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Research Article

Aqueous Cocoa Powder Extract Moderates Physical, Biochemical, Hematological Parameters and Total Antioxidant Capacity in Alloxan Diabetic Wistar Albino Rat

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Abstract

Complications from diabetes mellitus demand for increase research on the disease. Forty eight Albino rats (randomly grouped into six) were used for the study. Diabetes was induced using 120 mg kg^{-1} b.wt., alloxan and different doses of the extract were administered orally for 40 days except in the diabetic untreated group. A weekly body weight measurement was also done. On day 40 fasting plasma glucose, total protein, albumin, urea, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) were determined using standard methods. Results showed an initial weight loss in all the administered doses in the early weeks of extract administration and this improves as the effect of the extract became established in the later weeks. There was a 15.2, 7.4, 19, 11, 3.6 and 11.4% decrease, respectively in 200, 300, 500, 10000 mg kg^{-1} treated groups; normoglycemic and diabetic untreated group. Also, a non-significant ($p>0.05$) change in the plasma albumin, creatinine, urea, red blood cell and hemoglobin; a mixed significant change in plasma aspartate aminotransferase, alanine aminotransferase, total protein and globulin, a significant ($p<0.05$) decrease in plasma glucose and malondialdehyde and a significant increase in plasma total antioxidant capacity, white blood cells and lymphocytes were observed in the extract treated and normoglycemic control groups in comparison to the diabetic untreated group. Conclusively, ingestion of aqueous cocoa powder modifies the characteristic body weight changes, biochemical and hematological parameters in alloxan diabetes.

Key words: Diabetes mellitus, alloxan, weight, biochemical parameters, total antioxidant capacity

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In the recent years attention had shifted to the use of phytochemicals with chemotherapeutic and chemopreventive properties obtainable from plants for the management of chronic diseases like diabetes mellitus. Diabetes mellitus is a disorder of glucose metabolism characterized by polyuria, polyphagia, polydipsia and weight loss (Olooto *et al*., 2014). These characteristic features are reflections of the hyperglycemic state which is either due to insufficiency or deficient secretion or action of endogenous insulin (Maritim *et al*., 2003). The notable insulin deficiency in diabetes mellitus leads to glucose accumulation in the blood vessels and its deficiency at tissue level for cellular oxidation and energy generation, ketoacidosis from high ketone levels in the body and dehydration from polyuria.

Diabetes is a major world-wide health problem predisposing individuals with the disease to serious morbidity and markedly increased cardiovascular mortality (which causes up to 80% of deaths in people with diabetes) and mortality related to development of neuropathy and retinopathy (IDF., 2005).

Notable biochemical changes in diabetes mellitus include hyperglycemia, hypercholesterolemia, high plasma LDL-C, high plasma VLDL-C and low plasma HDL-C (Susanti *et al*., 2010). These biochemical changes in diabetes is associated with increased vascular damage thereby causing coronary artery, cerebrovascular and peripheral vascular diseases, blindness, amputations and end stage renal disease (IDF., 2005). The vascular damage is secondary to increased hyperglycemia-induced free radical generation and biochemical changes in the plasma involving insulin-driven metabolism of carbohydrate, lipid and protein. Complications from diabetes mellitus are manifestations of poor glycemic control arising from mismanagement of the disease condition. Adjustment in lifestyle, dietary modification, exercises and drugs (sulfonylureas, biguanides, α-amylase inhibitors, etc.) are important in the management of the disease (Bennett *et al*., 2011).

Considering the etiologic role of oxidative stress in diabetes, administration of plant materials rich in antioxidants (such as flavonols) will be of importance in the management of the disease. Also, with the perceived side effects of drugs which are of about the same chance for all diabetic medications, rise in global poverty, drug unavailability, non-drug procurement, non-drug compliance and drug resistance, there is the need to search for relatively cheaper and readily available plant materials with little or no side effect in the management of diabetes mellitus (Muthu *et al*., 2006; Iwu and Wootton, 2002). This study thus focused on the likely physical, biochemical and hematological changes that may occur upon the administration of aqueous cocoa powder extract for the management of alloxan diabetic Albino rats.

