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Research paper

Hepatoprotective activity of the methanolic leaf extract of *Moringa oleifera (Lam)* against chemically-induced Liver Toxicity

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Abstract

Aim: *Moringa oleifera* commonly known as "miracle plant" possesses enormous nutritional and medicinal properties. It is used in traditional medicine in treating many ailments, including liver disorders. Though some works on hepatoprotective effects have been done (Mishra et al., 2011), the present study aims at evaluating the methanolic leaf extract (MLE) of *Moringa oleifera* harvested in Ewet Housing Estate in Uyo; AkwaIbom State in South-South Nigeria.

Materials and Methods: The Carbon tetrachloride (CCl_4) model was employed throughout this investigation (Dongare et al., 2013). Briefly, thirty animals were randomly divided into six groups of five animals in each group. Group I served as normal control and was administered 10ml/kg normal saline. Group II was the toxic control and received 3 ml/kg of CCl_4. Group III served as the reference control and received 100mg/kg silymarin while groups IV, V and VI received 9, 18 and 27 mg/kg of MLE respectively. Phytochemical screening, acute toxicity tests as well as free radical scavenging effects using DPPH (in vitro) were also carried out.

Results: Preliminary phytochemical tests revealed the presence of tannins, flavonoids, alkaloids, anthraquinones, saponins, terpenes, phlobatanins and cardiac glycosides. The acute toxicity investigations showed that MLE LD_{50} was 90 mg/kg. In the hepatoprotective studies, liver function tests (LFT) revealed a significant ($p<0.05$) protective effect when compared with silymarin. The histopathological studies also provided supportive evidence for the protective effects of MLE. The DPPH studies showed that MLE has antioxidant property.

Conclusion: It can be concluded based on findings from this study that the MLE of *Moringa oleifera* possesses antioxidant and hepatoprotective activities in a dose-dependent manner and safe for oral administration.

Keywords: *Antioxidant; carbon tetrachloride; hepatitis; necrosis; silymarin.*

1. Introduction

The liver is the main organ where exogenous chemicals are metabolized and eventually excreted. Owing to its strategic anatomical location, it is exposed to many xenobiotics including therapeutic agents (Chatterjee, 2000). This can cause liver dysfunction, cell injury and even organ failure.

About 20,000 deaths recorded every year have been ascribed to liver disorders (Gupta et al., 2006). Treatment options for common liver diseases such as liver cirrhosis, fatty liver and chronic hepatitis are problematic. Physicians and patients are in need of effective therapeutic agents with low incidence of side effects. Liver disease is still a worldwide health problem. Unfortunately, conventional or synthetic drugs used for the treatment of liver diseases are inadequate and sometimes can have serious side effects (Gupta, 2006). In spite of tremendous advances in modern medicine, no effective drugs are available, which stimulate liver functions and offer protection to the liver from the damage or help to regenerate hepatic cells (Panda, 2009). In the absence of a reliable liver-protective drug in modern medicine, a study on this "wonder plant" *Moringa oleifera* is pertinent.

The plant kingdom represents a rich store house of organic compounds, many of which have been used for medicinal purposes and could serve as lead for the development of novel agents having good efficacy in various pathological disorders in the coming years (Goyal et al., 2007). This suggests that, in principle, most plants have a potential medicinal value. Medicinal plants have been considered as important therapeutic aids for alleviating ailments of humankind. About 80% of the world population relies on the use of traditional medicine, which is predominantly based on plant materials (Singh et al., 2013). Traditional medicine practitioners in various parts around the world have used Moringa in their herbal medicine repertoire for ailments ranging from gastrointestinal problems, to veneral diseases and inflammations as well as to treat liver and renal disorders, etc., thus earning the plant names such as "miracle tree" "wonder tree" "mother best friend" (Ganatra et al., 2012).

2. Materials and methods

2.1. Plant collection and identification

Fresh leaves of *Moringa oleifera* were collected from Ewet Housing Estate in Uyo Local Government Area of Akwa Ibom State, Nigeria in November, 2014. The plant material was identified and authenticated by Dr. (Mrs.) Margaret Bassey, a plant

Taxonomist in the Department of Botany and Ecological Studies, University of Uyo, Uyo. A voucher specimen A (50)i was deposited in the herbarium of the Faculty of Pharmacy, University of Uyo.

