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Cinchonain Ib isolated from *Eriobotrya japonica* induces insulin secretion *in vitro* and *in vivo*

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ABSTRACT

Aims of the study: Eriobotrya japonica leaves had been used traditionally for the treatment of diabetes mellitus by immersing the dried leaves in a hot water drink. Few studies have shown the hypoglycemic effect of *Eriobotrya japonica* using crude alcoholic extract and isolated methanolic compounds. These studies proposed that the mechanism of action could be by stimulating the β-islets of Langerhans to secrete insulin, however with no scientific evidence.
Methods: Eriobotrya japonica water extract (EJWE) and the compounds derived from it: cinchonain Ib, procyanidin B-2, chlorogenic acid and epicatechin, were tested for their effects on insulin secretion from INS-1 cells and following oral administration in rats.
Results: The present study showed that EJWE increased significantly ($p < 0.05$) insulin secretion from INS-1 cells in dose-dependent manner. Oral administration of EJWE at 230 mg/kg to rats, however, decreased plasma insulin level for as long as 240 min post-administration and caused a transient drop of blood glucose at 15 and 30 min post-administration. On the other hand, cinchonain Ib enhanced significantly ($p < 0.05$) insulin secretion from INS-1 cells, whereas epicatechin inhibited significantly ($p < 0.05$) insulin secretion from INS-1 cells. In addition, cinchonain Ib enhanced significantly (150%: $p < 0.05$) plasma insulin level in rats for as long as 240 min after 108 mg/kg oral administration but did not induce any change in blood glucose level.
Conclusion: These data indicate that cinchonain Ib has an insulinotropic effect and suggest the possible use of cinchonain Ib for managing type 2 diabetes.

1. Introduction

Diabetes mellitus is a chronic metabolic disease affecting 4% of the population worldwide (Gaster and Hirsch, 1998; De Fronzo, 1999). This percentage is expected to increase as the incidence of type 2 diabetes which is affecting 85–90% of the diabetic population is increasing dramatically (Gaster and Hirsch, 1998; De Fronzo, 1999). Type 2 diabetes is characterized by abnormal insulin response to glucose and insulin resistance of target peripheral tissues (Yki-Jarvinen, 1994; De Fronzo, 1999) and is considered a major health problem because of its high frequency and long duration especially when accompanied with its chronic complications (Gaster and Hirsch, 1998; De Fronzo, 1999). Patients with type 2 diabetes often fail to achieve glycemic control with oral anti-diabetic drugs. In addition, these drugs have adverse effects that limit their usage. Therefore, searching for new treatments with similar efficacy but with less adverse events is still warranted.

Eriobotrya japonica LINDL (Rosaceae) leaves had been used traditionally for the treatment of diabetes mellitus (Roman-Ramos et al., 1991). The traditional use of *Eriobotrya japonica* is based on immersing the dried leaves in a hot water drink. Few studies have shown the hypoglycemic effect *Eriobotrya japonica* using crude alcoholic extract and isolated methanolic compounds (Noreen et al., 1988; De Tommasi et al., 1991; Li et al., 2007; Chen et al., 2008). These studies proposed that the mechanism of action could be by stimulating the β-islets of Langerhans to secrete insulin (De Tommasi et al., 1991), however with no scientific evidence. The aims of the present study were: firstly, to evaluate the hypoglycemic and insulinotropic effects of *Eriobotrya japonica* as whole water extract (EJWE) secondly, to isolate the compounds from the EJWE and to elucidate their structures and thirdly, to evaluate the insulinotropic and hypoglycemic effect of each of the isolated compounds.

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2. Materials and methods

2.1. Plant material

Eriobotrya japonica leaves were collected from Tarek area (Jordan; 06/2000) and identified in comparison with authentic material deposited at the Herbarium of the Institut für Pharmazeutische Biologie und Phytochemie, Münster-Germany under PBMS18.

