

PROTECTIVE EFFECT OF *PHYLLANTHUS POLYPHYLLUS* ON ACETAMINOPHEN INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

Methanolic extract of *Phyllanthus polyphyllus* was evaluated for hepatoprotective and antioxidant activities in rats. The plant extract (200 and 300 mg/kg, p.o.) showed a remarkable hepatoprotective and antioxidant activity against acetaminophen induced hepatotoxicity as judged from the serum marker enzymes and antioxidant levels in liver tissues. Acetaminophen induced a significant rise in aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total bilirubin, gamma glutamate transpeptidase (GGTP), lipid peroxidase (LPO) with a reduction of total protein, superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione S-transferase (GST). Treatment of rats with different doses of plant extract (200 and 300 mg/kg) significantly ($P < 0.001$) altered serum marker enzymes and antioxidant levels to near normal against acetaminophen treated rats. The activity of the extract at dose of 300 mg/kg was comparable to the standard drug, silymarin (50 mg/kg, p.o.). Histopathological changes of liver sample were compared with respective control. Results indicate the hepatoprotective and antioxidant properties of *Phyllanthus polyphyllus* against acetaminophen-induced hepatotoxicity in rats.

Keywords: *Phyllanthus polyphyllus*; acetaminophen; biochemical parameters; antioxidants; lipid peroxidation; histopathology.

INTRODUCTION

Herbal medicines have recently attracted much attention as alternative medicines useful for treating or preventing life style related disorders and relatively very little knowledge is available about their mode of action. There has been a growing interest in the analysis of plant products which has stimulated intense research on their potential health benefits. Liver, the key organ of metabolism and excretion has an immense task of detoxification of xenobiotics, environmental pollutants and chemotherapeutic agents. Hence, this organ is subjected to variety of diseases and disorders. Several hundred plants have been examined for use in a wide variety of liver disorders. Antioxidants play an important role in inhibiting and scavenging free radicals and thus providing protection against infections and degenerative diseases.

Phyllanthus polyphyllus Linn (Euphorbiaceae) is a deciduous shrub or small tree found mostly in hill areas of South India and Ceylon. It is popularly known as Sirunelli in Tamil. Leaves are traditionally used for liver diseases by tribes of Kolli hills, Tamilnadu, India (Gamble, 1993; Mathew, 1983). The phytochemical studies of the plant have revealed the presence of benzenoid, 4-O-methyl gallic acid, together with three aryl naphthalide lignans, namely phyllamyricin, justicidin B and diphyllin. Its extract shows dose dependent inhibition

of inflammatory mediators such as LPS/INF- γ stimulated by peritoneal exuded macrophages (Rao *et al.*, 2006), monoacylated triterpene arabinosides and terpenes found have cytotoxic activity against human cancer cell lines (Youkwan *et al.*, 2005). The present study is aimed to evaluate the hepatoprotective and antioxidant activity of methanol extract of the leaves of *Phyllanthus polyphyllus* against acetaminophen induced hepatotoxicity in rats.

MATERIALS AND METHODS

Plant material and extraction: Leaves of *Phyllanthus polyphyllus* were collected in the month of December 2005 at Kolli hills, Salem District and authenticated by Dr. G. Murthy, Botanical Survey of India, Coimbatore, Tamilnadu, India. A voucher specimen (EPP-03) has been kept in our laboratory for future reference. The leaves were dried in the shade and pulverized. The powder was treated with petroleum ether for dewaxing as well as to remove chlorophyll. The powder was then packed into soxhlet apparatus and subjected to hot continuous percolation using methanol (95% v/v) as solvent. The extract was concentrated under vacuum and dried in a vacuum desiccator (yield 4.1 % w/w) and then suspended in 5% gum acacia for hepatoprotective studies.

Animals: Male Wistar rats (100-125 g) and Swiss albino mice (20-25 g) were procured from Tamilnadu Veterinary College, Chennai, India. They were housed in microcolon boxes with standard laboratory diet and water

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ad libitum. The study was conducted after obtaining Institutional animal ethical committee clearance.

Chemicals: Acetaminophen was purchased from Lupin Ltd., Mumbai, India. Silymarin (Micro labs, Hosur, India), 1-chloro-2,4-dinitro benzoic acid (CDNB), 5,5-dithio-bis-2-nitro benzoic acid (DTNB) and reduced glutathione (GSH) were supplied by Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Thiobarbituric acid was purchased from E-Merck, India. All other chemicals used were of analytical grade.

