



# Pronuciferine and nuciferine inhibit lipogenesis in 3T3-L1 adipocytes by activating the AMPK signaling pathway



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## ABSTRACT

**Aims:** *Nelumbo nucifera* (Gaertn.) leaves are used widely in modulating obesity in traditional Chinese medicine. Our previous work demonstrated that aporphine alkaloids from it increased the glucose consumption in mature 3T3-L1 adipocytes. However, the underlying mechanisms of this increase remain unclear. Here we investigated the modulating effects of pronuciferine and nuciferine on lipogenesis and glucose uptake in insulin resistant 3T3-L1 adipocytes *in vitro*.

**Main methods:** Insulin resistant 3T3-L1 mature adipocytes were induced with dexamethasone, 3-isobutylmethylxanthine and insulin. The lipid droplets and the intracellular triglyceride contents in mature adipocytes were detected by Oil red O staining and colorimetry respectively. The glucose uptake was measured with a fluorescent deoxyglucose analog (2-NBDG). The glucose transporter type 4 (GLUT-4) expression was measured by fluorescent-immunohistochemistry and the activation of 5'-AMP-activated protein kinase (AMPK) was detected by its alpha subunit phosphorylation.

**Key findings:** Both nuciferine and pronuciferine treatments significantly decreased the lipid droplets and the intracellular triglyceride contents but increased the glucose uptake in the insulin resistant 3T3-L1 adipocytes. Furthermore, both pronuciferine and nuciferine showed the ability to up-regulate the expression of GLUT4, triggering the phosphorylation of AMPK in mature 3T3-L1 adipocytes, although pronuciferine exhibited a more powerful effect compared to nuciferine.

**Significance:** In summary, all the results demonstrate that pronuciferine and nuciferine ameliorate the glucose and lipid metabolism in insulin-resistant 3T3-L1 adipocytes, which might be due to the activation of the AMPK signaling pathway.

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## 1. Introduction

Type 2 diabetes mellitus (T2DM) has been steadily increasing and become one of the most serious health problems worldwide. T2DM is characterized by an abnormal high blood glucose level as a result of insulin resistance (IR) and/or insulin deficiency [17]. IR, a hallmark of T2DM, is a condition in which cells fail to respond to insulin. The body produces insulin, but the cells in the body become resistant to insulin and are unable to use it effectively, leading to a high blood sugar concentration. The development of IR is associated with multiple factors. Indeed, it is becoming increasingly clear that genetic, pathophysiological, habitual and environmental factors may be involved [19]. Based on extensive experimental evidence, it has been shown that dysfunctions of glucose and lipid metabolism in adipose tissue have a direct impact on glucose homeostasis [6]. The glucose transporter GLUT4 is the

insulin-sensitive glucose transporter whose main role is to provide the insulin-stimulated glucose uptake by adipose tissue and skeletal muscle [13]. It has been reported that reduced gene expression in the adipocytes of this glucose transporter is directly correlated to the development of insulin resistance and thereby increases the risk of developing diabetes [1]. The adenosine monophosphate-activated protein kinase (AMPK) is the key modulator in energy homeostasis [9], and its activation resulted in beneficial effects on metabolic disorder such as T2DM and obesity [33]. Some anti-diabetic drugs such as metformin and rosiglitazone could activate the AMPK signaling pathway [5]. In addition, the activation of AMPK enhances GLUT4 translocation into the plasma membrane of 3T3-L1 adipocytes [32].

The lotus (*Nelumbo nucifera* Gaertn., Nymphaeaceae) is an aquatic perennial angiosperm that is widely cultivated in eastern Asia, especially in China. The fruit, seeds, roots, and leaves of the lotus are widely used by people as food and drink [23]. Moreover, lotus leaves are extensively used as a traditional Chinese medical herb. Several studies reported that lotus leaves are rich in flavonoids and benzyloisoquinoline alkaloids [34].

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Indeed, the lotus possesses various pharmacological and physiological activities, including antioxidant [14], hepatoprotective [27], anti-diarrheal [18], antiviral [8], and immunomodulatory [15] activities. Thus, the matured leaf is usually used as a functional food and as a medicine in Asia. Some herbal formulas containing lotus leaves in traditional Chinese medicine had been used for the modulation of glucose and lipid metabolism [31,30]. *N. nucifera* leaf extract could impair digestion, inhibited absorption of lipids and carbohydrates, accelerated lipid metabolism and up-regulated energy expenditure [21]. Ahn and his colleagues found that some of the megastigmanes from lotus leaves could inhibit pancreatic lipase and adipocyte differentiation which might contribute to its anti-obesity efficiency [2].

