

Antioxidants (Article)

Comparison of Efficacies of Different Jakyakgamcho-tang Extracts on H₂O₂-Induced C2C12 Cell Viability

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Abstract: Oxidative stress is a major contributor to muscle aging and loss of muscle tissue. Jakyakgamcho-tang has been used in traditional Eastern medicine to treat muscle pain. Here, we compared various solvent-based Jakyakgamcho-tang extracts in terms of their effects against hydrogen peroxide-induced oxidative stress in murine C2C12 skeletal muscle cells. Total phenolic content and total flavonoid content in 30% ethanol extracts of Jakyakgamcho-tang were higher than those of water extracts of Jakyakgamcho-tang. Ethanol extracts of Jakyakgamcho-tang had stronger antioxidant and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid and 2,2'-diphenyl-1-picrylhydrazyl-scavenging activity than water extracts of Jakyakgamcho-tang. The ethanol extract of Jakyakgamcho-tang inhibited peroxide-induced cell viability and intracellular reactive oxygen species generation more effectively than the water extract of Jakyakgamcho-tang in a dose-dependent manner. These results suggest that the ethanol extract of Jakyakgamcho-tang is relatively more efficacious at protecting against oxidative stress-induced muscle cell death because it prevents reactive oxygen species generation in C2C12 cells. Moreover, the current study indicated that the effective dose of the ethanol extract of Jakyakgamcho-tang required to alleviate muscle pain might be lower than that required for Jakyakgamcho-tang.

Keywords: antioxidant; C2C12 cell; Jakyakgamcho-tang; muscle atrophy; oxidative stress

1. Introduction

Sarcopenia or aging-associated muscle loss affects 10% of all adults aged over 50 years. Muscle aging is characterized by a decline in function and a loss of tissue. Oxidative stress and chronic inflammation are the main mechanisms of skeletal muscle senescence and are associated with increased protein breakdown, decreased protein synthesis, mitochondrial dysfunction, and apoptosis [1, 2]. Oxidative stress induces muscle aging. Reactive oxygen species (ROS) are naturally and constantly formed during normal cellular activity [3]. Hydroxyl radical ($\cdot\text{OH}$), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2) cause oxidative damage to cells.

Herbs are sources of important remedies and supplements used in the traditional medicine of Eastern Asia. Nearly 65% of the global population uses herbs as part of their primary health care modality [4]. About 25% of all modern medicines such as aspirin and ephedrine were derived from plants [5]. Natural antioxidants occur in herbal medicines and foods. Several studies have assessed the impacts of supplement and nutrients on muscle strength, mass, and physical performance [6]. Dietary antioxidants such as vitamins C and E inhibited oxidation, induced antioxidant enzymes, and improved positive work function in

the chronically loaded muscles of aged rats [7]. Vitamin C regenerates vitamin E in cell membranes and the latter inhibits free radical production [6]. The free radical-scavenging abilities of natural products have been evaluated via in vitro assays of their scavenging activities against ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), superoxide, and hydroxyl and nitric oxide radicals as well as their influences on lipid peroxidation levels and antioxidant enzyme activation [8].

Jakyakgamcho-tang (JGT, Shaoyao-gancao-tang in Chinese; Shakuyaku-kanzo-to in Japanese) is a herbal medicine widely prescribed in traditional Eastern medicine that is used to alleviate muscle pain. JGT consists of *Paeonia lactiflora* Pallas radix (root) and *Glycyrrhiza uralensis* Fisch radix and rhizome. In a clinical trial, JGT reduced muscle pain and improved muscle damage after exercise [9]. JGT and its major component *Paeonia lactiflora* have potent anti-glycation properties [10]. Glycation is a spontaneous nonenzymatic reaction of free reducing sugars and amino groups with protein and lipids in the blood and tissues. Glycation is highly accelerated under oxidative stress and is implicated in the pathogenesis of diabetes and age-related diseases.

