# Stimulation of Glucose Consumption in 3T3-L1 Adipocytes by Triterpenoids from *Cyclocarya paliurus* Leaves

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**Abstract:** *Cyclocarya paliurus* is a unique plant native to China, tea made from the leaves of this plant known for its hypoglycaemic effects. To further explore its hypoglycaemic property, total triterpenoids from *Cyclocarya paliurus* leaves (CPTT) were extracted by an ultrasound-assisted method and successively purified by extraction, macroporous resin adsorption, and decolorization. The total triterpenoid content of CPTT was as high as 86.3%. The content of ursolic acid, oleanolic acid, maslinic acid, corosolic acid, and betulinic acid in CPTT were 19.35%, 17.38%, 7.53%, 4.59%, and 1.42%, respectively. No obvious change in glucose consumption was observed when 3T3-L1 pre-adipocytes were treated with CPTT in the basal condition, but the consumption significantly increased after simultaneous stimulation with 10 nM insulin. For mature adipocytes, CPTT increased glucose consumption significantly in both basal and insulin-stimulated conditions. For dexamethasone-induced insulin-resistant 3T3-L1 adipocytes, CPTT effectively improved insulin sensitivity and enhanced glucose consumption in a dose-dependent manner, with the optimal effect observed at 25 μg/mL. These results suggest that triterpenoids from *Cyclocarya paliurus* leaves can effectively increase glucose consumption in adipocytes and therefore have a potential application in both the food and pharmaceutical industries.

**Key words:** *Cyclocarya paliurus*; glucose consumption; insulin-resistant; triterpenoid; 3T3-L1 adipocytes

**Article No. :** 1673-9078(2014)8-31-37

# 青钱柳叶总三萜刺激 3T3-L1 脂肪细胞的葡萄糖消耗

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**摘要：**青钱柳为我国特有植物，以其叶制成的茶叶具有较好的降血糖效果。为了进一步研究其降糖作用，以超声辅助法提取青钱柳叶总三萜，经萃取、大孔树脂吸附、脱色等纯化后纯度达 86.3%，其中熊果酸、齐墩果酸、山楂酸、科罗索酸和白桦脂酸的含量分别为 19.35%、17.38%、7.53%、4.59%和 1.42%。对于 3T3-L1 前脂肪细胞，纯化后的青钱柳叶总三萜（CPTT）在基础状态下对葡萄糖消耗没有明显的影响，但在同时添加 10 nM 胰岛素进行刺激时能显著提高葡萄糖消耗；对于成熟的脂肪细胞，无论是在基础状态下还是胰岛素刺激状态下，CPTT 均能显著促进其葡萄糖消耗；对于由地塞米松诱导的胰岛素抵抗 3T3-L1 脂肪细胞，CPTT 能有效地改善其胰岛素敏感性，提高葡萄糖消耗，且呈剂量-效应关系，以 25 μg/mL 的效果最佳。以上研究结果表明，CPTT 能促进脂肪细胞的葡萄糖消耗，在食品和药品领域有很好的应用前景。

**关键词：**青钱柳；葡萄糖消耗；胰岛素抵抗；三萜化合物；3T3-L1 脂肪细胞

*Cyclocarya paliurus* (Batal.) Iljinskaja (*C. paliurus*), a plant endemic to central China, is commonly known as Jin-Qian-Liu for its clusters of fruits which look like old Chinese copper coins. Previous reports have shown that *C. paliurus* leaves contain various functional compounds such as triterpenoids, flavonoids, polysaccharides, and trace elements. Leaves of *C. paliurus* therefore have several kinds of health care functions and therapeutic effects[1~3]. The tea processed from *C. paliurus* leaves is called “magical tea” for its beneficial health care effects as well as “sweet tea” for its sweet taste and is now

收稿日期：2014-03-31

基金项目：国家自然科学基金资助项目(31060213 和31260368)；江西省自然科学基金项目(20132BAB204004)

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popular in some places in China [4]. This tea is the first FDA-approved health tea processed in China. A clinical research study on *C. paliurus* tea conducted from 1986 to 1992 in China showed that the rate of hypoglycemic effect of this tea was as high as 90%. These effects were attributed to the functional compounds including steroids, saponins, and trace elements [5].