2.2. Extraction and isolation

Air-dried material (1 kg) was exhaustively extracted with boiled water (10 l) and the combined extracts were evaporated *in vacuo* to 1.5 l, filtered and concentrated. Successive extractions with ethyl acetate (7.5 l) gave on evaporation of solvent solid of 10 g ethyl acetate (EA) phase. The remaining H_2O-phase (WP) was evaporated to dryness (g). 8.0 of the EA-phase were subjected to CC on Sephadex LH-20 (5.5 cm × 68 cm) with EtOH–H_2O (6 l), EtOH–MeOH 1:1 (7 l), MeOH (3 l) and acetone–H_2O 7:3 (4 l) to give 8 fractions. Fraction 3 (3800–4200 ml, 1.3 g) was subjected to chromatography on MCI-gel CHP 20 P (25 mm × 250 mm) with a 10–80% MeOH linear gradient (17 ml/frs.) to afford epicatechin (subfrs. 91–107, 450 mg). Fraction 4 (4200–4770 ml, 2.2 g) was separated on MCI-gel with the same gradient as above to afford epicatechin-(4β → 8)-epicatechin (procyanidin B-2; subfrs. 90–105, 270 mg). Fraction 6 (5600–6700 ml, 1.8 g) was separated on MCI-gel to afford chlorogenic acid subfrs. 87–115 (120 mg). Cinchonain Ib was achieved from fraction 8 (9200–9700 ml, 550 mg) followed by MCI-gel chromatography (subfrs. 41–49, 47 mg). All compounds were identified as free phenolic compounds like cinchonain Ib and chlorogenic acid or after acetylation by their physical data (NMR, MS, and CD) and by comparison with authentic samples and published values (Jung et al., 1999; Ploss et al., 2001; Beltrame et al., 2006).

2.3. Analytical procedure

NMR spectra were recorded for chlorogenic acid and cinchonain Ib (Fig. 1) in CD_3OD and for epicatechin peracetate and procyanidin peracetate in $CDCl_3$ on a Varian Mercury 400 plus (400 MHz) relative to CH_3OH or $CHCl_3$. ^{13}C NMR were recorded at 100 MHz. CD data were obtained in MeOH on a Jasco J 600. MALDI-TOF mass spectrometer: LAZARUS II (home built), N2-laser (LSI VSL337ND) 337 nm, 3 ns pulse width, focus diameter 0.1 mm, 16 kV acceleration voltage, 1 m drift length, data logging with LeCroy9450A, 2.5 ns sampling time and expected mass accuracy ±0.1%, sample preparation: acetylated compounds (epicatechin and procyanidin B-2) were deposited from a solution in $CHCl_3$ on a thin layer of 2,5-dihydroxybenzoic acid (DHB) crystals. Chlorogenic acid and cinchonain Ib were deposited from a solution of MeOH. Analytical TLC was carried out on aluminum sheets (Kieselgel 60 F_{254}, 0.2 mm, Merck) using ethyl acetate–water–formic acid (90:5:5). The compounds were visualized by spraying with vanillin–HCl reagent or by 1% ethanolic $FeCl_3$ solution. Acetylation was performed in pyridine–acetic acid anhydride (1:1.2) at ambient temp for 24 h.

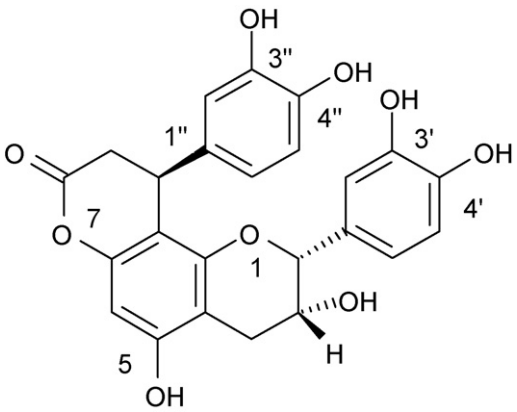

Fig. 1. Structure of cinchonain Ib.

2.4. Cell culture

Rat insulinoma cell line, INS-1 cells, were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin. Cells were grown in micro-well for 4–6 days to reach confluence (cell density in half-confluence ~1–2 × 10^6 cells/ml) (Verspohl, 2002; Verspohl et al., 2005). Prior to the experiment, cells were washed three times and incubated in Krebs-Ringer buffer containing 10 mM HEPES and 5% bovine serum albumin (KRBH). When measuring insulin secretion, half-confluent cells were incubated for 90 min at 37 °C, in the presence or absence of the extract or compounds diluted in the KBRH buffer containing 3.0 or 5.6 mM glucose, followed by collection the media-containing insulin. The level of insulin release at 5.6 mM glucose was normalized to 100% (Verspohl, 2002; Verspohl et al., 2005).

