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Fruits of *Rosa laevigata* and its bio-active principal sitostenone facilitate glucose uptake and insulin sensitivity in hepatic cells *via* AMPK/ PPAR-γ activation

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ABSTRACT

Background: Fruits of *Rosa laevigata* Michx is commonly used in traditional Chinese medicine for treating of spleen deficiency, chronic diarrhea, and chronic urinary tract infection. Recently, fruits of *R. laevigate* have been shown to have renal protective effects in diabetic rats. However, up to now, no studies have been reported on the anti-hyperglycemic or anti-diabetic effect of *R. laevigata* or its derived compounds.
Purpose: Therefore, this study investigated the anti-diabetic effect of ethanol extract of *R. laevigata* (EtRL) fruits, its derivate sub-fractions, and pure compounds in terms of glucose-lowering effect on hyperglycemia-induced hepatic cells *in vitro*.
Methods: We investigated the anti-hyperglycemic effect of EtRL and its derivate sub-fractions (water, *n*-butanol, ethyl acetate, and *n*-hexane), and its major bioactive compound, sitostenone, in normal and high glucose-induced insulin-resistant hepatic HepG2 cells using *in vitro* DNS glucose uptake and Western blotting assays.
Results: Treatment with EtRL and its derivate sub-fractions significantly increased glucose uptake by hepatic cells. Besides, co-treatment with insulin further improved glucose uptake in insulin-resistant cells. Notably, compared with all soluble fractions, the *n*-hexane sub-fraction displayed the strongest activity in the context of glucose consumption. Furthermore, sitostenone, a primary bioactive compound was isolated from *n*-hexane sub-fraction by bioactivity-guided fractionation procedure. Sitostenone treatment significantly increased glucose uptake, whereas there was no further increase when co-treatment with insulin. Indeed, sitostenone significantly increased glucose uptake and promote insulin sensitivity in insulin-resistant cells. Further analysis revealed that sitostenone activates proteins involved in the insulin signal transduction pathway, including insulin receptor substrate-1 (IRS-1), AKT, and glycogen synthase kinase 3 β (GSK3β). It was also found that sitostenone provoked glucose uptake in insulin-resistant cells *via* peroxisome proliferator-activated receptor-γ (PPAR-γ) and AMP-activated protein kinase (AMPK) activation, which facilitates up-regulation of glucose transporters, GLUT2 and GLUT4 in the cell membrane and down-regulation of Forkhead box protein O1 (FOXO1) in the nucleus.
Conclusion: This study demonstrating that sitostenone, a steroid-like compound from fruits of *R. laevigata* has a promising ability to promote glucose uptake and repairs insulin resistance in hepatic cells.

Introduction

Diabetes mellitus is a chronic disease caused by insufficient insulin or insulin resistance in the human body. Diabetes patients often cause blindness, kidney failure, heart disease, stroke, and other diseases and lead to lower limb amputation (Tabish, 2007). In all patients with diabetes, about 90% of cases belong to type-2 diabetes (WHO, 2020). According to World Health Organization report, the number of patients with diabetes increased from 108 million to 422 million between 1980 and 2014 (WHO, 2020). Among them, the prevalence of diabetes among people over 18 increased from 4.7% to 8.5%. In 2016, diabetes caused 1.6 million deaths directly, and it has been listed as one of the four major

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non-communicable diseases (WHO, 2020).

At present, the drugs' mechanism for treating diabetes is reducing the body's absorption of glucose, promoting insulin secretion, using insulin sensitizers, and direct injection of insulin. However, these drugs also bring many side effects while improving diabetes, such as α-glucosidase inhibitors that inhibit polysaccharides decomposition in the small intestine and block sugars absorption which can cause gastrointestinal discomfort under long-term use (Derosa and Maffioli, 2012). α-Glucosidase inhibitors-associated side effects include diarrhea, sulfonylureas, glibenclamide, which stimulates pancreatic β-cells to secrete insulin, which increase mortality risk from cardiovascular diseases (Derosa and Maffioli, 2012). Thiazolidinedione (TZDs) that increases insulin sensitivity can cause weight gain and edema (Ko et al., 2017). Besides, high levels of insulin in circulation increase various forms of cancer incidence (Dankner et al., 2012; Orgel and Mittelman, 2013). Therefore, it is an important research topic to find compounds that improve diabetic conditions or treat diabetes with minimal side effects.

Oxidative stress plays a pivotal role in the pathogenesis of type-2 diabetes and its complications (Giacco and Brownlee, 2010). When the imbalance between excessive production of reactive oxygen species and a limited number of endogenous anti-oxidants that this damage can become debilitatinged and cumulative. Various factors involved in oxidative stress control, among them the detoxifying enzymes including catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), hemeoxygenase-1 (HO-1), and NAD(P)H quinone dehydrogenase-1 (NQO-1) are the first line of defense. The variation in the levels of these enzymes makes the tissue susceptible to oxidative stress and leading to the development of diabetic complications. In such conditions, exogenous anti-oxidants has a remarkable role (Birben et al., 2012). Indeed, herbal extracts and natural products are potent anti-oxidants, which counterpart the activity of the endogenous anti-oxidant defense. Accumulating evidence suggests that plant polyphenols possess high anti-oxidant activity through direct free radical scavenging or activating the endogenous anti-oxidant defense mechanism (Pandey and Rizvi, 2009). Additionally, researchers have identified that inflammation is strongly associated with diabetes. The levels of pro-inflammatory molecules, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6 were often found higher in people with type-2 diabetes compared to people without diabetes. Indeed, abnormal inflammation alters insulin's activity and contributes to disease progression. Alternatively, when body becomes less sensitive to insulin, in such condition called insulin resistance leads to inflammation (Xie and Du, 2011). A vast array of phytochemicals derived from medicinal plants or herbs exhibited notable anti-inflammatory properties *in vitro* and *in vivo* and these phytoconstituents play a functional role in lowering blood glucose and controlling inflammation or limiting pro-inflammatory molecules production and breaking insulin resistance (Kong et al., 2021).

Rosa laevigata (Rosaceae), also known as Cherokee rose, is a fragrant rose native to southern China and Taiwan. The fruits of *R. laevigata* are widely used as a functional food in Asian countries to prepare marmalade, garnishes, fruit pies, and wines (Zhan et al., 2020). In traditional Chinese medicine, used the fruits to treat arterial sclerosis, urinary incontinence, chronic cough, and menstrual irregularities (Tao et al., 2016). Pharmacological studies have revealed that *R. laevigata* possessed various bio-pharmacological activities, including hypolipidemic (Zhang et al., 2020); immunomodulatory (Zhan et al., 2020); hepato-protection (Dong et al., 2018); neuroprotection (Liu et al., 2018) and cardio-protection (Luo et al., 2016). These biological activities are attributed to the presence of various bioactive compounds, including rutin, quercetin, kaempferol, luteolin, apigenin, liquiritigenin, isorhamnetin, chlorogenic acid, 4-hydroxy-3-methoxybenzoic acid, kaempferide-3-*O*-glucoside, quercetin-3-rhamnoside, isorhamnetin-3-*O*-β-rutinoside, and so on (Li et al., 2012; Yan et al., 2013; Zeng et al., 2011). A previous study has demonstrated that 18 polyphenol compounds were isolated from the fruits of *R. laevigata*, among them 15 compounds exhibited strong anti-oxidant activity in DPPH and FRAP assays, and β-carotene linoleate model system (Li et al., 2012). Another study has found that fruits of *R. laevigata* possessed strong anti-inflammatory activity as evidenced by a significant reduction in particulate matter-10 (PM-10)-induced expression of pro-inflammatory genes including TNF-α, IL-1β, IL-6, IL-13, and IL-17 in human lung epithelial cells (Ko et al., 2020). Since, the fruits of *R. laevigata* possessed strong anti-oxidant and anti-inflammatory properties, we hypothesize that fruits of *R. laevigata* and its derived pure compounds may have anti-diabetic effect. Also, so far, there is no study has been conducted on the anti-diabetic activity of *R. laevigata* or its derived pure compounds. Therefore, this study explored the anti-diabetic properties of ethanol extract of *R. laevigata* fruits and its bioactivity compounds.

Materials and methods

Plant materials

Fresh fruits of *R. laevigata* were collected from the Hui-Sun Experimental Forest Station of National Chung Hsing University (Nantou County, Taiwan) in July 2015. The species was identified and confirmed by Prof Yen-Hsueh Tseng (Department of Forestry, National Chung Hsing University). The voucher specimen (YHT0021 [TCF]) was deposited in the herbarium of the same university.

