

Amelioration of insulin resistance by Rk₁ + Rg₅ complex under endoplasmic reticulum stress conditions

Shree Priya Ponnuraj, Fayeza Siraj, Sera Kang, Hae Yong Noh, Jin-Woo Min, Yeon-Ju Kim, Deok-Chun Yang

Department of Oriental Medicinal Materials and Processing, Kyung Hee University, Suwon, Korea

Submitted: 21-01-2014

Revised: 11-03-2014

Published: 06-08-2014

ABSTRACT

Background: Diabetes mellitus is a metabolic syndrome exaggerated by stress conditions. Endoplasmic reticulum stress (ERS) impairs the insulin signaling pathway making the diabetic conditions worsen. Pharmacological agents are supplied externally to overcome this malfunction. Ginsenosides from *Panax ginseng* C.A Meyer possesses many pharmacological properties and are used for the treatment of diabetes. **Objective:** To investigate the effects of the Rk₁ + Rg₅ complex on the amelioration of insulin resistance in 3T3-L1 cells under endoplasmic reticulum stress conditions. **Materials and Methods:** Heat-processed ginseng extracts are found to contain many pharmacologically active ginsenosides. Among them Rk₁ + Rg₅ is found to be present in higher concentrations than the other minor ginsenosides. The Rk₁ + Rg₅ complex was tested for its effect in the 3T3-L1 insulin-resistant model and subjected to the MTT assay, glucose oxidase assay and gene expression studies using RT-PCR and real-time PCR under endoplasmic reticulum stress conditions. **Results:** Rk₁ + Rg₅ treatment is found to increase the glucose uptake into the cells when compared to that of a positive control (tunicamycin treatment group, TM). Further we have analyzed the role at gene expression level. The Rk₁ + Rg₅ complex was found to show an effect on the IGF 2R receptor, CHOP-10, and C/EBP gene at a particular treated concentration (50 μM). Moreover, stress condition (about 50% decreases) was overcome by the ginsenoside treatments at 50 μM. **Conclusion:** The present results showed that under endoplasmic reticulum stress conditions Rk₁ + Rg₅ complex exhibits a potential protective role in insulin-resistant 3T3-L1 cells.

Keywords: 3T3-L1, dexamethasone, diabetes, insulin, insulin resistance, ginsenosides

INTRODUCTION

Insulin resistance refers to the incompatible state of the cells in taking up the glucose. Insulin lowers blood glucose levels by facilitating glucose uptake into peripheral organs such as the liver, fat and skeletal muscles. Under insulin-resistant conditions, these organs are found to be functioning improperly.^[1] Endoplasmic reticulum stress is the potential contributor in increasing the disease conditions, ER stress triggers transcriptional factors in the protection of the cells from the stress situations. Some of them include CHOP-10, XBP1 splicing, GRP 78. CHOP-10/GADD 153, a member in the family of CCAAT/enhancer-binding proteins. It

includes other components such as α, β and γ C/EBP. One of the members C/EBP β is known to interact with CHOP-10 protein, which in turn leads to C/EBP α in increasing Glut-4 translocation. Some reports states that C/EBP β and δ are very essential for adipocyte differentiation and insulin-stimulated glucose uptake; it is also possible that they may be the therapeutic targets to treat diabetes mellitus. In addition, ER stress, by the up regulation of CHOP, impairs resistin transcription in adipocytes related to insulin resistance.^[2,3] The insulin signaling pathway is initiated via insulin binding on the IGF receptor (insulin-like growth factor receptor) of the effector cells. In diabetic conditions, the insulin binding and the downstream signaling pathway are reported to be malfunctioning and in 3T3-L1 cells (effector cells) the insulin signaling pathway was found to be decreased making diabetic conditions complicated. ER stress reduces the insulin signaling pathway retaining the glucose transport.^[4-6] Ginseng, a well-known medical herb famous for its pharmacological properties, overcomes

Access this article online

Website:
www.phcogres.com

DOI: 10.4103/0974-8490.138257

Quick Response Code:



Address for correspondence:

