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Standardization of *Rehmannia glutinosa* (Gaertn.) DC. steam processing and evaluation of its chemical, anti-oxidant, and anti-inflammatory properties

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Abstract

Rehmannia glutinosa (Gaertn.) DC., belonging to the family Scrophulariaceae, is an important medicinal herb cultivated in East Asia. Traditionally, *R. glutinosa* is steam processed to increase its efficacy in treating various ailments such as diabetes, hematinic deficiencies and adrenal disorder. However, standardization of processed *R. glutinosa* is highly needed to increase its quality to fulfill global market demand that is safe and possess high level of efficacy. Therefore, this study aimed to optimize the *R. glutinosa* steam processing methods by evaluating some key parameters such as steaming temperature, number of steaming times, steaming duration, and additive supplementation. *R. glutinosa* samples were steam processed at different temperatures (100 °C, 110 °C, and 120 °C), various steaming times (1 to 5 times), several steaming duration (1 to 4 h), and additives supplementation (rice wine, 5% EtOH, 10% EtOH, 20% EtOH, 30% EtOH, and 40% EtOH). As the result, 2 h, 3 replications, and supplementation with 20% EtOH at 120 °C were identified as the optimal conditions for *R. glutinosa* steam processing. Optimized processed *R. glutinosa* (SPRR 20%EtOH) resulted in significantly higher content of 5-HMF (7648.60 ± 150.08 μg/g) and iso-verbacoside (203.80 ± 10.72 μg/g) compared with unprocessed *R. glutinosa* (UPR). Compared to those of other samples, SPRR 20% EtOH samples had higher total flavonoid (55.36 ± 1.68 mg/g) and phenolic (69.24 ± 4.56 mg/g) contents and stronger DPPH antioxidant activity (56%). Furthermore, SPRR 20% EtOH had excellent anti-inflammatory activity, as evidenced by the suppression of inducible nitric oxide synthase (iNOS) caused by activation of nuclear factor-κB (NF-κB) through p-p65 pathway in LPS-stimulated RAW 264.7 cells. These findings will provide a basis towards industrialization of *R. glutinosa* processing technology that will be very helpful for oriental medication field.

Keywords *Rehmannia glutinosa* roots steam processing, Anti-oxidant activity, Anti-inflammatory activity, Herbal medicine processing, 5-hydroxymethyl-2-furaldehyde (5-HMF)

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Introduction

Rehmannia glutinosa (Gaertn.) DC. (Scrophulariaceae) is an herb with oriental medicine applications such as for treating diabetes, hematinic deficiencies and adrenal disorder [1]. It has been cultivated in China, Japan, and Korea for over 1000 years [2]. Depending on the processing method, *R. glutinosa* roots are classified into three types for medicinal purposes, fresh roots (Rehmanniae Radix Crudus), dried roots (Rehmanniae Radix), and processed roots (Rehmanniae Radix Preparata) [3]. Fresh and dried roots affect the *Yin*, decrease heat from the blood, and increase body fluids and salivation, while steamed roots are mainly used to treat *Yang* deficiencies, facilitating nourishment and bone marrow improvement [1, 4].

About 140 compounds have been isolated from unprocessed *R. glutinosa,* including monoterpenoids, triterpenes, phenyl-ethyl alcohol glycosides, phenolic, ligans, flavonoids, and polysaccharides [5–7]. Among these, the majority of the constituents are iridoid glycosides (catalpol and harpagide) and phenylethanoid glycosides (verbacoside and iso-verbacoside). Recent pharmacological studies have reported that *R. glutinosa* possesses anti-oxidant, hypoglycemic, anti-inflammatory, anti-cancer, and immunoregulatory effects [7]. In addition, processed *R. glutinosa* roots protect against aging, oxidation, and cancer and improve marrow and essence [8]. However, steam processing could alter the contents of some compounds, thereby affecting pharmacological activity [9].

According to the Korean Pharmacopoeia, processed *R. glutinosa* roots (Rehmanniae Radix Preparata) are required to have a 5-hydroxymethyl-2-furaldehyde (5-HMF) content of greater than 0.1% to guarantee quality [10, 11]. Moreover, the verbascoside content, according to the Chinese Pharmacopoeia, is required to be $\geq$ 0.20% in processed *R. glutinosa* roots [12]. Therefore, it's important to develop *R. glutinosa* processing condition that meet those criteria to maintain its quality and increase the efficacy. However, this could be started by choosing high quality *R. glutinosa* raw material. In the previous study, we developed high quality *R. glutinosa* seedlings trough in vitro culture approach (Korea patent no. 10-1881305) and its productivity was verified in the larger outdoor field [13]. Moreover, our team had also developed a high quality of adventitious roots of *R. glutinosa* for industrial applications [14]. These materials can be used to produce high quality of processed *R. glutinosa* roots.

However, standardization of processed *R. glutinosa* root is highly needed to increase its quality to fulfill global market demand that is safe and possess high level of efficacy. Therefore, this study aimed to optimize the steam processing conditions by assessing some key parameters such as steaming temperature, number of steaming times, steaming duration, and additives supplementation. In addition, we evaluated its marker compounds (catalpol, 5-HMF, verbascoside, and iso-verbascoside) composition, total phenolic and flavonoid content, anti-oxidant and anti-inflammatory activities to verify the efficacy of processed *R. glutinosa* roots under optimized steaming conditions.

Materials and methods

Materials and reagents

Raw roots of *R. glutinosa* were collected from Geumsan Farm in Chungcheongnam-do province, Korea. To prevent decay, the roots were dried in an oven at 60 °C for 48 h. Before and after processing, the moisture content of each sample was measured using a moisture analyzer balance (Kett Electric Laboratory, Tokyo, Japan). Chromatography grade water, acetonitrile, and methanol (Merck, Darmstadt Germany) were used for high-performance liquid chromatography (HPLC). 1.1-diphenyl-2picrylhydrazyl (DPPH), gallic acid, and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibody of COX-2 and iNOS were supplied by Cell Signaling (Denvers, MA, USA), NF-κB and all other western luminal reagents were purchased from Santa Cruz.

