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Research Article

Chrysophyllum albidum Reversed Mitochondrial Dysfunction and Dyslipidemia in Diabetic Wistar Rats

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Abstract

This study investigated *in vitro* and *in vivo* effect of methanol extract of *Chrysopyllum albidum* (*C. albidum*) stem bark on mitochondrial membrane permeability transition pore (MMPTP) opening, lipid peroxidation (LPO) and blood lipids in diabetic male Wistar rats. Normal male rats (5) were studied *in vitro.* Forty rats (160-180g), divided into four groups of 10 animals: Normal control, Diabetic untreated, Diabetic treated and Normal treated were used for *in vivo* study. Diabetes was induced with a single intraperitoneal injection of alloxan (120mg/kg) in overnight fasted rats. Animals were treated with methanol extract of *C. albidum* (500mg/kg) orally for 4weeks. The liver was excised for mitochondrial isolation from five animals per group under light ether anaesthesia after two and four weeks of treatment. The MMPTP opening, LPO, total cholesterol (TC), triglyceride (TG), High density lipoprotein-cholesterol (HDL-c), Low density lipoprotein-cholesterol (LDL-c), Very low Density Lipoprotein-cholesterol (VLDL-c) and blood glucose were assessed in all groups. Data obtained were analyzed using one way ANOVA and significance considered at $P<0.05$. The *in vitro* study showed that the extract did not induce MMPTP opening while it inhibited LPO at all concentrations. The *in vivo* study showed that the extract significantly inhibited MMPTP opening, reduced TC, TG, LDL-c and VLDL-c (80.20±22.81, 47.75± 13.73, 50.67±7.59 and 9.55±2.75 mg/dl respectively) while it increased HDL-c (9.70±1.66mg/dl) compared to normal and diabetic untreated. Methanol extract of *Chrysophyllum albidum* inhibited lipid peroxidation, reversed MMPTP opening, ameliorated hyperglycaemia and dyslipidemia induced by diabetes mellitus.

Keyword: *Chrysophyllum albidum,* diabetes, mitochondrial membrane permeability transition pore, lipid Profile

INTRODUCTION

Diabetes mellitus is a clinical syndrome characterised by hyperglycaemia due to absolute or relative deficiency of insulin and/or insulin resistance (WHO and IDF 2013). Evidences emerging have linked mitochondrial pore opening with diabetes (Lowel and Shulman, 2005). Diabetes-induced mitochondrial dysfunction subsequently causes increase in free radical production, impaired antioxidant capabilities and dys-regulation of mitochondrial membrane permeability transition pore (MMPTP) opening which are related to the onset, progression and pathological consequences of diabetes (Rolo and Palmeira, 2006; Adrienne *et al*., 2010). Therapeutic interventions had provided different classes of drugs with well demonstrated mechanisms of actions for the management of diabetes (Kirkham, *et al*., 2009; *Farsi et al.,* 2014). However, challenges in terms of cost, availability of medications and adverse effects have limited their usage thus promoting exploration of safe alternatives (WHO, 2007; Agabegi and Agebegi, 2008). A number of medicinal plants with claimed hypoglycaemic potentials are currently been explored and screened for their therapeutic potentials for the management of diabetes mellitus. One of such medicinal plants is *Chrysophyllum albidum* (African Star apple) which has much ethno-medicinal significance.

Various parts of *Chrysophyllum albidum* plant (root, stem bark and seed cotyledon) have been reported to possess beneficial effects in management and treatment of diabetes mellitus (Olorunnisola *et al*., 2008; Onyeka *et al*., 2013), clearance of malaria parasites (Adewoye *et al*., 2010; 2011) and prevention of red blood cell haemolysis (Adewoye *et al*., 2012). Despite the reported beneficial effect of *C. albidum*, possible mechanism of action is poorly elucidated. This study therefore investigated the effect of methanol extract of *C. albidum* stem bark on MMPTP opening, lipid membrane peroxidation, dyslipidemia and hyperglycemia in alloxan-induced diabetic male Wistar rats.

MATERIAL AND METHODS

Animals and experimental design: Forty-five adult male Wistar rats (160-180) were used for *in vitro and in vivo* experiments. The animals were acclimatized under standard laboratory conditions for two weeks and maintained on standard rat chow (Kesmac Feeds and Agric Consult, Nigeria) with free access to water *ad libitum.* Five animals were studied *in vitro* while forty animals were randomly grouped into four of ten animals each: Normal control fed with rat chows and allowed free access to water), diabetic untreated, diabetic treated with 500mg/kg *p*.o. methanol extract of *Chrysophylum*

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albidum) and normal treated with 500mg/kg *p.o.* methanol extract of *C. albidum*. All experiments were carried out according to the ethical procedures of the University of Ibadan for the use of experimental animals.

