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INFLUENCE OF *CALOTROPIS PROCERA* AQUEOUS LEAF EXTRACT ON HISTOLOGY AND MORPHOLOGY OF LIVER, KIDNEY AND RED BLOOD CELLS IN ALLOXAN-INDUCED DIABETIC RATS

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ABSTRACT

Diabetes is becoming the third killer disease of mankind, after cancer and cardiovascular diseases because of its high prevalence, morbidity and mortality. Diabetes complications are burden on vital organs in the body. Nephropathy and liver failure are common among diabetic patients. In view of this, influence of aqueous extract of Calotropis procera leaf on histology and morphology of liver, kidney and red blood cells in alloxan-induced diabetic rats was investigated in the present study. Alloxan induction resulted in 5-fold elevation of blood glucose level in administered groups B to F when compared to non-induced group A. The photomicrograph of the untreated diabetic group B showed degenerated liver tissue and loss of integrity, narrowed Bowman capsule with disarrayed glomeruli and disrupted red blood cell (crenation) with reduction in RBC size. The extract at higher doses particularly 100 mg/kg b.w. extract significantly lowered ($p<0.05$) the elevated values back to normal (19.7 to 3.3 mmol/L). The extract at higher doses restored the integrity and organization of the atrophied organs and also ameliorated deformility in red blood cells (RBC) rheology as evident from photomicrographs of administered groups (C, D, E and F). In the present study, the extract repaired scarred organs and also offered protection and restoration to red blood cells in administered groups when compared to non-administered group B.

Introduction

Diabetes mellitus is a worldwide disease that resulted from derangement in carbohydrates metabolism. It is a disease of the poor and the rich because the poor weeps while the rich cries for lack of cure for diabetes but management.

In Nigeria, several studies on the cause(s) and survival of the household disease have been carried out. Ajiboso *et al.,* (2016) and Ajiboso *et al.,* (2018) have reported the nature of Nigerians' diet which is mainly carbohydrates as the cause and at the same time means of survival of the populace from diabetes mellitus. Carbohydrates foods such as rice, maize, wheat products and cassava are staple foods in Nigeria. These foods and their products are daily consumed in Nigerian homes, popularly served in ceremonies, hawked around the communities and also means of livelihood among the populace.

Diabetes mellitus is associated with short and long terms complications such as polydipsia, polyphagia, polyuria, retinopathy, nephropathy, neuropathy, weight loss, fatigue and organs atrophy (Ajiboso, 2014; Njagi *et al.,* 2015). At each stage of clinical manifestations of these complications, organs in the body are partially or fully compromised leading to dysfunction and inactivity. However, a long time untreated diabetes may lead to atrophy of some organs in the body and such organs include liver, kidney, pancreas and heart.

Infiltrations and exacerbation of some liver and kidney function markers such as glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase, creatinine, urea, total protein and albumin have been reported in diabetic subjects (Ajiboso, 2014). Infiltration and high concentration of these markers in blood may be linked to damaged organs.

Problem of damaged or compromised organs is common among diabetic patients and most often organ transplanting is suggested at the instance of clinical manifestation, the organ transplanting is costly and could only be afforded by the rich, and the question is what will be the fate of the poor? Thus, the need to intensify research effort on histology and morphology of organs in the diabetic becomes imperative; such effort includes sourcing alternative treatment and prevention of organ atrophy from medicinal plants.

Aqueous extract of *Calotropis procera* plant have been reported to possess anti-diabetic property (Ajiboso *et al.,* 2016). The various parts of the plant have been reported to possess medicinal properties (Larhsini *et al.,* 1997); several studies have also substantiated its acclaimed uses as a potent medicinal plant in Nigeria folklore traditional medicine (Ajagbonna et al., Ajiboso and Tarfa, 2018).

It is therefore the interest of the present study to determine the influence of *Calotropis procera* aqueous leaf extract on histology and morphology of liver, kidney and red blood cells in alloxan-induced diabetic rat.

Materials and methods

Plant Material

Fresh leaves of *Calotropis procera* were collected in August 2017 when rainfall was at its peak from the garden behind Pharmaceutical Sciences Department, Bingham University Karu, Nasarawa State, Nigeria. Taxonomic identification and authentication of the plant were done using standard botanical monographs at Plant Section of Federal Polytechnic Bida Nigeria; voucher specimen with number 95007 was deposited at the herbarium unit.

