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Polyphenol-Rich Extract of Fermented Chili Pepper Alleviates Insulin Resistance in HepG2 Cells via Regulating INSR, PTP1B, PPAR-γ, and AMPK Pathways

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Abstract: Fermented *Capsicum frutescens* L. is a well-known traditional food ingredient in China with a variety of potential nutritional functions due to the increased content of polyphenolic compounds during the fermentation process. This study aimed to investigate the ameliorative effect of fermented chili peppers (FCP) on insulin resistance and the potential mechanism of action. HepG2 cells were treated with 5×10^{-6} mol/L insulin for 12 h to establish the insulin resistance model. The results showed that the ethanol extract of FCP (1 mg/mL), rather than non-FCP extract, significantly increased glucose consumption in insulin-resistant HepG2 cells, which was at least partly attributed to an increase in polyphenolic compounds after fermentation, including kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, and apigenin. Molecular docking analysis suggested that these five significantly increased polyphenolic compounds in FCP could partially and effectively interact with the key amino acid residues of four key insulin resistance-related receptors (INSR, PTP1B, PPAR-γ, and AMPK). In conclusion, the fermentation process enhanced or even conferred a pronounced anti-insulin resistance effect on chili peppers, and the increased polyphenolic compounds in chili pepper had synergistic effects in modulating the INSR, PTP1B, PPAR-γ, and AMPK pathways to regulate the destruction of glucose consumption.

Keywords: fermented *Capsicum frutescens* L.; polyphenolic compounds; insulin resistance; in vitro

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1. Introduction

Insulin resistance is the condition of declined responsiveness of insulin-targeting tissues such as liver, adipose tissue, and skeletal muscle, which is a key pathogenic factor of various metabolic diseases, such as fatty liver, cardiovascular diseases, and type 2 diabetes mellitus (T2DM) [1]. Insulin is a peptide hormone secreted by β cells of islets, which can maintain normal blood glucose levels by regulating the metabolism of whole-body carbohydrates, proteins, and fats in the body [2]. Once insulin resistance occurs, the key protein receptors (e.g., insulin receptor, INSR; adenosine 5′-monophosphate-activated protein kinase, AMPK; protein tyrosine phosphatase 1B, PTP1B; and peroxisome proliferator-activated receptor gamma, PPAR-γ) associated with insulin resistance in our body (especially in the liver, skeletal muscle, and adipose tissue) do not adequately respond to normal levels of insulin, which in turn disrupts the function of insulin in maintaining glucose homeostasis [3–6]. Thus, persistent insulin resistance could lead to the elevation of blood glucose, the compensatory increase of insulin secretion (hyperinsulinemia), and the increase of islet burden and β-cells failure, which eventually lead to T2DM [7]. Since T2DM is characterized

by the abnormality of glucolipid metabolism due to the decreased responsiveness or sensitivity of insulin metabolic action [8], improving insulin resistance is one of the effective ways to relieve T2DM.

Some drugs such as metformin, rosiglitazone, and pioglitazone are used clinically to alleviate insulin resistance, but these chemicals may have some side effects in long-term medication. Currently, both rosiglitazone and pioglitazone are excluded from EASD/ADA 2022 guidelines due to their strong side effects. However, they are still allowed for clinical use in China until 10 January 2023. For insulin resistance, sodium-glucose cotransporter-2 (SGLT-2) inhibitors and glucagon-like peptide-1 receptor agonists (GLP-1 RAs) have shown satisfactory efficacy in patients with T2DM because they can improve β-cell function and enhance insulin sensitivity [9]. In addition, the exploration of natural phytochemicals from the food matrix with fewer side effects to ameliorate insulin resistance through dietary intervention is also a new strategy to alleviate insulin resistance. The pathogenic mechanisms of insulin resistance are multiple. In addition to genetic defects (e.g., mutations and polymorphisms in insulin receptors, glucose transporters, and insulin signal transduction-related signaling proteins), some acquired factors (e.g., obesity, inflammation, and oxidative stress) can also contribute to the development of insulin resistance [4,10]. It has been reported that oxidative stress is a main inducible factor of insulin resistance because the oxidative stress is closely related to other factors causing insulin resistance, such as inflammation and obesity [11,12]. Therefore, foods rich in phytochemicals with antioxidant properties are a potential dietary intervention to alleviate insulin resistance.

Chili peppers (*Capsicum* spp.) are the most widely grown vegetable and spice crop in the world and are very popular in China, especially in southwest China, such as the Sichuan, Guizhou, and Yunnan provinces. It has been found that chili peppers are rich in bioactive substances, mainly including capsaicin, phenolic compounds, carotenoids, amino acid, and vitamins, which confer capsicum with potential functional activities [13,14]. In addition to being a source of spiciness, flavor, and food additive, chili peppers are also used for medicinal purposes due to their antioxidant [15], anti-obesity [16], and anti-inflammation activities [17]. Since chili peppers need to be grown in a suitable environment and season, to ensure the consumption of chili peppers in different seasons, various processing methods, such as fermentation, drying, or sauce making, are often used to extend their shelf life. Fermentation is one of the common food pre-processing methods that can improve the taste, flavor, and nutritional value of fruits and vegetables. A prior study reported that fermentation can effectively improve the bioavailability of polyphenols in foods [18]. Since chili peppers are rich in phenolic compounds, it can be hypothesized that the bioavailability of polyphenols in chili peppers would be significantly enhanced during fermentation. Our previous study suggested that the content of total phenolic compounds in fermented pepper extract (FCP) was significantly higher than that of non-FCP extract, which conferred FCP with superior antioxidant activity [19].