MATERIALS AND METHODS

Commercially available Nigerian pure cocoa powder obtained from appetizing food company Ibadan was used for the study.

Experimental design: The study was longitudinal and conducted using 48 adult female Wistar strain Albino rats. The rats were relatively healthy and non-gravid. The animals were randomly selected into six groups, each consisting of eight rats. Diabetes was induced using 120 mg kg^{-1} b.wt., alloxan (Olooto *et al*., 2014) administered intraperitoneally into all the rats excluding the normoglycemic controls. Diabetes mellitus status was confirmed after 48 h of alloxan administration and treatment with the aqueous cocoa powder extract was commenced immediately except in the diabetic untreated control group. The room temperature of 25-28°C and the natural cycle of 12 h daylight and night were employed during the study.

The different doses (200, 300, 500 and 1000 mg kg^{-1}) of the aqueous cocoa powder extract were freshly prepared daily and administered orally once daily for 40 days in a constant volume. The rats were fed with normal rat chow and water was given *ad libitum* for the whole research period. Each dose was administered in a single dose using oral cannula after overnight fasting.

Preparation of animals: The animals were randomly selected and arranged into six groups using the different doses of aqueous cocoa powder (200, 300, 500 and 1000 mg kg^{-1}), diabetic untreated and normoglycemic control groups. The animals were tail-marked for individual and group identification and kept acclimatized to the laboratory conditions for one week before diabetes induction. The care of the animals was done in accordance with the U.S. Public Health Service Guidelines (NRC., 2011).

Specimen collection, storage and processing: Fasting blood sample was collected from the tail vein on daily basis to determine the blood glucose concentration using accu-chek glucometer. The collected blood was applied directly onto the glucometer strip as specified by the manufacturer. At the expiration of forty days experimental period, fasting blood was

then collected by cardiac puncture (using diethyl ether as anesthetic agent) into heparinized bottle for biochemical studies and EDTA bottle for hematological studies.

Method of assay: Plasma total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea and creatinine levels were determined using the standard methods described by Doumas *et al*. (1971), Kingsley (1939), Reitman and Frankel (1957), Schumann and Klauke (2003), Wybenga *et al*. (1971) and Henry *et al*. (1974), respectively. The plasma globulin was computed as the difference between total protein and albumin. Total Antioxidant Capacity (TAC) was determined by the method described by Koracevic *et al*. (2001), lipid peroxidation was determined using the method described by Varshney and Kale (1990) and *in vitro* α-amylase inhibition study was done based on the starch iodine test using the method described by Xiao *et al*. (2006). Hematological study was done using a hematology automated analyzer (Swelab alpha hematology analyzer, Sweden).

Data analysis: The statistical analysis was done using SPSS software version 21. Descriptive statistic and bar chart representations were used to describe and represent variables. ANOVA was used to compare differences in mean between more than two groups. The level of statistical difference was set at $p<0.05$.

RESULTS

Result from this study shows a reduction in body weight of the rats in the early weeks of the study which increases In later weeks across groups in the extract treated and normoglycemic control groups (Table 1). There was a 15.2, 7.4, 19, 11, 3.6 and 11.4% decrease, respectively in 200, 300, 500 and 1000 mg kg^{-1} treated groups; normoglycemic and diabetic untreated group (Table 2).

Considering plasma biochemical parameters, a non-significant change was observed in the plasma albumin ($p = 0.53$, 0.88, 0.06, 0.79 and 0.46) and creatinine ($p = 0.84$, 0.28, 0.92, 0.11 and 0.83), respectively at 200, 300, 500 and 1000 mg kg^{-1} dose and normoglycemic groups when compared with the diabetic untreated group. While, a mixed significant change in plasma urea ($p = 0.004$, 0.14, 0.11, 0.12 and 0.78), AST ($p = 0.39$, 0.37, 0.00, 0.30 and 0.08), ALT ($p = 0.05$, 0.07, 0.39, 0.04 and 0.39), total protein ($p = 0.98$, 0.11, 0.01, 0.02 and 0.03) and globulin ($p = 0.06$, 0.02, 0.00, 0.58 and 0.01) was observed in the extract treated and normoglycemic control groups in comparison to the diabetic untreated group, a significant decrease in plasma glucose ($p = 0.000$, 0.000, 0.000, 0.000 and 0.000) and malondialdehyde (MDA) ($p = 0.000$, 0.000, 0.000, 0.000 and 0.000) and a significant increase in plasma TAC ($p = 0.002$, 0.000, 0.000, 0.000 and 0.000) was noted amongst the groups (Table 3).

