

Pancreatic and extra-pancreatic effects of the traditional anti-diabetic plant, *Medicago sativa* (lucerne)

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Medicago sativa (lucerne) is used as a traditional plant treatment of diabetes. In the present study, administration of lucerne in the diet (62.5 g/kg) and drinking water (2.5 g/l) reduced the hyperglycaemia of streptozotocin-diabetic mice. An aqueous extract of lucerne (1 mg/ml) stimulated 2-deoxy-glucose transport (1.8-fold), glucose oxidation (1.7-fold) and incorporation of glucose into glycogen (1.6-fold) in mouse abdominal muscle. In acute 20 min tests, 0.25–1 mg/ml aqueous extract of lucerne evoked a stepwise 2.5–6.3-fold stimulation of insulin secretion from the BRIN-BD11 pancreatic B-cell line. This effect was abolished by 0.5 mM-diazoxide, and prior exposure to extract did not affect subsequent stimulation of insulin secretion by 10 mM-L-alanine, thereby negating a detrimental effect on cell viability. The effect of extract was potentiated by 16.7 mM-glucose and by 1 mM-3-isobutyl-1-methylxanthine. L-Alanine (10 mM) and a depolarizing concentration of KCl (25 mM) did not augment the insulin-releasing activity of lucerne. Activity of the extract was found to be heat stable and largely acetone insoluble, and was enhanced by exposure to acid and alkali (0.1 M-HCl and NaOH) but decreased 25 % with dialysis to remove components with molecular mass < 2000 Da. Sequential extraction with solvents revealed insulin-releasing activity in both methanol and water fractions indicating a cumulative effect of more than one extract constituent. The results demonstrate the presence of antihyperglycaemic, insulin-releasing and insulin-like activity in the traditional antidiabetic plant, *Medicago sativa*.

Lucerne: Insulin: Diabetes mellitus

Traditional plant remedies continue to have widespread use for the treatment of diabetes, predominantly in countries where modern medicines are not readily available (Bailey & Day, 1989). However, few have received scientific or medical scrutiny and the World Health Organization (1980) has recommended that traditional plant treatments for diabetes warrant further evaluation.

Leaves of *Medicago sativa* (lucerne) are used traditionally as a tea to treat diabetes in South Africa (Lust, 1986). The use in human subjects of lucerne as an antidiabetic agent has, at least in part, been attributed to its relatively high Mn content (Lewis, 1949; Rubenstein *et al.* 1962*a,b*). Recent studies in streptozotocin-diabetic mice have confirmed the antihyperglycaemic efficacy of *Medicago sativa* (Swanston-Flatt *et al.* 1990) although no significant effect was observed in normal (non-diabetic) mice.

The present study was undertaken to investigate the mechanism(s) responsible for the anti-hyperglycaemic effect of *Medicago sativa*. Glucose transport and metabolism in mouse (abdominal) skeletal muscle, and insulin secretion by clonal BRIN-BD11 cells were determined during *in vitro* incubation with an aqueous extract of *Medicago sativa*.

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MATERIALS AND METHODS

Plant material

Dried lucerne leaves from a retail source in Birmingham, West Midlands, were homogenized to a fine powder and stored at room temperature ($20 \pm 2^\circ$) in opaque screwtop jars until use. An aqueous extract of lucerne was prepared by immersion of the powdered material at 25 g/l in boiling (distilled) water, and infusion for 15 min. The suspension was filtered (Whatman no. 1 filter paper) and the soluble extract was stored at -20° . For consumption as drinking fluid, the extract was diluted tenfold with tap water (2.5 g/l). For *in vitro* studies, portions of extract were brought to dryness under vacuum (Savant speedvac; Savant Instrumentation Incorp., Framingdale, NY, USA) and reconstituted in incubation buffer. To account for possible differences in potency, test and control incubations within a single experiment using isolated muscle or BRIN-BD11 cells were always conducted using the same batch of extract. This allowed for variation in potency of different batches of extract apparent in the same insulin release experiments.

