



Erythrocyte stability, membrane protective and haematological activities of *Newbouldia laevis* in alloxan – induced diabetic rats

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Abstract

The high prevalence rate of diabetes mellitus (DM) in the developing world and its attendant high cost on healthcare have necessitated search for cheaper, effective and readily available alternative therapies in plants. One of such plants used in Nigeria is *Newbouldia laevis* (P. Beauv) (NLE). Its effect on erythrocyte fragility, membrane stability and haematological parameters in alloxan-induced diabetic rats for 21 days showed that *Newbouldia laevis* at 250 mg/kg reduced erythrocyte haemolysis to 11.08±2.50 % while Vitamin C (Ascorbic acid 200 mg/kg) reduced the haemolysis by 10.87±2.16 %. Glibenclamide (2 mg/kg) a standard oral antidiabetic drug reduced the haemolysis to 22.52±3.50 % all at the NaCl concentration of 0.85 %. It also demonstrated its ability to protect the liver, kidney and the pancreas especially at the dose rate of 250 mg/kg against alloxan-induced diabetic membrane destruction. It dose-dependently decreased the packed cell volume (PCV) from 43.67±7.34 % at the dose of 62.5 mg/kg to 33.64±6.34 % at the dose of 125.0 mg/kg and 28.33±3.67 % at the dose of 250 mg/kg. Haemoglobin concentration (Hb), reduced from 14.57±2.43 % at the dose of 62.5 mg/kg to 9.43±1.20 % at 250 mg/kg. But at the same time, it dose-dependently increased the white blood cell count (WBC) from 4.13±0.83 x 10³ at the dose of 62.5 mg/kg to 6.26±1.3 x 10³ at the dose of 250.0 mg/kg. In conclusion, *Newbouldia laevis* at 250 mg/kg has erythrocyte and membrane protection ability in alloxan-induced diabetic rats comparable to Vitamin C and glibenclamide, but has variable effects on haematological parameters that are within the normal ranges in diabetic rats.

Keywords: Diabetes, Erythrocytes, Haematology, Membrane stability, *Newbouldia laevis*

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Introduction

Diabetes mellitus (DM) is defined as a pathophysiologic condition in which there is excessive glucose in the blood due to disturbance in homeostasis of carbohydrate, protein and lipid metabolism regulated by the hormone insulin (Dewanjee *et al.*, 2008; Rakesh *et al.*, 2010). Blood glucose remains high in DM either because the body does not produce enough insulin or because cells of the body do not respond properly to the insulin that is produced (Oliveira *et al.*, 2008). This can also be referred to as insulin resistance (Turner *et al.*, 1998). The primary defect in glucose metabolism results in widespread multi-organ complications that ultimately encompass virtually every system of the body (Wadker *et al.*, 2008). The lack of absorption of glucose leads to

hypoglycaemia at the cellular level while there is hyperglycaemia in the blood/plasma. The body now breaks down the stores of fats and proteins to make glucose and ketones in the liver and this results to loss of weight in both humans and other animals (Szudelski, 2001). The breakdown of protein and decreased protein synthesis lead to general lethargy, hypoalbumina and decreased gamma globulins. The end result is negative energy balance, weight loss, increase susceptibility to infections and delayed wound healing. Hyperglycaemia cause both macro and microvascular damages to the cardiovascular system and some important organs like the kidney and retina (Tayyab *et al.*, 2012).

The currently estimated worldwide prevalence of DM is 8.3% and is projected to be 9.9% in the next 20 years (Daniel *et al.*, 2011; Staykova *et al.*, 2014). According to WHO (2013) about 377 million people had DM worldwide in 2013 and about 3.4 million died from consequences of high blood sugar in 2004. In Africa about 10.4 million people were reported to have DM in 2008 and this figure is expected to rise to 18.7 million by 2025 (Hamman, 2008). In Nigeria, with a population of about 160 million people (NPC, 2014) about 6 million people are reported to have full blown DM in Nigeria (NDF, 2014).