In our previous study, we successfully purified 2-hydroxy-1-methoxyaporphine, pronuciferine, nuciferine and roemerine from the crude extract of lotus leaves by high-speed counter-current chromatography in one step [16]. We also found that 2-hydroxy-1-methoxyaporphine and pronuciferine increased glucose consumption in 3T3-L1 adipocytes. However, little is known about the mechanisms of action of aporphine alkaloids from lotus leaves on glucose and lipid metabolism. In the present study we investigated the effects of pronuciferine and nuciferine (Fig. 1) on glucose uptake and lipid accumulation and their modulation on AMPK activation in insulin resistant 3T3-L1 adipocytes *in vitro*.

## 2. Materials and methods

### 2.1. Materials

Pronuciferine and nuciferine were purified as previously described [16]. 3T3-L1 cells were obtained from the cell bank of the Institute of Biochemistry and Cell Biology of Shanghai (China). Phosphate buffered saline (PBS), DMEM medium, calf serum (CS), fetal bovine serum (FBS), penicillin–streptomycin solution and trypsin–EDTA solution were purchased from GIBCO Co. Inc. (Beijing Representative Office, Beijing, China). 3-Isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), insulin, Oil-red O and 2-NBDG were purchased from Sigma-Aldrich Co. Inc. (Milwaukee, WI, USA). Antibodies specific for polyclonal AMPK, p-AMPK (p-AMPK $\alpha$ 1/2, Thr 172) and GLUT4 and the monoclonal  $\beta$ -actin antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The HRP-labeled goat anti-rabbit IgG antibody and FITC-labeled goat anti-rabbit IgG were purchased from Boster Biotech Co., Ltd. (Wuhan, China).

### 2.2. 3T3-L1 pre-adipocyte culture

3T3-L1 preadipocytes were cultured in DMEM medium with 10% CS, 1.5 g/L sodium bicarbonate and 1% penicillin–streptomycin solution. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and the medium was replaced every other day until confluence.

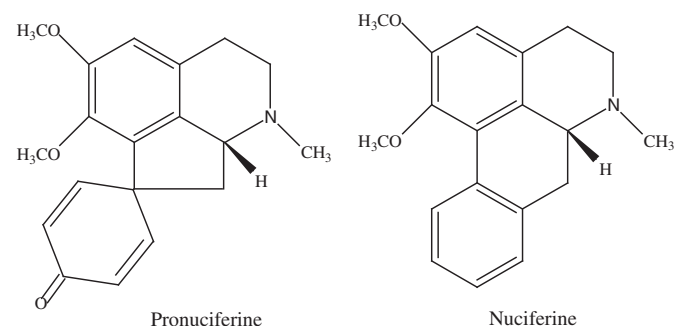


Fig. 1. Chemical structure of pronuciferine and nuciferine.

### 2.3. Generation of mature adipocytes and insulin-resistant adipocyte induction

Mature adipocytes were induced in 3T3-L1 cells as described previously [16]. Briefly, 2 day post-confluent pre-adipocytes (day 0) were cultured in 10% FBS and high glucose (30 mM) DMEM medium containing 0.5 mmol/L isobutylmethylxanthine (IBMX), 1  $\mu$ mol/L dexamethasone and 10  $\mu$ g/mL insulin for 2 days. The cells were further incubated in the growth medium containing 1  $\mu$ g/mL insulin for additional 2 days. Thereafter, cells were maintained in insulin free DMEM with 10% FBS, which was changed every 2 days until the cells were fully differentiated into mature adipocytes. Over 95% of the pre-adipocytes differentiated into mature adipocytes by day 8, as determined by Oil-red O staining.

Insulin-resistant adipocytes were induced in 3T3-L1 mature adipocytes according to reference [3]. The differentiated 3T3-L1 mature adipocytes were treated with 1  $\mu$ mol/L dexamethasone in high glucose DMEM for another 3 days.