2.5. Animals

Wister rats (males and females) weighing 250–300 g were used. The animals were housed at 22 °C with a 12 h light/dark cycle and fed a standard pellet diet with tap water *ad libitum*. The use of animals was conducted in accordance with the University of Münster Protocol.

2.6. Extract/compounds administration and blood sampling

Each extract concentration and or compounds were diluted in saline and administered orally into rats (Verspohl, 2002). Following anaesthetization of rats with halothane (4%), blood was drawn by retrobulbar technique and collected into chilled tubes containing heparin at several time points (0, 15, 30, 60, 120, and 240 min). Plasma was obtained following centrifugation, glucose levels were assayed and the rest of the plasma was kept frozen at −20 °C until insulin assay was performed (Verspohl, 2002; Verspohl et al., 2005).

2.7. Blood glucose and insulin assays

Blood glucose was determined by the enzymatic hexokinase method (Boehringer-Mannheim, Germany) while insulin (cell-secreting insulin and rat plasma insulin) concentrations were assayed with a radioimmunoassay using rat insulin as a standard (mono-^{125}I-Tyr A^{14})–porcine insulin (Avantis, Frankfurt, Germany) labeled compound and anti-insulin (Linco, St Louis, USA) (Verspohl, 2002; Verspohl et al., 2005). The basal plasma insulin levels were in the range of 6–14 μU/ml.

2.8. Statistical analysis

Multiple comparisons of means were tested by one-way ANOVA followed by a post hoc Newman–Keuls test. p value of <0.05 is considered significant.

3. Results

Eriobotrya japonica water extract (EJWE) increased significantly ($p < 0.05$) insulin secretion from INS-1 cells in dose-dependent manner (Fig. 2). The maximum percentage of insulin increase was approximately of 260% of the basal level. This level was reached by

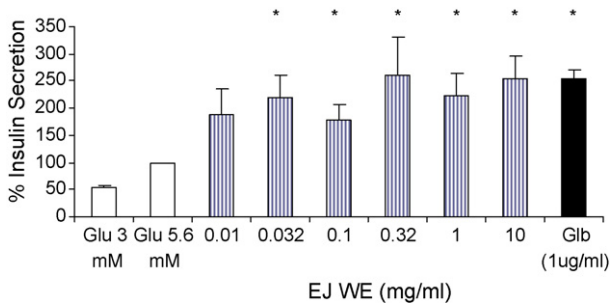

Fig. 2. The effect of EJWE on insulin secretion from INS-1 cell line. Glucose level was adjusted to 5.6 mM and represents 100% of insulin secretion. Increasing concentrations of EJWE extract revealed a significant increase (*$p < 0.05$) in insulin secretion in a dose-dependent manner. The bars represent the mean ± S.E. of four independent experiments.

0.32 mg/ml of EJWE and was similar to 1 μg/ml of glibenclamide, an insulinotropic drug. Oral administration of EJWE at 230 mg/kg to rats, however, decreased plasma insulin level for as long as 240 min post-administration (Fig. 3a) and caused a transient drop of blood glucose at 15 and 30 min post-administration (Fig. 3b).

Several compounds were isolated from EJWE and tested for their *in vitro* and *in vivo* insulin secretion. These compounds were cinchonain Ib, procyanidin B-2, chlorogenic acid and epicatechin. The major portion isolated from EJWE was the epicatechin, whereas cinchonain Ib (Fig. 1), procyanidin B-2, and chlorogenic acid were available at lower amounts.

The induction of insulin by the above isolated compounds from INS-1 cells in the presence of 5.6 mM glucose concentration is presented in Table 1. Cinchonain Ib increased significantly ($p < 0.05$) insulin secretion from INS-1 cells and in dose-dependent manner, whereas epicatechin decreased significantly ($p < 0.05$) insulin release and in dose-dependent manner. On the other hand, chlorogenic acid has no insulinotropic effect, while procyanidin B-2 resulted in apparent decrease in insulin secretion but with no statistical significance (Table 1).