Dr. Deok-Chun Yang, Department of Oriental Medicinal Materials and Processing, 1, Seocheondong, Giheunggu, Yongin Si, Gyeonggi-do, 446-701, Korea.
E-mail: deokchunyang@yahoo.co.kr

the stress situations by regulating the insulin signaling pathway and glucose uptake in the insulin-resistant organs.^[7,8] Pharmacological agents, such as an external supply of IGF growth factors or the agents mimicking the function of IGF growth factors; and insulin acting on the IGF signaling receptors, are available for overcoming stress situations and affected insulin signaling pathways.^[9] IGF receptor (a kind of glucocorticoid receptor) malfunctioning is found to be ameliorated by ginsenosides. Among the well-known ginsenosides, Rg₁, Rh₁ and C-K are reported to show an effect on overcoming the insulin resistance.^[10,11] Heat processing of ginseng is a technological approach to increase the amount of pharmacologically active ginsenosides. Heat processed ginseng extracts are found to be rich in the Rk₁+Rg₅ complex. The Rk₁+Rg₅ complex is minor ginsenosides obtained from PPD type of ginsenosides by repeated heat treatment and have been reported to show multi therapeutic properties. It exhibits radical scavenging activity of about 68% by the DPPH method.^[12,13] Sun ginsengs containing Rk₁ and Rg₅ as its components are known to be tumor preventer, aging reliever and kidney protector.^[14] Here in this study, we focus on the Rk₁+Rg₅ complex's effect on the insulin resistance phenomenon using the effector cells, adipocytes under endoplasmic reticulum stress. Reports of ginsenosides mediating the adipogenic pathways have been published elsewhere.^[15,16] In this attempt we are reporting under endoplasmic reticulum stress conditions Rk₁+Rg₅ complex's efficacy on reverting the insulin-resistant conditions of the adipocytes.

MATERIALS AND METHODS

Cell culture and reagents

3T3-L1 cells were cultured in DMEM/High glucose at 10% BCS and 1% P/S (Penicillin/Streptomycin) for 2 days to reach the confluence. These cells were later immersed in differentiation media containing 10% BCS, 1% P/S, 0.5 mM IBMX, 5 µg/mL insulin and 250 nM dexamethasone. After culturing for 2 days the cells were then immersed in insulin-containing media (5 µg/mL) alone. In the following days, the cells were cultured in media containing 100 nM dexamethasone, making them insulin resistant. Later ER stress induction was carried out using tunicamycin at 2 µg/mL. The effect of ginsenosides was checked by treating them and incubating at 37°C in humidified CO₂ incubators (5% CO₂).^[17] The Rk₁ + Rg₅ complex used in the present study has been obtained by repeated heat treatment of ginseng root, kindly provided by Ginseng resource bank, South Korea.^[12]

Cell viability assay

The effect of the Rk₁+Rg₅ complex on the viability of the cells used in the study was analyzed by using MTT assay as per the previous reports. In brief, about 2 × 10⁵ cells were plated into 96-well plates. After incubation at 37°C for 24 hours in a humidified incubator, the cells were treated with tunicamycin (2 µg/mL) and ginsenosides at varying concentrations for 24 hours. After this incubation, the MTT solution at 1 mg/mL was added to each well. Cells were then incubated at 37°C for a further 3 hours and the optical density was measured using a micro plate reader (Biotek, USA) at 570 and 630 nm. The cell viability was determined based on the absorbance values at 570 and 630 nm.^[18]

Glucose uptake assays

The glucose uptake from the media was analyzed by using the glucose oxidase reagent (Sigma G3660). Briefly, cells were differentiated using the above media (4500 mg/L of glucose) and then treated with stress agent and ginsenosides. The media was collected and incubated with glucose oxidase at 1 µM for 1.5 hours at 37°C and then with dianoisidine reagent at 37°C for 30 minutes. The absorbance was taken spectrophotometrically at 540 nm and the amount of glucose left in the medium is calculated from the standard glucose, thus giving the glucose utilization under stress and ginsenoside treatment.^[19]

