



**IN VITRO AND IN VIVO ANTIOXIDANT ACTIVITY AND HEPATOPROTECTIVE
POTENTIAL OF MORINGA PTERIGOSPERMAGAERTN. LEAF AGAINST CCL₄
INDUCED HEPATOTOXICITY**

Shah Anuttara*, Patil Darshana² and Patil Avinash¹

¹Department of Botany, Birla College, Kalyan- 421304, (M.S.), India.

²Smt. C.H.M. College, Ulhasnagar- 421003, (M.S.), India.

*Corresponding Author: Shah Anuttara

Department of Botany, Birla College, Kalyan- 421304, (M.S.), India.

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ABSTRACT

This study aims to investigate the *in vitro* and *in vivo* antioxidant activity, hepatoprotective effect of *Moringa pterigosperma* Gaertn. *in vitro* antioxidant activity of *Moringa pterigosperma* Gaertn. leaves were investigated by using standard established assays. Mice with CCl₄ induced liver injury were used to assess the antioxidant potential and hepatoprotective effect of aqueous slurry of *Moringa pterigosperma* Gaertn. leaves. Biochemical markers like SGOT, SGPT, ALP and Total Protein content were determined from blood serum. Antioxidant markers such as GSH, CAT, SOD and LPO were analyzed from liver homogenate using standard procedures. *Moringa pterigosperma* Gaertn. leaf extract exhibits good antioxidant potential *in vitro*. The *in vivo* results indicated that, treatment with aqueous slurry of *Moringa pterigosperma* Gaertn. leaf exhibited significant reduction in the levels of SGOT, SGPT and ALP in all groups. Out of various dosages, it was found that aqueous slurry of *Moringa pterigosperma* Gaertn. leaf administered at 750 mg / kg b.w. was most effective, as it maximally reduced LPO of liver with parallel increase in cellular antioxidants. Results were comparable with Silymarin, used as modern drug control. Findings from this study indicated hepatoprotective potential as well as significant antioxidant activity of *Moringa pterigosperma* Gaertn. leaf.

KEYWORDS: *Moringa*, CCl₄, hepatoprotective activity, antioxidant activity, *in vitro*, *in vivo*.

1. INTRODUCTION

Since long time, there was an increased interest within the role of oxygen- free radicals, normally known as "Reactive Oxygen Species" (ROS) and "Reactive Nitrogen Species" (RNS) (Halliwell and Gutteridge, 1999). ROS or RNS are regarded to play a dual role in biological systems, given that they can be either dangerous or beneficial to living beings. (Valko et al., 2004). The molecular basis of many illnesses is thought to involve oxidative pressure due to unfastened radicals (Rice- Evans, 2004). Cell damage due to free radicals appears to be a primary contributor in growing older, cancer, cardiovascular diseases, compromised immune system and so on. (Langseth, 1993; Halliwell, 1994). Liver is a prime organ attacked by ROS (Sanchez- Valle et al., 2012). Oxidative stress is considered as the vital purpose of liver damage in several liver disorders (Medina and Moreno-Otero, 2005).

Protective nature of numerous natural and synthetic antioxidants towards hepatotoxicity had been mentioned by many researchers (Hafez and Darwish, 1997; Carbonari et al., 2006; Lin et al., 2008). *M.*

pterigosperma Gaertn. has antioxidant galore (Chumark et.al., 2008 and Kumar et.al., 2013). Virtually almost all parts of this plant including the leaves, roots, seeds, bark, fruit and immature pods possesses anti inflammatory, diuretic, antihypertensive, cholesterol lowering, antioxidant activity (Paliwal et al., 2011; Sharma et al., 2011). More studies should be achieved to discover the efficacy of herbal antioxidants (Madhavi and Sakunkhe, 1995). Therefore, the present work was carried out in deliberate attempts to evaluate the *in vitro* and *in vivo* antioxidant potential of *Moringa pterigosperma* Gaertn. Leaf, against CCl₄ triggered hepatotoxicity in swiss albino mice.