Since food-derived polyphenols have a protective effect against oxidative stress and diabetes, which are the trigger factor and cause of insulin resistance, respectively [20–22], we speculated that polyphenol-rich FCP extract (E) has an ameliorative effect on insulin resistance. Therefore, in this study, we firstly constructed insulin resistant HepG2 cells using high glucose and high insulin to assess the ameliorative effect of FCPE on insulin resistance. Then, the key phenolic compounds in FCP that potentially play a key role in ameliorating insulin resistance were evaluated. Moreover, the interactions between these phenolic compounds in FCP and key target proteins associated with insulin resistance (INSR, PTP1B, PPAR-γ, and AMPK) were analyzed using molecular docking methods to speculate on their potential mechanisms of action. The results of this study will confirm the effect of FCP in alleviating insulin resistance and further indicate that FCP is a selective dietary ingredient for patients with insulin resistance, which is conducive to promoting the development of the FCP industry.

2. Materials and Methods

2.1. Chemical and Reagents

High-glucose Dulbecco's Modified Eagle's Medium (HG-DMEM) and phosphate buffered solution (PBS) were purchased from HyClone (Logan, UT, USA). Penicillin-streptomycin solution (100×) and trypsin-EDTA solution were supplied by Biosharp (Biosharp, Hefei, Anhui, China). The bicinchoninic acid (BCA) protein assay kit and phenylmethanesulfonyl fluoride (PMSF) were obtained from Beyotime Biotechnology (Shanghai, China). A glucose assay kit was provided by Jiancheng Bioengineering Institute (Nan Jing, Jiangsu, China). Cell proliferation and cytotoxicity assay kit [3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide, MTT] was purchased from Biosynthesis Biotechnology Co. Ltd. (Beijing, China). Rosiglitazone and recombinant human insulin were supplied by Solarbio Science & Technology Co. Ltd. (Beijing, China). Fetal bovine serum (FBS) was purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). Dimethyl sulfoxide (DMSO) was supplied by Jindong Tianzhen Precision Chemical Reagent Factory (Tianjin, China). Polyphenolic compounds including chlorogenic acid, luteolin, caffeic acid, isoschaftoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, and apigenin were purchased from Cdmust Biotechnology Co. Ltd. (Chengdu, Sichuan, China).

2.2. Preparation and Identification of FCP Extract (E)

FCP (fermented for 42 days) and non-FCP were supplied by Hongbin Green Food Group Co. Ltd. (Jianshui, Yunnan, China). The preparation and identification of FCPE were referred to in our previous study [19]. Briefly, the FCP samples were processed including: (1) preparation of crude FCPE by lyophilization and powdering of FCP, ultrasonic-assisted extraction in 80% ethanol (200 W, 20 °C, 30 min), rotary evaporation to remove organic solvents, and then lyophilizing the crude extraction solution; (2) purification of crude FCPE using AB-8 macroporous resin; and (3) identification of FCPE using ultrahigh-performance liquid chromatography (UHPLC). To better represent the effect of fermentation on FCP, we prepared non-fermented FCP extract (non-FCPE) using the same extraction procedure.

The chromatogram of polyphenols in FCPE is shown in Figure S1. A total of 12 polyphenolic compounds were separated and identified in FCPE. As shown in Table S1, compared with non-FCPE, 6 compounds were significantly increased in FCPE (non-FCPE vs. FCPE), including kaempferol-3-*O*-rutinoside (149.88 ± 4.44 vs. 1095.74 ± 46.70 μg/g), caffeic acid (99.01 ± 6.72 vs. 1047.54 ± 53.57 μg/g), luteolin *C*-[pentosyl]-glucoside (10.55 ± 0.87 vs. 137.25 ± 17.51 μg/g), kaempferol-3-*O*-glucoside (19.29 ± 0.39 vs. 24.00 ± 0.56 μg/g), luteolin (0.00 ± 0.00 vs. 159.17 ± 2.51 μg/g), and apigenin (15.55 ± 0.04 vs. 35.26 ± 0.36 μg/g). Except for luteolin *C*-[pentosyl]-glucoside (no standard sample), the other 5 significantly increased compounds were used to assess the ameliorative effect on insulin resistance in vitro.

2.3. Cell Culture and Cell Viability Assay

HepG2 cells were obtained from the cell bank of the Kunming Institute of Zoology (Chinese Academy of Sciences, Kunming, Yunnan). The cells were cultured in HG-DMEM supplemented with 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) and kept in an atmosphere with 5% CO_2 at 37 °C.

Cell viability was measured using an MTT assay. In brief, HepG2 cells were seeded into 96-well plates at a density of ~1×10^4 cells/well and incubated for 24 h. Subsequently, the cells were treated with different samples (dissolved in the normal medium with different concentrations): insulin (5×10^{-5}, 5×10^{-6}, 5×10^{-7}, 5×10^{-8}, and 5×10^{-9} mol/L), rosiglitazone (1×10^{-4}, 1×10^{-5}, 1×10^{-6}, and 1×10^{-7} mol/L), FCPE (1, 3, 5, and 7 mg/mL), and different polyphenol compounds (luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, caffeic acid, and apigenin at different concentrations of 20, 40, and 60 μmol/mL). After treatment for 24 h, each well was gently washed three times with PBS, and then 150 μL MTT solution (0.5 mg/mL) was added and incubated in darkness

for 4 h. Upon termination, the supernatant was removed and replaced with 150 µL DMSO and shaken for 5 min. The absorbance was measured at 570 nm using a SpectraMax M5 Multi-Mode Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA)

2.4. Insulin-Resistance Induction

The insulin-resistant HepG2 cell model was established according to the previous study [23] with some minor modifications. Briefly, 2 mL HepG2 cell suspension (~5×10^4 cells/mL) was incubated in 6-well plates for 24 h under normal media and conditions. After removing the supernatant and washing it with PBS, the cells were then cultured in the medium without FBS for 24 h. Subsequently, the medium was replaced with fresh FBS-free medium containing 1×10^{-6} or 5×10^{-6} mol/L insulin and incubated for 12 or 24 h. Finally, the optimum insulin concentration and incubation time for the model of insulin resistance were obtained by analyzing the glucose consumption. Cells cultured in normal media and conditions without any special treatment were used as controls. To assess whether the insulin-resistant HepG2 cell model was successfully constructed, normal HepG2 cells cultured under normal medium and conditions without any special treatment were used as controls.