Animal studies

Male mice derived from a colony maintained at Aston University, Birmingham, West Midlands (Flatt & Bailey, 1981) were used at 21–24 weeks of age. The mice were housed in an air-conditioned room at $22 \pm 2^\circ$ with a lighting schedule of 12 h light (08.00–20.00 hours) and 12 h dark. A standard pellet diet (Mouse Breeding Diet, Pilsbury Ltd, Birmingham, West Midlands) and tap water were supplied *ad libitum*. The experimental procedure for *in vivo* studies was similar to that previously described (Swanston-Flatt *et al.* 1990). Lucerne was incorporated into the diet (62.5 g/kg) and drinking water (2.5 g/l) of groups of six mice 5 d before and after intraperitoneal administration of streptozotocin (STZ; Sigma Chemical Co., Poole, Dorset) at 200 mg/kg body weight in 0.1 M-sodium citrate buffer (pH 4.5). Daily measures of body weight, food intake and fluid intake were made. Non-fasting blood samples obtained from the cut tail-tip of conscious mice were collected on days 12 and 20 for plasma glucose analysis (Stevens, 1971). Groups of six normal mice and six STZ-treated mice which received unsupplemented diet and drinking water *ad libitum* were used as controls.

Glucose transport and glucose metabolism in vitro

Recently weaned non-fasting male mice (3–5 weeks) were killed by cervical dislocation and squares of abdominal muscle (approximately 10–20 mg) were prepared. Incubations were performed using Krebs–Ringer bicarbonate buffer supplemented with 20 g/l insulin-free bovine serum albumin (KRB-BSA; 118 mM-NaCl, 25 mM-NaHCO₃, 5 mM-KCl, 1.28 mM-CaCl₂, 1.18 mM-MgSO₄, 1.17 mM-KH₂PO₄). Flasks sealed with rubber stoppers (Subaseal; Gallenkamp, London) were gently shaken throughout incubation and gassed with O₂–CO₂ (95 : 5, v/v) for 15–20 min before and for the initial 5 min of incubation.

To determine glucose uptake, muscle squares were incubated at 30° for 30 min in 1 ml KRB-BSA supplemented with 2 mM-sodium pyruvate, 3.7 kBq/ml 2-deoxy-D-[1-³H]glucose, 0.37 kBq/ml L-[1-¹⁴C]glucose (Amersham, Bucks.) in the presence and absence of 10^{-8} M-human insulin (Sigma) and 1 mg/ml extract of lucerne. After incubation, tissue was removed, blotted, hydrolysed in 0.5 ml 1 M-NaOH (85° , 1 h) and counted for ³H and ¹⁴C radioactivity in HiSafe II scintillant (Fisons, Loughborough, Leics.). The extracellular fluid volume of the muscle was determined from the amount of the non-transported L-

[1-¹⁴C]glucose, and this was taken into account in the calculation of tissue 2-deoxy-D-[1-³H]glucose uptake, expressed as disintegrations/min (dpm)/mg wet weight muscle per h.

Oxidative glucose metabolism to CO₂ and incorporation of glucose into glycogen were determined by incubation of muscle at 37° for 60 min in 1 ml KRB-BSA supplemented with 10 mM-glucose, 18.5 kBq/ml D-[U-¹⁴C]glucose (Amersham Life Science, Bucks.) in the presence and absence of 10⁻⁸ M-human insulin and 1 mg/ml extract of lucerne. After incubation, 0.1 ml 1 M-NaOH was carefully injected through the rubber stopper onto a corrugated filter-paper plug (Whatman no. 1) in a centre well. Flasks were then placed on ice for 5 min after which the tissue was removed for glycogen analysis. Flasks were quickly re-stoppered and 0.1 ml 3 M-HClO₄ was injected into the incubation medium and allowed to stand for 1 h at room temperature. ¹⁴C radioactivity of the filter paper was then counted in scintillant. CO₂ production was expressed as nmol CO₂/mg wet weight muscle per h. The incorporation of glucose into glycogen was determined by hydrolysis of the tissue with 1 ml 1 M-KOH (85°, 1 h). Once cooled, glycogen was precipitated twice with ethanol (950 ml/l) followed by centrifugation. The resultant glycogen pellet was hydrolysed to glucose using 1 ml 1 M-H₂SO₄ (85°, 2 h), allowed to cool and neutralized with 5 M-NaOH. A 0.5 ml portion was added to scintillant and counted for ¹⁴C radioactivity. Incorporation of glucose into glycogen was expressed as nmol glucose equivalents/mg wet weight muscle per h.