2. MATERIALS AND METHODS

2.1 Chemicals

All reagents used were of analytical grade. Carbon tetra chloride (CCl₄, GR grade) was procured from, Merck Specialities Pvt. Ltd., Mumbai, Silybon Suspension (as standard Silymarin 100 mg) was procured from Apollo chemists, Kalyan, India. Sodium carbonate (Na₂CO₃), Folin Cioalceu reagent, Catechol, Aluminium chloride (AlCl₃), Potassium Acetate, Quercetin, H₂SO₄, Sodium

dihydrogen phosphate (NaH_2PO_4), Ammonium molybdate, Ascorbic acid, Potassium ferricyanide, Trichloro acetic acid (TCA), Ferric chloride (FeCl_3), 1,1-Diphenyl- 1,2- picryl Hydrazyl (DPPH), 2,4,6-tripyridyl-s-triazine (TPTZ), Sodium nitroprusside (SNP), Sulphanilamide, N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), nitro blue tetrazolium (NBT), reduced nicotinamide adenine dinucleotide (NADH), phenazine methosulfate (PMS), H_2SO_4 , Thio barbituric acid (TBA), Glutathione (GHS), 2,2 dipyridyl, ortho phosphoric acid (H_3PO_4) etc. were purchased from Hi-Media, Mumbai.

2.2 Plant materials

Plant material (leaf) of *M. pterigosperma* Gaertn. was collected from Katai area, Dombivali, District Thane, Maharashtra, India. Collected plant material was sent for authentication at Agharkar Research Institute, Pune. The sample was carefully segregated, cleaned and shade dried to constant weight, powdered, and was stored in air tight container for further use.

2.3 Preparation of plant extract for *in vitro* assay

The dried powder material of leaf was soaked in distilled water for 10-12 hours (overnight) and kept on mechanical shaker, filtered through Whatman Paper No. 42 to get an aqueous extract of *M. pterigosperma* Gaertn. leaf (AEMPL).

2.4. Determination of total phenolic and flavonoid content

The total phenolic content of different parts of *M. pterigosperma* leaf aqueous extract was measured using spectrophotometric Folin-Ciocalteu method (Sadashivam and Manickam, 2008). The results were expressed as mg of pyrocatechol equivalents / gm of dry extract. The flavonoid content was measured using modified spectrophotometric method of Chang *et al.*, 2002.

2.5 *In vitro* Antioxidant activity

2.5.1. DPPH radical scavenging assay

2 ml of extract was added to 2 ml of 0.02 M phosphate buffer (pH 6) and 2 ml of 0.2 mM DPPH. The mixture was mixed thoroughly and left at room temperature for 30 minutes. Absorbance was measured at 517 nm (Chen *et al.*, 2007). The capability to scavenge the DPPH radical was calculated using following equation.

$$\text{DPPH radical \% scavenging} = \frac{A_{\text{con}} - A_{\text{test}}}{A_{\text{con}}} \times 100$$

Where;

A_{con} = Absorbance of control

A_{test} = Absorbance of extract or standard.

2.5.2 Nitric oxide radical scavenging assay

5 mM SNP in 0.1 M PBS (pH 7.4) was mixed with different concentrations of the extract and incubated at 25 °C for 150 min. to this Griess reagent was added. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and

subsequent coupling with NEDD was read at 546 nm and referred to the absorbance of standard solution of ascorbic acid treated in the same way with Griess reagent (Ilavarasn *et al.*, 2005). The percentage inhibition or scavenging activity of nitric oxide radical was calculated by aforementioned equation for DPPH.

2.5.3. Super oxide anion scavenging assay

Measurement of superoxide anion scavenging activity of *M. pterigosperma* Gaertn. leaf extract was based on the method described by Liu (1997) and Shivkumar *et al* (2006) with slight modifications. Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of nitro blue tetrazolium (NBT). 1 ml of 100 mM NBT solution, 1 ml of 677 μM reduced NADH and 0.5 ml of extract were mixed and the reaction was initiated with addition of 100 μl of 60 μM PMS solution. The reaction mixture was incubated at 25 °C for 5 minutes, and then the absorbance was measured at 560 nm in a spectrophotometer. L-Ascorbic acid was used as a control/standard. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. The percentage inhibition of superoxide anion generated was calculated using the aforementioned formula for DPPH.

