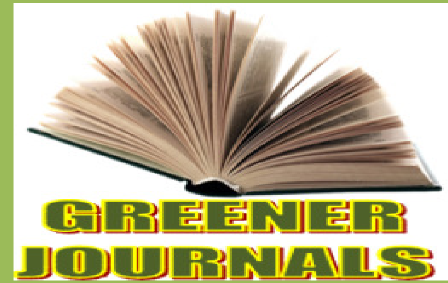

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Dare Christianah Adebimpe
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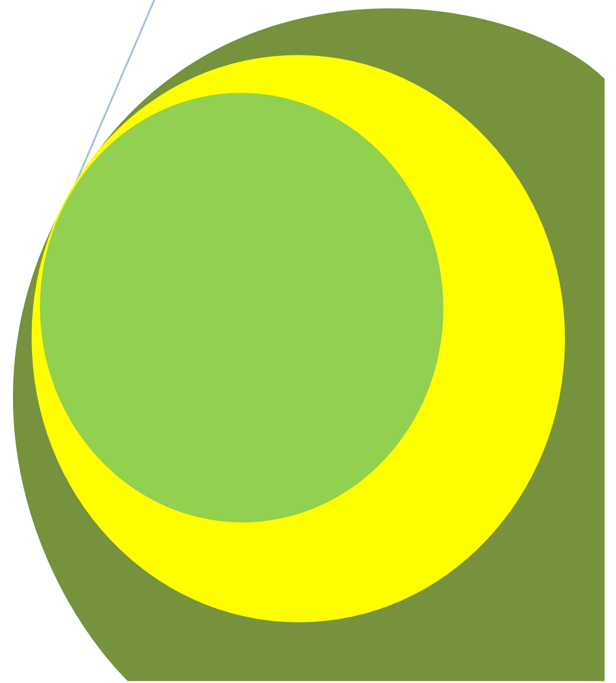

Research Article

Studies on the Effect of Polyphenol of Cocoa (*Theobroma cacao* L.) Seeds on Specific Carbohydrate-Degrading Enzymes in Streptozotocin-Induced Diabetic Rats

Dare Christianah Adebimpe, Onwumelu Rowland Nzeribe and Oyedapo Oluboade Oluokun*

Department of Biochemistry, Faculty of Science, Obafemi Awolowo University, Ile-Ife.

*Corresponding Author's Email: ooyedapo@yahoo.co.uk/ ooyedapo@oauife.edu.ng

ABSTRACT

This study focused on the investigation of the anti-hyperglycaemic potentials of polyphenols extracted from fermented and unfermented *Theobroma cacao* seeds in Wistar rats with a view to investigating the use of cocoa tea in the management of hyperglycaemic conditions. Fresh seeds of cocoa were collected, divided into two groups, a group was sun dried immediately for 7 days and used as unfermented seeds. The other group was fermented traditionally for 5 days; sun dried for 7 days and used as fermented seeds. The seeds (fermented and unfermented) were then ground to powder. The aqueous extracts of the seeds (fermented and fermented) were prepared, phytochemically screened; polyphenols were extracted with 80% (v/v) acetone from both extracts and assayed for α-amylase and α-glucosidase activities. The polyphenols were termed fermented (FP) and unfermented (UP) respectively. Preliminary assays showed that the polyphenol of the fermented *T. cacao* seeds exhibited a higher α-amylase inhibitory potential of 99.25 ± 2.24 % at 400 µg/ ml (IC_{50} 142.62 ± 6.03 µg/ ml) compared to the unfermented polyphenol 83.30 ± 6.25 % at 400 µg/ ml (IC_{50} 188.56 ± 15.32 µg/ ml), as such FP was used for further studies. The effects of the fermented polyphenols (150 mg/kg bwt and 300 mg//kg bwt) on total cholesterol, triacylglycerols, and α-amylase and α-glucosidase activities were examined in diabetic and non-diabetic rats. The results of the findings revealed that 150 mg/kg bwt fermented polyphenol proved to be more potent and efficient in reducing the activities of the carbohydrate-degrading enzymes, thus more anti-hyperglycaemic.

Keywords: Anti-hyperglycaemia, polyphenols, *Theobroma cacao,* α-amylase, α-glucosidase, carbohydrate-degrading enzymes.

INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous metabolic disorder characterized by hyperglycaemia resulting from defective insulin secretion, resistance to insulin action or both (Gavin *et al.,* 1997). In diabetes mellitus, oxidative stress seems mainly to be due to an increased production of free radicals and/or a sharp reduction of antioxidant defences (Young *et al.,* 1992; Thompson and Lee, 1993; Low *et al.,* 1997). Oxygen-derived free radicals have been implicated in the pathophysiology of various disease states, including diabetes mellitus (Giugliano *et al.,* 1996). It is well known that superoxide anion is the primary radical formed by the reduction of molecular oxygen that may lead to secondary radicals or reactive oxygen species (ROS) such as hydrogen peroxide and hydroxyl radical (Grisham and McCord, 1986) suggested to be involved in the pathogenesis and progression of diabetic tissue damage. On the other hand, there is evidence that diabetes induces changes in the activities of antioxidant enzymes in various tissues (Oberley, 1988). Diabetes mellitus is characterized by increased generation of glycoxidation products associated with the advanced oxidative stress (Mullarkey *et al.,* 1990). The presence of higher glucose or glycated protein concentration enhances lipid peroxidation (Kawamura *et al.,* 1994) and reversely, lipid peroxides may increase the extent of advanced glycation end-products (Hicks *et al.,* 1989). Hyperglycaemia is a widely known cause of enhanced plasma free radical concentrations. Free radical production caused by hyperglycaemia may occur via at least four different routes; namely (i) increased polyol/sorbitol pathway flux; (ii) increased intracellular formation of advanced glycation end-products (AGEs); (iii) increased flux through hexosamine pathway; and (iv) activation of protein kinase C (Hammes *et al*., 1997; Brownlee, 2001).