Optimization of processing methods

Dried *R. glutinosa* roots were cut into 20 mm slices, soaked in water for 16 h, and followed by steam processing (Fig. 1). All samples were subjected to steam processing (KSP-240 L, Kyungseo E&P, South Korea) at various temperatures (100 °C, 110 °C, and 120 °C), various steaming times (1 to 5 times), several steaming durations (1 to 4 h), and supplementation with various additives (distilled water, rice wine, and different concentration of ethyl alcohol [5%, 10%, 20%, 30%, and 40%]). All processed samples were dried in an oven at 60 °C for 48 h. For further study, all processed samples were pulverized with a grinder (250G New Type Pulverizing Machine, Model RT-N04-2 V, Taiwan) and filtered through a 60-mesh sieve to make sample powder. A tenfold volume of 70% methanol was added to the sample powder (v/w), and the mixture was subjected to ultrasonic extraction for 1 h. Finally, three types of samples were obtained for further experiments: unprocessed *R.*

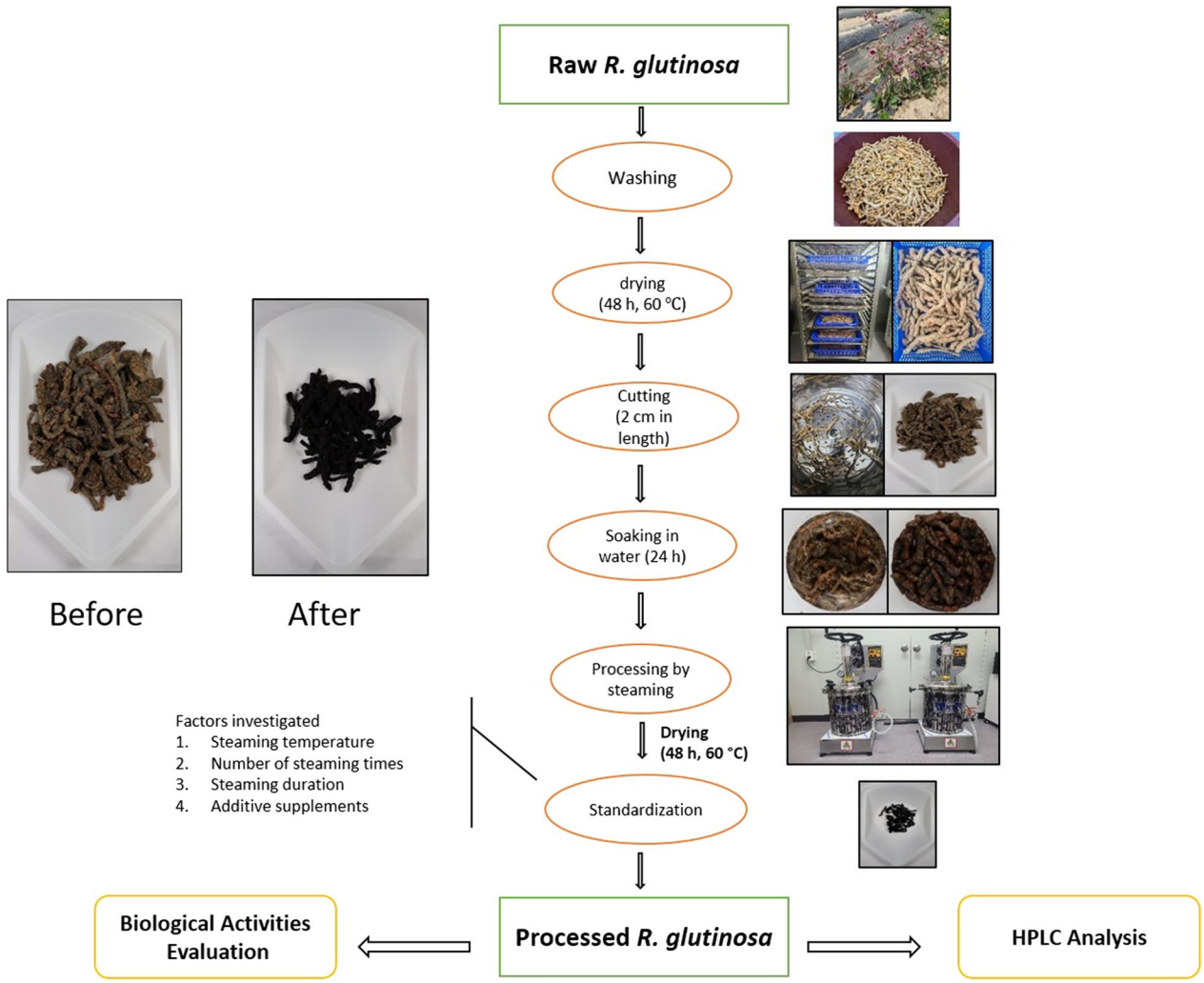

Fig. 1 The overall schematic diagram of *R. glutinosa* processing method by steaming treatment

glutinosa (UPR), processed *R. glutinosa* (PRR), and supplemented processed *R. glutinosa* (SPRR).

Measurement of R. glutinosa moisture content

The moisture content of all *R. glutinosa* samples was determined using the Kett FD-720 infrared moisture analyzer (Kett Electric Laboratory, Tokyo, Japan). The measurement was performed following previously described study [15]. Two grams of all samples were placed to the weighing dish, then the instrument automatically calculated the moisture content of the *R. glutinosa* at setting temperature 105 °C. The results showed as percentages (%).

Preparation and analysis of samples

HPLC analysis conditions and parameters for constituent analysis

Chromatography was performed following the National Institute of Food and Drug Safety Evaluation of Oriental Medicine guideline. HPLC was performed using the Waters Alliance 2695 HPLC system (Milford, MA, USA) equipped with a Capcell Pak UG120 C18 analytical column (250 × 4.6 mm, 5 µm; Shiseido, Japan) using water (A) and acetonitrile (B) as the mobile phase. The gradient elution was as follows: 0–15 min, 95% A; 15–25 min, 85% A; 25–45 min, 70% A; 45–60 min, 95% A. Absorbance was detected at 205 and 280 nm and the column was kept at 25 °C. The injection volume was 10 µL, and the flow rate was set at 0.8 mL/min.

Fourier transform near-infrared spectroscopy (FT-NIR) analysis

FT-NIR was performed following previously described methods [16]. Absorbance spectra for ground *R. glutinosa* in a glass vial (5 mm internal diameter) were collected using the TANGO FT-NIR spectrometer (Billerica, MA, USA) with the InGaAs detector, a broadband light source (50 W), interferometer, and a quartz halogen lamp. TANGO FT-NIR was completely controlled using OPUS (version 6.5). The spectra for *R. glutinosa* samples were detected using three positions per sample. The spectral conditions for TANGO FT-NIR were a resolution of 16 cm, mirror speed of 0.9494 cm/s, and spectral range of 800 to 2500 nm. To avoid surface interaction and the penetration of light into samples, incidence angles were set to 75°.

