Insulinotropic action of *Citrullus colocynthis* seed extracts in rat pancreatic islets

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Abstract. The present study aimed to investigate the direct *in vitro* effects of several distinct *Citrullus colocynthis* seed extracts on glucose-stimulated insulin release from pancreatic islets isolated from rats. Six extracts were tested, a crude aqueous, defatted aqueous, ethyl acetate, H_2O-methanol and n-butanol extract and an extract containing a major component (fraction A) identified by gel chromatography in the ethyl acetate, n-butanol and H_2O-methanol extracts. Under selected experimental conditions, the majority of extracts exhibited a positive insulinotropic action, at least when tested in the presence of 8.3 mM D-glucose. The concentration-response correlation observed with distinct extracts revealed the participation of distinct chemical compounds, including compounds with an inhibitory insulinotropic potential, in the modulation of the insulin secretory response to D-glucose. The results of the present study are relevant for further investigations which aim to identify compounds exhibiting positive insulinotropic actions. These agents may be suitable for the treatment of human diabetic subjects.

Introduction

Citrullus colocynthis belongs to the *cucurbitaceae* family and is a well-recognized plant in traditional medicine. The plant has been previously utilized in rural areas as a purgative, anti-diabetic, insecticide and antitumoral agent (1). In a previous study, the beneficial long-term effects of *Citrullus colocynthis* seed extracts on glucose homeostasis and body weight maintenance were documented in streptozotocin-induced diabetic rats (1).

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The aim of the present study was to explore the direct *in vitro* effects of several distinct *Citrullus colocynthis* seed extracts on glucose-stimulated insulin release from rat isolated pancreatic islets.

Materials and methods

Citrullus colocynthis extracts. Six extracts from *Citrullus colocynthis* seeds were tested; a crude aqueous, defatted aqueous, ethyl acetate, H_2O-methanol and n-butanol extract and an extract containing the major component of the ethyl acetate, n-butanol and H_2O-methanol extracts, named fraction A.

Extract preparation. The preparation of the crude aqueous and defatted extracts was reported in a previous study (1). For the hydromethanolic extract, 50 g of seeds was ground and degreased in hexane. This material was heated and stirred 3 times for 3 h in a H_2O-methanol mixture (30/70). Following filtration and centrifugation, the recovered solution was divided into 2 volumes; one was solidified to form a hygroscopic red-orange residue (H_2O-methanol extract; 4.5% dry matter). The second volume underwent liquid-liquid extraction 3 times with ethyl acetate and n-butanol, for the preparation of the ethyl acetate (orange powder; 1.1% dry matter) and n-butanol (brown powder; 1.2% dry matter) extracts, respectively.

Thin layer chromatography. Following separation by thin layer chromatography, ethyl acetate, n-butanol and H_2O-methanol extracts revealed the presence of a major single spot. Fractionation of these extracts on a column silica gel, using the elution system methanol/water (80/20), enabled material collection from these fractions (observed under UV light at 254 and 336 nm), which, following solidification, was referred to as fraction A (217 mg).

Analysis of insulin release. For measurement of insulin release, groups of 8 islets prepared by the collagenase method (2) were incubated for 90 min at 37˚C in 1.0 ml salt-balanced medium (3) containing 2.5 mg/ml bovine serum albumin and equilibrated against a mixture of O_2-CO_2 (95-5, v-v). The insulin content of the incubation media was measured by radioimmunoassay (4). The present study was approved by

Table I. Relative values for insulin output ± SEM (%) in the presence of aqueous extract.

	D-glucose (mM)			
Aqueous extract (μg/ml)	2.8	8.3	16.7	Pooled data
Nil	100.0±5.6 (28)	100.0±5.7 (30)	100.0±5.5(24)	100.0±3.2(82)
45	125.6±6.4 (29)[i]	136.5±12.5 (30)[g]	117.1±5.6 (27)[f]	126.7±5.2 (86)[i]
135	112.3±9.1 (28)[c]	85.6±3.4 (30)[f]	100.9±7.4 (30)[b]	99.3±4.1 (88)[a]
450	81.3±5.1 (30)[g]	80.3±2.9 (30)[h]	87.2±5.7 (28)[e]	82.8±2.7 (88)[i]
4,500	5.7±1.7 (30)[i]	2.2±1.2 (30)[i]	0.0±0.0 (30)[i]	2.6±0.7 (90)[i]

[a]P>0.98; [b]P>0.90; [c]P>0.2; [d]P>0.18; [e]P>0.12; [f]P<0.04; [g]P<0.02; [h]P<0.003; [i]P<0.001.

the Commission d'Ethique et du Bien-Etre Animal (Faculty of Medicine, Brussels Free University, Brussels, Belgium).

