Ameliorative effects of *Cnidoscolus aconitifolius* on alloxan toxicity in Wistar rats

* Azeez OI[1], Oyagbemi AA[1], Oyeyemi MO[2], Odetola AA[3]

1. Department of Veterinary Physiology, Biochemistry and Pharmacology, University of Ibadan, Ibadan, Nigeria.
2. Department of Veterinary Surgery and Reproduction, University of Ibadan, Ibadan, Nigeria.
3. Department of Biochemistry, College of Medicine, University of Ibadan, Ibadan, Nigeria.

Abstract

Background: Diabetes has been associated with several complications occasioned by oxidative stress. Thus, in treatment of the condition, these complications must also be taken into consideration. This study evaluates the effect of *Cnidoscolus aconitifolius* complications of diabetes induced by alloxan, on haematology and sperm morphometry using the Wistar rats.
Methods: Diabetes was induced in 25 rats using alloxan. The diabetic rats were then divided into five groups B-F consisting of five rats per group. Groups C-E were administered with 100mg/kg, 500mg/kg and 1000mg/kg of ethanolic leaves extract of *Cnidoscolus aconitifolius,* respectively, for four weeks post treatment with alloxan, while group F received Chlorpropamide (Diabenes®, Pfizer). The diabetic rats in group B were not treated while group A served as the non diabetic control.
Result: Following treatment with alloxan, there was anaemia, thrombocytopenia and leucopenia, while the sperm count, motility and live/dead ratio were significantly reduced. Sperm morphological abnormalities and erythrocyte osmotic fragility also increased significantly. Following treatment of alloxan treated-rats with the extract, there were significant increases in the PCV, RBC, Hb, WBC, MCV and the platelet values. Erythrocyte osmotic fragility, sperm count, motility and live/dead ratio also improved significantly.
Conclusion: *Cnidoscolus aconitifolius* extract was found to ameliorate the effects of alloxan induced diabetes on the haematology but not on the abnormal sperm morphometry in rats.
Keywords: Haematological parameters, osmotic fragility, sperm morphometry, *Cnidoscolus aconitifolius*, chlorpropamide
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Introduction

Diabetes mellitus is considered to be one of a rank of free radical diseases. The existence of hyperglycemia produces increased oxidative stress (OS) via non-enzymatic glycation, glucose autoxidation, and alterations in polyol pathway activity with subsequent influences on the whole organism.[1] Macromolecules such as found in the extracellular matrix, lipoproteins, and deoxyribonucleic acid usually constitute the targets for free radicals in diabetes mellitus. This oxidative stress is involved in the pathophysiology of diabetes complications. The chronic hyperglycemic status also favors glycation reactions (irreversible glucose binding on protein amino groups), thereby leading to increased generation of advanced glycation end-products (AGEs).[2] Involvement of oxidative stress and advanced glycation end-products in diabetes complications is the basis of the development of adjunct therapies with antioxidant and advanced end products (AGEs) formation inhibitors. For example, Vitamin C and E have been shown to reduce considerably, the elaboration of markers of oxidative changes and subsequently reduced DNA damage in diabetic children,[1] while ramipril and aminoguanidine (ACE inhibitors) were found to reduce the rate of elaboration of nitrotyrosine, a marker of protein oxidation in Streptozocin-induced diabetic animals, via inhibition of the receptor for AGEs, gene expression of the membrane-bound NADPH oxidase subunit gp91phox, and nuclear transcription factor-κB which were all increased by diabetes mellitus.[3]

***Corresponding author:**
Dr. Odunayo I. Azeez
Department of Veterinary Physiology
Biochemistry and Pharmacology
University of Ibadan
Ibadan, Nigeria
Tel: +234 803 728 44 22
Fax: 02-8103043
E mail: odunayo.azeez@mail.ui.edu.ng; odunayoazeez@yahoo.com.

