ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 8, Issue, 3, pp. 15894-15897, March, 2017

International Journal of Recent Scientific Research

DOI: 10.24327/IJRSR

Research Article

IN VITRO CYTOTOXICITY AND GLUCOSE UPTAKE ACTIVITY OF CHRYSIN, A FLAVONE IN L6 CELL LINES

Anitha Saravana Kumar* and Rajadurai M

Department of Biochemistry, Muthayammal College of Arts & Science, Rasipuram - 637 408

DOI: http://dx.doi.org/10.24327/ijrsr.2017.0803.0028

ARTICLE INFO

Article History:

Received 05th December, 2016
Received in revised form 21st January, 2016
Accepted 06th February, 2017
Published online 28th March, 2017

Key Words:

Diabetes, Chrysin,
Glucose uptake,
PPARγ, GLUT4

ABSTRACT

Management of type-II diabetes mellitus (T2DM) includes the removal of glucose in blood by stimulating the glucose uptake into peripheral tissues. In relation to DM, recent researchers have demonstrated the beneficial effects of flavonoids, this study was designed to examine the effects of chrysin, a flavone, on glucose uptake into L6 muscle cell line, and to investigate the molecular mechanisms involved in glucose uptake through the expression studies of GLUT4 and PPARγ using L6 cell lines. The cytotoxicity was also evaluated by MTT assay. The results revealed that the chrysin showed the minimal cytotoxicity and the better glucose uptake potential by cell lines over control. The gene expression studies strongly evidenced that the chrysin activates PPARγ thereby increasing the expression of glucose transporter GLUT4.

INTRODUCTION

According to statistics, the greatest increase in the prevalence of type-II diabetes (T2DM) is expected to occur in Asia and Africa by 2030 (Wild *et al*). It is estimated that the number of people with DM will rise from 381.8 million in 2013 to 591.9 million in 2035 (Guariguata *et al*., 2014). In T2DM, there is a malfunction in the insulin-mediated transport of glucose into peripheral tissues, which leads to glucose intolerance followed by a compensatory rise in insulin in the circulation contributing significantly to the pathogenesis of the disease (Lau *et al*., 2011). In the maintenance of whole body glucose homeostasis, skeletal muscle plays a significant role and is responsible for more than 80% of the disposal of postprandial glucose (Chiu *et al*., 2011). Insulin-stimulated glucose transport from blood into skeletal muscle and adipose tissues is the major cellular mechanism for disposal of an exogenous glucose load. Main focus in the treatment of diabetes is on the postprandial lowering of glucose. For the management of T2DM, sufficient numbers of drugs are available commercially, but many of them are out of reach for a significant proportion of the population and many of them have side effects. The use of medicinal plants and their phytochemicals for treating T2DM is not just a search for safer alternatives to pharmaceuticals, which transiently lower the blood glucose.

Based on the number of experimental animal studies, numerous health-promoting properties are being attributed to flavonoids. Many diets are rich in polyphenolic compounds and are consumed daily, having a relatively long half-life with minimum side effects and is easily absorbed in the intestine after injestion. Chrysin, a 5,7-dihydroxyflavone (Figure 1.1) is widely distributed in many plants such as passiflora, pinacaeae, *Pinus aristata*, *Prunus domestica*, silver linden and is also found in honey, bee pollen and bee propolis (glue). Fruits, vegetables, and beverages contain low concentration of chrysin (Pichichero *et al*., 2010; Harminder and Chaudhary, 2011). It is also found in *Oroxylum indicum* or Indian trumpet flower (Deka *et al*., 2013). It has many different biological activities and pharmacological effects on human health such as antiviral, anticancer, bactericidal, anti-inflammatory properties, antiallergic, antimutagenic, antianxiolytic and antioxidant effects (Cho *et al*., 2004).

Hence the present study was aimed to screen the cytotoxicity of the chrysin by MTT assay, to evaluate their glucose uptake by L6 muscle cell line and to investigate the mechanism of action of chrysin in glucose uptake through the analysis of PPARγ and GLUT4 gene expression by RT-PCR in L6 muscle cell line.

