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Nephroprotective Effects of *Balanites aegyptiaca* (*l.*) *delile* Stem-Bark Extract and Fractions against Diclofenac-Induced Nephrotoxicity in Rats

Ogbonnaya Mba[1]*, Ilodigwe E. Emeka[1], Ihekwereme C. Peter[1], Erhirhie E. Oghenesuvwe[2], Amedu J. Osaremen[1], Ajagu Nnenna[3]

[1]*Department of Pharmacoloy and Toxicology, Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Nigeria*
[2]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Chukwuemeka Odumegwu Ojukwu University, Igbariam, Nigeria*
[3]*Department of Pharmacology and Toxicology, Faculty of Pharmaceutical Sciences, Enugu State University of Science and Technology, Enugu, Nigeria*

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ABSTRACT

Balanites aegyptiaca (*B. aegyptiaca*) is a medicinal plant locally used in Northern Nigeria against kidney-related diseases, but no scientific proof yet. The study evaluated the nephroprotective effects of *B. aegyptiaca* stem-bark extract and fractions. Wistar rats were divided into six (6) groups of six animals each as follows: normal control, induced control, Silymarin (positive control, 100 mg/kg) and *B. aegyptiaca* extract (250, 500 and 1000 mg/kg). Nephrotoxicity was induced in all groups except normal control by oral administration of Diclofenac sodium (15 mg/kg/day, p.o). Treatments were administered orally concomitantly with Diclofenac sodium for 15 days. On the 16th day, Wistar rats were sacrificed and blood samples were collected for the estimation of serum levels of creatinine, urea, total protein, albumin, malondialdehyde (MDA), Catalase (CAT), Superoxide dismutase (SOD) and reduced glutathione (GSH) levels. N-hexane, ethyl acetate and n - butanol fractions were also tested alongside the extract at 250 and 500 mg/kg. Kidneys were harvested for histopathological analysis. N- Butanol fraction at 500 mg/kg significantly ($p<0.05$) decreased the elevated serum levels of MDA, urea and creatinine and significantly ($p<0.05$) increased the serum levels of albumin, SOD, CAT and reduced GSH. Ethyl acetate fraction (250 mg/kg) also caused significant changes on the altered serum levels of creatinine, urea, total protein and albumin and oxidative stress biomarkers. Histopathological analysis showed that 15 mg/kg Diclofenac sodium caused nephrotoxicity while the extract and fractions ameliorated the nephrotoxic effects. From this study, stem-bark extract and fractions of *B. aegyptiaca* were protective against Diclofenac induced nephrotoxicity.

***Keywords*:** *Balanites aegyptiaca*, Nephroprotective, Diclofenac sodium, Renal biomarkers, Antioxidant parameters.

Introduction

Globally, an estimated 5-10 million people die annually from kidney-related diseases according to World Health Organization.[1] In sub-Saharan Africa, the estimation is alarming in view of the fact that the global prevalence of maintenance dialysis has doubled since 1990 and the renal replacement therapy was accessed to be about 1.8 million people worldwide in 2004.[2] In Nigeria, the peak prevalence of kidney disease especially the chronic kidney disease (CKD) is between the first 10 to 50 years of life, thus contributing to manpower shortage and economic wastage. Age, hypertension, obesity, diabetes mellitus and prolonged use of non-steroidal anti-inflammatory drugs (NSAID) have been identified as risk factors for chronic kidney disease.[3]

The kidney is one of the vital organs in the body that functions to maintain homeostasis, regulates salts and water balance (osmoregulation) and acid-base balance; cleaning and secreting metabolites like urea, uric acid, creatinine and minerals from the blood and removal of nitrogenous wastes along with water as urine.[4]Diclofenac is one of the most commonly used non-steroidal anti-inflammatory drugs (NSAIDs) worldwide which can easily be sourced over the counter with an estimated usage of 30 million daily.[5] This drug exerts anti-inflammatory, analgesic and anti-pyretic effects through the suppression of prostaglandin (PG) synthesis, by inhibiting the enzyme cyclooxygenase (COX).[6] The kidneys are important targets for unwarranted clinical events associated with the use of non-steroidal anti-inflammatory drugs such as diclofenac among others.[7] The frequent and overuse of diclofenac to treat common ailments such as fever, pain and inflammation can actually lead to renal pathologies such as acute renal failure (ARF), chronic interstitial nephritis and nephrotic syndrome.[8] Available medications for the treatment of end-stage renal disease (ESRD) are hemodialysis and transplantation which have proven to be expensive and not accessible to most people in rural areas as they are time consuming and possess adverse side effects. Hence, there has been a continuous search for drugs that can protect the kidney from toxins (that can affect the normal functions of renal cells) and be used as adjunct in treating various kidney-related diseases.[9]

World Health Organization has encouraged the use of medicinal herbs for prevention and treatment of various diseases due to its affordability, abundance and little or no side effects.[8] *B. aegyptiaca*) is a medicinal plant used mainly in Northern Nigeria. It is commonly known as desert date, "Aduwa" in Hausa, "Enyi-ndimmuo in Igbo and "Tanni" in Fulani. It belongs to the family of Zygophyllaceae and it can be found in different habitats, tolerating a wide variety of soil types, from sand to heavy clay and climatic moisture levels.[10] *B. aegyptiaca* is widely used in folk medicine in the treatment of various

*Corresponding author. E mail: **mbabte@yahoo.com**
Tel: 07038908868

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ailments such as jaundice, intestinal worm infection, wounds, malaria, syphilis, epilepsy, dysentery, constipation and diarrhea, hemorrhoid, stomach ache, asthma and fever.[10] In East Africa, *B. aegyptiaca* is widely used as an anti-helminthic.[11] The root is used in various folk medicines for the treatment of abdominal pain and as a purgative.[10] The root, bark, kernel and the fruit have been shown to be lethal to mollusks.[11] In the Forfure community in Adamawa State, Nigeria, the stem-bark is macerated in water or alcohol for 2-3 days after which it is taken twice daily for kidney stones, edema, and other related diseases. The use of this plant in protecting kidney-related diseases in folklore medicine has not been scientifically proven. The study validated the traditional use of *B. aegyptiaca* in kidney disease.

