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Original Research Article

Sanggenon C alleviates palmitic acid-induced insulin resistance in HepG2 cells via AMPK pathway

Lan Shou[1], Lingling Zhou[2], Jinhua Hu[2], Qianru Zhu[3], Hong Luo[4]*

[1]School of Public Health, Hangzhou Normal University, Hangzhou, Zhejiang 311121, [2]Department of Endocrinology, Affiliated Hospital of Hangzhou Normal University, Hangzhou, Zhejiang 310015, [3]School of Traditional Chinese Medicine, Macau University of Science and Technology, Macao Special Administrative Region 999078, [4]Department of Medicine, Hangzhou Normal University, Hangzhou, Zhejiang 311121, China

For correspondence: **Email: Luohong_668@163.com; **Tel:** +86-13588453966*

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Abstract

Purpose: *To investigate the potential role of sanggenon C alleviating in insulin resistance.*
Methods: *HepG2 cell line was incubated with increasing concentrations of sanggenon C at 1, 5, 10, 15 or 20 μM for 4 h. to induce cytotoxicity, and then further incubated with 100 μM palmitic acid to induce insulin resistance. HepG2 cells without sanggenon C and palmitic acid treatment servered as control group. Glucose uptake was determined by measuring 2-NBDG (2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]-D-glucose) fluorescence intensity using a microplate reader. Oil Red O staining was used to assess intracellular lipid accumulation, while oxidative stress was evaluated by enzyme-linked immunosorbent assay (ELISA).*
Results: *Palmitic acid significantly decreased glucose uptake and increased intracellular lipid accumulation in HepG2 ($p < 0.01$), while sanggenon C enhanced t glucose uptake and lowered lipid accumulation in insulin-resistant HepG2 ($p < 0.01$). Sanggenon C significantly attenuated palmitic acid-induced increase in p-insulin receptor substrate 1 (p-IRS1), as well as decrease in p-AKT and p-FOXO1 ($p < 0.01$). Palmitic acid also induced oxidative stress in HepG2 through the up-regulation of reactive oxygen species (ROS) and malondialdehyde (MDA), as well as the down-regulation of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px). However, sanggenon C reduced ROS and MDA levels ($p < 0.05$), and enhanced SOD and GSH-Px in insulin-resistant HepG2 ($p < 0.05$). However, sanggenon C significantly increased p-AMP-activated protein kinase (p-AMPK) levels and p-ACC (acetyl-CoA carboxylase) in insulin-resistant HepG2 ($p < 0.01$).*
Conclusion: *Sanggenon C lowers oxidative stress and ameliorates lipid accumulation thereby alleviating palmitic acid-induced insulin-resistant HepG2 cells via activation of AMPK pathway, thus suggesting that it is a potential strategy for overcoming insulin resistance.*

Keywords: *Sanggenon C, Oxidative stress, Lipid accumulation, Palmitic acid, Insulin resistance, HepG2, AMPK*

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INTRODUCTION

Type-2 diabetes mellitus is a metabolic disorder with numerous complications, including nephropathy, blindness, and retinopathy [1]. Resistance to insulin action is hallmarks of type 2 diabetes mellitus [2]. Insulin resistance leads to high blood glucose, and ectopic lipid

accumulation in peripheral tissues and the liver, thus contributing to type 2 diabetes mellitus [2]. Emerging evidence has shown that amelioration of insulin resistance attenuated metabolic disorder in patients with type 2 diabetes mellitus [3]. Therefore, promising anti-insulin resistance strategies are urgently needed for the disease.

Fatty acids repress insulin signaling, reduce glycogen synthesis and glucose uptake, and enhances the accumulation of lipid metabolites in the liver, thus contributing to insulin resistance [4]. Long-term exposure to saturated free fatty acids has been widely used in the induction of insulin resistance [5].