Determination of anti-oxidant activity

DPPH assay

A DPPH assay was performed as described previously [17]. Briefly, 1 mL of sample extract (at various concentrations) was mixed with 1 mL of 20 mM DPPH ethanol solution, followed by incubation at 37 °C for 30 min. The absorbance of the mixture was measured at 517 nm (SpectraMax i3x; Molecular Devices, Wokingham, UK) and absolute ethanol was used as a positive control. The scavenging capacity of DPPH radicals was calculated using the following formula:

$$\text{DPPH radical scavenging activity (\%)} = \left[1-\left(A_{\text{sample}} - A_{\text{empty}}\right)/A_{\text{control}}\right] \times 100.$$

Determination of total polyphenols

Total polyphenols were evaluated using Folin-Ciocateu reagent according to previously described methods [18], with modifications. Pulverized sample extraction was performed by using 1 g and 70% methanol with centrifugation for 10 min at 8000 × *g* at 4 °C. The supernatant was harvested for analyses. A 500 µL aliquot of each sample was mixed with 500 µL of the Folin-Ciocalteu reagent. After 3 min, 500 µL of 10% sodium carbonate (Na_2CO_3) were added and incubated in dark conditions at 25 °C for 1 h. Absorbance was measured at 725 nm using the SpectraMax i3x (Molecular Devices). Gallic acid was used to generate a standard curve for quantification.

Determination of total flavonoids

Total flavonoids were evaluated by an aluminum chloride colorimetric assay as described previously [19], with modifications. The 70% methanolic extract of steamed root (100 µL) was added to 1 mL of diethylene glycol and 10 µL of sodium hydroxide (NaOH). After incubation at 37 °C for 1 h, absorbance was measured at 420 nm (SpectraMax i3x; Molecular Devices). Rutin was used to generate standard curves for quantification.

Determination of anti-inflammatory activity

Cell culture

Cells were cultured according to previously described methods [20]. The RAW 264.7 macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% (v/v) streptomycin/penicillin and 10% fetal bovine serum at 37 °C with a 5% CO_2 atmosphere. Cells were seeded at 3×10^5 cells/mL in 96-well plates. Cells were then treated with *R. glutinosa* at various concentrations and further cultured with or without LPS (1 µg/mL) for 24 h at 37 °C.

Cell viability assay

RAW 264.7 macrophages cell lines was cultured in Dulbecoo's Modified Eagle Medium (DMEM) (Gibco, Invitrogen) added with 1% (v/v) penicillin and 10% fetal bovine serum (FBS), followed by incubation at 37 °C with a 5% CO_2 atmosphere. The RAW 264.7 macrophages were treated with all *R. glutinosa* extracts at concentration of 200 µg/ml followed by culture with or without LPS (1 µg/mL) for 24 h at 37 °C. The determination of the cell lines proliferation on the substrates was done using cell counting kit assay (CCK-8, Dojindo, Kumamoto, Japan). The 96-well plate was used to seed the samples (2×10^5 cells/mL density per well). ELISA plate reader was used to measure the absorbance at 490 nm.

Measurement of NO levels

NO levels were measured according to previously described methods [21], with modifications. RAW 264.7 cells (2×10^5 cells/mL) were plated in 6-well plates. After cell adhesion, various *R. glutinosa* extracts in PBS were treated with or without LPS (6×10^3 U/mL). The supernatant was harvested to measure NO after treatment for 24 h. Nitrite accumulation in the LPS-induced RAW 264.7 macrophages was measured as an indicator of NO by the Griess reaction method.

Western blot analysis

The nuclear and cytosolic fractions of the RAW264.7 cells were obtained following a previously reported method

[22]. Raw264.7 macrophages (2×10^6 cells/mL) were pretreated with *R. glutinosa* extracts before the addition of LPS (1 μg/mL) for stimulation in 24-well plates. After 16 h, proteins were extracted by RIPA buffer. The proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% (w/v) skim milk, and the blots were incubated for overnight at 4 °C with primary antibodies (1:1000) for iNOS, COX-2, phospho-NF-κB, and β-actin. Thereafter, secondary antibodies (1:10,000) were incubated with the membrane for 2 h. Bands were evaluated using western blotting luminal reagent and images were obtained using a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA).

Statistical data analysis

All experimental data were evaluated by analysis of variance (ANOVA) with Dunnett's post hoc tests or Duncan's multiple range tests using Prism (Graph Pad v 5. 03). Differences were considered statistically significant at $p \leq 0.05$.

Results and discussion

Effect of steam processing on R. glutinosa moisture content

R. glutinosa is a perishable material due to high moisture and sugar contents [23]. To maintain its quality, we therefore investigated the differences in the moisture content between unprocessed *R. glutinosa* (fresh and dried UPR) and processed *R. glutinosa* (PRR) (Fig. 2). The average water contents excluding other constituents were 77% for fresh UPR, 12% for dried UPR, and 0.1% for PRR (Fig. 2b). Total moisture was reduced by about 75% during processing from fresh to dried UPR and by