Gene Expression studies using RT-PCR

Total RNA was isolated from the cells using a Qiagen RNeasy Mini Kit. The mRNA expression levels of the genes CHOP-10, C/EBP β and IGF-2R were determined by RT-PCR analysis of total RNA by using one-step RT-PCR kit with gene specific primers as given in Table 1 (included as supplementary). The intensity of the band has been calculated using Image J Software, USA. The Glut-4 gene has been quantified for 40 cycles

Table 1: Primer list

| Primer | bp |
|-----------------------------------------|-----|
| IRS-2 F-CATCGACTTGTCCCATCA | 151 |
| IRS-2 R-CCCATCCTCAAGGTCAAAGG | |
| PDX-1 F-AGGAAAACAAGAGGACCCGTACT | 88 |
| PDX-1 R-CGGGAGATGTATTTGTTAAATAAGAATTC | |
| IGF-2R F-TATCAACATCTGCCAGCCAC | 189 |
| IGF-2R R-CTGTCCGGCTAAGCAATGAGT | |
| CHOP-10 F-AACAGAGGTCACACGCACAT | 150 |
| CHOP-10 R-ACTTTCCGCTCGTTCTCCTG | |
| C/EBP beta F-GCAACACACGTGTAAGTGTG | 212 |
| C/EBP beta R-ATGCTCGAAACGGAAAAGGT | |
| Glut 4F-CTAGATCCCGGAGAGCCTT | 133 |
| Glut 4R-AATAGGGTATAGGGTCCGGG | |
| Beta actin F-ATGAAGTGTGACGTTGACATCC | 196 |
| Beta actin R-CCTAGAAGCATTTCGGGTGCCAGATG | |

IRS=Insulin Receptor Substrate; PDX=Pancreatic and duodenal homeobox-1; CHOP=C/EBP homologous protein-10; C/EBP-β=CCAATT/enhancer binding protein beta

by the real-time SYBR green dye and analyzed using the Rotor-Gene 6000 real-time rotary analyzer (Corbett Life Science, Australia).

Statistical analysis

All data are expressed as mean \pm S.E. The statistical significance between means of the independent groups was analyzed using the Student's *t*-test and a *P* value less than 0.05 and 0.001 was considered to be statistically significant.

RESULTS AND DISCUSSIONS

Cell viability and dose effect of the Rk₁ + Rg₅ complex

The cells were treated with Rk₁+Rg₅ complex in increasing concentration and these were found to be non-toxic until 100 micromolar concentrations were reached. When treated with tunicamycin at 2 μ g/mL, this was found to cause stress to the insulin-resistant cells and at the same time by treating them with the Rk₁+Rg₅ complex the stress was found to be overcome in increasing concentrations [Figures 1 and 2]. Similar studies with other natural bioactive compounds and other ginsenosides have been reported by many other researchers with Rg₃ and Re.^[20]

Rk₁ + Rg₅ effect on insulin-stimulated glucose uptake

The media used to incubate cells with the ginsenosides and stress agents at 37°C was analyzed for the glucose uptake in to the cells. Cells treated with incubation medium (Glucose concentration 25 mM, 10% BCS, 1% P/S) are used as a control. Cells incubated with tunicamycin are used as a positive control. From Figure 3, it can be interpreted that the amount of glucose left in the medium is high for the cells under tunicamycin treatment when compared to that of cells incubated with the incubation medium alone. Parallel treatment with the Rk₁+Rg₅ in increasing concentrations is found to show a decrease in the glucose amount remaining in the medium where the Rk₁+Rg₅ complex aids in the glucose transport via Glut-4 translocation protein. The amount of glucose has been calculated based on the glucose standard used and the insulin-treated cells. A similar phenomenon has been shown by natural components like 3 β -taraxerol, *Mangifera indica* ethyl acetate extract and ginsenosides such as Rg₁, Compound K, Rb₁.^[21,22]

Gene expression

As a next step we were interested in analyzing the protective role of the Rk₁+Rg₅ complex in 3T3-L1 cells under endoplasmic reticulum (ER) stress at concentrations of 25 to 100 μ M. Induction of CHOP-10 is involved in apoptosis of beta cells under ER stress.^[23] CHOP-10, whose expression increased by tunicamycin treatment, was found to shown a decrease in expression by

