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Research Article

Antifatigue Potential of Loquat Leaf Extract in Physical Stress in C2C12 Myotubes and *In Vivo* Models

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Recent studies suggest that oxidative stress could be one of the mechanisms contributing to fatigue. The purpose of the present study was to determine the antifatigue potential of loquat leaf (*Eriobotrya japonica* Lindl., EJ) and its component, chlorogenic acid (CA) in C2C12 myotubes and treadmill stress test (TST), and forced swimming test (FST) in animal models. EJ and CA reduced levels of metabolic factors associated with fatigue and enhanced catalytic properties of superoxide dismutase and catalase in C2C12 myotubes. In the FST and TST, EJ and CA decreased immobility time and prolonged exhaustion time. The administration of EJ and CA reduced the levels of the metabolic factors associated with fatigue and augmented the levels of the substances which relieve fatigue. These results prove that EJ alleviates fatigue by increasing antioxidative activity. Therefore, we suggest that EJ may be a potential candidate for management of fatigue as a health functional food.

1. Introduction

Fatigue is a term used to describe a decrease in physical performance and is related to an increase in the actual/perceived difficulty in performing a task or exercise. It is a common symptom of many physical, neurological, and psychiatric disorders, including depression, human immunodeficiency virus infection, Parkinson's disease, cancer, multiple sclerosis, aging, and other potential physical illnesses [1]. Fatigue can be categorized into three types, namely, pathological, physiological, and psychological fatigue [2]. Pathological fatigue is often experienced by people with chronic illnesses such as those with liver dysfunction, diabetes, and gastrointestinal diseases and can severely impair functional activity and quality of life [3]. Physiological fatigue is a state of reduced mental or physical capability which occurs due to the accumulation of metabolic products in the blood which induces fatigue [2]. Psychological fatigue occurs due to emotional conflicts, anxiety, or boredom [3]. Currently, there are several theories regarding the mechanisms which cause fatigue. The metabolic causes of fatigue include a decrease in phosphocreatine levels, accumulation of protons (acidemia), depletion of glycogen in the muscles and decrease in blood glucose concentrations, and increase in specific amino acids in the plasma [4]. Also, fatigue is caused by the accumulation of lactate through the activation of lactate dehydrogenase (LDH) and the production of cortisol by the adrenocorticotropic hormone in the anterior pituitary gland, whereas it is improved by energy production through glycolysis and the citrate cycle which is activated by hexokinase and citrate synthase. A rapidly emerging concept that has recently

received attention is the role of the free radicals in fatigue and the benefits of antioxidants in relieving fatigue [5, 6]. The increased oxidative stress during excessive exercise induces fatigue by increasing the release of free radicals and inflammatory cytokines [7, 8]. Therefore, it can be inferred that symptoms of fatigue can improve through the consumption of antioxidants that increase superoxide dismutase (SOD) and catalase activities and regulate inflammatory cytokines.

Therapeutic drugs used to relieve fatigue provide temporary benefits and are also associated with side effects. The antifatigue effect of natural antioxidants has been reported in [9]. Therefore, components of natural products are worth investigating, as they are associated with fewer side effects and could be effective in treating or preventing fatigue.

In traditional medicine, loquat leaf (*Eriobotrya japonica* Lindl., EJ) is used to treat inflammatory diseases [10] and has neuroprotective, antiallergic, antioxidant, and antiarthritic properties [11–14]. Furthermore, EJ contains many antioxidants such as chlorogenic acid (CA), quercetin-3-sambubioside, euscaphic acid, and ursolic acid [15]. In a study by Jung et al. [15], EJ and CA demonstrated antioxidative and antiinflammatory properties. Therefore, in this study, we hypothesized that EJ might exhibit antifatigue potential through its antioxidative actions. We used the C2C12 myotubes, the treadmill stress test (TST), and the forced swimming test (FST) to investigate the antifatigue potential of EJ and CA and its mechanism. These results can be utilized as basic experimental data for the development of health nutraceuticals and herbal medicines to combat fatigue.

2. Materials and Methods

2.1. Preparation of EJ Extracts. EJ extracts were provided by COSMAX NBT, INC. (Seongnam, Republic of Korea). The EJ solution was extracted using water (EJ water extract, WEJ) or ethanol (EJ ethanol extract, EEJ). Each of the extracted solutions was filtered, concentrated, mixed with dextrin, and spray-dried.

2.2. Ultrahigh Performance Liquid Chromatography (UHPLC) and Mass Spectrometry (MS) Analysis. Liquid chromatography/MS (LC/MS) analyses were carried out using an LTQ Orbitrap XL Fourier transform mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) coupled to an Accelar ultrahigh pressure liquid chromatography system (Thermo Fisher Scientific, Inc.). The chromatographic separation of metabolites was carried out using an ACQUITY UPLC® BEH C_{18} column and mobile phases A (water with 0.1% formic acid) and B (acetonitrile with 0.1% formic acid). Each compound was detected with a photodiode array at $200 \sim 500$ nm. The MS analysis was performed with polarity switching with the following parameters for the MS/MS scan: m/z range of 150–1,500. High-resolution mass spectra were acquired on the LTQ Orbitrap XL (Supplementary Figure 1). To determine the Kovats retention indices, we proceeded as follows: a sample of $2 \mu l$ of a mixture

of a standard solution of linear alkanes C8–C20 in chromatograph gas was injected into the same chromatographic column with the same conditions of analysis as in the case of basil volatile oil. After confirmation (based on MS spectra) of the linear alkane structures separated by the gas chromatographic analysis, we determined the retention time for each component of the mixture (Supplementary Table 1).

2.3. C2C12 Myoblasts Cell Culture. Murine myoblast C2C12 cells were grown in Dulbecco's modified eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum and antibiotics at 37°C in a 5% CO_2 atmosphere. To differentiate the C2C12 cells, 80% of the confluent culture was replaced with DMEM containing 2% horse serum, and the medium was replaced with fresh medium every other day for 4 days.

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay. The MTT tetrazolium assay was used to check cytotoxicity by analyzing the intracellular metabolic activity in which MTT is converted into a purple formazan product. The differentiated C2C12 cells (3×10^5/well) were plated on a 24-well plate, stabilized for 30 min, and treated with WEJ, EEJ, and CA for 24 h at 37°C. After changing to a fresh medium, the plates were treated with MTT (0.5 mg/ml) for 4 h. Insoluble formazan crystals were solubilized in dimethyl sulfoxide. The amount of formazan was quantitated by absorbance at 570 nm using an enzyme-linked immunosorbent assay (ELISA) reader.

2.5. ELISA. The produced contents of cytokines were quantitated by the ELISA method [16].

2.6. Animals. The ICR mice (male, 4 weeks old), purchased from the Dae–Han Experimental Animal Center (Chungbuk, Republic of Korea), were stabilized in individual cages for one week at room temperature (20–23°C), with 50–60% humidity and a 12 h light and 12 h dark cycle. The mice were treated in accordance with the current law and animal care standards provided in the Guide for the care and use of laboratory animals approved by the Animal Ethics Committee of Kyung Hee University (KHUASP (SE)-20-263).

