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Effects of Lactic Acid Bacteria Fermentation on the Bioactive Composition and Anti-Hepatic Steatosis Activity of *Citrus taiwanica* Peel

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Abstract This study on *Citrus taiwanica*, which is an endemic species of Taiwan and elucidate the major flavonoid compounds of this species and provide. Using HPLC-MS/MS-ESI analysis, we investigates the change of ten flavonoid compounds in *Citrus taiwanica* peel (CTP) and fermented *Citrus taiwanica* peel (FCTP) with Lactic acid bacteria. Naringin and hesperidin are the abundant flavonoid compounds in CTP. The FCTP contained hesperetin, hesperetin 7-O-glucoside, naringenin 7-0-glucoside and naringenin. After fermentation, the concentration of aglycones in FCTP were significantly increased, while the concentration of hesperidin and naringin were decreased. FCTP reduced lipid accumulation by 42.4% in OA-treated HepG2 cell, relative to the oleic acid-treated control group. These findings demonstrate that FCTP possess lipid-lowering activity in HepG2 cells, supporting their potential application as functional ingredients for the prevention of hepatic steatosis and the improvement of metabolic health. This study has demonstrated that fermentation is a useful way to treat agriculture waste to become valuable materials and effective strategy to release phenolic compounds and increase antioxidant activity due to the enzymes effect produced from lactic acid bacteria.

Keywords: *Citrus taiwanica, hepatic steatosis, lactic acid bacteria*

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1. Introduction

Lipid accumulation caused by hyperlipidemia and could be led to non-alcoholic fatty liver disease (NAFLD). NAFLD affecting more than 25 % of the adult population, is currently the most common liver disease in Taiwan [1,2,3]. Several studies have demonstrated the pharmacological properties of citrus flavonoids in various health benefits, including anti-obesity, gut health-promoting effects, reduces the risk of NAFLD, hepatoprotective effect and therapeutic potentials in regulating lipid metabolisms [4,5]. Hesperidin, found abundantly in orange peels and various citrus fruits, exhibits promising potential in reducing risk factors associated with cardiovascular disease and has been shown to have anti-inflammatory properties [6]. Naringin, a flavonoid found in citrus fruits, offers various health benefits due to its antioxidant, anti-inflammatory, and potential anticancer properties. It may also play a role in managing metabolic syndrome, cardiovascular health, and even neurological disorders [7].

Citrus peels is major waste from food processing and the cheap source of the medicinally flavonoids [8]. Some studies have been demonstrated, the Citrus fermented with lactic acid bacteria could be increase the bioavailability and bioactivity of flavonoid compounds by modifying their chemical structures [9,10]. *Citrus taiwanica* is an endemic species of Taiwan and classified belonging to the sour orange group [11]. The fruit of the *C. taiwanica* is the largest among Taiwan's wild oranges. The thick peel is similar to the peel of the grapefruit is the characteristic (Figure 1). In addition, the fruit is very sour and unpalatable, therefore, this species is already endangered in the wild. However, very little research on the application of this species for human health care [12] and it was not reported the active ingredients effect on lipid accumulation. In this experiment, a fast HPLC-ESI-MS/MS with multiple reaction monitoring mode method was used for simultaneous quantitation the changes in flavonoids of before and after fermentation of Taiwan Citrus.

In addition, the direct anti-steatotic effects of fermented

Citrus taiwanica peel preparations remain largely underexplored. To investigate this, the present study evaluated the anti-steatotic potential of fermented *C. taiwanica* peel, using an oleic acid-induced steatosis model in HepG2 cells—a well-established in vitro system for studying intracellular lipid accumulation [13,14].The findings may provide new evidence supporting the development of citrus-derived fermented ingredients as functional food components or complementary strategies for improving hepatic lipid homeostasis and managing NAFLD.

