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Asparagus adscendens (Shweta musali) stimulates insulin secretion, insulin action and inhibits starch digestion

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Diabetes mellitus is a complex metabolic disease characterised by glucose overproduction and under-utilisation. As the incidence of diabetes expands rapidly across the globe there is an urgent need to expand the range of effective treatments. Higher plants such as *Asparagus adscendens* provide therapeutic opportunities and a rich source of potential antidiabetic agents. In the present study an aqueous extract of *Asparagus adscendens* was shown to induce a significant non-toxic 19–248 % increase in glucose-dependent insulinotropic actions ($P<0{\cdot}001$) in the clonal pancreatic β cell line, BRIN-BD11. In addition, the extract produced an 81 % ($P<0{\cdot}0001$) increase in glucose uptake in 3T3-L1 adipocytes. *Asparagus adscendens* also produced a 21 % ($P<0{\cdot}001$) decrease in starch digestion *in vitro*. The present study has revealed the presence of insulinotropic, insulin-enhancing activity and inhibitory effects on starch digestion in *Asparagus adscendens*. The former actions are dependent on the active principle(s) in the plant being absorbed intact. Future work assessing its use as a dietary adjunct or as a source of active components may provide new opportunities for the treatment of diabetes.

***Asparagus adscendens*: Diabetes: Insulin**

Diabetes mellitus is a complex metabolic disorder in which the control of blood glucose is of paramount importance (De Fronzo *et al.* 1992; Lilloja *et al.* 1993). Regimes that counter hyperglycaemia, including diet, oral anti-diabetic drugs and insulin form the cornerstone of available therapy (Clark, 1998; Laws, 2001). Through attempting to restore a near normal metabolic environment, such treatments help decrease the incidence of the long-term complications of the disease (Mandrup-Poulsen, 1998). Despite their usefulness, however, these treatments do not normalise blood glucose levels nor prevent the risk of developing diabetic complications (UKPDS, 1995; Amos *et al.* 1997). As the incidence of diabetes increases rapidly across the globe there is an urgent need to expand the range of effective palliatives available to sufferers.

Man has long turned to plants as a source of new and innovative medicines (Day, 1990). In 1980, 75 % of the world's population relied mainly upon plant medicines (Weragoda, 1980). Until recently this figure was mainly confined to developing regions. However, the recent explosion in the area of herbal medicine (MacLennan *et al.* 1996; Astin, 1998; Brevoort, 1998) has led to a renaissance of nutritional, clinical and scientific interest in the potential of plant treatments for diabetes across the world (Bailey & Day, 1989; Swanston-Flatt *et al.* 1991*a*,*b*; Gray & Flatt, 1997; Oubre *et al.* 1997).

Asparagus adscendens is commonly referred to as Shweta musali in India and Sutaid musk in Pakistan. According to Indian folklore its origins can be traced back to the oldest mountain ranges in India. Anecdotal uses of *Asparagus adscendens* include the treatment of diarrhoea, dysentery and general debility, it is also known as a galacatagogue, demulcent and tonic (Shinwari & Khan, 2000). The present study is the first to consider the anti-diabetic potential of *Asparagus adscendens*, and has investigated its effect on glucose uptake and insulin secretion at cellular level, and on starch digestion *in vitro*.

Materials and methods

Plant material

Dried rhizome of Shweta musali (*Asparagus adscendens*) was obtained from a commercial supplier in Delhi, India, during the winter season. Rhizome was homogenised to a fine powder and stored in opaque screw-top jars at room temperature (20 ± 2°C) until use. 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. Aliquots (1 ml) of the filtered plant solution were brought to dryness under vacuum (Savant Speedvac; Savant Instrumentation Inc., New York, USA). Dried fractions were stored at −20°C until required. Fractions were reconstituted in incubation buffer for subsequent experiments as required.

Insulin secretion in vitro

Insulin release was determined using monolayers of BRIN-BD11 clonal pancreatic cells (McClenaghan *et al.* 1996). This cell line is

Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; IBMX, 3-isobutyl-1-methylxanthine; KRB buffer, Krebs Ringer bicarbonate buffer.

