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Research Article

Antrodia cinnamomea Extract Attenuates Cisplatin-Induced Muscle Atrophy, Apoptosis, and Cell Growth Suppression

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Antrodia cinnamomea (AC), a potential medicinal fungus which possesses anti-inflammatory and anticancer activities, has been previously reported to be able to ameliorate muscle wasting in cisplatin-treated lung tumor-bearing mice via AC extract. However, whether AC extract modulates muscle cell differentiation, apoptosis, and cell cycle progression remains unclear. Here, we show that the ethanol extract of AC (EEAC) significantly restored cisplatin-reduced quadricep mass in mice. EEAC attenuated cisplatin/gemcitabine (C/G)-suppressed elongated myotube formation, which is differentiated from C2C12 cells. Moreover, EEAC synergized with C/G to inhibit cell growth of LLC1 cells, whereas EEAC attenuated C/G-reduced proliferation of C2C12 cells. Although EEAC protected C/G-induced apoptosis of both LLC1 and C2C12, EEAC suppressed cyclin D expression in LLC1 while partially restoring C/G-reduced cyclin D level in C2C12 cells. Finally, as p53 and p21 participate in inducing skeletal muscle atrophy, we found that C/G induced p53 and p21 expression in C2C12 cells but with its effect significantly attenuated by EEAC. Our findings indicate that AC extract is a potential natural agent for attenuating cisplatin-induced muscle atrophy. *Practical Applications*. Muscle atrophy is one of the major side effects caused by chemotherapy. In addition to inducing cell death, chemotherapeutic agents inhibit cell growth of both cancer and normal cells as well. Our current findings indicated that AC extract attenuates cisplatin-induced muscle wasting and apoptosis of C2C12 cells. AC extract is a potential dietary supplement used for ameliorating chemotherapy-induced muscle atrophy.

1. Introduction

Cancer patients receiving chemotherapy suffer from side effects of nausea, diarrhea, anorexia, and extensive loss of skeletal muscle mass. Among these symptoms, muscle wasting is a hallmark of cachexia induced by chemotherapeutic drugs [1, 2]. The antineoplastic drugs, cisplatin and other platinum-based drugs, are widely used in cancer treatment. Platinum-DNA adducts formed in cisplatin-treated cells induce cytotoxicity [3]. Despite killing cancer cells, cisplatin-induced muscle atrophy is reported to be caused by modulating IGF-1 signaling, proinflammatory cytokines, oxidative stresses, autophagy, abnormal proteolysis through ubiquitin-proteasome pathway, and increase of apoptosis as well as cell cycle arrest [4, 5].

Apoptotic cells share several common features including cell shrinkage, nuclear fragmentation, chromatin condensation, and DNA fragmentation [6–8]. The intrinsic pathway of apoptosis is associated with mitochondria dysfunction and cytochrome c released into cytosol from mitochondria. Cytochrome c activates apoptotic protease activating factor 1 (Apaf-1) complex, consequently stimulating downstream caspase cascades, including cleaving and activating procaspase-3 and poly (ADP-ribose) polymerase (PARP) to induce apoptosis [6–8].

A cell cycle is a series of events that occur when a cell proliferates, which includes the process of DNA replication and division into two individual cells. It contains four stages, G1/S/G2/M phases, and these stages are regulated by various factors including cyclins, cyclin-dependent kinases (cdk), cdk modulators, tumor suppressor proteins, and transcriptional factors [9–11]. The activation of the p53 tumor suppressor contributes to the induction of cell growth arrest gene $p21^{Cip1/Waf1}$ [12]. It was recognized that p53 and p21 are crucial factors in modulating cell cycle arrest and apoptosis [12]. An earlier study indicated that p53 participates in apoptosis during unloading-induced muscle atrophy [13]. Chronic activation of p53 induces muscle atrophy consistent with sarcopenia in aging muscle [14]. Limb immobilization induces muscle wasting and increases p53 expression, whereas the knockout p53 gene is partially resistant to immobilization-induced muscle atrophy [15]. In addition, it has been demonstrated that p21 was specifically upregulated by myostatin, a negative regulator of muscle growth in myoblasts [16], and mRNA levels of both myostatin and p21 were increased in cisplatin-treated mice [5]. These results reveal that the p53/p21 signal cascade plays a crucial role in inducing muscle loss.

Antrodia cinnamomea (AC, synonyms: *Antrodia camphorata*; *Taiwanofungus camphoratus*) is a potential medicinal fungus that grows in the inner cavity of *Cinnamomum kanehirai,* an endemic plant of Taiwan [17, 18]. AC is considered to have potential properties for anticancer activity [18–25], anti-inflammation [26], antioxidative stresses [27–29], and attenuating alcoholic liver injury [18, 30]. These protective characters come from the mycelium or fruiting bodies of AC through water or alcohol extraction [30, 31] and unique triterpenoids isolated from AC [32]. In addition, earlier studies indicated that AC extract possesses potential anticachexia and antifatigue effects in chemotherapeutic drug-treated mice [33, 34]. However, the molecular mechanism by which AC attenuates chemotherapeutic drug-induced muscle wasting remains unclear. In this study, we established a cisplatin-induced sarcopenia mice model and found that ethanol extracts of AC (EEAC) have a protective effect on the attenuation of cisplatin-caused muscle wasting. We further demonstrated that EEAC ameliorates cisplatin/gemcitabine (C/G)-suppressed myotube formation and attenuates both C/G-reduced cell proliferation and apoptosis of C2C12 cells. Finally, we found that C/G increased p53 and p21 levels, but this effect was significantly attenuated by EEAC in C2C12 cells. Taken together, our findings provide evidence that AC extract can be used as a potential dietary supplement used for ameliorating chemotherapy-induced muscle atrophy.