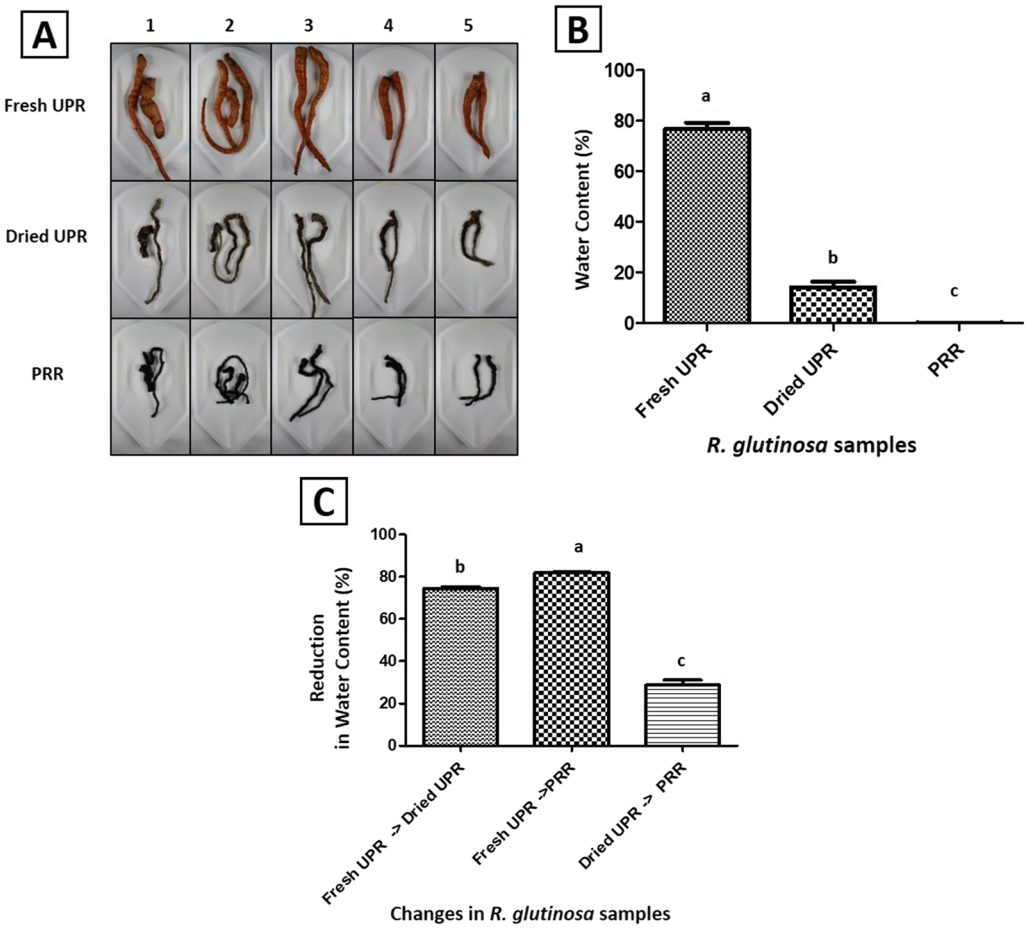

Fig. 2 The changes in biomass root morphology and water content of *R. glutinosa* samples. **a** Changes in root morphology appearance of *R. glutinosa* samples. **b** The water content of *R. glutinosa* samples. **c**. Water content loss of *R. glutinosa* samples during processing. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

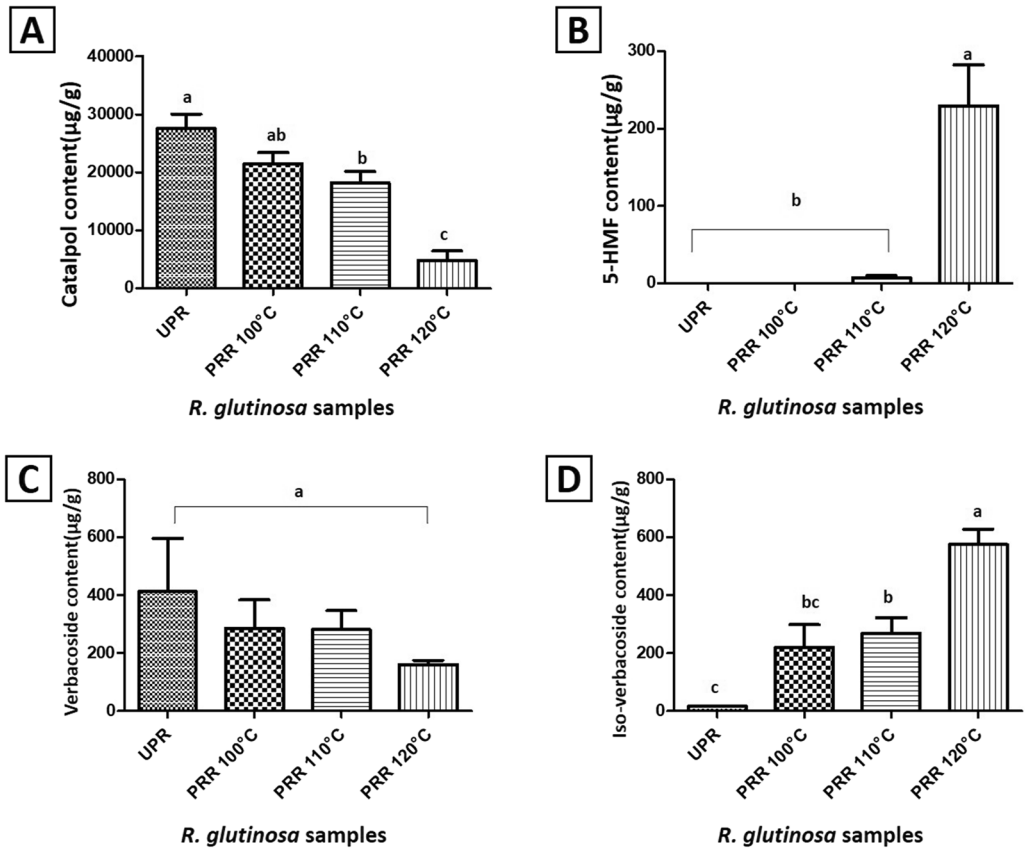

Fig. 3 Effect of different steaming temperature (100 ℃, 110 ℃, and 120 ℃) on marker compounds content of *R. glutinosa* samples. **a** Catalpol content of *R. glutinosa* samples. **b** 5-HMF content of *R. glutinosa* samples. **c** Verbacoside content of *R. glutinosa* samples. **d** Iso-verbacoside content of *R. glutinosa* samples. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

an additional 29% from dried UPR to PRR. These results indicated that most of the total water (i.e., 80%) is lost during all stage of processing (Fig. 2c). Previous analysis have revealed that during long-term storage, samples with a moisture content of less than 12% show minimal quality changes, including changes in appearance and texture [24]. However, traditional practice to dry *R. glutinosa* samples is not really suitable to maintain its quality as they applied sun drying that can easily get contaminated, thereby reducing quality [25]. Therefore, to maintain high quality, it is essential to apply modern methods for drying [26].

Effect of steam processing on marker compounds content

Steaming temperature, number of steaming times, steaming duration, and additives were selected as the main factors for processed *R. glutinosa* standardization. An analysis of steaming temperature showed that 120 °C resulted in significantly higher 5-HMF (229.52 ± 74.85 μg/g) and iso-verbascoside (575.05 ± 74.90 μg/g) contents compared to 100 °C and 110 °C (Fig. 3). Moreover, steaming temperature of 120 °C also dramatically reduced the level of catalpol (4836.43 ± 2295.44 μg/g) and verbacoside (160.77 ± 20.49 μg/g) compared with 100 °C and 110 °C (Fig. 3). Therefore, 120 °C is the optimum temperature for steam processing of *R. glutinosa* roots.

