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Research Article

A novel protocol for batch-separating gintonin-enriched, polysaccharide-enriched, and crude ginsenoside-containing fractions from *Panax* ginseng

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ABSTRACT

Background: Ginseng contains three active components: ginsenosides, gintonin, and polysaccharides. After the separation of 1 of the 3 ingredient fractions, other fractions are usually discarded as waste. In this study, we developed a simple and effective method, called the ginpolin protocol, to separate gintonin-enriched fraction (GEF), ginseng polysaccharide fraction (GPF), and crude ginseng saponin fraction (cGSF).

Methods: Dried ginseng (1 kg) was extracted using 70% ethanol (EtOH). The extract was water fractionated to obtain a water-insoluble precipitate (GEF). The upper layer after GEF separation was precipitated with 80% EtOH for GPF preparation, and the remaining upper layer was vacuum dried to obtain cGSF.

Results: The yields of GEF, GPF, and cGSF were 14.8, 54.2, and 185.3 g, respectively, from 333 g EtOH extract. We quantified the active ingredients of 3 fractions: L-arginine, galacturonic acid, ginsenosides, glucuronic acid, lysophosphatidic acid (LPA), phosphatidic acid (PA), and polyphenols. The order of the LPA, PA, and polyphenol content was GEF > cGSF > GPF. The order of L-arginine and galacturonic acid was GPF >> GEF = cGSF. Interestingly, GEF contained a high amount of ginsenoside Rb1, whereas cGSF contained more ginsenoside Rg1. GEF and cGSF, but not GPF, induced intracellular $[Ca^{2+}]_i$ transient with antiplatelet activity. The order of antioxidant activity was GPF > GEF = cGSF. Immunological activities (related to nitric oxide production, phagocytosis, and IL-6 and TNF-α release) were, in order, GPF > GEF = cGSF. The neuroprotective ability (against reactive oxygen species) order was GEF > cGSP > GPF.

Conclusion: We developed a novel ginpolin protocol to isolate 3 fractions in batches and determined that each fraction has distinct biological effects.

1. Introduction

For thousands of years, ginseng, as an herbal tonic, has been used for activating brain function and resisting diseases and stress; it has anti-fatigue, anti-cancer, anti-diabetes, and other beneficial

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physiological and pharmacological functions [1]. Korea, China, and Japan have been using ginseng for over 100 years. Ginseng is one of the most valuable medicinal herbs [2,3].

Ginseng contains diverse active ingredients, and dedicated researchers have isolated the active ingredients of ginseng and studied their biological functions. Ginsenosides are representative ingredients in ginseng; they were thought to be responsible for its efficacy for many years before other functional ingredients were found [4,5]. For example, ginseng polysaccharides activate immune systems [6,7]. The active ingredient responsible for the immune activation is the acidic polysaccharide component of ginseng [8]. Pyo et al isolated a novel glycolipoprotein that elicits $[Ca^{2+}]_i$ transient, a second messenger, in neuronal and non-neuronal cells, which was not induced by ginsenosides and ginseng polysaccharides [9]. Subsequently, Hwang et al named it a gintonin and found that it contains high amounts of LPAs that function as exogenous ligands for G protein-coupled lysophosphatidic acid (LPA) receptor [10]. Thus, gintonin elicits $[Ca^{2+}]_i$ transients in neuronal and non-neuronal cells via $G\alpha_{q/11}$-coupled LPA receptors, and gintonin-mediated $[Ca^{2+}]_i$ transients are a biological basis for diverse ginseng functionality [11]. Thus, ginseng contains a G protein-coupled receptor ligand like that of other medicinal plants. In addition to other minor components of ginseng, the 3 aforementioned components are the representative bioactive components of ginseng [2].

Ginsenosides were the first known ginseng components; thus, crude ginseng total saponin fraction (cGSF) has been prepared using methanol and butanol extraction from ginseng, owing to the limited yield of pure ginsenosides, complexity of the extraction process, and high cost of pure ginsenosides [12–16]. Furthermore, the limitations of using organic solvents such as methanol and butanol for preparing crude ginseng total saponin fraction are not acceptable for direct human application. Therefore, the use of cGSF isolated from organic solvents is limited to only research applications, and it has not been applied in humans [17]. Moreover, most of the other bioactive substances, except cGSF, were discarded because organic solvent was used for the separation step [17].

For polysaccharide separation, ginseng is also extracted using water, ethanol, butanol, or methanol, and the precipitated fraction is eliminated [7]. Next, the supernatant is precipitated with ethanol, and the precipitate is used as crude ginseng polysaccharide fraction (GPF). This method also has a limitation, as the remaining part (after separation of the GPF) cannot be further utilized [7].

Gintonin was first isolated using methanol and butanol extraction and anion exchange column chromatography, and a subsequent gintonin-enriched fraction (GEF) was prepared for the mass production of edible gintonin using only ethanol and water [9]. However, this method for GEF preparation from ginseng also has a limitation: other ginseng substances, such as ginseng ginsenosides and ginseng polysaccharides, were not retrieved after GEF separation [18].

Obtaining qualified ginseng products requires cultivating the ginseng for at least 4 to 6 years; thus, the production cost of ginseng is relatively higher than that of other natural herbal medicinal products [19]. Despite the high production cost of ginseng, most of its bioactive compounds are discarded without further processing after separating 1 ginseng fraction. Therefore, ginseng waste prevention processes such as recycling and the retrieval of ginseng components are necessary. However, a separation method for the 3 representative fractions simultaneously has not been developed.

In this study, we developed a “ginpolin” protocol that sequentially separates GEF, GPF, and cGSF using only ethanol and water. The origin of the term “ginpolin” is as follows; *gin*, *pol*, and *in* are derived from *gin*tonin, ginseng *po*lysaccharides, and ginseng sapon*in*, respectively.

2. Methods

2.1. Materials preparation

Four-year-old Korean White ginseng (Korea Ginseng Cooperation, Daejon, Korea) was purchased from a local ginseng market. Ginseng marcs were obtained from local ginseng product manufacturers. All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). 1-Linoleoyl-2-hydroxy-sn-glycero-3-phosphate (LPA C18:2) was purchased from Echelon (Salt Lake City, UT, USA), and 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphate (PA 16:0-18:2) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Ethanol was purchased from Korea Ethanol Supplies Company (Seoul, Republic of Korea).