The activities of xanthine oxidase, a superoxide-generating enzyme have also been shown to increase in the liver and plasma of diabetic animals. The increase in plasma xanthine oxidase activity may be explained by the increase in the hepatic release of this enzyme which can be completely inhibited by allopurinol, an inhibitor of xanthine oxidase. Thus, treatment with allopurinol decreases oxidative stress in type 1 diabetic patients: hemoglobin glycation, glutathione oxidation, and the increase in lipid peroxidation are prevented.[4] This may also prevent late onset vascular complications in diabetic mellitus, since xanthine oxidase produced from vessel wall under the influence of heparin is inhibited by allopurinaol.

In as much as oxidative stress has been implicated in the major complications of diabetes mellitus, including retinopathy, nephropathy, neuropathy and accelerated coronary artery disease[5], anaemia[6,7], increased erythrocyte osmotic fragility[8,] oligospermia and abnormal sperm morphometry.[9] It then follows that the use of therapeutic or prophylactic agents that reduced oxidative damage in diabetes mellitus will inadvertently reduce these complications.

Many plants have been shown to possess antidiabetic and hypoglycemic properties by lowering the blood glucose levels and reducing the various complications associated with diabetes. For example, *Artemisia sphaerocephala* (Krasch seed) has been used successfully in the treatment of alloxan induced diabetes in rats[10]. Similarly, *Azadirachta indica* leaf extract has also been shown to lower the levels of cholesterol and lipids in Streptozotocin induced diabetic rats[11] while *Eruca sativa* seeds oil has currently been used in the treatment of diabetes, because of its antioxidant properties[12]. The ameliorative effects of *Murraya koenigii* (curry leaf) on hyperlipidemia associated with diabetes mellitus has also been reported.[13]

Alloxan, a toxic glucose analogue, has been used in the induction of diabetes mellitus in experimental animals. It is so called because it inhibits glucose induced insulin secretion in the pancreatic B cells;[14] and selectively destroys the B cells as it accumulates in these cells via Glut 2 glucose transporters.[15] Within the cell thereafter, it generates hydroxyl radicals from its reaction with the intracellular thiols (glutathione) to form dialuric acid. Subsequent auto oxidation of dialuric acid generates superoxide radicals, hydrogen peroxide as well as hydroxyl radicals. The hydroxyl radical so formed is then responsible for destruction of the B cells and the ensuing insulin dependent diabetes.[14,15]

Cnidoscolus aconitifolius is a perennial shrub of the Family *Euphorbiaceae* commonly found in the tropics. It is commonly eaten as vegetable in soup condiment in South Western Nigeria where it is called Iyana Ipaja. It has been demonstrated to contain phenols, saponins, cardiac glycosides and Phlobatannin[16]. High fiber content and antibacterial activities of this plant have been reported[19]. Apart from the antibacterial activities,[16] the ameliorative effect of *Cnidoscolus aconitifolius* on anaemia and increased erythrocyte osmotic fragility induced by protein energy malnutrition (PEM) has been reported[17] while its antidiabetic property has also been elucidated.

The present study was aimed at determining the ameliorative effects of ethanolic extract of *Cnidoscolus aconitifolius* on the haematological parameters, erythrocyte osmotic fragility and sperm morphometry in alloxan induced diabetes mellitus in male wistar rats. Since alloxan toxicity has been associated with anaemia, increased erythrocyte fragility and abnormal sperm morphometry which are complications of diabetes mellitus, the ameliorative effects of this plant on these complications of diabetes therefore need to be evaluated.

Method

Plant Material

Collection of Plant Materials

Fresh matured leaves of *Cnidoscolus aconitifolius (Euphorbiaceae)* were collected at the University Teaching Hospital, College of Medicine, and Ibadan and were identified and authenticated at department of Botany and Microbiology, University of Ibadan. It was then deposited at the Forestry Research Institute of Nigeria (FRIN) with voucher no: FHI 107727. The leaves were air-dried, reduced to powder and kept in airtight containers until the time of use.

