ORIGINAL RESEARCH ARTICLE

Gas Chromatography–Mass Spectrometry Analysis of *Jatropha tanjorensis* Tannin-rich Leaf Extract and Its Hepatoprotective Effect on Methotrexate-induced Liver Injury in Rats

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Abstract

Drug-induced toxicity frequently involves the liver due to the its crucial function in drug metabolism. This study investigates the bioactive components and hepatoprotective effects of tannin-rich extract of *Jatropha tanjorensis* leaves on methotrexate (MTX)-induced liver injury in Wistar rats. Thirty rats were divided into six groups: A positive control group, a negative control group treated with MTX (5 mg/kg bodyweight), a standard drug group receiving silymarin (100 mg/kg), and three experimental groups administered 100, 200, and 400 mg/kg of the extract daily for 14 days. Gas chromatography–mass spectrometry (GC–MS) analysis identified key bioactive compounds, including chlorogenic acid, caffeic acid, ferulic acid, resveratrol, rutin, ursolic acid, and rosmarinic acid. Biochemical assays showed significant elevations in alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), bilirubin, and malondialdehyde (MDA) levels in the MTX-only group, which were significantly reduced by extract treatment ($p < 0.05$). The MTX group exhibited decreased albumin and hepatic antioxidant enzyme activities [catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px)], which were restored by the extract. The albumin/globulin ratio of the extract-treated group improved significantly compared to the negative control. Histopathological analysis revealed that MTX-induced severe liver damage, including necrosis, which was ameliorated by the extract, restoring hepatic architecture. These findings suggest that *J. tanjorensis* tannin-rich extract, rich in antioxidant and anti-inflammatory compounds, offers significant hepatoprotective potential against MTX-induced liver injury.

Keywords: Cytoprotection, Hepatotoxicity, Inflammation, *Jatropha*, Liver histology, Oxidative stress.

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Introduction

Drug-induced toxicity frequently involves the liver due to the liver's crucial function in drug metabolism. The multistep process of drug-induced liver injury (DILI) includes both direct drug injury and the accompanying activation of pathways of inflammation. Liver injury is often triggered by the parent drug or, more commonly, by its metabolites generated during early metabolic processing. These harmful metabolites are typically produced through Phase I metabolism, primarily involving the cytochrome P450 (CYP450) enzyme family, which exhibits genetic variability. However, conjugative phase II metabolism may also produce the hazardous chemicals.[1] The adverse effect of the drug then spreads through ensuing cell stress, mitochondrial inhibition, and/or certain immunological responses. Drug-induced liver injury accounts for 60% of acute liver failure and 5–10% mortality.[2]

A folic acid antagonist known as methotrexate (MTX) is frequently used as a chemotherapeutic medication to treat several malignant stages, including acute lymphoblastic leukemia, as well as a number of inflammatory illnesses.[3] Adverse effects of MTX may involve hypersensitivity-related lung inflammation, neurotoxicity affecting both the central and peripheral nervous systems, impairment of hepatic and gastrointestinal functions, as well as hematological disorders.[4] The toxicity associated with MTX is believed to result from a complex interplay of various factors, including dosage regimen, duration of therapy, individual patient susceptibility, underlying disease type, and the influence of genetic and molecular mechanisms related to apoptosis.[5]

Methotrexate-induced hepatotoxicity (MIH) is thought to be caused through signaling pathways associated to inflammation and stress.[6] To prevent and treat MIH, therapeutic approaches focused at reducing oxidative stress and the accompanying inflammation as well as increasing cellular antioxidants can be investigated.

According to recent research, adding natural phytoactive substances with antioxidant qualities to a diet may protect the liver from MIH.[7,8]

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Conflict of interest: None

Jatropha tanjorensis belongs to the "Euphorbiaceae" family and is commonly cultivated in Nigeria.[9] In several regions of Nigeria, its leaf is often eaten as a vegetable. It is referred to as "hospital too far," "lapalapa," and "asibiti kusa" informally.[10] *J. tanjorensis* leaves are particularly abundant in flavonoids and phenols, both free and bound.[11] Studies on the free and bound phenolic extracts of *J. tanjorensis* leaves' *in vitro* antioxidant activities revealed that the extracts prevented the hepatic and cerebral lipid peroxidation (LPO) process from being triggered by Fe^{2+}.[12] This research aimed to evaluate the constituents of methanol extract of *J. tanjorensis* leaf and to assess as its potential hepatoprotective effect against liver damage induced by MTX in albino rats.

Materials and Methods

Collection and Preparation of Plant Sample

All experimental procedures involving animals were conducted in accordance with standard ethical guidelines for animal research. Ethical approval was obtained from the Research Ethics Committee of Federal University of Technology Minna, Nigeria (Approval No.: 000058EAU). Fresh, healthy leaves of *J. tanjorensis* were collected from the Bosso area (latitude: 9.6522, longitude: 6.5261) in Minna, Niger State, Nigeria. The plant was identified and assigned voucher number FUT/PLB/EUPH/003 at the Federal University of Technology, Minna Herbarium.

Tannin Extraction

The collected leaf sample was shade-dried and pulverized in a Wiley mill. An amount of 400 g of the powdered material was agitated continuously at a speed of 230 rpm for 11 hours at ambient temperature (22 ± 2°C) with 70% methanol (4 L) solvent for extraction. Extraction with 70% methanol was chosen for its proven efficiency in extracting tannins, offering optimal polarity to dissolve both hydrophilic and moderately lipophilic compounds. Khoddami et al.[13] reported that 70% methanol yielded 20–30% more tannins than pure methanol or ethanol, with better reproducibility.