*Corresponding author: ***Anitha Saravana Kumar***
Department of Biochemistry, Muthayammal College of Arts & Science, Rasipuram – 637 408

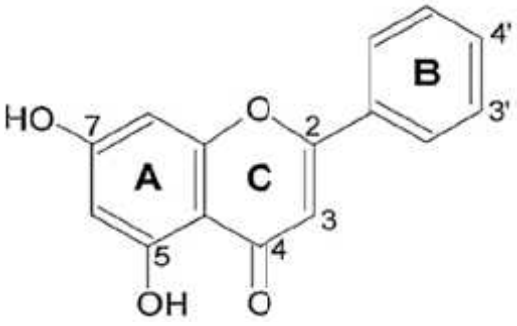

Figure 1 Structure of chrysin (5,7-Dihydroxyflavone)

MATERIALS AND METHODS

Chemicals

3-(4, 5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Bovine Serum Albumin (BSA), D-glucose, Dulbecco's Modified Eagle's Medium (DMEM), Metformin and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA and antibiotics were from Hi-Media Laboratories Ltd., Mumbai. Insulin (Nova Nordisk, 40 IU/ml) was purchased from a local drug store. Dimethyl Sulfoxide (DMSO) and Propanol were from E. Merck Ltd., Mumbai, India.

In vitro Glucose uptake study in L6 muscle cell line

Cell lines and Culture medium

L-6 (Rat, Skeletal muscle) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of L-6 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml) and amphotericin B (5 mg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For *in vitro* studies, each weighed chrysin were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay

Principle: The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells were found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 ml of different test concentrations of chrysin were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 ml of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 ml of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of chrysin needed to inhibit cell growth by 50% (CTC50) values are generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of individual test growth}}{\text{Mean OD of control group}} \right] X\ 100$$

***In vitro* glucose uptake assay** (Imamura *et al.*, 2003; Yap *et al.*, 2007)

Glucose uptake activity of chrysin was determined in differentiated L6 cells. In brief, the 24 h cell cultures with 70-80% confluency in 40mm petri plates were allowed to differentiate by maintaining in DMEM with 2% FBS for 4-6 days. The extent of differentiation was established by observing multinucleation of cells. The differentiated cells were serum starved over night and at the time of experiment, cells were washed with HEPES buffered Krebs Ringer Phosphate solution (KRP buffer) once and incubated with KRP buffer with 0.1% BSA for 30 min at 37°C. Cells were treated with different non-toxic concentrations of chrysin and standard drugs for 30 min along with negative controls at 37°C. 20µl of D-glucose solution was added simultaneously to each well and incubated at 37°C for 30 min. After incubation, the uptake of the glucose was terminated by aspiration of solutions from wells and washing thrice with ice-cold KRP buffer solution. Cells were lysed with 0.1M NaOH solution and an aliquot of cell lysates were used to measure the cell-associated glucose. The glucose levels in cell lysates were measured using glucose assay kit (Biovision Inc., USA). Three independent experimental values in duplicates were taken to determine the percentage enhancement of glucose uptake over controls.

Gene expression studies of GLUT4 and PPARγ- In vitro Assay

Reverse transcriptase – Polymerase chain reaction (RT-PCR)

To investigate the mechanism of action of chrysin glucose uptake, treated cells were analyzed for PPARγ and GLUT4 gene expression by Reverse transcriptase PCR (RT-PCR) as described previously (Armoni *et al.*, 2005). In brief, L6 cells