Recent studies have shown that many natural triterpenoids such as ursolic acid (UA), oleanolic acid (OA), and betulinic acid (BA) have diverse pharmacological and biological activities [6~8], rendering them promising as drugs, foods, and cosmetics. Alqahtani et al. [9] summarized the pentacyclic triterpenoids including the oleanane, ursane, and lupane groups in traditional medicine for the treatment of diabetes and diabetic complications, and elaborated its apparent effects on glucose absorption, glucose uptake, insulin secretion, diabetic vascular dysfunction, retinopathy, and nephropathy. According to the report of Wang et al. [10], total triterpenoids from *Psidium guajava* leaves decrease the level of blood glucose and lipids in diabetic rats.

Based on the analysis of the above-mentioned studies, whether the triterpenoids in *C. paliurus* leaves could stimulate glucose consumption and are the principal functional compounds become the main point to study. In this paper, the triterpenoids in *C. paliurus* leaves were extracted by ultrasonication and successively purified by extraction, macroporous resin adsorption, and decoloration. The stimulant effects on glucose consumption of purified total triterpenoids from *C. paliurus* leaves (CPTT) were investigated using a 3T3-L1 preadipocyte, insulin-sensitive, and insulin-resistant adipocyte model.

## 1 Materials and methods

### 1.1 Materials

The leaves of wild *C. Paliurus* were collected in Xiushui county, Jiangxi province, China, in July and dried under vacuum at 60 ℃. All samples were ground into fine powder in a mill before extraction. 3T3-L1 preadipocytes were purchased from KeyGEN Biotech Co., Ltd. (Nanjing, China).

### 1.2 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM, powder, high glucose, with 1 mM pyruvate and 4 mM L-Glutamine, cat. no. 12800-017) and pancreatin (0.25%, cat. no. 25200056) were purchased from Gibco Invitrogen Corporation. Porcine insulin (≥27 UPS unit/mg, cat no. I5500) and 3-isobutyl-1-methylxanthine (IBMX, HPLC ≥99%, lot no. 1416650V) were purchased from Sigma. Dexamethasone (DEX, HPLC ≥98%, cat. no. D8040), 3-(4, 5- dimethylthiazol-2-yl) -2,5 - diphenyltetrazolium bromide (MTT, ultra-pure grade, cat. no. M9190), dimethyl sulfoxide (DMSO, for cell culture), penicillin (USP grade), and streptomycin (USP grade) were purchased from Solarbio Co., Ltd. (Beijing, China). Glucose Assay Kit (GOD-POD) was purchased from Biosino Bio-Technology and Science Incorporation (Beijing, China). Rosiglitazone (HPLC ≥98%, lot no. 365485774363) was purchased from Shanghai DEMO Medical Tech Co., Ltd. (Shanghai, China). Ursolic acid, OA, BA, corosolic acid (CA), and maslinic acid (MA) (HPLC purity ≥ 95%) were all purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China).

### 1.3 Extraction and purification of CPTT

The dried powder of *C. paliurus* leaves was extracted twice with 65% ethanol (20 mL per gram) at 60 ℃ for 30 min in an ultrasonic extractor (HF-2.5B, Beijing Hong Xiang Long Biotechnology Developing Co., Ltd., China). The concentrated extract was decolored and degreased twice by extraction of petroleum ether and subsequently extracted twice by water-saturated n-butanol. The crude total triterpenoids purified by extraction was adsorbed using AB-8 macroporous resin in a chromatographic column and total triterpenoids was obtained from the 95% ethanol elution fraction. The total triterpenoids was redissolved in acetic ether and decolored using acticarbon by gentle boiling for 5 min. Purified total triterpenoids from *C. paliurus* was obtained after volatilization of acetic ether, ground into powder, and stored at -20 ℃ prior to use.