Sanggenon C is a bioactive flavonoid from root bark of *Cortex Mori*, and it possesses various biological capabilities, including immune-modulatory, antithrombotic, antiviral, antimicrobial, anti-inflammatory, and anticancer capabilitiies [6]. For example, Sanggenon C promotes activation of mitochondrial pathway in order to induce colon cancer cell apoptosis [6].

In diabetes, Sanggenon C can exert biological activities in the amelioration of diabetes through the regulation of α-glucosidase activity [7]. Since snggenon C has been found to be a potential inhibitor of α-glucosidase [8], it was hypothesized that sanggenon C might exert anti-diabetic effect, thereby attenuating insulin resistance.

Hence, the effects of sanggenon C on insulin resistance and oxidative stress in insulin-resistant HepG2 cells were investigated in this work in order to elucidate its probable mechanism of action.

EXPERIMENTAL

Cell culture and treatment

HepG2 was cultured in Dulbecco's modified Eagle's medium (Hyclone, Victoria, Australia) with 10 % fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA) and then incubated in a 37 ℃ incubator with 5 % CO_2. To induce cytotoxicity, HepG2 cells were incubated with increasing concentrations (1, 5, 10, 15 or 20 μM of sanggenon C (Chengdu Mansite Biotech Co. Ltd; Chengdu, China) for 4 h. To induce insulin resistance, HepG2 cells in serum-free medium were pretreated with 1, 5, or 10 μM sanggenon C for 4 h, and then incubated with 100 μM palmitic acid (Sigma-Aldrich) for another 24 h [9]. HepG2 cells without sanggenon C and palmitic acid treatment were served as control group.

Determination of cell viability and glucose uptake

HepG2 cells were seeded into a 96-well plate, and incubated with 1, 5, 10, 15 or 20 μM sanggenon C for 4 h; the cells were then cultured at 37 ℃ for another 24 h. Cell Counting Kit-8 (CCK-8) solution (Beyotime, Beijing, China) was added into the cells, and absorbance at 450 nm was measured spectrophotometrically in a microplate reader.

For glucose uptake studies, HepG2 cells was pretreated with 1, 5, or 10 μM sanggenon C for 4 h, and then incubated with 100 μM palmitic acid for another 24 h. Insulin-resistant HepG2 cells were incubated in serum-free medium with 1 nM insulin and 25 mM D-glucose for 3 h. Glucose oxidase/peroxidase reagent (Sigma-Aldrich) was used to determine glucose content. Absorbance at 505 nm was measured. HepG2 cells were also treated with 50 nM 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)-amino]-D-glucose (2-NBDG, Sigma-Aldrich) for 30 min after treatment with palmitic acid and sanggenon C, and then fluorescence intensity was determined in a microplate reader (emission at 535 nm and excitation at 485 nm).

Oil Red O Staining

HepG2 cells, after incubating with sanggenon C for 4 h and palmitic acid for 24 h were fixed in 4 % paraformaldehyde for 30 min, and washed using 60 % isopropanol. The cells were stained with Oil Red O solution (Sigma-Aldrich) for 1 h, and then examined under a microscope. Lipid content was determined spectrophotometrically at 510 nm.

Determination of oxidative stress

HepG2 cells were incubated with 10 μM dichloro-dihydro-fluorescein diacetate for 30 min. Immunofluorescence was observed under a laser confocal microscope, while fluorescence was measured in a microplate reader (emission at 550 nm, excitation at 488 nm). Lipid Peroxidation (MDA) assay kit (Sigma-Aldrich) was used to determine MDA level, while SOD and GSH-Px activities were determined using ELISA kits (Sigma-Aldrich).

Western blot

HepG2 was lysed in radioimmunoprecipitation assay buffer (Beyotime), and the isolated proteins were separated by 10 % SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked, and then probed with

specific antibodies: anti-p-IRS-1 anti-IRS-1 (1:2000), anti-p-AKT and anti-AKT (1:2500), anti-p21, anti-p-FOXO1 and anti-FOXO1 (1:3000), anti-p-ACC and anti-ACC (1:3500), anti-p-AMPK and anti-AMPK (1:4000), anti-GAPDH (1:4500).