Extraction of Plant Material

Air-dried powder (1kg) of fresh matured *Cnidoscolus aconitifolius* leaves were extracted by percolation at room temperature with 70% ethanol. The leaf extract was then concentrated under reduced pressure (bath temp. 50^{o}C) and finally defatted with n-hexane. The filtrate was evaporated to dryness. The dry mass yield was 62.5g from 1kg of air-dried and powdered leaves. The plant extract was not soluble in water,

so, was dissolved in corn oil which served as solvent for the extract.

Animals

Male albino rats of the Wistar strain (100-250g; 4-8 weeks old) were maintained under controlled conditions of light (12-h light/dark cycle) and temperature (30 ± 1°C). Rat chow (Vital feeds Nigeria, Ltd, Nigeria) and water were provided *ad libitum* throughout the period of 28 days which the experiment lasted.

Induction of Diabetes

Diabetes was induced in the rats after an overnight fasting by intra-peritoneal injection of 100mg/kg body weight of Alloxan monohydrate (5% W/V), freshly dissolved in physiological saline before use at 10g/0.75L. The diabetes state was assessed by measuring the non-fasting blood glucose concentration 72 h after Alloxan treatment. The rats with blood glucose above 200mg/dl or 11mm/l and signs of polyuria, polydypsia, polyphagia, weight loss, glucosuria and hyperglycemia were selected for the experiment[21].

Treatment groups

The diabetic animals were randomly classified into five groups (B-E) of five rats per group. Diabetes was not induced in Group A which served as the control. They however received 2.0ml of sterile normal saline. Group B received Alloxan alone while groups C-E received ethanolic extract of *Cnidoscolus aconitifolius* (orally) at varying dosages of 100mg/kg body weight, 500mg/kg body weight and 1,000mg/kg body weight respectivelyon a daily basis for 28 days, while group F received a standard oral hypoglycemic agent; Chlorpropamide (Diabenes®, 25mg/kg body weight) in physiological saline as vehicle for the same period. The ethanolic extracts of *Cnidoscolus aconitifolius* were re-dissolved in corn oil and administered with oral canula.

Blood sample analysis

Blood sample collection

The animals were anaesthetized with ether after which blood was collected from the retro orbital venous plexus for determination of the haematological parameters. Blood glucose was monitored weekly using glucometer (Acucheck advantage II).

Determination of Haematological Parameters

The red blood cell (RBC) was counted with by haemocytometer, the packed cell volume (PCV) by the microhaematocrit method and the haemoglobin (Hb) concentration by the cyanmethaemoglobin method. The mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated from the values obtained for RBC, PCV and Hb.[22] The total white blood cell count was determined manually using the improved Neubauer haemocytometer while the differential leucocytes counts were determined by morphological identification and counting of hundred leucocytes in Giemsa stained smears of each blood sample; these are expressed as percentages of the total WBC from which the absolute leucocyte counts were calculated.

Erythrocyte osmotic fragility

Erythrocyte osmotic fragility was determined according to the method described by Oyewale[23]. Briefly described, 0.02 ml of blood was added to tubes containing increasing concentration of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4 (0, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8, and 0.9%). The tubes were gently mixed and incubated at room temperature (29°C) for 30 minutes. The content in each tube was then centrifuged at 1500 rev/ min for 10 minutes and supernatant decanted. Optical density of the supernatant was determined spectrophotometrically at 540nm using SM22PC Spectrophotometer (Surgienfield Instruments, England). Haemolysis in each tube was expressed as a percentage, taking haemolysis in distilled water (0% NaCl) as 100%.

Semen examination

Sperm motility

Sperm motility was determined according to the method described by Zemjanis[24]. Briefly, a drop of semen was collected from the caudal epididymis onto a glass slide. Sodium citrate buffer (2.9%) was then added to the semen and mixed until the desired dilution was obtained. Motility (%) was then evaluated microscopically within 2-4 minutes of semen collection.

Sperm count

The total spermatozoa in the caudal epididymal sperm sample was counted using the improved Neubauer haemocytometer slide[25]

Sperm morphological abnormalities and percentage live/dead ratio

Sperm suspension for morphological examination was placed on another slide and stained with Wells and Awa stain containing 0.2g of eosin and 0.6g of fast green stains dissolved in 10 and 20 ml of distilled water respectively. 18 ml of ethanol was then added to the mixture. Live/dead ratio on the other hand was determined using 1% eosin and 5% nigrosine in 3% Sodium citrate[26].

