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Effects of Fermented Lemon Peel by *Lactobacillus plantarum* PM-A87 Protects Skeletal Muscle Cell

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Abstract This study investigates the effect of lemon peel fermented with *Lactobacillus plantarum* PM-A0087 on the contents of total polyphenols, total flavonoids and eriocitrin. Results show that eriocitrin content of fermented lemon peel was significantly higher than unfermented, and had effective capacity of scavenging for superoxide radical and correlated with flavonoid component increased. Treatment with the lemon fermented product did not affect cell viability at concentrations up to 10 mg/mL. The purpose of this study was to evaluate the efficacy of fermented lemon peel in protecting murine skeletal muscle cells from H_2O_2 and dexamethasone induced oxidative stress. The present results indicate that fermented lemon peel protects C2C12 myotubes against peroxide-induced oxidative stress. Myotubes that had atrophied due to dexamethason exposure exhibited a notable increase in diameter upon treatment with the fermented lemon peel at a 2 mg/mL concentration. These findings indicate the potential of the lemon fermented product in countering dexamethason-induced muscle atrophy in C2C12 myotubes.

Keywords: *lemon peel, eriocitrin, C2C12 myoblast, Sarcopenie*

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1. Introduction

Sarcopenia is a degenerative disease that involves progressive loss of skeletal muscle mass with age. However, no suitable therapeutic drugs are presently useful [1]. The aim of this study was to evaluate the effects of fermented lemon peel by *Lactobacillus plantarum* PM-A87 in protecting murine skeletal muscle cells (C2C12 myoblas) from dexamethasone induced oxidative stress.

Flavonoids with different health characteristic including antioxidant, anti-obesity, anti-diabetes, and anti-inflammation [2]. Recent study demonstrate various flavonoids, such as rutin, nobiletin, eriocitrin, quercetin and epicatechin have a positive effect on skeletal muscle health in vitro and in mouse models [3]. Epicatechin increasing Myf5 and decreased myostatin in skeletal muscle of aged mice [4]. Previous studies have reported that eriocitrin of lemon suppresses disuse muscle atrophy by reducing oxidative stress [5]. Therefore, eriocitrin maybe the main active substance beneficial to muscle health. The amount of eriocitrin in lemon is richest in citrus species. Lemon peel are generated during processing and accounts for 50% of the total dry weight of lemon [6]. Because of the high amount of minerals and dietary fiber, lemon peel should be suitable substrates for probiotic growth [7]. Additionally, the reuse of agricultural by-products through fermentation plays an important role due to its sustainable benefits. Fermentation increases the biochemical and activity compounds of the substrate, enhance the production of bioactive compounds after fermentation process [8]. In our preliminary study, we found similar results, fermented lemon peel by *L. plantarum* PM-A87 significantly increased eriocitrin levels, it was hypothesized that the fermented lemon peel may protects skeletal muscle cell. In this study, dexamethasone induced muscle cells were treated with fermented lemon peel to understand the anti-sarcopenia potential of fermented lemon peel.

2. Materials and Methods

2.1. Chemicals and Reagents

Eroicitrin, Folin–Ciocalteau's, 1,1-diphenyl-2-picrylhydrazyl (DPPH), Methanol and formic acid were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Dexamethasone (DEX), hydrogen peroxide (H_2O_2), Giemsa stain, trypsin-EDTA, and trypan blue were purchased from

Sigma Chemical Co. (St. Louis, MO, USA). The fetal bovine serum (FBS) was purchased from Cytiva (Marlborough, MA, USA). The Antibiotic-Antimycotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B), Dulbecco's modified Eagle's medium (DMEM), and phosphate buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 10 N NaOH solution were purchased from APOLO biochemical Inc. (New York, NY, USA). ROS Assay Kit was purchased from Dojindo Molecular Technologies (Kumamoto, Japan).