Figure 1. The appearance of Citrus taiwanica

2. Materials and Methods

2.1. Chemicals and Reagents

Hesperidin, hesperetin, eriocitrin, naringenin, naringin, narirutin, nobiletin, tangeretin, hesperetin 7-O-glucoside, naringenin 7-0-glucoside, MS-grade formic acid HPLC grade methanol were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA), isopropanol, Oil Red O, and trypsin-EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bezafibrate was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from Cytiva (Marlborough, MA, USA). The 100× Antibiotic-Antimycotic (10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL Amphotericin B), 100× non-essential amino acids solution (NEAA), phosphate-buffered saline (PBS), and sodium pyruvate were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and 10 N NaOH solution were purchased from APOLO Biochemical Inc. (Rochester, NY, USA). Formalin was purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of Fermentation Taiwan Citrus Peel

Citrus taiwanica peel were provided from Miaoli District Agricultural Research and Extension Station Council of Agriculture. Dried Citrus peel (CTP) powder was mixed with pure water and treated with Viscozyme for hydrolysis. *Lactobacillus plantarum* PM-A87 (BCRC910475) and *Lactobacillus acidophilus* PM-A0002 (BCRC 910308) with hydrolysed citrus peel co-fermentation at 37°C in an incubator for 144 hrs. Centrifuge the fermentation broth for 3 minutes at 5000 rpm to obtain the supernatant and then dried into a powder (Fermented *Citrus taiwanica* peel, FCTP).

2.3. HPLC-ESI-MS/MS Analysis of CTP and FCTP

The *Citrus taiwanica* peel (CTP) and Fermented *Citrus taiwanica* peel (FCTP) samples dissolved in methanol for flavonoids analysis and filtered through a 0.22 µm before injection. 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 a C18 column (150×4.0 mm I.D, 5 µm, Agilent, USA). 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, 95–65% (0–3 min), 65–45% (3–6 min), 45–0% (6–9.5 min), 0–65% (9.5–12 min), 65–95% (12–15min). The column temperature was fixed at 35°C, the flow rate was set 1 mL/min, and injection volume was 2 µL. The electrospray negative mode was selected as an ion source for hesperidin, hesperetin, eriocitrin, naringenin, naringin, narirutin, hesperetin 7-O-glucoside, naringenin 7-0-glucoside detection. The positive electrospray mode was selected as an ion source for nobiletin and tangeretin detection. The quantification was performed in multiple reactions monitoring (MRM). The optimized ESI source parameters were as follows: ion spray voltage, -4500 V for negative mode and 4500 V for positive mode; nitrogen nebulizer gas pressure, 50 psi; nitrogen curtain gas pressure, 11 psi; heater temperature, 480 °C; collisionally activated dissociation (CAD) gas, 11 psi. The precursor-to-product ion transitions were m/z 609/301, m/z 301/151, m/z 595.3/287, m/z 287/151, m/z 271/151, m/z 579/459.5, m/z 579/271, m/z 463/301, m/z 433/271, m/z 403/373 and m/z 373/343.2 for hesperidin, hesperetin, eriocitrin, naringenin, naringin, narirutin, hesperetin 7-O-glucoside, naringenin 7-0-glucoside, nobiletin and tangeretin, respectively. Their optimized declustering potentials (DP) and collision energies (CE) were listed on Table 1. All data acquisition and processing were performed using Analyst 1.7.3 software (AB SCIEX, Concord, ON, Canada). The peak area of each component in the samples was acquired from its chromatogram and the abundance of each compound was calculated from its corresponding calibration curve. Experiments were conducted in triplicate and the resulting data was represented as mg/g.

Table 1. MRM tansitions, declustering potential (DP) and collision energy (CE) of ten signature compounds

Compound	MRM transition (m/z)	DP(V)	CE(eV)
hesperidin	609 >301	-115	-46
hesperetin	301 > 151	-90	-20
eriocitrin	595.5 > 287	-29	-23
hesperetin 7-O-glucoside	463 >301	-86	-20
naringenin	271>151	-85	-23
naringin	579 >459.5	-111	-35
narirutin	579 >271	-109	-39
nobiletin	403 > 373	105	37
tangeretin	373> 343.2	89	36
naringenin 7-0-glucoside	433> 271	-80	-18

2.4. Determination of Total Phenolic Content (TPC)

The TPC were measured using Folin–Ciocalteau methods. Pipette 100 µL standard gallic acid (10-400 µg/mL) and samples were mixed with 1 mL of 2 % sodium carbonate. After 15 min incubation, 100 µL of 0.5 N Folin–Ciocalteus reagent was added to each test tube and allowed to stand for 25 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).

