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Terminalia bellirica stimulates the secretion and action of insulin and inhibits starch digestion and protein glycation *in vitro*

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Traditional plant treatments have been used throughout the world for the therapy of diabetes mellitus. The aim of the present study was to investigate the efficacy and mode of action of *Terminalia bellirica* used traditionally for the treatment of diabetes in India. *T. bellirica* aqueous extract stimulated basal insulin output and potentiated glucose-stimulated insulin secretion concentration-dependently in the clonal pancreatic β-cell line, BRIN-BD11 ($P<0{\cdot}001$). The insulin-secretory activity of the plant extract was abolished in the absence of extracellular Ca^{2+} and by inhibitors of cellular Ca^{2+} uptake, diazoxide and verapamil ($P<0{\cdot}001$; n 8). Furthermore, the extract did not increase insulin secretion in depolarised cells and did not further augment insulin secretion triggered by tolbutamide or glibenclamide. *T. bellirica* extract also displayed insulin-mimetic activity and enhanced insulin-stimulated glucose uptake in 3T3-L1 adipocytes by 300 %. At higher concentrations, the extract also produced a 10–50 % ($P<0{\cdot}001$) decrease in starch digestion *in vitro* and inhibited protein glycation ($P<0{\cdot}001$). The present study has revealed that components in *T. bellirica* extract stimulate insulin secretion, enhance insulin action and inhibit both protein glycation and starch digestion. The former actions are dependent on the active principle(s) in the plant being absorbed intact. Future work assessing the use of *T. bellirica* as a dietary adjunct or as a source of active anti-diabetic agents may provide new opportunities for the treatment of diabetes.

***Terminalia bellirica*: Insulin: Diabetes**

Type 2 diabetes is characterised by deranged metabolism and inappropriate hyperglycaemia, resulting from defects in the secretion and cellular action of insulin. Treatments aimed at enhancing β-cell function and reducing insulin resistance are therefore key to improving metabolic control and retarding the development of diabetic complications. At least 250 million individuals worldwide suffer diabetes and it is estimated that by 2030 this number will double[1]. Diabetes is one of the top five most significant diseases in the developed world. Based on WHO recommendations, anti-diabetic agents of plant origin are important for use in traditional medicine[2]. Man has long turned to plants as a source of readily available and innovative medicines[3]. It is estimated that at least 75 % of the world's population relies significantly on plant medicines[4]. These considerations have led to a renaissance of nutritional, clinical and scientific interest in the potential of plant treatments from across the world for diabetes therapy[5–7]. Recent studies have explored the insulinotropic effects of aqueous extracts of traditional plants on perfused pancreas and similar effects were observed by *in vitro* testing of clonal pancreatic β-cell lines[6,7].

The fruit of *Terminalia bellirica* has been used in traditional medicine for the treatment of anaemia, asthma, cancer, colic, constipation, diarrhoea, dysuria, headache, hypertension, inflammation and rheumatism[8]. Several studies have shown that the fruit contains termilignan, thannilignan, 7-hydroxy-3′,4′-(methylenedioxy) flavone, anolignan B, gallic acid, ellagic acid, β-sitosterol, arjungenin, belleric acid, bellericoside and cannogenol 3-*O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-L-rhamnopyranoside[9–10].

T. bellirica has many reported medicinal applications. The fruits of *T. bellirica* together with *T. chebula* and *Embilica officinalis* have been used traditionally for the treatment of diabetes and have been investigated for their anti-diabetic and antioxidant activities[11]. These studies have demonstrated that oral administration of a single dose of *T. bellirica* extract (100 mg/kg body weight) reduced blood glucose in normal and alloxan-treated (120 mg/kg) diabetic rats within 4 h. Furthermore, daily administration of extract for up to 11 d significantly reduced glucose from day 7 onwards[11]. Aqueous extracts of the bark or fruits of *T. bellirica* have also been used for a variety of ailments in humans, including diarrhoea, leucoderma, impotence, bronchitis, cold, cholera, respiratory tract infections, anaemia, haemorrhoids, eye infections, as a brain tonic and as a diuretic to remove kidney stones[12–14].

The aim of the present study was to investigate the anti-diabetic actions of *T. bellirica* fruit extract on insulin secretion and glucose uptake at the cellular level. Furthermore, possible effects on protein glycation and starch digestion were examined *in vitro*.

