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PHYTOCHEMICAL INVESTIGATION AND HEPATOPROTECTIVE POTENTIAL OF *MARSILEA MINUTA* LINN. LEAF EXTRACT AGAINST CCL_4-INDUCED HEPATIC DAMAGE IN WISTAR ALBINO RATS

Sharon Kujur, Renu Das* and Dheeraj Ahirwar

School of Pharmacy, Chouksey Engineering College, Bilaspur (C.G.), 495004, India

**Corresponding author E-mail*: renudas67@gmail.com

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Abstract:

This study evaluated the phytochemical profile, extraction yields, and hepatoprotective efficacy of Marsilea minuta Linn. leaf extracts against carbon tetrachloride (CCl4)-induced liver injury in experimental rat models. Extraction yields varied across solvents, with water yielding the highest recovery (5.68 g/100 g) and petroleum ether the lowest (1.32 g/100 g). Phytochemical quantification indicated that the ethyl alcohol extract contained the highest total phenolic content (19.39 ± 0.07 mg GAE/g), whereas methyl alcohol yielded the highest total flavonoid content (15.34 ± 0.07 mg QE/g). Assessment of serum biomarker enzymes—alanine aminotransferase (ALT), aspartate aminotransferase (AST), and albumin (ALB)—demonstrated substantial reduction in CCl4-induced hepatic damage. Overall, M. minuta leaves exhibit potent hepatoprotective properties, likely driven by high phenolic and flavonoid concentrations. However, due to potential risks at elevated doses, further safety evaluations and pharmacological profiling are warranted to fully establish its therapeutic window.

Keywords: *Marsilea minuta, Hepatoprotective Activity, Phytochemical Analysis, Carbon Tetrachloride, Phenolic Content, Flavonoids.*

1. Introduction

The liver is a major organ involved in metabolic processes and plays a vital role in detoxifying various harmful substances that are either ingested or produced during the digestion and absorption of nutrients. The liver also performs important functions related to the storage and secretion of various substances. It helps regulate, synthesize, store, and release essential proteins, nutrients, and other biochemical compounds. In addition, it contributes significantly to the removal and elimination of toxic and unwanted substances from the body (1). Liver diseases are among the main causes of morbidity and mortality universally. Drug-induced liver injury (DILI) has been one of the very familiar causative factors that presents a major clinical challenge. The symptoms of drug-induced hepatotoxicity are extremely variable, ranging from an asymptomatic rise of liver enzymes to acute liver

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failure (2). The pathogenesis of DILI involves genetic, metabolic, and immune factors. Despite difficulties in classification, DILI can be divided into three categories that share some common features as well as differences (3). Damage to the liver can develop as a result of exposure to toxic substances including alcohol, chemicals, overdose of antibiotics, and in certain clinical disorders (4, 5). One of the molecular mechanisms in chlorpyrifos (CPF) intoxication is the overproduction of free radicals, which in turn may induce toxicities across various organs (6). The hepatotoxicity of carbon tetrachloride (CCl4) is primarily mediated through metabolic activation, oxidative stress, and inflammatory pathways, which collectively lead to hepatocyte necrosis and progressive liver dysfunction.