2.2. Plant Material and Fermentation

Eureka lemons (*Citrus limon*), a Citrus variety, were purchased by local farmers (Pingtung County, Taiwan). *Lactobacillus plantarum* PM-A87 (BCRC910475) is a self-isolating strain and stored at -80 ℃. Weigh an appropriate amount of lemon peel and glucose to prepare the fermentation culture medium. Add 10 % of *L. plantarum* PM-A0087 bacterial liquid pre-cultured for 24 hours, mix it with the fermentation culture medium, and co-fermentation at 35°C in an incubator for 72 hours. Centrifuge the fermentation broth for 5 minutes at 3000 rpm to obtain the supernatant and then dried into a powder (fermented lemon peel, FLP).

2.3. Determination of Total Phenolic and Flavonoid

2.3.1. Total Phenolic Content (TPC)

The TPC were measured using Folin–Ciocalteau methods [9]. Pipette 100 µL standard gallic acid (10-500 µg/mL) or the fermented lemon peel samples were mixed with 1 mL of 2 % sodium carbonate. After 10 min incubation, 100 µL of 0.5 N Folin–Ciocalteus reagent was added to each test tube and allowed to stand for 30 min in the dark at room temperature. The absorbance of the mixture was detected at 750 nm in an automated microplate reader (Tecan Infinite 200 Pro, Switzerland)). TPC was expressed as mg gallic acid equivalents per gram of dried weight (mg GAE/g DW).

2.3.2. Total Flavonoid Content (TFC)

The TFC were measured using the method of Kim [10] with some modifications. Each sample (200 µL) or a quercetin standard (10-1000 µg/mL) was mixed with distilled water and 5 % $NaNO_2$ (75 µL). After 6 min incubation, 100 µL of 10 % $AlCl_3$ was added to each test tube and kept for 10 min in the dark. The absorbance of the mixture was detected at 420 nm in an automated microplate reader (Tecan Infinite 200 Pro, Switzerland). TFC was reported as mg quercetin equivalents per gram of dried weight (mg QE/g DW).

2.4. HPLC-ESI-MS/MS Analysis of the Fermented Lemon Peel

HPLC-ESI-MS/MS analysis was performed using an Nexera XR-20A system (Shimadzu 8045, Kyoto, Japan) coupled to an API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystem, Foster City, CA, USA). Chromatographic separation was performed on an Aglient C18 column (150×4.0 mm I.D, 5 µm; Waters, Ireland). The mobile phase consisted of 0.1 % formic acid aqueous solution (solution A) and acetonitrile (solution B) and a gradient elution program was set as follows: solution A, 90–60% (0–3.5 min), 60–40% (3.5–6 min), 40–0% (6–9 min), 0–40% (9–10min) and 40–60% (10–12 min), 50–90% (12–15min). The column temperature was fixed at 40°C, the flow rate was set 0.5 mL/min, and injection volume was 2 µL. The electrospray negative mode was selected as an ion source for eriocitrin detection. The quantification was performed in multiple reactions monitoring (MRM) analysis model. The optimized ESI source parameters were as follows: ion spray voltage, -4500 V; nitrogen nebulizer gas pressure, 50 psi; nitrogen curtain gas pressure, 12 psi; heater temperature, 450 °C; collisionally activated dissociation (CAD) gas, 10 psi. The precursor-to-product ion transitions were m/z 595/163. All data acquisition and processing were performed using Analyst 1.6.3 software (AB SCIEX, Concord, ON, Canada).

2.5. Antioxidant Activity

DPPH radical scavenging activity was measured according to a Li et al. [11] with slight modifications. Briefly, 100 µL of each extract solution were mixed with 0.2 mM DPPH solution in methanol (100 µL). The mixture was vortexed and incubated in the dark at room temperature for 15 min. The absorbance was recorded at 517 nm in an automated microplate reader. The data were expressed as the half-maximal inhibitory concentration (IC_{50}), which is the concentration of sample that inhibits 50 % of the DPPH radical.

2.6. C2C12 Myoblast Culture and Differentiation

Mouse C2C12 myoblasts were purchased from the cell bank of BCRC (Hsinchu, Taiwan). For maintenance of C2C12 myoblasts, the cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Amphotericin B, in a humidified 5% CO2 incubator at 37°C. The growth medium was refreshed at least every 2 days. For the induction of C2C12 myoblast cells to differentiate into myotubes, C2C12 myoblasts at 90% confluence were transferred to differentiation medium (DMEM containing 2% horse serum), and then cultured for 6 days. The growth medium was changed every 2 days.