The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody (1:4000), and visualized using enhanced chemiluminescence (Sigma-Aldrich). All the antibodies used were acquired from Abcam (Cambridge, MA, USA).

Statistical analysis

Experiments were carried out in triplicate and the data are expressed as mean ± SEM, and were analyzed by Student's t-test or one-way analysis of variance using SPSS software, version 11.5. $P < 0.05$ was considered statistically significant.

RESULTS

Sanggenon C enhances glucose uptake in insulin-resistant HepG2 cells

HepG2 was pretreated with sanggenon C (Figure 1 A) before incubation with palmitic acid. Sanggenon C at 10 μM did not affect the cell viability of HepG2, while 15 and 20 μM sanggenon C significantly reduced cell viability ($p < 0.01$; Figure 1 A). Treatment with palmitic acid increased glucose content (Figure 1 B), and decreased 2-NBDG fluorescence intensity (Figure 1 C) in HepG2.

However, sanggenon C reduced glucose content (Figure 1 B), and enhanced fluorescence intensity in insulin-resistant HepG2 cells ($p < 0.01$; Figure 1 C), suggesting that sanggenon C enhanced glucose uptake by attenuating insulin resistance.

Sanggenon C regulates proteins involved in insulin resistance

Proteins involved in insulin resistance, including FOXO1, AKT and IRS-1, were not affected by sanggenon C in insulin-resistant HepG2 (Figure 2 A). Palmitic acid significantly induced an increase in p-IRS-1 (Figure 2 A and B) ($p < 0.001$), and a decrease of p-AKT (Figure 2 A and C) and p-FOXO1 (Figure 2 A and D) ($p < 0.01$) in HepG2. Moreover, sanggenon C reduced p-IRS-1 (Figure 2 A and B), enhanced p-AKT (Figure 2 A and C) and p-FOXO1 (Figure 2 A and D) in insulin-resistant HepG2 cells in order to alleviate the insulin resistance.

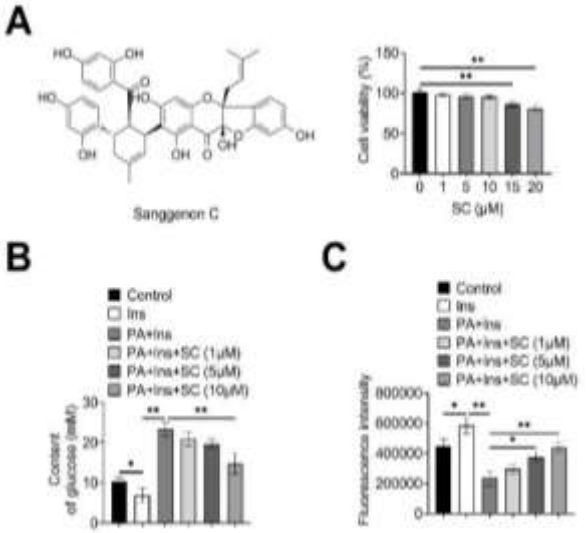

Figure 1: Sanggenon C enhanced glucose uptake in insulin-resistant HepG2. (A) Sanggenon C < 10 μM did not affect cell viability of HepG2, while 15 or 20 μM sanggenon C reduced cell viability. (B) Sanggenon C reduced glucose content in insulin-resistant HepG2 in a dosage-dependent way. (C) Sanggenon C enhanced the fluorescence intensity in insulin-resistant HepG2 in a dosage-dependent way. *$P < 0.05$, **$p < 0.01$. Control: cells without sanggenon C and palmitic acid; Ins: insulin-resistant, palmitic acid-induced HepG2; PA+Ins+SC: palmitic acid-induced HepG2 was treated with 0, 1, 5, or 10 μM sanggenon C.

