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# Hypoglycaemic properties of Malaysian cocoa (*Theobroma cacao*) polyphenols-rich extract

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**Abstract :** The objective of the study was to investigate the hypoglycaemic properties of Malaysian cocoa (*Theobroma cacao*) polyphenols extract *in-vivo* and insulin sensitivity *in-vitro*. Cocoa extract (CE) (containing 190 - 286 mg total polyphenol per gram extract) was prepared from fermented and roasted (140°C, 20 min) beans by extracting with 80% ethanol in the ratio of 1 to 10. For the *in-vivo* study, the CE was administered in three dosages (1%, 2%, and 3%) to groups of normal and diabetic rats for a period of 4 weeks by force-feeding. Results showed that dosages of 1% and 3% CE significantly reduced ($p < 0.05$) plasma glucose levels in the diabetic rats. An *in-vitro* study (BRIN-BD11 cell lines) was used to evaluate the effect of CE on insulin sensitivity. The results demonstrated that CE at a concentration of 0.1 mg/ml significantly increased ($p < 0.05$) insulin level compared to the control. The results of this study showed that Malaysian cocoa polyphenol extract have the potential of being an insulin-mimetic agent. Further studies are on-going to elucidate the underlying mechanisms of polyphenols present in CE that contribute to the reduction of plasma glucose levels and insulin mimicking activity.

**Keywords: Cocoa beans, diabetes, hypoglycaemic, polyphenol-rich extract**

## INTRODUCTION

Diabetes mellitus is a non-communicable disease, which is considered one of the five leading causes of death in the world today. Recently, the search for appropriate hypoglycaemic agents has focused on plants used in traditional medicine, partly because of leads provided by traditional medicine to natural products with potentials of being better treatments compared to currently used drugs (Lu *et al.*, 2002; Kar *et al.*, 2003; Jang *et al.*, 2003). Drugs such as sulphonylureas, lead to higher risk of hypoglycaemia, and metformin brings a higher risk of lactic acidosis. Due to the side effects of these drugs, many studies have been conducted to explore natural products derived from plants which have potential hypoglycaemic effects (Kamtchouing *et al.*, 1998; Jayakar and Suresh, 2003; Ladeji *et al.*, 2003; Maghrani *et al.*, 2003).

Besides traditional medicinal plants, cocoa beans were thought to have fearsome magical powers by the Mayas and were carefully used in rituals, religious ceremonies and healings by priests. The Mayas used cocoa medicinally as a treatment for fever, coughs and even to help ease discomfort during pregnancy. After the 16th century conquest of Central America by Spain, Cortes introduced cocoa to Europe, where it was typically viewed as a healthy and nutritious beverage (Dillinger *et al.*, 2000). Cocoa beans were recently recognized as a rich source of polyphenols, specifically procyanidins. Zumbe (1998) reported that total polyphenol content of the cocoa bean is about 6-8% by weight of the dry bean. Some of the earliest studies on procyanidins also established that the major flavanoids of cocoa bean are catechin, epicatechin, the dimers [epicatechin-(4β®8)-catechin (procyanidin B-l) and epicatechin-(4β®8)-epicatechin (procyanidin B-2)] and the trimer [epicatechin-$(4\beta\text{®}8)_2$ epicatechin (procyanidin C-l)] (Porter *et al.*, 1991; Osakabe *et al.*, 1998). Upon fermentation and roasting, these procyanidins are converted to the largely insoluble red-brown material (tannin) resulting in the characteristic colour and taste of chocolate (Pettipher, 1986).
Polyphenols have been a choice of research interest for decades, mostly because of their antioxidant properties (Wiseman *et al.*, 1997; Sanbogi *et al.*, 1998; Netzel *et al.*, 2003). The consumption of flavanoids, including those found in cocoa and other cocoa

