ALLEVIATION HARMFUL EFFECTS OF NON-ALCOHOLIC FATTY LIVER DISEASE WITH Phyllanthus niruri EXTRACT IN ALBINO RATS

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Abstract: Non-alcoholic fatty liver disease (NAFLD) is a serious global health problem that is strongly linked to insulin resistance, obesity, and oxidative stress. The goal of current work was to inspect the hepatoprotective and hypolipidemic effects of Phyllanthus niruri ethanolic extract (PNEE) on high fat hyperlipidemic diet –induced rat’s fatty liver. A unique high-fat diet (HFD) composed of coconut oil (7.3%), beef tallow (7.3%), safflower oil (0.4%) and cholesterol (1.5%) were used in the present study for induction of NAFLD or hyperlipidemia in male Albino rats. Forty-eight clinically healthy male Albino rats were equally divided into four groups: the first served as control. The second group, rats administered ethanol extract of Phyllanthus niruri (PN) (250 mg/kg b.wt.); while the third group was rats fed diet supplemented high fat concentrated (HFCD); and the fourth was rats received combination of HFCD and PN (HFCD+PN). All treatments were given daily by oral route for 105 days. All treatments were given daily by oral route for 105 days. Treatment of hyperlipidemic rats by PNEE resulted in reduction of serum liver enzymes, and improvement in proteins and lipids profile, as well as pancreatic function tests, in comparison with hyperlipidemic rats (gp.3) which showed severe biochemical alterations. This was confirmed by histopathological findings in the liver. It could be concluded that PNEE treatment has a strong hepatoprotective impact against fatty alterations in animal models, suggesting that PNEE could be used as a promising agent to prevent fatty liver disease.

Key words: Nonalcoholic fatty liver; liver enzymes; insulin; glucose; HOMA-β

Introduction

Non-alcoholic fatty liver disease (NAFLD) is currently the world’s common liver disease. It is demarcated by a liver triglyceride concentration of more than 5% and a histological hallmark of simple steatosis, with no signs of alcohol abuse (1). NAFLD is a common disorder characterized by increased liver fat (hepatic steatosis), which can lead to inflammation in the form of non-alcoholic steatohepatitis (NASH) and fibrosis (2). Improvement in NAFLD was frequently linked to weight loss and the reduction of insulin resistance (3). The Middle East (32%) and South America (30%) have the highest prevalence rates, while Africa (13%) has the lowest. However, certain subpopulations, such as the highly obese (90 percent) and type 2 diabetic patients (76 percent), have significantly higher prevalence rates (4). Fatty liver disease (FLD), also known as fatty liver or hepatic steatosis, is a disorder in which hepatocytes accumulate excessive amounts of lipids. When fat metabolism is disrupted, the fat can accumulate in the liver in excessive amounts, resulting in a fatty liver (5). Depending on share of alcohol in the etiology of FLD, it is characterized as alcoholic steatosis (alcoholic FLD) or NAFLD (6). Hyperlipidemia causes health hazards in many countries around the world as obesity, myocardial localized necrosis, atherosclerosis,
sort 2 diabetes, degenerative joint infection, and circulatory malady, and 10%-30% of adults suffer from NAFLD due to obesity on overall average (7). The most prevalent cause of chronic liver disease worldwide is NAFLD, and its prevalence continues to increase NAFLD, closely associated with insulin resistance, obesity and oxidative stress is one of the main global health concerns (8).

Herbal medicine has gained popularity in the recent years because of their safety, efficiency, and cost effectiveness. Recently, auxiliary plant metabolites (phytochemicals) have been broadly explored as a source of restorative operators (9). An alternative therapy with lesser side effects would be useful, one of which is the extract from Phyllanthus niruri (P. niruri) herb. Phyllanthus niruri (PN), a typical member of family Euphorbiaceae, has curative properties due to the presence of bioactive phytochemicals (alkaloids, terpenoids, lignans, polyphenols, tannins, coumarins and saponins). Saponins and alkaloids are active components in the extract of PN that can lower cholesterol and fat levels (10). Moreover, several evidence suggest that the extracts of PN possess a prevalent sort of pharmacological deeds like antimicrobial, antioxidant, anti-cancer, anti-inflammatory, hepatoprotective, lipid lowering action, anti-diabetic, and anti-fungal action (11). Therefore, this study was aimed to investigate the hepatoprotective and hypolipidemic effects of Phyllanthus niruri ethanolic extract (PNEE) against non-alcoholic fatty liver disease induced by high fat hyperlipidemic diet.