### 2.4. Analysis of glucose uptake

The glucose uptake in insulin-resistant mature adipocytes was measured using the fluorescent glucose analog 2-NBDG according to Laffitte's method [10] and modified as previously reported [22]. Briefly, differentiated 3T3-L1 mature adipocytes were gently seeded into 96-well plates ( $1 \times 10^4$  cells/well). After being attached, the cells were maintained in high glucose (30 mM) DMEM with 1.0  $\mu$ mol/L dexamethasone for 24 h. Next, the cells were treated with nuciferine (1 or 2 mg/L), pronuciferine (1 or 2 mg/L) and rosiglitazone (0.35 mg/L) for 48 h respectively, in which the DMEM medium contains 1.0  $\mu$ mol/L dexamethasone. Subsequently, the medium was changed to serum-free DMEM containing 10  $\mu$ mol/L 2-NBDG and 1.0  $\mu$ mol/L insulin. After incubation for 1 h, cells were washed thrice with PBS and the resultant fluorescence was measured (excitation at 485/20 nm and emission at 540/20 nm) using a fluorescent micro-plate reader (Biotek Synergy 2). Values of fluorescence minus background were used for subsequent data analysis.

### 2.5. Oil-red O staining and triglyceride assay

For Oil-red O staining, 3T3-L1 pre-adipocytes were differentiated as mentioned above on Lab-Tek® chambered cover glasses (Nalge Nunc International, Naperville, IL, USA). After induction of insulin resistance and pretreatment of nuciferine (2 mg/L), pronuciferine (2 mg/L) and rosiglitazone (0.35 mg/L) respectively, 3T3-L1 mature adipocytes were washed gently with PBS and fixed with 10% neutral formalin. The cells were permeated using 0.5% Triton-X 100 and stained with filtered Oil-red O solution (60% isopropanol and 40% water) for 30 min. Next, the cells were washed 2–5 times with dH<sub>2</sub>O to remove the excess Oil-red O solution and incubated in Hematoxylin solution for approximately 1 min. The plates were rinsed with dH<sub>2</sub>O and dried. Digital photographs of the stained cells were obtained using a Nikon digital imaging system.

To analyze the content of intracellular triglycerides, the cells were cultured in 12-well plates, washed twice in ice-cold PBS, and harvested in ice-cold lysis buffer (0.1% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris, pH = 8). Total triglyceride content was measured in the lysates using an assay kit and the cellular protein was measured using the BCA protein assay kit (both assay kits were purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The results were expressed as mg of triglyceride per gram of cellular protein.

### 2.6. Western blotting analysis of AMPK

Western blotting was performed according to standard procedures. Firstly, the 3T3-L1 adipocytes were seeded in 6-well plates and differentiated as described in the previous paragraphs. After the treatment

with 1  $\mu\text{mol/L}$  dexamethasone for 24 h, nuciferine (2 mg/L), pronuciferine (2 mg/L) and rosiglitazone (0.35 mg/L) were respectively added in DMEM for additional 2 days, in which the medium also contains 1.0  $\mu\text{mol/L}$  dexamethasone. Next, the cells were lysed in lysis buffer (50 mM Tris-HCl (pH 8.0), 0.4% NP-40, 120 mM NaCl, 1.5 mM  $\text{MgCl}_2$ , 0.1% SDS, 2 mM PMSF, 80 mg/ml leupeptin, 3 mM NaF and 1 mM DTT). Cell lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a polyvinylidene fluoride (PVDF) membrane, blocked with 5% skim milk and incubated overnight with total or phosphorylated AMPK (p-AMPK) primary antibodies (1:1000) (Cell Signaling Technology) at 4 °C, followed by the HRP-conjugated secondary antibody (1:500) for 2 h at room temperature. Finally, immunoreactive proteins were detected using an enhanced chemiluminescent ECL assay kit (Millipore Corp., USA) according to the manufacturer's instructions. The photographs were visualized, and measured by densitometry using a ChemiDoc XRS digital imaging system and Multi-Analyst software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

