In vitro Enhancement of Pancreatic β Cells MIN6 Proliferation by Insulinotropic *Gymnema sylvestre* Aqueous Extracts: Evidence-based Regenerative Therapeutic Capacity of a Medicinal Herb

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VK, FUA and YB designed the study and wrote protocol. Author VK performed the statistical analysis. All authors were responsible for the coordination of the overall study, including: The study design, collaboration amongst investigators, data analysis, and manuscript preparation. Authors SM, RN and HSM managed samples for subsequent biological analyses and all technical work involved. Authors VK, FUA and YB wrote first draft. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Aim: To show that *Gymnema sylvestre* (Roxb.) Asclepiadaceae not only has antidiabetic propensities, but it most likely works by regeneration of pancreatic β cells which is imperative in anti-obesity-diabetes therapeutic applications of medicinal plants.
Study Design: The present study design investigated the effects of *G. sylvestre* leaves crude

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aqueous extracts (AEs), traditionally utilized in diabetes treatment, on the pancreatic β-cell MIN6 proliferation and insulin secretion and extrapancreatic dietary carbohydrate and lipid digestion

Place and Duration of Study: Faculty of Pharmacy, The University of Jordan, 2008-2012.

Results: Comparable to GLP-1 (500 nM) pancreatic proliferative capacity; *G. sylvestre* AE concentrations (0.01 and 0.1 mg/mL) induced MIN6 monolayers expansion by respective 130.3% and 127.4% (P<0.001 *vs.* spontaneous control). Like L-alanine (10 mM) insulinotropic efficacy and without exerting cytotoxicity, glucose-stimulated insulin secretion was potentiated by *G. sylvestre* AEs (5, 10 and 25 mg/mL) (711.0%, 843.0% and 906.5%, respectively, P<0.001 vs. basal control). The potent plants' insulin secretory bioactivities were abolished in the depleted Ca^{2+} conditions (P<0.001). Similar to orlistat antilipolytic efficacy, pancreatic lipase IC_{50} value for *G. sylvestre* AEs was 106.3±7.2 µg/mL. Unlike acarbose (100 µg/mL) dual inhibition of α-amylase/α-glucosidase, *G. sylvestre* AE was inactive at used doses. Dissimilar to guar gum (50 mg/mL) diffusional hindrance in a simple dialysis model, *G. sylvestre* AEs (10, 25 and 50 mg/mL) proved inactive. This *in vitro* ineffectiveness was mirrored in respective *in vivo* oral carbohydrate tolerance tests in overnight fasting normoglycemic rats.

Conclusion: This evaluation has revealed that *G. sylvestre* leaves AEs augmented β-cell expansion and potentiated glucose-evoked Ca^{2+}-regulated insulin secretion; combined with impressive antilipolytic activity. These actions depend on the bioactive water soluble phyto-principles intact absorption *in vivo*. Future directives may assess the potential of *G. sylvestre* as a new alternative for anti-obesity-diabetes pharmacotherapy and prevention.

1. INTRODUCTION

Diabetes is a chronic and progressive metabolic disease characterized by impaired β-cell function, and reduced insulin sensitivity and secretion [1,2]. It is recognized as a worldwide major health problem as well as in Jordan [3]. Oral hypoglycaemic sulphonylureas have found widespread application in drug therapy of type 2 diabetes, mainly due to a direct stimulation of insulin secretion [4]. Nevertheless, chronic sulphonylureas therapy tends to progressively fail following direct desensitization and decline in beta cell K_{ATP} channel activity [5]. This further dwells upon the need for discovery of novel insulinotropic entities from medicinal plants reputed for their antidiabetic efficacies within traditional ethnomedicine practices. Literature surveys demonstrate the benefit of several ethnobotanicals as antidiabetic agents evaluated in the form of their crude extracts and/or isolated pure compounds with varying degrees of hypoglycemic or antihyperglycemic bioactivities [6,7]. Evidently, multiple medicinal and edible herbs, either indigenous or imported, were promoted locally for diabetes traditional medicine. These were closely linked to appreciable prevalence of herbal use among diabetes patients on conventional medicaments in Jordan [8]. Principally, *G. sylvestre* along with its phytochemistry have been long recognized for their antidiabetic pharmacological properties in multiple traditional medicine systems [9-14]. In both type 1 and type 2 diabetes; beta cell mass and function are decreased to varying degrees. Therefore, islet cell replacement or regeneration therapy may thus offer therapeutic benefit to people with diabetes [15-17]. It is well accepted that diabetes regenerative therapeutics can be obtained from plants with proliferative capacity to induce expansion and rejuvenation of pre-existing pancreatic β-cells [18-27].

Accordingly, to observe the possible changes in the proliferation, the MIN6 cells were incubated over 48 h in the presence and absence of *G. sylvestre* AEs. Furthermore, to test the possible extrapancreatic role of *G. sylvestre* AEs in viscosity-based delay and/or minimizing in postprandial hyperglycaemia and postprandial hyperinsulinemia; a simple glucose dialysis model was considered [28]. Finally as for its physiological insulin secretagogue activities, at the submaximal stimulatory glucose concentration (5.6 mM), the anticipated potentiating of a Ca^{2+}-regulated glucose-stimulated insulin secretion by *G. sylvestre* AEs was critically evaluated [29-31]. Possible *in vitro* effects of *G. sylvestre* on pancreatic α-amylase/α-glucosidase and intestinal triacylglycerol lipase were investigated [32]. Besides, complementary supportive acute *in vivo* testing was adopted. In effect, these investigations aimed to scientifically justify the

antidiabetic use of the selected medicinal herb *G. sylvestre*; a practice maintained in folk medicine for generations.

2. MATERIALS AND METHODS

2.1 Equipments, Chemicals and Biochemicals

Dulbecco Modified Eagle Medium (DMEM) containing 25 mM glucose was obtained from Invitrogen (USA). ELISA jumbo kit for rat high insulin was purchased from ALPCO (USA) and MTT assay kit from Promega (USA). The assays were performed according to manufacturers' instructions. Unless stated otherwise, all reagents and chemicals of analytical grades were from Sigma (Dorset, UK). Dialysis tubing Spectra/Por® 7 Biotech Regenerated Cellulose (RC) membranes, MWCO 2000 was purchased from Spectrum Europe B.V, Breda, Netherlands. Falcon tube 50 mL was obtained from Iwaki Scitech Division, Japan. Shaking incubator was from LabTech®, Daihan LabTech Co., LTD. (Korea). D (+) glucose was procured from Riedel-deHaen, Seize (Germany). Glucose GOD-PAP (glucose oxidase - para-aminophenazone) kit was obtained from BioLabo Reagents, France. In UV determinations UV-VIS spectrophotometer (Ultraviolet – Visible) from SpectroScan 80D (UK) was used. Sonicator (Bandelin Sonorex, Bandelin electronics, Germany), rotary evaporator (Laborota 4000-efficient, Heidolph, Germany), and Accu-Chek® Active Glucose meter (Roche, Germany) were also used.

2.2 Plant Material and Preparation

Leaves crude aqueous extracts (AEs) of *G. sylvestre* (Roxb.) Asclepiadaceae were procured as tabulated capsules from Himalaya Drug Company, Banglore (India). Voucher specimens were deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy-The University of Jordan. For *in vitro* and *in vivo* testing, each 10 g of the capsules content were refluxed with 100 mL tap water for 15 min and kept overnight. After filtering twice, the volume of the filtered solution was increased to 100 mL with tap water to obtain 10% (equivalent to 100 mg/1mL) crude aqueous solutions [33].