Animals

Adult *Rattus norvegicus* rats (male and female) of mean weight 85.00±00.21g were obtained from animal house – Medblock of Bingham University Karu Nigeria. the rats were kept at the animal house and fed at *ad libitum* with standard pellet Mix and water throughout the period of the experiment.

Glucometer and Assay kit Bayer ContourTM TS blood glucose kit was a product of Bayer Consumer Care AG, Postfash, Basel, Switzerland.

Drug and Chemicals

Alloxan monohydrate was a product of Explicit Chemicals PVT, Ltd., Pune, India. Metformin was a product of NWP Springville, Illinois, USA. All other chemicals were products of Sigma-Aldrich CHEME Gmbh, Steinhelm Germany. The chemicals were prepared in glass distilled water unless otherwise stated.

Methods

Preparation of aqueous extract and induction of diabetes

The methods described by Yakubu et al., (2010) were used to prepare the extract and induce diabetes.

Animal grouping

30 rats (5 normal, 25 alloxan induced-diabetic rats) were distributed into six groups (A-F) of five rats each after diabetes had been confirmed. Calculated amount of the residue was weighed and constituted in distilled water to give the required doses of 25, 50 and 100 mg/kg body weight to groups D, E and F respectively while groups A, B and C were non-diabetic, distilled water treated diabetic and metformin treated diabetic rats respectively. The doses

used in this study were as obtained from the ethno-botanical survey carried out on the plant within our locality.

Treatment was administered orally with feeding bottle to respective groups. Preliminary studies conducted by Yakubu et al., (2010) revealed that the diabetic untreated rats could survive up till the 12th day; therefore this experiment was terminated on the 10th day. The rats were handled in accordance with the guidelines of European Convention for the protection of vertebrate animals and other scientific purposes -ETS-123 (European Treaty Series, 2005).

Histopathological examination

Tissue collection and fixation

Perfusion fixation was done through the heart with 20 ml of phosphate buffered saline (PBS) for 5 minutes to drain out whole body blood. The organs (liver and kidney) were placed in the vials containing NBF fixative for 12 hours in the Fumehood (LABTECH, Namyangju, Korea). After that, 250 ml of neutral buffer formalin (NBF) fixative was perfused for 30 minutes to fix the organs. After perfusion fixation, liver and kidney were excised. The kidneys were cut into longitudinal and transverse sections (vertical and horizontal).

Tissue processing was carried out according to the procedure described by Border *et al.*, (1990). The procedures described by Bancroft and Gamble (2007) were used for tissue embedding and sectioning.

Haematoxylin & Eosin (H&E) staining

Slides of liver and kidney sections were selected for staining from all experimental groups. At first, all the slides were kept in xylene for 15 minutes to deparaffinise the sections. After that, the sections were rehydrated with decreasing concentrations of 2-propanol, i.e. 100%, 90% and 70% for 2 minutes each. The slides were washed with deionised water for 10 minutes. The staining was done with Mayer's Hemalaum solution (ROTH, Karlstruhe, Germany) for 3 minutes and then washed with running tap for 10 minutes. After that freshly prepared acidified eosin G was added to sections with the help of dropper and left for 1 minute. The slides were washed with deionised water and then dehydrated with increasing concentrations of 2-propanol, i.e. 70%, 90% and 100% for few seconds by just dipping the slides from both sides. The slides were made clear in xylene for 2 minutes and finally mounted with mounting media (DPX, MERCK, Darmstadt, Germany) using clean cover slips. The slides were thereafter dried at room temperature and then observed under light microscope (Nikon 90*i*) at a magnification of x400.

Tissue examination

The stained images were observed using Nikon 90*i* Microscope (Nikon, Tokyo, Japan) equipped with a Nikon DXM-1200C digital camera. Images were processed using Adobe photoshop 7.0 and saved in jpeg format.

Morphological examination

Examination of red blood cell morphology (shape and size) was carried out according to the procedure described by Sood (1999).