UPR and PRR samples were investigated for the presence of catalpol, 5-HMF, verbascoside, and iso-verbascoside influenced by number of steaming times and steaming duration (Fig. 4). *R. glutinosa* samples were subjected to steaming for 1 h, 2 h, and 4 h with five replications. The results showed that samples processed by steaming for 1 h with five replications, 2 h with three replications, and 4 h with two replications met the minimal Korean Pharmacopoeia criteria for 5-HMF content

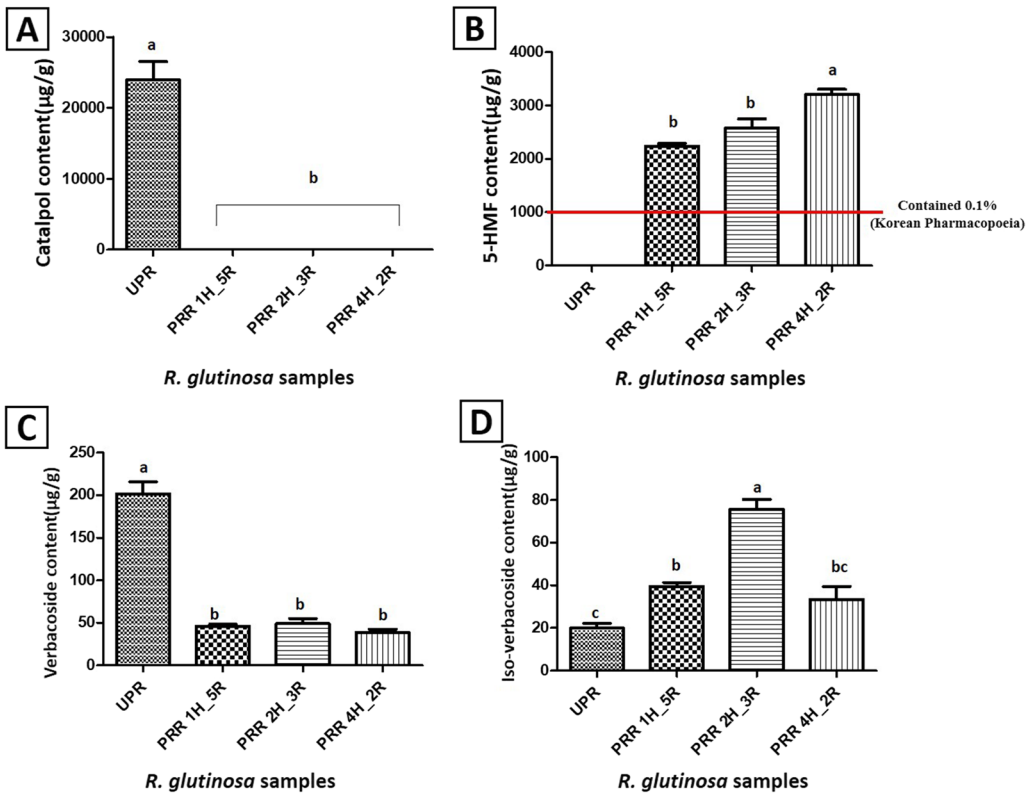

Fig. 4 Effect of different steaming times (five times) and steaming duration (1 h, 2 h, and 4 h) on marker compounds content of *R. glutinosa* samples. **a** Catalpol content of *R. glutinosa* samples. **b** 5-HMF content of *R. glutinosa* samples. **c** Verbacoside content of *R. glutinosa* samples. **d** Iso-verbacoside content of *R. glutinosa* samples. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

(above 0.1%) [27]. Furthermore, we decided to choose steaming for 2 h with three replications as the most optimal condition as it's produced highest content of verbacoside (48.98 ± 12.40 μg/g) and iso-verbacoside (52.09 ± 29.38 μg/g) (Fig. 4c, d). Although steaming for 4 h with two replications yielded the highest content of 5-HMF among all samples but surplus 5-HMF could induce cell toxicity [28]. In addition, steaming for 4 h with two replications showed lower iso-verbacoside content compared to other samples.

Figure 5 shows the effect of additive supplementation on marker compound content of *R. glutinosa* root samples. The results revealed that 5-HMF, verbascoside, and iso-verbascoside contents were higher in SPRR samples compared with UPR and PRR (Fig. 5). However, all SPRR samples met the criteria established by the Korean and Chinese Pharmacopoeia (verbascoside content > 0.02% and 5-HMF content > 0.1%). Moreover, among SPRR samples, supplementation with 20% EtOH resulted in the highest verbascoside (119.88 ± 5.88 μg/g) and iso-verbascoside contents (203.80 ± 10.72 μg/g). Hence, 20% EtOH is the best additive supplement for processed *R. glutinosa*. In addition, our optimized processing method (SPRR 20% EtOH) had been evaluated by Korea Ministry of Food and Drug Safety resulted in high quality of processed *R. glutinosa* roots with 0.5% 5-HMF content (Additional file 1: Fig. S1).

FT-NIR analysis

FT-NIR spectroscopy, a non-destructive chemical evaluation technique, is used industrially for the rapid characterization and identification of the chemical composition of materials [29]. The FT-NIR spectrum provides information on the major chemical bonds constituting the chemical composition of plant samples [30]. The result showed that UPR consist of eight spectrum peaks while PRR and SPRR both have seven peaks (Fig. 6a). The spectrum peaks of all samples were observed between 9000 and 4000 cm^{-1} of wavenumbers range. UPR showed different pattern of spectra compared with processed *R. glutinosa* samples (PRR and SPRR). This possibly happened due to the change of chemical composition during

