Sumerianz Journal of Biotechnology, 2021, Vol. 4, No. 3, pp. 114-125
ISSN(e): 2617-3050, ISSN(p): 2617-3123
Website: https://www.sumerianz.com
DOI: https://doi.org/10.47752/sjb.43.114.125

Original Article Open Access

In vitro Antioxidant and *In vivo* Hepatocurative and Nephrocurative Activities of Aqueous Leaf Extract of *Newbouldia laevis* in Albino Rats

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Article History
Received: May 27, 2021
Revised: July 13, 2021
Accepted: September 8, 2021
Published: September 11, 2021

Abstract

The *in vitro* antioxidant activity and *in vivo* hepatocurative and nephrocurative potential of *Newbouldia laevis* aqueous leaf extract (NLALE) was evaluated. The study used 30 male, albino rats (*Rattus norvegicus*) weighing 180 ± 20 g, of which 25 were intoxicated by oral administration of a single dose of diclofenac (100 mg/kg b. wt.). Animals were treated by oral administration of silymarin (200 mg/kg b. wt.), furosemide (1.5 mg/kg b. wt.) and NLALE (200 mg/kg and 400 mg/kg b. wt.) for seven consecutive days before animals were sacrificed on the 8th day and serum/plasma was analyzed for biochemical markers of hepatotoxicity and nephrotoxicity. Phytochemical screening of NLALE revealed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, steroids and tannins. The extract scavenged DPPH radical, reduced Fe^{3+} and inhibited TBARs in comparable manner to ascorbic acid *in vitro*. NLALE also attenuated diclofenac-induced liver and kidney intoxication as indicated by the significantly ($p<0.05$) reduced levels of serum biomarkers of hepatotoxicity: ALT, AST, bilirubin, but increased total protein levels and nephrotoxicity: urea, creatinine, Na^+ and K^+. The observed effects are dose dependent as the 400 mg/kg b. wt. appeared to be more potent than the 200 mg/kg b. wt. dose. It may be concluded from this study that *Newbouldia laevis* leaf has ameliorative effect against diclofenac-induced hepatotoxicity and nephrotoxicity probably through antioxidative mechanism and the curative claim and the folkloric use of the plant in the treatment of liver and kidney diseases have been scientifically validated.

Keywords: *Newbouldia laevis;* Hepatotoxicity; Hepatocurative; Nephrotoxicity; Nephrocurative; Antioxidant activity; Diclofenac.

1. Introduction

The liver is one of the most important body organs; it links the digestive tract and general circulation and plays a central role in nutrient metabolism, synthesis of functional proteins and detoxification of drugs and chemicals [1]. Liver is subjected to various diseases, which may be metabolic, toxin or chemical-induced tissue inflammation or viral hepatitis B or C-associated cirrhosis and carcinomas [2]. Liver diseases are among the global health problems; in which, liver cirrhosis is the ninth leading cause of death in western nations and constitute a major public health threat in developing countries [3, 4]. Liver disease accounts for approximately 2 million deaths per year worldwide [5] and drugs are one potential cause of liver disease. Drug-induced liver disease accounts for as much as 20% of acute liver failure in paediatric populations and a similar percentage of adults with acute liver failure [6].

The kidney is an essential organ in human body, receives an abundant blood flow of cardiac output and eliminates foreign substances and metabolic waste products from the blood into the urine [7] via glomerular filtration, tubular reabsorption and tubular secretion [8, 9]. The glomerulus and renal tubules are naturally exposed to high concentrations of metabolites as well as drugs and xenobiotics, therefore, making them vulnerable to toxicity [10]. Drug-induced nephrotoxicity (DIN) is one of the most common causes of acute kidney injury (AKI); various agents exert nephrotoxic effects through different pathogenic mechanisms. Aminoglycoside antibiotics, chemotherapeutic agents, radio contrast media, and nonsteroidal anti-inflammatory drugs (NSAIDs) are among common nephrotoxic agents [11]. There are several mechanisms for DIN including drug-induced oxidative stress, inflammation, increased apoptosis, and direct cytotoxicity to the tubular epithelial cells [12]. Recent studies have increased insight into the subcellular mechanisms of drug-induced AKI that include direct cellular toxicity and immune-mediated effects [13]. AKI is associated with increased morbidity and mortality in undeveloped and developing countries and a growing problem with untoward economic and medical consequences [14, 15].