Looking at the effect of the aqueous cocoa powder extract on the activity of alpha amylase enzyme, the extract was observed to inhibit activities of the enzyme. The least inhibition (45%) was observed at a dose of 0.3 mg mL^{-1} and the maximum inhibition (70%) was observed at a dose of 1.5 mg mL^{-1}. Meanwhile, the IC_{50} value was determined to be 1.05 mg mL^{-1} (representing the concentration of the extract containing inhibitor that inhibited 50% of the alpha amylase activity).

Table 1: Changes in body weight amongst the extract treated, diabetic untreated and normoglycemia control rats

Dose	Initial (g)	Wk 1 (g)	Wk 2 (g)	Wk 3 (g)	Wk 4 (g)	Wk 5 (g)	Wk 6 (g)
200 mg kg^{-1}	230.5±9.3	202.8±7.4	194.4±10.5	181.0±9.5	182.6±8.3	191.0±8.2	195.4±9.0
300 mg kg^{-1}	241.5±9.8	2173±10.8	213.0±11.0	218.6±10	220.3±10.4	222.6±10.4	223.6±10.4
500 mg kg^{-1}	254.3±10.3	207.0±9.9	206.0±9.6	192.8±8.7	190.8±7.5	195.4±6	205.9±10.6
1000 mg kg^{-1}	264.5±12.3	227.3±11.5	214.9±14.4	219.3±15.3	215.9±17.8	207.5±14	205.9±14.5
Normoglycemia	268.0±18.5	249.3±9.9	250.3±6.8	253.5±6.8	256.1±6.8	255.3±7.2	258.4±7.2
Diabetic control	250.0±11.2	227.3±15.5	225.5±15.2	239.9±25.5	222.5±16.8	219.6±15.6	222.5±15.1

Statistically significant at $p<0.05$, Wk: Week

Table 2: Percentage differences in body weight amongst the extract treated, diabetic untreated and control rats

	Initial weight (g)	Final weight (g)	Weight changes (g)	Weight changes (%)
200 mg kg^{-1}	230.5	195.4	35.1	15.2
300 mg kg^{-1}	241.5	223.6	17.9	7.4
500 mg kg^{-1}	254.3	205.9	48.4	19.0
1000 mg kg^{-1}	250.0	222.5	27.5	11.0
Normoglycemia	268.0	258.4	9.6	3.6
Diabetic untreated	250.0	222.5	28.5	11.4

Table 3: Biochemical parameters amongst the extract treated, diabetic untreated and normoglycemic control groups

Dose/Parameters	200 mg	300 mg	500 mg	1000 mg	Diabetic untreated	Normoglycemic control	F-value	p-value
Glucose (mg dL^{-1})	311.9±5.2*	183.6±5.6*	239.8±6.9*	271.70±6.4*	336.3±3.9	81.60±0.6*	247.75	0.000
Total protein (mg dL^{-1})	8.3±0.47	8.8±0.41*	8.7±0.29*	7.70±0.49	7.5±0.11	8.60±0.24*	2.65	0.02
Albumin (mg dL^{-1})	3.5±0.26	3.7±0.03	3.1±0.30	3.60±0.24	3.7±0.31	3.46±0.2	0.82	0.56
Globulin (mg dL^{-1})	4.8±0.45	5.2±0.41*	5.7±0.31*	4.10±0.50	3.8±0.34	5.18±0.34*	3.66	0.004
AST (IU L^{-1})	58.4±5.11	58.5±6.32	75.0±5.29*	46.90±3.78	53.1±3.69	42.50±1.74	6.97	0.000
ALT (IU L^{-1})	32.9±7.99	31.6±2.50	25.8±5.41	33.40±2.70*	20.5±2.29	25.80±3.69	1.32	0.266
Urea (mg dL^{-1})	99.3±15.32*	77.5±6.26	79.1±13.15	78.40±11.89	56.1±3.38	52.10±2.80	3.15	0.011
Creatinine (mg dL^{-1})	0.8±0.23	1.1±0.37	0.8±0.19	1.30±0.37	0.8±0.04	0.70±0.21	1.04	0.409
MDA (nmol mL^{-1})	3.1±0.07*	2.9±0.05*	2.2±0.14*	2.70±0.09*	4.5±0.13	2.10±0.05*	64.27	0.000
TAC (nM L^{-1})	0.3±0.02*	0.4±0.01*	0.4±0.01*	0.50±0.02*	0.2±0.00	0.80±0.05*	76.44	0.000

