Modulation of pancreatic MIN6 insulin secretion and proliferation, and extrapancreatic glucose absorption with Achillea santolina, Eryngium creticum and Pistacia atlantica extracts: in vitro evaluation.

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Abstract

Objective: The present in vitro studies aimed to investigate the pancreatic and extrapancreatic effects of crude aqueous extracts (AE) of Achillea santolina L, Eryngium creticum Lam, and Pistacia atlantica Desf utilized in Jordan diabetes ethnomedicine.

Methods: Bioassays of β-cell proliferation and insulin secretion as well as glucose diffusion as possible modes of action were recruited.

Results: Similar to L-alanine insulinotropic efficacy in MIN6 β-cell, glucose-stimulated Ca²⁺ regulated insulin secretion was potentiated by AE of E.creticum (0.01 mg/ml) and P.atlantica (0.01, 0.1 and 0.5 mg/ml). A.santolina AE, however, was found ineffective. Comparable to glucagon-like peptide-1-enhanced β-cell proliferation in 2-day treatment wells, a dose dependent augmentation of bromodeoxyuridine incorporation was obtained with the A.santolina AE (0.05-1 mg/ml), and E.creticum AE (0.1, 0.5 and 1 mg/ml). P.atlantica concentrations lacked pancreatic proliferative capacity. While A.santolina and E.creticum AE proved inactive, P.atlantica inhibited dose dependently overnight glucose movement in vitro, as effectively as guar gum diffusional hindrance in a simple glucose dialysis model.

Conclusion: Current findings signify the in vitro diverse therapeutic antidiabetes properties of the selected medicinal plants. Future directives may assess the use of A.santolina, E.creticum and P.atlantica as new potential sources of functional foods or nutraceuticals or active leads into diabetes type 2 pharmacotherapy.

Key words:
Achillea santolina; Eryngium creticum; Jordan; Glucose dialysis; MIN6 β-cells; Pistacia atlantica

Introduction

It is projected that the worldwide prevalence of diabetes is likely to increase. At least 250 million individuals worldwide suffer diabetes and it is estimated that by 2030 this number will double [1]. Increases in complications will undoubtedly follow increasing prevalence of diabetes. The current therapies, namely sulphonylureas, biguanides and insulin sensitizers (thiazolidinediones), only partially compensate for metabolic derangements seen in diabetes and do not correct the fundamental biochemical lesions [2]. Ethnobotanical and ethnopharmacological research is very crucial in the development of drugs from natural sources [3]. This can be coupled to the unmet need for the medicinal plants and phytopharmaceuticals with scientifically proven antidiabetic efficacy comparable to orthodox medicine. Based on International Diabetes Federation data, Jordan has the ninth highest prevalence of diabetes in Middle Eastern and North Africa (MENA) countries and stands at 10.1% [4]. Several studies indicated that the prevalence of type 2 diabetes and impaired fasting glycemia in Jordan is increasing [5-7].

On the other hand, ethnopharmacological studies and surveys confirmed that there is an appreciable prevalence of herbal use among patients with diabetes in Jordan [8, 9]. These surveys based on the information from the herbalists and inhabitants indicated that Achillea santolina L (Asteraceae), Eryngium creticum Lam (Apiaceae) and Pistacia atlantica Desf (Anacardiaceae) are used in Jordan as antidiabetic plants. These selected plants are preferably consumed as infusions [10, 11]. Literature illustrates that A.santolina had a high hypoglycaemic activity, mainly attributed to its antioxidant potential [12, 13] in streptozotocin (STZ) diabetic rats. Evidently, E.creticum aqueous decoction was recognised for its hypoglycemic effects in normal and diabetic rats [14]. Also, remarkable and diverse potencies of P.atlantica Desf were reviewed elsewhere [15-17].

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For the first time, the insulin secretory efficacies of these selected medicinal plant species, used either traditionally or assumed promising in treating diabetes in Jordan, are tested using well-characterized glucose responsive insulin releasing pancreatic MIN6 β-cells. Parallel cytotoxicity studies are undertaken with plant incubations to rule out any undesirable non-physiological insulin leakage from MIN6 cells in acute incubation wells. For the plant extracts proven significantly efficacious and safe, investigating physiological regulation of their insulin stimulatory mechanisms are considered. Another possible avenue to explore is the β-cell regenerative capacity of the selected plants. Accordingly, cell proliferation assays have been recruited to investigate the changes in the proliferation of MIN6 cells incubated with plant extracts on chronic bases. Also, an extrapancreatic mode of action has been explored with a simple model of glucose dialysis overnight. Taken together, due to the good acceptance of herbal drugs among population, potent plants and phytopharmaceuticals with demonstrated scientific and clinical efficacy could become a suitable adjuvant therapy or excellent therapeutic alternative to current medication in the integrated management of diabetes.