Statistical Analysis

All results obtained were statistically analyzed for significance using one-way ANOVA in Graphpad Prism version 4.00, 2003 statistical software. Parameters from the treated samples were compared with those of the diabetic rats using Dunnett analysis. Results are expressed as mean ± SD while $P < 0.05$ was considered to be significant.

Results

Haematology

The effects of alloxan induced diabetes on haematological parameters and erythrocytes osmotic fragility was investigated and compared with similar parameters of normal non diabetic rats as shown in Table 1. Following induction of diabetes in the rats, most of the haematological parameters were significantly reduced ($P<0.01$) with the exception of the differential lymphocytes and neutrophil counts which was significantly higher ($P<0.01$) than values obtained in normal non diabetic rats. There was microcytic hypochromic anemia following significant fall ($P<0.01$) in the packed cell volume (PCV), haemoglobin (Hb) concentration and red blood cell (RBC) count. The mean corpuscular volume (MCV), and mean corpuscular haemoglobin (MCH) were also significantly lower ($P<0.05$) in the diabetic rats than in the normal non diabetic ones. Leucopenia and thrombocytopenia were also apparent in the diabetic rats as indicated by the reduction in total white blood cell (WBC) count and platelets count which decreased significantly ($P<0.001$ and $P<0.01$ respectively) in the diabetic rats.

As shown in Fig 1, the erythrocytes osmotic fragility of the diabetic rats was higher at 0.0%, 0.1% and at 0.5% NaCl concentration but showed lower erythrocyte fragility at 0.3% NaCl concentration. However, the differences were not statistically significant.

Table 1: Haematological parameters of alloxan induced diabetic rats following treatment with ethanolic extract of *Cnidoscolus aconitifolius* and chlorpropamide for four weeks

Haematological Parameters	Group A Non diabetic rats	Group B Diabetic rats before treatment	Group C treated with 100mg/kg C.a	Group D treated with 500mg/kg C.a	Group E treated with 1000mg/kg C.a	Group F treated with Chlorpropamide
PCV (%)	43.00± 1.90**	28.80± 10.08	42.50± 2.05	42.50± 6.00	42.00± 2.38	43.60± 2.61**
HB (g/dl)	14.62± 0.28**	9.54± 3.39	13.95± 1.01**	13.17± 1.35*	13.55± 0.49**	13.90± 0.62**
RBC ($x10^6/\mu L$)	7.11± 0.09**	5.03± 1.00	7.25± 1.05**	6.77± 0.59	6.93± 0.21*	7.10± 0.37**
MCV (fl)	60.52± 2.65*	57.18± 2.49	60.10± 2.10	61.27± 3.98	62.13*± 5.02	61.66± 2.27**
MCH (pg)	20.74± 1.07	18.95± 1.92	19.48± 1.50	19.47± 0.63	19.68± 1.16	19.60± 0.75
MCHC (g/dl)	34.01± 1.32	33.13± 1.63	32.60± 1.49	31.77± 1.10	31.75± 1.14	31.82± 0.61
WBC ($x10^3/\mu L$)	8.22± 0.92	5.76± 0.72	10.83± 4.35	3.96± 2.32	13.45± 0.93**	13.90± 0.62*
Platelets ($/\mu L$)	177± 0.14	109± 0.11	602.80± 124**	569.30± 249**	622.50± 104**	499.80± 104**
Lymphocytes %	66.00± 4.21	85.20± 1.23	71.50± 12.40	58.00± 14.53	76.25± 9.18	64.60± 16.41
Neutrophils (%)	17.17± 1.42	35.00± 11.0	28.87± 12.40	42.00± 14.53	23.75± 9.18	52.60± 22.96

Values are expressed as mean ± SD. Number of animals in parenthesis. Asterisks indicate significant difference from Group B. *P<0.05, **P<0.01. Number of animals in parenthesis.