Acute toxicity: The acute toxicity of the extract of *P. polyphyllus* was evaluated in mice using the up and down procedure (OECD, 2001). Mice received alcohol extract at various doses (500-2000 mg/kg) orally by gavage. The animals were observed for toxic symptoms continuously for the first 4 h after dosing. Finally, the number of survivors was noted after 24 h.

Experimental design

Rats were divided into five groups, each group consisting of six animals.

- Group I: Controls received the vehicle viz. normal saline (2 ml/kg).
- Group II: Received acetaminophen (750 mg/kg p.o.) (Hiroshini, *et al.*, 1987) at every 72 h for 10 days.
- Group III: Received silymarin 50 mg/kg p.o. for 10 days and simultaneously administered acetaminophen 750 mg/kg body wt. every 72 h.
- Group IV: Received alcohol extract of *P. polyphyllus* 200 mg/kg p.o. for 10 days and simultaneously administered acetaminophen 750 mg/kg body wt. every 72 h.
- Group V: Received alcohol extract of *P. polyphyllus* 300 mg/kg p.o. for 10 days and simultaneously administered acetaminophen 750 mg/kg body wt. every 72 h.

At the end of experimental period, all the animals were sacrificed by cervical decapitation. Blood samples were collected, allowed to clot. Serum was separated by centrifuging at 2500 rpm for 15 min and analyzed for various biochemical parameters.

Assessment of liver function

Biochemical parameters i.e., aspartate amino transferase (AST) (Reitman and Frankel, 1957) alanine amino transferase (ALT) (Reitman and Frankel, 1957), alkaline phosphatase (ALP) (Kind and King, 1954), γ -glutamate transpeptidase (GGTP) (Szasz, 1969), total bilirubin (Mallay and Evelyn, 1937) and total protein (Lowry *et al.*, 1951) were analyzed according to the reported

methods. The liver was removed, weighed and morphological changes were observed. A 10% of liver homogenate was used for antioxidant studies such as lipid peroxidation (LPO) (Devasagayam and Tarachand, 1987), superoxide dismutase (SOD) (Marklund and Marklund, 1974), catalase (Sinha, 1971), glutathione peroxidase (GPx) (Rotruck *et al.*, 1973) and glutathione S-transferase (GST) (Habig *et al.*, 1974). A portion of liver was fixed in 10% formalin for histopathological studies.

Histopathological studies: Liver slices fixed for 12 hrs in Bouin's solution were processed for paraffin embedding following standard micro techniques (Galigher and Kozloff, 1971). 5 μ m sections of liver stained with alum haematoxylin and eosin, were observed microscopically for histopathological changes.

Statistical analysis: The values were expressed as mean $\pm$ SEM. Statistical analysis was performed by one way analysis of variance (ANOVA) followed by Tukey multiple comparison test and data on liver weight variations were analyzed using Student's 't' test. P values < 0.05 were considered as significant.

RESULTS

The effect of *P. polyphyllus* on serum marker enzymes are presented in table 1. The levels of serum AST, ALT, ALP, total bilirubin, GGTP were markedly elevated and that of protein decreased in acetaminophen treated animals, indicating liver damage. Administration of *P. polyphyllus* extract at the doses of 200 and 300 mg/kg remarkably prevented acetaminophen-induced hepatotoxicity in a dose dependent manner.

Analysis of LPO levels by thiobarbituric acid reaction showed a significant ($P < 0.001$) increase in the acetaminophen treated rats. Treatment with *P. polyphyllus* (200 mg/kg and 300 mg/kg) significantly ($P < 0.001$) prevented the increase in LPO level which was brought to near normal. The effect of *P. polyphyllus* was comparable with that of standard drug silymarin (table 2).

Acetaminophen treatment caused a significant ($P < 0.001$) decrease in the level of SOD, catalase, GPx and GST in liver tissue when compared with control group (table 2). The treatment of *P. polyphyllus* at the doses of 200 and 300 mg/kg resulted in a significant increase of SOD, catalase, GPx and GST when compared to acetaminophen treated rats. The liver of silymarin treated animals also showed a significant increase in antioxidant enzymes levels compared to acetaminophen treated rats.

