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Nephroprotective Effect of *Phoenix reclinata* Total Crude Root Extract on Tenofovir Induced Kidney Damage in Wistar Albino Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author CBN designed the study and did the experiments. Author KI performed the statistical analysis and reviewed the manuscript. Author AT contributed to the protocol and data analysis. Author GT managed literature searches and reviewed the manuscript. Author JK designed the study and reviewed the manuscript. Author CDK contributed to the protocol, supervised the work and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Tenofovir (TDF), used in combination with other antiretroviral agents, is an effective therapy for HIV infection. However, prolonged use of this drug is limited by a life threatening nephrotoxicity. *Phoenix reclinata* is a common palm species native to tropical Africa and is commonly recommended by traditional herbalists as a remedy for kidney disease. However, scientific evidence for its nephroprotective effects is nonexistent. In this study, we investigated the nephroprotective effect of *Phoenix reclinata* total crude extract on TDF induced kidney damage using Wistar albino rats. Six groups of Wistar albino rats (N=8) were utilized to test the total crude root extract of *Phoenix reclinata.* Three groups received 600 mg/kg of tenofovir with concurrent administration of plant extract at; 200 mg/kg, 400 mg/kg and 800 mg/kg by gavage tube. The

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negative control group received only tenofovir, the positive control received tenofovir and calcitol with the normal control receiving only distilled water. After 21 days blood was collected by cardiac puncture for serum urea, creatinine, sodium, potassium and chlorine assays. In addition, kidney tissue sections were taken for histopathological analysis.
Our results revealed that oral administration of TDF resulted in significant elevation ($p< 0.05$) of serum creatinine (57.33±6.61 µmol/L), urea (7.33±0.82 mmol/L), sodium (157.2±5.07 mmol/L), potassium (14.29±2.96 mmol/L) and chlorine (107.9±6.16 mmol/L). Histopathological findings observed included severe proximal tubular necrosis, glomerular and renal tubular degeneration tending to necrosis, lymphocyte infiltration and intertubular hemorrhages. These effects were significantly reversed with the administration of the extract in a dose dependent manner. The effects observed in the 800 mg/kg of extract were comparable to those of the calcitriol group. Our study demonstrated for the first time the protective effect of *Phoenix reclinata* on TDF induced nephrotoxicity. Future studies on structural elucidation of the bioactive compounds responsible for the nephroprotective effective and these utilizing pure extracts are recommended.

Keywords: Phoenix reclinata; tenofovir; nephrotoxicity; Senegal date palm.

ABBREVIATIONS

HIV : Human Immuno Deficiency Virus
AIDS : Acquired Immuno Deficiency Syndrome
HAART : Highly Active Antiretroviral Therapy
TDF : Tenofovir Disoproxil Fumurate
GFR : Glomerular Filtration Rate
CrCl : Creatinine Clearance
sCr : Serum creatinine

1. INTRODUCTION

Tenofovir, used in combination with other antiretroviral agents, is an effective therapy for HIV infection [1]. Tenofovir disoproxil fumurate (TDF), a prodrug for tenofovir has been one of the most prescribed antiretroviral agents in formulation with other antiretroviral drugs such as truvada and atripla [2]. Indeed, the cumulative patient TDF exposure in Europe and North America was estimated to be 455,392 persons-years, up to the end of year 2007 [3].

A benign renal profile for tenofovir and numerous cases of kidney injury supported by large clinical studies and post marketing data have raised concern for nephrotoxic potential. Experimental animal models have demonstrated tenofovir mitochondrial DNA depletion and mitochondrial toxicity [4]. Proximal tubular cell transporters favor accumulation of tenofovir leading to mitochondrial DNA replication inhibition [5,6]. This mitochondrial injury impairs molecular transport leading to further accumulation of tenofovir in the renal tubules that impacts on vitamin D activation and urinary acidification [7]. Studies by Herlitz et al. [8] among human subjects demonstrated tenofovir related nephrotoxicity, revealing toxic acute tubular necrosis with distinctive proximal tubular eosinophilic inclusions. The toxicity of tenofovir is compartmental as a result of combined tenofovir elimination by glomerular filtration and proximal tubular secretion [1]. Likewise, the presence of polymorphisms at genes (ABCC2 and ABCC4) coding for transporter proteins involved in tenofovir elimination is also associated with tubular renal damage [9]. Management of tenofovir associated kidney damage is usually achieved through tenofovir therapy discontinuation [10], dialysis, transplantation and use of Angiotensin Converting Enzyme inhibitors (enalapril) to slow glomerular filtration rate decline [11] among others. However, the few conventional drugs on the market for managing kidney damage are not without side effects, are expensive and not easily accessible in the developing world.