Material and methods

High-Fat Diet Preparation

The high fat hyperlipidemic diet (HFHD) was prepared by mixing the normal diet with 7.3% coconut oil, 7.3% beef tallow, 0.4% safflower oil, and 1.5% cholesterol for 105 days according to Stangl et al. (12).

Phyllanthus niruri extract preparation

Phyllanthus niruri (PN) leaves were obtained from the herbal market, Zagazig city, Sharkia Governorate, Egypt. The plant was identified with the aid of staff members at the Botany Department, Faculty of Agriculture, Cairo University, Egypt. The leaves were dried at room temperature for two weeks and powdered in a mixer grinder and exposed to extraction procedures, according to reported methods (13). The powdered plant material (3 kg) was extracted with one liter of 95% ethanol for 48 h by successive macerations three times. The resulting mixture was filtered using filter paper (Whatman No. 1) and the solvents were removed using Rota Vapor Apparatus (Switzerland) to yield viscous residues of 268 g equal 8.93% from total powdered plant material. The dried mass of P. niruri was diluted with distilled water before starting the experiments and given orally at dose of 250 mg/kg b.wt. according to Amin et al. (14) with some modifications.

Experimental animals

Forty-eight male albino rats of two months old (120 g average body weight) were bought from the Animal Laboratory House, Helwan, Egypt. All animals were kept in clean metal cages beneath sanitary conditions on stable ration and water was supplied ad-libitum. Rats were kept under hygienic conditions (12h light/dark cycles, 23±2 °C temperature and 44% humidity) during the experimental period. All animals were acclimatized for 2 weeks before starting the experiment. This work’s methodology obeyed with the ethical criteria for the Care and Utilize of Laboratory Animals in Scientific Investigations established by the Animal Welfare and Research Ethics Committee of Fac. of Vet. Med., Zagazig University, Egypt.

Experimental design

Forty-eight male albino rats were randomly alienated into equal four groups: group fed only basal diet (control), group administered ethanol extract of PN at a dose of 250 mg/kg b.wt. dissolved in 1 mL of distilled water (PN), group supplemented with high fat concentrated diet (HFCD); and finally, group received combination of HFCD and PN (HFCD+PN) in the same aforementioned doses. All administrations were given by oral route daily for 105 days.

Blood sampling

At the terminus (105 days), samples of blood (7 rats/group) were gathered from the retro-orbital venous plexus of fasted rats into clean, dry, and labeled centrifuge tubes (El Nasr Company, Egypt) without anticoagulant. The blood was left
to clot and clear serum was obtained by centrifuga-
tion at 3000 rpm for 15 min, then aspirated gen-
tly using sterile Pasteur pipettes and transferred to
dry, sterile, and labeled stoppered vials for bio-
chemical analysis.

**Biochemical assays**

All measurements were determined calorimetr-
ically utilizing commercial kits (Biomerieux, Egypt)
and spectrophotometer, Germany.

**Hepatic markers evaluation**

The activity of serum alanine aminotransferase
(ALT) and aspartate aminotransferase (AST) were
determined according to Reitman and Frankel
(15); while serum alkaline phosphatase (ALP) was
measured according to Masrour and Mahjoub
(16). The serum bilirubin levels were estimated ac-
cording to the method of Jendrassik (17). Serum
total protein (TP), and albumin levels were deter-
mined using colorimetric methods (18, 19), respec-
tively. Globulin level was calculated from subtra-
tion of the values of albumin from those of TP (19).

**Complement estimation**

Serum complement (C3) level was determined
using colorimetric methods (20).

**Serum lipids analysis**

Total cholesterol (TC), triglyceride (TG) and
HDL-C concentrations were determined accord-
ning to former methods (21-23) respectively. The
serum VLDL-C and LDL-C were calculated math-
ematically according to described equation of Fri-
dewald (24).

