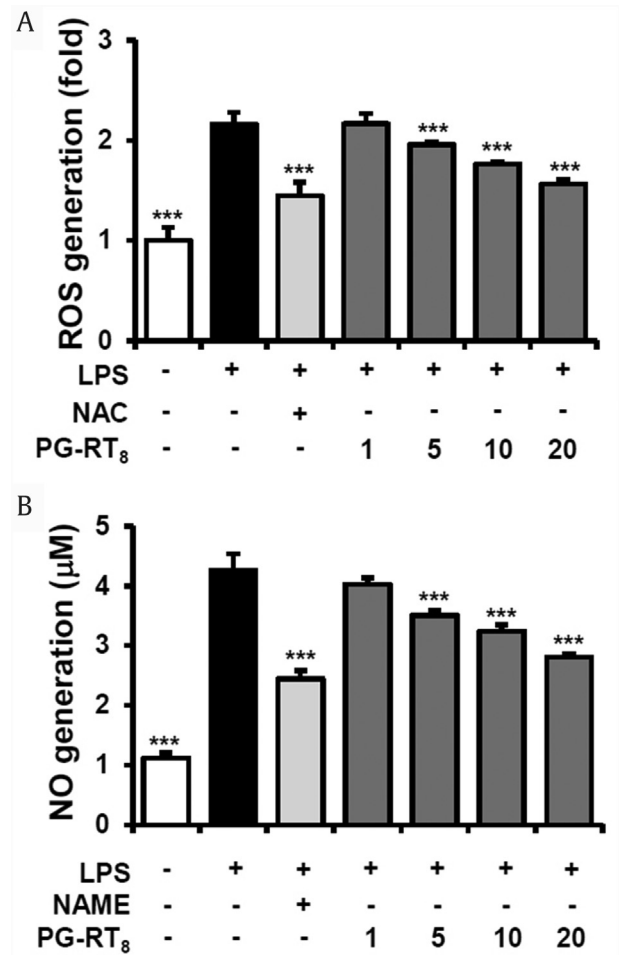


**Fig. 6.** PG-RT$_8$ decreases ROS and NO levels in LPS-stimulated RAW264.7 macrophages. (A) RAW264.7 macrophages were treated with N-acetyl-L-cysteine (NAC; 1 mM) or PG-RT$_8$ (1, 5, 10, and 20 μM) for 1 h. Then, cells were treated with LPS (10 ng/ml) for 30 minutes. The relative levels of ROS were measured as described in the Section 2. (B) RAW264.7 macrophages were treated with Nω-nitro-L-arginine methyl ester (L-NAME; 10 μM) or PG-RT$_8$ (1, 5, 10, and 20 μM) for 1 h. The cells were then treated with LPS (10 ng/mL) for an additional 1 h. NO accumulation in the medium was assessed by the Griess reaction, as described in the Section 2. ***P < 0.001 vs. LPS. LPS, lipopolysaccharide; NO, nitric oxide; PG-RT$_8$, pseudoginsenoside RT$_8$; ROS, reactive oxygen species.

### 3.5. Pseudoginsenoside RT₈ reduced the production of NO and ROS in a dose-dependent manner

Proinflammatory cytokines mediate the recruitment of activated immune cells to inflamed tissues. The effector immune cells build up a cytotoxic condition to get rid of invading pathogens by producing high levels of toxic molecules such as NO and ROS. Finally, such excessive reactive oxygen and nitrogen species lead to tissue damage [12]. As mentioned previously, NO is generated by the catalytic action of iNOS, and both are involved as proinflammatory mediators. Considering these factors, ROS and NO production was evaluated in the presence of PG-RT₈ using LPS-stimulated RAW264.7 macrophages.

The production of ROS and NO in RAW264.7 macrophages decreased with increasing doses of PG-RT₈ (Fig. 6), which was consistent with the iNOS gene expression pattern (Fig. 4). N-acetylcysteine (NAC) has been used as a positive control when measuring the antiinflammatory effects of PG-RT₈. In this regard, it is noteworthy that 20 μM of PG-RT₈ was as effective in inhibiting ROS production as 1 mM of NAC ($P < 0.001$). Furthermore, 20 μM of PG-RT₈ inhibited NO production ($P < 0.001$) to a level comparable with Nω-nitro-L-arginine methyl ester (10 μM), a well-known nitric oxide synthase inhibitor. These results indicate that PG-RT₈ possesses multiple and strong antiinflammatory properties and has a pharmaceutical potential as an antiinflammatory agent.

## 4. Conclusion

A phytochemical study of *P. ginseng* seeds led to the isolation of a novel ginsenoside, namely pseudoginsenoside RT₈, as well as 15 known compounds. The yield of G-Re was the highest among the isolated ginsenosides, which suggests that G-Re may be a characteristic ginsenoside in *P. ginseng* seeds. The seven isolated ginsenosides and three steroidal saponins were evaluated for their antiinflammatory activities using LPS-stimulated RAW264.7 macrophages and THP-1 human monocyte leukemia cells. A novel compound, PG-RT₈, showed the most potent antiinflammatory activities, attenuating the LPS-mediated induction of proinflammatory genes (IL-1β, IL-6, iNOS COX2, and MMP-9) in a dose-dependent manner. In addition, PG-RT₈ strongly suppressed ROS and NO production. These findings indicate that PG-RT₈ has a pharmaceutical potential as an antiinflammatory agent and that *P. ginseng* seeds are also a good natural source for discovering novel bioactive molecules.

## Conflicts of interests

None of authors declare conflicts of interest.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://doi.org/10.1016/j.jgr.2018.11.001.

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