The sample was passed through a membrane filter with a pore size of 0.45 µm to remove particulates. The resulting filtrate was transferred into a round-bottom flask and subjected to solvent removal under reduced pressure using a rotary evaporator. The concentrated extract was subsequently dried in a vacuum oven maintained at 50°C until a solid residue was obtained.[14]

Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

The tannin-rich extract of *J. tanjorensis* was subjected to GC–MS analysis at Bato Chemical Laboratory, Lagos, Nigeria. The analysis was performed using a TRACE GC Ultragas chromatograph (Thermo Scientific Corp., Waltham, MA, USA) coupled to an ISQ Single Quadrupole Mass Spectrometer. The chromatographic separation was achieved on a TG-5MS capillary column (30 m length × 0.25 mm internal diameter, 0.25 µm film thickness). Helium served as the gas carrier at a constant flow rate of 1.0 mL/min, and the injection was made in split mode with a ratio of 1:10.

The temperature program began at 50°C (held for 3 minutes), followed by a gradual increase of 5°C per minute up to 300°C, which was maintained for an additional 20 minutes. The temperature of the injector and detector was set at 28°C. Each sample (1 µL), diluted at a 1:10 ratio in hexane (v/v), was introduced into the system. Ionization of compounds was performed via electron impact (EI) at 70 eV, and mass spectra were recorded within the m/z range of 40–450.

Experimental Design

Thirty male Wistar rats, weighing between 150 and 200 gm, were housed in groups of five per cage. Male rats were selected to avoid confounding effects of estrous cycle variations in females, which can influence drug metabolism (e.g., CYP450 enzyme activity) and hepatotoxicity outcomes.[15] This ensures uniformity in responses to MTX and the tested extract.

The rats used were 8–10 weeks old, an age at which they are physiologically mature with stable liver function—ideal for toxicological studies and free from age-related hepatic changes.[16]

Prior to the experiment, the rats underwent a 15-day acclimation period under controlled laboratory conditions, including a 12-hour light/dark cycle and a stable room temperature of 24 ± 2°C. Food and water were provided ad libitum throughout the study period. Body weights were recorded on day one and again at the conclusion of the 14-day experimental period. Subsequently, the rats were randomly allocated into six distinct experimental groups.

All groups, except group I (positive control), were intraperitoneally injected with MTX at a dosage of 5 mg per kilogram of body weight on the first day. Group II (negative control) received no further treatment. Group III (MTX + silymarin) received an additional oral dose of 100 mg/kg body weight of silymarin daily for 14 days. Meanwhile, groups IV, V, and VI were administered daily oral doses of *J. tanjorensis* extract at 100, 200, and 400 mg/kg body weight, respectively, for 14 days. During this period, group I received 0.5 mL of distilled water daily.

Blood and Tissue Collection

Animals were euthanized by cervical dislocation on the 15th day, and blood samples were collected into heparinized tubes. After being left at room temperature (29°C) for 20 minutes, the collected blood samples were subjected to centrifugation at 3,000 revolutions per minute for 10 minutes to isolate the plasma, which was subsequently stored at –20°C pending biochemical evaluations. Liver tissue samples from each animal were immediately immersed in a 10% formalin solution and refrigerated for subsequent histopathological examination.

Biochemical Investigations

Experimental rats were euthanized by cervical dislocation, and a cardiac puncture was used to obtain a blood sample. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), and total protein concentrations were quantified using enzyme-based assay kits obtained from Vitro Scientific (Germany), following the manufacturer's protocols.

Evaluation of *In Vivo* Antioxidant Activity

Tissue samples were homogenized in chilled phosphate buffer (50 mM, pH: 7.4) and subsequently centrifuged at 800 rpm for 10 minutes at 4°C. The supernatant obtained was then utilized to determine levels of LPO, the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px). Lipid peroxidation was assessed at 532 nm using a UV-visible spectrophotometer and expressed as moles of malondialdehyde (MDA) per 100 mg of protein (Sevanian and Ursini, 2000). Catalase activity was assessed using the method described by Beers and Sizer.[17] The activity was measured at an absorbance of 240 nm using a UV-visible spectrophotometer and expressed as units of H_2O_2 per milligram of tissue. Superoxide dismutase levels

were determined following the protocol outlined by Sagu et al.[18] The results were expressed as SOD activity/mg of tissue and were measured at 480 nm against a blank. By measuring absorbance at 412 nm against a blank, the Ellman method was used to calculate GSH-Px.[19] The percentage of inhibition was computed.

Histological Evaluation of Liver Samples

The liver tissues were preserved in 10% neutral-buffered formalin for 24 hours, followed by dehydration through a series of increasing ethanol concentrations. After dehydration, the samples were cleared with xylene and embedded in paraffin wax. Using a microtome, thin sections approximately 4 µm thick were obtained, mounted on slides, and stained with hematoxylin and eosin (H&E). The prepared slides were then examined microscopically under a light microscope and captured using a digital camera.[20]

Results

Investigation of the Components of *J. tanjorensis* Tannin-rich Extract Using GC–MS Analysis

The GC–MS analysis of the tannin-rich extract from *J. tanjorensis* leaves, as presented in Tables 1A and B, identified 18 compounds. Among these, Hesperidin was the most abundant, followed by vitexin, ferulic acid, jathrophane, and rutin. Identification of the compounds was achieved by analyzing their retention times and mass spectral fragmentation profiles in comparison with those of established reference standards analyzed under identical conditions. (Fig. 1, Tables 1A and B).[21–23]