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Diclofenac is the most significant and extensively used NSAID that was approved for clinical use in treating several rheumatic diseases and as analgesic, antipyretic and anti-inflammatory agent, both as a prescription drug and over the counter purchases [16, 17]. Like other NSAIDs, long term use of diclofenac has been associated with a small but significant incidence of hepatotoxicity ranging from mild, asymptomatic, reversible increase in liver function tests to jaundice and hepatitis, including several reports of fatal hepatitis [17-19]. The mechanism of diclofenac hepatotoxicity involves covalent protein modification by reactive metabolites [20, 21], oxidative stress generation by peroxidase-catalyzed reaction [22, 23] and mitochondrial injury propagation by reactive oxygen species [24, 25]. Diclofenac is a non-specific inhibitor of cyclooxygenase (COX) enzymes [26] thereby decreasing the synthesis of prostaglandin and thromboxane. COX-catalyzed prostaglandin productions are involved in regulating tubuloglomerular feedback and further resulted in afferent arteriolar vasoconstriction. In the states of decreased renal blood flow, the prostaglandin can protect against renal ischemia by antagonizing renal vasoconstriction. Administration of NSAIDs in this condition may disturb the balance of activity between renal vasoconstriction and vasodilation thus promotes renal ischemia with loss of glomerular filtration [27].COX-1 and 2 are essentially expressed in the kidney, their inhibition was suggested to cause renal ischemia leading to renal damage [28]. However, it was reported that diclofenac was one of the most common causes of drug-induced kidney injury and seemed to induce nephrotoxicity even in patients with normal baseline kidney function [29]. The mechanisms underlying the nephrotoxicity of diclofenac include but are not limited to non-selective COX inhibition and direct renal tubular cytotoxicity [30, 31].

Newbouldia laevis (Bignonicaceae) is commonly known as “African border tree” or boundary tree [32]. It is called “Aduruku” in Hausa; “Ogirisi” in Igbo and “Akoko” in Yoruba languages of Nigeria [33]. A medium sized angiosperm that grows to a height of about 7 – 15 metres and more usually a shrub of 2 - 3 metres, with many stemmed forming clumps of gnarled branches. It has short branches, coarsely toothed leaflets as well as purple and white flowers [34]. *Newbouldia laevis* is used by traditional medical practitioners in West Africa to treat several diseases including diarrhea, dysentery, some sexually transmitted diseases or used as anthelmintic [35]. Moreover, herbalists in Adamawa state, northeastern Nigeria administer *Newbouldia laevis* leaf extract to liver and kidney disease patients. However, the antimalarial [36], antidiabetic/anti-hyperglycemic [37-40], antioxidant [41] and hematological [42] activities of the plant have previously been reported. There is dearth of information on the curative potential of *Newbouldia laevis* on drug-induced tissue toxicity in the open scientific literature. Therefore, the present study was carried out to determine the *in vitro* antioxidant activity and *in vivo* curative capacity of *Newbouldia laevis* aqueous leaf extract (NLALE) on diclofenac-induced liver and kidney toxicity in albino rats (*Rattus norvegicus*) to scientifically validate the hepatocurative and nephrocurative claim and the folkloric use of the plant in the treatment of liver and kidney diseases.

2. Materials and Methods

2.1. Experimental Animals

Thirty (30) male albino rats (*Rattus norvegicus*) were purchased from the animal farm, National Veterinary Research Institute Vom, Plateau State, Nigeria with an initial mean body weight of 98.45 ± 10.74 g. The animals were housed and maintained in plastic laboratory rat cages in temperature and humidity controlled room (temperature: 25 ± 2 °C, humidity: 60 ± 5%, 12-hour light/dark cycle). Moreover, all the animals were fed with a commercial rat diet (Vital Feeds, Jos, Nigeria) and drinking water *ad libitum*, were allowed to acclimatize for two weeks, and attained a weight of 180 ± 20 g before they were used for the experiment. A standard protocol according to the guidelines of the Good Laboratory Practice (GLP) regulations of WHO as well as the rules and regulations of experimental animal ethics committee of Modibbo Adama University of Technology (M. A. U. Tech) Yola, Nigeria was strictly adhered to.

2.2. Drugs and Chemicals

Diclofenac (Voltaren Retard) was purchased from Novartis Pharma Stein (Switzerland), Silymarin was purchased from Micro Labs Ltd., (India), Furosemide (RENIX) from Fredun Pharmaceuticals Limited (India). Alanine aminotransferase (ALT), Aspartate transaminase (AST), Bilirubin, Total protein, Urea and Creatinine assay kits were purchased from Randox Laboratories (U.K). Sodium and Potassium Elyte kit 3 was purchased from Crest Biosystems, (India). All other chemicals used were of analytical grade.

2.3. Preparation of Plant Sample

The matured fresh leaves of *Newbouldia laevis* was collected in the dry season from Girei town, Girei Local Government area of Adamawa State. The leaf was identified and authenticated at the Department of Plant Science M. A. U. Tech. Yola, Adamawa State, Nigeria. The leaves was washed and air-dried under a shade for a period of two weeks after which they were pulverized into fine powder using an electric grinding machine.

2.4. Preparation of Aqueous Extract

Aqueous leaf extract of *Newbouldia laevis* was prepared as described by Oluduro and Aderiye [43]. Exactly 100 g of the powdered leaf of *Newbouldia laevis* was soaked in 600 ml distilled water at ambient temperature for three days and filtered using Whatman filter paper No. 1. The bulk filtrate was reduced in vacuum at 14˚ C. The solid residue was stored at low temperature until it is needed.

2.5. Qualitative Phytochemical Analysis

The aqueous leaf extract of *Newbouldia laevis* was subjected to qualitative analysis for various phytoconstituents by observing characteristics color change using standard procedures described by Trease and Evans [44] and Sofowora [45].