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Materials and methods

Plant material preparation

Dried fruits of *T. bellirica* (Gaertn.) Roxb. (family: Combretaceae) were procured from a commercial supplier in Delhi, and available in Europe (batch T/002080) from Top-Op (Foods) Ltd (Stanmore, Middlesex, UK). For *in vitro* work, a decoction was prepared by bringing 25 g/l of material to the boil in water. Once boiling, the suspension was removed from the heat and allowed to infuse over 15 min. The suspension was filtered (Whatman no. 1 filter paper) and the volume adjusted so the final concentration was 25 g/l. Samples, 1 ml, of the filtered plant solution were brought to dryness under vacuum (Savant Speed-vac; Savant Instrumentation, Inc., Farmingdale, NY, USA). Dried fractions were stored at −20°C until required. Fractions were reconstituted in incubation buffer for subsequent experiments as required.

Insulin secretion

Insulin release was determined using monolayers of BRIN-BD11 clonal pancreatic cells[15]. BRIN-BD11 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 tissue culture medium containing 11·1 mM-glucose, 10 % fetal calf serum and antibiotics (50 000 IU penicillin–streptomycin/litre), and maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % air. At 24 h before acute experiments, cells were harvested and seeded in twenty-four-well plates at a density of $1{\cdot}0 \times 10^5$ cells per well. Following overnight attachment, the culture medium was removed and cells were preincubated for 40 min at 37°C with 1 ml Krebs–Ringer bicarbonate buffer supplemented with 1·1 mM-glucose and 1 % bovine serum albumin. Subsequent test incubations were performed for 20 min at 5·6 mM-glucose using a similar buffer supplemented with the aqueous plant extract and the agents indicated in the figures. Samples were stored at −20°C for subsequent insulin RIA[16]. Cell viability was assessed after 20 min incubation using a modified neutral red assay as described previously[17].

Adipocyte differentiation and cellular glucose uptake

3T3-L1 fibroblasts obtained from the American Type Culture Collection (Manassas, VA, USA) were used to determine glucose uptake[18]. Cells (passages 5–10) were seeded in twelve-well plates at a density of $1{\cdot}0 \times 10^5$ cells per well, maintained at 37 ± 2°C with 5 % CO_2 and fed every 2 d with Dulbecco's modified Eagle's medium supplemented with penicillin (50 U/ml), streptomycin (50 μl/ml) and fetal bovine serum (10 %, v/v). Adipocyte differentiation was initiated as described in detail elsewhere by the addition of insulin (1 μg/ml), 0·5 mM-3-isobutyl-1-methylxanthine and 0·25 μM-dexamethasone[17]. Before acute tests, cells were incubated in serum-free Dulbecco's modified Eagle's medium for 2–3 h to establish basal glucose uptake. Cellular glucose uptake was determined for 15 min at 37°C using Krebs–Ringer bicarbonate buffer supplemented with ^{3}H-labelled 2-deoxyglucose (18·5 kBq (0·5 μCi)/well), 50 mM-glucose, insulin and other test agents as indicated in the figures. Hexose uptake was terminated after 5 min by three rapid washes with ice-cold PBS, after which cells were detached by the addition of 0·1 % SDS and subsequently lysed. Scintillation fluid was added to solubilised cell suspensions and mixed thoroughly. Radioactivity was measured on a Wallac 1409 Scintillation Counter (Wallac, Turku, Finland).

Starch digestion

To assess *in vitro* starch digestion, 100 mg soluble starch (Sigma-Aldrich, St Louis, MO, USA) was dissolved in 3 ml distilled water in the absence and presence of plant extract or acarbose (50 μg/ml) (Bayer AG, Leverkusen, Germany) as a positive control. Then 40 μl of 0·01 % heat-stable α-amylase (from *Bacillus leicheniformis*; Sigma-Aldrich) was added. After incubation at 80°C for 20 min, the mixture was diluted to 10 ml and 1 ml was incubated with 2 ml of 0·1 M-sodium acetate buffer (pH 4·75) and 30 μl of 0·1 % amyloglucosidase from Rhizopus mold (Sigma-Aldrich) for 30 min at 60°C. Glucose released was measured on the Analox GM9 glucose analyser (Analox Instruments, London, UK).

Protein glycation

A simple *in vitro* system was employed to assess protein glycation based on the use of insulin as a model substrate[19]. In brief, 100 μl human insulin (1 mg/ml) was incubated in 10 mM-sodium phosphate buffer (pH 7·4) with 220 mM-D-glucose, plant extract or aminoguanidine (positive control) for 24 h. Sodium cyanoborohydride was added and the reaction was stopped by the addition of 0·5 M-acetic acid. Glycated and non-glycated insulin was separated and quantified using reversed-phase HPLC[19].

