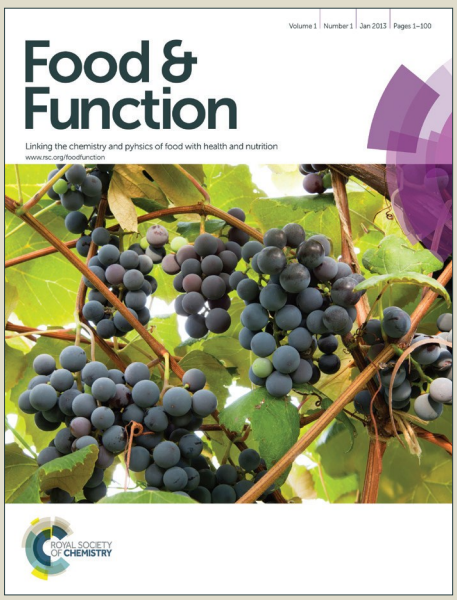
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

The potential beneficial effects of phenolic compounds isolated from *A*. Ledeb on hepatic HepG2 insulin-resistant cells

Antidiabetic effect of an unusual plant as tea combinations

Hui Teng[#], Lei Chen[*#], Hongbo Song[*]

College of Food Science, Fujian Agriculture and Forestry University, Fuzhou, Fujian 350002, China

*Corresponding author:

Hongbo Song, sghgbode@163.com

Lei Chen, afred521@163.com

Telephone: 86-591-83756316

Fax: 86-591-83756316

[#]these authors equal to this work

Abstract

Agrimonia pilosa Ledeb (AP) has already been applied in practice for the treatment of different disorders and are available to access without the provision of medical prescription. The present study aims at investigating the effect of bioactive compounds isolated from AP on the improvement of insulin resistance, figuring about the mechanism in insulin-responsive cell lines. Five compounds were isolated from AP using column chromatography, including agrimonolide (**K1**), desmethylagrimonolide (**K2**), tormentic acid (**K3**), ursolic acid (**K4**), and quercetin (**K5**) . Glucose metabolism was evaluated in insulin-resistance HepG2 cells. Ursolic acid had the strongest activity among all isolated compounds with the lowering value of 71.5% (1.24 mM glucose in DMEM) and 71.7% (1.23 mM) respectively, when compared to the control. **K1** consisting of **K2** effectively increased insulin-mediated glycogen level in heptocytes. At concentration level of 20 μM, **K2** significantly elevated hepatic glucokinase (GK) activity (3.2 U/min/mg protein), followed by **K1** (3.0 U/min/mg protein). Both of them significantly increased (p<0.05) the GK activity as compared to the control. On the same line, **K2** and **K1** caused a significant reduction of glucose-6-phosphatase (G6Pase) activity and a significant change in Phosphoenolpyruvate carboxykinase (PEPCK) activity. In summary, bioactive compounds in AP may play an important role in regulating glucose metabolism in insulin-resistance HepG2 cells and could be developed as a promising natural material for diabetes prevention and treatment.

Keywords: phenolic compounds/ glucokinase/ glucose-6-phosphatase/ Phosphoenol-pyruvate carboxykinase/ HepG2

1 Introduction

Fasting hyperglycemia, the hallmark of diabetes mellitus, is primarily the result of

excess production of glucose by the liver. Besides, skeletal muscle is the major site of glucose uptake in the postprandial state in humans. Fasting and insulin-stimulated glucose oxidation, glucose storage, and muscle glycogen synthase activation were all fully normalized during hyperglycemia in the diabetic patients [1, 2]. Researchers have assessed short-term physiological regulation in skeletal muscle and adipose tissues by excess nutrient and fasting to modulate glucose transport and mitochondrial oxidation [3, 4]. Hepatic glucose production is the balance between the fluxes through glucokinase (GK) and glucose-6-phosphatase (G6Pase). GK is markedly decreased, but G6Pase activity is markedly increased in insulin-deficient diabetic [1]. Phosphoenolpyruvate carboxykinase (PEPCK), a member of lyase family, is functional in the metabolic pathway of gluconeogenesis [2]. These observations suggest that these enzymes are important regulators in diabetes. Glycemia is a parameter over which the organism establishes tight control. Insulin negatively regulates transcription of genes involved in hepatic glucose production, such as those encoding (PEPCK) insulin growth factor-binding protein-1 (IGFBP-1), and G6Pase, via regulation of various transcription factors that bind to the insulin-response unit [5]. Therefore, identifying agents that can inhibit hepatic gluconeogenesis via insulin- independent signaling may provide new therapeutic options to curtail the elevated gluconeogenesis caused by insulin resistance in type-2 diabetes.

Based on this, there has been a growing interest in flavonoids, which are widely distributed in plants and ingested by humans, due to their antioxidative, mild estrogenic, and hypolipidemic activity [6]. Several flavonoids have also been shown to exert an effect on glucose transport, the insulin-receptor function, and peroxisome proliferators-activated receptor (PPAR) activation. Such phenolicsinclude quercetin [7], ascorbic acid [8], apigenin, chrysin, and kaempferol [9], all of which play essential roles in diabetes. Insulin resistance is a fundamental aspect of the etiology of type-2 diabetes and it is also linked to a wide array of other pathophysiologic sequelae

including hypertension and hyperlipidemia [10]. These impairments in insulin action play an important role not only in the development of hyperglycaemia of non-insulin dependent diabetes but also in the pathogenesis of long term complications. Insulin-sensitive tissues, such as liver, fat, and muscle are typically involved in regulating whole body fuel metabolism [11].

The species of AP, belonging to the *Rosaceae*, are listed in the Chinese Pharmacopoeia as an astringent hemostatic for treating various kinds of bleeding [12]. Pharmacological studies have reported that AP have broad biological properties, such as antioxidant [13], nitric oxide scavenging [14], acetylcholinesterase [15] and α-glucosidase inhibitory activities [16]. Traditionally, AP was used widely including prevention of Non-Small Cell Lung Cancer (NSCLC), promoting blood circulation, arresting bleeding [17]. They are therefore favorable uses alone or in combination with traditional Chinese Medicines (shepherdspurse, 40 g; green tea, 6 g) for the treatment of various disease. As discussed previously[18], intake of AP for 2 h after meal, the blood glucose level was significantly down-regulated by promoting insulin secretion or increasing conversion of glucose. AP contains numerous biologically active compounds, such as quercetin, quercitrin, hyperoside, taxifoliol, luteolin-7-*O*-beta-D-glucopyranoside and rutin. The antidiabetic potential and our ongoing interest in bioactive natural compounds prompted us to investigate this plant. To the author's knowledge there are no systematic studies on the molecular structure-activity mechanism, thus, the present study was designed to separate the compounds in AP, and evaluate the antihyperglycemic effect of these compounds in HepG2 cells.