Extraction and purification

Air-dried *R. laevigata* fruits (3.87 kg) were extracted with 70% EtOH (30 L) at ambient temperature and concentrated under vacuum to yield the EtOH extract (EtRL; 412.3 g). Based on a previous work, the EtRL extract was partitioned between *n*-hexane, ethyl acetate, and *n*-butanol with water to give *n*-hexane-soluble (19.5 g), ethyl acetate-soluble (20.5 g), *n*-butanol-soluble (150.3 g), and water-soluble (53.8 g) fractions (Li et al., 2008). HPLC further separated the *n*-hexane-soluble fraction with a mixture of ethyl acetate and *n*-hexane solution. 0 to 5 min, ethyl acetate:*n*-hexane = 5: 95; 10 to 15 min, ethyl acetate: *n*-hexane = 10 : 90; 20 to 30 min ethyl acetate : *n*-hexane = 15 : 85 at a flow rate of 3 mL/min. The wavelength of the UV detector set at 254 nm, and the obtained compounds were analyzed by LC-MS and 400 MHz NMR. A bioactive compound, sitostenone, was isolated at the retention of 23 to 25 min following bioactivity-guided isolation protocol. The structure of the compound was analyzed by electrospray ionization (ESI) and LC-MS was used to detect the molecular weight (m/z) of the compound and its fragments. For structural identification, the compound was dissolved in deuterated chloroform (d-chloroform; $CDCl_3$) for detection, and the hydrogen nucleus (^{1}H NMR) or carbon nucleus (^{13}C NMR) in the compound was detected under a magnetic field. The difference in resonance frequency between tetramethylsilane and tetramethylsilane (called chemical shift, and expressed in δ value (ppm)) was analyzed. Alternatively, 2D-NMR analysis, such as distortionless enhancement by polarization transfer (DEPT) , heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) was performed to determine the chemical structure of the sample. The spectral data of sitostenone was correspondent to a previous report (Li et al., 2008).

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco/Invitrogen (Carlsbad, CA). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and metformin were purchased from Sigma–Aldrich (St. Louis, CA). Antibodies against AKT, IRS-1, PPARγ, GSK3β, FOXO1, GLUT4, phospho-IRS-1^{Tyr895}, phospho-AKT^{Ser473}, phospho-GSK3β^{Ser9}, phospho-AMPKα^{T172}, and HRP-conjugated goat

anti-mouse IgG were obtained from Cell Signaling Technology (Denvers, MA). Antibodies against AMPKα and HRP-conjugated goat anti-rabbit IgG were purchased from Millipore (Temecula, CA). The antibody against β-actin was purchased from Santa Cruz Biotechnology (Dallas, TX). The antibody against GLUT2 was bought from Proteintech (Rosemont, IL). All other chemicals were of the highest grade commercially available and supplied either by Merck (Darmstadt, Germany) or Sigma.

Cell culture and cell viability assay

Human hepatocellular carcinoma (HepG2) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in low-glucose Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% feral bovine serum (FBS), 1 mM sodium pyruvate, glucose (5.5 mM), penicillin (100 U/mL) and streptomycin (100 μg/mL). Cells were grown in 10-cm culture dishes and incubated in a humidified atmosphere containing 5% CO_2 at 37°C. For cell viability assay, HepG2 cells were treated with increasing concentrations of EtRL (20, 40, and 80 μg/mL), soluble fractions (10, 20 and 40 μg/mL) or sitostenone (10, 20, 25, 30, 50, 100, 250, 500, 750, 1000 μM). All the test samples were dissolved in dimethyl sulfoxide and an equal volume of DMSO (0.1%) was used as vehicle control. Cell viability was assessed by MTT colorimetric assay as described previously (Senthil Kumar et al., 2012).

Protein extraction and Western blotting

HepG2 cells (1×10^6 cells/dish) were seeded in 6-cm dish and incubated overnight, after culture media were replaced with serum-free medium for 24 h. Then the cells were treated with sitostenone or metformin (2 mM) in the presence or absence of insulin (100 nM) for 6-24 h. Cells were lysed with either mammalian protein extraction reagent (M-PER) or radio-immuno precipitation assay (RIPA) buffer (Pierce Biotechnology, Rockford, IL). Protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein samples (60-100 μg) were separated by 8-12% SDS-PAGE and the separated proteins were transferred onto polyvinylidene fluoride (PVDF) membrane overnight. The transferred protein membranes were blocked with 5% nonfat skim milk for 30 min, followed by incubation with specific primary antibodies overnight, and then incubated with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse or anti-goat antibodies for 2 h. Immunoblots were developed with the enhanced chemiluminescence reagents (Advansta Inc., San Jose, CA), images were captured by ChemiDoc XRS$^+$ docking system, and the protein bands were quantified using Imagelab software (Bio-Rad).

Glucose uptake assay

HepG2 cells were seeded into 48-well cell culture plate with a cell density of 1.5×10^5 cells/well in low glucose (5.5 mM) DMEM containing 10% FBS. The culture plate was incubated for overnight to ensure cell attachment. Then the cells were replaced with serum-free low glucose DMEM. After cells were starved for 24 h, cells were treated with samples for 6 h. Insulin or metformin was served as positive drug control. Glucose levels in the culture supernatant were measured using commercially available glucose assay kit (Abcam, Cambridge, UK).

Insulin resistance model

HepG2 cells were seeded into 48-well cell culture plate with a cell density of 1.5×10^5 cells/well in low glucose (5.5 mM) DMEM containing 10% FBS. The culture plate was incubated for overnight to ensure cell attachment. Then the cells were replaced with serum-free high glucose (30 mM) DMEM for 24 h to induce the cells to produce insulin resistance. After insulin resistance induction, the cells were washed with PBS to remove excess glucose, and then the subsequent experiments were performed.

Determination of reducing sugar content

3,5-Dinitrosalicylic acid (DNS) can react with reducing sugars or other compounds with reducing ability to form 3-amino-5-nitrosalicylic acid, which can be absorbed in a maximum absorption of a wavelength of 540 nm as described previously (Garriga et al., 2017). After cell treatment, the culture supernatant was deprived. To measure the glucose concentration in the culture medium, 200 μL of the culture medium and 200 μL of DNS reagent was added to the 1.5 mL eppendorf and the mixture was heated at 100°C for 5 min. After cooling to room temperature, the absorbance of the supernatant was measured at 540 nm (OD_{540}) using ELISA micro-plate reader. The relative glucose consumption in cells is calculated according to the following formula. Glucose consumption (%) = $[1-(A-B)/B] \times 100$; were as A is the OD_{540} of medium of cell culture, B is the OD_{540} of medium without cell.

Statistical analysis

Data are expressed as mean ± SD. All data were analyzed using the statistical software Graphpad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA). Statistical analysis was performed using one-way ANOVA followed by Dunnett's test for multiple comparison. *P* values of less than 0.05*, 0.01**, and 0.001*** were considered statistically significant for the control *vs* samples treatment groups. *P* value of less than 0.001^{Δ} was considered statistically significant for the control *vs* high glucose (insulin-resistant) group. *P* value of less than 0.001^{Φ} was considered statistically significant for the control *vs* insulin alone treatment group.

Results and discussion

Many studies are confirming that *Rosaceae* plants can improve the symptoms of diabetes. For example, *Malus toringoides* can improve the symptoms of diabetes in rodents by reducing blood glucose and increasing insulin (Li et al., 2014); *Agrimonia pilosa* reduces high-fat diet-induced inflammation in rats through regulating blood sugar balance (Jang et al., 2020); the pentacyclic triterpenoids from *Eriobotrya japonica* break the insulin resistance and reduce fat accumulation in high-fat diet fed mice (Shih et al., 2010); phenolic compounds from *Prunus avium* exhibited blood sugar lowering, anti-oxidation, α-amylase, and α-glucosidase inhibitory activity, and increase in glucose uptake and insulin secretion (Noratto et al., 2018); also, phenolic compounds of strawberries and cranberries can increase insulin sensitivity (Paquette et al., 2017). These studies provided a strong base that the *Rosaceae* plants have great potential for the develpment of anti-diabetic agents . Additionally, the role of oxidative stress and inflammation in the pathogenesis of type-2 diabetes and its complications were confirmed by interventional studies. It has been shown that Chinese medicinal herbs reduce the incidence of type-2 diabetes and its vascular complications partially *via* anti-inflammatory mechanisms. Xie and Du (2011) systematically listed most frequently used Chinese herbs including, *Radix astragali, Radix rehmanniae, Radix trichosanthis, Panax ginseng, Fructus schisandrae, Radix ophiopogonis, Rhizoma anemarrhenae, Radix puerariae, Fructus lycii, Poria sp, Rhizoma coptidis, Rhizoma dioscoreae, Rhizoma polygonati, Radix salviae miltiorrhizae, Radix glycyrrhizae, Semen trigonellae, Momordica charantia, Allium sativum, Opuntia stricta, Aloe vera, Cortex Cinnamomi, Rhizoma Curcumae longae* for the treatment of diabetes through its anti-inflammatory action. These herbs exhibit anti-inflammatory effects in various *in vitro* and *in vivo* models by inhibiting the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), IL-1β, IL-6, and TNF-α, while also reducing production of NO and PGE_2. Their anti-inflammatory effects is

attributed to the inactivation of their corresponding transcriptional factors including, nuclear factor -κB (NF-κB) and/or mitogen-activated protein kinases (MAPKs). Morvaridzadeh et al (2020) systematically reviewed and reported that supplementation of ginger, one of the common ingredients in Chinese herbal medicine markedly reduced C-reactive protein (CRP) and TNF-α levels, but not IL-6 in diabetic patients compared to non-diabetic patients. Additionally, ginger supplementation was found to be reduce blood sugar and ameliorate blood lipids through its anti-oxidant property. Another detailed analysis indicating that lemon balm intake significantly reduced blood pressure and inflammatory markers, including CRP, whereas serum lipid profile and glycemic parameters were not significantly altered by lemon balm (Heshmati et al., 2020). A recent study has shown that *R. laevigata* inhibits expression levels of inflammatory genes, including IL-1β, IL-6, IL-13, IL-17, and TNF-α in PM-10-challanged human lung epithelial cells (Ko et al., 2020). In this study, we demonstrated that ethanol extract of *R. laevigata* (EtRL) fruits, its derivate sub-fractions, and its major bioactive compound sitostenone promote glucose uptake and repairs insulin resistance in hyperglycemia-induced hepatic cells *in vitro*.

