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In vitro Modulation of Pancreatic Insulin Secretion and Extra Pancreatic Insulin Action, Enzymatic Starch Digestion and Protein Glycation by *Terminalia chebula* Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VK, PRF and YHAW designed the study and wrote protocol. Author VK evaluated biological analyses and performed the statistical analysis. All authors wrote first draft and managed samples for analyses. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aim: Traditional plant treatments have been used throughout the world for the therapy of diabetes mellitus.
Study Design: Using multiple *in vitro* models; this study was designed to investigate the efficacy and mode of action of *Terminalia chebula* Retz. (*Combretaceae*) used traditionally for treatment of diabetes.
Place and Duration of Study: School of Biomedical Sciences**,** University of Ulster, 2001-2004.
Results: *T. chebula* aqueous extract stimulated basal insulin output and potentiated glucose-stimulated insulin secretion concentration-dependently in the clonal pancreatic beta cell line, BRIN-BD11 ($p<0.001$). The insulin secretory activity of plant extract was abolished in the absence of extracellular Ca^{2+} and by inhibitors of cellular Ca^{2+} uptake, diazoxide and verapamil, ($p<0.001$). Furthermore, the extract increased insulin secretion in depolarised cells and augmented insulin secretion triggered by IBMX, but not by tolbutamide or glibenclamide. *T. chebula* extract did not display insulin mimetic activity but

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it enhanced insulin-stimulated glucose transport in 3T3 L1 adipocytes by 280% ($p<0.001$). At (0.5-5.0mg/mL) concentrations, the extract also produced 22-84% ($p<0.001$) decrease in starch digestion *In vitro* and inhibited protein glycation ($p<0.001$) at 1mg/ml aqueous extract.

Conclusion: This study has revealed that water soluble bioactive principles in *T.chebula* extract stimulate insulin secretion, enhance insulin action and inhibit both protein glycation and starch digestion. The former actions are dependent on the bioeffective component(s) in the plant being absorbed intact. Future work assessing the use of *Terminalia chebula* as dietary adjunct or as a source of active antidiabetic agents may provide new opportunities for the treatment of diabetes.

Keywords: Terminilia chebula Retz. (Combretaceae); insulin secretion; insulin action; starch digestion; peptide glycation.

1. INTRODUCTION

Diabetes mellitus is a syndrome, initially characterized by a loss of glucose homeostasis resulting from defects in insulin secretion and insulin action, resulting in impaired metabolism of glucose and other energy-yielding fuels [1]. Despite the great strides that have been made in the understanding and management of diabetes, the disease and disease related complications are increasing unabated [2]. In 2013; 385 million people have diabetes; and by 2035 this will rise to 592 million [3]. Increases in complications will undoubtedly follow increasing prevalence of diabetes. The current therapies –sulphonylureas, biguanides and insulin sensitizers (thiazolidinediones) GLP1 mimetics and inhibitors of DPPIV and SGLT2-only partially compensate for metabolic derangements seen in diabetes and do not correct the fundamental biochemical lesions [4]. Remedies from medicinal plants are used with success to treat this disease in spite of the presence of known antidiabetic medicine in the pharmaceutical market [5-7]. Based on the WHO recommendations hypoglycemic agents of plant origin used in traditional medicine are important [8]. The attributed anti hyperglycemic effects of these plants are due to their ability to restore the secretory function of pancreatic β-cells and/or smooth out fluctuations in circulating glucose levels following alleviation of peripheral resistance inhibition of the intestinal absorption of rising postprandial glucose. Recent studies showed that aqueous extracts of agrimony (*Agrimony eupatoria*) eucalyptus (*Eucalyptus globulus*), edible mushroom (*Agaricus campestris*), coriander (*Coriandrum sativum*), lucerne (*Medicago sativa)*, mistletoe (*Viscum album)* and elder (*Sambucus nigra*) enhanced insulin secretion and mimicked the effect of insulin on glucose metabolism *In vitro* [9-15] with a striking dose dependent inhibition of *In vitro* insulin glycation.