Plant-derived polyphenols are historically a significant and natural constituent of the human diet. Diets rich in flavonoids (a class of polyphenols) have been associated with a reduced risk of cardiovascular disease (Zhu *et al*., 2002). Generally, the consumption of cocoa powder (22 g daily in trials) or dark chocolate (16 g daily

in trials) leads to improved cholesterol ratios, with a higher ratio of high density lipoproteins (HDL) to low density lipoproteins (LDL) (Baba *et al.,* 2007). A higher ratio of HDL to LDL is associated with a lower risk for heart disease.

The systemic effects of dietary polyphenols depend largely on the synergistic action that polyphenols may exert after entering circulation, and are affected by other constituents present in the diet as well as endogenous factors (Liu, 2003; Lila, 2007). Maintenance of glucose homeostasis is of utmost importance to human physiology, being under strict hormonal control. Failure of this control results in the metabolic syndrome, a multi-symptom disorder of energy homeostasis encompassing obesity, hyperglycaemia, impaired glucose tolerance, hypertension and dyslipidaemia (Eckel *et al.,* 2005).

Polyphenols affect glycaemia through different mechanisms, including the inhibition of glucose absorption in the gut or of its uptake by peripheral tissues (Matsui *et al.,* 2002). The inhibition of intestinal glucosidases and glucose transporter by polyphenols has been studied (Matsui *et al.,* 2001). In this study, the inhibitory potentials of polyphenols of *Theobroma cacao* seeds on α-amylase and α-glucosidase enzymes were investigated, providing an alternative in the treatment of hyperglycaemia related conditions.

MATERIALS AND METHODS

Materials

Collection of Cocoa Pods

Fresh and ripe cocoa pods were collected from a cocoa plantation at Babajakan village in Ayedaade Local Government Area of Osun State, Nigeria. The plant materials were identified and authenticated at IFE Herbarium where the specimen copy was deposited and identification number (IFE172418) was collected.

Chemicals and Reagents

All chemicals and reagents used for this study were of analytical grade and purchased from various sources. Alpha-amylase, *p*-nitrophenyl-α-D-glucopyranoside (pNPG), Streptozotocin were obtained from Sigma Fine Chemical Limited, while acetone, hydrochloric acid, hydrogen peroxide, sodium potassium tartarate, sodium chloride, sodium dihydrogen phosphate, disodium hydrogen orthophosphate, copper tetraoxosulphate (vi) salt, sodium hydroxide and others were obtained from British Drug House (BDH) Chemical Limited, Poole, England. Assay kits for total cholesterol, triacylglycerols, total protein, albumin and bilirubin were purchased from Randox Laboratories Ltd., UK.

Experimental Animals

Thirty-five (35) healthy Wistar rats (*Rattus norvegicus*) of both sexes were purchased from Animal House, Faculty of Health Sciences, Obafemi Awolowo University, Ile-Ife. They were maintained under standard Animal House conditions, fed with commercial rat chow (Ladokun Feeds, Ibadan) and allowed water *ad libitum*. Fasted animals were deprived food for at least 16 hours, but allowed free access to water. All animals were carefully monitored and maintained in accordance with ethical recommendations of Nigerian Veterinary Science.

METHODS

Collection of Cocoa Seeds

The pods were opened with a cutlass and by hitting the pods against each other. The seeds were collected into clean containers and divided into two portions. One portion was used as “unfermented” and sun dried for 7 days. The other portion was fermented traditionally by wrapping the seeds in fresh banana leaves and kept in the dark cupboard for 5 days. The fermented seeds were then sun dried for 7 days.

Preparation of Fermented and Unfermented Cocoa Seed Extracts.

The dried seeds (fermented and unfermented, 213 g each) were powdered in a warring blender and defatted using chloroform: isopropanol (3:2 v/v) until the supernatant became colourless. The defatted seed was then extracted with distilled water for 24 hr and concentrated to dryness on a rotary evaporator (Edward High Vacuum Pump, Crawley England) under reduced pressure at 45 ℃ to give dark-brown residues termed “unfermented and fermented aqueous extracts”.

Phytochemical Screening

Aqueous extracts of fermented and unfermented cocoa seeds were screened for the presence of secondary metabolites such as flavonoids, alkaloids, tannins, saponins, anthraquinones, terpenoids according to a procedure that was based on those of Oyedapo *et al*. (1999), Trease and Evans (2002) and Sofowora (2006).

Extraction of Polyphenol

The extraction of polyphenol from the defatted aqueous extracts of fermented and unfermented cocoa seeds was carried out according to the procedure described by Natsume *et al.* (2000). The aqueous extracts of fermented (20 g) and unfermented (20 g) cacao seed were extracted 3 times with a 100-fold volume of 80 % (v/v) acetone at 25 ℃. The filtrates were concentrated on a rotatory evaporator under reduced pressure at 45 ℃ to yield a dry brownish residue which was termed “fermented polyphenol” (FP) and dark-brownish residue termed “unfermented polyphenol” (UP) extracts respectively. Phytochemical screening, thin layer chromatography (tlc), staining with specific detection reagent (methanolic $AlCl_3$) together with chemical tests with 2, 4 dinitrophenyl hydrazine revealed that 80% (v/v) fractions contained mainly polyphenol (Akinwunmi and Oyedapo, 2013).