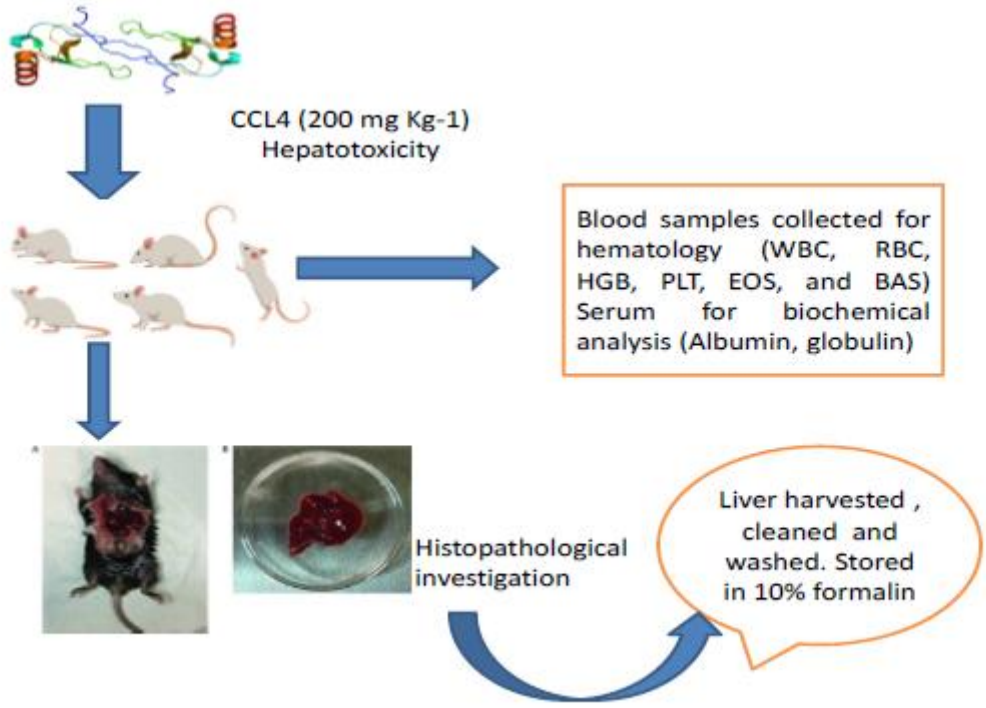


Figure 1: Phytochemical investigation and hepatoprotective potential of *Marsilea minuta* Linn. leaf extract against CCl4-induced hepatic damage in Wistar albino rats

Metabolic activation: CCl4 undergoes bioactivation in the liver by cytochrome P450 enzymes, especially CYP2E1. This metabolism generates trichloromethyl free radicals (•CCl3), which further react with molecular oxygen to form trichloromethylperoxy radicals (•OOCCl3) (7). These highly reactive radicals initiate a cascade of oxidative damage within hepatocytes.

Oxidative stress: The free radicals attack polyunsaturated fatty acids in hepatocyte membranes, leading to lipid peroxidation and the formation of toxic by-products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). These disrupt membrane integrity, impair enzyme function, and alter mitochondrial permeability (8). The oxidative burden overwhelms endogenous antioxidant defenses, including glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT), resulting in hepatocyte injury and necrosis.



Figure 2: Mechanism of action of CCl4-induced hepatotoxicity

1.1 Origin and distribution

Marsilea minuta Linn. (MML), commonly known as water clover, originates from India (Tripura), Malaysia, and the Philippines. It is a species of aquatic fern that is native to tropical and subtropical regions of Asia and is commonly found in wetland habitats, including ponds, marshes, and paddy fields.

1.2 Agro-ecology

Water clover thrives in subtropical climates. *Marsilea minuta* Linn. is a hydrophytic, aquatic or semi-aquatic fern that thrives in moist and waterlogged environments. The plant grows well under warm and humid climatic conditions, particularly in tropical and subtropical regions. It prefers moist to saturated soils and can tolerate periodic flooding. It generally grows in sunny to partially shaded locations, with adequate moisture availability.

MML is known by a variety of names in different cultures: *susnisak* in Tripura (India) and *tapak itik* in Malaysia. The plant is called *sunishnak* in Hindi. This variation in naming illustrates the widespread value and importance of *Marsilea minuta* across various geographical regions.

The taxonomic classification of *Marsilea minuta* Linn. is tabulated in Table 1.

Table 1: Taxonomic classification of *Marsilea minuta* Linn.

