

Protective effect of polyphenol-rich extract prepared from Malaysian cocoa (*Theobroma cacao*) on glucose levels and lipid profiles in streptozotocin-induced diabetic rats

Azli Mohd Mokhtar Ruzaidi,^{1,2} Maleyki Mhd Jalil Abbe,¹ Ismail Amin,^{1*} Abdul Ghani Nawalyah¹ and Hamid Muhajir³

¹Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

²Biotechnology Research Institute, Universiti Malaysia Sabah, 88999 Kota Kinabalu, Sabah, Malaysia

³Department of Microbiology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

Abstract

BACKGROUND: Cocoa beans are used for preparing cocoa liquor and cocoa powder, which are the main ingredients of cocoa-based products. Previous studies have reported the health benefits of cocoa polyphenols in reducing the risk of cardiovascular diseases. However, there is no report on the efficacy of cocoa polyphenols on diabetes mellitus. Therefore this study was designed to evaluate the protective effect of cocoa polyphenol-rich extract (CE) on glucose levels and lipid profiles in streptozotocin (STZ)-induced diabetic rats. Male Sprague-Dawley rats were divided into diabetic control, diabetic CE and diabetic glibenclamide groups.

RESULTS: Three different dosages of CE (10, 20 and 30 mg per 100 g body weight) were administered orally once a day for 1 week before STZ injection and for 3 weeks thereafter. The results showed that CE could normalise the body weight loss caused by STZ. In the 20 mg CE-pretreated group there was a 143% increase in plasma glucose levels, compared with a 226% increase in diabetic control rats. CE could also normalise total cholesterol, triglycerides and high-density lipoprotein cholesterol at the end of the experiment compared with the baseline.

CONCLUSION: The present study suggests that pretreatment with CE from roasted cocoa beans could prevent the development of diabetes induced by STZ injection in rats.

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Keywords: *Theobroma cacao*; cocoa extract; glucose levels; hypoglycaemic

INTRODUCTION

There are several ways of preventing diabetes and/or controlling its progression. Public and professional awareness of the risk factors and symptoms of diabetes is an important step towards its prevention and control. There is increasing demand by patients for natural products with antihyperglycaemic activity owing to the side effects associated with insulin and oral hypoglycaemic drugs.^{1,2} Therefore it has become necessary to look for an economical as well as a therapeutically effective use of natural products in prevention and treatment, especially in developing and underdeveloped countries.

The search for safer and more effective compounds to protect β -cells from inflammatory destruction is still in progress. Several compounds such as

metallothionein, nicotinamide and (–)-epicatechin have been reported to inhibit the diabetogenic action of streptozotocin (STZ) or alloxan in animal studies.^{3,4} Palm Vitee (palm oil vitamin E) has a protective action against the toxic inflammation caused by STZ.⁵

Cocoa beans have been reported to be a rich source of polyphenols, especially (–)-epicatechin. Some of the earliest studies established that the major flavanoids in cocoa beans were catechin, epicatechin, the dimers epicatechin-(4 β → 8)-catechin (procyanidin B-1) and epicatechin-(4 β → 8)-epicatechin (procyanidin B-2) and the trimer [epicatechin-(4 β → 8)]₂-epicatechin (procyanidin C-1).^{6,7} In a previous study we showed that a diet containing cocoa polyphenol-rich extract reduced the glucose levels and lipid profiles in STZ-induced diabetic rats.⁸ In similar work,

* Correspondence to: Ismail Amin, Department of Nutrition and Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

E-mail: amin@medic.upm.edu.my

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Osakabe *et al.*⁹ demonstrated that proanthocyanidins derived from cocoa inhibited diabetes-induced cataract formation. However, limited research has been conducted on the protective effect of cocoa polyphenol-rich extract (CE) against STZ diabetogenic action. Therefore the present study was focused on evaluating the protective action of CE against the destruction of insulin-producing β -cells of the pancreas in STZ-induced diabetic rats.

