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Evaluation of GLP-1 Secretion Enhancement by Fermented Taiwan Citrus Peel in STC-1 Cells

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Abstract This study using HPLC tandem mass investigates the contents of main flavonoid compounds in fermented peel of Taiwan Citrus with Lactic acid bacteria. The results showed hesperidin, nobiletin and tangeretin were the main flavonoid compounds in fermented peel of *Citrus taiwanica* (FCT). In contrast, eriocitrin and hesperidin were most abundant bioactivity compounds in fermented peel of *Citrus limon* (FLP07). In this study, we investigated the effect of fermented citrus peel (FCT and FLP07) on Glucagon-like peptide-1(GLP-1) secretion in STC-1 cells, a well-established enteroendocrine cell model. The results showed FCT and FLP07 at all tested concentrations induced significantly higher GLP-1 secretion and suggest that FCT and FLP07 are more potent than serotonin in stimulating GLP-1 release, highlighting their potential as functional ingredients for metabolic health applications.

Keywords: *fermented citrus peel, flavonoid, Glucagon-like peptide-1, metabolic associated fatty liver disease*

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1. Introduction

Metabolic associated fatty liver disease (MAFLD) formerly known as non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease. Affecting more than 25 % of the adult population, MAFLD is currently the most common liver disease in East Asia, especially in Taiwan [1,2,3]. MAFLD is associated with increased mortality, particularly due to non-alcoholic steatohepatitis (NASH) and liver-related events [4]. The inflammation and liver damage of NASH can cause fibrosis and may lead to cirrhosis and liver cancer [5]. In additional, Glucagon-like peptide-1 (GLP-1) is an incretin hormone that plays a crucial role in glucose homeostasis by stimulating insulin secretion, inhibiting glucagon release, delaying gastric emptying [6] and can increase liver fatty acid oxidation and insulin sensitivity by binding to GLP-1receptor , thereby improving NAFLD [7]. GLP-1 presents a novel therapeutic approach against NAFLD by decreasing lipogenesis, and improving hepatic glucose metabolism [8].

Lemons, grapefruit, mandarins and oranges are the main citrus fruits. These belong to the genus Citrus in the family Rutaceae [9]. Due to Citrus species medicinal activities contributed from flavonoid compounds. There are several major flavonoids of citrus species including hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, nobiletin and tangeretin, which are abundant in different citrus fruit peels [9,10,11]. Several studies have demonstrated the pharmacological properties of citrus flavonoids in various health benefits, including anti-diabetic, anti-obesity, and gut health-promoting effects [12,13,14]. Some studies revealed the protective role of bioactives present in Citrus flavonoids against chronic metabolic conditions and dietary eriocitrin ameliorates diet-induced hepatic steatosis with activation of mitochondrial biogenesis [15]. Recently it has been found that flavonoids are capable of attenuating the process of NAFLD by improving lipid metabolism and a mixture of citrus flavonoids (eriocitrin, hesperidin, naringin, and didymin), which reduces glycemia and increases glucagon-like peptide-1 [16,17,18]. Citrus and lemon peels is main waste from food processing and could be considered as a cheap source of the medicinally important flavonoids [9]. Fermentation has been shown to enhance the bioavailability and bioactivity of plant-derived compounds by modifying their chemical structures and increasing the production of bioactive metabolites [19]. Some studies have been demonstrated, the Citrus fermented with lactic acid bacteria can enhance the levels of bioactive compounds and can potentially influence the bioactive constituents of citrus by-products [20].

Citrus taiwanica is an endangered citrus species from Taiwan, was classified as an independent species

belonging to the sour orange group [21]. However, to the best of our knowledge, the fermentation product of flavonoids from *Citrus taiwanica* have never been evaluated. In this study, a simple and fast liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) with a MRM mode method was developed for simultaneous quantitation of various flavonoids of fermented Taiwan Citrus peel.

Due to the clinical use of synthetic GLP-1 analogs is often constrained by high cost and potential adverse effects, including gastrointestinal discomfort and nausea. Therefore, identifying natural compounds that can enhance endogenous GLP-1 secretion has gained increasing attention as an alternative strategy for developing novel pharmaceuticals and functional foods targeting metabolic health improvement [22,23].