Materials and Methods

Plant collection and authentication
Fresh stembark of *B. aegyptiaca (Linn) Delile* (Desert date) were collected from Forfure district in Adamawa State, Nigeria with the help of a Herbalist at about 6:30 pm in the evening, on 24th May, 2019, and the plant material was authenticated by Dr. Egungbo, A. J. of Forest Herbarium, Ibadan (FHI) with voucher No. 112537.

Equipment
Visible Spectrophotometer (721G, Zhejiang Top Cloud-Agri Technology Co., Ltd., China), refrigerator (Haier Thermocool), electronic weighing balance (WANT Balance Instrument Co. Ltd, China), centrifuge (Filtertech, Inc. Manilius, NY), water bath (Bio Technics, Mumbai, India).

Chemicals and reagents
Diclofenac sodium 100 mg (Voltaren by Norvatis, Swissland). Silymarin 150 mg and Dimethyl sulfoxide (DMSO), sodium chloride and sodium hydroxide, ethanol, ethyl acetate, n-butanol, n-hexane and hydrogen peroxide (Guangdong Guanghua Sci., Tech. Co., Ltd., China), Standard kits for creatinine, urea, total protein and albumin (Randox Laboratories Ltd, UK), DTNB (dithio-bis-nitrobenzoic acid) (Central Drug House (P) Ltd., New Delhi, India), epinephrine,Thiobarbituric acids (Loba Chemie Pvt Ltd., India).

Experimental animals
Wistar rats (100 - 150) g of either sex obtained from the animal house of the Faculty of Pharmaceutical Sciences, Nnamdi Azikiwe University, Awka, Anambra State, Nigeria were used. The animals were housed under standard environmental conditions and allowed to acclimatize for 7 days. The rats were fed with Growers finisher (Grand Cereals Ltd., Onitsha, Anambra State) and given access to drinking water *ad libitum*. All experiments were performed in compliance with institutional and international policies governing the human and ethical treatment of experimental animals as contained in the United States National Institute for Health Guidelines (1985) as approved by the Faculty of Pharmaceutical Sciences Postgraduate Research committee.

Preparation of ethanol extract
The stembarks were properly air-dried at room temperature for 14 days. Thereafter, they were ground into powder using an electric mill and sieved (size 2 mm) to obtain fine powder. One kilogram (1 kg) of the fine powder was macerated in 4 L of 80% ethanol for 72 hrs. The mixture was filtered using Whatman's filter paper No1 and the filtrate concentrated under reduced pressure using a rotary evaporator at 40°C.[11]

Fractionation of the extract
A portion of the extract (70 g) was reconstituted in 250 ml distilled water and successively fractionated in separating funnel using three (3) solvents (n-hexane, ethyl acetate and n-butanol) in increasing order of polarity to yield n-hexane, ethyl acetate and n-butanol fractions respectively. Each fraction was concentrated to dryness using a water bath at a temperature of 40°C and stored in the refrigerator prior to the experiment.[13]

Acute toxicity study
The mean lethal dose (LD_{50}) study was done using the method described by Erhirhie *et al*.[14] Animals were fasted overnight prior to the study and was carried out in two phases. A total of thirteen (13) Wistar rats of either sex were used to determine the lethal median dose, LD_{50} of the extract. Nine (9) animals that were used in the first phase were grouped into three (3) groups of three animals each. Each group received 10, 100, and 1000 mg/kg of the extract. They were observed for 24 h to 72 h for signs of toxicity and mortality. The second phase involved four (4) groups of one animal each. Animals were treated with higher doses, 2000, 3000, 4000 and 5000 mg/kg of the extract and were observed for another 24 hrs. to 72 hrs. for any abnormal behavioral changes and mortality.
The LD_{50} of the extract was calculated using the formula;

$LD_{50} = (\sqrt{D0} * D100)$ …… equation 1
Where D0 = highest dose that produced no mortality
D100 = lowest dose that produced mortality

Phytochemical analysis
The test was carried out according to the procedures described by Trease and Evans.[15] Ten percent (10%) preparation of the extract and fractions in distilled water were considered as the test samples. Distilled water was used as a negative control throughout the phytochemical tests.

Measurement of body weight of rats
The body weights of the rats were evaluated weekly and recorded using sensitive digital weighing balance according to Patrick-Iwuanyanwu *et al*.,.[16] The mean body weight and percentage (%) changes in the body weight were determined using the formula:

$$\text{Percentage (\%) change} = \frac{[Fbw - Ibw]}{Fbw} \times 100 \ldots\ldots\ldots\ldots \text{ equation 2}$$

Where Fbw = final mean body weight
Ibw = initial mean body weight

Experimental design
Preliminary study
The effects of the standard drug (Silymarin 100 mg/kg) and stem-bark extract of *B. aegyptiaca* were screened on Diclofenac-induced nephrotoxicity model in Wistar rats. Thirty-six (36) rats of either sex (100-150) g were randomly selected into six (6) groups of six (6) rats each and treated orally with the extract in 5% dimethyl sulfoxide (DMSO).