2.3 Experimental Animals

The study was undertaken in the Experimental Animal Laboratory of the Faculty of Medicine, The University of Jordan. All animals were housed, fed and treated in accordance with The University of Jordan ethical guidelines for animal protection. Experimental approval was obtained from the Scientific Research Council at the Deanship of Academic Research and the Faculty of Pharmacy (registration number 218/2007-2008). Before testing, the rats were kept for one week to be acclimatized under the standard laboratory conditions. Throughout the experimentation period, healthy female Sprague/Dawley rats weighing 200-250 g were housed in single cages and were given proper pellet diet and water *ad libitum.* Also, rats were 14 h-fasted before *in vivo* blood glucose determination. Blood glucose levels from cut tail tips were determined using Accu-Chek® Active Glucose meter.

2.4 Oral Starch Tolerance Test (OSTT)

In this line of investigations; rats were divided into 5 groups (n=5-8 rats per group). At -30 min, fasting glycemia levels were evaluated and instantly acarbose (3 mg/Kg b.wt) or *G. sylvestre* AEs in doses 125, 250 and 500 mg/Kg b.wt were administered orally via 2 mL intra-gastric intubations under mild anesthesia. Control untreated animals were given tap water (2 mL/rat). At 0 min, all rats of five groups (plant extracts and acarbose) were given corn starch 3 g/Kg b.wt following the fasting blood glucose determination. Later evaluations of glycemia took place at 45, 90, and 135 min from 0 min [33].

2.5 Enzymatic Starch Hydrolysis *In vitro*

Acarbose (1000 µg/ mL) was as the reference drug [34] *vs.* control (tap water only) samples. A concentration range (1-50 mg/mL, n=3) of *G. sylvestre* AEs was assayed for comparable inhibitory efficacy of α-amylase and α-glucosidase.

2.6 Oral Glucose Tolerance Test (OGTT)

Rats were divided into 6 groups (n = 5-8 rats per group). The OGTT was carried out as the above protocol for OSTT except for glucose (3 g/Kg b.wt) utilization instead of starch and metformin (300 mg/Kg b.wt) and glipizide (600 µg/Kg b.wt) treatments as positive controls instead of acarbose [33].

2.7 Glucose Movement *In vitro*

Glucose solution (0.22M in 0.15 M NaCl) was added to a dialysis tubing (10 cm x 11 mm). The

tubing was sealed at both ends, and dialysed against 45 mL of 0.15 M NaCl in 50 mL tube overnight. The optimum temperature for maximum glucose diffusion was established at 37ºC. Gentle shaking is used as well in a shaking incubator to simulate the effect of intestinal contractions on intestinal glucose absorption [35]. The end point of glucose diffusion equilibrium (glucose diffusion into the external solution) was found by measuring the external solution glucose content in dialysate at 0, 3, 6, 18 and 24 h time intervals. Glucose concentrations were measured in duplicates per time point-sample. The assay was internally controlled using 5 mM glucose solutions prepared alongside the experimental glucose samples. Guar gum 50 mg/mL or plant AEs 10, 25 and 50 mg/mL in 0.22 M glucose in triplicates were dialysed against 0.15 M NaCl overnight at 37ºC with gentle shaking and a parallel plant-free (negative) control was included [28].

2.8 Pancreatic β-cell Viability and Proliferation Assays *in vitro*

Cell viability was assessed by a MTT kit. MIN6 cells were subcultured on 96-well plates (10,000 cells/well) in growth medium containing different concentrations of *G. sylvestre* AEs (0.01-0.1 mg/mL, n=4). After 48 h of incubation, the effects of the plant extracts on cell viability were evaluated according to kit's manufacturer instructions. Proliferation of MIN6 cells was evaluated with a colorimetric ELISA- based BrdU incorporation kit (Roche, Germany). In brief, MIN6 cells (young passage) were seeded onto 96well plate (10,000 cell/well density) and left to adhere overnight. They were serum starved for 24 h before 48 h treatments with *G. sylvestre* AEs concentrations (0.01-0.1 mg/mL) or GLP-1 500 nM [22] (n=3). BrdU dye 10 μM/well was added to culture medium of different treatment wells and incubated for the second 24 h of the 48 h-chronic incubation time. The rest of the assay was performed in accordance with manufacturer instructions.

2.9 MIN6 Cell Culture and Insulin Secretion Static Incubation Experiments

Pancreatic β-cells MIN6 (passage 39-45) were maintained in DMEM containing 15% Foetal Bovine Serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL L-glutamate, and 5 μl/L β-mercaptoethanol in a 37ºC humidified atmosphere with 95% air and 5% CO_2. The culture medium was changed every 48-72 h [36]. In testing the Glucose Stimulated Insulin Secretion (GSIS) from MIN6, cells were cultured in 96-well plates at density 50,000 cell/well until 80% confluent. On the day of experiment, growth medium was removed and the cells were washed with Phosphate-Buffered Saline (PBS). Cells were preincubated for 1 h at 37ºC in 5% CO_2 in HEPES-balanced Krebs-Ringer phosphate buffer (KRH) composed of (in mM) 129 NaCl, 5 NaHCO$_3$, 4.8 KCl, 1.2 KH$_2$PO$_4$, 1.2 MgSO$_4$, 10 HEPES, 2.5 CaCl$_2$ and 0.1% BSA (pH 7.4, NaOH) supplemented with 1.1 mM glucose. Incubation medium was removed, and the cells were washed once in glucose free KRH. Subsequent test incubations in 5.6 mM glucose - KRH alone (untreated negative control) or supplemented with appropriate treatments (L-alanine 10 mM or *G. sylvestre* AEs 0.01-25 mg/mL, n=4) were performed for another 1h. L-alanine was the reference robust and powerful stimulant of insulin secretion from pancreatic beta cells [28-30]. Cell viability was assessed post 1 h-acute incubations using MTT assay. When investigating the effects of extracellular Ca^{2+}-free incubations on pancreatic GSIS, 2.5 mM CaCl$_2$ was removed from KRH buffer preparations, so that cells were incubated in a Ca^{2+}-free KRH in the same panel of GSIS studies described previously. For all experiments, incubation medium was collected and stored at -20ºC for a subsequent ELISA determination of the amount of secreted mouse insulin.

2.10 Preparation of *G. sylvestre* AEs and Orlistat for *In vitro* Pancreatic Lipase (PL) Activity Assay and Spectrophotometric Quantification of PL Inhibition by Test Extracts

Each of the tested AEs was initially dissolved in Tris-HCl buffer (2.5 mM (Promega, USA), pH 7.4 with 2.5 mM NaCl) to give five initial stock solutions of 6.25-100 mg/mL. Subsequently, 20 μL aliquot of each stock solution was used in the reaction mixture to give a final concentration range 125-2000 μg/mL (n=3). Extracts were prepared according to the traditional indications of use, thus dimethylsulfoxide (DMSO) or any other organic solvent; even to the minimum concentration, was avoided [37]. Finally, orlistat, the reference drug (in DMSO; 1 mg/mL), was prepared in six different stock solutions with a concentration range of 0.625 - 20 μg/ mL [38]. Thereafter, 20 μL aliquot of each stock solution was used in the reaction mixture to give a final

concentration range of 0.0125 – 0.4 µg/ mL. According to Bustanji et al. [39], *In vitro* enzymatic PL activity was assayed. Subsequent determinations were undertaken for the tested extracts in comparison to control evaluations, to calculate the concentration required for PL 50% inhibition (IC_{50}).

