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POLYPHENOL-RICH LEAF EXTRACT OF *Moringa oleifera Lam.* ATTENUATES NEPHROTOXOCITY AND OXIDATIVE STRESS IN CARBON TETRACHLORIDE (CCl_4)-TREATED ALBINO RATS

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Abstract

This study evaluated the effect of polyphenol-rich leaf extract of *Moringa oleifera* (PRE-*M. o*) in carbon tetrachloride (CCl_4)-induced nephrotoxic rats. Thirty-six (36) male albino rats were randomly divided into 6 groups. Groups one to six received 1mL/kg olive oil (normal group), 1mL/kg CCl_4 (toxicity control group), N-acetyl cysteine with 1mL/kg CCl_4, 50mg/kg PRE-*M. o* with 1mL/kg CCl_4, 100mg/kg PRE-*M. o* with 1mL/kg CCl_4 and 250mg/kg PRE-*M. o* with 1mL/kg CCl_4. PRE-*M o* was administered orally, once daily for seven days while CCl_4 prepared in olive oil was administered intraperitoneally on day seven. Twenty-four hours after, animals were sacrificed and Kidneys were dissected for histology, kidney homogenates were prepared and blood samples were collected for biochemical and antioxidant parameters. Effects were investigated using biochemical indices of renal function and oxidative stress. Total phenol, which was quantified in the extract and expressed as mgGAE/g increased with increasing concentrations of the extract. CCl_4 caused a significant increase in serum creatinine, urea and uric acid concentrations as well as a significant decrease in total protein and albumin. Malondialdehyde increased while Glutathione (GSH) and superoxide dismutase (SOD) decreased significantly in the CCl_4-treated rats. Histopathological study revealed altered glomerular and tubular architecture with noticeable interstitial haemorrhage and infiltration of inflammatory cells in kidney of rats exposed to CCL_4. Treatment with PRE-*M. o* was able to significantly revert all altered parameters which showed that PRE-*M. o* can restore altered kidney function and ameliorate oxidative stress.

Keywords: Nephroprotective, Medicinal plant, *Moringa oleifera*, Polyphenol, Oxidative stress, Carbon tetrachloride

Introduction

The kidneys are such vital organs whose functionality directly influences overall human health. The kidneys' specific detoxification and excretion functions makes them practically vulnerable to damage by toxicants (Kim and Moon, 2012). The structural and functional integrity of organs/tissues remains impaired by continuous exposure to various xenobiotics triggering tissue damages and inflammations (Hasegawa *et al.*, 1995). Carbon tetrachloride (CCl_4) is one of many importantly used experimental models for free radical production, promoting tissues (brain, liver, lung, kidneys, blood etc.) damage (Kumar *et*

al., 2005; Khan and Ahmed, 2009). The pathological lesions occurring in CCl_4-treated experimental animals happens as a result of the covalent binding of CCl_4 metabolites ($CCl_3^{\cdot}$, $CCl_3OO^{\cdot\cdot}$) to cellular proteins leading to events' cascade which appreciates to cellular necrosis due to concurrent membrane lipid peroxidation (Recknagel *et al.*, 1989). Trichloromethyl radical species react with protein thiols as well as the sulhydryl group of glutathione (GSH) consequently diminishing the cellular antioxidant system and increasing its vulnerability to progressive damage by hydroxyl radicals and superoxide species (Jadeja *et al.*, 2011; Vitaglione *et al.*, 2004).