Group 1 (Normal control) received 10 ml distilled water and served as an untreated group.
Group 2 (Induced control) received 15 mg kg^{-1} Diclofenac
Group 3 (Standard control) received Silymarin 100 mg kg^{-1}
Groups 4, 5 and 6 of the animals were treated with 1/20th, 1/10th and 1/5th of the LD_{50} as low, medium and high doses of the extract respectively[17] for 15 days. Nephrotoxicity was induced by oral administration of 15 mg/kg Diclofenac sodium in 5% DMSO as a vehicle for 15 days[18] and on the last day, blood samples were collected from retro-orbital venous plexus of the rats for the estimation of renal function biomarkers (creatinine, albumin, urea and total protein) and lipid peroxidation parameter (MDA). The rats were sacrificed, and blood samples were collected via cardiac puncture, into plain tubes for biochemical assays.

Fractionation study
After the preliminary study, the fractions (n-hexane, ethyl acetate and butanol) of the extract were screened for nephroprotective activity alongside the effective dose of the crude extract. Sixty-six (66) rats were divided into eleven (11) groups of six (6) rats each. Half of the effective dose (dose 1) and the effective dose (dose 2) obtained from preliminary study of the extract were selected and used for the fraction and treated orally in 5% DMSO for 15 days. Grouping was done as follows:

Group 1 Normal control (non-induced control) received 10 ml distilled water
Group 2 Induced control, received 15 mg/kg Diclofenac
Group 3 Standard control (Silymarin 100 mg/kg)
Group 4 250 mg/kg crude extracts (half of effective dose, dose 1)
Group 5 500 mg/kg crude extract (effective dose, dose 2)
Group 6 250 mg/kg ethylacetate fraction
Group 7 500 mg/kg ethylacetate fraction
Group 8 250 mg/kg butanol fraction
Group 9 500 mg/kg butanol fraction
Group 10 250 mg/kg n-hexane fraction
Group 11 500 mg/kg n-hexane fraction

On the last day, animals were sacrificed after anesthetizing with chloroform and blood samples were collected and stored for the estimation of renal biomarkers (albumin, creatinine, total protein and urea) and markers of oxidative stress (lipid peroxidation (MDA), catalase, superoxide dismutase and reduced glutathione).

Biochemical assays
The blood samples were allowed to clot and centrifuged at 3000 rpm at 30^0C for 15 minutes and separated serum was used for the following biochemical assays using commercially available kits from Randox Laboratories Ltd. UK, as previously reported: Creatinin,[19] Urea,[20] Albumin, [21] and Total protein[22]. All the renal markers were measured according to the manufacturer's protocols.

Biochemical assays of markers of oxidative stress
MDA content was determined according to the earlier method reported by Aydin.[23] CAT activity was assayed from the rate of decomposition of H_2O_2 by the method of Sinha.[24] SOD activity was estimated using the method previously reported by Assady *et al*.[25] Reduced GSH activity was determined when reacted with dithiobisnitrobenzoic acid (DTNB) to give a compound that absorbs at 412 nm as previously reported by Moretti *et al*..[26]

Histopathological studies
At the end of the experiment, animals were sacrificed, and their kidneys were carefully dissected out en bloc for histopathology. After rinsing in normal saline, sections were taken from each harvested kidney, fixed in 10% formalin, dehydrated in gradual ethanol (50-100%), cleared in xylene and embedded in paraffin wax. The 5-6µm cut sections were prepared and stained with haematoxylin and eosin (H-E) dye for microscopic observation of histopathological changes.[27]

Statistical analysis
All results were expressed as Mean ± standard error of mean (Mean ± SEM). Comparisons of groups with the controls were performed using One-way-analysis of variance (ANOVA) with SPPS version 20. The significant differences between groups were considered at ($p < 0.05$) level.