Terminalia chebula Retz. (*combretaceae)* has multiple reported medicinal properties. Decoction of bark is used to treat sores in mouth and constipation and as cardio tonic. The fruit pulp is also a constituent of the 'triphala' [16]. *T. chebula* was reported to be purgative [17], blood coagulant, used to cure asthma, urinary disorders, heart disease [18], burns, anemia, pimples, diarrhea, dysentery, and eye infections [19], *T. chebula* was found to prevent microsomal lipid peroxidation with strong antioxidant properties [20]. Moreover, *T. chebula* extracts harbored constituents with promising antimutagenic and anticancerous potential [21]. Comparable to glibenclamide, the chloroform extract of *T. chebula* seeds produced dose-dependent reductions in blood glucose of diabetic rats in a short term study; it also produced significant reductions in blood glucose in a long term study [22]. An updated comprehensive appraisal of *T. chebula* pharmacological and medicinal propensities is reported [23].

As pharmacological antidiabetic properties were proven for *Terminalia bellirica* of the same genus [24]; the present study was undertaken to investigate the antidiabetic efficacies of *T. chebula* extract on insulin secretion and action at the cellular level. Furthermore, possible extrapancreatic effects on protein glycation and starch digestion were examined *In vitro*, to elucidate the speculated mechanisms responsible for its reported antihyperglycemic effects.

2. MATERIALS AND METHOD

2.1 Cell Lines, Chemicals and Biochemicals

3T3-L1 fibroblasts were obtained from the American Type Culture Collection ((ATCC) Virginia, USA). Filter paper no.1 (Whatman), vacuum dryer (Savant Speedvac, Savant Instrumentation Incorporation, NY (USA) were used in extract preparations. Wallac 1409 Scintillation Counter was from Wallac, Turke (Finland). Analox GM9 Glucose analyzer was from Analox Instruments (London, UK). Acarbose was obtained from Bayer AG (Germany). Unless stated otherwise, all other reagents were purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA).

2.2 Plant Material

Dried fruits of *Terminalia chebula* Retz. (Family: *Combretaceae*) were procured from a commercial supplier in Delhi, India, during the winter season, and available from Top-Op (Foods) Ltd (Stanmore, Middlesex, UK). Voucher specimens are preserved in Diabetes Research Group, School of Biomedical Sciences, University of Ulster**.** Fruit was homogenised to a fine powder and stored in opaque screw-top jars at room temperature (20°C±2°C) until use. For *In vitro* work, a decoction was prepared by bringing 25g/L of material to the boil in water. Once boiling, the suspension was removed from the heat and allowed to infuse over 15 minutes. The suspension was filtered and the volume was adjusted so that the final concentration was 25g/L. Sample aliquots of 1mL of the filtered plant solution were brought to dryness under vacuum. Dried fractions were stored at –20°C until required. Fractions were reconstituted in incubation buffer for subsequent experiments as required.

2.3 Insulin Secretion

Insulin release was determined using monolayers of BRIN BD11 clonal pancreatic cells [25]. BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11.1mmol glucose/L, 10% fetal calf serum and antibiotics (50,000IU penicillin-streptomycin/L), and maintained at 37°C in an atmosphere of 5% CO_2 and 95% air. Twenty-four hours prior to acute experiments, cells were harvested and seeded in 24 - well plates at a density of $1.0X10^5$ cells per well. Following overnight attachment, culture medium was removed and cells were preincubated for 40 min at 37°C with 1mL of Krebs Ringer Bicarbonate (KRB) buffer supplemented with 1.1mM glucose and 1% bovine serum albumin (BSA). Subsequent test incubations were performed for 20 min at 5.6mM glucose using similar buffer supplemented with dried fruit aqueous extracts of *T. chebula* (DFAETC) and the agents indicated in Figures. Samples were stored at –20°C for subsequent insulin radioimmunoassay [26]. Cell viability was assessed using a modified neutral red assay as described previously [27].

2.4 Adipocyte Differentiation and Cellular Glucose Transport

Rat 3T3 L1 fibroblasts were used to determine glucose transport [28]. Cells (passages 5-10) were seeded in 12-well plates at a density of $1.0X10^5$ cells per well, maintained at 37°C±2°C with 5% CO_2 and fed every 2 days with DMEM supplemented with penicillin (50U/mL), streptomycin (50µL/mL) and foetal bovine serum (10%v/v). Adipocyte differentiation was initiated as described in detail elsewhere by the addition of 1µg/mL insulin, 0.5mM IBMX and 0.25µM dexamethosone [27]. Prior to acute tests, cells were incubated in serum free DMEM for 2-3 hours to establish basal glucose transport. Cellular glucose transport was determined for 15min at 37°C using KRB buffer supplemented with tritiated 2-deoxyglucose (0.5µCi/well), 50mM glucose, insulin and other test agents as well as DFAETC as indicated in the Figures. Hexose transport was terminated after 5 minutes by 3 rapid washes with ice-cold PBS, after which cells were detached by the addition of 0.1% sodium dodecyl sulphate (SDS) and subsequently lysed. Scintillation fluid was added to solubilised cell suspensions and mixed thoroughly. Radioactivity was measured on a scintillation counter.