2.5. Glucose Consumption

The insulin-resistant HepG2 cell model was first established in 96-well plates as described above (Section 2.4). After removing the supernatant and washing in PBS, cells were subsequently treated with different samples (dissolved in the normal medium) for 24 h including rosiglitazone (5×10^{-6} mol/L), FCPE (1 mg/mL), and different standard solutions of polyphenol compounds (luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, caffeic acid, and apigenin, 20 µmol/mL). To assess the ability of the different treated samples to alleviate the glucose consumption of insulin-resistant cells, we used normal HepG2 cells cultured in normal medium and conditions without any special treatment as controls.

2.6. Action Mechanism Analysis by Molecular Docking

The interactions between polyphenol compounds (luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, caffeic acid, and apigenin) and insulin resistance-related proteins (INSR, AMPK, PTP1B, and PPAR-γ) were analyzed by molecular docking using SYBYL-X 2.1.1 software (Tripos Inc., St. Louis, MO, USA). The structures of the polyphenol compounds used in this study were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/, accessed on 1 November 2021) as PDB files. The crystal structures of insulin resistance-related proteins (INSR, PDB ID: 1IR3; AMPK, PDB ID: 2V8Q; PTP1B, PDB ID: 2QBS; and PPAR-γ, PDB ID: 2PRG) were obtained from the Protein Data Bank (http://www.rcsb.org/pdb, accessed on 1 November 2021). The specific analytical method of molecular docking was referred to in the previous study [19]. Before the docking between molecule and the polyphenol compound ligands, the structures of the insulin resistance-related proteins were pretreated to expose their active sites, including the removal of crystal water molecules, metal ions, and self-ligands, and the addition of hydrogen atoms and charges.

2.7. Statistical Analysis

Experimental data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. Significant differences between groups were determined by Student's *t*-test or one-way ANOVA followed by Tukey's test for multiple comparisons using Origin 2021 software (OriginLab Crop., Northampton, MA, USA). A *p*-value less than 0.05 suggested statistical significance.

3. Results

3.1. Effects of FCPE on Cell Viability of HepG2 Cells

To examine the cytotoxicity of FCPE on HepG2 cells, the cell viability was measured by MTT assay (Figure 1A). Compared with the control group, the results showed that 1 or 3 mg/mL FCPE had no cytotoxic effect on HepG2 cells, and FCPE at 1 mg/mL even had a positive effect on them. However, the cell viability was significantly reduced when treated with high doses of FCPE (5 and 7 mg/mL). In the non-FCPE treatment, just the low doses (1 mg/mL) had no significant negative effect on the viability of HepG2 cells. Therefore, 1 mg/mL FCPE was selected to analyze its anti-insulin-resistant effect in vitro.

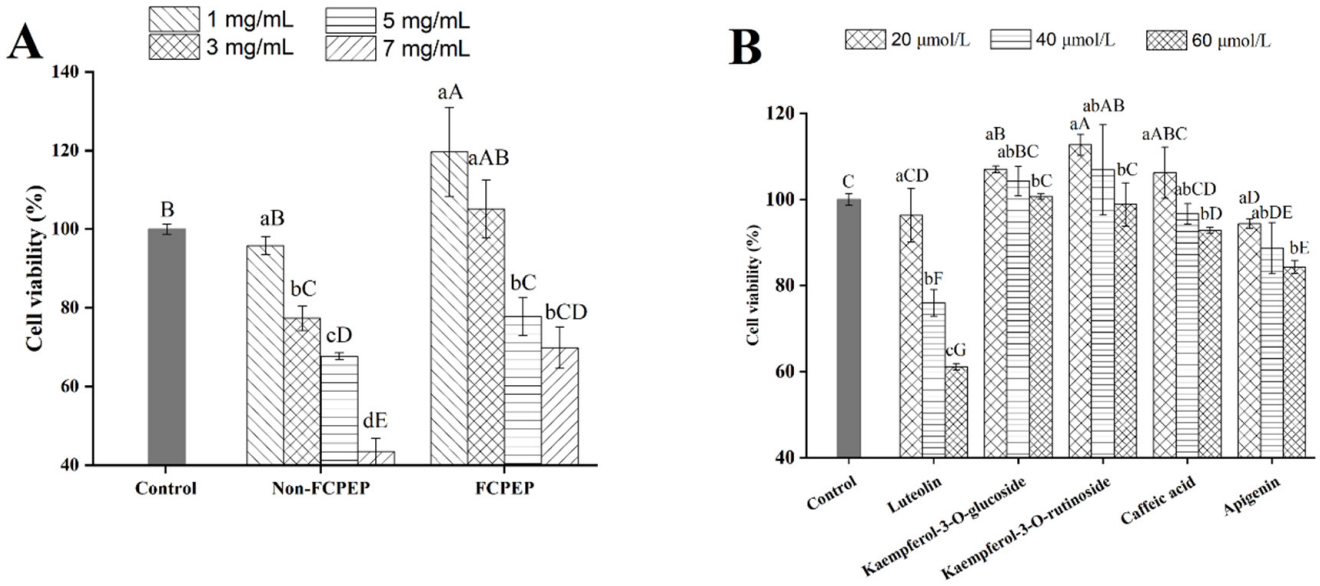

Figure 1. Effect of (**A**) fermented chili pepper extract and (**B**) specific polyphenol compounds (including kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, and apigenin) on HepG2 cell viability. Different lowercase letters above bars represent statistically significant differences between the same treatment sample under different treatment concentrations ($p < 0.05$), while different uppercase letters above bars represent statistically significant differences among all treatments ($p < 0.05$).

