



Research Article

MODULATION OF INSULIN RECEPTOR SUBSTRATE-1 AND GLUCOSE TRANSPORTER 4 EXPRESSIONS IN MOUSE C2C12 MYOBLAST TREATED WITH *CYNANCHUM WILFORDII* AND *PHLOMIS UMBROSA* EXTRACTS

Enkhbaatar Batjargal¹, Chiranjit Ghosh¹, Oliver D. Abanto², Seung Hak Yang³, Kwon-Taek Yi⁴ and Seong-Gu Hwang¹

¹Department of Animal Life and Environmental Science, Hankyong National University, 456-749, Republic of Korea

²University of the Philippines Los Banos, College, Laguna, The Philippines.

³Animal Environment Division, National Institute of Animal Science, R.D.A., Gyeonggi 441-706, Korea

⁴Naturalendo Tech Co., Ltd., Bundang-gu, Sungnam-shi, Gyeonggi-do, 463-400, Republic of Korea

(Received: 11 November 2012; Accepted: 22 November, 2012; Published: 29 December, 2012)

Corresponding Author's email: sghwang@hknu.ac.kr

Abstract: *Phlomis umbrosa* and *Cynanchum wilfordii* were reported to have several immunogenic properties. **Problem statement:** Our present study was focused on determining the effect of the water extracts of *Phlomis umbrosa* (PU) and *Cynanchum wilfordii* (CW) in enhancing insulin receptor substrate 1(IRS-1), glucose transporter 4(GLUT4) expressions and cell proliferation in C2C12 myoblast. **Approach:** Mouse C2C12 cells were treated with 100, 250, and 500 ug/ml of each extract and the cell viability was measured by WST assay. The IRS-1 and GLUT4 mRNA and proteins expressions were determined by reverse transcriptase PCR and western blot respectively. **Results:** PU and CW have no cytotoxic effects on the growth of C2C12 cells. The expressions of IRS-1 and GLUT4 proteins were up-regulated following PU and CW treatment. Moreover, both extracts also increases cellular glucose uptake *in vivo*. **Conclusion:** Our finding reveals that the PU and CW stimulates IRS-1 and GLUT4 expressions in C2C12 cells. PU and CW also increased glucose uptake on *in vivo* model. These results suggest that PU and CW can be potential therapeutic agent for the management of diabetes.

Key words: *Cynanchum wilfordii*, *Phlomis umbrosa*, IRS-1, GLUT4, C2C12 myoblast.

1. INTRODUCTION

Type 2 diabetes has become a common health back draws in majority of our population now a days. It arises mainly due to insulin resistance. When the pancreatic β cell insulin secretion fails to compensate for peripheral insulin resistance, it leads to the type 2 diabetes¹. However study also suggests that diabetes begins with skeletal muscle insulin resistance².

Insulin receptor substrate 1(IRS-1) and glucose transporter 4 (GLUT4) plays a vital role in the insulin mediated glucose uptake pathway. Binding of tyrosine phosphorylated IRS-1 to phosphatidylinositol 3 kinase (PI-3 K) is an important event in insulin signaling pathway leading to insulin stimulated glucose uptake. Low level of IRS-1 and a decrease in its tyrosine phosphorylation have reported in 30 percent of subject at high risk for type 2 diabetes³. The expression of GLUT4 also profoundly affects whole body insulin action and consequently glucose and lipid metabolism^{4,5}.