were treated with chrysin along with standard drug insulin in DMEM with 2% FBS at 37°C with 5% CO_2 for 24 h. After incubation, media was aspirated and total RNA was extracted from cells using total RNA isolation reagent (TRIR). The isolated RNA was quantified on denaturing agarose gel and was subjected to DNase I treatment. Total RNA and oligo dT were used for cDNA synthesis by reverse transcriptase. PCR amplification was carried out using specific primers. The primers used were as follows: Glut-4 sense 5'-CGG GAC GTG GAG CTG GCC GAG GAG - 3'; antisense-5'- CCC CCT CAG CAG CGA GTG A -3'; PPARγ sense 5'-GGA TTC ATG ACC AGG GAG TTC CTC-3'; anti-sense, 5'-GCG GTC TCC ACT GAG AAT AAT GAC-3'; and GAPDH sense, 5'- CCA CCC ATG GCA AAT TCC ATG GCA-3'; anti- sense, 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'. For PPAR gene amplification, the PCR reaction was carried out for 35 cycles, with each cycle comprising a denaturation for 20 sec at 95°C, annealing for 30 sec at 60°C and elongation for 40 sec at 72°C. For GLUT4 gene amplification, the PCR reaction was carried out for 30 cycles with each cycle comprising a denaturation for 45 sec at 95°C, annealing for 1 min at 65°C and elongation for 1 min at 72°C. The final extension was carried out at 72°C for 10 min. GAPDH was used as internal control in each case. The final PCR products were resolved on 1.5% agarose gel stained with ethidium bromide and photographed under exposure to UV light.

Statistical evaluation

The data were expressed as mean ± SEM. Statistical comparisons were performed by one-way ANOVA followed by Turkey's post-test using Graph Pad Prism version 4.0.

RESULTS

The cytotoxicity of chrysin was evaluated by MTT assay and the results were shown in Figure 2 Chrysin has CTC50 value of >100 µg/ml. Glucose utilization in L6 cell lines was studied and the results were given in Figure 3. The results revealed that chrysin enhance glucose uptake by 71.1±0.46% overt control at 100 µg/ml dose. Results were compared with injectable antidiabetic drug insulin (1 IU/ml) to confirm any synergistic effect with chrysin, but results indicate that chrysin does not have any synergistic effect with insulin. Chrysin showed to have potent activity in enhancing the glucose uptake in L6 myotubes with percentage of glucose uptake of 71.1±0.46 and 20.2±4.33 % over the control in L6 myotubes.

GLUT4 and PPARγ expression is down-regulated when there is a relative insulin deficiency in diabetic rats. In accordance with *in vitro* gene expression analysis, Figure 4 shows that the significant insulin stimulated GLUT4 and PPARγ gene expression.

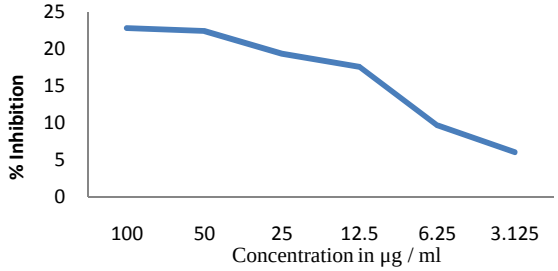

Figure 2 Cytotoxicity of chrysin against L6 cell line by MTT assay

Cells treated with chrysin (100 µg/ml) also significantly induced expression of GLUT4 and PPARγ to the same extent as insulin.

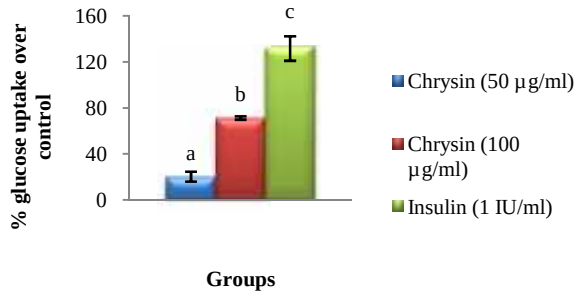

Figure 3 Effect of chrysin on glucose uptake in L6 cell line

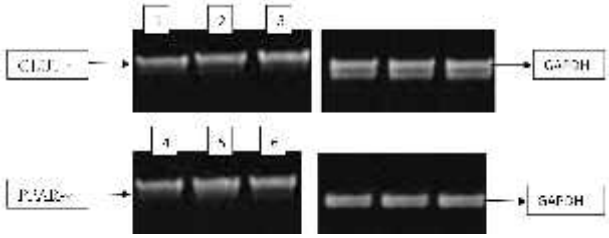

Figure 4 Effect of chrysin on GLUT4 and PPARγ gene expression

Lane: 1-Untreated
Lane: 2- Chrysin 100 µg/ml
Lane: 3- Insulin 1 IU/ml
Lane: 4- Untreated
Lane: 5- Insulin 1 IU/ml
Lane: 6- Chrysin 100 µg/ml