Morphological observations showed an increased size and enlargement of the liver in acetaminophen treated groups. These changes were reversed by treatment with silymarin and also *P. polyphyllus* at the doses tested (fig 1).

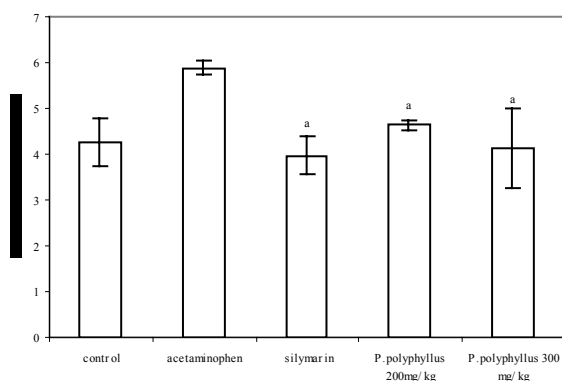


Fig. 1: Effect of *P. polyphyllus* on liver weight variation of acetaminophen induced hepatotoxicity in rats.

N = 6; Values are expressed as mean ± SEM

^aP<0.001 Vs Acetaminophen

Data were analyzed by using Student's 't' test

Histopathological studies, showed acetaminophen to produced extensive vascular degenerative changes and centrilobular necrosis in hepatocytes. Treatment with different doses of *P. polyphyllus* extract produced mild

degenerative changes and absence of centrilobular necrosis when compared with control (fig. 2). All these results indicate a hepatoprotective potential of the extract.

DISCUSSION

Acetaminophen (Paracetamol) a widely used antipyretic-analgesic drug produces acute hepatic damage on accidental overdosage. It is established that, a fraction of acetaminophen is converted via the cytochrome P₄₅₀ pathway to a highly toxic metabolite; N-acetyl-p-benzoquinamine (NAPQI) (Dahlin *et al.*, 1984) which is normally conjugated with glutathione and excreted in urine. Overdose of acetaminophen depletes glutathione stores, leading to accumulation of NAPQI, mitochondrial dysfunction (Parmar, and Kandakar, 1995) and the development of acute hepatic necrosis. Several P₄₅₀ enzymes are known to play an important role in APAP bioactivation to NAPQI. P₄₅₀ 2E1 have been suggested to be primary enzymes for acetaminophen bioactivation in liver microsomes (Raucy *et al.*, 1989). Studies demonstrated that acetaminophen induced hepatotoxicity can be modulated by substances that influence P₄₅₀ activity (Mitchell *et al.*, 1973).

