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Biochemical Studies on the Effects of Polyphenols from Fermented and Unfermented Acetone Extracts of Cocoa (*Theobroma cacao* L.) Seeds on Antioxidant Enzymes of Streptozotocin-Induced Diabetic Rats

Dare, C. A., Onwumelu, R. N. and Oyedapo, O. O.
Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, 220005, Nigeria.

ABSTRACT: This study was carried out to investigate both the *in vivo* and *in vitro* antioxidant effects of polyphenols extracted from fermented and unfermented *Theobroma cacao* seeds with a view to using the extract in the management of hyperglycaemia-related conditions. Phytochemical screening of the aqueous extracts, total phenol and flavonoid quantifications were carried out using standard methods. DPPH free radicals scavenging, lipid peroxidation, reducing power and membrane stabilizing activities of the fermented (FP) and unfermented (UP) polyphenols were carried out *in vitro.* The effect of FP (which was the most potent from the *in vitro* assays) on antioxidant enzymes and liver function indices (150 mg/kg bwt and 300 mg/kg bwt) were investigated in diabetic and non-diabetic rats, with 2 mg/ kg bwt glimepiride as the reference drug. The FP and UP activities for DPPH radical scavenging were 93.72 ± 0.00 % and 77.69 ± 1.67 % at 50 µg/ml (IC_{50} 23.94 ± 0.005/31.07 ± 0.570 µg/ml) which compared favourably with the reference chemical ascorbic acid (96.35 ± 0.03 %, IC_{50} 17.43 ± 0.70 µg/ml), reducing power 1.17 ± 0.066 and 0.85 ± 0.023 at 300 µg/ml (Ascorbic acid 2.10 ± .008), lipid peroxidation 63.06 ± 4.17 % ,IC_{50} 0.469 ± 0.080 mg/ml and 70.45 ± 2.18 %, IC_{50}0.489 ± 0.039 mg/ ml at 1000 µg/ml (with reference to BHT 63.20 ± 4.55 %, IC_{50} 0.68 ± 0.064 mg/ml) and membrane stabilizing potential 33.25 ± 3.41 % and 28.17 ± 0.08 % at 350 µg/ml (with reference to ibuprofen 43.73 ± 2.98 %). The antioxidant and anti-diabetic activities of acetone extract of the fermented cocoa seeds on experimental rats compared favourably with the reference drug. However, the effects on antioxidant enzymes and liver function tests revealed that the 150 mg/kg bwt of the extract was quite effective.

KEYWORDS: Polyphenols, *Theobroma cacao*, Free radicals, Antioxidant enzymes, Liver function indices**.**

1.0 Introduction

Diabetes mellitus, often simply referred to as diabetes, is a group of chronic metabolic diseases characterised by high blood sugar, either due to inability to produce enough insulin, or because cells do not respond to the insulin that is produced (Bell, 1991). The disease which is the seventh leading cause of death in the world has affected more than120 million people world-wide, which is estimated to increase to 220 million by 2020 (Kumar and Clark, 2002). The available therapies are expensive with associated adverse side-effects (Codario, 2005). Over the past few years, the antioxidant and health-promoting properties of cocoa (*Theobroma cacao* L.) and cocoa-related products have been thoroughly investigated. Oyedapo *et al.* (2004) and Falade *et al. (*2005) have demonstrated that extracts of root-bark of *T. cacao* exhibited potent and appreciable red blood stabilizing potentials against heat and hypotonic induced lyses. Also the herbal decoctions of *T. cacao* root-bark, *Sorghum bicolor*, (L.) and *Hibiscus sabdariffa*, (Linn.) stabilized the red blood cells from stress injuries and in the management and treatment of severe and moderate anaemia.

Theobroma cacao, known commonly as cocoa, is a small size evergreen tree, with a height of 4-8 m. It is a native of the deep tropical region of the North and South America. In 1737, the cocoa tree was named *Theobroma cacao* which refers to the mythical background of the tree literally means "cocoa, food of the gods"

Corresponding Author
Tel.: +2348034038146;
E-mail: ooyedapo@oauife.edu.ng

(Dillinger *et al*., 2000). The cocoa tree (family *Sterculiaceae*) is a major source of cocoa powder, chocolate and cocoa butter all of which are produced from the seeds (Anega and Gianfagne, 1994). Cocoa had long been identified as a polyphenols-rich food. The main polyphenol in cocoa or known as cacao was first identified by Ultée and van Dorsen in 1909. The crystalline compound they discovered, with empirical formula $C_{16}H_{16}O_6$, was called "Kakaool". This was later purified to be $C_{15}H_{14}O_6$ (Freudenberg *et al*., 1932). Daily cocoa extract administration prevented the overproduction of free radicals after heat exposure and thus protect from cognitive impairments (Rozan *et al*., 2006). Polyphenols are good antioxidants (Zhu *et al*., 2002). An antioxidant is a substance that inhibits oxidation or reactions promoted by oxygen and peroxides, and that include many held to protect the living body from the deleterious effects of free radicals.

Numerous studies demonstrated that oxidative stress, mediated mainly by hyperglycaemia-induced generation of free radicals, contributes to the development and progression of diabetes and related contributions, it became clear that ameliorating oxidative stress through treatment with antioxidants might be an effective strategy for reducing diabetic complications (Giugliano *et al.,*1996; Ceriello, 2003; Ceriello and Motz, 2004). Oxidative stress in general term can be defined as excessive formation and/or insufficient removal of highly reactive molecules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Turko *et al.,* 2001; Maritim *et al*., 2003). It has also been proposed that reduction of glucose to sorbitol by NADPH consumes NADPH. As NADPH is required for regenerating reduced glutathione (GSH), this could induce or exacerbate intracellular oxidative stress. Decreased levels of GSH have in fact been found in the lenses of transgenic mice that overexpress aldose reductase, and this is the most likely mechanism by which increased flux through the polyol pathway has deleterious consequences (Lee and Chung, 1999). ROS can stimulate oxidation of low-density lipoprotein (LDL) and ox-LDL, which are not recognized by the LDL receptor. These are then taken up by scavenger receptors in macrophages leading to foam cell formation and atherosclerotic plaques (Boullier *et al.,* 2001; Taniyama and Griendling, 2003). Polyphenols, widely distributed in plant foods, are the main antioxidant-active fraction of cocoa. Phenolics of cocoa have been reported in many studies as bioactive compounds with potential health benefits for various chronic diseases, including inflammation, cardiovascular ailments, neurodegenerative disorders and possibly cancer (Carnesecchi *et al.*, 2002; Noé *et al.,* 2004). This study reports *in vitro* anti-oxidant and anti-inflammatory potentials of polyphenol of *T. cacao* seeds and the biological anti-diabetic potentials of the polyphenol on streptozotocin-induced diabetic rats. This was with a view to evaluating the usefulness of the *cacao* products in the treatment and management of diabetes and related conditions.