Several studies were done on the use of natural extract for different health treatments and it seems to be very beneficial these days. Recently,

several extracts from Chinese medicinal herbs were found to be modulating immune response and hormone secretion in human and animals, examples of these are *Cynanchum wilfordii* and *Phlomis umbrosa*. Gagaminine a steroidal alkaloid isolated from *C.wilfordii* were found to be a potent natural antioxidant and useful for clinical tests⁶. Wilfoside K1N is a polyoxypregnane glycoside isolated from *C.wilfordii* (Asclepiadaceae) may have strong anti-angiogenic and anti-invasive activities both *in vitro* and *in vivo*⁷. On the other hand extracts of *P. umbrosa* were found to have beneficial effect on treating allergic diseases^{8,9}. However it was reported that *Polygonum multiflorum* which is a Chinese counterpart of *C.wilfordii* contains emodin derivative compound 10 and the emodin has been found to have glucose uptake regulating properties in 3T3-L1 cells¹¹. Preliminary studies reveal that these extract has got several immunogenic properties. But there is no previous report regarding the effect of this extract on glucose uptake regulation and cell proliferation.

In order to evaluate the possible effects of the CW and PU on glucose uptake regulation the

IRS-1 and GLUT4 expression levels were evaluated on cellular level together with cell proliferation study using C2C12 myoblast cells.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

The cell culture media and reagents such as: Dulbecco's Modified Eagle medium (DMEM), Fetal Bovine Serum (FBS), and Dulbecco's Phosphate Buffered Saline (DPBS); were all purchased from Invitrogen Corporation, Auckland, New Zealand (Cat. Nos: 12100-038, 26140-079, 21600-051, respectively). The Penicillin/Streptomycin (PS) antibiotic was obtained from Lonza, Walkersville, MD, USA (Cat. No. 17-602E). The Quick Cell Proliferation Assay Kit II was purchased from BioVision Research Products, CA, USA (Cat. No. K302-2500). Trizol reagent used in RNA preparation was acquired from Invitrogen Corporation, Carlsbad, CA, USA (Cat. No. 15596-026). The actin primary antibody used was purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA (Cat. No. sc-8432,); GLUT4 antibody was from Abcam, Cambridge, UK (Cat. No. ab35826,); and IRS-1 primary antibody was from Bioriginal International, Saco, ME, USA (Cat. No. K88102R). All secondary antibodies and Enhanced Chemiluminescence Kit were acquired from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA. The other chemicals used were analytical grade and were purchased from Sigma Co., St. Louis, MO, USA

2.2 Animals and Cell Lines

The male Sprague-Dawley rats used in this study were purchased from Charles River Laboratories International, Inc., Wilmington, MA, USA. The C2C12 myoblast cells were acquired from American Type Culture Collection (ATCC), Rockville, CT, USA.

2.3 Preparation of Plant Materials

The *C.wilfordii* and *P.umbrosa* root powder originated from China and supplied by Dawheung Trading Co. Ltd. The two plant powders were prepared in similar process according to the product descriptions. Fifty grams of powdered roots of *C.wilfordii* or *P.umbrosa* was wrapped in a filter cloth. Extracts were obtained by 500 ml water (1:10 sample-water ratio) maintained at 95 to 100°C for 6 hrs. The extracts were dried in a liquid evaporator. The dried extract recovery per gram powder was quantified.

2.4 Blood Glucose Regulation Study in vivo

Twelve (3-week-old) Sprague-Dawley male rats were randomly assigned to three treatment groups: (1) control group, (2) CW group, (3) PU

group. Rats under treatment were gavaged daily with 200 mg/kg of the plant extract samples for 4 weeks; whereas the control group was gavaged by the same volume of water. After 4 week of treatment and after 17 hrs of fasting, rats were anaesthetized with diethyl ether. Blood samples were collected from the inferior vena cava and transferred to heparinized tube, and then the rats were sacrificed. Blood samples were centrifuged for 10 mins at 3,000 rpm. Blood serum glucose was then analyzed. All animals handling and management practices are in accordance with guidelines of Animal Welfare Committee of Hankyong National University, Korea.

2.5 Cell Culture Condition

The study followed the culture condition for C2C12 cells as described by ATCC. Briefly, C2C12 cells were cultured in DMEM supplemented with 10% (v/v) FBS and 1% PS. Cells were incubated at 37°C humidified atmosphere with 5% (v/v) CO₂. All cell incubations were done in this condition unless specified. The medium was changed every 48 hrs until 70 to 80% confluent has been reached. From there, C2C12 cells were either sub-cultured or cultured in treatment conditions.