**Serum pancreatic markers**

Serum glucose was estimated approving to pre-
viously standard method (25). Serum insulin con-
centration was measured by radioimmunoassay
method using a commercially available DSL-1600
insulin kit (Inchinnan, UK) according to Bates
(26). Homeostasis model assessment-β (HOMA-
β) and those for insulin resistance (HOMA-IR)
was calculated conferring to Matthews et al. (27).

**Histopathological investigation**

At the end of experiment, specimen from the
liver was immediately excised and fixed in formali-
lin (10%). Paraffin sections of 5 µm thickness
were prepared and stained with hematoxylin and
cosin (H& E) stains for histopathological exami-
nation (28).

**Statistical analysis**

All data were analyzed by one- way ANOVA
to test the variances among control and different
treated groups using SPSS program version 16.
The comparison of means among the groups was
performed with Duncan’s multiple range tests
(29). The results were reported as Mean ± SE and
the values were considered significant at P ≤ 0.05.

**Results**

**Biochemical results**

As displayed in Figure 1, serum ALT, AST and
ALP activities were non- statistically differed in
rats treated with ethanol extract of PN than con-
trol group (P ≥ 0.05), while fatty liver group given
high fat concentrated diet (HFCD) showed the
highest levels of hepatic enzymes. Co-administra-
tion of HFCD+ PN exhibited significant diminu-
tion in the liver enzymes compared to HFCD
group (P ≤ 0.05). Serum bilirubin (total, direct,
and indirect) levels revealed no change between
all rat’s groups (P ≥ 0.05).

As shown in Figure 2, serum levels of total pro-
tein, albumin, and A/G ratio had a significantly
declined in rats fed HFCD, while serum globulin
and C3 levels were significantly augmented com-
pared to control (P≤ 0.05). However, administra-
tion of PN ethanol extract only produced no effect
on total protein, globulin and C3 levels but caused
a significant increase in level of albumin and A/G
ratio related to control. Treatment of hyper-
lipidemic rats with combination of HFCD+PN
resulted in a significant improvement in the levels
of proteinogram in parallel to HFCD group.
As shown in Figure 3, the results showed that PN-treated group revealed non-significant changes in serum levels of lipid profile compared to control group ($P \geq 0.05$). Rat's group with fatty liver (HFCD) displayed a significant increase in serum TC, TG, VLDL-C and LDL-C, with exception of HDL-C which markedly declined in matched with the control ($P \leq 0.05$). However, administration of HFCD+PN combination exhibited marked reduction in the lipid profile than non-treated fatty liver group (HFCD), but the values of lipid profile in HFCD+PN treated group remained significantly higher than control and PN normal groups ($P \leq 0.05$).
Alleviation harmful effects of non-alcoholic fatty liver disease with *Phyllanthus niruri* extract in albino rats

**Figure 3:** The changes in TC, TG, HDL-c, VLDL-c and LDL-c parameters in control, treated rats with ethanol extract of *Phyllanthus niruri* (PN), high fat concentrated diet (HFCD) and combination of HFCD +PN (n=7/group).

**Figure 4:** show the changes in the levels of glucose, insulin, HOMA-β, and HOMA-IR in control, treated rats with ethanol extract of *Phyllanthus niruri* (PN), high fat concentrated diet (HFCD) and combination of HFCD +PN (n=7/group).

As shown in Figure 4, the results showed that hyperlipidemic group (HFCD) exhibited marked augmentation in serum glucose, insulin, HOMA-IR, and HOMA-β levels compared to control group. While HFCD+PN combination showed marked reduction in the levels of glucose, insulin, HOMA-IR compared to non-treated fatty liver group (P ≤ 0.05), but the value of HOMA-β was non-significantly changed compared to HFCD group (P ≥ 0.05).