2. Materials and Methods

2.1. EEAC and Reagents. EEAC used in this study was kindly provided by the Balay Biotechnology Corporation, Taipei, Taiwan. The components of EEAC were analyzed and confirmed by the HPLC system consisting of a Spectra SYSTEM P1000 quipped with Spectra SYSTE AS3000 autosampler, Surveyor PDA plus detector, and a reverse-phase column (C18, 4.6×250 mm, Thermo, BDS HYPERSIL). The marker triterpenoids standards of ACF, including antcin A, B, C, H, K, dehydrosulphurenic acid, and dehydroeburicoic acid, were used as external standards to identify the specific components in EEAC. The quantitative LC-MS/MS analysis to determine the concentrations of 7 marker triterpenoids in EEAC was performed by ABM International Lab Inc. (New Taipei City, Taiwan). cis-Diammineplatinum (II) dichloride (cisplatin), gemcitabine hydrochloride, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Experimental Animals. All experimental procedures performed in this study conform to the guidelines and regulations approved by the Institutional Animal Care and Use Committee of the National Health Research Institutes (NHRI-IACUC-108120-A) of Taiwan. 8- to 10-week-old C57BL/6 mice were purchased from the National Laboratory Animal Center and housed in a specific pathogen-free facility of the National Health Research Institutes of Taiwan. Mice were randomized into four groups including control (CTR, $n = 6$), EEAC ($n = 6$), cisplatin alone (cisplatin, $n = 6$), and EEAC combined with cisplatin (EEAC + cisplatin, $n = 6$). Mice were fed with EEAC (30 mg/kg body weight) by oral gavage once daily for 8 days. At day 3, muscle atrophy was induced by intraperitoneal injection of cisplatin (40 mg/kg body weight) once daily for 6 days [35]. Control mice were injected with DMSO diluted in an equal volume of phosphate-buffered saline solution. The mice were sacrificed at day 8, and the mass of the quadriceps femoris was measured after harvesting.

2.3. Cell Culture and C2C12 Differentiation. LLC1 mouse Lewis lung carcinoma cell line and mouse myoblast C2C12 cells were obtained from the ATCC (Rockville, Maryland, USA). Both cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Invitrogen-Gibco) and penicillin-streptomycin (100 U/ml, Invitrogen-Gibco). For differentiation of C2C12 myoblasts into myotubes, confluence C2C12 cells were incubated in differentiation medium (DMEM containing 2% horse serum and penicillin-streptomycin) with EEAC and/or C/G, and the medium was changed once every day for six days. Differentiated C2C12 cells were fixed and permeabilized with iced methanol (Sigma-Aldrich) and then incubated in 50 nM NH_4Cl (Sigma-Aldrich) for 15 min. Fixed cells were incubated with MHC antibody (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight followed by incubation with HRP-conjugated secondary antibody (Thermo Fisher Scientific). The expression of MHC was visualized with a DAB substrate kit (Thermo Fisher Scientific). The nuclei were stained with hematoxylin (Sigma-Aldrich), and the values of fusion index were indicated by the ratio of MHC-positive nuclei number to total nuclei number of total cells.

2.4. Western Blot Analysis. For determination of protein levels, cells were harvested and lysed by iced RIPA buffer (Millipore, Temecula, CA, USA) containing protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Lysates were centrifuged at 15,000 rpm for 10 minutes. Protein concentrations of supernatant were determined using Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were loaded on gradient SDS-PAGE gels. Gels were transferred to 0.22 μM NC membranes (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and then probed with specific antibodies against cleaved caspase-3, cyclin D, p53, p21 (Cell Signaling, Danvers, MA, USA), and β-actin (Sigma-Aldrich). The membranes were immersed in 0.1% PBST containing horseradish peroxidase-conjugated secondary antibodies (Cell Signaling), and the protein levels were determined using enhanced chemiluminescence reagents (Millipore).

2.5. Real-Time Quantitative PCR (RT-qPCR). Total RNA was isolated by RNAzol® RT (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's protocol, and cDNA was synthesized using the ABI RT Kit (ABI Applied Biosystems, Waltham, MA, USA). The cDNA was applied to real-time quantitative PCR instruments (ABI ViiA 7, ABI Applied Biosystems) using SYBR Green (Kapa Biosystems, Woburn, MA, USA). Primer sequences for qPCR are used in this study of p53 (F: GAGGCCGGCTCT GAGTATACC; R: GGCAGGCACAAACACGAACC);p21 (F: CCAATCCTGGTGATGTCCGA; R: AGTCAAAGT TCCACCGTTCTCG); and GAPDH (F: CGCTCTCTGCTC CTCCTGTT; R: CCATGGTGTCTGAGCGATGT). All PCRs were performed in triplicate. Raw data were analyzed by using QuantStudio™ Real-Time PCR Software. The quantity of the specific genes was normalized with GAPDH of the same sample. The fold change of ΔΔCt was determined as the ratio compared to each control sample.