2.11 Statistical Analysis

Results are presented as mean ± S.E.M (Standard Error of the mean) (n=3-4 determinations). Statistical differences between different treatment groups and respective controls were evaluated using ANOVA (one way analysis of variance) followed by Dunnett post test (GraphPad Prism Software 3.02, USA). Results were significantly different if $P < 0.05$ and highly significantly different if $P < 0.01$ and $P < 0.001$.

3. RESULTS AND DISCUSSION

3.1 Lack of Extrapancreatic Modulation of Enzymatic Starch Digestion, Glucose Movement, or Oral Carbohydrate Tolerance Tests by *G. sylvestre* AEs

New approaches to the prevention/modulation of postprandial hyperglycemia may emerge from the therapeutic use of α-amylase and α-glucosidase inhibitors [33,34]. Hence, the present *in vivo* and *in vitro* experiments evaluate the acute (up to 165 min carbohydrate tolerance testing in normal animal models) postprandial antihyperglycemic activity of the *G. sylvestre* with claimed antidiabetic activity exploring the enzymatic starch digestion and glucose diffusion modalities as possible antidiabetes action modes. Fig. 1a demonstrates that at -30 min time point acarbose 3 mg/Kg b.wt administration reduced highly significantly the starch induced hyperglycemia at 45, 90, and 135 min, thus evoking highly substantial reduction (p<0.001) of the overall glycemic excursion compared to controls. *G. sylvestre* treatment groups, nevertheless, did not minimize effectively the overall glycemic excursion versus the control and drug treated animals (the same figure). Fig. 1b confirms the *in vitro* lack of the effect of *G. sylvestre* AEs on enzymatic starch digestion. Fig. 2a displays that 30 min pre-glucose-load treatments with metformin (300 mg/Kg b.wt) and glipizide (0.6 mg/Kg b.wt) minimized highly markedly (p<0.001) the overall glycemic excursions in OGTTs compared to control normal

rats. It further highlights the substantial (p<0.001) antihyperglycemic efficacies of both oral antidiabetic therapeutics at 45, 90, and 135 min following sugar load. Oral administration of *G. sylvestre* AEs had no marked improvement of glucose tolerance AUCs, unlike the therapeutic efficacies of metformin or glipizide *vs.* control glucose-only fed rats. Fig. 2b illustrates that using a simple diffusion model,; mean AUC, for the viscous water-soluble gel forming guar gum (50 mg/mL) was decreased highly distinctly by 30.9±2.5% (p<0.001, n=3) compared to overnight negative control. Guar is a natural oral antidiabetic and classical positive control [22]. *G. sylvestre* AEs lacked any statistically substantial glucose diffusional hindrances into external solution across dialysis membrane (with respective 5.9±0.5% and 1.9±0.34% AUC reductions, p>0.05 *vs.* controls' AUCs, the same figure).

3.2 Pancreatic β-cell Monolayers' Expansion Modulated by *G. sylvestre* AEs

Approximately 80% of the investigated traditional plants used for the treatment of diabetes demonstrated some antidiabetic activity; thus plants can represent a vast pool of potentially useful dietary supplements for improving blood glucose control and preventing diabetes long-term complications [21-23,29-30,33,34,40]. Compared to control untreated cells, the MTT method revealed that 48h post seeding *G. sylvestre* AEs at doses 0.01-0.1 mg/ml preserved cell integrity (Table 1). A colorimetric immunoassay of BrdU-incorporation into MIN6 β-cell genome was recruited to ascertain proliferative principles of chronic plants treatments. Fig. 3 demonstrates that GLP-1 (500 nM) highly significantly promoted a maximal extent of BrdU incorporation by 1.2 folds (P<0.001) in comparison to basal BrdU incorporation (spontaneous control). Comparable to GLP-1 proliferative properties, *G. sylvestre* AE 0.01 and 0.1 mg/ml augmented BrdU incorporation by 1.3 folds equally (P<0.01 *vs.* basal controls, the same figure).

Obviously, *G. sylvestre* not only was ascribed antidiabetic property, but also it works most likely by regeneration of the pancreatic β-cells which is very imperative in light of damaged pancreatic islets in diabetes patient. In both type 1 and type 2 diabetes; insufficient numbers of insulin-producing beta cells are a major cause of

defective control of blood glucose and its complications [41,42]. Accordingly, therapies that increase functional beta-cell mass may offer a cure for diabetes. Efforts to achieve this goal explore several directions. Based on the realization that beta cells are capable of significant proliferation throughout adult life, the enhanced proliferation of beta cells *in vivo* or *in vitro* is pursued as a strategy for regenerative medicine for diabetes [43]. This study clearly links our substantially outstanding *in vitro* outcomes with the extensively reported in *vivo* regenerative outcomes for *G. Ssylvestre* [9-12, 44-46] in diabetes animal models and patients.

Thus it provides potentially promising avenue in the design and implications of regenerative therapies aimed at increasing β-cell replication and mass in patients with diabetes [47]. Basically, *G. sylvestre* is an interesting source for antioxidant phenolic phytochemicals that can be useful for various therapeutic applications [48].

Therefore it effactually could protect islets of Langerhans against diabetogenic alloxan- and STZ- induced oxidative insult [49,50] in animal models of the chronic disease course. This may have taken place via enhancing natural cellular antioxidant system [51].

Table 1. Lack of modulatory effects of *G. sylvestre* AEs (0.01, 0.05 and 0.1 mg/mL) on the viability of pancreatic β-cells MIN6 in 48 h post seeding as measured by MTT kit

Treatment	MIN6 viability (as % control)
Control incubations (plant free)	99.5±10.4
G. sylvestre AE (mg/mL)	
0.01	99.0±8.5
0.05	115.4±7.2
0.1	96.0±25.9

Each result (as % Control) indicates the mean ± S.E.M of four independent experiments
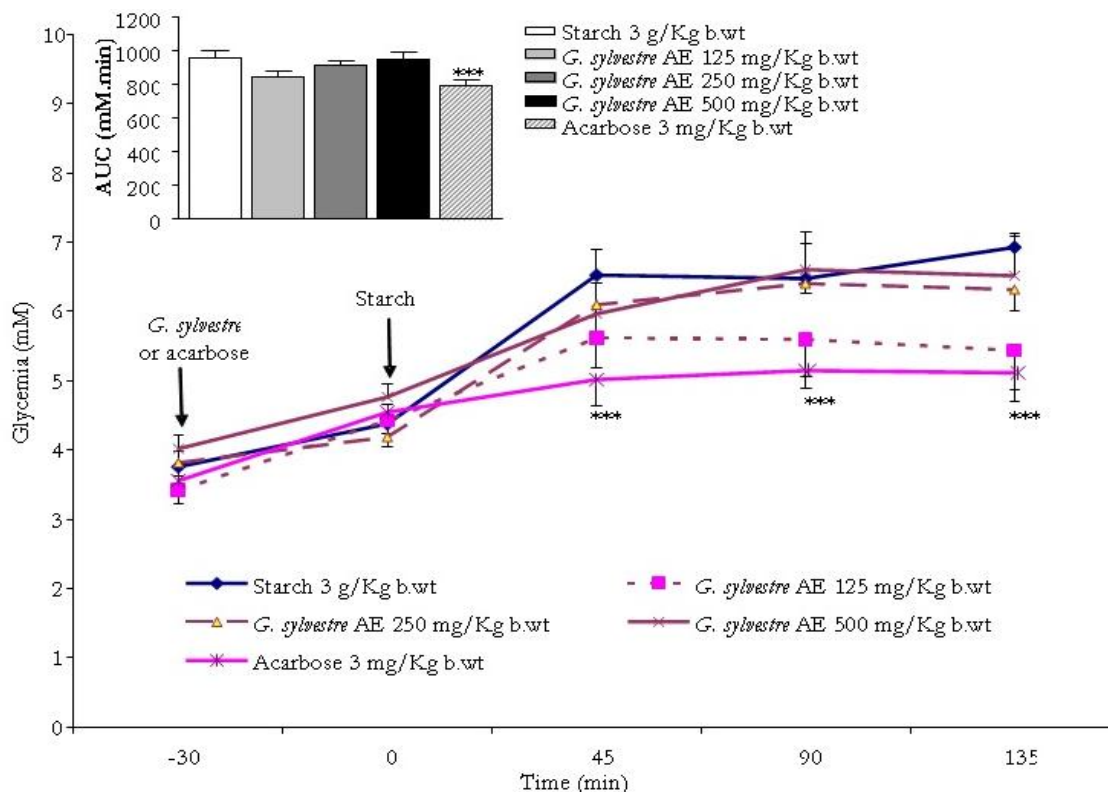

