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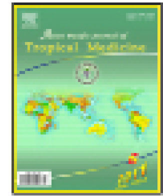

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Protective effects of *Phyllanthus acidus* (L.) Skeels leaf extracts on acetaminophen and thioacetamide induced hepatic injuries in Wistar rats

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ABSTRACT

Objective: To investigate and compare the hepatoprotective effects of crude ethanolic and aqueous extracts of *Phyllanthus acidus* (L.) Skeels (*P. acidus)* leaves on acetaminophen (APAP) and thioacetamide (TAA) induced liver toxicity in wistar rats. Silymarin was the reference hepatoprotective agent. **Methods:** In two different sets of experiments, the *P. acidus* extracts (200 and 400 mg/kg, body weight) and silymarin (100 mg/kg, body weight) were given orally for 7 days and a single dose of APAP (2 g/kg, per oral) or TAA (100 mg/kg, subcutaneous) were given to rats. The level of serum aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total bilirubin and total protein were monitored to assess hepatotoxicity and hepatoprotection. **Results:** APAP or TAA administration caused severe hepatic damage in rats as evident from significant rise in serum AST, ALT, ALP, total bilirubin and concurrent depletion in total serum protein. The *P. acidus* extracts and silymarin prevented the toxic effects of APAP or TAA on the above serum parameters indicating the hepatoprotective action. The aqueous extract was found to be more potent than the corresponding ethanolic extract against both toxicants. The phenolic and flavonoid content (175.02±4.35 and 74.68±1.28, respectively) and 2,2-diphenyl-1-picrylhydrazil (DPPH) [IC_{50} = (33.2±0.31) μ g/mL] scavenging potential was found maximum with aqueous extract as compared to ethanolic extract. **Conclusions:** The results of present study suggests that the aqueous extract of *P. acidus* leaves has significant hepatoprotective activity on APAP and TAA induced hepatotoxicity, which might be associate with its high phenolic and flavonoid content and antioxidant properties.

1. Introduction

Phyllanthus acidus (L.) Skeels (Euphorbiaceae) (*P. acidus*), commonly known as harfarauri or star gooseberry, is a widely distributed plant in India and other Asian countries. It is about 4-6 m high with obliquely ovate acute and distichous thin leaves. The leaf is analgesic, antipyretic, antirheumatic and cures jaundice, small pox, itching and gum infection. Traditionally it is used as liver tonic and blood purifier[1, 2]. The leaf decoction is used by tribal healers of Chittagong Hill Tracts region of Bangladesh to treat liver disease[3]. An aqueous extract of leaf is reported to have remarkable antiviral[4] and anti cystic fibrosis properties[5]. Studies with methanolic extract of *P. acidus* leaf have shown protection against carbon tetrachloride induced hepatotoxicity in rats[6]. Some important chemical constituents of the leaf include kaempferol, hypogallic acid, gallic acid, quercetin, and adenosine[5, 6].

The aim of present study was to investigate and compare the hepatoprotective effects of crude ethanolic and aqueous extracts of *P. acidus* leaves on acetaminophen (APAP) and thioacetamide (TAA) induced acute liver toxicity in rats. The protective effects were compared with silymarin, a well known hepatoprotective against APAP and TAA induced hepatotoxicity[7].

2. Material and methods

2.1. Chemicals and drugs

Acetaminophen, and 2,2-diphenyl-1-picrylhydrazil (DPPH) were purchased from Sigma Chemical Co. (St.

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Louis, MO, USA). Thioacetamide was obtained from Loba Chemie, Mumbai. Silymarin was obtained as a gift sample from Serum International Ltd., Pune, India. Aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), bilirubin and protein estimation kits were procured from Span Diagnostics, Surat, India. All other chemicals and reagents used were of highest commercially available purity and purchased from Qualigens, India.

2.2. Plant material

The fresh leaves of *P. acidus* were collected in May-June, 2008 from University (Dr. H. S. Gour University) campus, Sagar (India) and authenticated in Botany Department of University, where a voucher specimen (No.Bot/H/4322) has been preserved for future reference. The leaves were shade dried, coarsely powdered and stored for further use.

