OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Comparative Effects of *Phyllanthus niruri* and *Plantago major* in Carbon Tetrachloride Intoxicated Rats

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Abstract | Liver damage is recognized as a severe global health issue. *Phyllanthus niruri* (PN) and *Plantago major* (PM) are herbal plants that are supposed to have hepatoprotective properties. This study aimed to compare the effects of both medicinal plant extracts on rats intoxicated with carbon tetrachloride (CCl_4). A total of 60 male albino rats were equally distributed in six groups. The first group received purified water and was kept as a control. The second and third groups were given oral PN and PM (500 mg/kg/day) for 31 days, respectively. The fourth group was intraperitoneally injected with CCl_4 (2 ml/kg/day) on days 15 and 16 of the experiment. The fifth and sixth groups received oral PN and PM (500 mg/kg/day), respectively, for 31 days and were injected with CCl_4 on days 15 and 16. On days 17 and 32 of the trial, liver specimens were gathered for estimation of malondialdehyde (MDA), antioxidants, apoptotic markers, cytokines, and histopathological changes. Our results revealed a significantly increased MDA, caspase-3, p53, tumor necrosis factor-α, interleukin (IL)-1β, IL-18 and, IL-10, and a significant decline in glutathione and superoxide dismutase in the CCl_4 group. However, intoxicated groups treated with PN and PM showed marked improvement in the measured parameters. Therefore, PN and PM have antioxidant and anti-inflammatory effects, especially PM, which showed better improvement than PN against CCl_4 hepatotoxicity in rats.

Keywords | *Phyllanthus niruri*, *Plantago major*, carbon tetrachloride, hepatotoxicity, histopathology, apoptotic markers

Received | March 24, 2022; **Accepted** | May 08, 2022; **Published** | June 18, 2022
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Citation | Khamis A, Abdalla O, Hashem M, Abdelnaeim N (2022). Comparative effects of *Phyllanthus niruri* and *Plantago major* in carbon tetrachloride intoxicated rats. Adv. Anim. Vet. Sci. 10(7):1444-1450.
DOI | https://dx.doi.org/10.17582/journal.aavs/2022/10.7.1444.1450
ISSN (Online) | 2307-8316

INTRODUCTION

The liver is vital for metabolizing and detoxifying medicines, environmental toxins, microbial metabolites, and certain pharmaceutical preparations, which are the leading causes of hepatic injury worldwide (Upadhyay et al., 2008; Pandit et al., 2012). Carbon tetrachloride (CCl_4) is a xenobiotic that is commonly utilized to initiate hepatic tissue damage in laboratory animals (El-Sayed et al., 2015). In the liver, cytochrome P450 transformed CCl_4 into highly reactive metabolites, resulting in an excess of free radicals and a drop in hepatic antioxidant markers (Kanter et al., 2005). Consequently, reducing the free radicals could combat hepatotoxicity (Zhang et al., 2015). Several studies have proved the antioxidant properties of food-derived phenolic compounds, which protect the liver against hepatotoxicity (Liu et al., 2014). Hence, herbal alternatives have received much consideration as safe treatments for this issue (Ezzat et al., 2020).

Phyllanthus niruri (PN) is a plant related to the Euphorbiaceae family that possesses various medicinal properties, such as antiulcer, antitumor, anticarcinogenic, hypolipidemic, antiviral, and antioxidant activities (Baskaran et al., 2010). Many disorders can be treated with PN, including dyspepsia, diuretics, jaundice, hyperglycemia,

and kidney stones removal (Bagalkotkar et al., 2006). PN is an effective treatment for gallstones and kidney stones (Chughtai, 2016).

*Plantago major (*PM*)* belongs to the Plantaginaceae family (Nazarizadeh et al., 2013). PM contains a variety of bioactive chemicals, including flavonoids, pectin, terpenoids, iridoid, tannins, and glycosides that have antioxidant and anti-inflammatory properties (Zubair et al., 2011; Nazarizadeh et al., 2013). Previous studies discovered that this herb has other therapeutic properties, such as hepatoprotective (Hussan et al., 2015a), immunomodulating, and anticarcinogenic activities (Zubair et al., 2011).