Fig. 1a. Lack of modulatory postprandial antihyperglycemic effects of *G. sylvestre* (AEs) concentrations in mg/Kg b.wt on oral starch tolerance over 165 min and AUC in normoglycemic overnight fasting rats
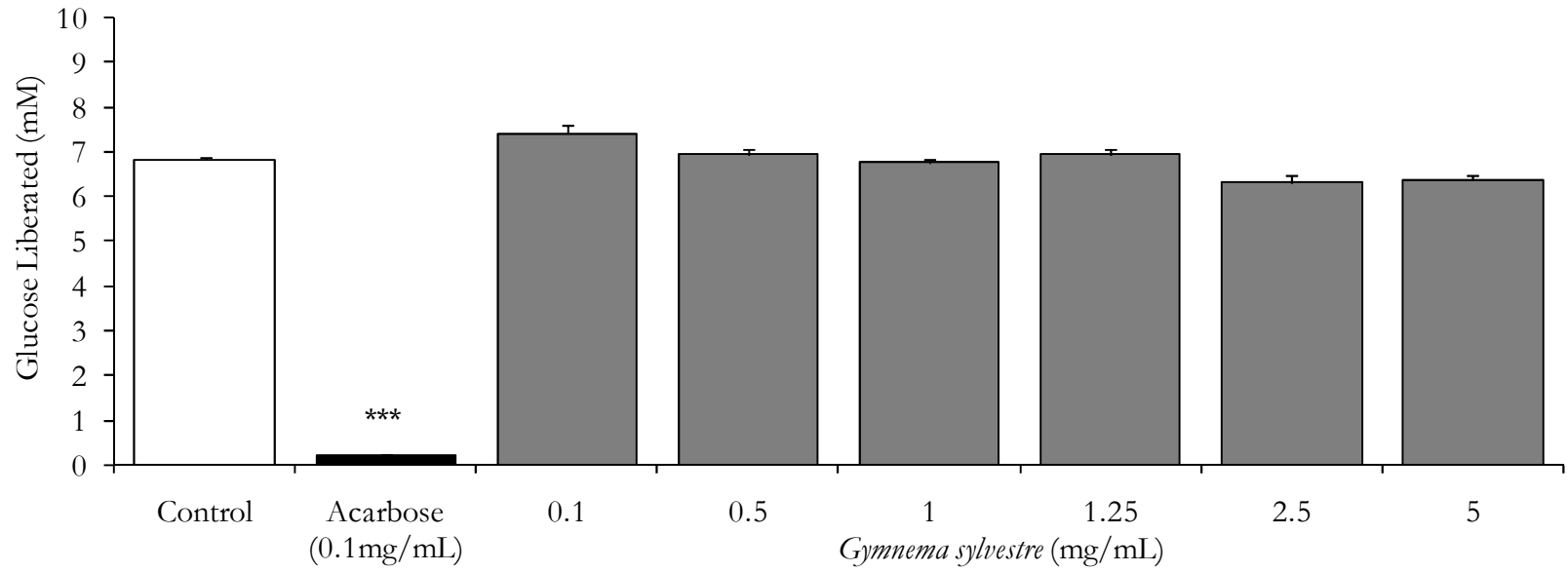**P<0.05 and ***P<0.001 compared to control untreated animals, as determined by unpaired sample t-test*

Fig. 1b. Lack of *in vitro* inhibitory effects of *G. sylvestre* (AEs) (mg/mL) on starch digestion enzymes α-amylase and α-glucosidase
*Results are mean ± SEM (n = 3 independent replicates). ***P<0.001 compared to control (drug-free or plant-free) incubations, as determined by unpaired sample t-test*
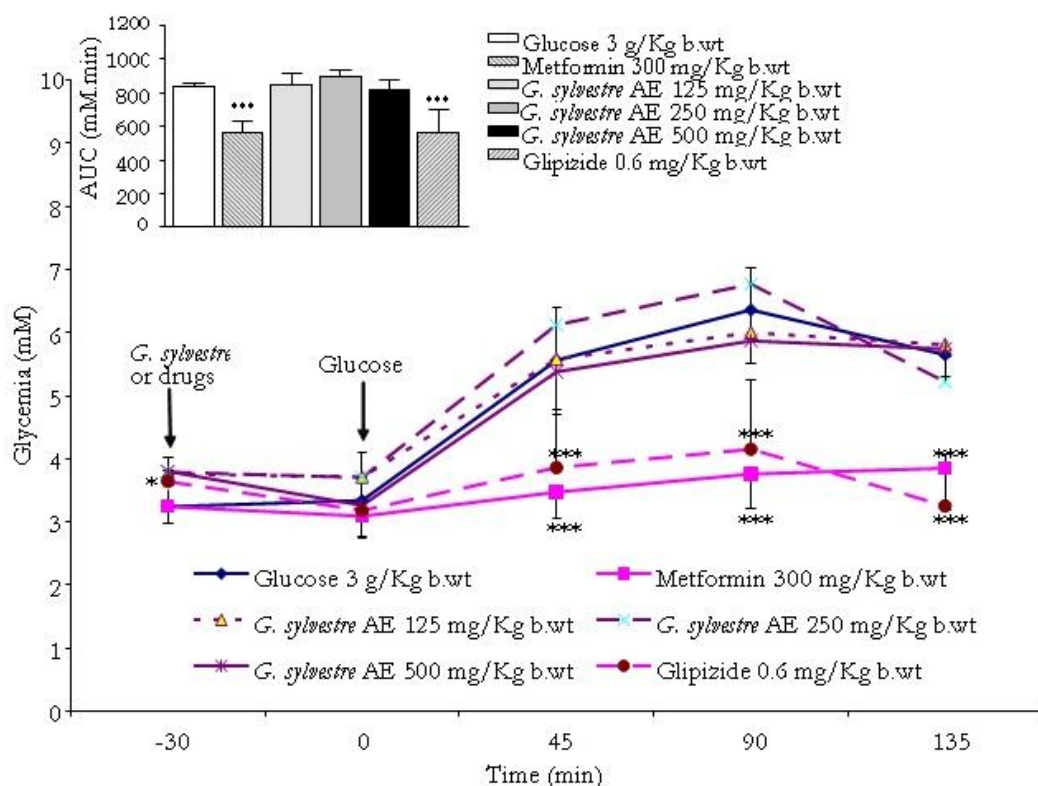
Fig. 2a. Lack of modulatory postprandial antihyperglycemic effects of *G. sylvestre* (AEs) concentrations in mg/Kg b.wt on oral glucose tolerance over 165 min and AUC in normoglycemic overnight fasting rats

*P<0.05 and ***P<0.001 compared to control untreated animals, as determined by unpaired sample t-test

3.3 Glucose-Dependent Modulation of Insulin Secretion in Pancreatic β-cell by *G. sylvestre* AE is Mediated by Ca^{2+} Dependency

A key feature of type 2 DM is that glucose fails to stimulate an adequate release of insulin from pancreatic β-cells. The stimulatory effect is mediated via the pancreatic beta cell K_{ATP} channel [52]. Binding of sulphonylureas leads to K_{ATP} channel closure, evoking membrane depolarization and subsequent opening of VOCCs (voltage operated Ca^{2+}-channels), followed by elevation of $[Ca^{2+}]i$ (intracellular Ca^{2+} concentrations) due to increased Ca^{2+} influx, ultimately leading to exocytosis of insulin from secretory granules [52].