2.6 Cell Viability Assay

The effects of CW and PU on C2C12 cell proliferation were determined using protocols described by the manufacturer of Quick Cell Proliferation assay Kit II (WST Assay) with some modifications. Briefly, 100µl suspensions of C2C12 cells were seeded at 96-well plate at cell density of 1×10^3 cells/well. Cells were allowed to adhere at the bottom of the culture dish for 3 hrs. Then, 10µl of the treatment extracts in different dilutions were supplied to the cells to achieve medium concentrations of 0, 100, 250, and 500 µg/ml. The cells were further incubated for 24 h. Then, 10µl WST reagent was added into the cell suspension and further incubated for 3 hrs. Optical density was measured using micro plate reader (Tecan A-5002, Tecan Group Ltd., Grodig, Austria) at 450 nm wavelength with reference wavelength of 650 nm.

2.7 RNA Isolation and RT-PCR Analysis

The C2C12 cells were seeded in 100-mm culture dish at cell density of 1×10^5 cells/ml using the usual DMEM growth medium as discussed earlier. Cells were allowed to adhere at the bottom of the culture dish for 3 hrs. The medium was replaced with starvation medium containing DMEM supplemented with 2% FBS and 1% PS and further incubated for 6 hrs. Sample plant extracts were supplemented to the cells at medium concentrations of 0, 100, 250 and 500µg/ml, then incubated for another 24 hrs. To harvest RNA, medium was discarded and cells were washed

twice with cold 1X DPBS. Total RNA was extracted with 1 ml trizol reagent. The RNA isolation was conducted using trizol protocol. The RNA concentration was measured at 260 nm and 280nm absorbance wavelength. The integrity of the RNA was checked by visual inspection of the two ribosomal RNAs 28S and 18S in an agarose gel. The cDNA was prepared by incubating 1 µg of total RNA in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, RNase inhibitors with 250 units of avian myeloblastosis virus-reverse transcriptase, 1 µM dNTP, and random primer (0.05 µM) for 60 min at 37°C. The cDNA fragment was amplified by PCR using the following primers: IRS-1 forward: 5'-CCC ACA GCA GAT CAT TAA CC-3', and IRS-1 reverse: 5'-AGA GAC GAA GAT GCT GGT GC-3'; GAPDH forward: 5'-CCA TCA ACG ACC CCT TCA T-3', and GAPDH reverse: 5'-GTC CTC AGT GTA GCC CAA GA-3'. The PCR was initiated in a thermal cycle programmed at 95°C for 5 min, 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min and amplified for 25 cycles. The amplified products were visualized on 1.5% agarose gels.

2.8 Measurement of IRS-1 and GLUT4 Protein Expressions Using Western Blot Analysis

The C2C12 cells were cultured and treated similarly with those used in Western blot analysis. To harvest proteins, the medium was discarded, cells were washed twice with cold 1X DPBS, treated with 400 µl protein lysis buffer per dish,

and then scraped using cell scraper. Lysates were then vortexed and centrifuged at 10,000 rpm for 5 min at 4°C. The aqueous fraction (protein sample) was transferred to a new tube and stored at -20°C prior to further analysis. Proteins were separated by 8% SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose transfer membrane (Protran, Whatman GmbH, Dassel, Germany). Membranes were incubated for 2 hrs with primary antibodies, followed by 1 hr incubation with horseradish peroxidase-conjugated secondary antibodies. The blots were detected using an Enhanced chemiluminescence's Kit.

2.9 Statistical analysis

All quantitative data are representative of at least three independent experiments. Quantitative data are presented as means ±SD and ANOVA was used to calculate the statistical significance of the differences using Duncan test for multiple comparisons of observed means ($P < 0.05$) using SAS 9.2 statistical software.