Traditional Chinese and Oriental herbal medicines comprise water extracts. Recently, ethanol or a mixture of ethanol and water has been used as an extraction solvent for pharmaceuticals and dietary supplements. The Korea Food & Drug Administration either exempts or requires minimum toxicity test data for the approval of Oriental herbal medicines when the ethanol content in the extraction solvent is $\leq 30\%$ and the balance is water.

Here, we analyzed and evaluated the antioxidant properties of water extracts and 30% ethanol extracts of JGT. The JGT solvent extract with the highest antioxidant efficacy was then selected and its ability to prevent H₂O₂-induced cell death in murine C2C12 skeletal muscle cells was assessed.

2. Materials and Methods

2.1. Preparation of 30% ethanol and water extracts of Jakyakgamcho-tang (JGT-E and JGT-W, respectively)

Paeonia lactiflora and *Glycyrrhiza uralensis* roots were purchased from an oriental herbal market (Omniherb, GyeongsangBuk-Do, Republic of Korea) (<http://www.omniherb.com/>) that only handles herbs certified by the Korean Pharmacopoeia. The Jakyakgamcho-tang was prepared using a 2:1 (w/w) ratio of *P. lactiflora* root to *G. uralensis* root. The JGT extracts were prepared by accurately weighing out 15 g of *P. lactiflora* root and 7.5 g of *G. uralensis* root and by mixing them according to the Oriental Medicine Advanced Searching Integrated System (<http://oasis.kiom.re.kr>) of the Korea Institute of Oriental Medicine. Distilled water or 30% (v/v) ethanol was added to the roots and the mixture was extracted for 1 h in a reflux extractor (MS-DM607; M-TOPS, Seoul, Korea). The extracts were then filtered, evaporated under reduced pressure in a rotary evaporator (N-1200A; Eyela, Tokyo, Japan) at 50°C, and freeze-dried (FDU-2100; Eyela, Tokyo, Japan) at -80°C for 72 h to obtain a 30% (v/v) ethanol extract (JGT-E; yield 39.6%) or water extract (JGT-W; yield 45.8%). For ultra-performance liquid chromatography (UPLC), 20 mg of each extract was dissolved in 2 mL dimethyl sulfoxide (DMSO) and diluted tenfold with distilled water. The solutions were passed through a 0.45- μ m syringe filter (Whatman; Clifton, NJ, USA) before injection.

2.2. UPLC-quadrupole time-of-flight mass spectrometry (UPLC-Q-ToF/MS) analysis

UPLC was performed in a Waters Acuity UPLC System (Waters Corp., Milford, MA, USA) coupled to a Q-ToF Premier ESI/Mass Spectrometer (Q-ToF Premier™, Waters Corp., Milford, MA, USA). Three-microliter aliquots per sample were injected into a BEH C18 column (100 mm x 2.1 mm; i.d. 1.7 μ m) at a flow rate of 0.4 mL min⁻¹ and eluted with a

chromatographic gradient comprising the mobile phases A (water containing 0.1% (v/v) formic acid) and B (acetonitrile containing 0.1% (v/v) formic acid). The linear gradient was optimized as follows: 0 min, 10% B; 0–9 min, 10%–20% B; 9–11 min, 20%–30% B; 11–12 min, 30%–90% B; 12–13 min, 90%–100% B; 13–14 min, 100% B; and 14–15 min, 10% B. The Q-ToF was operated in negative ion mode under the following conditions: capillary voltage, 2.3 kV; cone voltage, 50 V; source temperature, 110°C; and desolvation temperature, 350°C. A sprayer with a leucine-enkephalin reference solution ($[M-H]^-$ m/z 554.2615) served as the lock mass. Full scan data and MS/MS spectra were collected with MassLynx 4.1 software (Waters Corp., Milford, MA, USA).