Results and Discussion

The nephroprotective effects of *B. aegyptiaca* stem-bark extract and fractions against Diclofenac induced-nephrotoxic rats was evaluated by estimating biochemical, anti-oxidative parameters and renal histopathology. The result of the toxicological study of the extract of *B. aegyptiaca* showed that the extract was not toxic at 5000 mg/kg and without any clinical signs during the period of observation, suggesting that it may be safe following single-dose administration. This is in line with the finding from an earlier study Ugwah *et al*[10] which estimated the LD_{50} of the stem-bark extract to be above 5000 mg/kg.
The phytochemicals found to be present in the stem-bark extract and fractions were saponins, flavonoids and tannins. They were abundantly present in the crude extract, n-hexane and ethyl acetate fractions but in small amounts in butanol fraction. Alkaloids were moderately high in n-hexane fraction but in small concentrations in crude extract, ethyl acetate and butanol fractions. Other bioactive molecules present are terpenoids, glycosides, steroids and proteins, carbohydrates, fats and oil (Table 1). This is in agreement with Farrid *et al.*[28] and Ibrahim[29] who stated that *B. aegyptiaca* extract possesses saponins, flavonoids, and tannins in abundant concentrations and other bioactive molecules in moderately high concentrations. Tannins have been reported to possess vasodilatory activity[30] and as a renal vasodilator it can improve the glomerular filtration rate (GFR) and urine output. The presence of tannins in the stem-bark extract of *B. aegyptiaca* suggests its ethno-medicinal use as diuretics in Northern part of Nigeria. Hence, terpenoids, tannins and flavonoids present in the extract and fractions may have contributed to the protection from renal damage induced by Diclofenac by their antioxidant and vasodilatory as well as diuretic actions.
The result of changes in body weight of rats treated with *B. aegyptiaca* extract and fractions is presented in Table 2. There was a reduction in body weight of animals by 13.01% in diclofenac-induced nephrotoxic group compared to non-induced control and other groups and gain weight minimally. Minimal weight loss of 3.17% and 3.51% were observed in the 500 mg/kg extract and butanol fraction treated groups respectively. Higher level of weight loss in diclofenac control (without any treatment) may be attributed to nephrotoxic effect of Diclofenac, which has been reported in other studies.[31]
Nephrotoxicity is one of the most notable side effects and therapeutic limitations of analgesic drugs including Diclofenac.[31] Oxidative stress-related renal disease, induction of renal tubular necrosis, glomerular damage and renal inflammation are some of the major incidences implicated in analgesia induced nephrotoxicity.[32] The stem-bark extract and fractions were investigated for its further development as a natural medicinal agent for renal health. Nephrotoxicity caused by Diclofenac may result from its accumulation in the mitochondria of renal epithelial cells and induction of ROS primarily by decreasing the activity of antioxidant enzymes and depleting intracellular concentration of GSH and also causing the peroxidation of membrane lipids.[33] Similar observation was made in Cisplatin-induced nephrotoxicity.[34]
The result of the pilot study to determine the level of nephrotoxicity caused by diclofenac is shown in Table 3. There was a significant elevation in serum level of urea, malondialdehydes and creatinine and significant decrease in serum levels of total protein and albumin showing that at 15 mg/kg of diclofenac was nephrotoxic in rats.
The effects of *B. aegyptiaca* crude extract on serum total protein, albumin, creatinine, and urea are presented in Table 4.

Table 1: Phytochemical Analysis of the extract and fractions of *B. aegyptiaca*

Phytochemicals	Crude extract	n-hexane fraction	Ethyl acetate fraction	Butanol fraction
Saponins	+	+	+	+
Tannins	+	+	+	+
Reducing Sugars	+	+	+	+
Flavonoids	+	+	+	+
Alkaloids	+	+	+	+
Glycosides	+	+	+	-
Steroids	+	+	+	+
Fats and oils	+	+	+	+
Proteins	+	+	-	-
Acidic compounds	-	-	-	-
Terpenoids	+	+	+	+
Anthraquinones	+	-	+	-

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Table 2: Effects of *Balanites aegyptiaca extract and fractions on* body weight of diclofenac- induced nephrotoxic rats

Treatment (mg/kg)	Initial body weight (g)	Final body weight (g)	Weight change (%)
Normal control	137.40 ± 3.80	140.80 ± 3.97	2.41
Induced control	129.80 ± 0.91	134.50 ± 1.89	-13.01
Silymarin, 100	129.80 ± 2.40	131.80 ± 3.46	1.52
Extract, 250	133.20 ± 2.99	134.00 ± 2.98	0.60
Extract, 500	122.00 ± 0.91	118.25 ± 6.56	-3.17
Ethyl acetate fraction, 250	126.75 ± 1.18	128.50 ± 4.57	1.36
Ethyl acetate fraction, 500	130.80 ± .56	132.60 ± 4.27	1.36
Butanol fraction, 250	126.40 ± .69	126.60 ± 4.03	0.16
Butanol fraction, 500	125.25 ± 2.21	121.00 ± 7.10	-3.51
N-hexane fraction, 250	128.60 ± 2.38	131.00 ± 4.79	1.83
N-hexane fraction, 500	123.60 ± 2.06	125.60 ± 2.40	1.59

Values are presented as mean ± Standard error of mean (SEM), n = 6.

Table 3: Nephrotoxic effects of Diclofenac-induced rats

	Albumin (g/dL)	Creatinine (g/dL)	Urea (g/dL)	Total protein (g/dL)	MDA (mmol/dL)
Control group	3.61 ± 0.26	0.75 ± 0.13	17.89 ± 5.00	4.30 ± 0.60	2.66 ± 0.28
Induced group	2.07 ± 0.40	2.48 ± 0.17*	30.89 ± 0.30*	2.90 ± 0.00*	4.20 ± 0.14*

Values are presented as mean ± Standard error of mean (SEM), n = 6. *P< 0.05 is significantly different from control group.

Table 4: Effects of *Balanites aegyptiaca Del.* stem-bark crude extract on serum Biochemical renal markers

Treatment (mg/kg)	Total protein (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)	Albumin (g/dl)
Normal control	7.15 ± 0.25	1.02 ± 0.14	21.47 ± 2.00	3.89 ± 0.18
Induced control	4.46 ± 0.39*	2.93 ± 0.16*	29.44 ± 3.64	2.51 ± 0.29*
Silymarin,100 mg/kg	6.23 ± 0.22[a]	1.10 ± 0.16	21.09 ± 2.51*	3.49 ± 0.12*[a]
Extract, 250	6.05 ± 0.58[a]	2.04 ± 0.18	23.15 ± 4.46*	3.38 ± 0.17[a]
Extract, 500	6.73 ± 0.37[a]	1.02 ± 0.04*[a]	20.99 ± 0.04*	3.73 ± 0.13[a]
Extract, 1000	7.22 ± 0.42[a]	1.04 ± 0.21	21.05 ± 2.63*[a]	3.71 ± 0.03*[a]

Values are presented as mean ± Standard error of mean (SEM). N = 6; *P < 0.05: Significantly different from groups 1 (Normal control). [a]P < 0.05: Significantly different from groups 2 (Induced control).