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products have been associated with reduction in the risk of heart diseases, having potentially beneficial effects on inflammatory activity, and being cancer-protective agents (Borchers *et al.*, 2000). Recent research showed the ability of cocoa to reduce platelet activation *in vivo*, which can cause atherosclerosis (Rein *et al.*, 2000). Oxidative stress is well known to be an important factor leading to chronic disease such as diabetes mellitus and atherosclerosis. Sabu *et al.* (2002) reported that administration of green tea polyphenols was found to reduce oxidative stress and serum glucose tolerance in alloxan-diabetic rats. A similar finding was reported by Ceriello *et al.* (2001) for red wine in humans. Our previous study on oral glucose tolerance test (OGTT) of diabetic rats showed that cocoa powder extract could reduce blood glucose levels (Amin *et al.*, 2004). However, limited studies have been done on the hypoglycaemic effect of polyphenol-rich cocoa extract. Therefore, this study was designed to assess the effectiveness of polyphenol-rich cocoa extract in reducing hyperglycaemia based on *in-vivo* (animal) and *in-vitro* studies.

## MATERIALS AND METHODS

### *Preparation of extract from cocoa beans*

Fermented and dried Malaysian cocoa beans were purchased from KL-Kepong Cocoa Products Sdn. Bhd., Port Klang, Selangor, Malaysia. The beans were roasted using an air-oven for 20 min at 140ºC (Jinap *et al.*, 1998). After cooling to room temperature, the beans were deshelled using a cocoa breaker (Limprimita, John Gordon and Co., UK). The broken nibs were ground, then defatted with petroleum-ether (b.p 40-60ºC) using a Soxhlet apparatus in order to remove cocoa butter. The defatted sample was air-dried to remove solvent residues. The extract was prepared by extracting the defatted powder with 80% (v/v) ethanol for 2 hr. The ethanol residue was removed from the extract using a rotary evaporator (Buchi Rotavor R-200, Switzerland) under reduced pressure for 20 min at 70ºC and the sample was then lyophilised.

### *Determination of total phenolic content*

The total phenolic content was determined according to the Folin-Ciocalteu method (Velioglu *et al.*, 1998). Briefly, the cocoa extract was dissolved in 80% (v/v) ethanol and then centrifuged (Rotofix 32, Hettich Zentrifugen, Germany) at 1000 x g for 15 min. A total of 100 µl of the supernatant was mixed with 0.75 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min. Then, 0.75 ml of sodium carbonate (60 g/l) solution was added to the mixture. This was allowed to stand at room temperature for 90 min and the absorbance was read at 725 nm using a UV-Vis spectrophotometer (Anthelie Advanced 5, Secomam, France). A standard calibration curve was obtained from 0.02 - 0.12 mg/ml of (-)-epicatechin (Sigma Co., St. Louis, USA). Results were expressed as epicatechin equivalents in milligrams per gram extract.

### *Animal study*

Ninety male (n = 90) Sprague-Dawley rats were purchased from Syarikat Usaha Cahaya Sdn. Bhd., Batu Caves, Selangor, Malaysia. This study was approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The rats were housed in individual plastic cages with stainless steel covers and kept at room temperature (24-28°C) under 12 h dark-light cycle. Rats were allowed free access to their respective diets and water. All rats were allowed 1 week to adapt to the environment before the treatments were administered. The experiment was carried out for 4 weeks, and body weights and blood glucose levels were recorded weekly. The rats were divided into nine groups each consisting of ten rats as follows:

Group 1: Normal rats administered saline solution
Group 2: Normal rats administered 1% CE
Group 3: Normal rats administered 2% CE
Group 4: Normal rats administered 3% CE
Group 5: Diabetic rats administered saline solution
Group 6: Diabetic rats administered 1% CE
Group 7: Diabetic rats administered 2% CE
Group 8: Diabetic rats administered 3% CE
Group 9: Diabetic rats administered glybenclamide (100 mg/ml in saline solution)

The three dosages (1%, 2%, and 3%) of CE were selected based on our previous study (Ruzaidi *et al.*, 2005). CE was suspended in 0.9% (w/v) saline solution, then administered daily (1 ml/ 100 g body weight) to the experimental rats by direct stomach intubation, using a force-feeding needle. The rats were administered with their respective treatments after induction with diabetes using streptozotocin (STZ).