2.3. Preparation of extracts

2.3.1. Preparation of ethanolic extract

The leaf powder (400 g) was sequentially extracted with petroleum ether (60-80 ℃) and ethanol (95% v/v) till complete exhaustion using Soxhlet apparatus. The ethanolic extracts were filtered and the filtrate was evaporated to dryness at 50 ℃ under reduced pressure in a rotary evaporator. About 14 g of dried ethanolic extract was obtained from 400 g of dried leaf powder (3.5% w/w). The extract was uniformly suspended in 2% aqueous gum acacia prior to experiment.

2.3.2. Preparation of aqueous extract

500 g leaf powder was subjected to hot water extraction for 4 h at 80 ℃ and then filtered. The filtrate was evaporated to dryness to get the water extract. The yield of extract was 33 g from 500 g of leaf powder (6.6% w/w). This crude extract was re-suspended in water prior to experiment.

2.4. Preliminary phytochemical screening

Preliminary phytochemical analysis on plant extracts was performed using the following chemicals and reagents: flavonoids (Mg metal and HCl), phenolics ($FeCl_3$), protein and amino acid (Millon's and Ninhydrin reagent), alkaloids (Mayer and Dragendorff's reagent), saponins (Foam test), phytosterols and triterpenoids (Liebermann- Burchard Test) and glycosides (NaCl and Fehling's solution A and B) [8].

2.5. Animals

Wistar albino rats (200-220 g) and Swiss albino mice (20-25 g) of either sex were used for the studies. The animals were grouped and housed in polyacrylic cages with not more than six per cage and maintained under standard laboratory conditions of temperature [(25±2) ℃] and relative humidity [(55±5) %] with dark and light cycle (12/12 hours). They were acclimatized to laboratory conditions for 7 days before commencing the experiment and allowed for free access to standard pellet diet (Him Feed H.P., Agro industries) and water ad libitum. Animal studies were approved by the Institutional Animal Ethics Committee (379/01/ab/CPCSEA) and conducted as per the regulations of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.6. Acute oral toxicity studies

The acute oral toxicity studies of *P. acidus* extracts were carried out as per OECD guidelines[9]. On the basis of these studies, the oral doses of 200 and 400 mg/kg, bw were selected for the *in vivo* experiments.

2.7. Total phenol and flavonoid content determination

The total phenol content of plant extracts was determined by Folin-Ciocalteau method[10]. The total phenolic content was expressed as milligrams of gallic acid equivalents/g extract (mg GAE/g of dry mass).

The flavonoid content of extracts was estimated as quercetin equivalent[11]. The total flavonoid content was expressed in milligrams of quercetin equivalents/g of extract (mg QE/g of dry mass).

2.8. DPPH scavenging assay

The free radial scavenging (antioxidant) property of extracts was determined as bleaching of the stable DPPH radical[12]. Ascorbic acid, a well known antioxidant was used as a positive control.

2.9. APAP- induced hepatotoxicity

The experiment was conducted according to the method described previously[13]. Rats were randomly divided into 7 groups, each consisting of 6 rats. Group Ⅰ served as normal control and received distilled water (1 mL/kg, po.) daily for 7 days and 50% aqueous sucrose (10 mL/kg, po.) on days 5. Group Ⅱ served as hepatotoxicity (APAP) control and received distilled water (1 mL/kg, po.) daily for 7 days and received APAP (2 g/kg, po.) as suspension in 50% aqueous sucrose solution on days 5. Group Ⅲ was treated with silymarin (100 mg/kg, po.) daily for 7 days and received APAP (2 g/kg, po.) on days 5, 30 min after the administration of silymarin. Groups Ⅳ-Ⅴ and Ⅵ-Ⅶ were treated with ethanolic and aqueous extracts at two doses (200 and 400 mg/kg, po.), respectively, for 7 days and received APAP (2 g/kg, po.) on days 5, 30 min after administration of extracts.

2.10. TAA- induced hepatotoxicity

Rats were randomly divided into 7 groups, each consisting of 6 rats. Group Ⅰ served as normal control and received distilled water (1 mL/kg, po.) daily for 7 days. Group Ⅱ served as hepatotoxicity (TAA) control and received distilled water (1 mL/kg, po.) daily for 7 days and received TAA (100 mg/kg, sc.) as 2% w/v solution in distilled water on days 6[14].