2.6. Quantitative Determination of Phenols and Flavonoids

2.6.1. Determination of Total Phenols

The total phenolic content of the leaf extract was determined using the Folin-Colcalteu method as modified by Dewanto, *et al.* [46]. Briefly, 0.5 ml of deionized water and 125 µl of Folin– Colcalteu reagent were added to 125 µl of the suitably diluted sample extract. The mixture was allowed to stand for 6 minutes before adding 1.25 ml of 7% aqueous Na_2CO_3 solution. The mixture was then allowed to stand for additional 90 minutes before taking the absorbance at 760 nm. The amount of total phenolics was expressed as gallic acid equivalents (GAE, mg gallic acid/g sample) through the calibration curve of gallic acid.

2.6.2. Determination of Total Flavonoids

The total flavonoids content was determined using a colorimetric method described by Dewanto, *et al.* [46]. To 0.25 ml of the suitably diluted sample, 75 µl of 5% $NaNO_2$ solution, 0.150 ml of freshly prepared 10% $AlCl_3$ solution, and 0.5 ml of 1 M NaOH solution were added. The final volume was then adjusted to 2.5 ml with deionized water. The mixture was allowed to stand for 5 minutes and the absorption measured at 510 nm against the same mixture, without the sample, as blank. The amount of total flavonoids was expressed as quercetin equivalents (QE, mg Quercetin/g sample) through the calibration curve of quercetin.

2.7. Antioxidant Activity Assays

2.7.1. DPPH (2, 2'-diphenyl-1-picrylhydrazyl) Assay

The free radical scavenging activity of NLALE was determined *in vitro* by 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) assay according to the standard method described by Braca, *et al.* [47]. Initially, 3 ml of 0.004% DPPH in methanol was mixed with 1 ml of various concentrations (100, 80, 60, 40, 20 µg/ml) of NLALE separately. Mixtures were incubated for 30 minutes at room temperature in a dark place. The absorbance of the mixtures in the samples were measured using a spectrophotometer at 517 nm against methanol as blank. The percentage of radical scavenging activities of the samples were evaluated compared with a control (3 ml DPPH solution and 1 ml methanol). Each sample was measured in triplicate and the average was calculated. The percentage of radical scavenging activity (RSA) was calculated using the following formula:

% RSA = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control, and A_1 is the absorbance of samples after 30 minutes. The free radical scavenging activity of the plant extracts was expressed as IC_{50}. The IC_{50} value is defined as the concentration (in µg/ml) of a sample that inhibits 50% of the DPPH radical.

2.7.2. FRAP (Ferric Reducing Antioxidant Power) Assay

The reducing power was based on Fe (III) to Fe (II) transformation in the presence of the solvent as described by Fejes, *et al.* [48]. The Fe (II) can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Exactly 2 ml of various concentrations (20, 40, 60, 80, 100 µg/ml) of the sample were mixed with 2 ml of phosphate buffer (0.2 M, pH 6.6) and 2 ml of potassium ferricyanide (10 mg/ml). The mixture was incubated at 50°C for 20 min followed by addition of 2 ml of trichloroacetic acid (100 mg/l). The mixture was centrifuged at 3000 rpm for 10 min to collect the upper layer of the solution. A volume of 2 ml from each of the mixture earlier mentioned was mixed with 2 ml of distilled water and 0.4 ml of 0.1% (w/v) fresh ferric chloride. After 10 min reaction, the absorbance was measured at 700 nm. Each sample was measured in triplicate, the average was calculated and the reducing power of NLALE was expressed as IC_{50}.

2.7.3. TBARS (Thiobarbituric Acid Reactive Substances) Assay

Thiobarbituric acid reactive substances (TBARS) assay was performed by method described by Aazza, *et al.* [49]. Egg yolk homogenates were used as a lipid-rich medium obtained as described by [50]. Briefly, 100 µl of egg yolk [(10% w/v) in KCl (1.15%)] was added into test tubes labeled 'sample' and 'standard', 50 µl of extract and standard vitamin E in ethanol (10 - 1000 µg/ ml) were added to the test tubes respectively. Then, 300 µl of 20% acetic acid (pH 3.5) and 300 µl of 0.8% (w/v) TBA in 1.1% (w/v) sodium dodecyl sulphate (SDS) were added. The resulting mixture was vortexed and heated at 95°C for 60 minutes. After cooling, at room temperature, 750 µl of butan-1-ol was added to each tube; the contents of the tubes were stirred and centrifuged at 3000 rpm for 10 minutes. The upper organic layer was transferred to 96-well microtitre plate and absorbance was measured at 532 nm using an ELISA plate reader. All of the values were based on the percentage antioxidant index (AI%), whereby the control was completely peroxidized and each sample demonstrated a degree of change; the percentage inhibition was calculated using the formula (1- T/C) × 100, where C is the absorbance value of the fully oxidized control and T is the absorbance of the test sample. The antioxidant capacity was determined from three replicates and IC_{50} values were determined. Same amount of deionized water was used as the control.

