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# Beneficial effects of WON-21 on the symptoms of a hangover and identification of active compounds: experimental studies on antioxidant, anti-inflammation, and alcohol-metabolizing enzymes

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## Abstract

Many hangover cure products containing natural ingredients that are also effective against alcohol-related liver damage or improve liver function have recently become available. In addition to curing liver damage, antioxidants, anti-inflammatory agents, and blood ethanol reduction aids are emerging as relief targets that reduce hangover symptoms. We investigated the ameliorating effect of WON-21 herbal medicinal products by studying the mixing ratio of oriental medicine concept with respect to antioxidant potential, anti-inflammation, and aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) enzyme activities. WON-21 and its components exerted antioxidant and anti-inflammatory effects. Rutin, taxifolin, and quercetin showed superior antioxidant effects compared to the other components. WON-12 effectively reduced iNOS and COX-2 in LPS-stimulated macrophages. Quercetin and apigenin were 2 compounds effective for the inhibition of iNOS and COX-2. WON-21 and quercetin also significantly increased the activities of ALDH and ADH enzymes in a concentration-dependent manner.

**Keywords** Hangover, Blood ethanol, Antioxidant, Anti-inflammation

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## Introduction

Hangovers are symptoms that can occur after consuming alcohol. The exact mechanism of hangovers is not fully understood; however, several factors are thought to contribute to their onset [1]. Alcohol is a diuretic that increases urine production and can lead to dehydration [2]. Dehydration can cause headaches, fatigue [3], and a dry mouth, which are common symptoms of hangovers. Alcohol can cause inflammation, leading to headaches, body aches, and a general feeling of malaise [4, 5]. When alcohol is metabolized in the liver, it is converted into acetaldehyde, a toxic substance that can cause headaches, nausea, and vomiting. Alcohol can disrupt blood sugar levels, cause hypoglycemia (low blood sugar), and contribute to symptoms such as weakness, dizziness, and shakiness. Alcohol can disrupt the normal sleep cycle, leading to fatigue and irritability the next day [1, 6].

Overall, hangovers are thought to be caused by a combination of these factors, and the severity of symptoms can vary depending on factors such as the amount and type of alcohol consumed, tolerance, and hydration levels [1]. There is no known cure for hangovers. However, drinking plenty of water, resting, and avoiding alcohol can help alleviate symptoms. Several substances are commonly used to reduce hangover symptoms. Drinking water can alleviate dehydration, which is a common cause of hangovers. Water can also help flush out toxins and improve overall hydration levels. Electrolytes such as sodium, potassium, and magnesium can help replenish body fluids and electrolytes, which can be depleted by drinking alcohol [7]. Sports drinks and electrolyte supplements may also be helpful in this regard. Nonsteroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen can help reduce inflammation, relieve headaches, and also alleviate body aches that are common during hangovers [8]. Antioxidants, such as vitamins C and E, can help neutralize free radicals in the body, which can be produced by alcohol metabolism and contribute to inflammation and tissue damage. Food and supplements rich in antioxidants may be helpful in this regard. *N*-acetylcysteine (NAC) can help replenish glutathione stores, a powerful antioxidant important for detoxification. NAC may also help reduce inflammation and improve liver function after alcohol consumption [9, 10].

The mechanisms of action of substances that reduce the hangover symptoms are diverse and primarily aim to address the underlying causes of hangovers such as dehydration, inflammation, and oxidative stress. Recently, many hangover cure products containing natural ingredients that are effective against alcohol-related liver damage or improved liver function have been made available in the market. In addition to curing liver damage, antioxidant and anti-inflammatory effects as well as blood ethanol reduction, are emerging as targets for relieving hangover symptoms [11, 12].

Galhwahajung-tang and Daekumeumja are 2 important herbal prescriptions for relieving a hangover after consuming too much alcohol [13, 14]. WON-21 is a mixture of Galhwahajung-tang, Daekumeumja, and herbal medicines reported to be beneficial for relieving the symptoms of hangovers [15–17]. In this study, we investigated the efficacy of WON-21, a herbal medicinal product developed by using the mixing ratio based on the oriental medicine concept, in terms of its antioxidant, anti-inflammatory, and enzyme activities of ADH and ALDH.

## Materials and methods

### Preparation of WON-21 water extract

As shown in Table 1, the WON-21 water extract, composed of 18 ingredients, was manufactured at the Korea Institute of Oriental Medicine (KIOM). Briefly, 3 L distilled water was added to 18 mixed materials (270 g), and they were extracted using a reflux condenser at 100 °C for 2 h. After cooling to room temperature, the extract was centrifuged at 4000 rpm for 30 min, filtered using a vacuum pump and Whatman filter paper No. 2 (150 mm), and finally freeze–dried (FD8518, IlShinBioBase, Dongducheon, Korea). The amount of lyophilized sample was 110.0 g (yield 40.7%).

### Chemicals

The 12 standard compounds (Fig. 1) were purchased from specialized manufactures as follows: 3′-hydroxypuerarin (CAS No. 117060-54-5, catalog No. CFN90680, purity, 99.8%), 3′-methoxypuerarin (CAS No. 117047-07-1, catalog No. CFN90780, purity: 99.2%) and quercetin (CAS No. 117-39-5, catalog no. CFN99272, purity, 99.2%) from ChemFaces Biochemical Co., Ltd. (Wuhan, China); Chlorogenic acid (CAS No. 327-97-9, catalog No. 109240010, purity, 99.6%) from Acros Organics (Pittsburgh, PA, USA); puerarin (CAS No. 3681-99-0, catalog No. 165-22001, purity, 99.8%) and daidzin (CAS No. 552-66-9, catalog No. 309-05161, purity, 98.0%) from Fujifilm Wako Pure Chemical Co. (Osaka, Japan); rutin (CAS No. 153-18-4, catalog No. 89270, purity, 97.2%) from Phyto-Lab GmbH & Co. KG (Vestenbergsgreuth, Germany); hyperoside (CAS No. 482-36-0; Catalog No. BP0753, purity: 98.7%), hesperidin (CAS No. 520-26-3, catalog No. BP0725, purity 98.6), and apigenin (CAS No. 520-36-5, catalog No. BP0177, purity, 98.1%) from Chengdu Biopurify Phytochemicals (Chengdu, China); taxifolin (CAS No. 480-18-2, caltalog No. 156745, purity, 98.0%) from ICN Biomedicals Inc. (Aurora, OH, USA); naringin (CAS No. 10236-42-7, catalog No. 71162, purity, 95.0%) from Merck KGaA (Darmastadt, Germany). The solvents, distilled water (CAS No. 7732-18-5, catalog No. 4218-88),

**Table 1** Composition of WON-21

| Scientific name | Latin name | Amount (g) |
|---|---|---|
| *Hovenia dulcis* Thunb. | Hoveniae Semen seu Fructus | 15 |
| *Pueraria lobata* Ohwi | Puerariae Flos | 15 |
| *Citrus unshiu* Markovich | Citri Unshius Pericarpium | 5 |
| *Curcuma longa* L. | Curcumae Longae Rhizoma | 5 |
| Lycium chinensis Miller | Lycii Fructus | 5 |
| *Cirisum japonicum* DC. Var. *ussuriense* (Regel) Kitamura | Cirsii Radis | 5 |
| *Crataegus pinnatifida* Bunge | Crataegi Fructus | 4 |
| *Poria cocos* Wolf | Poria Sclerotium | 4 |
| *Acanthopanax sessiliflorum* (Rupr. Et Maxim.) Seem. | Acanthopanacis Cortex | 2.5 |
| *Amomum villosum* Loureiro | Amomi Fructus | 2.5 |
| Fermented *Oryza sativa* seeds with *Monascus purpureus* | | 2.5 |
| *Cervus elaphus* L. | Cervi Parvum Cornu | 2.5 |
| *Hordeum vulgare* L. var. *hexastichon* Aschers | Hordei Fructus Germinatus | 2 |
| *Atractylodes japonica* Koidz. | Atractylodis Rhizoma Alba | 2 |
| *Dolichos lablab* L. | Dolichoris Semen | 2 |
| *Raphanus sativus* L. | Raphani Semen | 2 |
| *Atractylodes chinensis* Koida. | Atractylodis Rhizoma | 2 |
| *Agastache rugosa* (Fisher et Meyer) O. Kuntze | Agastachis Herba | 2 |
| | Total | 80 |

acetonitrile (CAS No. 75-05-8, catalog No. 9107-88, purity, ≥ 99.9%), and methanol (CAS No. 67-56-1, catalog No. 9093-88, purity, ≥ 99.9%), and formic acid (CAS No. 64-18-6, catalog No. 5330020050, purity, 98.0–100.0%) were all HPLC grade and were purchased from J.T. Baker (Phillipsburg, NJ, USA) and Merck KGaA (Darmstadt, Germany), respectively.