Newbouldia laevis (NLE) (P. Beauv) Seeman ex Bureau also known as "Border" or "Boundary tree" belongs to the family *Bignoniaceae* and has a genus of one species (Keay, 1989). It is widely used in traditional medicine in Nigeria and other West African countries to treat fever, ear aches, chest pain, convulsion and epilepsy in children (Keay, 1989; Burkil, 1994; Tanko *et al.*, 2008). The stem bark is used for treating skin infections while the roots are used to treat arthritis, general body pain and diarrhoea (NNMDA, 2006; Owolabi *et al.*, 2011). However, Tiv traditional medical practitioners in central Nigeria use the leaves of *Newbouldia laevis* to treat disease conditions that result in frequent urination and sweet urine (Bosha, 2015). The high prevalence rate of DM in the developing world and its attendant high cost on healthcare have necessitated searches for cheaper, effective and readily available alternative therapies in plants. Therefore, this growing interest in herbal medicine demands concise information on the effects of these herbal preparations on haematological parameters as well as the membrane integrity of erythrocytes because the function of an erythrocyte depends on the maintenance and stability of its membrane. It is known that in DM there are increased oxidative stress and free radical production which affect membrane stability of erythrocytes and can damage cellular molecules like proteins and lipids (Sadighara, 2009). Free radicals are also involved in the induction of DM by alloxan and streptozotocin through the destruction of β -cells (Szudelski, 2001). There are reports that NLE contain lots of antioxidants which are known to scavenge on the free radicals and can slow down the process of most chronic and metabolic diseases including DM (Ogunlana & Ogunlana, 2008). However, there is paucity of information on the effect of NLE on the erythrocyte membrane stability as quantified by erythrocyte osmotic fragility. Therefore, the aim of the work was to assess the protective effect of NLE on erythrocyte membrane as well as other organ membranes in diabetic rats.

Materials and Methods

Experimental animals and management

A total of forty-eight (48) adult wistar rats of both sexes with a weight ranging from 120.00 g-152.00 g were used for the study. The animals were fed with pelleted growers mash. The animals were allowed to acclimatize to the environment two weeks before the experiment commenced. They were housed in steel cages in houses with ambient temperature of 27-35°C and a lighting period of about 12 h day and night and a relative humidity of 40-60 %.

Experimental design

One (1 kg) of the leaves of *Newbouldia laevis* (NLE) were collected and identified by Mr. Terry Waya with a specimen number UAM/FHM/205 deposited at the Forestry herbarium at the University of Agriculture, Makurdi. The leaves were grinded and extracted by cold maceration using 80 % methanol. Rotary evaporator (Rotavapor-R-215) was used to concentrate and dry the extract *in vacuo* and the percentage yield was calculated. Forty-eight rats were randomly divided into 6 groups of eight animals per group. DM was induced by single intraperitoneal administration of 150 mg/kg of alloxan monohydrate (Acrose, New Jersey USA), as described by Szudelski (2001). The rats were observed until diabetes was established on the sixth day with the presence of fasting blood sugar (FBS) \geq 7.0 mMol, and other clinical signs which included glycosuria, frequent urination and loss of weight (WHO,1980). The FBS was measured using Accu-check Advantage II[®] (Roche Diagnostics, New Jersey USA). The rats were then treated as follows: Group 1 served as negative control and was given 10 ml/kg distilled water being the vehicle in which NLE was dissolved. Group 2 received 2 mg/kg of glibenclamide (M. P. Biomedicals Inc France), a reference antidiabetic drug while groups 3, 4 and 5 were given 62.5, 125.0 and 250.0 mg/kg of *Newbouldia laevis* leaves (NLE) respectively (Bosha, 2015). Group 6 was given vitamin C 200 mg/kg (Hopkin & William, England) (Chervyenkov *et al.*, 1977). The treatment with distilled water, reference drug, vitamin C and the extract doses were administered orally for 21 days through intragastric tube (Bosha, 2015). The FBS and weight of the rats were measured on days 0, 1, 7, 14 and 21. On the last day of treatment (day 21), 2 ml of blood samples were collected from the heart under light chloroform anaesthesia for haematological studies.

Determination of haematological parameters

Packed cell volume was determined using microhaematocrit method, haemoglobin

concentration (Hb) using the cyanomethaemoglobin method, total red blood cells (RBC) count, total leukocyte (WBC) count were determined using haemocytometric method (Coles, 1986).

Determination of erythrocyte osmotic fragility

Erythrocyte fragility test (OFT) was carried out as originally described by Parpart *et al.* (1947). It requires the preparation of a series of hypotonic solutions with NaCl content ranging from 0.1% to 0.9% to which 0.05 ml of fresh blood is added. The absorbance was read at 540 nm using distilled water as blank. This procedure was carried out for all the samples and the percentage (%) haemolysis was calculated using the formula;

$$\text{Percentage (\% Haemolysis)} = \frac{\text{Abs (test)}}{\text{Abs (distilled H}_2\text{O)}} \times 100$$

Where Abs (test) is absorbance of test

Abs (distilled H₂O) is absorbance of distilled water.