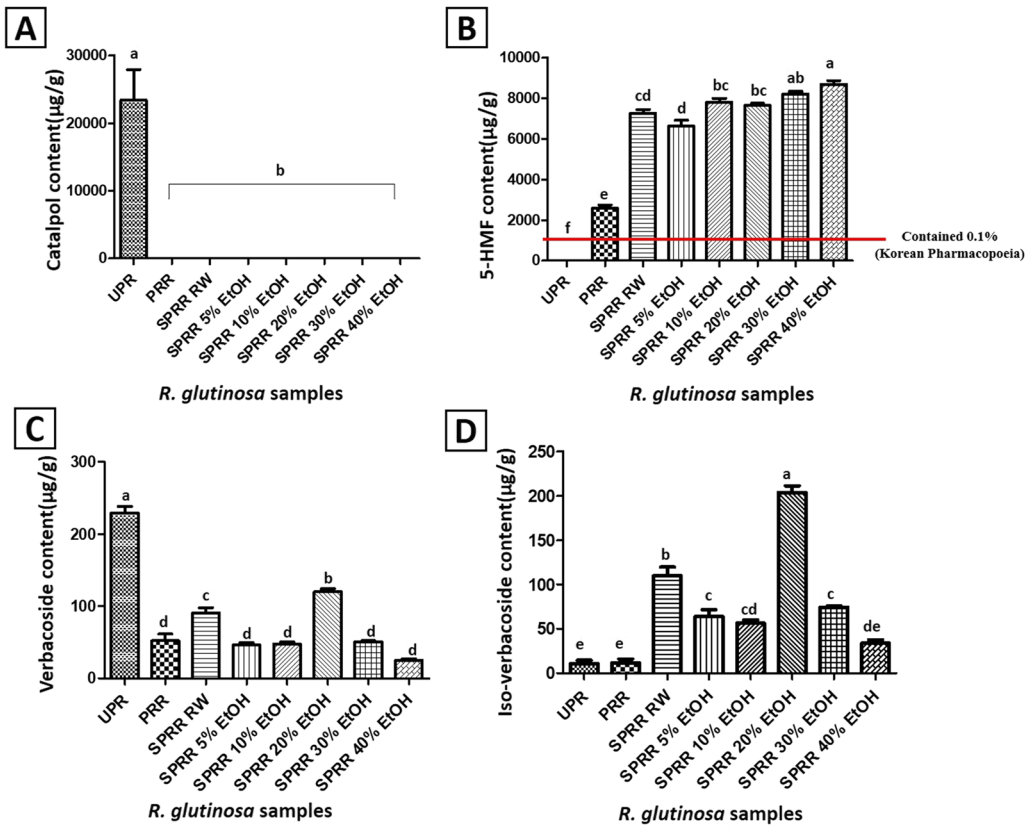

Fig. 5 Effect of different additive supplements (rice wine, 5% EtOH, 10% EtOH, 20% EtOH, 30% EtOH, and 40% EtOH) on marker compounds content of *R. glutinosa* samples. **a** Catalpol content of *R. glutinosa* samples. **b** 5-HMF content of *R. glutinosa* samples. **c** Verbacoside content of *R. glutinosa* samples. **d** Iso-verbacoside content of *R. glutinosa* samples. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*, *SPRR* Supplemented Processed *R. glutinosa*, *RW* Rice wine. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

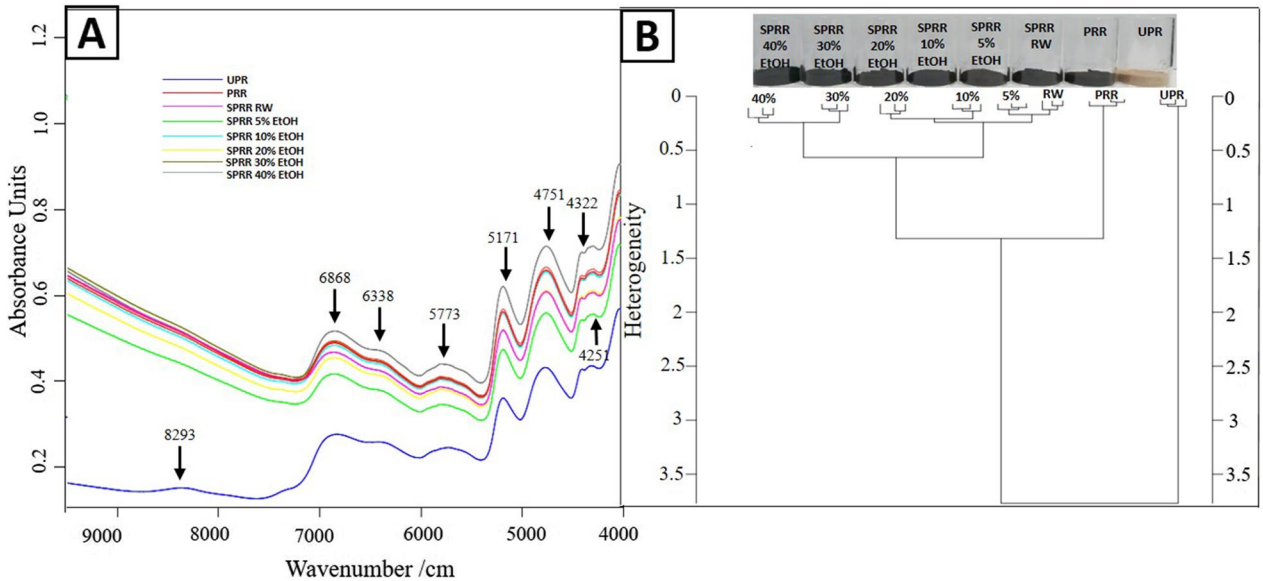

Fig. 6 The fourier transform near-infrared (FT-NIR) spectroscopy of characterization all *R. glutinosa* samples. **a** FT-NIR spectra of analysed *R. glutinosa* samples. **b** Dendogram of analysed FT-NIR showing the heterogeneity of all *R. glutinosa* samples. **UPR* Unprocessed *R. glutinosa*, *PRR*, Processed *R. glutinosa*, *SPR*, Supplemented Processed *R. glutinosa*, *RW* Rice wine

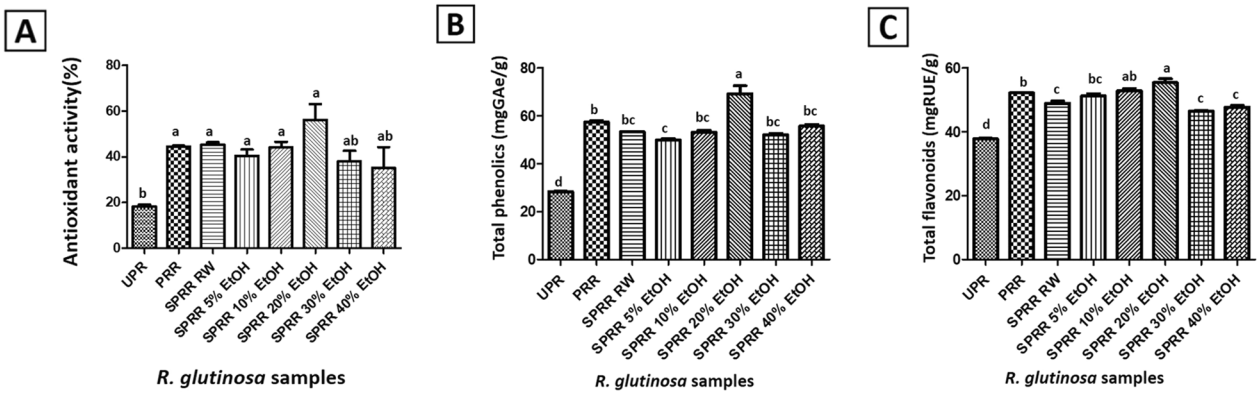

Fig. 7 Percentage of DPPH antioxidant activity, total phenolic, and total flavonoid content of all *R. glutinosa* sample extracts. **a** Antioxidant activity of *R. glutinosa* samples. **b** Total phenolics content of of *R. glutinosa* samples. **c** Total flavonoids content of *R. glutinosa* samples. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*, *SPRR* Supplemented Processed *R. glutinosa*, *RW* Rice wine. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

steam processing [31]. For instance, high temperature of steam changed some active compound composition such as decreased content of catalpol and verbacoside and increased content of 5-HMF from unprocessed to processed *R. glutinosa* (Fig. 5). Meanwhile, PRR and SPRR have similar pattern of FT-NIR spectra indicating the homogeneity of certain functional classes of chemicals within these materials.