This study aimed to assess the effects of PN and PM on the redox state, apoptotic markers, pro-inflammatory, and anti-inflammatory cytokines, as well as histopathological examination in CCl_4-intoxicated rats.

MATERIALS AND METHODS

Chemicals

CCl_4 was procured from Merck (Darmstadt, Germany). The kits used to assess oxidative stress and antioxidant biomarkers were bought from Biodiagnostic Co. (Giza, Egypt). Caspase-3 and tumor protein P53 were evaluated using the kits from MyBioSource (San Diego, USA). Cytokine kits were acquired from Boster Biological Technology (California, U.S.A).

Preparation of plant extracts

PN was obtained from Madurai (Tamil Nadu, India) and PM from the canal banks of the Nile delta (Egypt). The dried aerial parts of each plant (1500 g) were turned into powder using a mortar and ceramic grinder. Cold extraction was performed on the dried plant matter by soaking it in 70% ethanol for 2 days at ambient temperature with discontinuous shaking. Afterward, it was filtered using Whatman filter paper No. 1 (125 mm). The filtrate was dried in an oven at 40 °C for 2-3 hours daily to obtain a semi-solid mass. The dried extract was then weighed and stored in container at 4 °C (Nofal et al., 2016; Ezzat et al., 2020).

Animals and experimental design

Sixty healthy albino male rats (150–180 g B.Wt) were purchased from the Laboratory Animal House of the Faculty of Veterinary Medicine, Suez Canal University in Egypt. The animals were kept in separate, clean, and disinfected metal cages (10 rats per cage) with a 12-hour light/darkness cycle and a constant temperature of approximately 25 °C. Water and food were regularly given to the animals. The experimental scheme was accepted by the Research Ethical Committee of the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (approval no. 2019015). All possible measures were taken to reduce rat suffering.

All rats were given a 2-week acclimatization period before the start of the study. Six equal groups of rats were formed. The first group received purified water and was kept as a control, while the second group was given PN (500 mg/kg B.Wt) daily by stomach tube for 31 days (Muhammad et al., 2020). The PM was given to the third group at a dosage of 500 mg/kg B.Wt every day by stomach tube for 31 days (Eldesoky et al., 2018). The fourth group (CCl_4) intraperitoneally received 2 ml/kg B.Wt/day of CCl_4 (Venkatesh et al., 2010) dissolved in olive oil (1:1) on days 15 and 16 of the trial. The fifth and sixth groups were given 500 mg/kg B.Wt of PN and PM extracts, respectively, by stomach tube each day, followed by CCl_4 injections on days 15 and 16, and then PN and PM extracts for another 2 weeks.

Tissue sampling

The rats were sedated with isoflurane and then euthanized, and liver tissues were extracted for redox state, apoptotic markers, cytokines, and histological analyses on days 17 and 32 of the trial. One gram of liver sample was collected and homogenized with 5–10 ml of chilled buffer. The homogenate was centrifuged for 30 min at 5000 rpm to eliminate cell debris. The supernatant was stored at -80 °C to assess the malondialdehyde (MDA) levels, reduced glutathione (GSH), and superoxide dismutase (SOD) levels, in addition to apoptotic markers (caspase-3 and tumor protein P53), and cytokines (tumor necrosis factor [TNF]-α, interleukin [IL-]-1β, IL-18, and IL-10). Liver samples from each group were also taken and preserved in 10% formalin for histological evaluation.

Assay of hepatic MDA and antioxidant markers

MDA was assessed in the liver homogenate according to Ohkawa et al. (1979). GSH and SOD levels were determined as described by Beutler et al. (1963) and Nishikimi et al. (1972), respectively.

Apoptotic markers and cytokine assessment

The levels of caspase-3, tumor protein P53 (P53), TNF-α, IL-1, IL18, and IL10 in tissue homogenates were determined using an enzyme-linked immunosorbent assay as reported by Somade et al. (2020).