Erythrocyte osmotic fragility curve was obtained by plotting percentage haemolysis against the saline concentrations.

Histopathology

Some of the rats (3 per group) were later sacrificed under chloroform anaesthesia at the end of the experiment and the pancreas, liver and kidney were collected in bijoux bottles and preserved in 10 % formalin for histopathological studies (Bancroft & Stevens (1977). Sections were prepared and examined with a light microscope (Olympus) under various magnifications (x10, x40, x100 and oil immersion). Photomicrographs of lesions were taken with an Olympus photomicroscope for

observation and documentation of histopathological lesions. All applicable international, national and institutional guidelines for the care and use of laboratory animals were followed as described by NRC (1996).

Statistical analysis

The data collected for this experiment were expressed as mean \pm SEM (standard error of mean). Significance was tested at 5% level among groups using one-way analysis of variance (ANOVA). Least significant difference (LSD) and Duncan test were used to detect significant differences between means of treatment and control groups as well as among treatment groups with the help of Statistical Package for Social Sciences (SPSS) version 17.0. Figures, linegraphs and tables were used for data presentation.

Results

The lowest PCV value was obtained in the highest dose of NLE (group 5) with a value of 28.33 ± 3.67 %, however, the highest significant value ($p < 0.05$) was obtained in the lowest dose (group 3) with a value of 43.67 ± 7.34 %. The same trend occurred for Hb, MCV and MCH, while the highest value of $12.35 \pm 4.0 \times 10^6/\mu\text{l}$ of total RBC count was obtained in the glibenclamide, (group 2). For the WBC, the lowest value of $3.06 \pm 0.6 \times 10^3/\mu\text{l}$ was obtained in the negative treatment (group 1), while the highest value of $6.26 \pm 1.3 \times 10^3/\mu\text{l}$ was seen in the 250.0 mg/kg treatment (group 5) (Table 1).

The effect of NLE on erythrocyte osmotic fragility is presented in Figure 1. The minimum haemolysis of 11.08 ± 2.50 % was observed at 0.85 % NaCl concentration in 250.0 mg/kg NLE (group 5) rats.

Table 1: Effect of *Newbouldia laevis* extract on haematological parameters of alloxan- induced diabetic rats

Group	Treatment	PCV (%)	Hb (mg/dl)	RBC (X10 ⁶)	WBC (X10 ³)	MCV (ferntolitre)	MCH (picogramme)	MCHC (g/dl)
1	Dist H ₂ O (10ml/kg)	35.67 \pm 0.34	12.0 \pm 0.15	10.72 \pm 2.5	3.06 \pm 0.6	34.89 \pm 11.2	11.64 \pm 0.25	33.36 \pm 0.05
2	Glibenclamide (2mg/kg)	40.33 \pm 0.34*	13.43 \pm 0.13*	12.35 \pm 4.0*	3.60 \pm 0.6*	35.09 \pm 3.5	11.71 \pm 0.35	33.30 \pm 0.08
3	NLE (62.5mg/kg)	43.67 \pm 7.34*	14.57 \pm 2.43*	9.87 \pm 1.23	4.13 \pm 0.83*	45.72 \pm 2.64*	11.91 \pm 0.23	33.36 \pm 0.05
4	NLE (125mg/kg)	33.64 \pm 6.34	11.23 \pm 2.3	11.21 \pm 1.34	5.40 \pm 2.5*	29.78 \pm 2.64*	9.94 \pm 0.45	33.38 \pm 0.16
5	NLE (250mg/kg)	28.33 \pm 3.67*	9.43 \pm 1.2*	10.06 \pm 2.7	6.26 \pm 1.3**	29.29 \pm 4.2*	9.74 \pm 0.50	33.28 \pm 0.16
6	Vitamin C (200mg/kg)	40.23 \pm 2.06*	13.50 \pm 1.23*	11.50 \pm 2.34	4.05 \pm 0.9*	37.12 \pm 525	10.17 \pm 0.85	33.36 \pm 0.21