Assay of *In vitro* α-amylase Inhibition

Assay of α-amylase inhibition was carried out according to the method of Nickavar *et al.* (2008). Typically, 1.0 ml of different concentrations of polyphenols from fermented and unfermented cocoa seeds (100, 200, 300, 400, 500 µg/ ml) were mixed with 1.0 ml of enzyme solution (0.001 g of α-amylase in 100 ml of 20 mM sodium phosphate buffer, pH 6.9, containing 6.7 mM NaCl) in a test tube and incubated for 30 min at 25 ℃. 1.0 ml of starch solution (0.25 g soluble potato starch in 50.0 ml glass distilled water) was mixed with 1.0 ml of this mixture and incubated at 25 ℃ for 3 min. 1.0 ml of the colour reagent [96 mM 3,5-dinitrosalicylic acid (20.0 ml), 5.31 M sodium potassium tartarate in 2 M NaOH (8.0 ml) and deionized water (2.0 ml)] was added. The tubes were tightly closed and placed into an 85 ℃ water bath. After 15 min., the reaction mixture was removed from the water bath, cooled and diluted with 9.0 ml distilled water. The intensity of blue colour was measured at 540 nm against the blank. Individual blank was prepared for correcting the background absorbance by adding the colour reagent solution prior to the addition of starch solution. Plant polyphenol was replaced with 1.0 ml distilled water for control. The inhibition percentage of α-amylase was calculated from the expression:

$$I_{\alpha\text{-amylase}}\% = 100 \times \frac{\Delta A_{Control} - \Delta A_{Sample}}{\Delta A_{Control}}$$

$$\Delta A_{Control} = A_{Test} - A_{Blank}$$

$$\Delta A_{Sample} = A_{Test} - A_{Blank}$$

The $I_{\alpha\text{-amylase}}\%$ was plotted against the sample concentration and a logarithmic regression curve was established in order to calculate the IC_{50} value.

Induction of Diabetes with Streptozotocin (STZ)

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg bwt of streptozotocin in 0.1 M sodium citrate buffer, pH 4.5 to twenty overnight fasted rats of the total thirty-five rats. Diabetes was confirmed in animals after 48 hr of streptozotocin injection with a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany) and a compatible test strip, making use of blood samples from the tail vein of the rats. A blood glucose range of 200-300 mg/ dL was used for the experiment. The animals were allowed free access to food and water for 21 days after which all the thirty-five animals were divided into 7 groups of 5 animals (n = 5). Every treatment was given orally by gavage. The body weight and blood glucose concentrations of the animals were taken on a weekly basis throughout the experimental period. Animals were treated with the polyphenol from fermented cocoa seeds (FP) based on its higher activities over the unfermented from previous *in vitro* assays.

Grouping and Administration of Extract

Group I: Normal rats received only distilled water.
Group II: Normal rats administered with 150 mg/ kg bwt FP.
Group III: Normal rats administered with 300 mg/ kg bwt FP.
Group IV: Diabetic rats received only distilled water.
Group V: Diabetic rats administered with 150 mg/ kg bwt FP.
Group VI: Diabetic rats administered with 300 mg/ kg bwt FP.
Group VII: Diabetic rats administered with 2 mg/ kg (bwt) glimepiride (Positive control).

Animals were treated consistently (daily) and regularly for 21 days and observed for behavioral change(s) during experimental period. Glucose level and body weight of the experimental animals were determined on days 0, 7, 14 and 21 using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany) and a compatible test strip.

Sacrifice of Animals and Collection of Tissues

On the 22nd day the animals were put to sleep by chloroform anaesthesia and cut open with clean sterile pair of scissors. The blood was collected by cardiac puncture into heparined tubes containing anticoagulant (3.8 % trisodium citrate). The livers were surgically removed and immediately perfused in heparinized saline (0.85 % NaCl), wrapped with foil paper and then stored frozen. Also, the pancreases were surgically removed and perfused in normal saline and kept in 10 % formal-saline for histopathological study.

Preparation of Blood Plasma and Liver Homogenates (Post mitochondrial Fractions)

The blood collected by cardiac puncture into heparinised tubes containing anticoagulant was centrifuged at 3000 rpm for 10 min. The preparation of blood plasma, erythrocytes and liver *homogenates was carried out as earlier reported (Olagunju *et al*., 2000; Oyedapo *et al*., 2004). The plasma was collected and kept frozen for analyses (α-amylase and α-glucosidase assays, total protein, total cholesterol and triacylglycerols estimations).

Estimation of Plasma Total Cholesterol Concentration

The plasma total cholesterol estimation was carried out according to the method of Richmond (1973) and Roeschlau *et al*. (1974) using Randox Diagnostic kit. The plasma (10 µl) was mixed with 1.0 ml of reagent. Ten µl distilled water and standard cholesterol (195 mg/ dL) replaced the plasma for blank and standard respectively. This was mixed and incubated at 25 ℃ for 5 min. The absorbance was read at 500 nm within 60 min. against reagent blank.

Concentration of cholesterol in sample was calculated using the equation:

Cholesterol conc. (mg/dL) = $\frac{\Delta A_{sample}}{\Delta A_{standard}}$ x Conc. of standard (195 mg/ dL)

Estimation of Plasma Triacylglycerol Concentration

Plasma triaclyglycerol estimation was carried out according to the procedure described by Tietz (1990) using Randox Diagnostic kit. The plasma (10 µl) was mixed with 1.0 ml of reagent (one vial of R1b reconstituted with 15.0 ml of R1a). 10 µl distilled water and standard replaced the plasma for blank and standard respectively. This was mixed and incubated at 25 ℃ for 5 min. The absorbance was read at 546 nm within 60 min. against reagent blank.

Concentration of triacylglycerol in sample was calculated using the equation:

Triacylglycerol conc. (mg/dL) = $\frac{\Delta A_{sample}}{\Delta A_{standard}}$ x Conc. of standard (196 mg/ dL).