### *Induction of diabetes*

Streptozotocin (STZ) (Sigma Co., St. Louis, USA) was used to induce diabetes. After overnight fasting, rats were injected intravenously with 45 mg/kg body weight of STZ dissolved in 0.05 M citrate buffer,

pH 4.5. Normal rats were injected with the same volume of 0.05 M citrate buffer. Three days after STZ injection, blood was collected from the tail milking using a Blood Glucose Sensor (MediSense Optium, Abbot Laboratories, USA) to ensure that the rats had diabetes. Only rats with fasting blood glucose of 15 mmol/l and above were included in this study.

***Determination of plasma glucose levels***

At 0 and 4 weeks of the experiment, 5 ml blood was collected from all rats through cardiac puncture and placed into a vacutainer tube. Then, the samples were centrifuged (Universal 32®, Hettich Zentrifugen, Germany) for 10 min at 1000 x g at room temperature. The supernatant was collected and kept at -20°C for determination of plasma glucose levels using a Chemistry Analyzer (Hitachi 902 Automatic Analyzer, Japan).

***Measurement of insulin secretion***

For measurement of insulin-release from cell monolayers, BRIN-BD11 cells were seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of 2.5 x $10^5$ cells per well in RPMI-1640 medium and allowed to attach during overnight incubation (37ºC; 5% $CO_2$). After washing with PBS buffer, pre-incubations were performed using the Krebs-Ringer bicarbonate buffer pH 7.4 for 40 min (37ºC; 5% $CO_2$). Then, the cells were incubated at 37ºC under 5% $CO_2$ with KRB buffer (pH 7.4) supplemented with different concentrations of CE. Following 30 min of incubation, the aliquots were removed from each well, then centrifuged at 1000 x g for 1 min at room temperature (Centrifuge 5414, Eppendorf, Germany), and supernatants were kept at -20ºC before determination using the ELISA (Mercodia, Uppsala, Sweden).

***Statistical analysis***

Data are expressed as means ± S.E.M. One-way ANOVA was used for statistical analysis. Duncan's multiple range test was used to determine any significant differences between the means at 5% level of probability.

## RESULTS

Normal and diabetic rats experiment were carried out separately which helps to explain why the initial body weight of normal and diabetic rats were significantly different. At the beginning of experiments, all groups consisted 10 rats. However, about half of the diabetic-induced rats died after STZ injection due to extreme hyperglycaemia.

Table 1 shows the effect of CE on body weights in normal and diabetic rats. Body weights of all normal rats increased significantly ($p < 0.05$) at the end of the experiment (week 4). No significant differences in body-weight changes were observed among the groups of normal rats. On the other hand, there was significant reduction ($p < 0.05$) in body weight of DC, DCE1, DCE2 and DG groups in diabetic rats. The weight loss in DC, DCE1, DCE2 and DG groups at the end of the experiment was 31%, 17%, 24% and 20% compared to week 0, respectively. The loss in body weights were not significantly different in the DCE3 group compared to their initial weights. In addition, the mean body weight in the DCE3 group was significantly higher ($p < 0.05$) compared to that of DC, DCE2 and DG groups.