2.3. Determination of total phenolic content (TPC) and total flavonoid content (TFC) in JGT-W and JGT-E

The total phenolic content (TPC) in various extracts of JGT and its constituent herbs were determined by a modified Folin-Ciocalteu method [11]. Briefly, 0.125 mL of each extract was transferred to a test tube containing 1.8 mL Folin-Ciocalteu reagent. After 5 min, 1.2 mL of 15% (w/v) Na_2CO_3 was added and the mixture was kept in the dark at 25°C for 2 h. Absorbance was measured at 765 nm in a Multi-Mode Microplate Reader SpectraMax M2 (Molecular Devices, Sunnyvale, CA, USA). A linear calibration curve ($R^2 = 0.998$) was plotted using gallic acid and the results were expressed in mg gallic acid equivalents (GAE) per g extract sample.

Total flavonoid content (TFC) was determined by an aluminum chloride colorimetric assay [11]. Quantification was expressed by reporting the absorbance in the quercetin calibration graph used as the flavonoid standard ($R^2 = 0.999$). A 1-mL sample of prepared extract or standard was mixed with 4 mL distilled water and 0.3 mL of 5% (w/v) NaNO_2 . After 5 min and 1 min, respectively, 0.3 mL of 10% (w/v) AlCl_3 and 2 mL of 1 M NaOH were added. Then 2.4 mL distilled water was added and the mixture was shaken. The resultant pink color was read in a Multi-Mode Microplate Reader Spectra Max M2 at 510 nm and the results were expressed in mg quercetin equivalents (QE) per g extract sample.

2.4. Scavenging of 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

ABTS-scavenging activity was assessed according to a modified version of the method described by Re et al. [12]. ABTS (7.0 mM) and potassium persulfate (2.45 mM) were mixed in water and stored at room temperature for 12 h in the dark to produce $\text{ABTS}^{\cdot+}$. The aqueous $\text{ABTS}^{\cdot+}$ solution was then diluted to an absorbance of 1.50 at 405 nm. Various concentrations of each extract solution (1 mL) were added to 2 mL diluted $\text{ABTS}^{\cdot+}$ solution and the absorbance were measured at 405 nm. 2.5. 2,2'-diphenyl-1-picrylhydrazyl (DPPH) free radical assay

Water and 30% (v/v) ethanol extracts of the *P. lactiflora* and *G. uralensis* roots ($50 \mu\text{g mL}^{-1}$, $100 \mu\text{g mL}^{-1}$, $250 \mu\text{g mL}^{-1}$, $500 \mu\text{g mL}^{-1}$, and $1,000 \mu\text{g mL}^{-1}$) were prepared for the DPPH assay. The DPPH-scavenging activity of the extracts was measured according to a previously reported method [8]. One-milliliter aliquots of the various concentrations of the extract solutions were added to two milliliters of a DPPH-methanol solution ($5 \text{ mg } 100 \text{ mL}^{-1}$). Decrease in absorbance at 517 nm was measured with a Multi-Mode Microplate Reader Spectra Max M2. The DPPH-scavenging activity (%) was calculated according to the following equation:

$$\text{DPPH scavenging activity (\%)} = \left[1 - \frac{A_{\text{sample}}}{A_0} \right] \times 100 \quad (2)$$

where A_{sample} is the steady-state absorbance of the sample solution and A_0 is the absorbance of the DPPH solution before addition of the extract.

2.6. Cell culture

The immortalized mouse myoblast cell line C2C12 was purchased from the American Type Culture Collection (ATCC CRL-1772; Manassas, VA, USA) and cultured in the Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (w/v) penicillin-streptomycin (P/S) at 37°C in a humidified 5% CO₂ incubator as per previously described methods [13]. To induce myotube formation, the media were replaced with differentiation media (DMEM with 2% (v/v) FBS and 1% (w/v) P/S) and cultured for an additional 7 d.

2.5. Cell viability assay in H₂O₂-induced C2C12 cells

C2C12 viability was determined by a cell counting kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Kumamoto, Japan). C2C12 cells (0.5×10^4 cells/well) were seeded in each well of a 96-well plate containing DMEM plus 10% (v/v) FBS and incubated for 24 h. After attachment, the cells were pretreated with JGT extract in serum-free medium for 24 h and then with 250 μ M H₂O₂ for 3 h. After incubation, 10 μ L CCK-8 was added to each well and the plate was incubated at 37°C and 5% CO₂ in a humidified incubator for 2 h. Absorbance was measured at 450 nm in a Multi-Detection Microplate Reader (Synergy HT; BioTek, Winooski, VT, USA).