*Statistically significant at p<0.05

Table 4: Hematological parameters amongst extract treated, diabetic untreated and normoglycemic control groups

Dose/Parameters	200 mg kg^{-1}	300 mg kg^{-1}	500 mg kg^{-1}	1000 mg kg^{-1}	Diabetic untreated	Normoglycemic control	F-value	p-value
TRBC (cells per milliliter)	6.8±0.33	8.7±0.02*	7.2±0.1	6.02±0.5*	7.6±0.4	7.4±0.15	30.59	0.000
HGB (g dL^{-1})	13.4±0.82	13.9±0.1	13.8±0.1	13.60±0.04	14.9±0.7	13.9±0.09	38.80	0.000
TWBC (cells per milliliter)	7.4±0.35*	7.2±0.1	7.3±1.1	7.60±0.8	8.2±1.5	7.1±0.28	10.06	0.000
Lymphocyte (%)	81.2±2.2*	78.4±0.1	76.1±1.9	56.40±10.1	57.0±3.8	81.4±1.95	7.63	0.000

*Statistically significant at p<0.05

Results of hematological parameters showed a non-significant reduction in the red blood cells count (p = 0.27, 0.002, 0.459, 0.005 and 0.905) and hemoglobin levels (p = 0.415, 0.000, 0.210, 0.656 and 0.132) in all the administered doses (except 300 mg kg^{-1}) as compared to the diabetic untreated group (Table 4). A relative leukocytosis was also observed as evidenced by slightly higher average Total White Blood Cell (TWBC) in the diabetic untreated group in comparison to the extract treated and normoglycemic groups. Also, a mixed statistically significant (p = 0.000, 0.001, 0.003, 0.922 and 0.000) increase in blood lymphocyte was noted in the extract treated and normoglycemic groups in comparison with diabetic untreated group (Table 4).

DISCUSSION

An objective means of measuring improvements in diabetes mellitus may involve weight monitoring which is expected to improve as the condition is controlled. From this study, it was observed that oral administration of all the various doses of cocoa powder extract reduces body weight of the rats at the end of the forty days treatment in comparison to the initial weight of both the treated and control groups (Table 1). The characteristic diabetic weight change was revealed by high percentage weight loss in the diabetic untreated group (22.2%) as compared with the normoglycemic group (1.32%). The extract at a dose of 300 mg kg^{-1} thus showed better improvement in the hyperglycemic condition as revealed by the weight change (7.4%).

The trend in weight changes was an initial weight loss in the early weeks of extract administration which improves as the effect of the extract became established in the later weeks of administration (Table 1). This was observed in all the administered doses and was due to the effect of diabetes mellitus in the early weeks and modulating effect of the extract on body carbohydrate and lipid metabolism in the later weeks. The observed weight changes in the earlier weeks support the earlier reported emaciating weight changes in diabetic rats (Braslasu *et al*., 2007). It is a reflection of interplay between the compensatory biochemical changes in carbohydrate (gluconeogenesis, glycogenolysis), lipids (lipolysis, lipoprotein oxidation), body proteins and associated metabolic changes (ketoacidosis, cellular dehydration).