**Table 1:** Effect of cocoa extract (ce) on body weight of rats

| Group | n | Body weight (g) | |
|---|---|---|---|
| | | week 0 | week 4 |
| Normal control (NC) | 8 | 184.4 ± 5.7b | 279.8 ± 8.0a |
| Normal + 1% CE (NCE1) | 8 | 182.2 ± 6.2b | 282.6 ± 7.3a |
| Normal + 2% CE (NCE2) | 9 | 184.1 ± 7.3b | 277.9 ± 12.0a |
| Normal + 3% CE (NCE3) | 10 | 180.4 ± 7.5b | 265.1 ± 12.0a |
| Diabetes control (DC) | 6 | 256.4 ± 12.1a | 176.4 ± 18.3b |
| Diabetes + 1% CE (DCE1) | 4 | 254.5 ± 10.6a | 212.5 ± 2.5b |
| Diabetes + 2%CE (DCE2) | 5 | 249.0 ± 12.1a | 189.0 ± 23.7b |
| Diabetes + 3% CE (DCE3) | 5 | 254.7 ± 10.8a | 241.8 ± 26.1a |
| Diabetes + Glibenclamide (DG) | 4 | 248.6 ± 10.2a | 197.8 ± 7.1b |

Values were expressed as means ± S.E.M. Different letters in a column indicate significant differences ($p < 0.05$). Body weights were measured every day at 9.00 am

Initial plasma glucose level of normal rats was in the range of 3.1 - 8.2 mmol/l. The mean plasma glucose level was significantly increased (about 400%) in diabetic groups as compared to normal groups after 3 days STZ injection. In normal rats, no significant differences in glucose levels were observed between week 0 and 4 (Figure 1). For diabetic rats, plasma glucose levels decreased significantly ($p < 0.05$) after feeding with 1% and 3% CE (DCE1 and DCE3) at the end of experiment (Figure 2). The reduction in percentage plasma glucose levels in DCE1 and DCE3 groups was about 47% and 57%, respectively at the end of experiment. Plasma glucose levels in DCE2 and DG groups tend to be lower compared to DC, but it was not significantly different.

Figure 3 shows insulin secretion of BRIN-BD11 rat pancreatic cell-lines at various concentrations of CE. In this study, five concentrations (2.0, 1.0, 0.5, 0.1 and 0.05 mg/ml) of CE were used. The of 0.1 mg/ml CE showed a significant increase ($p < 0.05$) in insulin secretion compared to the control. BRIN-BD11 cells treated with 2.0 mg/ml CE was significantly lower ($p < 0.05$) compared to the control in insulin secretion.

The results showed that a range from 0.05–0.1 mg/ml CE is capable of enhancing basal insulin secretion (at 2 mmol/l glucose) about 9-15% compared to the untreated BRIN-BD11 cell lines (control). However, insulin secretion at concentrations of 0.5, 1.0 and 2.0 mg/ml was lower compared to the control.

## DISCUSSION

Very limited published data is available on hypoglycaemic effect of cocoa products based on *in-vitro* or *in-vivo* studies. This study was initiated to investigate the effect of CE on glucose levels using *in-vitro* (cell culture) and *in-vivo* (animal models) methods. For diabetic models, STZ was used to induce diabetic conditions in rats. Elsner *et al.* (2000) suggested that STZ induces hyperglycaemia by damaging DNA in the nuclei of pancreatic β-cells through alkylation, leading to an increase in poly (ADP-ribose) synthase. The increase in this enzyme activity results in a drastic decrease in nicotinamide adenine dinucleotide (NAD) concentrations of the β-cells, then a decrease in the number of β-cells and death of the cells. All these changes may induce dysfunction of the pancreas in insulin secretion. According to Thulesen *et al.* (1997), STZ transported into β-cells through glucose transporter GLUT-2 located on their cell membranes, and this will injure the mitochondria. This will inevitably lead to a reduction of ATP generation through electron transport system and an increase in ADP concentrations. Subsequent degradation of ADP provides hypoxanthine, a substrate of xanthine oxidase (XOD). When XOD reaction takes place in β-cells, oxygen radicals which are free radicals will be produced, resulting in cell damage and the onset of the diabetic conditions.