Nephrotic syndrome has long been treated in China by utilizing herbal medicine, *Astragalus mongholic* and *Angelica sinesis* associated with anti-fibrotic effects similar to those of an Angiotensin Converting Enzyme inhibitors - enalapril [12]. The wild date palm or Senegal date palm (*Phoenix reclinata, reclinata*-Latin, reclining) is a species of flowering plant in the palm family (*Arecaceae*) native to tropical Africa. Palm species have been included among raw lists of plants used for specific ailments in the latest studies in African traditional medicines [13]. Decoctions from *Phoenix reclinata* roots have been used to correct epilepsy [14], manage impotence and erectile dysfunction [15-17], relieving stomach ache and colic in Senegal [18] and as a remedy for pleurisy [19]. The roots have also been used in traditional veterinary medicine to induce oestral cycle [20] and to treat foot rot in

sheep and goats when mixed with *Arctotis arctotoides* leaves [21].

Studies from Cameroon indicate that *Phoenix reclinata* might have kidney cleaning effects [22]. Similarly, in Uganda *Phoenix reclinata* is currently being used by traditional medicine practitioners and claimed to be effective in the management of kidney disease. However, its efficacy in the management of nephrotoxicity has not been evaluated. We therefore investigate in this study the nephroprotective effect of *Phoenix reclinata* on tenofovir induced nephrotoxicity in Wistar albino rats. These results will form a basis for further pharmacological and toxicological research about *Phoenix reclinata*.

2. MATERIALS AND METHODS

2.1 Experimental Design

We carried out an experimental study to investigate the nephroprotective effect of *Phoenix reclinata* total crude root extract on tenofovir disoproxil fumurate, TDF (Cipla, India) induced kidney damage using Wistar albino rats. A total of 48 Wistar albino rats of approximately 8 weeks of age with a mean weight of 162.3 ± 18.7 g were housed in cages for a period of two weeks to allow acclimatization in a controlled environment. In all cases, rats were treated according to the international guidelines on the use of Laboratory animals in biomedical research [23]. Rats were randomly divided into six groups with eight animals per group and treated as follows: Three groups received 600 mg/kg of TDF with concurrent administration of plant extract at; 200 mg/kg, 400 mg/kg and 800 mg/kg by gastric lavage. The negative control received only 600 mg/kg tenofovir, the positive control received 600 mg/kg tenofovir and 0.05 µg/kg Calcitrol, with the normal control given only distilled water. After 21 days, rats were anaesthetized with diethyl ether and about 3 mls of blood collected by cardiac puncture into EDTA vacutainers. Blood was, centrifuged at 3000 g for 15 mins and serum stored at -20°C for further analysis within 6 days. In addition kidney tissue sections were taken for histopathological descriptions.

2.2 Plant Material and Phytochemical Analysis

Plant material collection and authentication was done at the Makerere University herbarium unit (00° 20` 10″N, 32° 33` 57″E) and assigned a collector number (01, NCB). The roots of *Phoenix reclinata* were cleaned and shed dried for 2 weeks in a solar drier before being coarsely ground using a mortar and pestle for extraction purpose. Five hundred grams (500 g) of ground material was then evenly packed into clean containers and 700 ml of ether added to effect extraction of the organic compounds in the roots. The set up was stoppered and left to stand for three days with constant agitation after which period the extract was filtered into a clean container for concentration using a rotary evaporator (Buchi, Switzerland). The same procedure was applied to the residue with 700 ml of methanol for three days to enable extraction of aqueous compounds after which it was discarded. The two dry extracts (ether and methanol) obtained were then mixed together and placed in a hot air oven (Narang medical Limited, India) to remove any residual moisture. The total crude extract of *Phoenix reclinata* was screened for the presence of various phytoconstituents such as alkaloids, tannins, anthraquinones, coumarin derivatives, steroid glycosides, reducing sugars and saponins based on the methods as previously described by Harbone [24].

