Clinicobiochemical Effects of *Phyllanthus niruri* and *Plantago major* on CCl₄ Intoxicated Rat Model

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Abstract

Carbon tetrachloride (CCl₄) is a well-known hepatotoxin that is widely used to induce acute toxic liver injury. *Phyllanthus niruri* (PN) and *Plantago major* (PM) have hepatoprotective properties. The purpose of our study was to evaluate the protective effects of PN and PM on CCl₄-intoxicated rats. Sixty male Albino rats were randomly assigned into six equal groups. The first group was kept as a control and received purified water. The second and third groups were administered PN and PM (500 mg/kg/day) orally for 31 days, respectively. The fourth group was injected intraperitoneally with CCl₄ (2 ml/kg/day) on days 15 and 16 of the experiment. The fifth and sixth groups were given PN and PM, and were injected with CCl₄. On days 17 and 32 of the experiment, five rats were assembled randomly from each group, and blood samples were collected for analyzing serum biochemical parameters including liver enzymes, total bilirubin, direct and indirect bilirubin, proteinogram, and lipid profile. Our results revealed a significant increase in ALT, AST, ALP, GGT, total bilirubin, direct and indirect bilirubin, total cholesterol, total triglyceride, LDL-c and VLDL and a significant decline in total protein, albumin, and HDL in the CCl₄ group. However, PN+CCl₄ group significantly improved liver enzymes, bilirubin, albumin, total cholesterol, total triglyceride, LDL-c and VLDL. While the PM+CCl₄ group revealed improvement in AST, ALP, total cholesterol, total triglyceride, LDL-c and VLDL. Finally, it could be concluded that the PN and PM have beneficial effects against CCl₄-induced hepatotoxicity in rats.

Keywords: carbon tetrachloride, *Phyllanthus niruri, Plantago major*, hepatotoxicity, rats.
Introduction
The liver has a lot of functions, including carbohydrate, protein, and fat metabolism, detoxification, secretion, and storage. Therefore, maintaining a healthy liver is critical for animal health and longevity (Pandit et al., 2012). However, its function is usually affected by xenobiotics. Excessive or chronic exposure to xenobiotics leads to cirrhosis or malignant diseases (Okoli et al., 2011). Carbon tetrachloride (CCl₄) is a powerful hepatotoxin, and is widely used to induce hepatic injury in animal models (Dong et al., 2013). The CCl₄ toxicity causes huge production of free radicals and inflammation, which affects the structural and functional membrane of hepatocytes (Sun et al., 2018). Synthetic medications used to treat liver problems are ineffective and can have major negative effects in some cases. Therefore, there is a growing interest to evaluate the traditional herbal plants that claim to have hepatoprotective properties (Baranisrinivasan et al., 2009).

*Phyllanthus niruri* (PN) is related to the family of Euphorbiaceae and is used traditionally for its medicinal properties (Barros et al., 2006). Therapeutically, PN has been used to cure gastrointestinal and genitourinary tract disorders (Karuna et al., 2009). Previous studies found that the PN is hypolipidemic (Khanna et al., 2002b), hepatoprotective (Amin et al., 2013), anticarcinogenic (Rajeshkumar et al., 2002), anti-inflammatory (Obidike et al., 2010), antioxidant and anti-diabetic (Okoli et al., 2011).

*Plantago major* (PM) is from the Plantaginaceae family (Nazarizadeh et al., 2013). The PM has various therapeutic properties such as antiulcerogenic, anti-inflammatory, and immunomodulating and antioxidant properties, as well as anticarcinogenic activities (Zubair et al., 2011).

The aim of our research was to evaluate the effects of *Phyllanthus niruri* and *Plantago major* regarding the serum biochemical parameters including liver enzymes, bilirubin levels, proteinogram and lipid profile against CCl₄ intoxication in rats.

Materials and methods

Chemicals
Carbon tetrachloride was obtained from Merck Company (Germany). The kits used to assess the biochemical parameters were bought from Roche Diagnostics GmbH, (Germany). All the parameters were analyzed using automated Cobas C 311 analyzer (Tokyo, Japan).

Plant extract
PN was gathered from Madurai (Tamil Nadu, India), and PM from the canal banks of Nile delta (Egypt). Using a mortar ceramic grinder, the dried aerial parts of each plant (1500 g) were slightly powdered. These dried plants material was exposed to cold extraction by soaking it in 70% ethanol for two days at room temperature with irregular shaking. Then, it was subjected to filtration.
using Whatmann® filter paper No. 1 (125 mm). After that, the filtrate was evaporated in an oven at 40 °C for 2-3 hours every day to get a semi-solid material. Then, the dried extract was weighed, placed in a container, and stored at 4 °C (Nofal et al., 2016; Ezzat et al., 2020).