Effect of Tannin-rich Extract of *J. tanjorensis* Leaf on Serum ALT Activity in MTX-induced Liver Injury in Rats

Figure 2 presents the effect of the tannin-rich extract of *J. tanjorensis* leaves on serum ALT activity in MIH in rats. Serum ALT activity was markedly elevated ($p < 0.05$) in the negative control group in comparison with the positive control, silymarin-treated, and *J. tanjorensis*-treated groups. However, no significant differences in ALT levels were observed among the positive control, silymarin, and 400 mg/kg *J. tanjorensis*-treated groups. The administration of the tannin-rich extract effectively protected against MTX-induced ALT elevation in a dose-dependent manner.

Effect of Tannin-rich Extract of *J. tanjorensis* Leaf on Serum AST Activity in MTX-induced Liver Injury in Rats

Figure 3 illustrates the effect of the tannin-rich extract of *J. tanjorensis* leaves on serum AST activity in MIH in rats. A marked increase ($p < 0.05$) in serum AST activity was observed in the negative control

Table 1A: Gas chromatography–mass spectrometry analysis of *J. tanjorensis* tannin-rich leaf extract

Component	*Retention*	*Area*	*Molecular weight gm/mol*	*Molecular formula*	*Hepatoprotective mechanism*	*Reference*
Solvent	0.466	91582.4930				
Chlorogenic acid	7.016	415.0820	354.31	$C_{16}H_{18}O_9$	Antioxidant, reduces oxidative stress and inflammation	24
Casbene	7.566	67.8040	272.5	$C_{20}H_{32}$	Anti-inflammatory properties	25
Daphnane	8.250	57.4720	315.5	$C_{22}H_{37}N$	Potential cytoprotective effects	26
Tigliane	9.133	1178.9900	274.5	$C_{20}H_{34}$	Modulates immune response	27
Podocarpene	9.816	143.7430	272.5	$C_{20}H_{32}$	Antioxidant activity	28
Caffeic acid	10.983	734.3130	180.16	$C_9H_8O_4$	Enhances Nrf2 pathway, boosts glutathione levels	29
P-Coumaric acid	11.283	590.7320	164.16	$C_9H_8O_3$	Reduces lipid peroxidation and liver enzyme levels	30
Catechin	11.550	566.8210	290.27	$C_{15}H_{14}O_6$	Potent free radical scavenger, protects against fibrosis	31
Resveratrol	12.350	1914.4210	228.25	$C_{14}H_{12}O_3$	Activates SIRT1, reduces hepatic steatosis and inflammation	32

Table 1B: Gas chromatography–mass spectrometry analysis of *J. tanjorensis* tannin-rich leaf extract

Component	*Retention*	*Area*	*Molecular weight gm/mol*	*Molecular formula*	*Hepatoprotective mechanism*	*Reference*
Rhamnofolane	12.650	346.2210	372.50	$C_{22}H_{28}O_5$	Potential anti-fibrotic effects	33
Ursolic acid	13.416	1361.1820	456.7	$C_{30}H_{48}O_3$	Anti-apoptotic, reduces ALT/AST, enhances antioxidant enzymes	34
Rosmarinic acid	13.966	234.9090	360.3	$C_{18}H_{16}O_8$	Suppresses TGF-β1, prevents collagen deposition	35
Kaempferol	14.233	1726.5310	286.24	$C_{15}H_{10}O_6$	Modulates PI3K/Akt pathway, protects hepatocytes	36
Rutin	15.216	1671.4050	610.5	$C_{27}H_{30}O_{16}$	Chelates iron, prevents lipid peroxidation, strengthens capillaries	37
Jatrophane	15.866	5305.5820	699.7	$C_{36}H_{45}NO_{13}$	Potential P-glycoprotein modulation affecting drug metabolism	38
Ferulic acid	16.366	451.0290	194.18	$C_{10}H_{10}O_4$	Upregulates HO-1, reduces necroinflammation	39
Vitexin	16.816	183.4015	432.4	$C_{21}H_{20}O_{10}$	AMPK activation, improves lipid metabolism	40
Hesperidin	17.150	1212.1165	610.6	$C_{28}H_{34}O_{15}$	Reduces TNF-α and IL-6, protects against ethanol-induced injury	41