### 2.7. Immunofluorescence analysis of GLUT4

GLUT4 on the cell surface was assessed using immunofluorescence staining by a confocal laser-scanning microscope (CLSM, Olympus Fluoview FV1000, Japan). 3T3-L1 cells were grown and differentiated on glass coverslips as described in the previous paragraphs. After the insulin resistance protocol and the appropriate compound pre-treatments as described in Section 2.3, the cells in the coverslips were washed gently with PBS and fixed with 10% neutral formalin. The cells were then blocked with 5% skim milk and incubated overnight with the primary rabbit GLUT4 antibody (1:1000) at 4 °C, followed by incubation with the secondary antibody FITC-conjugated anti-rabbit IgG (1:500) for 2 h at room temperature. After washing for 3 times with PBS, the nuclei were stained by DAPI solution (10  $\mu\text{g/mL}$ ) for 3 min and the images were obtained by CLSM. Graphs were semi-quantified by imaging densitometry and analyzed by the "Image-Pro Plus 6.0" software. Ten representative independent views were selected and analyzed for each treatment in each independent experiment. Data are

shown as intensity optical density (IOD) compared to the mature adipocyte group in which IOD was considered as 100%.

### 2.8. Statistical analysis

Data were shown as means  $\pm$  SD from three independent experiments. Statistical analysis was performed by one way ANOVA or Student's *t*-test using the statistical analysis software SPSS 18.0.  $P < 0.05$  was considered statistically significant.