Histopathology

Liver tissue specimens were fixed for at least 1 day in 10% formalin. All fixed samples were dehydrated before embedding in paraffin. Then thin paraffin sections were prepared and stained with hematoxylin and eosin as reported by Bancroft et al. (1996).

Statistical analysis

The Statistical Package for the Social Science software version 20.0 was used to collect data and perform a one-way analysis of variance. Then, Tukey's multiple comparison test was used to compare the means. *P*-values of ≤0.05 were considered significant. The results were presented as means ± standard errors (Landau and Everitt, 2003).

RESULTS

Hepatic MDA and antioxidant biomarkers

Table 1 shows the effects of different treatments on the hepatic MDA and antioxidant biomarkers (GSH and SOD) on days 17 and 32 of the experiment. The groups treated with PN and PM showed no significant changes in the MDA levels compared with the control group. However, in the CCl_4 intoxicated group, CCl_4 administration resulted in a considerable rise compared to the control. Meanwhile, in the PN-CCl_4 and PM-CCl_4 groups, the pretreatment with PN or PM caused a marked decrease in liver MDA content compared to the CCl_4 group. The hepatic GSH did not display any considerable changes in the PN and PM groups compared with the control group. However, the CCl_4 intoxicated group showed a substantial decrease in GSH compared to the control. The PN-CCl_4 and PM-CCl_4 groups showed numerical elevations compared with the CCl_4 group. The PN and PM groups did not show any significant changes in the SOD levels compared to the control. SOD dramatically declined in the CCl_4 compared to the control. On day 17, the PN-CCl_4 and PM-CCl_4 groups revealed a numerical increase in the SOD level compared to the CCl_4 group. However, on day 32, SOD numerically increased in the PN-CCl_4 group, while it dramatically increased in the PM-CCl_4 group compared with the CCl_4 group.

Tissue apoptotic markers

The effects of different treatments on apoptotic markers on days 17 and 32 of the experiment are shown in Figure 1. The liver apoptotic marker concentrations (caspase-3 and P53) in the PN and PM groups were not significantly different from those in the control group. Conversely, the CCl_4 intoxication significantly raised the apoptotic markers in the CCl_4 group compared with the control. On day 17 of the experiment, the PN-CCl_4 and PM-CCl_4 groups exhibited a marked decrease in caspase-3 and P53 compared to the CCl_4 group. However, on day 32 of the experiment, caspase-3 numerically decreased in the PN-CCl_4 group but dramatically decreased in the PM-CCl_4 group compared to the CCl_4 group. P53 significantly decreased in both the PN-CCl_4 and PM-CCl_4 groups compared to the CCl_4 group.