Group Ⅲ was treated with silymarin (100 mg/kg, po.) daily for 7 days and received TAA (100 mg/kg, sc.) on days 6, 30 min after administration of silymarin. Groups Ⅳ-Ⅴ and Ⅵ-Ⅶ were treated with ethanolic and water extracts at two doses of 200 and 400 (mg/kg, po.), respectively, for 7 days and received TAA (100 mg/kg, sc.) on days 6, 30 min after the administration of extracts.

2.11. Biochemical assay

On the 7th day, under ether anesthesia, blood samples were taken from retro-arbitral plexus of rats. The blood samples were allowed to clot for 30 min and serum was separated by centrifugation at 3 000 rpm at 4 ℃.

The activities of serum ALT and AST[15] and ALP[16] were determined. Total bilirubin[16] and protein content[17] were also determined in serum to assess the acute hepatic injuries.

2.12. Statistical analysis

The results of study are expressed as mean±standard error mean (SEM) and statistically analyzed using one-way ANOVA followed by Dunnett's test for comparison with normal control group and hepatotoxin control group. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Preliminary phytochemical analysis

On preliminary phytochemical analysis, the ethanolic extracts of *P. acidus* showed the presence of flavonoids, glycosides, phenolic compounds, phytosterols and saponins, whereas the aqueous extract revealed the presence of flavonoids, glycosides, phenolic compounds, proteins, amino acids, carbohydrates and saponins.

3.2. Acute oral toxicity study

In acute oral toxicity studies, the *P. acidus* extracts did not show any sign and symptoms of toxicity and mortality up to 2 000 mg/kg dose, considered relatively safe.

3.3. Total phenolic and flavonoid content

The phenolic and flavonoid contents were found maximum in aqueous extract (175.02±4.35 and 74.68±1.28, respectively) than compare to ethanolic extract (96.05±2.51 and 59.25±1.09, respectively).

3.4. DPPH scavenging activity

The aqueous extract of *P. acidus* has better scavenging property [IC_{50}=(33.2±0.31) μg/mL] than ethanolic extract [IC_{50} =(69.6±0.23) μg/mL]. The IC_{50} value of ascorbic acid (reference antioxidant) was found to be (21.8±0.34) μg/mL.

3.5. Effect of *P. acidus* extracts on APAP- induced hepatotoxicity

As shown in Table 1, APAP control rats (group Ⅱ) showed significant increase in serum AST (2.7 fold), ALT (2.9 fold), ALP (2.4 fold) and bilirubin (6.3 fold) levels and a significant decrease in serum total protein (1.52 fold) in comparison with normal control rats. On the other hand, administration of both extracts of *P. acidus* at two dose levels (200 and 400 mg/kg, po.) attenuated the increased levels and caused a subsequent recovery towards normalization. Pretreatment of rats with aqueous extract (400 mg/kg, po.) showed highly significant activity (P<0.01 and 0.001) almost like that of silymarin (100 mg/kg, po.) (Table 1).

3.6. Effect of *P. acidus* extracts on TAA induced hepatotoxicity

The results of hepatoprotective effect of *P. acidus* leaf extracts on TAA-intoxicated rats are shown in Table 2. In TAA control rats, a marked increase in serum AST (2.7 fold), ALT (3 fold), ALP (2 fold) and total bilirubin (5 fold) was observed in comparison with normal control rats.

Table 1

Effect of *P. acidus* leaf extracts and silymarin on serum markers on acetaminophen (APAP, 2 g/kg, po.)- induced hepatotoxicity in Wistar rat.