Recent studies suggest that fermented plant products can influence gut hormone secretion and improve metabolic health, making them promising candidates for the development of functional ingredients in nutraceuticals and pharmaceuticals [24,25]. However, the effect of fermented citrus peel on GLP-1 secretion remains unexplored. In this study, we investigated the effect of fermented citrus peel (FCT and FLP07) on GLP-1 secretion in STC-1 cells, a well-established enteroendocrine cell model. The findings of this study may provide insights into the potential use of fermented citrus peel in the development of functional foods or therapeutic agents for metabolic disorders.

2. Materials and Methods

2.1. Chemicals and Reagents

Hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, nobiletin, tangeretin and methanol purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypsin-EDTA was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The fetal bovine serum (FBS) was purchased from Cytiva (Marlborough, MA, USA). The bovine serum albumin (BSA) was purchased from Roche (Basel, Switzerland). 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) with high glucose (4.5 g/L), DMEM with low glucose (1 g/L), and phosphate-buffered saline (PBS) were purchased from Gibco (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), and 10 N NaOH solution were purchased from APOLO biochemical Inc. (Rochester, NY, USA). The Human GLP-1 ELISA Kit was purchased from Merck Millipore (Burlington, MA, USA).

2.2. Plant Material and Fermentation

Citrus taiwanica peel were collected from Miaoli County and Eureka lemons (*Citrus limon*) peel purchased from organic farm (Pingtung County, Taiwan). Citrus peel powder was mixed with pure water and treated with Viscozyme (40◦C, 20 h) for hydrolysis. To inactivate the enzymes, the reaction mixture was heated in a boiling water. *Lactobacillus plantarum* PM-A87 (BCRC910475), *Lactobacillus acidophilus* PM-A0002 (BCRC 910308) and *Lactobacillus reuteri* BR101 (BCRC 910512) is a self-isolating strain and stored at -80°C. Weigh an appropriate amount of citrus peel and glucose to prepare the fermentation culture medium. Add Lactic acid bacterial liquid pre-cultured for 24 hours, mix it with the fermentation culture medium, and co-fermentation at 35°C in an incubator for 168 hrs. Centrifuge the fermentation broth for 5 minutes at 3000 rpm to obtain the supernatant and then dried into a powder (Fermented *Citrus taiwanica* peel, FCT; fermented lemon peel, FLP07).

2.3. HPLC-ESI-MS/MS Analysis of FCT and FLP07

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, 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 30°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, eriodictyol, naringenin, naringin and narirutin 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, 450 °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 403/373 and m/z 373/343.2 for hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, 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 FCT and FLP07 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.

2.4. STC-1 Cell Culture

STC-1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution at 37°C in a 5% CO_2 incubator. Cells were passaged at 80%

confluency by washing with PBS, treating with trypsin-EDTA, and neutralizing with fresh culture medium before reseeding.

2.5. Preparation of Solutions Using Fermented Citrus Peel Powder

FCT and FLP07 powders were dissolved in serum-free low-glucose DMEM to an initial concentration of 62.5 mg/mL. The pH was adjusted to 7.0–7.5 using 10 N NaOH. The solution was then centrifuged at 2,000 ×g for 5 min at room temperature, and the supernatant was sterilized by filtration through a 0.22 μm syringe filter (Merck Millipore, Burlington, MA, USA). The final working concentrations of FCT and FLP07 were prepared by serial dilution in serum-free low-glucose DMEM to 25, 12.5, 6.25, 3.13, 1.57, 0.78, and 0.39 mg/mL.

2.6. Cell Viability Assay (MTT Assay)

STC-1 cells were seeded in 96-well plates at a density of 3×10^4 cells per well in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution and incubated for 48 hours. The culture medium was then replaced with 200 μL of serum-free low-glucose DMEM for 3 hours. Subsequently, the medium was removed, and cells were treated with various concentrations of FCT or FLP07 in serum-free low-glucose DMEM for 1 hour.Cell viability was assessed using the MTT assay. After incubation with MTT reagent for 90 minutes, formazan crystals were dissolved in DMSO, and absorbance at 570 nm was measured using an Infinite® M200 PRO microplate reader (Tecan, Switzerland). Cell viability (%) was calculated using the following formula: [(ASample-ABlank) / (AControl-ABlank)] × 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 FCT or FLP07-treated cells with MTT solution; (ii) the blank group, where OD_{570} values were obtained from wells containing only serum-free low-glucose DMEM and MTT solution; and (iii) the control group, where OD_{570} values were obtained from wells containing cells and MTT solution without treatment.