MATERIALS AND METHODS

Preparation of ethanolic extract

Raw (fermented and dried) Malaysian cocoa beans were purchased from KL-Kepong Cocoa Products Sdn. Bhd. (Port Klang, Selangor, Malaysia). The beans were roasted in an air oven for 20 min at 140 °C.¹⁰ After cooling to room temperature, the roasted beans were deshelled using a cocoa breaker (Limprimita, John Gordon & Co., Laneashire UK). The cotyledons were ground and defatted with petroleum ether (b.p. 40–60 °C) in a Soxhlet apparatus. The defatted cotyledons were air dried to remove the solvent residue. The extract was prepared by treating the defatted powder with 80% (v/v) ethanol for 2 h. The ethanol residue was removed from the extract using a rotary evaporator (Rotavor R-200, Büchi, Flawil, Switzerland) for 20 min at 70 °C and the resulting extract was lyophilised. This ethanolic extract was considered to be cocoa polyphenol-rich⁸ and was used for total phenolic determination and the animal study.

Determination of total phenolics

Total phenolic content was estimated according to the Folin–Ciocalteu assay.¹¹ Briefly, CE was dissolved in 80% (v/v) ethanol and centrifuged (Rotofix 32, Hettich Zentrifugen, Tuttlingen, Germany) at 1000 $\times g$ for 15 min. Following centrifugation, 100 μ L of the supernatant was mixed with 0.75 mL of Folin–Ciocalteu reagent (previously diluted 1:10 with distilled water) and allowed to stand at room temperature for 5 min. Sodium carbonate solution (0.75 mL) was then added to the mixture. After standing for a further 90 min at room temperature, the absorbance at 725 nm was recorded using a UV–visible spectrophotometer (Anthelie Advanced 5, Secomam, Ales, France). A standard calibration curve was constructed using 0.02–0.12 mg mL⁻¹ (-)-epicatechin (Sigma, St Louis, MO, USA). Results were expressed as mg epicatechin equivalents g⁻¹ extract.

Animal study

Preparation of animals

This study has been approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Fifty male Sprague–Dawley rats (200–350 g initial weight) were purchased from Syarikat Usaha Cahaya Sdn. Bhd.

(Batu Caves, Selangor, Malaysia). The rats were housed in individual plastic cages with stainless steel covers and kept at room temperature (24–28 °C) under a 12/12 h dark/light cycle. Animals were allowed free access to their respective diets and water. All rats were allowed 7 days to adapt to the environment before being given the treatment. The experiment was conducted for 28 days. Body weights, food intakes and blood glucose levels were recorded weekly. The rats were divided into five groups, each consisting of ten rats ($n = 10$):

- group 1 – diabetic rats administered normal saline (DC);
- group 2 – diabetic rats administered 10 mg mL⁻¹ CE (DCE1);
- group 3 – diabetic rats administered 20 mg mL⁻¹ CE (DCE2);
- group 4 – diabetic rats administered 30 mg mL⁻¹ CE (DCE3);
- group 5 – diabetic rats administered 100 mg mL⁻¹ glibenclamide (DG).

CE (10, 20 and 30 mg mL⁻¹) and glibenclamide (100 mg mL⁻¹) were suspended in 0.9% (w/v) normal saline and given daily (1 mL per 100 g body weight) to the experimental rats by gastric intubation using a force-feeding needle. The animals were given CE once daily for 7 days before STZ injection and for 21 days thereafter. At day 7 the rats were given CE 1 h before STZ injection.

Induction of diabetes

STZ (Sigma) was used for inducing diabetes in the rats at day 7. After overnight fasting, the rats were injected intravenously with 45 mg kg⁻¹ body weight of STZ dissolved in 0.05 mol L⁻¹ citrate buffer (pH 4.5). Rats injected with the same volume of 0.05 mol L⁻¹ citrate buffer served as the control group.

Determination of glucose levels and lipid profiles

At days 0, 9 and 28 of the experiment, 5 mL of blood was collected from the abdominal aorta of each animal, placed in a Vacutainer tube and centrifuged (Universal 32®, Hettich Zentrifugen) at 1000 $\times g$ for 10 min at room temperature. The supernatant was collected and kept at -20 °C for further analysis. Plasma glucose levels and lipid profiles were measured using a chemistry analyser (Automatic Analyser 902, Hitachi, Tokyo, Japan).