Cytotoxicity of EtRL on hepatic cells

Before the experiment, the EtRL's cytotoxicity against human hepatoma HepG2 cells was determined by MTT colorimetric assay. As shown in Figure 1, treatment with either EtRL or its derivative soluble fractions, including water, *n*-butanol, ethyl acetate, and *n*-hexane does not show cytotoxicity against HepG2 cells up to a treatment concentration of 80 μg/mL. The observed cell survival rates were 101.5%, 106.8%, 100.8%, 107.8%, and 106%, by EtRL (Fig. 1A), water (Fig. 1B), *n*-butanol (Fig. 1C), ethyl acetate (Fig. 1D), and *n*-hexane (Fig. 1E) fractions, respectively, indicating that the ethanol extract and the soluble fractions are not cytotoxic to HepG2 cells. Cells exposed to plumbagin, a known cytotoxic drug, significantly induced cell death in HepG2 cells (Fig. 1F). These data are consistent with other observations that aqueous extract of *R. laevigata* does not show cytotoxicity to HepG2 cells (Ko et al., 2015), human lung epithelial cells (Lee et al., 2020), and human lens epithelial cells (Liu et al., 2015) up to a maximum concentration of 300 μg/mL, 1000 mg/mL and 10 mg/mL, respectively.

EtRL and its soluble fractions promote glucose uptake in hepatic cells

Type-2 diabetes is characterized by insufficient secretion of insulin from β-cells of the pancreatic islets resulted in hyperglycemia (glucose accumulation in the circulation). The most direct way to improve the symptoms of hyperglycemia is to inject insulin to encourage the target cells, such as muscle, fat and liver cells to uptake glucose to achieve the purpose of lowering blood sugar (Cantley and Ashcroft, 2015). However, several *in vitro* and *in vivo* experiments revealed that high insulin concentrations can also result in impaired insulin action in targeted tissues, a condition called insulin resistance (Wilcox, 2005). Therefore, any chemical agent with similar insulin function can be used to replace insulin and also reduce insulin-associated complications. Many plant extracts, and phytochemicals were reported to mimic insulin (Broadhurst et al., 2000; Patel et al., 2012; Teoh and Das, 2018). A previous study by Zhou et al. (2012) reported that the aqueous extract of *R. laevigata* protects rat's kidneys from streptozotocin-induced oxidative stress and diabetic nephropathy through the inhibition of the NF-κB signaling pathway. Another study by Liu et al. (2015) revealed that aqueous extract of *R. laevigata* protects human lens epithelial cells from hyperglycemia-induced oxidative stress *via* up-regulation of Nrf-2-mediated anti-oxidant genes *in vitro*. However, there is no report indicating the insulin-mimetic effect of *R. laevigata* or its derived compounds. Thus, this study investigated whether the ethanol extract of *R. laevigata* has an insulin-like ability to promote glucose uptake.

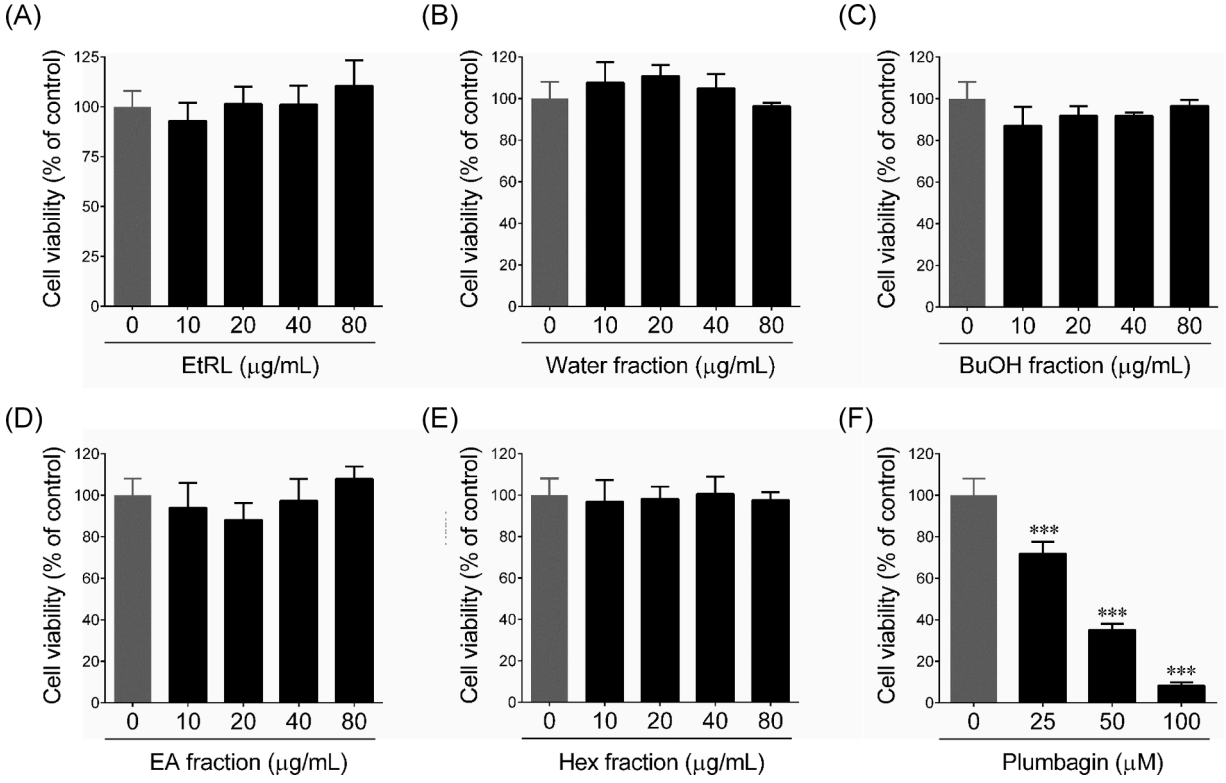

Fig. 1. Cytotoxic effects of ethanol extract of *R. laevigata* fruits (EtRL) and its derivative sub-fractions on human hepatic cells. HepG2 cells were incubated with increasing concentrations of (10, 20, 40 and 80 μg/mL) EtRL (A), and its souluble fractions, including water (B), *n*-butanol (C), ethyl acetate (D), *n*-nexane (E) and a positive drug control plumbagin (25, 50 and 100 μM) for 24 h. The cell survival was analyzed by MTT colorimetric assay. Values represent the mean ± SD of three independent experiments. Statistical significance ***$P < 0.001$ was compared to control *vs.* sample treatment groups.