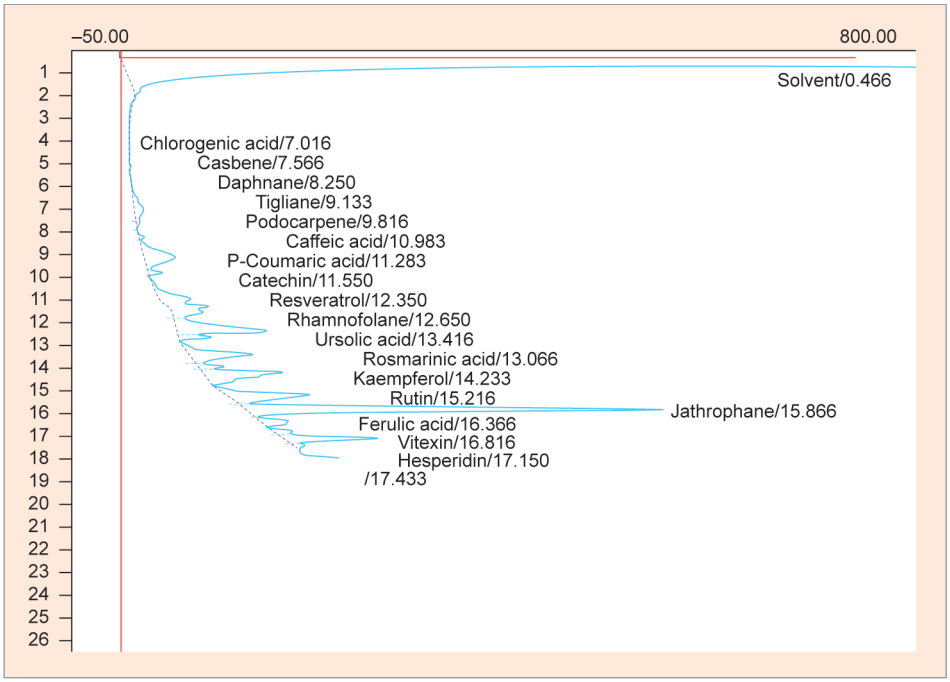

Fig. 1: Gas chromatography–mass spectrometry chromatogram of *J. tanjorensis* tannin-rich leaf extract

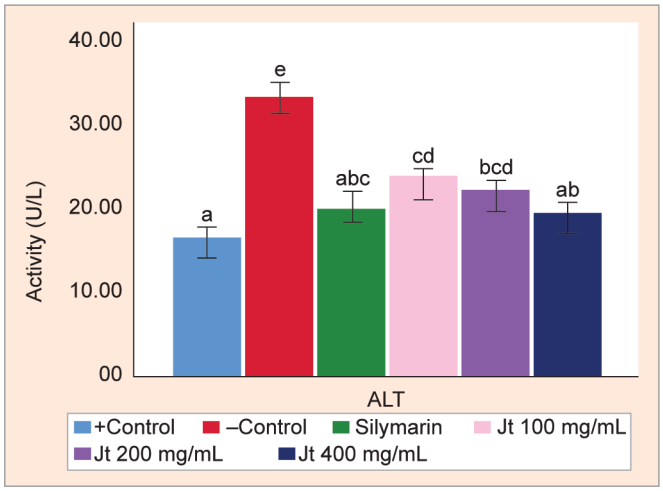

Fig. 2: Effect of tannin-rich extract of *J. tanjorensis* leaf on serum ALT activity in MTX-induced liver injury in rats. Bars are expressed as the mean of five values ± standard error of mean. Bars with different superscripts are significantly different at $p < 0.05$ Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin –Control: 5 mg/kg bw of MTX +Control: 0.5 mL distilled water

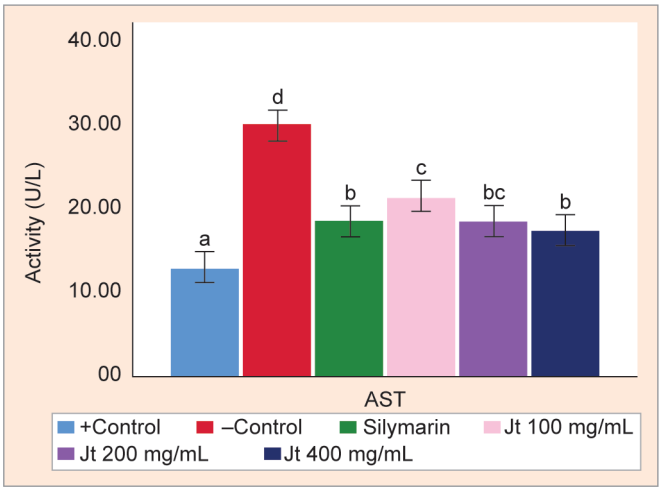

Fig. 3: Effect of tannin-rich extract of *J. tanjorensis* leaf on serum AST activity in MTX-induced liver injury in rats. Bars are expressed as the mean of five values ± standard error of the mean. Bars with different superscripts are significantly different at $p < 0.05$ Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin –Control: 5 mg/kg bw of MTX +Control: 0.5 mL distilled water

group in comparison with the positive control, silymarin-treated, and *J. tanjorensis*-treated groups. Notably, no significant difference in AST activity was detected between the silymarin group and the groups treated with 200 mg/kg and 400 mg/kg bodyweight of the extract. The administration of the tannin-rich extract effectively mitigated the MTX-induced elevation in AST activity in a dose-dependent manner.

Effect of Tannin-rich Extract of *J. tanjorensis* Leaf on Serum ALP Activity of MTX-induced Liver Injury in Rats

Figure 4 illustrates the effect of the tannin-rich extract of *J. tanjorensis* leaves on serum ALP activity in MIH in rats. Serum ALP activity was markedly elevated ($p < 0.05$) in the negative control group in comparison with the positive control, silymarin-treated,

