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Actions of the traditional anti-diabetic plant, *Agrimony eupatoria* (agrimony): effects on hyperglycaemia, cellular glucose metabolism and insulin secretion

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Agrimony eupatoria (agrimony) has been documented as a traditional treatment of diabetes. Here, the effects of dietary administration of agrimony on streptozotocin (STZ)-diabetic mice and on *in vitro* glucose uptake and glucose metabolism, and on insulin secretion by BRIN-BD11 cells were investigated. Agrimony incorporated into the diet (62·5 g/kg) and drinking water (2·5 g/l) countered the weight loss, polydipsia, hyperphagia and hyperglycaemia of STZ-diabetic mice. Aqueous extract of agrimony (1 mg/ml) stimulated 2-deoxy-glucose transport (1·4-fold), glucose oxidation (1·4-fold) and incorporation of glucose into glycogen (2·0-fold) in mouse abdominal muscle comparable with 0·1 μM-insulin. In acute 20 min tests, 0·25–1 mg/ml aqueous extract of agrimony evoked a stepwise 1·9–3·8-fold stimulation of insulin secretion from the BRIN-BD11 pancreatic B-cell line. This effect was abolished by 0·5 mM-diazoxide and previous exposure to extract did not adversely affect subsequent stimulation of insulin secretion by 10 mM-L-alanine, thereby indicating that there was no detrimental effect of the extract on cell viability. The effect of extract was glucose-independent and was not evident in BRIN-BD11 cells exposed to a depolarizing concentration of KCl. The ability of agrimony extract to enhance insulin secretion was dependent on use of heat during extract preparation. These results demonstrate the presence of antihyperglycaemic, insulin-releasing and insulin-like activity in *Agrimony eupatoria*.

Agrimony: Insulin: Diabetes: Traditional plant therapies

Before the introduction of insulin in 1922, the treatment of diabetes mellitus relied heavily on dietary measures, which included the use of traditional plant therapies. Many traditional plant treatments for diabetes exist (Bailey & Day, 1989; Swanston-Flatt *et al.* 1991; Gray & Flatt, 1997*a*) but few have received scientific or medical scrutiny. The World Health Organization (1980) has recommended accordingly that traditional plant treatments for diabetes warrant further evaluation. *Agrimony eupatoria* (agrimony, cocklebur, stickwort) is indigenous to Europe, and has been traditionally utilized for its anti-diabetic properties (Lewis, 1949). *Agrimony eupatoria* reduced polydipsia and countered the early hyperglycaemia of streptozotocin (STZ)-treated mice (Swanston-Flatt *et al.* 1990).

An anti-diabetic plant could exert a beneficial effect in the diabetic environment by improving or mimicking insulin action and/or by enhancing insulin secretion (Gray & Flatt, 1997*a*). To understand better the mechanisms involved, the present study has investigated actions of dietary agrimony in STZ-diabetic mice and effects of an aqueous extract of agrimony on glucose uptake and metabolism by isolated abdominal muscle and on insulin secretion by BRIN-BD11 cells.

Materials and methods

Plant material

Dried agrimony leaves were obtained from a commercial source (The Health Food Centre, Bull Ring Shopping Centre, Birmingham, UK). Leaves were homogenized to a fine powder and stored at room temperature (20 ± 2°) in opaque screwtop jars until used. Powdered agrimony was used for incorporation into the test animal diet. An aqueous extract of agrimony was prepared by a method of decoction: the powdered material was immersed at 25 g/l in cold distilled water, which was brought to the boil, removed from the heat and infused for 15 min. The suspension was filtered (Whatman no. 1) and the extract was stored at −20°.

Abbreviation: STZ, streptozotocin.

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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, incubations within a single experiment using isolated muscle or BRIN-BD11 cells were always conducted using the same batch of extract. This allowed for any variation in potency of different batches of extract.