Ethanol (CAS No. 4023-2304, Lot No. E3167WA1, HPLC solvent, 99.9%) was purchased from Daejung (Seoul, Korea), tert-butanol (CAS No. 19460, LOT No. NCCF5930, suitable for HPLC, 99.9%) was purchased from Sigma Aldrich (St. Louis, MO, United States), sodium chloride (saturated solution) was purchased from Sigma Aldrich. BD vacutainers were purchased from Becton Dickinson (Franklin Lakes, NJ, United States), and 20 mL gas chromatography (GC) vials were purchased from Thermo Fisher Scientific (Waltham, MA, United States).

#### HPLC analysis of WON-21

After adding 10 mL of 70% methanol to 100 mg of the lyophilized WON-21 sample, ultrasonic extraction was performed at room temperature for 60 min. Subsequently, the extract was subjected to 0.2 μm membrane filtration and used for further analysis. The prepared samples were analyzed under the conditions listed in Table 2, using a Shimadzu Prominence LC-20A system controlled by LCSolution (version 1.24) (Shimadzu Corporation, Kyoto, Japan).

#### DPPH radical scavenging activity

The concentration of the WON-21 and its components to be evaluated for antioxidant activity is prepared at twice the final concentration (0.1, 0.3, 1, and 2 mg/mL), and the 60 μM DPPH working solution by diluting the 1 mM DPPH stock (Glentham Life Sciences, UK, Cat. No. GX8745). Then, add the same amount of DPPH working solution and samples by 100 μL to each well. After incubation for 30 min at 25 °C, the absorbance value at 540 nm was measured using an E-Max microplate reader (Molecular Devices, LLC., San Jose, CA, United States). Antioxidant activity was calculated based on the untreated control.

#### Cell culture

The RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) (Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Carlsbad, MD, USA) and 1% penicillin–streptomycin (Invitrogen, Grand Island, NY, USA). The cells were maintained at 37 °C in a humidified atmosphere of 5% $CO_2$ and subcultured every 2 days.

#### Cell viability

RAW 264.7 cells were seeded in a 96-well plate at a density of $1 \times 10^5$ cells/well and grown for 24 h. The next day, cells were treated either with 0.5% dimethyl sulfoxide

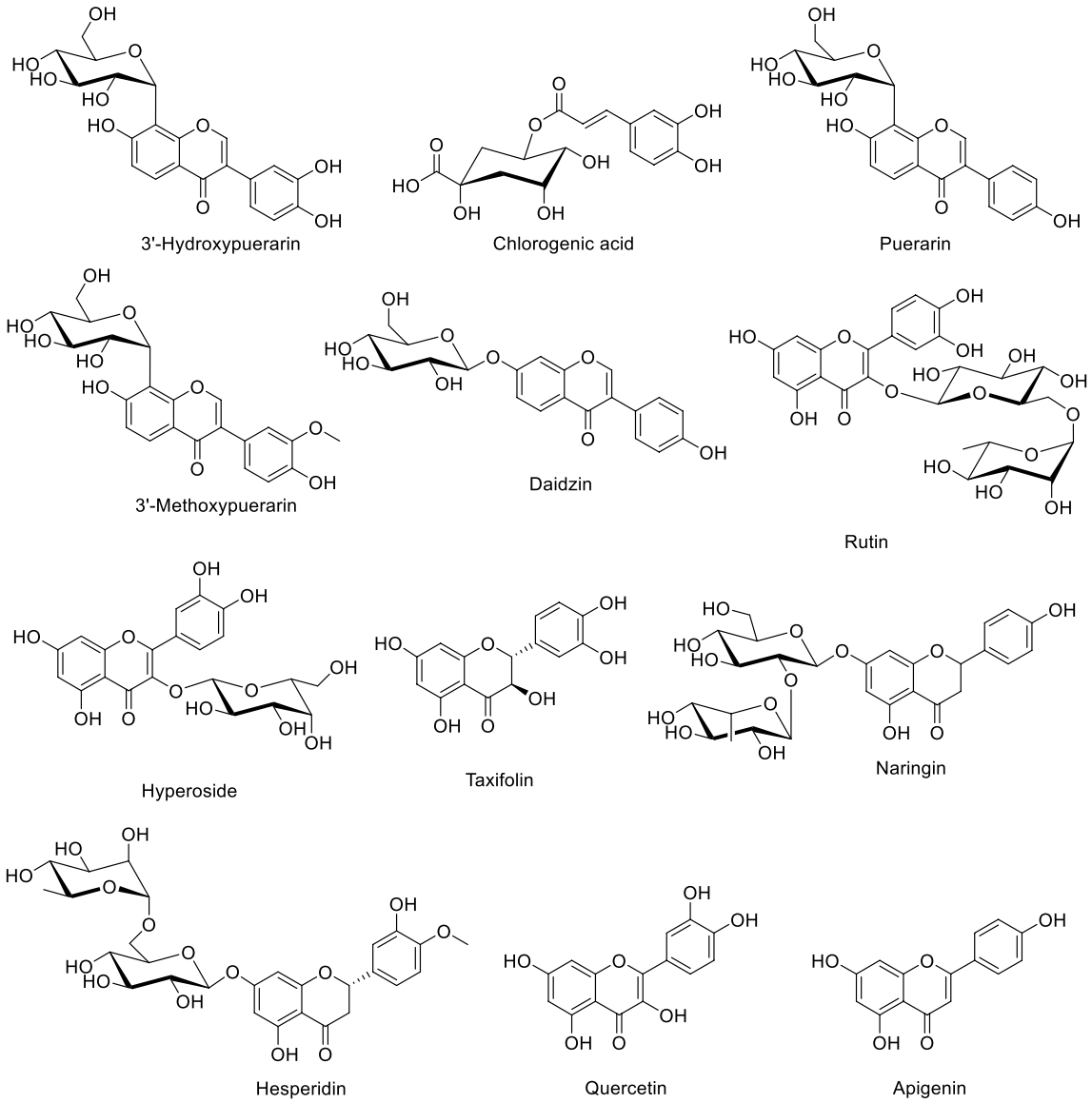


**Fig. 1** Chemical structures of 12 components detected in WON-21

(DMSO) (Sigma Aldrich), which served as a control, or with WON-21 at the concentrations of 0.1, 0.3, 1, or 2 mg/mL. After 24 h, 10 µL of EZ-Cytox reagent (DoGen, Seoul, Korea) was added to each well and incubated for 30 min. The cell viability was determined by measuring the change in absorbance at 450 nm using a PowerWave XS microplate reader (BioTek Instruments, Winooski, VT, USA) [18].

### Measurement of nitric oxide (NO) in RAW 246.7 cells

Nitric oxide production was evaluated by measuring nitrite concentration that accumulated in the culture medium. RAW264.7 cells were seeded in a 96-well plate at a density of $1 \times 10^5$ cells/well. The cells were pre-treated with WON-21 at the indicated concentrations for 2 h, followed by lipopolysaccharides (LPS) (1 µg/mL) for 24 h. The cell culture supernatant was collected and mixed with Griess reagent (supplemented with 1% sulfanilamide, 5% phosphoric acid, and 0.1% *N*-(1-naphthyl)-ethylenediamine) at a ratio of 1:1 (v/v) [19]. After incubation at room temperature for 15 min, the absorbance of the mixture was measured at 450 nm using a microplate reader. Nitrite concentration in the supernatant was calculated using a sodium nitrite standard reference curve.