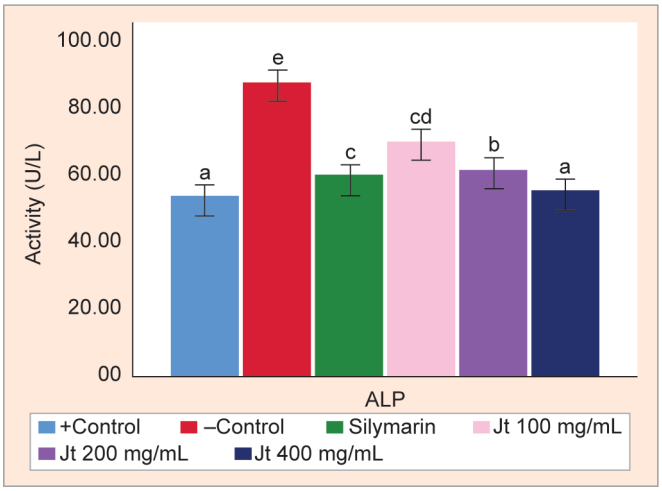

Fig. 4: Effect of tannin-rich extract of *J. tanjorensis* leaf on serum ALP activity in MTX-induced liver injury in rats. Bars are expressed as the mean of five values ± standard error of the mean. Bars with different superscripts are significantly different at $p < 0.05$ Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin –Control: 5 mg/kg bw of MTX +Control: 0.5 mL distilled water

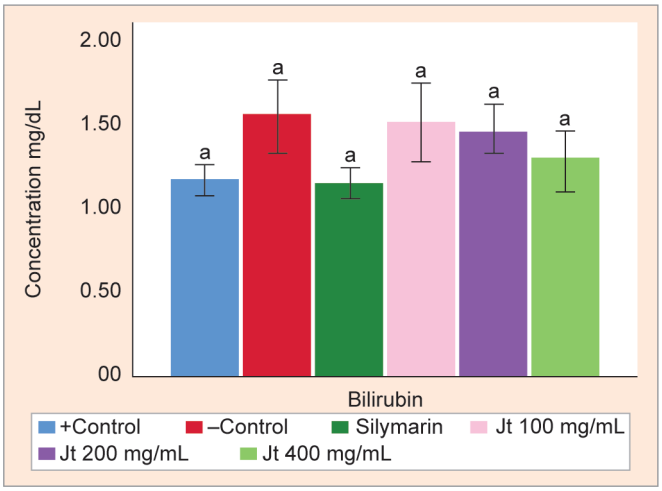

Fig. 5: Effect of phenol-rich extract of *J. tanjorensis* leaf on serum bilirubin in MTX-induced liver injury in rats. Bars are expressed as the mean of five values ± standard error of the mean. Bars with different superscripts are significantly different at $p < 0.05$ Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin –Control: 5 mg/kg bw of MTX +Control: 0.5 mL distilled water

Table 2: Bodyweight changes and liver/bodyweight ratio of the treatment groups

Treatment groups (mg/kg bodyweight)	*Initial bodyweight (g)*	*Final bodyweight (g)*	*Loss or gain (%)*	*Liver/Bodyweight (mg/100 g)*
Jt 100	219.42 ± 14.35	197.33 ± 12.20	–10.07	4.50 ± 0.43^{b}
Jt 200	211.44 ± 10.81	197.60 ± 8.63	–6.55	5.01 ± 0.27^{e}
Jt 400	221.54 ± 15.04	210.62 ± 10.86	–4.93	4.90 ± 0.43^{d}
Silymarin	226.00 ± 8.30	218.52 ± 6.74	–3.31	5.03 ± 0.04^{e}
–ve control	231.10 ± 12.24	198.41 ± 14.10	–14.15	4.39 ± 0.31^{a}
+ve control	209.21 ± 12.34	219.53 ± 8.22	4.93	5.04 ± 0.21^{e}

Values are expressed as the mean ± standard error of mean of five determinations ($n = 5$).

Different superscript alphabets in the same column indicate statistically significant difference at $p < 0.05$.

Note: The statement above suffices as it addresses the statistical significance of each parameter down a column. E.g. for "Liver/Bodyweight" the values that carry a different superscript alphabet imply that they differ significantly, $p < 0.05$.

Jt 200 and Silymarin group carry the same "e" superscript as the +control group and this implies no significant difference statistically at $p < 0.05$ when compared with the +control.

However, comparing Jt 100 and +control under "Liver/Bodyweight" column, Jt 100 carries a "b" superscript while +control carries "e" implying a statistically significant difference between the two groups

Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract; Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract; Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract; Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin; –Control: 5 mg/kg bw of MTX; +Control: 0.5 mL distilled water

and *J. tanjorensis*-treated groups. No significant differences in ALP activity were observed between the positive control group and the group treated with 400 mg/kg bodyweight of the extract. The administration of the tannin-rich extract of *J. tanjorensis* effectively mitigated MTX-induced ALP elevation in a dose-dependent manner.

Effect of Phenol-rich Extract of *J. tanjorensis* Leaf on Serum Bilirubin in MTX-induced Liver Injury in Rats

Figure 5 shows the effect of the phenol-rich extract of *J. tanjorensis* leaves on serum bilirubin concentration in MIH in rats. While the serum bilirubin concentration appeared higher in the negative control group, no statistically significant variations were observed among all the groups.

Bodyweight Changes and Liver/Bodyweight Ratio of the Treatment Groups

Table 2 presents the initial and final body weights of all groups. MTX administration caused a slight reduction in body weight in all MTX-treated groups, while the normal control group, which did not receive MTX, showed an increase in body weight. However, the liver-to-bodyweight ratios were not significantly different among the normal control, positive control, and the group treated with 200 mg/kg bodyweight of *J. tanjorensis* extract (Fig. 6).

