Nephroprotective activities of the aqueous seed extract of *Carica papaya* Linn. in carbon tetrachloride induced renal injured Wistar rats: a dose- and time-dependent study

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Abstract

In the present study, the dose related effect of the aqueous seed extract of *Carica papaya* Linn. extract (CP*E*) was evaluated by pre-treating three groups of rats (made up of six male rats per group) with 100 – 400 mg/kg body weight per oral of the extract for 7 days before challenging with 1.5 ml/kg body weight of 20% carbon tetrachloride in olive oil in addition to the untreated control and model control rats. Also, the time-course effect of 400 mg/kg per oral of the extract were determined at 3 hr pre-, 0 hr, 1 hr post-, 3 hr post-, and 6 hr post-CCl_4 induction, respectively, in addition to the untreated control and model control groups. After 72 hours, serum levels of uric acid, urea and creatinine of all study groups were measured using standard procedures. Histological studies of rat kidneys of all study groups were also done. Results showed that intraperitoneal injection of CCl_4 caused a significant ($p<0.001$) elevation in the serum levels of uric acid, urea and creatinine and induced histological features of severe tubulo-interstitial necrosis. However, elevations in the measured biochemical parameters were significantly ($p<0.05$, $p<0.01$ and $p<0.001$) attenuated in rats pre-treated with the graded oral doses of the extract, in dose related fashion. Maximum nephroprotection was offered by the extract at 400 mg/kg/day CP*E* which lasted up to 3 hours post-CCl_4 exposure and these biochemical evidences were corroborated by improvements in the renal histological lesions induced by CCl_4 intoxication. In conclusion, our study showed that CP*E* has nephroprotective effect on CCl_4 renal injured rats, an effect which could be mediated by any of the phytocomponents present in it via either antioxidant and/or free radical scavenging mechanism(s).

Keywords: *Carica papaya* Linn.; Aqueous seed extract; Nephroprotection; Carbon tetrachloride; Wistar rats.

Introduction

Carica papaya Linn. (Family: Caricaceae) is a perennial, herbaceous plant, with copious milky latex reaching to 6 - 10 meters tall. Its erect stem is about 30 cm thick and roughened with leaf scars (Duke, 1984). The unripe fruit is used traditionally among the Yoruba tribe of Nigeria for the treatment of various human and veterinary diseases including malaria, hypertension, diabetes mellitus, hypercholesterolaemia, jaundice, intestinal helminthiasis (Gill, 1992) and for the management of sickle cell anaemia (Ogunyemi *et al.*, 2008). Also, among the Yoruba herbalists, hot infusion of the seeds of the unripe, mature fruits of *Carica papaya* is employed in the local treatment of poison related renal and hepatic disorders. Based on the traditional use of *Carica papaya* seed in the treatment of poison related renal disease,

the present study was designed to investigate the effect of graded oral doses (100 - 400 mg/kg/day) and the time-course effect of the aqueous seed extract of mature unripe *Carica papaya* (CP*E*) in CCl_4 treated rats as a way of validating this folkloric use. The choice of the extract dose range employed in this was based on the result obtained from the orientation study earlier conducted.

Materials and Methods
Plant extract
Ten mature, unripe fruits of *Carica papaya* were collected from a cultivated Pawpaw Plantation at Oke-Afa, Isolo, Lagos State, Nigeria, in the first week of May, 2008. Plant identification and authentication had earlier been done by Olagunju *et al.* (1995). The pawpaw

fruits were cut into pieces and the wet seeds were separated out. These were then gently but thoroughly rinsed in tap water for two times and completely air-dried at room temperature for 4 weeks. The dried seeds were pulverized into fine powder using a new domestic mixer grinder (Kanchan Tycoon®, Kanchan International Limited Unit III, Daman, India).