2.8. Animal Groupings and Induction of Tissue Toxicity

Animals were randomly divided into six groups of five rats each, namely: Normal Control (NC), Disease Control (DC), Standard Control 1 - Toxicity + 100 mg/kg b. wt. of Silymarin (SC1), Standard Control 2- Toxicity + 1.5 mg/kg b. wt. of Furosemide (SC2), Toxicity + low dose (200 mg/kg BW) of *Newbouldia laevis* aqueous leaf extract (NLALE), (TNLAL), Toxicity + high dose (400 mg/kg BW) of NLALE (TNLAH). After two weeks acclimatization period, animals in DC, SC1, SC2, TNLAL and TNLAH were orally administered a single dose of 100 mg/kg b. wt. diclofenac (Voltaren) to induce hepatotoxicity and nephrotoxicity. While animals in NC were only supplied with, vehicle (Vital Feeds, Jos, Nigeria) and water only throughout the seven days experimental period.

At the end of the experimental period, animals were euthanized by halothane anesthesia and the whole blood of each animal was collected via cardiac puncture using a 10 ml syringe and 25 gauge needle after 24 hours of last treatment. The blood was collected in vacutainer tubes and centrifuged at 3000 rpm for 15 minutes, plasma and serum were separated and preserved for further analysis. The serum, plasma and whole blood were used for various biochemical analysis. The biochemical parameters determined were ALT, AST, Total bilirubin and Total protein, Urea, Creatinine and Electrolytes (Na^+ and K^+).

2.9. Determination of Alanine Aminotransferase (ALT)

Into test tubes labelled Sample blank and Sample, 500 µl each of solution 1 was pipetted and into the test tube, labelled Sample, 100 µl of serum was added, mixed and incubated in water bath at 37°C for exactly 30 minutes. Then 500 µl of solution 2 was added to both test tubes. Exactly 100 µl of distilled water was then added to the test tube labelled Sample blank, mixed and allowed to stand for 20 minutes at 20 - 25°C and finally 5000 µl of Sodium hydroxide was added to both test tubes labelled mixed and poured into cuvette. The absorbance of the sample was measured against sample blank after 5 minutes [51]. Enzyme activity and average absorbance difference per minute (ΔAbs/min) was also calculated using the following formula:

ALT (IU/L) = ΔAbs/min x TV x 1000/18.75 x LP x SV

Where ΔAbs/min = Average absorbance change per minute

1000 = Conversion of IU/ml to IU/L

TV = 1.025 = Total reaction volume (ml)

18.75 = Millimolar absorptivity of ρ-Nitrophenol

SV = 0.025 = Sample volume (ml)

LP = 1 = Light path in cm.

2.10. Determination of Aspartate Transaminase (AST)

Into test tubes labelled "Sample blank" and "Sample", 500 µl each of solution 1 was pipetted and into the test tube labelled Sample 100 µl of serum was added, mixed and incubated in water bath at 37°C for exactly 30 minutes. Then 500 µl of solution 2 was added to both test tubes and 100 µl of distilled water was then added to the test tube labelled Sample blank, mixed and allowed to stand for 20 minutes at 20 - 25°C and finally 5000 µl of Sodium hydroxide was added to both test tubes, mixed and poured into cuvettes. The absorbance of the sample was measured against sample blank after 5 minutes [51]. Enzyme activity and average absorbance difference per minute (ΔAbs/min) was also calculated using the following formula:

AST (IU/L) = ΔAbs/min x TV x 1000/18.75 x LP x SV

2.11. Determination of Serum Total Bilirubin

Serum total bilirubin was determined using the method describe by Jendrassik and Grof [52].

Into clean, dry test tubes, labelled "Blank" and "Test", 1.0 ml of total bilirubin reagent was added. To test tube labelled Test, 0.05 ml of total nitrite reagent was added, finally 0.1 ml of sample was added to both test tubes. The solutions was well mixed and incubated at room temperature for 10 minutes and the absorbance of test sample (A_T) against blank was immediately measured at 546 nm.

Calculation: Concentration of total bilirubin (mg/dl) = $A_T \times 13$

2.12. Determination of Serum Total Protein

Serum total protein was determine using biuret reaction as described by Tietz [53].

Into test tubes labelled blank, standard and sample, 0.02 ml of distilled water, standard (protein) and sample (serum) was added respectively. 1.0 ml of biuret reagent was added to all test tubes and the solutions was mixed and incubated for 30 minutes at 22°C before taking the absorbance of sample ($A_{(Sample)}$) and that of the standard ($A_{(Standard)}$) against the blank at 540 nm.

Total serum Proteins (in g/dl) = $\frac{A_{(Sample)}}{A_{(Standard)}}$ x Concentration of standard.

2.13. Determination of Serum Creatinine

Serum creatinine was determined using Jaffe Colorimetric method as described by Young [54].