2.5.4. Ferric reducing antioxidant potential (FRAP) Assay

The reducing ability of *M. pterigosperma* Gaertn. was determined by FRAP assay using method described by Benzie and Strain (1996) with slight modifications. The FRAP reagent was prepared as a mixture of 10 mM 2,4,6-tris(2pyridyl)-s-triazine (TPTZ) and 20 mM FeCl_3 . The freshly prepared FRAP reagent was incubated at 37 °C for 10 minutes. From this, 30 μl of a sample was mixed with 900 μl of the FRAP reagent and distilled water was added to make the volume to 1 ml. A blank sample was prepared in the same way as described above but saline solution was used instead of the extract. The reaction mixture was incubated at 37 °C for 30 minutes. Absorbance was measured at 593 nm against blank. The result of FRAP assay was expressed as mM FeSO_4 per gm of material (FRAP value). FRAP value of leaf extract was calculated and was compared to FRAP value of ascorbic acid, used as standard.

2.6. *In vivo* antioxidant and hepatoprotective activity

2.6.1. Preparation of aqueous slurry

Aqueous slurry of *M. pterigosperma* Gaertn. leaf was prepared by mixing accurately weighed leaf powder in distilled water. The animals were administered orally the required dose as per the body weights.

2.6.2. Test animals

The male Swiss albino mice weighing 25-30 gm were procured from Central Laboratory Animal House, Bombay Veterinary College, Parel, Mumbai. All the animals were housed in polypropylene cages with not more than six animals per cage. Animals were

maintained under controlled laboratory conditions at an ambient temperature of 25 ± 2 °C with light and dark cycle 12/12 hours. Prior to the experimentation all the animals were acclimatized for seven days under standard laboratory conditions. The guidelines given by Institutional Animals Ethical Committee (IAEC) regarding the maintenance and dissection of small animals were strictly followed. All the animals were allowed the free access to the standard pellet diet (Amrut) and the water was allowed *ad libitum* under strict hygienic conditions.

2.6.3. Safety evaluation

Safety of *M. pterigosperma* Gaertn. leaves was carried out as per OECD guidelines (No 420, fixed dose

procedure). Mice were fasted overnight and orally administered with aqueous slurry of leaf (2000 mg / kg b.w.). The animals were observed individually during the first 30 mins for all reflexes, periodically during first 48 hrs. with special attention given during first hour and first 4 hours (short term toxicity) and thereafter daily for next 14 days (long term toxicity) for change in general behavior and clinical symptoms like change in skin and fur texture, ptosis, breathing problems, excessive salivation, gait, stool condition etc. daily body weight, food and water consumption was recorded and the results were compared with control administered with distilled water.

2.6.4. CCl₄ induced oxidative hepatotoxicity

The mice were randomly divided into 7 groups based on their body weight. Each group contains 6 animals.

Group I	Normal Control
Group II	CCl ₄ (0.5 ml/kg body weight + Liquid paraffin 0.5 ml/kg b.w.)
Group III	Natural recovery
Group IV	Modern drug control treated with Silymarin (100 mg/kg b.w.)
Group V	Treated with aqueous slurry of <i>Moringa pterigosperma</i> Gaertn. leaf powder (Low dose; 100 mg/ kg b.w.)
Group VI	Treated with aqueous slurry of <i>Moringa pterigosperma</i> Gaertn. leaf powder (Intermediate dose; 500 mg/ kg b.w.)
Group VII	Treated with aqueous slurry of <i>Moringa pterigosperma</i> Gaertn. leaf powder (High dose; 750 mg/ kg b.w.)

Male Swiss Albino Mice were kept on fasting overnight. CCl₄ (0.5 ml/kg body weight + Liquid paraffin 0.5 ml/kg body weight) was administered to group no. II to VII by sub cutaneous route on first day of the study. Animals from group no. V to VII were treated with respective dose of aqueous slurry daily and one-hour post induction on first day. Animals from group no. IV were treated with standard drug Silymarin daily and one-hour post induction on first day. Animals from group I, II, IV, V, VI and VII were sacrificed on 4th day of the study (72 Hrs. after dosing) and animals from group III were sacrificed on 7th day for the comparative evaluation of natural recovery in the study. Daily record of body weight, food and water consumption was maintained.