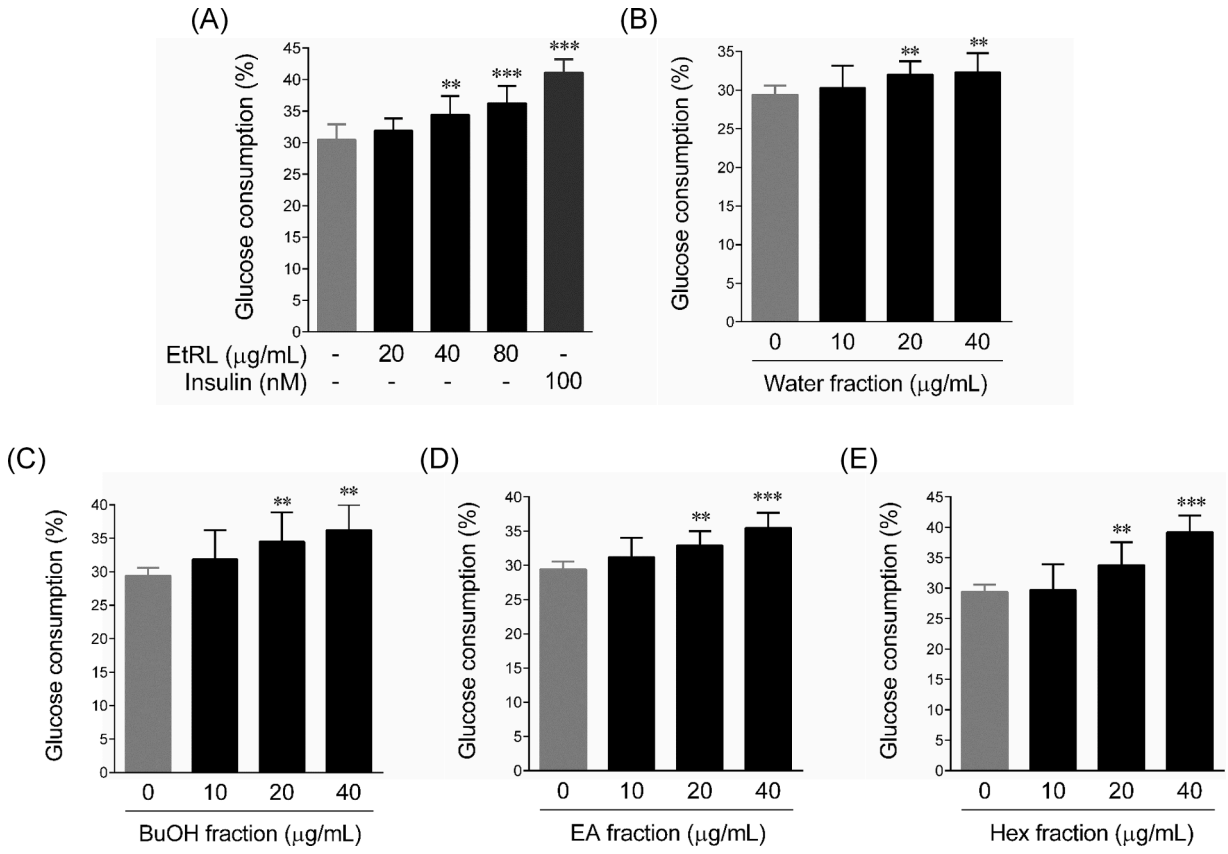

Fig. 2. The ethanol extract of *R. laevigata* fruits (EtRL) and its derivative souluble fractions promote glucose uptake in hepatic cells. (A) HepG2 cells were treated with different concentrations of EtRL (20, 40 and 80 μg/mL) for 6 h or various concentrations of (10, 20, 40 μg/mL) EtRL soluble fractions, such as water (B), *n*-butanol (C), ethyl acetate (D), and *n*-hexane for 6 h. 100 nM of insulin was subjected as positive control. After treatment, culture supernatant was collected and glucose concentration was tested by DNS method. Values represent the mean ± SD of three independent experiments. Statistical significance $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to control *vs.* sample treatment groups.

Glucose concentration in the culture media was determined by DNS assay. As shown in Figure 2A, a significant increase in glucose uptake was observed after treatment with EtRL for 6 h. Compared to that of basal uptake (30.5%), cells incubated with 20, 40, and 80 μg/mL of EtRL increased glucose uptake to 31.2%, 34.4% and 36.2%, respectively. Alternatively, cells exposed to 100 nM insulin significantly increased glucose uptake to 41.1%. These data strongly suggest that EtRL can promote glucose uptake as well as a mimic insulin.

Next, to further evaluate the glucose uptake activity of EtRL-derived soluble fractions, cells were incubated with increasing concentrations of water, *n*-butanol, ethyl acetate, and *n*-hexane fractions for 6 h, and the glucose concentration in the culture media was determined by DNS assay. Results showed that the basal glucose consumption by the control was noted as 29.4%, while treatment with water (Fig. 2B), *n*-butanol (Fig. 2C), ethyl acetate (Fig. 2D), and *n*-hexane (Fig. 2E) fractions significantly increased glucose uptake in HepG2 cells. Particularly, cells exposed to high dosages of ethyl acetate and *n*-hexane fractions (40 μg/mL) increased glucose uptake to 35.5% and 39.2%, respectively. Notably, the effect of glucose uptake by *n*-hexane fraction was comparable to that of the insulin group, which showed an increase of 39.3%.

EtRL and its soluble fractions promote glucose uptake insulin-resistant hepatic cells

Insulin-resistant glucose uptake is a prominent hallmark of type-2 diabetes, characterized by reducing sensitivity or responsiveness of muscle, liver, and adipose tissue to the metabolic actions of insulin in human and experimental models of diabetes in rodents (Czech, 2017). This defect is evident *in vitro* as reduced glucose uptake responds to insulin resistance in hepatic cells (Leclercq et al., 2007). It was well demonstrated that hepatic cells were exposed to high glucose provoking insulin resistance *in vitro* (Boonloh et al., 2015). Therefore, next, we sought to examine whether EtRL and its derived soluble fractions could improve glucose uptake in insulin-resistant hepatic cells. Insulin resistance in HepG2 cells was induced by high concentration of glucose (30 mM) for 24 h; then the cells were washed with PBS and incubated with increasing concentrations of EtRL or its derived soluble fractions for 6 h. Glucose concentration in the culture media was determined by DNS assay. The result showed that a significant decrease in glucose consumption was noted in insulin-resistant HepG2 cells compared with control cells (Fig. 3A). This result indicates that high glucose concentration can produce insulin resistance in HepG2 cells, and this assay can be used to determine insulin impedance *in vitro*. This result is also well correlated with other observations that 30 mM glucose induces insulin resistance in HepG2 cells (Xuguang et al., 2019).

Next, we found that insulin treatment significantly increased glucose uptake in insulin-resistant cells. In contrast, a profound increase in glucose uptake was noted when cells were dose-dependently co-incubated with EtRL (Fig. 3A). Compared with the insulin resistance group (25.5%), glucose consumption was significantly provoked in high doses of EtRL (40 and 80 μg/mL) treated cells. The effect of EtRL on glucose uptake in insulin-resistant cells was highly comparable to that of metformin, a biguanide agent that reduces hyperinsulinaemia and improved hepatic insulin resistance (Li et al., 2019). This result strongly suggests that it can improve insulin resistance in human hepatic cells.

Next, we examined the effect of EtRL derived soluble fractions on glucose uptake in insulin-resistant cells. The result showed that compared with insulin-resistant cells, co-treatment of insulin and water

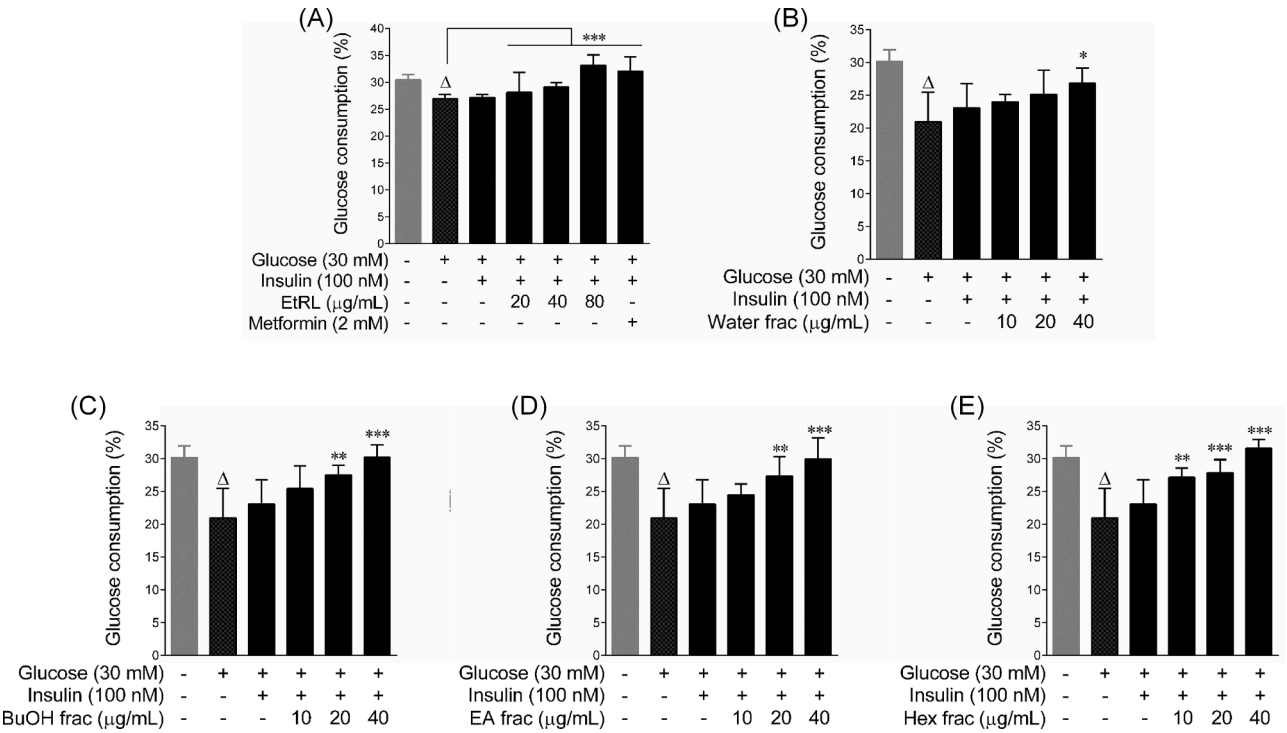

Fig. 3. The ethanol extract of *R. laevigata* fruits (EtRL) and its derivative soluble fractions promote glucose uptake in insulin-resistant hepatic cells. To induce insulin-resistant, HepG2 cells were pre-incubated with 30 mM of D-glucose for 24 h, then the cells were treated with different concentrations of (20, 40, and 80 μg/mL) EtRL (A) for 6 h or various concentrations (10, 20, and 40 μg/mL) of EtRL soluble fractions, such as water (B), *n*-butanol (C), ethyl acetate (D), and *n*-hexane for 6 h with or without 100 nM of insulin. 2 mM of metformin was utilized as positive drug control. After treatment, culture supernatant was collected and glucose concentration was tested by DNS method. Values represent the mean ± SD of three independent experiments. Statistical significance $^{\Delta}p < 0.001$ compared to control *vs.* high glucose treatment (insulin-resistant) group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to insulin-resistant *vs.* sample treatment groups.