Oral administration of 108, 188, and 225 mg/kg of cinchonain Ib, chlorogenic acid and procyanidin B-2, respectively, induced

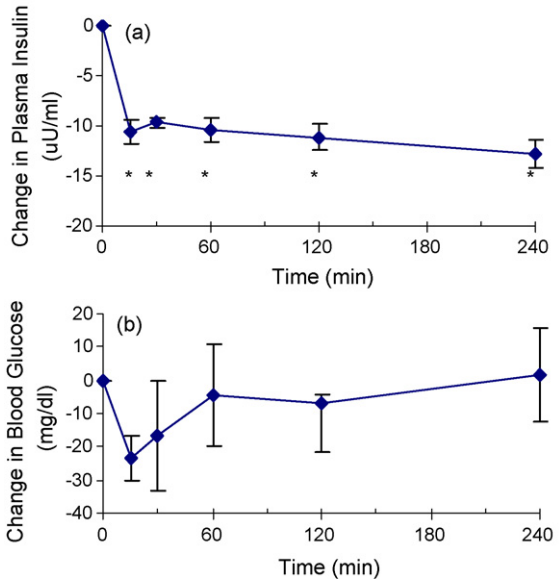

Fig. 3. Oral administration of 230 mg/kg of EJWE caused a significant (*$p < 0.05$) drop in plasma insulin and it remained for 240 min (a) but induced only a transient drop in glucose level at 15–30 min (b). Each point represents the mean ± S.E. of four independent experiments.

Table 1
The effect of compounds isolated from EJWE on glucose-mediated insulin secretion from INS-1 cells *in vitro*. Each value represents the mean percentage of insulin ± S.E.M. of three independent experiments.

Condition	Concentration (μM)	Insulin secretion (%)	Effect[a]
Glucose 3.0 mM	–	53 ± 4	
Glucose 5.6 mM	–	100 ± 0	
Glucose 5.6 mM	Cinchonain Ib		Enhancing
	0.01	122 ± 18	
	0.032	127 ± 27	
	0.1	109 ± 9	
	0.32	214 ± 27	
Glucose 5.6 mM	Procyanidin B-2		No Effect
	0.01	119 ± 19	
	0.032	113 ± 17	
	0.1	83 ± 9	
	0.32	60 ± 12	
Glucose 5.6 mM	Chlorogenic acid		No Effect
	0.01	115 ± 9	
	0.032	104 ± 19	
	0.1	111 ± 8	
	0.32	114 ± 25	
Glucose 5.6 mM	Epicatechin		Inhibition
	0.01	94 ± 15	
	0.032	78 ± 6	
	0.1	76 ± 4	
	0.32	57 ± 7	
Glucose 5.6 mM	Glibenclamide		Enhancing
	2 μM	177 ± 21	

[a] Statistically significant $p < 0.05$.

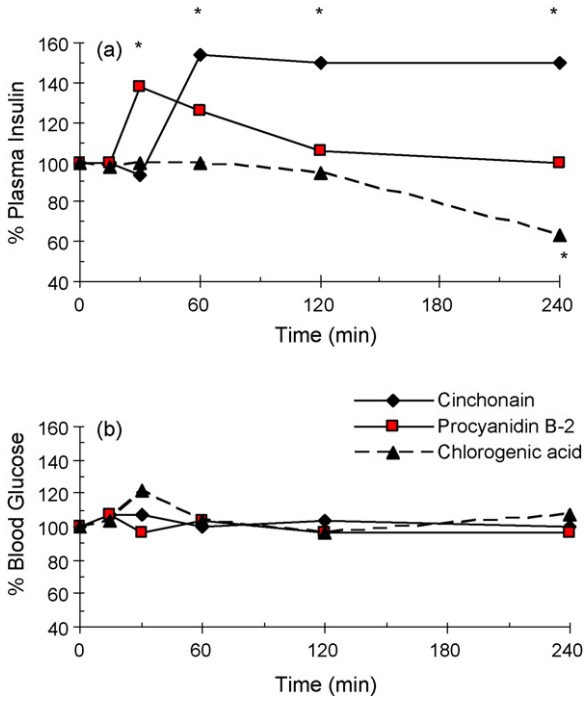

Fig. 4. The effect of an oral administration of 108, 188, and 225 mg/kg of cinchonain Ib, chlorogenic acid and procyanidin B-2, respectively, to rats. (a) Shows the effect of each compound on plasma insulin levels. Cinchonain Ib increased significantly (*$p < 0.05$) plasma insulin level, whereas epicatechin decreased significantly (*$p < 0.05$) plasma insulin level after 240 min following administration. (b) Shows the effect of each compound on blood glucose level. Each point represents the mean ± S.E. of three independent experiments.