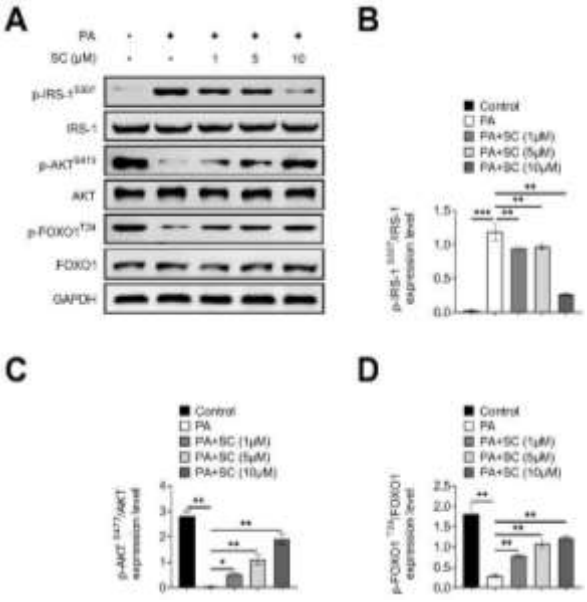

Figure 2: Sanggenon C regulated proteins involved in insulin resistance. (A) Sanggenon C reduced p-IRS-1 expression, enhanced p-AKT and p-FOXO1 in insulin-resistant HepG2. (B) Sanggenon C reduced p-IRS-1/IRS-1 ratio in insulin-resistant HepG2. (C) Sanggenon C enhanced p-AKT/AKT ratio in insulin-resistant HepG2. (D) Sanggenon C reduced enhanced p-FOXO1/FOXO1 ratio in insulin-resistant HepG2. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

Sanggenon C reduced lipid accumulation in insulin-resistant HepG2 cells

Oil Red O Staining showed that lipid accumulation in HepG2 was significantly increased by palmitic acid treatment ($p < 0.01$; Figure 3 A and B). However, sanggenon C reduced the lipid accumulation in insulin-resistant HepG2 (Figure 3 A and B), demonstrating that sanggenon C attenuated insulin resistance via down-regulation of lipid accumulation.

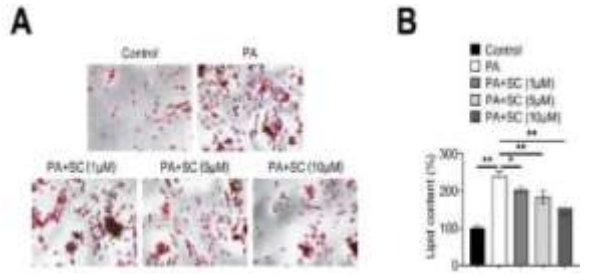

Figure 3: Sanggenon C reduced lipid accumulation in insulin-resistant HepG2. (A) Oil Red O Staining showed that sanggenon C reduced lipid accumulation in insulin-resistant HepG2 cells in a dose-dependent manner. (B) Lipid content in insulin-resistant HepG2 cells post-sanggenon C treatment. *$P < 0.05$, **$p < 0.01$

