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Curative Effect of *Spondias mombin* L. against Carbon Tetrachloride-Induced Liver Damage in Rat

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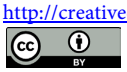

Abstract

Background: Oxidative stress is implicated in liver disease pathogenesis. Liver injuries are traditionally treated with stem bark from *Spondias mombin*. In our previous study, this plant demonstrated promising *in vitro* antioxidant activities. **Objective:** The purpose of this study was to evaluate the antioxidant and hepatocurative activity of methanolic extract of *S. mombin* (MESPM) through *in vivo* studies. **Methods:** Thirty rats were divided into five groups containing six rats each. Group I was the normal control, group II was the negative control and the other groups were experimental groups. Rats in groups II - V received, firstly, 2 mL/kg/day of carbon tetrachloride (CCl_4) intraperitoneally, for three days to induce oxidative stress and hepatotoxicity. On the following day, groups IV and V were administered MESPM orally for eleven consecutive days, respectively, at 200 and 400 mg/kg/day. Silymarin (50 mg/kg) was used as a standard (group II: positive control). Serum biochemical parameters and *in vivo* antioxidant activity were measured using standard procedures. Gross and histopathological studies were also performed. **Results:** CCl_4 induced oxidative stress was manifested by an increase in TBARS level as well as a decrease in FRAP level and DPPH radicals scavenging percentage compared to normal control ($P < 0.001$). Furthermore, CCl_4 hepatotoxicity caused significant increases in ALT, AST, ALP, bilirubin levels, with a decline in total proteins ($P < 0.001$). However, as a result of treatment with silymarin or MESPM (400 mg/kg), enzyme activities, bilirubina and total protein levels, were significantly reversed. Oxidative stress parameters were also restored. The macroscopic and histopathological changes of hepatocytes against CCl_4 confirmed that MESPM was effective. **Conclusion:** The results confirm MESPM's traditional use as a hepatocurative agent, probably due to

its antioxidant properties.

Keywords

Oxidative Stress, Hepatocurative, Antioxidant, *Spondias mombin*

1. Introduction

The liver is a major organ which plays a central role in many essential physiological processes, thus maintaining metabolic homeostasis, secretion, storage, and detoxification of diverse exogenous and endogenous xenobiotics [1]. Furthermore, the liver, due to its strategic location in the body, is continually exposed to various xenobiotics, environmental pollutants and chemotherapeutic agents which can lead to liver diseases, such as hepatitis, cirrhosis, alcohol liver disease, fatty liver, jaundice [2]. According to the World Health Organization [3], hepatitis B and C lead to chronic disease in hundreds of millions of people and are the most common cause of liver cirrhosis, liver cancer and viral hepatitis-related deaths. In Côte d'Ivoire, testing and treatment for hepatitis B and C are very limited, with prevalence estimates at 12% and 5%, respectively [4].

Oxidative stress, which is produced by an imbalance between free radical formation and cells' ability to neutralize them, has been implicated in the pathogenesis and pathophysiology of liver disease [5]-[7]. Therefore, antioxidants have a role in scavenging free radicals, inhibiting lipid peroxidation and by other mechanisms and protecting the hepatic cells from liver toxicity [8] [9]. During the past few decades, there has been increasing interest in naturally occurring antioxidants in view of the adverse side effects associated with synthetic antioxidants [10].

Spondias mombin is native to the humid plains of the Amazon rainforest and extends across Africa, tropical America, Brazil, the Caribbean and other tropical rainforests of the world [11]. In our preliminary works, an acute toxicity study demonstrated no significant toxicity of the methanolic extract of plant stem bark in a rat model. The methanolic extract of *S. mombin* also possessed antioxidant effects in *in vitro* studies [12] [13]. Moreover, in Côte d'Ivoire and other sub-Saharan countries, a decoction of the bark of *S. mombin* is used in the treatment [of liver diseases such as jaundice, severe jaundice, hepatitis and liver failure [14] [15].

We conducted this study in order to validate the traditional use of stem bark from *S. mombin* in liver diseases. A laboratory rat model was used to assess the hepatocurative potential and antioxidant properties of methanolic extract of *S. mombin* against carbon tetrachloride-induced oxidative stress and liver damage.

2. Materials and Methods

2.1. Plant Material

The stem bark of *Spondias mombin* was collected from Afféry (Region of Adzopé, south of Côte d'Ivoire). The plant was identified by her vernacular name and later

authenticated by the National Floristic Center, University Felix Houphouët-Boigny, Côte d'Ivoire. The collected plant material was air dried in darkness at room temperature (20˚C). Stem barks were cut up and stored in tight-sealed dark containers until needed.