3. RESULTS

3.1 Blood Glucose Regulation in vivo

The result of the present study showed that rats given with daily oral dose of 200 mg/kg body weight of PU and CW tended to have lower blood glucose level than the control group (Figure 1). This result suggested that blood glucose uptake in rat was up-regulated by PU and CW treatment.

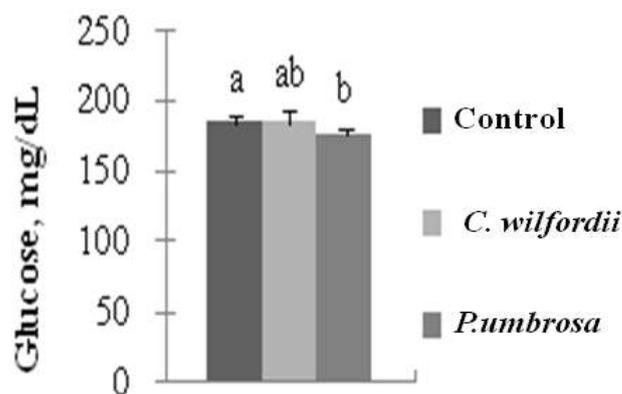


Figure 1. The effects of 200 mg/kg daily intake of CW and PU on plasma glucose level of rats. Each bar represents mean ±standard deviation of three independent experiments. Bars within the same panel with different letters are significantly different ($P < 0.05$).

3.2 C2C12 cells are viable upon PU and CW treatment

In this study we investigated the effect of PU and CW on the viability and cytotoxicity of C2C12 cells by WST assay. The C2C12 cells were treated with different concentrations of CW (0, 100, 250 and 500 µg/ml) and PU (0, 100, 250 and 500 µg/ml) for 24h respectively. The

concentrations of CW and UP and the duration of their treatment used in this study had no significant effect on the viability of C2C12 cells (Figure 2). Results were expressed as percentage of viability with respect to untreated group which were considered as 100% viable. Therefore, these concentrations of CW and UP were chosen for further experiments.

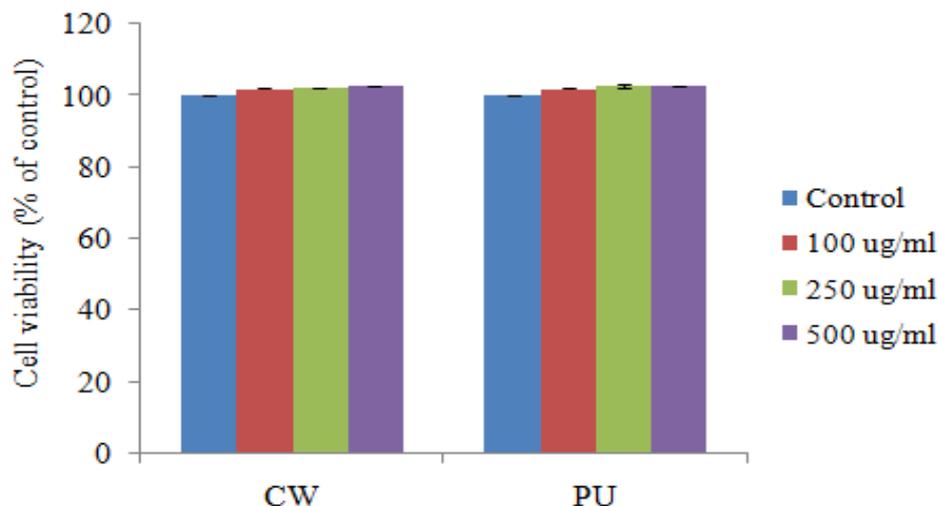


Figure 2. The effects of CW and UP in the viability of C2C12 cells. Briefly C2C12 cells were pretreated with different concentrations of CW (0, 100, 250 and 500 ug/ml) and PU (0, 100, 250 and 500 ug/ml) for 24h. Cell viability was determined using WST assay as described in section 2. Each column shows the mean \pm SEM of triplicate determinations.