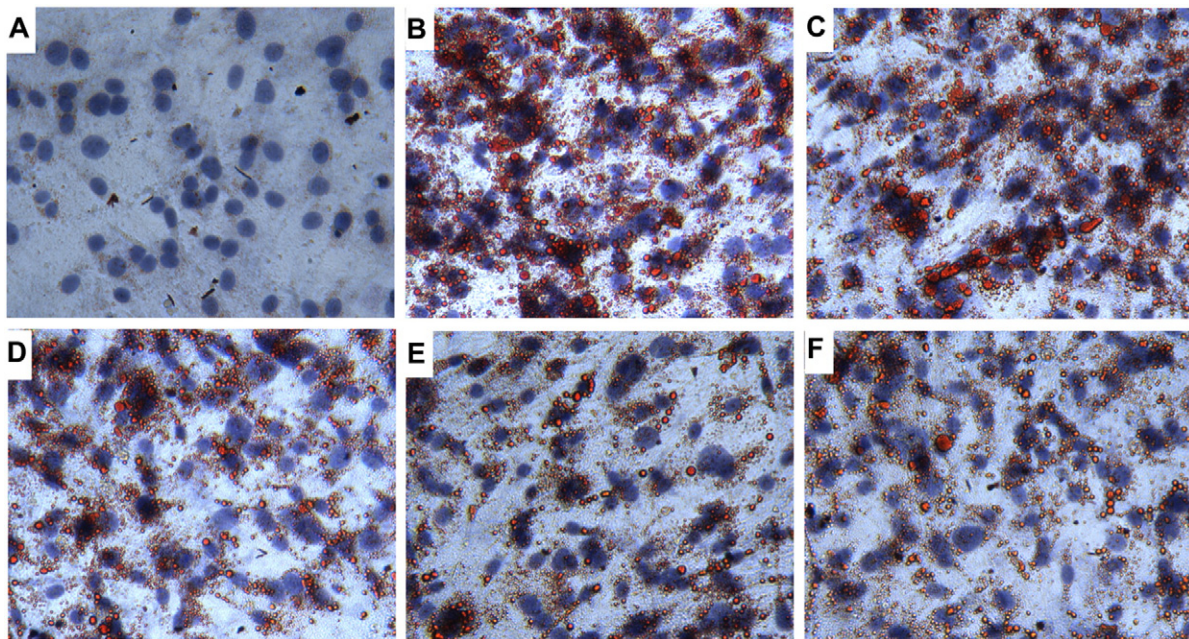
## 3. Results

### 3.1. Effects of pronuciferine and nuciferine on lipid droplet formation in 3T3-L1 adipocytes

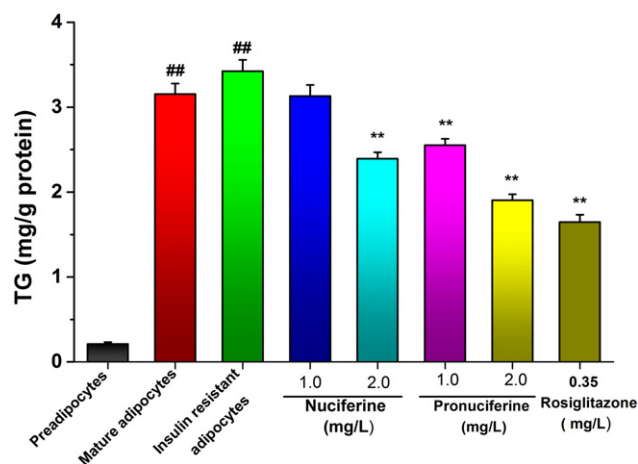
3T3-L1 cells were differentiated for 10 days as we previously reported [16]. Differentiated 3T3-L1 adipocytes were stained by Oil-red O. The results showed the obvious lipid droplet formation in differentiated 3T3-L1 cells (Fig. 2B) whereas none was found in undifferentiated 3T3-L1 cells (Fig. 2A). More big lipid droplets showed up in insulin resistant mature adipocytes (Fig. 2C). Both nuciferine (2 mg/L) (Fig. 2D) and pronuciferine (2 mg/L) (Fig. 2E) decreased the accumulation of lipid droplets as rosiglitazone (0.35 mg/L) did (Fig. 2F) in the differentiated mature 3T3-L1 adipocytes (Fig. 2).

### 3.2. Effects of pronuciferine and nuciferine on intracellular triglyceride (TG) content in 3T3-L1 adipocytes

Total lipid accumulation was quantified in 3T3-L1 adipocytes, as shown in Fig. 3. Differentiated 3T3-L1 adipocytes showed more lipid accumulation than the confluent 3T3-L1 pre-adipocytes, as we expected. To quantify the intracellular lipid content, TG levels in cell lysis solution were detected and the results showed the obvious decrease in the nuciferine, pronuciferine and rosiglitazone treated groups compared with the insulin-resistant adipocyte group (Fig. 3).



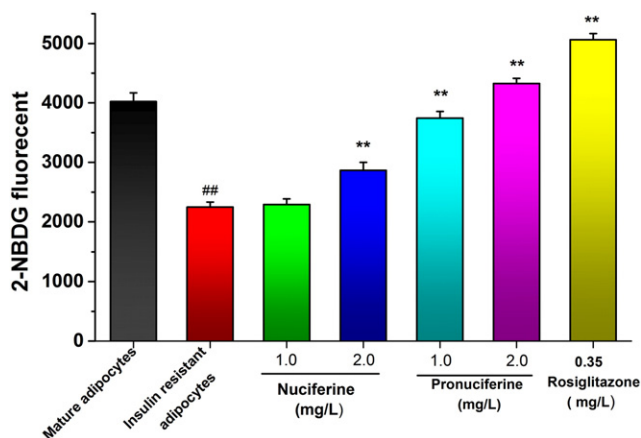
**Fig. 2.** Effects of nuciferine and pronuciferine on lipid droplet formation in 3T3-L1 differentiated mature adipocytes. Images of pre-adipocytes and mature adipocytes stained with Oil-red O and Hematoxylin (200 $\times$ ). A, 3T3-L1 undifferentiated pre-adipocytes; B, 3T3-L1 differentiated mature adipocytes; C, dexamethasone induced insulin-resistant mature adipocytes; D, E, and F, nuciferine (2 mg/L), pronuciferine (2 mg/L) and rosiglitazone (0.35 mg/L) treated insulin-resistant mature adipocytes, respectively.