Kingdom	Plantae
Phylum	Polypodiophyta
Division	Pteridophyta
Class	Equisetopsida C. Agardh
Subclass	Polypodiidae Cronquist, Takht. & W. Zimm
Order	Marsileales
Family	Marsileaceae Mirb.
Genus	*Marsilea*
Species	*Marsilea minuta L.*

Traditionally, the plant has been used in Ayurveda, Unani, and folk medicine for its hepatoprotective, diuretic, and general tonic properties (9). Various parts of the plant, including leaves, stems, and spores, have been reported to contain biologically active secondary metabolites such as flavonoids, terpenoids, alkaloids, glycosides, and phenolic compounds. These phytoconstituents exhibit multiple pharmacological activities, particularly antioxidant, anti-inflammatory, and hepatoprotective effects, which may counteract oxidative stress-induced damage and support hepatic tissue regeneration (10).

Recent studies have highlighted the potential of natural plant extracts as safer alternatives for managing chemical-induced hepatotoxicity. The bioactive compounds in *M. minuta*, especially flavonoids and phenolics, can scavenge free radicals, inhibit lipid peroxidation, and modulate inflammatory mediators, thereby protecting hepatocytes from toxic insults (11). Considering the increasing incidence of liver disorders and the limitations of current therapeutic approaches, exploring the hepatoprotective potential of *Marsilea minuta* offers a promising avenue for developing plant-based interventions with minimal side effects and broad pharmacological benefits. The practice of herb-herb combinations, otherwise known as polyherbal therapy, has existed for several years, but the scientific proof of their therapeutic benefits is wanting in the literature. Also, a hopeful effect of drug combination over a single drug in disease treatment has been reported (12). Therefore, this study was directed at comparing the hepatoprotective and antioxidant potential of ethanol extracts of *Marsilea minuta* leaves against CCl4-induced toxicity in rats.

2. Materials and methods

2.1 Drugs and chemicals

Silymarin and carbon tetrachloride (CCl4) were purchased from Sigma Chemicals, USA. Serum glutamate oxaloacetate transaminase (SGOT/AST), serum alkaline phosphatase (ALP), serum glutamate pyruvate transaminase (SGPT/ALT), serum total bilirubin (TB), albumin, and total protein diagnostic kits were purchased from RANDOX Laboratories Ltd., Ardmore, UK. All other chemicals used in this study were of analytical grade.

2.2 Collection and preparation of plant materials

The medicinal plant materials of *Marsilea minuta* leaves were collected from hilly regions and surrounding areas near Bartunga, Tripura and West Bengal. The plant material was authenticated by Dr. Ashwini Kumar Dixit, Department of Botany, Guru Ghasidas Central University, Bilaspur, Chhattisgarh (Ref. no.: Bot./GGV/2026/261). The plant material was dried, powdered, and stored in airtight containers for further studies.

2.3 Animals

The male albino rats (weighing 150–220 g) used for the study were obtained from the Anatomy Department of Delta State University, Abraka, Nigeria. They were fed grower's mash (Top Feed, Ltd., Sapele, Delta State) and water *ad libitum*. They were maintained in agreement with established guidelines on the care and wellbeing of research animals.

2.4 Animal grouping and treatment

The male albino rats were grouped into six groups (groups A–F) of five rats each and were treated orally for 14 days as follows:

- Group A: Animals + distilled water only (negative control).
- Group B: Animals + CCl4 in olive oil vehicle only (positive control).
- Group C: Animals + 200 mg/kg/day combined extract of *Marsilea minuta* (mixture of extracts in a 1:1 ratio).
- Group D: Animals + 200 mg/kg/day ethanol extract of *Marsilea minuta*.
- Group E: Animals + 400 mg/kg/day ethanol extract of *Marsilea minuta*.
- Group F: Animals + 100 mg/kg body weight (bw) of silymarin (standard control)

Treatment of the rats continued daily for 14 days, and animals in groups B–F were injected intraperitoneally with CCl4 in olive oil vehicle at a dosage of 1 mL/kg bw on the 14th day (30 minutes after the last extract/drug

treatment). The animals were fasted and sacrificed by cardiac puncture 24 hours after CCl4 administration to obtain blood serum and liver tissue for experimental assays.