2.3 Laboratory Evaluation of Renal Function

Quantification of serum creatinine, urea, phosphorous, calcium, sodium, potassium and chloride (mmol/L) were done as previously described [25]. All assays were done using an automated COBAS 6000 chemistry analyzer (Roche diagnostics, USA). At all times, machine calibration was achieved by running controls and standards.

2.4 Histopathological Analysis

Following surgical removal, the kidneys were immediately fixed in 10% formalin for 2 days. The organs were then transversely trimmed to include the renal cortex (since tenofovir-induced morphological abnormalities are mainly localized to the proximal tubules in the kidney [26]. The tissue sections were transferred into plastic cassettes and processed using a Histokinnette (Leica, Germany) as previously described by Spencer et al. [27]. The processed tissues were embedded in molten paraffin wax, blocked onto wooden blocks, trimmed and sectioned using a rotary microtome (Baired and Tatlock, London),

at 5 µm thickness. The sections were further floated out on a water bath (Leica, Germany, 1210) set at 44°C, dried and fixed onto glass slides in an oven set at 52°C for 48 hours. The sections were then subsequently deparaffinized, stained with hematoxylin and eosin (H&E) and later mounted using DPX (Distrene, Plasticizer and Xylene) in preparation for microscopic examination. The slides were later viewed using Carl Zeiss light microscope (Axiostar, Germany) in order to examine the kidney morphology and other pathological symptoms of nephrotoxicity in the different groups. Photomicrographs were taken with a mounted digital camera (PowerShot, China) using ZoomBrowser EX version 2 imaging software.

2.5 Data Analysis

Data analysis was done using Graphpad 6.0 statistical software. Values for each group were expressed as mean ± SEM. The differences between the levels of serum renal function parameters among the different groups were analyzed using a one-way ANOVA. Comparison between groups was done using Tukey multiple comparison test set at a significance level $\alpha = 0.05$.

2.6 Ethical Consideration

The Organization for Economic Co-operation and Development Environment Directorate (OECD) guidelines for testing of chemicals in laboratory animals were strictly adhered to [28]. The rats were maintained in a controlled environment under standard conditions of temperature (28 ± 2°C) and humidity with an alternating dark and light cycle. Animals were fed on commercially available pelleted rat chow and water ad libitum. Approval of this study was sought from the Institutional Review Board at the College of Veterinary Medicine Animal Resources and Biosecurity. All animals were euthanized and humanely sacrificed under di-ethyl ether, at the end of the experiment.

3. RESULTS

3.1 Induction of Tenofovir Disoproxil Fumurate (TDF) Nephrotoxicity

Nephrotoxicity was induced using 600 mg/kg body weight of TDF (Cipla, India) for 21 days, administered daily. After induction mortality was highest in the group receiving TDF alone (2, 40%) followed by the extract group at 200 mg/kg (1, 20%) and 400 mg/kg (1, 20%). No mortality was recorded for the extract group at 800 mg/kg (0, 0%), the positive control, calcitrol (Mega Life Sciences, Thailand) group (0, 0%) and the normal control (0, 0%) till the animals were sacrificed (Fig. 1). However, when survival curves were compared across groups, no significant differences were observed ($p = 0.25$). The predominant signs observed ranged from muscle wasting, anorexia, hypo-activity, asthenia, loose stool to poor fur content. Signs of ill health reduced downwards the extract groups at 200 mg/kg, 400 mg/kg, and 800 mg/kg and the calcitrol group as compared to the TDF group. There were no observable toxicity signs in the normal control treatment group.