To further investigate the specific active molecules that may have a potentially positive effect on promoting the anti-insulin-resistant effect of FCPE, the cytotoxicity of five significantly increased polyphenol compounds in FCPE was also evaluated on HepG2 cells (Figure 1B). The results showed that 20–60 μmol/L kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside had no significant negative effect on HepG2 cell viability, and they could promote the proliferation of HepG2 cells at low doses (especially kaempferol-3-*O*-rutinoside at 20–40 μmol/L). The low doses (20 μmol/L) of luteolin and caffeic acid also showed no significant cytotoxic effect on HepG2 cells. Although apigenin showed a significant negative effect on the viability of HepG2 cells, its inhibitory effect on cell viability at low doses (20 μmol/L) was still less than 10%. Therefore, the optimal treatment concentration of these polyphenol compounds was chosen to be 20 μmol/L for further study of their anti-insulin-resistant effects.

3.2. Insulin-Resistant HepG2 Cells Development

To establish an insulin-resistant HepG2 cell model, the cytotoxicity of insulin on HepG2 cells was first assessed (Figure 2A). The results showed that insulin at concentrations below 5×10^{-6} mol/L and rosiglitazone at concentrations below 1×10^{-6} mol/L had no significant negative effect on the viability of HepG2 cells, suggesting that treatment with insulin or rosiglitazone should be within these corresponding non-toxic concentration ranges.

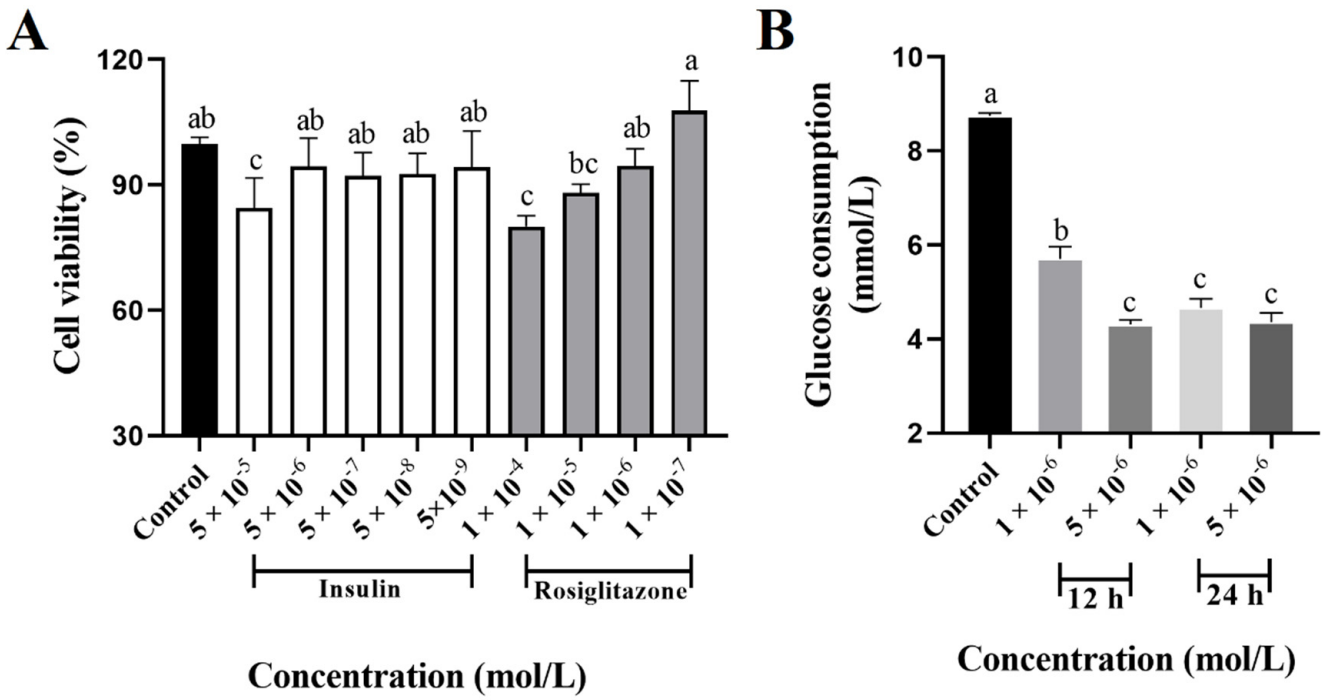

Figure 2. (**A**) The cytotoxicity of insulin on HepG2 cells and (**B**) the effect of different concentrations of insulin (1×10^{-6} and 5×10^{-6} mol/L) on glucose concentration at different time intervals (12 and 24 h). Different lowercase letters above bars represent statistically significant differences among all treatments ($p < 0.05$).

To obtain the optimal insulin concentration and treatment time for establishing an insulin-resistant HepG2 cell model, the effect of different concentrations of insulin (1×10^{-6} and 5×10^{-6} mol/L) on glucose concentration at different time intervals (12 and 24 h) was also investigated (Figure 2B). Significant reduction in the utilization of glucose consumption is an important manifestation of insulin resistance. The results showed that glucose consumption was significantly reduced in all insulin-treated HepG2 cells when compared to the control group (without insulin treatment), suggesting that the insulin-resistant HepG2 cell model was successfully established. In all insulin-treated groups, cells incubated with insulin at a concentration of 5×10^{-6} mol/L for 12 h showed the same minimum glucose consumption as those treated for 24 h with insulin at a concentration of 1×10^{-6} or 5×10^{-6} mol/L. Therefore, treatment with 5×10^{-6} mol/L insulin for 12 h was selected to induce insulin-resistant HepG2 cells as it could save the time of model induction.