Statistical analysis

All results are expressed as mean values with their standard errors for a given number of observations (n). Groups of data were compared statistically using the unpaired Student's t test. Results were considered significant if $P < 0{\cdot}05$.

Results

Insulin secretion studies

Insulin release from BRIN-BD11 cells was increased significantly in a dose-dependent manner by the aqueous extract of *T. bellirica* over the concentration range (0·5–10 mg/ml) (Fig. 1). Furthermore, L-alanine (10 mM) was used as a positive control and it significantly enhanced insulin secretion at 5·6 mM-glucose (7·3 (SEM 0·3) *v.* 1·2 (SEM 0·2) ng/10^6 cells per 20 min (control); $P < 0{\cdot}001$; n 8). Cell viability over 20 min incubations with the plant extract was unchanged at concentrations up to 5 mg/ml and was decreased by 10 % at 10 mg/ml (data not shown). The extract enhanced insulin secretion in the absence and presence of 16·7 mM-glucose. Both diazoxide (300 μM) and verapamil (50 μM) abolished *T. bellirica* (0·5 mg/ml)-induced insulin secretion (Fig. 2). Furthermore, the extract did not significantly increase insulin secretion in depolarised cells nor in cells stimulated with 200 μM-tolbutamide or 200 μM-glibenclamide (Fig. 2). In addition, the increase of the insulin release in depolarised

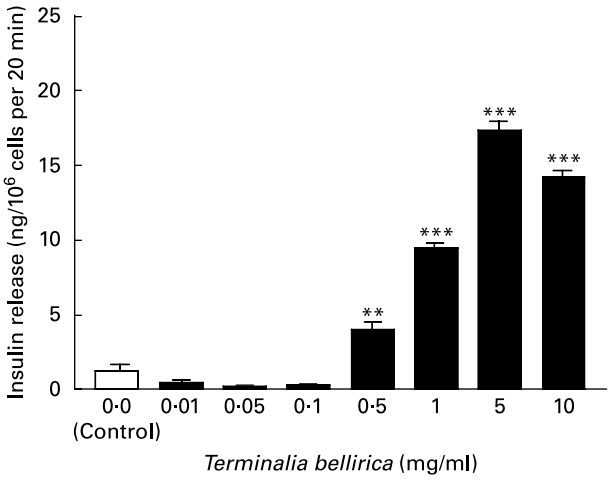

Fig. 1. Effect of *Terminalia bellirica* extract (■) compared with 5·6 mM-glucose (control) (□) on insulin release. Values are means of eight separate observations, with standard errors represented by vertical bars. Mean value was significantly different from that of the control treatment: ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.

cells (30 mM-KCl) was significantly less in the presence of the plant extract. The insulin-secretory activity of *T. bellirica* was abolished in the absence of Ca (Fig. 3).

Insulin action

The aqueous extract of *T. bellirica* (1 mg/ml) enhanced the basal [^{3}H]deoxyglucose uptake in a similar magnitude to 10^{-9} M-insulin ($P<0{\cdot}05$; Fig. 4). The combined actions of the extract and insulin further exceeded the effects of either alone ($P<0{\cdot}001$; Fig. 4).

Starch digestion

Incubation with the aqueous extract (10–50 mg/ml) resulted in a 10–78 % decrease in enzymic liberation of glucose from starch (Fig. 5). Using acarbose (1 mg/ml) as a positive control, glucose liberation from starch was inhibited by 95 % (4·7 (SEM 0·5) % glucose liberated compared with 99·6 (SEM 1·6) % for control; $P<0{\cdot}001$).