2.6.5. Biochemical determinations

Prior to the sacrifice, 2 ml blood was collected from retro orbital plexus in non-heparinized vials. From blood, serum was separated and biochemical parameters like ALT, AST, ALP, total protein, albumin and globulin were carried out using standard kits. At the end of study animals were sacrificed by CO₂ asphyxiation and gross necropsy was done and liver was collected in 10% formalin for Histopathology studies. Liver homogenate was prepared and the supernatant was used for the estimation of antioxidant enzymes Superoxide dismutase (SOD), Catalase and protein estimation. Glutathione (GSH) and Lipid peroxidation product named Malondialdehyde (MDA). All assays were performed using standard kits available.

2.7. Statistical analysis

All values were expressed as mean \pm SEM and statistically analyzed for significance using Dunnet's test, comparing normal group with other groups. Statistical significance was determined at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Determination of total phenolic and flavonoid content

Phenolic compounds which includes phenolic acids, polyphenols and flavonoids are commonly located in plants and have been mentioned to have multiple biological uses, inclusive of antioxidant activity (Brown and Rice -Evans, 1998). A strong correlation exists between phenolic content and anti-oxidative potential of the plant extract, a property attributed to the unfastened radical terminating ability of phenolic compounds (Shahidi and Wanasundara, 1992). The total phenolic and flavonoid content of *Moringa* leaf was 5.97 ± 0.32 mg Pyrocatechol equivalent/ gm of dry leaf and 3.96 ± 0.11 mg quercetin equivalent / gm of dry leaf respectively. The results show that aqueous extract of *Moringa* leaf has antioxidant potential.

3.2. In vitro assays

3.2.1. DPPH assay

DPPH is an especially stable radical compound broadly used to test free radical scavenging activity (Sanchez-Moreno, 2002). The free radical scavenging activity of DPPH is presented in fig 1. In DPPH assay, the IC₅₀ (the concentration required to scavenge 50% of radical)

values of leaf extract and standard Ascorbic acid was found to be $1168.94 \pm 2.50 \mu\text{g/ml}$ and $72.75 \pm 1.90 \mu\text{g/ml}$ respectively.

3.2.2. Nitric oxide radical scavenging assay

The plants exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite radical which was generated from SNP (sodium nitroprusside) at physiological pH in an aqueous environment. The antioxidant activity increased with an increase in concentration of the extracts reaching a plateau. Lower the IC₅₀ value better is the antioxidant potential of the sample. The IC₅₀ value of standard ascorbic acid and leaf was found to be $196.05 \pm 0.50 \mu\text{g/ml}$ and $1892.80 \pm 2.79 \mu\text{g/ml}$ respectively.

3.2.3. Super oxide anion scavenging assay

Superoxide anion is a weak oxidant produced throughout diverse biological reactions and is highly poisonous (Stief, 2003). Aqueous extract of *M. pterigosperma* leaf

exhibited dose dependent antioxidant activity. A decrease in absorbance indicated the antioxidant activity of the extracts which may be due to the inactivation or consumption of superoxide anion radicals produced in the reaction mixture. The IC₅₀ value of leaf extract was $2028.76 \pm 1.39 \mu\text{g/ml}$ and that of ascorbic acid was $68.65 \pm 1.30 \mu\text{g/ml}$.

3.2.4. Ferric reducing antioxidant potential (FRAP) assay

The reducing assay is a right way of measuring antioxidant capability. In FRAP non-enzymatic antioxidant reacts with pro-oxidants and inactivate them. In this assay, an easily reducible oxidant Fe (III) TPTZ is reduced to Fe (II) TPTZ (Szollosi and Varga, 2002).

The FRAP value of Ascorbic acid and leaf was found to be $1.75 \pm 0.05 \text{ mM FeSO}_4$ and $0.28 \pm 0.01 \text{ mM FeSO}_4/\text{gm}$ of leaf respectively.