**Table 2** Analytical conditions for simultaneous determination of 12 marker components in WON-21 sample by HPLC

| **Operation condition** | | | |
|---|---|---|---|
| Column | SunFire $C_{18}$ analytical column (250 mm × 4.6 mm, 5 μm) | | |
| Detector | PDA (250, 280, 290, 325, 335, 350, and 370 nm) | | |
| Flow rate | 1.0 mL/min | | |
| Injection volume | 10.0 μL | | |
| Column temperature | 40.0 °C | | |
| Mobile phase | A: 0.1% (v/v) aqueous formic acid<br>B: 0.1% (v/v) formic acid in acetonitrile | | |
| | **Time (min)** | **A (%)** | **B (%)** |
| Gradient elution | 0 | 95 | 5 |
| | 60 | 40 | 60 |
| | 70 | 40 | 60 |
| | 80 | 95 | 5 |
| | 90 | 95 | 5 |

*PDA* photo-diode array

#### Protein extraction and western blot analysis

The RAW 264.7 cells were seeded in 6-well plates at a density of $1 \times 10^7$ cells/well for 24 h. The cells were pretreated with WON-21 at the indicated concentrations for 2 h, followed by LPS (1 g/mL) for 24 h. Cells were collected and washed with Dulbecco's phosphate-buffered saline (DPBS) before being lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1× protease inhibitor cocktail and 1 mM sodium orthovanadate ($Na_3VO_4$) phosphatase inhibitor to obtain whole-cell extracts according to the manufacturer's instructions. The protein concentration of each whole-cell extract was determined using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, MA, USA). The equal protein amounts of each whole-cell extract (20 μg/lane) were separated by electrophoresis in a 10% sodium dodecyl sulfate–polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) transfer membranes. Epitope-specific primary antibodies included cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), phosphorylated nuclear factor kappa-light-chain-enhancer of activated B cells (p-NFκB), NFκB, and GAPDH, which were conjugated with secondary antibodies (Cell Signaling Technology, Boston, MA, USA), were used to label the target proteins. The bound antibodies were detected using Pierce™ ECL Advance Western Blotting Detection Reagents (Thermo Fisher Scientific) and visualized using a FUSION Solo Chemiluminescence System (PEQLAB Biotechnologie GmbH, Germany).

#### Measurement of tumor necrosis factor-α (TNF-α) production in RAW 264.7 cells

RAW 264.7 cells were plated in 24-well plates at a density of $4 \times 10^5$ cells/well and incubated for 24 h. Cells were pretreated to WON-21 at concentrations of 0.1, 0.3, 1, and 2 mg/mL for 2 h followed by 1 μg/mL LPS for 24 h. To determine tumor necrosis factor-alpha (TNF-α) production, the supernatant was obtained and assayed to quantify the levels of these cytokines using a TNF-α sandwich enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, CA, USA), according to the manufacturer's instructions.

#### Determination of aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) activities

The activities of ALDH (Sigma Aldrich, St. Louis, MO, USA, Cat. No. MAK082) and ADH (Sigma Aldrich, St. Louis, MO, USA, Cat. No. MAK053) were evaluated using the colorimetric assay kits. Experiments were performed by referring to the instructions included in each kit. WON-21 and quercetin were prepared at twice the final concentration. After preparing the reaction mix containing the enzyme and substrate, the sample and reaction mixture was dissolved in 50 μL each well and reacted at room temperature for 10 min. Then, after measuring at a wavelength of 450 nm using an E-Max microplate reader (Molecular Devices, LLC., San Jose, CA, United States), absorbance values were calculated based on the standard curve.

### Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM) from three independent experiments. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA, United States) with a one-way analysis of variance (ANOVA), followed by Tukey's test. Statistical significance was set at $p < 0.05$.

## Results

### HPLC analysis of WON-21

Twelve components were selected as marker compounds for WON-21 and were simultaneously analyzed in the WON-21 sample using HPLC. As shown in Additional file 1: Table S1, all markers showed excellent linearity, with a coefficient of determination of 0.9999–1.0000 within the tested ranges. Furthermore, the limit of detection (LOD) and limit of quantitation (LOQ) were calculated using the equations of $3.3 \times \sigma/S$ and $10 \times \sigma/S$, respectively. where $\sigma$ and $S$ represent the standard deviation of the $y$-intercept and the slope of the calibration curve in the regression equation of each compound. the LOD and LOQ in the tested linear ranges were calculated as 0.01–0.22 μg/mL and 0.03–0.65 μg/mL, respectively. For simultaneous quantification, the detection wavelength of each marker compound was determined using a photo-diode array detector (Additional file 1: Table S1). As a result of applying the established HPLC analytical method to the WON-21 sample, all markers were eluted within 40 min with a resolution of ≥ 4.0 and