Animal studies

Male mice derived from a colony maintained at Aston University, Birmingham, UK (Flatt & Bailey, 1981) were used at 21–24 weeks of age. Groups of two to three mice were housed in an air-conditioned room at 22 ± 2° with a lighting schedule of 12 h light (08.00–20.00 hours) and 12 h dark. Animals had free access to a standard pelleted diet (Mouse breeding diet; Pillsbury Ltd, Birmingham, UK) and tap water. The experimental procedure for *in vivo* studies was similar to that previously described (Gray & Flatt, 1997*b*). Agrimony was incorporated into the diet (62·5 g/kg) and drinking water (2·5 g/l) of a group of six mice 5 d before and subsequent to intraperitoneal administration of STZ (Sigma Chemical Co, Poole, Dorset, UK) at 200 mg/kg body weight in 0·1 M-sodium citrate buffer (pH 4·5). Daily measurements 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 at the same time (09.10–10.00 hours) for plasma glucose analysis (Stevens, 1971). Groups of eight normal mice and five STZ-treated mice with free access to unsupplemented diet and drinking water 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 pieces of abdominal muscle (approximately 10–20 mg) were prepared. Incubations were performed using Krebs-Ringer bicarbonate buffer (118 mM-NaCl, 25 mM-$NaHCO_3$, 5 mM-KCl, 1·28 mM-$CaCl_2$, 1·18 mM-$MgSO_4$, 1·17-mM KH_2PO_4) supplemented with insulin-free bovine serum albumin (20 g/l).

Glucose uptake was determined as described previously (Gray & Flatt, 1997*b*). In brief, muscle pieces were incubated at 30° for 30 min in 1 ml Krebs-Ringer bicarbonate buffer supplemented with insulin-free bovine serum albumin) and with 2 mM-sodium pyruvate, 3·70 kBq 2-deoxy-D-[1-^{3}H]glucose/ml, 0·37 kBq L-[1-^{14}C]glucose/ml in the presence and absence of 0·01 μM-human insulin and 1 mg extract of agrimony/ml (2 × 2 factorial design). After incubation, tissue was hydrolysed using 1 M-NaOH and counted for ^{3}H and ^{14}C radioactivity. The extracellular fluid volume of the muscle was determined from the amount of the non-transported L-[1-^{14}C]glucose, and this was taken into account in the calculation of tissue 2-deoxy-D-[1-^{3}H]glucose uptake, expressed as disintegrations/min per mg wet weight of muscle per h.

Oxidative glucose metabolism to CO_2 and incorporation of glucose into glycogen were determined as described previously (Gray & Flatt, 1997*b*). In brief, muscles were incubated at 37° for 60 min in 1 ml Krebs-Ringer bicarbonate buffer supplemented with insulin-free bovine serum albumin and with 10 mM-glucose, 18·50 kBq D-[U-^{14}C]glucose/ml in the presence and absence of 0·01 μM-human insulin and 1 mg extract of agrimony/ml (2 × 2 factorial design). Following incubation, CO_2 was captured onto a NaOH-saturated filter paper and muscles were removed for glycogen analysis (Gray & Flatt, 1997*b*). ^{14}C radioactivity of the filter paper was counted and CO_2 production was expressed as nmol CO_2/mg wet weight of muscle per h. The incorporation of glucose into glycogen was expressed as nmol glucose equivalents/mg wet weight of muscle per h. Total lactate output by muscle was determined by enzymic assay (Boehringer Mannheim, Mannheim, Germany) and expressed as nmol lactate/mg wet weight of 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 (McClenaghan *et al.* 1996; Gray & Flatt, 1997*b*). Cells were seeded at a concentration of 0·2 × 10^6 cells/well in twenty-four-well plates (Falcon, NJ, USA) cultured in RPMI-1640 containing 11·1 mM-glucose, 100 ml fetal calf serum/l and antibiotics (50 000 IU penicillin–streptomycin/l) to allow attachment overnight before acute tests. Cells were washed three times with Krebs-Ringer bicarbonate buffer (115 mM-NaCl, 4·7 mM-KCl, 1·28 mM-$CaCl_2$, 1·2 mM-KH_2PO_4, 1·2 mM-$MgSO_4$, 24 mM-$NaHCO_3$, 10 mM-Hepes (free acid), bovine serum albumin (1 g/l), 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 Krebs-Ringer bicarbonate buffer at 1·1 mM-glucose in the absence and presence of plant extract, diazoxide (an established opener of K^+-ATP channels) and other test agents. Following incubation, portions were removed from each well and stored at −20° for insulin assay (Flatt & Bailey, 1981).

To evaluate the importance of heat during extract preparation, aqueous extracts of agrimony were prepared by normal method of decoction (normal extract) or by cold infusion (cold extract; plant material placed in cold water, allowed to stand for 15 min then filtered as before). Modified aqueous extract was freshly reconstituted in Krebs-Ringer bicarbonate buffer and effect on insulin secretion evaluated at a concentration equivalent to 1 mg/ml compared with normal extract.

Statistical analyses

Data were evaluated using Student's unpaired *t* test, one-way 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 SD were significantly different (Bartlett's test for homogeneity of variances) data were transformed ($\log_{10}[\times]$).