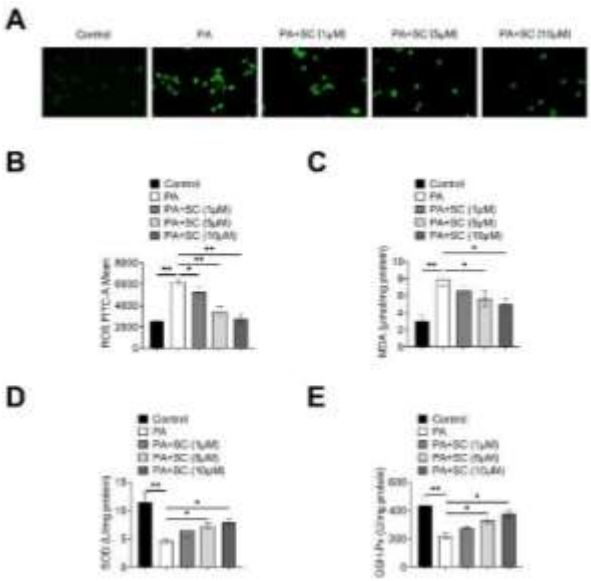

Figure 4: Sanggenon C reduced oxidative stress in insulin-resistant HepG2. (A) Sanggenon C reduced the ROS accumulation in insulin-resistant HepG2 in a dosage dependent way. (B) The relative fluorescence intensity of ROS in insulin-resistant HepG2 post Sanggenon C treatment. (C) Sanggenon C attenuated palmitic acid-induced increase of MDA in insulin-resistant HepG2. (D) Sanggenon C attenuated palmitic acid-induced decrease of SOD in insulin-resistant HepG2. (E) Sanggenon C attenuated palmitic acid-induced decrease of GSH-Px in insulin-resistant HepG2. *$P < 0.05$, **$p < 0.01$

Sanggenon C reduced oxidative stress in insulin-resistant HepG2

Palmitic acid induced up-regulation of ROS in HepG2 cells (Figure 4 A and B). Sanggenon C reduced ROS accumulation in insulin-resistant HepG2 (Figure 4 A and B). Moreover, sanggenon C significantly attenuated palmitic acid-induced increase in MDA activity (Figure 4 C), and decrease in SOD (Figure 4 D) and GSH-Px activities (Figure 4 E) ($p < 0.05$) in HepG2 cells, indicating anti-oxidant effect of sanggenon C against insulin resistance.

Sanggenon C promotes activation of AMPK signaling in insulin-resistant HepG2 cells

Sanggenon C did not affect proteins involved in AMPK signaling, including AMPK and ACC, in insulin-resistant HepG2 (Figure 5). However, sanggenon C elevated p-AMPK and p-ACC in insulin-resistant HepG2 (Figure 5), revealing that it promotes the activation of AMPK signaling in insulin resistance.

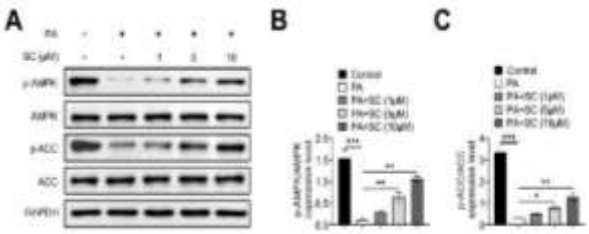

Figure 5: Sanggenon C promotes activation of AMPK signaling in insulin-resistant HepG2. Sanggenon C elevated p-AMPK and p-ACC expression in insulin-resistant HepG2. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$

DISCUSSION

The aqueous extract of *Cortex Mori* leaf reduces the phosphorylation of IRS1 and inflammation to ameliorate insulin resistance [10]. This study revealed that sanggenon C, a bioactive flavonoid of mulberry, exerted anti-diabetic effect through the suppression of insulin resistance and oxidative stress.

Palmitic acid induces decrease of glucose uptake in HepG2, and contributes to development of insulin resistance [11]. This study also revealed that palmitic acid induced an increase of glucose content and a decrease of 2-NBDG fluorescence intensity in HepG2 cells, thus promoting insulin resistance.