Basically, to evaluate the insulinotropic activity of plant extracts, submaximal stimulatory glucose concentration (5.6 mM) was used in the acute culture incubations. L-alanine 10 mM was used as a positive control and it enhanced substantially (p<0.05) GSIS in MIN6 by 307.3±34.6% (n=4) following 1 h-incubations, compared to untreated (5.6 mM glucose only) controls (Fig. 4). Exceedingly superior to L-alanine, G. *sylvestre* AEs concentrations 5-25 mg/mL potentiated GSIS in pancreatic MIN6 substantially by respective 711.1±39.4%, 848.0±13.8% and 906.5±44.3% (p<0.001 *vs.* basal control wells, Fig. 4). Significant increase of the MIN6 insulin release at lower concentrations was not detected. Cell viability, as checked by MTT assay, was unchanged over 1 h-incubations in effective concentrations' wells. Moreover the marked insulintropic trend of L-alanine was highly significantly abrogated in Ca^{2+} depleted KRH (195.7±31.6%, p<0.001), as compared to corresponding Ca^{2+} free glucose-only (negative control) wells. Comparably, *G. sylvestre* (5-25 mg/mL) insulinogenic efficacies were markedly ablated (p<0.001) following Ca^{2+} absence from acute plant incubations to 170.7±7.0%, 176.5±6.7% and 183.5±7.2%, respectively, compared to respective Ca^{2+}

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buffered conditions (Fig. 4). Explicitly, *G. sylvestre* extracts along with phyto-constituents are appraised for their pronounced insulintropic and insulinomimetic propensities *in vitro* and *in vivo* [53-58]. As indicated lately, combination therapy markedly improves glycaemic control, thus allowing for regimens design to specifically address the diabetes underlying abnormalities [1,2]. The substantial insulinogenic properties further complement the pancreatic proliferative efficacies illustrated of *G. sylvestre,* thereby collectively, advocating the plant as a potential source with functional properties for active leads into anti-diabetes pharmacology and/or its adjunctive therapeutic strategies.

3.4 *In vitro* Inhibition of PL by *G. sylvestre* AEs

Exceptionally, antiobesity qualities were attributed to *G. sylvestre* and its phytoprinciples in animal models [59-61]. Results of *G. sylvestre* AEs pancreatic triacylglycerol antilipase activity *in vitro* are shown in Fig. 5. Orlistat's PL-IC$_{50}$ value of 114.0±4.0 ng/mL, equivalent to 0.2±0.0 µM, is comparable to reported PL-IC$_{50}$ values elsewhere [38].
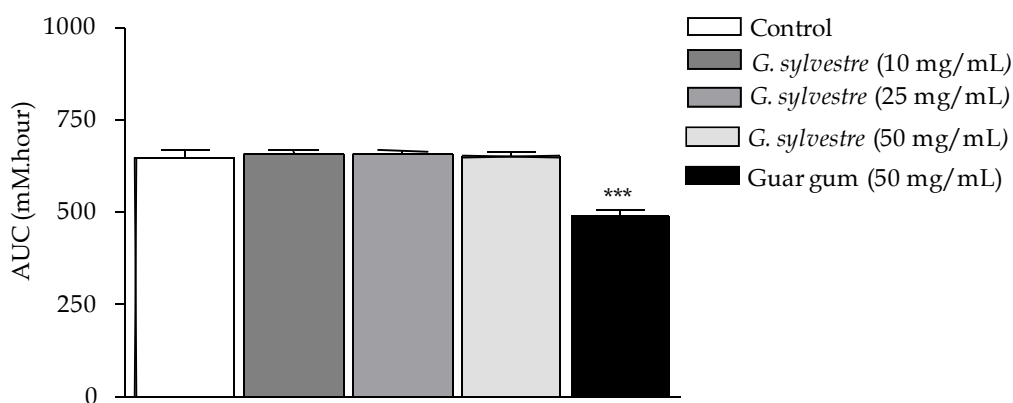

Fig. 2b. Lack of *in vitro* effects of *G. sylvestre* (AEs) (mg/mL) on the incremental AUC of 24h glucose movement
*Results are mean ± SEM (n = 3 independent replicates). ***P<0.001 compared to control (basal) incubations, as determined by unpaired sample t-test*
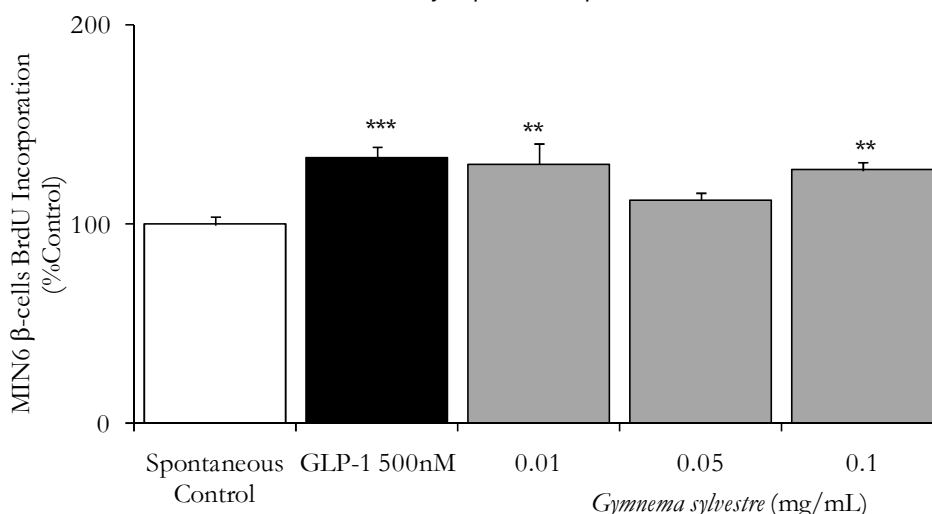