2.0 Materials and Methods

2.1 Collection of Cocoa Pods

Fresh and ripe cocoa pods were collected from a cocoa plantation at Babajakan village in Ayedaade Local Government, Osun State, Nigeria. The plant materials were identified and authenticated at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria where specimen copy was deposited and specimen number (IFE 172418) was collected.

2.2. Chemicals and Reagents

All chemicals and reagents used for this study were of analytical grade and purchased from various sources. L-Ascorbic acid, Thiobarbituric acid, DPPH (2,2-Diphenyl-1-picrylhydrazyl), Bovine Albumin, 2,6-Di-tert-butyl-4-methylphenol (BHT), Streptozotocin were obtained from Sigma Fine Chemical Limited, while inorganic solvents, salts and other reagents used were obtained from British Drug House (BDH) Chemical Limited, Poole, England. Assay kits for AST, ALT, total protein, albumin and bilirubin were purchased from Randox Laboratories Ltd., UK.

2.3 Experimental Animals

Thirty-five (35) healthy Wistar rats (*Rattus norvegicus*) of both sexes were purchased from the Animal House, Faculty of Health Sciences, Obafemi Awolowo University, Ile-Ife, Nigeria. 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 hr., but allowed free access to water. All animals were carefully monitored and maintained in accordance with ethical recommendations of Nigerian Veterinary Science.

2.4 Processing 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. The pulpy seeds of one portion were rinsed to remove pulp and sun-dried for 7 days.

2.4.1 Fermentation of Cocoa Seeds

The seeds in the second portion were fermented traditionally by wrapping the seeds in fresh banana leaves tightly and kept in the dark cupboard for 5 days. On the 6^{th} day, the fermented seeds were collected and sun-dried for 7 days.

2.4.2 Preparation of Unfermented and Fermented Cocoa Seed Extracts

The dried seeds (unfermented and fermented) were powdered in a warring blender and defatted using chloroform: isopropanol (3:2 v/v) mixture until the supernatants became colourless. The defatted materials were then extracted with distilled water for 24 hr and concentrated to dryness using Edman vacuum evaporator under reduced pressure at 45ºC to give a dark-brown residue termed "aqueous unfermented extract "and "aqueous fermented extract" respectively.

2.5 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).

2.6 Extraction of Polyphenol

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 and unfermented cacao seed were extracted 3 times with a 100-fold volume of 80 % (v/v) acetone at 25 °C. The hydroacetone solution contained almost all of the polyphenols. The acetone filtrate was concentrated using Edman vacuum evaporator under reduced pressure at 45ºC. The brownish residues from fermented and unfermented *T cacao* seeds were termed "polyphenol fermented" (FP) extract and "polyphenol unfermented" (UP) extract respectively. Phytochemical screening, thin layer chromatography and staining with specific detecting reagents (methanolic $AlCl_3$) and chemical tests with 2, 4, dinitrophenyl hydrazine revealed that 80% (v/v) acetone fractions/extracts contained mainly polyphenol of the seeds of *T. cacao* (Akinwunmi and Oyedapo, 2013).

2.7 Determination of Total Phenolic (TPH) Concentration

The total phenol contents in the aqueous and polyphenol extracts of both fermented and unfermented cocoa seeds were determined according to the Folin-Ciocalteu's reaction method of Singleton *et al.* (1999). The calibration curve of tannic acid was prepared by pipeting 1.0 ml of 10, 20, 30, 40, 50 µg/ml in triplicate into clean and dried test tubes. To each of the tubes was added 1.5 ml of Folin-Ciocalteu's phenol reagent (1:10) dilution. The reaction mixtures were incubated at room temperature for 5 minutes followed by the addition of 1.5 ml of 7.5 % (w/v) Na_2CO_3. The reaction mixtures were further incubated for 1 hr

30 minutes at room temperature. The absorbance was read at 725 nm against the reagent blank on Visible Spectrophotometer S23A, Gulfex Medical and Specific, England. The calibration curve was prepared by plotting the absorbance against tannic acid concentrations. The determination of phenol contents in the extracts of aqueous and acetone fermented and unfermented cocoa seeds were carried out by pipetting 0.1 ml and 0.2 ml of 0.1 mg/ml of the extracts in triplicates. The volumes of the extracts were adjusted to 1.0 ml with distilled water. The reaction was treated as described earlier. The absorbance was read at 725 nm against the reagent blank. The total phenol concentration was extrapolated from the calibration curve and expressed as mg tannic acid equivalent per g of extract (mg TAE/g extract).

2.8 Determination of Total Flavonoid (TFL) Concentration

The total flavonoid contents in the aqueous and polyphenol extracts of fermented and unfermented cocoa seeds were evaluated according to the Aluminum trichloride reaction method of Sun *et al*. (1999). The calibration curve of rutin was prepared by pipetting 1.0 ml of 20, 40, 60, 80 , 100 µg/ml rutin solution in triplicate into clean dried test tubes. To each of the tubes was added 0.3 ml of 5 % (w/v) $NaNO_2$, 0.3 ml of 10 % $AlCl_3$ and 2.0 ml of 4 % NaOH. The reaction mixture was incubated at room temperature for 15 minutes and the absorbance was read at 500 nm against the reagent blank. The calibration curve was prepared by plotting the absorbance against rutin concentrations. The determination of total flavonoid content in the aqueous and acetone extracts of fermented and unfermented cocoa seeds was done by pipetting 0.1 ml and 0.2ml of 0.1 mg/ml of the extracts. The volumes were adjusted to 1.0 ml with distilled water and treated as described earlier and the absorbance was then read at 500 nm against the reagent blank. The total flavonoid concentration was extrapolated from the calibration curve and expressed as mg rutin equivalent per g of extract (mg RE/g extract).