Results

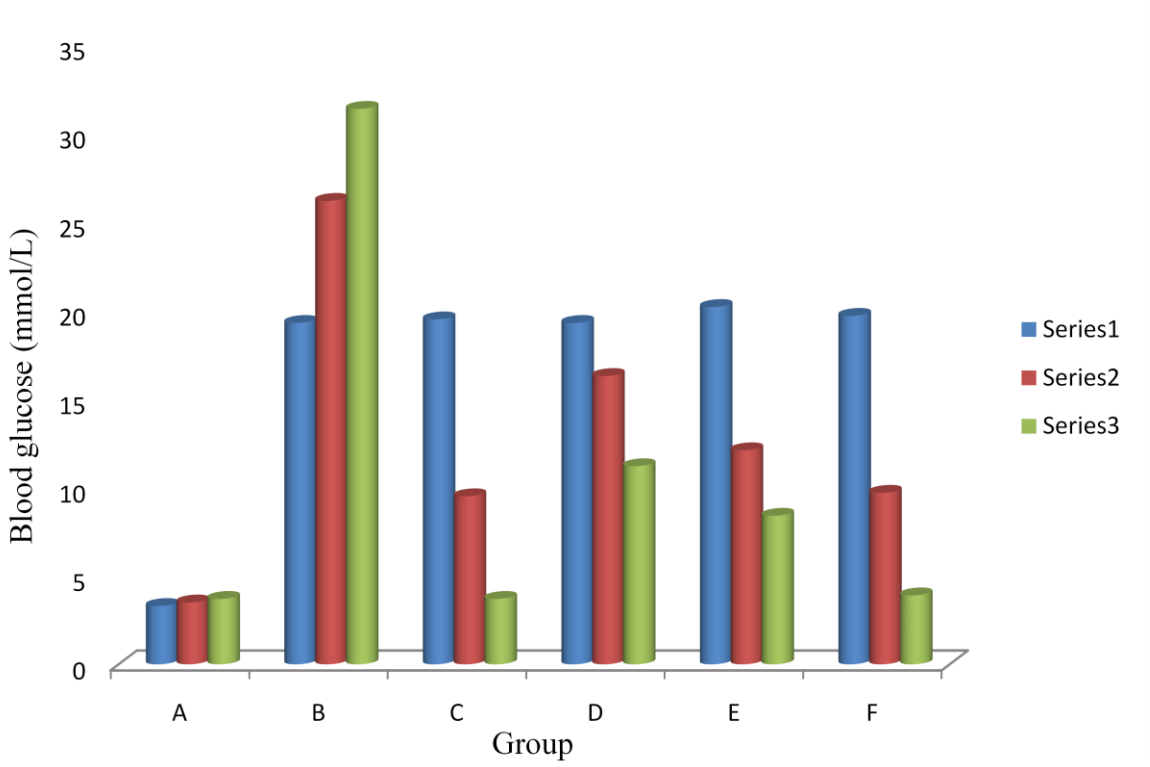
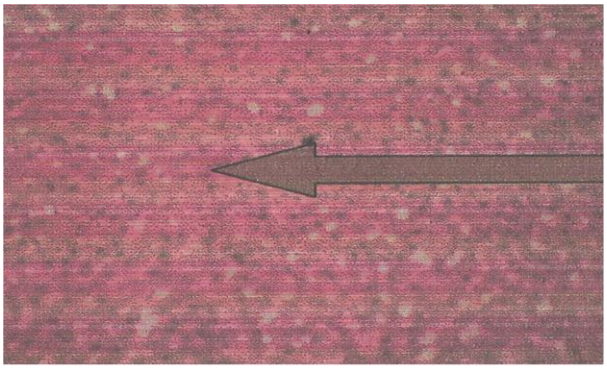


Figure 1: Graph of blood glucose levels of alloxanised rats before and after administration of aqueous extract of *Calotropis procera* leaf

Key: series 1= day 0; series 2= day 5; series 3= day10.

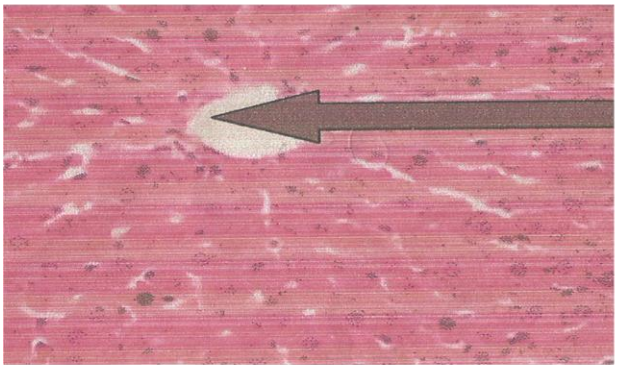
As shown in Figure 1, alloxan induction resulted in 5-fold elevation of blood glucose levels of administered groups (B to F). The non-induced group A administered distilled water only maintained slight increase in blood glucose level, this increase was not significant ($p<0.05$). The untreated diabetic rats in group B showed marked significant increase ($p<0.05$) in blood glucose level throughout the experimental period. However, administration of extract at different doses 25 mg/kg b.w., 50 mg/kg b.w. and 100 mg/kg b.w. to groups D, E and F