Analysis of lactate dehydrogenase (LDH). LDH output in the incubation medium and the final LDH content of the islets were measured by the CytoTox-ONE™ homogeneous membrane integrity assay (Promega Corp., Madison, WI, USA) and expressed as nmol of lactate converted to pyruvate per min and per 100 islets.

Statistical analysis. All results are presented as mean ± SEM with the number of individual observations (n) or degree of freedom (df). Statistical significance of differences between mean values was assessed using a Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Control insulin input. When measured within triplicate experiments, the control insulin output recorded in the absence of any extract was 27.3±1.4, 63.2±2.1 and 233.9±11.7 μU/islet/90 min (n=27-30) at 2.8, 8.3 and 16.7 mM D-glucose, respectively. The overall mean values, derived from separate experiments conducted at specific hexose concentrations was 56.5±2.5 (n=140) and 254.1±10.7 (n=54) μU/islet/90 min at 8.3 and 16.7 mM D-glucose, respectively. At 8.3 (P>0.21) and 16.7 mM D-glucose (P>0.23), no significant difference was identified between insulin output in the former and latter experiments.

Effect of aqueous extract on insulin output. At a concentration of 45 μg/ml, the aqueous extract was identified to significantly increase insulin output at all D-glucose concentrations (2.8, 8.3 and 16.7 mM). At 16.7 mM, insulin output was 279.3±18.2 and 325.4±13.2 μU/islet/90 min in the absence (n=24) and presence (n=27; P<0.05) of the aqueous extract, respectively. Relative to control insulin outputs observed at each D-glucose concentration (100.0±3.2%; n=82), results recorded at the same concentration of aqueous extract (45 μg/ml) were 126.7±5.2% (n=82; P<0.001). As demonstrated in Table I, a progressive decrease in insulin output was observed with higher concentrations of the aqueous extract. In the 45-450 μg/ml range, the decrease yielded, in semi-logarithmic coordinates, a slope of less pronounced incline than that identified at higher concentrations of the aqueous extract (Fig. 1).

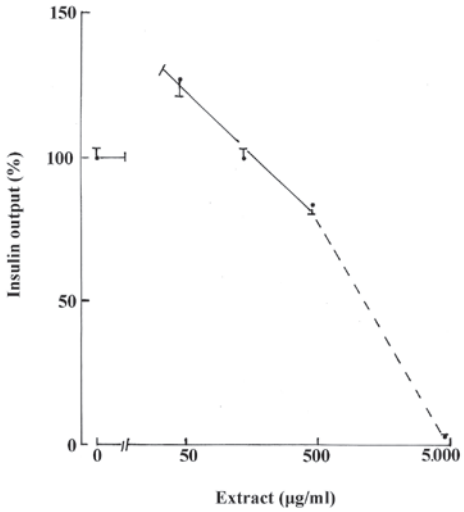

Figure 1. Relative values for insulin output (pooled data from Table I) with increasing concentrations of aqueous extract (logarithmic scale). Oblique solid line corresponds to the regression line.

Effect of aqueous extract on LDH content. Relative to the final LDH content of islets incubated for 90 min in the presence of 8.3 mM D-glucose (100.0±4.3%; n=4), the release of LDH in the incubation, after 30 and 90 min of incubation in the concomitant presence of KCN (2.0 mM) was >21.8±7.2% (n=3) and >33.1% (n=1), respectively. Incubations for 30 and 90 min in the absence of KCN revealed values corresponding to LDH release of 0.39±0.16% (n=4) and 0.82±0.23% (n=4). Release of LDH, following incubation in the presence of increasing concentrations of aqueous extract for 30 and 90 min, did not exceed 0.42±0.06 and 0.72±0.08, 0.86±0.11 and 0.81±0.06, and 0.66±0.18 and 0.81±0.11% at 50, 150 and 450 μg/ml, respectively (n=4 in all cases). Therefore, variations in insulin output evoked by the aqueous extract (45-450 μg/ml) in islets exposed to 8.3 mM D-glucose did not appear to correlate with a specific cytotoxic action.

Effect of defatted aqueous extract on insulin output. The defatted aqueous extract, when tested at 8.3 mM D-glucose,

Table II. Absolute values for insulin output (μU/islet/90 min) in the presence of ethyl acetate extract.