Insulin secretion in vitro

BRIN-BD11 cells, produced by electrofusion of immortal RINm5F cell with New England Deaconess Hospital rat pancreatic B-cell, were used to evaluate insulin secretion. The generation and basic characteristics of this glucose-responsive insulin secreting cell line have been described elsewhere (McClenaghan *et al.* 1996). BRIN-BD11 cells were cultured at 37° in a humidified atmosphere of CO₂ in air (50 ml/l) in RPMI-1640 containing 11.1 mM-glucose, 100 mg/ml fetal calf serum and antibiotics (50 000 IU/l penicillin-streptomycin). Cells were seeded at a concentration of 0.2 × 10⁶ cells/well in twenty-four-well plates (Falcon, NJ, USA) and allowed to attach overnight before acute tests. Wells were washed three times with Krebs-Ringer bicarbonate buffer (KRB; 115 mM-NaCl, 4.7 mM-KCl, 1.28 mM-CaCl₂, 1.2 mM-KH₂PO₄, 1.2 mM-MgSO₄, 24 mM-NaHCO₃, 10 mM-hepes-free acid, 1 g/l bovine serum albumin, 1.1 mM-glucose, pH 7.4) and preincubated for 40 min at 37°. Unless otherwise stated, cells were then incubated for 20 min with 1 ml KRB at 1.1 mM-glucose in the absence and presence of plant extract and other test agents. Portions were removed from each well, centrifuged (900 rev./min, 5 min, 4°) and stored at -20° for insulin assay (Flatt & Bailey, 1981). Concentrations of plant extract tested (0.25, 0.5, 1 mg/ml) did not influence the viability of BRIN-BD11 cells during the test period as evaluated by modified neutral red assay (Hunt *et al.* 1987).

To evaluate the nature of the insulin-releasing component(s) the aqueous extract of lucerne was subjected to heat, overnight dialysis, acid-alkali or acetone treatment. Heat: aqueous extract was boiled for 1 h immediately after preparation. Dialysis: aqueous extract was dialysed overnight (Spectra/Por molecular mass cut-off 2000 Da; Spectrum, Los Angeles, CA, USA) against deionized water (Milli-Q, Millipore Corp., Milford, MA, USA) at 4°. Acid-alkali treatment: portions of aqueous extract were added to 5 M-HCl or 5 M-NaOH to produce 0.1 M-HCl or 0.1 M-NaOH, allowed to stand at room temperature overnight, then neutralized. Acetone treatment: 1 ml of aqueous extract (1 mg/ml) was added to 10 ml ice-cold acetone, allowed to stand for 30 min and centrifuged

(1000 rev./min; 5 min) to obtain acetone-soluble and acetone-insoluble fractions. Portions of untreated extract and modified aqueous extracts were dried under vacuum. All modified aqueous extracts were freshly reconstituted in KRB and effects of insulin secretion at a concentration equivalent to 1 mg/ml compared with untreated extract.

In another series of experiments lucerne leaves were subjected to sequential extraction by increasingly polar solvents. Plant material (0.25 g) was placed in 5 ml hexane, agitated for 15 min, centrifuged (1200 rev./min; 5 min). The precipitate was dried under vacuum and extracted with a further 5 ml hexane and centrifuged as before. The extraction supernatant fractions were pooled, filtered (Whatman no. 1 filter paper) and the volume adjusted to 10 ml with hexane. The extraction precipitate (dried under vacuum) was subsequently extracted (as described earlier) with two 5 ml volumes of ethyl acetate, then methanol and finally with water. All extract fractions were freshly reconstituted in KRB and effects on insulin secretion at a concentration equivalent to 1 mg/ml compared with untreated extract.

Statistical analyses

Data were evaluated using Student's unpaired *t* test, one-way ANOVA or two-way ANOVA where appropriate. Groups were considered to be significantly different if $P < 0.05$. When a significant *F* value was obtained for ANOVA the differences between all pairs were tested using Student–Newman–Keuls multiple comparisons test. If standard deviations were significantly different (Bartlett's test for homogeneity of variances) data were transformed ($\log_{10}[x]$).

RESULTS

Studies in vivo

Compared with normal mice, STZ administration resulted in significant ($P < 0.05$) weight loss, polydipsia and hyperglycaemia (Table 1). Administration of lucerne in the diet and drinking water significantly decreased the hyperglycaemia by study day 12. Plasma glucose concentrations of STZ-treated mice receiving lucerne were comparable to those of normal mice at days 12 and 20 (Table 1).