respectively significantly lowered (at $p<0.05$) the blood glucose level in administered groups. The anti-hyperglycemic property of the extract as observed in the present study was dose-dependent with highest efficacy and potency in glucose mopping or scavenging ability shown by the 100 mg/kg b.w. of the extract, this dose of extract lowered the blood glucose in administered group to the range of values obtained for non- diabetic and metformin treated groups.



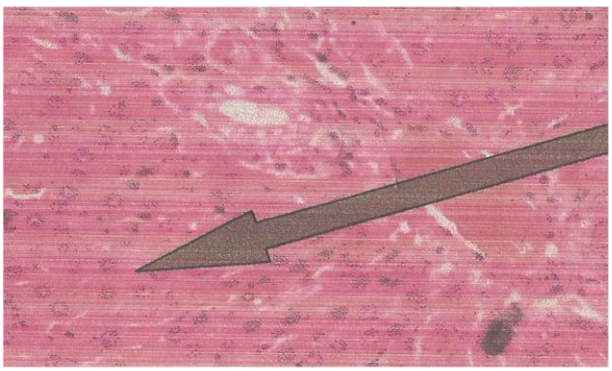
It is normal in appearance with normal liver tissues

Plate 1: Photomicrograph of cross section of liver of non-diabetic rat (x400;H&E)



Degenerated liver tissue and loss of integrity

Plate 2: Photomicrograph of cross section of liver of distilled water – treated diabetic rat (x400;H&E)



Intact liver tissue integrity

Plate 3: Photomicrograph of cross section of liver of metformin - treated diabetic rat (x400;H&E)

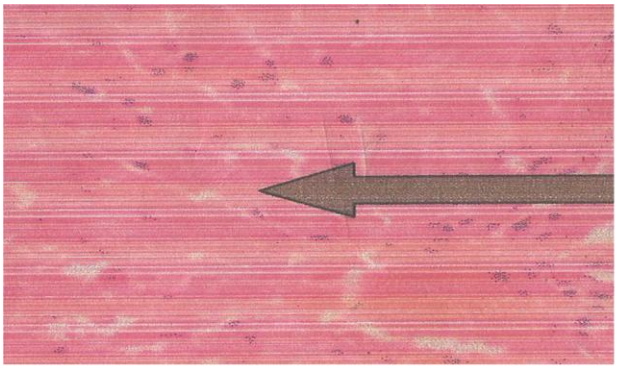


Plate 4: Photomicrograph of cross section of liver of 25mg/kg b.w. of extract-treated diabetic rat (x400;H&E)

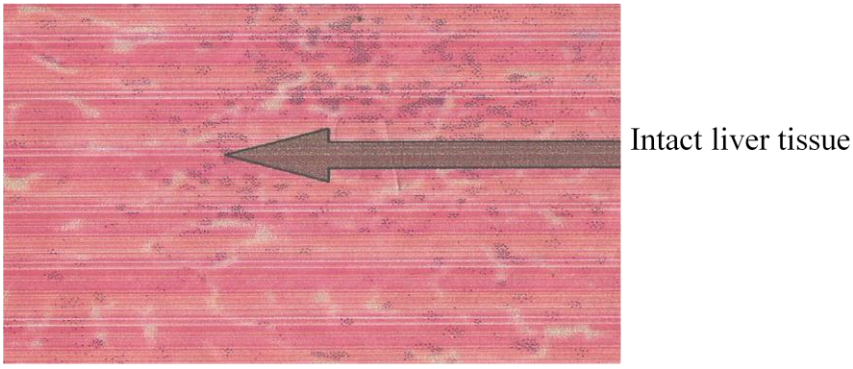


Plate 5: Photomicrograph of cross section of liver of 50mg/kg b.w. of extract-treated diabetic rat (x400;H&E)