3.3. Effects of FCPE and Phenolic Compounds on Glucose Consumption in Insulin-Resistant HepG2 Cells

Glucose consumption is an important indicator of the severity degree of insulin resistance. As shown in Figure 3A, the glucose consumption in the insulin-resistant HepG2 cells was significantly decreased compared with the control group, which suggested that insulin resistance occurred in the model group. Interestingly, the glucose consumption was significantly higher in the rosiglitazone and FCPE-treated groups compared with the model group, while there was no significant difference between the model and non-FCPE groups. These results suggested that FCPE, rather than non-FCPE, has an anti-insulin-resistant effect, which further indicated that the fermentation process could improve or even endow pepper with the improvement effect of insulin resistance. However, the glucose utilization in the FCPE-treated group was still lower than in the rosiglitazone-treated group.

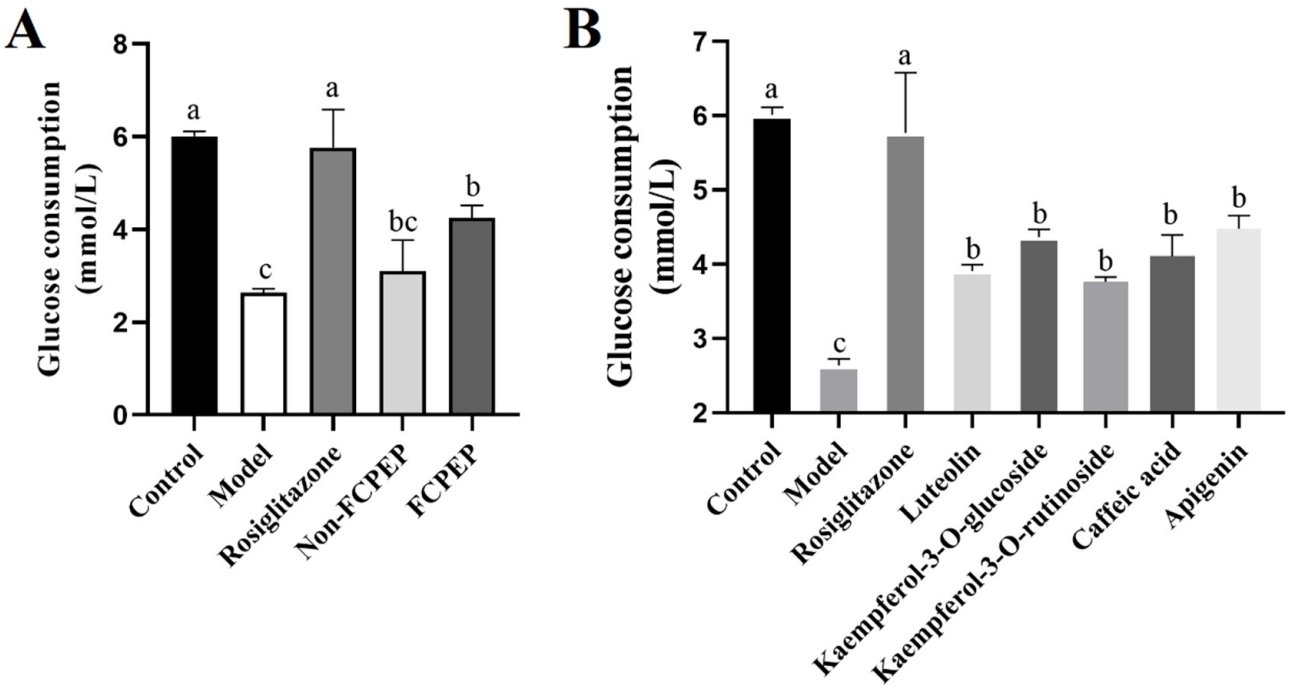

Figure 3. Effects of (**A**) fermented chili pepper extract and (**B**) specific polyphenol compounds (including kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, and apigenin) on glucose consumption in insulin-resistant HepG2 cells. Different lowercase letters above bars represent statistically significant differences among all treatments ($p < 0.05$).

To further explore specific phenolic compounds that may have potential alleviating effects on insulin resistance, we further investigated the effect of polyphenol compounds with increased contents in chili peppers during fermentation on glucose consumption in insulin-resistant HepG2 cells (Figure 3B). Compared with the control group (without insulin treatment), the glucose consumption in the insulin-resistant HepG2 cells was significantly decreased, while it was significantly reversed in the rosiglitazone- and phenolic compounds-treated groups. There was no significant difference in glucose consumption among all phenolic compounds treatment groups, indicating that the potential insulin-resistant effect of FCPE is the synergistic effect of multiple phenolic compounds, including luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, caffeic acid, and apigenin.

3.4. Molecular Docking Analysis of Phenolic Compounds with INSR, PTP1B, PPAR-γ, and AMPK

Molecular docking is an effective and rapid method to investigate the underlying binding sites between phytochemical compounds and enzymes, which will help to reveal the potential molecular mechanism between them. In this study, we further explore the interaction between polyphenol compounds (significantly increased in chili peppers after fermentation) and INSR, PTP1B, PPAR-γ, and AMPK using molecular docking. During molecular docking, *T*-score is an important index reflecting the affinity between the ligand and the corresponding receptor [24]. In this study, only *T*-scores higher than 4.5 were selected for further analysis, including the number and distance of hydrogen bonds, amino acid residues that can form hydrogen bonds with phenolic compounds, and the interaction between the ligands and the optimal docking conformation diagram of the receptor (Tables 1 and 2, and Figure 4).

Table 1. *T*-scores of phenolic compounds docking with four insulin resistance-related proteins.

Phenolic Compounds	INSR	PTP1B	PPAR-γ	AMPK
Luteolin	3.2183	3.7325	3.5430	4.3724
Kaempferol-3-*O*-glucoside	5.8579	5.3547	-	7.0769
Kaempferol-3-*O*-rutinoside	4.0451	6.3401	-	-
Caffeic acid	-	4.0343	-	3.3657
Apigenin	3.6774	3.4906	4.6780	6.293

INSR, insulin receptor; AMPK, adenosine 5′-monophosphate-activated protein kinase; PTP1B, protein tyrosine phosphatase 1B; PPAR-γ, peroxisome proliferator-activated receptor γ.