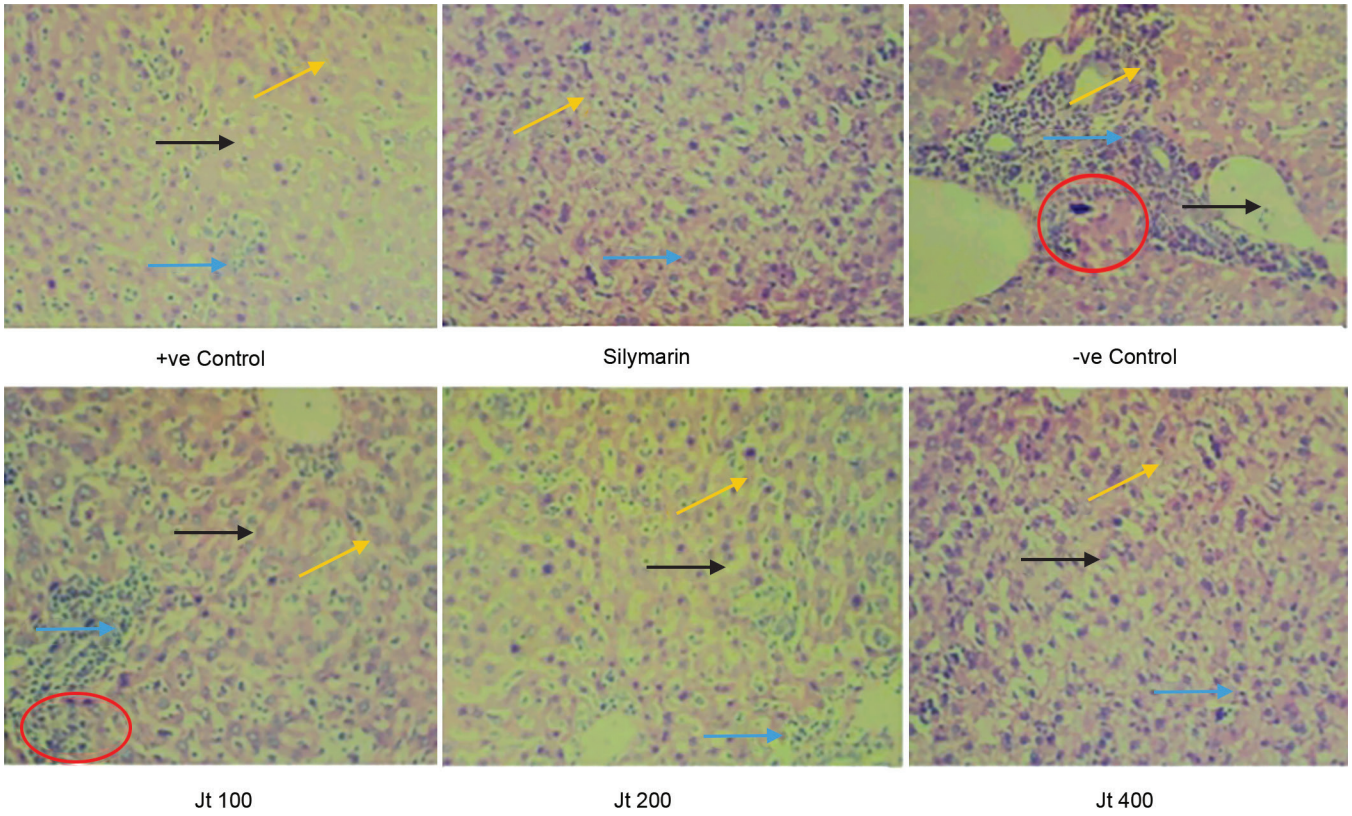

Fig. 6: Paraffin sections stained by hematoxylin and eosin (H&E, ×400) for histopathological examination of liver tissue of rats administered *J. tanjorensis* tannin-rich extract. –Control: 5 mg/kg bw of MTX Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin +Control: 0.5 mL distilled water Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract yellow arrow = portal vein blue arrow = hepatocytes black arrow = hepatocytes sinusoids red circle = necrosis

Table 3: Effect of tannin-rich extracts of *J. tanjorensis* leaf on serum total protein, albumin, and globulin in MTX-induced liver injury in rats

Sample (mg/kg bodyweight)	*Total Protein (g/dL)*	*Albumin (g/dL)*	*Globulin (g/dL)*	*Albumin/Globulin*
Jt 100	5.31 ± 1.02^{b}	2.47 ± 0.25^{b}	2.84 ± 0.64^{b}	0.87 ± 0.05^{bc}
Jt 200	5.56 ± 0.89^{c}	2.64 ± 0.63^{c}	2.92 ± 0.27^{c}	0.90 ± 0.07^{c}
Jt 400	6.44 ± 0.36^{f}	3.39 ± 0.43^{f}	3.05 ± 0.35^{d}	1.11 ± 0.06^{f}
Silymarin	6.54 ± 1.14^{f}	3.33 ± 0.57^{e}	3.21 ± 0.07^{f}	1.04 ± 0.05^{e}
–ve control	4.30 ± 0.54^{a}	1.72 ± 0.23^{a}	2.58 ± 0.26^{a}	0.67 ± 0.02^{a}
+ve control	7.68 ± 0.95^{h}	4.28 ± 0.32^{h}	3.40 ± 0.10^{g}	1.23 ± 0.04^{g}

Values are expressed as the mean ± standard error of mean of five determinations ($n = 5$).

Different superscript alphabets in the same column indicate statistically significant difference at $p < 0.05$.