2.9 Assay of DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the polyphenols of fermented and unfermented cocoa seeds was assayed according to the procedure of Blois (1985) as reported by Cakir *et al*. (2011). The serial dilution (1.0 ml) of 3.125-50 µg/ml of standard ascorbic acid and acetone extracts of fermented and unfermented cocoa seed was made in clean and dried test tubes in triplicates, after which 1.0 ml of 0.3 mM DPPH was added. The mixture was properly mixed by inversion and then incubated in a dark chamber for 30 minutes. The absorbance was read at 517 nm against the reagent blank. The percentage free radical scavenging activities of the standard and extracts were calculated from the percentage inhibition of DPPH using the expression:

$$I_{DPPH}\% = 100 \times \frac{\Delta Abs_{Control} - \Delta Abs_{Sample}}{Abs_{Control}}$$

$$\Delta Abc_{control} = Abs_{test} - Abs_{blank}$$

$$\Delta Abs_{sample} = Abs_{test} - Abs_{blank}$$

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

2.10 Assay of Reducing Power

The procedure of Oyaizu (1986) was employed for the assay of reducing power of polyphenols of fermented and unfermented cocoa seeds. The assay was based on the ability of the extracts to reduce Fe^{3+} to Fe^{2+}. Different concentrations (0-300 µg/ml) of the extracts (1.0 ml) were mixed with 2.5 ml of 0.2 M phosphate buffer, pH 6.6 and 2.5 ml of 1 % (w/v) potassium ferricyanide $[K_3Fe(CN)_6]$. The mixtures were incubated at 50 °C for 20 minutes after which the tubes were cooled. 2.5 ml of 10 % (w/v) tricholoroacetic acid was added to each reaction mixture, vigorously shaken and centrifuged at 704 g for 10 minutes. 2.5 ml of the supernatant was mixed with 2.5 ml distilled water and 0.5 ml $FeCl_3$. The absorbance was read at 700 nm after 10 minutes against the

reagent blank. Ascorbic acid was used as the standard. The absorbance obtained was plotted against the different concentrations.

2.11 Assay of Lipid Peroxidation

Lipid peroxidation assay was carried out on polyphenols of fermented and unfermented cocoa seeds according to the method of Ohkawa *et al.* (1999) as modified by Nabasree and Bratati (2004) with 2,6-Di-tert-butyl-4-methylphenol (BHT) as positive control. Typically, 0.5 ml of 10 % (v/v) egg yolk homogenate was added to 0.1ml of varying concentrations of the extract (1000, 500, 250, 125, 65.5, 31.25 µg/ml) in a test tube followed by the addition of 1ml distilled water. Then 50 µl of ascorbic acid (1mM) was added to the reaction mixture followed by 50µl of $FeSO_4$ (0.07 M) to induce lipid peroxidation. The mixture was vortexed and left undisturbed for 30 minutes at room temperature after which 1.5 ml of 20 % acetic acid and 1.5 ml of 0.8 % (w/v) thiobarbituric acid in 1.1 % sodium dodecyl sulphate were added. The resulting mixture was then heated in a water bath at 95 ℃ for 1 hr. After cooling, 4.0 ml of butan-1-ol was added to each tube and centrifuged at 4500 g for 10 minutes. The absorbance of the organic upper layer was read at 532 nm. The percentage inhibition of lipid peroxidation was calculated as:

$$\frac{A_o - A_1}{A_o} \times 100$$

where A_0 is the absorbance of control (absence of extract) and A_1 is the absorbance with extract samples.

2.12 Assay of Red Blood Cell Membrane Stability Activity

Determination of red blood cell membrane stability potential was carried out according to the modified procedure of Oyedapo *et al.* (2010). The assay mixture contained 1.0 ml of different concentrations of the polyphenols of fermented and unfermented cocoa seeds (0-350 µg/ml) pipetted into clean and dried test tubes and mixed with 1.0 ml of hyposaline and 0.5 ml of 0.15 M phosphate buffer, pH 7.6. 0.5 ml of 2 % (v/v) erythrocyte was added to give a final volume of 3.0 ml. The reaction mixture was incubated at 56 ℃ for 30 minutes. The tubes were cooled, removed and centrifuged at 704 g for 10 minutes. The control was the tube with zero concentration of extract. The supernatant was collected and the absorbance was read at 560 nm. The drug control lacked erythrocyte and was treated as above. Ibuprofen was used as reference drug.

Percentage inhibition of haemolysis by extract /drug = $100 - \left[\frac{Abs_{test} - Abs_{drug}}{Abs_{control} - Abs_{drug\ control}}\right] \times 100$.

2.13 Induction of Diabetes

Diabetes was induced by a single intraperitoneal injection of 60 mg/kg (bwt) of streptozotocin (dissolved in 0.1 M sodium citrate buffer, pH 4.5) to twenty overnight fasted rats of the thirty-five rats. Diabetes was confirmed in animals after 48 hr of 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. Animals with blood glucose concentration of 200-300 mg/ dL were used for the experiment. The animals were allowed free access to food (Ladokun Feeds, Ibadan, Nigeria) and water for 21 days. The thirty-five animals were divided into 7 groups of 5 animals (n = 5/ group). The animals were treated orally by gavage. The body weights and blood glucose concentrations of the animals were recorded weekly throughout the experimental period.

2.14 Grouping and Treatment of Animals

The study adopted the following experimental procedures. The animals were treated as follows:

Group I: Normal rats received only distilled water (General control)

Group II: Normal rats administered 150 mg/kg (bwt) FP

Group III: Normal rats administered 300 mg/kg (bwt) FP

Group IV: Diabetic rats received distilled water

only.
Group V: Diabetic rats administered 150 mg/kg (bwt) FP
Group VI: Diabetic rats administered 300 mg/kg (bwt) FP
Group VII: Diabetic rats administered 2 mg/kg (bwt) glimepiride (Positive control).
The animals were treated with the extract and reference drug once daily, consistently and regularly for 21 days and observed for any behavioural change(s) during experimental period. The glucose levels and body weights of the experimental animals were noted and recorded on days 0, 7, 14 and 21 using a digital glucometer (Accu-chek® Advantage, Roche Diagnostic, Germany).

2.15 Sacrifice of Animals and Collection of Tissues

On the 22nd day, the animals were put to sleep 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 liver was surgically removed and immediately perfused in heparinized saline (0.85 % NaCl), wrapped in foil paper and stored frozen.

2.16 Preparation of Plasma and Liver Homogenates (Post-mitochondrial Fractions)

The blood was centrifuged at 4500 g for 10 minutes on Bench Centrifuge Model 800D (Microfield Instrument, Essex, England, UK.). 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.

Exactly, 1 g of liver was thoroughly homogenized in 10 ml of freshly prepared phosphate buffer (100 mM, pH 6.8). The homogenate was then centrifuged at 704 g for 10 minutes using Bench Centrifuge Model 800D. The supernatants were decanted into clean tubes and used for protein, catalase, superoxide dismutase and GSH assays.

2.17 Biochemical Analyses

Alanine aminotransferase (ALT) was assayed as described by Reitman and Frankel (1957) in the exudates using a Sigma diagnostic kit. The assay mixture contained 50 µl plasma and 500 µl ALT substrate, followed by incubation at 37 °C for 1 hr. The colour reagent (0.5 ml) was added, followed by the addition of 5 ml of 0.4 M NaOH after 20 minutes. The plasma was replaced with 50 µl of distilled water for the blank. The amount of product formed was estimated from a calibration curve, after the absorbance was read at 505 nm. The activity was expressed as Units/ml.

Aspartate transaminase was also assayed as described previously, using AST substrate and incubation was for 30 minutes. The activity was also expressed as SF units/ ml.