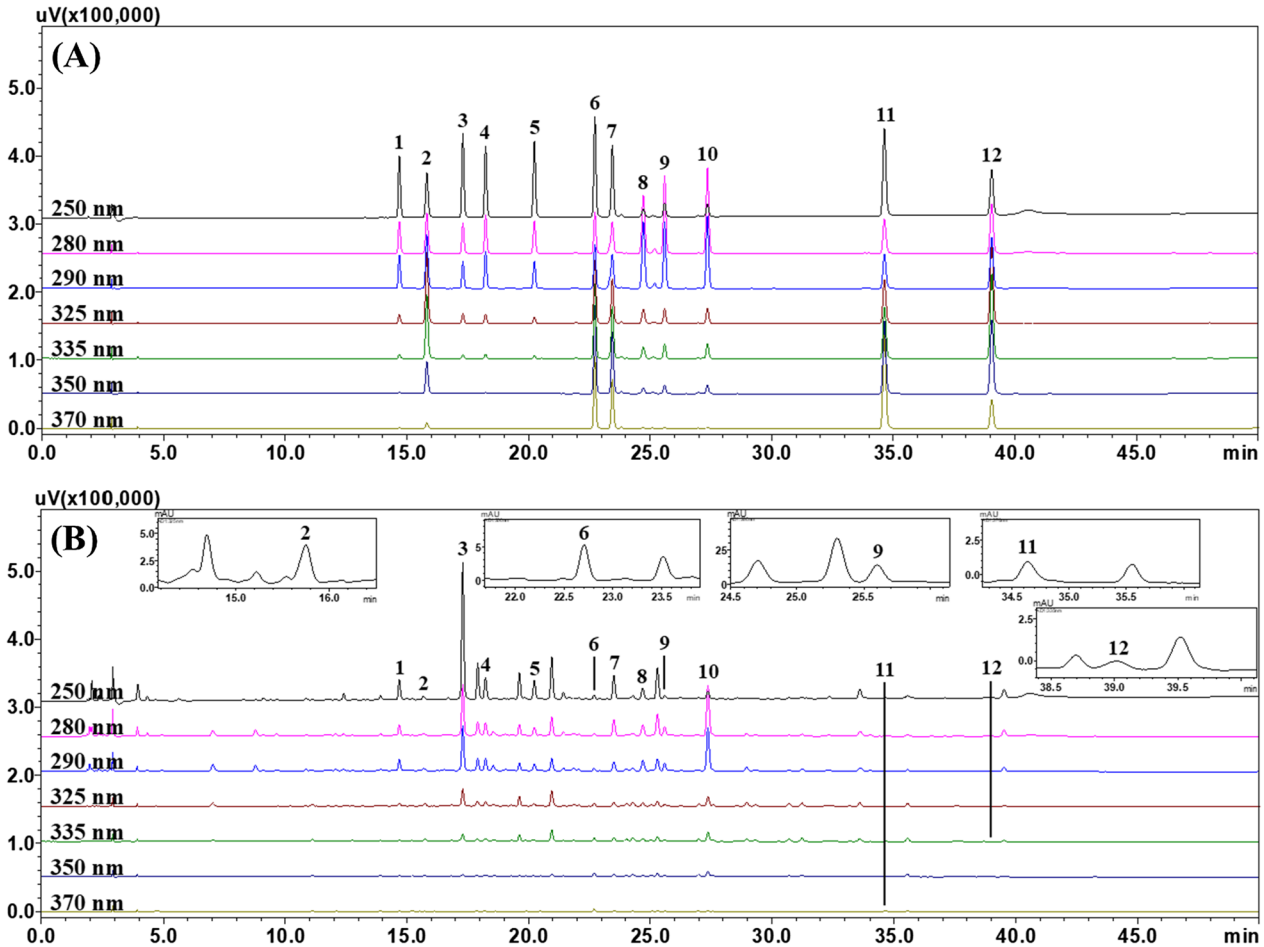


**Fig. 2** HPLC chromatograms of WON-21 and its ingredients. **A** Representative HPLC chromatograms of the standard solution of mixed markers at different wavelengths. **B** Representative HPLC chromatograms of the test solution of WON-21 water extract at different wavelengths. 3′-Hydroxypuerarin (1), chlorogenic acid (2), puerarin (3), 3′-methoxypuerarin (4), daidzin (5), rutin (6), hyperoside (7), taxifolin (8), narirutin (9), hesperidin (10), quercetin (11), and apigenin (12). The concentration of each compound in the standard solution (**A**) is 20.0 μg/mL for 3′-hydroxypuerarin, chlorogenic acid, puerarin, 3′-methoxypuerarin, daidzin, rutin, hyperoside, taxifolin, and apigenin, 30.0 μg/mL for narirutin and quercetin, and 50.0 μg/mL for hesperidin

were detected in an amount of 0.04–3.54 mg/g (Fig. 2 and Additional file 1: Tables S2 and S3). Among these components, hesperidin (a major compound of *C. unshiu*) and puerarin (a major compound of the *P. lobata*) were detected at 3.54 mg/g and 3.20 mg/g, respectively.

### Anti-oxidative effect of WON-21 and its components

DPPH is a stable free radical widely used to evaluate the antioxidant activities of natural extracts and pure compounds. In this study, the DPPH radical scavenging activity of WON-21 was 21.9%, 41.8%, 55.5%, and 60.4% at a concentration of 0.1, 0.3, 1, and 2 mg/mL compared with untreated control. The antioxidant activity of WON-21 increased significantly as the concentration increased in a dose-dependent manner (Table 3). Most components of WON-21 exerted antioxidant effects. In particular, IC50 values of 3'-hydroxypuerarin (16.3 μM), chlorogenic acid (16.5 μM), rutin (12.3 μM), hyperoside (18.5 μM), taxifolin (14.1 μM), and quercetin (15.4 μM) were statistically significant and were lower than 20 μM (Table 4). The result that the antioxidant effect of each compounds was slightly stronger than that of the extract seems to be resulted by the low concentration of active compounds in the extract.

### Effect of WON-21 on NO production in LPS-stimulated RAW 246.7 cells

To evaluate the effect of WON-21 on NO production in RAW 246.7 cells, non-cytotoxic concentrations were determined using cell viability assays. The cytotoxicity criterion was determined as 95% or less viability based on the 0.5% DMSO control. All tested concentrations of WON-21 had no cytotoxicity in RAW 264.7 cells (Fig. 3A). Effects of WON-21 on LPS-induced NO production in RAW 264.7 cells were confirmed. The WON-21 extracts significantly decreased LPS-induced NO produced by LPS stimulation by 33.1%, 47.4%, 78.1%, and 88.8% in a concentration-dependent manner as the concentration (0.1, 0.3, 1, and 2 mg/mL) of the extract increased (Fig. 3B).

### Effect of WON-21 on iNOS, COX-2, and NFκB expression in LPS-stimulated RAW 246.7 cells

NO production is directly related to the induction of iNOS, and COX-2 expression is related to pain during inflammation. Therefore, we examined the effects of WON-21 on LPS-induced iNOS and COX-2 stimulated by LPS. WON-21 decreased the expression of iNOS and COX-2 (Fig. 4A, B). iNOS protein expression levels significantly decreased by 15.8%, 25.9%, 50.6%, and 78.6% in

**Table 3** Antioxidant effect of WON-21 extracts

| Components | 0 mg/mL (%) | 0.1 mg/mL (%) | 0.3 mg/mL (%) | 1 mg/mL (%) | 2 mg/mL (%) | $IC_{50}$ (μg/mL) |
|---|---|---|---|---|---|---|
| WON-21 | 0.00 | 21.9 ± 1.53* | 41.8 ± 1.02* | 55.5 ± 2.83* | 60.4 ± 1.02* | 538.6 |

DPPH radical scavenging activity assay at various concentrations. $IC_{50}$ represents the concentration that exhibits 50% of antioxidant activity. Data presented the average of three independent experiments and was analyzed using one-way ANOVA with Tukey's post hoc

*P < 0.001 compared with untreated control

**Table 4** Antioxidant effect of components from WON-21 extract

| Components | 0 μM (%) | 10 μM (%) | 30 μM (%) | 100 μM (%) | $IC_{50}$ (μM) |
|---|---|---|---|---|---|
| 3′-Hydroxypuerarin | 0.00 | 48.9 ± 2.57* | 64.5 ± 2.59* | 74.6 ± 2.78* | 16.3 |
| Chlorogenic acid | 0.00 | 46.7 ± 1.78* | 75.9 ± 0.27* | 79.0 ± 1.23* | 16.5 |
| Puerarin | 0.00 | 1.1 ± 0.14 | 2.7 ± 0.76 | 3.1 ± 0.89 | > 100 |
| 3′-Methoxypuerarin | 0.00 | 14.6 ± 5.54* | 33.5 ± 2.33* | 52.4 ± 2.55* | 46.7 |
| Daidzin | 0.00 | 2.7 ± 0.80 | 6.7 ± 0.41 | 7.8 ± 0.96 | > 100 |
| Rutin | 0.00 | 49.9 ± 2.88* | 72.2 ± 0.00* | 73.2 ± 0.27* | 12.3 |
| Hyperoside | 0.00 | 43.2 ± 2.33* | 69.9 ± 4.06* | 76.7 ± 0.14* | 18.5 |
| Taxifolin | 0.00 | 48.2 ± 2.76* | 73.5 ± 2.30* | 78.3 ± 0.25* | 14.1 |
| Narirutin | 0.00 | 3.4 ± 1.67 | 5.0 ± 0.52 | 8.2 ± 1.43 | > 100 |
| Hesperidin | 0.00 | 2.1 ± 0.95 | 9.3 ± 0.58 | 18.6 ± 1.38 | > 100 |
| Quercetin | 0.00 | 48.0 ± 2.54* | 75.4 ± 3.14* | 76.1 ± 3.48* | 15.4 |
| Apigenin | 0.00 | 1.2 ± 1.51 | 4.0 ± 1.15 | 4.4 ± 0.63 | > 100 |

DPPH radical scavenging activity assay at various concentrations. $IC_{50}$ represents the concentration that exhibits 50% of antioxidant activity. Data presented the average of three independent experiments and was analyzed using one-way ANOVA with Tukey's post hoc

