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Anti-diabetic activities of theo broma cacao ethanolic seed extract on streptozotocin induced diabetic wistar rats

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Abstract
The study was aimed at investigating the antidiabetic activities of *Theobroma cocao* ethanolic seed extract on streptozotocin induced female wistar rats. A total of 40 rats were used and designated into six study groups of 8 rats per each group. Non-diabetic rats were administered distilled water, diabetic control group was induced with 60mg/kg of streptozotocin, diabetic treated group was treated with insulin while extracts treated groups were administered 75mg, 150mg and 300mg of *Theobroma cacao* seed extract respectively. Treatment was done orally for 14 consecutive days. Body weight indices and blood glucose levels were monitored during treatment and recorded. Animals treated with *Theobroma coaca* and insulin showed reduction in glucose concentration compared with diabetic control group. Heamtological indices significantly affected. There was a significant reduction in the low density lipoprotein of the treated groups compared with non-diabetic group, tricacylglyceerides was decreased, high density lipoprotein was not significantly increased in insulin treated group but it was increase significantly in the *Theobroma coaco* treated groups. Low density lipoprotein was significantly reduced in treated groups while it was raidsed in diabetic group. Serum enzymes ALT. AST and ALP were elevated in the STZ induced diabetic rats compared with non-diabetic control and there was a reduction in the liver enzymes os the treated groups. Serum albumin was increased in the diabetic rats , from this study , results show the *Theobroma cacao* seed extract lowers blood glucose level, improved serum enzymes and lipid profile activities in STZ Induced diabetic rats.

Keywords: Body weight indices, blood glucose level, serum enzymes heamatological indices and lipid profile

Introduction
Plants have been used by mankind as solutions to health problems, food etc, since the inception of the creation of man. Even the holy book of the Christian faith (The Holy Bible) encourages the use of plants as food and medicine (Genesis.1:29). All over the world the use of plants, for traditional medicine are well known. In places like Africa, Asia, some parts of Europe, uses of medicinal plants have gained so much popularity (Sharma *et al*., 1996) [12]
The use of medicinal plants as traditional medicine is made possible as a result of the presence of bioactive metabolite in plants which formed the basis of herbal medication (Sharma *et al*., 1996) [12]. Medicinal herbs used in indigenous medicines for the management of diabetes mellitus contain both organic and inorganic constituents (Sharma *et al*., 1996) [12]. Some of these inorganic trace elements possess anti-diabetic properties which accounts for the activity of medicinal plants (Akhere and Iyere, 2008) [1].
The *Theobroma cacao*, a diploid fruit tree species is the source of dried cocoa beans used as the main raw materials in the manufacture of chocolate, confectioneries and some cosmetic products. Although native to the humid tropical regions of the northern parts of South America and the northern parts of Central America, the largest cultivation of Cacao, an under-storey forest tree species takes place in West Africa and Central Africa. Originally designated as a member of sterculiaceae family (Pulseglove, 1974). *Theobroma cacao* was recently re-classified into the malvaceae plant family. Since its first introduction in the early 19th Century by the Portuguese and the Spaniards, the West and Central Africa regions have become the largest producer accounting for some 70% of the World's Cocoa output.
Theobroma cacao is a small (4-8m) (13-26feet) tall ever green in the family malvacea. It is grown in the tropics in a band between 10-20 degrees north and south of the equator sometimes called the 'Cocoa Belt'. The tree is often grown in the shades of other trees. It can also be as tall as 40 feet (12metres) and has fruits (Pods) which are more than 30cm long. The fruits may be brownish yellow to purple and contain 20-40 seeds. The Cacao theobroma belongs to the genus theobroma, part of the family sterculiaceae, more than twenty-two species are known but only one, *Theobroma cacao* or foods of the gods are produced for food. The Cacao tree is cultivated in many countries but today leading suppliers are Ivory coast, Ghana (14%), Cote d'ivoire (43% of global production), Nigeria (6%), Cameroon (5%). Others include Brazil, Ecuador, Dominican Republic etc.

Materials and methods
Experimental animals
Adult forty eight (48) female albino rats between 90-250g were purchased from a disease free stock of the University of Ibadan, Nigeria and used for the study. The rats were randomly assigned on the basis of their weight into six study groups of eight (8) rats each. Normal feeds and tap water

were given to the rats ad-libitum and food and water intake were noted. They were kept in plastic cages of 8 rats per cage, placed in a well-ventilated animal room of Joseph Ayo Babalola University at normal temperature of 30-35ºC. The cages were cleaned daily and the rats were treated according to the international guidelines for the care and use of laboratory animals (NIH, 2008). The animals were allowed for two weeks of acclimatization and their weights were measured before treatment commenced.