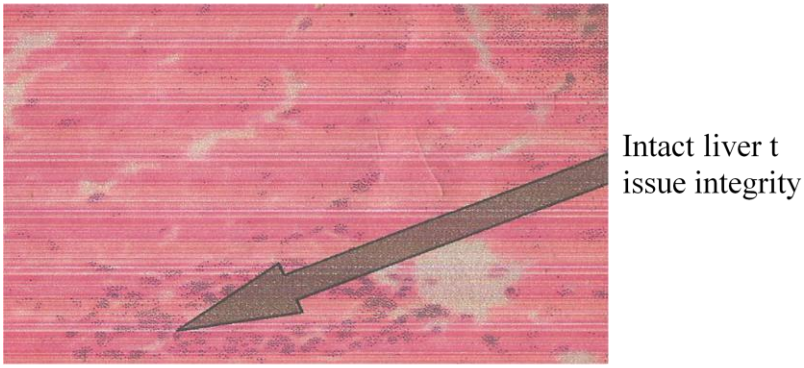


Plate 6: Photomicrograph of cross section of liver of 100mg/kg b.w. of extract treated diabetic rat (x400;H&E)

There was intact and normal liver tissue integrity in non-diabetic rats (Plate 1). There was loss of liver tissue integrity and the presence of severe degeneration in the liver of distilled water treated diabetic rats (Plate 2). Treatment with metformin (Plate 3) and 50 mg/kg and 100 mg/kg body weight of the extract (Plates 5 and 6) ameliorated the toxic effect of alloxan

in the liver. The severe degeneration caused by alloxan in the liver was mildly ameliorated in the treament groups except 25 mg/kg body weight of the extract treated group where loss of integrity and severe degeneration in liver was observed (Plate 4).

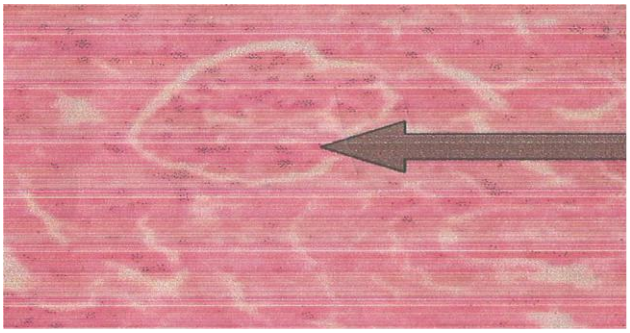


Plate 7: Photomicrograph of cross section of kidney of non-diabetic rat (x400;H&E)

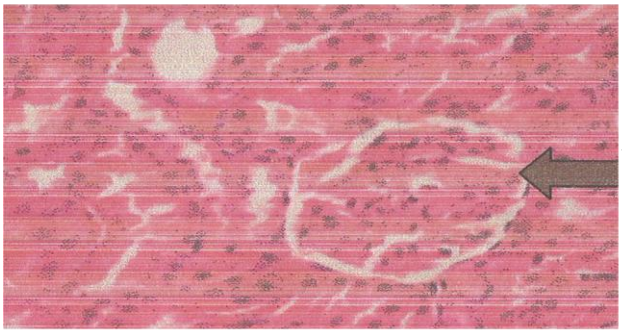


Plate 8: Photomicrograph of cross section of kidney of distilled water – treated diabetic rat (x400;H&E)