2.5. Determination of Total Flavonoid Content (TFC)

The TFC were measured using the method of Kim [15] with some modifications. The Samples and quercetin standard (20-1000 µg/mL) was mixed with distilled water and 5 % NaNO2 (70 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 425 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).

2.6. Antioxidant Activity

DPPH radical scavenging activity was measured according to a Li et al. [16] with slight modifications. Briefly, 120 µL of each sample solution were mixed with 0.25 mM DPPH solution in methanol. The mixture was incubated in the dark at room temperature for 30 min. The absorbance was recorded at 517 nm. 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.

2.7. HepG2 Cell Culture

Human hepatocellular carcinoma cells (HepG2) were purchased from the Bioresource Collection and Research Center (BCRC, Hsinchu, Taiwan). Cells were cultured in MEM supplemented with 10% FBS, 1% 100× Antibiotic-Antimycotic solution, 1% 100 mM sodium pyruvate, and 1% 100× NEAA at 37°C in a humidified incubator containing 5% CO_2. When cells reached approximately 80% confluency, they were washed with PBS, detached using trypsin-EDTA, neutralized with fresh culture medium, and reseeded into new culture vessels.

2.8. Preparation of Solutions Using Fermented Taiwan citrus Peel Powder

Fermented Taiwan citrus peel powder samples (FCTP) were dissolved in serum-free MEM to a stock concentration of 62.5 mg/mL. The pH of the solutions was adjusted to 7.0–8.0 using 10 N NaOH. The mixtures were then centrifuged at 2,330 ×g for 5 minutes at room temperature, and the resulting supernatants were sterilized by filtration through 0.22 µm syringe filters (Merck, USA). The sterile stock solutions were subsequently diluted in serum-free MEM to prepare working concentrations of 50, 25, 12.5, 6.25, 3.13, 1.57, 0.78, and 0.39 mg/mL for further experiments.

2.9. Cell Viability Assay (MTT Assay)

HepG2 cells were seeded in 96-well plates at a density of 1×10^3 cells/well and incubated overnight. Cells were then treated with serial dilutions of FCTP (ranging from 0.39 to 50 mg/mL) in culture medium containing 10% FBS and 1% Antibiotic-Antimycotic solution. After 24 hours of incubation, cell viability was assessed using the MTT assay. Fresh culture medium was mixed with PBS containing 5 mg/mL MTT reagent at a 5:1 ratio to prepare the MTT working solution. After removing the treatment medium, 120 µL of MTT working solution was added to each well and incubated for 2 hours. The supernatant solution was then discarded, and 100 µL of DMSO was added to dissolve the resulting formazan crystals. After incubation at 37°C for 15 minutes, absorbance was measured at 570 nm using an Infinite® M200 PRO microplate reader (Tecan, Männedorf, Switzerland). Cell viability (%) was calculated using the following formula: $[(A_{Sample}-A_{Blank}) / (A_{Control}-A_{Blank})] \times 100\%$, where 'A' represents absorbance at 570 nm. The experimental setup included three groups: (i) the sample group, where OD_{570} values were obtained from wells containing FCTP treated cells with MTT solution; (ii) the blank group, where OD_{570} values were obtained from wells containing only serum-free MEM and MTT solution; and (iii) the control group, where OD_{570} values were obtained from wells containing untreated cells with MTT solution.