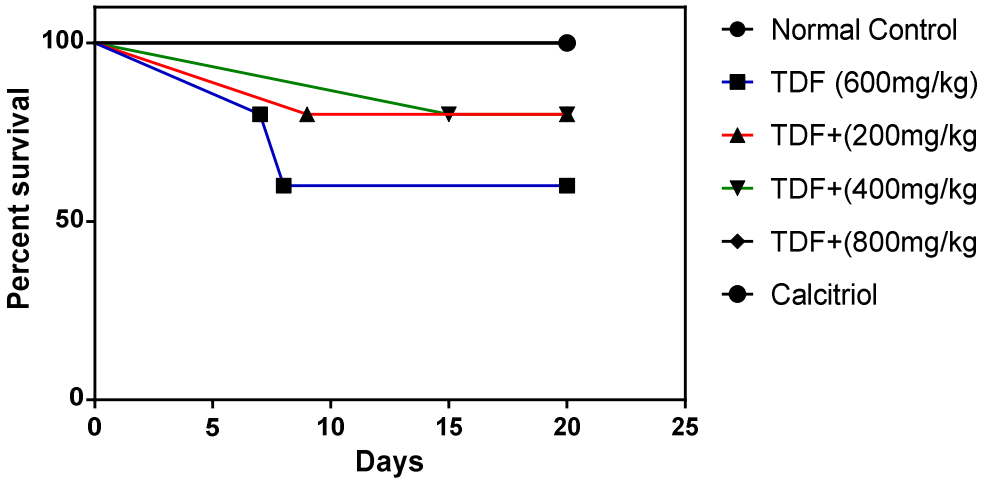

Fig. 1. Survival curves for the different treatment groups

3.2 Effect of *Phoenix reclinata* Total Crude Extract on TDF Induced Variations in Serum Biochemistry

The results revealed that treatment groups had a significant effect ($p = 0.03$, $F_{(5, 15)} = 3.2$) on serum creatinine levels. On comparison between groups, serum creatinine levels were significantly reduced in the TDF+800 mg/kg extract group (38.33±3.84 µmol/L) to levels comparable to those of the normal control (37.00±2.73 µmol/L) and the calcitorol group (36.00±2.73 µmol/L). No significant differences were observed in serum creatinine levels between the TDF group (57.33±6.61 µmol/L), the extract at 200 mg/kg (51.50±6.56 µmol/L) and 400 mg/kg (50.33±5.17 µmol/L). Similarly, serum urea levels significantly varied ($p=0.015$, $F_{(5, 11)} = 2.3$) across treatment groups. Significant reductions in serum urea levels were noted in the TDF+400 mg/kg (5.65±0.35 mmol/L) and TDF+800 mg/kg (5.13±0.12 mmol/L) groups to levels comparable to those of the normal control (5.55±0.35 mmol/L) and calcitrol groups (4.90±0.05 mmol/L). However, no significant differences in serum urea levels were revealed between the TDF group (7.33±0.82 mmol/L) and the extract at 200 mg/kg (6.00±0.54 mmol/L).

The results further showed a significant difference ($p= 0.0001$, $F_{(5, 19)} = 10.5$) in serum sodium levels across treatment groups. On comparison between groups, significant reductions in serum sodium levels of the TDF+400 mg/kg (135.5±1.52 mmol/L) and TDF+800 mg/kg (135.4±1.50 mmol/L) groups similar to those of the normal control (132.4±1.56 mmol/L) and calcitrol (132.7±1.03 mmol/L) groups were observed. There was no significant difference between the serum sodium levels in the TDF group (157.2±5.07 mmol/L) with those of the extract at 200 mg/kg (139.0±2.40 mmol/L). Likewise, the treatment showed a significant effect ($p=0.0003$, $F_{(5, 15)} = 9.7$) on serum potassium levels across groups. On comparison between groups, the levels of serum potassium were significantly reduced in the TDF+800 mg/kg group (9.88±0.45 mmol/L) to levels parallel to those of the normal control (8.48±0.67 mmol/L) and calcitrol (6.75±0.29 mmol/L) groups. No significant differences were observed in the serum potassium levels between the TDF group (14.29±2.96 mmol/L) and the extract groups at 200 mg/kg (10.65±0.79 mmol/L) and 400 mg/kg (10.05±0.63 mmol/L). On the contrary, serum chlorine levels did not significantly differ across treatment groups ($p=0.08$, $F_{(5, 18)} = 3.4$) (Table 1).