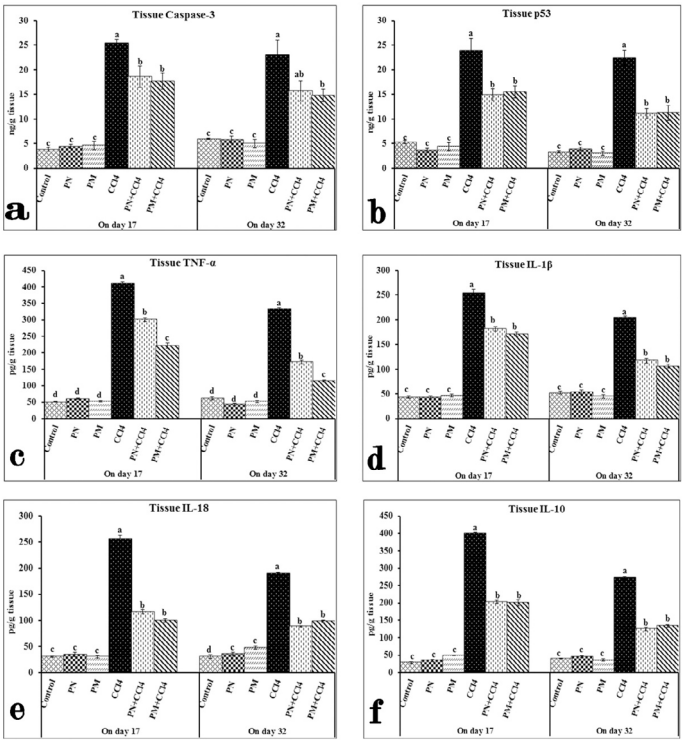

Figure 1: Protective effects of Phyllanthus niruri (PN) and Plantago major (PM) on CCl_4 intoxicated rats. (A) Tissue Caspase-3; (B) Tissue P53; (C) TNF-α; (D) IL-1β; (E) IL-18; (F) IL-10. Data show the mean ± SEM (n= 10). Columns with different superscripts refer to significant differences (P ≤ 0.05) between the groups.

Table 1: Hepatic malondialdehyde and antioxidant biomarkers in different animal groups at days 17 and 32 post-treatment.

Groups	Parameters					
	On day 17 post treatments			On day 32 post treatments		
	MDA (nmol /g tissue)	GSH (mmol /g tissue)	SOD (U /g tissue)	MDA (nmol /g tissue)	GSH (mmol /g tissue)	SOD (U /g tissue)
Control	5.47 ± 0.15^{c}	44.67 ± 1.45^{a}	67.33 ± 1.20^{ab}	5.27 ± 0.38^{c}	49.67 ± 0.88^{a}	69.67 ± 1.45^{a}
PN	6.10 ± 0.96^{c}	48.0 ± 1.73^{a}	77.33 ± 2.03^{a}	4.73 ± 0.74^{c}	48.33 ± 0.88^{a}	76.33 ± 2.03^{a}
PM	6.27 ± 0.95^{c}	48.33 ± 3.76^{a}	62.33 ± 2.60^{b}	5.43 ± 0.39^{c}	50.33 ± 1.45^{a}	73.33 ± 3.84^{a}
CCl_4	42.33 ± 3.76^{a}	11.70 ± 1.68^{b}	28.33 ± 0.88^{c}	25.33 ± 2.03^{a}	19.33 ± 0.88^{b}	34.0 ± 3.21^{c}
PN+ CCl_4	21.27 ± 0.93^{b}	21.27 ± 3.98^{b}	36.67 ± 2.60^{c}	14.17 ± 1.60^{b}	26.0 ± 2.52^{b}	39.33 ± 0.88^{bc}
PM+ CCl_4	19.63 ± 2.30^{b}	21.0 ± 2.21^{b}	35.67 ± 3.38^{c}	11.37 ± 0.78^{b}	25.67 ± 2.40^{b}	47.33 ± 0.33^{b}

Data are expressed as means ± SEM (n = 10). Mean values within the same column having different superscript letters are significant at *P* ≤ 0.05. PN: *Phyllanthus niruri*; PM: *Plantago major;* CCl_4: carbon tetrachloride.

Cytokines

Figure 1 reveals the influence of CCl_4 and different treatments on the pro-inflammatory cytokines (TNF-α, IL-1β, and IL18) and the anti-inflammatory cytokine (IL10) in the hepatic tissues of different animal groups on days 17 and 32 of the experiment. All the measured cytokines showed no significant variations in the PN and PM groups compared to the control group. However, intoxication with CCl_4 caused a considerable rise in the cytokines, compared to the control group. All cytokine levels in the PN-CCl_4 and PM-CCl_4 groups revealed a considerable decrease compared to the CCl_4 group.