**Histopathological findings**

Control rats showed normal gross picture of liver, macroscopically while microscopic examination revealed that hepatic lobular architecture was found to be normal, with hepatic cords of uniformly placed hepatocytes extending from a central vein surrounding a sinusoidal network (Figure 5a). Rats received ethanol extract of PN showed no gross lesions on 105th day. Microscopically, the hepatic tissue revealed a few lymphocytic aggregations in some portal areas and interstitial beside normal hepatic cells in all examined liver section were common (Figure 5b). Rats given HFCD showed enlarged yellowish colored liver macroscopically. Microscopic examination of the hepatic tissue showed intense microsteatosis (fatty changes) random distributed among different zones and involve all the hepatic lobules were seen in all examined liver section (Figure 5c).
In addition, all portal vascular walls had hyaline degeneration with endotheliosis, the majority of portal areas were highly infiltrated with leukocytes, mainly mononuclear cells and few hepatic cells displayed apoptosis or necrosis among degenerated cells. Rats treated with combination of HFCD + PN revealed normal size and color of liver on 105th day macroscopically. Microscopically, the majority of the hepatic parenchyma was apparently normal with a few scattered cells exhibited a few microsteatosis and few hepatic arterioles had hyalinized wall (Figure 5d).

Discussion

The NAFLD is defined as hepatic steatosis affecting more than or equal to 5% of the liver’s surface area without the presence of other disorders affecting the organ or the use of alcohol in hazardous amounts. The disease’s severity ranges from simple fat accumulation in the liver (known as hepatic steatosis) to more serious progressions like nonalcoholic steatohepatitis (30).

Regarding to changes in serum liver enzymes and bilirubin concentrations in the present study, the high fat concentrated diet (HFCD) administered rats markedly increased serum ALT, AST, and ALP activities in comparison with control group. ALT, AST, and ALP are the most common liver marker enzymes. ALT is present only in the cytoplasm, whereas AST is present in both the cytoplasm and mitochondria of hepatocytes. ALP is an enzyme that transports metabolites across cell membranes. ALT, AST, and ALP are also aminotransferases that could act as liver biomarkers.
The intake of HFCD can also lead to the fatty liver disease (31) with increase in serum enzymatic activities is related to hepatic parenchymal damage since ALT is released from mitochondrial and cytosolic localization from membranal sites, and cellular rupture allows the enzyme to escape into the blood (14). The raised serum liver enzymes such as ALT, AST, and ALP in hyperlipidemic rats compared to normally indicates necrosis of hepatocytes or hepatocellular injury that results in the leakage of transaminase and the elevation of serum ALP from a possible cholestasis, and this also can be attributed to the damage in the histological integrity of the hepatocytes (14, 32). Furthermore, hepatic damage may be caused by excess fat accumulation in hepatocytes by direct cellular cytotoxicity, with free fatty acids (FFAs), lipid peroxidation, mitochondrial dysfunction, oxidative stress, and cytokine-induced hepatotoxicity interfering, ultimately causing liver injury (33). The breakdown of lipid peroxides to more reactive lipid radicals may be a molecular mechanism for hepatic injury in the hyperlipidemic group, suggesting that lipid peroxidation is a plausible molecular mechanism for hepatic injury. Also, peroxidation of lipid causes liberation of ROS producing cell membrane damage with increased release and leakage out of these enzymes from liver cytosol into the blood stream (34). Consistent with earlier reports (35-38), the high-fat diet caused increased hepatic damage and a remarkable upsurge of enzymes activity in serum with pathological alterations. In addition, HFCD-induced hyperlipidemia in rats prominently augmented serum ALT, AST, TC, TG, LDL-C and hepatic MDA levels (32). *P. niruri* methanolic extract showed strong anti-angiogenic effects in rats and was able to ameliorate NAFLD with alleviation of serum lives enzymes and proteinogram (39).

Hyperlipidemic rats treated by ethanol extract of PN (HFCD+PN) showed a significant decrease and alleviation in the preceding liver enzymes matching to hyperlipidemic group (HFCD), this confirm that *P. niruri* has a significant hepatoprotective effect. The reduction seen in the levels of these enzymes in the treated rats hinted that the PN extract had stabilized the hepatocytes membrane and prevented the release of enzymes from liver into blood (14). Similar findings were previously obtained by others (40-45). Decreasing liver enzymes was related to polyphenolic compounds and flavonoids in PN which have relatively potent antioxidant effects that prevent lipid peroxidation, the free radical scavenging effect and improvement of antioxidant system in liver (46). *P. niruri*'s anti-NAFLD impact is most likely due to its antioxidant activity and owing to the presence of phenolic chemicals in PN preparation, which reduced oxidative stress by lowering lipid peroxidation and improved insulin signaling and β-oxidation (8). Treatment of hyperlipidemia in rats with PN extract (HFCD+PN) sheltered the liver from injury, and inhibited the production of liver enzyme markers, as well as effectively reduced the levels of TC and TG, this because of the crude leaf extract is shown to preserve the physiological integrity of the cells exposed to various stress (47). Rats received ethanol extract of PN only didn’t cause any alterations in liver enzymes, this indicate PN alone is safe and haven’t any deleterious effect on hepatic tissue and its function.