2.7. Cell Viability

MTT assay was utilized to evaluate the cytotoxic effects of the lemon fermented product. Initially, this product was dissolved in DMEM to achieve a concentration of 62.5 mg/mL. Subsequently, it was titrated with 10 N NaOH until the phenol red in the culture medium turned orange-red, indicating that the medium had reached a pH of 7.2±0.2. To fine-tune the concentration to 56.2 mg/mL, ultrapure water was added. The resultant solution was then sterilized through a 0.22 µm syringe filter (Merck, USA) for aseptic handling.

C2C12 myoblasts were seeded in 96-well plates at a density of 1.2 × 104 cells per well. Following a 24 h incubation period, the cells were treated with various concentrations of the lemon fermented product, ranging from 1 to 50 mg/mL, and incubated at 37°C. Subsequent to the 24 h treatment period, the cells were incubated with 0.5 mg/mL of MTT reagent for 1 h at 37°C. The medium was then discarded, and formazan crystals were dissolved in DMSO. The absorbance at 570 nm was measured using an Infinite® M200 PRO microplate reader (Tecan, Männedorf, Switzerland). Relative cell viability (%) was calculated using the formula: [(A Sample – A Blank) / (A Control – A Blank)] × 100%, where 'A' represents absorbance at 570 nm. The experimental setup included three groups: (i) the sample group, where the OD570 value was taken from wells containing lemon fermented product-treated cells and MTT solution; (ii) the blank group, where the OD570 value was taken only from wells containing medium and MTT solution; (iii) the control group, where the OD570 value was taken from wells containing cells and MTT solution without treatment. To assess the cytoprotective effect of the lemon fermented product against H_2O_2, C2C12 myoblasts were initially incubated in medium with various concentrations of the lemon fermented product, ranging from 1 to 5 mg/mL, for 24 h. Subsequently, the cells were exposed to FBS-free medium with 1 mM H_2O_2 for a duration of 2 h. To evaluate the protective efficacy of the lemon fermented product, an MTT assay was subsequently performed.

2.8. Measurement of Intracellular ROS

Intracellular ROS levels were measured using a ROS assay kit. C2C12 myoblasts were seeded in 96-well plates at a density of 1.2 × 104 cells per well. After a 24 h incubation, the cells were treated with various concentrations of fermented lemon peel (ranging from 1 to 5 mg/mL) and incubated at 37°C. Following this 24 h treatment period, the cells were washed twice with PBS. Subsequently, a DCFH-DA working solution was added to the cells in FBS-free medium and cultured at 37°C for 30 min, followed by a treatment with 1 mM H_2O_2 or medium alone for 1 h. After washing twice with PBS, the fluorescence (excitation/emission at 495 nm/525 nm), reflecting the ROS concentration, was analyzed using an Infinite® M200 PRO microplate reader. The ROS inhibition rate (%) was calculated using the formula: {1-[(L sample – L Blank) / (L Control – L Blank)]} × 100%, where 'L' represents fluorescence intensity. The experimental setup included three groups: (i) the sample group, where the fluorescence intensity was taken from wells containing lemon fermented product, DCFH-DA working solution and H_2O_2-treated cells; (ii) the blank group, where the fluorescence intensity was taken from wells containing DCFH-DA working solution and untreated cells; (iii) the control group, where the fluorescence intensity was taken from wells containing DCFH-DA working solution and H_2O_2-treated cells.