Into test tubes labelled sample, standard and blank, 1 ml of working reagent (Picric acid + NaOH) was added. One hundred (100) µl of sample (serum) was added to the test tube designated "Sample" and 100 µl of standard (Creatinine aqueous primary standard 2 mg/dl) was added to the test tube designated "Standard". The above preparation was properly mixed and allowed to stand for 30 seconds before absorbance A_1 was read and absorbance

A_2 was read after 90 seconds at 510 nm using 1 cm light path cuvette. Change in absorbance was calculated as $\Delta A = A_2 - A_1$. Serum creatinine concentration was calculated using the formula below;

Creatinine concentration (mg/dl) = $\frac{\Delta A\ Sample - \Delta A\ Blank}{\Delta A\ Standard - \Delta A\ Blank}$ x 2(Standard concentration)

2.14 Determination of Urea

Serum urea was determined using endpoint determination (Berthelot enzymatic reaction) as described by[55].

Into test tubes labelled sample, standard and blank, 1000 µl of working solution (Buffer + vial enzyme reagent) was added. 10 µl of sample (serum) was added into test tube labelled "Sample" and 10 µl of standard (Urea 50 mg/dl) was added into the test tube labelled "Standard". The preparations was properly mixed and incubated for 10 minutes at 25°C. 1000 µl of Hypochlorite solution was added to all three test tubes mixed and incubated again for 10 minutes at 25°C before absorbance was read at 580 nm. Serum urea concentration was calculated thus, as;

$\frac{A_{Sample}}{A_{Standard}}$ X Concentration of standard = Urea Concentration (mg/dl).

2.15. Determination of Electrolyte (K^+ and Na^+) Concentration in Serum

The concentrations of sodium and potassium ions were determined using Elyte kits using method described by Trinder [56].

2.15.1. Sodium

2.15.1.1. Precipitation

Into test tubes labelled as Standard and Test, 1.0 ml of precipitating reagent was pipetted. Followed by the addition of 0.02 ml of Na^+/K^+ standard and 0.02 ml of sample to the Standard and Test respectively. The solutions was mixed well and left to stand at room temperature for 5 minutes, shaking well intermittently. The solution was centrifuged at 2500 rpm to obtain a clear supernatant.

2.15.1.2. Colour Development

Three test tubes labelled as Blank, Standard, and Test were used. 1.0 ml of acid reagent was added into all three test tubes, followed by addition of 0.02 ml of supernatant from step 1 to Standard and Test and 0.02 ml of precipitating reagent to blank. 0.1 ml of colour reagent was then added to all the test tubes, the solutions was well mixed and incubated at room temperature for 5 minutes. The absorbance of the Blank (A_B), Standard (A_S) and Test Sample (A_T) were measured, against distilled water within 15 minutes at 530 nm. Sodium concentration was calculated using the formula:

Sodium concentration (mmol/l) = $\frac{A_{B-A_T}}{A_{B-A_S}}$ X 150

2.15.2. Potassium

Into test tubes labelled as Blank, Standard and Test, 1.0 ml of potassium reagent was added. Then 0.02 ml of deionized water, Na^+/K^+ standard and sample (serum) were added to Blank, Standard and Test respectively. The solutions were mixed well and incubated at room temperature for 5 minutes. The absorbance of the Standard (A_S), and Test Sample (A_T) were measured against Blank, at 630 nm, within 15 minutes. Potassium concentration was calculated using the formula:

Potassium concentration (mmol/l) = $\frac{A_T}{A_S}$ X 5

2.16. Statistical Analysis

The data obtained was analyzed using R. Statistical software Package version 3.4.4. The results were expressed as mean ± standard error of mean SEM. Data was analyzed using one-way analysis of variance (ANOVA); followed by the differences in mean values using Duncan's multiple comparison test. P value of <0.05 was considered as statistically significant.

3. Results

The qualitative phytochemical screening of NLALE showed the presence of alkaloids, flavonoids, glycosides, phenols, saponins, steroids, tannins, and terpenoids (Table 1) and the extract have significantly (P>0.05) high phenols and flavonoids content (Table 2).

Percentage inhibition of DPPH radical is presented in table 3. The results showed that NLALE significantly ($p<0.05$) scavenged DPPH radical in a comparable manner to ascorbic acid. In addition, the activity increases with increase in concentration.

The antioxidant capacity of NLALE was estimated for its ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). The FRAP value is significantly ($p < 0.05$) high and showed an increase in chelating activity with increase in drug concentration; however, NLALE have significantly ($p < 0.05$) lower activity when compared with ascorbic acid (Table 4).

NLALE is also found to be capable of inhibiting lipid peroxidation in dose dependent manner (Table 5). However, NLALE showed significantly ($P < 0.05$) lower TBARs inhibitory potential compared to the standard (Ascorbic acid).

The effects of NLALE on levels of liver marker enzymes (ALT, and AST) and proteins (Total protein and bilirubin) are shown in table 6. The levels of ALT, AST and bilirubin were elevated significantly ($P < 0.05$) and the level of total protein was lowered significantly ($P < 0.05$) owing to the hepatotoxicity induced by diclofenac in treated rats when compared with rats in normal control group. However, oral treatment with NLALE significantly ($P < 0.05$) lowered the levels of ALT, AST and bilirubin and significantly ($P < 0.05$), but increased the level of total protein when compared with animals in normal control group.