Additionally, Ward's algorithm was applied to cluster all spectra produced from FT-NIR analysis (Fig. 6b). According to Ward's algorithm grouping, processed *R. glutinosa* samples (PRR and SPRR) display more homogeneity among themselves than unprocessed *R. glutinosa* (UPR). Moreover, all SPRR samples indicated more similar heterogeneity compared to the rest of the samples. The similar heterogeneity recorded in the dendrogram of the respective grouped samples of *R. glutinosa* roots was caused by higher similarity between the distinct near-IR spectra of these samples [32]. This suggests a close chemical phylogenetic relationship between the samples.

Effect of steam processing on antioxidant activity

The antioxidant activity level all *R. glutinosa* samples were evaluated by a DPPH assay. As shown in Fig. 7a, all of processed *R. glutinosa* (PRR and SPRR) have significantly higher antioxidant activity when compared with that in unprocessed sample (UPR) (18%). Previous studies have shown that heat treatment causes chemical changes, such as changes in Maillard reaction products, including 5-HMF production. In this regard, an increase in 5-HMF may have contributed to the increase in anti-oxidant capacity detected by the DPPH scavenging assay [31]. In addition, among SPRR samples, supplementation with 20% EtOH (SPRR 20%EtOH) showed highest antioxidant activity. This is correlated with its high content of verbacoside and iso-verbacoside compared to other SPRR samples (Fig. 5c, d). Several previous reports showed that verbacoside and iso-verbacoside are good antioxidant agents [33].

Effect of steam processing on total flavonoids and phenolic contents

The total phenolic and flavonoid contents of all *R. glutinosa* samples are presented in Fig. 7b, c, respectively. The results indicated that steaming process clearly influences phenolic and flavonoid contents. Compared with unprocessed sample (UPR), all processed samples (PRR and SPRR) showed higher total phenolic and flavonoids contents. Many reports have said that heat treatment could increase the total phenolic and flavonoids content in the plants [34]. Among all SPRR samples, supplementation with 20% EtOH significantly increased the total phenolic and flavonoids content of 69.24 ± 4.56 mg/g and 55.36 ± 1.68 mg/g, respectively. This result is in line with the level of antioxidant activity where optimum result obtained by supplementation with 20% EtOH. Therefore, the increased in total phenolic and flavonoids content could also level up the antioxidant activity. Flavonoids and phenolic compounds are widely used in Oriental medicine and food products due to their capability in promoting antioxidant activity [35].

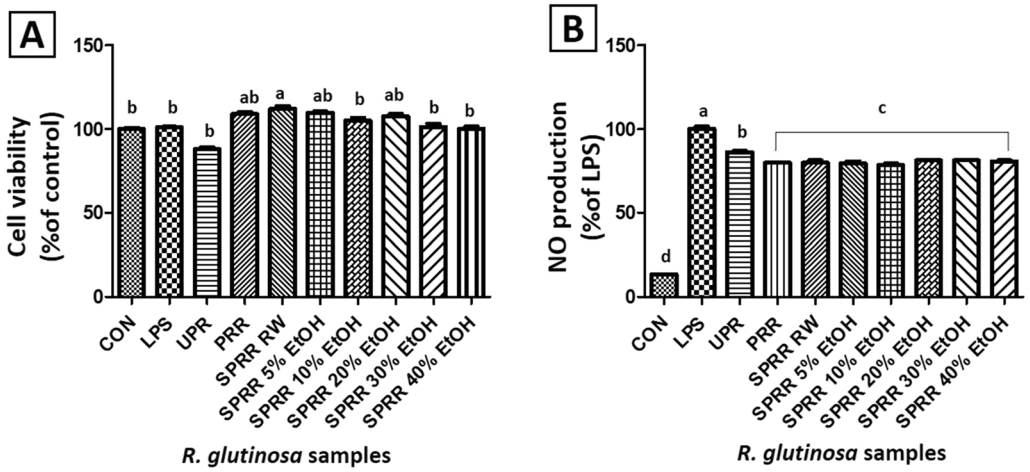

Fig. 8 Effect of *R.glutinosa* samples extracts (200 μg/ml) on cell viability and nitric oxide (NO) production in LPS-stimulated macrophages cell lines. **a** Cell viability of LPS-stimulated macrophages treated with *R. glutinosa* extracts. **b** Inhibition of NO production by all *R. glutinosa* sample extracts. **UPR* Unprocessed *R. glutinosa*, *PRR* Processed *R. glutinosa*, *SPRR* Supplemented Processed *R. glutinosa*, *RW* Rice wine. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

Effect of steam processing on anti-inflammatory activity

We tested the effects of *R. glutinosa* extracts on cell viability to detect cytotoxicity in RAW264.7 cells by the CCK assay. As shown in Fig. 8a, all *R. glutinosa* samples at 200 μg/mL did not cause cell death. This is consistent with previous research showing that unprocessed and processed *R. glutinosa* do not exert cytotoxicity [36]. Therefore, all *R. glutinosa* samples at concentration of 200 μg/mL is considered safe for further study and applications.

NO is a free radical synthesized in tissues and cells. Low NO levels contribute to homeostasis, while high NO levels are associated with inflammation and various diseases. Thus, NO levels are an indicator of anti-inflammatory activity [37]. NO production was distinctly diminished after LPS-induced macrophages were treated with all *R. glutinosa* extracts. Moreover, processed *R. glutinosa* showed higher inhibition activity on NO production (81%) compared with unprocessed *R. glutinosa* (86%) (Fig. 8). This is consistent with their effect on antioxidant activity where processed *R. glutinosa* showing stronger effect than unprocessed *R. glutinosa*. This could be related with the increased level of some active compounds (5-HMF, iso-verbascoside, flavonoid, and phenolics) in processed *R. glutinosa*. However, there were no significant differences in NO inhibition effect among processed *R. glutinosa* samples. Due to its high 5-HMF, verbacoside, and iso-verbacoside contents, SPRR 20% EtOH was chosen for further western blot analysis to uncover its molecular mechanism of action for anti-inflammatory activity based on NO suppression ability. Several previous studies have also reported anti-inflammatory potential of 5-HMF, verbascoside and iso-verbascoside [33].