Extensive studies have been conducted on the alleviation of oxidative stress by nature-based antioxidants (Taylor, 2009; Toldy *et al.*, 2005) as safer alternatives to chemical-based antioxidants, most of which are not without side effects (Rao *et al.,* 2006). *Moringa oleifera* Lam., native to India and Pakistan, as well as other middle eastern countries (Anwar *et al.*, 2007; Sahay *et al.,* 2017; Oyeyinka and Oyeyinka, 2018), is now widely cultivated in various regions of the world due to its unique features such as drought resistance (Chukwuebuka, 2015; Gupta *et al.*, 2018), immense therapeutic values (Natsir *et al.*, 2019; Oguntibeju *et al.*, 2019) and applications in nutritional science (Oyeyinka and Oyeyinka, 2018; Karim *et al.*, 2013). This plant has been reported for its various pharmacological properties which includes antioxidative property (Oguntibeju *et al.*, 2020), hepatoprotective activity (Omotosho *et al.*, 2015), antidiabetic property (Muzumbukilwa *et al.*, 2019), anticancer property (Tiloke *et al.,* 2018), anti-inflammatory property (Koheil *et al.*, 2011), nephroprotective property (Padayachee and Baijnath, 2020) and many more. Protective properties of plants such as *Moringa oleifera* against diseases and metabolic disorders are largely due to the presence of natural antioxidants (Didunyemi *et al.*, 2020; Sulaiman and Balachandran, 2012) which are isolated from polyphenols such as phenolic acids, flavonoids, anthocyanins etc. (Vonghirundecha *et al*, 2022). Polyphenols are reportedly potent in the scavenging of free radicals (Ningappa *et al.*, 2008), as such very promising in the management of free radical-induced organ dysfunctions within the body systems, hence the need to evaluate specifically the polyphenolic extract of *Moringa oleifera* leaf in carbon tetrachloride-exposed rats.

Materials and Methods

Experimental Animals.

Thirty-six (36) male albino rats (Rattus novergicus) weighing about 100 - 150g were used for the experiment. Before the commencement of the experiment, the animals were acclimatized for 1 week under standard conditions of temperature, relative humidity and light/night cycles with free access to commercial rat chow and water ad libitum. Ethical clearance with the number OAUSTECH/ETHC-BCH/2022/003 was obtained for the study protocol having met the requirement of the institutional ethical review committee

Plant Materials

Fresh leaves of *Moringa oleifera* plant were collected from a farm located at Doctor's Road in Oktitipupa town, in the southwestern part of Ondo State, Nigeria, Africa, with the geographical coordinates being $6^{0}32'53''$ North, $4^{0}41'56''$ East. Identification and authentication were done in the herbarium unit of the Department of Botany, Olusegun Agagu University of Science and Technology (OAUSTECH) Okitipupa, Ondo State, Nigeria with the voucher number OAUSTECH/BH002/VN774. The leaves were air dried at room temperature for 2 weeks and blended with an electric blender to a coarse

powder.

Chemicals

All used assay kits are products of Randox company, United Kingdom. Methanol, Ethyl acetate n-Hexane and other reagent used are of analytical grade and prepared in glass distilled water.

Preparation of Polyphenol Rich Extract of *Moringa oleifera* (PRE-*M. o*)

The method of Lin *et al.* (2005) was used for the preparation of Polyphenol Rich Extract of *Moringa oleifera* (PRE-*M. o*). Briefly 100 g of powdered leaves were extracted three times with methanol (300 mL) at 50°C for 3 hours. Samples were filtered following each extraction and solvent was removed from the combined extracts with the help of a vacuum rotary evaporator. The residue was then dissolved in 500 ml of water (50°C) and extracted with 200 ml hexane to remove some of the pigments. The resulting aqueous phase was extracted three times with 180 mL ethyl acetate (which was evaporated under reduced pressure). The residue was re-dissolved in 250 mL water and lyophilized to obtain approximately 3.840g of the extract. Yield was stored at -20°C before use.

Determination of Oral Lethal Dose (LD50) of PRE-*M. o*

The oral LD_{50} of PRE-*M. o* was determined according to the method of Lorke (1983). Nine animals were used for the first phase and 3 animals for the second phase. In the first phase of the experiment, nine rats were divided into 3 groups of 3 rats each and were administered PRE-*M. o* at doses of 10, 100 and 1000 mg/kg orally and were observed for 24 h for signs of toxicity. In the second phase, 3 rats were divided into 3 groups of one rat each and were administered PRE-*M. o* at 1600, 2700 and 5000 mg/kg, orally and were also examined for 24 h.