Animals and experimental design
Sixty apparently healthy Wister male rats (150–180 g B.Wt) were obtained from the Laboratory Animal House of the Faculty of Veterinary Medicine, Suez Canal University in Egypt. The animals were housed in isolated hygienic metal cages and divided equally into six groups (10 rats per cage) with a 12-hour light/darkness cycle and a constant temperature of 25 °C. The animals were supplemented regularly with water and food. The experimental design was approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (approval no. 2019015). To alleviate animal suffering, all possible measures were taken.

The rats were accustomed for two weeks before the beginning of the study to ensure normal growth, adaptation, and behavior. Rats were kept in their receptive groups for 32 days, observed closely every day and weighted every week. The first group was given purified water and kept as a control, while the second group received orally PN (500 mg/kg B.Wt) daily by stomach tube for 31 days (Muhammad et al., 2020). The third group administered PM orally at a dose of 500 mg/kg B.Wt every day by stomach tube for 31 days (Eldesoky et al., 2018). On days 15 and 16 of the experiment, the fourth group was injected intraperitoneally with CCl₄ at a dosage of 2 ml/kg B.Wt/day (Venkatesh et al., 2010a) dissolved in olive oil (1:1). The fifth and sixth groups received 500 mg/kg B.Wt of PN and PM extracts by stomach tube every day, followed by CCl₄ injections on days 15 and 16, then PN and PM extracts for another two weeks.

Blood sampling
On days 17 and 32 of the experiment, five rats were randomly gathered from each group. The rats were sedated with isoflurane and blood samples were drawn from the heart. The blood samples were placed in plain centrifuge tubes, left in a slope position at room temperature to clot and centrifuged at 3000 rpm for 10 min for separation of serum. The clear serum was carefully collected and kept frozen at -20°C until used for serum biochemical analysis.

Liver enzymes and bilirubin
The activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured according to Bergmeyer et al. (1986) and ECCLS. (1989) respectively. Alkaline phosphatase (ALP) activity was assessed according to Tietz et al. (1983). Gamma glutamine transferase (GGT) activity was estimated as
described by Szasz (1974). Total and direct bilirubin levels were measured as mentioned by Wahlefeld (1972) and Malloy and Evelyn (1937) respectively.

### Serum proteinogram
Total proteins level was evaluated according to Weichselbaum (1946). Albumin level was assessed according to Doumas et al. (1971). Globulins was calculated as mentioned by Watson (1966).

### Lipid profile
Total cholesterol was measured according to Allain et al. (1974). Triglyceride was determined according to Siedel et al. (1993). HDL-c was assessed as described by Matsuzaki et al. (1996). VLDL-c and LDL were calculated according to Wilson et al. (1985) and Friedewald et al. (1972) respectively.

### Statistical analysis
The SPSS software package version 20.0 was used to collect data and perform a one-way analysis of variance (ANOVA). Then, Tukey’s multiple comparison test was used to compare the means, when a P ≤0.05 was considered as a significant level. The results were presented as means ± standard errors (Landau and Everitt, 2003).

### Results
Figure 1 shows the effects of CCl₄ and different treatments on the hepatic enzyme activities. The PN and PM groups showed no significant changes in the activities of liver enzymes (ALT, AST, ALP, and GGT) compared to the control. However, these parameters were considerably higher in CCl₄-intoxicated rats than control on days 17 and 32 of the experiment. The PN+CCl₄ group exhibited a significant reduction in all the parameters on days 17 and 32 compared to the CCl₄ group, except GGT, which only showed a significant reduction on day 32 of the trial. The PM+CCl₄ group demonstrated a decrease in liver enzymes on day 17 of the experiment, with the exception of GGT level, which did not alter when compared to the CCl₄ group. Conversely, on day 32 of the experiment, this group showed a significant decrease in AST and ALP and did not reveal significant differences in ALT and GGT compared to the CCl₄ group.

Figure 2 reveals the influence of CCl₄ and different treatments on the levels of bilirubin and the proteinogram. The PN and PM groups showed a non-significant change in the levels of bilirubin, total protein, albumin, and globulin when compared to the control group. However, the CCl₄ group revealed a substantial rise in the bilirubin levels and a reduction in the total protein compared to the control on days 17 and 32 of the experiment. The albumin level decreased dramatically on day 17 of the experiment and did not show a significant change on day 32 of the experiment compared to the control. The globulin level did not reveal any
significant change on days 17 and 32 of the experiment. The PN+CCl₄ exhibited a significant reduction in the total, direct, and indirect bilirubin and did not change significantly in the proteinogram. The PM+CCl₄ groups showed a significant decrease in the total bilirubin level and did not show significant variations in the other parameters compared to the CCl₄ group.