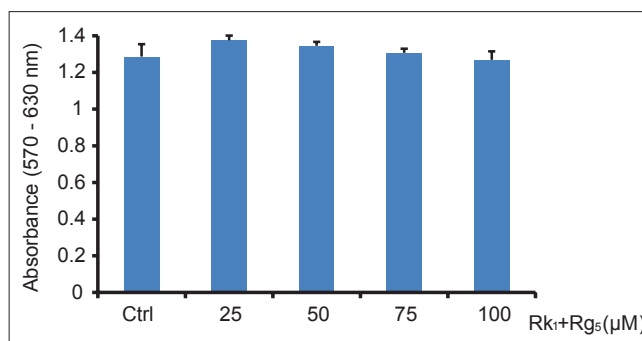


Figure 1: Effect of Rk₁+Rg₅ on insulin-resistant 3T3-L1 cells. Each bar represents the average of three independent experiments Mean \pm S.E. The data have been statistically analyzed

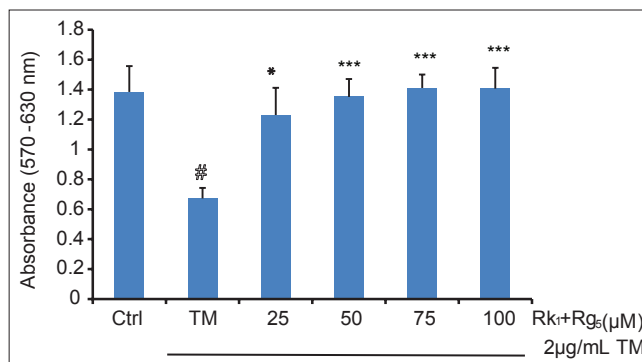


Figure 2: Effect of Rk₁+Rg₅ on insulin-resistant 3T3-L1 cells under tunicamycin (TM) stress treatment. Each bar represents the average of three independent experiments Mean \pm S.E. ****P*<0.001, **P*<0.05 significant levels relative to the tunicamycin treatment group, #*P*<0.001 significant levels relative to the untreated group

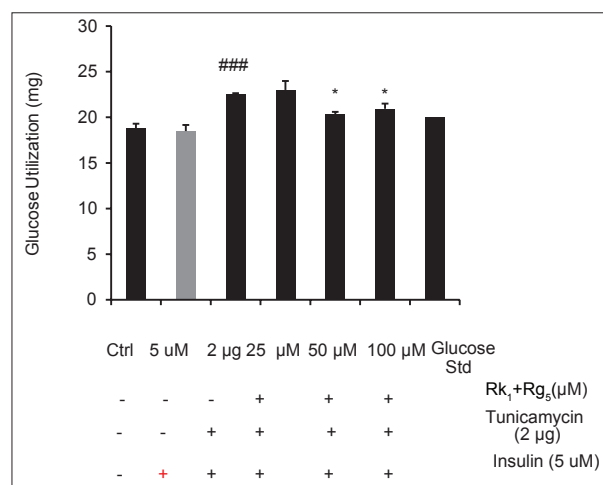


Figure 3: Glucose remaining in the media after stress and ginsenosides treatment. Each bar represents average of three independent experiments Mean \pm S.E. **P*<0.05 compared to stress treatment control (positive control). ###*P*<0.001 compared to that of the untreated control group

Rk₁ + Rg₅ treatment at 100 μ M concentration [Figure 4]. This has been found to be similar with that of another gene expression XBP1 splicing (data not shown), where