### 1.4 Triterpenoid determination by HPLC

The content of UA, OA, BA, CA, and MA in CPTT was determined by HPLC. The separation was performed on an RP-$C_{18}$ column (Waters Symmetry, 4.6 × 250 mm, 5 μm) at 40 ℃ with a mobile phase velocity of 0.85 mL/min, which consisted of 90% methanol (HPLC-grade)

and 10% water containing 0.2% acetic acid. The separation was monitored at 210 nm by a dual λ ultraviolet detector (Waters 2487). Purified total triterpenoids were dissolved in 1 mL methanol (HPLC-grade) and filtered through a syringe filter (0.22 μm, Millipore) before injection. Identification of CPTT was performed by comparing the retention time with that of authentic triterpenoids. The regression equation for each triterpenoid quantification was as follows: UA: y = 280334x - 78652 ($R^2$ = 0.9998); OA: y = 443418x - 104447 ($R^2$ = 0.9988); BA: y = 358166x + 117867 ($R^2$ = 0.9979); CA: y = 481808x – 12262 ($R^2$ = 0.9915); MA: y = 365574x – 53989 ($R^2$ = 0.9983). The letter *y* denotes the peak area under the curve (AUC) and *x* stands the weight (μg) of UA, OA, BA, CA, and MA, respectively.

### 1.5 Determination of total triterpenoids

The content of total triterpenoids in CPTT was determined by colorimetry of vanillic aldehyde - perchloric acid [2].

### 1.6 Culture and differentiation of 3T3-L1 preadipocytes

3T3-L1 preadipocytes were seeded in culture plates and cultured in DMEM with high glucose (25 mM), antibiotics (penicillin 60 μg/mL and streptomycin 100 μg/mL), and 10% fetal calf serum (FCS) at 37 ℃ under a humidified 5% $CO_2$ atmosphere. Cell differentiation was induced on the second day after confluence (defined as day 0). Preadipocytes were stimulated with differentiation induction medium that contained 0.5 mM IBMX, 0.25 μM DEX, and 5 μg/mL insulin for 2 days, followed by an incubation in DMEM supplemented with 5 μg/mL insulin and 10% FCS for another 2 days. Subsequently, cells were cultured in DMEM containing antibiotics and 10% FCS until day 8, at which time 90% of the preadipocytes were differentiated into mature adipocytes as characterized by the accumulation of lipid droplets in the cytoplasm.

### 1.7 Development and validation of an insulin-resistant model

An insulin-resistant model was established by DEX induction. Differentiated adipocytes were induced by 1 μM DEX for 48 h and subsequently cultured in DMEM containing 10% FCS with or without 10 nM insulin for 24 h. The glucose concentration of the culture medium was measured using a standard glucose assay kit based on the GOD-POD method (Biosino Bio-Technology and Science Incorporation, Beijing, China). Cells of which glucose consumption had decreased significantly compared with the non-induced control were considered insulin-resistant cells. The drug rosiglitazone was used as a positive control to validate the insulin-resistant model and to confirm that a suitable insulin-sensitizing agent could reverse the effects of this model.

### 1.8 Effects of CPTT on glucose consumption of 3T3-L1 preadipocytes, insulin-sensitive, and insulin-resistant adipocytes

Preadipocytes, insulin-sensitive adipocytes (i.e. differentiated adipocytes), and insulin-resistant adipocytes were treated with CPTT for 24 h, followed by incubation in DMEM containing antibiotics, 10% FCS, and CPTT with or without 10 nM insulin for another 24 h. Glucose consumption on the second day was determined using the GOD-POD glucose assay kit. The effects of CPTT on glucose consumption were analyzed by comparison with the control.