The effects of CCl₄ and different treatments on the lipid profile are shown in figure 3. The PN and PM groups did not show any significant variations compared to the control group. On the other hand, the CCl₄ group revealed significant elevations in the serum triglycerides, total cholesterol, LDL-c, and VLDL, while, it showed a marked reduction in HDL-c compared to the control group. On day 17 of the experiment, the PN+ CCl₄ and PM+ CCl₄ groups showed non-significant changes in the lipid profile compared to the CCl₄ group. However, on day 32 of the experiment, the PN+ CCl₄ and PM+ CCl₄ groups exhibited significant reductions in the serum triglycerides, total cholesterol, LDL-c, and VLDL. But there were no significant differences in HDL-c compared to the CCl₄ group.

![Figure 1](image)

**Figure 1.** Protective effects of *Phyllanthus niruri* (PN) and *Plantago major* (PM) on CCl₄-intoxicated rats. (A) ALT; (B) AST; (C) ALP; (D) GGT. Data shows the mean ± SEM (n=10). Columns with different superscripts refer to significant differences (P ≤ 0.05) between the groups.
Figure 2. Protective effects of *Phyllanthus niruri* (PN) and *Plantago major* (PM) on CCl₄-intoxicated rats. (A) Total bilirubin; (B) Direct bilirubin; (C) Indirect bilirubin; (D) Total proteins; (E) Albumin; (F) Globulins. Data shows the mean ± SEM (n=10). Columns with different superscripts refer to significant differences (P ≤ 0.05) between the groups.
Figure 3. Protective effects of *Phyllanthus niruri* (PN) and *Plantago major* (PM) on *CCl*$_4$-intoxicated rats. (A) Total cholesterol; (B) Triglycerides; (C) HDL-c; (D) LDL-c; (E) VLDL. Data shows the mean ± SEM (n=10). Columns with different superscripts refer to significant differences (P ≤ 0.05) between the groups.

**Discussion**

Inside the body, *CCl*$_4$ is transformed by cytochrome P450 into free radicals (*CCl*$_3$). These free radicals combine with oxygen to form trichloromethylperoxy radicals (*CCl*$_3$OO) and reactive oxygen species, which induce lipid peroxidation and damage polyunsaturated fatty acids, especially those linked to phospholipids (*Jain et al., 2008; Li et al., 2011*). As a result, cell integrity is compromised, and ALT and AST are released into the bloodstream, causing apoptosis and necrosis (*Yang et al., 2018*). The extended increases in ALT in the circulation until day 32 could be due to increased ALT production in regenerating liver tissue. Other factors that can raise ALT activity include cholestatic lesions that indicate a problem with bile flow (*York, 2017*). The serum ALP, GGT, and total bilirubin levels were dramatically elevated due to biliary lesions or intrahepatic cholestasis, which happens in the bile canaliculi and bile ductules. As a result of these lesions, certain membrane-bound hepatic enzymes, such as ALP and GGT, may release (*Latimer et al., 2003*). Our results are in agreement with *Ullah et al. (2020)*, who reported a significant increase in ALT, AST, ALP, and total bilirubin in *CCl*$_4$ intoxicated mice. Furthermore, our results came in line with *Venkatesh et al. (2010b)*, who revealed an increase in the activity of ALP and total bilirubin level after
administering CCl₄ (2ml/kg) in rats. However, the PN+CCl₄ group exhibited a considerable improvement in all these parameters compared to the CCl₄ group. These findings came in accordance with Ezzat et al. (2020), who reported a decrease in ALT, AST, ALP, and bilirubin levels due to using PN. The PM+CCl₄ group showed significant decrease in AST and ALP enzymes and numerical reduction in ALT, GGT and bilirubin levels compared to the CCl₄ group. Our findings are in consistent with Eldesoky et al. (2018), who revealed a decrease in ALT, AST, ALP, GGT and bilirubin levels due to using PM in rats. These findings could be attributed to the ability of the PM to preserve the hepatocyte membrane integrity in CCl₄ exposed rats by lowering the production of reactive CCl₄ metabolites (Eldeoky et al., 2018).