3.3 Effect of *Phoenix reclinata* Total Crude Extract on Histology of the Kidney

Histopathological examination of sections from the kidney cortex in the normal control group revealed normal kidney architecture with visible renal corpuscles and convoluted tubules (Fig. 2, Plate 1). Severe proximal tubular necrosis, glomerular and renal tubular degeneration tending to necrosis, lymphocyte infiltration and intertubular hemorrhages provided histopathological evidence of tissue injury in the tenofovir treated group (Fig. 2, Plate 2). However, these effects were significantly reversed with administration of *Phoenix reclinata* total crude extract in a dose dependent manner (Fig. 2, Plates 3, 4 and 5). Likewise, the co-administration of tenofovir and *Phoenix reclinata* total crude extract at 800 mg/kg body weight (Fig. 2: Plate 5) alleviated observed histopathological changes to levels comparable to those of the positive control group (Fig. 2, Plate 6).

Table 1. Effect of total crude extract on serum biochemical parameters

Treatment groups	Creatinine (mmol/L)	Urea (mmol/L)	Sodium (mmol/L)	Potassium (mmol/L)	Chlorine (mmol/L)
Distilled water (2 ml)	37.00±2.73^{b}	5.55±0.35^{b}	132.4±1.56^{b}	8.48±0.67^{b}	97.84±2.16^{a}
TDF (600 mg/kg)	57.33±6.61^{a}	7.33±0.82^{a}	157.2±5.07^{a}	14.29±2.96^{a}	107.9±6.16^{a}
TDF+200 mg/kg extract	51.50±6.56^{a}	6.00±0.54^{a}	139.0±2.40^{a}	10.65±0.79^{a}	95.13±1.76^{a}
TDF+400 mg/kg extract	50.33±5.17^{a}	5.65±0.35^{b}	135.5±1.52^{b}	10.05±0.63^{a}	94.63±1.30^{a}
TDF+800 mg/kg extract	38.33±3.84^{b}	5.13±0.12^{b}	135.4±1.50^{b}	9.88±0.45^{b}	94.60±1.22^{a}
TDF+Calcitriol	36.00±2.73^{b}	4.90±0.05^{b}	132.7±1.03^{b}	6.75±0.29^{b}	93.72±1.00^{a}

Lower case letters ($^{a, b}$) indicate significant differences when compared across treatment groups for each analyte. Results were expressed as mean ± SEM. TDF; Tenofovir disoproxil fumurate