Experimental design

36 male albino rats weighing 100 – 150g were randomly divided into six groups (n = 6) and treated as follows;

Group 1, Normal control received 1 ml distilled water daily + olive oil on day 7

Group 2, Negative control group, received 1ml distilled water daily + 1.5mL/kg "CCl_4 on day 7

Group 3, received 50 mg/kg PRE-*M. o* daily + 1.5mL/kg "CCL_4on day 7

Group 4, 100 mg/kg PRE-*M. o* daily + 1.5mL/kg "CCL4on day 7

Group 5, 250 mg/kg PRE-*M. o* daily + 1.5mL/kg "CCL4on day 7

Group 6, received 100 mg/kg *b. w* N-acetyl cysteine daily + 1.5mL/kg "CCl_4on day 7

Distilled water and PRE-*M. o* was administered orally while olive oil / CCl_4 in olive oil were administered intraperitoneally.

Preparation of Serum and Tissue Homogenate

After the 7th day of the experiment, the animals were fasted for 24 hours. Thereafter, the animals were anaesthetized under light ether, and blood samples were collected by cardiac puncture method into sample tubes and then centrifuged at 3000x g for 10 minutes to obtain serum and stored at -20°C for further analysis. After blood sample collection, the animals were sacrificed and kidney tissue was harvested for biochemical and histopathological studies. kidney tissue (1 g) was homogenized in nine volumes of phosphate buffer and centrifuged at 12 000 g for 30 min at 4°C. The supernatant was collected and used for the estimation of MDA, SOD and GSH (Jollow *et al.*, 1974). The kidney samples from different groups were preserved in 10% buffered formalin.

Determination of total phenolic content

The total phenolic contents of the extracts were measured using the Folin-Ciocalteau colourimetric method described by Saeed *et al.*, (2012), with gallic acid as the standard. This method relies on the transfer of electrons in an alkaline medium from phenolic

compounds to form a blue chromophore constituted by a phosphotungstic/ phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds. Basically, 0.2 ml of extract was mixed with 1ml of 10 % Folin-Ciocalteau phenol reagent. After 4 min, 0.8 ml of 7.5% saturated Na_2CO_3 solution was added and the mixture was left standing for 2 hours. Absorbance was measured at 765 nm and the amount of total polyphenols in different extracts were expressed as mg of gallic acid equivalent (GAE)/g of extract **(**Saeed *et al.*, 2012)

Total Flavonoid Content

Flavonoids were quantified using the aluminum chloride reagent ($AlCl_3$) method. The flavonoids content was expressed as quercetin equivalents (QE). Briefly, 1 ml of extract, dissolved in corresponding solvent was added to 1 ml of $AlCl_3$ (2% in methanol). After incubation at room temperature for 10 min, absorbance was measured at 430 nm. (Ayoola *et al.*, 2008).

Assessment of Kidney Damage

kidney damage was assessed by the estimation of serum creatinine, Urea and Uric acid and Total protein using commercially available test kits (Randox, UK). Kidney superoxide dismutase (SOD) was done by method of Misra *et al.* (1989), lipid peroxidation (MDA quantification) by method of Mihara & Uchiyama (1978) and glutathione level (GSH) was assessed by method of Beutler (1963). Histopathological assessment of kidney damage was done by studying the hematoxylin and eosin-stained slides of kidney tissue (Avwioro, 2010).

Statistical analysis

Results are expressed as the mean ± SD, Each experiment was performed in triplicates. Statistical differences between the groups and the controls were done by one-way analysis of variance (ANOVA) and Duncan's multiple ranges test using GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The criterion for statistical significance was $*P < 0.05$

Results

Acute Toxicity Study

The acute lethal study of PRE-*M. o* on rats (Table 1) shows that no animal died within 24 hours after treatment with the extract and the LD_{50} was greater than 5000 mg/kg *b. w*. No physical signs of extract toxicity were also observed among the animals.

Table 1: result of oral acute toxicity test of PRE-*M. o*

No. of Rats	Dose (mg/kg *b.w*)	No. of Deaths after 24 hrs
First Phase		
3	10	0/3
3	100	0/3
3	1000	0/3
Control		
3	0	0/3
Second phase		
1	1300	0/1
1	2700	0/1
1	5000	0/1

Total phenol and total flavonoid quantification

Table 2: Total phenol and flavonoid content of Polyphenol-rich leaf extract of *Moringa oleifera*

Conc. (µ g/ml)	100	250	500	1000
Total phenol (mgGAE/g)	17.91±0.13	38.54±0.65	45.15±0.75	70.53±4.78
Total flavonoid (mgQE/g)	281.10±9.45	472.52±11.61	736.04±10.17	1107.31±16.06

Values represent Mean ± standard deviation of total phenol and total flavonoids of the Polyphenol-rich leaf extract of *Moringa oleifera*. Assay was done in triplicates for each of the tested concentrations.