Chemicals
Streptozotocin, chloroform, ethanol and other chemicals were obtained from Fam-lab Nigeria Limited and Lixok-k chemicals, Akure respectively. Alanine transaminase (ALT), Aspartate transaminase (AST), Serum albumin were obtained from Randox Laboratories Limited, UK. Johnson one touch glucometer kit was purchased from De-shalom pharmacy, Ilesa, Osun state, Nigeria. All other chemicals used were of analytical grade. De-ionised and distilled water was also used during the experimental process.

Source of Drug
Insulin injection (Randox laboratories, UK) marketed by May and Baker, was obtained from a registered pharmacist in De-shalom pharmacy, Ilesa, Osun state, Nigeria and used for the study.

Plant Materials and Identification
Fresh and ripe pods of *Theobroma cacao* were obtained from cocoa trees at a plantation in Ile-Ife, Osun State, Nigeria. The seeds were extracted, and samples taken to the department of Botany, Obafemi Awolowo University (O.A.U) for identification and authentication.

Preparation of Cacao Seed Extracts
Theobroma cacao seeds were extracted, sorted out, and air dried under shade for 4 weeks. The dried materials were grounded using mechanical grinder and weighed in a weighing balance. The powder form was kept in airtight container and stored at 4ºC until when needed for further analysis.

2. Extraction Procedures
Ethanolic extraction
150g of the powdered extract was soaked in 98% of ethanol in 250ml at room temperature for 72 hours after which the mixture was filtered. The filtrate was placed in centrifuge at 5000r.p.m for 30 minutes. The centrifuged sample formed 2 layers; the upper layer was then poured inside another beaker. It was boiled at a very high temperature using a water bath. During the boiling process, it was observed that the sample formed a crystal structure in a foaming form. The resulting filtrate was air-dried at room temperature into petri dishes. The residue obtained was weighed and kept in air-tight container at 4ºC. Percentage yield was determined from the weight of the dried sample.

Phytochemical Screening of Extract
Basic phytochemical screening is the method employed to test the presence of certain biologically active compounds in plants e.g. tannins, saponins, antraquinone, flavonoids, cardiac glycosides, steroids, phenolics, cardenolides and denolides. The methods are as follows (A.O.A.C, 2001):

Animal Maintenance
The rats were kept in wooden cages barred with steel nets and housed in an environmentally controlled room temperature: 30.0±0.6ºC, 50-60% relative humidity with a 12 hour day and night cycle and they were constantly fed and supplied with water. Their surroundings were also cleaned to maintain proper hygiene. The animals had free access to food and water and were treated according to the international guidelines for the care and use of laboratory animals (NIH, 1985). The animals were allowed to acclimatize for two weeks before commencement of the practical.

Induction of Diabetes
Diabetes mellitus was induced by single intraperitoneal dose of 60mg/kg of streptozotocin (Sigma chemicals, St Louis USA) dissolved in 0.1M fresh cold citrate buffer at a pH of 4.5 into 12 hours overnight fasted rats (Burcelin *et al.*, 1995). After 3 days, fasting blood sugar levels were monitored with a glucometer (Accu check active, Roche Diagnostics GmbH, Germany) and the rats having fasting blood glucose levels more than 200mg/dl were isolated, classified diabetic and used for experimentation.

Experimental Design
The grouping and treatment given to the rats in each groups are as follows; Group A**:** Designated as NDC consisted of non-diabetic control rats administered 1ml of distilled water. Group B: Designated as DC consisted of diabetic control rats administered 1ml of streptozotocin. Group C: Designated as DO consisted of diabetic rats administered 1ml of orthodox drug (insulin). Group D: Designated as DLB1 consisted of diabetic rats administered 75mg of *Theobroma cacao seed* extract. Group E: Designated as DLB2 consisted of diabetic rats administered 150mg of *theobroma cacao seed* extract.
Group F: Designated as DLB3 consisted of diabetic rats administered 300mg of T*heobroma cacao* seed extract.

Sacrifice of the Animals
At the end of the experimental period, rats in each study group were fasted overnight and sacrificed under anesthesia by cervical dislocation.