The effects of NLALE on serum markers of nephrotoxicity in diclofenac-induced animals is shown in table 7. The results showed that there is a significant ($p<0.05$) increase in serum levels of urea, creatinine, sodium and potassium ions in the diclofenac treated group when compared to the normal control. However, treatment with furosemide significantly ($p<0.05$) reduced the elevated level to near normal. Moreover, oral treatment with 200 mg/kg b. wt. of NLALE significantly ($p>0.05$) decreased the levels of the serum markers of nephrotoxicity in rats. The results also showed that a greater activity is achieved with oral treatment with 400 mg/kg b. wt. of NLALE which also significantly ($p>0.05$) reduced the serum levels of the biomarkers near the values obtained from the standard drug.

Table-1. Qualitative phytochemical screening of aqueous leaf extract of *Newbouldia laevis*

Phytochemical	Aqueous Extract
Alkaloids	+
Flavonoids	+
Steroids	+
Terpenoids	+
Tannins	+
Saponins	+
Glycosides	+
Phenols	+

KEY: + indicates active component is present

Table-2. Quantitative Determination of Total Phenols and Flavonoids of *Newbouldia laevis* Aqueous Leaf Extract

Phytochemical	Concentration
Total phenols	$16.65 \pm 1.20^{*}$
Total flavonoids	$22.30 \pm 2.40^{\#}$

Values are expressed as mean ± SEM of triplicate determinations.
Superscripts: * = mg Gallic acid equivalent/g extract powder; # = mg Quercetin equivalent/g extract powder

Table-3. DPPH Radical Scavenging Activity (% inhibition) of *Newbouldia laevis* Aqueous Leaf Extract

Concentration (µg/ml)	Ascorbic acid	NLALE
20	24.24 ± 0.03	10.75 ± 2.20
40	40.80 ± 2.30	17.46 ± 3.04
60	36.69 ± 3.62	24.22 ± 2.10
80	48.70 ± 1.50	35.29 ± 3.55
100	62.72 ± 3.42	48.20 ± 4.20
IC_{50}	11.20	19.32

Values are expressed as mean ± standard error of mean (SEM), (n=3).

Table-4. Ferric Reducing Antioxidant Power (FRAP) of Aqueous Leaf Extract of *Newbouldia laevis*

Concentration (µg/ml)	Ascorbic acid	NLALE
20	38.21 ± 2.25	32.15 ± 1.54
40	46.27 ± 4.10	36.20 ± 2.35
60	62.31 ± 3.34	48.53 ± 2.19
80	78.35 ± 2.54	56.44 ± 2.41
100	85.38 ± 4.30	67.42 ± 1.64
IC_{50}	22.34	43.06

KEY: Values are expressed as mean ± standard error of mean (SEM); (n=3)

Table-5. Percentage Inhibition of TBARS of Aqueous Leaf Extract of *Newbouldia laevis*

Concentration (mg/ml)	Ascorbic acid	NLALE
20	64.24 ± 1.24	50.84 ± 3.44
40	73.26 ± 3.51	48.42 ± 2.43
60	81.19 ± 5.62	56.91 ± 4.08
80	86.25 ± 3.10	64.72 ± 2.47
100	91.19 ± 4.52	68.32 ± 4.50
IC_{50}	41.50	52.42

KEY: Values are expressed as mean ± standard error of mean (SEM); (n=3).

Table-6. Effects of Aqueous Leaf Extract of *Newbouldia laevis* on the Activity of Liver markers of Hepatotoxicity

Groups	AST (IU/L)	ALT (IU/L)	Total Protein (g/dl)	Bilirubin (mg/dl)
NC	68.60 ± 5.1	36.40 ± 2.2	8.20 ± 3.0	1.78 ± 0.08
DC	125.20 ± 4.2 ^{a}	98.00 ± 3.2 ^{a}	3.80 ± 3.7 ^{c}	6.32 ± 0.15 ^{a}
SC1	72.40 ± 1.1^{ab}	42.80 ± 1.9 ^{ab}	6.60 ± 3.8 ^{cd}	2.14 ± 0.19 ^{ab}
TNLAL	89.40 ± 2.7 ^{ab}	78.40 ± 1.4 ^{ab}	4.20 ± 5.9 ^{cd}	3.08 ± 0.39 ^{ab}
TNLAH	81.80 ± 3.0 ^{ab}	54.40 ± 3.5 ^{ab}	5.90 ± 2.1 ^{cd}	2.84 ± 0. 94^{ab}

Values are expressed as mean ± standard error of mean (SEM); with n = 5.
Superscripts: a = significantly ($p < 0.05$) higher compared to normal control; b = significantly ($p < 0.05$) lower compared to disease control; c = significantly ($p < 0.05$) lower compared to normal control; d = significantly ($p < 0.05$) higher compared to disease control

Table-7. Effects of Aqueous Leaf Extract of *Newbouldia Laevis* on Serum Markers of Nephrotoxicity