Assay of Alpha-amylase Activity

Alpha-amylase activity was assayed according to the method of Bernfeld (1951) as reported by Katoh *et al.* (1997). The reducing groups released from starch were measured by the reduction of 3,5-dinitrosalicylic acid. One unit of enzyme releases from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 25 ℃ and pH 6.9 under the specified conditions. 50 µl starch solution (at 25 ℃) was added to 50 µl of plasma and incubated at room temperature for 3 min. 100 µl dinitrosalicylic acid colour reagent was added and incubated in a boiling water bath for 5 min. This was cooled to room temperature and 1.0 ml distilled water was added. The absorbance was read at 540 nm against the blank. The amount of maltose released was obtained from standard calibration curve.

Enzyme units/mg = $\frac{\text{micromoles maltose released}}{\text{mg of enxyme in reaction x 3 min}}$

Assay of Alpha-glucosidase Activity

The alpha-glucosidase assay was carried out according to the method of Whitson *et al.* (2010). Typically, plasma (20 µl) was mixed with 500 µl of 67 mM phosphate buffer, pH 6.8 and 20 µl GSH (3 mM). The reaction mixture was allowed to equilibrate at room temperature followed by the addition of 50 µl p-nitrophenyl-α-D-glucopyranoside (10 mM pNPG). This was mixed and incubated at 37 ℃ for 20 min. 200 µl of the mixture was added to 800 µl Na_2CO_3. The absorbance of the mixture was read at 400 nm against the blank. The activity of the enzyme was calculated from the expression:

$$\text{Units/ml enzyme} = \frac{A_{test} - A_{blank} \times 10 \times 0.59 \times df}{18.3 \times 20 \times 1 \times 0.2}$$

0.59 = Volume (in millilitres) of reaction mixture
df = Dilution factor
18.3 = Millimolar extinction coefficient of p-Nitrophenol at 400nm
20 = Time (in minutes) of the assay
10 = Volume (in millilitres) of Colorimetric Determination
1 = Volume (in millilitres) of reaction mix used in the colorimetric determination

Histopathology

The histological parameters were studied at Mr. Gbela's laboratory at Oluwalose Quarters, Road 7, Ile-Ife and Department of Anatomy and Cell Biology, Obafemi Awolowo University, Ile-Ife under the supervision of Dr. David A. Ofusori.

Statistical Analysis

The results were expressed as Mean ± SEM, n = 3 readings for *in vitro*, n = 5 rats for *in vivo*. Differences between mean values of control and treated were determined by One-way ANOVA followed by Tukey multiple comparison test using GraphPad Prism 5. Differences were considered to be significant at $P<0.05$.

RESULTS AND DISCUSSION

In this present study, the following symptoms were observed in diabetic rats: polyuria (excessive urination), loss of weight, polydipsia (excessive thirst), and weakness, formation of cataracts on the eyes of diabetic rats, polyphagia (excessive hunger), scattered fur and prolonged healing. It was observed that the wounds on the tails of diabetic rats did not heal up while the wounds of non-diabetic rats healed up before subsequent week. In Plates 1a and 1b are the photographs of non-diabetic rat and diabetic rats, showing that the rats were truly diabetic before the commencement of the treatment with the polyphenols.

In Table 1 is the summary of the phytochemical constituents in the aqueous extracts of fermented and unfermented *T. cacao* seeds. It was observed that both extracts contained the same phytochemicals which included tannins, saponins, flavonoids, anthraquinones amongst other phytochemicals. A variety of polyphenols have been shown to inhibit α-amylase and α-glucosidase activities *in vitro*. The inhibitory polyphenols include flavonoids (anthocyanins, catechins, flavanones, flavonols, flavones and isoflavones), phenolic acids and tannins (proanthocyanidins and ellagitannins). Insulin secretion by the pancreas involves numerous reactions which are potential targets for the action of polyphenols (Kim *et al*., 2000; Iwai *et al*., 2006; Tadera *et al*., 2006). Studies have implicated phytochemicals (flavonoids, sterols, terpenes and tannins) in the management of diabetic and inflammatory disorders (Mohamed, 2008; 2013). Moreover, ethanolic extracts of *Blighia sapida* (Kazeem *et al*., 2013), *Desmodium pulchellum* barks (Noor *et al*., 2013) as well as polyherbal combinations of certain medicinal plants (Patil *et al*., 2012; Marles and Farnsworth, 1995) have been demonstrated to exhibit potent and appreciable hypoglycaemic activities in experimental animals.

Table 1: Summary of Phytochemicals in the Aqueous Extracts of Fermented and Unfermented *T. cacao* Seeds

Phytochemicals	Aqueous Unfermented	Aqueous Fermented
Flavonoids	+++	+++
Alkaloids	+	+
Cardiac glycosides	+	+
Tannins	++	++
Saponins	++	++
Anthraquinones	+	+
Steroids	-	-
Xanthoproteins	+	+
Triterpenes	-	-

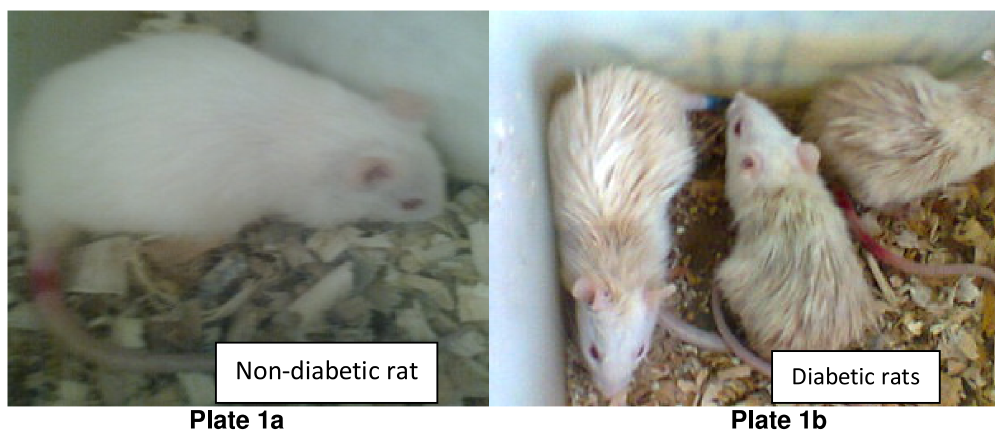

Plate 1a **Plate 1b**

Plate 1: Photographs of Non-Diabetic Rat and Diabetic Rats Revealing the Status of the Experimental Rats.