2.6. Statistical Analysis. Data were analyzed by Student's *t*-test between different groups, with $p < 0.05$ considered statistically significant.

3. Results

3.1. EEAC Ameliorated the Cisplatin-Induced Muscle Loss in Mice. The quality and compositions of EEAC used in this study were confirmed and analyzed by HPLC. EEAC contains 7 marker triterpenoid ingredients of AC including antcin A (at 74.7 min), B (at 64.4 and 65.4 min), C (at 42.2 and 45.7 min), H (at 45.0 min), K (at 20.1 and 21.0 min), dehydrosulphurenic acid (at 57.4 min), and dehydroeburicoic acid (at 84.9 min). The amounts of antcin A, antcin B, antcin C, antcin H, antcin K, dehydroeburicoic acid, and dehydrosulphurenic acid were abundantly detected in EEAC (Table 1).

To evaluate the protective efficacy of EEAC on chemotherapeutic drugs-induced muscle wasting, we established a mice muscle atrophy model caused by cisplatin. C57BL/6 mice (8–10 weeks old) were fed with EEAC (30 mg/kg) or ethanol control by oral gavage once daily for 8 days. At day 3, mice were intraperitoneally injected with cisplatin (40 mg/ml) once daily for 6 days [35] (illustrated scheme in Figure 1(a)). Injection of cisplatin dramatically reduced the body weight of mice, whereas oral gavage of EEAC has no significant effect on the fluctuation of body weight. On day 8, the mice were sacrificed, and the mass of quadriceps femoris was measured. Cisplatin injection significantly reduced the mass of mouse quadriceps when compared with the control and EEAC groups (Figure 1(b)). Intriguingly, EEAC treatment ameliorates cisplatin-induced muscle loss of the quadriceps (Figure 1(b)). These results reveal that EEAC may play a protective role in chemotherapy-induced muscle atrophy.

Table 1: The marker triterpenoid in EEAC measured by LC-MS/MS.

Triterpenoids	LC/MS quantitative result (ppm)
Antcin A	3123.0
Antcin B	3418.5
Antcin C	34327.0
Antcin H	10332.5
Antcin K	29308.0
Dehydroeburicoic acid	3229.5
Dehydrosulphurenic acid	28682.5
Total	112421.0

3.2. EEAC Impaired C/G-Suppressed Myotube Formation of C2C12 Cells. To investigate the effect of EEAC and chemotherapeutic drugs-regulated myotube formation, mouse myoblast C2C12 cells were treated with 2% horse serum for 6 days to differentiate into myotubes (representative image in Figure 2(a)). Myosin heavy chain (MHC)-positive multinucleated cells were examined by immunohistochemistry staining with its specific antibody, and the nuclei were stained with hematoxylin of differentiated elongated myotubes (Figure 2(b)). The values of the fusion index demonstrating the capability of myotube formation were compared within differently treated groups (control, EEAC, C/G, and EEAC + C/G). We found that chemotherapeutic drugs C/G inhibited myotube formation whereas EEAC possessed no significant effect on myoblast (Figures 2(a)–2(c)). Intriguingly, C/G-suppressed myotube formation was ameliorated by EEAC treatment (Figure 2(c)).

3.3. Differential Effects of EEAC on C/G-Regulated Cell Proliferation and Apoptosis in LLC1 and C2C12 Cells. To investigate the suppressive effects of C/G on myoblasts and cancer cells, C2C12 and LLC1 cells were treated with C/G and/or EEAC for 24 hours, and the viable cell number was calculated by 0.4% trypan blue staining (Figure 3(a)). EEAC partially reduced cell numbers of LLC1, but this reduction was not significant on C2C12 cells (Figure 3(b)). EEAC synergized with C/G to reduce the cell number of LLC1 cells, but EEAC attenuated the suppressive effect caused by C/G on C2C12 cells (Figure 3(b)). We then examined whether