2.5 Starch Digestion

To assess *In vitro* starch digestion, 100mg of soluble starch was dissolved in 3mL of distilled water in the absence and presence of DFAETC or acarbose 1000µg/mL as a positive control. Then 40µL of 0.01% heat stable α-amylase (from *Bacillus leicheniformis)* was added. After incubation at 80°C for 20 minutes, the mixture was diluted to 10mL and 1mL was incubated with 2mL of 0.1M sodium acetate buffer (pH 4.75) and 30µL of 0.1% amyloglucosidase from *Rhizopus* mold for 30 minutes at 60°C. Glucose released was measured on the glucose analyzer.

2.6 Protein Glycation

A simple *In vitro* system was employed to assess protein glycation based on the use of insulin as a model substrate [29]. In brief 100µL of human insulin (1mg/ml) was incubated in 10mM sodium phosphate buffer (pH 7.4) with 220mM D-glucose, DFAETC or aminoguanidine 44mM (positive control) for 24h. Sodium cyanoborohydride was added and the reaction was stopped by addition of 0.5M acetic acid. Glycated and non-glycated insulin were separated and quantified using reversed-phase high performance liquid chromatography [29].

2.7 Statistical Analysis

All results are expressed as mean±S.E.M (Standard Error of the Mean) for a given number of independent observations (n). Groups of data were compared statistically using unpaired Student's t test. Results were considered significant if $p<0.05$.

3. RESULTS

3.1 Insulin Secretion Studies

Insulin release from BRIN BD11 cells was increased significantly in a dose-dependent manner over DFAETC concentration gradient (0.01-25mg/mL) in the presence of 5.6mM glucose (Fig. 1). The minimum effective concentration of DFAETC was at 1mg/mL and

above (2.8-18 fold increase in insulin release ($p<0.001$) Fig. 1). Cell viability remained unchanged up to 10mg/mL (data not shown). Extract initiated/augmented insulin secretion in the absence and presence of 16.7mM glucose highly significantly ($p<0.001$, Fig. 2). Verapamil and diazoxide significantly reduced the glucose-stimulated insulin releasing effect of DFAETC by 37% ($p<0.001$) and 43% ($p<0.001$), respectively. Moreover, insulin secretion in the presence of 3-isobutyl-1-methylxanthine (IBMX) and 5.6mM glucose was significantly enhanced 2.3-fold ($p<0.01$) by DFAETC. In addition, the extract has significantly increased insulin secretion in depolarised cells but not in cells stimulated with 200µM tolbutamide or 200µM glibenclamide (Fig. 2). The insulin stimulatory effects of DFAETC (5 and 10mg/mL) were reduced by 60% ($p<0.001$) and 15% ($p<0.05$), in Ca^{2+} free conditions (Fig. 3). However, DFAETC (10mg/mL) seemed to exert part of its stimulatory effect on insulin release in a Ca^{2+}-independent manner.