Assay of L-γ-Glutamyltransferase (GGT) Activity was carried out with Randox diagnostic kits following the principle described by Szasz (1969). The GGT activity was calculated from the expression:

GGT activity (U/ L) = 1158 x ΔA 405 nm/ min

The total protein concentration in plasma and liver homogenates was carried pout using Randox diagnostic kits according to Flack and Woollen (1984) using the expression.

$$\text{Total Protein Concentration (g/dL)} = \frac{A_{test} - A_{blank}}{A_{standard} - A_{blank}} \times \text{Conc of Standard (5.95 g/dL)}$$

The concentration of plasma albumin was determined according to the method described by Chawla (1999) using Randox Diagnostic kit. The albumin concentration was calculated from this expression:

$$\text{Albumin Concentration (g/dL)} = \frac{A_{sample} - A_{blank}}{A_{standard} - A_{blank}} \times \text{Conc of Standard (4.68 g/dL)}$$

Reduced glutathione level in the liver was estimated according to the method of Moron *et al*. (1979). Liver GSH level was extrapolated from the glutathione standard curve and the values were expressed in µg GSH/ ml sample. The liver glutathione peroxidase activity was

estimated as a measure of the reduced glutathione present in the liver homogenate. The enzymatic activities of catalase superoxide dismutase (SOD) were carried out according to the method of Sinha (1972) and Misra and Fridovich (1972) respectively.

2.18 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 animals were determined by One-way ANOVA followed by Tukey multiple comparison test using GraphPad Prism 5. Differences were considered to be significant if $P<0.05$.

3.0 Results and Discussion

The phytochemical screening of the aqueous extracts of both fermented and unfermented *T. cacao* seeds revealed that both extracts contained tannins, saponins, flavonoids, alkaloids, xanthoproteins, cardiac glycosides and anthraquinones (Table 1). Phytochemicals, especially polyphenols, have received increasing attention because of their biological activities (Cho *et al.*, 2003) such as anti-oxidation, anti-inflammation, anti-aging, as well as inhibition of angiogenesis. Flavonoids have been reported to possess a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, hepatoprotective, antithrombic, antiviral, anticarcinogenic, vasodilatory and anti-diabetic actions. Many of these biological functions have been attributed to the free radical scavenging and antioxidant activities of these compounds (Raj and Shalini, 1999; Marles and Farnsworth, 1995; Middleton *et al.*, 2000; Williams *et al.*, 2004; Soobrattee *et al.*, 2005; Bode and Oyedapo, 2011).

The phenolics (tannins, anthraquinones etc.) and flavonoid contents of both aqueous and acetone extracts of both fermented and unfermented *T. cacao* seeds are presented in Table 2. It was observed that phenolic content of acetone extracts of both fermented and unfermented were very high which implied that acetone extracts contained exclusively phenolic compounds (polyphenols). This observation was in agreement with earlier reports (Natsume *et al.*, 2000; Bode and Oyedapo, 2011; Akinwunmi and Oyedapo, 2013) that phenolics (polyphenols and flavonoids) are exclusively extracted with ethylacetate and acetone.

Analysis of the aqueous and acetone extracts of the fermented and unfermented cocoa seeds revealed a high flavonoid and phenolic contents with the acetone extracts (polyphenol) having greater content. This was in agreement with earlier observations of Natsume *et al.* (2000). The flavonoid and phenolic contents of unfermented was higher than that of the fermented. This could be as a result of the observation of Hansen *et al. (*1998) that during fermentation, polyphenols diffuse from their storage cells and undergo oxidation to become condensed high molecular compounds mostly insoluble tannins.

DPPH is a stable, organic free radical extensively used to evaluate scavenging activity of antioxidants because it is sensitive enough to detect active ingredients at low concentrations. In this study, a dose-response relationship and concentration-dependent found in DPPH radical scavenging activity acetone extracts of both fermented and unfermented cocoa seeds implied that as the concentration increased, the activity increased (Figure 1).

IC_{50} values are employed to evaluate the potency of extracts or purified phytochemicals as inhibitors of a particular enzyme when compared with that of a reference compound (Burlingham and Widlanski, 2003). The IC_{50} values of the extracts showed that they are excellent electron donor and will require small amount of the extract to bring about 50% inhibition of DPPH radical. The polyphenol of the fermented and unfermented extracts exhibited excellent free radical scavenging activity, with the fermented better and also compared favourably with the ascorbic acid standard (Table 3 and Figure 1). The high contents of flavonoid and phenolic in the extracts could be responsible for their high antioxidant activities in being excellent hydrogen donors. Phenols and flavonoids have several oxygen atoms which are electron-rich centres that served effectively in neutralising

free radicals by donating electrons to them (Skerget *et al.,* 2005; Talukdar, 2013).

Lipid peroxidation is a very important process in free radical pathology quantified by the levels of malondialdehyde, a lipid peroxidation product, as an index (Cheeseman and Holley, 1993). Increased lipid peroxidation impairs membrane activity by virtue of decreasing membrane fluidity, altering the activity of membrane-bound enzymes and receptors. The products of lipid peroxidation are injurious to most of the cells in the body, and are associated with a variety of diseases such as

Table 1: Phytochemical constituents of aqueous extracts of fermented and unfermented *T. cacao* seeds

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

+ = present, - + absent

Table 2: Total flavonoid and total phenolic contents of extracts of fermented and unfermented *T. cacao* seeds

Samples	**Total Flavonoid (mg RTE/g extract)**	**Total Phenolic (mg TAE/g extract)**
Aqueous Unfermented	601.17 ± 11.77	382.61 ± 3.50
Aqueous Fermented	523.49 ± 9.95	358.11 ± 1.75
Acetone Unfermented	1266.67 ± 51.88	1055.02 ± 105.34
Acetone Fermented	1090.20 ± 51.88	986.74 ± 73.29