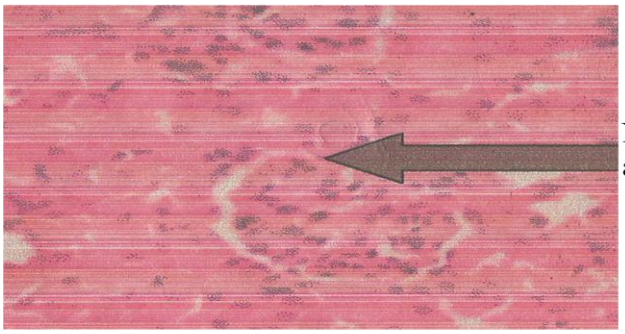
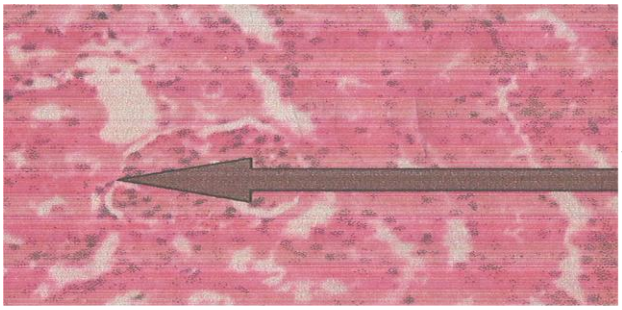
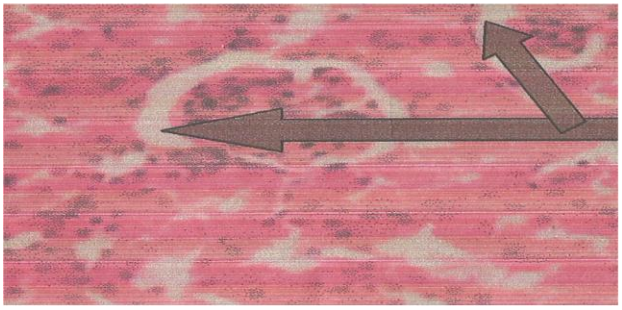


Plate 9: Photomicrograph of cross section of kidney of metformin - treated diabetic rat (x400;H&E)



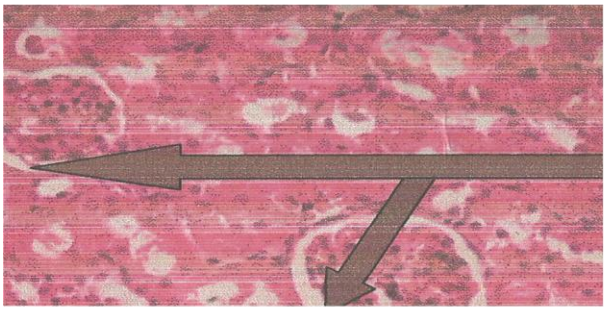
Narrowed Bowman capsules and scattered glomerulus

Plate 10: Photomicrograph of cross section of kidney of 25mg/kg b.w. of extract-treated diabetic rat (x400;H&E)



Wide Bowman capsules and scattered glomerulus

Plate 11: Photomicrograph of cross section of kidney of 50mg/kg b.w. of extract-treated diabetic rat (x400;H&E)

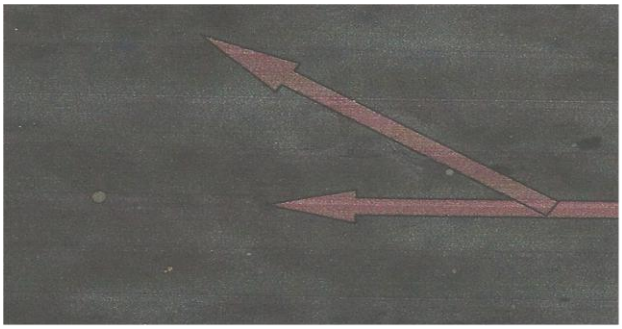


Wide Bowman capsules and organised glomerulus

Plate 12: Photomicrograph of cross section of kidney of 100mg/kg b.w. of extract treated diabetic rat (x400;H&E)

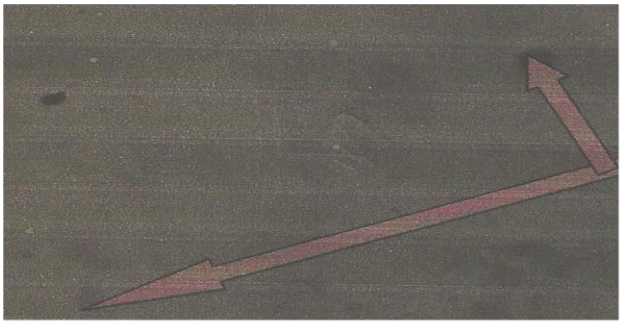
There was presence of wide Bowman's space which separates the glomerulus from the inner lining of the Bowman capsule in the kidney of the non-diabetic rats. It also showed perfect organisation in the kidney (Plate 7). The architecture of the kidney was extensively disrupted and alteration of the Bowman space in the Bowman capsule in the kidney of distilled water treated diabetic rat, this was accompanied by deposition of glycogen in the kidney (Plate 8). Treatment with aqueous extract of *Calotropis procera* leaf ameliorated these adverse observations in the kidney. This depicted by restoration of Bowman's space and ordered nature of the Bowman capsule at 100 mg/kg body weight of the extract. However, glycogen

deposits were still observed in the kidney of the extract – treated rats compared to the distilled water – treated diabetic rat (Plates 11 and 12). This result obtained for the extract – treated rat was comparable to that of metformin – treated rats (Plate 9).