Table 1 also shows the haematological parameters of diabetic rats following treatment with 100mg/kg, 500mg/kg and 1000mg/kg of ethanolic extract of *Cnidoscolus aconitifolius*. There was restoration of almost all the haematological parameters after four weeks of treatment with the extract and a standard antidiabetic drug (Chlorpropamide). The PCV, Hb concentration, RBC count and the MCV values were restored to the values in non diabetic rats.

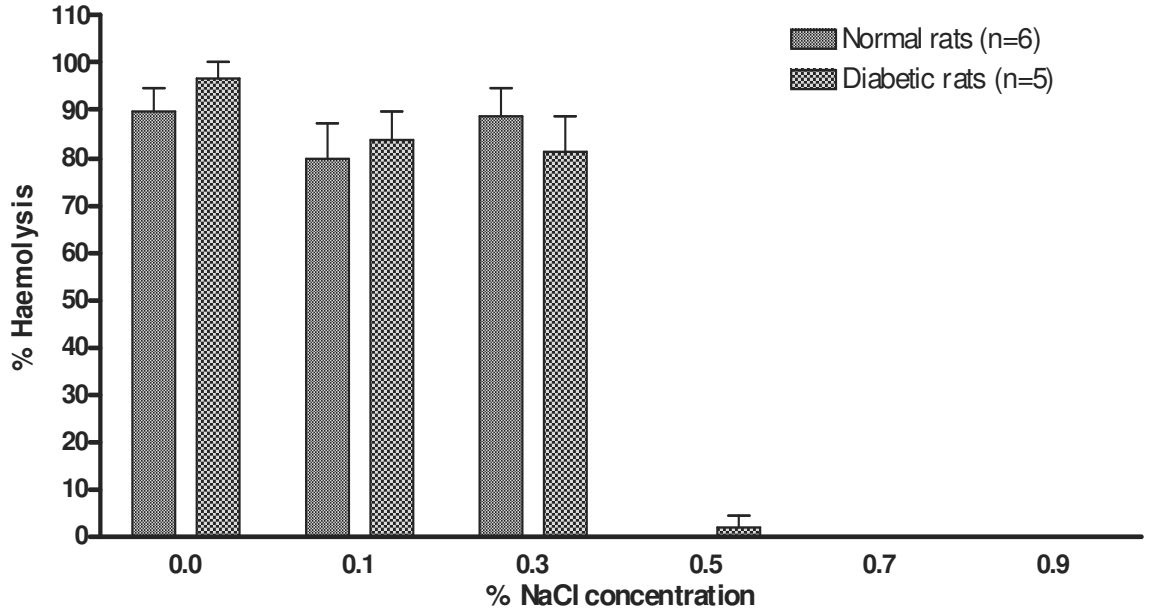
After treatment, leucopenia (reduced total WBC count) associated with diabetes was significantly restored in rats that were given 1000mg/kg of the extract and Chlorpropamide ($P<0.01$ and $P<0.5$ respectively). Similarly, lymphocytosis and

neutrophilia that were observed also abated following treatment of the diabetic rats with the extract and Chlorpropamide (Table 1). The total platelets count of the treated rats were also significantly higher (P<0.01) in all the four groups of rats that were treated with the extract than that of the diabetic rats. The restorative effects of 500mg/kg of the extract was however significantly lower (P<0.01) than that of either 1000mg/kg of *C. aconitifolius* or Chlorpropamide. Similarly, WBC count in rats treated with 500mg/kg extract was also lower (P<0.05) than the WBC count obtained in rats that were treated with 100mg/kg of the extract.