Table 2. Hydrogen bonds formed between four insulin resistance-related proteins and phenolic compounds.

Insulin Resistance-Related Proteins	Phenolic Compounds	*T*-Score	Number of Hydrogen Bonds	Amino Acid Residue	Average Hydrogen Bond Distance (Å)
INSR	kaempferol-3-*O*-glucoside	5.8579	7	Ser1006, Glu1043, Arg1136, Met1153, Asp1150	2.055
PTP1B	kaempferol-3-*O*-rutinoside	6.3401	6	Arg24, Asp48, Arg254, Gly259	2.287
	kaempferol-3-*O*-glucoside	5.3547	8	Tyr20, Arg24, Ser28, Lys36, Asp48, Gln262	2.158
PPAR-γ	Apigenin	4.6780	3	Gln410, Gly395	1.96
AMPK	Apigenin	6.2390	10	Lys46, Val48, Gly67, Arg69, Ala70, Tyr164, Ile165, Thr167	2.131
	kaempferol-3-*O*-glucoside	7.0769	10	Lys46, Val48, Ala70, Arg69, Tyr164, Thr167	1.99

Phenolic compounds that significantly increased in chili peppers after fermentation. Only the *T*-score of phenolic compounds docking with four insulin resistance-related proteins > 4.5 is shown. INSR, insulin receptor; AMPK, adenosine 5′-monophosphate (AMP)-activated protein kinase; PTP1B, protein tyrosine phosphatase 1B; PPAR-γ, peroxisome proliferator-activated receptor gamma.

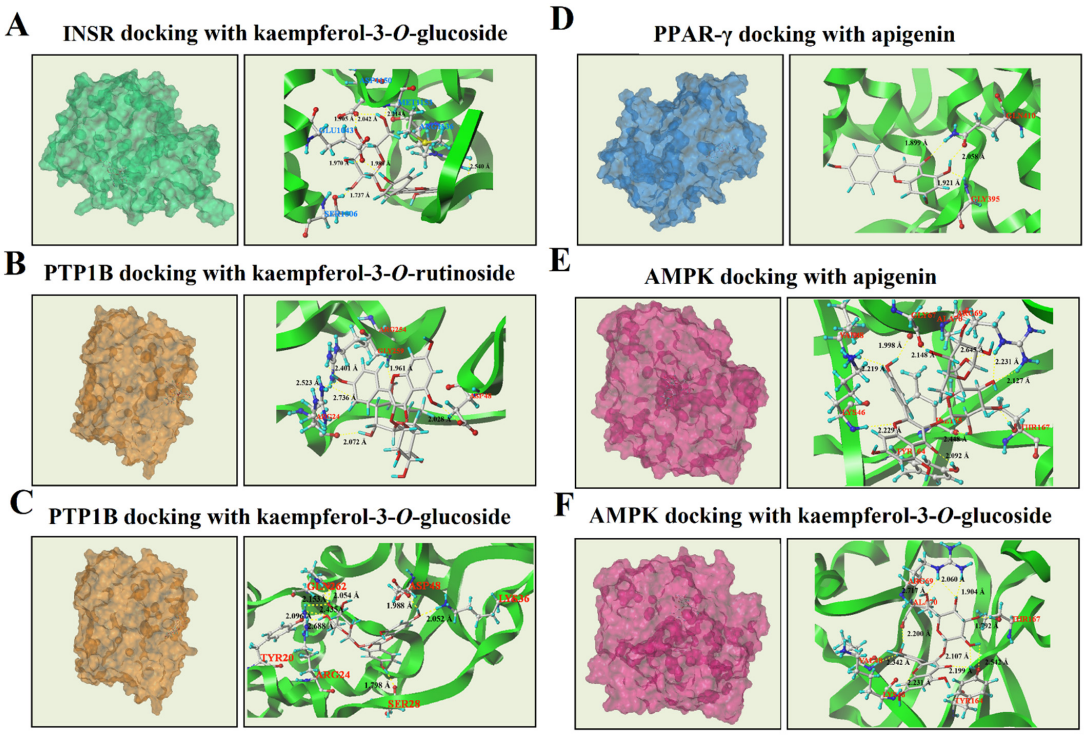

Figure 4. Molecular docking analysis of phenolic compounds with INSR, PTP1B, PPAR-γ, and AMPK. (**A**), kaempferol-3-O-glucoside docking with INSR; (**B**), kaempferol-3-*O*-rutinoside docking with PTP1B; (**C**), kaempferol-3-*O*-glucoside docking with PTP1B; (**D**), apigenin docking with PPAR-γ; (**E**), apigenin docking with AMPK; (**F**), kaempferol-3-*O*-glucoside docking with AMPK.

3.4.1. Molecular Docking Results of INSR

Except for caffeic acid, the *T*-scores of luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, and apigenin with INSR were 3.2183, 5.8579, 4.0451, and 3.6774, respectively (Table 1). These results suggested that kaempferol-3-O-glucoside in FCP had the strongest affinity with INSR. The interaction between kaempferol-3-*O*-glucoside and the optimal docking conformation of INSR is shown in Figure 4A. Results showed that kaempferol-3-*O*-glucoside formed seven hydrogen bonds with Ser1006, Glu1043, Arg1136, Met1153, and Asp1150 amino acid residues of INSR, and the average hydrogen bond distance of these five amino acid residues was 2.055 Å (Table 2).