	D-glucose (mM), mean ± SEM (%)		
Ethyl acetate extract (μg/ml)	2.8	8.3	16.7
Nil	27.3±1.4 (27)	63.2±2.1 (30)	233.9±11.7 (30)
25	25.4±1.5 (30)[a]	72.1±2.2 (29)[b]	286.8±13.3 (30)[c]

[a]P>0.33; [b]P<0.006; [c]P<0.005.

yielded results comparable with those recorded with the untreated aqueous extract. At 50 μg/ml, the defatted aqueous extract increased insulin release to 126.0±12.2% (n=20) compared with the corresponding control value (100.0±6.5%; n=20), the former percentage was not identified as significantly different to the untreated aqueous extract (136.5±12.5%; n=30; P>0.55). However, at higher concentrations (150-450 μg/ml) of the defatted aqueous extract, islet insulin output expressed relative to control data was identified as significantly higher (102.5±4.7%; n=40; P<0.001) than that found with the untreated aqueous extract (83.0±2.3%; n=60).

Effect of ethyl acetate extract on insulin output. At 25 μg/ml, the ethyl acetate extract was identified to significantly increase insulin output (P<0.02) in the presence of 8.3 or 16.7 mM D-glucose (Table II). At these concentrations of D-glucose, relative magnitudes of increments were calculated as 14.1±4.7 (df=57) and 24.2±7.0% (df=58), respectively. These increments were not identified to differ significantly (P≥0.13) from those recorded at identical concentrations of D-glucose in the presence of aqueous extract tested at a 45-μg/ml concentration, i.e., 36.5±13.7 (df=58) and 17.1±7.9% (df=49) at 8.3 and 16.7 mM D-glucose, respectively.

Effect of H_2O-methanol extract on insulin output. In the presence of 8.3 mM D-glucose, the H_2O-methanol extract, tested at concentrations of 56, 167 and 500 μg/ml, caused a progressive increase in insulin output (Fig. 2). Observed increases were only identified to be significant at H_2O-methanol extract concentrations of 167 and 500 μg/ml (P≤0.04).

Effect of n-butanol extract on insulin output. The n-butanol extract was tested in the presence of 8.3 mM D-glucose at increasing concentrations over a 9-fold range (40, 116 and 350 μg/ml). At the lowest concentration of n-butanol extract (40 μg/ml), insulin release was decreased (P<0.05) to 79.6±7.2% (n=20) of the corresponding mean values in the absence of the extract (100.0±6.5%; n=20). When the concentration of n-butanol was increased to 116 and 350 μg/ml, a modest progressive increase in insulin output was observed. However, the insulin output measured at 350 μg/ml n-butanol extract (97.0±11.2%; n=20) was not identified as significantly different (P>0.8) from the mean corresponding control value (Fig. 2).

Effect of fraction A extract on insulin output. Fraction A extract (0.5 mg/ml) treatment resulted in an increased insulin output from islets incubated in the presence of 8.3 mM D-glucose to 171.2±12.3% (n=20; P<0.001) compared with control (100.0±8.2%; n=20). The relative magnitude of the latter increase (71.2±14.8%; df=38) was not identified as significantly different (P>0.41) from that recorded at the same concentration (0.5 mg/ml) of the H_2O-methanol extract at 8.3 mM D-glucose (55.4±12.4%; df=38). However, insulin output in the presence of fraction A did exceed (P<0.04) the relative magnitude of the increase in insulin output caused by the aqueous extracts (32.3±9.8%; df=98) tested at a lower concentration (0.05 mg/ml) in the presence of 8.3 mM D-glucose. A 4-fold increase in the concentration of fraction A extract up to 2.0 mg/ml decreased insulin output to 70.7±9.4% (n=20; P<0.025) of control (100.0±8.2%; n=20). The output of insulin in the presence of 2.0 mg/ml fraction A extract was 40.5±4.4% (n=20; P<0.001) of the mean corresponding value identified within the same experiment(s) in the presence of 0.5 mg/ml fraction A extract (100.0±5.1%; n=20). This decrease was more pronounced (P<0.01) than that recorded at the same concentration of D-glucose (8.3 mM), when the concentration of aqueous extract underwent a 9- to

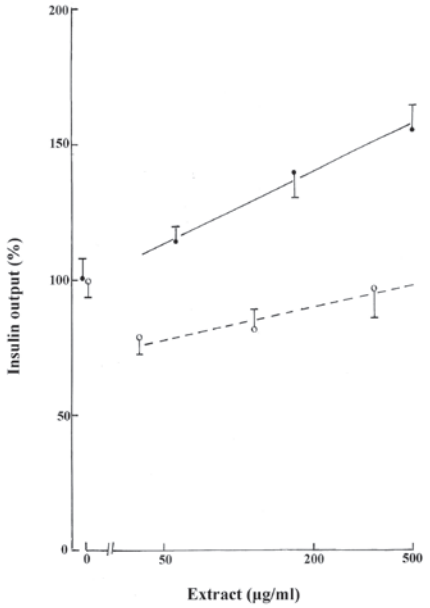

Figure 2. Relative values for insulin output (expressed relative to control) at increasing concentrations of n-butanol (open circles and dotted line) or H_2O-methanol extracts (closed circles and solid lines). Mean values (± SEM) refer to 20 individual determinations. Oblique line corresponds to the regression line.