Statistical analysis

Data were expressed as mean \pm standard error of mean (SEM). One-way analysis of variance (ANOVA) was applied to determine differences between groups. Duncan's multiple range test was used to find significant differences among means. Results were considered significantly different at the level of $P < 0.05$.

RESULTS

The initial body weights of rats were in the range 200–350 g. The body weights of each group of rats were not significantly different before STZ injection (Table 1). At 21 days after STZ injection the body weights of DC, DCE1, DCE3 and DG rats were significantly decreased ($P < 0.05$) compared with their initial weights. However, there was no significant decrease in body weight in the DCE2 group.

All rats exhibited a decrease in body weight gain after STZ injection at week 1 (Fig. 1). The body weights of DC and DG rats were drastically decreased at week 2. However, in the CE-pretreated groups (DCE1, DCE2 and DCE3) the body weight loss was much lower compared with the DC group at weeks 2, 3 and 4, though there was no significant difference.

Figure 2 shows the protective effect of CE on plasma glucose levels in STZ-induced diabetic rats. At 2 days after STZ injection, i.e. at day 9, glucose levels increased significantly ($P < 0.05$) in all groups compared with the initial glucose levels. In the CE-pretreated groups (DCE1, DCE2 and DCE3) and the glibenclamide-pretreated group (DG) the increase was significantly lower ($P < 0.05$) compared with the DC group at day 9. The increments in glucose levels in the DC, DCE1, DCE2, DCE3 and DG groups were 226, 163, 143, 156 and 148% respectively. There was no significant increase in glucose levels in treated rats at the end of the study (day 28) compared with day 9, except for the DCE3 group. Only the DCE2 group showed a significant decrease ($P < 0.05$) in glucose

Table 1. Effect of cocoa extract (CE) on body weight of rats

Group	Body weight (g) ^a	
	Initial	Final
Diabetic control (DC)	328.6 ± 13.7b	222.0 ± 38.0a
Diabetic + 10 mg CE (DCE1)	325.8 ± 13.4b	251.3 ± 26.3a
Diabetic + 20 mg CE (DCE2)	323.1 ± 13.3b	280.3 ± 29.2ab
Diabetic + 30 mg CE (DCE3)	320.8 ± 11.8b	251.0 ± 8.2a
Diabetic + glibenclamide (DG)	313.4 ± 19.3b	224.0 ± 26.9a

^a Body weights were measured weekly. Values are expressed as mean ± SEM. Different letters indicate significant differences ($P < 0.05$).

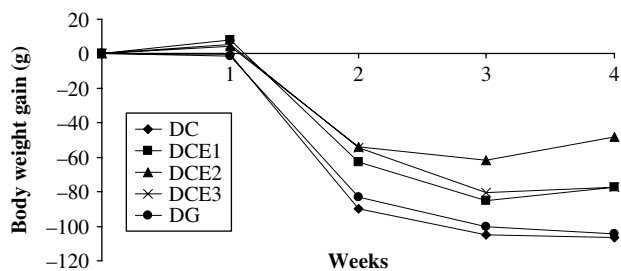


Figure 1. Pattern of body weight in control rats and those pretreated with cocoa extract (CE) and glibenclamide: DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. The coefficient of variation for all data was less than 24%. Body weights were measured weekly.

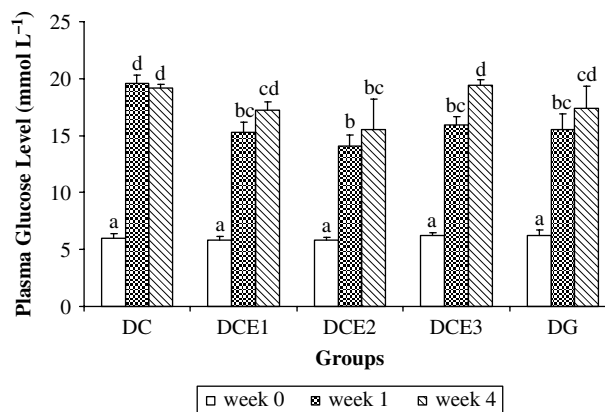


Figure 2. Plasma glucose levels of diabetic rats pretreated with cocoa extract (CE): DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. Values with different letters are significantly different ($P < 0.05$) between groups and weeks.

levels compared with the DC group at the end of the experiment (day 28).