A transient hyperglycemia usually followed ingestion of carbohydrate meal and this is adequately managed by prompt pancreatic response as measured by plasma insulin activity. The blood glucose will later revert back to the pre-prandial level by the interplay between insulin activity and that of counter regulatory hormones. Diabetes is associated with chronic hyperglycemia which further worsens the oxidative stress and further increase metabolic pressure on the body due to the need to counter the stress using endogenous antioxidants. A significant (p<0.05) reduction in plasma glucose was observed following oral administration of different doses of aqueous cocoa powder extract in the extract treated as compared to diabetic untreated groups (Table 3).

The anti-hyperglycemic effect of the extract was earlier reported to peak at 120 min of administration of the extract (Olooto *et al*., 2014). The mechanism involved in the

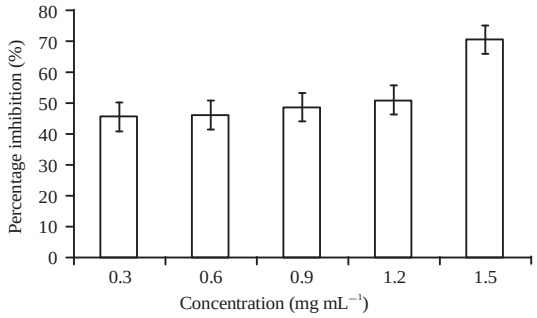

Fig. 1: Inhibition of alpha amylase activity by different doses of aqueous cocoa powder extract

anti-hyperglycemic effect of the extract was discovered from this study to be inhibition of α-amylase enzyme activities (Fig. 1) amongst other mechanisms. The least inhibition of the activity of α-amylase enzyme (45%) was seen at a dose of 0.3mg/ml while the maximum inhibition (70%) was observed at a dose of 1.5 mg mL^{-1}. Alpha-Amylase is an important enzyme in the digestion of carbohydrate. It catalyzes the initial step in the hydrolysis of starch to α-limit dextrins, maltotriose and maltose and finally to glucose. Thus, inhibition of this enzyme retards starch digestion and controls plasma glucose level in diabetes (Tarling *et al*., 2008). Other possible mechanisms may include arrest of further destruction of the remaining β-cells in the islet by the flavonols therein present thereby allowing other phytochemicals present in the powder to induce regenerative activities. Also, flavonols have been reported to cause increase in β-cells (Chakravarthy *et al*., 1980) and this is probably due to the presence of some stable cells in the islets with regenerating ability (De Fronzo *et al*., 1997).

The plasma level of proteins revealed a non-significant ($p>0.05$) decrease in the plasma albumin and a mixed significant increase in plasma total protein and globulin concentrations amongst the extract treated and normoglycemic control groups when compared with diabetic untreated group (Table 3). This finding probably suggests that the aqueous extract modulates the synthetic function of the liver.

Also, a mixed significant increase in plasma ALT and AST activities were observed amongst the treated and normoglycemic groups as compared to the diabetic untreated group (Table 3). This probably indicates an associated hepatic dysfunction in alloxan diabetics. Supporting this finding was the reported case of liver necrosis in diabetic patients by Larcan *et al*. (1979). The observed increase in plasma activities of AST and ALT in some doses may be mainly due to loss of hepatocellular integrity and consequent leakage of these enzymes from the liver cytosol into the blood stream, which gives an indication of the hepatotoxic effect of alloxan and probably a component of the cocoa powder. High plasma AST level may also be from other disease conditions of the heart, muscle, kidney, brain and red blood cells.

Result from this study also showed a significantly high levels ($p<0.05$) of urea in the 200 mg kg^{-1} concentrations while it was not significantly ($p>0.05$) high in 300, 500 and 1000 mg kg^{-1} doses and diabetic untreated group as compared to normoglycemic control group (Table 3). The non-significant changes observed in 300, 500 and 1000 mg kg^{-1} concentrations may be due to the protective function of antioxidants which are present at a higher concentration in those doses than in the 200 mg kg^{-1} dose. However, there was no significant change ($p>0.05$) in the plasma creatinine levels amongst all the doses administered. High plasma urea and creatinine levels were considered as markers of renal dysfunction and in this case may probably reflect the nephrotoxicity of the administered alloxan alone or a function of the synergic renal complication from both alloxan and diabetes mellitus in which case the hyperglycemia induces high plasma urea and creatinine levels (Almdal and Vilstup, 1988).