(Fig. 3B) or *n*-butanol (Fig. 3C) or ethyl acetate (Fig. 3D) or *n*-hexane (Figure 3E) soluble fractions significantly improved glucose uptake in insulin-resistant HepG2 cells. These fractions increased glucose consumption from 24.5% to 26.9%, 30.2%, 29.9%, and 31.6%, respectively. Indeed, all the fractions significantly improved insulin resistant. The *n*-hexane soluble fraction produced high effect, which is close to the metformin, a positive drug control, improved insulin resistance by provoking increased glucose uptake (31.1%).

Separation, identification and quantification of sitostenone

To obtain a major active compounds from the *n*-hexane-soluble fraction, HPLC was performed to separate the compounds in the soluble *n*-hexane fraction. The isolated compounds were tested their biological activity based on DNS glucose uptake assay. Among them, a compound appearing in the retention time between 23 and 25 min was found to have potent glucose uptake activity. We further purified and obtained a colorless solid component. The molecular ion peak measured by ESI-MS is $[M+1]^+$ m/z 413, and the molecular weight of the compound is 412 along with NMR analysis, the compound was identified as sitotenone (Li et al., 2008) (Fig. 4A). To quantify the amount of sitostenone in *R. laevigata*, further quantitative analysis of sitostenone was conducted. According to the quantitative results, it was found that 1 g of the EtRL containing 4.9 mg of sitostenone (0.49%; w/w), *i.e.* 1 kg of *R. laevigata*'

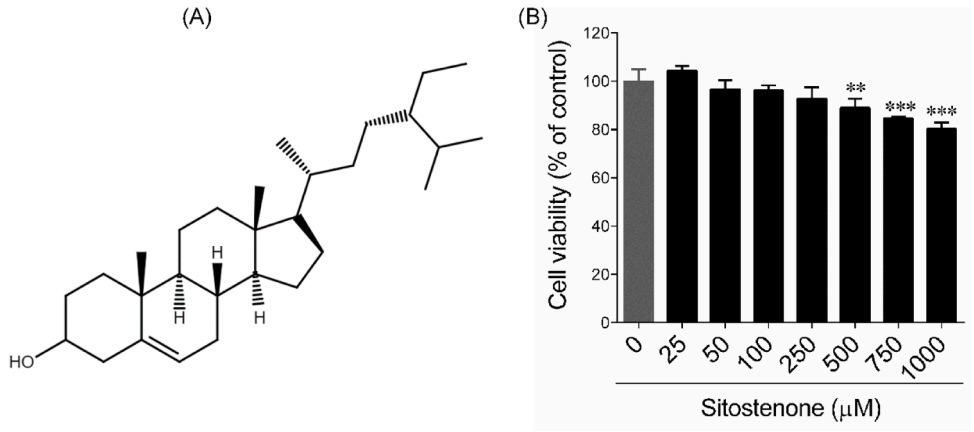

Fig. 4. Chemical structure and cytotoxic effect of sitostenone on human hepatic cells. (A) Chemical structure of sitostenone. (B) HepG2 cells were incubated with increasing doses of sitostenone (25, 50, 100, 250, 500, 750, and 1000 μM) for 24 h. The cell survival was analyzed by MTT assay. Values represent the mean ± SD of three independent experiments. Statistical significance $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to control *vs.* sample treatment groups.

fruits contain 518 mg sitostenone.

The cytotoxic effect of sitostenone on hepatic cells

To determine the cytotoxicity of sitostenone, HepG2 cells were incubated with increasing concentrations of sitostenone for 24 h, the cell viability was determined by MTT colorimetric assay. As shown in Figure 4B, treatment with sitostenone (25, 50, 100, 250, 500, 750, and 1000 μM) for 24 h has no significant cytotoxicity in HepG2 cells, the cell survival rates were noted as 104.38%, 96.7%, 96.3%, 92.8%, 89.0%, 84.6%, and 80.4%, respectively. The cell survival rate was significantly reduced over a concentration of 500 μM, however the cell survival rate is still greater than 80% at a concentration of 1000 μM. It was previously demonstrated that when the cell viability is above 80%, the drug can be thought to have no significant cytotoxicity on cell growth (López-García et al., 2014). Therefore, we concluded that sitostenone has no significant cytotoxicity toward HepG2 cells.

Sitostenone promotes glucose uptake in hepatic cells

Phytosterols are bioactive natural products, *e.g.* β-sitosterol, campesterol, and stigmasterols are abundant plant sterols with a structure similar to cholesterol (Trautwein and Demonty, 2007). Phytosterols are characterized by an extra one or two carbon substituents on the side-chain, attached at C–24. For example, 4–methyl and 24–ethyl conjugate at C-24 of cholesterol and produce campesterol and sitosterol, respectively (Tapiero et al., 2003). Phytosterols are highly regarded for their cholesterol-lowering effect by competitively blocking cholesterol absorption from the intestinal lumen (Tapiero et al., 2003). Tanaka et al. (2006) reported five sterols (lophenol, 24-methyl-lophenol, 24-ethyl-lophenol, cycloartanol, and 24-methylene-cycloartanol) from *Aloe vera* gel reduced blood glucose in the diabetic mice. A recent study by Ramalingam et al. (2020) found that treatment with β-sitosterol significantly reduced the plasma glucose level in high-fat diet and streptozotocin-induced diabetic mice. Many studies have reported bio-pharmaceutical activities, including the anti-diabetic activity of phytosterols (Miras-Moreno et al., 2016). However, no study indicates the anti-diabetic properties of phyto-stenones, which are the byproducts of sterol degradation (Dai et al., 2005). The result from bioactivity-guided fractionation analysis revealed that sitostenone possessed significant glucose-lowering effect *in vitro*. Briefly, HepG2 cells were incubated with increasing concentrations of sitostenone (10, 20, and 30 μM) for 6 h, then the glucose level in the culture media was analyzed by the DNS method. As shown in Figure 5A, treatment with sitostenone significantly promoted glucose uptake from 30.5% to 33.7%, 35.8%, and 37.3% by 10, 20, and 30 μM, respectively. The degree of glucose consumption by 30 μM sitostenone was highly comparable with the insulin treatment group (40.0%). This data provides