2.2. Preparation of extract

The leaves were sun dried for seven days. The dried leaves were pulverized to powder, weighed and macerated in 60% methanol for 72 hours. The filtrate obtained was concentrated and evaporated to dryness using water bath. The dried extract was preserved in the refrigerator until required for use. The yield of the extract was calculated to be 10.93% w/w.

2.3. Phytochemical screening of extract

The crude methanol extracts were subjected to phytochemical screening using standard procedures (Trease and Evans, 2002).

2.4. Determination of Median Lethal Dose

The Up-and-Down procedure (UDP)-OECD TG 425 was used to determine the LD_{50}. This method uses death as an endpoint, but doses animals one at a time to see if the dose needs to be put up or down to achieve an estimate of the LD_{50} therefore, giving the minimum number of animals a lethal dose of the test substance (Paramveer, 2010). In the Up-and-Down procedure, animals are dosed one at a time. If an animal survives, the dose for the next animal is increased; if it dies, the dose is decreased. Each animal was observed for 24 h. briefly, doses of the extract (20-2000 mg/kg) were administered intraperitoneally (i.p) to the mice and signs of physical toxicity were observed. The LD_{50} was determined from this. Swiss albino mice were used for this study.

2.5. Experimental design

a) Hepatoprotective studies:

Thirty (30) animals were randomly divided into six (6) groups with five (5) animals in each group. They were treated as described below:

Group I (Normal Control): Normal saline (10ml/kg) was administered orally for eight (8) days.

Group II (Toxic Control): Normal saline (10ml/kg) was administered orally for seven (7) days. On the eighth day, (3ml/kg) of CCl_4 was administered intraperitoneally (i.p).

Group III (Reference Control): Silymarin (100mg/kg) was administered orally for seven (7) days. On the eighth day, (3ml/kg) of CCl_4 was administered intraperitoneally (i.p).

Groups IV, Group V and Group VI received 9, 18 and 27mg/kg of MLE orally for seven (7) days. On the eighth day, (3ml/kg) of CCl_4 were administered intraperitoneally (i.p).

On the ninth day and 24 hours after the CCl_4 administration, each animal was weighed and then sacrificed under chloroform anesthesia. Blood samples were collected for biochemical analysis. Liver tissues were obtained for histopathological analysis.

b) Antioxidant Studies

The free-radicalscavenging activity of *Moringa oleifera* methanolic leaf extracts was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Blois (1958) with slight modification. Briefly, 2ml of DPPH solution (0.004% in methanol) was incubated with 2ml of extract at various concentrations (20-100 µg/ml). The reaction mixture was shaken and incubated at room temperature for 30 minutes in the dark. The control was prepared as above without extract. The absorbance of the solution was measured at 517nm against a blank. Vitamin C (ascorbic acid) was used as reference standard. The DPPH radical scavenging activity was calculated according to the following equation.

$$(\%\ \text{inhibition}) = \{(A_0 - A_1) / A_0\} \times 100$$

Where A_0 was the absorbance of the control reaction (DPPH) alone and A_1 was the absorbance in the presence of the sample (extract or vitamin C).

The IC_{50} value denotes the concentration of sample required to scavenge 50% of DPPH free radicals.

2.6. Statistical analysis

Data were expressed as mean ± SEM. Statistical comparisons between groups were performed using analysis of variance (ANOVA). Differences between Means were determined by Tukey-Kramer pair-wise comparison test at a level of significance $p<0.05$.

3. Results

The phytochemical screening results show that *Moringa oleifera* leaf extract contains flavonoids, tannins, saponins, alkaloids, phlobatanins, terpenes and cardiac glycocides. Free and combined anthraquinones were absent. They are as presented in table 1.

The median lethal dose (LD_{50}) in mice following oral administration was calculated to be 90 mg/kg. The physical signs of toxicity included writhing, decreased respiratory rate, decreased motor activity and death within 24 h.