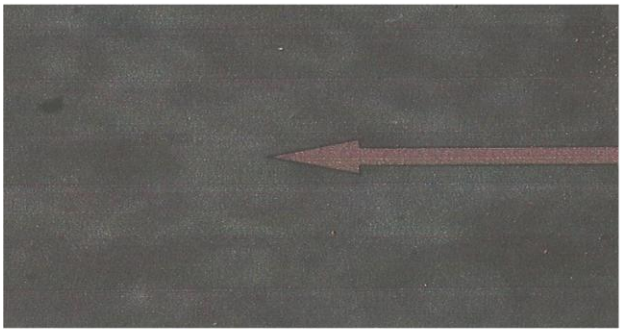
Film is normal with normal red blood cell in shape and size

Plate 13: Photomicrograph of red blood cell of non-diabetic rat (x400;H&E)



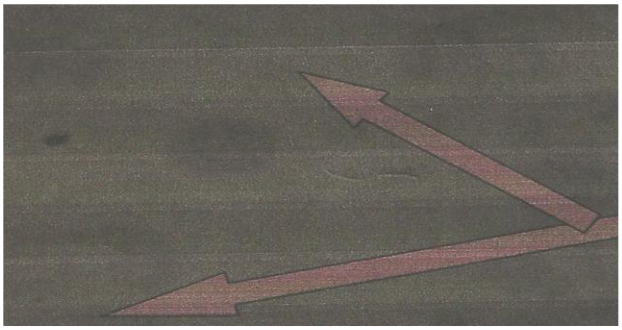
Film is normal with disrupted red blood cell and reduction in RBC size

Plate 14: Photomicrograph of red blood cell of distilled water – treated diabetic rat (x400;H&E)



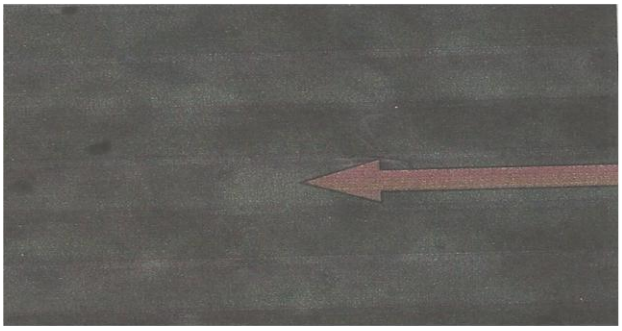
Film is normal with normal red blood cell in shape and size

Plate 15: Photomicrograph of red blood cell of metformin– treated diabetic rat (x400;H&E)



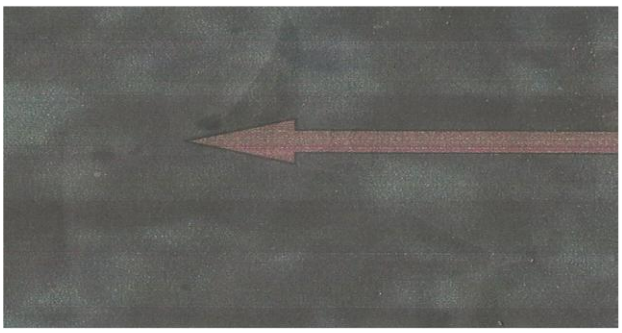
Film shows slight disruption of red blood cell and reduction in size

Plate 16: Photomicrograph of red blood cell of 25mg/kg b.w. of extract-treated diabetic rat (x400;H&E)



Film is normal with normal red blood cell in shape and size

Plate 17: Photomicrograph of red blood cell of 50mg/kg b.w. of extract-treated diabetic rat (x400;H&E)



Film is normal with normal red blood cell in shape and size

Plate 18: Photomicrograph of red blood cell of 100mg/kg b.w. of extract-treated diabetic rat (x400;H&E)

Induction of diabetes using alloxan adversely affected the morphology of the red blood cell membrane in diabetic rats and caused hemolysis. There were reduced red blood cells count and disrupted red blood cell shape in distilled water treated rats (Plate 14) compared to non-diabetic rats in which intact and normal red blood cell shape and size were observed (Plate 13). Treatment with higher doses of the extract particularly 100 mg/kg body weight of the extract reversed these toxic effects of alloxan and restored the normal size and shape of the

cells in a manner similar to metformin (Plates 15, 17 and 18). However, 25 mg/kg body weight of the extract could not ameliorate these adverse effects (Plate 16).