The dried aqueous seed extract of the unripe mature fruits of *Carica papaya* was obtained as follows: 40 g of the powdered dried seeds of *Carica papaya* was boiled in 500 ml of distilled water for 30 minutes, after which it was rapidly filtered through a piece of clean cotton gauze. The filtrate was allowed to cool at room temperature for 4 hours after which it was completely oven-dried at a preset temperature of 40 °C, producing a fine, aromatic, and chocolate colour solid residue. The procedure was repeated 4 times with this same quantity of solvent [yield: 22.0 ± 0.5% (w/w)]. The dry residues obtained were weighed, pooled and stored in air- and water-proof container kept in a refrigerator at 4 ºC. From this stock, fresh preparation was made whenever required.

Experimental animals
Male Wistar rats weighing between 150 and 180 g were used for investigating the nephroprotective effect *in vivo* on CCl_4-induced nephrotoxic rats. All animals were conditioned in standard metallic cages (6 rats per cage), fed standard laboratory diet (Ladokun Animal Feeds, Ibadan, Nigeria) *ad libitum* and allowed free access to drinking water. The animals were also kept in 12:12 hour light/dark cycle. The experimental rats were handled in strict compliance with international guidelines as prescribed by the Canadian Council on the Care and Use of Laboratory Animals in Biomedical Research (1984).

Determination of the dose-related protective effect of the extract
To determine the dose of CP*E* necessary for the maximum nephroprotection, three different groups of Wistar rats were separately treated with 100 mg/kg, 200 mg/kg and 400 mg/kg of CP*E* in addition to the untreated control and model control groups, for 7 days prior to single intraperitoneal CCl_4 treatment at a dose of 1.5 ml/kg body weight of 20% CCl_4 in olive oil. Seventy-two hours post-induction, the

rats were sacrificed under inhaled diethyl ether anaesthesia.

Determination of the time-course effect of the extract
To determine the time required for the CP*E* to exhibit nephroprotection, seven groups of rats were separately treated with the extract at 400 mg/kg of CP*E* at 3 hours pre-, 0 hour, 1 hour post-, 3 hours post- and 6 hours post-CCl_4 induction, respectively.

Experimental induction of CCl_4 nephrotoxicity
CCl_4-induced acute renal injury was initiated by intraperitoneal injection of 1.5 ml/kg of 20% CCl_4 (Merck, Darmstadt, Germany) dissolved in olive oil (Roberts Laboratories Limited, Belton, England) as described by Lu *et al.* (2002).

For the oral graded dose model, CCl_4 was intraperitoneally injected into groups II – V fasted rats 24 hours after the last oral dose of CP*E* on the 7th day while group I rats (untreated control) received 10 ml/kg oral route for 7 days before intraperitoneal injection of 1 ml/kg of distilled water. Rat feed and potable water were further withheld for additional 4 hours before the rats were allowed free access to feed and water. Similarly, for the time course model, same dose of the nephrotoxicant was administered intraperitoneally except that the toxicant was injected at specified time intervals of 3 hours pre-, 0 hour, 1 hour post-, 3 hours post- and 6 hours post-CCl_4 induction.

Assessment of serum uric acid, urea and creatinine
Blood samples, obtained directly from the heart chamber of the anaesthetized rats were kept at the temperature of 4 ºC for 6 hours before they were centrifuged using Uniscope Laboratory Centrifuge (Model SM 902B, Surgifriend Medicals, England, U.K.) at 3000 rpm at same temperature for 20 minutes in order to separate the sera. The urea and creatinine levels in all the sample sera were estimated by modified methods based on diacetylmonoxime reaction (Marsh *et al.*, 1965) and Jaffe's reaction (Biod and Sirota, 1948), respectively, on standard diagnostic test kits (Randox Laboratories, Crumlin, U.K.) on Automated Clinical System (Sychron Clinical System®, model: CX5 PRO) (Beckman Coulter Inc., Galway, Ireland).