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derived from electrofusion of NEDH (New England Deaconess Hospital) rat pancreatic β cells and rat insulinoma RINm5F cells. BRIN-BD11 cells were grown in RPMI-1640 tissue culture medium containing 11·1 mmol glucose/l, 10 % fetal calf serum and antibiotics (50,000 IU penicillin–streptomycin/l), and maintained at 37°C in an atmosphere of 5 % CO_2 and 95 % air. Prior (24 h) to 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, culture medium was removed and cells were preincubated with 1 ml Krebs Ringer bicarbonate (KRB) buffer for 40 min at 37°C. KRB buffer contained 115 mM-NaCl, 4·7 mM-KCl, 1·28 mM-$CaCl_2$, 1·2 mM-$MgSO_4$, 1·2 mM-KH_2PO_4, 25 mM-HEPES and 8·4 (w/v) $NaHCO_3$ (pH 7·4 with NaOH) supplemented with 1·1 mM-glucose and 1 % bovine serum albumin. Test solution consisted of KRB buffer supplemented with glucose, aqueous plant extract and various modulators of insulin secretion such as diazoxide (K^+-ATP channel opener), verapamil (voltage-dependent Ca^{2+} channel blocker), 3-isobutyl-1-methylxanthine (IBMX; phosphodiesterase inhibitor, increasing cellular cyclic AMP), KCl (inducing membrane depolarisation and Ca^{2+} influx) and tolbutamide (binds to sulphonylurea receptor component of K^+-ATP channel leading to closure, depolarisation and consequent Ca^{2+} influx). All tests were performed at 5·6 mM-glucose unless specified. Cells were incubated for 20 min at 37°C with test solution, after which a 900 μl aliquot was collected from each well and stored at −20°C for subsequent determination of insulin concentration (Flatt & Bailey, 1981). Cell viability was subsequently assessed using a modified neutral red assay (Hunt *et al.*, 1987). Following 20 min incubation with test agent, cells were washed three times with KRB buffer, and incubated for 2 h with 1 ml neutral red solution (50 μl neutral red solution dissolved in 50 μl dimethyl sulphoxide) and made up to 200 ml with KRB. After washing (as described earlier), 1 ml distilled water–ethanol–glacial acetic acid (49:50:1) was added and plates were gently agitated for 15 min. Absorbance of each well was read at 540 nm and means and their standard errors were calculated. Results were expressed as a percentage of control (incubations performed in the absence of test agent) giving percentage cell viability after 20 min exposure to test agent.

Intracellular calcium concentration and membrane potential studies

Changes in membrane potential and intracellular calcium concentration ($[Ca^{2+}]_i$) were determined fluorimetrically (Miguel *et al.* 2003) using monolayers of BRIN-BD11 cells. The fluorescent probes are internalised by living cells during preincubation and emit characteristic fluoresence in relation to changes in cellular membrane potential or $[Ca^{2+}]_i$. Cells were seeded into ninety-six-well plates (black walls, clear bottom; Costar, Roskilde, Denmark) at a density of $1{\cdot}0 \times 10^5$ viable cells per well and allowed to attach overnight in culture. The cells were washed once with KRB buffer (115 mM-NaCl, 4·7 mM-KCl, 1·28 mM-$CaCl_2$, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 10 mM-$NaHCO_3$ and 0·1 % (w/v) bovine serum albumin, pH 7·4) supplemented with 5·6 mM-glucose, 20 mM-HEPES and 500 μM-probenecid. The cells were incubated at 37°C for 1 h with either membrane potential assay kit or Ca^{2+} assay kit (Molecular Devices), prepared with the same washing buffer, to a final volume of 200μl. Fluorimetric data were acquired using a FlexStation scanning fluorimeter with integrated fluid transfer workstation (Molecular Devices). Excitation, emission and cut-off filter were set to 530, 565 and 550 nm for membrane potential, and 485, 525 and 515 nm for Ca^{2+}. The FlexStation was set to run for 10 min, collecting data at a 2·5 s interval (six readings per well). Test solutions (50 μl at 5-fold concentration) were transferred at 60 s from the start of readings at a rate of 78 ml/s.