DISCUSSION

The usage of animal cell culture as models of human disease is an integral part of scientific research, providing taxonomic equivalents resembling human physiology (Skelin *et al*., 2010). Consistency and reproducibility of results that can be obtained from a batch of clonal cells is the major advantage of using cell culture (Ulrich *et al*., 2002). L6 is a well-established skeletal muscle cell line and signify a good model for studying glucose uptake, because they have been used extensively to elucidate the mechanism of glucose uptake in muscle (Gupta *et al*., 2010).

Skeletal muscle is the primary site responsible for postprandial glucose use. Moreover, it is the most abundant tissue in the whole body, and thus, the skeletal muscle is more important to maintain normal glucose level (Lalitha and Palani 2015). In skeletal muscle, GLUT4 mediated glucose transport is a major rate-limiting step in glucose metabolism. Impaired glucose transport in skeletal muscle leads to impaired whole body glucose uptake and contributes to the development of T2DM (Dachani *et al*., 2012).

GLUT4 is the major glucose transporter expressed in insulin responsive skeletal muscle cells, where they respond to an acute insulin change by translocating GLUT4 rapidly from an intracellular membrane storage site to the plasma membrane (Gupta *et al*., 2010). Expression and translocation defect of GLUT4 has been reported to be the primary metabolic abnormality in diabetic skeletal muscle (Kong *et al*., 2013).

There are several possible molecular mechanisms which lead to increased glucose uptake into cells, of which the well-

characterized in skeletal muscle is translocation of glucose transporter GLUT4 (Oberg *et al*., 2011). Previous studies have reported whole-body glucose uptake as a linear function of GLUT4 expression in skeletal muscle (Mingrone *et al*., 2002). The activation of PPARγ also enhances insulin sensitization and promotes glucose metabolism by increasing the expression of glucose transporter GLUT4 (Arora *et al*., 2010).

CONCLUSION

Using a L6 rat skeletal muscle cell line, we have demonstrated here a direct relationship between PPARγ activation by chrysin and glucose uptake in muscle cells. The increase in expression of GLUT4 and PPARγ in chrysin treated cells may result from the augmented glucose uptake by L6 muscle cell line. This concludes the contribution of the normoglycemic effect of chrysin.

References

Armoni, M., Harel, C., Bar-Yoseph, F., Milo, S., Karieli, E., (2005). Free fatty acids repress the GLUT4 gene expression in cardiac muscle via novel response elements. *J Biol Chem.* 280(41): 34786-34789.

Arora, M.K., Reddy, K., Balakumar, P., (2010). The low dose combination of fenofibrate and rosiglitazone halts the progression of diabetes-induced experimental nephropathy. *Eur J Pharmacol*. 636: 137-144.

Chiu, T.T., Jensen, T.E., Sylow, L., Richter, E.A., Klip, A., (2011). Rac1 signalling towards GLUT4/glucose uptake in skeletal muscle. *Cell Signal*. 23: 1546-1554.

Cho, H., Yun, C.W. Park, W.K., Kong, J.Y., Kim, K.S. Park, Y. Lee, S. Kim, B.K., (2004). Modulation of the activity of pro-inflammatory enzymes, COX-2 and iNOS, by chrysin derivatives. *Pharmacol Res.* 49: 37-43.

Dachani,S.R., Avanapu, S.R., Ananth, P.H., (2013). *In vitro* antioxidant and glucose uptake effect of Trichodesma indicum in L6 cell lines. *J pharm Bio Sci.* 3 (4): 810-819.

Deka, D.C., Kumar, V., Prasad, C., Kumar, K., Gogoi, B.J, Singh, B.J., Srivastava, R.B., (2013). *Oroxylum indicum*- a medicinal plant of North East India: An overview of its nutritional, remedial, and prophylactic properties. *J Pharm Sci*. 3(1): 104-112.