2 Materials and methods

2.1 Chemical and regent

Human insulin, PD98059, palmitate, anthrone reagent, anthrone, and badford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cell Lysis Solution: Tris

0.121 g, NaCl 0.876 g, EDTA 0.029 g, PMSF (Phenylmethanesulfonyl fluoride) 0.017 g, SDS 0.1 g, sodium deoxycholate 0.1 g to 100 mL D.W (adjust pH to 7.4). Culture medium: High (4500 mmol/L) and low glucose (1100 mmol/L) DMEM and fetal bovine serum (FBS) obtained from Hyclone (Logan, UT, USA). The cell homogenate buffer: Tris 6.057 g, KCl 6 g, $EDTANa_2$ 0.931 g, 0.25% Triton-100 750 μL to 500 mL D. W (adjust pH to 7.4).

2.2 Extraction, isolation and identification of compounds

Extraction solvents, including ethylacetate (EtOAc), methanol (MeOH), n-hexane and n-butanol (n-BuOH) were purchased from Duksan Chemical. α-Glucosidase and p-nitrophenyl-α-D-glucopyranoside were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Nuclear Magnetic Resonance (NMR) spectra were obtained on a Bruker AV-500 using MeOD with TMS spectrometer as internal standard. Chemical shifts (d) are expressed in ppm relative to TMS (spectrum parameters: scan numbers; 8, acquisition time; 3.1719 s, original points count; 32,768, sweep width; 10330.58 Hz). Optical density (OD) was read with a microplate reader ((Power Wave XS, Bio-Tek Instrument, Winooski, VT). HPLC (Jasco, Tokyo, Japan) on ZORBAX Eclipse XDB-C18 (4.6 cm × 250 mm, 5 μm, Agilent, Santa Clara, CA, USA) column, associated with UV-visible detector (Jasco, Tokyo, Japan). The isolation of pure products was carried out via column (silica gel 60, 70-230 mesh, Merck) or thin layer (silica gel 60 GF254, Merck) chromatography.

2.3 Insulin-resistant HepG2 cell model

Human HepG2 cells were grown in DMEM-high medium supplemented with 10% fetal bovine serum (FBS) and the following antibiotics: gentamicin, penicillin and streptomycin (50 mg/L). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Insulin-resistant cell model was induced according to the previous

method [10]. After confluence, cells (1×10^5 cells/well) were cultured in 96-well cluster plates in high-glucose (4500 mg/L) DMEM supplemented with 10% FBS for 24 h, and then the cells were treated with 10^{-7} mol/L insulin in serum-free and phenol red-free low-glucose (1100 mg/L) DMEM for 24 h. After that, 5×10^{-7} mol/L insulin was stimulated, in order to make the cells adapt to the process from high to low glucose DMEM, the cells were washed 4 times with low-glucose DMEM (pH4, low-glucose conditions can lead to a rapid decline in cell viability) and twice with PBS, and then serum-free and phenol red-free high-glucose DMEM with compounds or extract in different concentrations (no cytotoxic concentrations) were added and incubated for 4 h. Before experiments, HepG2 cells were serum-starved for 24 h in DMEM low-glucose medium. After serum starvation, the cells were treated with or without different concentrations of samples or metformin for 4 h.

2.4 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

To assess cell viability, culture media from cells exposed to the different compounds were tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially as described by previous study [12]. The HepG2 cells were seeded on 96 multi-well plates at 1×10^5 cells/well and cultured for 24 h, medium was changed every other day. After incubation for further 24 h, 25 μL of MTT solution (5 mg/mL in phosphate-buffered saline, pH 7.4) was added to each well and further incubated at 37°C for 4 h. Upon termination, the supernatant was aspirated and the MTT formazan, formed by metabolically viable cells, was dissolved in DMSO, 150 μL) by mixing on a gyratory shaker for 30 min, and the plates were scanned at 540 nm.

2.5 Glucose consumption and glycogen content

Glucose content in culture medium was detected by the glucose oxidizes/peroxides (GOD-POD) method using a glucose assay kit (Sigma, St. Louis, MO) by following

the manufacturer's instructions. Glucose consumption was calculated using the starting glucose concentration in culture medium (1 mg/mL) minus the glucose concentration measured at the end of experiment. Glycogen content of cells was determined by the anthrone reagent. The amount of blue compound generated by the reaction was assayed at 620 nm. The protein content of the collected HepG2 cells was quantified with Bradford reagent. Values were then presented in the ratio of glycogen/protein.

2.6 Glucokinase (GK) activity assay

Cells (1×10^5 cells/well) were lysed in 500 μL of homogenized buffer consisting of 50 mM triethanolamine hydrochloride (pH 7.4), 100 mM KCl, 1 mM dithiothreitol, 5% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A and1 μg/ml leupeptin. Homogenates were collected in Eppendorf tubes, incubated on ice with PEG 25% for 10 min, and then centrifuged at 10, 000 × g for 15 min under 4 °C. Glucose phosphorylating activities were measured by an enzymatic method measuring the production of NADPH from $NADP^+$ in the presence of glucose 6-phosphate dehydrogenase (G6PDH) with either 100 mM glucose or 0.5 mM glucose, to distinguish HK from GK activity. The assay buffer contained 100 mM riethanolamine hydrochloride (pH 7.4), 40 mM $MgCl_2$, 200 mM KCl, 2 mM dithiothreitol, 0.2% bovine serum albumin, 5 mM ATP, 1 mM NADP, and 30 units/ml of G6PDH. The phosphorylating capacity obtained at 0.5 mM glucose is considered the hexokinase activity, whereas the subtraction of activity measured at 100 mM glucose from the activity measured at 0.5 mM glucose is considered the glucokinase activity of the extract. Glucose phosphorylating activity was taken as the increase in NADPH absorbance measured at 340 nm after 3 min at 25 °C, expressed in milliunits per mg of protein. Protein concentration was measured using the Bradford method (Bio-Rad, Laboratory) with bovine serum albumin as standard.

190

191 **2.7 Measurement of G6Pase activity**

192 G6Pase activity was determined in cell extracts prepared from cultured hepatocytes
193 by an enzymatic method [19]. Incubation was stopped by placing the cells (1×10^5
194 cells/well) on ice followed by centrifugation at 4°C for 60 s at 12000rpm × g. The
195 supernatant was removed, and the cells were re-suspended in 300 μL of
196 homogenization buffer (50 mm Tris-HCl (pH 7.5), 250 mm sucrose and 0.2 mm
197 EDTA) and homogenized using a glass/Tefl on homogenizer (Wheaton Science, NJ,
198 USA). For the G6Pase assay, the microsomal fraction was prepared as follows: the
199 homogenate was centrifuged at 20000 × g for 20 min at 4°C, and then
200 ultra-centrifuged at 12000 × g for 1 h at 4°C [20]. The resultant sediments were
201 dissolved in 0.1 mL of homogenization buffer and stored at -80°C until use. The
202 microsomal suspension was incubated with 14 mM glucose-6-phosphate and 50 mM
203 Tris-cacodylate (pH 6.5) in a final volume of 100 μL at 37°C for 1 h. To determine
204 the amount of inorganic phosphate formed during the reaction, 300 μL of stop
205 solution containing 0.42% ammonium molybdate in 1 N H_2SO_4 and 10% ascorbic
206 acid (6:1, v/v) was added. After incubation at 45°C for 20 min, the optical density at
207 340 nm was measured. Enzyme activity was expressed as the molar value of
208 phosphate hydrolysed by 1 mg of microsomal protein per hour.