Reagents: The reagents used were of analytical grade and were obtained from Sigma Chemical Co, USA and BDH Chemicals Ltd, England.

Collection and identification of plant materials: Fresh stem bark of *C. albidum* was collected, identified and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria with authentication number FHI 107514.

Extract preparation: Fresh stem bark of *C. albidum* collected was air-dried at room temperature and grounded using a dry electric mill before subjected to extraction. Methanol extraction of *C. albidum* was done using the defatting method employed by Adewoye *et al.,* 2012. The methanol extract was evaporated to dryness *in-vacuo* and dried extract was stored at 4^0C until use.

Induction of diabetes: Diabetes was induced by a single intraperitoneal injection of Alloxan (120 mg/kg) (Szkudelski, 2001) to overnight fasted rats. Diabetes was confirmed 72 hours post alloxan injection using Accu-Chek glucometer (Roche diagnostics, Germany). Animals with constant blood glucose level above 250mg/dl were recruited into the diabetic groups.

Isolation of rat liver mitochondria: Liver mitochondrion was isolated using the method of Johnson and Lardy (1967). Animals were sacrificed after two and four weeks of treatment with light ether anaesthesia and then by cervical dislocation. Liver was excised from each animal, weighed and homogenized on ice in 9X buffer (containing 210mM Mannitol, 70mM Sucrose, 5mM HEPES-KOH, 1mM EGTA in 100ml of distilled water, pH 7.4) using Potter Elvehjem glass homogenizer. Homogenates obtained were centrifuged at 2300rpm for 15mins to remove nuclear debris. Supernatant obtained after washing homogenates twice with homogenizing buffer was centrifuged at 13000rpm for 10minutes to obtain the mitochondrial pellets. The pellets were washed twice in washing buffer (containing 210mM Mannitol, 70mM sucrose, 5mM HEPES-KOH, 0.5% BSA in 100ml of distilled water, pH 7.4) at 12000rpm for 10minutes. The mitochondria obtained were immediately re-suspended in appropriate volume of MSH buffer (210mM Mannitol, 70mM sucrose and 5mM HEPE-KOH in 100ml of distilled water, pH 7.4) and dispensed into Eppendorf tubes kept on ice. The samples were frozen until use.

Determination of mitochondrial protein: Mitochondrial protein was determined according to the method of Lowry *et al*. (1957) using bovine serum albumin as standard.

***In vitro* MMPTP assay:** Effect of *C. albidum* on MMPTP opening was assessed using the method of Lapidus and Sokolove (1993). Mitochondrial (0.4 mg protein per ml) was pre-incubated in the presence of 0.8µM rotenone in a swelling buffer (containing 210mM Mannitol, 70mM sucrose and 5mM HEPES-KOH in 100ml of distilled water, pH 7.4) and varying concentrations of methanol extract of *C. albidum* stem bark (40, 80, 120, 200, 280, and 360 µg/ml) at 27ºC for 3minutes. Absorbance at 540nm was taken every 30seconds for 120minutes after 30seconds of adding sodium succinate. Spermine (4mM) and $CaCl_2$ (3µM) were used as inhibitor and source of triggering agent ($Ca2^+$) respectively.

In vivo* MMPTP assay*: Mitochondrial membrane permeability transition pore opening was assessed *in vivo* according to the method of Lapidus and Sokolove (1993). Mitochondrial fraction (0.4mg protein/ml) were pre-incubated in the presence of 0.8µM rotenone in a medium containing swelling buffer (containing 210mM Mannitol, 70mM sucrose and 5mM HEPES-KOH in 100ml of distilled water, pH 7.4) for 3minutes at 27ºC prior to the addition of 120µM $Cacl_2$. After 30s, 5mM succinate was added and MMPTP was quantified at 540nm every 30s for 12minutes.