Erythrocyte osmotic fragility

The erythrocyte osmotic fragility of the diabetic rats treated with Chlorpropamide was significantly higher (P<0.05) at 0.5% NaCl concentration than that of either Group A (normal control) or Group B (Diabetic rats). It was also higher (P<0.05) than that of diabetic rats treated with 500mg/kg of *Cnidoscolus aconitifolius* extract. Similarly, at 0.9% NaCl, the erythrocyte osmotic fragility of rats treated with Chlorpropamide was higher than that of either the normal control (P<0.01) or the diabetic rats (P<0.05). It was also higher (P<0.01) than that of the rats treated with 100mg/kg and 500mg/kg (Group C and D) as well as those treated with 1000mg/kg of the extract (P<0.05 respectively).

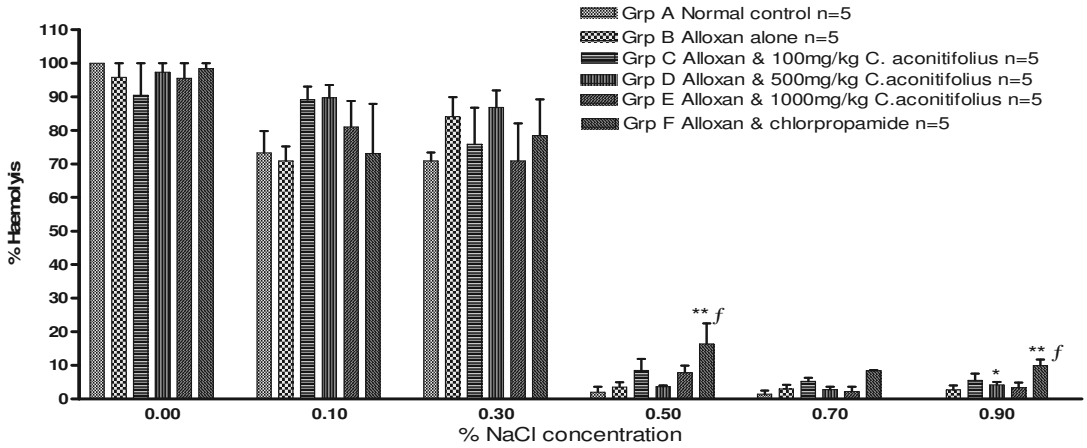
Fig 1. Erythrocyte osmotic fragility of normal and alloxan induced diabetic rats before treatment. Values are means while vertical bars represent SD. n is the number of animals.