209 **2.8 Phosphoenolpyruvate carboxykinase (PEPCK) activity**

210 PEPCK was measured using the $NaHCO_3$ fixation assay as described by Noce and
211 Utter [21] and Burcelin *et al* [22] with some modifications. Briefly, after removing the
212 culture medium, each well (1×10^5 cells/well) was scraped into 500 μL of reaction
213 buffer containing 150 μmoL Tris/acetate (pH 7.2), 5 μmoL sodium
214 inosine-diphosphate, 10 μmoL $MnCl_2$, 250 μmoL KCl, 10 mM DTT, 2 mM
215 glutathione and 400 μmoL $KHCO_3$. The reaction began by the addition of 10 μL of
216 0.4 M phosphoenolpyruvate and the process was terminated after 10 min of

incubation at 25°C by the addition of 1 ml of 6 N HCl and by placing the tube on ice. The reaction mixture for PEP carboxykinase contained up to 0-008 unit of enzyme, 100 µmoL of imidazole-HCl buffer, pH 6.6, 2 umoles of $MnCl_2$, 1umole of GSH, 1.25 µmoL of sodium IDP, 50 µM of $KHCO_3$, 2.5 µmoL of NADH, 1.5 µmoL of PEP and 2 U of malate dehydrogenase in a volume of 1 mL. The final pH was 7.1. The optical density was then measured at 340 nm.

2.9 IC_{50} determination

Solutions of compounds were made in DMSO. The IC_{50} of each of the above mentioned compounds was performed subsequently [23] as described previously, in the presence of increasing concentrations of the above mentioned compounds. The IC_{50} values were calculated from the plots of log concentration for inhibitor concentration versus percentage inhibition curves by using Sigma Plot 10.0 (IL, USA). The inhibitory concentration 50% (IC_{50}) was calculated from the Prism dose–response curve obtained by plotting the percentage of inhibition versus the concentrations.

2.10 Statistical analysis

Statistical analyses were performed by Statistical Analysis System (SAS, Cary, NC). Data were subjected to the analysis of variance (ANOVA), specifically the normal distribution of the residuals and the homogeneity of variance was tested by means of the Shapiro-Wilk's, and the Levene's tests respectively. In cases where statistical significant differences were identified, the dependent variables were compared using Duncan's multiple range tests at $p < 0.05$.

3. Results and discussion

3.1 Identification of isolated compounds

The identity of compounds were established by comparing their ^{1}H and ^{13}C NMR

chemical shifts and proton coupling constants in $CDCl_3$ or DMSO-d_6 with those reported previously in the literature. The molecular formulas of compounds **K1** and **K2** were assigned as $C_{18}H_{18}O_5$ and $C_{17}H_{16}O_5$ respectively, from LRMS and NMR data. Compound **K1** has one additional methyl group with respect to **K2**. Compound **K3** was an ursane-type triterpene. The ^{1}H-NMR spectrums of **K3** showed the presence of seven singlet methyls, two doublet methyls, two carbinol methine protons assignable to H-2 (δ 3.83) and H-3 (δ 3.36), and one vinylic proton (δ 5.29) on C-12. Splitting pattern (J=9.4 Hz) of the H-3 in ^{1}H-NMR spectrum indicated the axial interaction with H-2. Each of the coupling constant of H-2 could explain the axial-axial (J=11.2 Hz) and axial-equatorial (J = 3.6 Hz) coupling with corresponding geminal protons at C-1. Furthermore, ^{13}C NMR data of **K3** showed a good agreement with reported data of tormentic acid. The ^{1}H-NMR and ^{13}C NMR chemical shifts showed that compound **K4** was ursolic acid, which was in agreement with published data. Compound **K5** was obtained as yellow powder with the molecular formula of $C_{15}H_{10}O_7$,being identified as quercetin as its data were in agreement with published data [24]. In addition, different compounds normally possess specific chromatographic behavior and UV visible spectral characteristics. Thus, Compounds isolated from AP and tested in this study were systematically identified and confirmed by RP-HPLC with UV detector. HPLC profiles of the representative constituents from AP are shown in Figure 1 and their quantification are summarized in Table 1. . K5 and K2 were the primary compounds in AP extract with the highest content of 9.88 mg/g d.w and 6.22 mg/g d.w, respectively. Recovery in the analytical method was also studied by adding three increasing amounts of each target compound to the extract to cover the expected range of concentrations and then analyzing each one in triplicate. Recovery results in Table 1 confirmed that the mean recoveries of all tested components were satisfactory with values all above 94.8%.

3.2 Glucose uptake and glycogen level in insulin resistant HepG2 cells

Our previous study [23] has verified that a significant decrease ($P < 0.05$) of extracellular glucose occurred after incubation of HepG2 cell with 5×10^{-7} mol/L insulin for 24 h Figure 2A indicated that the effect of compounds isolated from AP (**K1-K5**) on insulin resistance was comparable to that of metformin, which is a biguanide agent able to reduce hyperinsulinemia and improve hepatic insulin resistance. The extract showed the strongest effect with the lowering value of 82.1%, at the 100 μg/mL concentration. As a positive control, metformin exhibited comparatively a weaker effect with the lowering value of 70.8%, at the 20 μM concentration. The relevance of the concentrations selected based on damage degree of HepG2 cell after being treated with either extract or compounds according to the MTT cytotoxicity results. It is worth to mention that concentrations used in the study are not far below realistic and within the range recommended for in vitro studies. For instance, relative study had used eriodictyol (5 and 25 μM) to enhance insulin-stimulated glucose uptake in HepG2 cells [24]. An array of concentrations between 1 and 10 μM of 7-*O*-methylaromadendrin (flavonoid) have been reported significantly stimulating insulin-induced glucose uptake in HepG2 cells [25]. **K5** and kaempferol have been revealed showing conflicts in regulating insulin-stimulated glucose uptake in adipocytes at the doses of 5, 10, and 20 μM [26]. Indeed, several reports have also shown that flavonoids, such as galangin, kaempferol, **K5**, and myricetin, at concentration level of 5 μM, modulating glucose uptake in HepG2 cell model [27]. Altogether, these suggested that recommended range for HepG2 cells was 1-50 μM.