10-fold increase from 45-50 to 450 μg/ml. In this case the output of insulin averaged at the highest of these concentrations 69.5±2.9% (n=50; P<0.001) of that recorded within the same experiment(s) at the lower concentration of the aqueous extracts (100.0±5.2%; n=50). Therefore, despite a 2.25- to 2.50-fold higher increase in extract concentration, the relative magnitude of the decrease in insulin output was 2-fold lower (P<0.01) with aqueous (30.5±6.0%; df=98) than with the fraction A extract (59.5±6.7%; df=38).

Discussion

The present study demonstrates that under selected experimental conditions, the majority of extracts examined in this study (aqueous, ethyl acetate, H_2O-methanol and fraction A) exhibit positive insulinotropic actions. In the case of the aqueous extract, a positive insulinotropic action was observed with 2.8, 8.3 and 16.7 mM D-glucose. However, in the presence of ethyl acetate extract a significant increase in insulin output was only observed at 8.3 and 16.7 mM D-glucose. H_2O-methanol, n-butanol and fraction A extracts were only tested at 8.3 mM hexose.

When the concentration of the extract was progressively increased, positive insulinotropic action was often reduced and glucose-stimulated insulin secretion was inhibited. Specifically, this was observed in the presence of the aqueous extract at 2.8, 8.3 and 16.7 mM and fraction A at 8.3 mM D-glucose.

An explanation for this dual effect may involve participation of distinct chemical compounds in the modulation of the insulinotropic action of D-glucose and may differ between distinct extracts. For example, inhibition of glucose-stimulated insulin output at a low concentration (40 μg/ml) was only identified in the n-butanol extract and inhibition was reduced at higher concentrations of the extract (116 and 350 μg/ml).

The following observations are consistent with this hypothesis. Firstly, the relative magnitude of the highest increment in insulin output differed with distinct extracts. At 8.3 mM D-glucose, the relative magnitude was 71.2±14.8, 55.4±12.4, 31.2±9.8 and 24.1±7.6% in the presence of fraction A, H_2O-methanol, aqueous and ethyl acetate, respectively. At the highest concentration of the n-butanol extract, the output of insulin recorded at 8.3 mM D-glucose was even 3.0±12.9% lower than the corresponding control value. This may be due to data referring to distinct concentrations of each extract. Secondly and more convincingly, at almost identical concentrations (0.45-0.50 mg/ml) of various extracts and D-glucose (8.3 mM), the relative magnitude of the changes in insulin output ranged from positive values of 71.2±14.8 and 55.4±12.4% and a negative value of 12.1±6.1% with fraction A, H_2O-methanol and aqueous extracts, respectively. These variations in secretory response to distinct extracts tested over a comparable concentration range is further demonstrated in Fig. 2. In addition, the relative magnitude of the decrease in insulin output in response to increased extract concentration was variable, as illustrated by comparison between the aqueous or fraction A extracts.

The present results must be compared with those previously reported by Nmila *et al* (5). The group analyzed a basic fraction obtained by ion exchange chromatography of an H_2O-isopropanol extract, which was prepared following hexane delipidation of a crude *Citrullus colocynthis* seed powdered extract. Increased pancreatic flow and insulin release was observed at 0.1 mg/ml basic fraction in rat isolated pancreas perfused in the presence of 8.3 mM D-glucose. The authors hypothesized that this effect is associated with insulinotropic amino acids in the extract, including leucine, isoleucine and phenylalanine. However, β-(pyrazol-1-yl-)-L-alanine, a major amino acid present in *Citrullus colocynthis* seeds and specific members of the *cucurbitaceae* family, is not involved in the stimulation of insulin secretion (5). Phytochemical testing of extracts of the present study revealed that the hydromethanolic and ethyl acetate extracts exhibit higher levels of polyphenols and flavonoids than aqueous and n-butanol extracts, particularly quercetin and myrcetin (unpublished data). In previous studies, exposure of rat isolated islets to specific flavonoids, including (-)epicathechin or quercetin, resulted in a 44-70% increase in insulin release (6). It was argued that such flavonoids may act on islet function, at least in part, via alteration of Ca^{2+} fluxes and cyclic nucleotide metabolism (6).

In conclusion, the present study provides a preliminary insight into the chemical compounds responsible for the positive and negative insulinotropic actions of *Citrullus colocynthis* seed extracts and may prove useful for the identification of molecules suitable for the prevention and/or correction of diabetes mellitus in human subjects.

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