There was significant (p<0.05) reduction in the levels of total protein and albumin as well as significant (p<0.05) elevation in levels of creatinine and urea in Diclofenac-induced groups when compared with normal control (untreated groups). Treatment with the standard drug (Silymarin, 100 mg/kg) and extract (250, 500 and 1000 mg/kg), caused significant (p < 0.05) increases in the levels of total protein and albumin and reduction in serum levels of creatinine and urea.

Diclofenac sodium is an analgesic which exerts its function by preventing the conversion of arachidonic acid to prostaglandins by reversibly inhibiting cyclooxygenase enzymes in the renal cells.[5] The inhibition of prostaglandin synthesis is the initiating factor for the pathological changes in kidney injury.[35] This results in reduction in renal functions, thus, causing accumulation of urea and creatinine as well as reduced levels of serum albumin and total protein.[36]

The results of the effects of *B. aegyptiaca* stem-bark crude extract on serum malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and reduced glutathione (GSH) is presented in Table 5. There were significant (p < 0.05) increases in serum levels of MDA and decreased levels of serum SOD, CAT and GSH in diclofenac-induced group (positive control) when compared to normal untreated group (Negative control). Treatment with standard drug (Silymarin 100 mg/kg) and the crude extract (250, 500 and 1000 mg/kg) significantly (p < 0.05) elevated the serum levels of SOD, CAT and reduced GSH and decreased the serum level of MDA towards normal when compared to induced untreated (positive) group.

The increased lipid peroxidation parameter, malondialdehyde suggests that Diclofenac may cause an increase in reactive oxygen species (ROS) in the renal cortex, thus, increased cellular oxidative stress and decreased intracellular antioxidant activities of the cell.[37] The significantly (P < 0.05) high serum levels of creatinine and urea in Diclofenac sodium-induced groups when compared to normal (negative control) non-induced groups substantiate the fact that there was renal injury.

The effects of *B. aegyptiaca* extract and fractions on serum renal biomarkers (total protein, albumin, creatinine and urea) are presented in Table 6. There was significant (p<0.05) increases in serum levels of creatinine and urea in diclofenac induced group when compared to normal (negative) group. The serum levels of total protein were reduced, but the reduction was significant (p<0.05) in serum levels of albumin. However, treatment with the extract 500 mg/kg, standard drug (Silymarin 100 mg/kg) and butanol fraction (500 mg/kg) significantly (p< 0.05) decreased the serum levels of urea and creatinine in Diclofenac induced group (positive control) when compared to normal control. The extract 500 mg/kg, butanol fraction 500 mg/kg and the silymarin 100 mg/kg significantly (p>0.05) increased the serum level albumin. The ethyl acetate fraction 250 mg/kg and n-hexane fraction 500 mg/kg also caused a significant (p< 0.05) reduction in serum creatinine and urea levels increased the levels of total protein and albumin when compared to positive control groups.

Chinnappan *et al*[27] also showed that herbal extract of *Eurycoma longifolia* decreased the elevated serum levels of renal biomarkers in NSAID induced hepato-reno- toxicity. The significant decrease ($p<0.05$) in serum levels of albumin and total proteins in Diclofenac induced groups when compared to normal control may be due to arylation of proteins by 5-hydroxyl diclofenac which is involved in reduced renal function. The n-hexane and ethyl acetate fractions could not exert significant increase to the serum albumin when compared to silymarin (100 mg/kg) treated group. However, the extract 500 mg/kg and butanol fraction 500 mg/kg significantly ($p<0.05$) increased the levels of serum albumin which were decreased in Diclofenac-induced group. This is in agreement with El-ashmawy[18] which showed that treatment with 15 mg/kg Diclofenac caused an increase in serum levels of creatinine, urea and malondialdehydes as well as decreases in serum levels of albumin and total protein. The effects of *B. aegyptiaca* extracts and fractions on serum antioxidant parameters (MDA, SOD, GSH, and CAT) are presented in Table 7. Treatment with Diclofenac significantly ($p<0.05$) elevated the levels of malondialdehyde and decreased the serum concentration of reduced GSH, Superoxide dismutase, and catalase when compared with the normal untreated groups. Treatment with Silymarin significantly reduced MDA levels while increasing GSH, SOD, and CAT levels when compared to Diclofenac induced group. The higher doses (500 mg/kg) of the extract, butanol and n-hexane fractions reduced the MDA levels while increasing GSH, SOD and CAT when compared with Diclofenac-induced group (positive control). This is similar to the result obtained in the extract suggesting that the fraction derived from the extract also possess nephroprotective activities.

Secondary metabolites such as alkaloids, saponins, flavonoids, tannins, steroids, and terpenoids found in the extract and fractions may have contributed to reduction in the amount of crystals produced in the calcium oxalate metastable solution, thereby inhibiting various processes of nucleation and aggregation of the stones involved in the calcium oxalate stone formation.[38] This supports the use of stem-bark extract of *B. aegyptiaca* to treat kidney stones by the local populace. The reduction in serum creatinine and urea suggests that the extract and fractions (especially the butanol fraction) possess bioactive molecules which could improve renal functions by normalizing deranged renal biomarkers of kidney injury caused by Diclofenac. This is in agreement with earlier studies.[39,40] which showed that the *B. aegyptiaca* extract caused reduction in elevated levels of creatinine and blood urea nitrogen in CCl_4 induced hepatotoxicity.