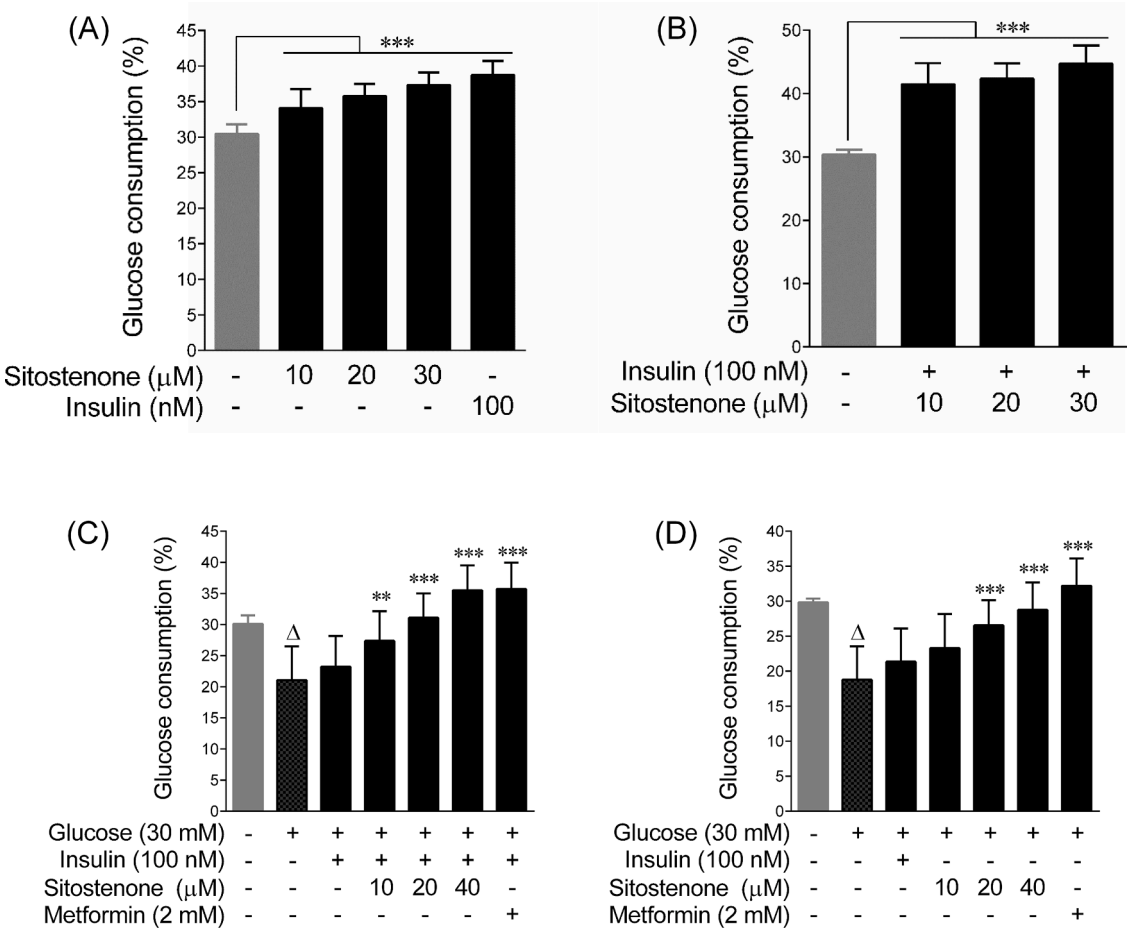

Fig. 5. Sitostenone promotes the glucose consumption in normal and insulin-resitant hepatic cells. (A) HepG2 cells were treated with sitostenone (10, 20, and 30 μM) or insulin (100 nM) for 6 h. (B) Cells were incubated with a combination of sitostenone and 100 nM insulin for 6 h. (C) To induce insulin-resistant, cells were pre-incubated with 30 mM D-glucose for 24 h, then the cells were treated with different concentrations of sitostnone or metformin in the presence of insulin for 6 h. (D) Insulin-resistant cells were incubated with various concentrations of sitostenone or 2 mM of metformin for 6 h. After treatment, culture supernatant was collected and glucose concentration was tested by DNS method. Values represent the mean ± SD of three independent experiments. Statistical significance $^{\Delta}p < 0.001$ compared to control *vs.* high glucose treatment (insulin-resistant) group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to insulin-resistant *vs.* sample treatment groups.

positive feedback that under normal physiological conditions, sitostenone mimics insulin and provokes glucose consumption in hepatic cells. Next, we compared the glucose uptake ability of sitostenone along with insulin. Interestingly, compared with control groups, sitostenone co-treatment with insulin remarkably increased glucose uptake from 30.1% to 41.5%, 42.3%, and 44.7% by 10, 20, and 30 μM sitostenone with insulin, respectively (Fig. 5B). However, the glucose uptake was not statistically significant from sitostenone alone versus co-treatment with insulin. It is speculated that under normal conditions, sitostenone slightly inhibits insulin's ability to promote glucose consumption failed to increase insulin sensitivity in hepatic cells.

Sitostenone promotes glucose uptake in insulin-resistant hepatic cells

The above result indicates that sitostenone failed to increase insulin sensitivity under normal condition, whereas it significantly promotes glucose uptake. To further clarify this mechanism, the glucose uptake ability of sitostenone was determined under insulin-resistant condition. As shown in Figure 5C, glucose consumption in control cells was observed as 30.1%, which was markedly increased to 41.6% after the addition of 100 nM insulin. Alternatively, glucose consumption by the control group was noted as 21.1% under insulin-resistant conditions. Although insulin treatment failed to provoke glucose uptake in insulin-resistant cells, glucose consumption was noted as 23.2%, which is significantly lower than that of un-induced and insulin-treated groups. Interestingly, when the insulin-resistant cells were co-incubated with 10, 20, and 30 μM sitosterone and 100 nM insulin significantly increased glucose uptake from 21.1% to 27.4%, 31.1%, and 35.5%, respectively. The 30 μM sitostenone-mediated glucose uptake in insulin-resistant cells was highly comparable with the positive drug control metformin (35.7%). These data strongly suggest that sitostenone can promote glucose uptake in insulin-resistant cells.

To further explore the role of sitostenone on glucose uptake in insulin-resistant cells without the addition of insulin, the insulin-resistant hepatic cells were incubated with increasing concentrations of sitostenone for 6 h, and then the contents of glucose in the culture medium were determined. As shown in Figure 5D, compared with insulin-resistant control cells, treatment with sitostenone markedly improved glucose uptake from 21.4% to 23.3%, 26.6%, and 28.8% by 10, 20, and 30 μM, respectively. Indeed, 20 and 30 μM sitostenone significantly provoked glucose in insulin-resistant cells, which is similar to the positive drug control metformin (32.2%). These data strongly suggest that sitostenone can simultaneously promote glucose consumption and increase insulin sensitivity in hepatic cells.

Sitostenone up-regulates IRS-1 and AKT activation in hepatic cells

It has been well demonstrated that activation of insulin signaling pathway, which includes phosphorylation of insulin receptor substrate-1 (IRS-1) and PI3K/AKT are the critical regulators of glucose uptake, protein synthesis, and gene expression in many cell types, including liver, muscle, and adipocytes (Boucher et al., 2014). Therefore, to further elucidate the molecular mechanism of the effect of sitostenone in hepatic cells, we determined the IRS-1 and AKT phosphorylation levels using Western blotting. As shown in Figure 6A, the control cells had a lower expression of phosphorylated IRS-1^{Y895}, while treatment with sitostenone significantly increased IRS-phosphorylation. Indeed, treatment with 20 μM sitostenone exhibited the most vital IRS-1 activation then that of 30 μM sitostenone or 100 nM of insulin-treated cells. Additionally, sitostenone significantly and dose-dependently activate the AKT^{Ser473}, a down-stream regulator IRS-1 in hepatic cells (Fig. 6B). These data are consistent with other observations that β-sitosterol, a relative compound to sitostenone activates IRS-1 and AKT in sucrose-induced diabetic mice (Babu et al., 2020).

Sitostenone up-regulates IRS-1, AKT, and GSK3β activation in insulin-resistant hepatic cells

It has become increasingly apparent that chronic accumulation of glucose in the bloodstream (hyperglycemia) played a crucial role in the impairment of insulin-stimulated glucose uptake in obesity and type-2 diabetic patients (Czech, 2017). Hepatic cells exposed to high glucose-impaired glycogen biosynthesis and glucose usage (Huang et al., 2015). Furthermore, prolonged exposure of D-glucose wound the insulin signaling molecules by decreasing the phosphorylation of IRS-1 and AKT and down-regulate the expression and membrane export of GLUT-4, which eventually resulted in the defect of glucose uptake, in such a condition called insulin-resistance (Boucher et al., 2014; Huang et al., 2015). Consistent with the above studies, our results also demonstrated that 30 mM D-glucose significantly reduced insulin-induced phosphorylation of IRS-1 and AKT levels in hepatic cells. To further observe the effect of sittostenone on insulin signaling molecules under insulin resistance conditions, we examined the phosphorylation of IRS-1 and its down-stream molecule AKT in high glucose-induced insulin-resistant hepatic cells. As shown in Figure 7A, treatment with sitostenone significantly recovered the phosphorylation of IRS-1under stimulation with insulin. The total level of IRS-1 was also increased dramatically by sitotenone in a dose-dependent manner. Besides, treatment with sitostenone significantly prevented the diminution in the phosphorylation of

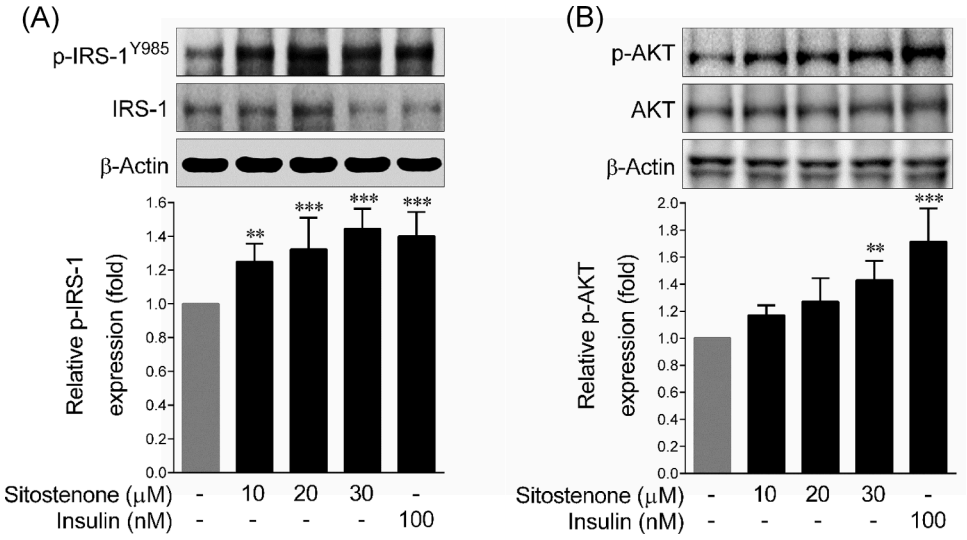

Fig. 6. Sitostenone promotes phosphorylation of IRS-1 and AKT in hepatic cells. Cells were treated with various doses of sitostenone or 100 nM of insulin for 10 min. Western blot analysis was performed to determine total and phosphorylated protein levels of IRS-1 (A) and AKT (B). β-actin was served as an internal control. Histogram indicate relative protein expression of the corresponding immunoblot. The phosphorylated levels of IRS-1 and AKT were normalized with their corresponding total levels. Values represent the mean ± SD of three independent experiments. Statistical significance $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to control *vs.* sample treatment groups.