To verify the efficacy of optimized processed *R. glutinosa* (SPRR 20% EtOH) on anti-inflammatory activity, RAW 264.7 pro-inflammatory factors (iNOS, NF- κB) and cytokine (COX-2) release was evaluated by western blot [38]. The increase in iNOS protein expression levels in LPS-stimulated macrophages is related to a substantial increase in NO production, and iNOS acts as an important regulator of inflammation and immune defense [39]. The result showed that processed *R. glutinosa* supplemented with 20% EtOH (SPRR 20% EtOH) strongly inhibited iNOS expression to levels in untreated samples (Fig. 9a). The suppressive effect of SPRR 20% EtOH samples was concentration-dependent, in which a high concentration (i.e., 200 μg/mL) resulted in a significantly higher inhibition value than that of a low concentration (100 μg/mL). However, levels of pro-inflammatory COX-2 were not affected by SPRR 20% EtOH samples (Fig. 9b). This indicates that the ability of processed *R. glutinosa* samples to induce anti-inflammatory only through the inhibtion of iNOS expression.

The activation of NF-κB has a critical role in the induction of the inflammatory factors COX-2 and iNOS [40]. The nuclear transcription factor NF-κB is a key mediator of inflammation and plays an important role in cytokine expression [41]. Various factors acting upstream of NF-κB activation, including NH2-terminal kinase (JNK), extracellular signal-regulated protein kinase (ERK), mitogen-activated protein kinase (MAPK), and

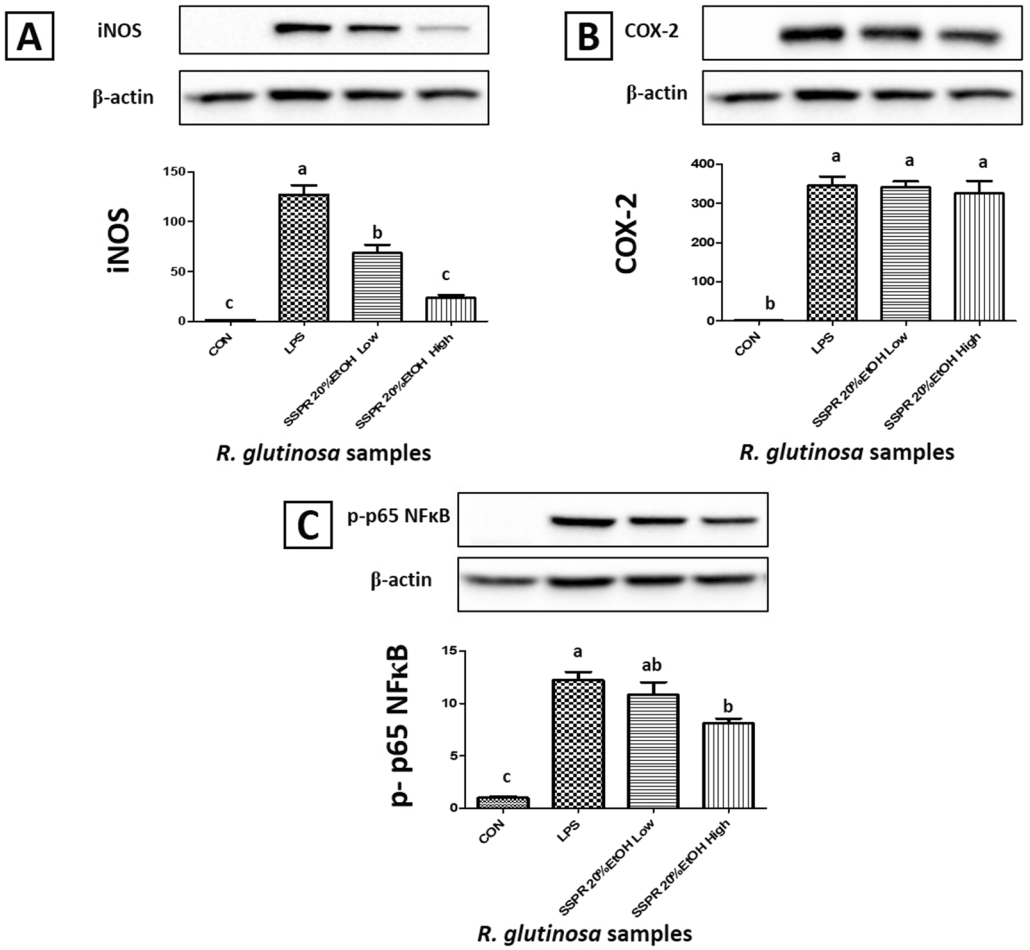

Fig. 9 Inhibition effect of processed *R.glutinosa* sample extracts on the expression of iNOS, COX-2, and NFκB in LPS-stimulated macrophages. **a** Effect of processed *R.glutinosa* extracts on iNOS expression. **b** Effect of processed *R.glutinosa* extracts on COX-2 expression. **c** Effect of processed R.glutinosa extracts on NF-κB expression. **SPRR* Supplemented Processed *R. glutinosa*; RW. Values are presented as means ± standard deviation. Same letters are not significantly different by Tukey's test and p = 0.05

p-65 phosphorylation (p65) [40, 42]. The NF-κB levels through p-p65 pathway in LPS-stimulated RAW264.7 macrophages and SPRR 20% EtOH samples are summarized in Fig. 9c. Processed *R. glutinosa* supplemented with 20% EtOH significantly inhibited p-p65 expression in a concentration-dependent manner (100 and 200 μg/mL). These results indicated that processed *R. glutinosa* supplemented with 20% EtOH exerts anti-inflammatory effects by the inhibition of iNOS via the NF-κB activation. Moreover, these results support those of previous studies demonstrating that polysaccharide derivatives such as 5-HMF isolated from steam processed *R. glutinosa* effectively suppress the expression of pro-inflammatory factors [43]. Moreover, the increase in iso-verbascoside after steam processing could contribute to the anti-inflammatory effect [44].

Abbreviations

UPR	Unprocessed *R. glutinosa*
PRR	Processed *R. glutinosa*
SPRR	Supplemented processed *R. glutinosa*
5-HMF	5-Hydroxymethyl-2-furaldehyde
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
NF-κB	Nuclear factor-kappa B
LPS	Lipopolysaccharide
COX-2	Cyclooxygenase-2
HPLC	High-performance liquid chromatography
DPPH	1.1-Diphenyl-2picrylhydrazyl
FT-NIR	Fourier transform near-infrared spectroscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00773-7.

Additional file 1: Figure S1. Certificate of analysis of optimized processed *R. glutinosa* (SPRR 20% EtOH) accredited by Korea ministry of Food and Drug Safety.

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Author contributions
YC and ER are co-first authors. YC: Conceptualization, methodology, data collection and curation, writing original draft. ER: Data supervision, review, and manuscript editing. HHN: Western blotting supervision. AL: HPLC supervision. JHP: Anti-inflammatory study supervision. BCM: Project administration. YK: Supervision, project administration, review and editing. All authors read and approved the final manuscript.

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Availability of data and materials
The data will be made available by the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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