Additionally, our previous study confirmed that agrimonolide did not show any cytotoxicity up to concentration of 80 μM [28]. Previous studies evaluated various concentrations (1, 10, 25, 50 μM) of triterpenoids for their ability to enhance glucose uptake in L6 rat skeletal muscle cells [29] and insulin-resistant human HepG2 Cells [30].

Besides, ursolic acid (**K4),** a plant triterpenoid, was reported to increase glucose uptake in insulin-stimulated HepG2 cells at 12.5 and 25 μM doses [31]. Based on these reports, in order to further understand the potential roles of 5 tested compounds from AP in glucose metabolism, regulate glucose consumption ability of them was investigated in HepG2 cells using the same concentration range. Among all the tested compounds, **K4** had the strongest activity with the lowering value of 71.5% (1.24 mM glucose in DMEM) as compared to the control and had no significant difference with metformin (70.5%). **Other isolated compounds from AP** (K1, K2, K3, and K5) showed feeblish activities with the values of 62, 59, 59 and 66%, respectively. Several studies have reported that blood glucose and lipid levels in DM rats can be decreased after the treatment of quercetin, ferulic acid, groenlandicine and berberrubine, [32, 33, 34], and declared that low-dose was more pronounced than high dose. Such observations showed for the first time the association of **K4** with glucose consumption in cultured cells and indicated that **K1** and **K2** might be better than the others, as far as the anti-diabetic activity is concerned. Similar to previous research [35], PD98059 was taken as positive control in present study. As shown in Fig. 2B, stimulation of PD98059 resulted in a decrease in glycogen content，but compound **K1** and **K2** effectively increased insulin-mediated glycogen level in heptocytes. Glycogen level from different precursors and its degradation are known to be dependent on nutritional and hormonal factors, principally glucose and insulin. **K4**, **K1** consisting of **K2** or metformin treatments significantly reduced the concentration of glucose within the medium of previously starved cells when compared to the control. The starved cells were cultured for 4 h in DMEM medium and adjusted to 1000 mg/mL glucose concentration. As a result, metformin as well as these compounds showed the significantly greater effect ($p < 0.05$). The plasma glucose-lowering action of metformin is most partly due to improvement of hepatic insulin resistance, leading to reduction in hepatic glucose production, which mainly due to a decrease in

gluconeogenesis [36]. The decreased glucose concentration in the culture medium could be the result of either increased glucose uptake or reduced gluconeogenesis. The reduction in glucose concentration in the culture medium of cells treated with **K4**, **K1** and **K2** is consistent with a metformin-mediated decrease in gluconeogenesis. However, **K1** and **K2** could act via increasing glucose uptake, or reducing gluconeogenesis, or react as both mechanisms. To determine the cytotoxicity of isolated compounds on HepG2 cells, MTT assay were performed (Fig. 2C), and the MTT assay showed a survival rate of ≥90% for all the concentrations tested (Fig. 2C). Results also showed that the HepG2 cells viability was negligibly affected at the concentrations of 20 μM. Besides, the cells treated with insulin (insulin resistant model) or without insulin (control) showed no morphology changes (Fig. 2D, E).

3.3 Hepatic enzyme activities in HepG2 cells

G6P lies at the beginning of both glycolysis and the pentose phosphate pathways. This enzyme and transporters are potential targets for antidiabetic therapy [37]. The inhibition of the G6P phosphohydrolase activity might be of help in the control of the hyperglycaemia present in diabetes. GK activity was significantly elevated, while G6Pase and PEPCK activities were markedly decreased by tested compounds when compared to the control group. As shown in Fig. 3A, at a concentration of 20 μM, **K2** showed the strongest GK activity (3.2 U/min/mg protein), followed by **K1** (3.0 U/min/mg protein), both of them significantly increased the GK activity compared to the control. The elevation of hepatic GK activity could increase the utilization of blood glucose for glycogen storage in the liver [7]. GK, the first enzyme in the glycolytic pathway, is induced by insulin and converts glucose to glucose-6-phosphate (G6P). G6P is metabolized through glycolysis, glycogen level, and via the pentose phosphate shunt. The evidences clearly confirmed that hesperidin and naringin significantly increased diabetic animal hepatic GK activity [38]. Our results

along with the reported results indicated that flavonoids significantly elevated hepatic GK activity as compared to suppressing the elevation of hepatic gluconeogenic G6Pase and PEPCK activities in IR HepG2 cells. GK is both an insulin-dependent and an insulin-sensitive enzyme and is almost completely inhibited or inactivated in the diabetic rat liver in the absence of insulin [39].GK insufficiency in diabetic rats can cause low utilization of glucose for energy production [40]. In contrast, increased GK activity leads to a decrease in blood glucose level by the utilization of glucose. In the same line, **K2** and **K1**caused a significant reduction of G6Pase activity (Fig. 3B) as compared to the control, and they also showed a significant change in PEPCK activity (Fig. 3C). In general, dose-dependent inhibitory effects of all tested compounds on G6Pase and PEPCK were observed. All the tested compounds had a G6Pase reduction above 20% (compare to control), at a concentration of 20 μM. **K2** exhibited the highest reductive effect of 77%, at the same concentration. A lower G6Pase reductive activity was observed in **K1** when compared with **K2**, while **K1** (65%) had a higher reductive effect on PEPCK than **K2** (54%). Insulin decreased the hepatic glucose output by activating glycogen level and glycolysis, and by inhibiting gluconeogesis [41]. As it can be seen in Table 2, there is a good agreement with the inhibitory percentages of G6Pase, PEPCK and the IC_{50} produced by the tested compounds. When comparing the IC_{50} values of various compounds, the potency order for the G6Pase inhibitory activity were as follows: **K2** > **K5** > **K1** > **K4** > **K3**, whereas the potency order for PEPCK were as follows: **K1** > **K4** > **K2** > **K3** > **K5**. Previous investigations revealed some flavonoids were able to either improve or inhibit insulin-stimulated glucose uptake in HepG2 cell model. Chlorogenic acid, a major component of coffee, was found to have a reversible linear competitive inhibitor of G6Pase in rat liver microsomes [42]. In addition to this effect on enzyme activity, Valentová et al. demonstrated that chlorogenic acid significantly enhanced GK activity in rat hepatoma cell lines [43]. Jung et al. also observed, in

C57BL/KsJ-db/db mice, that hepatic GK activity was significantly higher in the caffeic acid-supplemented group than the control group [44]. In agreement with the results presented in this study, the effect of quercetin (K5) on hepatic enzyme activities was also observed by Vessa et al [45]. Additionally, ursolic acid (K4) was previously demonstrated to show a significant effect on the GK activity increasing and G6Pase activity normalization in the diabetic rat, meanwhile, K4 supplementation significantly improved the G6Pase/GK ratio [46]. Recently, Shih et al. reported that loquat leaf extract which contain five triterpenes: tormentic acid, 44.30%; corosolic acid, 19.50%; maslinic acid, 14.65%; oleanolic acid, 1.60%; and ursolic acid, 5.30% could improve insulin sensitivity [47]. Tormentic acid was further proved to have abilities to reduce PEPCK and G6Pase activities [48]. According to the results, desmethylagrimonolide was more specific for G6Pase inhibition rather than PEPCK inhibition. The IC_{50} (concentration for 50% inhibition of enzymes) value of **K2 against** G6Pase was 2.2 μM, and **K1** was the most effective PEPCK inhibitor, whereas **K5** showed the least potent inhibitor among the tested compounds. The results showed that all of the tested compounds could increase the glucose consumption and stimulate glycogen level in HepG2 cells within certain concentrations, and no significant toxic effect was found in the HepG2 cells when the cells were treated with all isolated compounds at a concentration of 20 μM (Table 2).