Samreen *et al* [41], showed that alkaloids containing berberine is used in the treatment and removal of kidney stones and ameliorates proteinuria in type-2 diabetics, diabetic nephropathy and reduce oxidative stress in streptozotocin diabetic rats. This suggests that a high dose of the stem bark of *B. aegyptiaca* could minimize the toxic effects of diclofenac due to the presence of secondary metabolites such as alkaloids, flavonoids, tannins and saponins among others.

The decreased serum albumin may be due to albuminuria which is a clinical marker of diabetic nephropathy or glycation and might also be due to increased protein catabolism.[42] Albumin also acts as a major modulator of plasma oncotic pressure and functions to transport a variety of substances. Decrease in serum total protein and albumin of the kidney contents is a reflection of renal toxicity. The reductions of protein in Diclofenac intoxicated group indicate depletion in the protein reserve and thus suggest nephrotoxicity. Therefore, treatment of nephrotoxic rats with extract and fractions of *B. aegyptiaca* improved serum albumin levels. This may be due to restoration of blood glucose levels by the plant extract and fractions thereby reducing the intensity of protein glycation in the nephrotoxic animals. Saponins from fruits of *Solamm. xanthocarpium*, have been found to prevent the pathological changes caused by various lithogenic treatments such as damage to the renal function, oxidative stress, proteinuria and crystalluria.[43] Saponins also increase glycosaminoglycans, a macromolecule found in the urine and also speed up glomerular filtration.[44] The high concentration of saponins found in the extract and some of its fractions may be the reason for the local use of *B. aegyptiaca* in prevention and treatment of kidney stone caused by calcium oxalate crystals.

Free radicals generated by various mechanisms are important for biochemical processes in the body. The formation of reactive oxygen species in abnormal quantity leads to oxidative stress and it is counteracted by different endogenous anti-oxidant mechanisms. The cells can repair the damage directly or decrease the formation of free radicals by both enzymatic and non-enzymatic processes.[45] Lipid peroxidation has been postulated to be the destructive process of renal injury due to Diclofenac administration.[46] In the present study, the elevations in the levels of end products of lipid peroxidation, malondialdehyde (MDA) in the kidney of rats treated with Diclofenac suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanisms. Treatments with the fractions significantly ($p<0.05$) reduced the elevated serum levels of malondialdehyde (MDA) to towards normal normal. Thus, it may be possible that the mechanism of nephroprotection by the extract and fractions of *B. aegyptiaca* is due to its anti-oxidant effect. Flavonoids possess antioxidant and carcinogenic activities and it reduces pathological changes caused by toxins in the kidney by suppression of oxidative/nitrative stress and apoptosis.[47] Extracts containing flavonoids ameliorate tubular necrosis by diminishing p53 activation and PUMA-α expression as well as altering the Bax/Bcl-2 ratio, thereby enhancing the antioxidant enzyme activity which is responsible for its renoprotective properties.[43] On other hand, alkaloids, also regulate the expression of Bcl and Bax which help to explain their protective role against cell injuries involving mitochondrial pathway and provides the basis for their folkloric use in renal treatment.[48] The presence of these secondary metabolites in the *B.aegyptiaca* extract suggests its role in ameliorating the effects of kidney injuries caused by toxins. Earlier studies had revealed the hepatoprotective and antioxidant.[49] and cardioprotective[39] effects of *B. aegyptiaca*. Reduced glutathione (GSH) is an important intracellular buffer for oxidative stress and acts as a non-enzymatic antioxidant that reduces H_2O_2, hydroperoxides (ROOH) and drug toxicity.[50] The level of GSH depleted when animals were treated with Diclofenac sodium. The depleted level of GSH increased significantly ($p<0.05$) following the administration of *B. aegyptiaca* extract and butanol fraction at dose 500 mg/kg alongside the ethyl acetate fraction at 250 mg/kg whereas the n-hexane fraction was not significant when compared with the normal control. This is in agreement with earlier studies[28,28,39] which found that the leaves and fruits extracts of *B. aegyptiaca* caused a significant increase in the levels of plasma GSH.

Superoxide dismutase (SOD) and catalase (CAT) are enzymatic antioxidants widely distributed in renal tissues of animals that act to decompose hydrogen peroxide and protect the tissue from highly reactive hydroxyl radicals.[51] SOD is a key defense enzyme and catalyzes the dismutation of superoxide anions. CAT is a hemoprotein that catalyzes the reduction of hydrogen peroxide and able to prevent renal cells from reactive free radicals. Therefore, reduction in the activity of these enzymes may result in a number of deleterious effects due to the accumulation of superoxide radicals and H_2O_2. In the present study, the extract 500 mg/kg and butanol fraction 500 mg/kg significantly ($p<0.05$) restored the renal SOD and CAT activities. The ethyl acetate fraction 250 mg/kg that specifically caused an increase in the level of serum SOD and all the doses of n-hexane fractions (250 mg/kg and 500 mg/kg) significantly increased the serum levels of SOD and CAT. This might be due to varying degrees of bioactive molecules present in the extract and fractions of *B. aegyptiaca*. The increase in renal SOD and CAT by the stem-bark extract and fractions of *B. aegyptiaca* may be an indication that it could scavenge reactive free radicals, thus, reduces oxidative damage from the tissue and subsequently improves the activity of intracelluar antioxidant enzymes.[52] The presence of alkaloids in the extract may have increased the viability after diclofenac-induced nephrotoxicity of normal rat renal tubular epithelial cells. Alkaloids are believed to ameliorates drug-induced renal damage by suppressing the activity of N-acetyl-beta-D-glucosaminadase (NAG), a lysosomal enzyme that is constitutively expressed in the proximal kidney tubules.[53] Also, alkaloids containing emodin ameliorate cisplatin-induced apoptosis of rat renal cells *in vitro* by modulating adenosine monophosphate-activated protein kinase (AmpK) mechanistic target of rapamycin (mTOR) signaling pathways and activating autophage and *in vivo* by supressing *capase-3* and activity and apoptosis in renal tissues.[54]