Our results are confirmed by histological changes which showed intense microsteatosis, and fatty changes in the liver of rats on HFD-induced hyperlipidemia. All portal vascular walls had hyaline degeneration with endotheliosis. The majority of portal areas highly infiltrated with leukocytes mainly mononuclear cells. A few hepatic cells may develop apoptosis or necrosis among degenerated cells. Similar histopathological findings in the liver of hyperlipidemic rats were previously achieved (48-49). In addition, Al Zarzour et al. (8) displayed noticeable fat deposition with highest scores in steatosis, portal inflammation, hepatocyte ballooning and fibrosis of liver samples from HFD group. Moreover, the HFD group's liver sections revealed noteworthy lipid-droplet vacuoles in hepatocytes, hepatic cell disarray, necrotic alterations in the central lobes, and hepatic lobule destruction (32). However, hepatocytes in the hepatic tissue of rats treated with combination of HFCD+PN have returned to their normal state and a few scattered cells exhibited microsteatosis due to inhibit cholesterol biosynthesis in hepatocytes. *P. niruri* enhances the excretion of bile acids through feces and this contributes to regress the cholestetosisis in liver damage (41). Animals treated by (PN) didn’t cause any deterioration in hepatocyte. This may indicate PN is safe and haven’t any deleterious effect on hepatic tissue and its function.
The serum total, direct, and indirect bilirubin levels showed non-significant change in rats given HFCD; this indicated that common bile duct not affected by fatty liver in this study. Our results disagreed with other study (50). The difference may be due to the changes in feeding ration and duration. Treatment of HFCD-induced hyperlipidemic rats by PNEE resulted in non-significant changes in serum bilirubin concentrations, this agrees with former study (51) who reported no marked changes in serum total bilirubin in hyperlipemic rats given an aqueous leaf extract of PN.

Concerning serum protein profile, HFCD-induced hyperlipidemia displayed a diminution in TP, albumin, and A/G ratio matched with control. These results may be due to decreasing of capacity of protein synthesis by impaired liver cells. The hypoproteinemia and hypoalbuminemia may be due to hyperlipidemia induced by HFCD that causes fat penetration in hepatocytes, leading to hepato-cellular damage (52). This was agreed with aforementioned studies (37, 39, 45, 53) and disagreed with others (54). Hyperlipidemic rats treated with HFCD+PN combination displayed significant increase of total protein, albumin and A/G ratio comparing with non-treated HFD group, this may due to presence of bioactive phytochemicals (polyphenols) in P. niruri which have potent antioxidant effect on liver tissues (44). These findings suggesting that P. niruri supplementation causes no toxicity to the liver function and minimizes the injury caused by high-fat diet. Thus, it could be concluded that P. niruri administration had liver protective activity. Similar finding were previously obtained (86, 55, 56). Oral administration of Phyllanthus rheedia to streptozotocin-induced diabetic rats ensued a major lessening in blood glucose, and enzymes (ALT, AST and ALP) and improvement in lipid and proteins profile (57).

The restoration of these measures to near-normal values could be endorsed to inclusion of flavonoids in the alcoholic P. rheedia extract, which have been known to be hepatoprotective agents (58).

Rats treated with PN ethanolic extract only did not have any changes in serum proteins profile indicating no negative impact on protein synthesis and catabolism.

Concerning the results of serum complement (C3), HFCD received rats revealed a significant upsurge in C3 in comparison with control indicating fatty liver disease. Our results were in agreement with other reporters (59, 60). Moreover, Ursini et al. (61) reported that the increase in serum C3 is a potential predictor of NAFLD with good sensitivity and specificity. Co-administration of HFCD with ethanol extract of PN significant dwindling of C3, which may be due to the polyphenolic compound and flavonoids in PN extract that have antioxidant and anti-inflammatory properties. Previous study showed the same results (55).