* $p < 0.05$, ** $p < 0.01$ when compared to the negative control

Glibenclamide (group 2) showed haemolysis of 22.52 ± 3.50 % at the same NaCl concentration while Vit C, (group 6) also showed a haemolysis of 10.87 ± 2.16 % at the same level. The other NLE treatments 62.5 and 125.0 mg/kg showed minimal haemolysis of 18.50 ± 3.35 % and 24.55 ± 3.25 % respectively. Group I (negative control) showed a haemolysis of 78.88 ± 2.5 % at 0.85 % NaCl. This shows that all the doses of the NLE significantly ($p < 0.05$) decreased haemolysis in the treated diabetic rats and their effects were comparable to that of group 2 (glibenclamide). The highest dose (250.0 mg/kg) was comparable to the effect of group 6 (Vitamin C) at 0.7 and 0.85 NaCl concentrations. At 0.5 % NaCl concentration, group 1 (negative control) caused a haemolysis of 99.5 ± 0.5 % while group 2 (glibenclamide) at the same 0.5 % NaCl concentration caused a haemolysis of 81.05 ± 1.2 %. The NLE treated groups (62.5, 125.0 and 250.0 mg/kg) produced 73.91 ± 5.2 , 98.68 ± 7.25 and 87.89 ± 2.3 % haemolysis respectively. Group 6 (Vitamin C) induced 56.93 ± 3.25 % haemolysis at 0.5 % NaCl.

Histopathology

The photomicrograph of a section of the pancreas (Plate 1) showed severe vacuolation (white arrow) and decrease in pancreatic islet cell mass (black arrow). These effects are more prominent in negative control group (Group 1) and the lowest NLE dose (62.5mg/kg) (Group 3). Glibenclamide (2 mg/kg) showed almost normal pancreatic cell mass (Group 2) compared to group 6.

The photomicrograph of the liver, (Plate II) showed varying degrees of fatty degeneration of hepatocytes indicated by vacuolated cells (black arrow). It was more prominent in the negative control group (Group 1) and the lowest dose of NLE (62.5 mg/kg) (Group 3). The groups treated with NLE 125.0 mg/kg (Group 4) and 250.0 mg/kg (Group 5) showed less vacuolation. The group treated with glibenclamide (2 mg/kg)(Group 2) showed almost normal architecture of the liver with radiating cords of hepatocytes with pinkish cytoplasm (white arrow) compared to the normal vitamin C group (group 6).

Plate III is the photomicrograph of the kidney. The kidney showed dilated and degenerated renal tubules in all the treated groups (white arrow). The effect was more in the negative control group

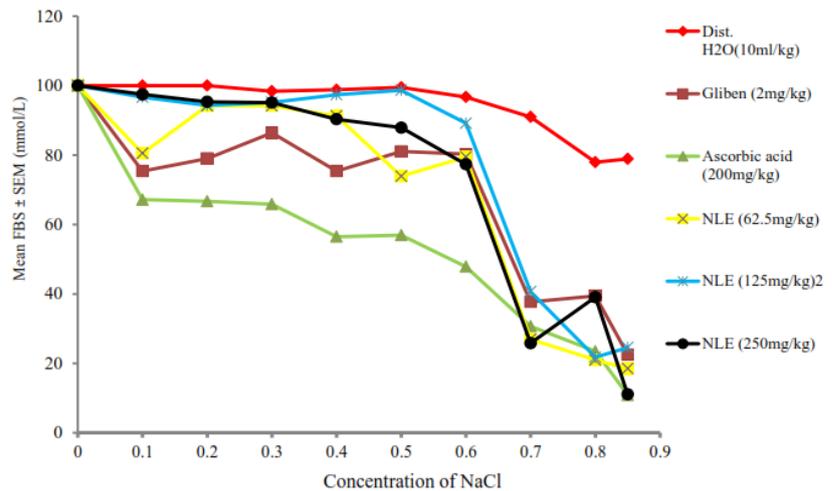


Figure 1: Effect of *Newbouldia laevis* extract on erythrocyte fragility of alloxan- induced diabetic rats

(Group 1) and the lowest NLE (62.5 mg/kg) treated group (Group 3). The groups treated with glibenclamide, 125.0 and 250.0 mg/kg NLE (Groups 2, 4 and 5) respectively did not show much signs of the degeneration of tubules.

Discussion

Haematological parameters are useful tools in evaluating the wellbeing of mammals as this determines oxygen carrying ability and ultimate delivery of oxygen to cells and tissues for metabolic activities (Pratt, 1985; Halim & Ali, 1996). Decrease in these values will affect oxygen utilization by cells and tissues. The result of this study showed decreased PCV, Hb and RBC count in group 1. But groups 2 and 3 showed significant ($p < 0.05$) increase (restoration) of these values except the RBC, while group 5 reversed the situation to the diabetic state (Table 1). It is postulated that non-enzymatic glycosylation of RBC membrane proteins increased in diabetes mellitus and correlated positively with hyperglycaemia (Kennedy & Baynes, 1984). Oxidation of glycosylated membrane proteins and hyperglycaemia cause an increase in production of lipid peroxidation, resulting in haemolysis of RBC (Crouch *et al.*, 1981). In DM also, the excess glucose present in the blood reacts with haemoglobin to form glycated haemoglobin (HbA1C). This leads to decrease in total haemoglobin level in alloxan-induced diabetic rats as reported by Sheela & Augusti, (1992). Increase in these three parameters PCV, Hb and RBC count by the low dose of NLE above group I (the control group) may be due to low level of lipid peroxidation, thereby maintaining the integrity of the membrane leading to decrease susceptibility of RBC to lysis and decrease in elevated glucose level. It has been documented that high lipid