*P < 0.001 compared with untreated control

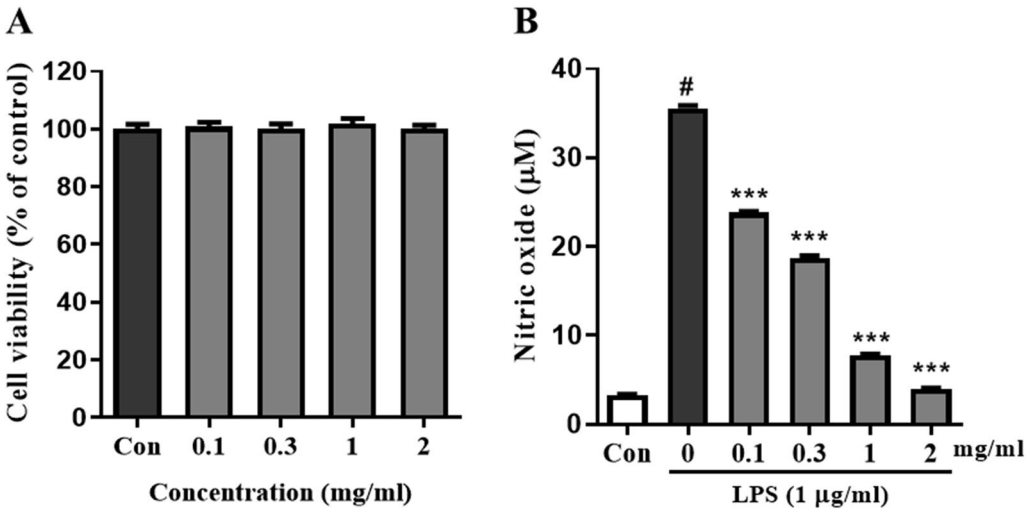


**Fig. 3** Effect of WON-21 on cell viability and nitric oxide (NO) inhibitory activity in RAW 264.7. Cells pretreated with the indicated concentration of WON-21 (**A**). The cells were stimulated with LPS to activate iNOS synthesis and eventually NO release (**B**). Data presented the average of three independent experiments and was analyzed using one-way ANOVA with Tukey's post hoc. $^{\#}p < 0.05$ compared with control; $^{***}p < 0.001$ compared with LPS-stimulated cells

a concentration-dependent manner. Additionally, COX-2 protein expression significantly decreased by 39.9% and 92.0% in a concentration-dependent manner at 1 and 2 mg/mL. NFκB is directly involved in inflammation and immune response, enabling nuclear translocation through phosphorylation and having a mechanism to regulate the subsequent inflammatory response. Furthermore, TNF-α is known as a pro-inflammatory factor and a factor that promotes an inflammatory response through tissue damage. Therefore, it was confirmed whether WON-21 affects the phosphorylation of NFκB and the production of TNF-α induced by LPS. WON-21 tended to inhibit NFκB phosphorylation and TNF-α production (Fig. 4A, C). In the case of NFκB phosphorylation, the protein expression level showed a tendency a decrease overall as the concentration increased and showed a significant decrease to 52.0% and 83.7% at 1 mg/mL and 2 mg/mL (Fig. 4A, B). Furthermore, the production of TNF-α also tended to decrease as the concentration increased and significantly decreased to 67.1% and 92.7% at 1 mg/mL and 2 mg/mL (Fig. 4C).

### Effect of compounds from WON-21 on NO production in LPS-stimulated RAW 246.7 cells

To evaluate the effect of components from WON-21 on NO production in RAW 246.7 cells, non-cytotoxic concentrations were determined using cell viability assays. The cytotoxicity criterion was determined as 95% or less viability based on the 0.5% DMSO control. Most of the tested concentrations of components exerted no cytotoxicity in RAW 264.7 cells (Fig. 5A–J). However, quercetin and apigenin showed toxicity at a high concentration of 100 μM (Fig. 5K, L), and the experimental conditions were followed except for toxic concentrations. Most components did not inhibit LPS-induced NO production in RAW 264.7 cells (Fig. 6A–G). Taxifolin (22.2%), narirutin (18.9%), and hesperidin (13.4%) slightly inhibited NO production at a concentration of 100 μM NO production (Fig. 6H–J). However, among the compounds, quercetin and apigenin showed a tendency to decrease NO produced by LPS stimulation in a concentration-dependent manner (Fig. 6K, L). Quercetin was significantly decreased by 19.9%, 49.4%, and 67.8% at 2, 10, and 30 μM concentrations. In addition, apigenin also significantly decreased by 47.9% and 85.6% at 10 and 30 μM concentrations.

### Effect of compounds from WOM-21 on iNOS, COX-2 in LPS-stimulated RAW 246.7 cells

We examined the effects of compounds from WON-21 on LPS-induced iNOS and COX-2 expressions stimulated by LPS. The iNOS protein expression level showed a concentration-dependent tendency to decrease by quercetin treatment and was significantly decreased to 50.3% at a high concentration of 30 μM. In addition, apigenin significantly decreased to 51.7% and 71.3% iNOS expression in a concentration-dependent manner at both 10 and 30 μM. COX-2 protein expression showed no change by quercetin treatment, whereas showed a concentration-dependent decrease by apigenin, and a significant decrease to 59.6% at a high concentration of 30 μM (Fig. 7).

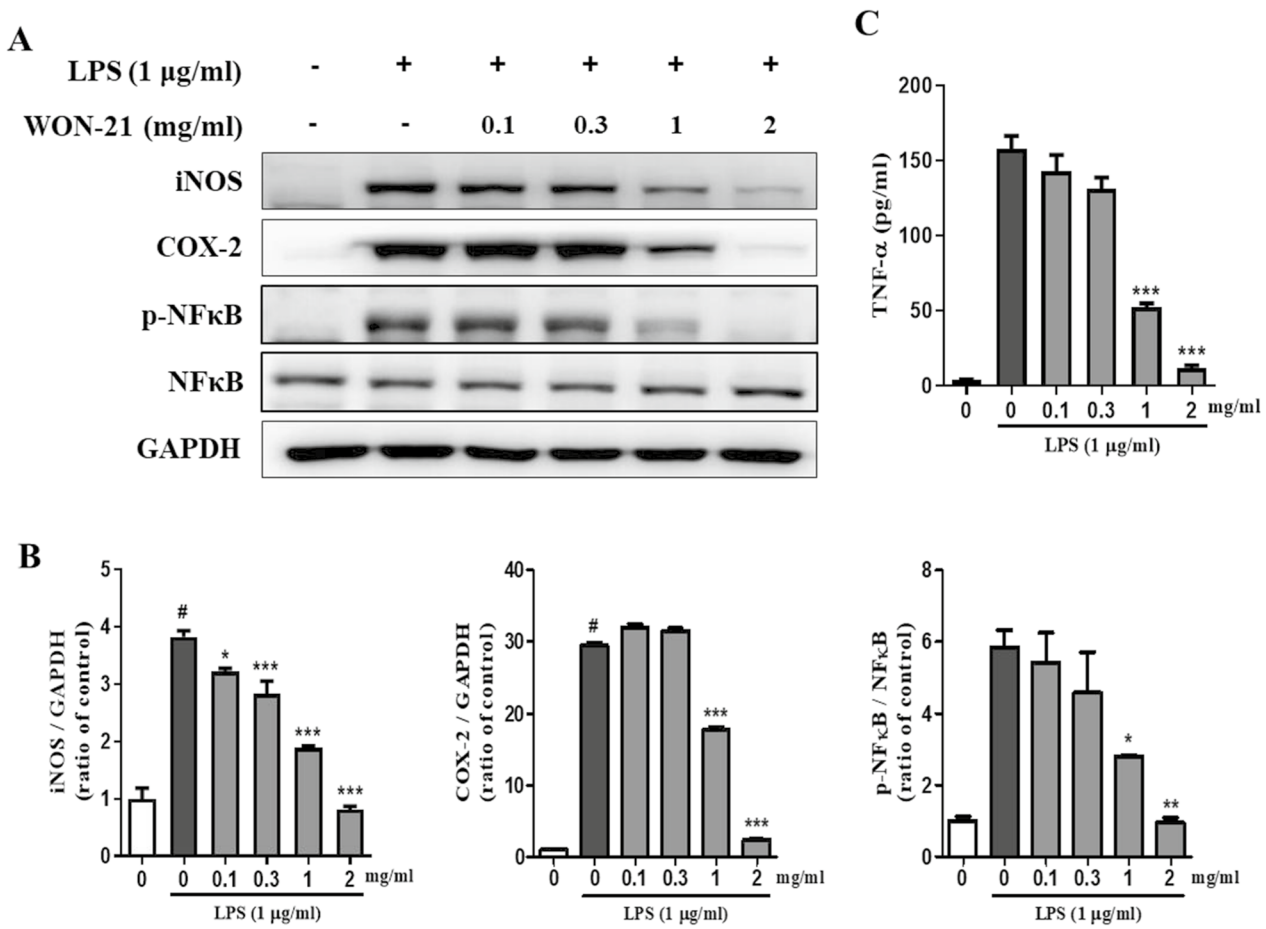


**Fig. 4** Effect of WON-21 on the expression of iNOS, COX-2, NFκB, and TNF-α levels in RAW 264.7. Representative western blot results and bar graphs show the expression and densitometry ratios of iNOS and COX-2 to GAPDH, and p- NFκB/ NFκB, respectively (**A**, **B**). ELISA measurement of TNF-α levels (**C**). Data presented the average of three independent ex-periments and was analyzed using one-way ANOVA with Tukey's post hoc. $^{\#}p < 0.05$ compared with control; $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ compared with LPS-stimulated cells. *iNOS* inducible nitric oxide synthase, *COX-2* cyclooxygenase-2, *NFκB* nuclear factor kappa-light-chain-enhancer of activated B cells, *TNF−α* tumor necrosis factor-α