2.5 Determination of serum biochemical parameters

Serum samples were used for hepatic function tests using standard diagnostic kits (RANDOX Laboratories Ltd., Ardmore, UK) in accordance with established methods: albumin (13), total bilirubin (14), total protein (15), alkaline phosphatase (16), alanine aminotransferase (17), and aspartate aminotransferase (18).

2.6 Determination of oxidative stress markers

The levels of endogenous antioxidants in liver tissue—superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GPx)—were estimated by the methods of Marklund (19), Sinha (20), Ellman (21), and Rotruck *et al.* (22), respectively. Lipid peroxidation by-product [malondialdehyde (MDA)] in liver tissue was quantified according to the method of (23).

2.7 Statistical analysis

Data are presented as mean ± standard deviation and were compared by one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test (for statistical significance) using GraphPad Prism (version 6.0). The level of statistical significance was established at $p < 0.05$.

3. Results

3.1 Biochemical changes in serum parameters

The effects of ethanol extracts of *Marsilea minuta* on the liver functioning of CCl4-intoxicated rats after 14 days of treatment are shown in Figures 1–6. A single dose of CCl4 administration significantly ($p < 0.05$) increased the levels of ALT, ALP, and AST in the positive control group compared to the negative control group. Also, a significant ($p < 0.05$) increase in the level of TB was observed in positive control animals compared to the negative control group. On the other hand, pre-treatment with either the plant extract or the standard drug significantly ($p < 0.05$) reduced the levels of serum hepatic enzymes (ALT, AST, and ALP) and TB when compared with the positive control group. The reduction in hepatic serum enzymes was observed in the following order: F > C > D > E (ALT); C/F > D > E (AST); F > C > E > D (ALP); and F > C/D/E (TB). However, lower levels of albumin and total protein were noticed in the positive control rats compared with the negative control group. Significantly ($p < 0.05$) higher levels of these two parameters were preserved in rats administered either the extracts or the standard drug, with the combined extract group (Group C/D) showing values for albumin and total protein near normal.

3.2 Lipid peroxidation level, glutathione, and glutathione peroxidase activities

The effects of *Marsilea minuta* extracts on lipid peroxidation (LPO) were measured by the formation of free MDA in liver tissue following exposure to CCl4 (Figure 3). Upon CCl4 administration, MDA levels increased significantly ($p < 0.05$; a***) in the positive control rats (approximately 3.86 nmol MDA/mg protein) compared to normal control rats (~1.25 nmol MDA/mg protein). However, treatment with either the extract or the standard drug significantly inhibited the formation of MDA in the treated groups, with silymarin (b***, ~1.48 nmol/mg protein), *M. minuta* low dose (200 mg/kg; b**, ~2.12 nmol/mg protein), and *M. minuta* high dose (400 mg/kg; b***, ~1.62 nmol/mg protein) displaying marked protection against peroxidative damage.

On the other hand, a significant ($p < 0.05$; a***) decrease in GSH concentration was observed in CCl4-treated rats (positive control: ~3.21 µmol/mg protein) compared with the negative control group (~8.52 µmol/mg protein). Administration of the ethanol extract of *Marsilea minuta* for 14 consecutive days afforded marked protection

against GSH depletion in the treated groups (Figure 4), maintaining concentrations at 6.12 µmol/mg protein (b**, low dose, 200 mg/kg) and 7.51 µmol/mg protein (b***, high dose, 400 mg/kg), comparable to standard silymarin (b***, ~7.92 µmol/mg protein).