Histopathological studies of the rat kidneys
After all the animals were sacrificed, postmortem examination was performed on all the identified and dissected rat kidneys. The kidneys were rinsed in normal saline and sections were taken from them. The renal tissue sections obtained were fixed in 10% neutral buffered formalin, dehydrated with 100% ethanol solution and embedded in paraffin. Tissue sections of 5 μm in thickness were prepared, stained with haematoxylin and eosin (H & E) and observed under a photomicroscope (Model N - 400ME, CEL-TECH Diagnostics, Hamburg, Germany).

Statistical analysis
Data were expressed as mean ± SEM of six observations and statistically assessed by two-way analysis of variance and the group means were compared using Student's t-test on statistical software program, SYSTAT 10.6. A probability of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered as significant.

Results
Dose-dependent preventive activity of CPE in the CCl_4 renal injured rats
Table 1 shows the effect of CP*E* on the serum levels of urea and creatinine in the CCl_4 injured rats. As shown in table 1, intraperitoneal injection of 1.5 ml/kg of 20% CCl_4 in olive oil caused significant ($p < 0.001$) rise in the serum levels of urea and creatinine as evident in the model control (group II rats). These elevations were significantly ($p < 0.05$, $p < 0.01$, $p < 0.001$) attenuated in rats pretreated with the extract in dose related fashion. However, maximum nephroprotection was offered by the 400 mg/kg of CP*E* pre-treatment.

Time-course protective activity of CPE in the CCl_4 renal injured rats
Table 2 shows the time-dependent preventive role of 400 mg/kg body weight/oral route of CP*E* against CCl_4 nephrotoxicity. As shown in table 2, CP*E* at the oral dose of 400 mg/kg body weight offered significant ($p < 0.05$) protection for up to 3 hours post-exposure to CCl_4 injection.

Table 1: Dose-dependent activities of 100 – 400 mg/kg of CP*E* on serum uric acid, urea and creatinine in CCl_4 renal injured rats.

Treatment groups	Serum concentration (mg/dl) of		
	Uric acid	Urea	Creatinine
I	2.9 ± 0.2	29.0 ± 3.8	0.9 ± 0.1
II	3.8 ± 0.2[a]	42.3 ± 3.5[c]	1.4 ± 0.2[a]
III	3.2 ± 0.2	33.3 ± 2.0[d]	1.0 ± 0.1
IV	3.2 ± 0.3[d]	29.0 ± 1.1[e]	1.1 ± 0.1
V	2.3 ± 0.1[f]	23.7 ± 1.4[f]	0.9 ± 0.2[d]

[a] and [c] represent significant increases at $p < 0.05$ and $p < 0.01$ when compared to group I (untreated control) values while [d, e] and [f] represent significant decreases at $p < 0.05$, $p < 0.01$ and $p < 0.001$ when compared with group II (model control) values.

I = 10 ml/kg per oral of DW + 1 ml/kg/IP of DW
II = 10 ml/kg per oral of DW + 1.5 ml/kg/IP of 20% CCl_4 in olive oil
III = 400 mg/kg of CP*E* + 1.5 ml/kg/IP of 20% CCl_4 in olive oil
IV = 200 mg/kg of CP*E* + 1.5 ml/kg/IP of 20% CCl_4 in olive oil
V = 400 mg/kg of CP*E* + 1.5 ml/kg/IP of 20% CCl_4 in olive oil
CP*E* = Aqueous seed extract of *Carica papaya*
IP = intraperitoneal route
CCl_4 = carbon tetrachloride
DW = distilled water

Table 2: Time-course activities of 400 mg/kg of CP*E* on serum uric acid, urea and creatinine in CCl$_4$ renal injured rats.