3.4 Correlations

The liver plays a key role in maintaining blood glucose concentration, through its ability to supply glucose to the circulation via glycogenolysis and gluconeogenesis and to remove glucose from the circulation to increase glycogen synthesis [49]. However, the hepatic insulin resistance is characterized by a reduced capacity of insulin to increase glycogen level and an impaired insulin signaling. Consequently, interventions to prevent insulin resistance are of great protective and therapeutic

interest. In order to determine the principles of glucose uptake and glycogen synthesis effect in HepG2, the correlation coefficient between these effects and hepatic enzyme activities were analyzed by means of SAS (SAS, Cary, NC). Using a bivariate correlation between glycogen and glucose consumption effect attributes with the key enzyme effect response variables, it was possible to observe correlations between these two activities by using different compounds at different concentrations. Besides , even through the correlation coefficient show statistically significant, it only display a trend on how the response variables are associated, thus, the coefficient of determination was also calculated. As shown in Fig. 4, the correlation coefficient between glycogen level and GK activity was recorded at 0.7897. This result revealed that the higher GK activity the compound has the more ability to increase insulin-mediated glycogen level in hepatocytes. Nonetheless, the correlation coefficient between glycogen level and G6Pase and PEPCK inhibition were 0.4479 and 0.4706 respectively, indicating these enzyme inhibitory effects also correlated with glycogen level (but not significantly). In a similar study, Park et al [50] reported that phenolic compounds supplement significantly elevated hepatic GK activity compared to the db/db group by 42% and 72%, respectively, while slightly inhibiting the elevation of G6Paseand PEPCK activities in the db/db mice. Tormentic acid, ursolic acid, and quercetin positively correlated to their GK activities, meanwhile, agrimonolide and desmethylagrimonolide were found to have significant correlations with the inhibitory effects on G6Pase and PEPCK.

4 Conclusions

Five compounds that were isolated from *A.* Ledeb stimulated glucose consumption of insulin-resistant HepG2 cells significantly, but they had no effect on cell proliferation. In addition, these compounds increased glycogen level by: lowering the activities of PEPCK and G6Pase, and constraining the gluconeogensis of insulin-resistant HepG2

cells to decrease the production of endogenous glucose; raising the glycogen content and as a result alleviating the condition of insulin-resistance distinctly. Collectively, these effects caused changes in biochemical processes that led to a net switch in the metabolic program of organism to fuel stores catabolism, which is an adaptation that may be of some benefit in the face of disorders characterized by insulin resistance.

Corresponding Author

Hongbo Song, sghgbode@163.com

Lei Chen, afred521@163.com

Telephone: 86-591-83756316

Fax: 86-591-83756316

Conflict of Interest

The authors declare that there are no conflicts of interest.

Abbreviations

Agrimonia pilosa Ledeb, AP. Dimethyl sulfoxidesulphoxide, DMSO. Foetal bovine serum, FBS. glucose 6-phosphate dehydrogenase, G6PDH. 2.4 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, MTT. Glucokinase, GK. Glucose-6-phosphatase, G6Pase. Phosphoenolpyruvate carboxykinase, PEPCK.

Acknowledgements

We gratefully acknowledge the improvement of English grammar contributed by Mfrekemfon Samuel Obot, College of Food Science, Fujian Agriculture and Forestry University. This research was supported by the Construction Project of Top University at Fujian Agriculture and Forestry University of China (Grant No. 612014042).

460

461 **References**

462 1. J. Ashmore and G. Weber, *Vitamins & Hormones*, 1959, **17**, 91-132.

463 2. K. E. Wellen and C. B. Thompson, *Nature reviews Molecular Cell Biology*,
464 2012, **13**, 270-276.

465 3. J. Liu, W. Zhang, G. C. Chuang, H. S. Hill, L. Tian, Y. Fu and W. T. Garvey,
466 *American Journal of Physiology-Endocrinology and Metabolism*, 2012, **303**,
467 908-916.

468 4. R. Z. Tom, P. M. Garcia-Roves, R. J. Sjögren, L. Q. Jiang, M. H. Holmström,
469 A. S. Deshmukh and J. R. Zierath, *Diabetes*, 2014, **63**, 1560-1571.

470 5. B. Desvergne, L. Michalik and W. Wahli, *Physiological Reviews*, 2006, **86**,
471 465-514.

472 6. S. J. Bhathena and M. T. Velasquez, *The American Journal of Clinical*
473 *Nutrition*, 2002, **76**, 1191-1201.

474 7. A. Shisheva and Y. Shechter, *Biochemistry*, 1992, **31**, 8059-8063.

475 8. J. Song, O. Kwon, S. Chen, R. Daruwala, P. Eck, J. B. Park and M. Levine,
476 *Journal of Biological Chemistry*, 2002, **277**, 15252-15260.

477 9. Y.-C. Liang, S.-H. Tsai, D.-C. Tsai, S.-Y. Lin-Shiau and J.-K. Lin, *FEBS*
478 *letters*, 2001, **496**, 12-18.

479 10. B. B. Kahn and J. S. Flier, *J Clin Invest*, 2000, **106**, 473-481.

480 11. D. LeRoith and O. Gavrilova, *Int J Biochem Cell B*, 2006, **38**, 904-912.

481 12. W. Xie, W. Wang, H. Su, D. Xing, Y. Pan and L. Du, *Comparative*
482 *Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 2006, **143**,
483 429-435.

484 13. L. Zhu, J. Tan, B. Wang, R. He, Y. Liu and C. Zheng, *Chemistry &*
485 *Biodiversity*, 2009, **6**, 1716-1726.

486 14. P.-J. Tsai, T.-H. Tsai, C.-H. Yu and S.-C. Ho, *Food and Chemical Toxicology*,

2007, **45**, 440-447.

15. M. Jung and M. Park, *Molecules*, 2007, **12**, 2130-2139.

16. L. Chen and Y. H. Kang, *Korean Journal of Plant Resources*, 2014, **27**, 642-649.

17. W. J. Du. Chinese Medicine Guide, 2008, **6**, 1872 (in chinese).

18 .L. A. Yang, X. Z. Qiao and F. X. Zhou, *Zhejiang Practical Medicine December*, 2005,**10**, 436 (in chinese).