2.7. GLP-1 Secretion Assay

STC-1 cells were seeded in 96-well plates at a density of 3×10^4 cells per well in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution and incubated for 48 hours. The culture medium was then replaced with 200 μL of serum-free low-glucose DMEM for 3 hours, followed by treatment with either serum-free low-glucose DMEM (control), 100 μM serotonin (positive control), or various concentrations of FCT or FLP07 for 1 hour. The culture supernatant was collected, centrifuged at 500 ×g for 5 minutes at room temperature, and GLP-1 levels were quantified using the Human GLP-1 ELISA Kit according to the manufacturer's instructions.

2.8. Statistical Analysis

All experiments were performed in triplicate, and data were expressed as mean ± standard deviation (SD). Statistical significance was assessed using one-way analysis of variance (ANOVA) followed by Dunnett's test in Minitab software (Minitab, LLC, State College, PA, USA). A p-value < 0.05 was considered statistically significant, while a p-value < 0.01 was considered highly significant.

3. Results

3.1. Analysis of Nine Signature Compounds by High-Performance Liquid Chromatography -Electro-Spray Ionization Mass Spectrometry with Multiple Reaction Monitoring

A High-Performance Liquid Chromatography Electro-Spray Ionization Mass Spectrometry with multiple reaction monitoring method was carried out for analysis the flavonoid compounds from Citrus taiwanica and lemon. The RP-C18 column (150×4.0 mm I.D, 5 μm; Agilent, USA) was selected for HPLC -ESI-MS analysis based on its good separation ability and within 15 min. With the ESI source of the mass spectrometer, hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, neohesperidin showed better sensitivity in the negative ion mode, but nobiletin and tangeretin exhibited a higher sensitivity in the positive ion mode (Figure 1). Given this discrepancy, a positive-negative conversion multiple reaction monitor was used to determine the content of the above nine compounds. 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 403/373 and m/z 373/343.2 for hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, nobiletin and tangeretin, respectively. Their optimized declustering potential (DP) and collision energies (CE) were list in Table 1.

Table 1. MRM tansitions, declustering potential (DP) and collision energy (CE) of nine 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
eriodictyol	287 >151	-81	-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

In this study, a simple sensitive LC-MS/MS method was used for the analysis of FCT and FLP07. The described HPLC-ESI-MS method was applied to analysis of FCT and FLP07 through simultaneous determination of flavonoid compounds.