Treatment groups	Serum concentration (mg/dl) of		
	Uric acid	Urea	Creatinine
I	2.9 ± 0.2	29.0 ± 3.8	0.9 ± 0.1
II	3.8 ± 0.2[a]	42.3 ± 3.5[c]	1.4 ± 0.2[b]
VIII	4.1 ± 0.3[a]	38.8 ± 3.3[a]	1.1 ± 0.1[b]
IX	2.9 ± 0.3[b]	28.8 ± 3.1[d]	0.6 ± 0.2[f]
X	3.4 ± 0.3	26.3 ± 1.5[e]	1.2 ± 0.1[d]
XI	3.0 ± 0.3[d]	34.5 ± 1.7[c]	1.0 ± 0.1[d]
XII	3.6 ± 0.6	42.3 ± 6.4[c]	1.5 ± 0.3[b]

[a] and [c] represent significant increases at $p < 0.05$ and $p < 0.001$, respectively, when compared to group I (untreated control) values while [d, e] and [f] represent significant decreases at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, when compared to group II (model control) values.

I = 10 ml/kg per oral of DW + 1 ml/kg/IP of DW
II = 10 ml/kg per oral of DW + 1.5 ml/kg/IP of 20% CCl$_4$ in olive oil
VIII = 400 mg/kg of CP*E* + 3 hr pre-CCl$_4$ induction
IX = 400 mg/kg of CP*E* + 0 hr of CCl$_4$ induction
X = 400 mg/kg of CP*E* + 1 hr post-CCl$_4$ induction
XI = 400 mg/kg of CP*E* + 3 hr post-CCl$_4$ induction
XII = 400 mg/kg of CP*E* + 6 hr post-CCl$_4$ induction
CP*E* = Aqueous seed extract of *Carica papaya*
IP = intraperitoneal route
CCl$_4$ = carbon tetrachloride
DW = distilled water

Histological studies of the dose- and time-dependent effects of CPE on CCl$_4$-*treated kidneys*
Figures 1, 2, 3, 4 and 5 are photomicrographs of sections of normal rat kidney, CCl$_4$-treated kidney, 100 mg/kg/day CP*E*, 200 mg/kg/day CP*E* and 400 mg/kg/day CP*E* pretreated rat kidneys, respectively. As shown in the photomicrographs, CCl$_4$ renal intoxication was associated with severe glomerular and tubulo-interstitial necrosis which was characterized by hydropic degeneration of the glomerular and tubular cells with complete obliteration of the tubular lumen (from hydropic degeneration and tubular casts) (figure 2) when compared to normal rat kidney (figure 1). However, oral pretreatments with 100 – 400 mg/kg/day

CP*E* ameliorated renal histological lesions in dose related fashion (figures 3 - 5) with the least renal architecturally-affected being the 400 mg/kg/day CP*E* treated group (figure 5).