2.2. Ginpolin protocol for extracting active ingredients from ginseng

Fig. 1 illustrates the novel ginpolin method for separating GEF, GPF, and crude ginseng saponin (cGSP) fractions. In brief, locally purchased white ginseng (1 kg) was ground into small pieces (>3 mm) and refluxed with 70% ethanol 4 times for 8 h at 80 °C. Ethanol extracts (333 g) were obtained as described in Fig. 1. The ethanol extract was dissolved in cold distilled water at a 1:10 ratio and stored at 4 °C for 24 h for precipitation. The supernatant and precipitate were separated by centrifugation (1977 g, 20 min), and the precipitate was freeze-dried and designated as GEF. The supernatant was separated again at a ratio of 1:4 (supernatant: 80% EtOH)

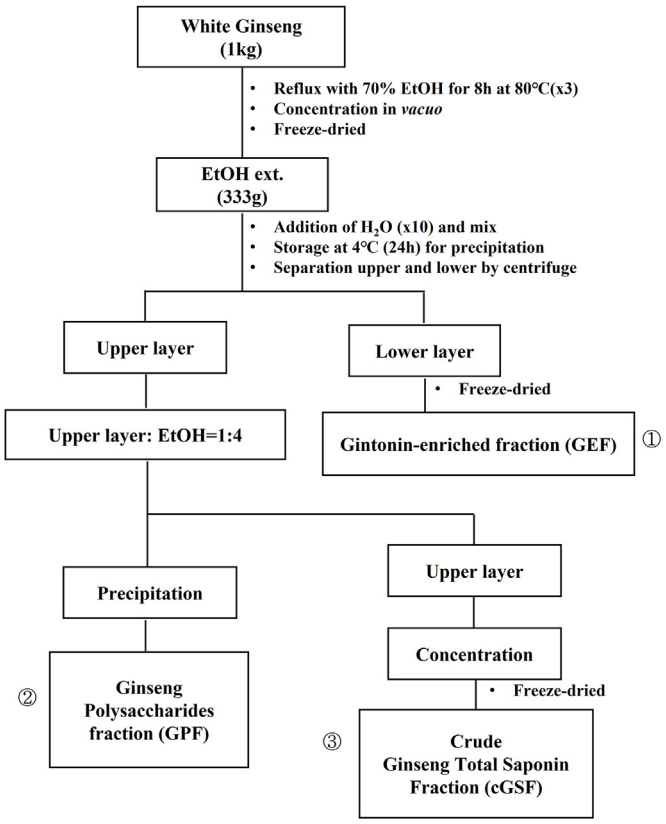

Fig. 1. Schematic methods for ginpolin protocol for 3 representative fractions from ginseng. A ginpolin protocol uses only edible ethanol and water. Ginseng was refluxed with 70% ethanol for 8 h at 80 °C 3 times; freeze-dried ethanol extract is dissolved in 10 times volume of water, precipitated, and separated by centrifugation. The resulting precipitate from water fractionation was designated GEF. The supernatant was mixed with ethanol in a 1:4 ratio, leading to ethanol precipitation to obtain GPF. The rest of the upper layer was concentrated via vacuum- and freeze-drying to obtain cGSF.

by yielding precipitate and designating GPF after freeze-drying. Supernatant in 80% EtOH was concentrated in vacuo, and this fraction was designated cGSF.

2.3. Quantitation of LPA $C_{18:2}$ and PA $C_{16:0-18:2}$ in GEF, GPF, and cGSF using LC-MS/MS

Stock solutions of LPAs and PAs were prepared and diluted in high-pressure liquid chromatography (HPLC) grade methanol (MeOH), and all other solutions were stored at 4 °C. LPAs and PAs were quantified by LC-MS/MS, using an Agilent series 1100 HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) and an API 2000 LC-MS/MS system (Applied Biosystems, Foster City, CA, USA) as previously reported [18]. All values are presented as mean ± relative standard deviation (%) from the 3 different samples of the 3 ginseng fractions.

2.4. Quantitation of ginsenosides in GEF, GPF, and cGSF

Ginsenoside quantification was performed by the Geumsan Ginseng & Herb Development Agency (Chungcheongnam-do, Republic of Korea). In brief, 1 g of sample was measured in a 50 mL tube, and 25 mL 70% (v/v) methanol was mixed by shaking for 15 min at 200 rpm. After centrifugation at 1600 × *g* for 10 min at 4 °C, the samples were filtered and diluted for analysis. The standard solution was prepared by mixing 10.2 mg ginsenoside (Chengdu Biopurify Phytochemicals Ltd. 98%, Chengdu, China) with 10 mL 70% (v/v) methanol and then diluting by 70% (v/v) methanol. Ginsenosides were quantified by high-performance liquid chromatography using an Agilent 1260 infinity HPLC/DAD (Agilent Technologies, Santa Clara, CA, USA) instrument at a wavelength of 203 nm. The ginsenosides were separated on an Accucore C18 column (2.6 μm, 3.0 × 50 mm, Thermo, MA, USA). The mobile phase comprised water (A) and acetonitrile (B). The isocratic pump mode was run at a flow rate of 0.8 mL/min, and 2 μL aliquots were injected into the column. The column temperature was maintained at 30 °C.

The content of ginsenosides was calculated as follows:

Ginsenoside content (mg/g) = C × (a/S) × b × 1/1000

C: each ginsenoside's concentration in the test sample (μg/mL)

A: total volume of the test sample (mL)

S: weighed test sample (g)

b: dilution factor

1/1000: unit conversion factor

2.5. Quantitation of acidic polysaccharides content in GEF, GPF, and cGSF

The acidic polysaccharide content in GPF was determined as follows: galacturonic acid or glucuronic acid as a standard marker was dissolved in distilled water as a 1 mg/mL stock solution and diluted to 0, 10, 20, 30, 40, and 50 μg/mL. Next, 20 μL diluted galacturonic acid or 100 mg/mL of GEF, GPF, or cGSF was mixed with 80 μL of distilled water. Subsequently, 50 μL 0.1% carbazol solution in 100% EtOH was kept at room temperature for 5 min and then mixed with 600 μL sulfuric acid. The reaction mixture was incubated at 85 °C for 15 min, and 100 μL of the mixture was transferred to a 96-well plate and read using a multiplate reader (Molecular Devices C., CA, USA) at 765 nm for galacturonic acid or at 525 nm for glucuronic acid. The measured optical density (OD) was used for the calculations. The R2 value of the calibration curve for the reference material was greater than 0.99.

2.6. Quantitation of polyphenolic component in GEF, GPF, and cGSF

Quantitative analysis of the phenol assay was performed using gallic acid as reference material. Gallic acid was measured at a concentration of 1 mg/mL in distilled water and diluted to solutions of 0, 10, 20, 30, 40, and 50 μg/mL. GEF, GPF, or cGSP were used to obtain 100 mg/mL stock solutions. Either diluted reference material or each fraction (80 μL) and Folin–Ciocalteu reagent (20 μL) were mixed and reacted for 5 min. Next, 2% sodium bicarbonate ($NaCO_3$) was added to the reaction mixture and incubated for 1 h at room temperature. The OD of each sample was measured at 765 nm using a multiplate reader (Molecular Devices, CA, USA). The R^2 value of the calibration curve for the reference material (gallic acid) was greater than 0.99.

The total gallic acid content was calculated using the following linear equation and was expressed as mg/g:

$A = 0.006X-0.0154$, $R^2 = 0.9905$

Where A is absorbance, and X is the amount of gallic acid in mg.

2.7. Free arginine analysis

Free L-arginine in each fraction, including GEF, GPF, and cGSF, was analyzed using an amino acid analyzer (SYKAM, No. S433; Sykam GmbH, Eresing, Germany). An amino acid standard stock solution type PH (Sykam Catalog No. S000031) at 10-fold dilution was used, yielding a final concentration of 100 nmol/mL. The samples were pretreated according to the application notes provided. In brief, 100 mg of each sample was measured, and 10 mL of distilled water was added; next, this solution was shaken at 25 °C for 3 h and centrifuged at 4000 rpm for 10 min at room temperature. Subsequently, the samples were filtered through a 0.22 μm filter and re-diluted with 5 mL sample dilution buffer. The samples (200 μL) were used for analysis. For chromatographic analysis, 3 different concentrations of lithium citrate buffer and regeneration solution were used as the mobile phase. The ninhydrin reagent was run using a separating pump with a flow rate of 0.35 mL/min. The column temperature was set to room temperature, and detection using a photometer was performed at 570 and 440 nm. The column (LCA K07/Li, 4.6 × 150 mm) was eluted by the 4 different aforementioned buffers, with a flow rate of 0.45 mL/min; the L-arginine content of the compounds is shown as milligrams per gram (mg/g) of sample extracts.