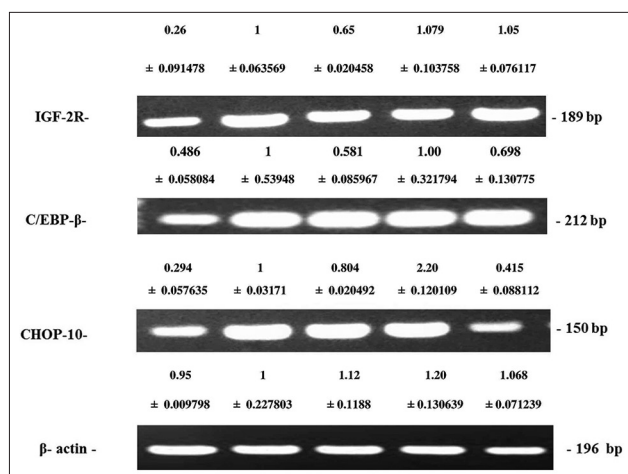


Figure 4: Effect of Rk_1+Rg_5 on the gene expression of signalling proteins: (a) IGF-2R, (b) C/EBP β , (c) CHOP-10, (d) β -actin. Number of cycle: 25, denaturation at 94°C, extension at 72°C. Intensity of the band has been calculated using Image J software and calibrated with the positive control and the adjusted density with that of the beta actin have been shown (Mean \pm S.E)

compound treatment at 100 μ M mediate the apoptotic pathway to combat the stress situations. In addition, C/EBP beta gene expression was increased by compound treatment (50 μ M, adjusted density of 1.00, Figure 4).^[24] Furthermore, from Figure 5, Glut-4 gene expression has been found to be increased by $Rk_1 + Rg_5$ treatment compared to that of the control and tunicamycin stress treatment group. One of the possible mechanisms may be that of CHOP-10 (25, 50 μ M) hetero-dimerizing with the C/EBP β gene, which has been reported to have a remarkable role in Glut-4 translocation.^[25] Thus from the above results, we can report that the ginsenoside $Rk_1 + Rg_5$ complex plays a role in making the insulin-resistant adipocytes to become sensitive to insulin. This acted by increasing C/EBP β at 25, 50 μ M concentrations and increasing the Glut-4 gene expression, thus overcoming the insulin resistance related to type 2 diabetes, obesity and endoplasmic reticulum stress.^[26] Many reports deal with the effective role of ginsenosides in overcoming an insulin-resistant state in the peripheral organs.^[27] The IGF-2R gene expressions have been found to be increased by TM (Tunicamycin). Importantly, treatment with $Rk_1 + Rg_5$ (μ M) was found to shown a greater increase in expression at 50 μ M than at 25 and 100 μ M (1.2 adjusted density) [Figure 4]. The IGF-2 protein is one of the proteins mimicking the function of insulin in the peripheral organs containing adipocytes.^[28] Pharmacological agents acting in a similar way to the IGF-2 protein and ginsenosides have also been reported. Some of them include Rh_2 , Compound K, Rg_1 , Rg_3 and berberine.^[29,30] Here we are reporting on the $Rk_1 + Rg_5$ complex mimicking the function of the IGF-2 protein in binding with that of

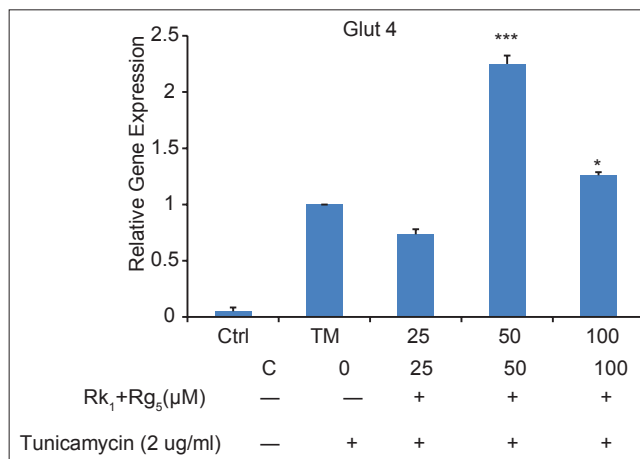


Figure 5: Effect of Rk_1+Rg_5 on Glut-4 gene expression under endoplasmic reticulum stress. Each bar represents the average of three independent experiments (Mean \pm S.E). *** P <0.001, * P <0.05 significant levels relative to the tunicamycin treatment group

the IGF-2 receptor under ER stress. On analysis of the anti-ER stress mechanism of the complex mixtures, it was found to have no effect on the downstream signaling of the IGF signaling pathway (IRS-2) (data not shown). However, it was found to have an effect on the IGF-2R receptor gene expression correlating with the Glut-4 gene expression maximum at 50 μ M. Similar results stating the role of Rg_5 acting as agonist have also been reported and are related to angiogenesis competing with IGF-1R.^[31]