Each value represented mean ± SEM, n = 3 determinations

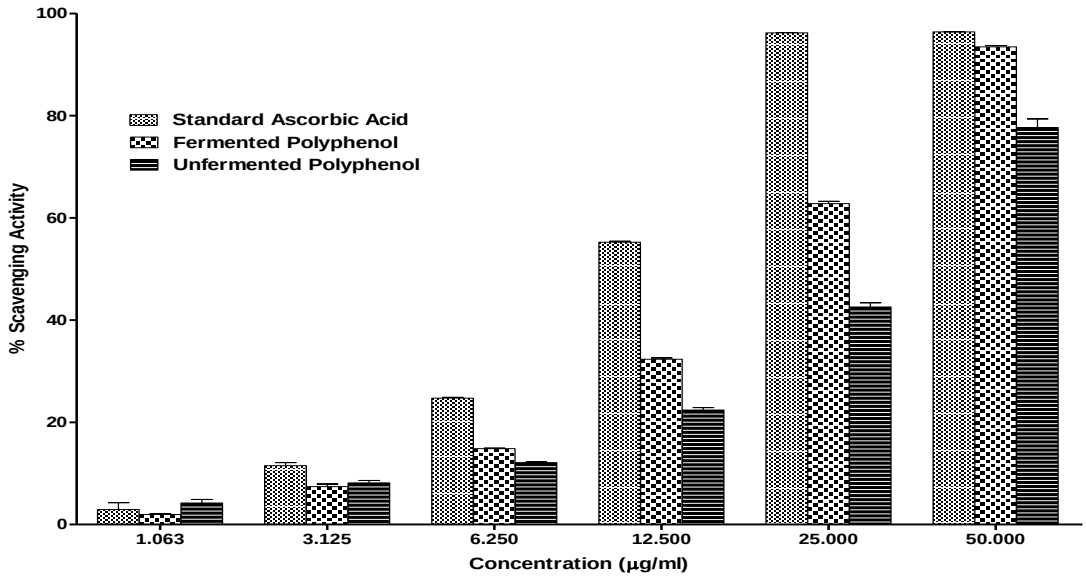

Figure 1: DPPH radical percentage scavenging activity
Each value represented mean ± SEM, n = 3 determinations

Table 3: IC_{50} of Fermented, Unfermented, Ascorbic Acid and 2,6-Di-tert- butyl-4-methylphenol

Fractions	IC_{50}	
	DPPH (µg/ ml)	Lipid Peroxidation (mg/ ml)
Fermented polyphenol	23.94± 0.005	0.469 ± 0.080
Unfermented polyphenol	31.07± 0.570	0.489 ± 0.039
Ascorbic Acid	17.43± 0.700	-
2,6-Di-tert- butyl-4-methylphenol (BHT)	-	0.680 ± 0.064

Each value represented mean ± SEM, n = 3 determinations

atherosclerosis and brain damage (Borek, 2001). The potency of the polyphenol to inhibit lipid peroxidation was measured as IC_{50} values i.e. the concentration of the extracts that brings about 50 % inhibition. The lower the IC_{50} value, the lower the concentration or amount of the extract required to bring about 50 % inhibition and the more potent an extract is. The fermented polyphenol extract of *T. cacao* seeds exhibited greatest potency over the unfermented and the standard BHT (Table 3). It implied that *T. cacao* polyphenols were even more potent than the standard drug BHT (Figure 2).

The ability of acetone extracts of fermented and unfermented *T. cacao* seed to stabilize red blood cell membrane was studied at varied concentrations (0 – 350 µg/ml) which involved stabilization of bovine red blood cell membrane against heat and hypotonicity-induced membrane lyses. The results revealed that fermented and unfermented acetone extracts of *T. cacao* seeds exerted maximum percentage membrane stability activities of 33.25 ± 3.41 % and 28.17 ± 0.08 % respectively (Figure 3). These activities compared moderately with that of ibuprofen (a non-steroidal anti-inflammatory drug) that exerted maximum percentage membrane stability activity of 43.73 ± 2.98 %.

The reducing ability of a compound generally depends on the presence of reductants (Duh *et al.,* 1999) which exhibit anti-oxidative potential by breaking the free-radical chain, donating a hydrogen atom (Gordon, 1990). The results of this study revealed that polyphenolic components of the acetone extracts of *T. cacao* seeds played an important role in scavenging of free radicals (Figure 4), and the scavenging activity was concentration dependent. The reducing capacity of compounds could serve as indicator of potential antioxidant properties (Meir *et al.,* 1995). The results revealed that the acetone extracts of *T. cacao* seeds (polyphenol) are electron donors and could react with free radicals, convert them to more stable products, and terminate radical chain reaction, with the fermented having greater reducing power than the unfermented.

The role of the liver in the pathogenesis of type 2 diabetes is attracting increasing attention. Fortunately, circulating concentrations of a number of variables appear to give insight into the extent of liver fat accumulation injury. Among these are γ-glutamyl transferase (GGT), alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Of the three, ALT is the most specific marker of liver pathology and appears to be the best marker of liver fat accumulation (Tiikkarrnen *et al*., 2003). The results of the study indicated a significant elevation in the levels of these enzymes in diabetic, untreated rats compared to normal rats. Interestingly, treatments with 150 mg/kg FP significantly lowered the elevated levels of these enzymes in the treated rats. Also, 300 mg/kg FP and 2 mg/kg glimepiride also brought about decrease in the level of these enzymes but not significantly in all cases. The increased plasma levels of AST and ALT in the plasma have been attributed to the damaged structural integrity of the liver, because these are cytoplasmic enzymes and are released into circulation after cellular damage (Recknagel *et al*., 1989). From the results (Table 4), it is obvious that STZ produced marked hepatic damage to the rats as evidenced by the elevation of serum ALT, AST and GGT. Then treatment with 150 mg/kg FP markedly reversed this liver damage as observed by the lowered serum levels of AST, ALT and GGT. The result therefore suggested that the extracts contained active principles that could possibly reduce or reverse hepatic damage. There was reduction in plasma total protein and albumin of diabetic control group when compared to normal control group. This is in agreement with hypoalbuminaemia observed in diabetes (Porte and Hatler, 1981). However, treatment with the polyphenols of fermented *T. cacao* seeds brought about mild amelioration of the adverse effects.

The body has an effective mechanism to prevent and neutralize the free radical induced damage. This is accomplished by a set of endogenous antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX). In this study, treatment of diabetic rats with 150 and 300 mg/kg FP resulted in increase in the activities of these enzymes especially in the 150 mg/kg FP group (Table 5). The increased activity of these antioxidant enzymes might be as a result of free

radicals generated by streptozotocin-induced diabetes and the scavenging activities of these enzymes (Szkudelski, 2001, Talukdar, 2013). It is possible that flavonoid stimulates antioxidant

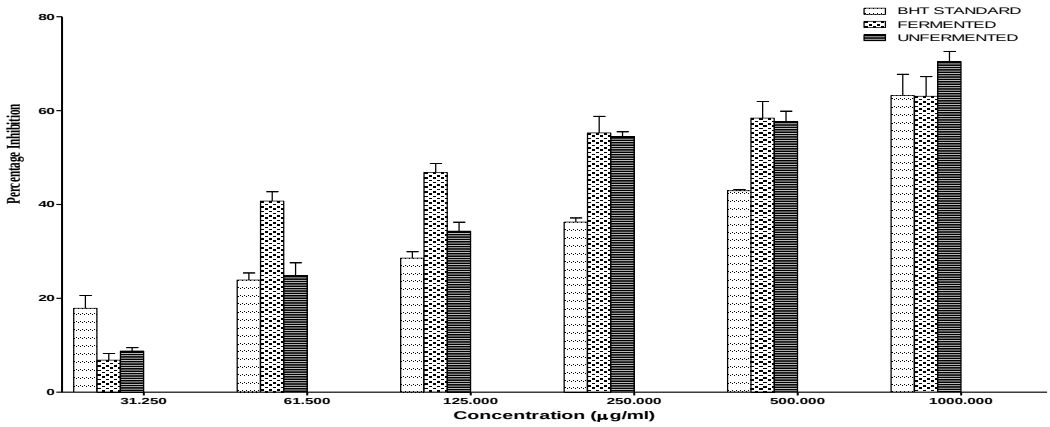

Fig 2: Percentage Inhibition of Lipid Peroxidation by Polyphenol of Fermented and Unfermented of *T. cacao* and BHT
Each value represented mean ± SEM, n = 3 determinations