2.6. Intracellular ROS measurements in H₂O₂-induced C2C12 cells

C2C12 cells were pretreated with JGT-E and JGT-W for 24 h and then subjected to 250 μ M H₂O₂ for 30 min. *N*-acetylcysteine (NAC, 1 mM) served as a positive control. Intracellular ROS levels were measured by the DCF-DA method, wherein the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA, Molecular Probes Inc., Eugene, OR, USA), is converted by intracellular esterases to 2',7'-dichlorodihydrofluorescein which, in turn, is oxidized by intracellular ROS to highly fluorescent 2',7'-dichlorofluorescein (DCF). The treated cells were then washed with the Hank's Balanced Salt Solution (HBSS) buffer and incubated in the dark for 30 min in HBSS buffer containing 50 μ M H₂DCF-DA. DCF fluorescence was measured in a Synergy HT spectrofluorometer (BioTek, Winooski, VT, USA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. All experiments were repeated at least thrice.

2.7. Statistical analysis

Data are means \pm SD or mean \pm SE. ANOVA with the Tukey's test was used for multiple treatment means comparisons in Prism v. 7.0 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Qualitative and quantitative analyses of JGT components by UPLC-PDA-ESI-QTOF-MS

As shown in Figs. 1 and 2, the major active compounds in JGT-E and JGT-W were albiflorin (1), paeoniflorin (2), liquiritin (3), pentagalloylglucose (4), isoliquiritin apioside (5), isoliquiritin (6), liquiritigenin (7), and glycyrrhizin (8). JGT-E contained 19%–53% more of the aforementioned constituents than those in JGT-W except for albiflorin (1) (Fig. 3).

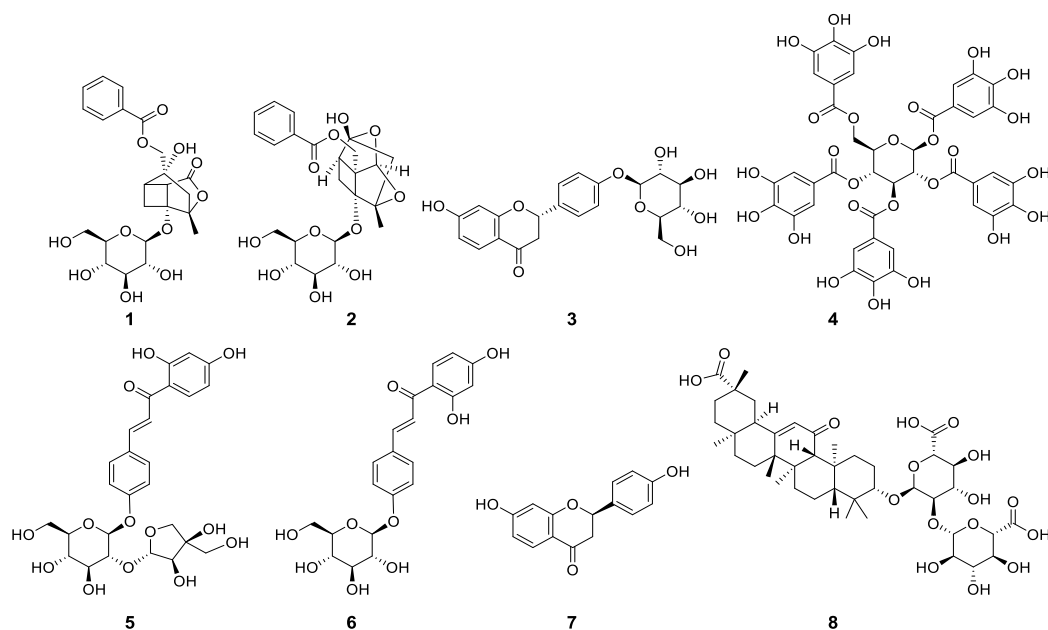


Fig. 1. Structures of albiflorin (1), paeoniflorin (2), liquiritin (3), pentagalloylglucose (4), isoliquiritin apioside (5), isoliquiritin (6), liquiritigenin (7), and glycyrrhizin (8).