2.7. FST. In the FST, time spent by the mice swimming or being immobile is measured and increased immobility time indicates fatigue. Mice were subjected to an FST initially, to determine the differences between the individual mice. The mice were divided into 7 groups based on the immobility time: normal, control (distilled water, D.W.), WEJ (25, 50, and 100 mg/kg), EEJ (50 mg/kg), and CA (1 mg/kg). According to the previous study [11], the concentrations of WEJ were determined to be 25, 50, and 100 mg/kg. The CA (1 mg/kg) concentration was also determined according to an earlier study [17]. WEJ, EEJ, and CA were orally administered to the mice daily for 28 days. The FST was then carried out on the last day as previously reported [18].

2.8. TST. The TST was conducted as previously reported [19]. Briefly, the treadmill speed was started at 0 m/min and slowly increased (10 m/min at 10 min, 16 m/min at 10 min, and 21 m/min at 10 min). On the 28th day, the mice were made to exercise until exhaustion (10 m/min at 5 min and 40 m/min at 30 min). The oral administration of D.W., WEJ, EEJ, and CA was carried out daily for 28 days.

2.9. Analysis of Fatigue-Related Biochemical Indicators in Serum, Muscle, or Liver. Blood, muscle (extensor digitorum longus (EDL), tibialis anterior (TA), soleus, and gastrocnemius muscles), or liver were collected immediately after performing FST and TST. The serum was obtained by centrifugation of blood of the mice, and muscle and liver samples were quickly collected. A glycogen colorimetric assay kit (Biovision Inc., Milpitas, California, USA) was used to measure the levels of glycogen. The levels of citrate synthase were analyzed using a commercially available kit (MyBiosource Inc., San Diego, California, USA). The levels of lactate, SOD, malondialdehyde (MDA), and catalase were quantitated using assay kits (DoGenBio, Seoul, South Korea). The levels of free fatty acids and cortisol were also quantitated using assay kits (Abcam, Cambridge, UK and Sigma-Aldrich Co., St. Louis, MO, USA), respectively. A DRI CHEM NX500 analyzer (Fujifilm, Tokyo, Japan) was used to quantify the levels of creatine kinase (CK), alanine transaminase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), glucose, and LDH.

2.10. Statistical Analysis. For all statistical analyses, the SPSS 12.0 (IBM Corp., Armonk, NY, USA) software was utilized. To verify the normality of the data, the Shapiro–Wilk test was used. An independent *t*-test was performed for a comparison between the normal (or blank) and control groups, and a one-way ANOVA test was performed for a comparison of three or more groups such as the control and drug groups. *In vivo* ($n = 5$/group) and *in vitro* data were expressed as mean-±standard error mean (SEM). *In vitro* data were indicated as the results of three independent experiments. A *P* value less than 0.05 meant that an effect was observed.

3. Results

3.1. Effect of EJ on Fatigue-Related Biochemical Indicators in H_2O_2-Stimulated C2C12 Myotubes. Initially, to investigate the antifatigue effects of EJ, the levels of fatigue-related biomarkers in H_2O_2-stimulated C2C12 myotubes were determined. The levels of LDH and CK were significantly augmented by H_2O_2 stimulation. However, the levels that were increased by H_2O_2 stimulation were remarkably decreased by treatment with WEJ, EEJ, or CA (Figures 1(a) and 1(b), $P < 0.05$). Glycogen levels were significantly augmented by treatment with WEJ, EEJ, or CA, compared with the levels seen with H_2O_2 stimulation (Figure 1(c), $P < 0.05$).

The inflammatory responses induced by the inflammatory cytokines are closely related to fatigue, and their high levels are indicative of chronic fatigue. In this study, H_2O_2 stimulation remarkably augmented the secretion of inflammatory cytokines, interleukin (IL)-6, and tumor necrosis factor (TNF)-α compared with the unstimulated cells. Treatment with WEJ, EEJ, or CA significantly decreased the levels of IL-6, and TNF-α increased by H_2O_2 stimulation (Figures 1(d) and 1(e), $P < 0.05$). WEJ dose-dependently regulated the fatigue-related biochemical indicators. Furthermore, WEJ, EEJ, or CA showed no cytotoxicity to C2C12 myotubes (Figure 1(f)).

3.2. Effect of EJ on Oxidation-Related Biochemical Indicators in H_2O_2-Stimulated C2C12 Myotubes. Oxidative stress is known to be one of the factors triggering fatigue. To investigate the antioxidative effect of EJ, the activities of SOD and catalase were analyzed using the respective assay kits. The stimulation with H_2O_2 significantly decreased the activities of SOD and catalase compared with unstimulated cells (Figures 2(a) and 2(b), $P < 0.05$). However, treatment with WEJ, EEJ, or CA showed a significant increase in the activities of SOD and catalase which were decreased by H_2O_2 (Figures 2(a) and 2(b), $P < 0.05$). In addition, WEJ remarkably increased the activities of SOD and catalase (Figures 2(a) and 2(b), $P < 0.05$). MDA is a direct indicator of oxidative stress and a biomarker of fatigue. In H_2O_2-stimulated C2C12 myotubes, WEJ, EEJ, or CA treatment decreased the levels of MDA significantly (Figure 2(c), $P < 0.05$).

3.3. Effect of EJ on Immobility and Exhaustion Time in Animal Models. Next, to investigate the antifatigue effect of EJ, we conducted experiments *in vivo* in mice [18]. In the FST, the immobility time of the mice administered WEJ, EEJ, or CA was significantly reduced compared with the control group at 28 days (Figure 3(a), $P < 0.05$). In addition, WEJ remarkably decreased the immobility time (Figure 3(a), $P < 0.05$). Furthermore, the antifatigue effect of EJ was also investigated by performing the TST. The time to exhaustion of the mice administered with WEJ, EEJ, or CA was significantly prolonged compared with the control group (Figure 3(b), $P < 0.05$). There was no significant change in the body weight of the WEJ, EEJ, or CA groups compared to the control group (Supplementary Figure 2).