Serum Creatinine results

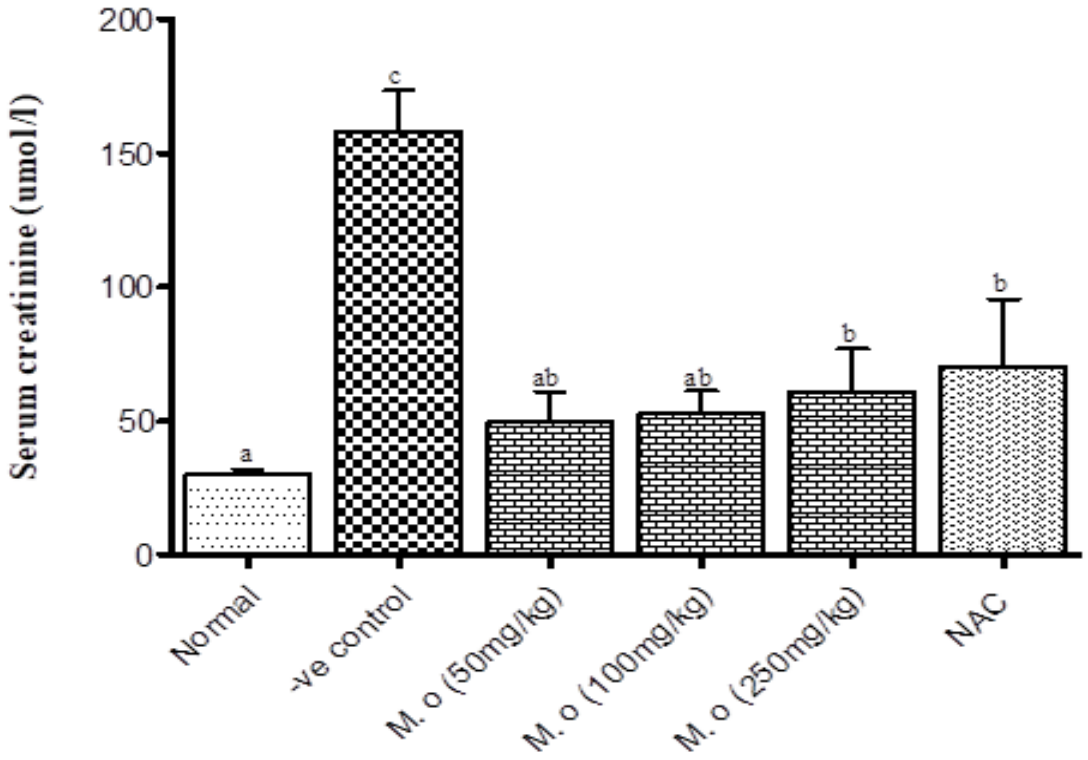

Figure 1: Serum creatinine concentrations of rats in Normal group, negative control group, Polyphenolic-rich leaf extract of *Moringa oleifera* (PRE-*M. o*)-treated groups and NAC-treated group. Bars represent mean ± standard deviation. Bar with a different alphabet is significantly different ($p<0.05$)

Urea, Uric acid, Total protein and Albumin results

Table 3: Serum Urea, Uric acid, Total protein and serum albumin concentrations of rats in the different experimental groups

Groups	Urea Conc. (mMol/l)	Uric acid (mg/dl)	Total Protein (g/dl)	Serum Albumin (g/dl)
Normal	7.75 ± 0.89 ^{b}	6.81 ± 0.83 ^{a}	7.61 ± 0.82 ^{bc}	3.38 ± 0.03 ^{b}
Negative control	17.99 ± 3.75^{c}	13.35 ± 1.83^{b}	3.72 ± 1.40 ^{a}	1.72 ± 0.77 ^{a}
PRE-*M. o* (50mg/kg)	7.16 ± 0.96 ^{b}	7.73 ± 3.26 ^{a}	8.34 ± 0.59 ^{c}	3.27 ± 0.18 ^{b}
PRE-*M. o* (100mg/kg)	6.76 ± 1.65 ^{b}	7.29 ± 2.31 ^{a}	5.94 ± 0.95 ^{bc}	2.94 ± 0.06 ^{b}
PRE-*M. o* (250mg/kg)	6.47 ± 1.48 ^{b}	6.13 ± 2.61 ^{a}	7.28 ± 2.26 ^{bc}	3.35 ± 0.59 ^{b}
NAC	2.82 ± 0.77 ^{a}	10.70 ± 0.17^{ab}	5.22 ± 0.57 ^{b}	3.35 ± 1.75 ^{b}