2.8. Cell culture

RAW264.7 and HT22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 units/mL penicillin at 37 °C with 5% CO_2. PC-3 cells were cultured in RPMI-1640 supplemented with 10 % (v/v) fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin.

2.9. Intracellular Ca^{2+} assay

Intracellular Ca^{2+} was measured in a Fura 2-loaded cell by an intracellular ion measurement system (RF-5300PC; Shimadzu Corporation, Japan). Specifically, Fura-2 loaded cells were diluted to a final concentration of 2 × 10^6 cells/mL and transferred to a polystyrene cuvette (Elkay Ultra–UV) as previously reported [10,20]. All values are presented as mean ± relative standard deviation (%) from the 3 different samples of the 3 ginseng fractions.

The ratio of absorbance values measured at 304 nm to 380 nm were converted to $[Ca^{2+}]_i$ using the formula by Grynkiewicz et al [21].

2.10. Cell viability assay

A colorimetric WST-8 kit was purchased from MONOBIO (Seoul, Republic of Korea). The viability of Raw264.7 cells was determined by the WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) assay, as described in the manufacturer's instructions. In brief, cells were seeded (1×10^5 cells per well) in 96–well plates in complete RPMI-1640 media and exposed to the indicated concentrations of ginsenoside (0.1, 0.3, 1, 3, 10, 30, 100, and 300 μg/mL) or LPS (2 μg/mL) for 24 h. Cells were then washed twice with 1X DPBS and placed in fresh medium with 10 μL Chromo-CKTM for 2 h. Absorbance was measured at 450 nm in a multiplate reader (SpectraMax ABS plus, Molecular Devices).

2.11. $ABTS^+$ radical scavenging activity (H_2O_2 specific test)

For the ABTS radical decolorization assay of each fraction (GEF, GPF, and cGSF), the procedure in Arnao et al was used, with minor modifications [22]. ABTS cation radicals were produced by mixing 14 mM ABTS and 4.9 mM potassium persulfate in distilled water. The 2 solutions were mixed in equal quantities, and the mixture was placed in the dark for 12-16 h before the experiment. The ABTS solution was further diluted with ethanol to adjust the OD value to 0.700 ± 0.02 at 734 nm. The supernatant of the 100 mg/mL ethanol solution was diluted after centrifugation at 3000 rpm for 10 min and filtration at a pore size of 0.22 μm. The doses evaluated were 0.3125, 0.625, 1.25, and 2.5 mg. All tests were performed in triplicate, and the inhibition of absorbance was calculated using the formula in Prior et al [23].

2.12. Neutral red uptake assay for macrophage phagocytosis

Raw264.7 cells were seeded in 96-well plates at density of 1×10^4 cells/well and treated with either 3 fractions (0.1, 0.3, 1, 3, 10, 30, 100, 300 μg/mL) at various doses or with LPS (2 μg/mL) as a control after 24 h in a 5% CO_2 humidified chamber at 37 °C. Dissolved in 10 mM PBS at 0.075% final concentration, 100 μL of neutral red solution (Neutral Red, Sigma-Aldrich, N4638-1G) was added and then incubated for 1 h. After supernatants were removed, the cells were washed twice, and 100 μL/well of EtOH: 0.01% (v/v) acetic acid (1:1) was added. The 96-well plate was then incubated O/N at RT, and the O.D. value at 540 nm was measured.

2.13. Cytokine (TNF-α and IL-6 analysis) release analysis

RAW264.7 cells were treated with GEF, GPF, or cGSF. Each sample was diluted with serum-free medium for treating cells at different concentrations (0.1, 0.3, 1, 3, 10, 30, 100, 300 μg/mL) and incubated at 37 °C in a 5% CO_2 humidified chamber for 24 h. The supernatant was collected to measure IL-6 and TNF-α levels using ELISA kits (BD Biosciences, San Diego, CA, USA and Invitrogen, San Diego, CA, USA). Cells were seeded at a density of 1×10^5 cells/mL in 96-well plates, treated with each of the 3 samples or LPS (2 μg/mL), and cultured for another 24 h. Each supernatant was centrifuged at 12,000 rpm for 3 min. For IL-6 analysis, 50 μL of supernatant was used and the reaction was followed by user's instructions. After the reaction process, the reaction was terminated by adding stop solution (50 μL), and the plate was read at 570 nm and 450 nm using a multiplate reader (SpectraMax ABS plus, Molecular Devices). For TNF-α analysis, 50 μL of supernatant was used under user's instructions. After the reaction was finished, O.D. at 450 nm was measured using a multiplate reader (SpectraMax ABS plus, Molecular Devices).

2.14. Platelet aggregation

Seven-week-old rats were used for platelet aggregation experiments [24]. The animals were anesthetized, blood was collected from the heart, and acid citrate dextrose was used as an anticoagulant. Collected blood samples were centrifuged at 1000 rpm for 7 min to obtain platelet-rich plasma (PRP); next, the PRP was again centrifuged at 2000 rpm for 7 min to yield pelleted platelets. The final washed platelets were obtained by suspension in Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.5 mM glucose, 2 mM KCl, 1 mM $MgCl_2$, and 0.3 mM $NaHPO_4$, pH 7.4) from pelleted platelets. An aggregometer (Chrono-log, Havertown, PA, USA)) was used for platelet aggregation tests. After adding 1 mM $CaCl_2$, 3 samples were mixed with cells containing platelets. For the induction of platelet aggregation, platelets were stirred for 1 min at 37 °C; next, the reaction with collagen (2.5 μg/mL) was induced for 5 min. The degree of aggregation was measured after 4 min.

2.15. Iodoacetic acid (IAA)-induced oxidative stress in HT22 cells

Cell viability and neuroprotection against IAA was measured using a WST-8-(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (XTT) assay according to the manufacturer's protocol as previously reported [31]. All values are presented as mean ± relative standard deviation (%) from the 3 different samples of the 3 ginseng fractions.

3. Results

3.1. Ginpolin protocol for a simultaneous GEF, GPF, and cGSF preparation using ethanol and H_2O from ginseng

When we prepared 3 fractions according to Fig. 1 procedure, the sum of the 3 fractions was approximately 254.3 g. The yield of each fraction was, in order, cGSF > GPF > GEF. Thus, the final yields of 3 active ingredients such as GEF, GPF, and cGSF were 1.48%, 5.42%, and 18.5%, respectively, with a total yield of 25.4% (Fig. 1). When we converted into % yield from the initial 70% ethanol extract (333 g), the final yield was as high as 77% (Fig. 1). This ginpolin protocol could be used to prepare 3 different physiologically and pharmacologically active ingredients from ginseng through several simple steps; this aspect is different from previously reported methods to prepare separately ginsenosides, gintonin, or polysaccharides from ginseng [25].