Adipocyte differentiation and glucose uptake in vitro

3T3-L1 fibroblasts obtained from the American Type Culture Collection (Manassas, Virginia, USA), were used to determine glucose uptake (Frost & Lane, 1985). Cells were seeded in twelve-well plates at a density of $1{\cdot}0 \times 10^5$ cells per well 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). Cells were maintained at 37 ± 2°C and 5 % CO_2. Adipocyte differentiation was initiated 2–3 d post-confluency by the addition of culture medium containing 1 μg/ml insulin, 0·5 mM-IBMX (in dimethyl sulphoxide) and 0·25 μM-dexamethosone (in ethanol). Cells were maintained in this medium for 3 d followed by a further 3 d in medium supplemented with insulin (1 μg/ml) alone. Following this period cells were returned to original medium until experiment (after 1–2 d). Cells were used at passages five to ten. On the morning of experiment, cells were incubated in serum-free Dulbecco's Modified Eagle's Medium for 2–3 h to establish basal glucose uptake. Cell monolayers were given three rapid washes with warmed PBS, prior to the addition of KRB buffer (116 mM-NaCl, 4·7 mM-KCl, 1·28 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 24 mM-$NaHCO_3$, 10 mM-HEPES, pH 7·4) for 15 min at 37°C. During the experiment, groups of cells (*n* 4) were incubated with and without aqueous plant extract (5 mg/ml), in the absence and presence of a stimulatory concentration of insulin (10^{-9} M). Extracts (5 mg/ml) were also tested in the presence of known enhancers of insulin action, metformin (1 mM), orthovanadate (500 μM) and molybdate (30 mM) and a further group of cells was incubated with insulin (10^{-6} M), which was considered the maximal stimulatory dose. Following 15 min exposure to test reagents, hexose uptake was initiated by the addition of 50 μl tritiated 2-deoxyglucose (0·5 μCi/well) plus glucose (50 mM final concentration). 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 % sodium dodecyl sulphate and subsequently lysed. Scintillation fluid (8 ml) was added to solublised cell suspensions and mixed thoroughly. Activity was measured on a Wallac 1409 Scintillation Counter (Wallac, Turke, Finland) and data are expressed as dpm.

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 (50 mg/ml). Heat-stable α-amylase (40 μl, 0·01 %; from *Bacillus leicheniformis*; Sigma-Aldrich) was added and the solution vortexed and placed in a water-bath at 80°C for 20 min. The mixture was then diluted to 10 ml with distilled water. To 1 ml of this solution, 2 ml 0·1 M-sodium acetate buffer (pH 4·75) and 30 μl 0·1 % amyloglucosidase from Rhizopus mould (Sigma-Aldrich) were added and placed in a water-bath at 60°C for 30 min. The

glucose released was measured on the Analox GM9 glucose analyser (Analox Instruments, London, UK). Incubations performed with 50 μg/ml acarbose (Glucobay®; Bayer AG, Leverkusen, Germany) served as a positive control. Experiments were performed in triplicate.

Statistical analysis

All results are expressed as means and their standard errors for a given number of observations (*n*). Groups of data were compared statistically using unpaired Student's *t*-test. When experiments included more than two test groups one-way ANOVA followed by Tukeys *post hoc* test was used. Calculations were performed using GraphPad Prism version 3·00 for Windows (GraphPad Software, San Diego, CA, USA). Results were considered significant if $P < 0·05$.

Results

Insulin secretion studies

Asparagus adscendens exerted a concentration-dependent stimulatory effect on insulin secretion in BRIN-BD11 cells at 5·6 mM-glucose (Fig. 1). Cell viability remained 100 (SEM 4·0) % and was not compromised even at the highest concentrations of extract (data not shown). Extract-enhanced insulin secretion was further augmented in the presence of 16·7 mM-glucose (Table 1). Insulin secretion was abolished when incubations were performed in calcium-free KRBB (Fig. 2). Both diazoxide (300 μM) and verapamil (50 μM) abolished *Asparagus adscendens* (5 mg/ml) induced insulin secretion (Table 1). Furthermore, the extract did not significantly increase insulin secretion in depolarised cells (Table 1). *Asparagus adscendens* (5 mg/ml) did not augment the insulin secretory activity of 200 μM-tolbutamide ($P = 0·09$; Table 1). Similarly, IBMX (100 μM) retained its full insulinotropic effect in the presence of the extract ($P = 0·91$; Table 1).