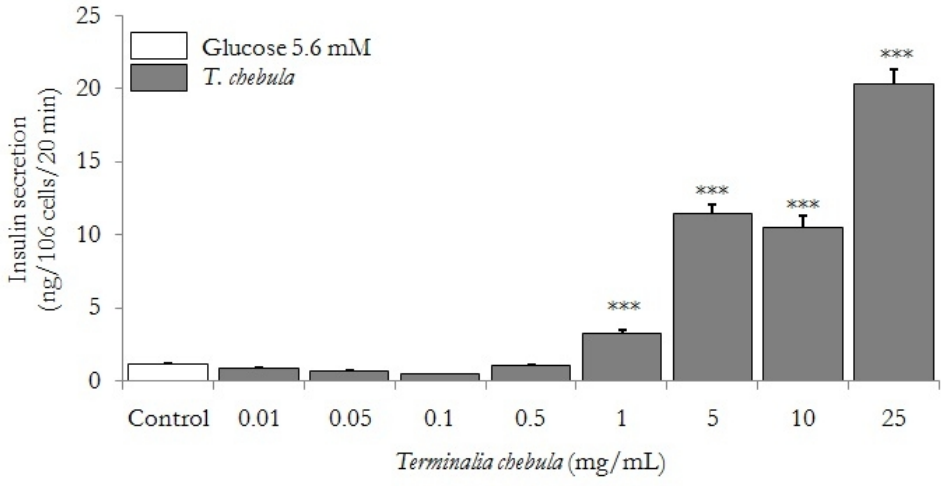

Fig. 1. Effects of DFAETC on insulin release *In vitro*

*Each bar indicates the mean±S.E.M of eight independent observations. ***P<0.001 compared to 5.6 mM glucose alone (control)*

3.2 Insulin Action

DFAETC (1mg/mL) by itself had no significant effect on basal 2-[^{3}H] deoxyglucose transport. Compared to 10^{-6}M insulin, DFAETC co-incubated with 10^{-9}M insulin, however, caused a marked 2.6-fold ($p<0.001$) increase in insulin-induced 2-[3H] deoxyglucose transport in 3T3L1 adipocytes (Fig. 4).

3.3 Starch Digestion

Using acarbose (1mg/mL) as a positive control, glucose liberation from starch was inhibited by 98.9% (1.1±0.5% glucose liberated compared with 99.6±1.6% for control, $p<0.001$). The inhibition of starch digestion achieved by DFAETC at 5.0mg/mL was 84% ($p<0.001$). Fig. 5 demonstrates the substantially significant ($p<0.001$) dose dependent inhibitory effects of DFAETC (0.5-5.0mg/mL) on starch digestion (22-84%).

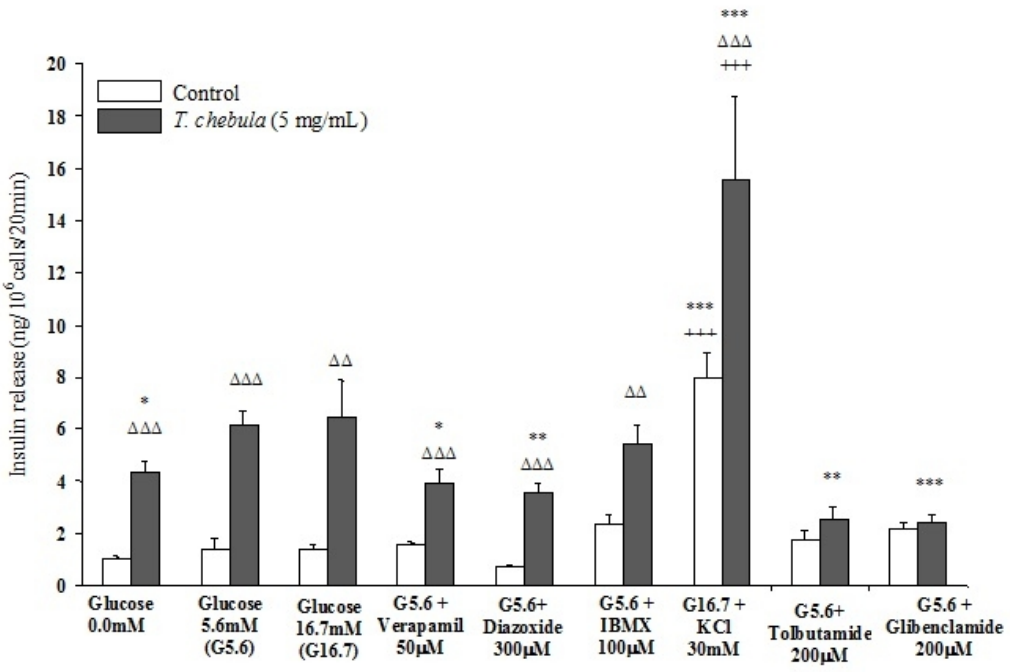

Fig. 2. Modulation of DFAETC-induced insulin secretion by established stimulators and inhibitors of beta cell function

*Each bar indicates the mean±S.E.M of eight independent observations.*P<0.05, **P<0.01, ***P<0.001 compared to glucose (control) in presence or absence of plant extract. $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared to the respective incubations in absence of plant extract. $^{+++}$P<0.001 compared to 16.7mM glucose in presence or absence of plant extract*