3.4. Effect of EJ on Metabolic Factors Associated with Fatigue after FST and TST. We investigated the antifatigue effects of EJ by analyzing metabolic factors associated with fatigue. Following exercise, the levels of lactate, LDH, MDA, or cortisol increased in the serum. The levels of lactate, LDH, MDA, and cortisol were quantitated in the serum of the mice administered with WEJ, EEJ, or CA after performing the FST and TST. As seen in Figure 4, the serum levels of lactate, LDH, MDA, and cortisol in the control group were remarkably augmented compared with the normal group ($P < 0.05$). The lactate, LDH, MDA, and cortisol levels in the serum of the mice administered WEJ or CA remarkably decreased significantly compared to the control group (Figure 4, $P < 0.05$). EEJ remarkably decreased the serum levels of LDH, MDA, and cortisol (Figure 4). The lactate levels were decreased by EEJ, but not significantly (Figure 4).
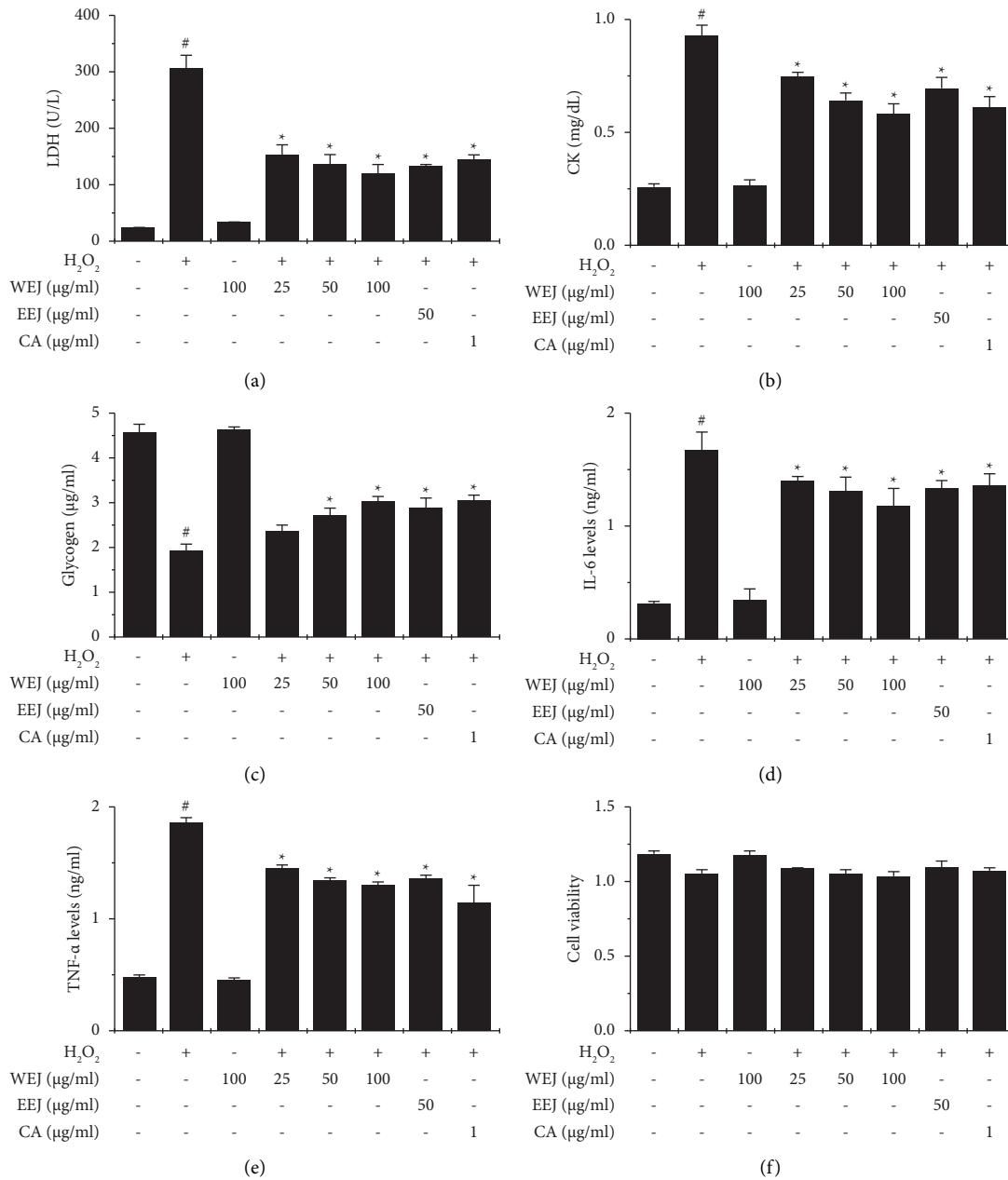
FIGURE 1: Effect of EJ on fatigue-related biochemical indicators in H_2O_2-stimulated C2C12 myotubes. C2C12 cells were differentiated by culture in DMEM containing 2% horse serum for 4 days. Differentiated C2C12 cells were treated with WEJ (25, 50, and 100 μg/ml), EEJ (50 μg/ml), or CA (1 μg/ml) and then stimulated with H_2O_2 (1 mM) for 24 h. Levels of (a) LDH, (b) CK, and (c) glycogen were measured. Levels of (d) IL-6 and (e) TNF-α were analyzed by the ELISA method. (f) Cell viability was measured by a MTT assay. Results show the mean ± SEM of data from three separate experiments with duplicate samples. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. $^{\#}P < 0.05$, significantly different from the unstimulated C2C12 myotubes. $^{*}P < 0.05$, significantly different from the H_2O_2-stimulated C2C12 myotubes.

The levels of fatigue-related blood biochemical indicators were measured after the FST and TST. The serum levels of BUN, ALT, AST, and CK in the control mice were remarkably augmented when compared with the normal group, but WEJ, EEJ, or CA-administration resulted in a significant decrease in these levels compared to the control group (Table 1, $P < 0.05$).

3.5. Effect of EJ on Factors Related to Improvement in Fatigue after the FST and TST. The glucose, free fatty acids, glycogen, and citrate synthase contents were measured after the FST and TST to investigate whether EJ increased the levels of the factors associated with improvement in fatigue. As shown in Figure 5(a), the glucose levels of the control group were remarkably augmented compared with the normal group.
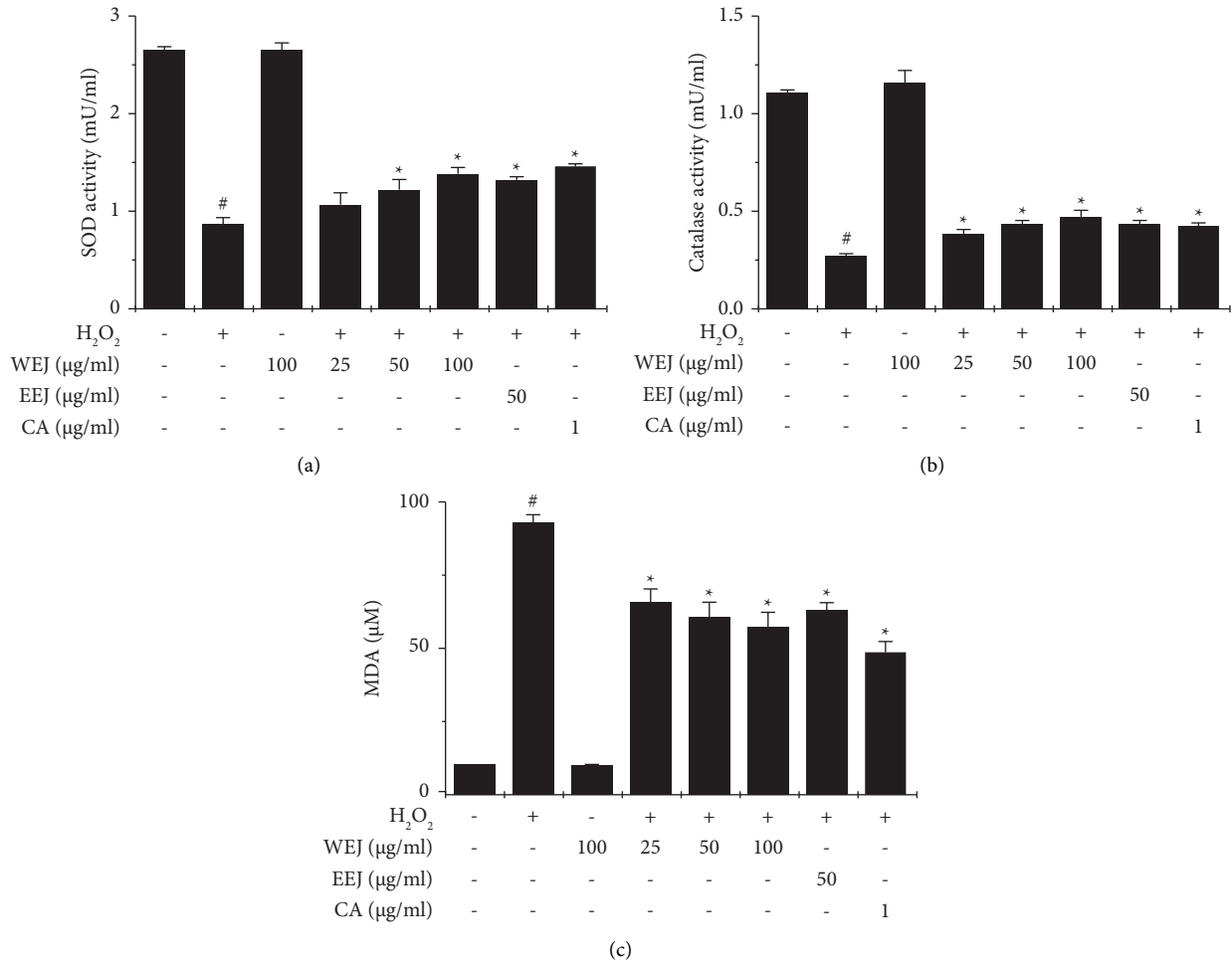
(a)