Francis, D., Rita, L., (1986). Rapid colorometric assay for cell growth and survival modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Methods.* 89: 271-277.

Guariguata L, Whiting DR, Hambleton I, Beagley J, Linnenkamp U, Shaw JE. Global estimates of diabetes prevalence for 2013 and projections for 2035. *Diabetes research and clinical practice*. 2014; 103: 137-49.

Gupta, R.N., Pareek, A., Suthar, M., Rathore G.S., Basniwal P.K., Jairr, D., (2010). Study of glucose uptake activity of *Helicteres isora Linn.* fruits in L-6 cell lines. *Int J Diab Dev Ctries.* 29(4): 170-173.

Harminder, S.V., Chaudhary, A.K., (2011). A review on the taxonomy, ethnobotany, chemistry and pharmacology of Oroxylum indicum Vent. *Indian J Pharm Sci*. 73: 483-490.

Imamura, H.T., Sekine, T., Murata, M., Takayama, K., Nakazawa, K., Nakagawa J., (2003). Stimulation of glucose uptake in muscle cells by prolonged treatment with scriptide, a histone deacetylase inhibitor. *Biosci Biotechnol Biochem.* 67: 1499-1506.

Kong, D., Song, G., Wang, C., Ma, H., Ren, L., Nie, Q., zhang, X., Gan, K., (2013). Overexpression of mitofusin 2 improves translocation of glucose transporter 4 in skeletal muscle of high-fat diet-fed rats through AMP-activated protein kinase signalling. *Mol Med Rep*. 8: 205-210.

Lalitha,R., and Palani, S. (2015). Effect of Alpha-Glucosidase Inhibitory property and glucose uptake assay in L6 cell line by *Griffithsia Pacifica kylin*.IOSR-JPBS. 10 (5): 53-57.

Lau, Fitzsimmons R.L., Pearen M.A., Watt M.J., Muscat G.E.O., (2011). Homozygous staggerer (sg/sg) mice display improved insulin sensitivity and enhanced glucose uptake in skeletal muscle. *Diabetologia*. 54: 1169-1180.

Mingrone, G., Rosa, G., Di Rocco, P., (2002). Skeletal muscle triglyceride lowering is associated with net improvement of insulin sensitivity, TNF-α reduction and GLUT4 expression enhancement. *Int J Obes Relat Metab Disord*. 26: 1165-1172.

Öberg, A.I., Yassin, K., Csikasz, R.I., Dehvari, N., Shabalina, I.G., Hutchinson, D.S., (2011). Shikonin increases glucose uptake in skeletal muscle cells and improves plasma glucose levels in diabetic Goto-Kakizaki rats. PLoS one 6(7): 1-16.

Pichichero, E., Cicconi, R., Mattei, M., Muzi, M.G., Canini, A., (2010). Acacia honey and chrysin reduce proliferation of melanoma cells through alterations in cell cycle progression. *Int J Oncol.* 37(4): 973-981.

Skelin, M., Rupnik, M., Cencic, A., (2010). Pancreatic β-cell lines and their applications in diabetes mellitus research. *ALTEX*. 27 (2/10):105-113.

Ulrich, A.B., Schmied, B.M., Standop, J. *et al*. (2002). Pancreatic cell lines: a review. *Pancreas.* 24(2): 111-120.

Wild, S., Roglic, G., Green, A., Sicree, R., King, H., (2004). A Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. *Diabetes Care.* (5): 1047-1053.

Yap, A., Nishiumi, S., Yoshida, K., Ashida, H., (2007). Rat L6 myotubes as an in vitro model system to study GLUT4-dependent glucose uptake stimulated by inositol derivatives. *Cytotechnology*. 55(2-3): 103-108.

****

How to cite this article:

Leslie C. Ravago *et al*.2017, Impact of Global Medical Service Trips on Physician Careers. *Int J Recent Sci Res.* 8(3), pp. 15826-15829.