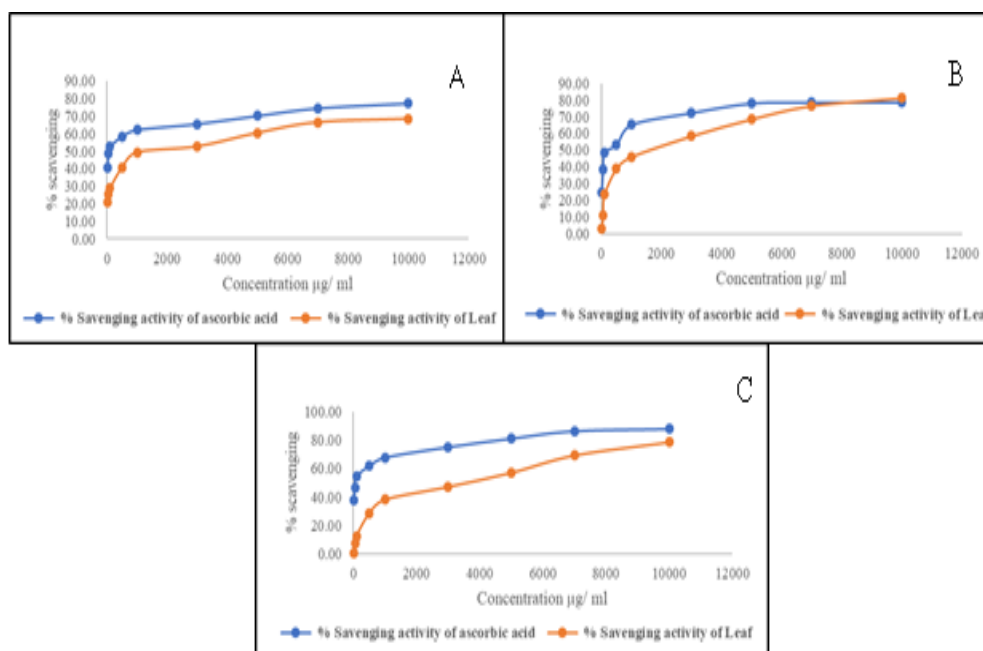


Figure 1: Antioxidant activity of *Moringa pterigosperma* Gaertn. aqueous leaf extract (ALE) as determined by (A) DPPH assay, (B) Nitric Oxide radical scavenging assay and (C) Super oxide anion scavenging assay.

3.3. In vivo assays

The CCl₄ triggered hepatotoxicity is extensively applied for the evaluation of the antioxidant potential of plant extracts (Bhathal et al., 1983; Weber et al., 2003; Awaad et al., 2006). In CCl₄ caused toxicity, CCl₄ is metabolized through cytochrome P450 monooxygenase assembly to supply trichloromethyl radical, which then reacts with oxygen to form trichloromethyl-peroxyl radical (Shenoy et al., 2001). CCl₄ metabolites react with polyunsaturated fatty acids and produce covalent adducts with lipids and proteins. This cause lipid peroxidation and destruction of cellular membranes with the ensuing liver injury (Clawson, 19889; Recknagel et al., 1989).

During hepatocellular harm, kinds of enzymes generally located in the cytosol are released into the blood stream. Their quantification in plasma is a useful biomarker of the extent and sort of hepatocellular harm (Pari and Murugan, 2004). Hepatocellular necrosis results in elevation of the serum marker enzymes, which might be released from liver into blood (Shenoy et al., 2002).

In the present study CCl₄ induction increased levels of all the liver markers like ALT, AST and ALP. whereas treatment with aqueous slurry of *M. pterigosperma* Gaertn. leaf exhibited significant decrease in all these marker enzymes (Figure 2 A). Levels of AST, ALT and ALP significantly decreased in all the groups after administration of aqueous slurry of *M. pterigosperma*

leaf. Total protein levels significantly fall after administration of CCl_4 but it increases after administration of *M. pterigosperma* leaf slurry. Total protein, albumin and globulin levels increase in group III and IV when compared with group II (Figure 2 B).

SOD and CAT have important capabilities in protection mechanisms in opposition to the harmful outcomes of reactive oxygen species and free radicals in living beings. GSH content is another crucial parameter that reveals oxidative damage in liver and kidney. TBARS are cytotoxic byproducts which are hallmarks of lipid peroxidation (Huang et al., 2010).

Determination of *in vivo* antioxidant activity by assessing SOD, CAT and GSH is represented in figure 2 C. Antioxidant enzymes SOD and CAT were significantly reduced by CCl_4 intoxication, besides reduction of GSH. Lipid peroxidation increase significantly after CCl_4 induction. All these changes were significantly reversed after treatment with *M. pterigosperma* leaf aqueous slurry in dose dependent manner (Figure 2 D). Aqueous slurry at 750 mg/ kg b.w. was found to be more effective in reducing the harmful effect caused to liver after CCl_4 induction.