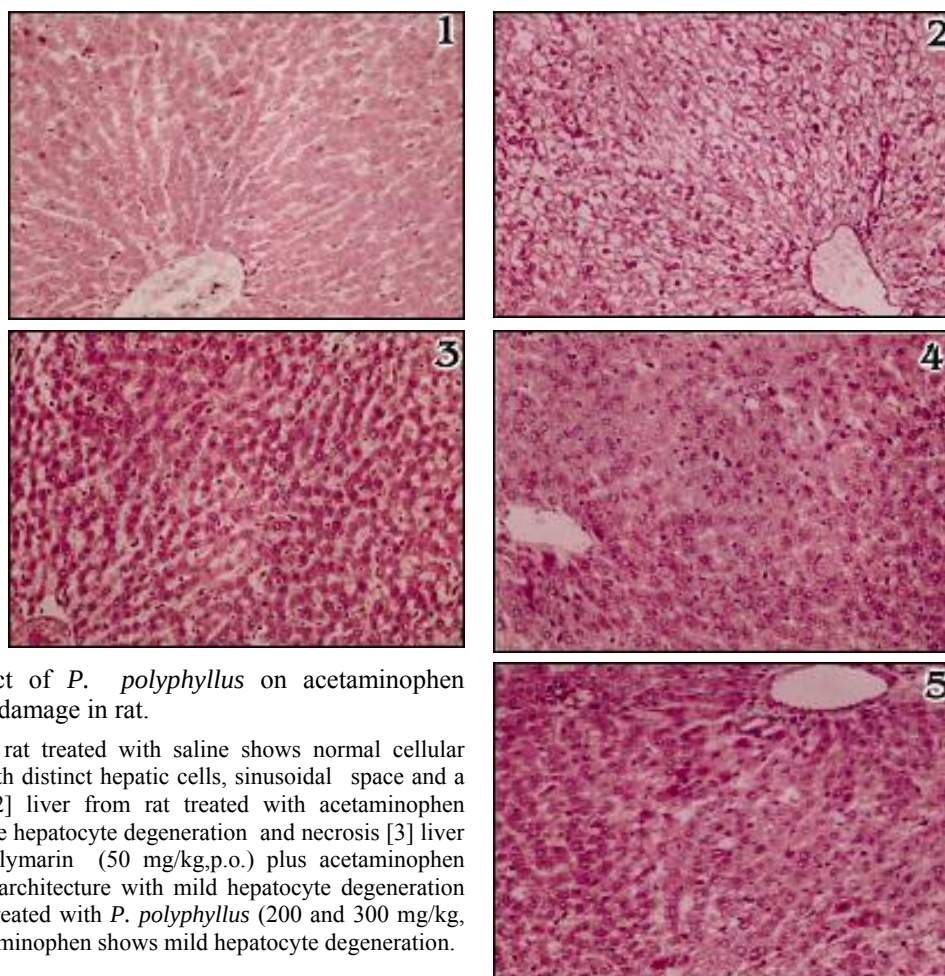


Fig. 2: Effect of *P. polyphyllus* on acetaminophen induced liver damage in rat.

[1] liver from rat treated with saline shows normal cellular architecture with distinct hepatic cells, sinusoidal space and a central vein [2] liver from rat treated with acetaminophen exhibited severe hepatocyte degeneration and necrosis [3] liver treated with silymarin (50 mg/kg,p.o.) plus acetaminophen shows normal architecture with mild hepatocyte degeneration [4 & 5] liver treated with *P. polyphyllus* (200 and 300 mg/kg, p.o.) plus acetaminophen shows mild hepatocyte degeneration.

Table 1: Effect of *Phyllanthus polyphyllus* on biochemical parameters in acetaminophen induced hepatotoxicity in rats.

Treatment	Dose (mg/kg)	AST U/L	ALT U/L	ALP U/L	Total bilirubin mg%	Total Protein mg%	GGTP U/L
Control	-	157.66 ± 4.6	74.2 ± 2.92	188.4 ± 3.16	0.8 ± 0.05	8.13 ± 1.4	26.01 ± 1.10
Acetaminophen	750	227.5 ± 6.8 ^a	176 ± 4.7 ^a	578 ± 8.9 ^a	1.1 ± 0.08 ^b	6.35 ± 0.35	62.1 ± 2.48 ^a
Silymarin	50	151.4 ± 6.63 ^c	89.2 ± 3.6 ^c	228.4 ± 5.42 ^{d, c}	0.72 ± 0.03 ^c	8.12 ± 0.56	35.3 ± 1.78 ^c
<i>P. polyphyllus</i> extract	200	217.5 ± 4.64 ^c	122 ± 2.5 ^{a, c}	414 ± 12.89 ^{a, c}	0.65 ± 0.06 ^c	9.22 ± 0.31	39.6 ± 1.08 ^{b, c}
	300	174.25 ± 6.73 ^a	107 ± 5.42 ^{b, c}	299.75 ± 11.89 ^{a, c}	0.7 ± 0.04 ^c	8.62 ± 0.96	31.5 ± 3.61 ^c

N=6; Values are expressed as mean ± SEM; ^aP< 0.001; ^bP< 0.01; ^dP< 0.05 Vs Control; ^cP< 0.001 Vs Acetaminophen
Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.

Table 2: Effect of *Phyllanthus polyphyllus* on antioxidants level in acetaminophen induced hepatotoxicity in rats.