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Table 5: Effects of *Balanites aegyptiaca Del* (stembark) crude extract on serum biochemical markers of oxidative stress

Treatment (mg/kg)	MDA (mmol/ml)	SOD (U/ml)	CAT (ug/mg)	Reduced GS (ug/mg)
Normal control	2.19 ± 0.27	7.67 ± 1.25	102.00 ± 1.17	8.55 ± 0.68
Induced control	4.88 ± 0.14*	5.83 ± 0.33*	90.89 ± 0.40*	5.05 ± 0.11*
Silymarin,100 mg/kg	2.60 ± 0.46*	8.92 ± 0.80[a]	99.09 ± 5.19*	6.66 ± 0.60
Extract, 250	2.11 ± 0.21*[a]	11.25 ± 1.72	85.81 ± 5.03	5.40 ± 0.41[a]
Extract, 500	1.623 ± 0.01*[a]	9.17 ± 0.83*	101.00 ± 0.04*[a]	7.55 ± 0.02*[a]
Extract, 1000	2.26 ± 0.21*[a]	22.50 ± 2.85*[a]	97.58 ± 7.20	4.69 ± 0.54[a]

Values are presented as mean ± Standard error of mean (SEM). n= 6; *P<0.05: Significantly different from groups 1 (Negative Control). [a]P < 0.05: Significantly different from groups 2 (Positive control).

Table 6: Effects of *Balanites aegyptiaca extract and fractions* on renal biomarkers (total protein, albumin, creatinine, and urea)

Treatment (mg/kg)	Total protein (g/dl)	Albumin (g/dl)	Creatinine (mg/dl)	Urea (mg/dl)
Normal control	4.43 ± 0.33	2.79 ± 0.13	1.02 ± 0.08	32.79 ± 2.75
Induced control	3.56 ± 0.31	1.26 ± 0.01*	2.74 ± 0.03*	42.80 ± 0.02*
Silymarin, 100 mg/kg	4.32 ± 0.75	2.48 ± 0.19	1.50 ± 0.02	31.16 ± 4.25
Extract, 250	4.24 ± 0.60	2.86 ± 0.11	0.88 ± 0.12	44.75 ± 2.07
Extract, 500	4.37 ± 0.47	2.57 ± 0.05*[a]	1.22 ± 0.04*	29.86 ± 0.03*
Ethyl acetate fraction,250	4.74 ± 0.42	2.26 ± 0.61	1.52 ± 0.56	47.00 ± 3.78
Ethyl acetate fraction,500	4.71 ± 0.55	2.60 ± 0.16	1.87 ± 0.21	39.41 ± 4.16
Butanol fraction, 250	4.51 ± 0.31	3.11 ± 0.39	1.80 ± 0.28	39.95 ± 7.46
Butanol fraction, 500	4.17 ± 0.28	2.75 ± 0.04*[a]	1.65 ± 0.02*[a]	31.99 ± 0.01*[a]
N-hexane fraction, 250	4.50 ± 0.13	3.00 ± 0.25	2.34 ± 0.06	38.96 ± 3.94
N-hexane fraction, 500	4.63 ± 0.33	2.05 ± 0.25	2.25 ± 0.07	38.96 ± 3.94

Values are presented as mean ± Standard error of mean (SEM). N = 6; *p < 0.05: significantly different from negative control. [a]p < 0.05: significantly different from Diclofenac induced group (positive control).

Table 7: Effects of *B. aegyptiaca* (stembark) extract and fractions on antioxidant parameters of Diclofenac-induced nephrotoxic rats

Treatment (mg/kg)	GSH (ug/mg)	MDA (nmol/ml)	SOD (U/ml)	CAT (Ku/l)
Normal control	1.72 ± 0.54	2.55 ± 0.69	14.70 ± 0.85	51.36 ± 9.72
Induced control	1.23 ± 0.09	4.93 ± 0.51	11.45 ± 0.64	38.23 ± 4.06
Sylimarin, 100 mg/kg	1.68 ± 1.04	2.47 ± 0.54	14.38 ± 0.24	49.25 ± 2.18
Extract, 250	2.54 ± 0.78	3.51 ± 0.82[a]	13.58 ± 1.58	48.42 ± 3.40
Extract, 500	1.59 ± 0.23	2.40 ± 1.33[a]	14.23 ± 1.11	51.11 ± 1.46[a]
Ethyl acetate fraction, 250	1.71 ± 0.42	2.74 ± 0.05*	14.28 ± 0.02*[a]	46.76 ± 1.15
Ethyl acetate fraction, 500	1.66 ± 0.32	2.53 ± 0.57	14.14 ± 1.10	50.25 ± 4.12
Butanol fraction, 250	2.22 ± 0.55	3.38 ± 0.88[a]	10.29 ± 1.77*	51.84 ± 10.11
Butanol fraction, 500	1.66 ± 0.04*[a]	2.46 ± 0.02*[a]	12.83 ± 0.01*	49.04 ± 0.01*[a]
N-hexane fraction, 250	1.99 ± 0.03*[a]	4.14 ± 1.08[a]	10.31 ± 0.01*	45.51 ± 1.89[a]
N-hexane fraction, 500	1.31 ± 0.86	2.54 ± 0.04**	2.54 ± 0.04**	48.63 ± 2.45

Values are presented as mean ± Standard error of mean (SEM). N = 6; *p < 0.05: Significantly different from negative control. [a]p<0.05: significantly different from induced control (positive control).