2.2. Chemicals

Carbon tetrachloride (CCl_4) and sylimarin were obtained, respectively, from Merck (Darmstadt, Germany) and Sigma Aldrich (St Louis, MO, USA).

2.3. Preparation of Extract

One hundred grams (100 g) of washed, air-dried powdered stem of the plants were extracted with 1.5 L of methanol at room temperature for 48 hours with stirring at intervals. The methanolic solution obtained was filtered using a Buckner funnel and Whatman No 1. It was concentrated to dryness at 40˚C using a rotary evaporator under reduced pressure. The dried extracts were stored at 4˚C for subsequent analysis. The methanolic extract of the stem bark of *Spondias mombin* was named MESPM.

2.4. Experimental Design

Albino rats (130 - 170 g) of both sexes were used for the experiment and resided in the animal rearing house of Ecole Normale Supérieure d'Abidjan (Côte d'Ivoire). They were provided with tap water and regular food with maintained 12 h day and night cycle, at a room temperature of 27˚C ± 1˚C. All experiments were performed in accordance with regulations on experimental animal administration issued by the Ethical Guidelines of the University Committee on Animal Resources (Côte d'Ivoire). Doses of the plant were selected based on our previous acute toxicity study [12]. Thirty animals were divided into 5 groups consisting of 6 animals each (**Figure 1**). Group I served as a normal control group and received any treatment for fourteen (14) days. Group II served as a negative control group, receiving, intraperitoneally (i.p), only 2 mL/kg/day of 30% CCl_4 dissolved in liquid paraffin, on days 1, 2 and 3. Group III referred as a positive control group, was injected with CCl_4 (day 1, 2 and 3), and orally received silymarin (50 mg/kg/day) for eleven (11) consecutive days. The experimental groups IV and V, after intoxication with 30% CCl_4, the first 3 days, were given, orally, 200 mg/kg/day and 400 mg/kg/day of MESPM, respectively for 11 consecutive days.

Twenty-four hours after the last treatment *i.e.*, on 15th day, animals were anaesthetized using light ether anesthesia and blood was collected from the retro orbital plexus. The blood samples were allowed to clot and the serum was separated by centrifugation at 2500 rpm for 15 min at 37˚C and kept at –4˚C. Furthermore, the liver was rapidly excised, rinsed in ice-cold saline, and was fixed in 10% formalin solution for histopathological evaluation while a 10% w/v homogenate was prepared using 0.15M KCl, centrifuged at 3000 g for 20 min at 4˚C. This supernatant was ultimately used to assess parameters.

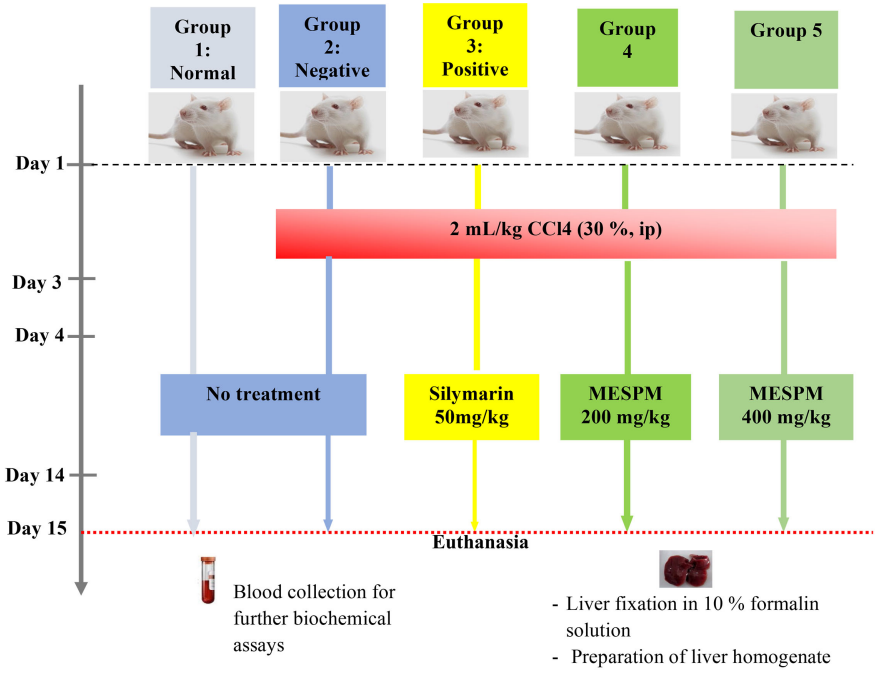

Figure 1. Experimental Design. The animals were distributed into five groups, consisting of 6 animals each. Oxidative stress and liver damage were induced by administering 2 mL/kg/day, i.p., of CCl_4 diluted in liquid paraffin.