Sanggenon C protected HepG2 cells against palmitic acid-induced insulin resistance through the decrease in glucose content and the increase of fluorescence intensity. Insulin signaling is

implicated in the pathogenesis of insulin resistance through the IRS/AKT/FOXO1 pathway [12]. Palmitic acid induced the phosphorylation of IRS-1, inhibited the downstream insulin signaling through inactivation of PI3K/AKT/FOXO1 signaling, and mediated transcription of genes involved in glycogen synthesis [13]. Sanggenon C reduced IRS-1 phosphorylation, and enhanced AKT and FOXO1 phosphorylation, so as to down-regulate lipid accumulation in insulin-resistant HepG2, thus ameliorating insulin resistance. Moreover, α-glucosidase is important for glucose uptake, and inhibitors of α-glucosidase are regarded as anti-diabetic drugs, which ameliorate diabetic complications [14]. Sanggenon C has been found to be a potential inhibitor of α-glucosidase [8], and therefore might inhibit the activity of α-glucosidase to attenuate insulin resistance.

Excess glucose and lipid accumulation promotes the production of oxidants and oxidative stress, and diminishes glucose transport activity and insulin signaling elements during the development of insulin resistance [15]. Sanggenon C has been reported to exert anti-oxidant effects on cerebral ischemia-reperfusion injury [16]. Here, Sanggenon C attenuated palmitic acid-induced increase of MDA and ROS, and a decrease of SOD and GSH-Px in HepG2 cells, thus showing anti-oxidant effects against insulin resistance.

AMPK signaling is a key regulator in fatty acid oxidation, triglycerides, adipogenesis, cholesterol synthesis and gluconeogenesis in the liver [17]. AMPK also activates autophagy, suppresses oxidative stress, and inflammation, thus participating in insulin resistance [17]. The blockage of AMPK aggravates insulin resistance [18], while activation of AMPK contributes to the attenuation of palmitic acid-induced insulin resistance and oxidative stress [19]. Sanggenon C enhanced the activation of AMPK and reduced hypoxia-induced injury in cardiomyocyte [20]. This study showed that sanggenon C elevated p-AMPK and p-ACC in insulin-resistant HepG2 cells, thus alleviating insulin resistance.

CONCLUSION

Sanggenon C exerts anti-oxidant effects against palmitic acid-induced HepG2 cells, and also ameliorates insulin resistance through the activation of AMPK signaling. Thus, sanggenon C is a promising agent for insulin resistance. However, the role of sanggenon C in insulin-resistant animal models should be investigated in further research.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lan Shou and Hong Luo designed the study and carried it out; Lan Shou, Lingling Zhou, Jinhua Hu and Qianru Zhu supervised the data collection, as well as analyzed and interpreted the data; Lan Shou and Hong Luo prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

REFERENCES

1. *Tan X, Sun G, Yuan F, Qu L, Han A. Clinical effectiveness of a combination of metformin and ipragliflozin in the management of patients with type-2*

diabetes mellitus. Trop J Pharm Res 2021; 20(4): 783-788.