Semen examination

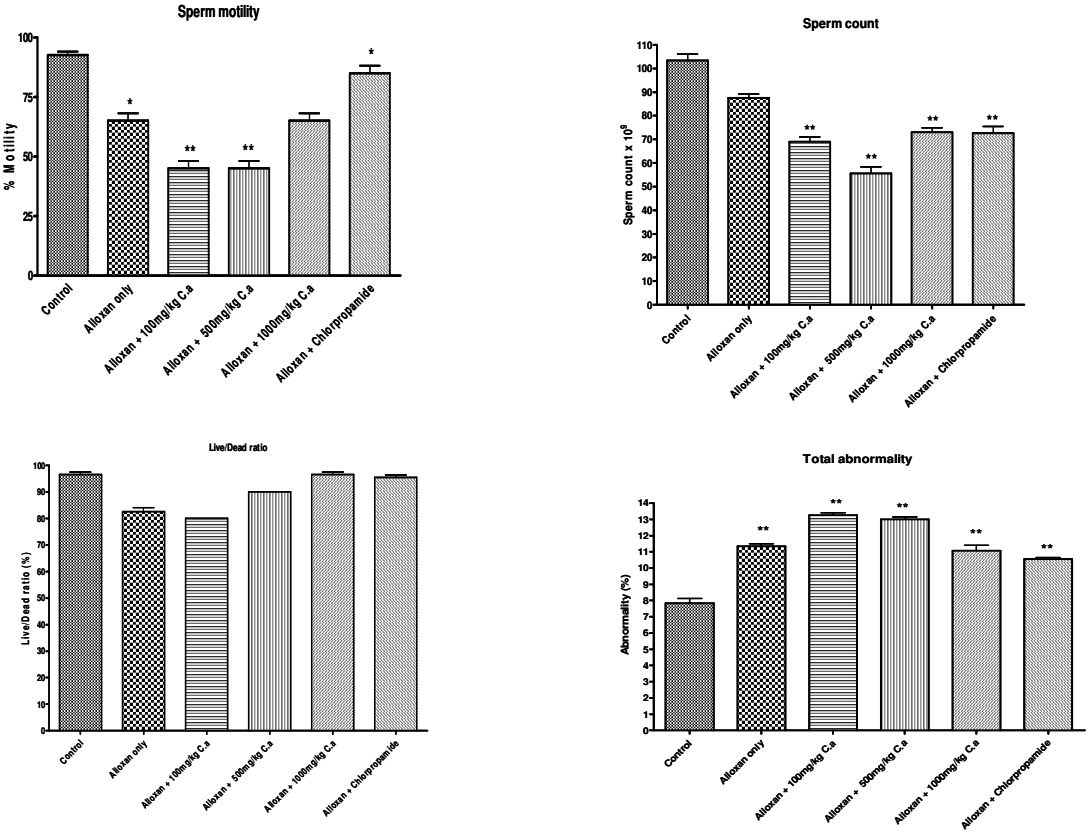
The effects of diabetes on sperm count, motility, and live dead ratio is shown in Figure 3. Following induction of diabetes with alloxan, there was a considerable fall (P<0.05) in the percentage sperm motility to 65% from the initial value of 92.5% observed in the normal non-diabetic group. Similarly, total sperm count and percentage live/dead ratio were also reduced significantly (P<0.01) from 96.5% ± 2.12 and 103.3 x $10^9/\mu L$ ± 6.10 in the normal rats to 82.5% ± 3.54 and 87.5 x $10^9/\mu L$ ± 3.54 respectively in the diabetic group. The use of 1000mg/kg of ethanolic extract of *Cnidoscolus aconitifolius* and Chlorpropamide however, exhibited ameliorative effects on the sperm motility, live/dead ratio and the total sperm count, while no significant ameliorative effect were seen in diabetic rats treated with 100 and 500mg/kg of the extract. Furthermore, sperm abnormalities increased following induction of diabetes with alloxan (Fig 3). But neither *Cnidoscolus aconitifolius* extract nor Chlorpropamide demonstrated any significant ameliorative effects on the sperm abnormalities in the diabetic rats.

Fig 2. Erythrocyte osmotic fragility of alloxan induced diabetic rats following treatment with ethanolic extract of *Cnidoscolus aconitifolius* and Chlorpropamide. Values are means while vertical bars represent SD. n=number of animals.



Asterisks indicate significant difference from the control *=P<0.05, **=P<0.01.
f indicates significant difference from group B at P<0.05

Fig 3: Effects of *Cnidoscolus aconitifolius* extract on sperm morphometry of alloxan treated rats. Asterisks indicate significant difference from the control. * P<0.05, **P<0.01



Discussion

Diabetes mellitus induced by alloxan from the above results (Table 1) is associated with anaemia of the microcytic hypochromic type as a result of the fall in the PCV, RBC, Hb and MCV values obtained in the diabetic rats. This might not be unconnected with both the effects of alloxan on rapidly dividing haemopoietic cells and suppression of haemopoiesis as a result of insulin deficiency occasioned by the selective destruction of the β cells in the Islets of Langerhans of the pancreas by alloxan[27, 18]. Significant increase in differential lymphocytes and neutrophils count in the diabetic rats must have also resulted from the stress induced by diabetes in accordance with stress induced lymphocytosis and neutrophilia in avian species[28]. El-Missiry and El Gindy[15] reported a decrease in insulin level, hyperglycemia, elevated total lipids, triglycerides and cholesterol, decreased high-density lipoprotein and hepatic glycogen contents and elevated hepatic glucose-6-phosphatase activity in the rats following a single dose injection of Alloxan (100 mg/kg). There was also an increase in the concentration of malondialdehyde and 4-hydroxynonenal in the liver as well as a significant decreased glutathione (GSH) content and superoxide dismutase activity in the liver of alloxan-diabetic rats. Shevchenko and Elfimov[7] also reported a decrease in haematocrit, haemoglobin, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration in glomectomized diabetic rats as a result of suppression of haemopoiesis in diabetes. Free radicals are also generated in diabetes[4, 29, 30] and this has been associated with increased erythrocytes osmotic fragility[31, 32]