The results of the biochemical analysis are presented in table 2 and figures 1 to 5. The administration of CCl_4 (3 ml/kg) evoked significant ($p<0.05$-0.001) elevations in serum levels of biomarker enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) when compared with the normal control. The pretreatment of rats with MLE (9, 18 and 27 mg/kg) significantly ($p<0.05$-0.001) mitigated these elevations in a dose-dependent manner. Similarly, pretreatment of rats with MLE (9, 18 and 27 mg/kg) attenuated the significant elevations ($p<0.05$-0.001) in total and conjugated bilirubin levels. The protection produced by 27 mg/kg of MLE was comparable to silymarin (100 mg/kg).

The effects of MLE on free radical scavenging activity using DPPH in vitro are presented in tables 3 and 4 as well as figure 6.

From the results the IC_{50} of the extract and vitamin C were determined to be 84.0 and 10.8 µg/ml respectively. The extract produced a concentration-dependent free radical scavenging activity.

The results of the activity of MLE on the histology of the liver are presented in Table 5. In the histopathological examination of the liver tissue the negative control showed normal architecture, no steatosis, mild portal inflammation, mild congested sinusoids, no necrosis and no fibrosis (Fig. 6) whereas the toxic control showed distorted architecture, focal necrosis, severe steatosis/portal inflammation with interphase hepatitis/congested sinusoids and no fibrosis (Fig. 7). Treatment with Silymarin 100 mg/kg showed improved architecture compared to the toxic control, decreased steatosis/necrosis/portal inflammation but no interphase hepatitis, mild to moderate congested sinusoids and no fibrosis (Fig. 8). Treatment with 9 mg/kg of extract showed mildly distorted architecture, mild focal necrosis/portal inflammation/congested sinusoids, mild to moderate steatosis and no fibrosis (Fig. 9). Treatment with 18 mg/kg of extract showed mildly distorted architecture, mild microvesicularsteatosis, moderate portal inflammation, mild congested sinusoids and no focal necrosis/fibrosis (Fig. 10). Treatment with 27 mg/kg of extract showed mildly distorted architecture, mild microvesicularsteatosis, mild congested sinusoids and no focal necrosis/portal inflammation/fibrosis (Fig. 11).

Table 1: Phytochemical Screening of Methanol Leaf Extract of Moringa Oleifera.

	Tests	Inference
1	Alkaloids	+++
2	Tannins	+++
3	Saponins	+
4	Flavonoids	++
5	Phlobatanins	++
6	Terpenes	++
7	Cardiac glycoside	++
8	Free & combine anthraquinones	-

Key: + Trace, ++ Positive, +++ stronglypositive, - Negative

Table 2: Effect of MLE on Biochemical Parameters

TREATMENT		PARAMETERS				
Groups	Doses	AST. IU/L	ALT. IU/L	ALP. IU/L	Total Bil. mMol/L	Conju. Bil. mMol/L
Normal control	10 ml/kg Normal saline (NS)	10.66±2.02	9.33±0.66	24.6±0.36	21.7±0.60	9.0±0.40
Toxic control	10 ml/kg NS + 3 ml/kg CCl_4	29.0±2.64^{b}	22.66±1.76^{c}	44.8±0.90^{c}	37.56±2.22^{c}	27.96±0.34^{c}
Ref. control	100 mg/kg Silymarin + 3ml/kg CCl_4	20.0±4.72^{nse}	10.0±2.51^{e}	25.0±0.45^{f}	24.2±0.01^{f}	8.6±0.96^{f}
MLE1	9 mg/kg + 3 ml/kg CCl_4	18.0±1.46^{nsd}	15.0±1.31^{d}	24.23±0.32^{f}	30.4±2.19^{d}	22.56±0.61^{ce}
MLE2	18 mg/kg + 3 ml/kg CCl_4	15.0±2.00^{npd}	14.33±1.66^{d}	23.66±0.72^{f}	27.3±1.76^{e}	15.92±1.34^{cf}
MLE3	27 mg/kg + 3 ml/kg CCl_4	11.33±3.66^{nse}	11.66±2.33^{e}	25.9±1.15^{f}	25.2±1.72^{f}	12.13±1.63^{cf}