### ALDH and ADH activity effect of WON-21 and Quercetin

Alcohol consumed in the body is metabolized to acetaldehyde by ADH, which is further metabolized through the action of the ALDH enzyme. Therefore, it is necessary to examine ALDH activity as well as ADH activity for the identification of hangover relief. As a result, the effects of WON-21 and quercetin on ADH and ALDH were confirmed. In the case of WON-21 extract (0, 2.5, 5, 10, 25, and 50 mg/mL), it was shown that the activities of ALDH and ADH enzymes were significantly increased in a concentration-dependent manner. In addition, quercetin (0, 0.06, 0.13, 0.25, 0.5, and 1 mM), one of the components of WON-21, also significantly increased the activity of ALDH and ADH enzymes in a concentration-dependent manner (Fig. 8).

## Discussion

Hangovers can result from excessive alcohol consumption, often leading to unpleasant symptoms such as headaches, fatigue, nausea, and dehydration [20]. Although many remedies are available for hangovers, natural products can be particularly effective in reducing the symptoms. Ginger reduces nausea and vomiting, which are common symptoms associated with hangovers [21]. It can be consumed as a tea, a supplement, or food. Turmeric contains curcumin, which exhibits anti-inflammatory properties. It can help reduce inflammation, which otherwise contributes to symptoms such as headaches [22]. Milk thistle has hepatoprotective effects [23]. As alcohol consumption can damage the liver, consuming milk thistle before or after drinking can help reduce

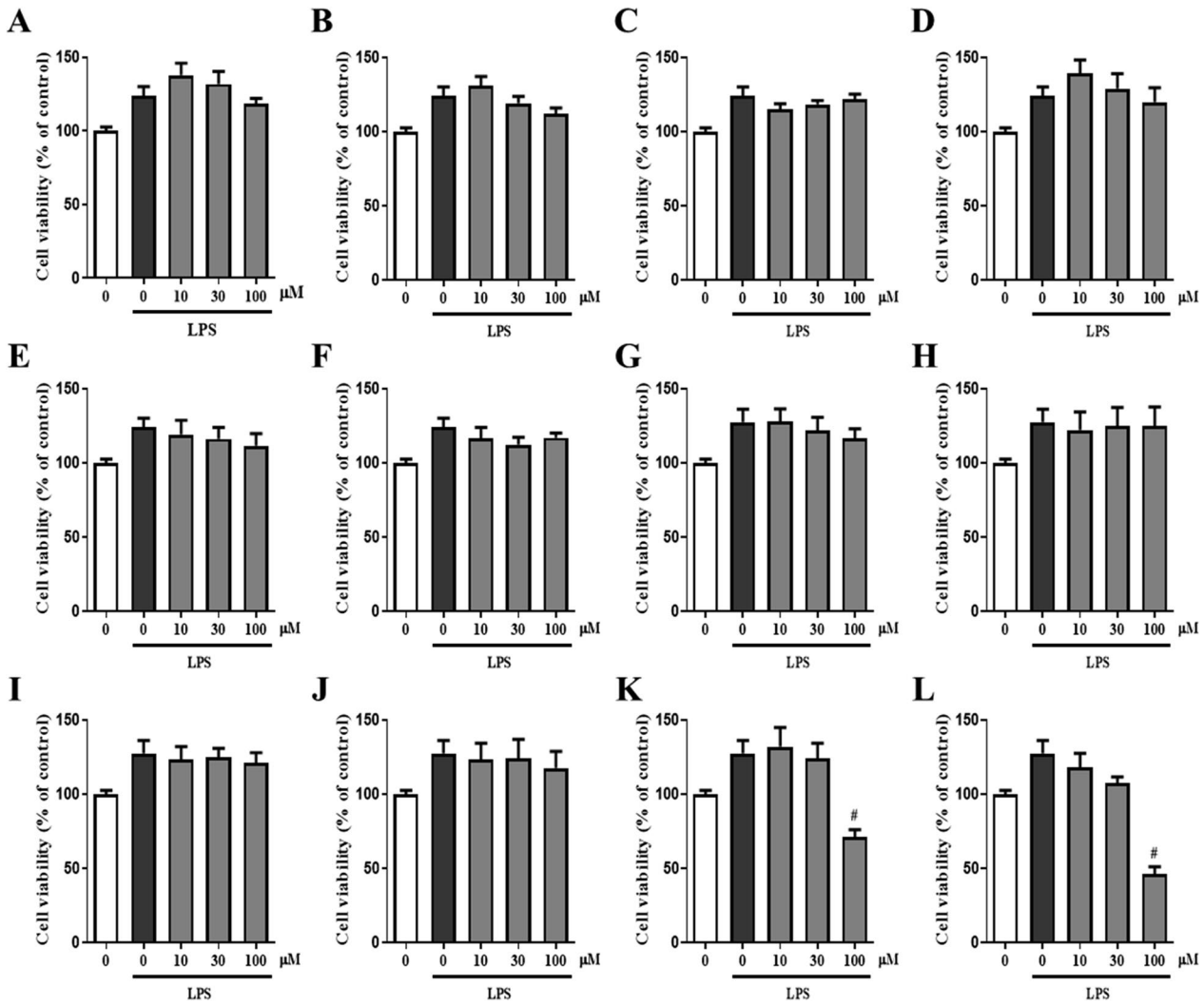


**Fig. 5** Comparison in cell viability of 12 components from WON-21 in Raw 264.7. Cells were pretreated with the indicated concentration of 12 components (**A**~**L**). the cells were stimulated with LPS to activate iNOS synthesis, and eventually NO release. Data presented the average of three independent experiments, and was analyzed using one-way ANOVA with Tukey's post hoc. $^{\#}p < 0.05$ compared with control. **A** 3′-hydroxypuerarin; **B** chlorogenic acid; **C** puerarin; **D** 3′-methoxypuerarin; **E** daidzin; **F** rutin; **G** hyperoside; **H** taxifolin; **I** narirutin; **J** hesperidin; **K** quercetin; **L** apigenin

symptom severity. Honey contains fructose, which helps metabolize alcohol more rapidly [24]. It can also help alleviate headaches and fatigue caused by hangovers. Prickly pear is a type of cactus shown to reduce inflammation and prevent dehydration [25, 26]. It can be consumed as a supplement or juice. The mechanisms of action of substances that reduce hangover symptoms diverse and are primarily aimed at addressing the underlying causes of hangovers, such as dehydration, inflammation, and oxidative stress. WON-21 used in our study was reported to be effective in relieving hangovers by mixing Galhwahajungtang, Daegeumeumja, and herbal medicines [15–17]. In this study, the efficacy of antioxidant, anti-inflammatory, ADH and ALDH enzymatic activity of WON-21, a herbal medicine developed by using the formulation based on the concept of oriental medicine, was investigated.