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Na^+ (mmol/l)	K^+ (mmol/l)
NC	19.86 ± 2.36	0.80 ± 0.2	139.0 ± 4.00	3.96 ± 0.40
DC	62.12 ± 0.92^{a}	4.20 ± 0.3^{a}	182.40 ± 2.50^{a}	9.28 ± 0.57^{a}
SC2	23.56 ± 0.93^{ab}	1.90 ± 2.7^{ab}	120.2 ± 3.0^{ab}	4.86 ± 0.47^{ab}
TNLAL	36.26 ±13.90^{ab}	2.92 ± 0.6^{ab}	133.2 ± 5.20^{ab}	8.76 ± 1.18^{ab}
TNLAH	29.40 ±14.77^{ab}	2.25 ± 1.6^{ab}	128.4 ± 11.4^{ab}	6.40 ± 1.35^{ab}

Values are expressed as mean ± standard error of mean (SEM); (n=5).
Superscripts: a= significantly ($p>0.05$) higher compared to normal; b= significantly ($p>0.05$) lower compared to negative control.

4. Discussion

The presence of alkaloids, flavonoids, steroids, terpenoids, tannins, saponins, glycosides, and phenols in the phytochemistry of NLALE (Table 1) is consistent with the findings of Chinyelu *et al*., (2017), but disagree with the findings of Salemcity, *et al.* [57]. Environmental factors influence the production of secondary metabolites in plants, the differences observed could be related to the fact that samples were collected in different regions and at different times of the year [58]. Total phenols and total flavonoids were quantitatively determined and the result of the present study (Table 2) showed that *Newbouldia laevis* has a considerably good phenols and flavonoids content, which are strong antioxidants that prevent the influence of free radicals and ROS, which are the basis of several chronic human diseases [59].

Free radicals are chemical entities that can co-exist with one or more unpaired electrons and their generation has extensive tissue damaging effect. Lipids, proteins and nucleic acids are all susceptible to attack by free radicals [60] . One of the mechanisms involved in antioxidant activity assay is the ability of a molecule to donate a hydrogen atom to a radical, and the propensity of the hydrogen donation is the critical factor involved in free radical scavenging [61].

DPPH assay measure the primary antioxidant activity of plant extracts because it is one of the most effective methods for evaluating the concentration of radical scavenging materials actively by a chain-breaking mechanism [62, 63] . DPPH is a stable nitrogen-centered free radical the color of which changes from purple/violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [64]. NLALE showed a concentration dependent DPPH radical scavenging activity with a calculated IC_{50} value of 19.32 µg/ml which can be compared with that of the standard (Ascorbic acid) which has an IC_{50} value of 11.20 µg/ml. The DPPH scavenging potential of NLALE may be attributed to the phenolic compounds present in the plant [65].

The ability of NLALE to reduce ferric ion (Fe^{3+}) to ferrous (Fe^{2+}) was determined by FRAP assay which measures the reducing capacity of a substance by increased sample absorbance based on the formed ferrous ions and the reducing capacity of the extract may serve as antioxidant activity [66, 67]. The reducing power of NLALE increased with increasing concentration in this study (Table 4) and the observed increase in absorbance is directly related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. The antioxidant activity observed can be attributed to various mechanisms, among which are the prevention of chain initiation, the binding of transition metal ion catalysts, decomposition of peroxides and the prevention of continued hydrogen abstraction [68].

Lipid peroxidation is widely recognized as primary toxicological event that is caused by the generation of free radicals from a variety of sources including organic hydroperoxides, redox cycling compounds and iron-containing compounds. TBARS are naturally present in biological specimens and include lipid hydroperoxides and aldehydes which increase in concentration as a response to oxidative stress [69, 70]. Oxidative stress causes damage to cells, some of which resulting from the formation of lipid hydroperoxides and aldehydes, such as malondialdehyde (MDA), substance known to be associated with the development of several diseases [71]. The TBARS assay results obtained in the present study indicate that NLALE has significantly good TBARS inhibition activity comparable to ascorbic acid (Table 5), which can be attributed to the presence of phenols which account for the largest part of the antioxidant activity of many plants [72], and other antioxidant phytochemicals that were positive in the phytochemistry that have been found useful in both preventive and curative medicine [73]. Phenolic compounds are famous powerful chain breaking antioxidants that have the ability to go into electron-donation reactions with oxidizing agents to form stable species [74] and thus inhibit or delay the oxidation of different biomolecules [75].

Liver is the main site of protein synthesis (especially albumin) and detoxification of bilirubin as well Muriel [76]. In this study, the levels of total protein and bilirubin were used to assess liver synthetic and detoxification capability respectively. The liver is continuously exposed to oxidative stress, which causes damage to cell components, such as proteins, lipids and nucleic acids and the release of free radicals is the main hepatotoxicity mechanism of toxicants [77, 78]. The administration of diclofenac to rats in DC group significantly ($P < 0.05$) increased serum levels of AST, ALT and total bilirubin and significantly ($P < 0.05$) but, decreased total protein level when compared to animals in NC group (Table 6), indicating liver injury or damage. The reduction in the total protein level is due to disruption and dissociation of polyribosomes on endoplasmic reticulum resulting in decrease in the biosynthesis of protein [79]. Similar studies [80, 81] reported that an increase in the level of tissue amino transferases (ALT and AST) is known to reflect the severity of liver injury. The increase in these liver parameters is clear indications of cellular leakage and loss of function and integrity of the membrane resulting from liver damage [82].