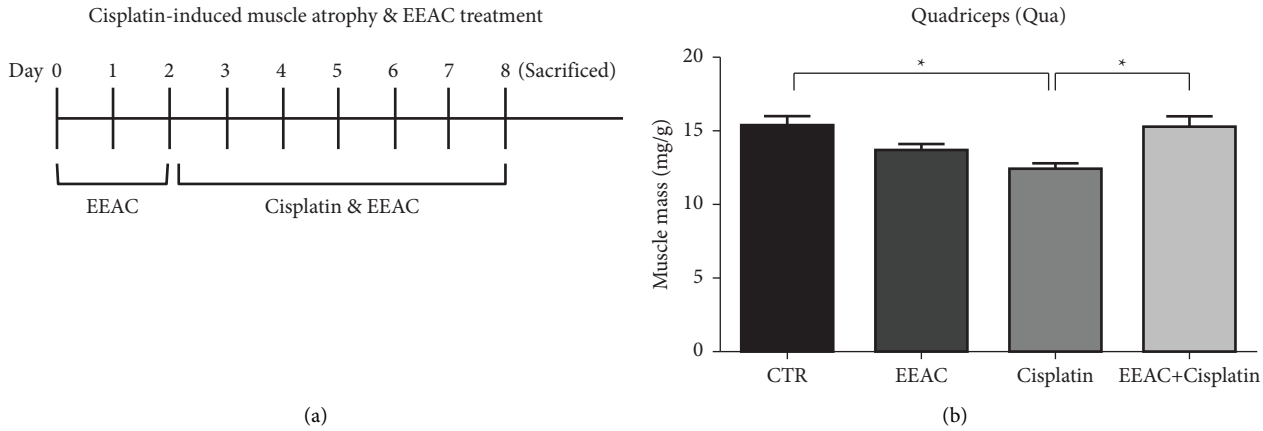

Figure 1: EEAC ameliorated the cisplatin-induced muscle loss in mice. (a) Scheme of the cisplatin-induced muscle atrophy and EEAC treatment in mice. C57BL/6J mice were fed with 30 mg/kg EEAC by oral gavage once daily for 8 days. Cisplatin (40 mg/kg) was intraperitoneally injected once daily since day 3. Mice were sacrificed on day 8. (b) The muscle mass (mg) was measured and normalized by body weight (g). *: A $p < 0.05$ was indicated when comparing the differences between two indicated variables.

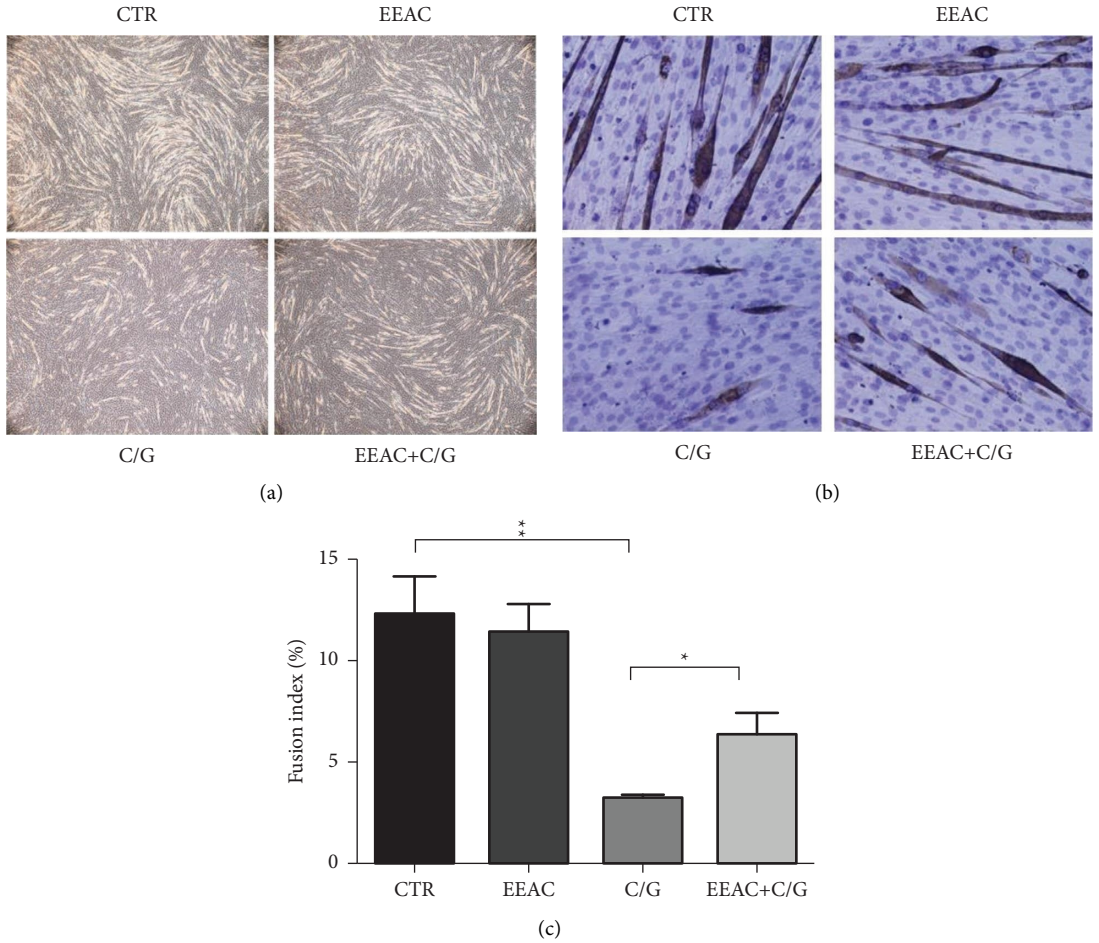

Figure 2: EEAC impaired C/G-suppressed myotube formation of C2C12 cells. C2C12 cells treated with control vehicle (CTR), EEAC (10 μg/ml), C/G (1 μM cisplatin and 1 μM gemcitabine), and EEAC combined with C/G (EEAC + C/G) were differentiated into elongated myotubes. (a) Representative images of C2C12 differentiated elongated myotubes. (b) Representative histological images of MHC-positive and multinucleated myotubes. (c) Fusion index was indicated as number of nuclei in MHC-positive myotubes divided by total number of nuclei. ** $p < 0.01$; * $p < 0.05$.