3.4 Glycation Studies

Amino guanidine (44mM) inhibited insulin glycation substantially by 81% ($p<0.001$). As shown in Fig. 6, DFAETC effected a significant 28±1.7% decrease in insulin glycation at 1mg/mL. However, at 10 and 25mg/mL, DFAETC increased % glycation of human insulin with 18% ($p<0.05$) and 32% ($p<0.001$) respectively above the basal % glycated insulin. RP-HPLC analysis of DFAETC with insulin alone in the absence of glucose revealed interfering peaks that co-eluted with nonglycated insulin fractions. Therefore no final conclusions could be drawn with this analysis method.

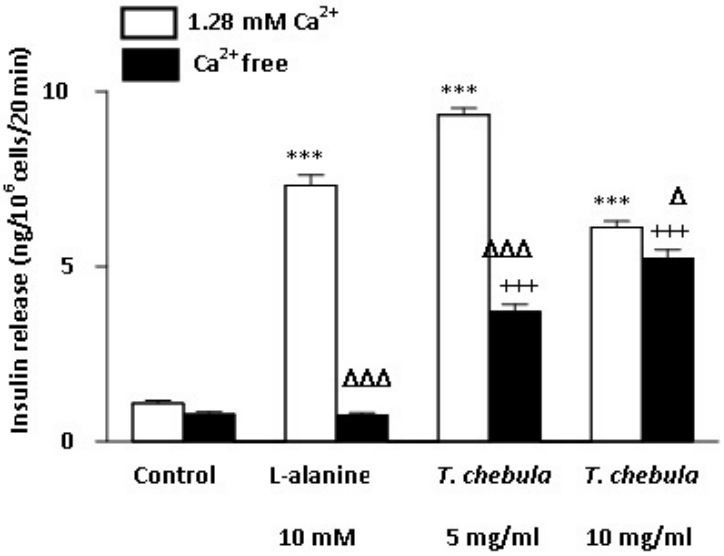

Fig. 3. Effects of DFAETC on insulin release in presence and absence of extracellular Ca^{2+}

*Each bar indicates the mean±S.E.M of eight independent observations. ***P<0.001 compared to 1.28 mM Ca^{2+}. $^{+++}$P<0.001 compared to respective Ca^{2+} free incubations. $^{\Delta}$P<0.05 and $^{\Delta\Delta\Delta}$P<0.001 compared to the respective compound in the presence of Ca^{2+}*

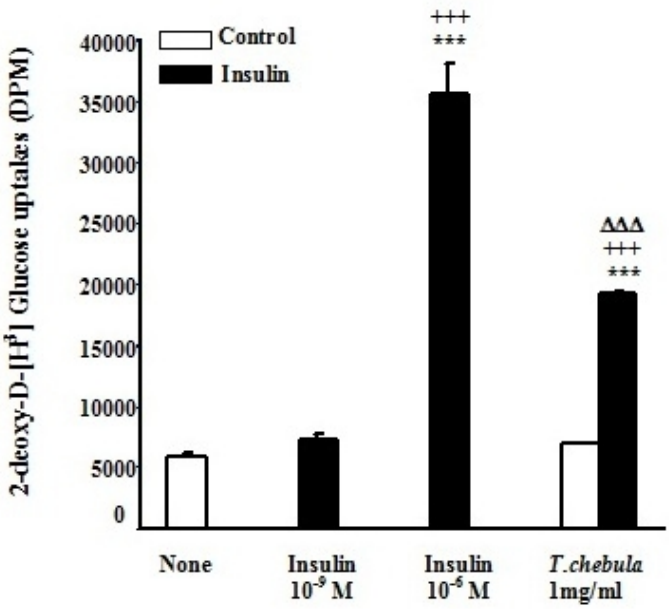

Fig. 4. Effects of DFAETC on 2-deoxy-D-[H^3] glucose transport *In vitro*.