Similarly, figures 6 - 10 represent the time-course effect of oral treatments in CCl$_4$-treated rat kidneys. As shown in the figures, 400 mg/kg/day CP*E* treatments at 0 hr, 1 hr post-, and 3 hr post- CCl$_4$ intoxication prevented marked renal histological lesions of hydropic degeneration of the glomerular and tubular cells (figures 7 – 9) when compared to those seen in the 3 hr pre-, and 6 hr post- CCl$_4$ induction treated groups (figures 6 and 10).
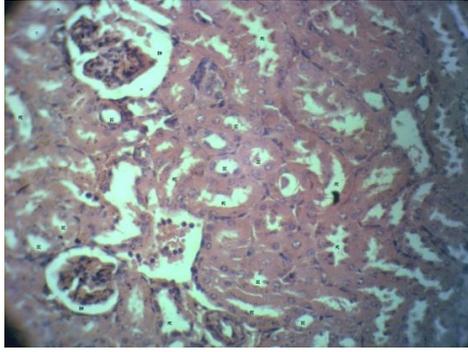
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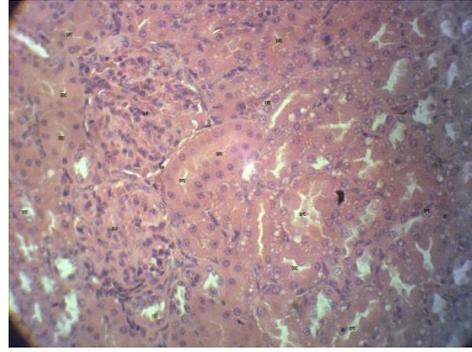
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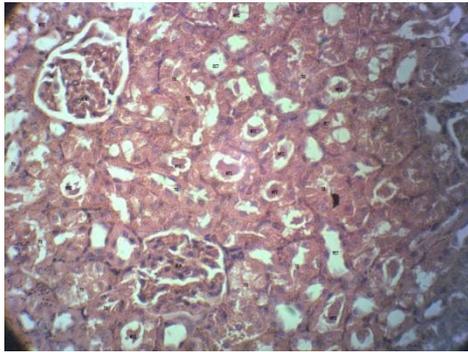
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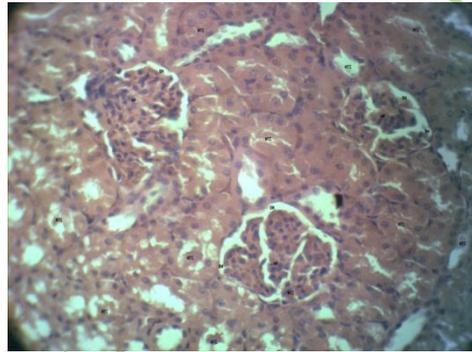
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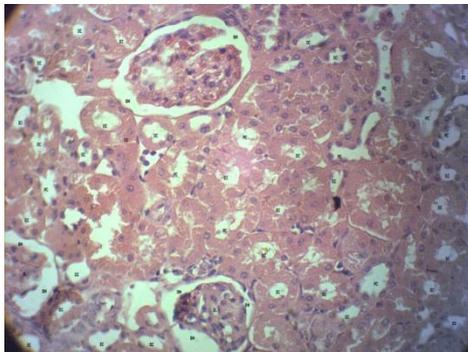
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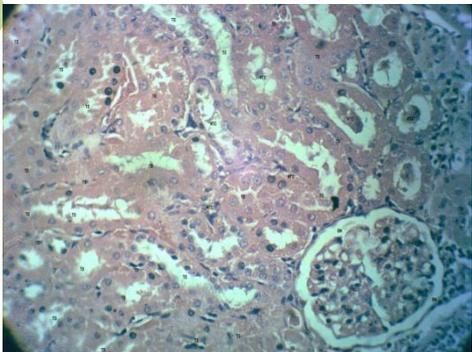
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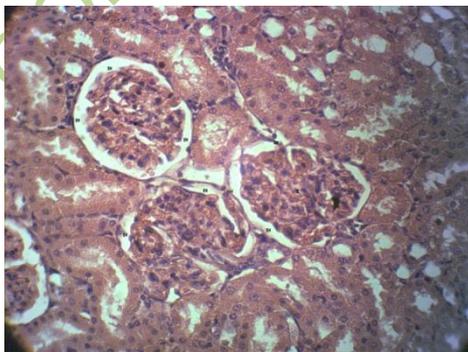
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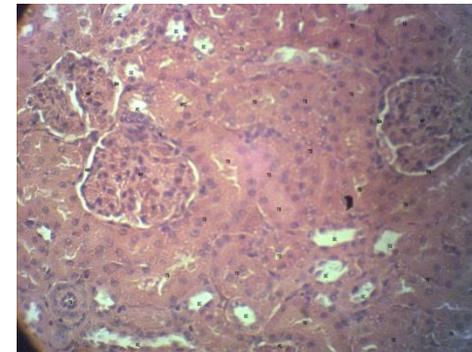
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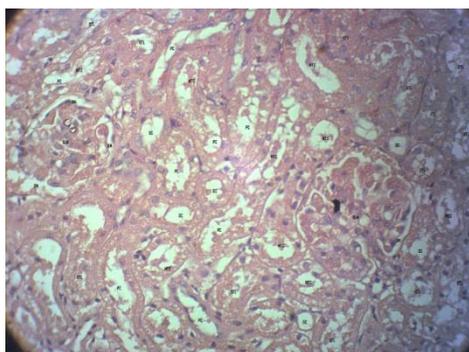
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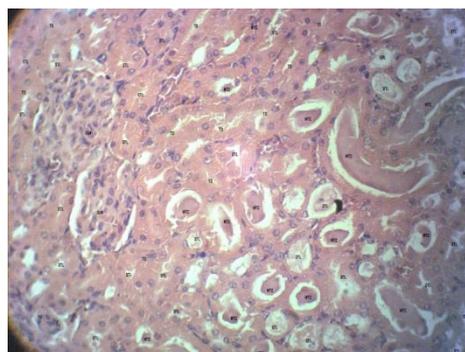
Figure 10