19. J. Louet, F. Chatelain, J. Decaux, E. Park, C. Kohl, T. Pineau, J. Girard and J. Pegorier, *Biochemistry Journal*, 2001, **354**, 189-197.

20. K. Aoki, T. Saito, S. Satoh, K. Mukasa, M. Kaneshiro, S. Kawasaki, A. Okamura and H. Sekihara, *Diabetes*, 1999, **48**, 1579-1585.

21. P. Noce and M. Utter, *Journal of Biological Chemistry*, 1975, **250**, 9099-9105.

22. R. Burcelin, M. Eddouks, J. Maury, J. Kande, R. Assan and J. Girard, *Diabetologia*, 1995, **38**, 283-290.

23. W. J. Arion, A. Lange and H. Walls, *Journal of Biological Chemistry*, 1980, **255**, 10387-10395.

24 W. Y. Zhang, J. J. Lee, Y. Kim, I. S. Kim, J. H. Han, S. G. Lee, C. S. Myung, *Journal of Agricultural and Food Chemistry*, 2012, **60**, 7652-7658

25 W. Y. Zhang, J. J. Lee, I. S. Kim, Y. Kim, J. S. Park, C. S. Myung, *Biological and Pharmaceutical Bulletin*, 2010, **33**, 1494-1499.

26 X. K. Fang, J. Gao, D. N. Zhu, *Life Science*, 2008, **82**, 615-622.

27 A. Kerimi, F. Jailani, G. Williamson, *Molecular Nutrition & Food Research*, 2015, **59**, 894-906.

28 L. Chen, H. Teng, T. Fang, J. Xiao, *Phytomedicines*, 2016, doi:10.1016/j.phymed.2016.03.016

29. J. Li, H. Jiang and R. Shi, *Natural Product Research*, 2009, **23**, 1378-1383.

30. Q. Huang, L. Chen, H. Teng, H. Song X. Wu, M. Xu, *Journal of Functional Foods*, 2015, http://dx.doi.org/10.1016/j.jff.2015.09.020

31 W. Guanliang, L. Jiahan, L.Di, W. Lin, Z. Dezeng, *Pharmacology and Clinics of Chinese Materia Medica*, 2015, *4*, 009.

32 M. S. Lee and P. T. Thuong, *Phytotherapy Research*, 2010, **24**, 49-53.

33 D. T. Tuan, N. B. Thu, N. X. Nhiem, T. M. Ngoc, N. Yim and K. Bae, *Bioorganic & Medicinal Chemistry Letters*, 2009, **19**, 5556-5559.

34 M. S. Balasubashini, R. Rukkumani and V. Menon, *Acta diabetologica*, 2003, **40**, 118-122.

35 Q. Lv, M. Si, Y. Yan, F. Luo, G. Hu, H. Wu, C. Sun, X. Li and K. Chen, *J Functional Foods*, 2014, **7**, 621-629.

36 P. Andújar-Plata, X. Pi-Sunyer and B. Laferrère, *Diabetes Research and Clinical Practice*, 2012, **95**, 1-9.

37 J. G. McCormack, N. Westergaard, M. Kristiansen, C. L. Brand and J. Lau, *Current Pharmaceutical Design*, 2001, **7**, 1451-1474.

38 P. Pu, D.-M. Gao, S. Mohamed, J. Chen, J. Zhang, X.-Y. Zhou, N.-J. Zhou, J. Xie and H. Jiang, *Arch Biochem Biophys*, 2012, **518**, 61-70.

39 D. Gupta, J. Raju and N. Z. Baquer, *Diabetes Research and Clinical Practice*, 1999, **46**, 1-7.

40 V. Vats, S. P. Yadav and J. K. Grover, *Journal of Ethnopharmacology*, 2003, **85**, 237-242.

41 J. D. McGarry, *Science*, 1992, **258**, 766-770.

42 W. J. Arion, W. K. Canfield, F. C. Ramos, M. L. Su, H. J. Burger, H. Hemmerle, G. Schubert, P. Below, A. W. Herling, *Archives of Biochemistry and Biophysics,* 1998, **351**, 279-285

43 K. Valentová, N. T. Truong, A. Moncion, I. De Waziers, J. Ulrichová, *Journal*

of Agricultural and Food Chemistry, 2007, **55**, 7726-7731.

44 U. J. Jung, M. K. Lee, Y. B. Park, S. M. Jeon, M. S. Choi, *Journal of Pharmacology and Experimental Therapy.* 2006, **318**, 476-483

45 M.Vessal, M. Hemmati, M. Vasei, *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 2003, **135**, 357-364.

46 S. M. Jang, M. J. Kim, M. S. Choi, E. Y. Kwon, M. K. Lee, *Metabolism*, 2010, **59**, 512-519.

47 C. C. Shih, J. L. Ciou, C. H. Lin, J. B. Wu, H. Y. Ho, *Molecules* 2013, **18**, 2726-2753.

48 J. B. Wu, Y. H. Kuo, C. H. Lin, H. Y. Ho, C. C. Shih, *Journal of Agricultural and Food Chemistry*, 2014, **62**, 10717-10726.

49 P. J. Klover and R. A. Mooney, *Intional Journal of Biochemical Cell B*, 2004, **36**, 753-758.

50 S. A. Park, M. S. Choi, S. Y. Cho, J. S. Seo, U. J. Jung, M. J. Kim and M. K. Lee. *Life Sciences*, 2006, **79**, 1207-1213.

Figure legends

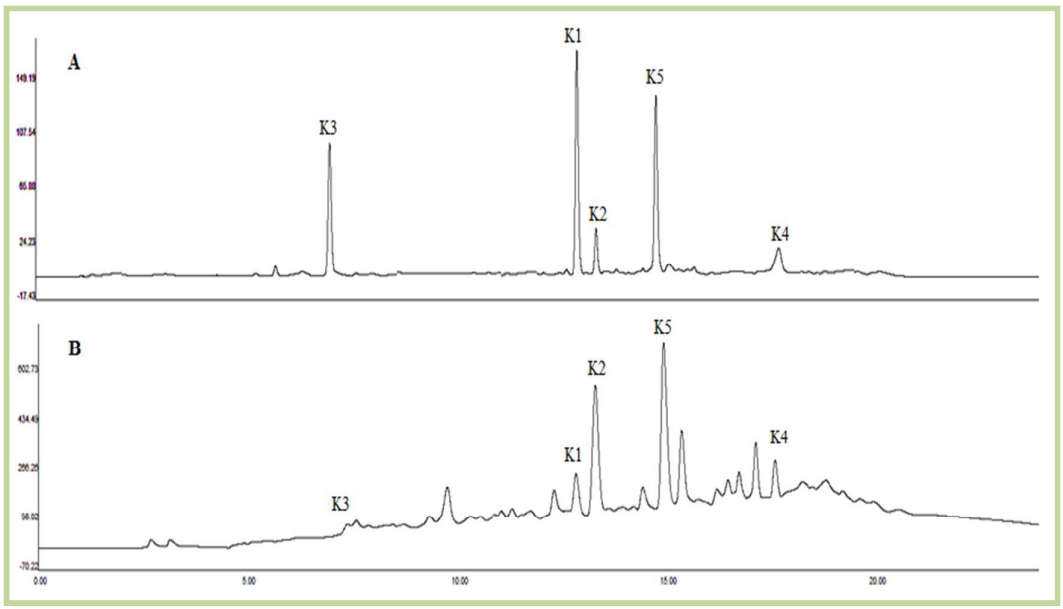
Fig. 1 HPLC profiles (280 nm) of isolated compounds and methanolic extracts of AP. K1 Agrimonolide; K2, Desmethylagrimonolide; K3, Tormentic acid; K4 Ursolic acid; K5, Quercetin.