3.4.2. Molecular Docking Results of PTP1B

The *T*-scores of luteolin, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-rutinoside, caffeic acid, and apigenin with PTP1B were 3.7325, 5.3547, 6.3401, 4.0343, and 3.4906, respectively (Table 1). Therefore, kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside are the two main phenolic compounds regulating PTP1B activity in FCP because they had the strongest affinity with PTP1B. Kaempferol-3-*O*-rutinoside formed six hydrogen bonds with four amino acid residues (Arg24, Asp48, Arg254, and Gly259) of PTP1B at the interaction site, and the average hydrogen bond distance was 2.055 Å (Figure 4B and Table 2). Kaempferol-3-*O*-glucoside formed eight hydrogen bonds with six amino acid residues (Tyr20, Arg24, Ser28, Lys36, Asp48, and Gln262) of PTP1B at the interaction site, and the average hydrogen bond distance was 2.158 Å (Figure 4C and Table 2).

3.4.3. Molecular Docking Results of PPAR-γ

Among the five significantly increased phenolic compounds in FCP, only luteolin and apigenin had *T*-scores when docking with PPAR-γ. Compared with the luteolin, the *T*-score of apigenin with PPAR-γ was higher (luteolin vs. apigenin, 3.5430 vs. 4.6780). Therefore, apigenin was the most likely phenolic compound that had a potential regulatory effect on PPAR-γ in the FCP. The interaction between apigenin and the optimal docking conformation of PPAR-γ is shown in Figure 4D. Results showed that apigenin formed three hydrogen bonds with two amino acid residues (Gln410 and Gly395) of the PPAR-γ receptor with an average hydrogen bond distance of 1.96 Å (Figure 4D and Table 2).

3.4.4. Molecular Docking Results of AMPK

Except for kaempferol-3-*O*-rutinoside, four other phenolic compounds increased in FCP had *T*-scores when docked with AMPK: luteolin (4.3724), kaempferol-3-*O*-glucoside (7.0769), caffeic acid (3.3657), and apigenin (6.293). Owing to apigenin and kaempferol-3-*O*-glucoside having the strongest affinity with AMPK, these two phenolic compounds were used for further docking analysis. The optimal docking conformation of apigenin and kaempferol-3-*O*-glucoside with AMPK is shown in Figure 4E,F. Ten hydrogen bonds were formed between apigenin and eight amino acid residues (Lys46, Val48, Gly67, Arg69, Ala70, Tyr164, Ile165, Thr167) of AMPK, and their average hydrogen bond distance was 2.131 Å (Figure 4E and Table 2). Similarly, 10 hydrogen bonds were also formed between kaempferol-3-*O*-glucoside and AMPK and 6 amino acid residues including Lys46, Val48, Ala70, Arg69, Tyr164 and Thr167, but their average hydrogen bond distance was 1.99 Å (Figure 4F and Table 2).

4. Discussion

Insulin resistance is defined as the reduced insulin sensitivity of the liver, skeletal muscle, and adipose tissue, and further causes glucose metabolism disorders and hyperglycemia, which was one of the major pathogenic mechanisms of T2DM [2,4]. Chili peppers, one of the famous vegetables in China, are rich in multiple bioactive phytochemicals, including antioxidant compounds [15]. In this study, 13 phenolic compounds were identified in chili peppers and 6 of them (kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, apigenin, and luteolin *C*-[pentosyl]-glucoside) were

significantly increased after fermentation (Figure S1 and Table S1). The aim of this study was to investigate the potential role of specific phenolic compounds in FCP in improving insulin resistance.

It has been reported that HepG2 cells could retain the glucose metabolism like normal hepatocytes, and it can be used to establish a model of insulin resistance induced by glucose and/or insulin [8,20,25]. In this study, an insulin-resistant HepG2 cell model was constructed using insulin in the HG-DMEM. A significant reduction in glucose consumption is an important manifestation of insulin resistance [10]. The results showed that the glucose consumption of the insulin-resistant model was significantly lower than that of the control group, suggesting that the insulin-resistant HepG2 cell model had been successfully established by 5×10^{-6} mol/L insulin for 12 h (Figure 2B). Compared to the insulin-resistant HepG2 cells constructed by Alaaeldin et al. [25] (induced by 0.005 μM insulin), the present study reduced the induction time from 24 h to 12 h.

Numerous studies have reported that antioxidant active substances derived from foods or food ingredients may alleviate insulin resistance by improving glucose metabolism and insulin sensitivity [20]. For example, the polyphenol-rich extract of Zhenjiang aromatic vinegar can increase glucose uptake and consumption in the high glucose-induced insulin-resistant HepG2 cell model [20]. Apple polyphenol extract showed improvement in insulin resistance in vitro and in vivo by improving insulin sensitivity [26]. Polyphenols extracted from pomegranate peel have beneficial effects in alleviating insulin resistance by improving insulin sensitivity and regulating glucose metabolism [27]. FCPE was rich in polyphenol compounds, and it has been found to have antioxidant effects in our previous study [19]. Therefore, we speculate that the ability of FCPE to alleviate insulin resistance may be partially related to its antioxidant capacity. In this study, treatment with FCPE also alleviated insulin resistance by promoting glucose consumption (Figure 3A). Interestingly, FCPE but not non-FCPE had a significant ameliorative effect on insulin resistance (Figure 3A), suggesting that the fermentation process improved or even conferred a significant anti-insulin-resistant effect on chili peppers. This may be due to the fermentation process promoting the release of phenolics from chili peppers.

Phenolic compounds are a common class of antioxidants in most plants and have a good ability to relieve insulin resistance [21,28]. The content of phenolic substances in chili pepper extract significantly increased after fermentation (Table S1). To explore the important role of specific phenolic compounds in FCP to attenuate insulin resistance, we further investigated the effect of five increased polyphenol compounds in FCP on glucose consumption in insulin-resistant HepG2 cells. The results showed that five tested polyphenol compounds increased glucose consumption in insulin-resistant HepG2 cells, and there was no significant difference between them (Figure 3B). Therefore, it can be speculated that the insulin resistance alleviating effect of FCP is at least a synergistic effect of these five phenolic compounds. Similarly, the crude extract of *Sonchus oleraceus* Linn was found to improve insulin sensitivity and glucose uptake, mainly attributed to the synergistic effect of caffeic acid and chlorogenic acid in its crude extract [29].