Hangovers are induced by various factors, such as dehydration, inflammation, and oxidative stress [1, 6]. Alcohol metabolism induces reactive oxygen species (ROS) production, which damages the cells and tissues [27]. In this study, WON-21 showed significant antioxidant activity in the DPPH assay, and most of the components in this extract showed high antioxidant efficacy (Tables 1 and 2). The antioxidant efficacy of each component showed higher activity than that of the WON-21 extract, and the reason for the lower effect in the extract is thought to be due to the difference in the

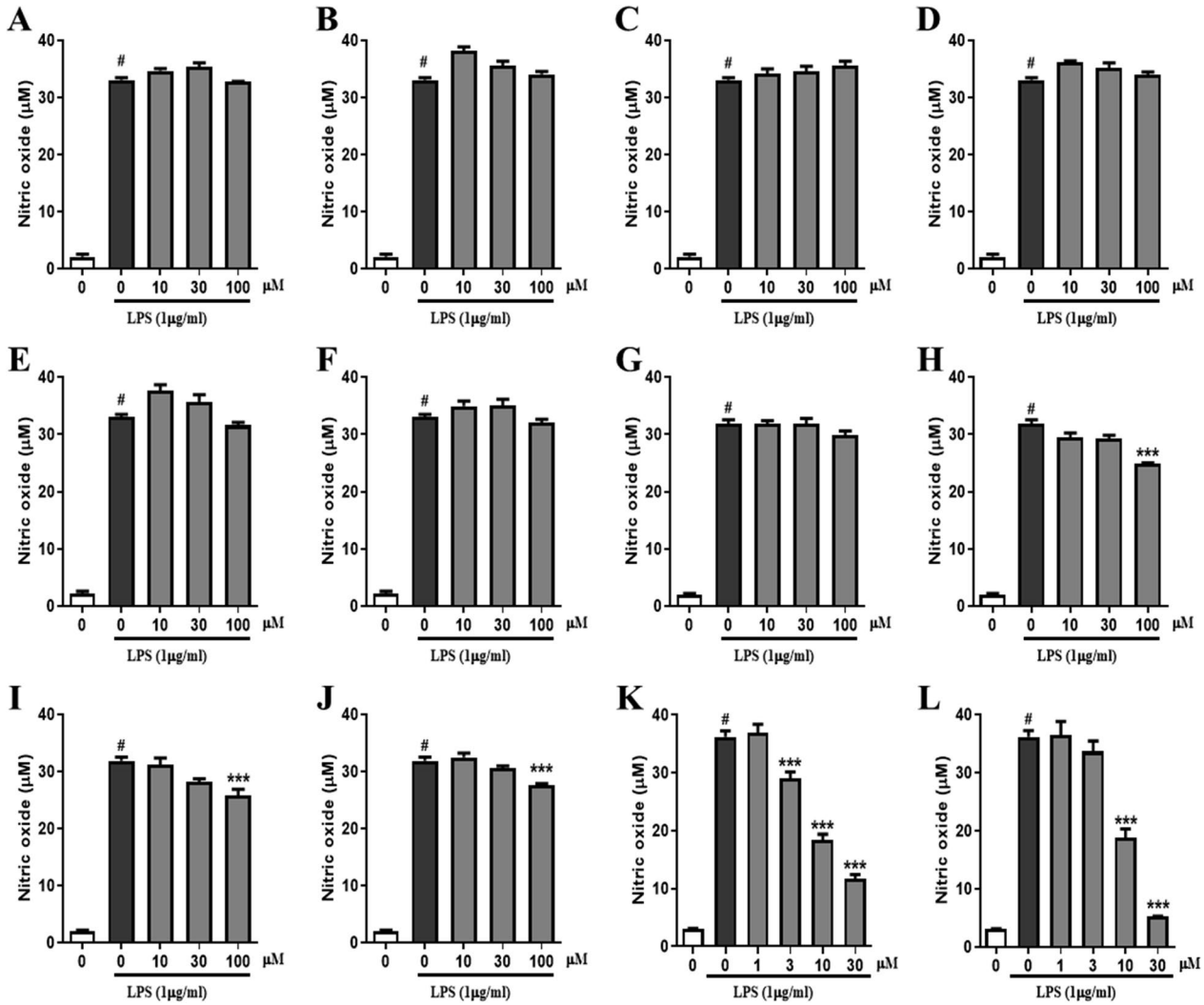


**Fig. 6** Comparison in NO inhibitory activity of 12 components from WON-21 in Raw 264.7. Cells were pretreated with the indicated concentration of 12 components (**A**~**L**). the cells were stimulated with LPS to activate iNOS synthesis, and eventually NO release. Data presented the average of three independent experiments, and was analyzed using one-way ANOVA with Tukey's post hoc. $^{\#}p < 0.05$ compared with control; $^{***}p < 0.001$ compared with LPS-stimulated cells. **A** 3′-hydroxypuerarin; **B** chlorogenic acid; **C** puerarin; **D** 3′-methoxypuerarin; **E** daidzin; **F** rutin; **G** hyperoside; **H** taxifolin; **I** narirutin; **J** hesperidin; **K** quercetin; **L** apigenin

content of the effective component. Nitric oxide (NO) is a molecule that plays a significant role in vasodilation and blood pressure regulation [28]. Alcohol consumption increases the level of NO in the body and is associated with hangovers, which cause symptoms such as headaches [29, 30]. COX-2 is an enzyme that produces inflammatory compounds called prostaglandins [31]. Alcohol consumption induces the production of COX-2 and increases inflammation, contributing to symptoms of muscle pain and headache [32, 33]. WON-21 significantly reduced NO production and COX-2 protein expression in LPS-stimulated RAW246.7 cells (Figs. 3, 4). Hangovers are symptoms that occur after alcohol consumption and include headache, dizziness, nausea, vomiting, and indigestion [34]. These symptoms are temporarily caused by incomplete alcohol metabolism, and alcohol intake triggers an inflammatory response to injury or tissue damage [35]. Alcohol intake increases the production of the pro-inflammatory cytokines IL-6 and TNF-α [5]; notably, TNF-α is related to the nausea symptoms of hangovers. TNF- α has been reported to regulate some physiological functions, such as appetite [36]. TNF- α acts as a trigger that stimulates nausea and vomiting by stimulating the vomiting center [37, 38]. WON-21 significantly decreased the activity of p-NFκB at concentrations of 1 and 2 mg/mL and reduced the