Fig. 2. Effects of isolated compounds on hepatic glucose consumption and glycogenolysis in insulin-resistant HepG2 cells. A: Effects of isolated compounds on glucose consumption in HepG2 cells, values followed by different lowercase letters within the same row are significant difference at $p < 0.05$. B: Changes of glycogen content in cells in all groups (Mean ± SD, n=5), values followed by different lowercase letters within the same row are significant difference at $p < 0.05$. * Significant difference from Blank. C: Cytostatic effect of HepG2 cells following 48 h treatment with 20 μM compounds and 100 μg/mL. Experiments were treated at least three times, and bars with different letters are significant different (p <0.05). Changes in morphology of cells following non-insulin (D) and insulin (E) treated HepG2 cells.

Fig. 3. Hepatic enzyme activities in HepG2 cells. Effects of isolated compounds on hepatic glucokinase (A), glucose-6-phosphatase (B), and Phosphoenolpyruvate carboxykinase (C) activities (Mean ± SD, n=5), values followed by different lowercase letters within the same row are significant difference at $p < 0.05$. * Significant difference from Blank.

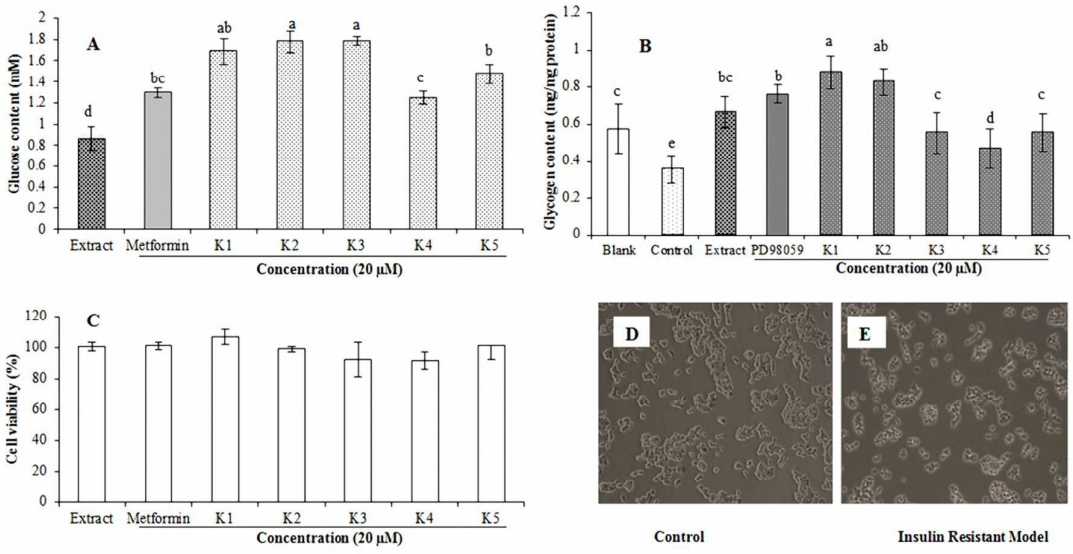
Fig. 4. Correlation coefficient between hepatic glucose consumption and glycogenolysis and enzyme activities in HepG2 cells. Correlation analysis was performed using Pearson's method (n=24).

583 **Figure 1**



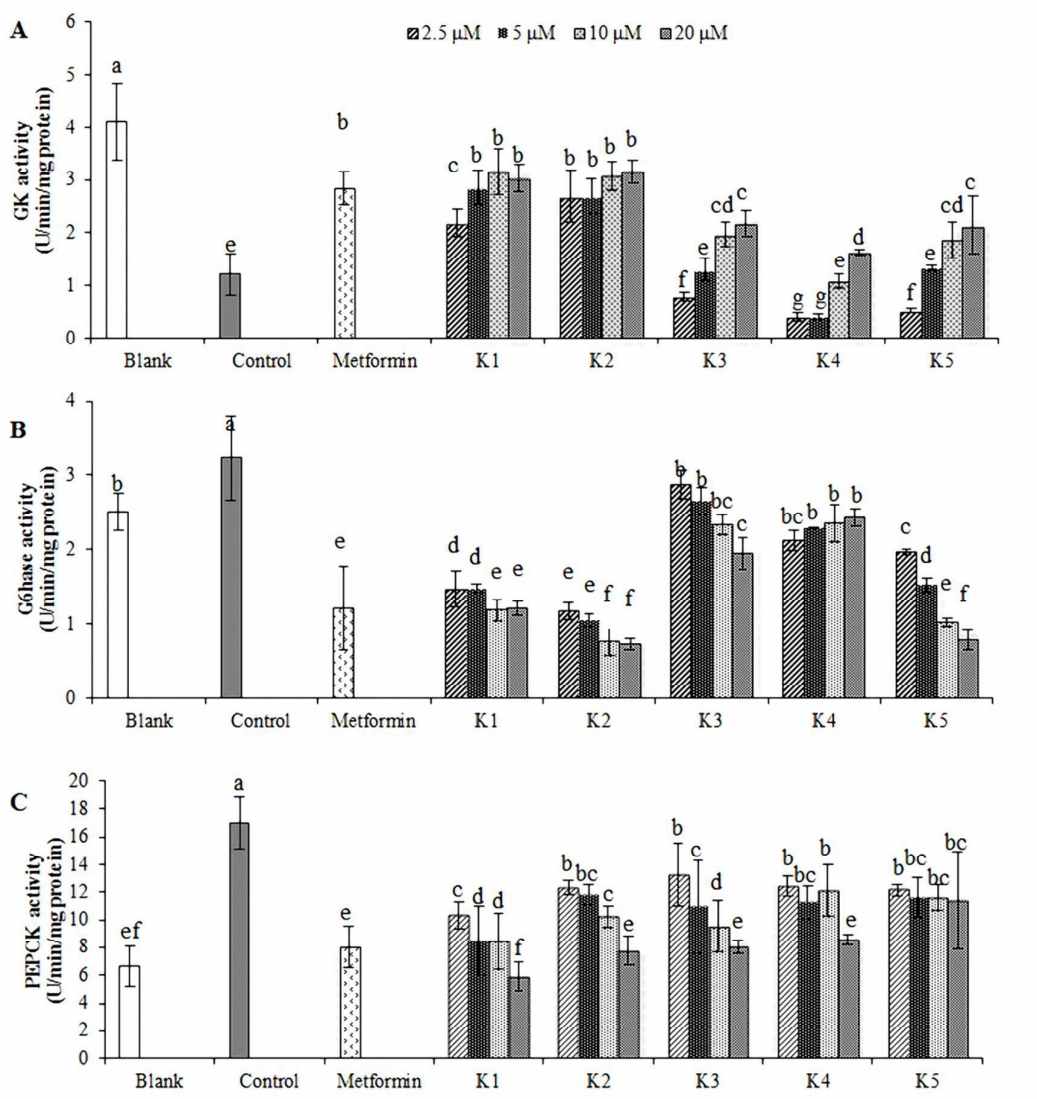
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600