The TAC is the summation of both enzymatic and non-enzymatic antioxidants present in the body while MDA measures extent of lipid peroxidation. During oxidative stress conditions like diabetes, there is an imbalance between free radical formation and body antioxidant protection consequent to increased utilization of endogenous antioxidants. The plasma TAC was determined to serve as a marker of oxidative stress. A significantly ($p<0.05$) high plasma TAC level was observed in all the aqueous cocoa powder treated groups in comparison to the diabetic untreated group in which plasma TAC level was low (Table 3). Similar reduced plasma TAC level was reported in human diabetes (Dordevic *et al*., 2008). This suggests a high degree of oxidative stress in diabetics and confirms the role of oxidative stress in diabetes mellitus as earlier reported (Hsu *et al*., 2006). The increase in plasma TAC level was observed to be dose dependent and corroborates the earlier report of Rein *et al*. (2000).

The higher plasma TAC in the extract treated groups is a reflection of the high flavonol content or stimulating effect of the extract on endogenous antioxidants which acts to scavenge free radicals generated by the hyperglycemia and the administered alloxan, in order to prevent oxidative stress development. The antioxidants act to neutralize the hyperglycemia-induced free radicals generated and thus inhibit the progression of diabetic complications.

The development and severity of diabetic complications (ketoacidosis, angiopathy, neuropthy, gangrene and retinopathy) are a measure of the degree of oxidative stress.

Treatment of diabetes with antioxidant-rich aqueous cocoa powder extract is expected to show anti-peroxidative effects by reducing MDA levels. There was a significantly ($p<0.05$) low plasma MDA level in the aqueous cocoa powder extract treated and normoglycemic groups as compared to high MDA levels in diabetic untreated group (Table 3). Similar high MDA level has been reported in the plasma, serum and many others tissues of diabetic patients (De Bandeira *et al*., 2012). The extract probably contains an effective chain-breaking antioxidant that protects polyunsaturated lipids from peroxidation by scavenging peroxyl radicals. The increase in lipid peroxidation (as evidenced by high MDA) is a reflection of decline in defense capacity of body antioxidants system (enzymatic and non-enzymatic) (Saddala *et al*., 2013).

Result of hematological parameters showed a non-significant ($p>0.05$) reduction in the red blood cells count and hemoglobin levels in all the administered doses (except 300 mg kg^{-1}) as compared to the diabetic untreated group (Table 4). This corroborates the earlier work of Abrokwah *et al*. (2009) and reflects poor absorption of non-heme iron from gastrointestinal tract (Reddy *et al*., 2006), hemolysis, or sepsis in rats. A relative leukocytosis was also observed as evidenced by slightly higher average Total White Blood Cell (TWBC) in the diabetic untreated group in comparison to the extract treated groups. This is due to the high levels of oxidative stress in diabetes mellitus which allows for resultant inflammatory reactions to occur in the pancreas and liver and this calls for the recruitment of macrophages to either kill invading microorganisms or neutralize their toxins (Sandhar *et al*., 2011). Also, a mixed statistically significant increase in blood lymphocyte was noted in the extract treated groups in comparison with diabetic untreated group (Table 4). The observed changes in lymphocyte count is a reflection of the immunomodulatory effects of the extract in rats (Campbell, 1996) and a compensatory hematological response to chronic inflammatory condition.

CONCLUSION

From the results of biochemical and hematological parameters obtained from this study, aqueous cocoa powder extract could be of importance in the management of diabetes mellitus. This borders on the anti-hyperglycemic and modulating effects of the aqueous extract on plasma biochemical and hematological parameters in alloxan diabetic rat. A better result may be achieved using de-caffeinated cocoa powder extract to manage diabetes mellitus. Hence, its use as adjuvant to other anti-hyperglycemic agents is important for the management of diabetes mellitus.

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