Materials and Methods

Chemicals and Biochemicals

Dulbecco Modified Eagle Medium (DMEM) containing 25 mM glucose was obtained from Invitrogen (Grand Island, NY, USA). ELISA jumbo kit for rat high insulin was purchased from ALPCO (Salem, NH, USA). MTT assay kit was purchased from Promega (Madison, WI, USA). The assays were performed according to manufacturer’s instructions. Unless stated otherwise, all reagents and chemicals were from Sigma (Dorset, UK). Dialysis tubing Spectra/Por® 7 Biotech RC (regenerated cellulose) membranes, MWCO 2000 was purchased from Spectrum Europe B.V. (Breda, The Netherlands). Coated analytical thin layer chromatography (TLC) plates were procured from Merck (Whitehouse Station, NJ, USA). Shaking incubator was from LabTech® (Daihan LabTech Co. Ltd., Namyangju-si, Korea). Glucose GOD-PAP kit was obtained from BioLabo Reagents (Maizy, France). In UV determinations UV-VIS spectrophotometer from SpectroScan 80D (Biotech Engineering Management Co. Ltd., UK) was used.

Plant Material

Fresh aerial parts of A. santolina [9 COMP-FMJ], E. creticum [8 UMBE-FMJ], and P. atlantica [2 ANAC-FMJ] leaves were collected from the Greater Amman area and from Zai, 50 km north of Amman in spring 2009. Collectively, they were taxonomically identified by Prof. Barakat Abu Irmaileh, Faculty of Agriculture-University of Jordan. Voucher specimens were deposited in the Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan. All collected fresh plant samples were cut into small pieces, air dried at room temperature and coarsely powdered.

Extraction

The crude aqueous extracts (AEs) were prepared by refluxing each 10 g of the dried coarsely powdered plant material with 100 ml tap water for 15 min and keeping the extract overnight. After filtering twice through filter paper, the volume of the filtered solution was increased to 100 ml with tap water to obtain 10% (equivalent to 100 mg/ml) crude aqueous solutions [15].

Phytochemical screening

Ethanolic extracts (10%) of each of the three plants were subjected to TLC examination for group determination of the secondary metabolites. Modified Dragendorff's reagent for alkaloids, ferric chloride reagent for phenolics, Naturstoff reagent for flavonoids, ethanolic KOH for coumarins and vanilline/sulphuric acid reagent for terpenoids were used. Solvent systems for the development of ready coated analytical TLC plates were selected according to Wagner and Bladt [18].

MIN6 Cell Culture

The widely used mouse insulinoma clonal pancreatic MIN6 β-cells [19] (passage 39-45) were maintained in DMEM containing 15% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml L-glutamate, and 5 μl/ml β-mercaptoethanol in a 37°C humidified atmosphere with 95% air and 5% CO₂. The culture medium was changed every 48-72 h.

Insulin secretion static incubation experiments

Glucose stimulated insulin secretion (GSIS) from MIN6 was determined using a static incubation protocol. MIN6 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 37°C in 5% CO₂ in HEPES-balanced Krebs-Ringer phosphate buffer (KRB) composed of (each in mM) 129 NaCl, 5 NaHCO₃, 4.8 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 10 HEPES, 2.5 CaCl₂ 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 plants’ AEs 0.01-25 mg/ml, n = 4 independent observations) were performed for another 1 h. Cell viability was assessed post 1 h-acute incubations using MTT assay. There was a modification to this standard protocol. When investigating the effects of extracellular Ca\(^{2+}\)-free incubations on pancreatic insulin secretion, 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 -20°C for a subsequent ELISA determination of the amount of secreted mouse insulin.

**Cell viability assay**

Cell viability was assessed by a MTT kit. MIN6 cells were subcultured on 96-well plates at a concentration of 10,000 cells/well in growth medium containing different aqueous concentrations of the selected plants (0.01-1 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 protocol.