2.9. Measurement of Myotube Diameter in C2C12 Myotubes After DEX and Fermented Lemon Peel (FLP) Treatment

C2C12 myoblasts were differentiated for 6 days, followed by treatment with 100 μM DEX and various concentrations of the fermented lemon peel (2 mg/mL and 5 mg/mL). After 24 h of incubation, the culture medium was removed and the cells were washed once with PBS. Subsequently, myotubes were fixed with 10% formalin solution for 10 min. After removing the liquid, Giemsa stain was added and the cells were left to stand for 2 h. Then, the liquid was removed and the cells were washed with ultrapure water, a total of 5 times. After air-drying, the myotubes and nuclei were observed using a Nikon Eclipse TS100 microscope (Avon, MA, USA), and images were captured with a Tekfar CCD camera (Taichung, Taiwan) at 40× magnification. A total of 100 myotube diameters were measured from at least 10 random fields using TekfarProVision software (Version 4.11; Taichung, Taiwan). The average myotube diameter was calculated as the median value of the measured values.

2.10. Statistical Analysis

All data are presented as the mean ± standard deviation (SD) from three independent experiments. Statistical analysis was conducted using a one-way analysis of variance (ANOVA) followed by Dunnett's test, utilizing IBM SPSS Statistics Version 20 (IBM Corp., Armonk, NY, USA). Differences from the control group were deemed statistically significant at a p-value of less than 0.05.

3. Results

3.1. The Content of Total Polyphenols, Total Flavonoids and Antioxidant Activity

The total phenolic content of fermented lemon peel were expressed as gallic acid equivalent. The total phenolic contents of unfermented and fermented lemon peel were 8.15 ± 0.23 mg and 20.46 ± 0.32 mg GAE/g (Table 1). The total flavonoids content of fermented lemon peel were expressed as quercetin equivalent. The total flavonoid contents of unfermented and fermented lemon peel were 2.71 ± 0.22 mg and 3.75 ± 0.45 mg GAE/g (Table 1). In this study, lemon peel was fermented with *L. Plantarum* A87 and the contents of total polyphenols from 8.15 to 20.46 mg GAE/g, respectively, while flavonoids varied from 2.71 to 3.75 mg QE/g DM. Fermentation is a useful way to treat agriculture waste to become valuable materials. Fermentation is one of the best processes to release phenolics because the enzymes effect produced from the probiotics [8]. Therefore, it is suggested that the increased amounts of total polyphenols and flavonoids in fermented lemon peel maybe caused by the improved release [12]. The radical scavenging activities of the fermented lemon peel were evaluated using DPPH radical scavenging assays. The unfermented and fermented lemon peel had IC50 values of 2.73 ± 0.27and 1.31 ± 0.16 mg/ml, respectively. The results of this study revealed that fermented lemon peel had effective capacity of scavenging superoxide radical and correlated with total phenolic and flavonoids content thus indicating its antioxidant potential. These may be due to the presence of a higher phenolic and flavonoids contents

in the fermented lemon peel, its play an important role as antioxidants in living organisms [13].

Table 1. Antioxidant activities and the contents of total phenolic, total flavonoid and Eriocitrin in fermented Lemon peel. Data values represent the mean ± SD (n = 3)

Group	Unfermented	Fermented
Total phenolic (mg/g)	8.15 ± 0.23 ^{c}	20.46 ± 0.32 ^{a}
Total flavonoid (mg/g)	2.71 ± 0.22 ^{b}	3.75 ± 0.45^{a}
DPPH radical scavenging activity, IC_{50} value (mg/ml)	2.73 ± 0.27^{b}	1.31 ± 0.16^{a}
Eriocitrin (mg/g)	0.64± 0.11^{c}	3.05± 0.29 ^{a}

3.2. Content of Eriocitrin Within Fermented Lemon Peel

Because Eriocitrin is a compound with potential bioactivity, therefore the content of Fermented Lemon Peel should be investigated [14]. In this study, we compared with unfermetation group, the fermented lemon peel contained markedly higher level (Table 1), revealing that fermentation of lemon peel by *L. plantarum* A87 is a promising process to raise Eriocitrin level.

3.3. Effects of the Fermented Lemon Peel on Cytotoxicity in C2C12 Myoblasts

The cytotoxicity of the lemon fermented product was assessed using the MTT assay. Treatment with the lemon fermented product did not affect cell viability at concentrations up to 10 mg/mL, as shown in Figure 1. However, cell viability decreased with treatments at concentrations higher than 10 mg/mL. Therefore, to determine the protective effect of the lemon fermented product against H_2O_2-induced cytotoxicity in C2C12 myoblasts, a concentration range of 1–5 mg/mL was used.