(b)

(c)

FIGURE 2: Effect of EJ on oxidation-related biochemical indicators in H_2O_2-stimulated C2C12 myotubes. C2C12 cells were differentiated by culture in DMEM containing 2% horse serum for 4 days. Differentiated C2C12 cells were treated with WEJ (25, 50, and 100 μg/ml), EEJ (50 μg/ml), or CA (1 μg/ml) and then stimulated with H_2O_2 (1 mM) for 24 h. Activities of (a) SOD, (b) catalase, and (c) levels of MDA were analyzed with each kit. Results show the mean ± SEM of data from three separate experiments with duplicate samples. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. #$P < 0.05$, significantly different from the unstimulated C2C12 myotubes. *$P < 0.05$, significantly different from the H_2O_2-stimulated C2C12 myotubes.
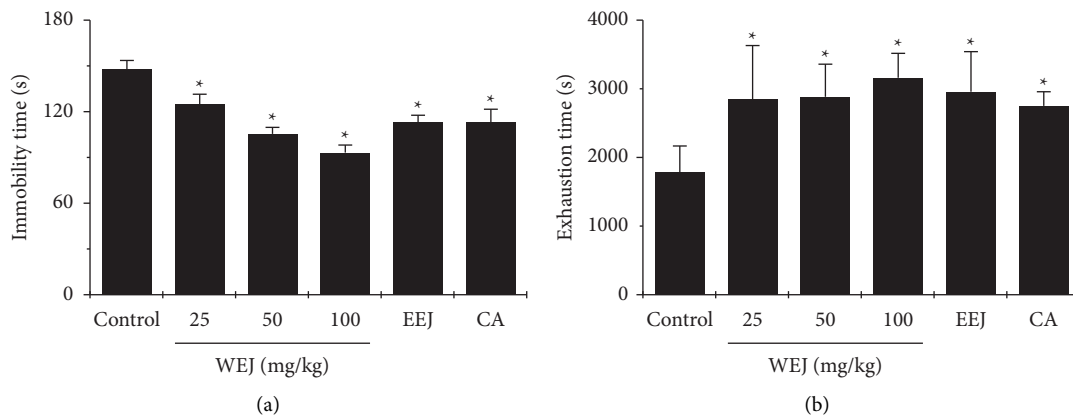

(a)

(b)

FIGURE 3: Effect of EJ on immobility and exhaustion time of fatigue animal models. (a) Immobility time in the FST and (b) exhaustion time in TST at 28 days. Values are means ± SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. *$P < 0.05$, significantly different from the control mice.
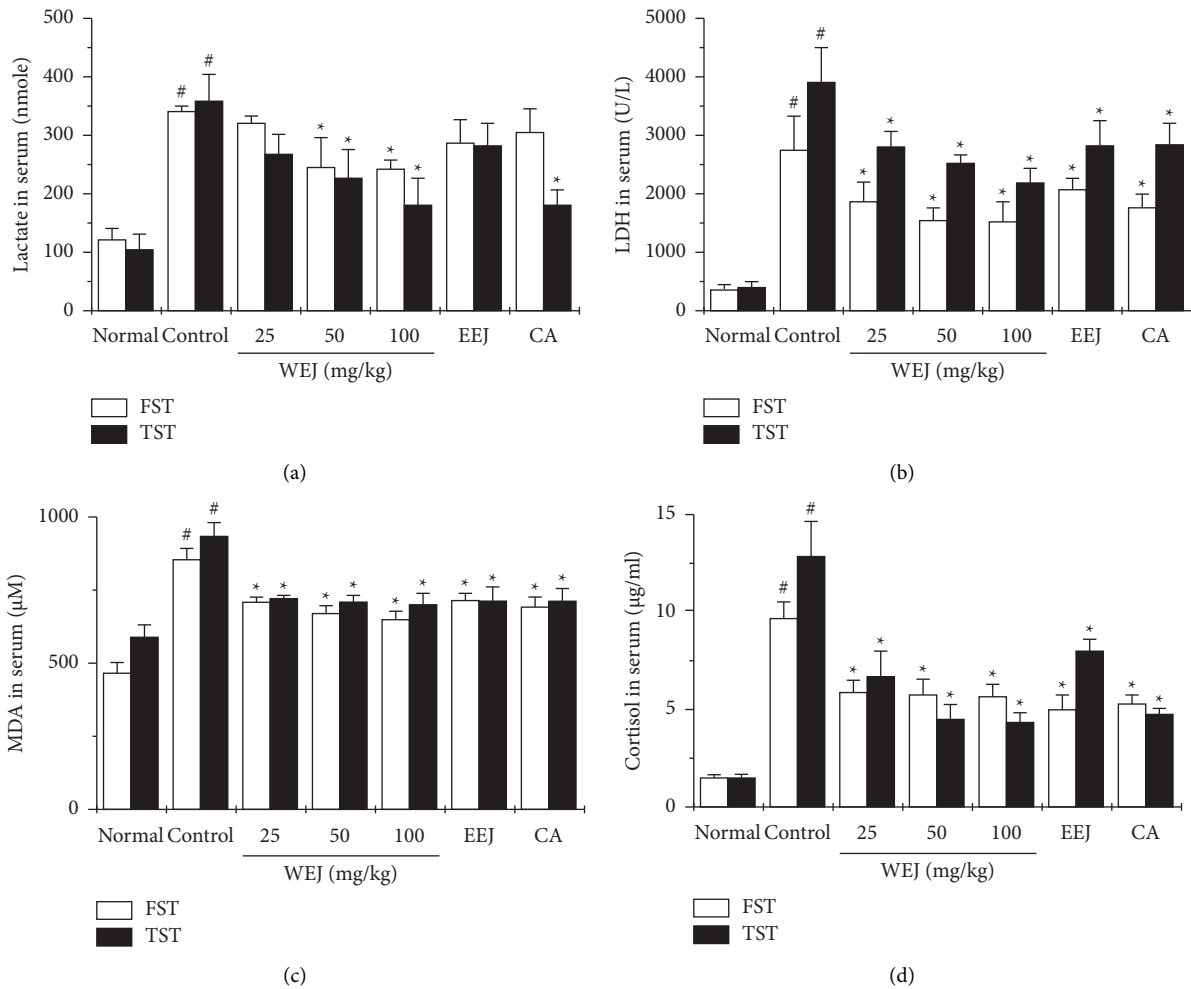
(a)