The plasma total cholesterol levels in all groups were significantly higher ($P < 0.05$) at day 9 after STZ injection compared with day 0 (Fig. 3). At the end of the experiment (day 28) the total cholesterol levels in all treated animals were normalised.

Figure 4 shows the effect of CE on plasma high-density lipoprotein (HDL) cholesterol levels in STZ-induced diabetic rats. After STZ injection, all rats exhibited a significant decrease ($P < 0.05$) in HDL cholesterol levels at day 9 compared with day 0. The reduction in HDL cholesterol levels was in the range 54–61%. Interestingly, HDL cholesterol levels were normalised in CE- and glibenclamide-pretreated rats at the end of the study. However, no significant change in plasma low-density lipoprotein (LDL) cholesterol levels was observed during the 4 week experimental period (Fig. 5).

There were significant increases ($P < 0.05$) in plasma triglyceride levels in DC, DCE1 and DG rats after STZ injection (Fig. 6). However, in the DCE2

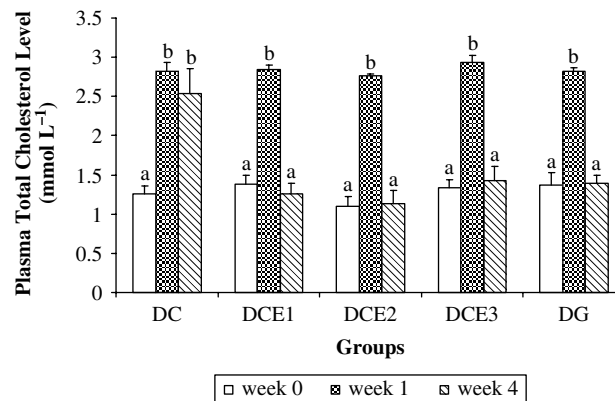


Figure 3. Plasma total cholesterol levels of diabetic rats pretreated with cocoa extract (CE): DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. Values with different letters are significantly different ($P < 0.05$) between groups and weeks.

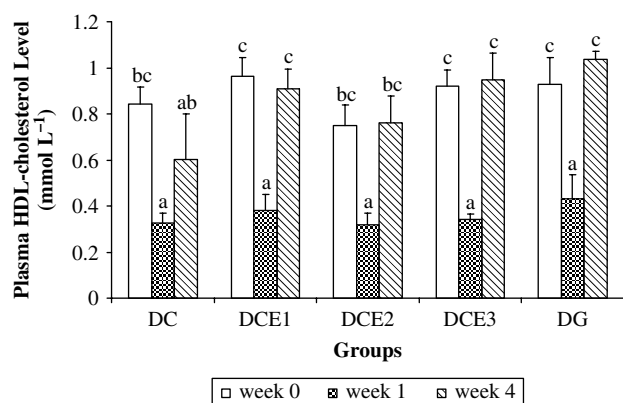


Figure 4. Plasma HDL cholesterol levels of diabetic rats pretreated with cocoa extract (CE): DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. Values with different letters are significantly different ($P < 0.05$) between groups and weeks.

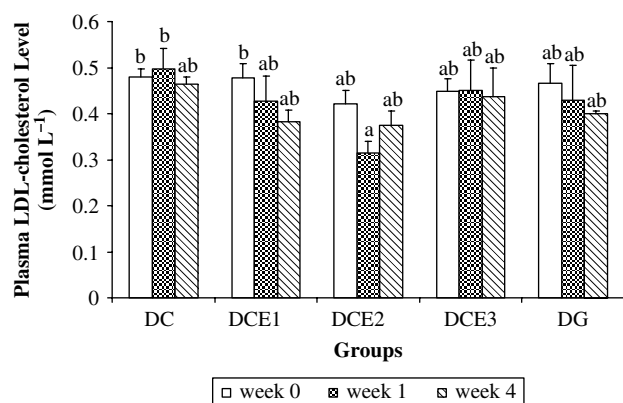


Figure 5. Plasma LDL cholesterol levels of diabetic rats pretreated with cocoa extract (CE): DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. Values with different letters are significantly different ($P < 0.05$) between groups and weeks.

and DCE3 groups the elevation was significantly lower ($P < 0.05$) compared with the DC group at day 9. At the end of the experiment a normalisation of plasma triglyceride levels was observed in CE- and glibenclamide-pretreated rats.