Serum lipid profile in the existing study were significantly augmented in rats fed HFCD-induced hyperlipidemia analogous to control group. Hypercholesterolemia, hypertriglyceridemia, in incorporation with high levels of VLDL-C and LDL-C and little HDL-C, is the hallmark of dyslipidemia or fatty liver disease (62). Fatty liver disease is intimately linked to oxidative stress and disturbances in lipid metabolism leading to hepatic lipid buildup, which affects different ROS generators, including mitochondria, endoplasmic reticulum, and NADPH oxidase (34). These results may be due to insulin resistance, hyperlipidemia and hepatic steatosis (63). Our results were in coordination with the earlier reports (33, 36, 37, 38, 64-66). We also determined that combination of HFCD and PN decreased and improved the abnormality of lipid profile in serum; this indicated the potential role of PN as anti-hyperlipidemic agent due to presence of bioactive phytochemicals (46). In cholesterol fed hyperlipemic rats, P. niruri could increase the level of HDL by increasing the activity of plasma lecithin: cholesterol acyltransferase (LCAT), which may contribute to the regulation of blood lipids. LCAT play a key role in lipoprotein metabolism and most of the lipoprotein changes are the outcome of primary abnormality owing to the liver diseases (41). Also, hyperlipidemic rats that received Psyllium husk ethanolic extract exhibited diminished lipogram and augmented HDL-C in parallel to the hyperlipidemic group (52). Other studies exhibited that flavonoids in P. niruri have a protective effect on LDL cholesterol to not to be oxidized (10, 67). These findings are similar to the former studies (37, 44). Feeding of hyperlipemic rats with P. niruri (HFCD+PN) resulted in more lipid lowering effect as it caused dropping in VLDL and LDL levels in rats (42).
Regarding pancreatic biomarkers in this study, rats given HFCD exhibited a high value of glucose, insulin, HOMA-β and HOMA-IR values. Hyperglycemia and hyperinsulinemia are caused by fatty acid-oxidation inhibition and lipogenesis promotion in the liver. HFCD is a major contributor to insulin resistance, which manifests as hyperinsulinemia, hyperlipidemia, hyperglycemia, and mitochondrial dysfunction (68). The reduced glucose removal in adipose tissue is mostly associated with obesity, which is usually connected to the development of hyperlipidemia and its complications. In addition, it is also caused by hyperglycemia, insulin resistance, hyperinsulinemia, and hyperlipidemia (69). Moreover, these findings could be explained by elevated cholesterol levels in β-cells, which cause lipotoxicity by lowering the expression of transcription factors necessary for β-cell survival (70). Furthermore, hyperlipidemia is also linked to the development of insulin resistance (71). Our findings support prior research that shows the HFCD can produce fast fat accumulation in the liver as well as an increase in insulin resistance (HOMA-IR values) (72). The increase of insulin resistance in the HFD group would cause oxidative stress in the liver by producing reactive oxygen species, inducing hepatic lipid peroxidation, and triggering inflammatory reactions that would promote fibrosis by activating hepatic stellate cells (HSC) (73, 74). Compensatory hyperinsulinemia can correct glucose levels in the blood, but it also causes changes in hepatic lipid metabolism, which leads to liver steatosis (75). Similar results were previously attained (76, 77). Hyperlipidemic rats treated with PN (HFCD+PN) revealed significant decrease and improvement in serum glucose, insulin, HOMA-IR and HOMA-β levels comparatively with HFCD group. These results may be due to antioxidant effect of bioactive phytochemicals of PN (47). The ethanol extract of *P. niruri* improved liver function and decreased serum levels of glucose, insulin concentrations, HOMA-IR and HOMA-β, indicating a significant decrease in insulin resistance (44).

**Conclusion**

Overall, we investigate the efficacy of *P. niruri* ethanolic extract (PNEE) on the hepatic and pancreatic alterations in high fat diet-induced hyperlipidemic rats as well as histopathology. The findings of this investigation revealed that PNEE possesses powerful hepatoprotective, anti-hyperlipidemia and anti-hyperglycemia properties. The administration of PNEE relieved the histopathological changes in the liver caused by hyperlipidemia.

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