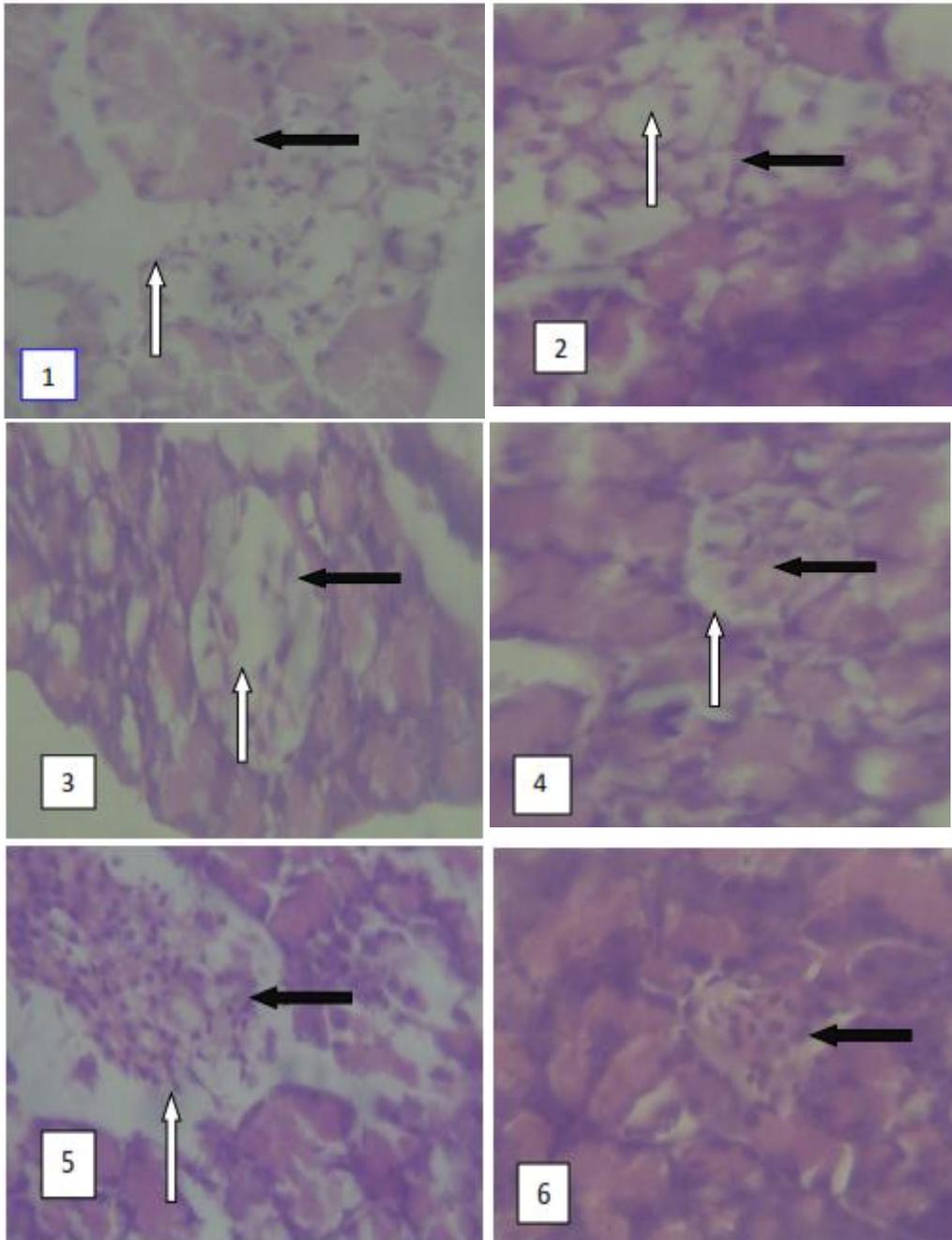


Plate I: Photomicrograph of sections of the pancreas from experimental rats showing severe vacuolation (white arrows) and decrease of pancreatic islet (black arrow) cells which is more prominent in the groups treated with distilled water (1) and 62.5mg/kg NLE (3) compared to group 2 (glibenclamide) and group 6 (Normal) which had intact islets. H and E x400

peroxidation by free radicals induces oxidative damage to erythrocytes (Adenkola & Onyeberechi, 2015). Another possible way this increase can occur is by stimulating erythropoiesis (Abu-Zaiton, 2010). Therefore higher doses of NLE might have an inhibitory effect on erythropoiesis resulting in the low values observed in this study.