2.10. Oil Red O Staining Assay for Intracellular Lipid Accumulation

To evaluate the inhibitory effects of fermented Taiwan citurs peel powder samples on lipid droplet accumulation in hepatocytes, HepG2 cells were seeded at a density of 2 × 10^5 cells/well in 24-well plates and incubated overnight. Following medium removal and PBS washing, 400 μL of serum-free MEM was added. Cells were then treated with 0.5 mM oleic acid and various concentrations of FCTP (ranging from 1.56 to 6.25 mg/mL). Control groups included a negative control (serum-free MEM only), a control (oleic acid without test substance), and a positive control (oleic acid plus 0.12 mM bezafibrate). After 24 hours of treatment, cells were washed and fixed with 0.5 mL 10% formalin for 1 hour, rinsed with ultrapure water, and incubated with 1 mL 60% isopropanol for 5 minutes before air-drying. Cells were then stained with 0.5 mL 0.5 mg/mL Oil Red O solution for 15 minutes and washed with ultrapure water. Microscopic images were captured using a Nikon microscope (Tokyo, Japan) equipped with a Tekfar camera system (Hsinchu, Taiwan). To quantify intracellular lipid content, stained cells were scanned using a flatbed scanner (Epson, Japan), then treated with 250 μL of 100% isopropanol and shaken at 20 rpm for 30 minutes. A 200 μL aliquot of the extracted dye was transferred to a 96-well plate and absorbance at 516 nm was measured using an Infinite® M200 PRO microplate reader. Lipid accumulation inhibition rate (%) was calculated using the following formula: {1 – [(ASample or positive control -Anegative control) / (AControl-Anegative control)] × 100%.

2.11. Statistical Analysis

All experiments were performed in triplicate, and the results are expressed as mean ± standard deviation (SD). Statistical significance was determined using one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test (Minitab LLC, State College, PA, USA). A *p*-value of < 0.05 was considered statistically significant, and $p < 0.01$ was considered highly significant.

3. Results and Discussion

3.1. Analysis of Flavnonoid Compounds of Taiwan Citrus Peel by High-Performance Liquid Chromatography -Electro-Spray Ionization Mass Spectrometry with Multiple Reaction Monitoring

Citrus taiwanica is an endemic species of Taiwan and classified belonging to the sour orange group. The contents of various flavonoid compounds in the *Citrus taiwanica* peel were in the range of 0.1-28 mg/g (Table 2). The previous study has demonstrated that naringin is the most abundant flavonoid in bitter orange peel and grapefruit peel, the content were in the range of 10.26 to 14.40 mg/g (peel dry basis) [17-20]. In this study, we found similar results that naringin and hesperidin are the abundant flavonoid compounds in *Citrus taiwanica* peel.

The flavonoid content in Citrus peel is much higher than other parts and mostly as glycosides [21,22]. Flavanone glycosides of citrus peel were transformed into corresponding aglycones by fermentation. In this study, *Lactobacillus plantarum* and *Lactobacillus acidophilus* be used to co-fermentation with citrus peel. The previous study demonstrated, some lactic acid bacteria such as *Lactobacillus plantarum*, showed high β-glucosidase activity during the fermentation process and allows release of bioactive aglycone compounds to improve the bioactivity of flavonoids [23]. *Lactobacillus acidophilus* is a probiotic bacteria that can produce α-L-rhamnosidase and is capable of cleaving the rhamnose from various flavonoid compounds to increase bioavailability [24]. The α-l-rhamnosidase converts hesperidin and naringin to hesperetin-7-O-glucoside and naringenin 7-0-glucoside respectively and β-glucosidase then hydrolyzes these glycosides to hesperetin and naringenin, respectively (Figure 2).

To identify the chemical profiles of the flavonoid glycosides and hydrolysis products, CTP and FCTP before and after bioconversion were characterized by HPLC-ESI-MS. The precursor-to-product ion transitions were m/z 609/301, m/z 301/151, m/z 287/151, m/z 271/151, m/z 579/459.5, m/z 463/301 and m/z 433/271 for hesperidin, hesperetin, naringenin, naringin, hesperetin 7-O-glucoside and naringenin 7-0-glucoside (Figure 3). After 144 hrs, the fermented Taiwan citrus peel (FCTP) contents of hesperetin, hesperetin 7-O-glucoside, naringenin 7-0-glucoside and naringenin were increased from not detected to 0.22 mg/g, 3.11 mg/g, 1.21 mg/g and 0.11 mg/g, respectively. The concentration of aglycones in FCTP were significantly increased, while the concentration of hesperidin and naringin were decreased (Table 2).