Results Represent Mean ± SEM
Values significant, n = 5
$^{a}p<0.05$; $^{b}p<0.01$; $^{e}p<0.001$ relative to control;
$^{d}p<0.05$; $^{e}p<0.01$; $^{f}p<0.001$ relative to CCl_4

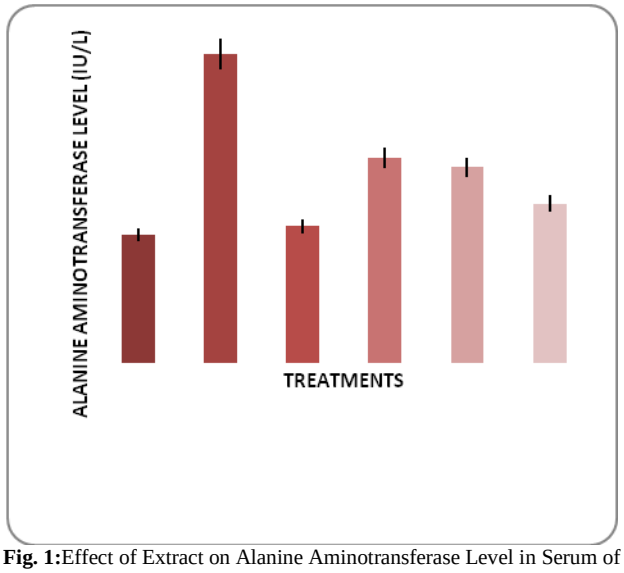

Fig. 1: Effect of Extract on Alanine Aminotransferase Level in Serum of Rats with Ccl_4-Induced Hepatotoxicity

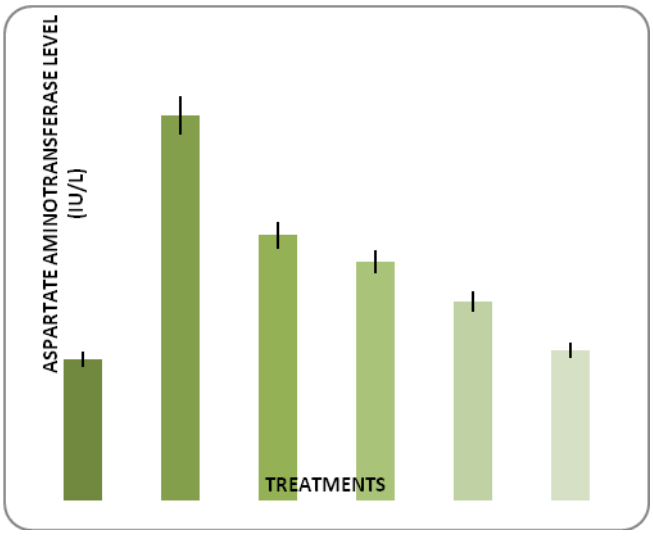

Fig.2: Effect of Extract on Aspartate Aminotransferase Level in Serum of Rats with Ccl_4-Induced Hepatotoxicity

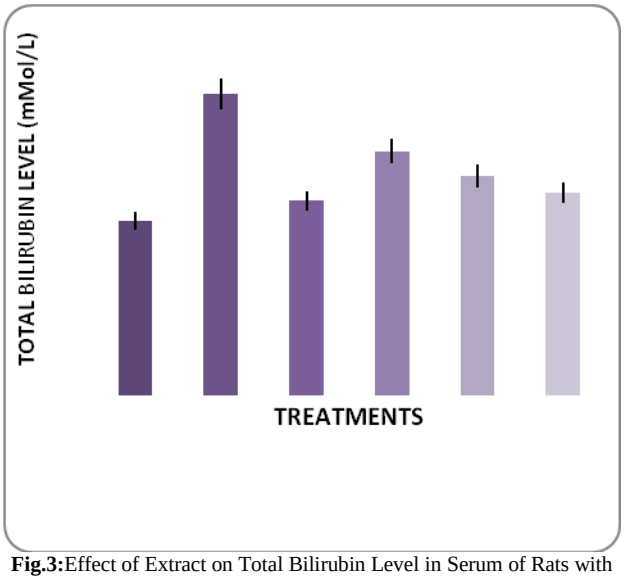

Fig.3: Effect of Extract on Total Bilirubin Level in Serum of Rats with Ccl_4- Induced Hepatotoxicity