**Cell proliferation assay**

Proliferation of MIN6 cells was evaluated with a colorimetric ELISA-based bromodeoxyuridine (BrdU) incorporation kit (Roche Diagnostics, Germany). In brief, MIN6 cells (young passage) were seeded onto 96-well plate at a 10,000 cell/well density and left to adhere overnight. They were serum starved for 24 h before 48 h treatments with plants aqueous concentrations (0.01-1 mg/ml) or glucagon-like peptide-1 (GLP-1) 500 nM, as indicated in bar graphs (n = 4). 10 μM BrdU dye/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 protocol instructions.

**Glucose movement in vitro**

Glucose solution 0.22 M in 0.15 M NaCl was added to a dialysis tubing (10 cm x 11 mm). This was kept wet, as drying may result in unrecoverable collapse of the pore structure. The tubing was sealed at both ends, and dialysed against 45 ml of 0.15 M NaCl in 50 ml tube overnight. Using the diffusion model described, the optimum temperature for maximum glucose diffusion was established at 37°C. Gentle shaking is used as well in a shaking incubator to simulate the effect of intestinal contractions on intestinal glucose absorption [20]. Thiebart-Fassy and Hervagault [21] reported that more vigorous stirring would have led to a decrease in the unstirred layer thickness, that is, to a decrease in the contribution of diffusional hindrance (whenever dietary fibres are included). 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.

To ascertain the viscosity-based diffusion hindrance of gel-forming dietary fibres, and hence, their postprandial glucose lowering efficacies in vitro, in the glucose movement model described above, guar 50 mg/ml, rutin 50 and 100 mg/ml or plant aqueous extract 10, 25 and 50 mg/ml in 0.22 M glucose in triplicates were dialysed against 0.15 M NaCl overnight at 37°C with gentle shaking and a parallel plant-free (negative) control was included [22].

**Statistical analysis**

The values are presented as mean ± S.E.M. (standard error of the mean) of 3-4 independent experiments. A.U.Cs (incremental Area Under 24 h-glucose Curves) were calculated by Graphpad Prism (version 3.02 for windows; GraphPad Software, San Diego, CA, USA). Statistical differences between control and different treatment groups and A.U.Cs were determined using Graphpad Prism one way analysis of variance (ANOVA) followed by Dunnett’s post hoc test whenever appropriate. Values were considered significantly different if p < 0.05.

**Results**

The results of phytochemical screening of the selected plants species are given in Table 1. Flavonoids, terpenoids, phenolics and coumarins were identified in all tested plants, while the presence of alkaloids could be hardly detected in any of the three.

**Glucose-dependent modulation of insulin secretion in pancreatic β-cell by plants’ AEs**

The mouse insulinoma MIN6 β-cell line was the cellular model to examine the in vitro effects of the *A.santolina*, *E.creticum* and *P.atlantica* AEs on pancreatic β-cell proliferation and insulin secretion. MIN6 is a well-characterised insulin-secreting cell line with a higher insulin content than other β-cell
lines, which retains the physiological regulation of insulin secretion and so it is a good experimental model of normal and/or impaired insulin secretion studies and mechanisms of action of insulin secretagogues [23].

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, a well-established nutritious amino acid and a classical insulin secretagogue [24, 25], was used as a positive control and it enhanced substantially (p < 0.05) GSIS in MIN6 by 178.5 ± 17.9% (n = 4) following 1 h-incubations, compared to untreated (glucose only) controls (Fig.1). With obvious unlikeness to L-alanine, A. santolina AE doses lacked any marked augmentation of MIN6 GSIS in acute treatment wells compared to controls, (Fig.1A). Nevertheless, comparable to L-alanine secretory capacity, E. creticum AE concentrations 0.01 mg/ml potentiated GSIS in pancreatic MIN6 substantially by 198.3 ± 22.1% (p < 0.05, Fig.1B). Significant increase of the MIN6 insulin release at higher concentrations was not detected. Except for high concentrations of E. creticum, cell viability, as checked by MTT assay, was unchanged over 1 h-incubations. Exceedingly superior to L-alanine, P. atlantica AE concentrations 0.01, 0.1 and 0.5 mg/ml potentiated GSIS in pancreatic MIN6 highly substantially (p < 0.001 vs basal controls) by respective 1117.2 ± 146%, 604.8 ± 92% and 732.6 ± 209%. Significant potentiation of the MIN6 insulin release at higher concentrations was not detected (Fig.1C). Cell viability was unaffected, negating against either plant inflected cytotoxicity.