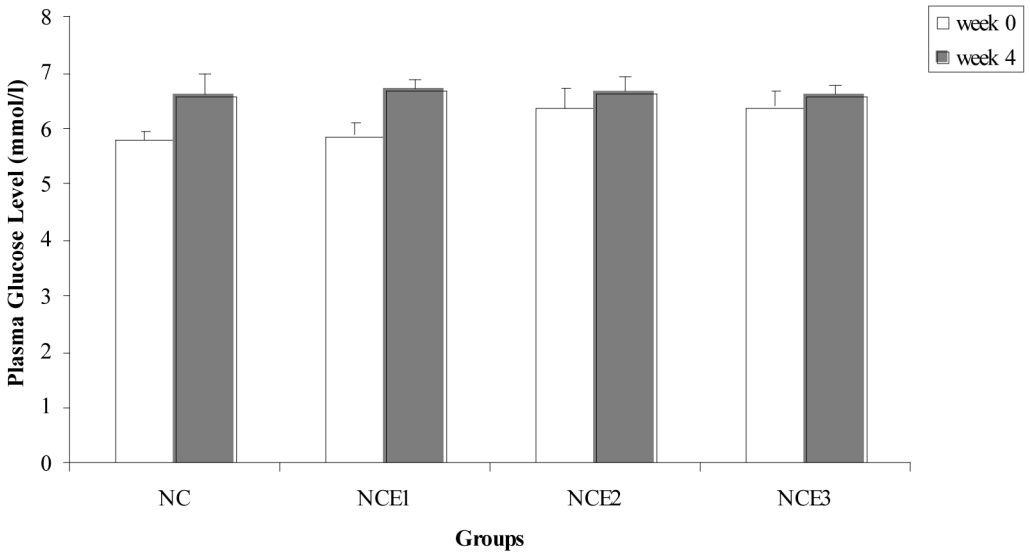


**Figure 1:** Plasma glucose levels of normal rats fed with cocoa extract (CE). No significant differences between week 0 and 4 for all groups. NC: Normal control (n = 8); NCE1: Normal + 1% CE (n = 8); NCE2: Normal + 2% CE (n = 9); NCE3: Normal + 3% CE (n = 10)

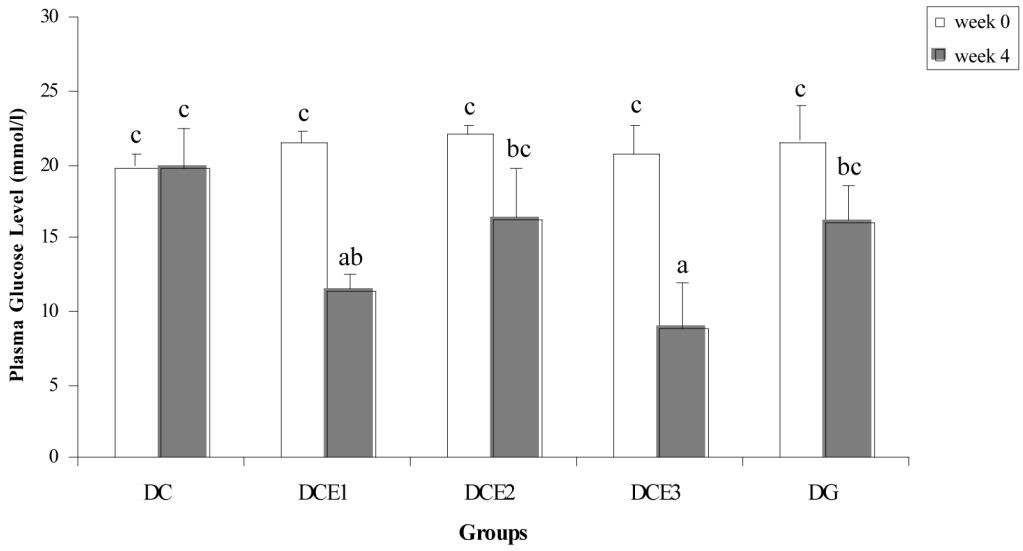


**Figure 2:** Plasma glucose levels of diabetic rats fed with cocoa extract (CE). Values with different superscripts were significantly different ($p < 0.05$). DC: Diabetes control (n =6); DCE1: Diabetes + 1% CE (n = 4); DCE2: Diabetes + 2% CE (n = 5); DCE3: Diabetes + 3% CE (n = 5); DG: Diabetes + glibenclamide (n = 4).