Serum Collection and Organ Extraction
After the rats have been sacrificed, 2-4ml of blood was collected from each rat and placed in specific sterile bottles (plain bottles for enzyme analysis and EDTA bottles for haematological indices). For enzyme analysis, the blood was allowed to stand for 30 minutes to clot and then centrifuge at 4000g for 15 minutes. The supernatant, which is the serum, was carefully decanted and was kept at 4ºC for further analysis. Other vital organs like the liver, kidneys, and the pancreas were also extracted and prepared.

Tissue Preparation
The liver and kidney obtained from each rat was removed upon sacrifice, blotted with tissue paper, freed of fat and extra tissue and weighed. They were then cut into small pieces and homogenized in ice-cold normal saline (1:4 w/v) using a mortar and pestle. The homogenate was centrifuged at 4,000g at 4ºC for 30 minutes in a centrifuge.

Biochemical analyses

Enzyme assay

Determination of l-alanine aminotransferase (ec. 2.6.1.2) activity

L-alanine aminotransferase (ALT) activity was estimated by the method of Reitman and Frankel (1957) [10]. The method measures spectrophotometric ally the intensity of the coloured hydrazine formed from the reaction of pyruvate with 2, 4-dinitrophenylhydrazine at 546 nm.

Principle

The assay is based on the following reaction of the enzyme;

Glutamate + Pyruvate ⇌ α-ketoglutarate + Alanine

The keto acid (pyruvate) reacts with 2, 4-dinitrophenyl hydrazine to form hydrazine.

Determination of l-aspartate aminotransferase (ec. 2.6.1.2) activity

L-aspartate aminotransferase (AST) activity was estimated by the method of Reitman and Frankel (1957) [10]. The method measures spectrophotometric ally the intensity of the coloured hydrazine formed from the reaction of pyruvate with 2, 4-dinitrophenylhydrazine at 546 nm.

Principle: The assay is based on the following reaction of the enzyme;

L-aspartate + α-ketoglutarate ⇌ Oxaloacetate + L-glutamate

Oxaloacetate produced by transamination activities of AST is spontaneously decarboxylated to pyruvate which then react with 2, 4-dinitrophenylhydrazine. The intensity of the red colour formed is a measure of transaminase activity. The reactions stopped by addition of 2.5ml 0.1 NaOH solution.

Determination of Alkaline Phosphatase Activity

Alkaline phosphatase activity was assayed according to the method described by Bassey *et al.* (1946) and modified by Wright and Plummer (1974) [13].

Determination of Serum Albumin

The method was described by Plummer (1979) [13] was used to determine the albumin concentration.

Lipid Profile

Total Cholesterol Determination

Cholesterol is measured enzymatically, in serum or plasma, in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction by products, H_2O_2 is quantitatively measure in a peroxidase catalyzed reaction that produces a colour. Absorbance is measured at 500nm. The colour intensity is proportional to cholesterol concentration. The reaction sequence is as follows:

$$\text{Cholesteryl ester} + H_2O \xrightarrow{\text{Cholesteryl ester hydrolase}} \text{Cholesterol} + \text{Fatty acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{Phenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 4H_2O$$

Triacylglycerol (Tag) Determination

Triacylglycerols are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produced glycerol. Glycerol is then oxidized using glycerol oxidase and H_2O_2, one of the reaction products, is measured as described above for cholesterol. Absorbance is measured at 500nm. The reaction sequence is as follows;

$$\text{Triglycerides} + 3H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + \text{Fatty acids}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-phosphate} + \text{ADP}$$

$$\text{Glycerol-3-phosphate} + O_2 \xrightarrow{\text{Glycerophosphate oxidase}} \text{Dihyroxyacetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-monoimino)-phenazone} + 2H_2O + HCl$$

High Density Cholesterol Determination

HDL was measured directly in serum. The basic principle of the method is as follows; the apoβ containing lipoproteins in the specimen reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoβ containing lipoproteins are thus effectively excluded from the assay and only HDL-cholesterol is detected under the assay conditions. The reagents were purchased from Roche/Boehringer-Mannheim Diagnostics. The method uses sulfated alpha-cyclodextrin in the presence of Mg^{2+}, which forms complexes with apoβ containing lipoproteins, polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement. The reactions are as follows;

1. Apoβ containing lipoproteins + α-cyclodextrin + Mg^{2+} + Dextran SO_4 Soluble non-reactive complexes with apoβ -containing lipoproteins.
2. HDL-cholesteryl esters $\xrightarrow{\text{PEG – cholesterol esterase}}$ HDL-unesterified cholesterol + fatty acid.
3. Unesterified cholesterol + O_2 $\xrightarrow{\text{PEG-cholesterol oxidase}}$ Cholestenone + H_2O_2
4. H_2O_2 + 5-aminophenazone + N-ethyl-N-(3-methylphenyl)-N-succinyl ethylene diamine + H_2O + H^+ peroxidase ⟶ Quinoneimine dye + H_2O

Absorbance was measured at 600nm.