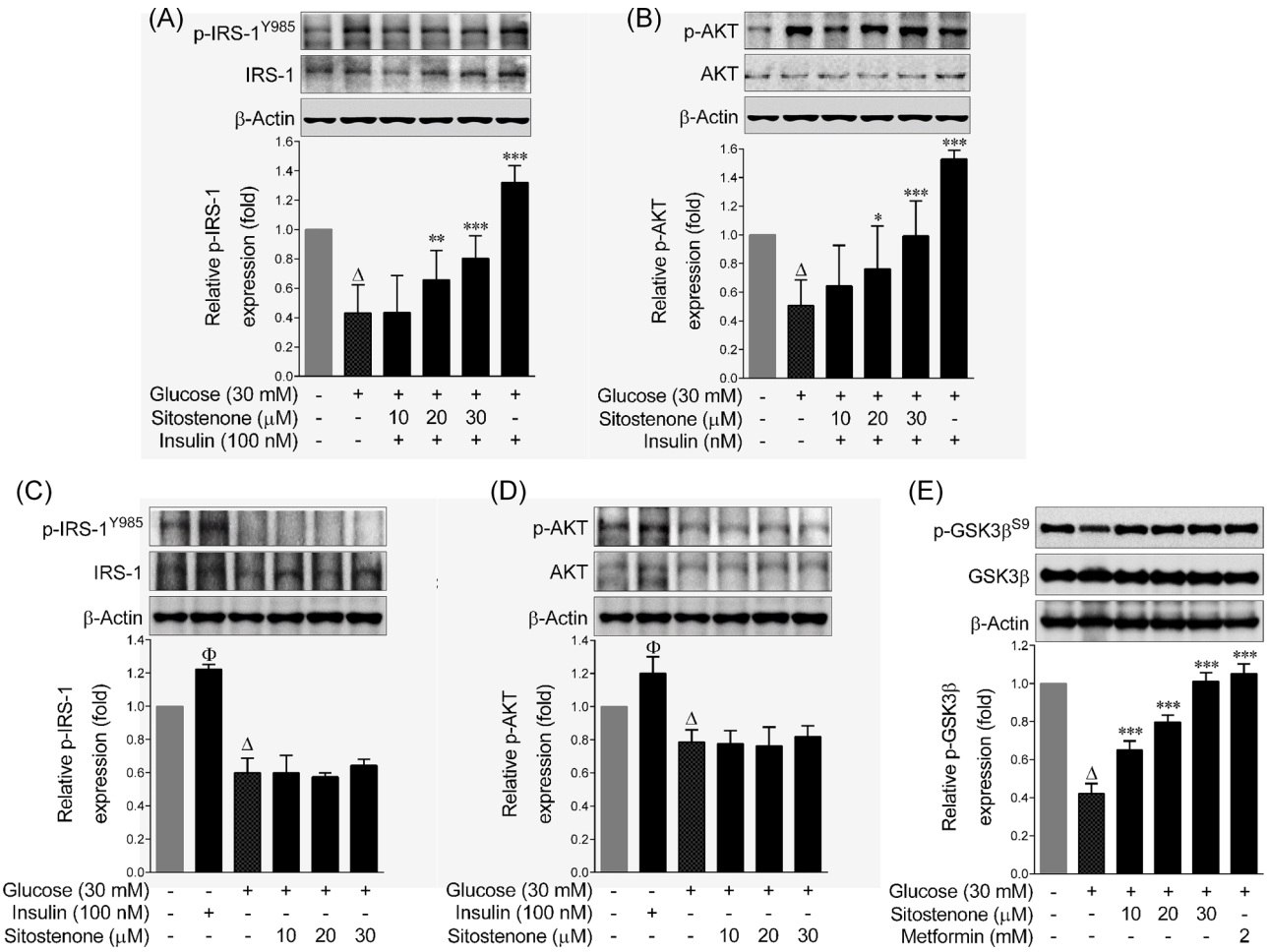

Fig. 7. Sitostenone promotes phosphorylation of IRS-1 and AKT in insulin-resistant hepatic cells. (A-E) HepG2 cells were exposed to high glucose (30 mM) for 24 h to produce insulin resistance, then the cells were treated with various doses of sitostenone along with or without100 nM of insulin for 10 min. Western blot analysis was performed to determine total and phosphorylated protein levels of IRS-1, AKT and GSK3β. β-actin was served as an internal control. Histogram indicate relative protein expression of the corresponding immunoblot. The phosphorylated levels of IRS-1, AKT and GSK3β were normalized with their corresponding total levels. Values represent the mean ± SD of three independent experiments. Statistical significance $^{\Phi}p < 0.001$ compared to control *vs.* insulin alone treatment group; $^{\Delta}p < 0.001$ compared to control *vs.* high glucose treatment (insulin-resistant) group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to insulin-resistant *vs.* sample treatment groups.

AKT caused by high glucose. In contrast, there was no significant impact on the total forms of IRS-1 and AKT levels (Fig. 7B). Furthermore, we found that treatment with sitostenone alone (without insulin) does not protect the high glucose-induced reduction of IRS-1 and AKT phosphorylation (Fig. 7C, D). Next, a down-stream target of AKT, the phosphorylation level of GSK3β, was determined. Compared with control, high glucose concentration significantly diminished GSK3β phosphorylation, whereas sitostenone treatment significantly provoked GSK3β phosphorylation in insulin-resistant cells. The restoration ability of sitostenone was identical to the effect of metformin (Fig. 7E). All these results indicate that sitostenone restrained the IRS-1 and AKT pathway inhibition, which constitutes a route in the insulin signaling pathway.

Sitostenone up-regulates PPAR-γ in insulin-resistant hepatic cells

Peroxisome proliferator-activated receptor-γ (PPAR-γ) is a member of the nuclear hormone receptor superfamily ligand-activated transcription factor that regulates cell growth, inflammation, lipid metabolism, and insulin sensitivity (Panchapakesan et al., 2004). The thiazolidinedione family of compounds that are now commonly used as insulin-sensitizing agents to improve glucose tolerance, enhancing insulin sensitivity, and restoring the function of β-cells in patients with type-2 diabetes (Soccio et al., 2014). The functional role of PPAR-γ in hepatic cells has been well characterized, with specific PPAR-γ activation to recover high glucose-induced insulin resistance and promotes glucose uptake (Kim and Ahn, 2004). Therefore, we examined whether the sitostenone-mediated increase in insulin sensitivity in hepatic cells was caused by PPAR-γ activation, the protein levels of PPAR-γ was determined by western blotting. As shown in Figure 8A, treatment with sitostenone significantly and dose-dependently increased PPAR-γ protein level in insulin-resistant cells. Compared with unstimulated control cells, a significant increase of PPAR-γ was found in high glucose-induced insulin-resistant cells. This result is consistent with other's observation that high glucose treatment resulted with PPAR-γ up-regulation in human kidney cells (Panchapakesan et al., 2004).