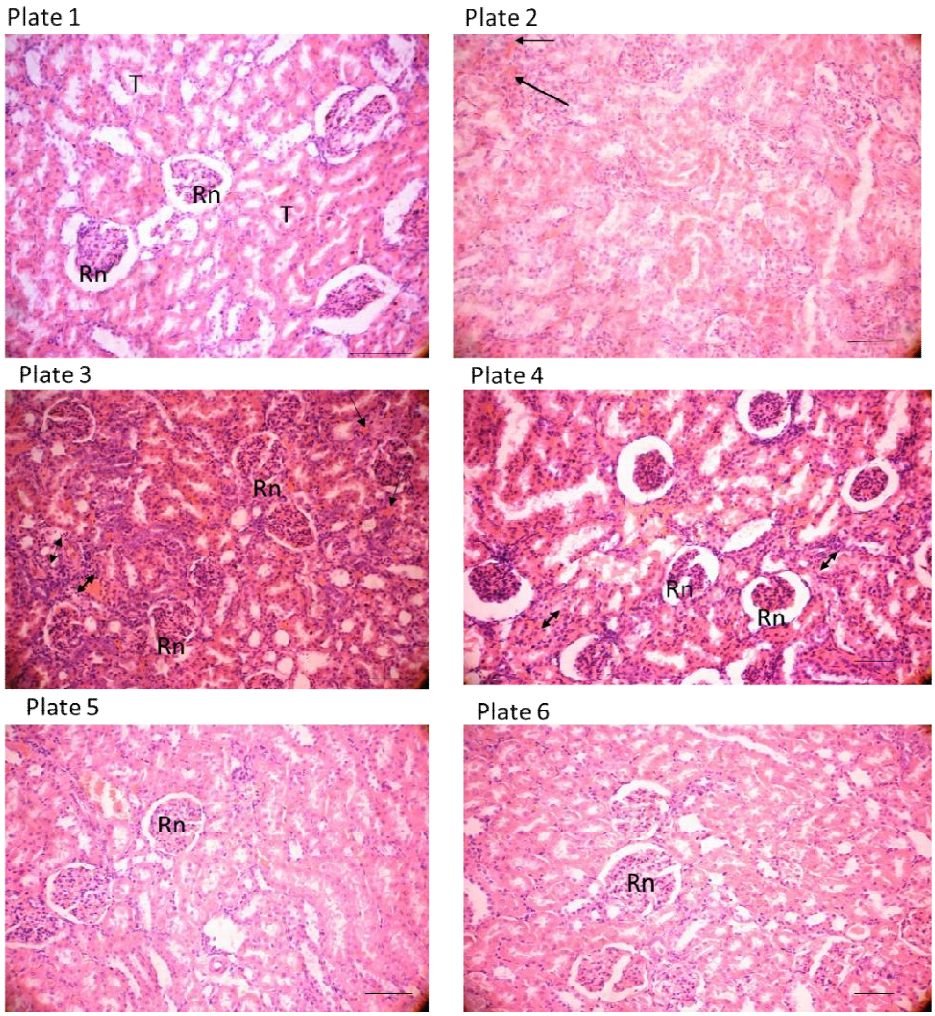

Fig. 2. Transverse section through the kidney showing histopathological changes. Plate 1 (normal control group) shows a normal kidney architecture, note the renal corpuscle (Rn) deep in the cortex, the proximal convoluted tubules (T) are also clearly discernible. In plates 2 (TDF group), plate 3 (TDF+200 mg/kg) and plate 4 (TDF+ 400 mg/kg), note the varying grades of nephrotoxicity with noticeable tissue hemorrhage (arrow heads), lymphocyte infiltration (double arrow heads), renal corpuscle atrophy and disintegration of the renal tubules. A normal kidney architecture comparable to that of the normal control (Plate 1) is noted in plate 5 (TDF + 800 mg/kg) and plate 6 (Calcitrol group). Scale bar is 25 µm

3.4 Phytochemical Constituents of *Phoenix reclinata* Total Crude Extract

Our results showed a significant presence of tannins (+++) and steroid glycosides (+++) in large amounts followed by moderate amounts of alkaloids (++), reducing sugars (++) and trace amounts of saponins (+) and anthraquinones (+). There were no detectable amounts of flavonoids (-) and carotenoids (-) present in the root extract (Table 2).

4. DISCUSSION

Tenofovir disoproxil fumurate (TDF), an oral ester prodrug for tenofovir has for some time been among the most prescribed antiretroviral agents in the developing world [2,3]. However, the effective use of TDF in the management of HIV-1infectionis hindered by severe and fatal nephrotoxicity associated with proximal tubular mitochondrial damage [5,29]. Tenofovir is administered as a combination (atripla) however,

Table 2. Photochemical constituents of *Phoenix reclinata* total crude extract

Phyto-constituents	Quantification	Qualification
Alkaloids	++	Yellowish white precipitate
Tannins	+++	Greenish black
Steroid glycosides	+++	Reddish brown ring
Saponins	+	Persistent foam for 15mins
Anthraquinones	+	Cherish red color
Flavonoids	-	No color change
Carotenoids	-	No color change
Reducing sugars	**++**	**Black red precipitate**

Key: +++; strongly present ++; moderately present +; weakly present -; absent

this combined therapy still poses a nephrotoxic risk to the patient as seen in HIV-infected antiretroviral-naïve partients starting TDF/emtricitabine +atazanavir/efavirenz/lopinavir [30]. *Phoenix reclinata*a palm species in the *Arecaceae* family is widely used in the management of gastrointestinal disturbances, female sterility, gonorrhea, impotence and epilepsy [31]. In Uganda *Phoenix reclinata* is used by traditional medicine practitioners and claimed to be effective in the management of kidney disease. To scientifically confirm these claims, we evaluated the nephroprotective effect of *Phoenix reclinata* total crude root extract on tenofovir induced nephrotoxicity in Wistar albino rats.