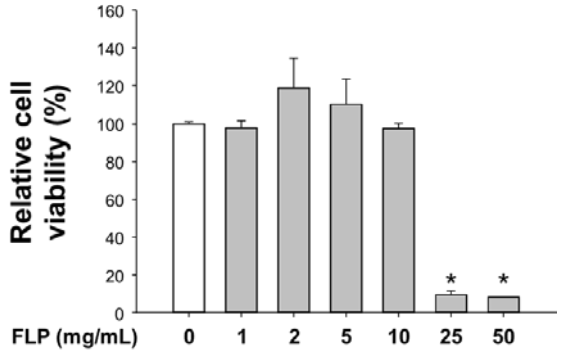

Figure 1. Effects of the fermented lemon peel (FLP) on the growth of C2C12 cells. The cells were treated with various concentrations of the FLP for 24 h, followed by the assessment of cell viability using an MTT reduction assay. *p < 0.05, significant difference compared with the control group.

3.4. Protective Effects of the Fermented Lemon Peel on H_2O_2-induced Cytotoxicity in C2C12 Myoblasts

To assess the cytoprotective effects of the lemon fermented product against H_2O_2-induced oxidative stress, C2C12 myoblasts were pre-treated with varying concentrations of the product for 24 hours prior to exposure to 1 mM H_2O_2 for 2 hours. Exposure to H_2O_2 alone led to a reduction in cell viability by approximately 21.9% compared to untreated control cells (Figure 2). Conversely, pre-treatment with the lemon fermented product at a concentration of 5 mg/mL significantly enhanced cell viability, offering the most pronounced protection against oxidative stress in C2C12 myoblasts.

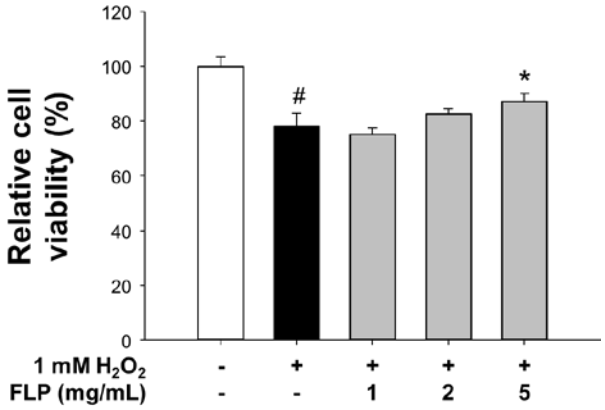

Figure 2. Cytoprotective effects of the fermented lemon peel (FLP) against H2O2-induced oxidative stress in C2C12 cells. #p < 0.05, significant difference compared with the control group. *p < 0.05, significant difference compared with the H2O2-treated group

3.5. Influence of the Fermented Lemon Peel on ROS Production in H_2O_2-treated C2C12 Myoblasts

Given that H_2O_2 cytotoxicity is predominantly mediated through oxidative stress, we explored the impact of the lemon fermented product on H_2O2-induced ROS accumulation by utilizing a ROS assay kit. Relative to the untreated control, intracellular ROS levels were substantially elevated in C2C12 cells following treatment with 1 mM H_2O_2 for 1 h, as depicted in Figure 3. Notably, pretreatment of the C2C12 cells with the lemon fermented product at concentrations of 5 mg/mL and 2 mg/mL resulted in a significant reduction in ROS generation, indicating that the lemon fermented product may trigger an antioxidative defense mechanism within the cells.