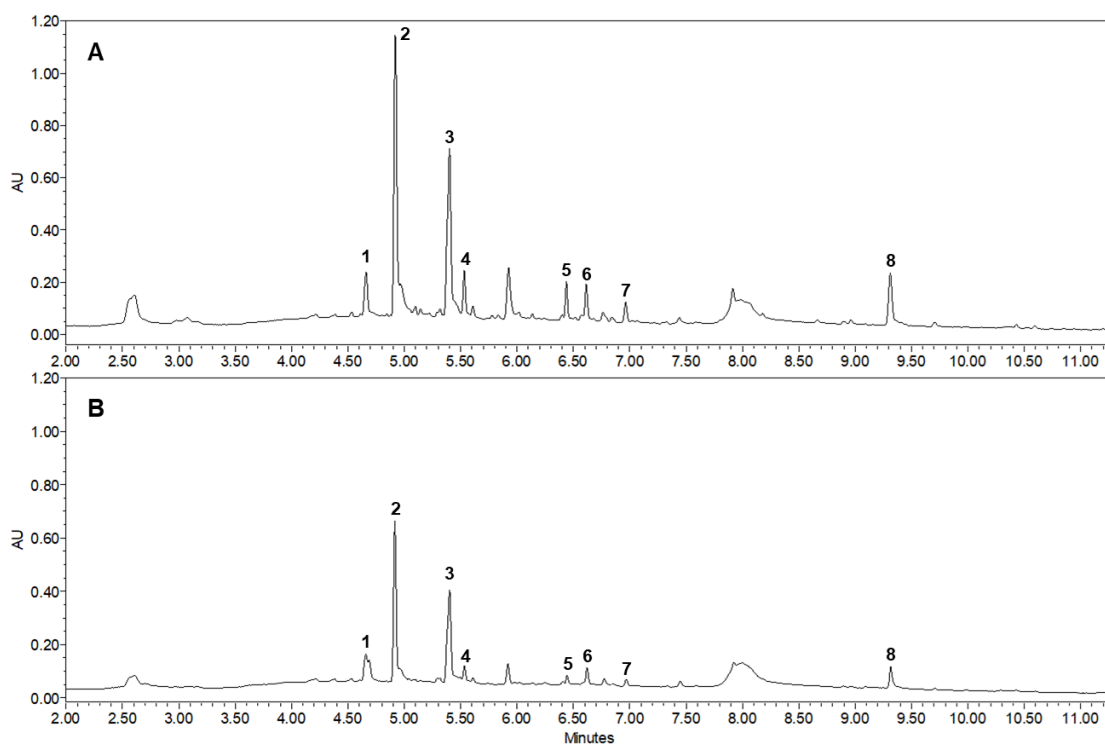


Fig. 2. UPLC-PDA profiles for JGT-E and JGT-W. (A) JGT-E, 30% (w/v) EtOH extract of Jakyakgamcho-tang, (B) JGT-W, water extract of Jakyakgamcho-tang. Albiflorin (1), paeoniflorin (2), liquiritin (3), pentagalloylglucose (4), isoliquiritin apioside (5), isoliquiritin (6), liquiritigenin (7), and glycyrrhizin (8).