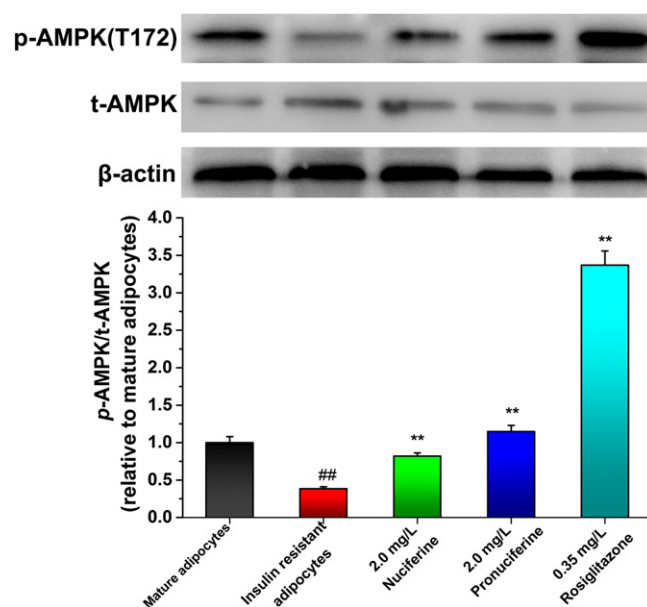
**Fig. 3.** Effects of nuciferine and pronuciferine on intracellular triglyceride (TG) content in 3T3-L1 differentiated mature adipocytes. Intracellular TG content in pre-adipocytes, differentiated 3T3-L1 mature adipocytes and insulin-resistant adipocytes was analyzed by colorimetric method. Data are shown as means  $\pm$  SD of three independent experiments with three replicates each ( $n = 3$ ).  $^{##}P < 0.01$  vs pre-adipocyte group;  $^{**}P < 0.01$  vs insulin-resistant mature adipocyte group.

### 3.3. Effects of pronuciferine and nuciferine on 2-NBDG uptake

The fluorescent deoxyglucose analog (2-NBDG) was used to measure the rates of glucose uptake to investigate the effects of pronuciferine and nuciferine on glucose uptake in the insulin resistant 3T3-L1 adipocytes. As shown in Fig. 4, rosiglitazone (0.35 mg/L) significantly increased insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Both pronuciferine and nuciferine enhanced the uptake of glucose in a concentration dependent manner compared with the insulin-resistant adipocyte group. At a concentration of 2 mg/L, the uptake of glucose in pronuciferine and nuciferine treated groups showed an increase of 27% and 92%, respectively, compared to the control group. In particular, the increase in 2-NBDG uptake in pronuciferine treated cells was almost equivalent to the increase after rosiglitazone treatment (125% increase). These results confirmed our previous study with the glucose oxidase method [16].



**Fig. 4.** Pronuciferine and nuciferine increased 2-NBDG uptake in 3T3-L1 adipocytes. Glucose uptake in 3T3-L1 adipocytes was calculated by the fluorescent intensity of the cells after incubation with 10  $\mu$ M 2-NBDG for 1 h. Data are shown as means  $\pm$  SD of three independent experiments with three replicates each ( $n = 3$ ).  $^{##}P < 0.01$  vs mature adipocyte group;  $^{**}P < 0.01$  vs insulin-resistant mature adipocyte group.



**Fig. 5.** Pronuciferine and nuciferine up-regulated AMPK phosphorylation in insulin-resistant adipocytes. Results are shown as means  $\pm$  SD of three independent experiments with three replicates each ( $n = 3$ ).  $^{##}P < 0.01$  vs mature adipocyte group;  $^{**}P < 0.01$  vs insulin-resistant adipocyte group.