Intracellular calcium concentration and membrane potential studies

Aqueous extract of *Asparagus adscendens* (5 mg/ml) generated depolarisation of the β cell. This produced a biphasic increase of $[Ca^{2+}]_i$, comprising a short sharp increase, followed by a gradual sustained increase (Fig. 3(A)). The initial increase in $[Ca^{2+}]_i$ largely declined before the addition of verapamil (50 μM), but addition of the voltage-dependent calcium channel antagonist had a small effect on $[Ca^{2+}]_i$ (Fig. 3(B)). Addition of diazoxide to cells previously exposed to extract produced a pronounced decrease in membrane potential level (Fig. 3(C)).

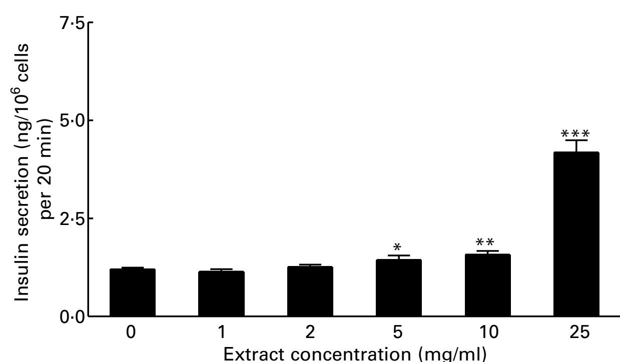

Fig. 1. Effects of aqueous extract of *Asparagus adscendens* on insulin secretion. Values are means with their standard errors depicted by vertical bars (*n* 7). For details of procedures, see p. 576. Mean values were significantly different from those of the control group (no extract): $^{**}P < 0·01$, $^{***}P < 0·001$.

Table 1. Effects of glucose, diazoxide, verapamil, 3-isobutyl-1-methylxanthine (IBMX), tolbutamide and depolarising conditions on the insulinotropic actions of aqueous extract of *Asparagus adscendens* (*aq*AA) in BRIN-BD11 cells (*n* 5–7)†

Test agent	Concentration (mmol/l)	Glucose (mmol/l)	*aq*AA (5 mg/ml)	Insulin secretion (ng/10^6 cells per 20 min)	
				Mean	SEM
None (control)	–	5·6	–	0·62	0·07
	–	5·6	+	1·41	0·11
	–	16·7	–	0·68	0·06
	–	16·7	+	4·53[abc]	0·35
Diazoxide	0·30	5·6	+	0·42	0·04
Verapamil	0·05	5·6	+	0·61	0·07
KCl	30	16·7	–	6·18[abc]	0·48
	30	16·7	+	7·68[abcd]	0·80
IBMX	0·10	5·6	–	1·27[d]	0·08
	0·10	5·6	+	2·99[a]	0·20
Tolbutamide	0·20	5·6	–	0·90	0·04
	0·20	5·6	+	1·51	0·22

[a,b,c,d] Mean values were significantly different ([a]$P < 0·001$ compared to 5·6 mM incubations performed in the absence of test agents, [b]$P < 0·001$ compared to 5·6 mM incubations performed in the presence of *aq*AA, [c]$P < 0·001$ compared to 16·7 mM incubations performed in the absence of test agents, [d]$P < 0·001$ compared to 16·7 mM incubations performed in the presence of *aq*AA; one-way ANOVA followed by Tukey's multiple comparison test).

† For details of procedures, see p. 576.

Glucose uptake

Asparagus adscendens (5 mg/ml) increased glucose uptake by 81 % in 3T3-L1 adipocytes ($P < 0·001$; Table 2). This effect was at least equivalent in magnitude to that of insulin (10^{-9} M). The combined actions of extract and insulin did not exceed the