3.3 *IRS-1* and *GLUT4* mRNA expressions

The CW extract significantly induced the mRNA expression of *IRS-1* in C2C12 cells at

concentration up to 500 ug/ml (Figure 3A). Meanwhile, the *IRS-1* mRNA expression was induced by only 100 ug/ml of PU (Figure 3B).

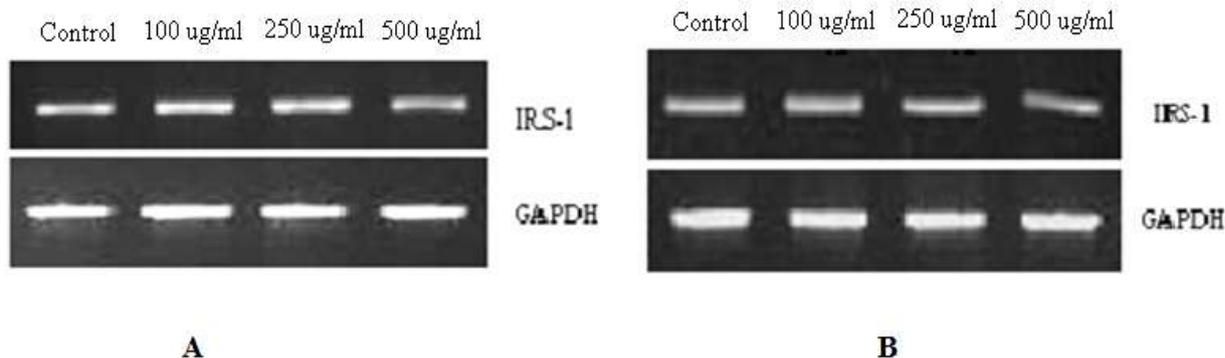


Figure 3. The effects of CW (A) and PU (B) on the mRNA expressions of *IRS-1* and *GAPDH* in C2C12 cells measured by RTPCR analysis.

3.4 *IRS-1* and *GLUT4* Protein expressions

In terms of *IRS-1* protein expression, the result showed that CW extract significantly induced *IRS-1* protein expression in C2C12 cells in dose-dependent and significant manner (Figure 4B). Concomitantly, *GLUT4* protein expression was induced by CW, but only at concentration up to 100 ug/ml (Figure 4A). Beyond 100 ug/ml, the

GLUT4 protein expression was inhibited. PU dose dependently stimulated *GLUT4* expression up to 250 ug/mL. However, at higher concentration *GLUT4* protein expression tended to decrease with PU treatment (Figure 5A). Whereas, *IRS-1* protein expression was stimulated by PU, but only at concentration up to 100 ug/mL (Figure 5B).