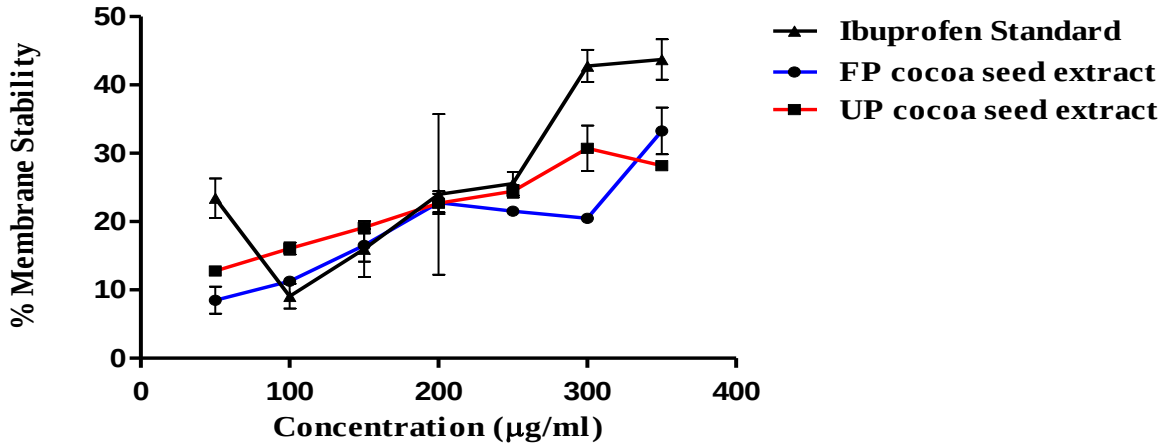

Fig 3: Membrane stabilizing profile of polyphenol from fermented, unfermented cocoa seed extract and Ibuprofen on erythrocytes exposed to heat and hypotonic-induced lyses

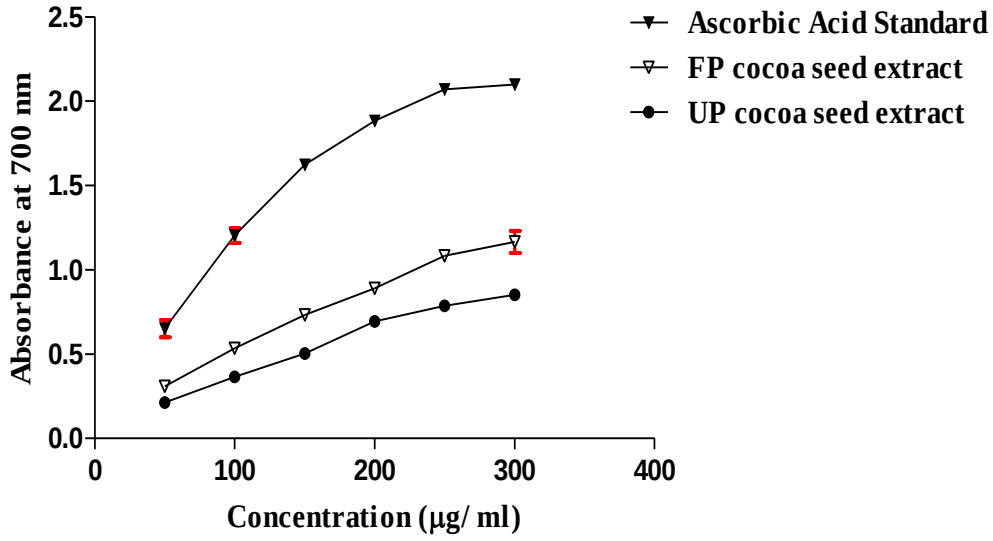

Fig 4: Reducing power of acetone extracts of fermented, unfermented cocoa seeds and ascorbic acid
Each value represented mean ± SEM, n = 3 determinations

Table 4: Effect of fermented polyphenol on some liver marker enzymes, total protein and albumin concentrations

Treatment	ALT (U/L)	AST (U/L)	GGT (U/L)	Total Protein (g/dL)	Albumin (g/dL)
General Control	29.68 ± 1.91	142.38 ± 8.28	3.13 ± 0.46	7.39 ± 0.16	4.20 ± 0.14
Normal Rats + 150 mg/kg FP	17.06 ± 1.44	70.40 ± 8.22*	4.21 ± 0.41	7.69 ± 0.51	4.28 ± 0.23
Normal Rats + 300 mg/kg FP	19.53 ± 3.97	47.66 ± 6.70*	3.82 ± 0.35	7.93 ± 0.23	4.38 ± 0.80
Diabetic Control	55.53 ± 5.92	221.63 ± 13.97	10.13 ± 0.75	6.17 ± 0.27	2.62 ± 0.16
Diabetic Rats + 150 mg/kg FP	28.14 ± 3.88 *	137.20 ± 8.06*	3.30 ± 1.80*	6.51 ± 0.17	3.07 ± 0.29
Diabetic Rats + 300 mg/kg FP	47.90 ± 4.18	188.33 ± 10.38*	8.05 ± 2.72	6.94 ± 0.33	3.46 ± 0.10
Diabetic Rats + 2 mg/kg Glimepiride	44.95 ± 1.16	169.75 ± 3.59*	7.40 ± 0.47	7.35 ± 0.22	3.63 ± 0.22

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.

Each value represented mean ± SEM, n = 3 determinations

Table 5: Effect of Fermented polyphenol on some antioxidant enzymes

Treatment	SOD (U/ mg)	CATALASE (U/ mg)	GSH (µg/ ml) GPx
General Control	1.85 ± 0.13	172.73 ± 0.07	0.28 ± 0.04
Normal Rats + 150 mg/kg FP	1.55 ± 0.05	190.78 ± 2.04*	0.30 ± 0.06
Normal Rats + 300 mg/kg FP	2.17 ± 0.17	183.14 ± 5.77	0.35 ± 0.04
Diabetic Control	1.65 ± 0.17	173.19 ± 0.30	0.24 ± 0.02
Diabetic Rats + 150 mg/kg FP	3.13 ± 0.13*	174.65 ± 1.65	0.43 ± 0.15
Diabetic Rats + 300 mg/kg FP	2.19 ± 0.12	172.53 ± 0.37	0.23 ± 0.03
Diabetic Rats + 2 mg/kg Glimepiride	1.72 ± 0.16	173.31 ± 0.49	0.16 ± 0.06

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 $P<0.05$.