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Figure 1: A sectional representation of normal rat kidney at x400 magnification (Haematoxylin and Eosin stain) showing normal glomeruli (**GL**) with an intact Bowman's capsule (**BM**), proximal convoluted (**CT**) and distal convoluted (**DT**) tubules

Figure 2: A representative section of CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing severe hydropic glomerular degeneration (**GLH**), obliterated proximal convoluted tubular lumen (**OPC**) and obliterated distal convoluted tubular lumen (**ODC**). The tubular lumens were completely obliterated and filled with fluid and casts

Figure 3: A representative section of 100 mg/kg/day CP*E* pretreated, CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing moderate hydropic glomerular (**GLM**) and tubular degenerations (**TD**). The tubular lumens were moderately filled with fluid and casts (**MTC**)

Figure 4: A sectional representation of 200 mg/kg/day CP*E* pretreated, CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing mesengial proliferation (**MP**) with thinning out of the Bowman's capsule (**BM**). There is mild tubular cast deposition (**MTC**) interposed with normal proximal convoluted tubule (**PC**) and distal convoluted tubule (**DC**)

Figure 5: A sectional representation of 400 mg/kg/day CP*E* pretreated, CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing normal glomeruli (**GL**) encapsulated by normal Bowman's capsule (**BM**). There is no obvious tubular cast deposition

Figure 6: A sectional representation of 400 mg/kg/day CP*E* 3 hr pretreated, CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing moderate tubular degeneration (**TD**) with normal glomeruli (**GL**) and Bowman's capsule (**BM**).

Figure 7: A sectional representation of 400 mg/kg/day CP*E* 0 hr pretreated, CCl$_4$–intoxicated rat kidney at x400 magnification (Haematoxylin and Eosin) showing normal glomeruli (**GL**) encapsulated in normal Bowman's capsule (**BM**). There is mild tubular degeneration (**TD**) and tubular cast deposition (**MTC**) interposed with normal proximal convoluted tubule (**PC**) and distal convoluted tubule (**DC**)

Figure 8: represents the kidney section (x400 magnification, Haematoxylin and Eosin stain) of 400 mg/kg/day CP*E* 1 hr post-CCl$_4$ treatment showing some degree of mesengial proliferation (**MP**) with mild hydropic tubular degeneration (**TD**) and mild tubular cast (**MTC**) interposed with normal proximal convoluted tubules (**PC**) and distal convoluted tubules (**DC**)

Figure 9: represents the kidney section (x 400 magnification, Haematoxylin and Eosin stain) of 400 mg/kg/day CP*E* 3 hr post-CCl$_4$ treatment showing moderate hydropic glomerular degeneration (**GLM**), tubular degeneration (**TD**) and moderate tubular cast (**MTC**) and tubular obliteration (**OTL**) interposed with normal proximal convoluted tubules (**PC**) and distal convoluted tubules (**DC**)

Figure 10: A section of rat kidney (x400 magnification, Haematoxylin and eosin stain) treated with 400 mg/kg/day CP*E* 6 hr post-CCl$_4$ treatment showing severe glomerular degeneration (**GLM**) and severe hydropic tubular degeneration (**TD**) and severe tubular cast (**MTC**) and obliteration of the tubular lumen (**OTL**)