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Table 1. Effects of agrimony, 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 groups of five to eight mice)

Variable	Control mice Mean	Control mice SEM	STZ-treated mice Mean	STZ-treated mice SEM	STZ-treated mice + agrimony Mean	STZ-treated mice + agrimony SEM
Body weight (g)						
Study day 0	43·8	0·91	40·3	2·24	41·2	2·39
Study day 12	45·8	1·20	35·4**	3·05	39·3*	2·93
Study day 20	45·8	0·93	35·7**	3·28	38·5*	2·74
Fluid intake (ml/d)						
Study day 0	5·3	0·46	5·9	0·40	5·2	0·45
Study day 12	5·7	0·67	11·2*	1·46	7·0	0·57
Study day 20	5·3	0·15	24·7***	0·50	10·4***†††	0·43
Food intake (g/d)						
Study day 0	3·6	0·62	4·0	0·41	3·2	0·52
Study day 12	3·9	0·23	4·0	0·69	3·9	0·06
Study day 20	4·2	0·15	6·1***	0·46	4·3††	0·12
Plasma glucose (mmol/l)						
Study day 12	6·3	0·50	14·7***	2·17	7·8†	1·60
Study day 20	5·1	0·52	15·9***	2·49	8·0†	1·44

‡ STZ was administered 4 d following the introduction of agrimony (day 0); for details see p. 110.
Mean values were significantly different from those for control mice: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.
Mean values were significantly different from those for STZ-treated mice receiving normal diet: †$P < 0{\cdot}05$, ††$P < 0{\cdot}01$, †††$P < 0{\cdot}001$.

Results

Studies in vivo

Compared with normal mice, STZ administration resulted in significant ($P < 0{\cdot}01$) weight loss, polydipsia, hyperphagia and hyperglycaemia (Table 1). Administration of agrimony in the diet and drinking water significantly decreased fluid and food intakes and the hyperglycaemia ($P < 0{\cdot}05$) by study day 12 to a level not significantly greater than that of normal mice (Table 1).

Glucose transport and glucose metabolism in vitro

Aqueous extract of agrimony (1 mg/ml) increased glucose uptake (1·4-fold), $^{14}CO_2$ production (1·4-fold), glycogenesis (2·0-fold) and total lactate output (1·4-fold) during incubations without insulin but did not significantly alter the stimulatory effect of 0·01 μM-insulin (Table 2).

Insulin secretion in vitro

Aqueous extract of agrimony (0·25–10 mg/ml) had a dose-dependent stimulatory effect on insulin secretion from BRIN-BD11 cells at 1·1 mM-glucose (Fig. 1). It was confirmed that these concentrations of extract did not influence the viability of BRIN-BD11 cells during the test period as evaluated by modified neutral red assay (Hunt *et al.* 1987). The presence of 0·5 mM-diazoxide inhibited the stimulatory effect of 0·5 mg/ml extract, indicating that enhancement of insulin release was not a mere consequence of cellular damage (Fig. 2(*a*)). Consistent with this view, previous exposure of BRIN-BD11 cells to 0·5 mg/ml extract for 20 min did not alter the subsequent insulin secretory response to 10 mM-L-alanine (Fig. 2(*b*)). The presence of high glucose (16.7 mM) did not augment the insulinotropic effect of 1 mg/ml extract (3·5 (SD 0·6) *v.* 3·1 (SD 0·3) ng/10^6 cells per 20 min for glucose alone, *n* 6). A depolarizing

Table 2. Effect of aqueous agrimony extract on glucose uptake and glucose metabolism by isolated mouse abdomen muscle during incubations in the absence and presence of 0·01 μM-insulin†

	Control			Insulin (0·01 μM)			Extract (1 mg/ml)			Insulin + extract		
	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*	Mean	SEM	*n*
Glucose uptake (dpm per mg per h)	343	41	6	497*	27	6	487*	17	6	563**	45	6
Glucose oxidation (nmol/mg per h)	0·32	0·04	6	0·52*	0·08	6	0·46*	0·04	6	0·58*	0·08	6
Incorporation of glucose into glycogen (nmol/mg per h)	0·22	0·03	6	0·40*	0·05	6	0·44*	0·07	6	0·64**	0·09	6
Total lactate output (nmol/mg per h)	28	2·8	5	45*	5·6	5	39*	3·1	5	44**	2·8	5

dpm, disintegrations/min.
Mean values were significantly different from those for control incubations in the absence of added insulin and extract: *$P < 0{\cdot}05$, **$P < 0{\cdot}01$.
† For details of agrimony extraction and incubation procedure, see pp. 109–110.