Assessment of lipid peroxidation level

A modified thiobarbituric acid reactive species (TBARS) assay of Ruberto *et al*. (2000) was used. Mitochondrial (2mg/ml protein) and varying concentrations of *C.* albidum (40, 80, 120, 200, 280, and 360 µg/ml) were added to each test tube and made up to 1ml with distilled water. The mixture was then incubated with 0.5ml of $FeSO_4$ (0.07M) for 30minutes at room temperature. Thereafter, 1.5ml of 20% acetic acid (pH 3.5) and 1.5ml of 0.8% (w/v) thiobarbituric acid in sodium dodecyl sulphate were added, vortexed and heated at 95ºC for 60mins. After cooling, 5.0ml of butan-1-ol were added to each tube and centrifuged at 3000rpm for 10min. Absorbance of the organic upper layer was measured at 532nm. Percentage inhibition of lipid peroxidation by the extract was calculated as

$$[1-(AC-AE) / AC] \times 100$$

Where AC is the absorbance value of the fully oxidized control and AE is the absorbance in the presence of extract.

Lipid profile assay: Lipid profile (total cholesterol – TC, triglyceride – TG, and High density lipoprotein-cholesterol – HDL-c) was assessed using Randox diagnostic assay kits (Randox Laboratories, UK). Low density lipoprotein-cholesterol (LDL-c) was estimated according to Freidewald *et al*. (1972) formula.

$$LDL= TC – (HDL + TG/5)$$

Statistical Analysis: Data were expressed as mean ±SEM and analyzed using one-way analysis of variance and Newman Keul's post-hoc test (Graph-pad 5.04, USA). Statistical significance was considered at $P < 0.05$

RESULTS

Treatment with the extract significantly ($P<0.05$) reduced blood glucose level in diabetic treated group by 43.86% and 70.06% after two and four weeks of treatment respectively (Table 1). However, the extract did not stimulate body weight gain in the diabetic treated group compared to diabetic untreated (Table 2).

Calcium-induced pore opening was reversed by spermine which shows the intactness of the mitochondrial used (Figure 1).

Table 1:
Effect of *C. albidum* stem bark on blood glucose level (mg/dl)

Groups	Day 1: 72 hours after alloxan injection	Day 7	Day 14	Day 21	Day 28
Control	98.18 ± 3.32	78.29±2.04	94.20 ± 3.88	83.40 ± 3.36	82.20±1.12
Diabetic untreated	523.00 ±19.03	463.60 ± 23.14*	454.20 ± 17.20*	445.20 ± 29.00*	435.50 ± 15.14
Diabetic treated	483.50 ± 24.34	291.38± 20.12*#	255.00 ± 15.04*#	157.80 ± 10.28*#	130.40 ± 3.02*#
Normal treated	105 ± 2.00	98.00±3.23	92.50±2.14	96.50 ± 4.74	95.50±2.12

Values expressed as mean ± SEM, n=5, P<0.05
* *statistically significant compared with the normal group*
# *statistically significant compared with the diabetic untreated group.*

Table 2:
Effect of *C. albidum* stem bark on body weight (g)

Groups	Day 1: 72 hours after alloxan injection	Day 7	Day 14	Day 21	Day 28
Control	173.71 ± 3.32	177.86 ± 2.04	186.67 ± 3.88	194.80 ± 3.36	197.44 ± 1.12
Diabetic untreated	179.00 ± 4.03	172.60 ± 1.14*	168.33 ± 1.12*	169.83 ± 29.00*	159.50± 1.14*
Diabetic treated	179.50 ± 4.34	181.38 ± 2.12	180.50 ± 3.04	178.80 ± 1.28	179.40 ± 1.02
Normal treated	175 ± 2.20	188.00±3.23	192.50±2.14	190.50 ± 4.74	198.50±2.12

Values expressed as mean ± SEM, n=5, P<0.05
* *statistically significant compared with the normal group*

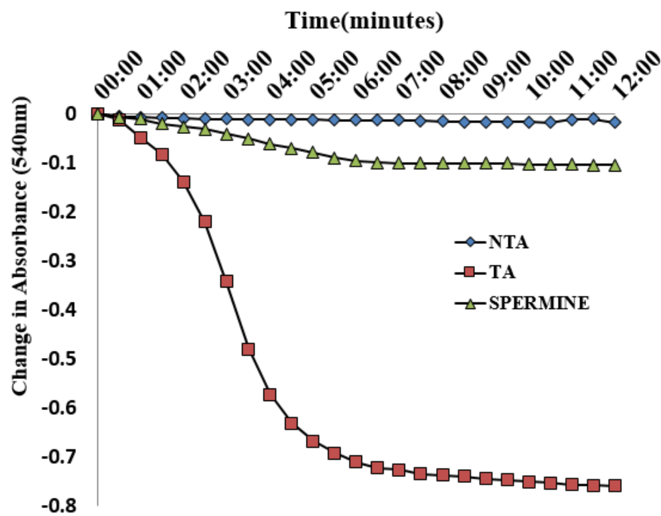

Figure 1:
Calcium-induced mitochondrial membrane permeability transition pore opening in normal rat liver and its reversal by spermine *in vitro* Values expressed as mean± SEM, n=5; *P<0.05.* NTA- no triggering agent (Control), TA- triggering agent (Ca^{2+}).