Fig. 3. Modulatory effects of *G. sylvestre* AE (0.01 – 0.1 mg/mL) on proliferation of pancreatic β-cells MIN6 in 48 h culture incubations as measured by a colorimetric ELISA-based BrdU incorporation kit
*Each bar indicates the mean ± S.E.M. of four independent determinations; *P<0.05 and ***P<0.001 compared to untreated (spontaneous) control conditions, as determined by unpaired sample t-test*
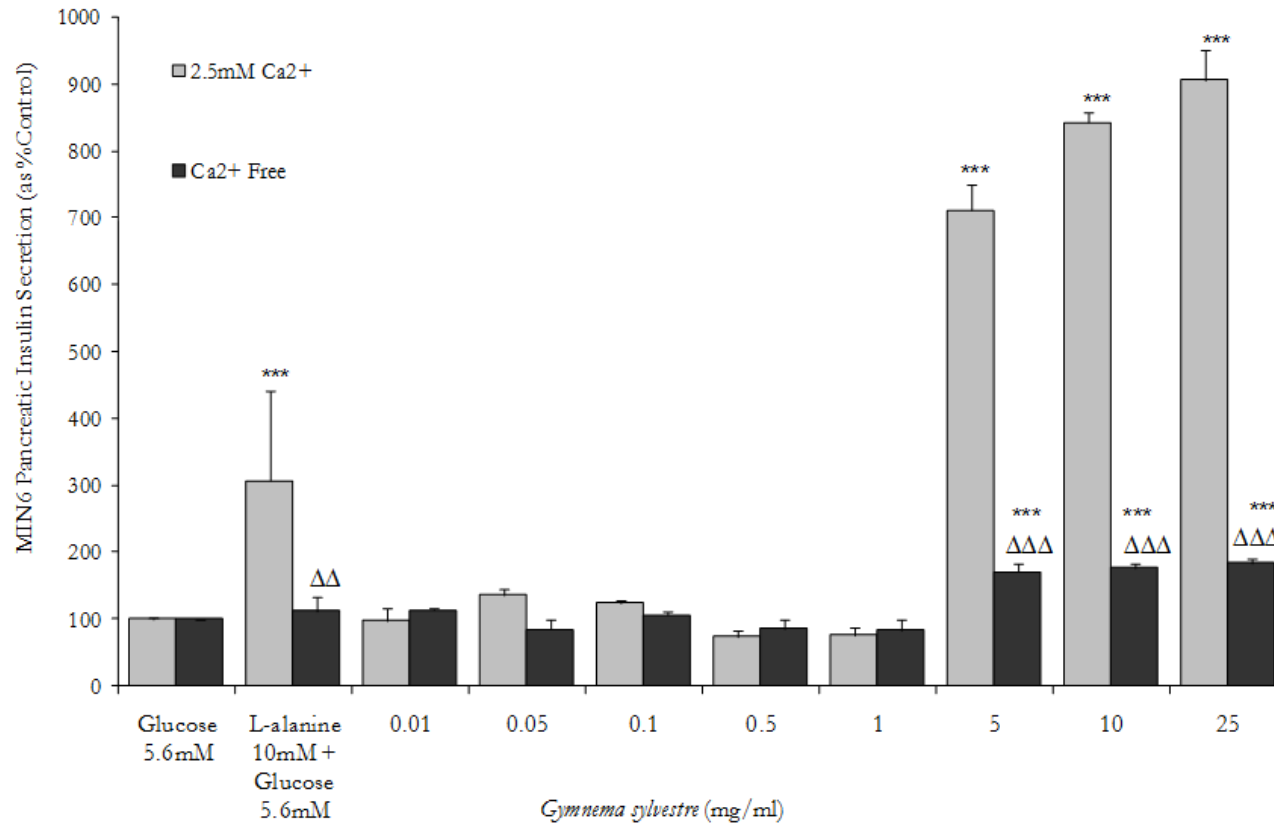
Fig. 4. Modulatory effects of *G. sylvestre* AEs (0.01 – 25 mg/mL) on secretory function of pancreatic MIN6 β-cells.
Such augmentation of GSIS (glucose stimulated insulin secretion) following acute 1h-treatments was evaluated by rat insulin ELISA.
***G. sylvestre* treatment wells were co-incubated in corresponding 5.6 mM glucose**
*Each bar indicates the mean ± S.E.M. of four determinations. ***P<0.001 compared to respective 5.6 mM glucose (basal) control wells as determined by unpaired t-test.;*
$^{\triangle\triangle}$*P<0.01 and* $^{\triangle\triangle\triangle}$*P<0.001   compared to respective treatment conditions in the presence of 2.5 mM Ca^{2+}*
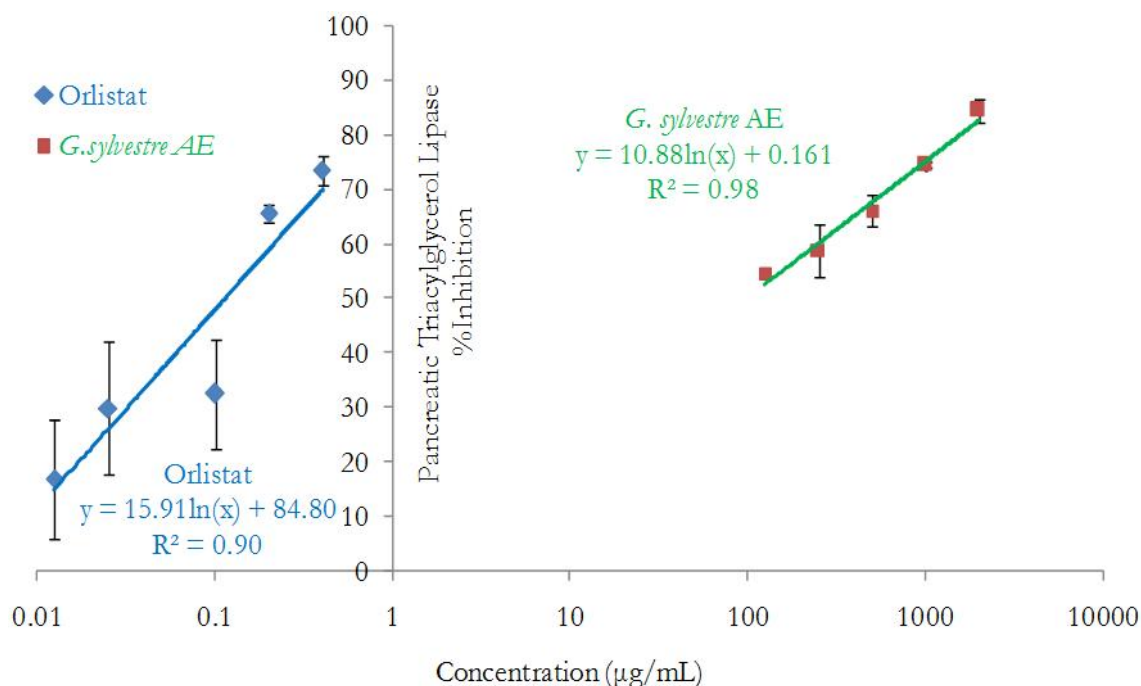
Fig. 5. *In vitro* inhibitory effects of *G. sylvestre* (AEs) as well as orlistat concentrations in µg/mL on Pancreatic Triacylglycerol Lipase Activity
Results are mean ± SEM (n = 3 independent replicates)

Comparable to orlistat performance, a marked concentration dependent PL inhibition trend was obtained per tested extracts (the same figure). *G. sylvestre* PL-IC$_{50}$ value for a minimum of triplicate determinations was 106.3±7.2 µg/mL. Thus *G. sylvestre* maybe be advocated as a slimming agent and, hence, a potential candidate for obesity-diabetes prevention and phytotherapy.

4. CONCLUSION

Unprecedentedly, inhibitors of α-amylase and/or α-glucosidase should be considered whenever postprandial hyperglycemia is the dominant metabolic abnormality. Our data indicate that *G. sylvestre* AEs could not improve glucose homeostasis via delaying carbohydrate digestion or absorption significantly but it was markedly proven as antilipolytic agent. Moreover, *G. sylvestre,* via induction of β-cell mass expansion, may ameliorate pancreatic dysfunction. Distinctly, *G. sylvestre* high concentrations potentiated Ca^{2+}-regulated glucose-evoked acute insulin secretion. However, further chronic subclinical testing is required to validate its clinical implementation as therapeutic agent for improvements in impaired peripheral carbohydrate tolerance and obesity-diabetes.