2. *Asiimwe D, Mauti GO, Kiconco R. Prevalence and Risk Factors Associated with Type 2 Diabetes in Elderly Patients Aged 45-80 Years at Kanungu District. Journal of Diabetes Research 2020; 2020: 5152146.*
3. *Liao E. Management of Type 2 Diabetes: New and Future Developments in Treatment. The American Journal of Medicine 2012; 125: S2–S3.*
4. *Zhang Q, Kong X, Yuan H, Guan H, Li Y, Niu Y. Mangiferin Improved Palmitate-Induced-Insulin Resistance by Promoting Free Fatty Acid Metabolism in HepG2 and C2C12 Cells via PPARα: Mangiferin Improved Insulin Resistance. Journal of diabetes research 2019; 2019: 2052675-2052675.*
5. *Malik SA, Acharya JD, Mehendale NK, Kamat SS, Ghaskadbi SS. Pterostilbene reverses palmitic acid mediated insulin resistance in HepG2 cells by reducing oxidative stress and triglyceride accumulation. Free Radical Research 2019; 53(7): 815-827.*
6. *Chen L-D, Liu Z-H, Zhang L-F, Yao J-N, Wang C-F. Sanggenon C induces apoptosis of colon cancer cells via inhibition of NO production, iNOS expression and ROS activation of the mitochondrial pathway. Oncology Reports 2017; 38(4): 2123-2131.*
7. *Hu T-G, Wen P, Linhardt R, Liao S-T, Wu H, Zou Y. Mulberry: A review of bioactive compounds and advanced processing technology. Trends in Food Science & Technology 2018; 83:*
8. *Chen Z, Du X, Yang Y, Cui X, Zhang Z, Li Y. Comparative study of chemical composition and active components against α-glucosidase of various medicinal parts of Morus alba L. Biomedical Chromatography 2018; 32(11): e4328.*
9. *Yang W, Zhao J, Zhao Y, Li W, Zhao L, Ren Y, Ou R, Xu Y. Hsa_circ_0048179 attenuates free fatty acid-induced steatosis via hsa_circ_0048179/miR-188-3p/GPX4 signaling. Aging (Albany NY) 2020; 12(23): 23996.*
10. *Tian S, Wang M, Liu C, Zhao H, Zhao B. Mulberry leaf reduces inflammation and insulin resistance in type 2 diabetic mice by TLRs and insulin Signalling pathway. BMC Complementary and Alternative Medicine 2019; 19(1): 326-326.*
11. *Huang F, Chen J, Wang J, Zhu P, Lin W. Palmitic Acid Induces MicroRNA-221 Expression to Decrease Glucose Uptake in HepG2 Cells via the PI3K/AKT/GLUT4 Pathway. BioMed Research International 2019; 2019(8171989-8171989.*
12. *Guo S. Molecular Basis of Insulin Resistance: The Role of IRS and Foxo1 in the Control of Diabetes Mellitus and Its Complications. Drug Discovery Today - Disease Mechanisms 2013; 10(1-2): e27-e33.*
13. *Zhang H, Ge Z, Tang S, Meng R, Bi Y, Zhu D. Erythropoietin ameliorates PA-induced insulin resistance through the IRS/AKT/FOXO1 and GSK-3β signaling pathway, and inhibits the inflammatory response in HepG2 cells. Mol Med Rep 2017; 16(2): 2295-2301.*
14. *Hossain U, Das AK, Ghosh S, Sil PC. An overview on the role of bioactive α-glucosidase inhibitors in ameliorating diabetic complications. Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association 2020; 145(111738-111738.*
15. *Henriksen EJ, Diamond-Stanic MK, Marchionne EM. Oxidative stress and the etiology of insulin resistance and type 2 diabetes. Free Radical Biology & Medicine 2011; 51(5): 993-999.*
16. *Zhao Y, Xu J. Sanggenon C Ameliorates Cerebral Ischemia-Reperfusion Injury by Inhibiting Inflammation and Oxidative Stress through Regulating RhoA-ROCK Signaling. Inflammation 2020; 43(4): 1476-1487.*
17. *Ruderman NB, Carling D, Prentki M, Cacicedo JM. AMPK, insulin resistance, and the metabolic syndrome. The Journal of Clinical Investigation 2013; 123(7): 2764-2772.*
18. *Mottillo EP, Desjardins EM, Crane JD, Smith BK, Green AE, Ducommun S, Henriksen TI, Rebalka IA, Razi A, Sakamoto K et al. Lack of Adipocyte AMPK Exacerbates Insulin Resistance and Hepatic Steatosis through Brown and Beige Adipose Tissue Function. Cell Metabolism 2016; 24(1): 118-129.*
19. *Kwak HJ, Choi H-E, Cheon HG. 5-LO inhibition ameliorates palmitic acid-induced ER stress, oxidative stress and insulin resistance via AMPK activation in murine myotubes. Scientific Reports 2017; 7(1): 5025.*
20. *Gu Y, Gao L, Chen Y, Xu Z, Yu K, Zhang D, Zhang G, Zhang X. Sanggenon C protects against cardiomyocyte hypoxia injury by increasing autophagy. Molecular Medicine Reports 2017; 16(6): 8130-8136.*