Methanol extract of *C. albidum* did not induce MMPTP opening *in vitro* at all concentrations compared to Ca^{2+}-induced pore opening (Figure 2) while Lipid peroxidation was inhibited *in vitro* at all concentrations of the extract treatment (Figure 3).

In vivo study showed that the extract did not induce MMPTP opening in normal treated rats(Figure 4) but significantly (*P<0.05*) reversed MMPTP opening in diabetic treated rats compared to diabetic untreated after two and four weeks of treatment (Figures 5 and 6).

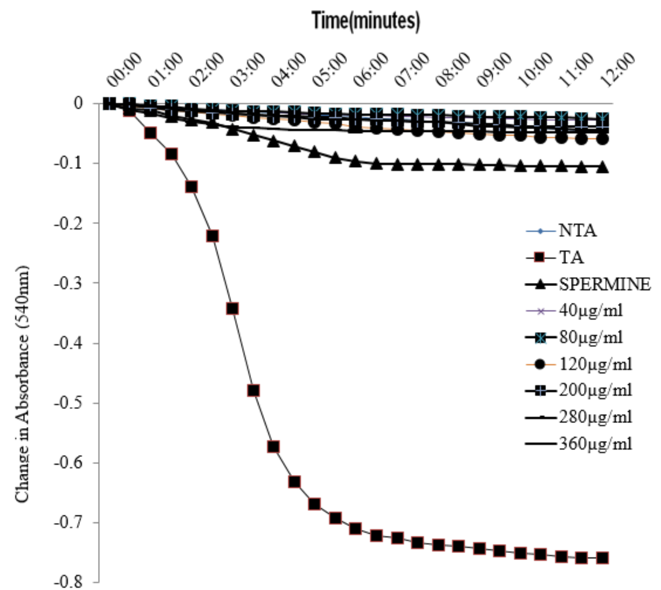

Figure 2:
Effect of methanol extract of *C. albidum* stem bark on rat liver mitochondrial membrane permeability transition pore opening *in vitro* . Values expressed as mean± SEM, n=5; *P<0.05*
NTA- No triggering agent (Control), TA- triggering agent (Ca^{2+}).

Treatment with the extract caused a significant (*P<0.05*) reduction in total cholesterol (80.20 ± 22.81 vs 188.67±11.17 mg/dl), triglycerides (47.75 ± 13.73 vs 166.75 ± 5.78) and LDL-c (49.99 ± 11.7 vs 141.16 ± 12.38 mg/dL) while it significantly increased HDL-c (50.67 ± 7.59 vs 9.70 ± 1.66 mg/dL) compared to diabetic untreated group (Table 3).

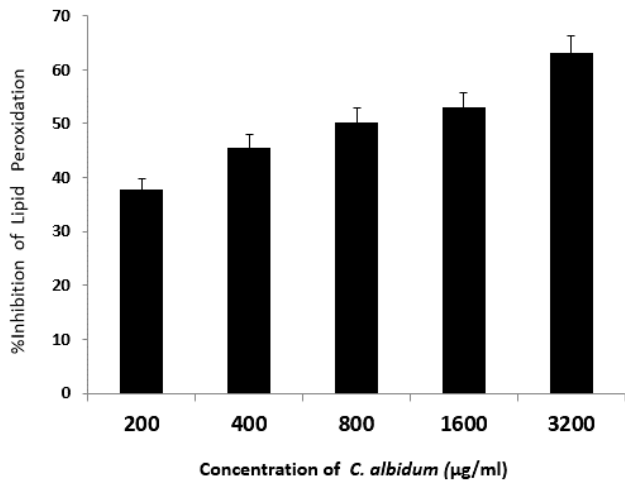

Figure 3:
Percentage inhibition of ferrous-induced lipid peroxidation by methanol extract of *C. albidum* stem bark
Values expressed as mean± SEM; *P<0.05*