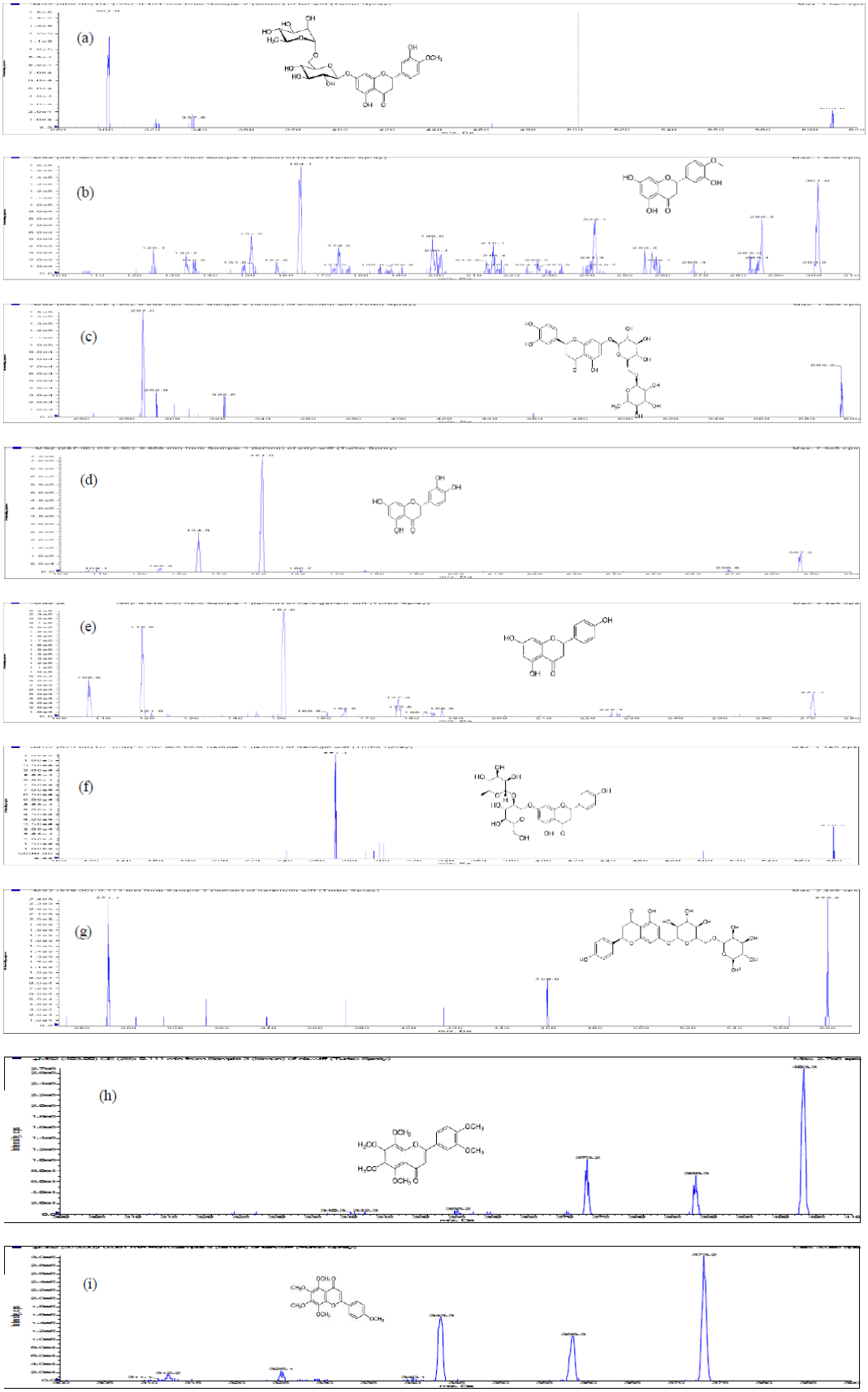

Figure 1. MS spectrum of (a)hesperidin, (b) hesperetin, (c) eriocitrin, (d) eriodictyol, (e) naringenin, (f) naringin, (g) narirutin, (h) nobiletin and (i) tangeretin

The quantitative analytical results (Table 2) indicated their contents distributed in these samples. The contents of hesperidin, hesperetin, eriocitrin, eriodictyol, naringenin, naringin, narirutin, neohesperidin, nobiletin and tangeretin in the samples were in the range of 0.03-23.5 mg/g. In this results, Hesperidin, nobiletin and tangeretin were the main flavonoid compounds in fermented peel of *Citrus taiwanica* (FCT). In additional, hesperidin, and eriocitrin

are the major flavonoid compounds in fermented Lemon peel (FLP07).

Table 2. The contents (mg/g) of the nine signature compounds in FCT and FLP07 (n=3)

Compound	FCT	FLP07
hesperidin	23.5	10
hesperetin	-	0.03
eriocitrin	-	5.4
eriodictyol	-	0.07
naringenin	-	-
naringin	-	-
narirutin	-	0.05
nobiletin	0.59	-
tangeretin	1.62	-

3.2. Cytotoxicity of Fermented Citrus Peel FCT and FLP07 in STC-1 Cells

MTT assay results demonstrated that FCT and FLP07, at concentrations up to 25 mg/mL, did not significantly affect STC-1 cell viability ($p > 0.05$), confirming their non-cytotoxicity (Figure 2). Based on these findings, subsequent experiments were conducted using non-cytotoxic concentrations of 25, 12.5, and 6.25 mg/mL to evaluate their effects on GLP-1 secretion in STC-1 cells.