The antidiabetic mechanism of polyphenolic compounds is also multifaceted, and one of them is the improvement of insulin resistance [21]. Numerous studies have reported that the molecular mechanism of anti-insulin-resistant effects of polyphenolics mainly include regulating the expressions of INSR, PTP1B, PPAR-γ, AMPK, glycogen synthase kinase 3 beta (GSK3β), phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt), and transporter type 4 (GLUT 4) [20,21,27,29–31]. For example, caffeic acid and chlorogenic acid can improve insulin resistance by preventing the inactivation of the PI3K/AKT pathway and reducing GLUT4 levels [29]. Vinegar extract rich in polyphenols can inhibit the expression of phosphorylated INSR-1 and activate the PI3K/AKT pathway [20]. Phenolic compounds (agrimonolide and desmethylagrimonolide) can improve glucose uptake by activating AMPK [30]. Molecular docking is a powerful tool used to investigate receptor–ligand interactions [32]. It has been commonly used to study the binding sites of polyphenolic compounds and proteins to reveal the underlying mechanisms [19,24]. INSR, PTP1B, PPAR-

γ, and AMPK play important roles in the insulin signaling pathway and are important targets for screening active substances that may alleviate insulin resistance. In this study, we further investigated the interaction of polyphenol compounds (five significantly increased in chili peppers after fermentation) with INSR, PTP1B, AMPK, and PPAR-γ using molecular docking. INSR can be activated by the phosphorylation of its tyrosine residues to regulate insulin sensitivity and glucose metabolic homeostasis [33,34]. This study showed that kaempferol-3-*O*-glucoside had the strongest affinity with INSR (Figure 4A and Table 2), suggesting that kaempferol-3-*O*-glucoside is the most likely polyphenol compound in FCP to ameliorate insulin resistance by regulating the expression of INSR. PTP1B can recognize the phosphorylated INSR and inactivate it, which played a negative role in the insulin signaling [31,35]. Our results showed that kaempferol-3-*O*-glucoside and kaempferol-3-*O*-rutinoside are the two most important phenolic compounds in FCP to regulate PTP1B activity, as they have the strongest affinity with PTP1B (Figure 4B,C and Table 2). AMPK is an enzyme that plays an important role in regulating glucose homeostasis [21]. Previous studies have shown that AMPK can promote glucose uptake and GLUT4 expression, regulate INRS phosphorylation and oxidative stress, and activate the PI3K/AKT signaling pathway [5,36,37]. Apigenin and kaempferol-3-*O*-glucoside in FCP were found to have the strongest affinity with AMPK (Figure 4E,F and Table 2). Naringin and naringenin can bind to the gamma subunit of AMPK and stimulate its phosphorylation, thereby increasing glucose uptake and alleviating insulin resistance [38]. PPAR-γ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors and its activation in hepatocytes can alleviate insulin resistance and promote glucose uptake [5,39]. The present study showed that apigenin is the most likely phenolic compound with potential PPAR-γ modulating effects in FCP (Figure 4D and Table 2). Similarly, apigenin was identified as a natural modulator to activate PPAR-γ [40].

Taken together, combined with the effects of phenolic substances in FCP on insulin resistance of HepG2 cells and the results of molecular docking, it can be inferred that the alleviation of insulin resistance by FCP is a synergistic effect of multiple polyphenolic compounds, including kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, and apigenin. These polyphenolic compounds, significantly increased in chili peppers after fermentation, were able to partially interact with INSR, PTP1B, PPAR-γ, and AMPK.

5. Conclusions

In this study, an insulin-resistant HepG2 cell model was successfully established using 5×10^{-6} mol/L insulin for 12 h induction in HG-DMEM. Compared with non-FCPE, FCPE increased glucose consumption in the established insulin-resistant HepG2 cell model, suggesting that the fermentation process enhanced or even conferred the ability of chili peppers to alleviate insulin resistance, mainly due to the increase of phenolic substances (including kaempferol-3-*O*-rutinoside, caffeic acid, kaempferol-3-*O*-glucoside, luteolin, and apigenin) in chili peppers during fermentation. Molecular docking analysis suggested that the ameliorative effect of FCP on insulin resistance is, at least, a synergistic effect of these significantly increased polyphenolic compounds in FCP, as they can lead to the activation of proteins related to insulin-resistant pathways including INSR, PTP1B, PPAR-γ, and AMPK, further regulating the disruption of glucose metabolism.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/fermentation9020084/s1, Figure S1: The representative chromatograms of (A) non-fermented chili pepper extract and (B) fermented chili pepper extract. The quantity of identified polyphenolic compounds and MS data are shown in Table S1; Table S1: The quantity of identified polyphenolic compounds and MS data of non-fermented chili pepper extract and fermented chili pepper extract.

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T.W. and M.L.; writing—original draft preparation, T.W.; writing—review and editing, L.Z.; visualization, S.C.; supervision, J.Y.; project administration, X.H.; funding acquisition, J.Y. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

AMPK: adenosine 5′-monophosphate-activated protein kinase; BCA: bicinchoninic acid; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; FCP: fermented chili peppers; FCPE: fermented chili pepper extract; GLUT 4: transporter type 4; GSK3β: glycogen synthase kinase 3 beta; HG-DMEM: high-glucose Dulbecco's Modified Eagle's Medium; INSR: insulin receptor; MTT: 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenytetrazoliumromide; PI3K: phosphatidylinositol 3-kinase; PPAR-γ: peroxisome proliferator-activated receptor gamma; PTP1B: protein tyrosine phosphatase 1B; T2DM: Type 2 Diabetes mellitus.

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