2.5. Assessment of *in Vivo* Antioxidant Potential

Three techniques were used to quantify the *in vivo* antioxidant activity in liver tissue and serum samples against CCl_4-induced oxidative stress in rats. TBARS (thiobarbituric acid reactive substances) level was assessed through the process of Ohkawa *et al.* [16]. The total antioxidant power was evaluated using FRAP (ferric reducing antioxidant power) assay developed by Benzie and Strain [17]. DPPH free radicals scavenging activity of serum samples was determined according to the protocol of Hasani *et al.* [18].

2.6. Biochemical Assays in Serum

To evaluate biochemical markers in liver injury, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total protein and total bilirubin were measured in plasma samples using an autoanalyzer (Roche/INTEGRA) and commercial assay kits (COBAS INTEGRA). The manufacturer's protocol was followed for all estimations.

2.7. Gross and Histopathological Studies

The livers of rats were macroscopically examined for signs of damage, followed by histopathological analysis of liver samples fixed in 10% formalin solution. Afterward, livers were dissected into approximately 5 mm-thick pieces. These sections were dehydrated in gradual ethanol concentration, cleared in xylene and finally embedded in paraffin wax. The paraffin embedded tissues were sectioned (4 μm to 5 μm) serially using a rotary microtome. Tissue slices were then stained

with eosin and hematoxylin for light microscopic observations.

2.8. Statistical Analysis

All data are presented as a mean with standard error (SEM). Graphpad Prism version 5.0 (San Diego, United States of America) was used to analyze the data. To analyze data sets, one-way analysis of variance (ANOVA) was used, and statistical analysis was performed using Tukey's multiple comparison. At $p < 0.05$, differences were considered statistically significant.

3. Results

3.1. Effect of Methanolic Extract of *S. mombin* on Oxidative Stress Parameters in CCl_4-Treated Rats

In this study, *in vivo* antioxidant activity of methanolic extract of *S. mombin* (MESPM) in serum and liver homogenate was assessed by measuring TBARS levels, FRAP values, and percentage inhibition of DPPH radicals. As shown in Table 1, administration of CCl_4 in rats caused higher level ($p < 0.001$) of TBARS in liver tissue by 312.10% compared to normal group. Also, a significant decrease ($p < 0.001$) in the FRAP values and percentage inhibition of DPPH radicals in serum, respectively, by around 47.08% and 68.30%, was observed in CCl_4 intoxicated group (negative control) compared to normal control. However, post-treatment, both with MESPM, at 400 mg/kg of body weight and silymarin (50 mg/kg body weight), in CCl_4-intoxicated rats, reduced significantly ($p < 0.001$) hepatic concentration of TBARS, respectively by 43.20% and 39.90%, whereas exhibited a significant increase ($p < 0.05$) of percentage inhibition of DPPH radicals as well as total antioxidant power, compared to negative control group without bringing them back to normal values. After treatment with MESPM (400 mg/kg of body weight) and silymarin, serum DPPH radical scavenging activity returned to normal levels, except for TBARS concentrations.

Table 1. Effect of treatment with methanolic extract of *S. mombin* (MESPM) on oxidative stress parameters in CCl_4 intoxicated rats.

Treatment	TBARS (nmol/g)	DPPH (%)	FRAP (µmol/L)
Normal control	161.2 ± 18.15***	32.12 ± 2.50**	422 ± 29.08***
Negative control	664.3 ± 60.48$^{\#\#\#}$	10.18 ± 2.19$^{\#\#\#}$	223.3 ± 15.44$^{\#\#\#}$
CCl_4 + silymarin (50 mg/kg)	399.3 ± 36.40$^{***\#\#}$	26.46 ± 4.14*	372.8 ± 20.23**
CCl_4 + MESPM (200 mg/kg)	489.9 ± 13.89$^{*\#\#\#}$	22.32 ± 3.69$^{\#}$	295.2 ±19.05$^{\#\#}$
CCl_4 + MESPM (400 mg/kg)	377.3 ± 29.40$^{***\#\#}$	25.89 ± 3.77*	326.1 ± 19.11$^{*\#}$

The values of the parameters studied are expressed as mean ± standard deviation, n = 6. The symbols (*#) represent statistical significance. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ denotes a significant difference between the negative control group and the other experimental groups. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ denotes a significant difference between the normal control group and the treatment groups.