### 3.4. Activation of the AMPK signaling pathway

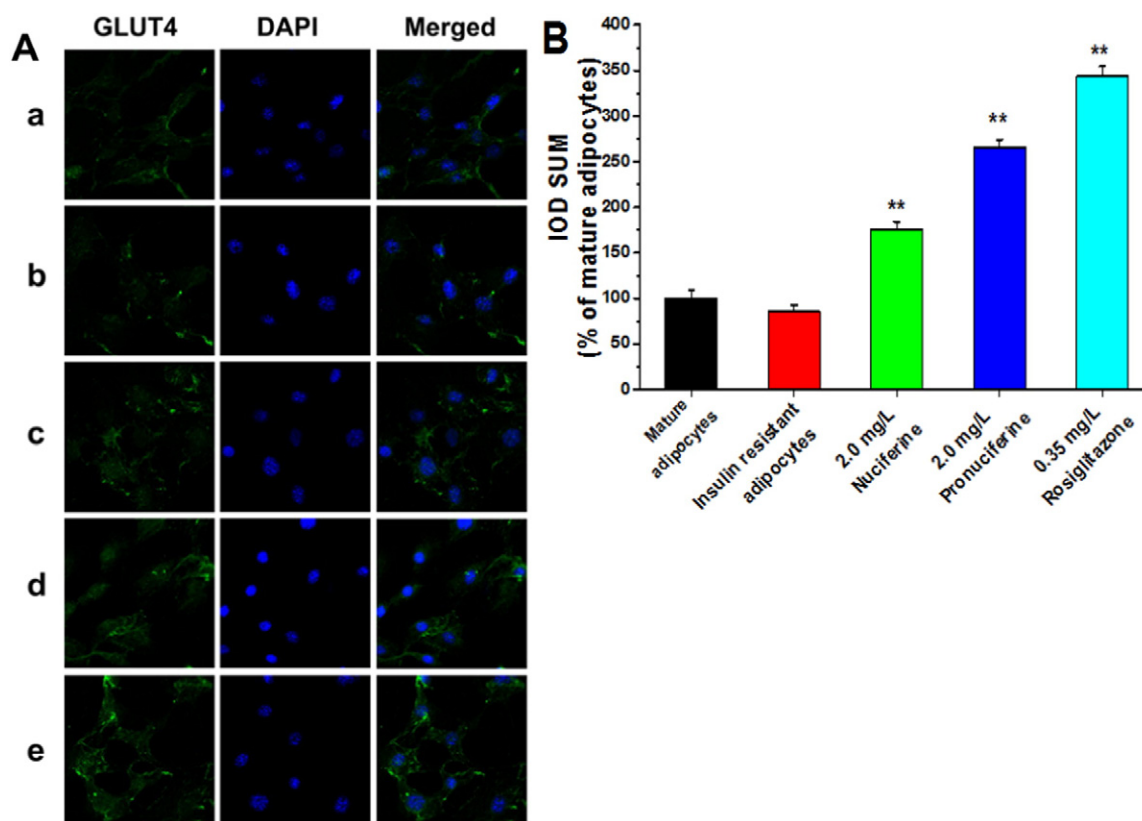
The activation of AMPK was analyzed by western blot to identify the molecular mechanism of action of pronuciferine and nuciferine (Fig. 5). Our results showed that the phosphorylation of AMPK was decreased in the insulin-resistant adipocyte group as compared to the 3T3-L1 pre-adipocyte group. Rosiglitazone 0.35 mg/L was used as a positive control, since it is able to activate the AMPK signaling pathway in 3T3-L1 adipocytes. Both pronuciferine and nuciferine treatments at 2 mg/L induced a statistically significant phosphorylation of AMPK, with pronuciferine showing a more remarkable effect.

### 3.5. Immunofluorescence analysis of GLUT4

GLUT4 expression in differentiated 3T3-L1 adipocytes was shown in Fig. 6. There was no obvious difference in the GLUT4 expression between the mature adipocytes and the insulin resistant mature adipocytes. Both the nuciferine (2.0 mg/L) and pronuciferine (2.0 mg/L) treatments significantly upregulated the GLUT4 expression as rosiglitazone (0.35 mg/L) did. The enhanced GLUT4 expression in the nuciferine, pronuciferine and rosiglitazone treated groups was 2.1, 3.1 and 4.0 fold higher, respectively, as compared to the insulin-resistant mature adipocyte group (Fig. 6B).

## 4. Discussion

Insulin resistance plays a key role in the development of T2DM and metabolic syndrome [28]. Several works over the past years have revealed that adipose tissue plays an important role in controlling whole-body glucose homeostasis in both normal and disease states [24]. Dysfunction of adipose tissue will result in insulin resistance [6]. Induced 3T3-L1 pre-adipocytes could develop into mature adipocytes, which are widely used to investigate the glucose and lipid metabolism in adipocytes *in vitro* [29]. Our previous study showed that the aporphine alkaloids from *N. nucifera* leaves could increase glucose consumption in 3T3-L1 adipocytes [16]. During our previous study we observed a modulating effect of the aporphine alkaloids on lipogenesis. Therefore, in the present work we further explored the effects of pronuciferine