CONSENT

It is not applicable.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors declare that there are no conflicts of interests.

REFERENCES

1. Cefalu WT. Pharmacotherapy for the treatment of patients with type 2 diabetes mellitus: Rationale and specific agents. Clin Pharmacol Ther. 2007;81(5):636-649.
2. Cefalu WT, Urquhart S. Clinical management strategies for type 2 diabetes. JAAPA. 2007;Suppl:9-14.
3. IDF. International Diabetes Federation. Diabetes Atlas 6th Edition; 2013.
4. Winkler G. The use of gliclazide in individualized sulfonylurea therapy. Orv Hetil. 2014;155(14):541-548.
5. Ball A, Flatt PR, McClenaghan NH. Alterations of insulin secretion following long-term manipulation of ATP-sensitive potassium channels by diazoxide and nateglinide. Biochem Pharmacol. 2005;69:59-63.
6. Afifi- Yazar FU, Kasabri V, Abu-Dahab R. Medicinal plants from Jordan in the treatment of diabetes: Traditional uses vs. *in vitro* and *in vivo* evaluations; Mini-review. Planta Medica. 2011;77(11):1210-1220.
7. Afifi FU, Kasabri V. Pharmacological and phytochemical appraisal of selected medicinal plants from Jordan with claimed antidiabetic activities. Sci Pharma. 2013;81:889-932
8. Wazaify M, Afifi FU, Al-Khateeb M, Ajlouni K. Complementary and alternative medicine use among Jordanian patients with diabetes. Complement Ther Clin Pract. 2011;17(2):71-75.
9. Shanmugasundaram ER, Gopinath KL, Radha Shanmugasundaram K, Rajendran VM. Possible regeneration of the islets of Langerhans in streptozotocin-diabetic rats given *Gymnema sylvestre* leaf extracts. J Ethnopharmacol. 1990;30(3):265-279.
10. Shanmugasundaram ER, Venkata-subrahmanyam M, Vijendran N, Shanmugasundaram KR. Effect of an isolate from *Gymnema sylvestre*, R. Br. In the control of diabetes mellitus and the associated pathological changes. Anc Sci Life. 1988;7(3-4):183-194.
11. Shanmugasundaram ER, Rajeswari G, Baskaran K, Rajesh Kumar BR, Radha Shanmugasundaram K, Kizar Ahmath B. Use *of Gymnema sylvestre* leaf extract in the control of blood glucose in insulin-

dependent diabetes mellitus. J Ethnopharmacol. 1990;30(3):281-294.
12. Khan MY, Azizi I, Bihari B, Kumar H, Roy M, Verma VK. A review-Phytomedicines used in treatment of diabetes. Int J Pharmacog. 2014;1(6):343-365.
13. Tiwari P, Mishra BN, Sangwan NS. Phytochemical and pharmacological properties of *Gymnema sylvestre*: An important medicinal plant. Biomed Res Int 2014; Article ID 830285. 2014;18.
14. Di Fabio G, Romanucci V, Zarrelli M, Giordano M, Zarrelli A. C-4 gem-dimethylated of *Gymnema sylvestre* and their pharmacological activities. Molecules. 2013;18(12):14892-14919.
15. Moss JB, Koustubhan P, Grenman M, Parsons MJ, Walter I, Moss LG. Regeneration of the pancreas in adult zebrafish. Diabetes. 2009;58(8):1844–1851.
16. Halban PA, German MS, Kahn SE, Weir GC. Current status of islet cell replacement and regeneration therapy. J Clin Endocrinol Metab. 2010; 95(3):1034–1043
17. Mellado-Gil JM, Cobo-Vuilleumier N, Gauthier BR. Islet β-cell mass preservation and regeneration in diabetes mellitus: four factors with potential therapeutic interest. J Transplant 2012; ID230870. 2012;9.
18. D'souza JJ, D'souza PP, Fazal F, Kumar A, Bhat HP, Baliga MS. Antidiabetic effects of the Indian indigenous fruit *Emblica officinalis* Gaertn: Active constituents and modes of action. Food Funct. 2014;5(4):635-644.
19. Eliakim-Ikechukwu CF, Obri AI. Histological changes in the pancreas following administration of ethanolic extract of *Alchornea cordifolia* leaf in alloxan-induced diabetic Wistar rats. Nig J Physiol Sci. 2009;24(2):153-155.
20. Ghosh S, Suryawanshi SA. Effect of *Vinca rosea* extracts in treatment of alloxan diabetes in male albino rats. Indian J Exp Biol. 2001;39(8):748–759.
21. Kasabri V, Abu-Dahab R, Afifi FU, Naffa R, Majdalawi L. Modulation of pancreatic MIN6 insulin secretion and proliferation and extrapancreatic glucose absorption with *Achillea santolina, Eryngium creticum* and *Pistacia atlantica* extracts: *In vitro* evaluation. J Exp Integ Med. 2012; 2(3):245-254.

22. Kasabri V, Abu-Dahab R, Afifi FU, Naffa R, Majdalawi L, Shawash H. *In vitro* effects of *Geranium graveolens*, *Sarcopoterium spinosum* and *Varthemia iphionoides* extracts on pancreatic MIN6 proliferation and insulin secretion and extrapancreatic glucose diffusion. Int J Diabetes Dev Countr. 2013;33(3):170-177.

23. Jelodar g, Mohsen M Shahram S. Effect of walnut leaf, coriander and pomegranate on blood glucose and histopathology of pancreas of alloxan induced diabetic rats. Afr J Trad CAM. 2007;4(3):299-305.

24. Nwangwa EK, Aloamaka CP. Regenerative effects of coconut water and coconut milk on the pancreatic β-cells and cyto-architecture in alloxan induced diabetic Wistar Albino rats. Am J Trop Med Public Health. 2011;1(3):137-146.

25. Mohammadi J, Naik PR. The histopathologic effects of *Morus alba* leaf extract on the pancreas of diabetic rats. Turk J Biol. 2012;36:211-216.

26. Singh N, Gupta M. Regeneration of β cells in islets of Langerhans of pancreas of alloxan diabetic rats by acetone of *Momordica charantia* (Linn.) (bitter gourd) fruits. Indian J Exp Biol. 2007;45:1055-1062.

27. Xiu LM, Miura AB, Yamamoto K, Kobayashi T, Song QH, Kitamura H, Cyong JC. Pancreatic islet regeneration by ephedrine in mice with streptozotocin-induced diabetes. Am J Chin Med. 2001; 29(3-4):493–500.

28. Gallagher AM, Flatt PR, Duggy G, Abdel-Wahab YHA. The effects of traditional antidiabetic plants on *In vitro* glucose diffusion. Nutr Res. 2003;23:413−424.

29. Kasabri V, Flatt PR, Abdel-Wahab YHA. *In vitro* modulation of pancreatic insulin secretion and extrapancreatic insulin action, enzymatic starch digestion and protein glycation by *Terminalia chebula* extracts. Eur J Med Plants. 2014; 4(7):771-782.

30. Kasabri V, Flatt PR, Abdel-Wahab YHA. *Emblica officinalis* stimulates the secretion and action of insulin and inhibits starch digestion and protein glycation *in vitro*. Eur J Med Plants. 2014;4(6):753-770.

31. Kasabri V, Flatt PR, Abdel-Wahab YHA. *In vitro* modulation of pancreatic insulin secretion, extrapancreatic insulin action and peptide glycation by *Curcuma longa*

aqueous extracts. J Exp Integr Med. 2014;4(3):1-7.