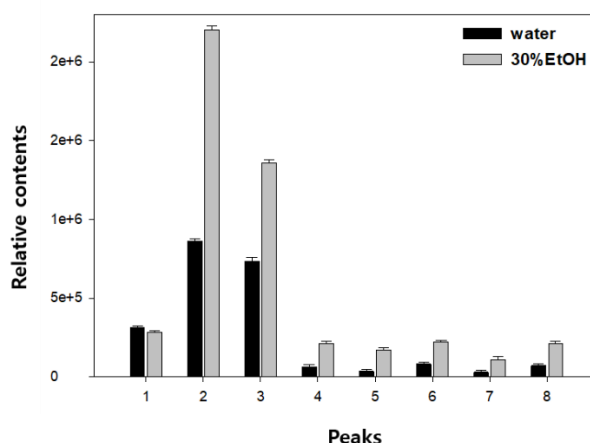


Fig. 3. Comparative changes in individual peak marker levels for two extraction solvents. Calculations were based on area of UPLC analysis. Mean \pm SD of measurements determined for three independent samples analyzed thrice.

3.2. Effects of JGT-E and JGT-W on ABTS- and DPPH-scavenging activities

Relative total phenolic content (TPC) and total flavonoid content (TFC) are shown in Table 1. The TPC and TFC content in the 30% (w/v) ethanol extracts of *P. lactiflora* and *G. uralensis* roots were higher than those in the water extracts. TPC and TFC in JGT-E were also higher than those in JGT-W.

Table 1. Total phenolic content (TPC) and total flavonoid content (TFC) in Jakyakgamcho-tang (JGT) and its constituents

Sample	TPC (mg GAE g ⁻¹)	TFC (mg QE g ⁻¹)
Water extract of <i>P. lactiflora</i> root	17.17 \pm 0.6	3.25 \pm 0.3
30% (w/v) ethanol extract of <i>P. lactiflora</i> root	29.70 \pm 1.0	8.11 \pm 0.1
Water extract of <i>G. uralensis</i> root	18.37 \pm 0.1	3.72 \pm 0.1
30% (w/v) ethanol extract of <i>G. uralensis</i> root	23.10 \pm 1.6	7.02 \pm 0.2
JGT-W	25.49 \pm 0.8	3.94 \pm 0.5
JGT-E	34.01 \pm 2.6	7.32 \pm 0.6

Data indicate mean \pm SD. All extracts were analyzed in triplicate.

Table 2 shows that the antioxidant properties of the water and 30% (w/v) EtOH extracts were evaluated by *in vitro* ABTS⁺-scavenging and DPPH-scavenging activity assays. The antioxidant activity of JGT-E was higher than that of JGT-W. The ABTS⁺- and DPPH-scavenging capacities JGT-E are commensurate with the phenolic compound content.

Table 2. Relative ABTS and DPPH activities of Jakyakgamcho-tang (JGT)

Samples	ABTS		DPPH	
	inhibition (%)	IC ₅₀ (μ g mL ⁻¹)	inhibition (%)	IC ₅₀ (μ g mL ⁻¹)
JGT-W	72.8 \pm 1.2	60.5 \pm 0.9	25.1 \pm 1.3	742.8 \pm 2.1

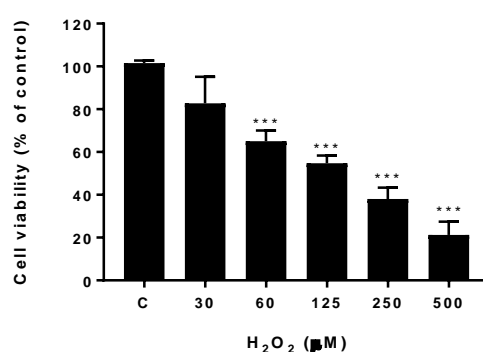
JGT-E	80.8 ± 1.1	34.2 ± 0.4	38.9 ± 1.1	436.1 ± 3.7
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Data indicate mean ± SD. All extracts were analyzed in triplicate.