### 1.9 Assessment of the cytotoxicity of CPTT

The cytotoxic effect of CPTT was assessed using the MTT assay. Briefly, after culturing for 48 h in DMEM containing a series of different concentration of CPTT, the medium was removed and the cells were washed twice with PBS. Fresh medium (200 μL) and MTT solution (20 μL, 5 mg/mL in PBS, pH 7.4) were then added to each well of a 96-well plate. Following 4 h of incubation, the medium was removed and the formazan crystals (reaction products of the MTT assay) were solubilized with 150 μL DMSO for 10 min. The amount of formazan was determined by the absorbance at 570 nm. The cytotoxicity of CPTT was expressed as percentage ratio of the sample's absorbance to that of the control.

### 1.10 Statistical analysis

All data were presented as the mean value ± standard deviation (n = 6). The statistical significance between the independent groups was analyzed using the statistical software DPS 6.55.

## 2 Results and discussion

### 2.1 Determination of triterpenoids in CPTT

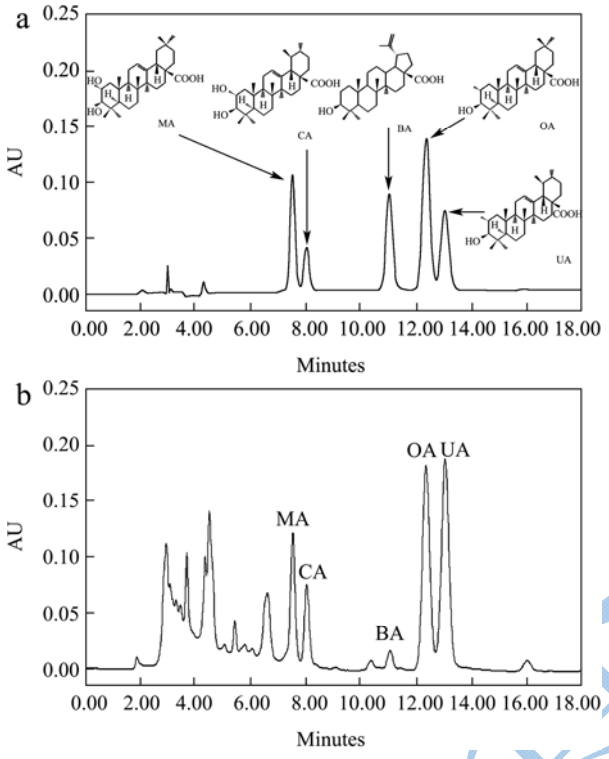


**Fig.1 HPLC chromatograms of five authentic triterpenoids and CPTT monitored at 210 nm**

Note: (a) The five authentic triterpenoids (UA, OA, BA, CA, and MA); (b) CPTT.

The purified total triterpenoids purified by extraction, macroporous resin adsorption, and decoloration was analyzed using HPLC to confirm its triterpenoid content. As shown in Fig. 1, five triterpenoids - UA, OA, MA, CA, and BA - were identified by the retention time compared with authentic triterpenoids. Ursolic acid was the dominant triterpenoid in CPTT, followed by OA, MA, CA, and BA with a content of 19.35%, 17.38%, 7.53%, 4.59%, and 1.42%, respectively. Total content of the above-mentioned five triterpenoids reached 50.27%. The content of total triterpenoids in CPTT was as high as 86.3%. Other components in CPTT (accounted for 13.7%) have not yet been identified and quantified. According to the HPLC chromatogram, most of those undetermined components were compounds with higher polarity and further studies on their effects on glucose consumption of 3T3-L1 adipocytes is needed.