It is interesting to note that all groups treated with standard Silymarin (100 mg/kg) in both preliminary and fractionation phases significantly restored the biochemical parameters measured, which is an evidence that Silymarin is a potent nephroprotective agent.[55]

The biochemical results were confirmed by histopathotological observation (Figures 1A, 1B, 2 and 3) which showed partial or complete destruction of large number of renal corpuscles with loss of glomerular tuft and dilated urinary space, cytoplasmic vacuolation and pyknotic nuclei with cellular debris in the lumina, dilatation of tubules with dense acidophilic casts in induced groups. This showed that Diclofenac orally treatment with the rats was responsible for renal necrosis or degeneration, tubular hyaline casts, intertubular hemorrhage, glomerular congestion and leukocyte infiltration of renal tissue. Treatment with *B. aegyptiaca* extract and fractions caused preservation of the glomeruli and the surrounding Bowman's capsule and mild swollen tubules. This may be due to the abundant bioactive

flavonoids and tannins in the extract and fractions. Flavonoids, possess reno-protective effects by inhibiting renal inflammation, inhibiting production of TNF.α, 1L-Iß 1l-6 and monocyte chemo-attractant protein (MCP)-1 and enhancing IL-10 production in serum and tissues.[56] Also, showed an improvement in renal oxidant status and a reduction in NF-$_k$B activation, inflammatory and apoptotic factors. On the other hand, tannins are implicated in ameliorating nephrotoxicity by increasing hemolysate and SOD levels in renal cells which lead to a reduction in lipid peroxidation of the cells.[57]

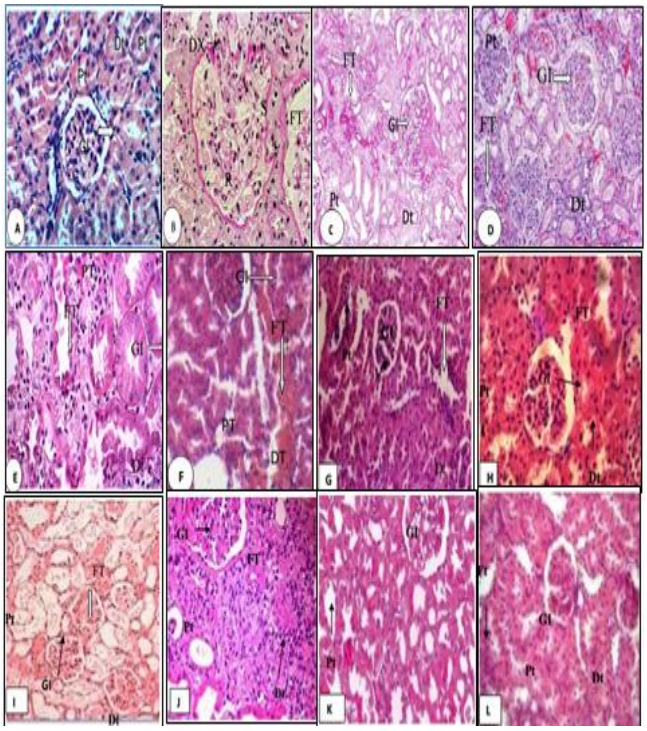

Figure 1: Photomicrograph of kidney sections of animals in;

Plat A: Normal rats, showing normal histological architecture by the kidney {renal corpuscles containing glomerulus (GI) and Bowman's space (arrow head), proximal tubules (PT) with narrow lumina and distal convulated tubules (DT) with wider lumina}.
Plate B: Diclofenac-induced nephrotoxic rats, showing destroyed renal corpuscles with loss of glomerular tufts (S), dilated urinary space (FT), cortical tubules with cytoplasmic vacuolation and pyknotic nuclei in the lumina (DX) and desquamation of their lining epithelium (RS).
Plates C, D and **E:** Rats treated with stem-bark extract (250, 500 mg/kg and 1000 mg/kg respectively), showing near normal or normal sizes of glomeruli (GI), normal squamous cell lining, Bowman's capsule with normal urinary space (FT) and renal tubules without pyknotic cells (Pt and Dt).
Plates F, G: Rats treated with ethyl acetate fraction (250 and 500 mg/kg),
Plates H and 1: Rats treated with butanol fraction (250 and 500 mg/kg)
Plates J and K: Rats treated with n-hexane fraction (250 and 500 mg/kg).
Plate L: rats treated with Silymarin (100 mg/kg)
Plates F-L showed renal corticles with normal sizes of glomeruli (GI), normal spaces (FT) and renal tubules without pyknotic cells (Pt and dt).
ic cells (Pt and dt).

Conclusion

The effects elicited by the extract and fractions of *B. aegyptiaca* might be due to its ability to activate antioxidant enzymes such as superoxide dismutase, catalase and reduced glutathione which could be the possible mechanism of action of the plant. Extract and fractions were found to have significant nephroprotective action. The extract and fractions were shown to possess important bioactive compounds such as flavonoids, tannins, alkaloids, saponins, terpenoids, steroids and glycosides. The bioactive metabolites may have contributed to the role of the plant in nephroprotections, and such support its uses as a remedy for renal damage.

Conflict of interest

The authors declare no conflict of interest

Author's Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating for the content of this article will be borne by them.

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