Glucose transport and glucose metabolism in vitro

Aqueous extract of lucerne (1 mg/ml) increased glucose uptake (1.8-fold), $^{14}\text{CO}_2$ production (1.7-fold), and glycogenesis (1.6-fold) during incubations without insulin but did not significantly alter the stimulatory effect of 10^{-8} M-insulin (Table 2).

Insulin secretion in vitro

Aqueous extract of lucerne (0.25–1 mg/ml) had a dose-dependent stimulatory effect on insulin secretion from BRIN-BD11 cells at 1.1 mM-glucose (Fig. 1). The presence of 0.5 mM-diazoxide inhibited the stimulatory effect of the extract indicating that enhancement of insulin release was not simply a consequence of cellular damage. Consistent with this view, prior exposure of BRIN-BD11 cells to 0.25–1 mg/ml extract for 20 min did not alter the subsequent 2.7–5.4-fold insulin secretory response to 10 mM-L-

Table 1. Effects of lucerne, administered in the diet (62.5 g/kg) and drinking water (2.5 g/l) on body weight, fluid intake, food intake and plasma glucose concentrations of streptozotocin (STZ)-treated mice[‡]

(Mean values with their standard errors for six mice per group)

	Normal mice		STZ mice		STZ mice + lucerne	
	Mean	SEM	Mean	SEM	Mean	SEM
Body weight (g)						
Study day 0	45.5	2.90	42.3	0.94	46.3	1.65
Study day 20	48.1	1.19	37.7**	2.38	38.8**	2.67
Fluid intake (ml/d)						
Study day 0	4.5	0.20	4.3	0.24	4.9	0.47
Study day 20	5.2	0.31	10.0***	0.50	10.6***	0.68
Food intake (g/d)						
Study day 0	4.4	0.49	4.4	0.43	3.9	0.61
Study day 20	5.0	0.40	4.9	0.40	4.0	0.49
Plasma glucose (mmol/l)						
Study day 12	6.6	0.45	13.5**	1.68	6.6††	0.49
Study day 20	9.9	0.74	22.3**	3.06	14.3	3.76

Mean values were significantly different from those for normal mice: ** $P < 0.01$; *** $P < 0.001$.

Mean value was significantly different from that for STZ-treated mice receiving the normal diet: †† $P < 0.01$.

‡ STZ was administered 4 d after the introduction of lucerne (day 0). For details of procedures, see p. 326.

alanine (results not shown). The insulin-releasing effect of 1 mg/ml extract was markedly potentiated by the presence of high (16.7 mM) glucose (Fig. 2(a)), whereas 10 mM-L-alanine did not significantly enhance the insulinotropic effect (Fig. 2(b)). The action of the extract (1 mg/ml) was also potentiated by 1 mM-3-isobutyl-1-methylxanthine (IBMX) (Fig. 2(c)) which increases cyclic AMP in insulin-secreting cells (Sharp, 1979). In the absence of added extract, a depolarizing concentration of KCl (25 mM) markedly enhanced the insulin response to 16.7 mM-glucose (Fig. 2(d)). However, depolarized BRIN-BD11 cells did not show a further insulin-releasing effect with lucerne extract.

Prolonged exposure to heat or dialysis did not significantly alter the insulin-releasing activity of plant extract (Table 3). Acid-exposed extract had greater insulin-releasing effects on BRIN-BD11 cells than alkali-exposed or untreated extract. Both acetone-insoluble and -soluble fractions of extract retained insulin enhancing effects, the action of the acetone-soluble fraction being reduced in comparison with the insoluble fraction (Table 3). Only methanol and water fractions, produced by sequential extraction of lucerne leaves, had significant insulin-releasing effects (6.8 (SE 0.73) and 6.8 (SE 0.95) ng/million cells per 20 min respectively; n 6) as compared with basal insulin release (1.4 (SE 0.10) ng/million cells per 20 min; n 6, $P < 0.001$) recorded in the absence of lucerne. Hexane and ethyl acetate fractions failed to alter basal insulin secretion by BRIN-BD11 cells.