3.2. Quantitation of LPA $C_{18:2}$ and phosphatidic acid (PA $C_{16:0\text{-}18:2}$) contents in GEF, GPF, or cGSF

We analyzed the LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ contents in GEF, GPF, and cGSF using LC-MS/MS [18]. The GEF fraction contained much

Table 1
Amount of LPA and PA in GEF, GPF, and cGSF From Ginseng (n = 3)

	LPA (μg/mg)	PA (μg/mg)
GEF	1.95 ± 0.11	10.60 ± 0.65
GPF	N.D.	N.D.
cGSF	0.66 ± 0.01	0.84 ± 0.07

N.D.; Not detected.
GEF, gintonin-enriched fraction; GPF, ginseng polysaccharide fraction; cGSF, crude ginseng total saponin fraction.

more LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ than the other 2 analytes did (Table 1). Thus, the proportion of cGSF LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ was approximately 30% of that of GEF analytes. However, LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ were rarely detected in GPF. This finding is consistent with GPF being water-soluble and GEF being water-insoluble, and we could completely separate LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ from GPF through ethanol precipitation. Thus, the LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ contents were in the following order: GEF > cGSF > GPF. In our earlier study, we showed that the yield of LPA $C_{18:2}$ and PA $C_{16:0\text{-}18:2}$ was 2.01 ± 5.61 μg/mg (0.20%) and 11.16 ± 0.95 μg/mg (1.12%), respectively, showing a slight difference but presenting a desired range of bioactive compounds such as GEF [18]. These results show that GEF is the main fraction that contains LPAs and PAs, as shown in the literature, and this finding is consistent with the following results of GEF-mediated $[Ca^{2+}]_i$ transient in the PC-3 cell line based on the LPA content.

3.3. Quantitation of ginsenosides in GEF, GPF, or cGSF

We also determined the amount of ginsenosides, such as Rb1, Rb2, Rb3, Rc, Rd, Re, Rf, Rg1, Rg2, and Rg3, in the 3 fractions. Notably, most of the ginsenosides existed in the GEF and cGSF fractions, whereas there was a trace of ginsenoside residues in the GPF fraction (Table 2). Surprisingly, we found that the content of protopanaxadiol ginsenosides, specifically the ginsenosides Rb1, Rb2, Rc, and Rd, in GEF, was higher than that in cGSF (Table 2). Particularly notable is that ginsenoside Rb1 was much more abundant than the other ginsenosides in GEF (Table 2). The order of contents was as follows: ginsenoside Rb1 > Rc > Rb2. Thus, protopanaxadiol ginsenosides were the most dominant ginsenosides in GEF, and protopanaxatriol ginsenosides, such as Rg1 and Re, were more abundant in cGSF than in the GEF fraction, in the following order: ginsenoside Rg1>Re. By contrast, only a trace amount of ginsenosides was detected in the GPF fraction (Table 2), indicating that the ethanol precipitation of the supernatant after the GEF for GPF preparation can exclude ginsenosides.

3.4. Quantitation of galacturonic acid, glucuronic acid, arginine, and gallic acid in GEF, GPF, or cGSF

Next, we determined the contents of galacturonic acid and glucuronic acid, which are reference chemicals that represent the acidic polysaccharide content of ginseng, in GEF, GPF, and cGSF, using a colorimetric method [26–28]. GPF contained twice as much galacturonic acid as GEF and cGSF (Fig. 2A). GEF and cGSF contained similar amounts of galacturonic acid. Thus, the order of galacturonic acid content was GPF > GEF = cGSP (Fig. 2A). GPF, GEF, and cGSF also showed a similar pattern of glucuronic acid content (Fig. 2B). Thus, the order of glucuronic acid content was similar to that of galacturonic acid and was as follows: GPF > GEF = cGSP (Fig. 2B). Arginine is an amino acid abundantly present in ginseng in its free or bound form, along with the other 23 amino acids found in ginseng [29,30]. Because arginine is the predominant amino acid in ginseng, its content was determined [18]. Arginine content analysis showed that GPF was 3.99-fold higher than that of GEF and cGSF (Fig. 2C). Next, we determined the polyphenolic compounds in the 3 fractions. Gallic acid was used as a total polyphenol reference chemical. The total polyphenolic content was, in order, GEF > cGSF > GPF (Fig. 2D). Notably, the content of gallic acid did not differ greatly among the fractions (Fig. 2D). However, GEF contained the highest level of total gallic acid, and GPF and cGSF fractions had similar amounts of gallic acid. These results show that water-soluble galacturonic acid, glucuronic acid, and arginine are the dominant ingredients in GPF.

3.5. Comparisons of $[Ca^{2+}]_i$ transient induction by GEF, GPF, or cGSF

Because gintonin contains LPAs and these LPAs induce $[Ca^{2+}]_i$ transients in cells that express endogenous LPA receptors, we next examined the effects of 3 factors on $[Ca^{2+}]_i$ transients using cells that endogenously express LPA receptors [10]. To achieve this objective, we used the PC-3 cell line, which expresses endogenous LPA receptor subtypes [31]. When the cells were first treated with 0.3 μg/mL of GEF, GPF, or cGSF, only GEF induced $[Ca^{2+}]_i$ transient but GPF and cGSF did not elicit any response (Fig. 3A). When the treatment concentrations were increased, GEF showed a dose-response induction of $[Ca^{2+}]_i$ transient, but GPF still did not elicit $[Ca^{2+}]_i$ transient, despite the concentrations being increased (Fig. 3B). Even though the cGSF fraction did not induce $[Ca^{2+}]_I$ transients to the same extent as the GEF fraction did, 1 μg/mL cGSF treatment elicited a $[Ca^{2+}]i$ transient showing a plateau state (Fig. 3B). The ability to induce $[Ca^{2+}]_i$ transients was GEF > cGSF; however, GPF did not elicit $[Ca^{2+}]_i$ transient induction (Fig. 3B). Because GPF does not contain LPAs (Table 1), these results show that the degree of $[Ca^{2+}]_i$ transient induction is closely proportional to the LPA $C_{18:2}$ content in the 3 fractions (Table 1). In addition, these results are consistent with other studies on $[Ca^{2+}]_i$ transients by gintonin or GEF, based on the intracellular Ca^{2+} mobilization being driven by the LPA-LPA receptor signaling pathway trigger [10,32].

3.6. Comparison of $ABTS^+$ radical scavenging effect of GEF, GPF, or cGSF and effect of GEF, GPF, or cGSF on collagen-induced platelet aggregation

Because a representative effect of ginseng is its antioxidant action, we first examined the free radical scavenging effect using GEF, GPF, or cGSF [33]. For this purpose, we assessed the degree of $ABTS^+$ radical removal by GEF, GPF, or cGSF. They removed $ABTS^+$ radicals in a dose-dependent manner (Fig. 4A). Among the 3 fractions, GPF showed the strongest $ABTS^+$ radical scavenging effect: more than a 2-fold higher removal effect than that of the other 2 fractions, i.e., GEF and cGSF (Fig. 4A). Notably, although all fractions showed dose-dependent $ABTS^+$ radical scavenging activity, only GPF and cGSF showed a statistically significant increase at 1.25 and 2.5 mg/mg. The order of the free radical scavenging effect was GPF > cGSF = GEF, and this finding is consistent with previous reports that determined that ginseng polysaccharides had antioxidant effects [34]. Ginseng saponins have been shown to attenuate platelet coagulation to smooth the blood flow [35,36]. In this study,

Table 2
Composition of Ginsenosides in GEF, GPF, and cGSF (mg/g) (n = 4)

	Rg1	Rb1	Rg3	Re	Rf	Rg2	Rc	Rb2	Rb3	Rd
GEF	6.54 ± 0.78	60.17 ± 10.02	1.94 ± 1.04	4.18 ± 0.04	4.53 ± 0.01	1.01 ± 0.06	24.45 ± 3.90	19.95 ± 2.14	2.56 ± 0.26	6.01 ± 0.99
GPF	0.14 ± 0.01	0.25 ± 0.04	*N.D.	*N.D.	*N.D.	*N.D.	*N.D.	*N.D.	*N.D.	*N.D.
cGSF	11.73 ± 0.50	15.04 ± 2.40	0.17 ± 0.12	8.04 ± 0.20	2.74 ± 0.25	0.29 ± 0.04	5.53 ± 1.58	3.76 ± 1.34	0.63 ± 0.01	1.48 ± 0.17

GEF, gintonin-enriched fraction; GPF, ginseng polysaccharide fraction; cGSF, crude ginseng total saponin fraction.
* N.D.; Not detected.