3.3. JGT-E and JGT-W protect C2C12 myocytes against H₂O₂-induced damage

To determine whether hydrogen peroxide (H₂O₂) induced C2C12 death, we subjected the cells to various H₂O₂ concentrations for 24 h (Fig. 4A). H₂O₂ decreased C2C12 viability in a dose-dependent manner. To assess the efficacies of JGT-E and JGT-W at preventing H₂O₂-induced C2C12 death, we pretreated the cells with JGT-E and JGT-W, subjected them to H₂O₂, and evaluated cell viability by CCK-8. Figure 3B shows that JGT-E and JGT-W preserve C2C12 viability in a dose-dependent manner.

(A)



(B)

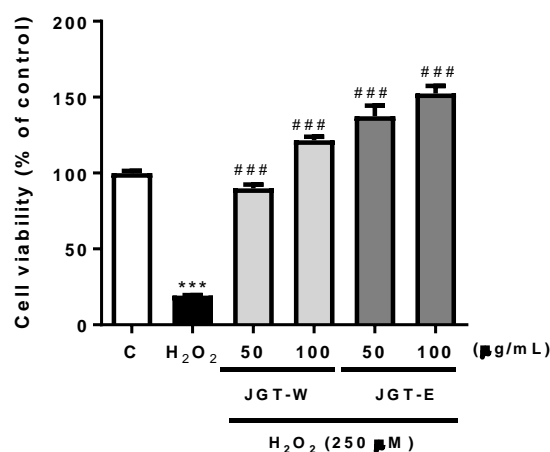


Fig. 4. Effects of JGT-W and JGT-E on viability of H₂O₂-treated C2C12 cells.

(A) H₂O₂-induced C2C12 death. (B) Protective effects of JGT-W and JGT-E in C2C12 against H₂O₂-induced cytotoxicity. Data are representative of three independent experiments and expressed as mean ± S.E.M. (n = 7). ****p* < 0.001 vs. Control; ###*p* < 0.001 vs. viability of H₂O₂-treated cells.

3.4. JGT-E and JGT-W protect C2C12 myocytes from H₂O₂-induced damage

The H₂O₂ treatment was cytotoxic to C2C12 but both JGT-W and JGT-E helped prevent cell death. To determine whether this protection was related to the antioxidant effects of JGT-W and JGT-E in C2C12, we evaluated whether JGT-W and JGT-E could inhibit intracellular ROS generation in H₂O₂-treated cells. Figure 5 shows that the ~fourfold increase in

intracellular ROS levels induced by H₂O₂ was significantly inhibited by JGT-W and JGT-E pretreatment in a dose-dependent manner. JGT-E reduced DCF fluorescence by > 27.75% (100 µg mL⁻¹) and 25.50% (50 µg mL⁻¹), respectively, compared to JGT-W. NAC pretreatment also attenuated H₂O₂-stimulated increase in intracellular ROS levels in C2C12.

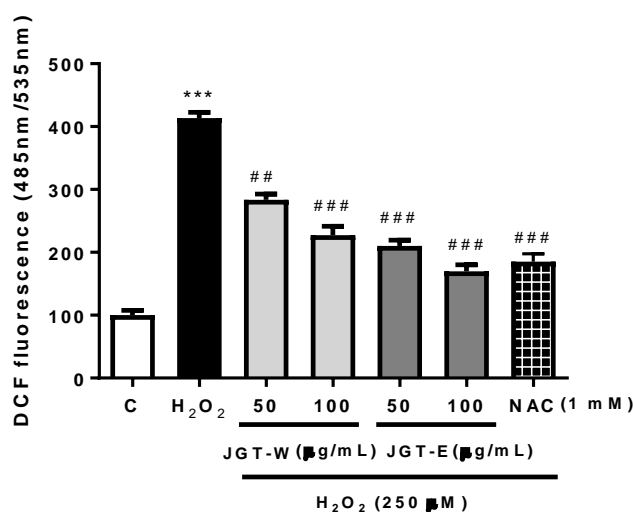


Fig. 5. Effects of JGT-W and JGT-E extracts on viability of H₂O₂-treated C2C12 cells. NAC was positive control. Data are representative of three independent experiments and expressed as mean ± S.E.M. (n = 7). ****p* < 0.001 vs. control; ##*p* < 0.01, ###*p* < 0.001 vs. viability of H₂O₂-treated cells.