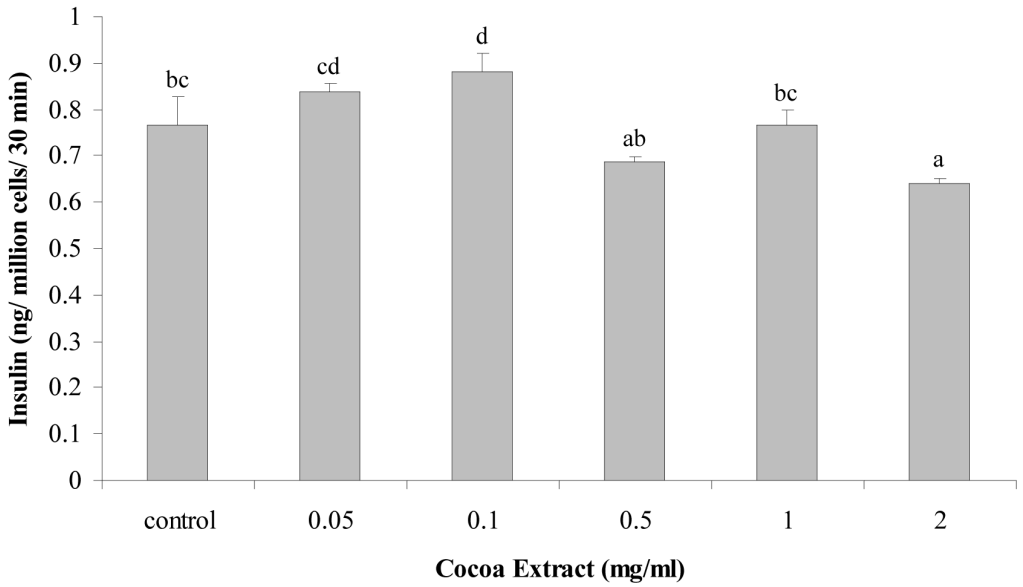


**Figure 3:** The effect of cocoa extract (CE) on *in-vitro* insulin secretion from BRIN-BD11 cell lines. The extract was prepared at concentrations of 0.05 to 2.0 mg/ml. Values are expressed as means ± S.E.M. of four replicate determinations. Values with different superscripts were significantly different ($p < 0.05$)

In this study, rats were administered orally (force-feeding) with their respective treatments daily. The results revealed that all normal rats showed an increase in body weights. It could be suggested that CE did not affect the growth of normal rats. In diabetic rats, a general decrease in body weight was observed after the injection of STZ. In the DCE3 group, however, no significant decrease was observed, suggesting that CE could normalize the weight loss caused by STZ. This study clearly demonstrated that oral administration of 1% and 3% CE exhibited a significant decrease ($p < 0.05$) in plasma glucose levels in STZ-induced diabetic rats compared to the control groups.