Table 2. The contents (mg/g) of the ten flavonoid compounds in CTP and FCTP (n=3)

Compound	CTP	FCTP
hesperidin	20.5± 0.11	11.2 ± 0.05
hesperetin	-	0.22 ± 0.01
eriocitrin	0.12 ± 0.05	0.15 ± 0.03
hesperetin 7-O-glucoside	-	3.11 ± 0.02
naringenin	-	0.11 ± 0.03
naringenin 7-0-glucoside	-	1.21 ± 0.02
naringin	28.2 ± 0.08	0.50 ± 0.07
narirutin	0.15 ± 0.03	0.12 ± 0.05
nobiletin	0.21 ± 0.09	0.10 ± 0.09
tangeretin	0.11 ± 0.06	0.51 ± 0.13

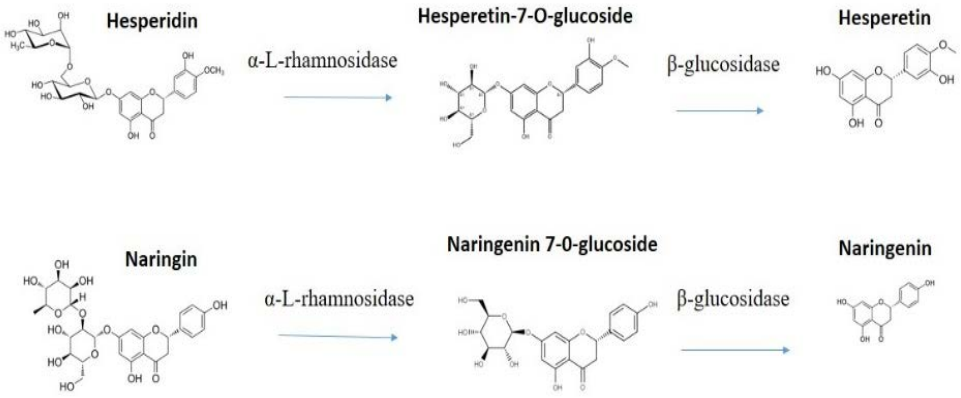

Figure 2. Degradation of Hesperidin and Naringin by α-L-rhamnosidase and β-D-glucosidase