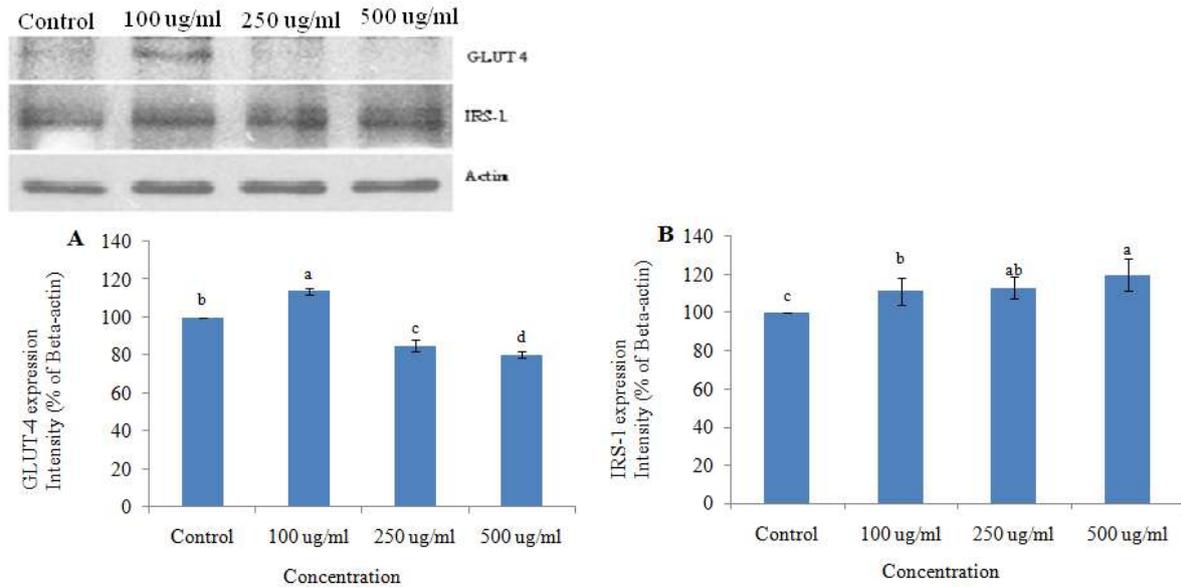


Figure 4. The effects of CW on the protein expression of GLUT4 and IRS-1 in C2C12 cells. C2C12 cells were pretreated with different concentrations of CW (0, 100, 250 and 500 ug/ml). Quantification of GLUT4 and IRS-1 proteins expression was performed by densitometric analysis. β -actin was used as an internal control. The values were expressed as a percentage of maximal band intensity of control cells. Data are the means \pm standard deviation of GLUT4/ β -actin (A) and IRS-1/ β -actin (B) of at least three separate experiments. Bars within the same panel with different letters are significantly different ($P < 0.05$).