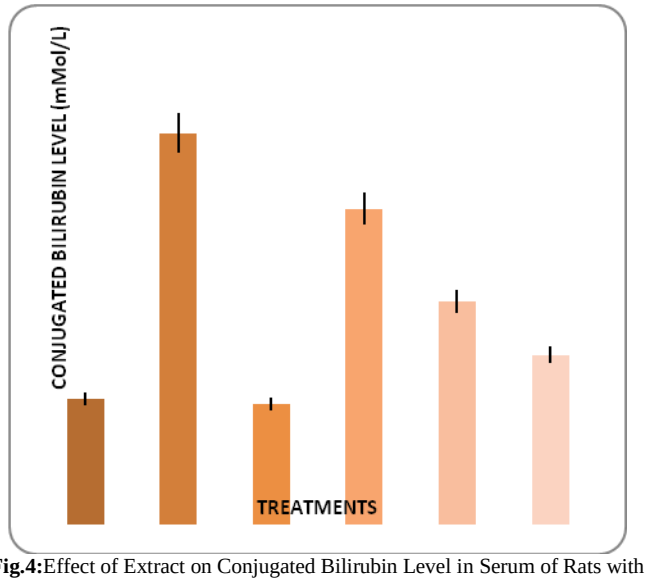

Fig.4: Effect of Extract on Conjugated Bilirubin Level in Serum of Rats with Ccl_4-Induced Hepatotoxicity

Table 3: DPPH Free Radical Scavenging Activity of the Methanolic Leaf Extract of Moringa Oleifera

S/No.	Concentration µg/ml	Absorbance	% Inhibition DPPH
1	20	0.841	41.02
2	40	0.772	45.86
3	60	0.753	47.19
4	80	0.729	48.88
5	100	0.664	53.43
	Blank	1.426	

Table 4: DPPH Free Radical Scavenging Activity of Vitamin C (Ascorbic Acid)

S/No.	Concentration µg/ml	Absorbance	% Inhibition DPPH
1	20	0.094	93.41
2	40	0.088	93.83
3	60	0.087	93.89
4	80	0.090	93.68
5	100	0.084	94.11
	Blank	1.426	