3.2. Effect of Methanolic Extract of *S. mombin* (MESPM) on Liver Enzymes, Total Bilirubin and Total Proteins against CCl_4 Induced Liver Toxicity in Rats

The effect of methanolic extract of stem barks of *S. mombin* (MESPM) on serum biochemical parameters in CCl_4-intoxicated rats is summarized in Table 2. The ALT, AST, ALP, and total bilirubin levels in the CCl_4-intoxicated group (negative control) were significantly raised ($p < 0.001$) in serum, respectively by 132.65%, 126.98%, 164.83%, and 465.21%, whereas total proteins were decreased (27.61%) as compared to normal control. Serum marker enzyme activity and bilirubin level in rats treated after CCl_4 intoxication with 200 mg/kg of body weight (except for AST) and 400 mg/kg of body weight of MESPM, are significantly reduced, while total proteins concentration increased compared to negative control. Furthermore, only rats treated with MESPM, at 400 mg/kg of body weight and silymarin (50 mg/kg of body) reversed these biochemical parameters, bringing them closer to normal control values ($p > 0.05$).

Table 2. Effect of treatment with methanolic extract of *S. mombin* on markers of liver damage in CCl_4 intoxicated rats.

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)	Bilirubin (g/L)	Total proteins (g/L)
Normal control	56.11 ± 6.43***	169.7 ±16.52***	85.60 ± 13.40***	0.46 ± 0.06***	70.80 ± 2.20***
Negative control	223.6 ± 32.74###	385.2 ± 33.74###	226.7 ± 49.59###	2.56 ± 0.37 ###	51.25 ± 2.75###
CCl_4 + silymarin (50 mg/kg)	70.17 ± 10.99***	224.4 ± 9.73**	77.00 ±14.75***	0.94 ± 0.34**	67.78 ± 3.42**
CCl_4 + MESPM (200 mg/kg)	137.4 ± 16.09*#	286.5 ± 34.48 #	164.0 ± 24.01*#	1.10 ± 0.34*	65.35 ± 1.31*
CCl_4 + MESPM (400 mg/kg)	64.72 ± 6.58***	213.8 ± 23.04***	110.5 ± 9.43**	1.01 ± 0.29*	66.75 ± 1.75**

The values of the parameters studied are expressed as mean ± standard deviation, n = 6. The symbols (*#) represent statistical significance. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ denotes a significant difference between the negative control group and the other experimental groups. #$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$ denotes a significant difference between the normal control group and the treatment groups.

3.3. Gross and Histopathological Observations

Gross necropsy and histopathological study were performed on the livers to observe any irregularities or anomalies on the structure. Figure 2 shows macroscopic observations. A normal control group liver displayed a reddish-brown color, a regular surface, and a normal anatomical structure. No lesions are visible to the naked eye (Figure 2(a)). According to Figure 2(b), liver treated with only CCl_4 showed an unregular surface with a rounded shape, dented by large cysts, suggesting severe hepatocellular damage. Its surface is dotted with white spots which are signs of necrosis. Post treatment of intoxicated liver with either the high dose of MESPM (400 mg/kg of body weight) and silymarin presented a pale liver color with minimal white spots and micronodules (Figures 2(d)-(e)). No serious lesions such as macronodules or cysts are detectable, indicating a regression of lesions induced by CCl_4.