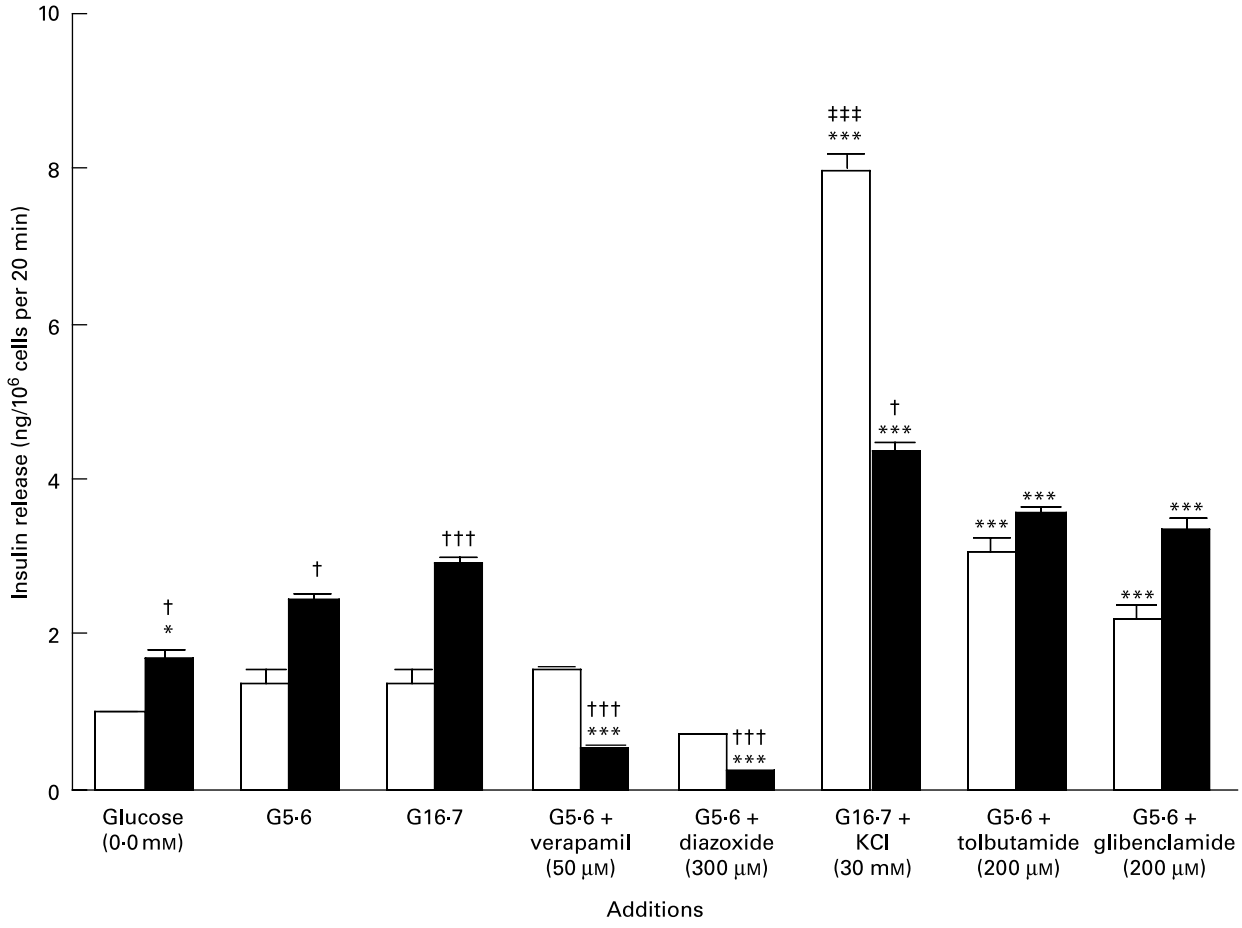

Fig. 2. Modulation of *Terminalia bellirica* extract-induced insulin secretion by established stimulators and inhibitors of β-cell function. (□), Control; (■), *T. bellirica* (0·5 mg/ml); G5·6, 5·6 mM-glucose; G16·7, 16·7 mM-glucose. Values are means of eight separate observations, with standard errors represented by vertical bars. Mean value was significantly different from that of the G5·6 control in the presence or absence of the plant extract: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$. Mean value was significantly different from that of the respective incubation in the absence of the plant extract: † $P<0{\cdot}05$, ††† $P<0{\cdot}001$. ‡‡‡ Mean value was significantly different from that of the 16·7 mM-glucose condition in the presence or absence of the plant extract ($P<0{\cdot}001$).