All the doses of NLE significantly ($p < 0.05$) and dose-dependently increased the WBC count over the period of 21 days. This similar finding was also reported by Kolawole & Akanji (2013), though they

used higher doses of *Newbouldia laevis* (500 mg/kg). This could possibly be due to the fact that administration of NLE may stimulate a cell-mediated immune response. However, more study is needed to elucidate this fact.

Generation of free radicals occurs during stress-induced disease conditions (Akinwande & Adebule, 2003) including DM (Szudelski, 2001). Also the constant exposure of RBC to high oxygen tension coupled with high level of iron and richness in polyunsaturated fatty acids (Kolanjiappan *et al.*

2002) and their inability to possess nucleus and other organelles (Doroevic *et al.*, 2008) have made it a centre of free radical attack, hence RBCs are frequently used to evaluate oxidative stress (Brzezinska-Slebozinska, 2003; Sadighara, 2009; Adenkola & Onyeberechi, 2015).

The finding in this study indicated that administration of NLE was able to reduce lysis of RBCs and thereby reduction in the state of haemolysis. This suggested that administration of NLE possesses some inherent properties that assist in maintaining the membrane integrity of RBCs.

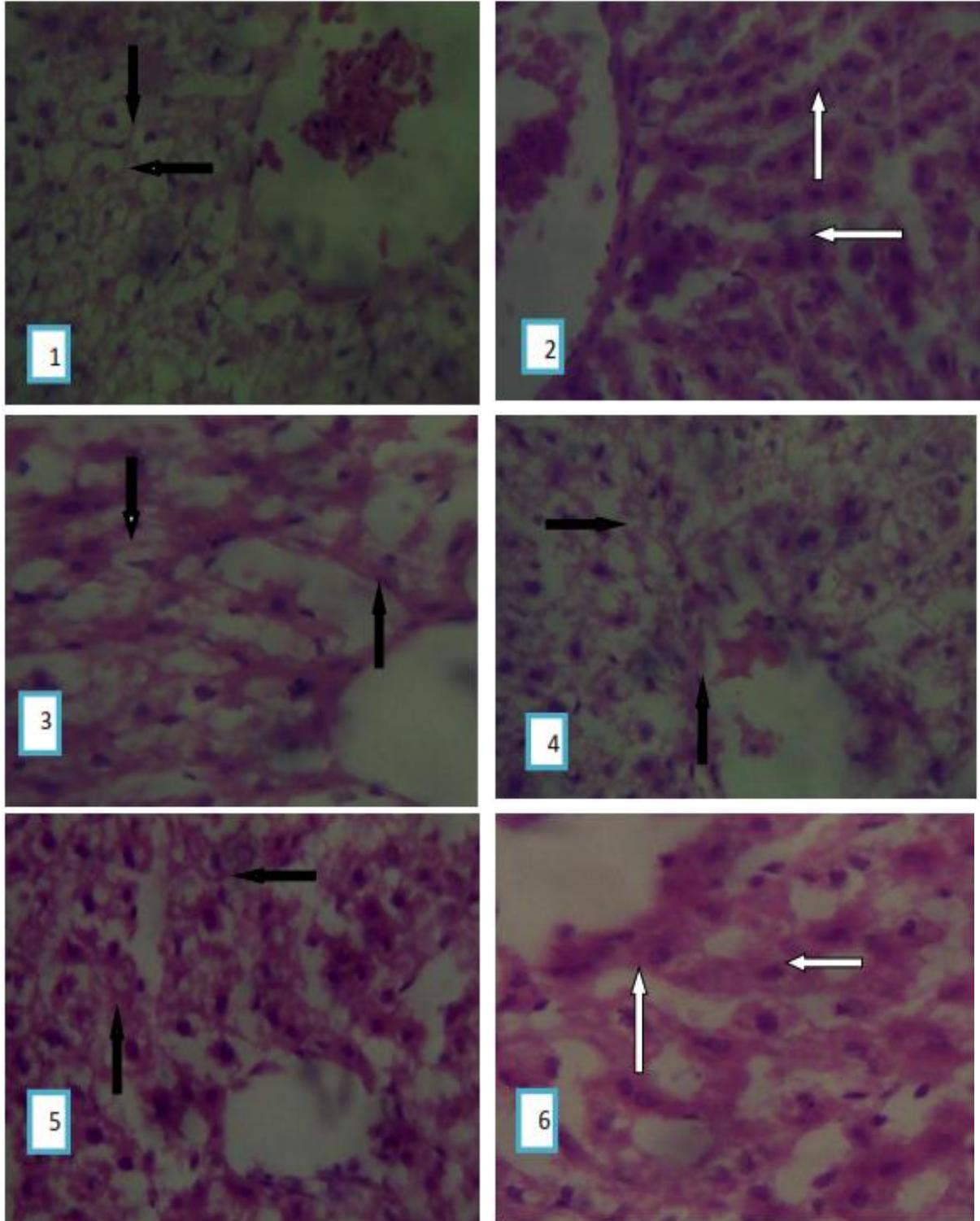


Plate II: Photomicrograph of liver sections from experimental rats after treatment showing varying degrees of fatty degeneration of hepatocytes (note the vacuolated cells-black arrows).This is more prominent in group treated with distilled water (1) and 62.5mg/kg NLE (3).See the near normal architecture of the liver in group 2 (glibenclamide) and group 6 (normal) as shown by the radiating cords of hepatocytes with pinkish cytoplasm (white arrows). H and E x400