DISCUSSION

The present study has confirmed that chronic administration of lucerne (62.5 g/kg diet, 2.5 g/l in place of drinking water) can reduce hyperglycaemia of STZ-diabetic mice (Swanston-Flatt *et al.* 1990). The antihyperglycaemic action may have been due in part to protection of B-cells from the cytotoxic action of STZ as evident by the slower fall of insulin concentrations (Swanston-Flatt *et al.* 1990). However this does not appear to

Table 2. *Effect of aqueous lucerne extract on glucose uptake and glucose metabolism by isolated mouse abdomen muscle during incubations in the absence and presence of 10^{-8} M-insulin*
(Mean values with their standard errors)

	Control			Insulin (10^{-8} M)			Extract (1 mg/ml)			Insulin + extract		
	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM	n	Mean	SEM
Glucose uptake (dpm/mg per h)	5	229	39	5	406*	65	4	408**	25	5	408*	60
Glucose oxidation (nmol/mg per h)	11	0.39	0.18	11	0.65*	0.09	11	0.65**	0.07	11	0.64*	0.11
Incorporation of glucose into glycogen (nmol/mg per h)	11	0.20	0.02	11	0.39**	0.06	11	0.32*	0.04	11	0.36**	0.46

Mean values were significantly different from those for control incubations: * $P < 0.05$, ** $P < 0.01$.

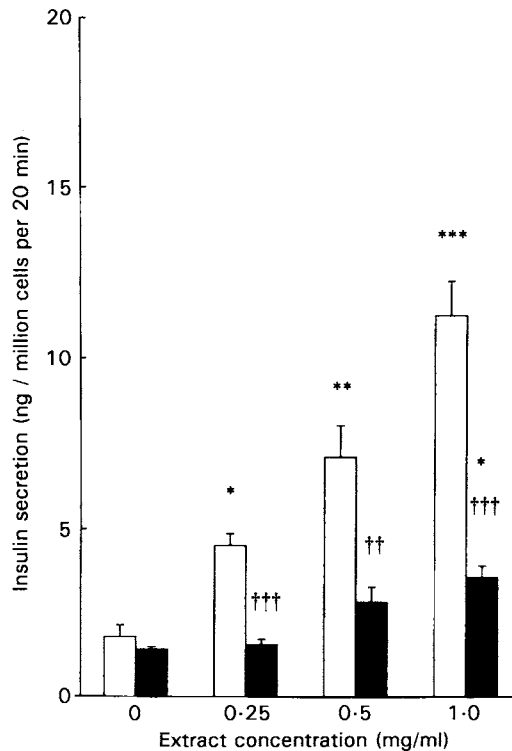


Fig. 1. Effects of aqueous extract of lucerne on insulin secretion in response to 1.1 mM-glucose by BRIN-BD11 cells in the absence (□) and presence (■) of 0.5 mM-diazoxide. Values are means for groups of six observations with their standard errors indicated by vertical bars. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with control incubations without extract; †† $P < 0.01$, ††† $P < 0.001$ compared with incubations at the same extract concentration without diazoxide.

account entirely for the glucose-lowering effect of lucerne since glucose concentrations were held down even when insulin concentrations were very low (Swanston-Flatt *et al.* 1990). This suggests that lucerne may have antihyperglycaemic metabolic actions. The present data demonstrate both extra-pancreatic and pancreatic effects of lucerne extract.

Using an isolated mouse skeletal-muscle preparation, aqueous extract of lucerne enhanced glucose transport and glucose metabolism to a similar magnitude to 10^{-8} M-insulin. Although this effect was observed in the absence of added insulin it does not preclude a possible involvement of residual insulin-receptor binding within the muscle preparation. However, the lack of potentiation in the presence of a high-concentration of insulin suggests that the extract is likely to act via pathways (at least terminally) that are utilized by insulin rather than entirely separate pathways. The effect of extract on glucose uptake differed from that of metformin which exerts its effects on glucose transport via insulin-mediated enhanced peripheral glucose uptake (Bailey & Pua, 1986; Prager *et al.* 1986).