DISCUSSION

STZ is a specific β -cell toxin and can be used to chemically induce hyperglycaemia in rats and mice. It is taken up by pancreatic β -cells via a glucose transporter (GLUT2) and causes alkylation of deoxyribonucleic acid (DNA).^{12,13} DNA damage induces activation of poly adenosine diphosphate (ADP)-ribosylation, a process that is more important for the diabetogenicity of STZ than DNA damage itself.¹⁴ Poly ADP-ribosylation leads to the depletion of cellular nicotinamide adenine dinucleotide (NAD⁺) and adenosine triphosphate (ATP).¹⁵ Enhanced ATP dephosphorylation after STZ treatment supplies a substrate for xanthine oxidase (XOD), resulting in the formation of superoxide radicals. Consequently,

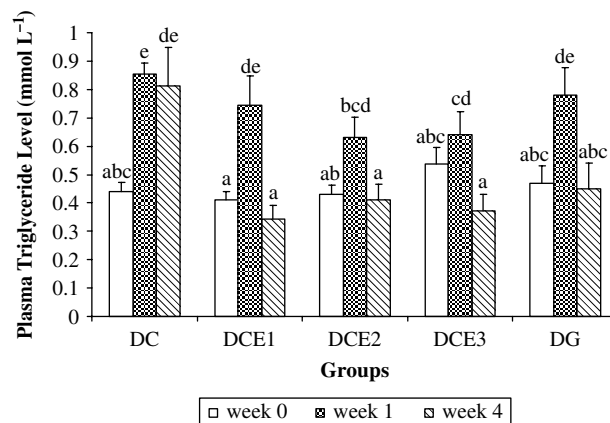


Figure 6. Plasma triglyceride levels of diabetic rats pretreated with cocoa extract (CE): DC, diabetic control; DCE1, diabetic + 10 mg CE; DCE2, diabetic + 20 mg CE; DCE3, diabetic + 30 mg CE; DG, diabetic + glibenclamide. Values with different letters are significantly different ($P < 0.05$) between groups and weeks.

hydrogen peroxide and hydroxyl radicals are also generated. Furthermore, STZ liberates toxic amounts of nitric oxide (NO), which inhibits aconitase activity and participates in DNA damage.¹⁵ As a result of the action of STZ, β -cells undergo destruction by necrosis.

In this study the total polyphenol content of CE was in the range 190–286 mg epicatechin equivalents g^{-1} extract. A previous study showed that cocoa beans are rich in polyphenols such as (–)-epicatechin, (+)-catechin, quercetin and procyanidin.¹⁶ To evaluate the protective effect of CE against STZ-induced diabetes in rats, CE (10, 20 and 30 mg mL^{-1}) was force-fed daily to the rats for 1 week before STZ injection. On the last day of pretreatment (day 7), CE was given to the rats 1 h before STZ injection. This procedure was based on the findings of Baba *et al.*,¹⁷ which indicated that (–)-epicatechin metabolite occurred at its maximum level in plasma between 30 and 60 min after rats were given a cocoa beverage. Our results showed that CE administration significantly lowered ($P < 0.05$) the hyperglycaemic action of STZ in the DCE2 group. Treatment with CE also seemed to prevent body weight loss and improve body weight to some extent. Thus pretreatment with CE could be effective in preventing the development of hyperglycaemia following STZ injection. Kamtchouing *et al.*¹⁸ also reported that Anacardiaceae (*Anacardium occidentale*) extract showed a protective effect against the diabetogenic action of STZ. Moreover, Gupta *et al.*¹⁹ showed that neem seed extract had a protective effect on the heart and erythrocytes of diabetic rats. It is suggested that CE may have reacted with or scavenged STZ. Superoxide dismutase (SOD) is an enzyme known to be part of the antioxidant defence system of cells and a scavenger of free radicals. Vucic *et al.*²⁰ reported that the activity of SOD is low in diabetes mellitus. CE may have acted by increasing the resistance of β -cells through activating SOD and scavenging free radicals caused by STZ. This scenario is supported by Sabu *et al.*,²¹ who found that green

tea polyphenols improved SOD levels in diabetic rats. The actual mechanisms of this pharmacological effect have yet to be determined.