Hypoglycaemic effects of some plants have been reported due to polyphenols (Maghrani *et al.*, 2003; Sachdewa and Khemani, 2003). Since cocoa beans is well known to be rich in polyphenols, it is possible that the hypoglycaemic effect could be related to these components. Polyphenols are reported to be the potential bioactive component for hypoglycaemic properties (Ivora *et al.*, 1989; Manickan *et al.*, 1997). These components were reported to demonstrate marked antioxidant activity. A significant reduction of plasma glucose levels in the group treated with CE may be due to the antioxidant properties of the CE. Antioxidant compounds are well known to possess free radical scavenging activity. Thus, it is suggested that glucose-lowering activity by CE could be due to polyphenols that inhibit or suppress the generation of free radical by STZ in diabetic rats. In addition, flavanoids have also been reported to regenerate damaged β-cells in alloxan diabetic rats (Chakravarty *et al.*, 1982). Therefore, it is postulated that CE could also regenerate or rejuvenate the damaged of β-cells cause by STZ.

Based on previous studies on hypoglycaemic effect of plants and herbs, several mechanisms of lowering blood glucose were proposed. One such mechanism of blood glucose lowering activity may be due to stimulation of peripheral glucose utilisation, especially in muscle and adipose tissue. Several medicinal plants have been reported to restore activity of key enzymes of glucose and glycogen metabolism which are strongly disturbed in STZ diabetic rats (Ugochukwu and Babady, 2003). Several studies on plants and herbs compounds have revealed that these compounds possess properties that mimick insulin action, preferably by interacting with the glycoprotein residues of the insulin receptor and enhanced glucose uptake by cells (Gray and Flatt, 1999) which is called insulin-like activity.

Insulin is essential for maintaining glucose homeostasis and regulating carbohydrate, lipid, and protein metabolism (Saltiel and Kahn, 2001). After a meal, blood glucose levels increase and insulin plays a major role in keeping blood glucose levels within a narrow range. The βcells of the pancreas respond to increasing glucose levels by releasing insulin into the blood. Insulin affects glucose turnover in many tissues. In liver, insulin inhibits glycogenolysis and gluconeogenesis (DeFronzo and Ferrannini, 1987). In skeletal muscle, which accounts for approximately 75 % of insulin-mediated glucose disposal after a glucose challenge (DeFronzo *et al.*, 1981; DeFronzo *et al.*, 1985), insulin promotes glucose uptake into cells.

It is strongly suggested that CE exhibited significant antihyperglycaemic activities in STZ-induced rats. However, the mechanism of the hypoglycaemic effect of the CE is not clear. Although pancreatic regeneration after partial resection or pancreatic injury has been demonstrated in animal models, whether regeneration occurs or not in human pancreas is still controversial. Studies by Tsiotos *et al.* (1999) revealed that human pancreas does not regenerate after partial anatomical resection. Yanardag *et al.* (2003) reported that plant therapy cannot regenerate βcells of the endocrine pancreas. Several published reports showed that antihyperglycaemic plants may affect circulating insulin levels (Esmaeili and Yazdanparast, 2004; Gray and Flatt, 1998). In the STZ-diabetic rat model, not all pancreatic ß-cells are destroyed, and the remaining cells can secrete a quantity of insulin sufficient to keep the animals alive for up to 2 months (Ozcelikay *et al.*, 1999). This study suggests that the mechanism of hypoglycaemic effect of CE could also be due to the surviving βcells that are still able to release more insulin.

Studies have shown that certain plants (*Agrimony eupatoria* and *Eucalyptus globules*) could improve insulin secretion using BRIN-BD11 cell lines (Gray and Flatt, 1998). Based on the possibility that CE might stimulate the release of insulin from βcells, BRIN-BD11 cell lines were used to evaluate the CE effect on insulin secretion. Incubations were performed with glucose-responsive BRIN-BD11 cells (McClenaghan *et al.*, 1996) to investigate the possible effects of CE on insulin secretion *in vitro*. The results showed that 0.1 mg/ml CE was able to stimulate insulin secretion in BRIN-BD11 cell lines.

## CONCLUSION

This study indicated that CE could reduce high blood glucose levels in diabetic rats through enhancing insulin secretion in partially damaged pancreas. Determination of insulin levels in plasma of diabetic rats would be important to support the finding. The mechanisms of this pharmacological effect have yet to be determined.

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