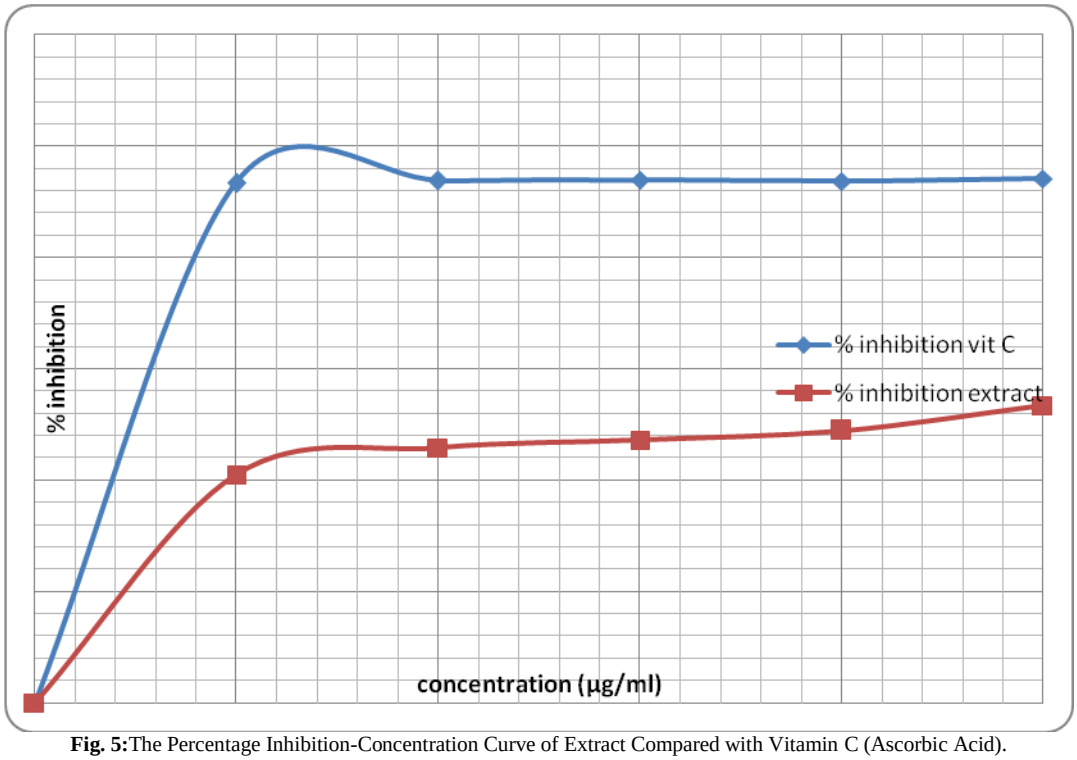

Fig. 5: The Percentage Inhibition-Concentration Curve of Extract Compared with Vitamin C (Ascorbic Acid).

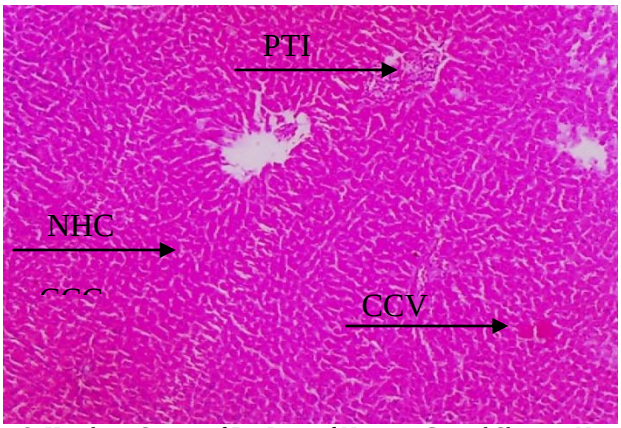

Fig. 6: Histologic Section of Rat Liver of Negative Control Showing Normal Hepatic Cells (NHC), Mild Congested Central Vein (CCV) and Mildly inflamed Portal Tract (IPT).

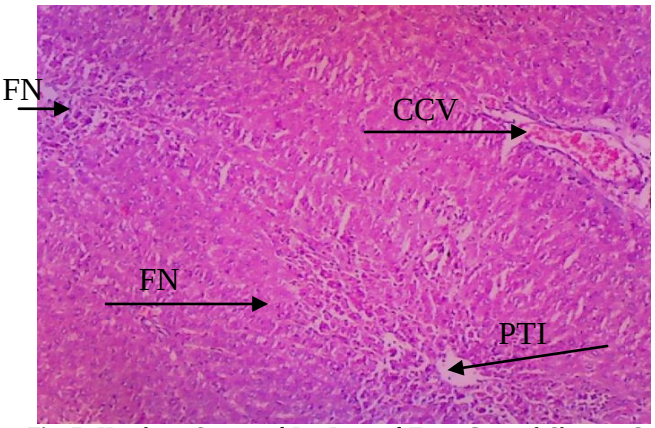

Fig. 7: Histologic Section of Rat Liver of Toxic Csontrol Showing Severely Distorted Architecture of the Liver by Severe Focal Necrosis (FN), Congested Central Vein (CCV) and Portal Tract Inflammation (PTI).

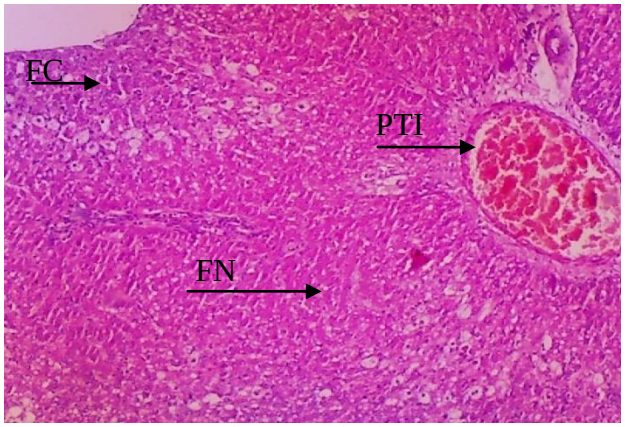

Fig. 8: Histologic Section of Rat Liver of Reference Control Showed Moderately Distorted Architecture of the Liver by Mild to Moderate Fatty Change (FC), Decreased Focal Necrosis (FN) and Portal Tract Inflammation (PTI).