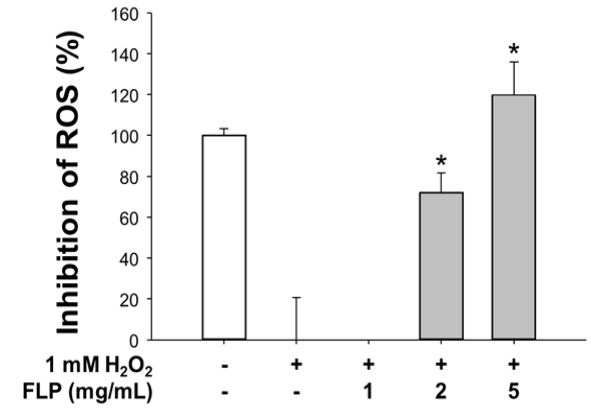

Figure 3. Inhibition of H_2O_2-induced ROS generation by the fermented lemon peel (FLP) in C2C12 cells. *p < 0.05, significant difference compared with the H_2O_2-treated group

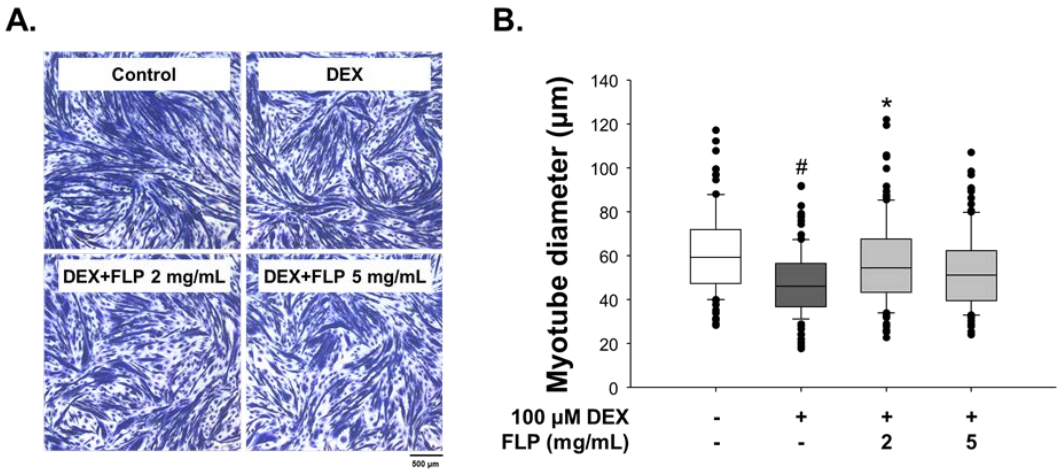

Figure 4. Effects of the fermented lemon peel on myotube diameter in DEX-stimulated C2C12 myotubes. (A) Representative images of C2C12 myotubes treated with 100 μM of DEX and the lemon fermented product. Fixed cells were reacted with the Giemsa stain. The scale bar represents 500 μm. (B) Comparison of myotube diameters among the four treatment groups. $^{\#}p < 0.05$, significant difference compared with the control group. $^{*}p < 0.05$, significant difference compared with the DEX-treated group

3.6. Fermented Lemon Peel Mitigates Dexamethasone-Induced Muscle Atrophy in C2C12 Myotubes

To ascertain if the lemon fermented product could mitigate the effects of DEX-induced myotube atrophy, C2C12 myotubes were subjected to Giemsa staining (Figure 4A). This revealed a significant reduction in myotube diameter following DEX treatment compared to the untreated control (Figure 4B). Conversely, myotubes that had atrophied due to DEX exposure exhibited a notable increase in diameter upon treatment with the lemon fermented product at a 2 mg/mL concentration. These findings indicate the potential of the lemon fermented product in countering DEX-induced muscle atrophy in C2C12 myotubes.

4. Conclusions

Sarcopenia has become a serious health problem in older adults. In this study, we revealed the potential of the fermented lemon peel in countering DEX-induced muscle atrophy in C2C12 myotubes. Fermented lemon peel contain rich flavonoids including eriocitrin, may have a great potential to treat sarcopenia.

Author Contributions

Conceptualization, design, screening, quality analysis, data extraction, data analysis, results interpretation, and manuscript preparation, C.-M.L., T.-Y., H, S-T.L, Z.-Y. J, W.-Z. H; study design, results interpretation, and manuscript check, J-P., W; C.-Y., Y, H.S. H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that there are no conflicts of interest.

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