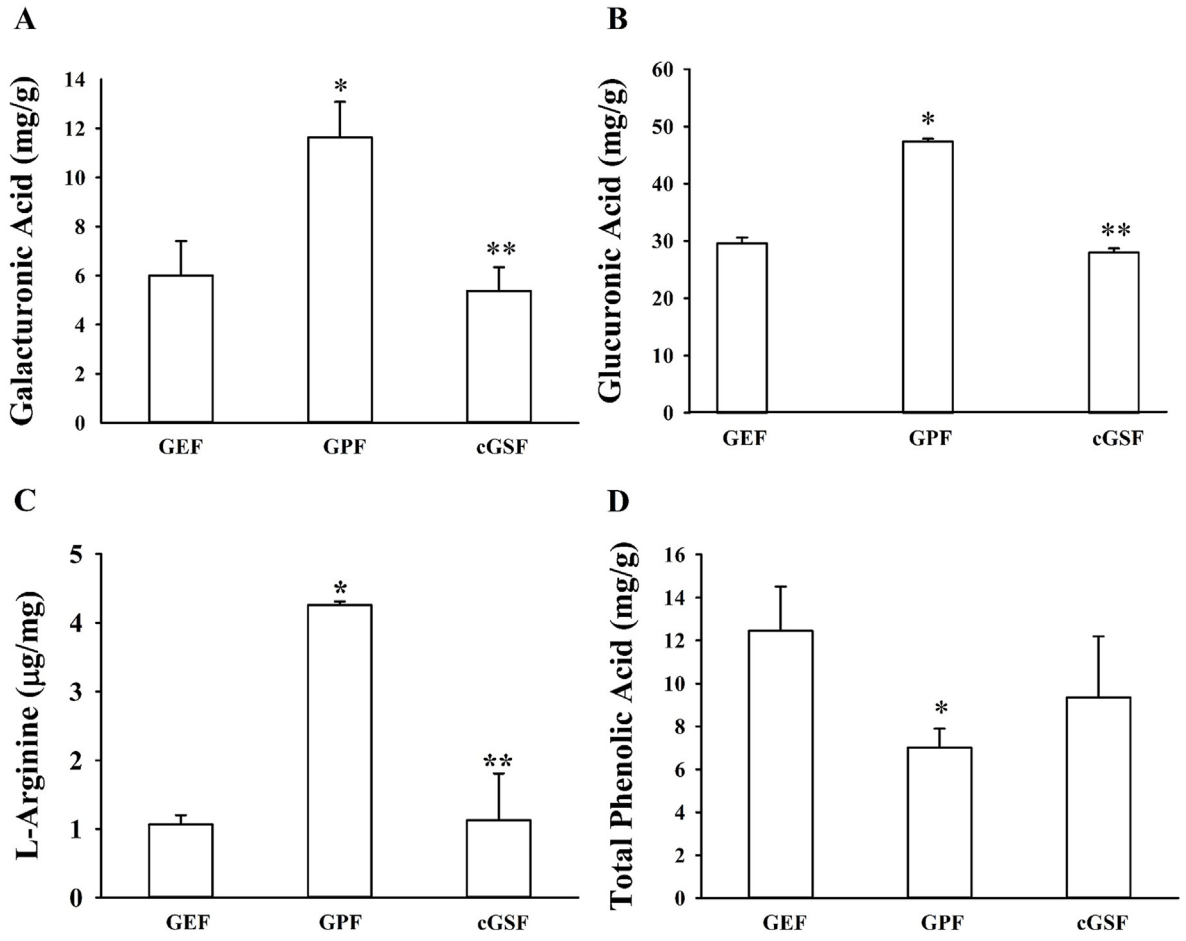

Fig. 2. Determination of contents of L-arginine, galacturonic acid, gallic acid, and glucuronic acid in each fraction. (A and B) Galacturonic acid and glucuronic acid are reference chemicals for acidic polysaccharides. GPF contains the most acidic polysaccharides component. Galacturonic acid and glucuronic acid contents are as follows: GPF > GEF > cGSF. *P < 0.05, GEF vs. GPF: **P < 0.05, GPF vs. cGSF. (C) L-arginine contents in GEF, GPF, and cGSF were measured. GPF fraction contains the highest level of L-arginine among the 3 fractions. Determination of L-arginine content is described in Materials and Methods. *P < 0.05, compared with GEF; **P < 0.05, compared with cGSF. (D) Gallic acid was used as a reference chemical for ginseng total polyphenols in GEF, GPF, and cGSF. Many ginseng polyphenols were detected in GEF. Gallic acid contents are as follows: GEF > cGSF > GPF. *P < 0.05, GEF vs. GPF: **P < 0.05, GPF vs. cGSF. Each graph shows mean ± SD of 3 independent experiments.