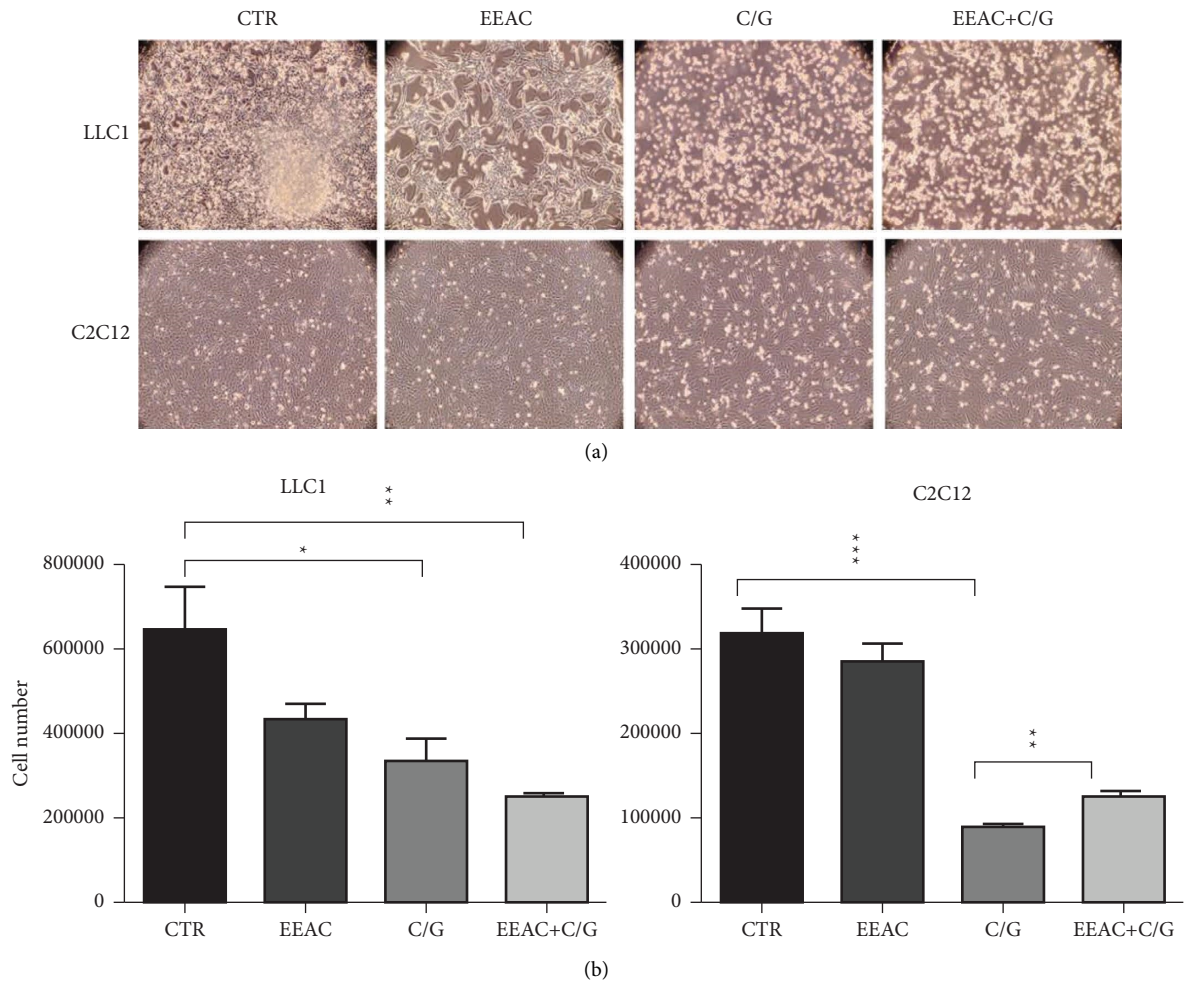

Figure 3: Differential effects of EEAC on C/G-reduced cell proliferation in LLC1 and C2C12 cells. (a) Representative images of LLC1 and C2C12 cells treated with control vehicle (CTR), EEAC (10 μg/ml), C/G (1 μM cisplatin and 1 μM gemcitabine), and EEAC combined with C/G (EEAC + C/G) for 24 hours. (b) EEAC synergized with C/G to reduce the number of LLC1 cells but attenuated the suppressive effect caused by C/G on C2C12 cells. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

EEAC modulates C/G-induced apoptosis and cell cycle progression in LLC1 and C2C12 cells by Western blot analysis. The level of cleaved caspase-3 was abundantly increased by C/G treatment, but this effect was significantly attenuated by EEAC in both LLC1 and C2C12 cells (Figure 4(a)). Intriguingly, EEAC inhibited cyclin D expression in LLC1 cells regardless of C/G treatment (Figure 4(b), upper panel). Meanwhile, EEAC applied alone had no effect, but EEAC impaired C/G-reduced cyclin D expression in C2C12 cells (Figure 4(b), lower panel). These results indicated that EEAC possesses differential effects on apoptosis and cell cycle repression in LLC1 and C2C12 cells.