Low Density Cholesterol Determination

Most of the circulating cholesterol is found in three major lipoprotein fractions, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density cholesterol (HDL).

[Total Cholesterol] = [VLDL-cholesterol] + [LDL-cholesterol] + [HDL-cholesterol]

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the relationship:

[LDL-cholesterol] = [Total Cholesterol] - [HDL-cholesterol] – [TG]/5

Where [TG]/5 is an estimate of VLDL-cholesterol and all values are expressed in mg/dL.

LDL carries most of the circulating cholesterol in man and when elevated contributes to the development of coronary

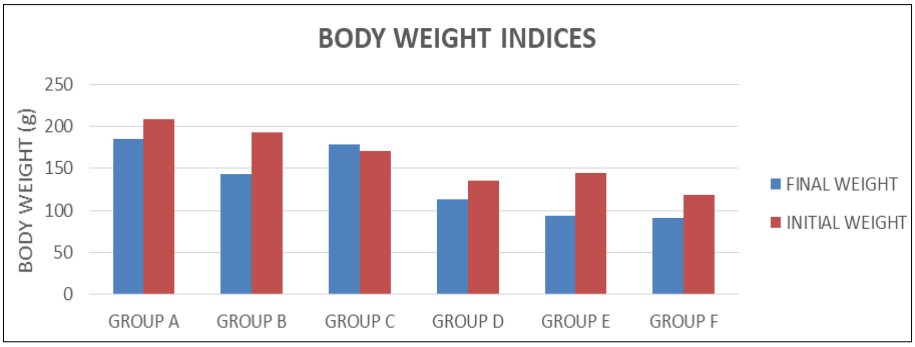
atherosclerosis. LDL-cholesterol is measured to assess risk for CHD and to follow the progress of patients being treated to lower LDL-cholesterol concentrations. Desirable levels of LDL-cholesterol are those below 130mg/dL in adults and 110mg/dL in children. In NHANES 2001-2002, LDL-cholesterol will be reported only for fasting participants > 5years of age.

Haematological investigation
Packed cell volume
Method: Micro haematocrit (Baker and Silverton, 2003)
Haemoglobin Estimation
Method: Cyanmethaemoglobin (Baker and Silverton, 2003)
Total White Blood Cell Count
Method: Bulk dilution (Ochei and Kolhatkar, 2007)
Histopathological Evaluation

Pieces of residual organs, i.e. liver, kidney and pancreas, were utilized for histopathological examination after the specimens were fixed in 10% formalin, dehydrated, embedded in paraffin, cut at 5 /-Lm and stained for histopathological examination with hematoxylin and eosin.

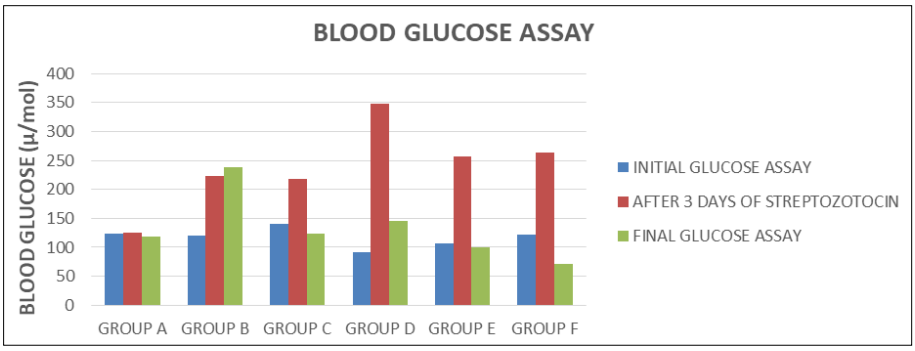
Statistical analysis
The data will be expressed as Mean value ± S.E.M (Standard error of the mean). All results will be mean of 8 data samples and the statistical analysis will be carried out using students't-test. The values will be considered at probability level of $p<0.05$. All the values of $p<0.05$ will be considered statistically significant.