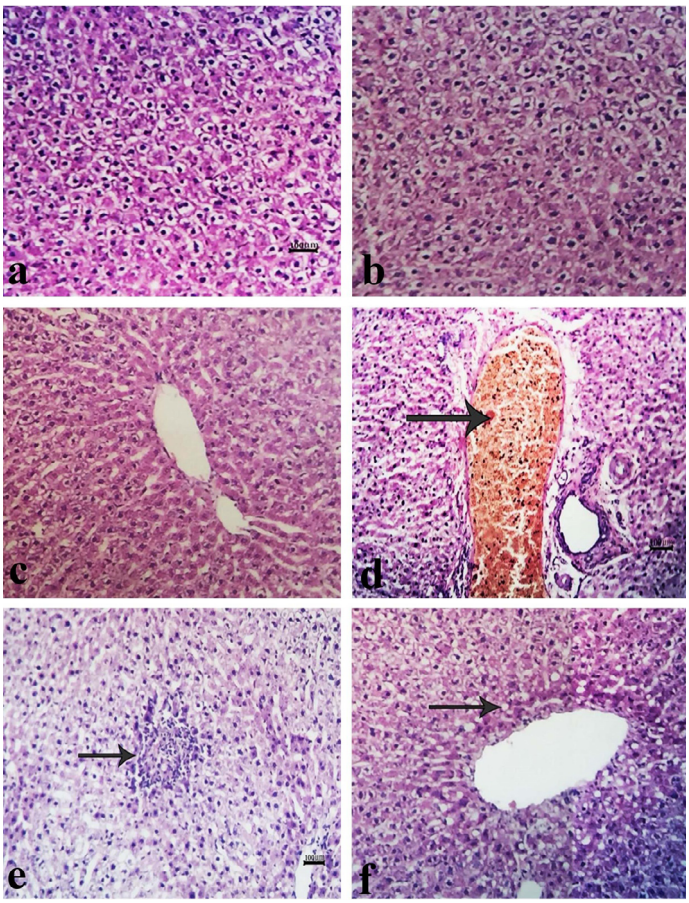

Figure 2: Liver sections of rats on day 17 of the experiment showing (a, b, c) normal hepatic tissue. Moderate blood vessel congestion (arrow), perivascular edema, bile duct hyperplasia, and degenerated hepatocytes (d). A mild focal area of fibroblastic proliferation (arrow) along with mild to moderate vacuolar degeneration of hepatocytes (e). Mild vacuolation of hepatocytes (arrow) (f). (a) control group; (b) *Phyllanthus niruri* (PN)group; (c) *Plantago major* (PM) group; (d) CCl_4 group; (e) PN-CCl_4 group; (f) PM-CCl_4 group.

Histopathology

The histopathological changes in separate groups on day 17 of the experiment are presented in Figure 2a-f. The livers of the control group revealed normal tissue architecture and cellular features. The livers of most rats that are given ethanolic PN extract exhibited normal hepatic parenchyma along with mild hepatic blood vessel congestion in a few cases. Hepatic tissue appeared to be normal in the PM group, with slight sinusoidal dilatation in some cases. Liver sections from the CCl_4 group, revealed diffuse hepatic cell vacuolation, a periductal proliferation of fibroblast cells, and von kupffer cells with mild perivascular edema and hepatic blood vessel congestion. Mild to moderate hyperplasia of bile ducts and focal necrosis of some hepatic cells were also observed. Liver sections in the PN-CCL_4 group exhibited a focal area of fibroblast and von kupffer cell proliferation with only a few mononuclear cell infiltrations and mild to moderate vacuolar degeneration of hepatocytes. Diffuse vacuolar degeneration was seen in the liver sections of the PM-CCL_4 group.