(b)

(c)

(d)

Figure 4: Effect of EJ on metabolic factors associated with fatigue in the serum after FST and TST. After the last FST and TST, serum samples were obtained from heart. Levels of (a) lactate, (b) LDH, (c) MDA, and (d) cortisol were measured with each kit. Values are the mean $\pm$ SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. [#]$P < 0.05$, significantly different from the normal mice. [*]$P < 0.05$, significantly different from the control mice.

Table 1: Effect of EJ on fatigue-causing factors in serum after FST and TST.

		Normal	Control	WEJ (25 mg/kg)	WEJ (50 mg/kg)	WEJ (100 mg/kg)	EEJ (50 mg/kg)	CA (1 mg/kg)
FST	BUN (mg/dL)	22.2 ± 1.22	68.34 ± 7.91[#]	54.08 ± 7.13[*]	53.93 ± 3.47[*]	46.8 ± 2.45[*]	44.64 ± 7.14[*]	33.24 ± 2.81[*]
	ALT (U/L)	31.4 ± 4.51	109.6 ± 10.69[#]	63.4 ± 7.7[*]	56 ± 9.22[*]	50.8 ± 2.38[*]	70.8 ± 10.33[*]	47 ± 7.18[*]
	AST (U/L)	104.2 ± 12.19	309 ± 19.65[#]	175.4 ± 16.8[*]	164.2 ± 14.07[*]	135.4 ± 8.26[*]	152.4 ± 12.34[*]	175.8 ± 16.25[*]
	CK (mg/dL)	0.19 ± 0.02	0.91 ± 0.06[#]	0.68 ± 0.03[*]	0.6 ± 0.04[*]	0.5 ± 0.03[*]	0.61 ± 0.06[*]	0.61 ± 0.06[*]
TST	BUN (mg/dL)	21.44 ± 3.49	86.4 ± 8.72[#]	53.04 ± 4.18[*]	48.06 ± 1.17[*]	31.96 ± 4.24[*]	33.74 ± 6.77[*]	33.12 ± 3.71[*]
	ALT (U/L)	31.8 ± 9.04	108 ± 6[#]	66.8 ± 8.32[*]	60.6 ± 5.13[*]	48.4 ± 6.73[*]	63 ± 11.47[*]	43.6 ± 13.2[*]
	AST (U/L)	70.6 ± 16.23	409.8 ± 59.58[#]	210.2 ± 21.25[*]	174 ± 9.3[*]	154 ± 14.54[*]	184.6 ± 33.23[*]	165 ± 30.88[*]
	CK (mg/dL)	0.13 ± 0.02	0.81 ± 0.08[#]	0.56 ± 0.03[*]	0.48 ± 0.02[*]	0.43 ± 0.01[*]	0.45 ± 0.02[*]	0.55 ± 0.02[*]

After the last FST and TST, serum samples were obtained from the heart. Levels of BUN, ALT, AST, and CK were analyzed with each kit. Values are the mean $\pm$ SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; CA, chlorogenic acid. [#]$P < 0.05$, significantly different from normal mice. [*]$P < 0.05$, significantly different from control mice.

However, the increased glucose levels seen in the control group were significantly lowered by the administration of WEJ, EEJ, or CA ($P < 0.05$). The free fatty acids contents in the serum and the glycogen and citrate synthase contents in the muscle were remarkably augmented by the administration of WEJ (50 and 100 mg/kg) compared to the control group (Figures 5(b)–5(d), $P < 0.05$). Administration of EEJ and CA significantly upregulated the free fatty acids and
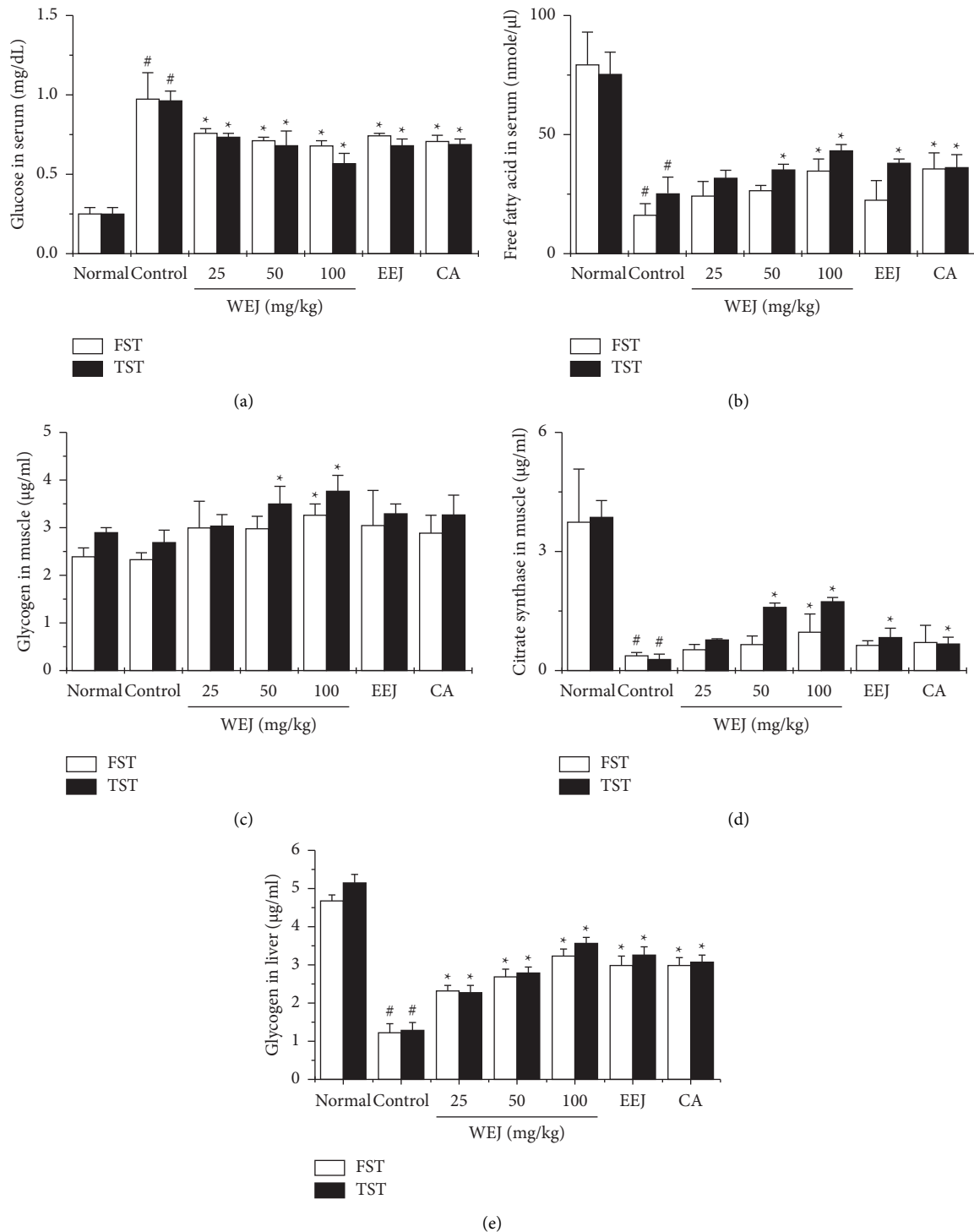
(a)