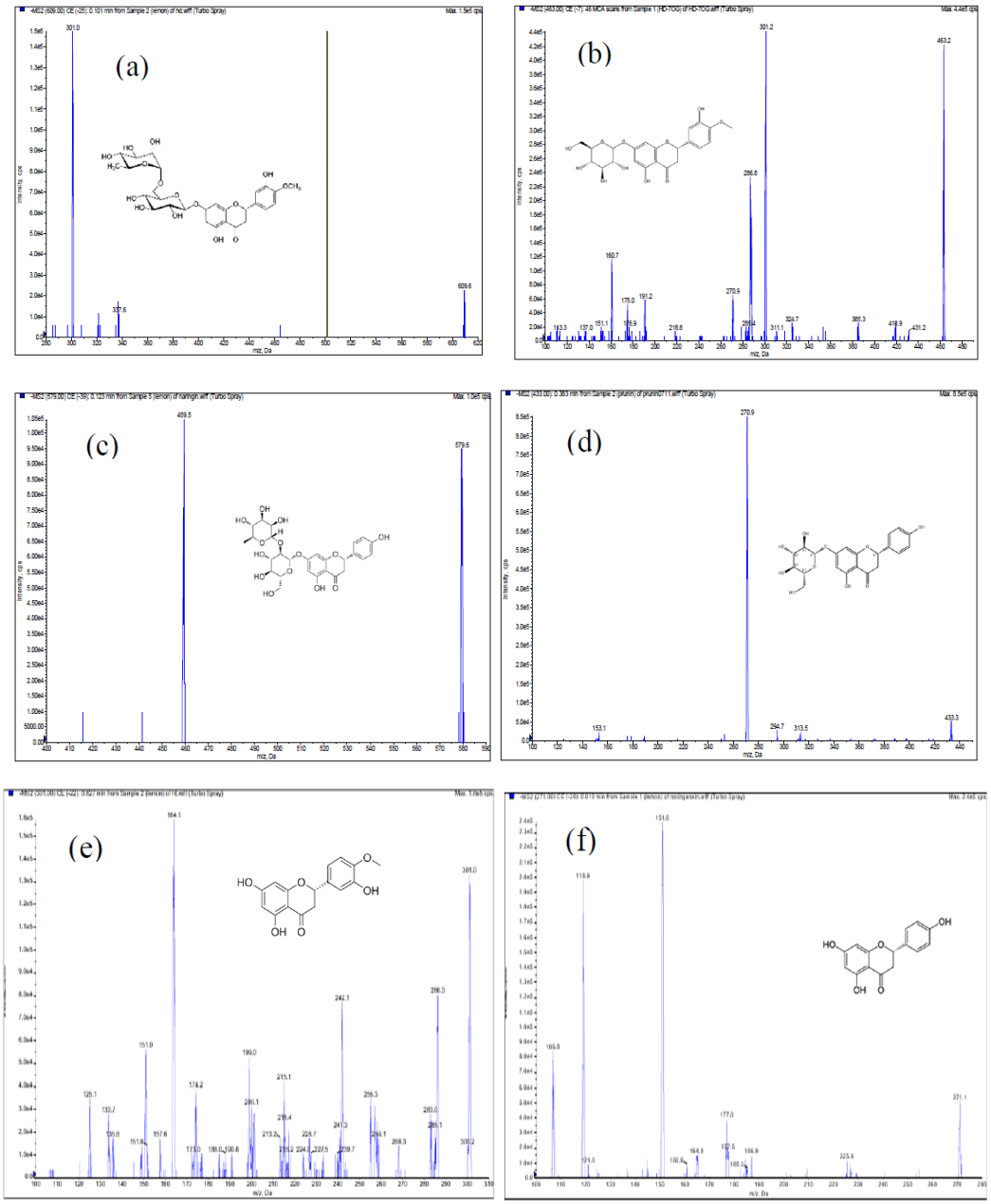

Figure 3. Typical chromatogram of flavonoid compounds (a) MS spectrum of hesperidin, (b) MS spectrum of hesperetin 7-O-glucosid, (c) MS spectrum of hesperetin, (d) MS spectrum of naringin, (e) MS spectrum of naringenin 7-0-glucosid. (f) MS spectrum of naringenin

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

The total phenolic content of CTP and FCTP were expressed as gallic acid equivalent. The total phenolic contents of CTP and FCTP were 18.5 ± 0.1 mg and 24.3 ± 0.1 mg GAE/g (Table 3). The total flavonoids content of CTP and FCTP were expressed as quercetin equivalent. The total flavonoid contents of CTP and FCTP were 10.6 ± 0.3 mg and 12.1 ± 0.4 mg GAE/g (Table 3). In present study, *C. taiwanic*a peel was fermented with *L. Plantarum* A87 and *Lactobacillus acidophilus* PM-A0002.The contents of total polyphenols from 18.5 to 24.3 mg GAE/g, while flavonoids varied from 10.6 to 12.1 mg QE/g DM. Fermentation is a useful way to treat agriculture waste to become valuable materials. Fermentation is one of the effective strategy to release phenolics compounds because the enzymes effect produced from the probiotics [25]. Therefore, it is suggested that the increased amounts of total polyphenols and flavonoids in fermented *C. taiwanica* peel maybe caused by the improved release [26]. The radical scavenging activities of the samples were evaluated using DPPH radical scavenging assays. The CTP and FCTP had IC_{50} values of 3.1 ± 0.2 and 2.2 ± 0.1 mg/ml, respectively. The results of this study revealed that FCTP had effective capacity of scavenging superoxide radical and correlated with total phenolic and flavonoids content thus indicating its antioxidant potential. This study demonstrated lactic acid bacteria fermentation is an effective processing method for increases bioavailability.