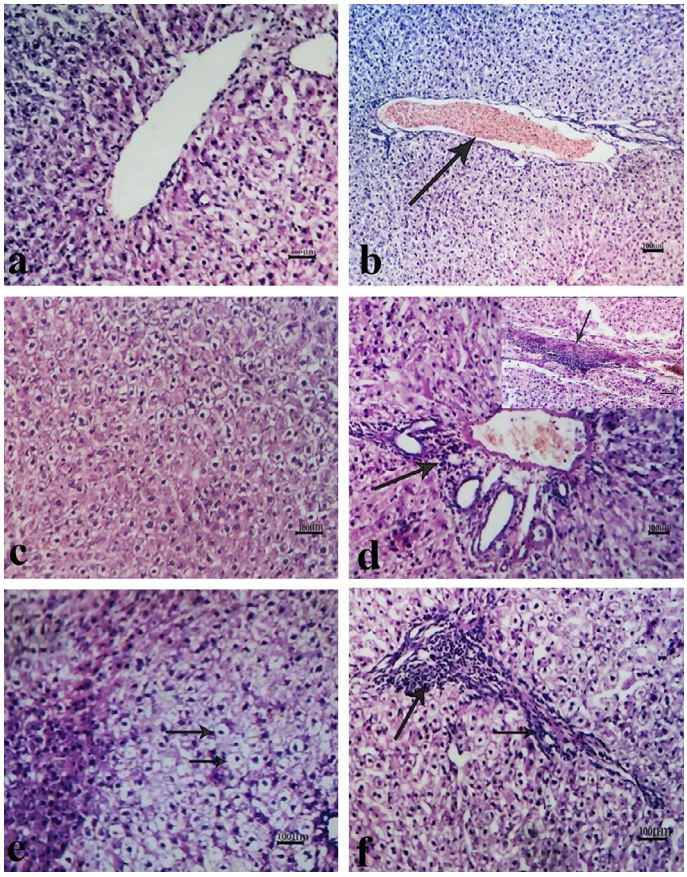

Figure 3: Liver sections of rats on day 32 of the experiment showing (a, b, c) fairly normal hepatic tissue in addition to mild congestion (b). Moderate congestion, bile duct hyperplasia (arrow), degenerated hepatocytes, and severe focal fibrosis (arrow in the window) (d). Mild vacuolation of hepatocytes (arrow) (e). A focal area of fibroblastic proliferation along with moderate hepatocytes vacuolation (arrow) (f). (a) control group; (b) *Phyllanthus niruri* (PN) group; (c) *Plantago major* (PM) group; (d) CCl_4 group; (e) PN-CCl_4 group; (f) PM-CCl_4 group.

Figure 3a-f illustrates the histopathological alterations in different groups on day 31 of the experiment. The liver of the normal group showed hepatic parenchyma with

regular tissue construction and cellular features. On a few occasions, liver sections from the PN group exhibited minor hepatic blood vessel congestion. The hepatic tissue architecture and cellular features in the liver of rats that were given PM ethanolic extract appeared to be normal. Contrastingly, the hepatic tissue from the CCl_4 group demonstrated perivascular edema, a focal area of coagulative necrosis, and hepatic blood vessel congestion. A considerably localized aggregation of proliferated fibroblasts and von kupffer cells were seen in some liver sections. However, the liver from the PN-CCL_4 group showed a focal region of hepatic vacuolation, which was characterized by hydropic degeneration. A mild focal area of proliferating fibroblasts was seen in a few cases in the PM-CCL_4 group, along with mildly dilated sinusoids and moderate vacuolation of hepatocytes.

DISCUSSION

The pathogenesis of liver disorders is thought to be influenced by oxidative stress (Siegel et al., 2014). Cytochrome P450 biotransforms CCl_4 into free radicals in the liver, causing membrane lipid breakdown and lipid peroxidation. Eventually, cell membrane integrity is lost, resulting in liver injury (Lee and Jeong, 2002). Our findings revealed that the CCl_4 administration induced a considerable rise in the hepatic MDA content and a marked drop in antioxidant biomarker concentrations (GSH and SOD). These findings matched those of Alayunt et al. (2019), who stated that rats intoxicated with CCl_4 had higher serum MDA levels and lower GSH concentrations. The PN-CCl_4 group exhibited a significant MDA level reduction and a numerical GSH and SOD elevation. Our findings supported previous studies, which found a decline in MDA and a rise in GSH and SOD after using PN (Manjrekar et al., 2008; Muhammad et al., 2020). This could be attributed to the efficacy of PN in liver disease treatment by inhibiting reactive oxygen species and lipid peroxidation (Gressner et al., 2007). Additionally, in the PM-CCl_4 group, MDA was significantly lower, whereas GSH was numerically higher and SOD was significantly higher. These investigations came in line with Hussan et al. (2015a), who reported an improved MDA and an increased in GSH and SOD after PM administration, which possesses antioxidant action due to the incidence of high phenolic compounds, flavonoids, alkaloids, terpenoids, and vitamin C levels.