*Each bar indicates the mean±S.E.M of four independent observations. ***P<0.001 compared with incubations in the absence of insulin. $^{+++}$P<0.001 compared to 10^{-9} M insulin alone. $^{\Delta\Delta\Delta}$P<0.001 compared to* DFAETC *incubations without insulin.*

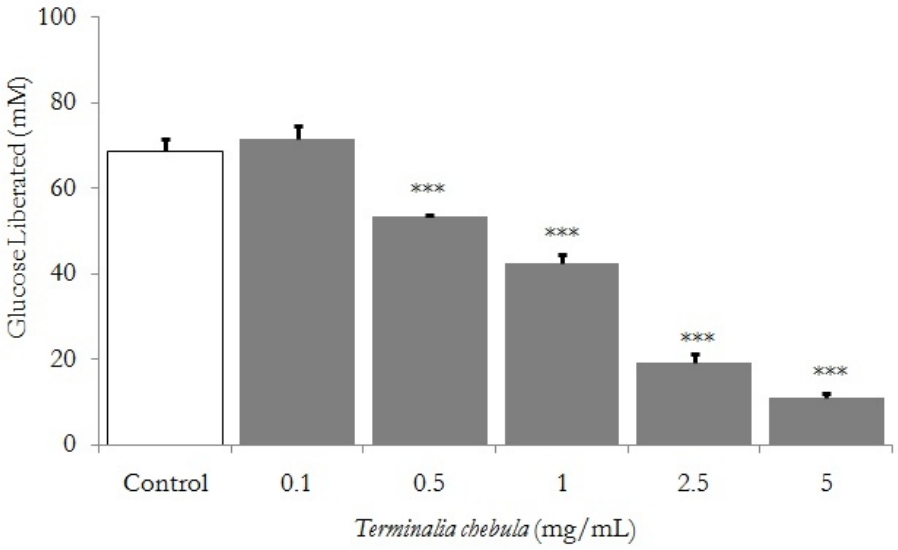

Fig. 5. Effects of DFAETC on enzymatic starch digestion *In vitro*
*Each bar indicates the mean±S.E.M of three independent observations. ***P<0.001 compared to glucose liberated in absence of plant extract*

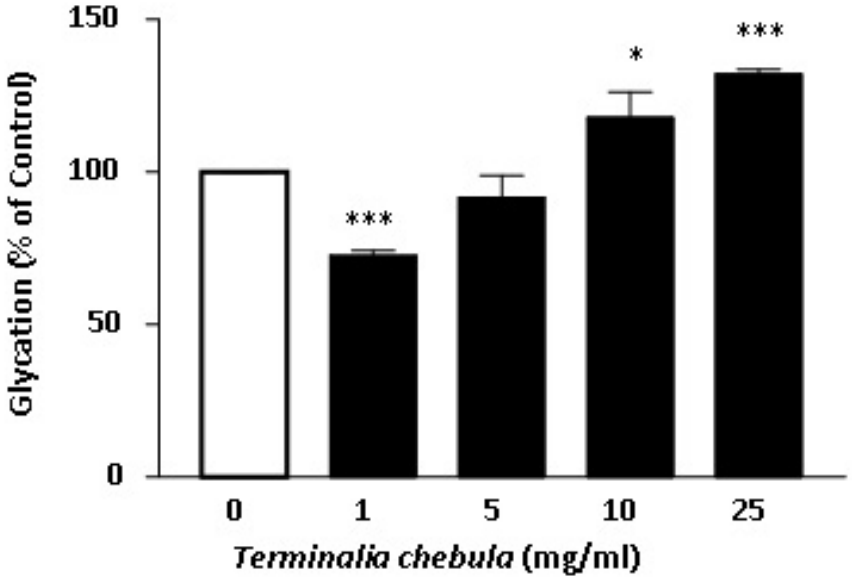

Fig. 6. Effects of DFAETC on protein glycation *In vitro*
*Each bar indicates the mean±S.E.M of three independent observations. *P<0.05 and***P<0.001 compared to glycation in the absence of plant extract*