### 2.2 Cytotoxicity of CPTT on 3T3-L1 preadipocytes, insulin-sensitive, and insulin-resistant adipocytes

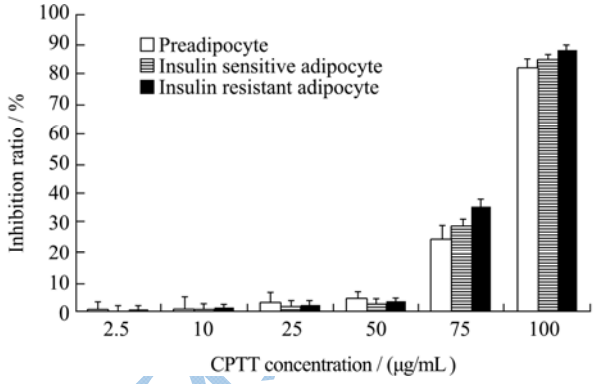


**Fig.2 The viability of preadipocytes, insulin-sensitive and insulin-resistant adipocytes treated by CPTT for 48 h**

To determine suitable CPTT doses for glucose consumption experiments, the cytotoxicity of CPTT on 3T3-L1 preadipocytes, insulin-sensitive, and insulin-resistant adipocytes was assessed using the MTT method after treatment with different concentrations of CPTT for 48 h. Results (Fig. 2) indicated that no cytotoxicity on preadipocytes, insulin-sensitive, and insulin-resistant adipocytes was observed when the concentration of CPTT was less than 50 μg/mL. However, the viability of the three kinds of cells was decreased significantly after the CPTT concentration was increased to 75 μg/mL. Furthermore, 48 h incubation with 100 μg/mL of CPTT resulted in apoptosis in the majority of the cells.

### 2.3 Validation of the DEX-induced insulin-resistant model

Insulin resistance is commonly described as defective utilization of glucose in insulin-targeted tissues. In view of its importance in diabetes mellitus, insulin resistance has been studied intensively not only in clinical cases but also in animal experiments and *in vitro* experiments. Nowadays, the DEX-induced insulin-resistant 3T3-L1 adipocyte model has been widely applied to investigate antidiabetic compounds. In this

paper, we used this model to study the stimulant effects of CPTT on glucose consumption. To validate the insulin-resistant model, glucose consumption analysis was performed after DEX induction for 48 h. Treatment with 1 μM DEX decreased glucose consumption by 11.38%, which was significantly lower than that of non-induced cells. A 10 nM insulin stimulation increased glucose consumption of these induced cells to only 4.36%, which was significantly lower than that of non-induced cells (11.22%). These results indicated that the insulin resistant model was induced by DEX. Our results showed that 3.6 μg/mL rosiglitazone increased glucose consumption of the insulin-resistant adipocytes by 14.08% in the presence of 10 nM insulin in DMEM medium, which suggested that the impaired glucose uptake could be reversed by rosiglitazone. Therefore, rosiglitazone was used as a positive control in this study to confirm the reversal effect of CPTT on insulin resistance.

## 2.4 Effects of CPTT on glucose consumption of 3T3-L1 preadipocytes

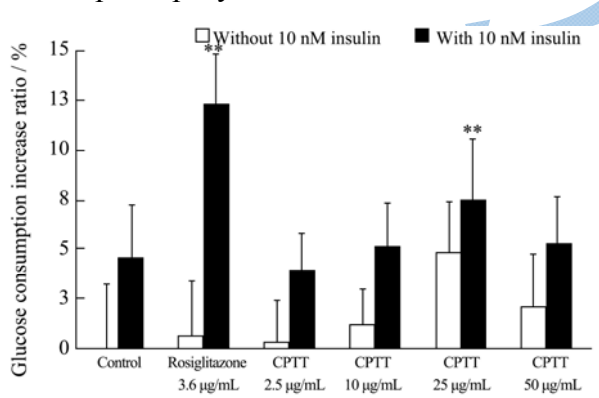


**Fig.3 Effects of CPTT and rosiglitazone on the glucose consumption of preadipocytes**

Note: (Increase ratio of glucose consumption (%) = (glucose consumption of treated group – glucose consumption of control group (without insulin)) / glucose consumption of control group (without insulin) * 100). The sign of ** indicates that this data is significantly different from that of the control group (without insulin) at $P \leq 0.01$.