Apoptosis is a basic cellular mechanism that helps prevent tumorigenesis by removing damaged cells in different physiological and pathological conditions (Somade et al., 2020). Our study revealed that, caspase 3 levels significantly increased in CCl_4- intoxicated rats, which may be due to oxidative stress and inflammatory induction (Hariri et al., 2010). Additionally, rats intoxicated with CCl_4 revealed a marked increase in the P53 level, which is accumulated in hepatocytes in liver diseases as a result of hepatocyte apoptosis (Liu et al., 2016). Our findings were coming in harmony with Guo et al. (2013), who reported that CCl_4 upregulates the p53 expression. However, the PN-CCl_4 and PM-CCl_4 groups exhibited a considerably decreased caspase-3 and p53 compared to the CCl_4 group. This decreased apoptotic markers indicated an improved hepatic injury.

Excessive inflammation is also an essential feature related to CCl_4-induced liver damage (Yu et al., 2014). The free radicals resulting from CCl_4 intoxication are also thought to generate the inflammatory response in the liver by activating macrophages, which then generate TNF and further pro-inflammatory cytokines (Li et al., 2021b). These cytokines, particularly TNF- and IL-1β, have a critical function in liver damage, by maintaining hepatic inflammation (Shin et al., 2013). Thereby, enhancing the antioxidant system as a key factor in preventing oxidative stress in the liver (Li et al., 2021a). Our study revealed considerably increased pro-inflammatory cytokines (TNF-α, IL-1β, and IL18) and anti-inflammatory cytokine (IL10) in the hepatic tissues of CCl_4-intoxicated rats. Our results agreed with Zhao et al. (2021), who found an increased TNF-α, IL-1β, and IL-18 blood levels in mice after CCl_4 intoxication. Additionally, our results were consistent with Ezzat et al. (2020), who revealed that CCl_4 intoxication significantly increased IL10. However, PN pretreatment significantly improved the measured cytokines in the CCl_4 intoxicated rats. This could be due to PN's anti-inflammatory qualities, as indicated by cytokine inhibition as a probable method of hepatoprotection, besides its antioxidant capabilities (Ezzat et al., 2020). Similarly, the PM-CCl_4 group exhibited significant cytokine amelioration due to the anti-inflammatory property of the PM (Hussan et al., 2015b). The cytokine results in different groups were confirmed by the histopathological examination. The hepatic tissues of the CCl_4 intoxicated rats revealed inflammation signs, such as localized proliferated fibroblast and von kupffer cell aggregation. The persistent inflammatory response has the potential to enhance tissue damage (Hussan et al., 2015b). However, the PN-CCl_4 and PM-CCl_4 groups showed considerable amelioration in the hepatic tissue, which could be attributed to inflammatory reaction inhibition.

In conclusion, CCl_4 produced severe toxicity in rats. PN and PM supplementation curtailed the toxic effects of CCl_4 as they exhibited markedly improved in antioxidant status, apoptotic markers, and inflammatory cytokines. Histopathological examination established the ameliorative potentials of both plant extracts as reflected in liver histoarchitecture restoration that was initially distorted by the toxicant. PM was more beneficial than PN. However,

both plants have potentials that can be exploited in liver disease management.

NOVELTY STATEMENT

This study was the first to demonstrate the ameliorative effect of *Phyllanthus niruri* and *Plantago major* on the levels of the apoptotic markers (caspase-3 and tumor protein P53) against carbon tetrachloride toxicity. This indicates that these herbal plants have an anti-apoptotic effect.

AUTHOR'S CONTRIBUTION

AK: Sample collection and lab analysis. NA: Write the manuscript. OA & MH: Idea, design and revision.

Conflict of interest

The authors have declared no conflict of interest.

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