In the current study, the erythrocyte osmotic fragility was higher at 0% and 0.1% NaCl concentration while the reverse was the case at 0.3% and 0.5% NaCl. This was contrary to previous observation on ameliorative effects of *Cnidoscolus aconitifolius* on anaemia and consistently high erythrocyte osmotic fragility in protein-energy malnourished rats[20]. Increased erythrocyte osmotic fragility has been observed to occur in diabetes patients as a result of persistent hyperglycemia in diabetes mellitus[12] leading to glucose auto-oxidation[3], peroxidation of the membrane lipid and other oxidative damage to the erythrocyte membrane.[32]

After treatment of the diabetic rats with ethanolic extracts of *Cnidoscolus aconitifolius,* there was complete restoration of the haematological parameters especially the PCV, Hb concentration, RBC and WBC count and the MCV to the values obtained in normal non diabetic rats (Table 1). The restorative effect of the extract on the haematological parameters was comparable to that of Chlorpropamide (a standard antidiabetic drug). Although, 1000mg/kg of the extract produced the highest restorative effects, the difference were however not significant (except on the total WBC count where its effects was significantly higher ($P<0.01$) than the effect of 500mg/kg of the extract). Several plant extracts are currently being investigated for their effects on diabetes control and treatment, for example *Azadirachta indica* leaf extract has been demonstrated to lower serum lipid in diabetes[14.] Hypoglycemic effect of *Artemisia sphaerocephala* (Krasch seed) polysaccharide in alloxan-induced diabetic rats has also been reported[13] while *Murraya koenigii* (curry leaf) has also been show to lower plasma lipid in alloxan induced diabetes in rats[16].

Some of these plant extracts have however been shown to increase erythrocytes osmotic fragility. *Hypericum perforatum* extract for example has been shown to increase erythrocyte osmotic fragility despite its anxiolytic, antiviral, wound healing, antimicrobial, analgesic, and anti-inflammatory effects[33]. Similarly, in this study as shown in Figure 2, the erythrocyte osmotic fragility of rats that were treated with the extract was slightly higher than that of the non diabetic control. But this was not as pronounced as the effects of Chlorpropamide on the erythrocyte osmotic fragility. At 0.5%, 0.7% and 0.9% NaCl concentration, the osmotic fragility of erythrocytes in the diabetic rats treated with Chlorpropamide was significantly higher than those of the control and the extract treated rats. This shows that the erythrocytes are less stable in hypotonic solution and might be an indication of membrane fluidity and intravascular haemolysis in subjects treated with Chlorpropamide.

The effects of diabetes on sperm motility, live/dead and sperm count were also investigated in the present study. In agreement with previous reports or observations, there was reduction in the sperm motility and count as well as the percentage live/dead ratio while the number of abnormal spermatozoa also increased considerably following induction of diabetes with alloxan[2, 3, 5], an indication of reduced fertility in the subjects[5]. This was attributed to damage of the secretory epithelia cells of the seminiferous tubules[3] probably due to oxidative damage from glucose auto-oxidation and excessive production of superoxide radicals and formation of advanced glycation end-products

associated with diabetes mellitus[4]. There was however some degree amelioration of these effects following treatment of the diabetic animals with the extract (especially 1000mg/kg) and the standard antidiabetic drug Chlorpropamide. The effect of the extract was particularly prominent on the percentage motility and the live/dead ratio of the spermatozoa but the ameliorative effect of Chlorpropamide was more prominent on the percentage motility than the effects of the extract (Fig 3). On the other hand, neither *Cnidoscolius aconitifolius* nor Chlorpropamide showed any significant ameliorative effects was on the various structural or morphological defects in the spermatozoa observed as a result of diabetes. This may probably be due to the short period of treatment of the diabetic rats with the extract.

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