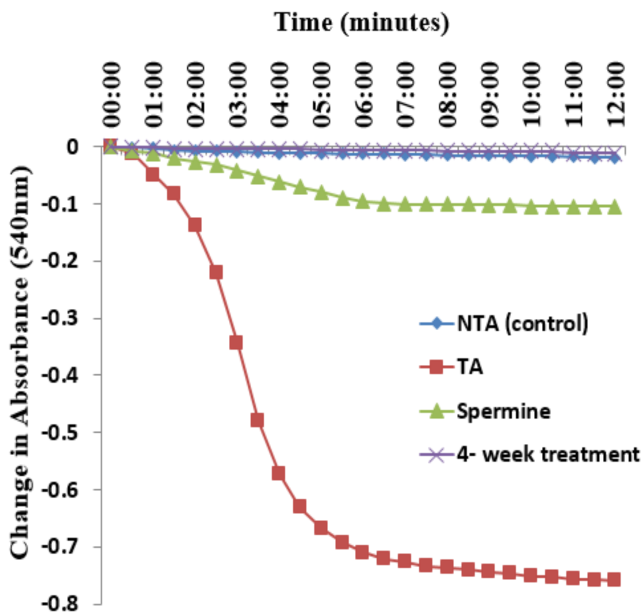

Figure 4: Effect of 4-week oral administration of *C. albidum* extract (500mg/kg bw) on MMPTP in normal rat liver. Values expressed as mean± SEM; *P<0.05*. NTA- Control, TA- Calcium

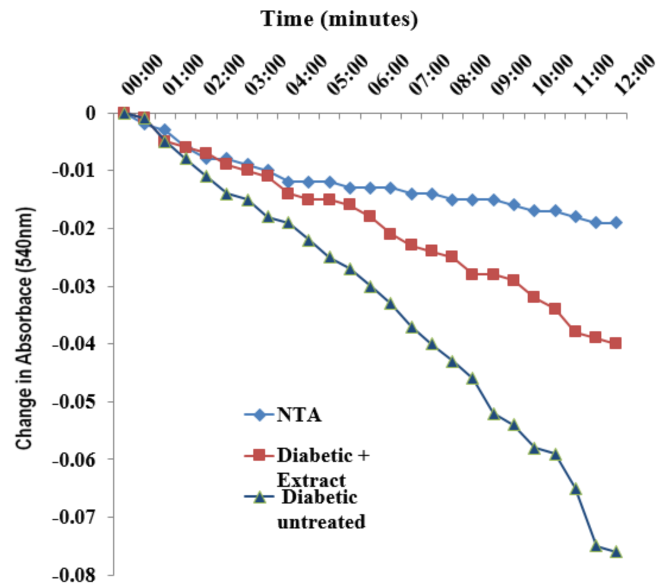

Figure 5:
Effect of 2-week oral administration of *C. albidum* (500mg/kg bw) on MMPTP in diabetic rat liver.
Values expressed as mean± SEM; *P<0.05*
NTA- Control, TA- Calcium

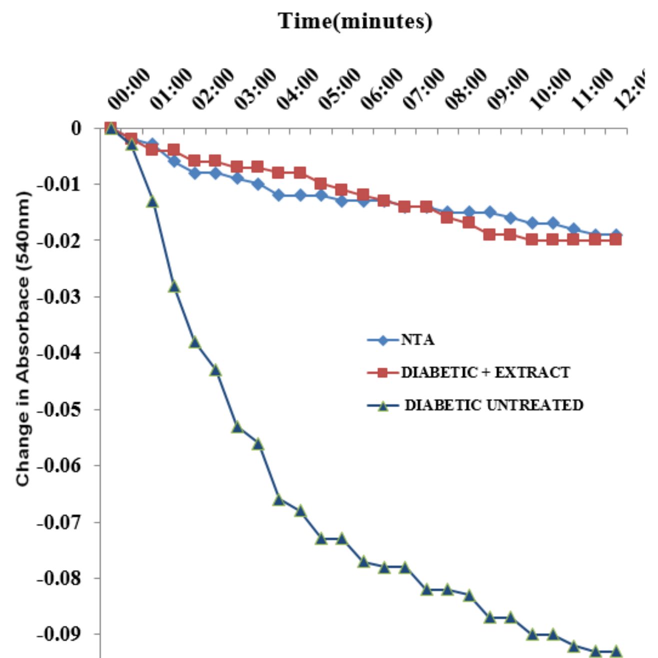

Figure 6:
Effect of 4-week oral administration of *C. albidum* (500mg/kg bw) on MMPTP in diabetic rat liver. Values expressed as mean± SEM; *P<0.05*; NTA- Control, TA- Calcium