4. DISCUSSION

DFAETC potentiated a dose dependent stimulation of glucose dependent insulin secretion at 5mg/mL and above. Removal of extracellular Ca^{2+} or cell viability studies argue against a simple cytotoxic action at the concentrations tested. The observations that verapamil decreased the ability of herbal extracts to stimulate insulin output further support the hypothesis that the insulinotropic actions of DFAETC are β-cell Ca^{2+} dependent. The inhibitory effects of diazoxide in the acute herbal incubations point to the involvement of K_{ATP} channel closure in the stimulatory actions of DFAETC. As noted for sulphonylureas, the acute stimulatory effects of DFAETC were evident in β-cells depolarized by 30 mM KCl and 16.7mM glucose, indicating K_{ATP} channel independent effects. In the presence of IBMX, DFAETC amplified synergistically insulin release, suggesting that cAMP potentiated DFAETC-evoked insulin release. These highly substantial findings can lend further weight to *In vivo* correlates. Comparable to tolbutamide and glibenclamide, *T. chebula* extracts (aqueous or ethanolic) produced highly significant improvement in oral glucose tolerance in streptozotocin diabetic rats and reduced elevated blood glucose significantly, with marked improvements in serum insulin levels as well as the HbA1c, in contrast to untreated diabetic animals [30-31]. Furthermore, the research teams indicated that the insulin release from pancreatic islets was nearly two times more than that in untreated animals [30], as *T. chebula* extracts have increased the number of secretory granules of remnant β-cells in streptozotocin induced diabetic rats in electron microscopic studies [31].

Further to the marked pancreatic bioactivities of DFAETC, the responses observed using plant/insulin dual treatments in differentiated 3T3-L1 adipocytes wells were greater than additive, increasing sensitivity to insulin by almost 3-fold. Comparing 1mg/mL aqueous plant extracts to maximal insulin (10^{-6}M), indicates synergism due to insulin sensitization potencies of DFAETC. Taken together, extracts of plants tested (1mg/mL) maybe useful for alleviation of insulin resistance and in the study of the pathways leading to glucose utilization. Future studies on plant extract bioactive principles are needed to assess the possible novelty of the mechanisms involved. New approaches to the prevention of postprandial hyperglycemia may emerge from the therapeutic use of α amylase or α glucosidase inhibitors. Acarbose is the classic competitively reversible inhibitor that diminishes the rate of catalysis by reducing the proportion of enzyme molecules bound to a substrate. It is a pseudotetrasaccharide, having a structural similarity to glucose, with an antihyperglycemic effect by inhibiting intestinal α-glucosidase activities [32], accompanied by a significant reduction in HbA1c [33].

In the optimized and validated enzymatic system, acarbose completely abolished glucose liberation from starch at a dose of 1000µg/mL. DFAETC effective doses (0.5-5.0mg/mL) were found to reduce glucose liberation from starch significantly. Chebulic acid, isolated from *T. chebula*, was proved most recently to be a potent reversible and non competitive inhibitor of α-glucosidase, further implying its use in managing type 2 diabetes [34]. Further extrapancreatic effects are ascribed to DFAETC, as it exhibited significant decrease in glycated insulin at 1mg/mL, but not the aqueous concentration gradient (5-50 mg/mL). However, the antioxidative-antiglycation link [35] may offer explanation, as *T. chebula* extracts proved to be strong antioxidants preventing microsomal lipid peroxidation [20,36], mainly attributed to the high phenolic content of *T. chebula* [37]. There has been much work done to elaborate on the etiology, prevention and treatment of diabetes related complications. The presence of AGEs –advanced glycation end products- is closely related to hyperglycemia and their pathobiochemistry could explain many changes observed in diabetes related complications [38]. In addition, insulin glycation in β-cells can contribute to

insulin resistance [39-41]. This is based on the fact that glycated insulin has reduced biological activity [42-44]. The main AGE inhibitor discovered is aminoguanidine [38]. This suggests that pharmacological intervention/ dietary measures/ herbal supplementations to prevent insulin glycation may provide an effective strategy for improving the blood glucose control and serve as a prophylactic therapy of diabetic complications.

5. CONCLUSIONS

Succinctly, this study has highlighted that DFAETC demonstrated highly significant dose dependent inhibitory effects on enzymatic starch digestion like acarbose. Despite the lack of insulin mimetic effect, DFAETC (1mg/mL) combined with 10^{-9} M insulin caused potentiation in insulin sensitivity in 3T3L1 fat cells. At pancreatic cellular levels, DFAETC initiated a stimulation of basal (no glucose) insulin release and potentiated glucose-evoked insulin output dose dependently. DFAETC 1mg/mL (only) had a significant inhibition of insulin glycation. Future work is required to purify and characterize the active components of DFAETC to bring forward potential novel agents for integrated diabetes management.

CONSENT

Not applicable.

COMPETING INTERESTS

The authors declare that they have no conflict of interest concerning this article.

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