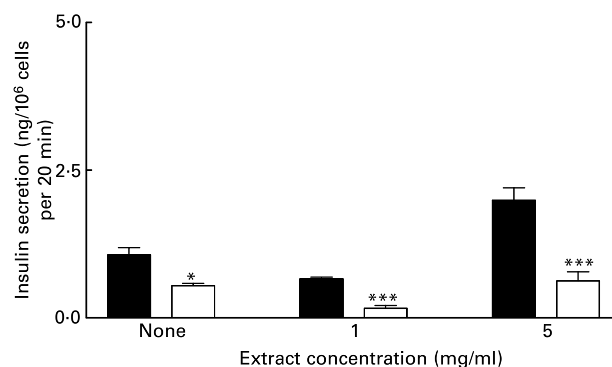

Fig. 2. Effects of Ca^{2+}-free conditions on the insulinotropic actions of *Asparagus adscendens*. BRIN-BD11 cells were incubated for 20 min in Krebs Ringer bicarbonate buffer with (■) and without (□) Ca^{2+}, in the absence and presence of aqueous extract of *Asparagus adscendens* (1 and 5 mg/ml). For details of procedures, see p. 576. Values are means with their standard errors depicted by vertical bars (*n* 6). Mean values were significantly different from those of the control groups (with Ca^{2+}): $^{*}P < 0·05$, $^{***}P < 0·001$.

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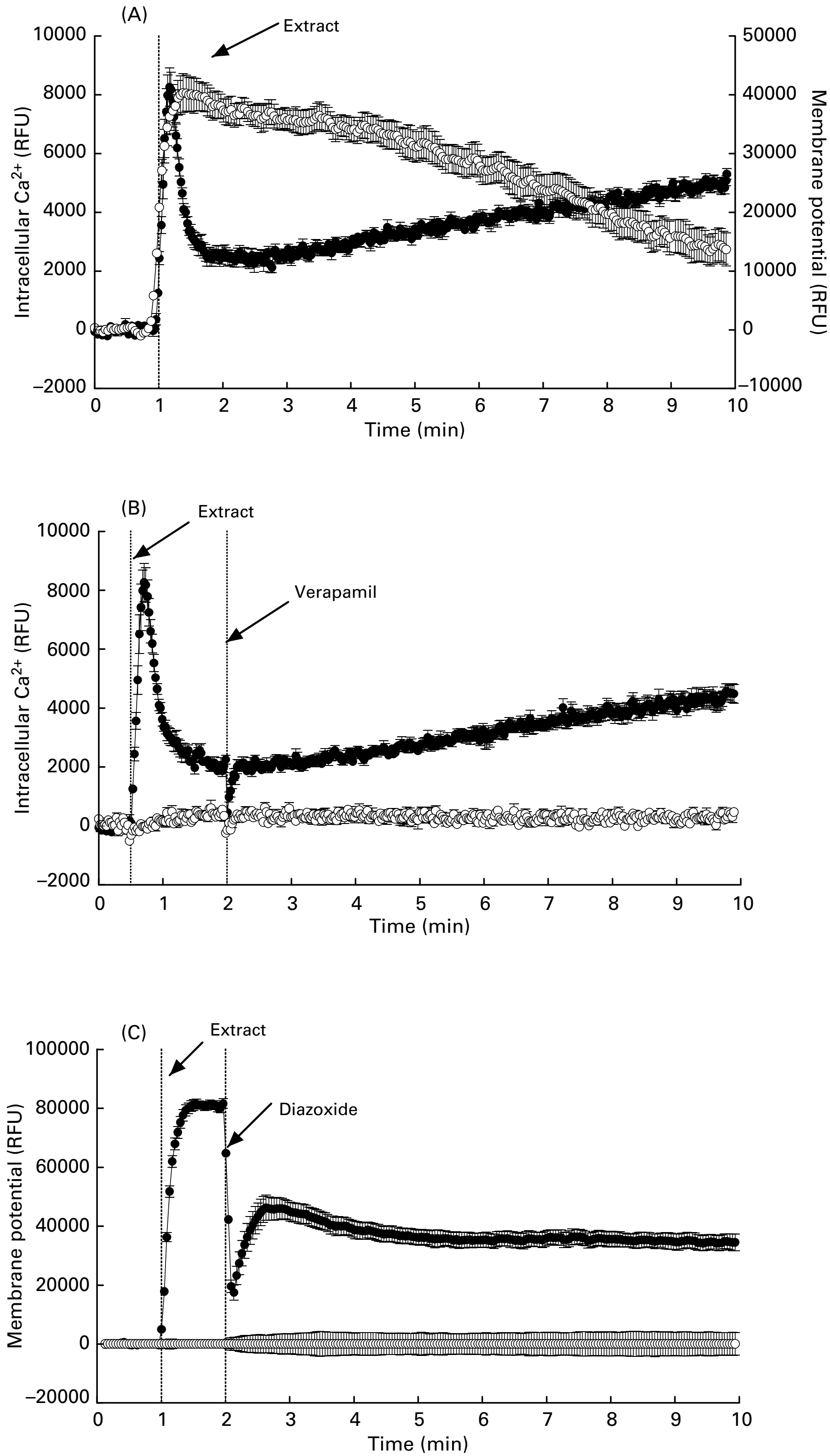