Neither rosiglitazone nor CPTT showed a significant effect on glucose consumption of 3T3-L1 preadipocytes under basal conditions ($p \geq 0.05$), but both increased the glucose uptake significantly ($p \leq 0.01$) after stimulation with 10 nM insulin (Fig. 3). These results indicated that glucose consumption of preadipocytes increased by 12.27% after treatment with 3.6 μg/mL rosiglitazone and stimulation with 10 nM insulin. However, only a 7.43% increase in glucose consumption was found after treatment with 25 μg/mL CPTT and 10 nM insulin, which is clearly lower than the result seen for rosiglitazone. To our knowledge, information on glucose consumption of 3T3-L1 preadipocytes is scarce and nothing has been reported yet on the effects of triterpenoids on glucose uptake of these cells. In this paper, we have investigated the effects of CPTT on glucose consumption of preadipocytes, but further studies on the mechanism underlying these effects and their significance for diabetes research and prevention are still needed.

## 2.5 Effects of CPTT on glucose consumption of insulin-sensitive adipocytes

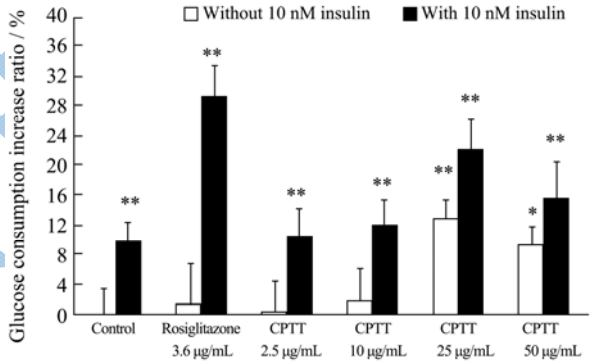


**Fig. 4 Effects of CPTT and rosiglitazone on the glucose consumption of insulin-sensitive adipocytes**

Notes: (Increase ratio of glucose consumption (%) = (glucose consumption of treated group – glucose consumption of control group (without insulin)) / glucose consumption of control group (without insulin) * 100). The sign of ** and * indicates that this data is significantly different from that of the control group (without insulin) at $P \leq 0.01$ and $P \leq 0.05$ respectively.

Purified total triterpenoids from *C. paliurus* significantly increased glucose consumption of insulin-sensitive adipocytes stimulated with 10 nM insulin ($p \leq 0.01$). A positive dose-effect relationship between the CPTT concentration and glucose consumption was found when the concentration of CPTT was increased from 2.5 μg/mL to 25 μg/mL, with a peak value of 25 μg/mL (Fig. 4). However, rosiglitazone showed a better effect (Fig. 4). It should be noted that,

without stimulation by insulin, rosiglitazone did not affect glucose consumption of the insulin-sensitive adipocytes ($p \geq 0.05$) significantly. In contrast, 25 μg/mL CPTT did have a significant effect ($p \leq 0.01$), which indicated that CPTT played a role in promoting glucose uptake by a mechanism different from that of rosiglitazone. *In vivo* experiments have indicated that UA could exhibit antidiabetic effects [11]. Ursolic acid and its derivatives enhanced insulin receptor phosphorylation in CHO/hIR cells by inhibiting protein tyrosine phosphatase 1B and stimulated glucose uptake in L6 myotubes [12]. Consequently, we thought that UA could play an important role in CPTT's stimulation of glucose consumption.