Table 3. Antioxidant activities and the contents of total phenolic, total flavonoid in CTP and FCTP. Data values represent the mean ± SD (n = 3)

Group	CTP	FCTP
Total phenolic (mg/g)	18.5 ± 0.1^{b}	24.3 ± 0.1^{a}
Total flavonoid(mg/g)	10.6 ± 0.3^{b}	12.1 ± 0.4^{a}
DPPH radical scavenging activity, IC_{50} value (mg/ml)	3.1 ± 0.2^{b}	2.2 ± 0.1^{a}

3.3. Effects of FCTP on HepG2 Cell Viability

The cytotoxicity of FCTP toward HepG2 cells was evaluated using the MTT assay. As shown in Figure 4, both FCTP significantly reduced cell viability at concentrations above 12.5 mg/mL. Therefore, subsequent experiments on intracellular lipid accumulation were conducted using lower concentrations of 6.25, 3.13, and 1.56 mg/mL.

3.4. Inhibitory Effects of FCTP on Intracellular Lipid Accumulation in HepG2 Cells

OA-induced steatosis in HepG2 cells may serve as an in vitro model for studying fatty liver disease. Oil Red O staining was performed to visualize lipid droplet accumulation in HepG2 cells following treatment with different concentrations of FCTP. Microscopic observations revealed a slight reduction in lipid droplet size after treatment with increasing concentrations (Figure 5). Quantitative analysis was carried out by extracting the bound dye and measuring absorbance at 516 nm. At 6.25 mg/mL, FCTP reduced lipid accumulation by 42.4%, relative to the oleic acid-treated control group (Figure 6). These findings demonstrate that FCTP possess lipid-lowering activity in HepG2 cells, supporting their potential application as functional ingredients for the prevention of hepatic steatosis and the improvement of metabolic health. In previous study has shown the *Citrus junos* peel extract reduced oleic acid-induced hepatic lipid accumulation in HepG2 cells [27]. These may be due to the presence of abundance phenolic and flavonoids contents in the fermented Taiwan citrus peel, its play an important role as antioxidants in living organisms [28]. In this study, we found similar results that fermented Taiwan citrus peel contain rich flavonoids including flavonoid glycosides, may have a great potential to anti-hepatic steatosis activity.

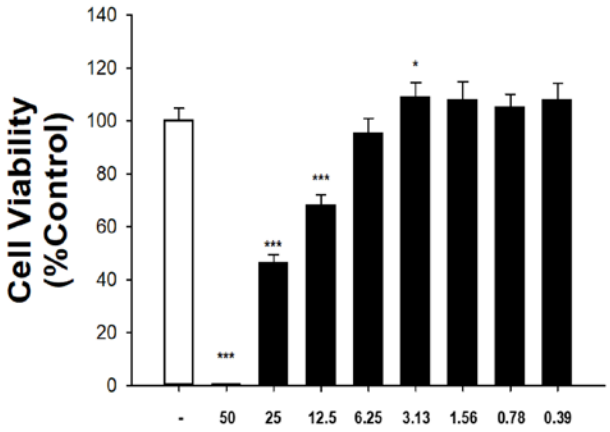

Figure 4. Effect of fermented Taiwan citrus peel powder samples FCTP on HepG2 cell viability. HepG2 cells were treated with various concentrations of FCTP (0.39–50 mg/mL) for 24 hours. Cell viability was evaluated using the MTT assay. Results are expressed as the percentage relative to the control group (untreated cells) and presented as mean ± SD (n = 3). Statistical significance compared to the control group is indicated as follows: $p \leq 0.05$ (*), $p \leq 0.001$ (***)