The selection of the dose of glibenclamide employed in this study was based on previous research by Nagappa *et al.*²² Glibenclamide is one of the most widely used orally active drugs (sulfonylureas) for the treatment of type 2 diabetes mellitus. The acute hypoglycaemic action of glibenclamide involves stimulation of insulin and inhibition of glucagon secretion.²³ However, glibenclamide is only effective when there are still surviving β -cells in the pancreas. In the present study, glibenclamide tended to lower plasma glucose levels, which may be due to activation of pancreatic β -cells to secrete insulin after a single administration of glibenclamide. It could be suggested that the diabetic rats in this study still have some surviving β -cells in the pancreas, though not sufficient to significantly decrease the plasma glucose levels.

Generally, diabetic models are also used to investigate successful treatments for hypercholesterolaemia.²⁴ Abnormalities in lipids and lipoproteins play key roles in the development and progression of atherosclerotic vascular diseases in type 1 diabetes mellitus.²⁵ The most common lipid abnormalities in diabetes mellitus are changes in plasma cholesterol and triglyceride levels, which certainly contribute to the development of cardiovascular diseases.²⁶ Hypercholesterolaemia and hypertriglyceridemia have been reported to occur in STZ-induced diabetic rats in several studies.^{24,27,28}

As to the protective role of CE against STZ action, rats pretreated with CE and glibenclamide did not seem to be protected from elevation of plasma cholesterol levels by STZ. However, after 3 weeks of further treatment with CE, total cholesterol levels decreased significantly ($P < 0.05$) and were normalised in diabetic rats. In contrast, elevation of triglyceride levels seemed to be significantly suppressed ($P < 0.05$) by pretreatment with 20 and 30 mg mL⁻¹ CE in diabetic rats. Thus the present study indicates that CE exhibits protective effects on triglyceride levels in STZ-induced diabetic rats.

This study suggests that polyphenols, the main components of CE, may be involved in the improvement of lipid profiles in diabetic rats. The main cause of elevated cholesterol and triglyceride levels in STZ-induced diabetic rats is insulin deficiency. It is well known that, under normal circumstances, insulin activates the enzyme lipoprotein lipase (LpL), which then hydrolyses very-low-density lipoprotein (VLDL) cholesterol.²⁹ However, in insulin-deficient diabetic rats, LpL is not activated, resulting in hypercholesterolaemia and hypertriglyceridemia. Our present study already suggests that the glucose-lowering effect of CE is due to the stimulation of insulin secretion in β -cells. This result is in agreement with a previous study which demonstrated that cocoa supplementation could increase postprandial insulin secretion and, to greater extent, improve insulin resistance.^{30,31}

Thus it is possible that the hypocholesterolaemic and hypotriglyceridemic effect of CE is also due to an increase in insulin secretion. Although LDL cholesterol levels did not seem to be affected by CE treatment, total cholesterol levels were normalised in diabetic rats after oral administration of CE. Therefore it can be suggested that CE might possess hypocholesterolaemic and hypotriglyceridemic activity in STZ-induced diabetic rats.

The most common abnormalities in humans with poorly controlled type 1 or 2 diabetes are hypertriglyceridemia and low HDL cholesterol levels.³² HDL is the smallest, densest lipoprotein with the lowest amount of triglyceride. Lower total cholesterol and higher HDL cholesterol levels represent a very desirable biochemical state for prevention of atherosclerosis and ischaemic conditions.³³ In its protective role against STZ action, CE pretreatment did not seem to prevent plasma HDL cholesterol levels from being reduced by STZ, but the levels were significantly enhanced ($P < 0.05$) after 3 weeks of further treatment with CE.

CONCLUSIONS

The underlying mechanisms responsible for the lack of a protective effect of CE on lipid profiles are not entirely understood and still to be determined. This study indicated that crude cocoa bean extract containing polyphenols and other components might not have a protective effect against hypercholesterolaemia, but it does exert a hypocholesterolaemic effect in STZ-induced diabetic rats.

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