Glucose-dependent modulation of insulin secretion in pancreatic β-cell by plants’ AEs is mediated by Ca²⁺ dependency

Changes in β-cell cytosolic Ca²⁺ concentrations, whether by an influx of extracellular Ca²⁺ or by release of Ca²⁺ from intracellular stores, are thought to be a primary trigger for the initiation of insulin exocytosis machinery. Fig.1B illustrates that the marked insulinotropic trend of L-alanine was highly significantly (54.9 ± 8.6%, p < 0.001) abolished in Ca²⁺ depleted KRH, as compared to corresponding Ca²⁺ free glucose-only (negative control) wells. Obviously, in Fig.1B, the insulin stimulatory effects of E. creticum AE (0.01-0.5 mg/ml) on the intracellular Ca²⁺ concentrations ([Ca²⁺])i) was completely abolished (p < 0.05-0.001) in the absence of extracellular Ca²⁺, as compared to respective Ca²⁺-supplemented wells. Consistent with L-alanine pancreatic physiology, the Ca²⁺ influx through voltage operated Ca²⁺ channels (VOCC) to sustain the [Ca²⁺]i increase in response

<table>
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<tr>
<th>Phytochemical screening of plants extracts</th>
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<tr>
<td><strong>Plant</strong></td>
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<tr>
<td>Alkaloids</td>
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<tr>
<td>Terpenoids</td>
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<td>Flavonoids</td>
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<td>Phenolics</td>
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<td>Coumarins</td>
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Figure 1. Modulatory effects of plants’ AEs (0.01-25 mg/ml) on function of MIN6 pancreatic β-cells. Such augmentation of GSIS following acute 1 h-treatments was evaluated by rat insulin ELISA. (A) Achillea santolina, (B) Eryngium creticum, (C) Pistacia atlantica. Plant AE treatment wells were co-incubated in corresponding 5.6 mM glucose. Each bar indicates the mean ± S.E.M. of four determinations; *p < 0.05 and **p < 0.001 compared to respective 5.6 mM glucose (negative) control wells; **p < 0.05 and ***p < 0.001 compared to respective treatment conditions in the presence of 2.5 mM Ca²⁺.
to *E. creticum* concentrations 1, 10 and 25 mg/ml was also decreased (p < 0.001 vs basal Ca\(^{2+}\) depleted glucose-only wells, Fig.1B). Comparably, *P. atlantica* insulingenic efficacies were markedly reduced (p < 0.001) following Ca\(^{2+}\) absence from acute plant incubations to 212 ± 92%, 181.2 ± 22% and 223 ± 23%, respectively, compared to respective Ca\(^{2+}\) buffered conditions (Fig.1C).

**Pancreatic β-cell viability/expansion modulation by plants’ AEs**

Compared to negative control untreated wells, the MTT method revealed that 48 h post seeding *A. santolina* AEs at doses 0.01-1 mg/ml had no viability-compromising properties on MIN6 cells. In parallel terms, *E. creticum* chronic treatments (0.01-1 mg/ml) proved non-cytotoxic to MIN6 wells. Interestingly, prolonged treatment with *P. atlantica* AEs (0.01-1 mg/ml), also preserved β-cell integrity (Table 2).

A colorimetric immunoassay of BrdU incorporation into MIN6 β-cell genome was recruited to ascertain proliferative principles of chronic plants treatments. The gut hormone GLP-1 agonists have been shown to stimulate the growth and differentiation of pancreatic cells, as well as to exert cytoprotective and antiapoptotic effects on β-cells [26]. Figure 2 demonstrate that GLP-1 (500 nM) highly significantly promoted a maximal extent of BrdU incorporation by 1.33 to 1.5 folds (p < 0.001, n = 4) in comparison to basal BrdU incorporation (spontaneous control).