Studies done by Abraham et al. [26] and Ramamoorthy et al. [32] revealed proximal tubular damage, proximal tubular dysfunction and extensive proximal tubular mitochondrial injury in Wister albino rats following TDF treatment at 600 mg/kg body weight for 5 weeks. This was in agreement with our study that confirmed TDF nephrotoxicity at a similar dose of 600 mg/kg (12 × clinical dose). However, nephrotoxicity was achieved sooner in our study after a treatment period of 21 days as evidenced by the significant elevation in serum biochemistry and kidney histopathology. Conversely, other doses as low as 50 mg/kg/day TDF (Adaramoye et al. [33] for 4 weeks and 100 mg/kg/day [29] for 8 weeks were claimed to be effective at inducing nephrotoxicity. However, according to Beisecker et al. [34] a dose of 300 mg/kg (6× human dose) was not effective in causing kidney tubulopathy even after 28 days.

The TDF-induced elevations of serum creatinine, urea, sodium, potassium and chlorine levels were significantly reduced by the concurrent administration of *Phoenix reclinata* total crude root extract at 200 mg/kg, 400 mg/kg and 800 mg/kg in a dose dependent manner. The regulation in serum biochemistry in this study was confirmed with histopathology. Amelioration of the observed histopathological TDF tubulopathy was dose dependent and most significant in the 800 mg/kg extract group to levels comparable to the calcitriol group. Related work by Al-Qarawi et al. [35] using *Phoenix dactylifera* also demonstrated a nephroprotective effect on gentamicin induced nephrotoxicity by significantly reducing elevated plasma creatinine, urea and successively reversing the tubular damage. Further studies by Salah et al. [36] and Arem et al. [37] also showed the nephroprotective effect of *Phoenix dactylifera* on dimethoate and tri-chloro acetic induced nephrotoxicity respectively.

Previous studies suggested antioxidant components such as vitamin E, melatonin and ascorbic acid as the basis for the nephroprotective effect of Phoenix *dactylifera* [33,32]. However, these were not investigated in our current study. We further investigated the phyto-chemistry of *Phoenix reclinata* and found higher amounts of tannins and steroid glycosides. Tannins are claimed to contain epigallocatechin-3-0-gallate, a predominant and effective component at decreasing urinary methyl-guanidine excretion in rats suggesting a hydroxyl radical scavenging action and also help to inhibit the progression of renal failure by scavenging radicals [38]. Indeed, Yokozawa et al. [39] showed a marked decrease in urea nitrogen, creatinine, methyl-guanidine and guanidine succinic acid levels with *Ephedraeherba*, *Vhebulaefructus* and *Geraniiherba* crude extracts containing tannins among other plant extracts without tannins in rats with renal failure. Similarly, in our study the high amounts of tannins in *Phoenix reclinata* might be responsible for its nephroprotective effect. Although the mode of action of most nephroprotective compounds is not clear, studies have shown that the most effective approach to

manage tenofovir induced nephrotoxicity is drug discontinuation [40,41] so as to avoid irreversible tubulointerstitial damage. Indeed, tenofovir has been shown to damage mitochondrial DNA and inhibit biogenesis through its accumulation in the proximal tubular cells [7]. Therefore, it is likely that *Phoenix reclinata* exhibits its nephroprotective effect by preventing tenofovir accumulation in the proximal tubular cells or by protecting tubular cells from injury. This similar nephroprotective mechanism has been reported for the drug, Probenecid when used to prevent proximal tubular toxicity against cidofovir [7]. However, the exact nephroprotective mechanism of *Phoenix reclinata* needs further investigation.

5. CONCLUSION

We have demonstrated in this study that the total crude root extract of *Phoenix reclinata* was able to ameliorate TDF induced nephrotoxicity in a dose dependent manner. At 800 mg/kg of extract, the nephroprotective effect was comparable to that of the control and Calcitrol groups. Structural elucidation of the bioactive compounds responsible for the nephroprotective role and the use of purified extracts are paramount in aiding future pharmacological investigation.

CONSENT

It is not applicable.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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