3.4. EEAC Attenuated C/G-Induced p53 *and* p21 *Expression of C2C12 Cells.* It has been reported that p53 and p21 play crucial factors in regulating cell cycle arrest, thereby promoting muscle atrophy. To elucidate whether p53 and p21 expressions are involved in EEAC and C/G-regulated muscle atrophy, we determined p53 and p21 expression in EEAC and C/G-treated C2C12 cells by Western blot analysis and qPCR. We found that C/G abundantly induced p53 and p21 expression in C2C12 cells. EEAC alone had no effect, but it significantly attenuated C/G-induced p53 and p21 expression of C2C12 cells (Western blot analysis in Figure 5(a); qPCR analysis in Figure 5(b)). These results suggest that EEAC protects C/G-induced muscle atrophy which is potentially mediated by regulating p53/ p21 signaling.

4. Discussion

Many studies had demonstrated that AC extract exhibits anticancer activity by reducing cancer cell proliferation and tumor growth. A sulfated galactoglucan isolated from AC and ethanol extract of AC fruiting bodies suppressed cancer cell migration by inhibiting the signaling pathways of metalloproteinases, ERK, JNK, p38, PI3K/Akt, and TGF/FAK/Slug in human lung cancer cells [20, 36, 37]. The ingredients isolated from AC, including 4-acetylantroquinonol B, antrocin, and antrodan, have been demonstrated to arrest cell proliferation and suppress cell migration and invasion of