Note: The statement above suffices as it addresses the statistical significance of each parameter down a column. E.g. for "Total protein" the values that carry a different superscript alphabet imply that they differ significantly, $p < 0.05$

Jt 400 and Silymarin carry the same "f" superscript and this implies the two values have no significant difference statistically at $p < 0.05$. However, when compared with +control which carries "h" as superscript, there exists a statistically significant difference

Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract; Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin; –Control: 5 mg/kg bw of MTX; +Control: 0.5 mL distilled water

Effect of Tannin-rich Extracts of *J. tanjorensis* Leaf on Serum Total Protein, Albumin, and Globulin in MTX-induced Liver Injury in Rats

Table 3 shows the effect of tannin-rich extracts of *J. tanjorensis* leaves on serum total protein, albumin, and globulin concentrations in MIH in rats. Serum total protein and albumin levels were significantly lower in the negative control group compared to the positive control, silymarin-treated, and *J. tanjorensis*-treated groups. Furthermore, no significant difference was observed in total protein parameters between the silymarin group and the group treated with 400 mg/kg bodyweight of the extract.

Effect of Tannin-rich Extracts of *J. tanjorensis* Leaf on Liver Antioxidant Indices in MTX-induced Liver Injury in Rats

Table 4 summarizes the effect of tannin-rich extracts of *J. tanjorensis* leaves on liver tissue CAT, SOD, GSH-Px, and MDA levels in MIH in rats.

Table 4: Effect of tannin-rich extracts of J. tanjorensis leaf on liver antioxidant indices in MTX-induced liver injury in rats

Sample (mg/kg bodyweight)	*CAT (U/mg protein)*	*SOD (U/mL)*	*MDA (μM)*	*GSH-Px (U/mg protein)*
Jt 100	5.96 ± 0.42^{b}	8.84 ± 0.80^{ab}	68.73 ± 2.55^{cd}	42.49 ± 1.54^{b}
Jt 200	8.75 ± 0.27^{c}	10.82 ± 0.87^{bc}	71.12 ± 1.78^{d}	44.00 ± 1.36^{bc}
Jt 400	8.54 ± 0.43^{c}	16.74 ± 1.19^{f}	64.11 ± 2.06^{bc}	43.76 ± 0.89^{bc}
Silymarin	8.62 ± 0.25^{c}	15.99 ± 0.87^{ef}	59.70 ± 2.01^{ab}	46.83 ± 1.06^{c}
−ve control	4.25 ± 0.15^{a}	7.63 ± 0.51^{a}	83.95 ± 1.84^{e}	31.33 ± 1.67^{a}
+ve control	11.68 ± 0.88^{d}	13.75 ± 0.97^{de}	55.16 ± 2.55^{a}	44.91 ± 1.08^{bc}

Values are expressed as the mean ± standard error of mean of five determinations ($n = 5$).

Different superscript alphabets in the same column indicate statistically significant difference at $p < 0.05$.

Note: The statement above suffices as it addresses the statistical significance of each parameter down a column.

Jt 100: 5 mg/kg bw of MTX + 100 mg/kg bw of *J. tanjorensis* tannin-rich extract; Jt 200: 5 mg/kg bw of MTX + 200 mg/kg bw of *J. tanjorensis* tannin-rich extract; Jt 400: 5 mg/kg bw of MTX + 400 mg/kg bw of *J. tanjorensis* tannin-rich extract; Silymarin: 5 mg/kg bw of MTX + 100 mg/kg bw of silymarin; −Control: 5 mg/kg bw of MTX; +Control: 0.5 mL distilled water

Catalase, SOD, and GSH-Px activities were significantly reduced in the negative control group compared to the *J. tanjorensis*-treated, silymarin-treated, and positive control groups. For GSH-Px activity, no significant difference was observed between the positive control, silymarin-treated group, and the group treated with 400 mg/kg bodyweight of the extract.

Methotrexate treatment significantly elevated MDA levels, as seen in the negative control group. However, administration of the tannin-rich extract effectively mitigated this effect, reducing MDA levels. No significant difference ($p > 0.05$) in CAT and MDA levels was observed between the 400 mg/kg bodyweight *J. tanjorensis*-treated group and the silymarin-treated group.

Histological Analysis of the Liver Tissues of Rats Administered with *J. Tanjorensis* Tannin-rich Extract

The histological analysis results are shown in Plate 4.2. Liver tissue examination under a light microscope revealed normal architecture in the positive control, silymarin-treated, and 400 mg/kg *J. tanjorensis*-treated groups. In contrast, the negative control group and the group treated with 100 mg/kg of the extract displayed sinusoidal enlargement, necrotic hepatocytes (highlighted with a red circle), and hepatocellular hemorrhage. Compared to the negative control, the group treated with 200 mg/kg of the extract showed a noticeable reduction in sinusoidal enlargement and hemorrhage, although mildly necrotic hepatocytes were still present. Meanwhile, the positive control, silymarin-treated, and 400 mg/kg *J. tanjorensis*-treated groups exhibited well-preserved liver architecture, including intact cords of hepatocytes, normal portal tracts, and a central vein.

Discussion

The protective effects of *J. tanjorensis* against MTX-induced hepatotoxicity can be attributed to several interrelated mechanisms, most notably its antioxidant, anti-inflammatory, and membrane-stabilizing properties.

In the current study, rats treated with MTX exhibited elevated levels of liver enzymes (AST, ALT, and ALP) and oxidative stress markers MDA, indicative of liver cell damage due to MTX-induced free radical production. *J. tanjorensis* administration significantly reduced these biomarkers, aligning with findings from other studies that report similar antioxidant effects of plant extracts rich in tannin and phenolic compounds.[24–43] Moreover, the recovery in SOD and GSH-Px levels observed in treated rats further supports the hypothesis that the extract mitigates oxidative stress by enhancing the body's endogenous antioxidant defense systems.