Oral treatment of rats with 200 mg/kg and 400 mg/ kg b. wt. NLALE results in significant ($P < 0.05$) decrease in the levels of AST, ALT, and total bilirubin and significant ($P < 0.05$) but, increased total protein levels. The decrease in the activity of these liver biomarkers suggests a curative effect [81]. This may be due to the observed antioxidant property of NLALE, since antioxidants prevent lipid peroxidation of biomembranes, thereby preventing the decline in the protein synthesis capacity, enhancing the detoxification power and reducing the leakage of intracellular enzymes of the liver probably through stabilizing endoplasmic reticulum or through regeneration of the liver architecture and improving the functions of the hepatic cells [83, 84]. Moreover, flavonoids, polyphenolic compounds and saponins have also been reported to exert hepatoprotective activity against Paracetamol-induced liver intoxication [85-87].

The kidney is extremely active in the synthesis and metabolism of prostaglandins which are responsible for the regulation of renal blood flow, glomerular filtration, modulation of rennin release, tubular ion transport, and water metabolism [88]. NSAIDs treatment causes disruption of renal physiology by decreasing renal blood flow and glomerular filtration rate and impairing potassium, sodium and water excretion [89, 90]. Glomerular filtration is the main way for excretion of water-soluble unbounded drugs such as diclofenac. However, Diclofenac prevents the synthesis of prostaglandins resulting in renal dysfunction and pathophysiological alterations [88]. Serum creatinine, urea and electrolytes (K^+ and Na^+) are considered reliable, indirect markers of renal function test parameters and profound alterations in the serum levels of these markers are diagnostic of nephropathy [91-93]. Nephrotoxicity was reliably induced by oral administration of a single dose of 100 mg/kg b. wt. of diclofenac to rats in this study, evident by markedly increased serum levels of creatinine, urea and electrolytes (K^+ and Na^+) in rats in the DC group compared to those in the NC group. The impaired glomerular filtration capacity of the kidney is biochemically demonstrated by reduced clearance of creatinine and urea as well as K^+ and Na^+ from the circulating blood resulting in increased serum levels, which are sensitive and dramatic indicators of glomerular filtration rate reduction and nephrotoxicity [94]. Our findings supports reports from previous studies that Diclofenac exerts toxicity on renal tissue [95-97]. Thus, the inhibition of renal prostaglandin synthesis coupled with tubular necrosis could be the mechanism responsible for the glomerular filtration rate reduction and increased levels of these markers following diclofenac administration.

The administration of NLALE 200 mg/kg and 400 mg/kg b. wt. doses significantly ($P < 0.05$) attenuated the rise in these renal injury markers and changes were more significant with 400 mg/kg than 200 mg/kg b. wt. dose (Table 7), indicating a dose dependent curative activity. The significant reduction of serum creatinine, urea, K^+ and Na^+ levels in the TNLAL and TNLAH groups compared to DC group demonstrated the ability of NLALE to restore kidney's excretory capacity, eventually normalizing creatinine and urea levels in the blood and the restoration of electrolytes homeostasis [98].

5. Conclusion

This study shows the curative effect of *Newbouldia laevis* against Diclofenac-induced hepatotoxicity and nephrotoxicity and lends credence to the ethno-pharmacological use of the plant in the treatment of liver and kidney diseases. Results obtained revealed that there was a dose-dependent amelioration of biomarkers of both liver and kidney injuries. Even though the ameliorative mechanisms of the plant extract is yet not elucidated, the observed antioxidant activity is one of the anticipated mechanisms. It is reasonable to suggest that the phytochemicals identified in this study may act individually or synergistically to produce the observed curative activities of NLALE. Additionally, the phytochemicals could maintain cell membrane stability or could protect cell membrane leakage upon damage by diclofenac as evidenced by a reduction in the liver and kidney biomarkers.

Acknowledgements

The authors thank Mal. Raji Lawal and Ahmed Ibrahim Hayatu for their technical assistance during the study.

Authors' Contributions

Augustine Elizabeth and Abubakar Umar Modibbo performed experiments and provided equipment and re-agents;

Mohammed Aliyu Sulaiman conducted the statistical analysis and wrote the manuscript;

Mahmoud Suleiman Jada made manuscript revisions. The authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

The animal study was conducted in strict compliance with the Animal Research Ethical Committee guide of the Modibbo Adama University of Technology Yola, Nigeria.

Competing Interests Disclaimer

I hereby declare that there is no competing interest on this manuscript and has not been considered or submitted for publication elsewhere. The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no competing interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. The research was also not funded by the producing company rather, it was funded by personal efforts of the authors.

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