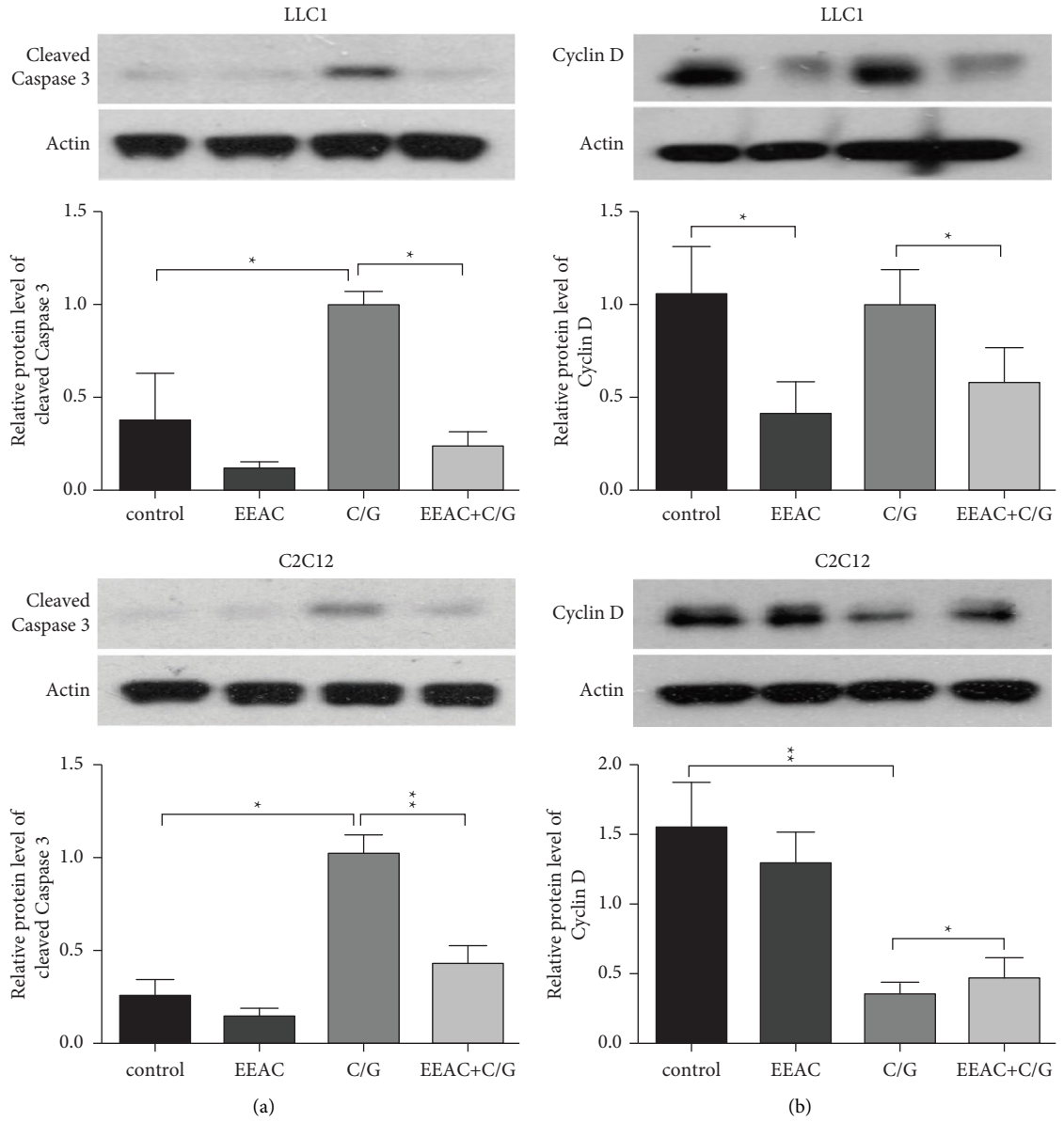

Figure 4: Differential effects of EEAC on C/G-induced apoptosis and cell cycle arrest in LLC1 and C2C12 cells. LLC1 and C2C12 cells were treated with control vehicle, EEAC (10 μg/ml), C/G (1 μM cisplatin and 1 μM gemcitabine), and EEAC combined with C/G (EEAC + C/G) for 24 hours. Levels of cleaved caspase-3 and cyclin D were determined by Western blotting analysis. (a) C/G increased caspase-3 cleavage and activation, while EEAC attenuated C/G-induced caspase-3 levels in both LLC1 and C2C12 cells. (b) EEAC suppressed cyclin D expression whereas C/G possesses no effect on LLC1 cells. In contrast, C/G decreased cyclin D expression in C2C12 cells, but it can be partially restored by EEAC. Lower panels, densitometric and statistical analysis. $^{**}p < 0.01$; $^{*}p < 0.05$.

hepatocellular carcinoma, lung carcinoma, bladder cancer, and breast cancer cells [32, 38–41]. Moreover, the anticancer effects of AC extract include synergizing chemotherapeutic drugs to increase apoptosis and cell cycle arrest of cancer cells, thereby enhancing the sensitivity of chemotherapy [19, 20, 25, 38, 42–44]. AC also enhances the chemotherapy sensitivity of 5-FU of colon cancer [22]. In addition, AC targets cancer-initiating cells by increasing cancer cell differentiation, thereby reducing cancer stemness and tumorigenesis by regulation of STAT3/Src signaling in neck squamous cell carcinoma cells and upregulation of miR-142-3p in colon cancer [22, 45].

Results from many studies reveal the potential molecular mechanism of how chemotherapeutic drugs induce cell cycle repression and apoptosis. It has been reported that cisplatin and gemcitabine cause cell cycle arrest and apoptosis through activation of JNK, p38, γ-histone H2AX, DNA damage response proteins Chk-1/Chk-2, p53, PARP, and caspase-3 in various cell types [46–50]. In this study, we focused on elucidating the effects of EEAC on chemotherapeutic drug-arrested cancer cells and induced muscle atrophy. Because muscle wasting is one of the common cachectic effects caused by chemotherapy, we investigated whether EEAC possesses antimuscle atrophy effects induced