DISCUSSION

Maintenance of normal blood glucose levels depends on a complex interconnection between the response of skeletal muscle and liver with glucose-stimulated secretion of insulin (Lowel and Shulman, 2005). Disorders with the muscle and liver response and/or insulin secretion result to hyperglycaemic condition (WHO and IDF, 2013). Findings of this study showed that methanol extract of *C. albidum* stem bark reversed hyperglycaemia but did not stimulate any weight gain in the diabetic treated (Tables 1 and 2). This observation is similar to the work of Olorunnisola *et al*. (2008) and *Onyeka et al*. (2013) who reported reduction in blood glucose level and no weight gain in *C. albidum* seed extract treated and root extract treated diabetic rats respectively. This probably indicates that the extract might not exert its hypoglycaemic effect through weight reduction.

According to Taylor *et al*. (2003) and Koshkin *et al*. (2004), mitochondria play a pivotal role in cellular secretion and response to insulin. It influences glucose-stimulated insulin secretion and nuclear encoded uncoupling proteins (UCPs) in beta cell toxicity (Rolo and Palmeira, 2006). Mitochondrial dysfunction exacerbates increase in free radical generation and Ca^{2+} imbalance implicated in many diseases including diabetes (Adrienne *et al*., 2010). Diabetes induces MMPTP dys-regulation which in turn promotes lipid membrane peroxidation, cell death and diabetes complications (Rimessi *et al*., 2008; Adrienne *et al*., 2010).

Table 3: Effect of methanol extract of *C. albidum* stem bark on lipid profile in normal and diabetic treated rats

Serum lipids (mg/dl)	Control	Diabetic Untreated	Diabetic treated
Total Serum Cholesterol	121.40 ± 8.43	188.67 ± 11.17*	80.20 ± 22.81#
Total Serum Triglycerides	86.69 ± 10.19	166.75 ± 5.78*	47.75± 13.73*#
High Density Lipoprotein	15.92 ± 1.61	9.70 ± 1.66	50.67 ± 7.59*#
Low Density Lipoprotein	88.08 ± 11.00	141.16 ± 12.38*	49.99 ± 11.76*#
Very Low Density Lipoprotein	17.34 ± 2.04	33.55 ± 1.25*	9.55 ± 2.75*#

Values expressed as mean± SEM, n=5. P<0.05
* *statistically significant compared with the normal group*
#*statistically significant compared with the diabetic untreated group*

In this study, *C. albidum* did not stimulate MMPTP opening *in vitro* and *in vivo* but reversed diabetes-induced MMPTP opening *in vivo* in diabetic group studied. This membrane stabilizing effect of *C. albidum* is similar to the report of Adewoye *et al*. (2012) on red blood cells in anaemic condition.

The inhibitory effect of *C. albidum* on MMPTP opening could as well be due to its physiochemical constituents like flavonoids, phenols and saponins with reported antioxidant activity (Prakash, 2001; Lou *et al*., 2007). Prakash (2001) and Adebayo *et al*. (2011) had earlier reported that compounds like polyphenols and flavonoids present in the plant are capable of scavenging free radicals. This was observed *in vitro* with *C. albidum* in a dose-dependent inhibition of lipid membrane peroxidation (Figure 3). Inhibition of lipid peroxidation that has been associated with diabetes (Halestrap, 2009) probably indicates that the extract could be useful in the prevention of pathogenesis of diabetes

According to Waugh and Grant (2010), lipid profile assessment can be used to predict risk of cardiovascular disorder in diabetes as lipid level is important to cardiovascular health. In this study, the observed dyslipidemia was reversed by the extract in diabetic animals (Table 3). Result obtained is similar to the report of Olorunnisola *et al.* (2008) on the effect of *C. albidum* seed extract on dyslipidemia.

Conclusion

The extract reversed MMPTP opening in diabetic rats, thus preventing cell death, inhibited lipid peroxidation, promoted good lipoproteins(HDL) and reduced bad lipoproteins(TC,TG, LDL and VLDL) thereby reversing the negative effects of diabetes mellitus induced in the male Wistar rats.

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