Table 2: Alpha-Amylase Inhibitory Activity IC_{50} Values (µg/ ml)

Sample	IC_{50} Value (µg/ ml)
Fermented Polyphenol	142.62 ± 6.03
Unfermented Polyphenol	188.56 ± 15.32

Each value represented the Mean ± SEM of n = 3 readings.

Naturally occuring α-amylase and α-glucosidase inhibitors from food-grade plant sources offer an attractive therapeutic approach to the treatment of post-prandial hyperglycaemia by decreasing glucose release from starch and delaying carbohydrate absorption by inhibiting the activity of the carbohydrate hydrolysing enzymes in the small intestine and may have potential for use in the treatment of diabetes mellitus and obesity. On the basis of the prevalence, the delay or inhibition of carbohydrate digestion would contribute to optimize a post-prandial blood glucose level (Gallaher and Schneeman, 1986; Murai *et al.,* 2002; Chiasson *et al.,* 2002). In Fig 1.0 is the summary of *in vitro* α-amylase inhibitory activity of the fermented and unfermented *T. cacao* polyphenol. It was observed that the polyphenols (fermented and unfermented) demonstrated an appreciable α-amylase inhibitory activity and was concentration-dependent. Moreover, the extracts exhibited low IC_{50} values which is an indication of their effectiveness as α-amylase inhibitors with the fermented polyphenol exerted better activity (142.62 ± 6.03 µg/ ml against 188.56 ± 15.32 µg/ ml) as shown in Table 2 - the lower the IC_{50} of a compound, the more effective or potent it is.

In Table 3 is the summary of variations in body weight of experimental animals in the course of the experiment. The normal control group had increase in weight from 132.30 ± 3.30 g (day 0) to 147.52 ± 6.16 g (week 6), an increase of about 11.50 %. On administration of extracts to the diabetic and non-diabetic groups in the third week, only the 300 mg/ kg FP non-diabetic dosing group and 2 mg/ kg glimepiride diabetic dosing groups had significant increase in weight (15.43 % and 15.27 % respectively).

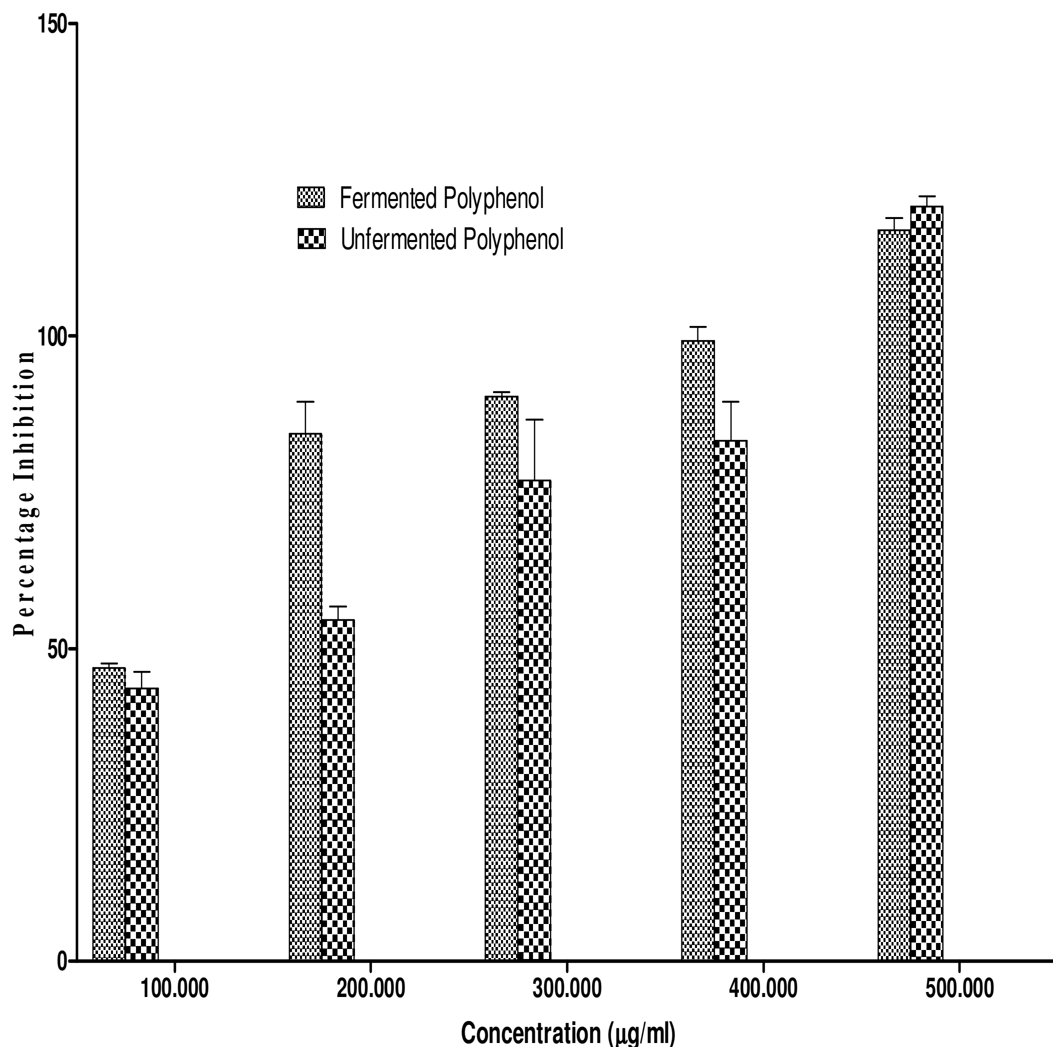

Figure 1: Alpha-amylase Inhibitory Activity of Fermented and Unfermented *T. cacao* Seeds Polyphenol.
Each value represented the Mean ± SEM of n = 3 readings.