CCl_4 induction also causes classical fatty liver, with increase in liver weight. Group II showed severe loss of hepatic architecture with zones of necrosis and fatty changes histopathologically (plate 1). Figure 2 E depicts the changes in liver weight.

The modern drug (Silymarin) control group were treated at 100 mg/ kg Silymarin showed marked recovery in all

the biochemical as well as antioxidant parameters. Histopathological findings supported the data as the treated animals showed near normal hepatic architecture with mild degree of necrosis which exhibits recovery and protective effect by the drug. Animals from group V to VII were orally treated with 100 mg/ kg b.w., 500 mg/ kg b.w. and 750 mg/ kg b.w. dose of aqueous slurry. the percentage of protection offered by the slurry is given in Table 1.

Histopathological results also showed marked recovery in dose dependent manner. Hepatic architecture was improved after treatment with the slurry. *M. pterigosperma* aqueous slurry at 750 mg/ kg b.w. dose showed better results than 100 mg/ kg b.w. and 500 mg/ kg b.w. doses (plate 2). The group III animals showed results almost similar to group II (CCl_4 induction group) (plate 1).

Percentage reduction data is given in table 1. Observations from recovery group shows that animals did not show significant reduction in liver markers and antioxidant enzymes even after extended recovery period of 7 days where as recovery rate is remarkable after treatment with aqueous slurry of *M. pterigosperma* leaf. Thus, the present study compares the activity of *M. pterigosperma* aqueous slurry at 100, 500 and 750 mg/ kg b.w. with the known hepatoprotectant drug, Silymarin in CCl_4 intoxicated mice. the results of the study confirm that the *Moringa pterigosperma* Gaertn. leaf exhibits good antioxidant activity *in vitro* and *in vivo* along with remarkable hepatoprotective potential.