The activity of glutathione peroxidase (GPx) and superoxide dismutase (SOD) in the different treated groups of rats is shown in Figure 5. A significant decrease ($p < 0.05$; a***) in SOD activity was observed for CCl4-treated rats (~2.38 U/mg protein) relative to normal control (~6.81 U/mg protein), while treatment with *Marsilea minuta* extract caused a significant, dose-dependent restoration (b*, low dose: ~4.98 U/mg protein; b***, high dose: ~5.82 U/mg protein) when compared with CCl4-treated rats. Similarly, a significant increase ($p < 0.05$; b***) was observed in the silymarin-treated group (~6.11 U/mg protein) compared with the positive control group.

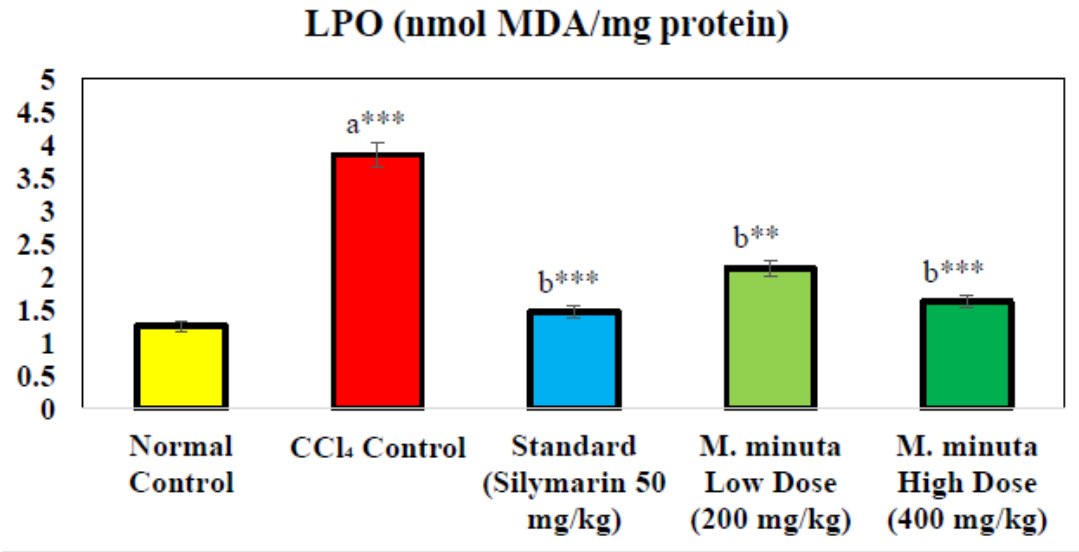


Figure 3: Effect of *Marsilea minuta* on oxidative stress parameters: lipid peroxidation (LPO, nmol MDA/mg protein) across experimental groups