Table 3: Effect of Polyphenol of Fermented *T. cacao* Seeds and Glimepiride on Body Weights (g) in Rats

Treatment / Duration	Grp I	Grp II	Grp III	Grp IV	Grp V	Grp VI	Grp VII
DAY 0	132.30 ± 3.30	144.72 ± 7.03	143.50 ± 8.88	124.23 ± 8.29	118.57 ± 6.59	131.98 ± 3.19	112.85 ± 7.35
1WK	138.92 ± 3.80	139.20 ± 7.30	133.40 ± 7.46	119.18 ± 3.89	111.30 ± 5.47	125.40 ± 2.93	109.85 ± 2.75
2WK	133.50 ± 4.38	146.34 ± 8.53	137.90 ± 9.24	124.80 ± 4.86	111.53 ± 3.91	117.90 ± 2.73	101.90 ± 1.60
3WK	145.68 ± 5.83	142.60 ± 7.68	135.43 ± 8.17	121.95 ± 4.64	112.43 ± 3.84	115.30 ± 2.83	100.55 ± 0.55
		TREATMENT			TREATMENT		
4WK	133.02 ± 5.72	154.00 ± 9.41	141.77 ± 3.73	119.23 ± 8.23	113.67 ± 2.68	120.40 ± 6.93	112.25 ± 0.95
5WK	145.52 ± 5.26	145.66 ± 9.52	146.80 ± 4.78	123.50 ± 7.29	96.00 ± 1.17	117.48 ± 3.05	113.30 ± 3.20
6WK	147.52 ± 6.16	147.06 ± 8.71	156.33 ± 5.73	118.88 ± 6.52	98.87 ± 1.01	115.38 ± 4.78	115.90 ± 0.80
% Change	11.50	3.13	15.43	-4.31	-12.06	0.07	15.27

Each value represented the Mean ± SEM of n = 5 rats.

Group I: Normal rats received only distilled water (General control), Group II: Normal rats administered with 150 mg/kg (bwt) FP, Group III: Normal rats administered with 300 mg/kg (bwt) FP, Group IV: Untreated diabetic rats (Diabetic control), Group V: Diabetic rats administered with 150 mg/kg (bwt) FP, Group VI: Diabetic rats administered with 300 mg/kg (bwt) FP, Group VII: Diabetic rats administered with 2 mg/kg (bwt) glimepiride (Positive control).

The increase in blood glucose concentration observed in all rats after a single intraperitoneal injection of 60 mg/ kg bwt streptozotocin confirmed the induction of diabetes in the rats. The diabetic rats treated with acetone extracts of fermented *T. cacao* seeds (150 mg/ kg bwt and 300 mg/ kg bwt) and standard glimepiride (2 mg/ kg bwt) responded positively to treatment. During the period of treatment, marked reduction in blood glucose concentrations was recorded for all treated diabetic animals. The treatment of diabetic rats with 150 mg/ kg FP and 300 mg/ kg FP resulted in a decrease in blood glucose concentration by 8.11 % and 9.13 % respectively. The diabetic rats treated with standard 2 mg/ kg glimepiride had the highest decrease of 17.32 % (Table 4). The anti-diabetic potentials of acetone extract of fermented *T. cacao* seeds could be attributable to the presence of flavonoids which are common in plants with known hypoglycaemic effect (Szkudelski, 2001).

Lipids play important role in pathogenesis of diabetes mellitus. Lipid abnormalities accompanying with atherosclerosis is the major cause of cardiovascular disease in diabetes. Therefore ideal treatment of diabetes, in addition to glycaemic control, should have a favourable effect on lipid profiles. The high levels of total cholesterol (TC) and low-density lipoprotein (LDL) are major coronary risk factors (Temme *et al.,* 2002). Further, studies suggested that triacylglycerols (TG) itself is independently related to coronary heart disease (Bainton *et al.,* 1992; El-harzmi and Warsy, 2001). The abnormalities in lipid metabolism could lead to elevation in the levels of plasma lipid and lipoprotein that in turn play an important role in occurrence of premature and severe atherosclerosis, which affects patients with diabetes (Ravi *et al.,* 2005). Hence, measurements of biochemical parameters are necessary to prevent cardiac complications in diabetes condition. In this study, treatment with different concentrations (150 mg/ kg and 300 mg/ kg) of FP showed significant reduction in TG level in the plasma of diabetic and non-diabetic rats. The standard glimepiride drug did not show significant decrease in TG level in the rat plasma. There was significant reduction in TC level in the serum of non-diabetic rats but no significant reduction was observed in the plasma of treated diabetic rats with the extracts and standard drug (Table 5). Studies have demonstrated that diabetes mellitus is accompanied by hyperglycaemia, hypercholesterolemic and hypertriacylglycemic conditions as a result of dysfunction of islet of Langerhans of the pancreas which is responsible for the production/secretion of insulin (Misra, 2009).