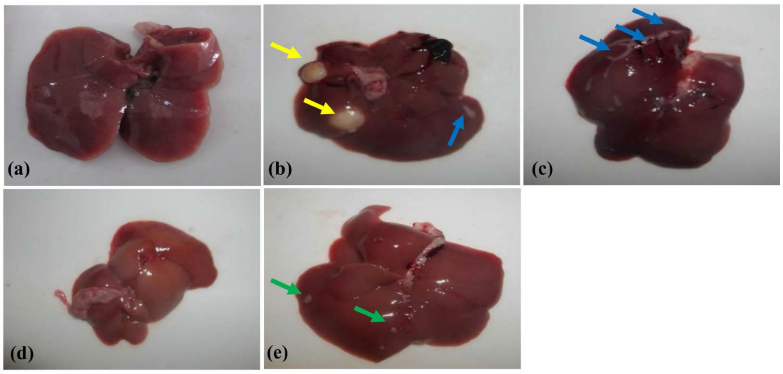

Figure 2. Macroscopic liver appearance of rats treated with methanolic extract of *S. mombin* (MESPM) and silymarin after CCl_4 intoxication. (a) Liver of normal control group; (b) Liver of negative control group; (c) Liver of rat poisoning with CCl_4 followed by treatment with 200 mg/kg of body weight of MESPM; (d) Liver intoxicated with CCl_4 and treated with 50 mg/kg of body weight of MESPM; (e) Liver intoxicated with CCl_4 and treated with 400 mg/kg of body weight of MESPM. Yellow arrows represent macronodules (cysts); blue arrows represent whitish spots of necrosis; green arrows display micronodules.

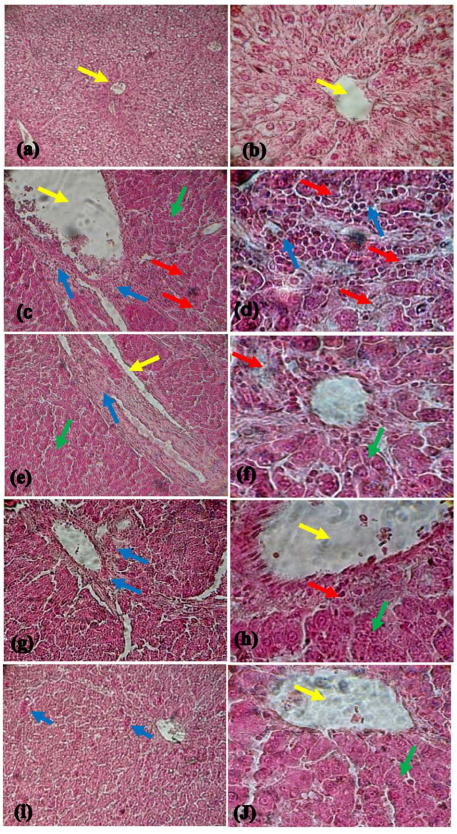

Figure 3. Hepatic histological analyses of the effect of methanolic extract of *S. mombin* and silymarin on CCl_4-induced liver injuries in rat. (a)-(b): Normal control group; (c)-(d) CCl_4 intoxicated liver tissue (negative control); (e)-(f): Positive control group (CCl_4 + silymarin); (g)-(h): CCl_4 + 200 mg/kg; (i)-(j): CCl_4 + 400 mg/kg. Hematoxylin & Eosin staining, (a), (c), (e), (g), (i): x 100; (b), (d), (f), (h), (j): x 400. The yellow arrow denotes central lobular vein dilatation while red arrows denote necrosis. The blue and green arrows represent respectively, inflammatory infiltrates and ballooning hepatocytes.

Histopathological examination of the liver section from the normal control group showed no abnormalities in hepatic cellular architecture (**Figure 3(a)**, **(Figure 3(b)**). Liver cells spaced by sinusoids were arranged radically from central vein. In the negative control group, a very significant dilatation of the centrilobular vein and numerous foci of inflammation around this vein is noted (**Figure 3(c)**). Liver tissue's trabecular organization is completely unrecognizable due to lysis and loss of cellular cohesion. Furthermore, necrotic lesions are present throughout the entire photomicrograph and significant inflammation foci are identified. In addition, hypercolored nuclei of hepatocytes are a sign of pyknosis (**Figure 3(d)**). The photomicrograph of the liver of rats intoxicated with CCl_4 then treated with 400 mg/kg of MESPM or silymarin indicated ballooned hepatocytes and moderate dilatation of the centrilobular vein are noted as well as relative lesions of necrosis and inflammation infiltrates (**Figures 3(g)-(j)**). Post-treatment with these doses generally resulted in a strong regression of CCl_4-induced hepatocyte lesions in rats compared to rats in negative controls.