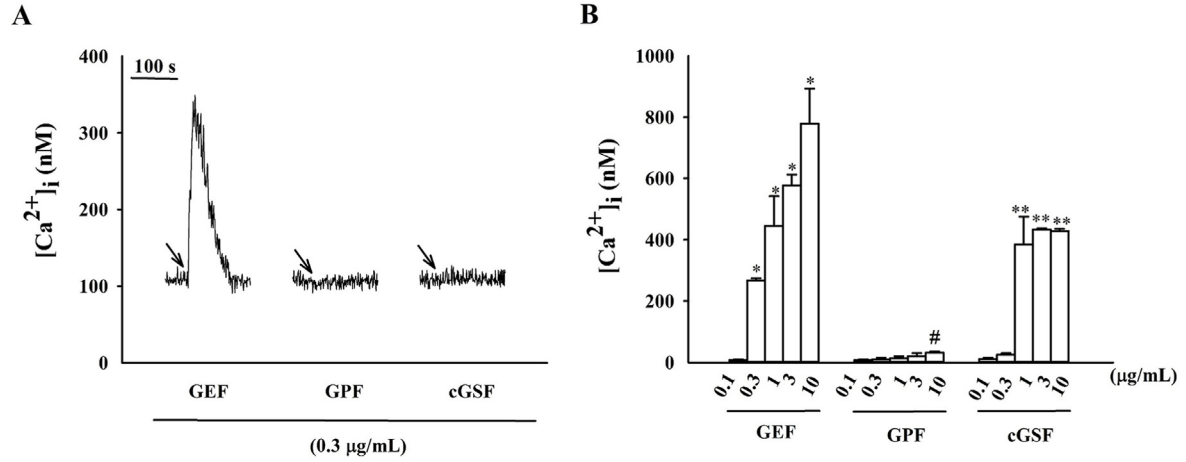

Fig. 3. Effects of GEF, GPF or cGSF on $[Ca^{2+}]_i$ transient. (A) Comparison of $[Ca^{2+}]_i$ transient in PC3 cell by GEF, GPF, or cGSF. The final concentration of each fraction was 0.3 μg/mL. (B) Histograms of $[Ca^{2+}]_i$ transient in PC3 cells induced by GEF, GPF, and cGSF (at different concentrations). Treatment concentrations are 0.1, 0.3, 1, 3, and 10 μg/mL. GEF exhibits the strongest $[Ca^{2+}]_i$ transient; cGSF elicited less $[Ca^{2+}]_i$ transient than GEF did. However, GPF had almost no effects on $[Ca^{2+}]_i$ transient. *P < 0.05, compared with GEF (0.1 μg/mL); #P < 0.05, compared with GPF (0.1 μg/mL); **P < 0.05, compared with cGSF (0.1 μg/mL). Each graph shows mean ± SD of 3 independent experiments.

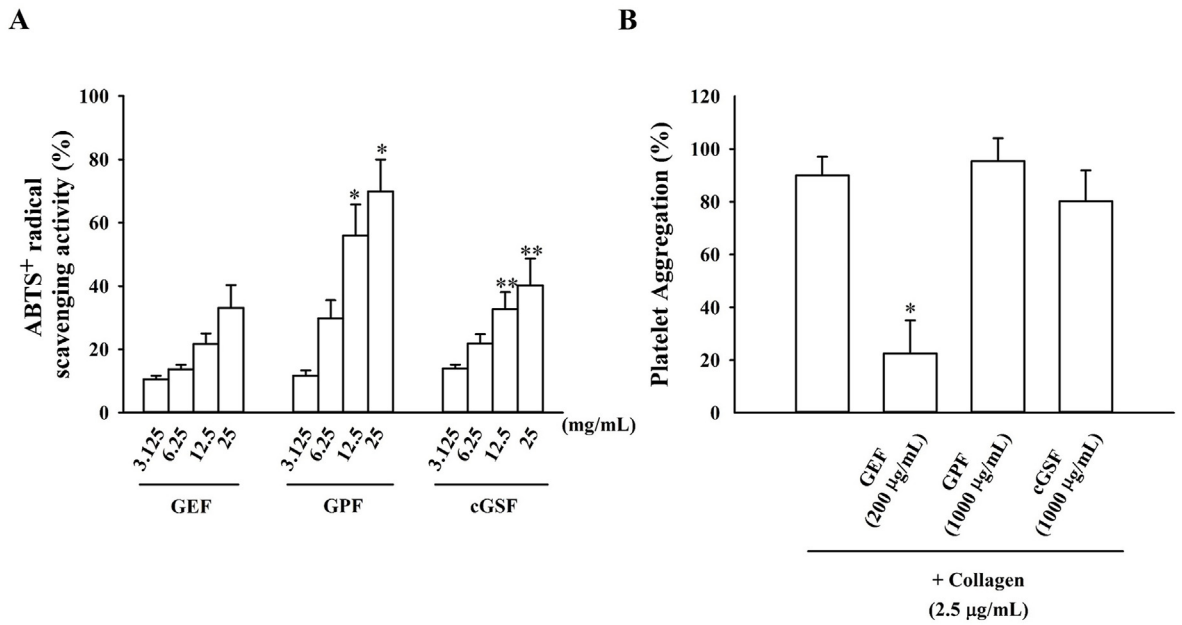

Fig. 4. ABTS $^{+}$ radical scavenging activity and platelet aggregation assay of 3 fractions. (A) ABTS^{+} radical scavenging activity was compared among GEF, GPF, and cGSF. Among the 3 fractions, GPF showed the strongest radical scavenging activity. GEF and cGSF showed a similar scavenging activity. *P < 0.05, compared with GPF 3.125 mg/mL; **P < 0.05, compared with cGSF 3.125 mg/mL. Each bar indicates mean ± S.D. (n = 4). For statistical analysis, one-way ANOVA was performed (P < 0.05). (B) The effect of GEF, GPF, or cGSF on platelet aggregation induced by collagen. Platelets (3×10^8/mL) were preincubated with or without GEF, GPF or cGSF in the presence of 1 mM $CaCl_2$ for 2 min at 37 °C. The platelet aggregation was then induced by 2.5 μg/mL collagen, and the extent of aggregation was measured. The aggregation reaction was terminated after 5 min, and the aggregation rate as a percentage was calculated. Each graph shows mean ± SD of 4 independent experiments. *P < 0.05 compared with the agonist control.

we examined the effects of GEF, GPF, and cGSF on the antiplatelet aggregation. As shown in Fig. 4B, only the GEF fraction inhibited platelet aggregation induced by collagen at the assessed concentration (P < 0.05) when collagen was used as a platelet-coagulating factor, whereas the GPF and cGSF fractions had no effect on collagen-induced platelet aggregation. In this regard, these results are consistent with those in the literature, and only GEF had an anticoagulant effect among the 3 independent fractions (Fig. 4B).

3.7. Comparisons of immune cell activation markers by GEF, GPF, or cGSF

Ginseng induces immune activation [37,38]. Hence, we examined the effects of GEF, GPF, or cGSF on immune cell activation. We utilized the mouse macrophages RAW264.7 cell line to determine the degree of nitric oxide (NO) production, IL-6 production, TNF-α release, and phagocytosis. Lipopolysaccharide (LPS) was used as a positive control for the induction of these markers. As shown in Fig. 5A, GPF showed the strongest NO production, whereas GEF and cGSF had weak or negligible effects on NO release. The effects of the 3 fractions on phagocytic activity were determined using a neutral red uptake assay. The phagocytic activity of macrophages was slightly but significantly increased after treatment with the 3 fractions. Notably, GPF increased phagocytic activity at low concentrations but decreased it at high concentrations. GEF showed phagocytic activity at 10 μg/mL, and cGSF showed phagocytic activity at more than 0.3 μg/mL. The effects of the 3 fractions on phagocytic activity were observed at different concentrations (Fig. 5B). In the IL-6 release test, GPF (300 μg/mL) increased IL-6 production in the RAW264.7 cell line, whereas GEF and cGSF inhibited IL-6 production (Fig. 5C). GPF increased TNF-α levels in a dose-dependent manner after treatment with GPF (Fig. 5D). However, GEF and cGSF did not significantly stimulate TNF-α production (Fig. 5D). LPS, the positive control, increased TNF-α production (Fig. 5D). These results show that GPF, rather than GPF and cGSF, had the dominant effect on TNF-α production among the 4 immune activation biomarkers.