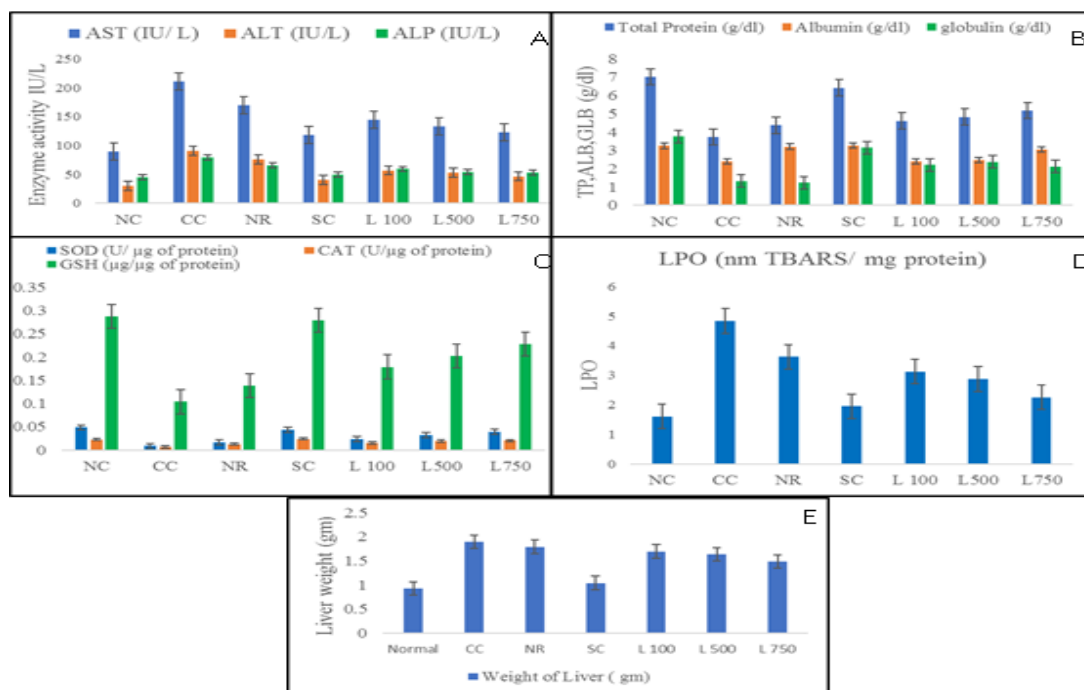


Figure 2: Effect of *Moringa pterigosperma* Gaertn. leaf aqueous slurry on (A) Levels of AST, ALT and ALP, (B) Total protein, Albumin and globulin, (c) Super oxide dismutase (SOD), catalase (CAT) and Glutathione (GSH), (D) Lipid peroxidation (LPO) and weight of liver in Liver of CCl_4 intoxicated mice.

NC= normal control, CC= CCl₄ control, NR= natural recovery, SC= Silymarin control, L100= leaf slurry at 100 mg/kg b.w., L500= leaf slurry at 500mg/kg b.w., L750= leaf slurry at 750 mg/kg b.w., AST= Aspartate transaminase, ALT= Alanine transaminase, ALP= Alkaline phosphatase.

Data presented is expressed as mean \pm SEM (n = 6) and experimental groups represented significant differences at $p < 0.05$ (one-way ANOVA followed by Dunnett's post hoc test).

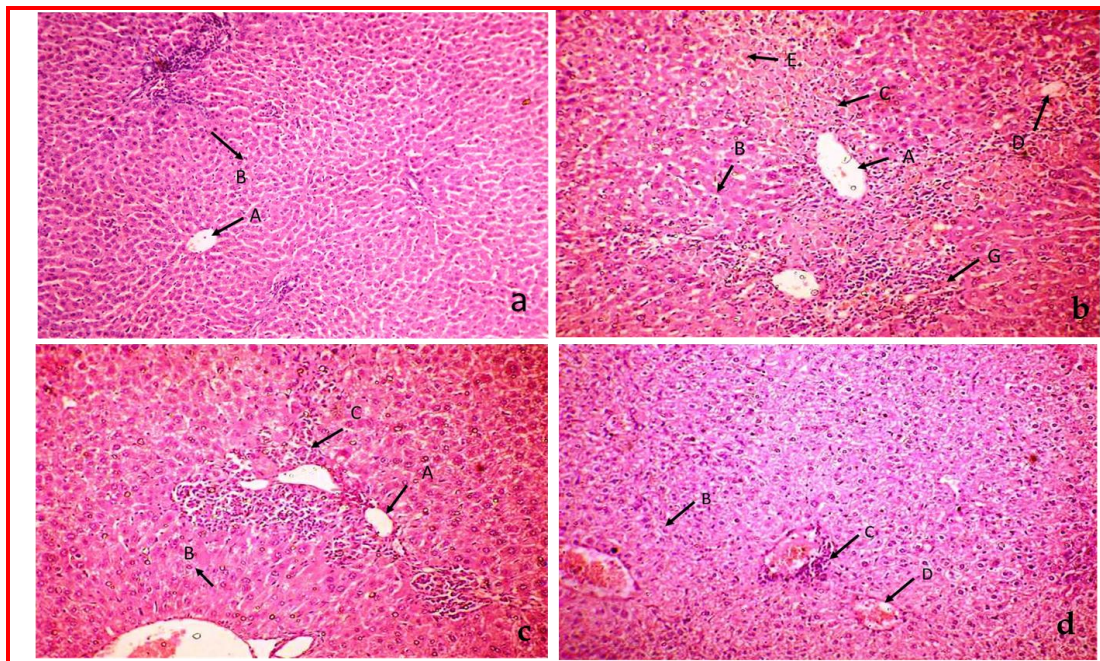


Plate 1: Histopathology of liver sections. a= Normal control group, b= CCl₄ control group. c= Natural recovery group, d= Silymarin control group.

A: Central vein, B: Hepatocytes, C: Centrilobular necrosis, D: Arteriole, E: Haemorrhage, G: Leukocytic infiltration.