Results



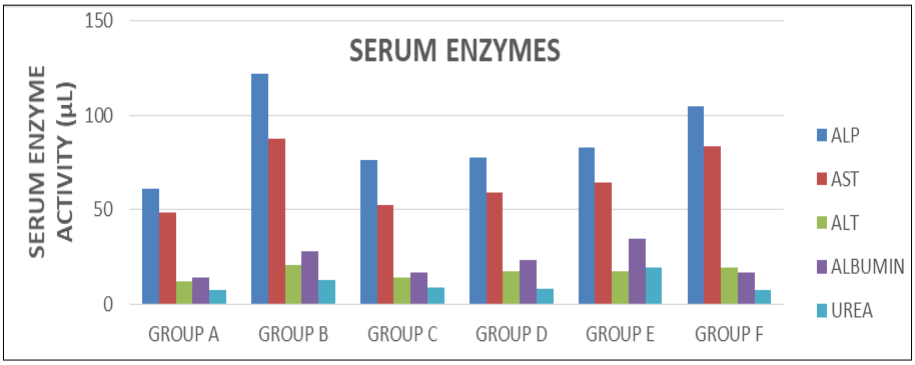
Values are expressed in Mean ±S.E.M

Fig 1: effect of *Theobroma coaco* ethanolic seed extract on body weight indices



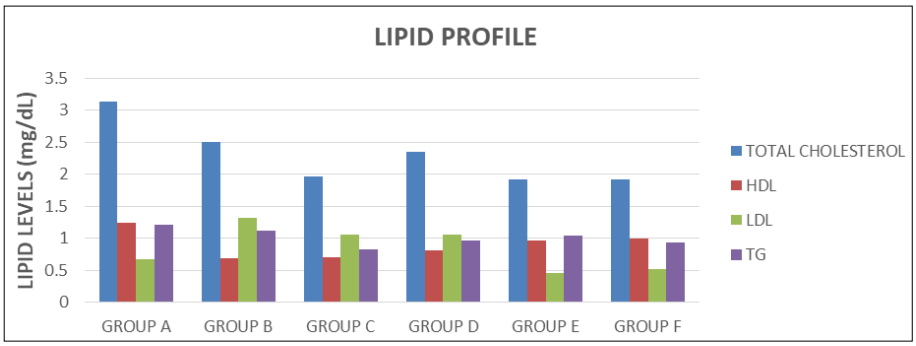
Values are expressed in Mean ±S.E.M

Fig 2: effect of *Theobroma coaco* ethanolic seed extract on blood glucose level



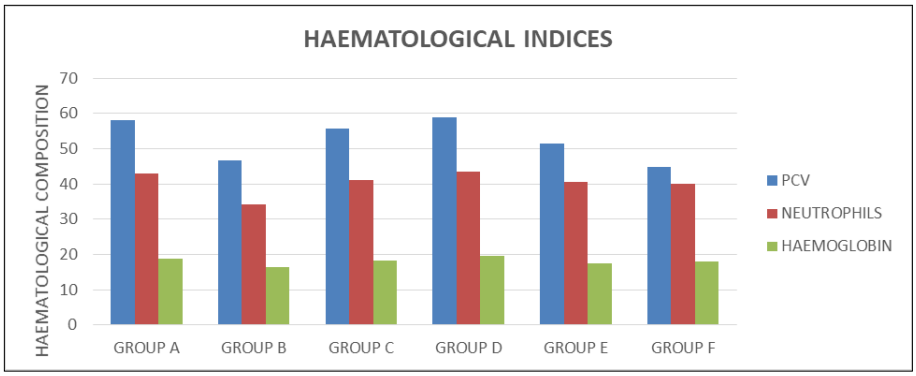
Values are expressed in Mean ±S.E.M

Fig 3: Effect of *Theobroma coaco* ethanolic seed extract on serum enzyme level



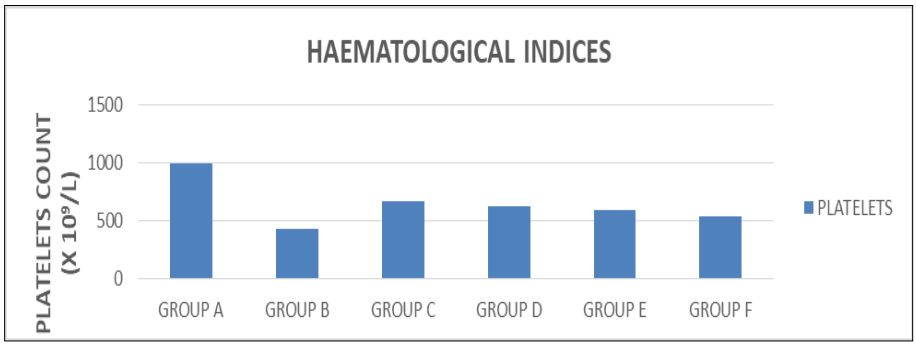
Values are expressed in Mean ±S.E.M

Fig 4: Effect of *Theobroma coaco* ethanolic seed extract on lipid profile



Values are expressed in Mean ±S.E.M

Fig 5: effect of *Theobroma coaco* ethanolic seed extract on HB, PVC and neutrophiles



Values are expressed in Mean ±S.E.M

Fig 6: effect of *Theobroma coaco* ethanolic seed extract on platelets level

Discussion and conclusion

Phytochemical screening of the extract revealed presence of cardiac glycoside , tannis, terpenoids and steroids and tested negative for flavonoids, saponnins, phenolics, alkaloids and the presence of the aforementioned phytochemicals is likely to be part of its anti-diabetic activities (Erdnna *et al.* , 2008).

There is also a significant reduction in body weight of diabetic control compared with the one treated with Insulin and all other diabetic groups and this is in consonance with loss of weight associated with diabetes as reported (Kumar and Clark, 2005) [4] (Preeti, 2013) [8]. The further decrease in weight of theobroma cacao seed extract treated group could be as a result of prevention of oxidation of low density lipo protein, hence cholesteroal level in the body (Alspach, 2007)

In addition, there is significant reduction in ($P<0.05$) the glucose level of the diabetic rats treated with the cacao seed extract and insulin but decrease in the extract treated group is marked compared to that of Insulin.

It is possible that the hypoglycaemia could be related to the polyphenols in which cocoa beans is well known to be rich in. Hypoglycemic effects of some plants have been reported due to polyphenols (Maghrani *et al*, 2003) [5] Polyphenols are reported to be the potential bioactive component for hypoglyceamic properties (Manickam *et al*, 1997) [6]