Comparably, a dose dependent augmentation of BrdU incorporation was obtained in the *A. santolina* AE (0.05-1 mg/ml) by 131.3 ± 9.3-144 ± 16.6%

**Table 2.** Modulatory effects of *Achillea santolina*, *Eryngium creticum* and *Pistacia atlantica* AEs (0.01-1 mg/ml) on the viability of pancreatic β-cells MIN6 in 48 h post seeding as measured by MTT kit

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MIN6 viability (as % control)</th>
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<tbody>
<tr>
<td>Control incubations (plant free)</td>
<td>99.5 ± 10.4</td>
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<tr>
<td><em>Achillea santolina</em> AE</td>
<td></td>
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<tr>
<td>0.01 mg/ml</td>
<td>100.4 ± 2.7</td>
</tr>
<tr>
<td>0.05 mg/ml</td>
<td>109.2 ± 1.7</td>
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<tr>
<td>0.1 mg/ml</td>
<td>95.9 ± 2.1</td>
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<tr>
<td>0.5 mg/ml</td>
<td>99.6 ± 1.8</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>93.2 ± 5.6</td>
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<tr>
<td><em>Eryngium creticum</em> AE</td>
<td></td>
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<tr>
<td>0.01 mg/ml</td>
<td>120.4 ± 2.7</td>
</tr>
<tr>
<td>0.05 mg/ml</td>
<td>112.9 ± 1.9</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>91.4 ± 7.2</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>92.0 ± 3.3</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>97.0 ± 1.8</td>
</tr>
<tr>
<td><em>Pistacia atlantica</em> AE</td>
<td></td>
</tr>
<tr>
<td>0.01 mg/ml</td>
<td>100.5 ± 6.62</td>
</tr>
<tr>
<td>0.05 mg/ml</td>
<td>130.8 ± 6</td>
</tr>
<tr>
<td>0.1 mg/ml</td>
<td>93.1 ± 3.1</td>
</tr>
<tr>
<td>0.5 mg/ml</td>
<td>94 ± 3.3</td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>124.8 ± 10</td>
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AE, aqueous extract; each result indicates the mean ± S.E.M. of four independent experiments.

Figure 2. Modulatory effects of plants’ AEs (0.01-1 mg/ml) on the proliferation of pancreatic β-cells MIN6 in 48 h culture incubations as measured by a colorimetric ELISA-based BrdU incorporation kit. (A) *Achillea santolina*, (B) *Eryngium creticum*, (C) *Pistacia atlantica*. Each bar indicates the mean ± S.E.M. of four independent determinations; *p < 0.05 and ***p < 0.001 compared to untreated control conditions.
(p < 0.05-0.001 vs controls, Fig.2A), thus inducing highly significantly pancreatic monolayers expansion. Similarly, highly significant β-cell proliferative efficacies were recognised for E.creticum AE 0.1, 0.5 and 1 mg/ml, promoting BrdU incorporation dose dependently by respective 1.31, 1.42 and 1.47 folds (p < 0.001 vs basal controls, Fig.2B). Prolonged pancreatic treatment with P.atlantica AE concentrations proved, however unlikely, ineffective (Fig.2C).

Extrapancreatic modulation of glucose movement in vitro by plants’ AEs

Using the simple diffusion model described, 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, Fig.3) compared to overnight negative control. Guar is a natural oral antidiabetic and classical positive control [27].

Equally effective, P.atlantica crude AE (10, 25 and 50 mg/ml) retarded dose dependently 24 h glucose efflux in vitro by respective 9.5 ± 1.8%, 17.5 ± 3.1% and 30.8 ± 2.4% decreases in AUC versus the controls (p < 0.05-0.001, Fig.3).

Rutin 50 and 100 mg/ml, an established phytoconstituent of Pstacica spp [28], performed relatively effectively (by 14.5 ± 6 and 19.6 ± 2.1% reductions in AUC, p < 0.001, Fig.3). Unlike expected, A.santolina and E.creticum AEs lacked any statistically substantial glucose diffusional hindrances into external solution across dialysis membrane (with respective 15.9 ± 3.85% and 11.9 ± 4.34% AUC reductions, p > 0.05 vs controls’ AUCs, Fig.3).

Discussion

A key feature of type 2 DM is that glucose fails to stimulate an adequate release of insulin from pancreatic β-cells. Eventually, the pancreatic β-cells fail to compensate for the raised glucose to achieve homeostasis, resulting in overt hyperglycemia. Insulinotropic sulphonylureas, such as tobutamide and glibenclamide, have found widespread application in drug therapy of type 2 diabetes, mainly due to a direct stimulation of insulin secretion, exerting a hypoglycemic action [29]. The stimulatory effect is mediated via the pancreatic beta cell K<sub>ATP</sub> channel [30]. Binding of sulphonylureas leads to K<sub>ATP</sub> channel closure, evoking membrane depolarization and subsequent opening of VOCC, followed by elevation of [Ca<sup>2+</sup>]i due to increased Ca<sup>2+</sup> influx, ultimately leading to exocytosis of insulin secretory granules [30].