(b)

(c)

(d)

(e)

Figure 5: Effect of EJ on glucose, free fatty acid, glycogen, and citrate synthase in the serum and muscle after the FST and TST. After the last FST and TST, serum and muscle samples were obtained from each mouse. Levels of (a) glucose and (b) free fatty acid in serum were analyzed by a DRI CHEM NX500 analyzer. Levels of (c) glycogen and (d) citrate synthase in muscle were measured with each kit. (e) Levels of glycogen in liver were measured with glycogen kit. Values are the mean ± SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. $^{\#}P < 0.05$, significantly different from the normal mice. $^{*}P < 0.05$, significantly different from the control mice.

citrate synthase contents compared to the control group after the FST or TST (Figures 5(b) and 5(d), $P < 0.05$). The glycogen content after the FST and TST was increased by EEJ and CA, but not significantly (Figure 5(c)). The glycogen contents in the liver were remarkably augmented by the administration of WEJ, EEJ, or CA compared to the control group (Figure 5(e), $P < 0.05$).

3.6. Effect of EJ on Fatigue-Related Inflammatory Cytokines in Serum after the FST and TST. To investigate whether EJ played a role in the down regulation of the inflammatory cytokines, the levels of IL-1β, IL-4, IL-6, and TNF-α in serum were analyzed using the ELISA method after the FST and TST. As seen in Figure 6, the serum levels of inflammatory cytokines in the control group were remarkably augmented compared with the normal group ($P < 0.05$). The serum levels of inflammatory cytokines in the mice administered WEJ, EEJ, or CA showed a significant decrease compared with the control group (Figure 6, $P < 0.05$).

3.7. Effect of EJ on SOD and Catalase Activities after the FST and TST. In the *in vitro* test, EJ augmented the activities of SOD and catalase (Figure 2). To confirm the antioxidative effect of EJ in the *in vivo* test, the SOD and catalase activities were analyzed after the FST and TST. As shown in Figures 7(a)–7(c), the SOD activities in the serum, liver, and muscle of the control mice were remarkably lower compared with the normal group ($P < 0.05$). However, the SOD activities in the serum, liver, and muscle of the WEJ or CA group were remarkably augmented compared with the control group after the FST and TST (Figures 7(a)–7(c), $P < 0.05$). The EEJ only increased the SOD activity after TST compared with the control group (Figures 7(a)–7(c)). Furthermore, the administration of WEJ, EEJ, or CA significantly upregulated the levels of catalase in the liver compared with the control group after the FST (Figure 7(d), $P < 0.05$). WEJ (100 mg/kg) significantly increased the catalase activity compared with the control group after the TST (Figure 7(d)).

4. Discussion

In this study, we demonstrated that EJ and CA have an antifatigue effect through their ability to decrease the levels of metabolic factors associated with fatigue and increase the levels of substances which play a role in improving fatigue in the C2C12 myotubes *in vitro* model and the FST and TST in the *in vivo* models.

The metabolic factors involved in the pathophysiology of fatigue include lactate, LDH, MDA, BUN, ALT, AST, CK, and cortisol, whereas recovery from fatigue is induced by glucose, free fatty acids, glycogen, and citrate synthase [4]. Accumulation of lactate by LDH reduces the pH of the blood and muscle tissue, damages various organs, and is one of the causes of fatigue [20]. During exercise, MDA increased by oxidative stress causes muscle damage and fatigue [21–23]. BUN is another sensitive index of fatigue status, and BUN

levels were found to increase significantly after excessive exercise [24]. Physiological changes which occur during fatigue are closely associated with increases in AST and ALT levels suggestive of liver failure [25]. The serum levels of CK are increased by exercise-induced muscle damage [26]. An increase in cortisol is associated with stress. During long-term exercise, the release of cortisol in the adrenal cortex is induced by an increase in the secretion of the adrenocorticotropic hormone in the anterior pituitary resulting in fatigue [27]. Fatigue is induced by a lack of energy due to mitochondrial dysfunction [28]. Therefore, energy storage and supply are important factors in exercise endurance. When exercising without an energy source (glucose, free fatty acids, and glycogen), physical fatigue increases, resulting in a significant reduction in endurance [4, 29]. A deficiency of citrate synthase could cause fatigue due to lactate accumulation in the blood, and a significant decrease in citrate synthase was shown in chronic fatigue syndrome (CFS) patients compared to healthy controls [30]. Many substances with antifatigue effects have been shown to decrease the levels of lactate, LDH, MDA, BUN, ALT, AST, CK, and cortisol and increase the levels of glycogen, free fatty acids, and citrate synthase [1–6, 18, 25]. Previous studies have shown that EJ improves muscle function and reduces muscle loss [31, 32]. In the present study, EJ and CA remarkably decreased the levels of lactate, LDH, MDA, BUN, ALT, AST, CK, and cortisol, whereas they augmented the glycogen, free fatty acids, and citrate synthase levels. Therefore, these results indicate that EJ and CA could have contributed to exercise performance by ameliorating fatigue.