3.8. Effects of 3 fractions on cell viability and cell protection from reactive oxygen species (ROS)-induced cell damages

Using the hippocampus-derived neuronal (HT-22) cell line, we tested cell viability and found that GPF and cGSF did not show cytotoxicity at the doses tested. Even concentrations higher than 300 μg/mL GPF did not show cytotoxicity (data not shown). By contrast, GEF damaged the cells at either 30 or 100 μg/mL, indicating that GEF might induce cell damage at high concentrations. Next, we examined the HT-22 protective effects against ROS of 3 different fractions. To achieve this objective, we used 3 μM iodoacetic acid (IAA), a type of ROS generator that blocks ATP generation in mitochondria and induces cell damage [31]. As shown in Fig. 6, cotreatment with GEF and IAA inhibited cell death in a dose-dependent manner, showing a similar result that gintonin is an attenuator of IAA-induced oxidative stress in HT-22 cells [31]. The cGSF and IAA cotreatment also slightly inhibited cell death compared with the IAA alone treatment group, but GPF and IAA cotreatment showed a much weaker effect on cell protection against IAA. Thus, the order of cell-protective effects against ROS-generating IAA was GEF > cGSF, but GPF had negligible effect. These results indicate that GEF exhibit potent protective effects against ROS.

4. Discussion

In this study, we developed a ginpolin protocol that guides the preparation of 3 representative fractions of ginseng: GEF, GPF, and cGSF. The ginpolin protocol is easy to prepare for GEF, GPF, and cGSF with ethanol and H_2O and does not use other organic solvents such as methanol or butanol, which are harmful to humans (e.g., inedible). Additionally, producing batches of 3 components using a

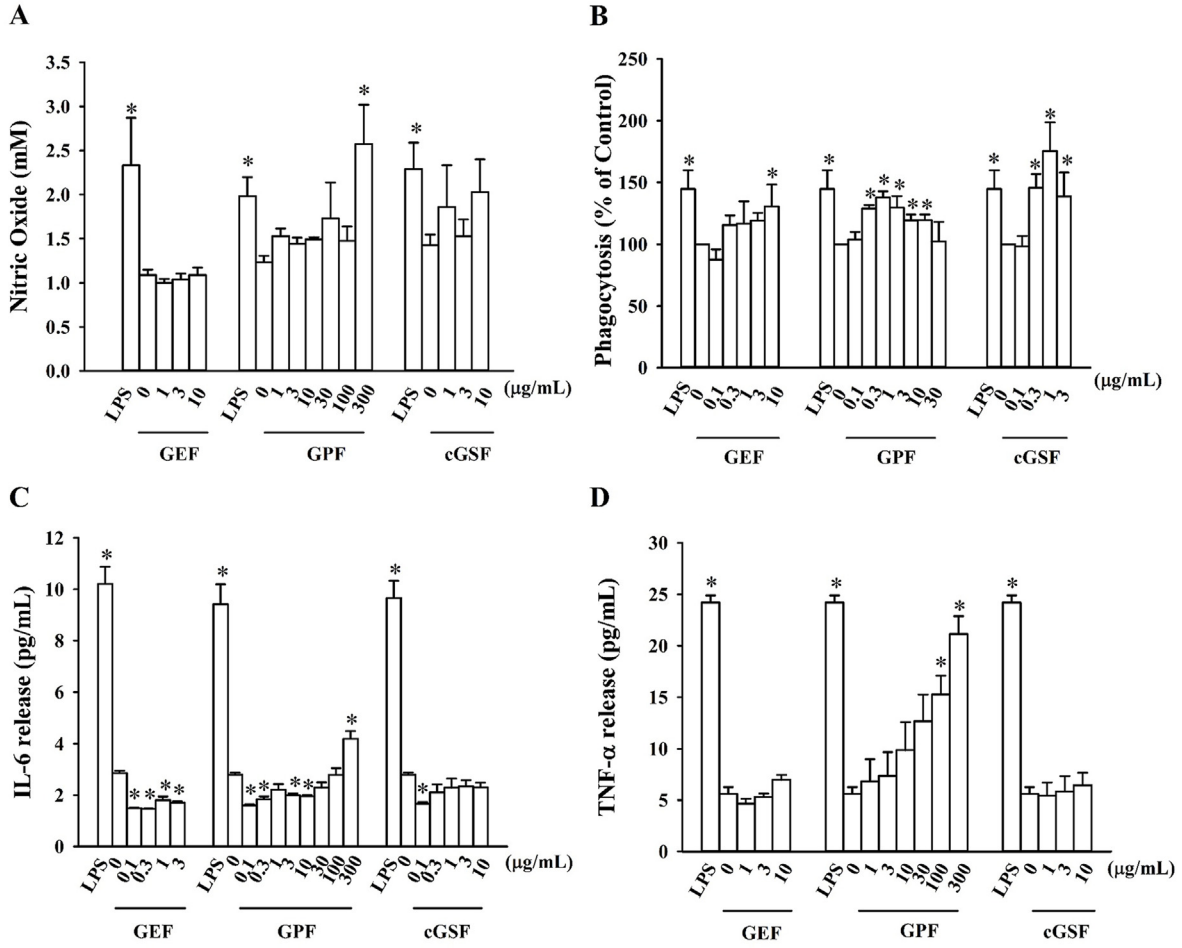

Fig. 5. Nitric oxide (NO), phagocytosis, and cytokine release assay by GEF, GPF, or cGSF. (A) NO production by 3 fractions was measured in RAW 264.7 cell line. NO level was significantly increased when treated with 300 μg/mL GPF. However, GEF and cGSF did not show significant increases in GEF and cGSF. (B) Degree of phagocytosis in RAW 264.7 cells using GEF, GPF, or cGSF was determined. (C) Measurement of released IL-6 in RAW 264.7 cell line. When RAW 264.7 cells were treated with GEF, GPF, or cGSF, the GEF and cGSF treatments showed a decreasing trend of IL-6 production in all concentrations, whereas the GPF treatment showed increasing trend of IL-6 production, which was significantly increased at 100 μg/mL. (D) Tumor necrosis factor (TNF)-α release was measured in RAW 264.7 cells. When RAW 264.7 cells were treated with GEF and cGSF, they did not stimulate TNF-α release. However, GPF stimulated TNF-α release in a dose-dependent manner. Each graph shows mean ± SD of 4 independent experiments. *P < 0.05, compared with LPS non-treatment; #P < 0.05 and **P < 0.01, compared with non-treatment of each fraction.