Liver membrane stability is crucial in the prevention of hepatocellular damage, particularly when exposed to hepatotoxic agents like MTX. In this study, the hepatoprotective effects of *J. tanjorensis* were also evidenced by histopathological analysis, which showed reduced cellular damage, less necrosis, and diminished inflammation in the liver tissue. This effect may be related to the membrane-stabilizing properties of the compounds identified in the extract, such as tannins and phytosterols, which are known to contribute to cell membrane integrity.[44,45]

Additionally, research by Omoboyowa[46] identified sterols in *J. tanjorensis* leaves that exhibit anti-inflammatory potential. The study employed red blood cell membrane stabilization assays to demonstrate the anti-inflammatory properties of these compounds, suggesting their role in maintaining cell membrane integrity. The regeneration of hepatocytes observed in histopathological sections may be facilitated by the activation of regenerative pathways influenced by these compounds.

The GC–MS analysis of *J. tanjorensis* extract revealed a broad spectrum of bioactive compounds known for their potential therapeutic effects. Among these, compounds such as chlorogenic acid, caffeic acid, ferulic acid, resveratrol, rutin, ursolic acid, rosmarinic acid, and vitexin have been associated with hepatoprotective effects, either directly or indirectly, by reducing oxidative stress, inflammation, and liver cell damage.

Chlorogenic acid (CGA) is a polyphenol known for its strong antioxidant activity and ability to suppress inflammatory responses. Studies have shown that chlorogenic acid can reduce oxidative stress and inflammation in the liver by increasing antioxidant enzyme levels such as SOD and glutathione (GSH).[47] Additionally, a review by Li et al.[48] discusses the mechanisms of CGA's anti-inflammatory and antioxidant activities, noting that CGA has been shown to upregulate antioxidant enzymes and enhance anti-inflammatory cytokines, while concurrently reducing indicators of oxidative stress and levels of pro-inflammatory cytokines. This further supports the role of CGA in protecting liver cells from oxidative damage and inflammation.

Caffeic acid has been shown to protect against liver injury in CCl4-induced hepatotoxicity models by enhancing antioxidant enzymes like SOD and GPx.[49]

A study by Roghani et al.[50] demonstrated that ferulic acid ameliorates liver dysfunction, oxidative stress, and inflammation in MTX-induced hepatotoxicity. The study found that ferulic acid administration led to significant reductions in oxidative stress markers and inflammatory cytokines in the liver. These findings suggest that ferulic acid's hepatoprotective effects are mediated through its antioxidant and anti-inflammatory properties.

Resveratrol and rutin are flavonoids known for their potent antioxidant, anti-inflammatory, and hepatoprotective effects. Rutin reduces oxidative stress and inflammation in CCl4-induced hepatotoxicity models, with significant improvements in liver function and histology.[51] Resveratrol, known for its anti-inflammatory effects, has been shown to reduce TNF-α and IL-6 levels in liver tissues and improve liver function markers in MTX-induced liver damage via inhibition of lipid peroxidation.[52] These compounds likely contribute to the hepatoprotective effects observed in the *J. tanjorensis* extract.

Ursolic acid and rosmarinic acid are known for their potent antioxidant and anti-inflammatory properties. A study by Gan et al.[53] demonstrated that ursolic acid ameliorates carbon tetrachloride (CCl_4)-induced liver fibrosis by reducing the expression of NADPH oxidases (NOXs) and reactive oxygen species (ROS) in hepatic cells. This reduction in oxidative stress contributes to decreased liver enzyme levels (including ALT, AST, and ALP), indicating hepatoprotection.[53] Rosmarinic acid has exhibited protective effects by modulating oxidative stress and improving liver function in MTX-induced hepatotoxicity.[54]

Vitexin has demonstrated hepatoprotective effects by reducing oxidative stress and modulating inflammatory markers in experimental models.[55] Additionally, a study by Noor et al.[56] investigated the hepatoprotective effect of vitexin against hepatotoxicity induced by cadmium in male rats. The findings revealed that vitexin administration improved liver function by enhancing antioxidant enzyme activities and reducing oxidative stress markers.

Conclusion

The bioactive compounds identified in the GC–MS analysis of *J. tanjorensis* tannin-rich extract exert their protective actions by reducing oxidative stress, modulating inflammation, stabilizing hepatocyte membranes, and promoting liver regeneration. The synergistic effect of these compounds in the plant extract likely accounts for its ability to ameliorate MIH in rats.

However, only male Wistar rats were used, which may limit applicability to females. The short study duration also limits insights into long-term efficacy and safety. Moreover, while protective effects were observed, the exact molecular mechanisms were not explored. Further studies are warranted to validate these findings in both sexes, over longer periods, and to elucidate the specific pathways involved.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Abdullahi S Yahaya conceptualized, conducted the laboratory study, data analysis and interpretation, and wrote the original draft. Hadiza L Muhammad conceptualized, designed and supervised the study, and was a major reviewer. Musa B Busari assisted with methodology, performed histological analysis and assisted in interpretation. Halimatu S Isah assisted with animal studies, lab work and study design. Hashim N Muhammad assisted with laboratory research and data analysis. Amina I Umar contributed to lab work, writing and editing. All authors read and approved the final manuscript.

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