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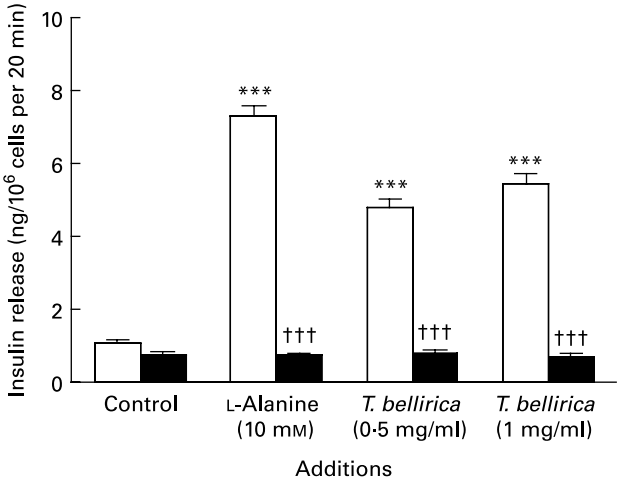

Fig. 3. Effects of *Terminalia bellirica* extract on insulin release in the presence of 1·28 mM-Ca^{2+} (□) and in the absence of Ca^{2+} (■). Values are means of eight separate observations, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the control in the presence of 1·28 mM-Ca^{2+} only ($P<0·001$). ††† Mean value was significantly different from that of the respective compound in the presence of 1·28 mM-Ca^{2+} ($P<0·001$).

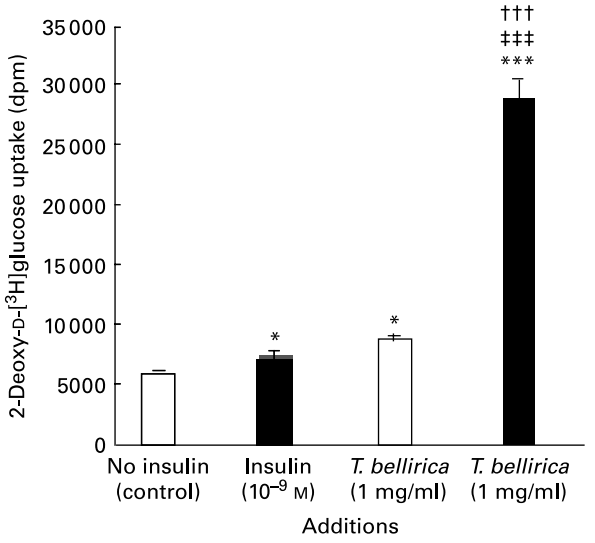

Fig. 4. Effects of *Terminalia bellirica* extract and the presence of 10^{-9} M-insulin (■) or the absence of insulin (□) on 2-deoxy-D-[^{3}H]glucose transport. Values are means of four separate observations, with standard errors represented by vertical bars. Mean value was significantly different from that of the no-insulin (control) condition: * $P<0·05$, *** $P<0·001$. ††† Mean value was significantly different from that of the *T. bellirica* incubation without insulin ($P<0·001$). ‡‡‡ Mean value was significantly different from that of the 10^{-9} M-insulin condition ($P<0·001$).

Glycation studies

The aqueous extract of *T. bellirica* exerted a partial concentration-dependent inhibition of insulin glycation (Fig. 6). At 25 and 50 mg/ml glycation was inhibited by 42–66 %. Aminoguanidine (50 mM) inhibited glycation by 75 % in this system.

Discussion

The aqueous extract of *T. bellirica* stimulated insulin secretion concentration-dependently from BRIN-BD11 cells at levels of 0·5 mg/ml and above. Cellular viability was not affected, confirming that the insulinotropic effect was not due to simple leakage of insulin from the cells. Depletion of extracellular Ca^{2+} or incubation with either verapamil (inhibitor of voltage-gated Ca^{2+} channels) or diazoxide (opener of K-ATP channels)[20] inhibited the stimulatory effect, suggesting the importance of Ca^{2+} uptake in the mode of action of the plant constituents. Consistent with this view, the stimulatory effects of agents that indirectly depolarise the cell by inhibiting K-ATP channels (the sulfonylureas tolbutamide and gilbenclamide) were not affected by the *T. bellirica* extract. However, the aqueous extract of *T. bellirica* partially attenuated the elevation in insulin secretion induced by the direct effect of the membrane-depolarising agent KCl.

In addition to the prominent β-cell-stimulatory effects, *T. bellirica* enhanced cellular glucose transport in differentiated 3T3-L1 adipocytes. At 1 mg/ml, its stimulatory actions were similar to those of insulin (10^{-9} M) alone. In addition, the combined actions of the extract and insulin exceeded the effects of either agent alone. The synergistic actions of insulin and *T. bellirica* extract on the enhancement of glucose uptake warrant further investigation to establish the dose–response relationship of these effects. Additional studies of the insulin-signalling pathway and effect of plant extract components are needed to assess the possible novelty of the mechanisms involved.