Glucose is the predominant energy source during exercise. Decreases in blood glucose levels lead to fatigue and impair endurance exercise [33]. Gibb et al. [34] reported that exercise-induced fatigue results in a decrease of glucose levels, while triglycerides are degraded and stored in the form of free fatty acids in the blood. In contrast, other researchers reported that the FST and TST increased the serum glucose levels and decreased serum free fatty acids, and that many substances with antifatigue effects decreased the glucose levels and increased the free fatty acids levels [4, 19, 35]. Also, the previous result indicated that blood glucose levels continued to rise steadily during exercise and gradually decreased to levels similar to the baseline during the recovery period (1-2 h after exercise cessation) [36]. In this study, blood was collected immediately after the cessation of FST and TST, and glucose levels increased in the control group, while EJ reduced glucose levels. Therefore, these results indicate that EJ can regulate the increase in glucose levels induced by exercise. Moreover, when exercise-induced stress increases, cortisol can supply the body with glucose, increasing blood glucose levels [37]. Exercise-induced exhaustion has been reported to decrease the availability of fatty acids and improve insulin sensitivity in glucose metabolism [38]. Rau et al. [39] reported that stress induces hyperglycemia and stress-induced hyperglycemia releases stress hormones and cytokines. Therefore, we speculate that EJ might alleviate stress-induced hyperglycemia. However, further investigation is necessary to
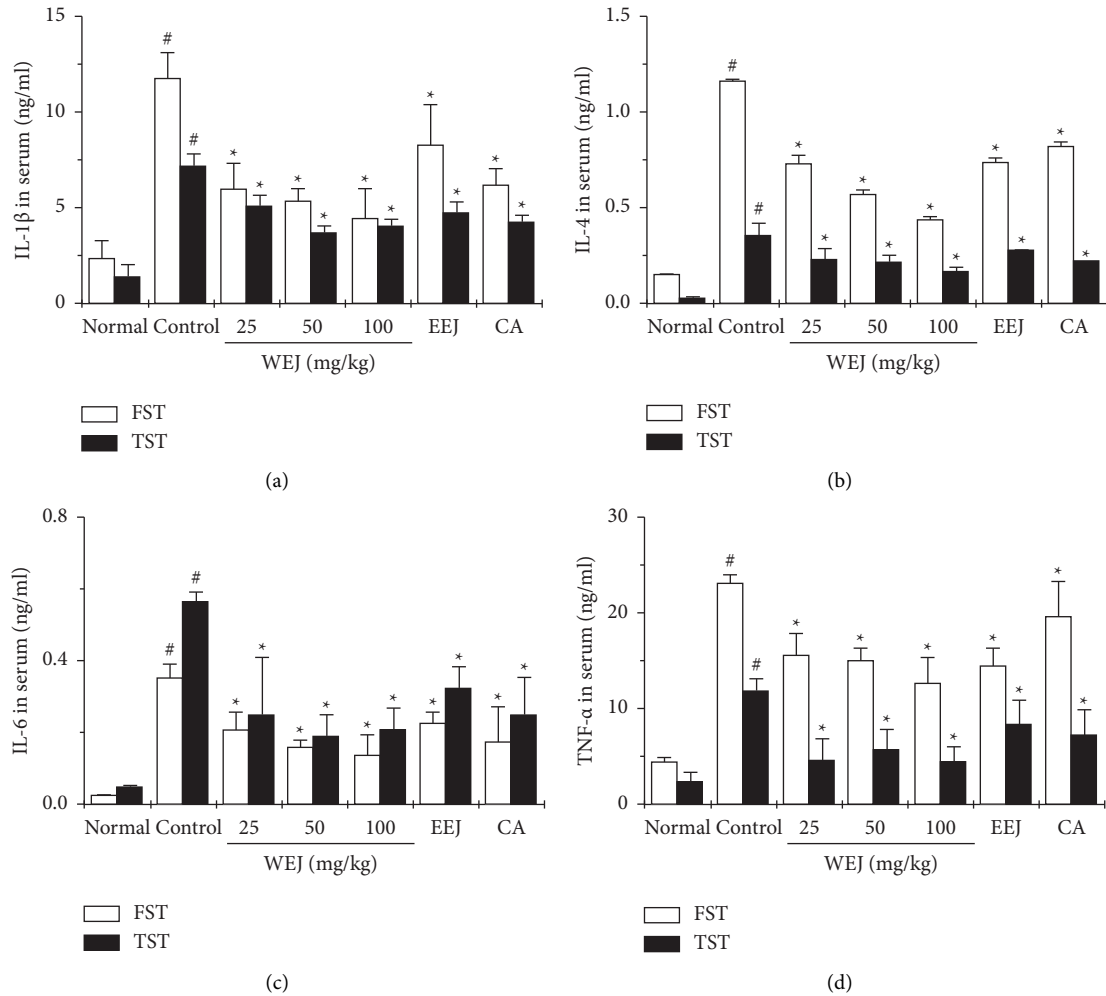
FIGURE 6: Effect of EJ on fatigue-related inflammatory cytokines in the serum after the FST and TST. After the last FST and TST, serum samples were obtained from heart. Levels of (a) IL-1β, (b) IL-4, (c) IL-6, and (d) TNF-α were analyzed by the ELISA method. Values are the mean ± SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. $^{\#}P < 0.05$, significantly different from the normal mice. $^{*}P < 0.05$, significantly different from the control mice.

determine whether EJ has an antihyperglycemic property in hyperglycemia animal models such as hyperglycemic mice or obese diabetic mice.

Oxidative stress causes mitochondrial dysfunction and cell damage, leading to inflammatory disorders, and is a major cause of fatigue [40]. In the CFS patients, the levels of antioxidants (SOD and catalase) were remarkably decreased, while there was an increase in the generation of reactive oxygen species (ROS) [41]. Excessive oxidative stress induces a rise in inflammatory markers, such as IL-1β, IL-4, IL-6, and TNF-α [42]. Blundell et al. [43] reported that the levels of inflammatory cytokines in CFS patients were augmented compared to the normal control. Oxidative stress after strenuous exercise resulted in increased TNF-α levels, resulting in muscle fatigue [29]. Therefore, substances that decrease the levels of inflammatory cytokines by increasing SOD and catalase activities could play a role in reducing fatigue [44]. *In vitro* and *in vivo* data indicate that CA has antioxidant activity and reduces oxidative stress in a variety of diseases [45]. According to Jiang et al. [46], CA reduces inflammation and oxidative stress while

also preventing cell damage. Moreover, CA reduced expression of IL-1β, TNF-α, and IL-6 via the inhibition of nuclear factor (NF)-κB activation, which in turn reduced rat liver fibrosis and inflammation [45].