Discussion

The 5-fold increase in blood glucose levels of alloxanised rats in the present study was in accordance and agreement with the report of Arika *et. al.,* (2016), who reported elevation of blood glucose level to 5-fold in alloxan-induced mice. The investigations of Ajiboso (2014), (2016) and (2018) at different period and season revealed repeated and consistent ameliorative property of aqueous extract of *Calotropis procera* leaf on elevated blood glucose resulting from alloxan induction.

The photomicrograph of liver histological studies reveals severely degenerated liver tissues in untreated diabetic rats. Exposure to alloxan has produced alterations in the hepatocytes, portal triads and sinusoids (Neyrinck, 2004). The alterations in the hepatocytes are mainly summarized as hydropic degeneration, cloudy swelling, fatty degeneration, portal and lobular infiltrate by chronic inflammatory cells and congestive dilated central veins (Reddy and Rao, 2006). The histological alterations may be an indication of injured hepatocytes due to alloxan toxicity (Neyrinck, 2004). The appearance of hepatocytes cytoplasmic degeneration and nuclear destruction may suggest that alloxan interacts with the antioxidant defense mechanism, leading to reactive oxygen species (ROS) generation. The ROS formed may induce stress in the hepatocytes to undergo atrophy and necrosis (Johar *et al.,* 2004). Degenerated liver tissues observed in distilled water – treated diabetic rats in this study explain the elevation of some of liver markers in the serum due to leakage from the damaged liver tissues. The regeneration of the liver tissue is an indication of protection offered to the organ by aqueous extract of *Calotropis procera* leaf in diabetic condition.

The histological studies of kidney of untreated diabetic rats showed clear signs of structural distortions that developed after a short period of hyperglycemia and was obvious even under the low magnification of light microscopy. It appears that the glomerulus is a principal site for the action of relative oxygen species leading to glomerulonephritis (Gwinner and Grone, 2000).

The structural changes in the glomeruli of the untreated diabetic rats are clear signs of glomerulonephritis and substantially compromise the filtration integrity of the glomeruli due to damage and dysfunction of endothelial cells (Schnachenberg, 2002) and podocytes (Susztak *et al.,* 2006). These structural and functional defects of the glomeruli may be

aggravated further by the glomerular cells own production of agents such as eicosanoids, chemokines cytokines, and growth factors, as well as proteases, which particularly damage and digest the glomerular filtration barrier (Ashraf *et al.*, 2004). In this study, hyperglycemia resulting from high level of glucose in the glomerular filtrate resulted to accumulation of glycogen in the kidney. These observed pathological changes were decreased in diabetic rats upon treatment with increased doses of aqueous extract of *Calotropis procera* leaf, particularly 100 mg/kg body weight of the extract. The glomeruli appeared normal and the cellular component arranged coupled with reduction in the glycogen molecules.

Red blood cells (RBCs) in diabetes have a more spherical shape than normal as evaluated by the spherical index (Deuticke, 2003). Diabetes is associated with alterations in RBC rheology; these observations suggest that the deformity of RBCs is shown in this disease. The reasons why RBCs are spherical in diabetic condition are multifactorial; these include modifications of RBCs membrane and reactive oxygen species (Mohanda and Chasis, 1993). Distilled water – treated diabetic rats showed reduction in serum antioxidant activity, and so may be more susceptible to free radical induced injury. The morphological examination of RBCs in this study which revealed crenation (disruption) of RBCs in distilled water – treated diabetic rats and 25 mg/kg body weight of the extract treated rats is caused by hyperglycemia resulting from alloxan toxicity. The amelioration of the deformity in RBC rheology by the extract at higher doses similar to that of metformin treatment is an indication that the extract can restore and also offer protection to the red blood cell morphology in diabetes.

Conclusion

As evident from the photomicrographs, the extract repaired scarred organs and also offered protection and restoration to red blood cells in administered groups when compared to non-administered group B.

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