Fig. 3. (A), Effects of *Asparagus adscendens* (5 mg/ml) on intracellular Ca^{2+}(●) and membrane potential (○). (B), Effects of verapamil (50 μM) on intracellular Ca^{2+} levels in cells previously exposed to extract (●) (○, control). (C), Effects of diazoxide (300 μM) on membrane potential of cells previously exposed to extract (●). Cells were exposed to test agents as indicated by the arrows. For details of procedures, see p. 576. Results were normalised with a control group that received 5·6 mM-glucose during the full experimental time. Values are means with their standard errors depicted by vertical bars (*n* 6). RFU, Relative Florescence Unit.

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Table 2. Effects of aqueous extract of *Asparagus adscendens* (*aq*AA) on glucose uptake in 3T3-L1 adipocytes (*n* 5–7)†

Test agent	Concentration (mmol/l)	Insulin (mol/l)	*aq*AA (5 mg/ml)	Insulin action ($dpm \times 10^3$)	
				Mean	SEM
None (control)	–	–	–	42·4	4·7
	–	–	+	76·7[a]	2·9
	–	10^{-9}	–	70·8[a]	3·1
	–	10^{-9}	+	83·7[a]	1·0
	–	10^{-6}	–	107·6[ab]	0·9
Metformin	1	10^{-9}	–	79·6[a]	3·2
	1	10^{-9}	+	73·5[a]	2·0
Orthovanadate	0·5	10^{-9}	–	89·2[a]	2·8
	0·5	10^{-9}	+	84·5[a]	9·6
Molybdate	30	10^{-9}	–	89·0[a]	4·2
	30	10^{-9}	+	89·7[a]	1·5

[a,b] Mean values were significantly different ([a]$P<0·001$ compared to incubations performed in the absence of test agents; [b]$P<0·001$ compared to incubations performed in the presence of *aq*AA; one-way ANOVA followed by Tukey's multiple comparison test).
† For details of procedures, see p. 576.

effect of either alone. The extract did not improve the actions of metformin, orthovanadate or molybdate when tested at 10^{-9} M-insulin (Table 2).

Starch digestion

Incubation with aqueous extract (50 mg/ml) resulted in a 21 % decrease in glucose liberated from starch ($P<0·001$; Table 3). When incubations were performed using 25 mg/ml extract the effect did not reach significance ($P=0·562$). Incubations performed with acarbose (50 mg/ml) served as a positive control and inhibited glucose liberation from starch by 95 % ($P<0·001$; Table 3). Furthermore, the extract exhibited only one-quarter of the inhibitory activity of acarbose.

Discussion

Insulin secretion

Aqueous extract of the powdered rhizome of *Asparagus adscendens*, also known as Shweta musali, stimulated insulin secretion from BRIN-BD11 cells at levels of 5 mg/ml and above. Acute incubation did not affect viability at any of the doses tested, as assessed by neutral red assay (Hunt *et al.* 1987), confirming that the insulinotropic effect was physiological. Incubation with diazoxide abolished the stimulatory effect of extract, indicating an effect mediated through K^+-ATP channels (Dunn & Peters, 1995). Modulation of Ca^{2+} handling by the β cell is an integral part of *Asparagus adscendens*-induced insulin secretion. This is confirmed by inhibition of secretion by verapamil, or removal of extracellular Ca^{2+}. Like glucose and sulphonylurea drugs, inclusion of IBMX enhanced insulin secretion in the presence of extract. Fluorimetric determination of membrane depolarisation revealed a sustained depolarising phase in response to extract. This alteration in membrane potential was accompanied by a biphasic increase in intracellular Ca^{2+}. The present findings are consistent with the profile of insulinotropic activity observed in the presence of *Asparagus adscendens*. Diazoxide had a marked effect on cellular fluorescence in the presence of extract; this corresponds to a repolarising effect, again implicating involvement of the K^+-ATP channel in the actions of extract.