4. Discussion

In aging, oxidative stress is associated with muscle protein loss or skeletal muscle atrophy [14]. Dietary supplementation with exogenous antioxidants such as Vitamins C and E and herbal medicines may exert health-promoting effects and may help to prevent muscle aging [15]. Jakyakgamcho-tang (JGT) consists of a 2:1 (w/w) mixture of *Paeonia lactiflora* and *Glycyrrhiza uralensis* roots and is used both clinically and pharmacologically in the treatment of muscle and acute abdominal pain and backache [16, 17]. Here, we analyzed the relative compositions and antioxidant efficacies of a water extract and a 30% (w/v) ethanol extract of JGT. We also assessed whether JGT prevented oxidative stress-induced death and ROS generation in skeletal muscle cells.

Plant phenolic compounds and flavonoids are secondary metabolites with various types of bioactivity and numerous health benefits. They have an aromatic ring bearing at least one hydroxyl group [11, 18]. Here, we analyzed and compared the composition and activity of water and ethanol extracts of Jakyakgamcho-tang (JGT-W and JGT-E, respectively). JGT-E had higher total phenolic and flavonoid content than those in an JGT-W. Plant phenolics and flavonoids scavenge free radicals and act as antioxidants [19]. JGT-E was relatively more effective at scavenging ABTS·⁺ and DPPH· and inhibiting intracellular ROS generation in skeletal muscle cells as it contained comparatively more TPC and TFC. Phenolic compounds strongly inhibited H₂O₂-induced apoptosis in HepG2 cells [20]. The levels of all measured phenolic compounds except albiflorin (1) were higher in JGT-E than JGT-W. Paeoniflorin (2) was the most abundant component and its concentration markedly differed between JGT-E and JGT-W. Paeoniflorin is a pinnae monoterpene glycoside with various pharmacological effects. It is anti-inflammatory and immunoregulatory in certain animal models of autoimmune disease such as rheumatoid arthritis (RA) and systemic lupus erythematosus

(SLE). It is also an antioxidant [21, 22]. Liquiritin (3) has antidepressant, antiviral, and antitumor efficacy [23-25]. Pentagalloylglucose (4) prevented acute lung injury in lipopolysaccharide-induced rat models and inhibited H⁺/K⁺-ATPase [26-28]. Isoliquiritin apioside (5) was antimetastatic and anti-angiogenic in epithelial and malignant cancer cells [29]. Isoliquiritin (6) presented with antifungal activity. Liquiritigenin (7) demonstrated efficacy against multidrug-resistant bacteria [30, 31]. Glycyrrhizin (8) showed antiviral and anti-inflammatory efficacy [32, 33].

The present study demonstrated that an ethanol-water solvent mixture was relatively more effective at extracting the bioactive components in JGT, thereby increasing its biological activity and reducing the dosage required for optimal efficacy. To the best of our knowledge, this is the first report on the mechanism and effectiveness of JGT against aging-related muscular atrophy.

5. Conclusions

The aim of this study was to compare the relative efficiencies of water and 30% (v/v) ethanol at extracting the antioxidant principles in the traditional herbal blend known as Jakyakgamcho-tang (JGT). The phenolic and flavonoid levels in the ethanol extract of JGT were higher than those in JGT-W. JGT-E had strong antioxidant activity and prevented oxidative stress-induced skeletal muscle cell death. The results of the present study suggest that low doses of JGT-E may help prevent skeletal muscle aging. Moreover, 30% (v/v) ethanol effectively extracts JGT and substantially enhances its antioxidant and other biological activities compared with traditional water extracts that have been prepared to reduce the dosages.

Author Contributions: Y.S.K. designed the *in vitro* cell experiment and wrote the manuscript; H.J.Y. confirmed the HPLC chromatogram and antioxidant assay; D-.S. K designed all experiments and reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Data Availability: All the data generated and analyzed in this study are mentioned in this manuscript.

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