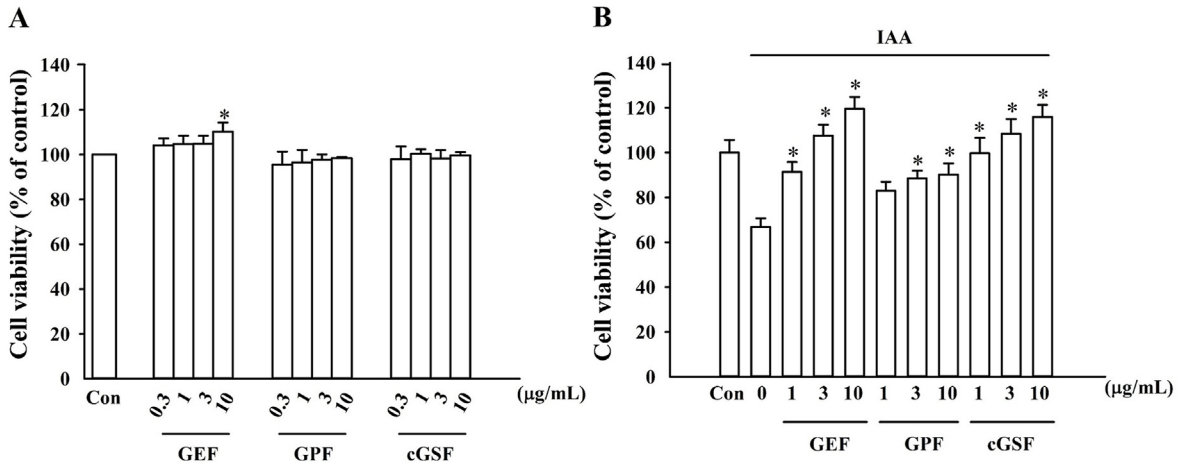

Fig. 6. Cell cytotoxicity and cell protection assay by GEF, GPF, or cGSF in HT22 cells. HT22 cells were seeded in a 96-well plate at a 1×10^4 cells/well density for assay. (A) HT22 cells were treated with GEF, GPF, or cGSF. GEF treatment (10 μg/mL) showed a significant increase. *P < 0.05, compared with non-treatment). However, the GPF or cGSF treatment group did not show a significant increase in cell viability. (B) Protective effect of GEF, GPF, or cGSF against iodoacetic acid (IAA), a ROS generator. IAA treatment induced HT22 cell damage, cotreatment of GEF and cGSF protected cells against IAA, and GPF showed cell-protective effects at high concentrations. *P < 0.05, compared with non-treatment of each fraction. Each graph shows mean ± SD of 3 independent experiments.

sequential procedure is possible. Thus, the ginpolin protocol enables the mass production of active components of ginseng by minimizing the loss of active ingredients of ginseng; unlike most of the methods in the literature, which have prepared 1 active fraction or ingredient of ginseng and discarded the other components as ginseng marc or waste. Because of all medicinal herbs ginseng is among the most expensive and has a long cultivation before harvest, the ginpolin protocol might be economical for ginseng products and applicable for maximizing the utilization of the individual active ingredients of ginseng.

GEF was first isolated using the ginpolin protocol after ethanol extraction and subsequent H_2O fractionation [39,40]. Chemical analysis of GEF compositions shows that GEF contains much more LPAs and PAs than GPF and cGSF do. LPAs in gintonin are responsible for the induction of $[Ca^{2+}]_I$ transients via the LPA receptor signaling pathway in cells, because GPF did not elicit $[Ca^{2+}]_i$ transients in the absence of LPAs (Fig. 3 and Table 1). In addition, cGSF elicited a $[Ca^{2+}]_i$ transient at higher concentrations than those of GEF, because cGSF contains less amount of LPAs than GEF does (Fig. 3 and Table 1). Notably, GEF contained a larger amount of ginsenoside Rb1 than the other 2 fractions (Table 2). The levels of other protopanaxadiol ginsenosides were also higher than those of the other fractions (Table 2). Why does GEF contain a larger amount of ginsenoside Rb1 (rather than the other ginsenosides) than the other 2 fractions? Based on this study and the literature, we cannot yet answer this question. A possibility is that ginsenoside Rb1 is the most abundant ginsenoside and that the physicochemical properties of ginsenoside Rb1 might be similar to those of LPAs and PAs. LPAs, PAs, and ginsenoside Rb1 are all amphiphilic because they contain both hydrophilic and hydrophobic portions in their chemical structures [11]. Therefore, they precipitated together during the water fractionation.

Second, GPF was isolated after GEF. GPF was obtained from the supernatant of the GEF water precipitate (Fig. 1). Notably, a characteristic of GPF was that it did not contain LPAs and PAs and contained only a trace amount of ginsenosides (Tables 1 and 2). Instead, GPF contains nearly two-fold galacturonic acid and glucuronic acid content (Fig. 2), which are marker molecules of acidic polysaccharides in ginseng, indicating that GPF contains acidic polysaccharides that are responsible for immune activation (Fig. 5). In addition, we found that GPF contained more arginine than GEF and cGSF, suggesting that ethanol-insoluble but water-soluble GPF contained water-soluble ginseng polysaccharides and arginine, whereas water-insoluble ginsenosides were not included in this fraction. Thus, ethanol precipitation after GEF preparation can be utilized as a method to assess the effects of arginine while excluding ginsenosides.

cGSF was the last fraction of the ginpolin protocol. cGSF was the most abundant among the 3 fractions. Although cGSF contains much lower amounts of protopanaxadiol ginsenosides, it contains approximately 40% more protopanaxatriol ginsenosides (Table 2). cGSF also contains one-third of LPAs and one-tenth of PAs, indicating that the ginpolin protocol does not completely collect LPAs and PAs into GEF, explaining the observation that cGSF also induced $[Ca^{2+}]_i$ transients (at higher concentrations than GEF) and protected cell ROS-induced cell damage (with less efficiency than GEF), which might originate from the presence of LPAs [31]. By contrast, the amounts of other minor components such as arginine, galacturonic acid, and gallic acid were similar to those of GEF.

In physiological and pharmacological tests, we found that GEF inhibited collagen-induced platelet aggregation, whereas GPF and cGSF had no effect (Fig. 4B). As ginsenosides have antiplatelet effects, determining which components (or fractions) exert antiplatelet activity is a worthwhile endeavor [41–43]. In this study, GEF, rather than GPF or cGSF, inhibited collagen-induced platelet aggregation. GEF might be the main component of ginseng responsible for the effects on antiplatelet activity, because GPF and cGSP had no effect even at much higher concentrations than GEF (Fig. 5). In a previous report, we showed that gintonin inhibits platelet aggregation by regulating the glycoprotein VI (GPVI) signaling pathway [35]. These results indicate that the inhibition of platelet aggregation might not be related to ginsenosides. In contrast, GPF showed more immune activation effects than the other 2 fractions. Notably, TNF-α production was more dominant than other biomarkers for immune activation (Fig. 5). These results are consistent with those in the literature that low-molecular-weight GPF significantly increased TNF-α production but not NO and IL-6 production [44]. GPF also showed more dominant antioxidant effects by scavenging free radicals than GEF and cGSP, although GPF exhibited a slight protective effect against ROS-induced cell damage (Fig. 4 and 6). Thus, GPF contains ginseng ingredients for boosting both the immune system and the antioxidant effects in addition to arginine. In addition, GEF induced neuronal cell damage at higher concentrations than the other 2 fractions did. GEF is a dominant component against ROS-induced cell death at low concentrations, because GPF had no effect on ROS-induced cell death and cGSF exhibited a slight attenuation against ROS-induced cell death (Fig. 6).

In this study, we showed that the ginpolin protocol is an easily performed method for the isolation of 3 independent fractions from ginseng. The chemical properties and cellular activities of the 3 fractions could be distinguished. The ginpolin protocol has at least 3 advantages over previously published methods for the individual separation of ginseng components. First, the ginpolin protocol minimizes the loss of the active components of ginseng as waste. Second, each fraction prepared according to the ginpolin protocol can be commercially applicable for ginseng-derived functional food manufacturing, separately or together, according to its purpose. Third, the ginpolin protocol enables the mass production of 3 different components using batch preparation. Ginpolin protocol can also be applied for recycling ginseng waste resources, such as ginseng stems, leaves, and fruit, in addition to Korean Red Ginseng marc, to produce a good yield of the aforementioned 3 fractions simultaneously. However, it might require further study for the ingredient standardization and validation of three fractions in future.

In conclusion, we developed a novel ginpolin protocol that can isolate active ingredients by batch without losing the active ingredients of ginseng and found that each fraction showed differential characteristics in its chemical properties and cellular actions.

Data availability

The data from this work will be made available upon request.

Declaration of competing interest

The authors declare that they have no conflict of interest regarding this publication.

Acknowledgment

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