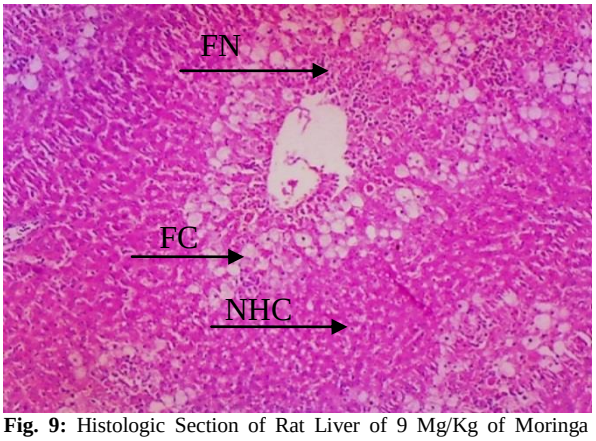

Fig. 9: Histologic Section of Rat Liver of 9 Mg/Kg of Moringa Leaf Extract Moderately Distorted Architecture by Mild Focal Necrosis (FN), Moderate Fatty Change (FC) And Numerous Hepatic Cells (NHC).

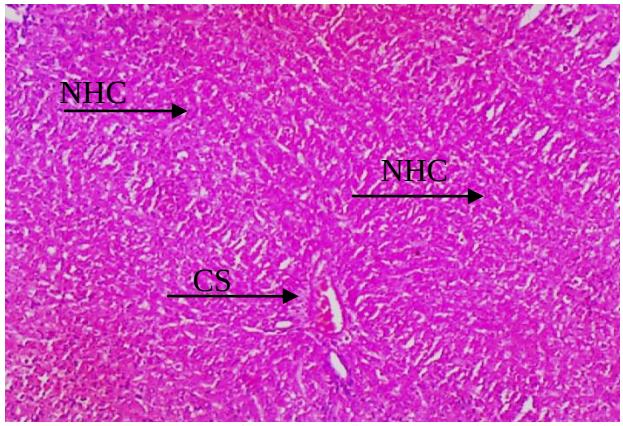

Fig. 10: Histologic Section of Rat Liver of 18 Mg/Kg of Moringa Leaf Extract Showed Almost Normal Architecture with Numerous Normal Hepatic Cells (NHC) and Congested Sinusoids (CS) as Well as Absence of Focal Necrosis and Fatty Changes.

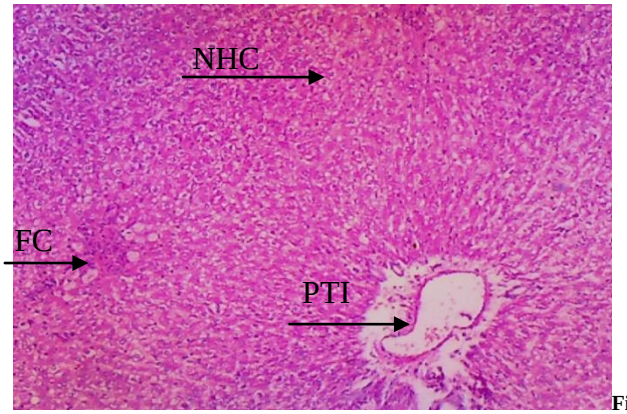

Fig. 11: Histologic Section of Rat Liver of 27 Mg/Kg of Moringa Leaf Extract Showed Mildly Distorted Architecture by Mild Fatty Changes (FC) and Portal Tract Inflammation (PTI) with Numerous Normal Hepatic Cells (NHC), as Well as Absence of Focal Necrosis.