The forkhead box O 1 (FOXO1), a nuclear transcription factor, which mediates the inhibitory action of insulin or insulin-like growth factor on key functions in diverse pathways, including cell metabolism, proliferation, differentiation, oxidative stress, cell survival as well as senescence, autophagy, and aging in mammals (Lee and Dong, 2017). The expression of FOXO1 in the nucleus was significantly increased after cells exposed to a high concentration of glucose, which is consistent with a previous study that described high glucose concentration forces FOXO1 in mesangial cells (Das et al., 2014). Interestingly, treatment with sitostenone significantly decreased FOXO1 expression in the nuclear fraction, and the FOXO1 inhibitory effect of 30 μM sitostenone was

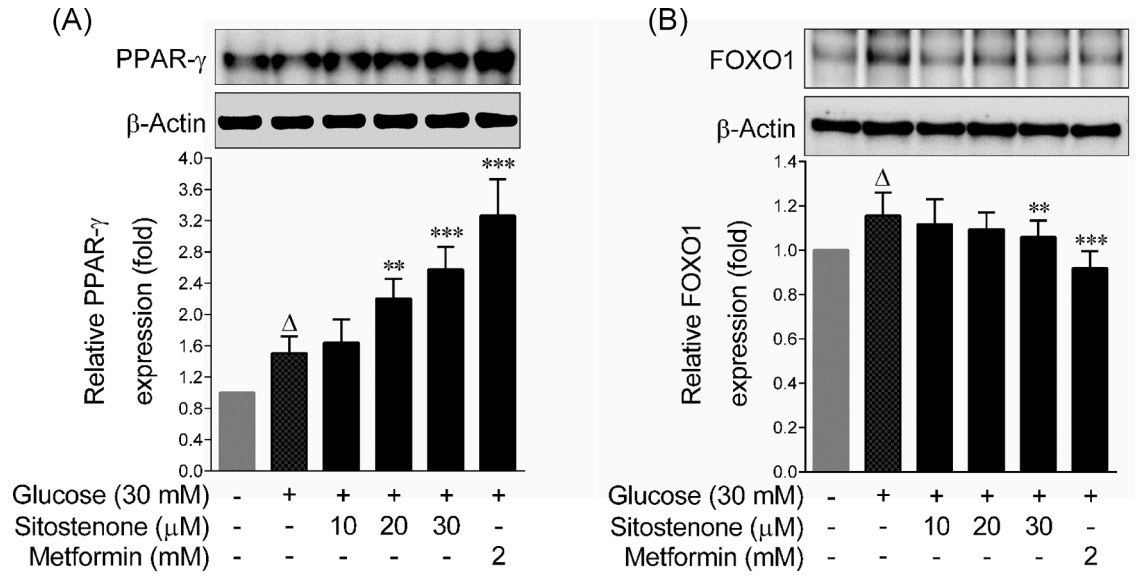

Fig. 8. Sitostenone up-regulates PPAR-γ and down-regulates FOXO1 expression in insulin-resistant hepatic cells. HepG2 cells were exposed to high glucose (30 mM) for 24 h to produce insulin resistance, then the cells were treated with various doses of sitostenone or 2 mM metformin for 6 h. Western blot analysis was performed to determine protein levels of PPAR-γ (A) and FOXO-1 (B). β-actin was served as an internal control. Histogram indicate relative protein expression of the corresponding immunoblot. Values represent the mean ± SD of three independent experiments. Statistical significance $^{\Delta}p < 0.001$ compared to control *vs.* high glucose treatment (insulin-resistant) group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to insulin-resistant *vs.* sample treatment groups.

highly comparable with metformin (Figure 8B). These data suggest that sitostenone-mediated improvement of glucose uptake and insulin resistance was associated with up-regulation of PPAR-γ and down-regulation of FOXO-1 in hepatic cells.

Sitostenone prevents high glucose-induced down-regulation AMPK activation in hepatic cells

5′ AMP-activated protein kinase (AMPK) is an energy-sensing enzyme activated when cellular energy levels are low, and it signals to stimulate glucose uptake, insulin sensitivity, fatty acid oxidation, and mitochondrial biogenesis in skeletal muscles, liver, and adipocytes. AMPK is down-regulated in humans and animals with diabetes, and the AMPK activation can improve insulin sensitivity and glucose uptake *via* glucose transporters such as GLUT2 and GLUT4 (Coughlan et al., 2014). Substantial evidence suggesting that numerous natural products activate AMPK, either directly or indirectly (Coughlan et al., 2014). Thus, in this study, the effect of sitostenone on the phosphorylation of AMPK was evaluated by Western blotting. In accordance with other's observation (Zang et al., 2004), hepatic cells exposed to high glucose significantly reduced AMPK phosphorylation compared with control cells, whereas sitostenone treatment significantly and dose-dependently restored the phosphorylation of AMPK in insulin-resistant hepatic cells (Fig. 9A). Notably, a sitostenone-mediated increase in AMPK phosphorylation was highly comparable with known anti-diabetic drug metformin.

Sitostenone restores high glucose-induced down-regulation GLUT2 and GLUT4 in hepatic cells

To further determine the effect of sitostenone on AMPK's down-

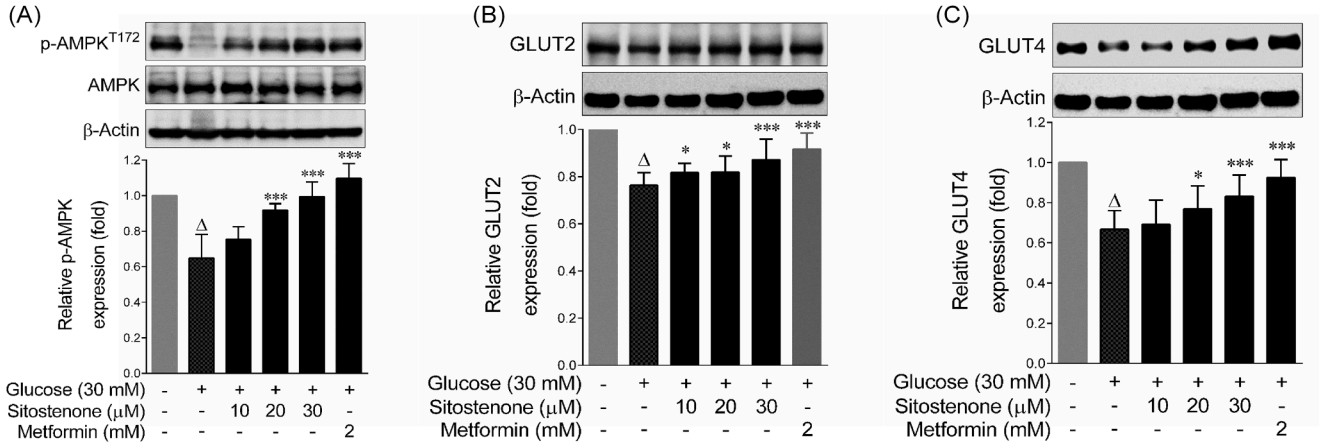

Fig. 9. Sitostenone activates AMPK and up-regulates GLUT2, GLUT4 levels in insulin-resistant hepatic cells. HepG2 cells were exposed to high glucose (30 mM) for 24 h to produce insulin-resistance, then the cells were treated with various doses of sitostenone or 2 mM metformin for 6 h. The membrane fraction was prepared and western blot was performed to determine total and phosphorylated level of AMPK (A) and the protein levels of GLUT2 (B) and GLUT4 (C). β-actin was served as an internal control. Histogram indicate relative protein expression of the corresponding immunoblot. The phosphorylated level of AMPK was normalized with its corresponding total levels. Values represent the mean ± SD of three independent experiments. Statistical significance $^{\Delta}p < 0.001$ compared to control *vs.* high glucose treatment (insulin-resistant) group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ were compared to insulin-resistant *vs.* sample treatment groups.

stream targets, such as GLUT2 and GLUT4, in insulin-resistant hepatic cells, cells were incubated with increasing concentrations of sitostenone and 2 mM metformin for 6 h. GLUT2 and GLUT4 expression levels in the membrane fraction was determined by Western blotting. Data demonstrated that compared with control cells, cells exposed to high glucose significantly reduced the protein expression levels of GLUT2 and GLUT4 in hepatic cells, whereas treatment with sitostenone significantly and dose-dependently restored GLUT2 and GLUT4 protein levels in the membrane (Fig. 9B). These results indicate that sitostenone promotes glucose uptake *via* up-regulation of GLUT2 and GLUT4 in the membrane through the AMPK pathway.

Conclusion

In this study, we first demonstrated that ethanol extract of *R. laevigata* fruits and its derivative sub-fractions promotes glucose uptake and alleviates hepatic insulin resistance, as they had low cytotoxicity in hepatic cells. Bioactivity-guided fractionation analysis revealed that sitostenone, a steroid-like compound exhibited a pronounceable effect on hepatic cells. As sitostenone treatment remarkably increased glucose uptake and insulin sensitivity in hepatic cells, which was seemingly mediated *via* IRS-1 and AKT cascade activation in normal hepatic cells. Also, sitostenone treatment restores insulin-induced phosphorylation of IRS-1 and AKT in high glucose-induced insulin-resistant cells. Furthermore, high glucose-induced dephosphorylation of IRS-1, AKT, GSK3β, and AMPK, and down-regulation of GLUT2 and GLUT4 were significantly restored by sitostenone. To conclude, this study strongly suggests the concept that activation GLUT2/4 by IRS-1-AKT *via* PPAR-γ/AMPK signaling pathways is involved in sitostenone-mediated improvement in insulin-resistant cells. However, further *in vivo* investigation of this activity is necessary to elaborate the mechanisms and permit full exploitation of its promise, specifically the role of altered signal transduction.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

Acknowledgment

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