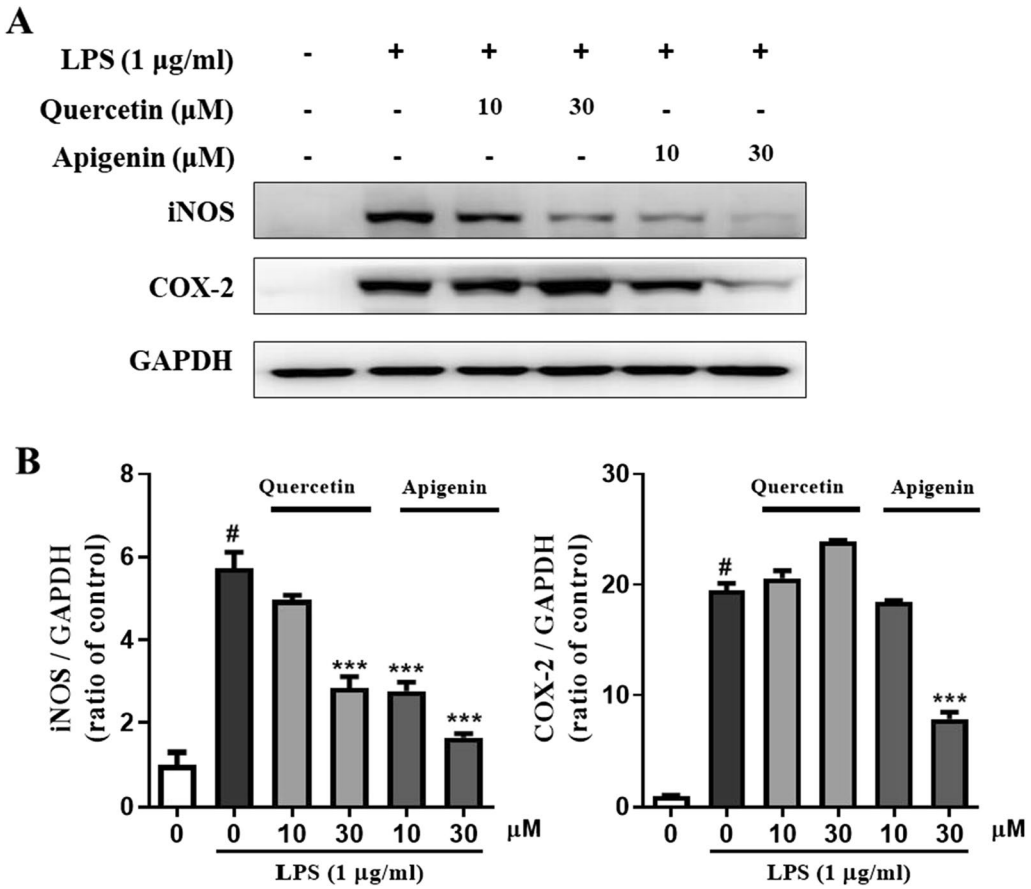


**Fig. 7** Effect of quercetin and apigenin on expression of iNOS and COX-2 in Raw 264.7 macrophages. Representative western blot (**A**) and bar graphs (**B**) show the expression and densitometry ratios of iNOS and COX-2 to GAPDH in control, respectively. Data presented the average of three independent experiments, and was analyzed using one-way ANOVA with Tukey's post hoc. $^{\#}p < 0.05$ compared with control; $^{*}p < 0.05$, $^{***}p < 0.001$ compared with LPS-stimulated cells. *iNOS* inducible nitric oxide synthase, *COX-2* cyclooxygenase-2

release of TNF-a (Fig. 4). In the comparison of inhibitory effects of the compounds of WON-21 on NO production, quercetin, and apigenin significantly decreased NO produced by LPS stimulation in a concentration-dependent manner. The increased iNOS protein expression by LPS was significantly decreased by quercetin and apigenin. COX-2 protein expression did not change by quercetin, but decreased significantly by apigenin. These results suggest that quercetin and apigenin from WON-21 are active compounds to inhibit anti-inflammatory factors such as cytokines as well as NO production. According to a recent study, the severity of hangovers was not related to acetaldehyde; however, the concentration and rapid elimination of ethanol in the blood were directly related to the improvement of hangovers [30, 35]. Alcohol ingested into the body is metabolized by ADH into acetaldehyde, which is further metabolized through the action of ALDH enzyme. Therefore, in order to identify relieving effect on hangover, it is necessary to test not only ADH activity but also ALDH activity. In this study, the effects of WON-21 and quercetin on the activities of ADH and ALDH were investigated. As shown in Fig. 8A, B, treatment with quercetin and WON-21 at various concentrations significantly increased the activities of ADH and ALDH.

Our experimental results suggest that WON-21 promotes the activities of ADH and ALDH enzymes that promote alcohol degradation and acetaldehyde degradation and has beneficial effects on improving hangover-induced headaches and some discomfort symptoms. In addition, the reason why such a result is effective at a rather high concentration is not only the extract by a specific combination of various herbal medicines rather than a single herbal medicine but also the low concentration of active ingredients because hot water extraction was performed compared to other extracts using organic solvents. Further research is needed on the results of direct alcohol metabolism and degradation using living organisms and the effect on antioxidant enzymes.

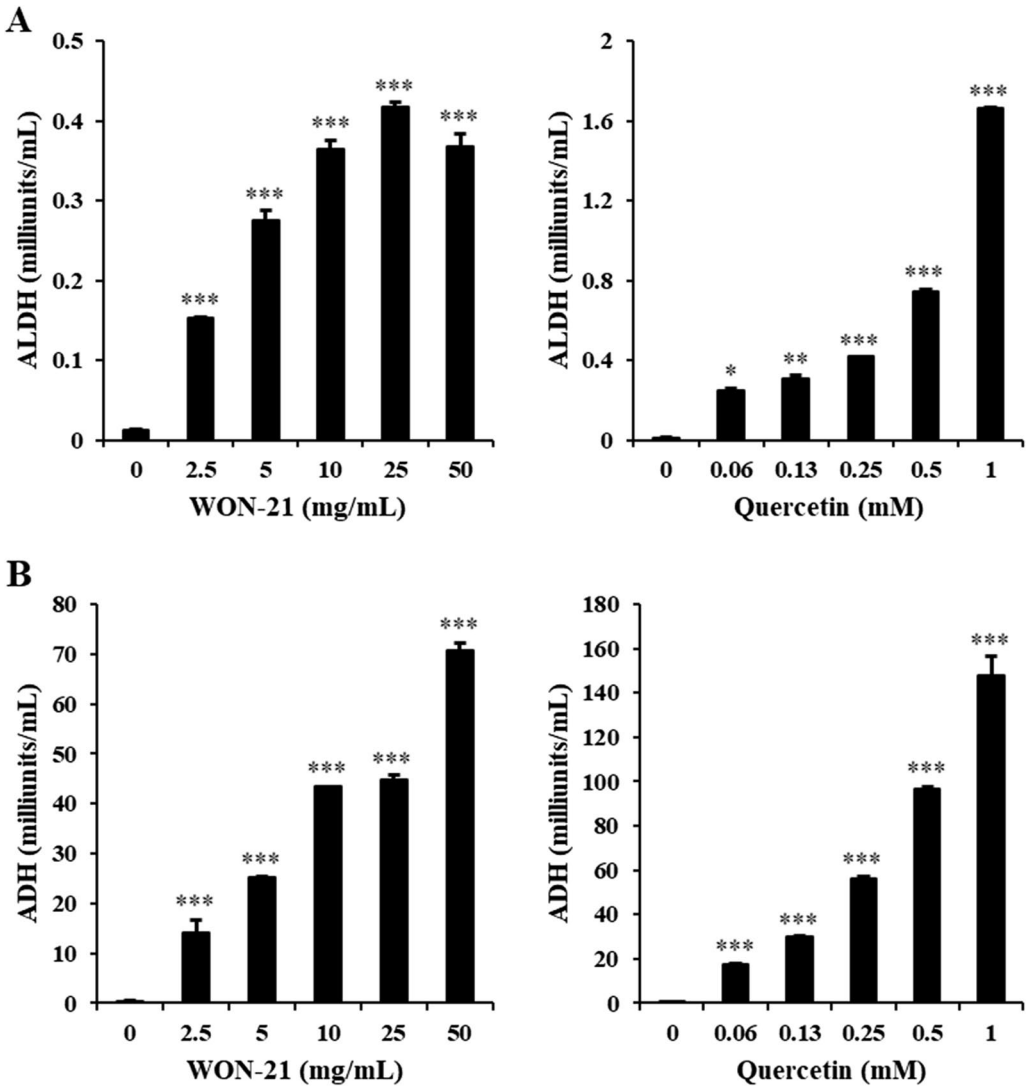


**Fig. 8** Effect of WON-21 on the aldehyde dehydrogenase (ALDH) and alcohol dehydrogenase (ADH) activities. The WON-21 was treated at the indicated concentrations. The activities of ADH and ALDH were measured as described in manufacturer's procedures. Data presented the average of three independent experiments and was analyzed using one-way ANOVA with Tukey's post hoc. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with control

## Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-023-00800-7.

**Additional file 1: Table S1.** Detection wavelength, linear range, regression equation, coefficient of determination ($r^2$), limit of detection (LOD), and limit of quantitation (LOQ)of 12 compounds. **Table S2.** Concentration of 12 compounds in Wonsanghwan water extract ($n = 3$). **Table S3.** Concentration of 12 compounds in WON-21 ($n = 3$).

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Not applicable.

**Author contributions**
Conceptualization: KSK and WH; methodology: JHL, JYB, JYP, and SK; formal analysis: JHL, SK, JP, I-HP and CSS; writing original draft preparation: JHL and WSH; writing review and editing: KSK and CSS; project administration: KSK; funding ac-quisition: KSK. All authors have read and agreed to the published version of the manuscript.

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**Availability of data and materials**
All data generated or analysed during this study are included in this published article and its Additional files.

## Declarations

**Competing interests**
The authors declare that they have no competing interests.

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