In conclusion, the $Rk_1 + Rg_5$ complex treatment increases IGF-2R receptor binding sites and facilitates the Glut 4 translocation, aiding in the glucose uptake by the peripheral organs, via the CHOP-mediated signaling pathway especially in adipocytes (in this study), thus ameliorating the insulin-sensitive state of the 3T3-L1 cells and making them responsive to insulin, and thereby combating the insulin resistance closely associated with type 2 diabetes under ER stress conditions.

ACKNOWLEDGEMENT

This research was supported by a grant from Korea institute of Planning and Evaluation for technology in Food, Agriculture, Forestry and Fisheries (#111035-03-1-SB010). This study was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, grant#: PJ00952903).

REFERENCES

- Schinner S, Scherbaum WA, Bornstein SR, Barthel A. Molecular Mechanisms of insulin resistance. *Diabet Med* 2005;22:674-82.
- Nakatani Y, Kaneto H, Kawamori D, Yoshiuchi K, Hatazaki M,

- Matsuoka TA, et al. Involvement of Endoplasmic reticulum stress in insulin resistance and diabetes. *J Biol Chem* 2005;280:847-51.
3. Flamment M, Hajduch E, Ferré P, Foufelle F. New Insights in to ER stress induced insulin resistance. *Trends Endocrinol Metab* 2012;23:381-90.
 4. Sakoda H, Ogihara T, Anai M, Funaki M, Inukai K, Katagiri H, et al. Dexamethasone-induced Insulin resistance in 3T3-L1 Adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes* 2000;49:1700-8.
 5. Srinivasan S, Ohsugi M, Liu Z, Fatrai S, Ernesto BM, Permutt MA. Endoplasmic reticulum stress-induced apoptosis is partly mediated by reduced insulin signalling through Phosphatidylinositol 3-Kinase/Akt and increased Glycogen Synthase Kinase-3 beta in Mouse Insulinoma cells. *Diabetes* 2004;54:968-75.
 6. Pessin JE, Saltiel AR. Signalling pathways in insulin action: Molecular targets of insulin resistance. *J Clin Invest* 2000; 106:165-9.
 7. Cheng JT. Merit of Ginseng in the improvement of Insulin resistance. *J Ginseng Res* 2010;34:155-9.
 8. Yuan HD, Kim JT, Kim SH, Chung SH. Ginseng and Diabetes: The Evidence from *In Vitro*, Animal and Human studies. *J Ginseng Res* 2012;36:27-39.
 9. Bailey CJ. Treating Insulin resistance: Future prospects. *Diab Vasc Dis Res* 2007;4:20-31.
 10. Du J, Cheng B, Zhu X, Ling C. Ginsenoside Rg₁, a novel glucocorticoid receptor agonist of plant origin, maintains glucocorticoid efficacy with reduced side effects. *J Immunol* 2011;187:942-50.
 11. Yoon SH, Han EJ, Sung JH, Chung SH. Anti-diabetic effects of Compound K versus metformin versus compound K metformin combination therapy in diabetic db/db mice. *Biol Pharm Bull* 2007;30:2196-200.
 12. Jin Y, Kim YJ, Jeon JN, Chao W, Min JW, Jung SY, et al. Changes of Ginsenosides and Physicochemical Properties in Ginseng by New 9 Repetitive Steaming and Drying Process. *Korean J Plant Res* 2012;25:473-81.
 13. Park IH, Kim NY, Han SB, Kim JM, Kwon SW, Kim HJ, et al. Three new dammarane glycosides from heat processed ginseng. *Arch Pharm Res* 2002;35:428-32.
 14. Lee HJ, Kim JH, Lee SY, Park JH, Hwang GS. Processed *Panax ginseng*, Sun Ginseng, Decreases oxidative Damage induced by *tert*-butyl Hydro peroxide via regulation of Antioxidant Enzyme and Anti-apoptotic molecules in HepG2 cells. *J Ginseng Res* 2012;36:248-55.