Nevertheless, chronic sulphonylureas therapy tends to progressively fail following direct desensitization and decline in beta cell K<sub>ATP</sub> channel activity [31, 32]. This lends further weight to the need for discovery of novel insulintropic entities from local medicinal plants reputed for their antidiabetic efficacies. Natural products have been a source of medical treatments for thousands of years, and plant-based systems continue to play an essential role in the primary health care of 80% of the underdeveloped and developing countries [33]. Approximately 80% of the investigated traditional plants used for the treatment of diabetes demonstrated some antidiabetic activity [34, 35].

The genus Achillea (Asteraceae) comprises more than 100 species which are mainly distributed in Eurasian northern hemisphere [36]. This genus is chemically characterized by the frequent accumulation of sesquiterpene lactones [37], alkamids [38], 3- or 7-glycosylated flavonoids [39] and free flavonoid aglycons [40]. With that in mind, numerous Chinese studies endorsed this genus for diversified ailments alleviation [41-46]. Most recently, potent immunomodulatory and anti-inflammatory potentialities were ascribed to A.santolina [47], thereby reasoning with its ethnopharmacological utility in Iranian traditional medicine. Furthermore, a favourable acute anti hyperglycemic trend for A.santolina bolus was reported in starch-fed normal rats, though it was deficient on any appreciable in vitro anti-α-amylase or anti-α-glucosidase activity [48]. In light of our current in vitro observations, A.santolina was also lacking any glucose diffusional hindrance properties or substantial acute potentiating of pancreatic MIN6 insulin secretory function. In other mechanistic studies, nevertheless, strong antioxidative potentiality was attributed to A.santolina [13] which mainly explained for the
high hypoglycemic activity of *A. santolina* in STZ-diabetic rats [12]. Most notably, *A. santolina* 2-day treatment could promote dose dependently highly significantly the pancreatic β-cell proliferation and monolayers expansion.

Belonging to a genus with more than 200 spp in Apiaceae (Umbelliferae) family, *Eryngium* species have been used traditionally for the treatment of a diversity of conditions [49-51]. *E. foetidum* L., marked in Eastern Asia for its strong coriander like taste and wide range of ethnomedicinal uses, is comprehensively reviewed [52]. *E. creticum*, on the other hand, is a perennial plant that grows in natural habitats of the Mediterranean region. Antioxidant activity [53] and antimutagenic properties [54] were attributed to *E. creticum*. Previous reports stressed the use of *E. creticum* for its hypoglycemic effects [55] in rat models. Especially important, a favourable acute antihyperglycemic trend was observed for *E. creticum* bolus treatment in starch-fed rats despite the lack of *in vitro* inhibitory activity of α-amylase and α-glucosidase [56] or glucose diffusional retardation effects, as of our present findings. In this line of *in vitro* pancreatic endocrinology studies, *E. creticum* could highly significantly potentiate MIN6 glucose stimulated insulin secretion. The reported acute antihyperglycemic effects [56] recognised for *E. creticum* can be evidently reasoned via its physiologically regulated pancreatic mode of action.