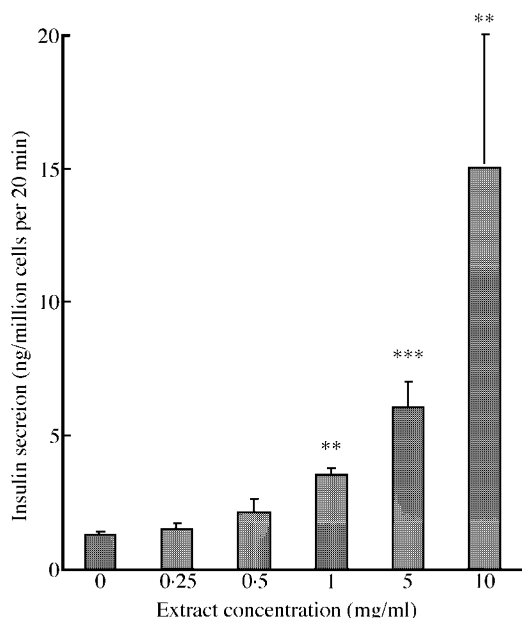

Fig. 1. Effects of aqueous extract of agrimony on insulin secretion by BRIN-BD11 cells. Values are means for groups of four to six observations with their standard errors indicated by vertical bars. Mean values were significantly different from those for control incubations without extract: ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

concentration of KCl (25 mM) increased insulin release at 16·7 mM-glucose (8·3 (SD 0·8) *v*. 3·1 (SD 0·3) ng/10^6 cells per 20 min for 16·7 mM-glucose alone, *n* 6; $P < 0{\cdot}01$). However, agrimony did not significantly enhance the insulin release further in depolarized BRIN-BD11 cells (8·3 (SD 0·8) *v*. 8·9 (SD 0·9) ng/10^6 cells per 20 min, *n* 6).

Only the normal extract of agrimony which was heated had significant insulin-releasing effects compared with basal insulin release recorded at 1·1 mM-glucose alone (1·8 (SD 0·1) and 1·3 (SD 0·1) ng/10^6 cells per 20 min respectively, *n* 6; $P < 0{\cdot}05$). Extract prepared by cold infusion (cold extract) failed to significantly alter basal insulin secretion by BRIN-BD11 cells (1·4 (SD 0·2) *v*. 1·3 (SD 0·1) ng/10^6 cells per 20 min, *n* 6).

Discussion

Chronic administration of agrimony has been demonstrated to reduce polydipsia and alleviate the hyperglycaemia of STZ-diabetic mice (Swanston-Flatt *et al.* 1990). The present studies confirm the anti-hyperglycaemic effect of agrimony and the ability of the plant to counter weight loss and hyperphagia in diabetes.

The mechanism of action of agrimony has been investigated by *in vitro* studies of glucose transport and metabolism by isolated muscle pieces. An aqueous extract of agrimony (1 mg/ml) enhanced glucose transport, glucose oxidation, glycogenesis and lactate release comparable with that evoked by 0·01 μM-insulin. The effect of extract on glucose uptake and metabolism differs from that of the