## 2.6 Effects of CPTT on glucose consumption of insulin-resistant adipocytes

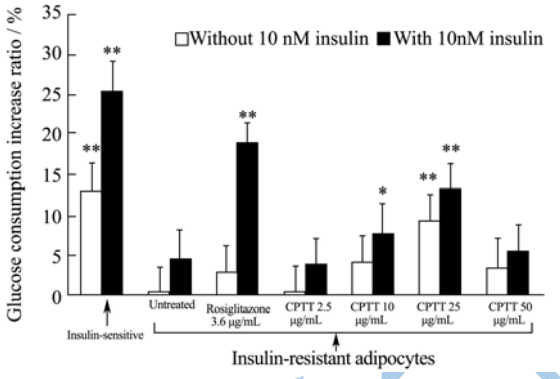


**Fig. 5 Effects of CPTT and rosiglitazone on the glucose consumption of insulin-resistant adipocytes**

Note: (Increase ratio of glucose consumption (%) = (glucose consumption of treated group – glucose consumption of insulin-resistant group (untreated and without insulin)) / glucose consumption of insulin-resistant group (untreated and without insulin) * 100). The sign of ** and * indicates that this data is significantly different from that of the insulin-resistant group (untreated and without insulin) at $P \leq 0.01$ and $P \leq 0.05$ respectively.

The DEX-induced insulin-resistant model in 3T3-L1 adipocytes is one of the most extensively used models in diabetes research. In the present paper, we used this model to study the reversal effect of CPTT on the inhibition of glucose consumption. Results (Fig. 5) suggested that the impaired glucose uptake was effectively restored by CPTT in a dose-dependent manner, with or without 10 nM insulin stimulation. The optimum restoration effect appeared at 25 μg/mL CPTT and glucose consumption increased by 9.14% and 13.12% under basal and 10 nM insulin-stimulated conditions, respectively. However, glucose consumption of the insulin-resistant cells treated with 3.6 μg/mL rosiglitazone and 10 nM insulin was still higher than that of cells treated with 25 μg/mL CPTT and 10 nM insulin. Although 3.6 μg/mL rosiglitazone increased glucose consumption by 19.06% compared with the model cells, no effect was observed without insulin stimulation. Moreover, both CPTT and rosiglitazone failed to restore glucose consumption to the normal level of the insulin-sensitive adipocytes.

It has been reported that the extract of *Eriobotrya japonica* leaves, which consisted of UA (8.7%), tormentic acid (8.2%), CA (5%), MA (2.4%), and OA (1.6%) (total content of the five triterpenoids were 25.9%), reversed insulin resistance of high-fat-fed mice [13]. Triterpenoids such as UA, OA, BA, and palbinone increased glucose uptake and glycogen synthesis via the activation of AMP-activated protein kinase (AMPK) in insulin-resistant human HepG2 cells [14]. Oleanolic acid promoted insulin signal transduction and gluconeogenesis in the insulin-resistant model, which might play a role in the phosphorylation of ERK [15]. Ursolic acid improved certain metabolic parameters of insulin resistance in C57BL/6 mice fed with a high-fat diet [16]. The above analysis indicate that natural triterpenoids could ameliorate insulin-resistance. Considering our triterpenoid content analysis results of CPTT (total amount of UA, OA, MA, CA, and BA accounted for 50.27%) and the analysis of the above-mentioned studies, we conclude that CPTT reversed insulin-resistance due to its five main triterpenoids.

## 3 Conclusions

Purified total triterpenoids was extracted and purified from *C. paliurus* leaves. The total content of five representative triterpenoids (UA, OA, MA, CA, and BA) was 50.27% according to HPLC analysis. The stimulant effects of CPTT on glucose consumption were investigated using the 3T3-L1 preadipocyte, insulin-sensitive, and insulin-resistant adipocyte model. Although CPTT had only a small effect on glucose consumption of preadipocytes under basal conditions, 25

μg·mL$^{-1}$ of CPTT with 10 nM insulin increased glucose uptake significantly ($p \leq 0.01$). A positive dose-effect relationship between the CPTT concentration and glucose consumption was found in both the insulin-sensitive and insulin-resistant adipocyte model, with the peak value of glucose consumption appearing at 25 μg/mL CPTT. Inhibition of glucose consumption of insulin-resistant 3T3-L1 adipocytes was effectively restored by CPTT. In conclusion, CPTT can effectively increase glucose consumption *in vitro* and this effect might be attributed to its main five triterpenoids.

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