Value represents mean ± standard deviation of three replicates of Serum Urea, Uric acid and Total protein concentrations of rats in Normal group, negative control group, Polyphenolic-rich leaf extract of *Moringa oleifera* (PRE-*M. o*)-treated groups and NAC-treated group. Value(s) with different superscript along the same column is/are significantly different ($p<0.05$).

Malondialdehyde, Superoxide dismutase and Reduced glutathione results

Table 4: Malondialdehyde (MDA), Superoxide dismutase (SOD) and Reduced glutathione (GSH) across the experimental groups.

GROUPS	LIPID PEROXIDATION (MDA) (nMol/ml)	SUPEROXIDE DISMUTASE (SOD) (U/ml)	GSH (mMol)
Normal	1.17 ± 0.09^{a}	224.03 ± 7.08^{b}	2.97 ± 0.05^{d}
Negative control	7.65 ± 1.40^{b}	122.43 ± 3.70^{a}	0.86 ± 0.11^{a}
PRE-*M. o* (50mg/kg)	1.03 ± 0.32^{a}	169.87 ± 14.20^{ab}	2.03 ± 0.06^{b}
PRE-*M. o* (100mg/kg)	0.94 ± 0.23^{a}	218.20 ± 9.27^{b}	2.33 ± 0.12^{c}
PRE-*M. o* (250mg/kg)	0.87 ± 0.07^{a}	205.87 ± 9.18^{b}	2.30 ± 0.46^{c}
NAC	1.05 ± 0.16^{a}	159.73 ± 4.20^{ab}	3.01 ± 0.16^{e}

Value represents the mean ± standard deviation of three replicates of MDA, SOD and GSH concentrations of rats in Normal group, negative control group, Polyphenolic-rich leaf extract of *Moringa oleifera* (PRE-*M. o*)-treated group and NAC-treated group. Value(s) with different superscript along the same column is/are significantly different ($p<0.05$).