Groups	Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)	Total serum protein (mg/dL)
Group Ⅰ	Normal (distilled water)	66.52± 0.69	36.54± 0.87	135.59± 3.32	0.36± 0.28	6.12± 0.09
Group Ⅱ	APAP (2 g/kg, po.)	178.89±1.98#	105.68±1.57 #	318.68± 6.08 #	2.28± 0.08#	4.02± 0.04 #
Group Ⅲ	Silymarin (100 mg/kg, po.)	81.67±1.29* (86.51)	43.68±1.87 *(89.67)	191.87± 9.68 *(69.26)	0.57± 0.06*(89.00)	6.25±0.15 *(106.19)
Group Ⅳ	Ethanolic extract (200 mg/kg, po.)	136.58±2.34 (37.65)	79.37±1.92 ☆(38.00)	272.63± 3.55 ☆(46.05)	1.34±0.04 ☆(46.95)	5.09±0.06☆(50.95)
Group Ⅴ	Ethanolic extract (400 mg/kg, po.)	109.35±1.57△ (61.89)	61.29±1.28 △(64.20)	223.54± 1.94 △(51.96)	1.16±0.05△(58.33)	5.28± 0.05△(60.00)
Group Ⅵ	Aqueous extract (200 mg/kg, po.)	107.64±0.97△ (63.40)	69.18±0.67 △(52.79)	242.87± 2.87 ☆(41.40)	1.09± 0.02△(61.97)	5.22± 0.01☆(57.14)
Group Ⅶ	Aqueous extract (400 mg/kg, po.)	96.85±1.18 * (73.00)	45.42± 0.81*(87.15)	198.54± 1.89 *(65.61)	0. 72±0.02 *(81.25)	5.95± 0.01*(91.90)
F -values		56.53	198.31	61.23	21.23	26.87

Values are mean ± SEM, n = 6 animals per group. #group Ⅱ (APAP control rats) compared with group Ⅰ (normal control rats) $P < 0.001$; * groups Ⅲ-Ⅳ (silymarin and plant extracts treated rats) compared with group Ⅱ (APAP control rats) $P < 0.001$; △groups Ⅲ-Ⅶ(silymarin and plant extracts treated rats) compared with group Ⅱ (APAP control rats) $P < 0.01$; ☆groups Ⅲ-Ⅶ (silymarin and plant extracts treated rats) compared with group Ⅱ(APAP control rats) $P < 0.05$.Values in the parenthesis indicate percent protection in individual biochemical parameters from their elevated values caused by the hepatoprotection. The percentage of the protection is calculated as 100 × (values of APAP control - values of sample) / (values of APAP control - values of normal control).

Table 2

Effect of *P. acidus* leaf extracts and silymarin on serum markers on thioacetamide (TAA, 100 mg/kg, sc.)– induced hepatotoxicity in Wistar rats

Groups	Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)	Total bilirubin (mg/dL)	Total serum protein (mg/dL)
Group Ⅰ	Normal (distilled water)	52.53± 0.35	32.43± 0.45	142.59± 2.16	0.42± 0.21	8.15± 0.06
Group Ⅱ	TAA (100 mg/kg, sc.)	138.94±1.27 #	98.63± 1.99 #	278.69± 5.04 #	2.13± 0.06 #	3.73± 0.05 #
Group Ⅲ	Silymarin (100 mg/kg, po.)	63.62±1.31 * (87.15)	49.79± 1.82 *(73.80)	165.62± 7.86 *(83.07)	0.45± 0.02 * (98.24)	8.22± 0.18 *(101.58)
Group Ⅳ	Ethanolic extract (200 mg/kg, po.)	106.52±1.67(37.51)	65.57± 1.25 ☆(49.90)	216.25± 4.37 ☆(45.87)	1.36± 0.05 ☆(45.02)	4.95± 0.24 ☆(27.66)
Group Ⅴ	Ethanolic extract (400 mg/kg, po.)	97.41±1.54 △(48.00)	58.32± 1.36 *(60.89)	198.68± 3.53 △(58.78)	1.12± 0.01△ (59.06)	5.62± 0.06 △(42.76)
Group Ⅵ	Aqueous extract (200 mg/kg, po.)	99.65±0.86 △(45.46)	66.15± 0.59 ☆(49.00)	201.81± 1.86 △ (56.48)	1.08± 0.01△ (61.40)	5.47± 0.01△(39.36)
Group Ⅶ	Aqueous extract (400 mg/kg, po.)	79.46±1.22 *(68.90)	52.84± 0.67 *(69.63)	176.54± 1.92 *(75.00)	0.82± 0.02 * (76.60)	6.53± 0.01*(56.56)
F -values		64.27	208.17	64.28	39.43	20.34

Values are mean ± SEM., n = 6 animals per group. #group Ⅱ (APAP control rats) compared with group I (normal control rats) $P < 0.001$; * groups Ⅲ–Ⅶ (silymarin and plant extracts treated rats) compared with group Ⅱ (APAP control rats) $P < 0.001$; △groups Ⅲ–Ⅶ (silymarin and plant extracts treated rats) compared with group Ⅱ (APAP control rats) $P < 0.01$; ☆groups Ⅲ–Ⅶ (silymarin and plant extracts treated rats) compared with group Ⅱ (APAP control rats) $P < 0.05$.Values in the parenthesis indicate percent protection in individual biochemical parameters from their elevated values caused by the hepatoprotection. The percentage of the protection is calculated as 100 × (values of APAP control – values of sample) / (values of APAP control – values of normal control).