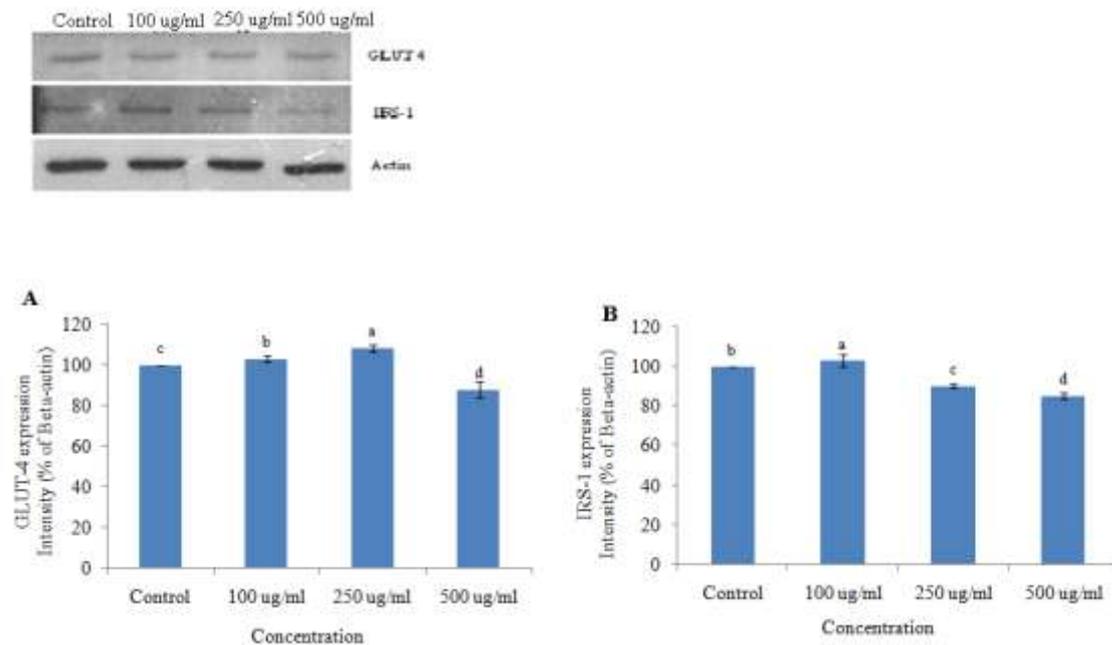


Figure 5. The effects of PU on the protein expression of GLUT4 and IRS-1 in C2C12 cells. C2C12 cells were pretreated with different concentrations of PU (0, 100, 250 and 500 ug/ml). Quantification of GLUT4 and IRS-1 proteins expression was performed by densitometric analysis. β -actin was used as an internal control. The values were expressed as a percentage of maximal band intensity of control cells. Data are the means \pm standard deviation of GLUT4/ β -actin (A) and IRS-1/ β -actin (B) of at least three separate experiments. Bars within the same panel with different letters are significantly different ($P < 0.05$).

4. DISCUSSION

The purpose of the present study was to determine the effects of PU and CW on IRS-1 and GLUT4 proteins and mRNA expressions. The ultimate goal of the study was to find potential additives that can lower blood glucose level by enhancing insulin sensitivity. The result of the present study demonstrated that the cellular expressions of IRS-1 somewhat correlates with blood glucose level in the *in vivo* study. Our data revealed that the increased expressions of IRS-1 up to 100 ug/mL and GLUT-4 up to 250 ug/mL in PU treated C2C12 cells coincide with the decrease in blood glucose level in treated rats. Similarly, the reductions of blood glucose in rats with CW treatment coincide with the expressions of IRS-1 and GLUT4 in C2C12 cells providing evidence for the modulation cellular glucose uptake.

The *C.wilfordii* has been reported to contain gagaminine compound that has antioxidant property⁶, and wilfoside K1N that has anti-angiogenic and anti-invasive properties⁷. However, it is first time in this paper that we reported the effect of *C.wilfordii* and *P.umbrosa* extracts on cellular glucose uptake regulation. The *Polygonum multiflorum*, which is the Chinese counterpart of *C.wilfordii*, has been reported to contain emodin derivative compounds¹⁰. Emodin has been found to have glucose uptake regulating properties in 3T3-L1 cells¹¹.

In order to evaluate the effects of PU and CW extracts on cell cytotoxicity, a C2C12 myoblast cell viability study was conducted. The concentrations of CW and PU and the duration of their treatment used in this study had no significant effect on the viability of C2C12 cells. The present study observed evidences that both CW and PU extracts play roles in enhancing insulin sensitivity as shown by increased expression of IRS-1 proteins. Furthermore, both CW and UP also significantly induced the expression of GLUT4 which serves a vital role in insulin mediated glucose transport.

In summary our studies have shown that the extract of CW induced the IRS-1 protein and mRNA expression up to 500 ug/mL and GLUT4 expression up to 100 ug/mL, and similarly the PU also induce IRS-1 expression up to 100 ug/mL, and GLUT4 expression up to 250 ug/mL. Considering the cell viability result we can conclude that both CW and PU extracts have got no toxic effect on the cells. Taken together, these findings are helpful in understanding the insulin sensitizing properties of these natural extracts and can be used as potential herbal component for the development of new anti-diabetic drugs. However, further supporting studies are required to evaluate these promising effects in animal and human models.

Acknowledgements

This work was partially supported by Korea Institute of Planning & Evaluation for Technology in Food, Agriculture, Forestry & Fisheries under the program entitled "Development of functional feed additive for the improvement of fattening and beef quality of castrated beef cattle by the natural extracts having estrogen-like action of phytoestrogen" (No. 506027-03) and also supported by the Naturalendo Tech Co., Ltd., Republic of Korea.

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