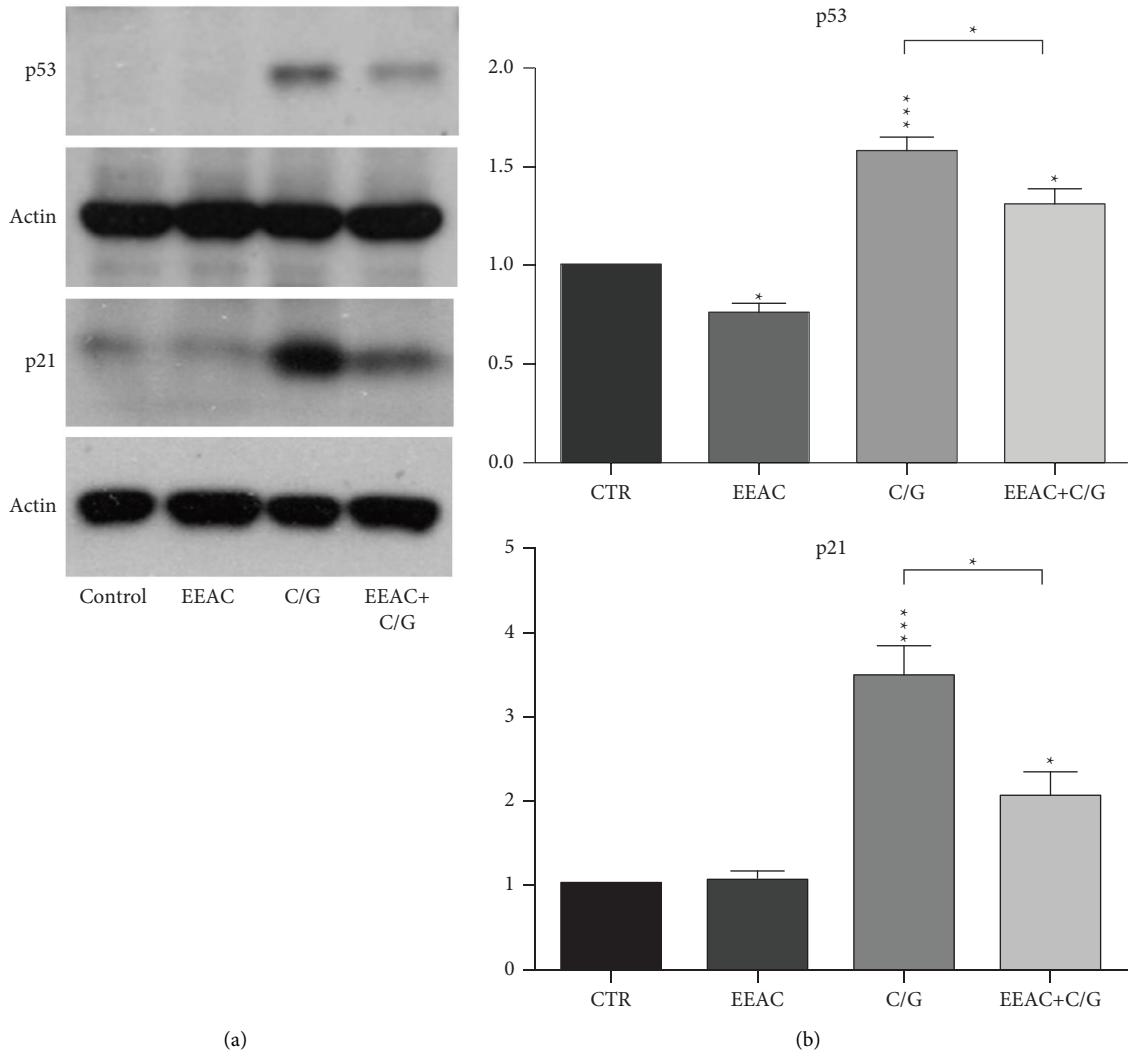

Figure 5: EEAC attenuated C/G-induced p53 and p21 expression of C2C12 cells. C2C12 cells were treated with control vehicle, EEAC (10 μg/ml), C/G (1 μM cisplatin and 1 μM gemcitabine), and EEAC combined with C/G (EEAC + C/G) for 24 hours. (a) Expressions of p53 and p21 were determined by Western blotting analysis. (b) mRNA levels of p53 and p21 were examined by qPCR. *** $p < 0.001$; * (or **) $p < 0.01$.

by C/G and its potential regulatory mechanism. We first found that cisplatin-induced mass loss of quadriceps femoris in mice was restored by EEAC (Figure 1(b)). This finding was supported by an earlier study which indicated that AC extract has an anticachectic effect in lung tumor-bearing mice with chemotherapy [45]. In addition, our findings indicated that C/G treatment increased levels of cleaved caspase-3 whereas EEAC attenuated this apoptotic effect in both LLC1 and C2C12 cells (Figure 4(a)). Intriguingly, EEAC partially restored C/G-reduced cyclin D expression in C2C12 but not in LLC1 cells (Figure 4(b)). These results indicated a differential regulatory mechanism of EEAC on cisplatin-induced apoptosis and cell cycle arrest between cancer and muscle myoblast cells.

In this study, we showed that EEAC attenuated chemotherapeutic drug-induced muscle atrophy in both *in vitro* and *in vivo* models. We found that C/G suppressed myotube differentiation and elongated myotube formation of C2C12 cells, whereas EEAC significantly restored this effect (Figure 2). Although EEAC protected C/G-induced apoptosis in both LLC1 and C2C12 cells, EEAC induced cell arrest of LLC1 cells but attenuated C/G-suppressed cyclin D expression in C2C12 cells (Figure 4(b)). In addition, we provide a potential mechanism of EEAC to protect C/G-induced muscle atrophy by inhibiting p53/p21 signaling. However, the mechanism behind how EEAC modulating p53/p21 activation abrogates C/G-induced muscle atrophy needs further investigation.

5. Conclusion

Chemotherapy or sarcopenia-induced muscle loss is repaired by myoblast proliferation and differentiation into myotubes. In this study, we provide evidence that AC extract attenuates cisplatin-induced muscle wasting, apoptosis, and cell cycle repression of C2C12 cells. AC extract is a potential natural supplement for attenuating chemotherapy-induced muscle atrophy.

Abbreviations

AC: *Antrodia cinnamomea*
C/G: Cisplatin/gemcitabine
CDK: Cyclin-dependent kinases
DMEM: Dulbecco's modified Eagle's medium
DMSO: Dimethyl sulfoxide
EEAC: Ethanol extract of AC
ERK: Extracellular signal-regulated kinase
FAK: Focal adhesion kinase
JNK: c-Jun N-terminal kinase
MHC: Myosin heavy chain
TGF: Tumor growth factor.

Data Availability

The data (including figures and table) used to support the findings of this study are included within the article.

Conflicts of Interest

All authors declare that there are no conflicts of interest.

Authors' Contributions

Shu-Man Liang and Chien-Liang Kuo contributed equally to this study.

Acknowledgments

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