Table 4: Effect of Polyphenol of Fermented *T. cacao* Seeds and Glimepiride on Blood Glucose Concentrations (mg/ dl) in Rats

Treatment / Duration	Grp I	Grp II	Grp III	Grp IV	Grp V	Grp VI	Grp VII
DAY 0	111.80 ± 6.70	109.40 ± 4.50	109.67 ± 4.63	89.25 ± 5.63	86.67 ± 5.46	96.50 ± 3.88	99.50 ± 8.50
INDUCTION				600.00 ± 0.00	577.33 ± 22.67	585.00 ± 15.00	600.00 ± 0.00
1WK	125.60 ± 2.62	87.20 ± 3.51	69.33 ± 4.49	600.00 ± 0.00	575.00 ± 23.03	572.25 ± 27.75	554.50 ± 77.50
2WK	101.80 ± 1.28	97.20 ± 6.27	95.33 ± 9.35	563.00 ± 37.00	583.00 ± 17.00	496.25 ± 36.75	517.00 ± 81.00
3WK	151.80 ± 2.15	81.60 ± 2.50	72.33 ± 6.06	572.25 ± 27.75	579.33 ± 20.67	600.00 ± 0.00	525.50 ± 106.50
		TREATMENT				TREATMENT	
4WK	94.40 ± 6.05	109.40 ± 3.64	94.00 ± 3.06	590.50 ± 9.50	524.33 ± 75.67	561.25 ± 22.01	600.00 ± 0.00
5WK	100.20 ± 5.16	87.80 ± 2.87	67.33 ± 3.84	506.25 ± 45.53	539.67 ± 51.13	525.25 ± 28.24	482.00 ± 43.00
6WK	115.60 ± 6.83	102.00 ± 3.18	109.00 ± 12.22	568.75 ± 31.25	532.33 ± 35.00	545.25 ± 31.66	434.50 ± 34.50
% Change	3.40	25.00	50.07	537.25	-8.11	-9.13	-17.32

Each value represented Mean ± SEM of n = 5 rats.

Group I: Normal rats received only distilled water (General control), Group II: Normal rats administered with 150 mg/kg (bwt) FP, Group III: Normal rats administered with 300 mg/kg (bwt) FP, Group IV: Untreated diabetic rats (Diabetic control), Group V: Diabetic rats administered with 150 mg/kg (bwt) FP, Group VI: Diabetic rats administered with 300 mg/kg (bwt) FP, Group VII: Diabetic rats administered with 2 mg/kg (bwt) glimepiride (Positive control).

Table 5: Effect of fermented *T. cacao* seeds polyphenol on Total Protein, Total Cholesterol and Triacylglycerols in Rats Plasma

Group	Total Cholesterol (mg/ dL)	Triacyglycerol (mg/ dL)	Total protein (g/ dL)
I	56.72 ± 1.72	40.32 ± 5.15	7.39 ± 0.16
II	24.13 ± 3.53*	21.73 ± 1.88 *	7.69 ± 0.51
III	31.02 ± 3.27 *	35.94 ± 8.17 *	7.93 ± 0.23
IV	62.52 ± 3.37	98.84 ± 11.19	6.17 ± 0.27
V	53.25 ± 1.95	34.72 ± 4.73 *	6.51 ± 0.17
VI	55.27 ± 3.65	58.13 ± 2.54 *	6.94 ± 0.33
VII	59.43 ± 3.05	81.65 ± 6.88	7.35 ± 0.22

Each value represented Mean ± SEM of n = 5 rats.

Non-diabetic treated groups are compared with normal control and diabetic treated groups are compared with diabetic control. The values with asterisk (*) are statistically significant at P<0.05. Group I: Normal rats received only distilled water (General control), Group II: Normal rats administered with 150 mg/ kg (bwt) FP, Group III: Normal rats administered with 300 mg/ kg (bwt) FP, Group IV: Untreated diabetic rats (Diabetic control), Group V: Diabetic rats administered with 150 mg/ kg (bwt) FP, Group VI: Diabetic rats administered with 300 mg/ kg (bwt) FP, Group VII: Diabetic rats administered with 2 mg/ kg (bwt) glimepiride (Positive control).

One of the therapeutic approaches for type 2 diabetes is to reduce the postprandial hyperglycaemia. Alpha amylase is an enzyme involved in the metabolism of carbohydrates. The enzyme degrades complex dietary carbohydrates to oligosaccharides and disaccharides which are ultimately converted into monosaccharide by α-glucosidase. The liberated glucose is then absorbed by the gut and results in postprandial hyperglycaemia. Inhibition of alpha amylase limits postprandial glucose levels by delaying the process of carbohydrate hydrolysis and absorption (David and Bell, 2004). Results from *in vivo* α-amylase and α-glucosidase assays in rats' plasma of treated groups revealed about 3 fold decrease in the activity of α-amylase in the diabetic rats treated with the extract and about 2 fold decrease in the activity of α-glucosidase in the treated diabetic rats (Table 6).

Table 6: Effect of Fermented Polyphenol on Plasma α-amylase and α-glucosidase Activities

Treatment	α-amylase (U/ml/min)	α-glucosidase (U/ml/min)
Grp I	594.52 ± 43.70	2.79 ± 0.17
Grp II	528.07 ± 46.17	7.05 ± 0.27 *
Grp III	399.39±3.92	3.90 ± 0.15
Grp IV	817.34 ± 414.03	12.30 ± 0.49
Grp V	289.51 ± 29.58 *	5.44 ± 0.20 *
Grp VI	274.63 ± 20.76 *	6.53 ± 0.50 *
Grp VII	271.85 ± 24.64	5.27 ± 0.33 *

Each value represented Mean ± SEM of n = 5 rats.