Figure 2



620 **Figure 3**

621



622

623

624

625

626

627

628

629

Figure. 4

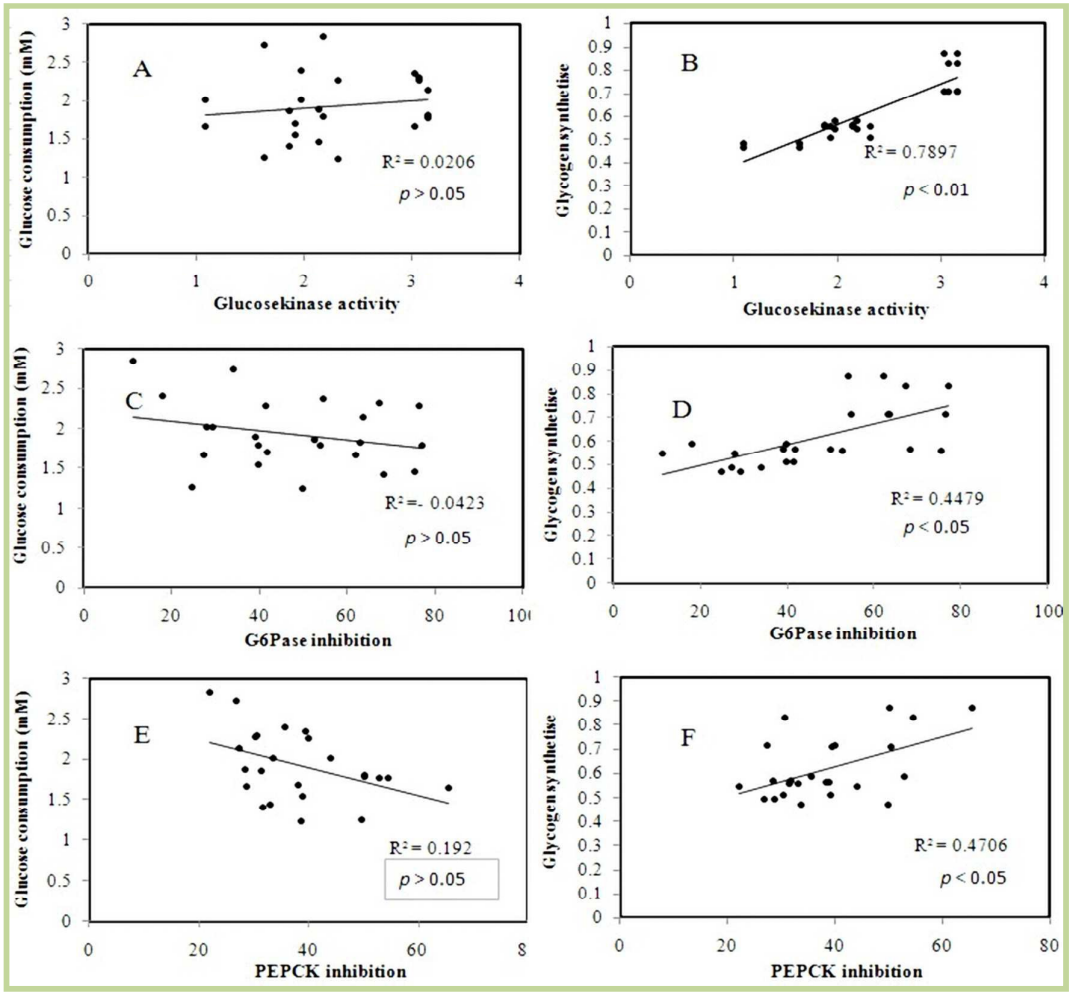


Table 1: HPLC profiles of isolated compounds in AP extract.

Compound	Content (mg/g d.w)	Recovery (%)	R.S.D. (%)
Agrimonolide (K1)	1.25 ± 0.16	95.6	1.2
Desmethylagrimonolide (K2)	6.26 ± 0.04	98.8	1.4
Tormentic acid (K3)	0.16 ± 0.11	102.6	1.5
Ursolic acid (K4)	1.35 ± 0.05	94.8	1.1
Quercetin (K5)	9.88± 0.32	96.3	1.6

The data presented is the average of three determinations. Recovery (%) = 100 × (amount found – original amount)/amount spiked.

669 Table 2: Effects of isolated compounds from AP on hepatic glucose-6-phosphatase
670 and PEPCK enzymes

	G6Pase		PEPCK enzyme		C.V
	Inhibition (%)	IC_{50}	Inhibition (%)	IC_{50}	%
Metformin (+)	66.3±1.6	12.4±1.6c	55.8±3.8	18.6±0.8d	92
Agrimonolide (K1)	62.1±2.9	11.6±0.8c	65.5±5.1	8.3±0.6e	85
Desmethylagrimonolide (K2)	77.0±3.5	2.2±0.3d	54.4±2.4	16.9±1.5d	88
Tormentic acid (K3)	39.8±2.5	25.6±2.2b	52.8±3.2	16.6±2.6d	95
Ursolic acid (K4)	24.8±1.2	33.2±1.4a	49.7±2.6	22.2±3.4c	99
Quercetin (K5)	75.4±3.2	2.9±0.1d	33.0±2.8	101.3±2.4a	98

671 The results are expressed as the percentage of inhibition in comparison with control at 20 μM
672 concentration. All values are the means of three different experiments ± standard deviation, and
673 IC_{50} expressed in μM. Different letters in the same column indicate statistical significance at $p <$
674 0.05. C.V: cell viability determined by MTT at 20 μM concentration

675
676
677
678
679
680
681
682
683
684
685
686
687