 15. Zhang Z, Li X, Lv W, Yang Y, Gao H, Yang J, et al. Ginsenoside Re Reduces insulin resistance through inhibition of c-Jun NH₂- Terminal Kinase and Nuclear Factor- κB. *Mol Endocrinol* 2008;22:186-95.
 16. Park S, Ahn IS, Kwon DY, Ko BS, Jun WK. Ginsenosides Rb₁ and Rg₃ suppress triglyceride accumulation in 3T3-L1 adipocytes and enhance beta cell insulin secretion and viability in MIN-6 beta cells via PKA-dependent pathways. *Biosci Biotechnol Biochem* 2008;72:2815-23.
 17. Sangeetha KN, Shilpa K, Kumari PJ, Lakshmi BS. Reversal of dexamethasone induced insulin resistance in 3T3-L1 adipocytes by 3β-taraxerol of *Mangifera indica*. *Phytomedicine* 2013;20:213-20.
 18. Kim WK, Song SY, Oh WK, Kaewsuwan S, Tran TL, Kim WS, et al. Wound Healing effect of ginsenoside Rd from leaves of *Panax ginseng* via cyclic AMP-dependent protein kinase pathway. *Eur J Pharmacol* 2013;702:285-93.
 19. Kumar BD, Krishnakumar K, Jaganathan SK, Mandal M. Effect of mangiferin and mahaimbine on glucose utilization in 3T3-L1 cell. *Pharmacogn Mag* 2013;9:72-5.
 20. Lee OH, Lee HH, Kim JH, Lee BY. Effect of ginsenosides Rg₃ and Re on glucose transport in mature 3T3-L1 adipocytes. *Phytother Res* 2011;25:768-73.
 21. Prabhakar PK, Doble M. Mechanism of action of natural products used in the treatment of Diabetes mellitus. *Chin J Integr Med* 2011;17:1-14.
 22. Kim SJ, Yuan HD, Chung SH. Ginsenoside Rg1 suppresses hepatic glucose production via AMP-activated protein kinase in HepG2 cells. *Biol Pharm Bull* 2010;33:325-8.
 23. Oyadomari S, Mori M. Roles of CHOP/GADD 153 in endoplasmic reticulum stress. *Cell Death Differ* 2004;11:381-9.
 24. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, et al. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 2003;18:3066-77.
 25. Newell FS, Su H, Tornqvist H, Whitehead JP, Prins JB, Hutley LJ. Characterization of the transcriptional and functional effects of fibroblast growth factor-1 on human Preadipocytes differentiation. *FASEB J* 2006;20:E2133-45.
 26. Miller RS, Diaczok D, Cooke DW. Repression of GLUT 4 expression by the endoplasmic reticulum stress response in 3T3-L1 adipocytes. *Biochem Biophys Res Commun* 2007;362:188-92.
 27. Leung KW, Wong AS. Pharmacology of ginsenosides: A literature review. *Chin Med* 2010;5:1-7.
 28. Wilcox G. Insulin and Insulin resistance. *Clin Biochem Rev* 2005;26:19-39.
 29. Xie JT, Mehendale S, Yuan CS. Ginseng and Diabetes. *Am J Chin Med* 2005;33:397-404.
 30. Sun Z, Lin Yi. Current Views on type 2 diabetes. *J Endocrinol* 2010;204:1-11.
 31. Kim YM. Rg₅ as a natural agonist of IGF-1R that regulates vascular homeostasis. At the Proceedings of the Joint Symposium of 2nd Asia-Pacific Vascular Biology and 10th Frontiers of Biomedical Sciences. May 2013.

Cite this article as: Ponnuraj SP, Siraj F, Kang S, Noh HY, Min J, Kim Y, et al. Amelioration of insulin resistance by Rk₁ + Rg₅ complex under endoplasmic reticulum stress conditions. *Phcog Res* 2014;6:292-6.

Source of Support: Nil, **Conflict of Interest:** None declared.