Histopathology result

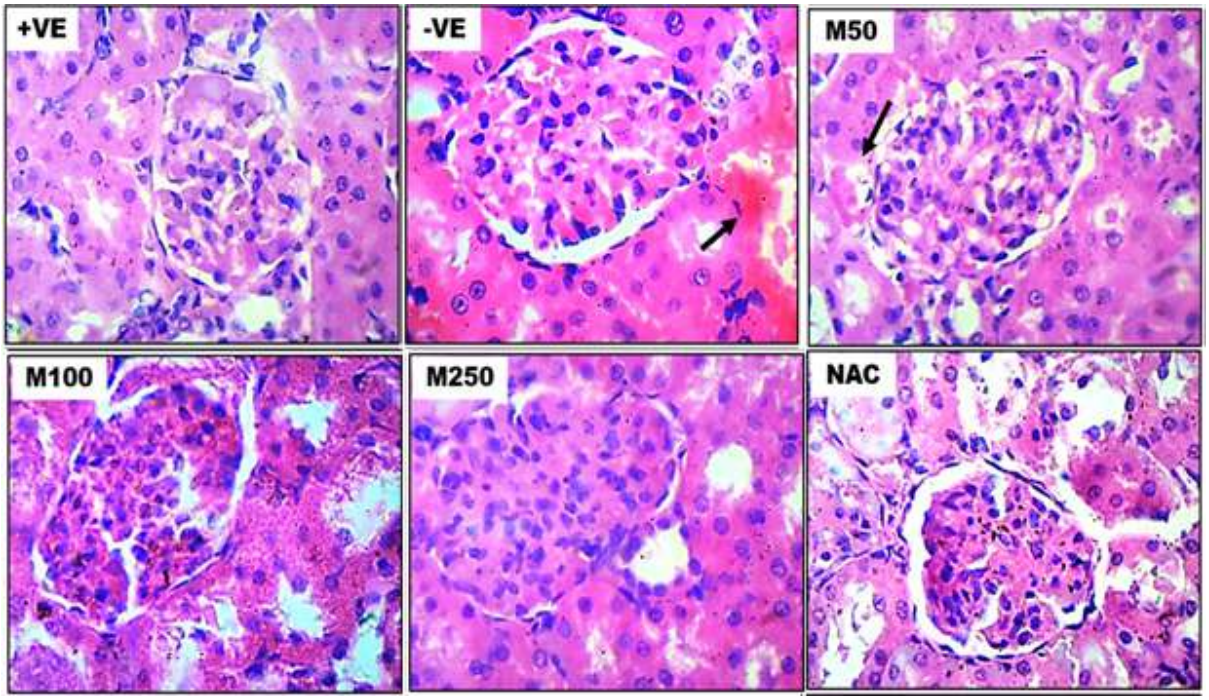

Figure 2: Micromorphological section of rat kidneys demonstrated by Haematoxylin and Eosin staining (**N**) Normal control group (**-ve**) CCl_4-treated group (**M50**) 50mg/kg *b.w* PRE-M. o-treated group (**M100**) 100mg/kg *b.w* PRE-*M. o*-treated group (**M250**) 250mg/kg *b.w* PRE-*M. o*-treated group and (**NAC**) N acetyl cysteine- or standard drug-treated group. Magnification (X400).

Discussion

Active plant constituents, such as phenolics confer antioxidative efficacies (Ayoola *et al.*, 2008) Phenolic compounds are good donors of hydrogen to active free radicals, owned to their phenolic hydroxyl group contents (Sawa *et al.*, 1999), hence breaking the chain reactions of oxidation of lipids (shadidi *et al.,* 1992) and proffering protection for cells and cellular components. Phenolic acids are reportedly efficient in mopping up or deactivating free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide (Erkan *et al.*, 2008; Valko *et al.*, 2006). Results of total phenolic contents (estimated in mgGAE/g of extract) obtained from the tested concentrations of the extract (PRE-*M. o*) exhibited a progressive increase in Total phenol content with increasing extract concentration (table 2). This finding further established and supports the reports of Anwar *et al.* (2007) and shows that *Moringa oleifera* plant is undoubtedly a good source of natural antioxidants with appreciable health benefits (Krawczyk *et al.*, 2022). Carbon tetrachloride had been adopted in studies as an experimental model to evaluate the nephroprotectivity of drugs and plant products (Ogeturk *et al.*, 2005; Safhi, 2018). CCl_4 toxicity constitutes the production of free radicals in many vital organs such as kidneys, liver, brain, lungs etc. The generated CCl_4 metabolites (free radicals) are trichloromethyl radical (CCl_3) and trichloromethyl peroxyl radical (CCl_3O_2) (Ali and Abdelaziz, 2014). These metabolites interact with many intracellular peptides, membrane lipids and DNA leading to the denaturation of proteins, peroxidation of lipid and oxidative damage of

DNA and consequentially, cell death (Hismiogullari *et al.*, 2015). Sanzgiri *et al.* (1997) reported a more even distribution of CCl_4 in the kidneys of CCl_4-treated rats than in the livers owned to a higher affinity of the kidney for CCl_4 and predominant presence of cytochrome p450 in the cortex of the kidneys. In this study, Intraperitoneal administration of 1mg/mL CCl_4 in the negative control group further established the pathologic characteristics attributable to CCl_4 intoxication by a significant ($p<0.05$) increase in serum creatinine (figure 1), urea and uric acid levels and a decrease in total protein and albumin concentrations (table 3) when compared to other experimental groups. However, oral administration of PRE-*M. o* at three concentration levels (50, 100 and 250mg/kg *b.w*) were able to ameliorate these alterations in serum creatinine, urea and uric acid levels as well as caused a significant ($p<0.05$) rise in serum albumin and total protein concentrations. Effects in the extract-treated groups were comparable statistically to normal and/or standard drug group(s). This may be due to the presence of high phenolic content in the extract (Ayoola *et al.*, 2008; Hegazy and Ibrahim, 2012; Didunyemi *et al.*, 2020) which directly influences the antioxidant capacities and free radical scavenging efficacy thus inhibiting renal damage. Soong and Barlow (2004) described Polyphenols as astute free radical scavengers and antioxidant agents from plants capable of neutralizing reactive CCl_4 metabolites, which impose deleterious effects on the kidney.