minor to significant changes in plasma insulin level (Fig. 4a). Cinchonain Ib increased (150%, $p < 0.05$) plasma insulin level in rats for as long as 240 min post-oral administration. Procyanidin B-2 caused a short-lived increase (30–60 min) in plasma insulin level whereas chlorogenic acid decreased plasma insulin level 4 h post-oral administration (Fig. 4a). No significant changes in blood glucose levels of rats were observed following oral administration of either compound (Fig. 4b).

4. Discussion

The aim of the present study was to investigate the mechanisms of anti-hyperglycemic effect of *Eriobotrya japonica*. *In vitro* EJWE increased insulin secretion and *in vivo* caused a transient decrease in blood glucose for up to 30 min in normal rats. However, this was not accompanied with an increase in plasma insulin level but rather a decrease of it for up to 240 min. This could be explained by that the content of EJWE can induce glucose-lowering effect independent of plasma insulin levels (Ryan et al., 1998; Vella et al., 2002). The difference in effects on insulin between in vitro and in vivo experiments may be speculated to be the result of higher bioavailability of inhibitory compounds of the water extract. It has to be mentioned that EJWE is rich in polysaccharides that revealed induction of proinflammatory cytokines *in vitro* and *in vivo* (Matalka et al., 2007); however following oral administration it did not increase glucose level.

Recently, Li et al. (2007) have shown that 70% of ethanolic extract of *Eriobotrya japonica*-induced hypoglycemic effect in alloxan-diabetic mice. It is known that alloxan induces partial damage to the β-cells of Langerhans. Cinchonain as one of the compounds isolated from *Eriobotrya japonica* (Ito et al., 2000) could be one of the reasons of this anti-hyperglycemic-induced effect. In addition and to the authors' knowledge, this is the first study to show that cinchonain Ib has an insulinotropic effect *in vitro* and *in vivo*. *In vitro*, cinchonain Ib at 0.3 μM increased insulin level similar to 2 μM of glibenclamide, a drug that stimulates insulin release. On the other hand epicatechin, another compound isolated from EJWE has a negative effect on insulin secretions. The amount of epicatechin isolated from EJWE is more than cinchonain Ib (data not shown). This could be the reason why plasma insulin decreased when EJWE is administered to normal rats. The latter finding regarding epicatechin, however, is different from the results previously published by Hii and Howell (1985) who have shown that epicatechin at 0.8 mM enhanced insulin secretion *in vitro* only in the presence of 20 mmol/l glucose. In the present study, the glucose concentration was kept at 5.6 mM and the maximum concentration of epicatechin used was 0.32 μM. These differences in the concentrations of glucose and epicatechin used could be the reason for such conflicting results.

In vivo, cinchonain Ib increased plasma insulin level but with no effect on plasma glucose level in normal rats. The reason for the absence of a hypoglycemic effect could be that no glucose challenge was introduced (Ryan et al., 1998; Vella et al., 2002; Verspohl et al., 2005). However, the possibility of an anti-hyperglycemic effect of cinchonain Ib cannot be ruled out. We can propose here that cinchonain Ib has a similar function to glucagon-like peptide 1 (GLP-1). GLP-1 is a potent stimulator of glucose-induced insulin release, stimulates insulin release in the fasting state, and decreases glucagon secretion. In previous studies by Ryan et al. (1998) and Vella et al. (2002), the authors have shown that in response to GLP-1 or GLP-1 analogue and while glucose levels remained constant, insulin levels increased indicating that GLP-1 does not augment insulin-mediated glucose uptake during euglycemia. Similarly, in normal rats cinchonain Ib increased plasma insulin but with no response on plasma glucose levels. Further studies in the above-mentioned directions are warranted to explain the above phenomenon.

The present study explains the effect of *Eriobotrya japonica* leaves in type 2 diabetes mellitus and introduces cinchonain Ib as an insulinotropic agent. Further studies are required to show the potential of cinchonain Ib as a natural oral agent for managing type 2 diabetes mellitus.

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