The muscles of a mouse include the EDL, TA, soleus, and gastrocnemius muscles [47, 48]. Among them, both EDL and soleus muscles are representative examples of fast-twitch and slow-twitch muscles [48]. Fast-twitch muscles are primarily utilized during short bursts of intense exercise, while slow-twitch muscles are predominantly used during prolonged or endurance activities [49]. The rate of glycogen accumulation varies depending on the muscle type such as fast-twitch and slow-twitch muscles [50]. Following exercise, the levels of citrate synthase decreased in the EDL muscles; on the contrary, the levels of citrate synthase increased in the soleus muscles [51]. During exercise, stanozolol administration induced a significant increase of SOD activity in both EDL and soleus muscles [52]. In the current study, we demonstrated that citrate synthase content and SOD activity decreased in the muscle tissues (EDL, TA, soleus, and gastrocnemius muscles)
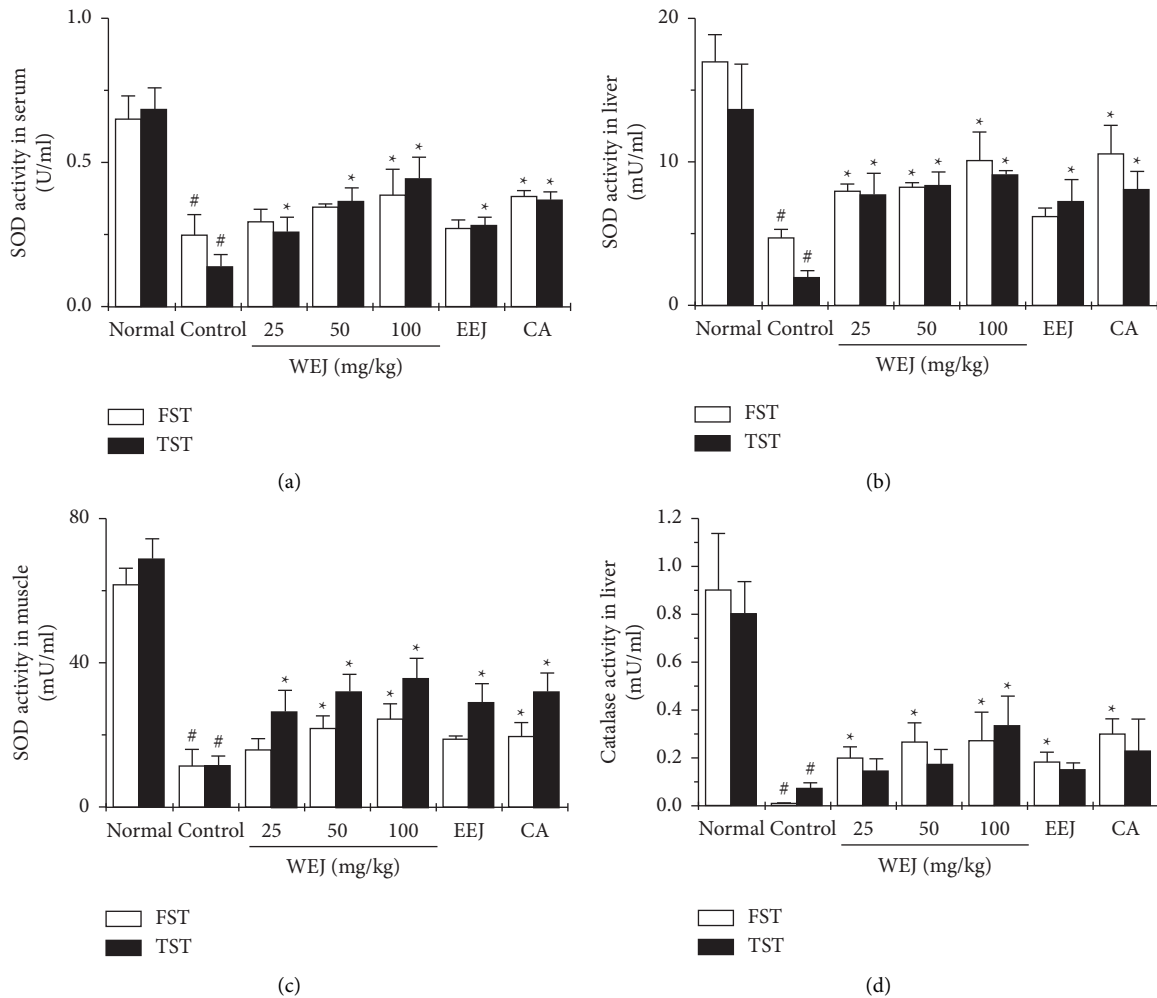
(a)

(b)

(c)

(d)

FIGURE 7: Effect of EJ on SOD activities in the serum, liver, and muscle after the FST and TST. After the last FST and TST, samples were analyzed for SOD and catalase activities. The activities of SOD in (a) serum, (b) liver, and (c) muscle. (d) The activity of catalase in liver. Values are the mean ± SEM. $n = 5$ per group. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. $^{\#}P < 0.05$, significantly different from the normal mice. $^{*}P < 0.05$, significantly different from the control mice.

of the control group, with no change in glycogen content. In contrast, EJ increased glycogen and citrate synthase contents and SOD activity within the muscle tissues (EDL, TA, soleus, and gastrocnemius muscles). Therefore, further research based on muscle tissue types should be conducted to elucidate the detailed effects of EJ on muscle biomarkers. In the CFS mice group, compared to the acute-exercise-treated mice group, hepatic glycogen levels significantly decreased, but muscle glycogen levels remained unchanged [53]. An antifatigue material, *Sarcodon imbricatus* significantly enhanced glycogen levels in the liver of acute-exercise-treated mice [53]. This response was similar to our research findings.

Published literature indicates that fatigue was one of the most common symptoms of COVID-19, and in many patients even after recovery. Fatigue caused by COVID-19 is pathological fatigue, and as mentioned above, the biomarkers for the pathophysiology of fatigue are similar. Therefore, we assume that EJ might have the ability to

alleviate pathological fatigue caused by COVID-19. However, because exercise-induced physiological fatigue can be distinguished from pathological and psychological fatigue, further research on pathological and psychological fatigue models should be conducted to confirm the improvement effect of EJ on COVID-19-induced pathological fatigue.

In the present study, administration of EJ and CA decreased the levels of IL-1β, IL-4, IL-6, and TNF-α and increased the activities of SOD and catalase in C2C12 myotubes and after FST and TST in animal models. Therefore, these data suggest that EJ and CA display antifatigue potential through their ability to increase antioxidative activity. Additionally, we confirm that CA plays an important role in fatigue by being an active component of EJ. In conclusion, this study showed that EJ and CA can significantly relieve excessive fatigue. Therefore, we suggest that EJ may have a potential therapeutic role in relieving fatigue in people suffering from the fatigue symptom.

Data Availability

The data used to support the findings of this study are included within the article.

Additional Points

Practical Applications. According to the results of several studies, fatigue ranked the highest among the long-term effects of infectious diseases such as coronavirus disease 2019 (COVID-19), and many people complained of fatigue even after recovery. We confirmed the antifatigue potential of loquat leaf (*Eriobotrya japonica* Lindl., EJ) in myotubes and animal models. Current findings suggest that EJ improved fatigue via increasing antioxidant and antiinflammatory activities. Based on these results, EJ may have a potential therapeutic role in relieving fatigue in people suffering from the fatigue symptom.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary Table 1: Kovats retention index of compound in EJ. Supplementary Figure 1: UHPLC-MS chromatograms of chlorogenic acid (CA) and euscaphic acid (EA) in EJ (Loquat leaf extract). (a) UHPLC chromatogram of EJ. (b) CA MS spectra of the peak. (c) EA MS spectra of the peak. Arrows indicated the CA or EA. Supplementary Figure 2: body weight changes in FST and TST. Body weights of mice were measured for 28 days. WEJ, EJ water extract; EEJ, EJ ethanol extract; and CA, chlorogenic acid. Data represent means ± SEM. (*Supplementary Materials*)

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