On day 17 of the investigation, the proteinogram revealed that CCl₄ intoxicated rats had significantly lower protein and albumin levels than the control rats. The severe hepatic lesions caused by CCl₄ intoxication may interfere with protein synthesis, resulting in hypoalbuminemia. Furthermore, lower serum albumin levels have been linked to active cirrhosis and biliary liver injury (Shukla and Bhatia, 2010). Our findings correspond with those of Eltahir et al. (2020), who found a substantial decrease in albumin levels in rats after CCl₄ injection (1 ml/kg) twice weekly for 6 weeks. In the PN+CCl₄ group, the serum total proteins and globulins levels numerically improved compared to the CCl₄ group. Our results came in agreement with Amin et al. (2012) who revealed increase in total proteins due to using PN and this may be attributed to the ability of PN to improve the ability of PN to improve the synthetic function of the liver and stabilizing the hepatocytes membranes. Similarly, in the PN+CCl₄ group, the serum total proteins and globulins levels numerically increased compared to the CCl₄ group. Eldeoky et al. (2018) reported an increase in total proteins due to using PM and this indicated the stabilization of endoplasmic reticulum that is responsible for protein synthesis.

The CCl₄ group revealed a significant increase in total cholesterol (TC), triglycerides (TG), LDL-c and VLDL, as well as a significant decline in HDL-c compared to the control group. Lipid profile changes are a causal factor for excessive lipid peroxidation (Makni et al., 2008) and oxidative stress (Tsimikas and Miller, 2011) that resulted from increase in ROS production and reduction in antioxidant enzymes. Our finding matched those of Althnaian et al. (2013) who found that intraperitoneal injection of CCl₄ (1 ml/kg) increased serum total cholesterol and triglyceride levels in rats. However, the co-treatment of PN+CCl₄ and PM+CCl₄ exhibited a considerable reduction in TC, TG, LDL-c, and VLDL compared to the
CCl4 group. Our results are in harmony with Ezzat et al. (2020), who concluded that using PN resulted in normalization of TC and TG, which is attributed to the antihyperlipidemic effect of the plant (Khanna et al., 2002a). Also, our findings agreed with Nofal et al. (2016), who found a decrease in TC and TG owing to using PM.

**Conclusion**

Supplementing with PN and PM improved liver enzymes and bilirubin levels, but PN was more beneficial than PM. Regarding to the proteinogram, they had a similar effect. The lipid profile was improved as a result of their efforts, but PM was more effective. Subsequently, both plants could aid in the treatment of liver illness.

**References**


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الملخص العربي
دراسات باثولوجية إكلينيكية مقارنة على المواد الواقية للكبد في الجرذان

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اُجريت هذه الدراسة على عدد ستين من ذكور الجرذان البيضاء لمقارنة التأثير الوقائي لنبات الأملج البولى ونبات لسان الحمل في الكبد. تم تقسيم الجرذان إلى ست مجموعات من النحو التالي: المجموعة الضابطة (المجموعة التثيقية) المجموعة الثانية التي أعطت الأملج البولى عن طريق الفم بجرعة 500 ملجم / كجم من وزن الجسم يوميا لمدة 30 يوما. المجموعة الثالثة التي تناولت لسان الحمل عن طريق الفم بجرعة 500 ملجم / كجم من وزن الجسم يوميا لمدة 30 يوما. المجموعة الرابعة (خليط) تحتت برابع كلوريد الكربون بجرعة 2 مل / كجم من وزن الجسم في اليومين 15 و 16 من التجربة. أما المجموعات الخامسة والسادسة فتم علاجهما بجرعات الأملج البولى ولسان الحمل متساويتين لمدة 30 يوما و 15 و 16 من التجربة تم حقن رابع كلوريد الكربون. تم تقييم القياسات في اليومين 17 و 32 من التجربة. تم تجميع عينات مصل الدم لتحليل البوسامي. وقد كشفت النتائج زيادة كبيرة في نشاط إنزيمات الكبد (الألانين الامينوترانسفيراز، الإسبرتات امينوترانسفيراز، الفوسفاتاز القلوي، والجالومين ترانسفيراز) ومستوى الصفراء الكلية ومستوى الدهون ومستوى الدهون.lower انخفاض كبير في إجمالي البروتينات والالبومين في مجموعة رابع كلوريد الكربون. بينما أظهرت النتائج برابع كلوريد الكربون. باستخدام الاملج البولى ولسان الحمل تحسن النتائج في هذه الفئات. ويمكن الاستنتاج أن الاملج البولى ولسان الحمل لهما تأثيرات واقية للكبد ولكن الاملج البولى أظهر تحسن أفضل من لسان الحمل ضد السمية الكبدية برابع كلوريد الكربون في الجرذان.

الكلمات الدالة: الأملج البولى- لسان الحمل- برابع كلوريد الكربون - السمية الكبدية - الجرذان.