Each value represented mean ± SEM, n = 5 determinations

activities by activating antioxidant enzymes to enhance their free radical scavenging capacity. It has been reported that rats treated with flavonoids recorded significantly enhanced activities of SOD, CAT and GPx (Pari and Amali, 2005; Vijayakumar *et al.*, 2008; Naik and Panda, 2008).

In conclusion, active ingredients with anti-hyperglycaemia potentials were discovered in the polyphenol extracts of fermented cocoa seeds which could be employed in the management of hyperglycaemic conditions/diseases.

Conflict of Interest: The authors wish to state categorically that there is no conflict of interest. The authors alone are responsible for the writing and content of the paper

References

Akinwunmi K. F. and Oyedapo O. O. (2013). Evaluation of antioxidant potentials of *Monodora myristica* (Gaertn) dunel seeds. African Journal of Food Science. 7(9): 317-324.

Anega, M. and Gianfagne, T. (1994). Cocoa. In: Encyclopedia of Agriculture Vol 1, 379-388.

Bell, G. I. (1991). Molecular defects in diabetes mellitus. Diabetes. 4: 413-422.

Blois, S. (1985). Anti-oxidant determination by use of stable free radicals. Nature. 29: 1199-1201.

Bode, S. O. and Oyedapo, O. O. (2011). Biological activities and phytoconstituents of the lower plant *Platycerium angolense* Welwex Hook. Journal of Medicinal Plants Research. 5(8): 1321-1329.

Boullier, A., Bird, D. A., Chang, M. K., Dennis, E. A., Friedman, P., Gillotre-Taylor, K., Horkko, S., Palinski, W., Quehenberger, O. and Shaw P. (2001). Scavenger receptors, oxidized LDL, and atherosclerosis. Annals of the New York Academy of Sciences. 947:214-222.

Burlingham, B. T. and Widlanski, T. S. (2003). An intuitive look at the relationship of K_i and IC_{50}: A more general use for the Dixon plot. Journal of Chemical Education. 80(2): 214.

Cakir, A., Mari, A., Yildinn, A., Duru, M. E., Harmender, M. and Kazaz, C. (2003). Isolation and characterization of anti-oxidant phenolic compounds from the aerial parts of *Hypericum hyssopifolium*, L. by activity guided fractionation. Journal of Ethnopharmacology. 87: 73-83.

Ceriello, A. (2003). New insights on oxidative stress and diabetic complications may lead to a "causal" antioxidant therapy. Diabetes Care. 26(5):1589-1596.

Ceriello, A. and Motz, E. (2004). Is oxidative stress the pathogenic mechanism underlying insulin resistance, diabetes, and cardiovascular disease? The common soil hypothesis revisited. Arteriosclerosis, Thrombosis, and Vascular Biology. 24(5):816-823.

Chawla, R. (1999). Biochemical tests for total

proteins, albumin and pyruvate. In: Practical Clinical Biochemistry Methods and Interpretations, 2nd Edition JAYPEE, pp. 107-134.

Cheeseman, K. H. and Holley, A. E. (1993). Measuring free radicals reaction *in vivo*. British Medical Bulletin 49: 491-505.

Cho, E. J., Yokozawa, T., Rhyu, D. Y., Kim, S. C., Shibahara, N., and Park, J. C. (2003). Study on the inhibitory effects to Korean medicinal plants and their main compounds on the 1, LDPPH Radical. Journal of Phytomedicine. 10: 544-551.

Clinical Medicine, WB Sunders, London. 2: 1069-1071.

Codario, R. A. (2005). Type 2 Diabetes, Pre-diabetes, and the Metabolic Syndrome: The primary care guide to diagnosis and management, Humana Press, Totowa, New Jersey. 75- 88.

Dillinger, T. L., Barriga, P., Escarcega, S., Jimenez, M., Lowe, D. S. and Grivetti, L. E. (2000). Food of the Gods: Cure for humanity? A cultural history of the medicinal and ritual use of chocolate. Journal of Nutrition. 130: 2057S-2072S.

Duh, P. D., Tu Y. Y., and Yen, G. C. (1999). Antioxidant activity of water extract of Harng Jyur (*Chrysanthemum morifolium* Ramat). Lebensmittel-Wissenschaft und-Technologie. 32(5): 269-277.

Flack, C. P. and Woollen, J. W. (1984). Clinical Chemistry. 30: 559.

Freudenberg, K., Cox, R. F. B. and Braun, E. (1932). The catechin of the cacao bean. Journal of the American Chemical Society. 54: 1913-1917.

Giugliano, D., Ceriello, A. and Paolisso, G. (1996). Oxidative stress and diabetic vascular complications. Diabetes Care 19(3):257-267.

Gordon, M. H. (1990). The mechanism of the antioxidant action *in vitro*. In: Food Antioxidants, B. J. E. Hudson, Ed., pp. 1-18.

Hansen, C. E., del Olmo, M. and Burri, C. (1998). Enzyme activities in cocoa beans during fermentation. Journal of Science Food & Agriculture 77: 273-281.

Kumar, P. and Clark, M. (2002). Diabetes mellitus and other disorders of metabolism. In: Clinical Medicine, WB Sunders, London. 2: 1069-1071.

Lee, A. Y. and Chung, S. S. (1999). Contributions of polyol pathway to oxidative stress in diabetic cataract. Federation of American Societies for Experimental Biology Journal. 13: 23-30.

Maritim, A. C., Sanders, R. A. and Watkins, J. B. 3rd. (2003). Diabetes, oxidative stress, and antioxidants: A review. Journal of Biochemical Molecular Toxicology. 17(1):24-38.

Marles, R. J. and Farnsworth, N. R. (1995). Antidiabetic plants and their active constituants. Phytomedicine 2: 137-189.

Meir, S., Kanner, J., Akiri, B. and Hadas, S. P. (1995). Determination and involvement of aqueous reducing compounds in oxidative defence systems of various senescing leaves. Journal of Agricultural Food Chemistry. 43: 1813-1817.

Middleton, E.J.R., Kandaswami, C. and Theoharides T.C. (2000). The effects of plant flavonoids on mammalian cells: implications for inflammation, heart diseases and cancer. Pharmacology Reviews. 52: 673-751.

Misra, H. P. and Fridovich, I. (1972). The role of superoxide anion in the auto-oxidation of epinephrine and a simple assay for superoxide dismutase. Biochemistry. 247(10): 3170-3175.