Using a simple *in vitro* test, consisting of the digestive enzymes α-amylase and α-glucosidase, the potential of *T. bellirica* to retard starch digestion was evaluated by its effect on glucose liberation. In this system, the established α-glucosidase inhibitor acarbose completely inhibited glucose liberation at the dose of 50 μg/ml. In contrast, the *T. bellirica* extract produced a significant 10–50 % reduction in starch digestion at concentrations of 10 mg/ml and above. Components of the extract responsible for this effect are unknown but it is noteworthy that several alkaloid compounds,

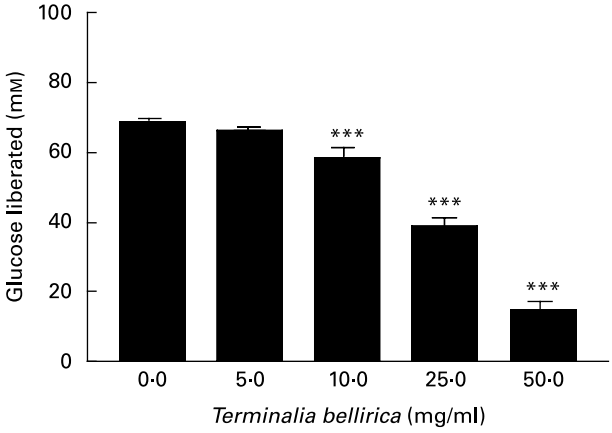

Fig. 5. Effects of *Terminalia bellirica* extract on starch digestion. Values are means of three separate observations, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the plant extract-absent condition ($P<0·001$).

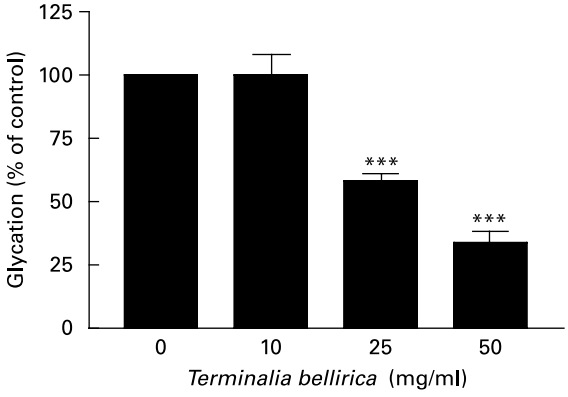

Fig. 6. Effects of *Terminalia bellirica* extract on protein glycation. Values are means of three separate observations, with standard errors represented by vertical bars. *** Mean value was significantly different from that of the plant extract-absent condition ($P<0{\cdot}001$).

including castonospermine from the seeds of *Castanosperum australe*, have established α-glucosidase-inhibitory action[3]. Moranoline (1-deoxynojirimycin), of which the therapeutic agent miglitol is a derivative, was originally isolated from mulberry root bark (*Mori cortex*)[21,22]. Several hydroxyflavonoid compounds have also been isolated from marjoram leaves, each exhibiting glucosidase-inhibitory activity[23].

In the final series of experiments, the ability of the *T. bellirica* extract to inhibit protein glycation was assessed using insulin as a model substrate[19]. It is also potentially interesting that insulin is normally glycated *in vivo* and that glycated insulin has reduced biological activity[24,25]. This raises the possibility that inhibition of protein glycation may have benefits in addition to serving as a prophylactic for diabetic complications. In this simple system, the classical inhibitor aminoguandine decreased insulin glycation by 80 %. Similarly, *T. bellirica* inhibited glycation by 42–66 % at 25–50 mg/ml. The effective concentration is substantially greater than that needed to influence the secretion and action of insulin, and therefore is less likely to reflect an important element of the anti-diabetic properties of this plant.

In conclusion, the present study has shown that an aqueous extract of fruit of *T. bellirica* stimulated both the secretion and action of insulin as well as inhibited starch digestion and protein glycation. The ability of plant constituents to influence these parameters in *vivo* depends entirely on soluble active principle(s) being absorbed via the gut. Further work is required to assess this and to isolate and characterise the active components to bring forward potential new agents for diabetic therapy.

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All authors have contributed to the conception and design of the experiments. V. K. and Y. H. A. A. W contributed to the experimental research. P. R. F. and Y. H. A. A. W contributed equally to the supervision of the research, analysis and preparation of the paper.

There is no conflict of interest to declare by any of the authors.

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