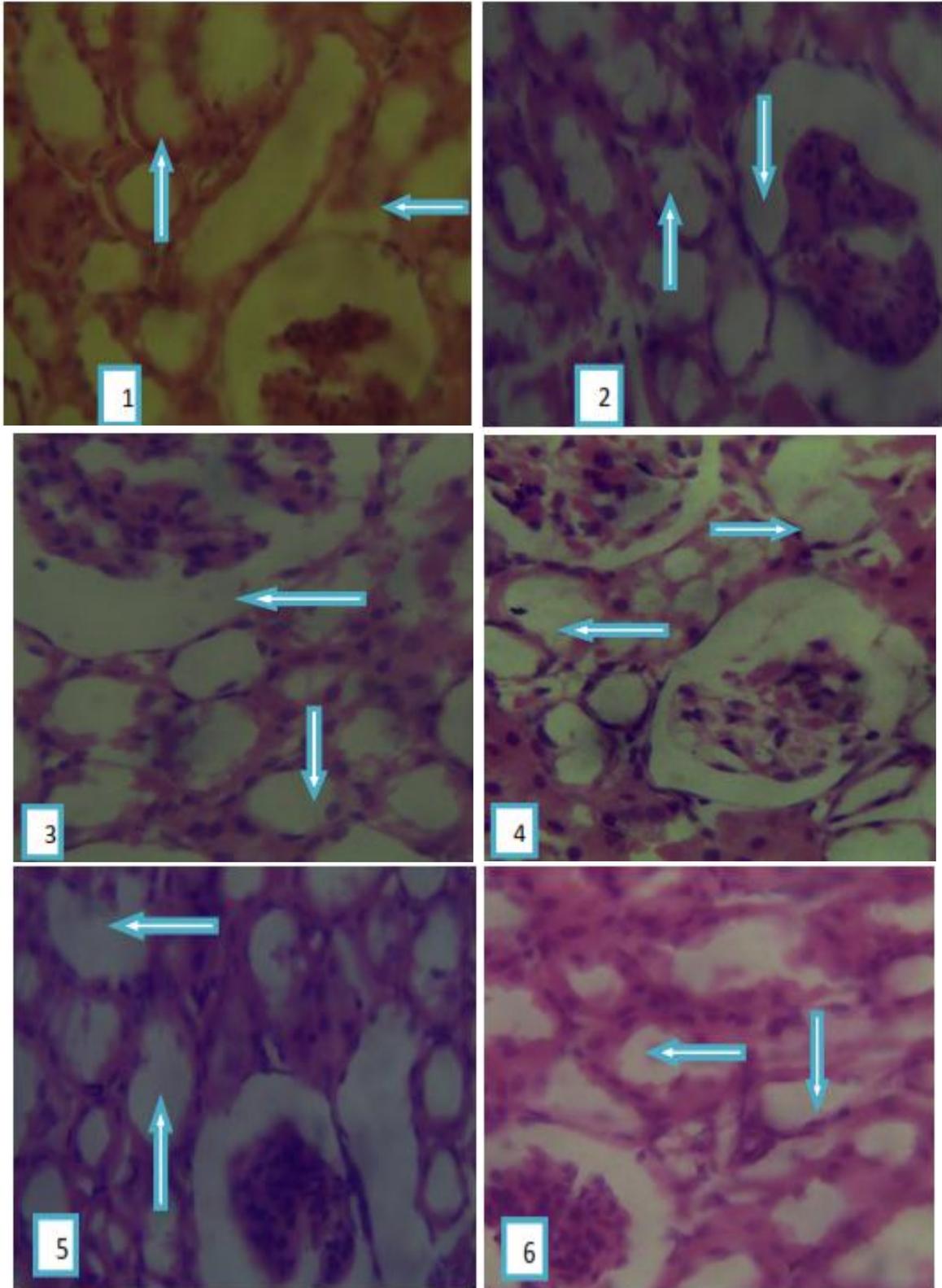


Plate III: Photomicrograph of kidney sections from experimental rats after treatment, showing dilated and degenerate renal tubules (arrows). This is more prominent in groups treated with distilled water (1) and 62.5mg/kg NLE (3) compared to group 2 (glibenclamide) and group 6 (Normal). H and E x400

Hence it could be said that NLE has membrane protective ability. Histopathological results of the pancreas showed degeneration of pancreatic islet cells due to the

necrotic action of alloxan monohydrate on the β – cells (plate I: 1 and 3). The degeneration resulted in the inability of the pancreas to produce and/or secrete adequate insulin that will control sugar

level therefore giving rise to DM, which is manifested by increased FBS in the untreated group of rats (Plate I. 1). These degenerative changes were seen to be ameliorated by NLE and glibenclamide administration followed by regeneration of some islet cells (Plate I: 2 and 5) with the attendant improved insulin production and reduction in FBS of the rats (Bosha, 2015). It has also been demonstrated that β -cells can regenerate from stem cells located in the pancreatic ducts or progenitor cells residing inside murine islets (Xu *et al.*, 2008). In extreme cases of β -cell loss, glucagon producing cells can differentiate into β -cells (Larsen, 2011). Metformin, a standard Biguanide oral antidiabetic also demonstrates this β -cell regeneration activity (Bailey 1992). The histopathology of the pancreas in this study showed the regeneration of β -cells or cell mass within the pancreas by higher NLE groups (4 and 5) perhaps, through a similar mechanism with metformin, but this is not yet clear. More studies are needed to elucidate the possible mechanism of β -cell regeneration. Many humoral factors have experimentally been shown to help in pancreatic cell regeneration (Frode & Medeiros, 2008).