32. Afifi FU, Kasabri V, Abu-Dahab R, Abaza IM. Chemical composition and *in vitro* studies of the essential oil and aqueous extract of *Pelargonium graveolens* growing in Jordan for hypoglycaemic and hypolipidemic properties. Eur J Med Plants. 2014;4(2): 220-233.

33. Kasabri V, Afifi FU, Hamdan I. Evaluation of the antihyperglycemic effects of four selected indigenous plants from Jordan used in traditional medicine. Pharm Biol. 2011;49(7):687-695.

34. Kasabri V, Afifi FU, Hamdan I. *In vitro* and *in vivo* antihyperglycemic effects of five selected indigenous plants from Jordan used in traditional medicine. J Ethnopharmacol. 2011;133:888-896.

35. Thiebart-Fassy I, Hervagault J. Combined effects of diffusional hindrances, electronic repulsion and product inhibition on the kinetic properties of a bound acid phosphatase. FEBS. 1993;334:89−94.

36. Miyazaki J, Araki K, Yamato E, Ikegami H, Asano T, Shibasaki Y, Oka Y, Yamamura K. Establishment of a pancreatic β cell line that retains glucose inducible insulin secretion: Special reference to expression of glucose transport forms. Endocrinology. 1990;127:126−132.

37. Gurbuz I, Ustu, O, Yesilada E, Sezik E, Kutsal O. Anti-ulcerogenic activity of some plants used as folk remedy in Turkey. J Ethnopharmacol. 2003;88(1):93-97.

38. Habtemariam S. The antiobesity potential of sigmoidin A. Pharma Biol. 2012;50: 1519-1522.

39. Bustanji Y, AlMasri I, Mohammad M, Hudai, M, Tawaha K, Tarazi H, AlKhatib H. Pancreatic lipase inhibition activities of Trilactone Terpenes of *Ginkgo biloba*. J Enz Inhibition Med Chem. 2011;26:453-459.

40. Harvey AL. The continuing value of natural products for drug discovery. Rev. Salud Anim. 2009;31(1):8-12.

41. List JF, Habener JF. Glucagon-like peptide 1 agonists and the development and growth of pancreatic β-cells. Am J Physiol Endocrinol Metab. 2004;286:875-881.

42. Sjoholm S. Liraglutide therapy for type 2 diabetes: Overcoming unmet needs. Pharmaceuticals. 2010;3:764‒781.

43. Porat S, Dor Y. New sources of pancreactic beta cells. Curr Diab Rep. 2007;7(4):304-308.

44. Ahmed AB, Rao AS, Rao MV. *In vitro* callus and *in vivo* leaf extract of *Gymnema sylvestre* stimulate β-cells regeneration and anti-diabetic activity in Wistar rats. Phytomedicine. 2010;17(13):1033-1039.

45. Baskaran K, Kizar Ahamath B, Radha Shanmugasundaram K, Shanmuga-sundaram ER Antidiabetic effect of a leaf extract from *Gymnema sylvestre* in non-insulin-dependent diabetes mellitus patients. J Ethnopharmacol. 1990; 30(3):295-300.

46. Wei JH, Zhen HS, Qiu Q, Chen J, Zhou F. Experimental study of hypoglycemic activity of conduritol A of stems of *Gymnema sylvestre*. Zhongguo Zhong Yao Za Zhi. 2008;33(24):2961-2965.

47. Hija A, Salpeter S, Klochendler A, Grimsby J, Brandeis M, Glaser B, Dor Y. G0-G1 Transition and the restriction point in pancreatic β-cells *in vivo*. Diabetes. 2014; 63(2):578-584.

48. Arun LB, Arunachalam AM, Arunachalam KD, Annamalai SK, Kumar KA. *In vivo* anti-ulcer, anti-stress, anti-allergic, and functional properties of gymnemic acid isolated from *Gymnema sylvestre* R Br. BMC Complement Altern Med. 2014; 14(1):70.

49. Ramkumar KM, Manjula C, Sankar L, Suriyanarayanan S, Rajaguru P. Potential in vitro antioxidant and protective effects of *Gymnema montanum* H. on alloxan-induced oxidative damage in pancreatic beta-cells, HIT-T15. Food Chem Toxicol. 2009;47(9):2246-2256.

50. Ramkumar KM, Lee AS, Krishnamurthi K, Devi SS, Chakrabarti T, Kang KP, Lee S, Kim W, Park SK, Lee NH, Rajaguru P. *Gymnema montanum* H. protects against alloxan-induced oxidative stress and apoptosis in pancreatic beta-cells. Cell Physiol Biochem. 2009;24(5-6):429-440.

51. Mujić A, Grdović N, Mujić I, Mihailović M, Živković J, Poznanović G, Vidakovic M. Antioxidative effects of phenolic extracts from chestnut leaves, catkins and spiny burs in streptozotocin-treated rat pancreatic β-cells. Food Chem. 2011;125: 841–849.

52. Ashcrof FM, Gribble FM. ATP-sensitive K+ channels and insulin secretion: their role in health and disease. Diabetologia. 1999; 42:903-919.

53. Persaud SJ, Al-Majed H, Raman A, Jones PM. *Gymnema sylvestre* stimulates insulin release *in vitro* by increased membrane permeability. J Endocrinol. 1999;163(2): 207-212.

54. Liu B, Asare-Anane H, Al-Romaiyan A, Huang G, Amiel SA, Jones PM, Persaud SJ. Characterization of the insulinotropic activity of an aqueous extract of *Gymnema sylvestre* in mouse beta-cells and human islets of Langerhans. Cell Physiol Biochem. 2009;23(1-3):125-132

55. Patel DK, Prasad SK, Kumar R, Hemalatha S. An overview on antidiabetic medicinal plants having insulin mimetic property. Asian Pac J Trop Biomed. 2012; 2(4):320-330.

56. Al-Romaiyan A, Liu B, Docherty R, Huang GC, Amiel S, Persaud SJ, Jones PM. Investigation of intracellular signaling cascades mediating stimulatory effect of a *Gymnema sylvestre* extract on insulin secretion from isolated mouse and human islets of Langerhans. Diabetes Obes Metab. 2012;14(12):1104-1113.

57. Al-Romaiyan A, King AJ, Persaud SJ, Jones PM. A novel extract of *Gymnema sylvestre* improves glucose tolerance *in vivo* and stimulates insulin secretion and synthesis *in vitro*. Phytother Res. 2013; 27(7):1006-1011.

58. Alqahtani A, Hamid K, Kam A, Wong KH, Abdelhak Z, Razmovski-Naumovski V, Chan K, Li KM, Groundwater PW, Li GQ. The pentacyclic triterpenoids in herbal medicines and their pharmacological activities in diabetes and diabetic complications. Curr Med Chem. 2013; 20(7):908-931.

59. Kumar V, Bhandari U, Tripathi CD, Khanna G. Evaluation of antiobesity and cardioprotective effect of *Gymnema sylvestre* extract in murine model. Indian J Pharmacol. 2012;44(5):607-613.

60. Kumar V, Bhandari U, Tripathi CD, Khanna G. Anti-obesity effect of *Gymnema sylvestre* extract on high fat diet-induced obesity in Wistar rats. Drug Res (Stuttg). 2013;63(12):625-632.

61. Bhansali S, Shafiq N, Pandhi P, Singh AP, Singh I, Singh PK, Sharma S, Malhotra S. Effect of a deacyl gymnemic acid on glucose homeostasis & metabolic parameters in a rat model of metabolic syndrome. Indian J Med Res. 2013; 137(6):1174-1179.

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