Incubations were performed with glucose-responsive BRIN-BD11 cells (McClenaghan *et al.* 1996) to investigate possible effects of aqueous extract of lucerne on insulin secretion *in vitro*. This revealed a stepwise dose-dependent stimulation of insulin secretion by lucerne extract at low (non-stimulatory) glucose concentration. Evaluation of cell viability using neutral-red assay and the insulin-releasing action of L-alanine following exposure of

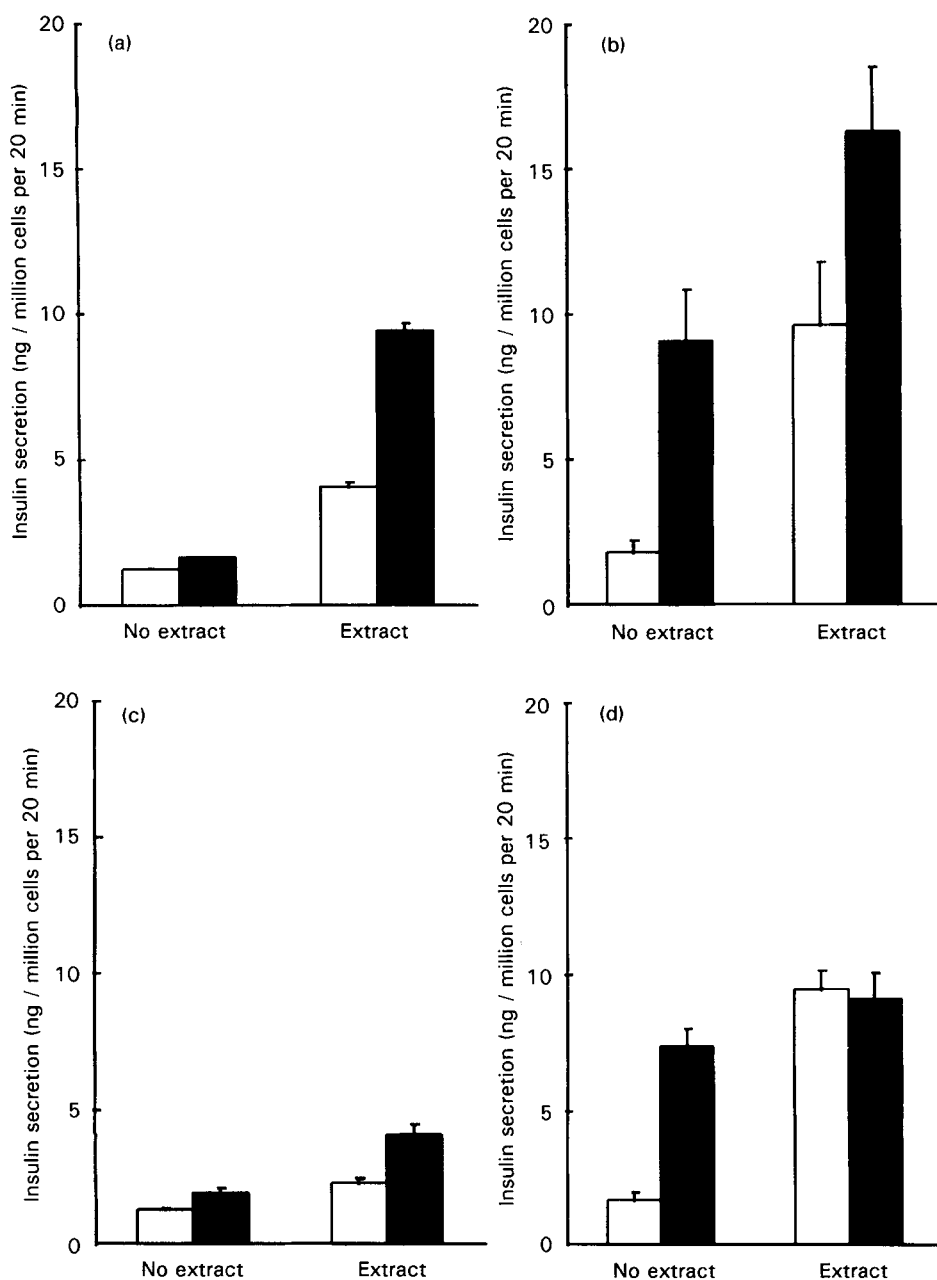


Fig. 2. Effects of (a) 1.1 mM-glucose (□, control) and 16.7 mM-glucose (■); (b) 1.1 mM-glucose (□, control) and 10 mM-L-alanine (■); (c) 1.1 mM-glucose (□, control) and 1 mM-3-isobutyl-1-methylxanthine (IBMX) (■); (d) 16.7 mM-glucose (□, control) and 25 mM-KCl (■), on the insulin releasing actions of aqueous extract (1 mg/ml) of lucerne. Values are means for groups of six observations with their standard errors indicated by vertical bars. Two-way ANOVA revealed (a) extract effect ($P < 0.001$), glucose effect ($P < 0.001$) and extract \times glucose interaction ($P = 0.002$); (b) extract effect ($P < 0.001$), L-alanine effect ($P < 0.001$) and extract \times L-alanine interaction ($P = 0.033$); (c) extract effect ($P < 0.001$); IBMX effect ($P < 0.001$) and extract \times IBMX interaction ($P = 0.012$); (d) extract effect ($P < 0.001$), KCl effect ($P < 0.001$) and extract \times KCl interaction ($P = 0.001$).

Table 3. Effect of heat, dialysis, acid-alkali treatment and acetone treatment on ability of aqueous extract of lucerne to enhance insulin secretion by BRIN-BD11 cells§

(Mean values with their standard errors for six observations)

Test	Insulin secretion (ng/10 ⁶ cells per 20 min)	
	Mean	SEM
Control (without extract)	1.58	0.09
Untreated extract	3.79***	0.26
Boiled extract	3.50***	0.44
Dialysed extract	2.84*	0.23
Acid-exposed extract	5.72*†	0.47
Alkali-exposed extract	4.75***†	0.21
Acetone-insoluble extract	3.26***	0.17
Acetone-soluble extract	2.40***†	0.29

Mean values were significantly different from those for control incubations: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

† Mean values were significantly different from those for incubations with acid-exposed extract, $P < 0.05$.

‡ Mean values were significantly different from those for incubations with untreated extract, $P < 0.05$.

§ For details of procedures, see pp. 326–328.