Moron, M. S., Depierre, J. W. and Manneryik, B. (1979). Levels of glutathione, glutathione reductase and glutathione-S-transferase activities in rat lung and liver. Biophysics Acta. 582:67-78.

Nabasree, D, and Bratati, D. (2004). Antioxidant activity of *Piper betle* L. leaf extract *in vitro.* Food Chemistry. 88(2): 219-224.

Naik S. R. and Panda V. S. (2008). Phytosome in rifampicin-induced liver injury in rats: Evidence of antioxidant activity. Fitoterapia.79: 439-445.

Natsume M., Osakabe N., Yamagishi M., Takizawa T., Nakamura T., Miyatake H., Hatano T. and Yoshida T. (2000). Analyses of polyphenols in cacao liquor, cocoa and chocolate by normal-phase and reversed-phase HPLC. Bioscience Biotechnology Biochemistry. 64(12): 2581-2587.

Noé, V., Penuelas, S., Lamuela-Raventos, R. M., Permanyer, J. and Ciudad, C. J. (2004). Epicatechin and a cocoa polyphenolic extract modulate gene expression in human Caco-2 cells. Journal of Nutrition. 134: 2509-2516.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry 95: 358.

Olagunju, J. A., Oyedapo, O.O., Onasanya, B. A., Oshoba, O. O., Adebayo, O. O., Eweje, O. and Sodehinde, A. B. (2000). Effects of isosaline extract of *Tetrapleura tetraptera* and *Olax subscorpioides* on certain biochemical parameters of albino rats. Pharmaceutical Biology. (USA) 38: 1-4.

Oyaizu, M. (1986). Studies on products of browning reaction prepared from glucosamine. Japanese Journal of Nutrition. 44: 307–315.

Oyedapo, O. O., Akinpelu, B. A. and Orefuwa S. O. (2004). Anti-inflammatory effect of *Theobroma cacao*, L. root extract. Journal of Tropical Medicinal Plants (Malaysia). 5(2): 161-166.

Oyedapo, O. O., Akinpelu, B. A., Akinwunmi, K. F.,

Adeyinka, M. O. and Sipeolu, F. O. (2010). Red blood cell membrane stabilizing potentials of extract of *Lantana camara* and its fractions. International Journal of Plant Physiology and Biochemistry. (2)4: 46-51.

Oyedapo, O. O., Sab, F. C., and Olagunju, J. A. (1999). Bioactivity of fresh leaves of *Lantana camara*. Biomedical letters 59: 175-183.

Pari, L. D. and Amali R. (2005). Protective role of tetrahydrocurcumin (THC) an active principle of turmeric on chloroquine induced hepatotoxicity in rats. Journal of Pharmaceutical Science. 8(1): 115-123.

Porte, D. J. and Halter, J. B. (1981). The endocrine pancreas and diabetes. In: Williams RH, editor. Text Book of Endocrinology. Philadelphia: W. B. Saunders Co., p. 715.

Raj, K. J. and Shalini, K. (1999). Flavonoids-a review of biological activities. Indian Drugs. 36: 668-676.

Recknagel, R. O., Glende, E.A. Jr., Dolak, J.A., Waller, R. L. (1989). Mechanisms of carbon tetrachloride toxicity. Pharmacology and Therapeutics. 43: 139-154.

Reitman, S. and Frankel, S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases," American Journal of Clinical Pathology 28: 56–63.

Rotruck, J. T., Pope, A. L., Ganther, H. E., Swanson, A. B., Hafeman, D.G. and Hoekstra, W.G. (1973). Selenium biochemical role as a component of glutathione peroxidase. Journal of Science. 179(73): 588-590.

Rozan, P., Hidalgo, S., Nejdi, A., Bisson, J. F., Lalonde, R. and Messaoudi, M. (2006). Preventive antioxidant effects of cocoa polyphenolic extract on free radical production and cognitive performances after heat exposure in Wistar rats. Journal of Food Science. 72: S203 - S206.

Singleton, V. L., Orthofera, R. and Lamuela, R. R. M. (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteaus reagent. Methods in Enzymology 299: 152-179.

Sinha. A.K. (1972). Colorimetric assay of catalase. Analytical Biochemistry. 47:398-394.

Skerget, M., Kotnik, P., Hadolin, M., Hras, A., Simonic, M. and Knez, Z. (2005). Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chemistry. 89: 191-198.

Sofowora, A. (2006). Phytochemical screening: In Medicinal plants and Traditional Medicine in Africa. Second Edition, Spectrum Books Limited Ibadan, Nigeria. pp. 150-153.

Soobrattea, M. A., Neergheen, V. S., Luximon-Ramma, A., Aruoma, O. I. and Bahorunn, T. (2005). Phenolics mecahnisms and actions. Mutation Research. 579: 200-213.

Sun, P. X., Yie, L. K., Zhang, Z. L., Hu, M. and Lu, L. (1999). Colometric determination of the total content of the flavonoids in epimedium capsules. Journal of Shenyang Pharmaceutical University. 16: 68-70.

Szasz, G. (1969). Reaction rate method for gama-glutamyl transeptidase activities in serum. Journal of Clinical Chemistry. 22: 205-210.

Szkudelski, T. (2001). The mechanism of alloxan and streptozotocin action in B cells of the rat pancreas. Physiological Research. 50(6): 537–546.

Talukdar, D. (2013). Antioxidant potential and type II diabetes rekated enzyme inhibition properties of raw and processed legumes in Indian Himalayas. Journal of Applied Pharmaceutical Science. 3(3): 13-19.

Taniyama, Y. and Griendling, K. K. (2003). Reactive oxygen species in the vasculature: Molecular and cellular mechanisms. Hypertension 42(6):1075-1081.

Tiikkarnen, M., Bergholm, R., Vehkavaara, S., Rissanen, A., Hakkinen, A. M., Jamminen, M., Teramo, K., Yki-Jarvinen, H. (2003). Effects of identical weight loss on body composition and features of insulin resistance in obese women with high and low liver fat content. Diabetes 52: 701-707.

Trease, G. and Evans, S.M. (2002). Pharmacognosy. 15th Edition. Bailer Tindal, London. pp. 23-67.

Turko, I. V., Marcondes, S. and Murad, F. (2001). Diabetes-associated nitration of tyrosine and inactivation of succinyl-CoA: 3-oxoacid CoA-transferase. American Journal of Physiology - Heart and Circulatory Physiology. 281(6): H2289-2294.

Vijayakumar, S., Presannakumar D. and Vijayalakshmi N. R. (2008). Antioxidant activity of banana flavonoids. Fitoterapia. 79: 279-282.

Williams, R. J., Spencer, J. P. E. and Rice-Evans, C. (2004). Flavonoids: antioxidants or signaling molecules? Free Radical Biology and Medicine. 36:838-849.