**Fig. 6.** Pronuciferine and nuciferine up-regulated the GLUT4 expression in insulin-resistant mature adipocytes. The GLUT4 expression was observed by confocal laser-scanning microscope (CLSM) after fluorescent immunocytochemical staining for GLUT4 in dexamethasone (1  $\mu\text{mol/L}$ ) induced insulin-resistant 3T3-L1 mature adipocytes (400 $\times$ ) (A). FITC labeled GLUT4 protein appeared on the cell membrane (green) and DAPI dye for fluorescent staining of DNA (blue). a, 3T3-L1 differentiated mature adipocytes; b, dexamethasone induced insulin-resistant mature adipocytes; and c, d, and e, nuciferine (2 mg/L), pronuciferine (2 mg/L) and rosiglitazone (0.35 mg/L) treated insulin-resistant mature adipocytes. CLSM graphs were semi-quantified by imaging densitometry and analyzed by the "Image-Pro Plus 6.0" software (B). Data are presented as the intensity optical density (IOD) compared to the mature adipocyte group that was considered as 100%. Results are shown as means  $\pm$  SD of three independent experiments with ten representative independent views each ( $n = 3$ ). \*\* $P < 0.01$  vs insulin-resistant mature adipocyte group.

and nuciferine on lipogenesis in 3T3-L1 differentiated mature adipocytes *in vitro* to discover and describe their mechanism of action. Our present results confirmed that the differentiated 3T3-L1 mature adipocytes accumulated lipid droplets and triglyceride. The insulin resistance induced by dexamethasone significantly reduced the glucose uptake in 3T3-L1 mature adipocytes. Both nuciferine and pronuciferine treatments reduced the triglyceride levels in insulin-resistant mature adipocytes. Since the glucose transporter is involved in the insulin resistance induced by dexamethasone [26] and the AMPK is the key modulator of glucose uptake in adipocytes [32], we investigated the effects of nuciferine and pronuciferine on the AMPK signal pathway activation and their ability to enhance GLUT4 translocation. The results showed that the phosphorylation level of AMPK and GLUT4 level in insulin-resistant adipocytes were significantly lower with respect to the level in the mature adipocytes. The nuciferine and pronuciferine treatments partially restore the AMPK phosphorylation and GLUT4 level, which indicates that the AMPK signal pathway might be involved in the lipogenesis inhibiting effects of the aporphine alkaloids.

The enzyme AMP-activated protein kinase (AMPK) represents an energy sensor and its activation due to modifications in the AMP/ATP ratio could increase the cellular ATP generation and reduce the ATP use for less critical processes. Stimulation of the AMPK is thus considered as a target approach to increase glucose uptake by some tissues and alleviate insulin resistance [25]. ADP and AMP could activate AMPK by promoting the phosphorylation of threonine 172 in the AMPK  $\alpha$  subunit [20]. Some natural compounds such as resveratrol [4] and berberine [12] could activate AMPK and possess beneficial metabolic effects in diabetic and insulin-resistant conditions. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), an AMPK activator, is able to inhibit the differentiation

of 3T3-L1 adipocytes, when added at an early phase of differentiation [7]. In addition, the AMPK activation mediates lipolysis in differentiated 3T3-L1 mature adipocytes induced by resveratrol [11]. These data indicate that the AMPK modulates not only pre-adipocyte differentiation but also lipolysis in mature adipocytes. In our present study insulin resistance in differentiated 3T3-L1 mature adipocytes was induced by dexamethasone and insulin. The nuciferine and pronuciferine treatments upregulated the glucose uptake while decreased the triglyceride content, which might be due to their ability on activating lipolysis. However, the mechanism responsible for these effects needs further studies.

In our present study we also found that pronuciferine resulted to be more effective in modulating lipogenesis and glucose uptake as well as the AMPK activation and GLUT4 expression compared to nuciferine at the same concentration in insulin-resistant mature adipocytes. Little is known on their structure–activity relationship. Therefore, it is important to further investigate the mechanisms of aporphine alkaloids on AMPK signal pathway activation since it represents a critical factor of substrate metabolic regulation.

## 5. Conclusions

In conclusion, our present study demonstrated that both pronuciferine and nuciferine significantly promoted the phosphorylation of AMPK and enhanced the expression of GLUT4, which finally lead to the stimulation of glucose uptake and the decrease of lipid accumulation. The effects of nuciferine were weaker, compared with the effects of pronuciferine at the same concentration, which might be related with their different chemical structures. In summary, our results indicated that aporphine alkaloids could ameliorate insulin resistance by

activating the AMPK signaling pathway and by up-regulation of the GLUT4 expression.

#### Conflict of interest statement

The authors declare that there are no conflicts of interest.

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