Non-diabetic treated groups are compared with normal control and diabetic treated groups are compared with diabetic control. The values with asterisk (*) are statistically significant at P<0.05. Group I: Normal rats received only distilled water (General control), Group II: Normal rats administered with 150 mg/ kg (bwt) FP, Group III: Normal rats administered with 300 mg/ kg (bwt) FP, Group IV: Untreated diabetic rats (Diabetic control), Group V: Diabetic rats administered with 150 mg/ kg (bwt) FP, Group VI: Diabetic rats administered with 300 mg/ kg (bwt) FP, Group VII: Diabetic rats administered with 2 mg/ kg (bwt) glimepiride (Positive control).

The pancreas is composed of clusters (acini) of cells (exocrine/parenchyma) that secret pancreatic juice. This contains a number of enzymes concerned in digestion. Interspersed among the acini are the islets of Langerhans (endocrine) which are isolated groups of cells that secrete the hormones glucagon (α-cells), insulin (β-cells), somatostatin and pancreatic polypeptide (δ-cells) into the bloodstream. Histological examination of pancreatic section showed normal arrangement of the islets of Langerhans of various sizes scattered throughout the exocrine (parenchyma) tissues of normal control and treated non-diabetic dosing groups. However, degenerated islet of Langerhans, atrophy and vacuolation and invasion of connective tissues in parenchyma of pancreatic islets were detected in diabetic control. These abnormalities have been corrected in 150 mg/ kg FP treated group and a sign of recovery was observed in the 300 mg/ kg FP treated group. The standard drug dosing group on the other hand did not reveal much difference from the diabetic control. The effect of flavonoids on pancreatic β-cells leading to their proliferation and secretion of more insulin have been proposed by Mahes and Menon (2004) and Sri-Balasubashini *et al.* (2004) as the mechanism by which they reduced hyperglycaemia caused by streptzotocin in diabetic rats.

In conclusion, the polyphenols of fermented cocoa seeds are excellent inhibitors of α-amylase and α-glucosidase enzymes (two major carbohydrate-degrading enzymes) that determine the concentration of post-prandial glucose, thus good candidate as anti-diabetic drug.

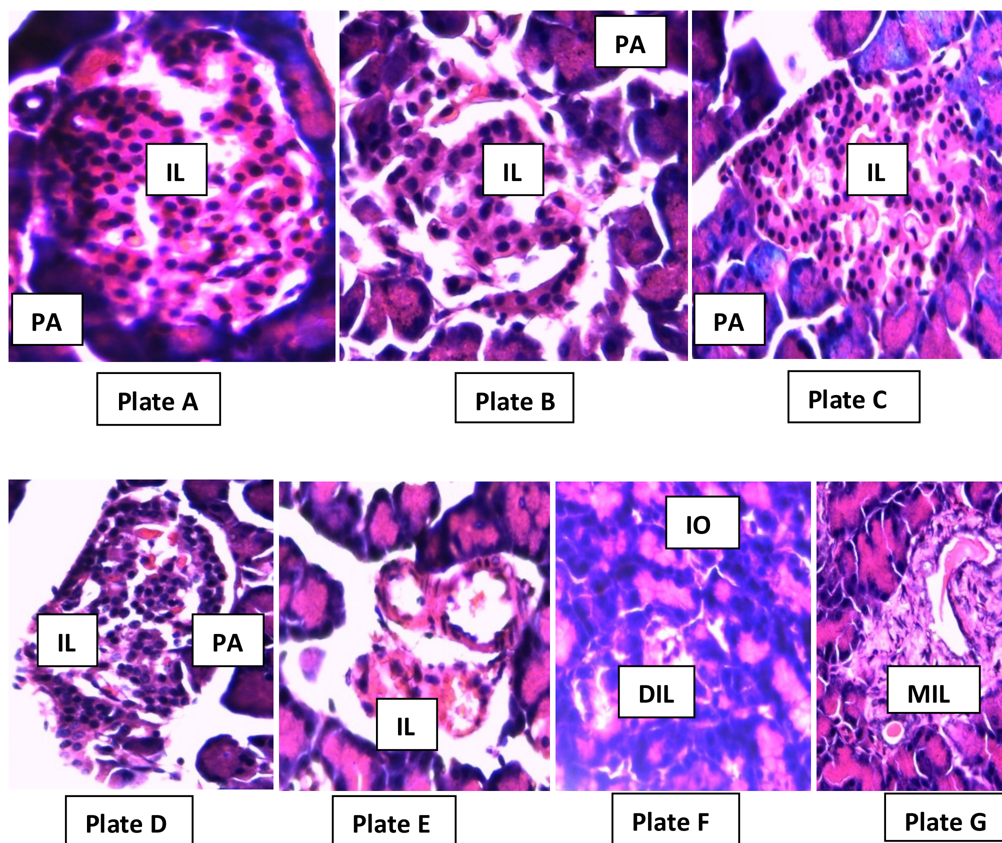

Plate A: Pancreas section showing normal appearance of the pancreatic acini (PA) and islet of Langerhans (IL) in the pancreas of Grp I (x 400) E & H. Plate B: Pancreatic section showing normal appearance of the islet of Langerhans (IL) in the pancreas of Grp II (x 400) E & H. Plate C: Pancreatic section showing normal appearance of the islet of Langerhans (IL) in the pancreas of 300 mg/ kg PF non-diabetic group (x 400) E & H. Plate D: Pancreas section showing normal appearance of the pancreatic acini (PA) and islet of Langerhans (IL) in the pancreas of Grp IV (x 400) E & H. Plate E: Pancreas section showing sign of recovery after degeneration of islet of Langerhans in the pancreas of Grp V (x 400) E & H. Plate F: Pancreas section showing degenerated islet of Langerhans (DIL) and interstitial oedema (IO) in the pancreas of Grp VI (x 400) E & H. Plate G: Pancreas section showing marked degeneration of the islet of Langerhans (MIL) in the pancreas of Grp VII (x 400) E & H.

Declaration of Interest:

There are no conflicts of interest amongst the authors.

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