Despite the fact there was no statistical significance observed following concurrent incubation of *Asparagus adscendens* with tolbutamide and IBMX, there was a trend in augmenting insulin secretion. Insulin secretion was greatly enhanced when incubations were performed at 16·7 mM-glucose, indicating the action of the extract was glucose-dependent. The extract failed to significantly enhance insulin secretion in depolarised cells, suggesting that it does not operate through K^+-ATP channel-independent mechanisms. The failure of verapamil to prevent biphasic Ca^{2+} increase and abolish insulin secretion may dispute the present finding, although differences in kinetics between the two types of experiment could be relevant.

Glucose uptake

The ability of *Asparagus adscendens* to stimulate cellular glucose transport was examined using 3T3-L1 adipocytes. 3T3-L1 adipocytes have been extensively used in studies investigating insulin-mediated glucose transport (Foyt *et al.* 2000) and displays all components of the insulin receptor and signal transduction cascade. *Asparagus adscendens* had a direct effect on glucose uptake in 3T3-L1 adipocytes. At a concentration of 5 mg/ml, its stimulatory actions were similar to that of insulin (10^{-9} M) alone. Although not statistically significant, the small number of experiments performed suggested an 18 % increase in *Asparagus adscendens*-induced insulin-mediated glucose uptake. However, the extract clearly failed to augment the effects of orthovanadate or molybdate. This is suggestive of a shared, at least in part, mechanism of action. The rapid effect of *Asparagus adscendens* on glucose uptake may involve an effect on the redistribution of glucose transporters. However, further work would be needed to clarify this.

Starch digestion

Using an *in vitro* model, consisting of the digestive enzymes α-amylase and α-glucosidase, the potential of *Asparagus adscendens* to retard starch digestion was assessed by its effect on glucose liberation from starch. Incubation with the therapeutic α-glucosidase inhibitor, acarbose, validated the method, showing complete inhibition of glucose release liberation at a dose of 50 mg/ml. *Asparagus adscendens* produced a significant 21 % reduction in starch digestion, representing inhibitory effects on α-amylase and/or α-glucosidase activity. Despite, the *in vitro* inhibitory effects of *Asparagus adscendens* on starch digestion, this could be partially weighed down *in vivo* by release of

Table 3. Effects of aqueous extract of *Asparagus adscendens* and acarbose on starch digestion (*n* 3)†

Test conditions	Glucose liberated (%)		Reduction (%)‡
	Mean	SEM	
Control	99·6	1·6	–
Asparagus adscendens	79·5	2·6	21***
Acarbose	4·7	0·5	95***

Mean values were significantly different from those of the control group: ***$P<0·001$.
† Aqueous extract of *Asparagus adscendens* (50 mg/ml) or acarbose (50 μg/ml) were incubated with 100 mg starch in the presence of digestive enzymes *in vitro* and the amount of glucose released was measured.
‡ Unsupplemented control incubations were considered 100 %.

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excessive amounts of amylase during the digestive process. Due to the lack of substantial, reliable information on the chemical composition of the extract, mechanistic actions are purely speculative. Possible explanations include the presence of alkaloids, which are commonly found in plants. Several alkaloid compounds, including castonospermine from the seeds of *Castanosperum australe* (Rhinehart *et al.* 1987), have demonstrated α-glucosidase inhibitory action (Day, 1990). 1-Deoxynojirimycin (moranoline), of which the therapeutic agent miglitol is a derivative, was originally isolated from mulberry root bark (*Mori cortex*) (Yoshikuni, 1998). A recent study has also identified a plant extract, which inhibits α-glucosidase enzymes (Kurihara *et al.* 2003). Several hydroxyflavonoid compounds have been isolated from marjoram leaves, each exhibiting glucosidase inhibitory activity (Kawabata *et al.* 2003).

In conclusion, the present study has shown that aqueous extract of *Asparagus adscendens* stimulates both the secretion and action of insulin as well as inhibiting starch digestion. The plant's ability to influence insulin secretion and action *in vivo* depends entirely on soluble active principle(s) in the extract being absorbed via the gut. Future work directed at the purification and characterisation of active components may reveal new agents for diabetic therapy.

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