A significant reduction of plasma glucose levels in the group treated with cacao extract may be due to the antioxidant properties of the cacao extract. Antioxidants compounds are well known to possess free radical scavenging activity.

Heamatological indices which include packed cell volume, white blood cell count, platelets counts are significantly reduced in diabetic control groups, but no significant reduction in the PCV of Insulin treated group and the *Theobroma cacao* seed extract treated group. The significance reduction is in line with literature stating that various hematological parameters and the immune system were disrupted during course of diabetes (Mansi and Lahham, 2008) [7]

There is significant reduction (P<0.05) in the cholesterol level of the *Theobroma cacao* treated diabetic rats in relation to the non-diabetic control, and also in Insulin treated group, triglycerides level is also significantly decreased in the treated groups, but no significant reduction in the Insulin treated group, High density lipoprotein (HDL-C) is not significantly increased in the Insulin treated group but significantly increased in the group treated with *Theobroma cacao* seed extract but as high dosage (150 mg and 300 mg/kg) and there is significant reduction in the LDL level of the treated diabetic group but no significant reduction in the diabetic rat with Insulin and this reduction the LDL level of *Theobroma cacao* treated diabetic rats substantiate the fact that treatment of hyperlipidaemia will benefit Diabetic Mellitus patients in decreasing heart disease (Sacks et al, 2002) [11].

Serum activities of ALT, AST and ALP were elevated in streptozotocin induced diabetic rats when compared to non-diabetic controls. Treatment of diabetic rats with 75mg, 150 mg/200mg/kg body weight significantly reduced the activity of these biomarkers with respect to control diabetic rats (P< 0.05). The serum album of the diabetic groups were significantly increased in comparison to Non-diabetic control groups at different dosage. It shows that inducement of diabetes with STZ and treatment with cacao extract has an effect on the synthetic functions of the liver as regards album production. It could be concluded that there are immunologic response as regards to the cocoa extract administered. Urea level was also observed to be increased in diabetic group and diabetic rats treated with 150mg/kg.

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