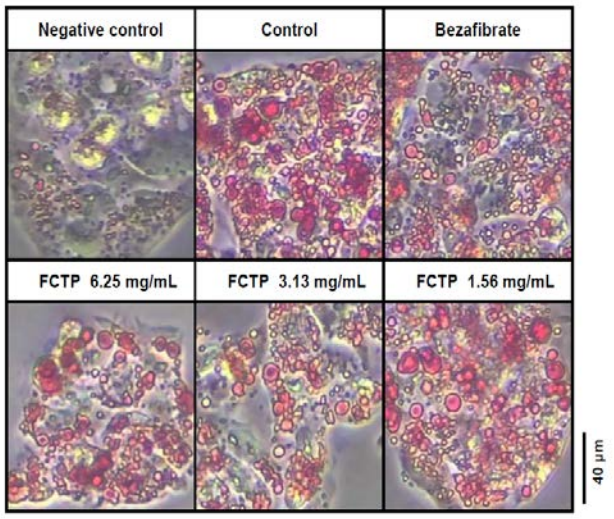

Figure 5. Microscopic observation of Oil Red O-stained lipid droplets in HepG2 cells. HepG2 cells were treated with FCTP at concentrations of 1.56, 3.13, and 6.25 mg/mL in the presence of 0.5 mM oleic acid. Images were taken under a microscope at 200× magnification

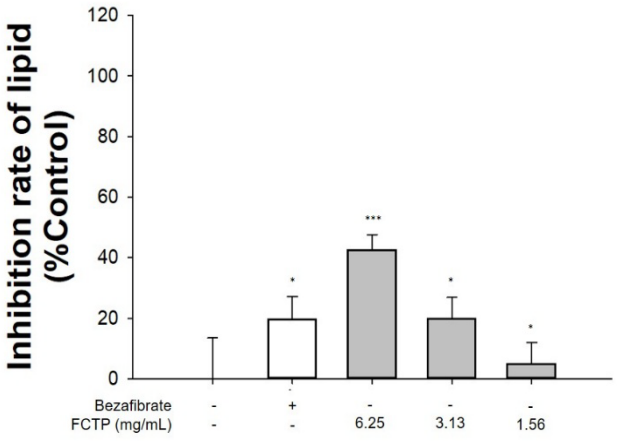

Figure 6. Inhibition rate of oleic acid-induced lipid accumulation in HepG2 cells after treatment with Taiwan citrus peel powder samples FCTP. HepG2 cells were treated with different concentrations of FCTP (1.56–6.25 mg/mL) in the presence of 0.5 mM oleic acid for 24 hours. Intracellular lipid accumulation was assessed by Oil Red O staining, followed by isopropanol extraction of the bound dye. Absorbance was measured at 516 nm to quantify lipid content, and inhibition rate was calculated relative to the oleic acid-treated control group. Data are presented as mean ± SD (n = 3). Statistical significance vs. oleic acid-treated group: $p \leq 0.05$ (*), $p \leq 0.001$ (***)

4. Conclusions

The Present study has demonstrated that fermentation is a useful way to treat agriculture waste to become valuable materials and effective strategy to release phenolic compounds and increase antioxidant activity due to the enzymes effect produced from lactic acid bacteria. The naringin and hesperidin were the most abundant flavonoid in *Citrus taiwanica* peel. After fermentation, the concentration of aglycones in citrus peel were significantly increased. Fermented *Citrus taiwanica* peel reduced lipid accumulation by 42.4% in OA-treated HepG2 cell, relative to the oleic acid-treated control group. These findings supporting their potential application for the prevention of hepatic steatosis and the improvement of metabolic health. However, further research is warranted to elucidate the mechanisms and to study these effects in animal models and human clinical trials.

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Author Contributions: Ho-shin Huang and Jyh-Perng Wang carried out all the experiments; Wen-Zheng Huang, Zhe-Yu Jiang, Si-Ting Lin, Chun-Mei Lu, Ting-Yuan Hsu designed all the experiments and analyzed the data; Ho-shin Huang and Jyh-Perng Wang wrote the manuscript.

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

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