Such elevated levels were substantially prevented in rats pretreated with *P. acidus* extracts. The aqueous extract (400 mg/kg, po.) showed remarkable protection on TAA hepatotoxicity but not as effective as the silymarin.

4. Discussion

APAP and TAA-induced hepatic injuries are commonly used screening models for new hepatoprotective drugs[18, 19]. APAP is a well known analgesic-antipyretic and is known to cause severe hepatic damage in humans and experimental animals with high doses. Its toxicity is attributed to formation of reactive metabolite viz. N-acetyl-p-benzoquinoneimine (NAPQI) by the action of cytochrome P-450 (CYP-450) enzyme system[20]. TAA is a potent hepatotoxic and hepatocarcinogenic compound, which is bioactivated by CYP-450 and/or flavin-containing monooxygenases system to form reactive metabolites viz. sulfene (sulfone) and sulfine (sulfoxide). These reactive metabolites initiate the generation of reactive oxygen species and leads to hepatocellular death via oxidative stress[21].

An indication of hepatic damage is leakage of cellular enzymes into the plasma. When liver cell membrane is damaged, a variety of cytosolic enzymes are released into the blood stream. Their estimation in serum is a useful quantitative marker of the extent and type of hepatocellular damage[22]. The elevated levels of AST and ALT in this study may be interpreted as a result of the hepatocytes damage or alterations in the membrane permeability indicating the severity of hepatocellular damage induced by APAP and TAA which is in accordance with previous reports[23, 24]. Pretreatment with *P. acidus* extracts attenuated the increased activities of these enzymes in serum. Recovery towards normalization suggests that *P. acidus* extracts prevent hepatic cellular damage, thereby, decreasing the enzyme leakage. In contrast, an increase in ALP activity and bilirubin level reflects the pathological alteration in biliary flow. Likewise their suppression in extract treated rats suggests the possibility of the extracts being able to stabilize biliary dysfunction.

On comparing the both extracts, aqueous extract was found to be stronger hepatoprotective than the corresponding ethanolic extract against both toxicants. Its hepatoprotective activity was comparable with that of silymarin. The DPPH scavenging activity was also found maximum with aqueous extract, which suggests that it could scavenge efficiently the free radicals generated during the APAP and TAA metabolism. In our preliminary phytochemical screening, the ethanolic extract revealed the presence of polyphenolic/saponins/steroids as major compounds; meanwhile the aqueous extract showed the presence of protein in addition to polyphenolics/saponins/flavonoids as major compounds. The protein isolated from *P. niruri* has been shown as a protective agent against xenobiotic induced hepatotoxicity[21, 25]. Phytochemical analysis also revealed the high content of phenolics and flavonoids in aqueous extract, which might be responsible for its stronger biological activities, because these phytochemicals were identified as strong antioxidants in other *Phyllanthus* species[26, 27].

The hepatoprotective and antioxidant properties of several *Phyllanthus* aqueous extracts have already been demonstrated[28-36]. The literature also documented the antioxidant and hepatoprotective value of gallic acid and quercetin[37,38]. The antioxidant property of *P. acidus* leaf extract against CCl_4-induced hepatotoxicity in mice has already been reported[7]. Thus, it appears that the hepatoprotection offered by *P. acidus* aqueous extract might be associated with such identified classes of compounds and antioxidative properties.

In conclusion, the results of the present study indicated that under experimental conditions, the aqueous extract of *P. acidus* leaves has significant hepatoprotective capacity against APAP and TAA induced hepatotoxicity. Further investigations on isolation and characterization of the active compounds responsible for the offered hepatoprotection by *P. acidus* aqueous extract are under way in our laboratory.

Conflict of interest statement

We declare that we have no conflict of interest.

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