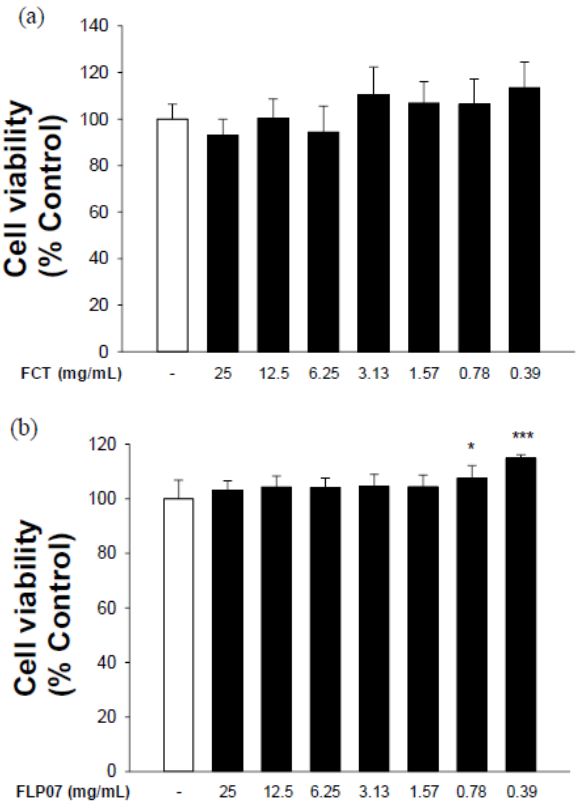

Figure 2. Effect of fermented Citrus peel FCT and FLP07 on STC-1 cell viability

STC-1 cells were treated with various concentrations of (a) FCT (0.39–25 mg/mL) and (b) FLP07 (0.39–25 mg/mL) for 1 hour, and cell viability was assessed using the MTT assay. Results are expressed as the percentage of the control (untreated cells) and presented as mean ± standard deviation (SD) from three independent experiments.

3.3. Effect of Fermented Citrus Peel FCT and FLP07 on GLP-1 Secretion in STC-1 Cells

ELISA analysis demonstrated that FCT and FLP07 significantly increased GLP-1 secretion in STC-1 cells in a dose-dependent manner (Figure 3). Treatment with FCT at 25 mg/mL resulted in the highest GLP-1 secretion (~2,000 pM, $p < 0.01$), corresponding to a 17.14-fold increase relative to the control group. Similarly, FLP07 at 25 mg/mL induced a GLP-1 secretion level of ~1,750 pM ($p < 0.01$), representing a 16.7-fold increase. Lower concentrations (12.5 and 6.25 mg/mL) of both compounds also significantly enhanced GLP-1 levels compared to the control. Compared to the 100 μM serotonin-treated group (~317 pM, $p < 0.05$), which exhibited only a 2.27-fold increase, FCT and FLP07 at all tested concentrations induced significantly higher GLP-1 secretion. These results suggest that FCT and FLP07 are more potent than serotonin in stimulating GLP-1 release, highlighting their potential as functional ingredients for metabolic health applications. This result is similar to previous studies that found that extracts rich in eriocitrin and hesperidin can increase glucagon-like peptide-1 [26,27].

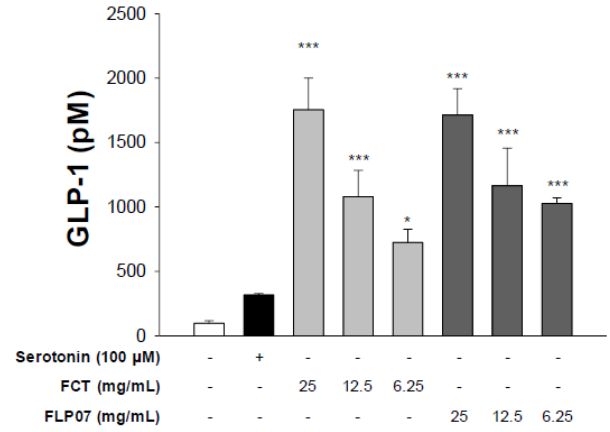

Figure 3. Effect of Fermented Citrus Peel FCT and FLP07 on GLP-1 Secretion in STC-1 Cells

STC-1 cells were treated with various concentrations of FCT or FLP07 (6.25–25 mg/mL) for 1 hour, and GLP-1 levels in the culture supernatant were measured using an ELISA assay. Serotonin (100 μM) was used as a positive control. Results are expressed as mean ± standard deviation (SD) from three independent experiments. Statistical significance was determined using one-way ANOVA, with $p \leq 0.05$ (*) and $p \leq 0.001$ (***) compared to the control group.

4. Conclusions

Metabolic associated fatty liver disease (MAFLD) has become the world's most common chronic liver Disease. In this study, the effect of fermented citrus peel on GLP-1 secretion in STC-1 cells was investigated. The results indicated that fermented citrus peel are more potent than serotonin in stimulating GLP-1 release and may be related to the rich contain of flavonoid compounds. This study shows the therapeutic potential of fermented citrus peel

in metabolic fatty liver disease by in vitro model. However, further work is necessary that to verify the effect through animal experiments and human clinical trials.

Author Contributions

Conceptualization, design, screening, quality analysis, data extraction, data analysis, results interpretation, and manuscript preparation, S-T.L, S-H. C, Z.-Y. J; study design, results interpretation, and manuscript check, C.-M.L., T.-Y., H, J.-P., W; 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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