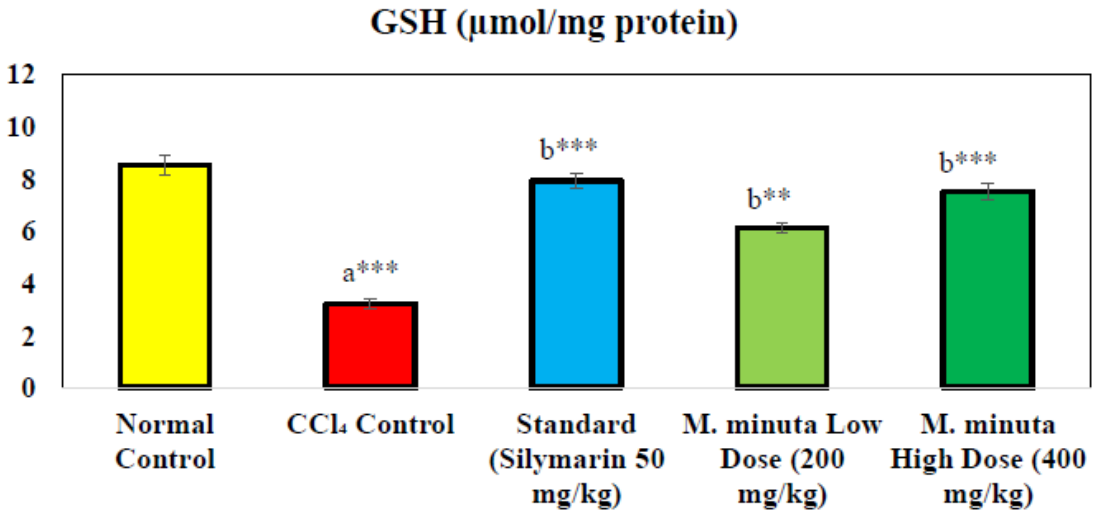


Figure 4: Effect of *Marsilea minuta* on oxidative stress parameters: reduced glutathione (GSH, µmol/mg protein) across experimental groups

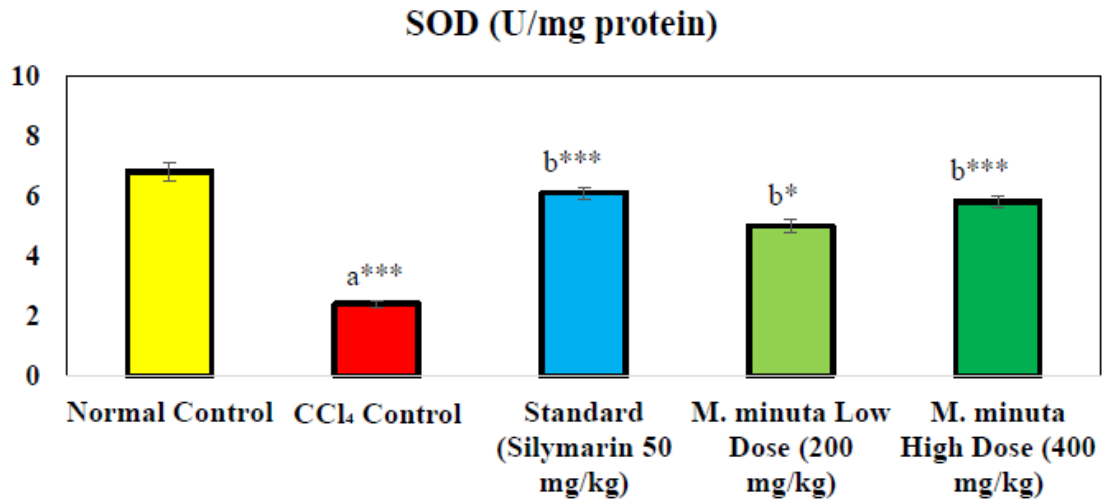


Figure 5: Effect of *Marsilea minuta* on oxidative stress parameters: superoxide dismutase (SOD, U/mg protein) across experimental groups

4. Discussion

Carbon tetrachloride (CCl4) is a toxic environmental substance found in both outdoor and indoor air, widely utilized in various industrial applications. Exposure to CCl4 can cause severe acute and chronic effects on the liver, kidneys, and central nervous system (CNS) in humans. Acute inhalation and oral exposure can lead to extensive liver damage (manifesting as hepatic swelling, tenderness, and jaundice) and kidney damage (nephritis, nephrosis, and proteinuria). CNS depression has also been reported (24) and was consistently corroborated in this research through biochemical and physiological evaluations.

The United States Environmental Protection Agency (EPA) has classified carbon tetrachloride (CCl4) as a probable human carcinogen (Group B2). Exposure to CCl4 has been intimately linked to severe hepatotoxicity, affecting various haematological parameters, including packed cell volume (PCV), erythrocyte count (RBC), white blood cell count (WBC), and platelet indices. However, treatment with *Marsilea minuta* leaf extract reversed the deleterious haematological alterations provoked by CCl4, demonstrating significant therapeutic restoration on WBC, RBC, and PCV levels. The extract's protective role in cellular proliferation and haematopoietic stabilization is consistent with previous research findings (25). The underlying cellular mechanism involves the potential for iron deposition within Kupffer cells, which catalyzes the generation of reactive oxygen species (ROS) via Fenton and Haber-Weiss reactions. These excessive ROS contribute directly to membrane lipid peroxidation, DNA oxidative damage, and potential neoplastic transformation. Despite this destructive pathway, the potent antioxidant properties of MML appear to decisively mitigate these effects, highlighting its therapeutic efficacy in protecting against CCl4-induced oxidative insults. MML's role in sustaining cellular proliferation aligns closely with the cytoprotective mechanisms described by (26).