Table 5: Effect of *Moringa oleifera* Leaf Extract on Histology of the Liver of Rats with Ccl_4-Induced Hepatotoxicity

	LIVER
GP1 (Negative Control): 10 ml/kg Normal saline only	Normal architecture. No steatosis. Mild Portal inflammation. Mild Congested sinusoids. No necrosis. No fibrosis. Distorted architecture.
GP2: 10 ml/kg of Normal saline + 3 ml/kg CCl_4 (Induction or Toxic control)	Focal necrosis Severe steatosis. Severe Portal inflammation with interphase hepatitis Severe Congested sinusoids. No fibrosis.
	Improving architecture compared to GP2

GP3: 100 mg/kg Silymarin + 3 ml/kg CCl_4 (Reference control)	Decreased steatosis. Decreased Portal inflammation but no interphase hepatitis. Mild to moderate Congested sinusoids. Decreased necrosis. No fibrosis. Mildly distorted architecture. Mild focal necrosis.
GP4: 9 mg/kg Extract + 3 ml/kg CCl_4 (Test I)	Mild to moderate steatosis in less than 25% of cases. Mild Portal inflammation. Mild Congested sinusoids. No fibrosis Mildly distorted architecture. Nil focal necrosis.
GP5: 18 mg/kg Extract + 3 ml/kg CCl_4 (Test II)	Mild Microvesicularsteatosis in less than 10%. Moderate Portal inflammation. Mild Congested sinusoids. No fibrosis Mildly distorted architecture. Nil focal necrosis.
GP6: 27 mg/kg Extract + 3 ml/kg CCl_4 (Test III).	Mild Microvesicularsteatosis in less than 10%. Nil Portal inflammation. Mild Congested sinusoids. No fibrosis
Remarks	The reference control has mild to moderate hepatoprotective properties. The hepatoprotective properties of the extract increase with increasing doses of the extract. The extract has appreciable protective hepatic effect at the three graded doses.

4. Discussion

This study was undertaken to evaluate the hepatoprotective effect of the methanolic leaf extract of *Moringa oleifera* in carbon tetrachloride (CCl_4)-induced liver injury in albino Wistar rats.

The phytochemical analysis of MLE showed that it contains flavonoids, tannins, saponins, alkaloids, phlebotomines, terpenes and cardiac glycosides. Free and combined anthraquinones were absent. This was similar to the result obtained by Mishra et al., 2011.

Assessment of liver function can be made by estimating the activities of serum ALT, AST, ALP and Bilirubin, which are enzymes originally present higher concentration within cytoplasm. When there is hepatopathy, these enzymes leak into the blood stream in conformity with the extent of liver damage (Suman et al., 2011). Bilirubin is one of the most useful clinical pointers to the severity of necrosis, and its accumulation is a measure of the binding, conjugation and excretory capacity of hepatocytes (Manokaran et al., 2008.

From the results of the biochemical analysis of this study, it was observed that administration of CCl_4 (3ml/kg) alone caused a significant increase ($p<0.05$) in serum levels of ALT, AST, ALP and bilirubin (total and conjugated). Treatment with MLE (9, 18 and 27mg/kg) reversed the altered levels to near normal, in a dose-dependent manner. It was compared with standard Silymarin (100mg/kg). This is similar to the work of Kshirsagar et al., 2009.

Results of histopathological studies provided supportive hepatoprotective evidence for biochemical analysis. Phytochemical analysis of the extract showed the presence of flavonoids. Flavonoids have been well documented as good hepatoprotective agents because they can effectively inhibit lipid peroxidation, scavenge free radicals and antioxidant enzyme activities (Potapovich et al., 2003). The DPPH free radical scavenging studies showed that MLE has antioxidant property. DPPH is a stable radical that has been used widely to evaluate the antioxidant activity of various natural products (Singh et al., 2011). In this study the 60% methanol leaf extract of *Moringa oleifera* gave a concentration-dependent free radical scavenging activity.

5. Conclusion

It can be concluded based on our findings that methanolic leaf extracts of *Moringa oleifera* possesses antioxidant and hepatoprotective activities in a dose - dependent manner and safe for oral administration.

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