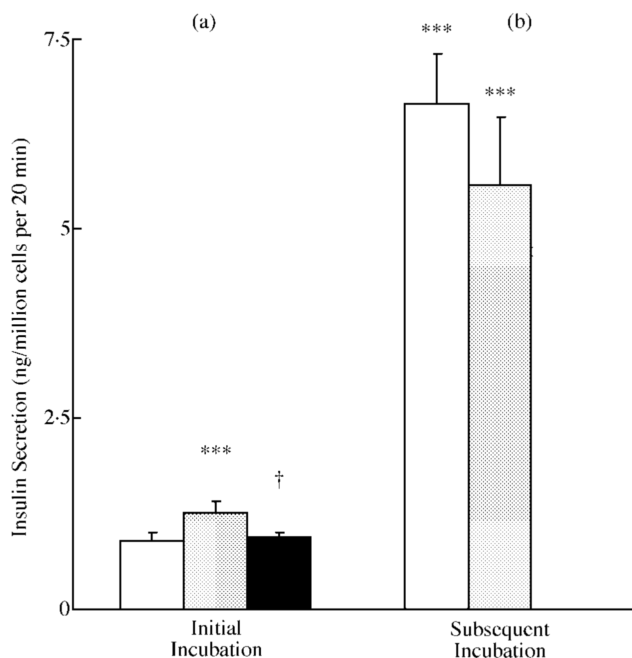

Fig. 2. (a) Effects of aqueous extract of agrimony (1 mg/ml) on insulin secretion by BRIN-BD11 cells to 1·1 mM-glucose ((□), control) in the absence (▨) or presence (■) of 0·5 mM-diazoxide. (b) Effects of 20 min previous exposure to 1·1 mM-glucose ((□), control) or extract (1 mg/ml, (▨)) on the subsequent insulin secretory response to 10 mM-L-alanine. Values are means for groups of six observations with their standard errors indicated by vertical bars. Mean values were significantly different from those for control in incubations without extract: *** $P < 0{\cdot}0001$. Mean values were significantly different from those for incubations with extract: † $P < 0{\cdot}05$.

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anti-hyperglycaemic drug metformin which exerts its effects on glucose uptake via insulin-mediated enhanced peripheral glucose uptake (Bailey & Puah, 1986; Prager *et al.* 1986). Insulin-free bovine serum albumin and disposable incubation vials negate the possibility of insulin contamination of muscle incubations.

Diazoxide inhibits glucose and sulphonylurea-induced insulin secretion by clamping open ATP-sensitive K^+ channels, thus preventing membrane depolarization and subsequent Ca^{2+} influx, two of the key initial steps in insulin secretion (Dunne *et al.* 1994). In the present studies, diazoxide abolished the insulin-releasing effects of agrimony extract on BRIN-BD11 cells. Agrimony extract is therefore likely to act at an early stage of the insulin secretory pathway before Ca^{2+} influx. However, the action of extract was not potentiated by glucose. These characteristics are reminiscent of sulphonylureas, but unlike this family of anti-diabetic drugs which appears to exert additional intracellular effects (Eliasson *et al.* 1996), agrimony extract did not augment insulin secretion from depolarized B-cells.

Cellular viability of BRIN-BD11 cells was maintained in the presence of agrimony extract (as determined by modified neutral red assay) and previous exposure to extract did not diminish subsequent cellular responses to L-alanine, providing further evidence that enhanced secretion of insulin by agrimony extract was not due to a detrimental cellular effect.

Fresh agrimony contains a glucoside alkaloid, traces of essential oil and organic acids, vitamin B_1, vitamin K, ascorbic acid, 15 mg/g of a triterpene (6 mg ursolic acid/g), and a derivative of α-amyrin (Duke, 1985). Agrimony leaf has been reported to contain 0·1–0·3 mg 'nicotinic acid' complex/g (Duke, 1985). Nicotinamide has been shown to ameliorate islet function in partially depancreatized rats and to afford protection against the B-cell cytotoxic actions of alloxan and STZ (Okamoto, 1992; Inoue *et al*. 1993). However, in these earlier studies nicotinamide was administered intraperitoneally and at doses up to 0·5 g/kg body weight. At lower levels, nicotinamide (10 mM) did not directly affect pancreatic B-cell function or cell replicatory rate (Sandler *et al*. 1993). The nicotinamide content of aqueous extract of agrimony was not determined. However, at the supplementation levels used in animal studies and at the levels of aqueous extract investigated *in vitro*, it is highly unlikely that such high concentrations were achieved. Indeed, the concentration of nicotinamide in aqueous extract used for *in vitro* testing is likely to be in the μmol/l range. Possible involvement of glucose tolerance factor which comprises two nicotinic acid molecules per Cr^{3+} as well as cysteine, glycine and possibly glutamic acid residues (Toepfer *et al.* 1977) is also highly unlikely. Thus agrimony is not noted for high Cr content and supplementation of the diet of diabetic mice with 50 g brewer's yeast (richest known source of glucose tolerance factor)/kg failed to improve glucose intolerance or insulin sensitivity over an 8-week period (Flatt *et al.* 1989).

These studies show that the anti-hyperglycaemic actions of agrimony are associated with the stimulation of insulin secretion and augmentation of glucose uptake and metabolism by muscle. Further studies are required to assess the additional possibility that agrimony affords protection against the cytotoxic action of STZ *in vivo*. Extraction of the component responsible for the enhancement of insulin secretion *in vitro* requires heat, and is thus heat-stable. In conclusion, agrimony represents a possible dietary adjunct for treatment of diabetes and a source of new oral hypoglycaemic agent(s). The properties and structure of these potentially useful natural products merit further investigation.

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