Oxidative stress/damage was investigated in the animals by assaying for the extent of lipid peroxidation (via quantification of malondialdehyde), superoxide dismutase (SOD) and reduced glutathione (GSH). MDA is a primary product of the peroxidation of membrane polyunsaturated fatty acids by reactive oxygen species (ROS), which by virtue of its reactivity is capable of interacting with various functional groups on proteins, lipoproteins and DNA thus forming adducts capable of mounting severe damage on cell structures, indicative of toxicological conditions (Jadoon and Malik, 2017). CCl_4 is one major of many toxicants reportedly capable of elevating MDA production and decreasing Superoxide dismutase (SOD) and Reduced glutathione (GSH) in experimental animals (Almundarij *et al.*, 2021) and the results obtained from CCl_4-treated groups in our experiment (table 4) is no exceptions. However, treatment with PRE-*M. o* was able to significantly ($p<0.05$) reduce MDA concentration in the kidney homogenates and the result compared well with the normal group and standard drug groups (table 4), this may be due to the ability of the components of the extract to donate hydrogen to unstable radicals thereby preventing them from harvesting electron from the membrane polyunsaturated fatty acids (PUFAs). Superoxide dismutase (SOD) is a biologically active substance capable of getting rid of harmful molecules generated during metabolic processes. It catalyzes the disproportionation of superoxide radicals to molecular oxygen and hydrogen peroxide, and hence is useful for protecting the cell against toxic products (Fridovich, 1997: Mccord and Fridovich, 1969). PRE-*M. o.* significantly alleviated the reduced SOD level caused by CCl_4 intoxication. The increase in SOD levels in the 50, 100 and 250mg/kg PRE-*M. o*-treated groups were not significantly different ($p<0.05$) from the control and standard drug groups. GSH (reduced glutathione) level is a reflection of the antioxidation efficiency in an animal. GSH act as cofactors for several detoxifying enzymes such as Glutathione peroxidase and Glutathione-S-transferase and helps to

maintain the redox balance of cells (Valko *et al.*, 2006). Reduction of GSH level obtained from the group administered with CCl_4 in the current study aligned with the reports of Makni *et al.* (2012), Khan and Siddique (2012) and Almundarij *et al.* (2021). Treatment with PRE-*M. o* exhibited significant protection against depleted GSH induced by CCl_4. Results of the antioxidant activities of the extract at all tested concentrations proved the antioxidant efficacy of the polyphenol-rich extract of *Moringa oleifera* and created an agreement with the rich phenolic content quantified in the extract. It shows that the extract can facilitate protection against oxidative stress induced by toxins.

The findings from the histological examinations of the kidneys conform with the results of the biochemical indices assayed in the animals. Intraperitoneal administration of CCl_4 in control group triggered glomerular and tubular lesions and this result is consistent with the reports of Dogukan *et al.* (2003) and Almundarij *et al.* (2021). The renal cortex of the rats shows glomeruli with some pyknotic-mesangial cells and wider capsular space as signs of collapsing glomeruli, the renal tubules appear congested and infiltrated with red inflammatory cells with some mild to severe vascular congestion (figure 2). These structural deformations promote renal dysfunction and of course, are a consequence of the production of free radicals by CCl_4. Notable structural restorations to near normal were observed in the groups treated with PRE-*M. o*. this may be due to the antioxidant efficacy of the extract (in scavenging free radicals produced by CCl_4 intoxication) as evident in the results of the biochemical assays.

Conclusion

From this study, we can conclude that the polyphenol-rich leaf extract of *Moringa oleifera* (PRE-*M. o*) is a promising source of antioxidants, capable of managing the deleterious effect of CCl_4 toxicity on kidneys and other vital organs and restoring organ functionality to near-normal. By extension, PRE-*M. o* can be an appreciable therapy for the management of various ailments with ROS involvement.

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