The histopathology of the liver also points to the membrane protective effect of the NLE as compared to the negative control (Plate II:1) which showed much fatty degeneration (vacuolation) compared to the less vacuolation in glibenclamide plate II: 2 and higher doses of NLE (Plate II. 5) and the normal liver (Plate II. 6).

The histopathology of the kidney showed degeneration of renal tubules which was more in the negative control group (group 1) compared to

the NLE and glibenclamide-treated groups. This also points to the protective effect of NLE on the tubules, which is the major site of attack by free radicals (Gillery *et al.*, 1989), in DM (Plate III). Kidneys are major organs of excretion and play a very important role in glucose metabolism. Their role in gluconeogenesis and glucose excretion helps in the development, maintenance and resolution of hyperglycaemia (Marsenic, 2009). The kidney takes up glucose from circulation and reabsorbs glucose from glomerular filtrate. When hyperglycaemia remains unchecked, the blood glucose level rises above the renal threshold and can be seen in the urine; a condition known as glycosuria and subsequently polyuria (Wadker *et al.*, 2008; Gerich, 2010). The excessive loss of water in urine (polyuria) is usually compensated by increased thirst and subsequent increased water consumption (polydipsia). This ensures serious burden on the kidney and can lead to kidney failure in DM patient if not checked. The kidney responds to noxious substances or injuries in various ways ranging from decreased waste elimination to cell death. Hamman (2010) reported that diabetics are 104 times more likely to have renal failure, 41 times more likely to have infection and 13 times more likely to die of cardiovascular disease than normal individuals. In this study, the highest dose of NLE was able to protect the pancreas, liver and the kidney of the diabetic rats (Plates I-III).

In conclusion, the administration of NLE at 250 mg/kg has membrane protective ability on the RBC, pancreas, liver and the kidney in alloxan-induced diabetic rats comparable to Vitamin C as seen in this study.

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