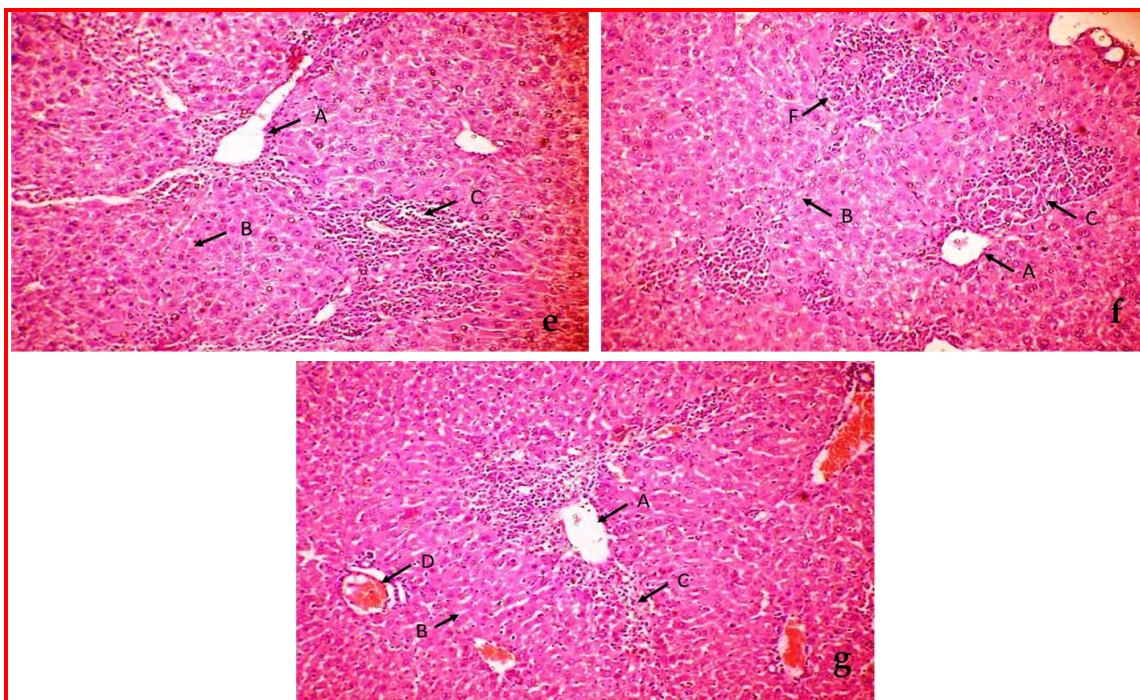


Plate 2: Histopathology of Liver sections. e= Leaf 100 mg/ kg b.w. group, f= Leaf 500 mg/ kg b.w. group, g= Leaf 750 mg/ kg b.w. group,

A: Central vein, B: Hepatocytes, C: Centrilobular necrosis, D: Arteriole, F: Portal triad.

Table 1: Biochemical estimation from serum.

Parameters Groups	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
NC	90 ± 1	29.667 ± 0.71	44.500 ± 1.20
CC	211.16 ± 0.87	90.833 ± 1.13	79.167 ± 0.94
NR % protection	33.98	25.34	39.90
SC % protection	77.03	82.83	85.58
L 100 % protection	54.61	55.59	57.21
L 500 % protection	64.65	62.40	73.56
L 750 % protection	73.18	72.48	75

Key words: AST= Aspartate transaminase, ALT= Alanine transaminase, ALP= Alkaline phosphatase. NC= normal control, CC= CCl₄ control, NR= natural recovery, SC= Silymarin control, L100= leaf slurry at 100 mg/kg b.w., L500= leaf slurry at 500mg/kg b.w., L750= leaf slurry at 750 mg/kg b.w.

*All values are Mean ± SEM

4. CONCLUSION

Findings of the present study indicate that aqueous slurry of *Moringa pterigosperma* Gaertn. Leaf exhibits antioxidant activity against CCl₄ induced hepatotoxicity. Antioxidant potential of *Moringa pterigosperma* may be due to its various phytochemicals acting synergistically. Extraction, isolation and characterization of active constituent attributing to the antioxidant potential can be carried out in order to develop more efficient natural drug. *Moringa* plant is native to India and it can become a great source of income if it's potential is fully exploited and utilised by carrying out more scientific studies. Further, such scientific studies might help industries in developing a nutraceutical product which can serve as a good dietary supplement and even a drug with multifarious effects may be developed which can serve humanity.

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