BRIN-BD11 cells to extract argue against a simple cytotoxic action. Inhibition of the stimulatory effects of the extract with diazoxide support this view. The established effects of diazoxide on the B-cell arising from activating K⁺-ATP channels (Trube *et al.* 1986) indicate involvement of closure of K⁺-ATP channels, membrane depolarization and Ca²⁺ influx in the stimulatory action of lucerne. The effect of the extract on insulin secretion was also potentiated by 16.7 mM-glucose and IBMX, suggesting that B-cell glucose metabolism and cyclic AMP generation are able to augment the insulinotropic stimulus. Consistent with this view, L-alanine which promotes insulin secretion through changes in Na⁺ transport (Yada, 1994), failed to affect the insulin-releasing action of lucerne. Furthermore, unlike the stimulatory effect of 16.7 mM-glucose alone, the action of lucerne extract was not augmented by further depolarization of the cell with 25 mM-KCl. This observation, like the inhibitory effect of diazoxide, suggests that the main action of lucerne extract was associated with strong depolarization of the B-cell plasma membrane. Such an effect is reminiscent of the hypoglycaemic sulfonylureas which promote insulin secretion by closure of B-cell K⁺-ATP channels and stimulation of Ca²⁺ influx (Schwanstecher & Panten, 1994).

Refinement of extract indicated that the active insulin-releasing component(s) was heat stable and was enhanced by previous exposure to an altered pH environment. Following overnight dialysis of extract, 75% of the insulin-releasing effect of aqueous plant extract was retained. Rubenstein *et al.* (1962a) described a single case of uncontrolled insulin-dependent diabetes mellitus which responded favourably to an aqueous extract of lucerne. On further investigation an aqueous solution of MnCl₂ administered orally produced a marked hypoglycaemia resembling that of the lucerne extract (Rubenstein *et al.* 1962a). However preliminary studies on several normal controls, insulin-dependent and non-insulin-dependent diabetic subjects failed to show any decrease in blood glucose levels following oral administration of aqueous MnCl₂ (Rubenstein *et al.* 1962b). The present findings discount a major role for smaller components including ions (e.g. Mn). The active constituent appears to be more acetone-insoluble than -soluble. Insulin-enhancing effects of lucerne were only retained in methanol and water fractions indicating active constituents that are polar in nature.

These results suggest that lucerne reduces hyperglycaemia via potentiation of insulin secretion and improvement of insulin action, possibly by the cumulative effect of more than one active constituent. Thus lucerne represents a possible dietary adjunct for the treatment of diabetes and a potential source for discovery of new agents with oral antihyperglycaemic activity.

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