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Discussion

Various environmental toxicants and clinically useful drugs, like acetaminophen and gentamicin, can cause severe organ toxicities through the metabolic activation to highly reactive free radicals including the superoxides and oxygen reactive species (Abraham and Wilfred, 1999). One of the most extensively studied of the environmental toxicants is carbon tetrachloride (CCl_4). CCl_4 is known to undergo reductive metabolism by CYP2E1 into a highly reactive trichloromethyl radical ($.CCl_3$) and phosgene that initiates lipid peroxidation, disrupts membrane integrity and causes cell death (Pohl *et al.*, 1984; Fadhel and Amran, 2002; Basu, 2003). Evidence suggests that various enzymatic and non-enzymatic systems have been developed by the cell to cope with the oxidative stress that is associated with reactive oxygen species (ROS) and other free radicals generated (Tom *et al.*, 1984). Literature shows that oxidative stress has been implicated in the aetiopathogenesis of CCl_4 organ injury and carcinogenesis (Slater, 1984; Stal and Olston, 2000). However, when the oxidative stress is overwhelming, the various inherent defence mechanisms (such as the antioxidant defence mechanisms, intracellular concentration of glutathione, superoxide dismutase (SOD) and catalase (CAT) activities become significantly impaired and insufficient (Szymonik-Lesiuk *et al.*, 2003).

As a measure of renal function status, serum urea and creatinine are often regarded as reliable markers (Adelman *et al.*, 1981). Thus, elevations in the serum concentrations of these markers are indicative of renal injury (Adelman *et al.*, 1981). In the present study, the dose- and time-dependent nephroprotective effect of the aqueous extract of the unripe seeds of *Carica papaya* were evaluated in CCl_4 induced acute nephrotoxic rats by assessing the effect of the extract pre-treatments on the serum uric acid, urea and creatinine in the CCl_4 injected renal injured rats. In addition, the associated histological lesions were studied. In the present study, it was observed that treatment with CCl_4 induced a significant elevation in the levels of serum uric acid, urea and creatinine within 72 hours of exposure to it. These biochemical alterations were corroborated by the histological findings of glomerular and tubulo-interstitial necrosis in the untreated model control group. However, daily pre-treatment with CP*E* for 7 days conferred nephroprotection on the CCl_4 renal injured rats in a dose-dependent fashion and 400 mg/kg dose offered maximum protection within 3 hours of exposure to it. Again, the histological findings of almost normal renal histological architecture corroborate the protection conferred by the extract within the stipulated time interval, especially at the maximum oral dose of 400 mg/kg/day of the extract. Although, the possible mechanism(s) of its protection against CCl_4 induced nephrotoxicity was not studied in the current study, it is possible that the protective effect of the extract is mediated through antioxidant and/or free radical scavenging activities. Literature has shown medicinal plants with nephroprotective properties to mediate their protection via antioxidant and/or free radical scavenging activities due to the high concentration of flavonoids and alkaloids they contain (Miller and Rice-Evans, 1997; Adeneye and Benebo, 2008). Equally, saponins have been reported to protect liver and kidneys against carbon tetrachloride intoxication (Jeong *et al.*, 1996). In addition, CP*E* has been reported to contain flavonoids, alkaloids, saponins and other active phytocomponents (Adeneye and Olagunju, In Press). Summing these facts, it is plausible for the alkaloid, flavonoid and saponin components of CP*E* to be responsible for the observed biological effects. These could constitute areas of future research. Again, the nephroprotection offered by the extract could be due to the presence of any of the phyto-principles contained in it.

In conclusion, it is proposed that the nephroprotective activities of the aqueous seed extract of the unripe, mature fruits of *Carica papaya* Linn. in carbon tetrachloride-induced nephrotoxicity may involve its antioxidant and/or oxidative free radical scavenging activities. Also, the results of this study have confirmed the rationale for the folkloric use of the aqueous seed extract of *Carica papaya* Linn. in the treatment of poison-related renal disorders.

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