4. Discussion

Liver is damaged by several agents such as chemicals including alcohol, drugs, and viruses [2]. In our investigation, carbon tetrachloride induced hepatotoxicity. The choice of this toxicant is motivated by the fact that it is commonly used as a model for the screening of anti-hepatotoxic and/or hepatoprotective activities of drugs [19]. Moreover, the liver damage caused by CCl_4 is similar to that produced by viral hepatitis [20]. Several reports demonstrated the implication of oxidative stress in the pathogenesis of liver diseases [5]-[7]. Therefore, antioxidants may protect against reactive free radicals caused by oxidative stress. In our previous work, methanolic extract of stem barks of *S. mombin* indicated a relative high content of total polyphenols (343.5 ± 6.44 mg gallic acid/g) and flavonoids (11.28 ± 0.45 mg quercetin/g) as well as strong *in vitro* antioxidant activities through different tests such as DPPH scavenging activity, inhibition of lipid peroxidation, and reducing power [12] [13]. In the present study, the *in vivo* antioxidant activity of MESPM against CCl_4 induced oxidative stress was evaluated. As an indicator of lipid peroxidation, TBARS reflects oxidative stress directly [21]. CCl_4 injections significantly increased the level of TBARS. In fact, CCl_4 induces hepatic damage by producing toxic reactive trichloromethyl and trichloromethyl peroxide radicals along with liver microsomes containing cytochrome P450 [22]. These reactive radicals further bind to unsaturated fatty acids in the membranes of hepatocytes, mitochondria and endoplasmic reticulum, inducing a chain lipid peroxidation process that leads first to damage and then to death of hepatocytes and intracellular structures [23]. After propagation of the peroxidation process, lipids are finally degraded into small molecules such as malondialdehyde (MDA) or 4-hydroxynonenal (HNE), which are highly reactive aldehydes that can form protein and DNA adducts [24] [25]. On the other hand, MESPM attenuated significantly the level of lipid peroxidation, suggesting a hepatocurative effect by decreasing

oxidative stress in the CCl_4-induced acute liver injury. A variety of other techniques have been developed to determine the antioxidant capacity of plasma, such as the ferric reducing antioxidant of plasma (FRAP) and the DPPH radical scavenging activity. In different clinical studies of oxidative stress-related pathological conditions, both methods are widely recognized and used [26] [27]. Data obtained by DPPH and FRAP assays indicate that administration of MESPM in intoxicated rats effectively inhibits oxidative stress by increasing DPPH radical scavenging percentage and FRAP level. The increase in plasma antioxidant capacity is probably attributed to the elevated levels of exogenous antioxidants acquired following treatment with *S. mombin* extract. The peroxidation of membrane lipids by CCl_4 is known to significantly alter their physical properties. In particular, peroxidation alters lipid-lipid interactions, ion gradients, membrane fluidity, and membrane permeability [28]. Disruption of the hepatocellular plasma membrane leads to the release of enzymes normally present in the cytosol into the blood circulation. Accordingly, liver transaminases (AST, ALT) and ALP activities were significantly increased in rats administered only with CCl_4 compared to the normal control group. Elevation of bilirubin and reduction of total protein levels were also observed in the CCl_4-treated group (negative control). The excretory function of the liver can be determined by blood bilirubin levels. It is known that abnormal increase in serum bilirubin indicates hepatobiliary disease and severe disturbance of hepatocellular architecture [29], while reduction in serum total proteins can be due to impaired protein synthesis due to liver tissue damage [30]. MESPM treatment in CCl_4-treated rats restores normal membrane integrity, recovers liver secretory and synthesis function by bringing these biochemical parameters closer to normal values. Histological examination supports previous biochemical findings. In CCl_4-treated rats, histopathological analysis revealed ballooning, degeneration of hepatocytes, inflammatory infiltration and severe hepatocellular necrosis. In comparison to normal control, oral administration of MESPM, especially at the highest dose (400 mg/kg), and silymarin restores the architecture of liver tissue.

5. Conclusion

Based on the results obtained in this study, it is concluded that the hepatocurative properties of the methanolic extract of *S. mombin* (MESPM) on carbon tetrachloride induced liver damages in rats were demonstrated by the restoration to normal values of the various biochemical parameters and histological abnormalities of liver tissues. The curative effects of *S. mombin* may be attributed to its antioxidant properties. Its traditional use as a liver injury treatment is also justified by these results. A further study is required to isolate the active principle of the plant that acts as an effective antihepatotoxic agent.

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Authors' Contributions

ANR B designed the experiments, supervised the study and wrote the first draft of the manuscript. AP D contributed to data analysis and interpretation. K K contributed to animal handling and experimentation. JD N critically reviewed the manuscript. All the authors contributed to revising the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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