*Pistacia atlantica* is among the frequently reported local plants with medicinal properties. It might mediate its antidiabetic action through a variety of mechanisms. In here, we could demonstrate that, despite lacking any marked enhancement of the pancreatic MIN6 proliferation in chronic culture conditions, *P. atlantica* augmented highly substantially acute β-cell insulin secretory efficacy. Also, *P. atlantica* exerted effectively dose dependent glucose diffusional retardation in the overnight simple glucose dialysis model. Similarly, it has been reported that *Trigonella foenum-graecum* (fenugreek) acts by delaying carbohydrate digestion and absorption, among its multiple antidiabetic modes of action [25]. Viscous dietary fibres have the dual effects of hampering the diffusion of glucose and postponing the absorption and digestion of carbohydrates [27, 57]. The validity of this glucose dialysis model allows the *in vitro* investigation of the postprandial serum glucose lowering mechanism of aqueous plant extracts. The results from our study are in agreement with reports stating decreases in absorption of glucose due to the presence of soluble fibres [58, 59], where the principal effect of adding soluble fibres to the diet was decreasing postprandial hyperglycaemia [60, 61]. So, the detected substantial enhancement of glucose tolerance in *P. atlantica*-fed rats [48] may be, at least in part, associated with the ability of its soluble/viscous components to reduce glucose diffusion. Obviously, strong dual anti-α-amylase and anti-α-glucosidase properties were recognised for *P. atlantica* AE [48], further indicating its potential use as “starch blocker” and, thus, an “effective antiobesity agent” [62]. Furthermore, coinciding with our findings, its richness with natural phenolic compounds [63] credited antioxidative qualities to *P. Atlantica* [64]. In our line of *in vitro* work, the AE of *P. atlantica* augmented GSIS in a concentration-independent fashion from pancreatic MIN6 cells, maximally enhancing insulin exocytosis at the lowest dose tested (0.01 mg/ml). Acutely depleted Ca^{2+} conditions manifested a reduction in the exocytotic machinery, which was partially accountable for the impairments in *P. atlantica* potentiating of regulated secretory response. Given the fact that the plasma glucose concentration usually changes between 5 and 8 mM under physiological conditions [65]; our results may therefore be physiologically relevant.

Considering the modulation of various drugs of insulin secretion, pancreatic MIN6 cells were used to characterize the insulin stimulatory effects and the physiologic mechanisms of glibenclamide [66], JTT-608 [67, 68] and KAD-1229 [69], thereby negating against toxic effects on β-cells. Similar to normal islets, pancreatic MIN6 β-cells exhibit the characteristics of glucose metabolism and glucose induced insulin secretion [70]. Given the linkage between cytosolic free [Ca^{2+}] increase and glucose metabolism in signalling insulin secretion [71], *E. creticum* evoked GSIS in MIN6 cells was shown to take place by modulating Ca^{2+} regulated exocytosis machinery. Remarkably, *E. creticum* augmented highly substantially the β-cell proliferation dose dependently. In parallel themes, *Gymnema sylvestre*, an Indian medicinal herb used in controlling diabetes, was proven for its insulinotrophic activity [72]. Moreover, *G. sylvestre* leaves extract doubled the islet number and β-cells number in STZ-treated rats, thereby, increasing serum insulin and reinstating glucose homeostasis by regeneration/repair of endocrine pancreas [73]. More recently, it has been reported that 30-day treatment of STZ-diabetic rats with *Nigella sativa* L. was associated with a partial regeneration/prolifera-
tion of pancreatic β-cells causing an increase in insulin secretion, thereby indicating the hypoglycemic potential of this plant [74].

The root cause of diabetes is associated with an absolute or relative decline in the number of functional β-cells. The preservation/restoration of mass is one of the most promising therapeutic strategies for this disease [75]. On one approach, diabetes-regenerative therapeutics are fundamentally based on the significant enhancement of self duplication capacity of pre-existing β-cells, rather than pluripotent stem cell differentiation [76, 77]. This can spur the body to copy existing functioning β-cells and ‘sweet talk’ the pancreas to create new ones. Thirdly, it may reveal the biological mechanisms regulating β-cell expansion, thus, providing new insights into novel therapeutic strategies and approaches to promote β-cell regeneration and cytoprotection. In this realm, A. santolina and E. creticum aqueous extracts may offer a potentially promising avenue for treatment of β-cells demise in diabetes. Hence, intensive chronic testing of plants inducing the pancreatic β-cell expansion will allow the emergence of safe and efficient cell replacement therapies [78]. Such an outcome may allow type 1 diabetes patients to regenerate residual remaining β-cells and regain control over blood sugar levels [77].

Succinctly, our data contribute to the evidence that E. creticum and P. atlantica can improve glucose homeostasis via proven insulin secretagogue and glucose absorption restrictive activity, respectively. Moreover, A. santolina along with E. creticum exerted β-cell mass expansion bioactivity, implying further restoration of pancreatic dysfunction. However, further chronic investigation is required to validate their use prior to clinical implementation as therapeutic agents for improvements in diabetes. The obtained results conclusively underline the possible health benefits and functional properties associated with their consumption, thereby qualifying for discovery of new orally active antidiabetic therapeutics.

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