This study systematically investigated the hepatotoxicity induced by CCl4 and the protective role afforded by MML. The results confirmed that MML treatment significantly reduced serum hepatic enzyme markers (ALT, AST, GGT, and ALP) that were elevated following CCl4 induction. Additionally, MML treatment improved renal enzymatic markers, including blood urea nitrogen (BUN) and creatinine clearance. These findings suggest that the bioactive phytochemicals present in MML possess profound ameliorative potential, protecting hepatic

parenchyma against CCl4-induced necrosis and cellular disruption. The results obtained in this study are consistent with earlier literature highlighting the therapeutic potential of MML in mitigating the adverse toxicological consequences of xenobiotic exposure (22, 23).

Furthermore, the study investigated the effects of carbon tetrachloride (CCl4) and *Marsilea minuta* leaf extract on tissue oxidative stress markers. The observed pattern corresponds with the findings reported by (24), which demonstrated progressive cellular damage in experimental models exposed to CCl4. Treatment with MML mitigated these hepatotoxic effects by suppressing CCl4-mediated radical activation and bolstering endogenous antioxidant enzyme capacity. Quantitative antioxidant assays—evaluating glutathione S-transferase (GST), glutathione peroxidase (GPx), reduced glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA)—revealed that MML-treated cohorts exhibited significant, concentration-dependent increases in antioxidant enzyme concentrations and enzymatic activity, attributed to the rich presence of bioactive secondary metabolites. In stark contrast, the untreated CCl4-intoxicated cohort suffered marked depletion of antioxidant defenses.

The study also examined pro-inflammatory cascades and established that CCl4 exposure triggered excessive production of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), accelerating oxidative stress, inflammatory infiltration, and structural tissue injury. However, pre-treatment with MML suppressed the activation of nuclear factor kappa B (NF-κB), a pivotal transcription factor regulating pro-inflammatory cytokine expression, thereby markedly decreasing the circulating and tissue concentrations of pro-inflammatory cytokines. These results demonstrate that MML exhibits concurrent anti-oxidative and anti-inflammatory properties in animal models of chemically induced oxidative stress and hepatotoxicity. Phytochemical screening of MML confirmed the presence of tannins, phenols, alkaloids, terpenoids, flavonoids, and steroids, which collectively display remarkable radical-scavenging capacities against reactive oxygen and nitrogen species. Overall, the findings demonstrate the multi-targeted ameliorating efficacy of MML against CCl4-induced oxidative stress and hepatotoxicity, attributable to its complementary phytoconstituents and robust antioxidant profile (24, 25).

5. Conclusion

In this study, acute liver damage caused by carbon tetrachloride (CCl4) was associated with pronounced lipid peroxidation and severe depletion of reduced glutathione and antioxidant enzymes in hepatic tissue. The levels of diagnostic serum biochemical markers, including ALT, ALP, and AST, were markedly elevated compared with normal physiological levels. Oral administration of *Marsilea minuta* leaf extract or the combined extract formulation at a dose of 200 mg/kg body weight significantly attenuated these elevated transaminase and phosphatase activities. Furthermore, the treatments prevented CCl4-induced lipid peroxidation and successfully restored endogenous antioxidant defenses near baseline values. Overall, these findings substantiate the pronounced hepatoprotective potential of *Marsilea minuta* extracts, with the combined extract formulation demonstrating the most promising protective efficacy among the evaluated regimens, supporting its traditional therapeutic use and warranting further translational investigation.

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