Treatment	Dose (mg/kg)	LPO	SOD	Catalase	GPx	GST
Control	-	7.85 ± 0.92	24.61 ± 1.68	51.29 ± 1.67	38.75 ± 1.96	0.38 ± 0.05
Acetaminophen	50	17.17 ± 1.14 ^a	18.05 ± 1.45 ^b	36.24 ± 1.35 ^a	16.82 ± 2.10 ^a	0.09 ± 0.02 ^a
Silymarin	50	11.01 ± 0.87 ^d	22.36 ± 1.18	48.29 ± 1.92 ^d	33.14 ± 1.45 ^{c, d}	0.25 ± 0.03 ^c
<i>P. polyphyllus</i> extract	200	6.54 ± 0.51 ^d	20.72 ± 1.39 ^a	36.24 ± 2.10 ^a	28.52 ± 2.45 ^d	0.48 ± 0.004 ^d
	300	7.51 ± 1.38 ^d	23.48 ± 1.04	45.17 ± 2.10 ^c	32.64 ± 1.76 ^d	0.39 ± 0.01 ^d

N = 6; Values are expressed as mean ± SEM; ^aP< 0.001; ^bP< 0.05; ^cP< 0.01 Vs Control; ^dP< 0.001; ^eP< 0.05 Vs Acetaminophen

Data were analyzed by using one way ANOVA followed by Tukey multiple comparison test.

LPO = μ moles of MDA/ min/mg protein

SOD = Units/min/mg protein

CAT = μ mole of H₂O₂ consumed/ min/mg protein

GPx = μ moles of GSH oxidized/min /mg protein

GST = μ moles of CDNB conjugation formed/min /mg protein

In the assessment of liver damage by acetaminophen the determination of enzyme levels such as AST, ALT is largely used. Necrosis or membrane damage releases the enzyme into circulation and hence it can be measured in the serum. High levels of AST indicates liver damage, such as that caused by viral hepatitis as well as cardiac infarction and muscle injury, AST catalyses the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore ALT is more specific to the liver, and is thus a better parameter for detecting liver injury. Elevated levels of serum enzymes are indicative of cellular leakage and loss of functional integrity of cell membrane in liver (Drotman and Lawhan, 1978). Serum ALP, bilirubin and total protein levels on other hand are related to the function of hepatic cell. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure (Muriel and Garcipiana, 1992).

Administration of acetaminophen caused a significant (P<0.001) elevation of enzyme levels such as AST, ALT, ALP, GGTP, total bilirubin and decrease in total protein when compared to control. There was a significant

(P<0.001) restoration of these enzyme levels on administration of the leaf extract in a dose dependent manner and also by silymarin at a dose of 50 mg/kg. The reversal of increased serum enzymes in acetaminophen-induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (Thabrew and Joice, 1987). Effective control of ALP, bilirubin and total protein levels points towards an early improvement in the secretory mechanism of the hepatic cells.

The efficacy of any hepatoprotective drug is dependent on its capacity of either reducing the harmful effect or restoring the normal hepatic physiology that has been distributed by a hepatotoxin. Both silymarin and the plant extract decreased acetaminophen induced elevated enzyme levels in tested groups, indicating the protection of structural integrity of hepatocytic cell membrane or regeneration of damaged liver cells.

The increase in LPO level in liver induced by acetaminophen suggests enhanced lipid peroxidation leading to tissue damage and failure of antioxidant defense mechanism to prevent formation of excessive free radicals. Treatment with *P. polyphyllus* significantly reverses these changes. Hence it is likely that the mechanism of hepatoprotection of *P. polyphyllus* is due to its antioxidant effect.

Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in liver injury (Curtis and Mortiz, 1972). SOD has been reported as one of the most important enzymes in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. In *P. polyphyllus* causes a significant increase in hepatic SOD activity and thus reduces reactive free radical induced oxidative damage to liver.

Catalase (CAT) is an enzymatic antioxidant widely distributed in all animal tissues, and the highest activity is found in the red cells and liver. CAT decomposes hydrogen peroxide and protects the tissues from highly reactive hydroxyl radicals (Chance and Greenstein, 1992). Therefore reduction in the activity of CAT may result in a number of deleterious effects due to the assimilation of superoxide radical and hydrogen peroxide. A higher dose (300 mg/kg) increases the level of CAT as produced by silymarin, the standard hepatoprotective drug.

Glutathione is one of the most abundant tripeptide, non-enzymatic biological antioxidant present in the liver. It removes free radical species such as hydrogen peroxide, superoxide radicals and maintains membrane protein thiols. Also it is substrate for glutathione peroxidase (GPx) (Prakash et al, 2001). Decreased level of GSH is associated with an enhanced lipid peroxidation in acetaminophen treated rats. Administration of *P. polyphyllus* significantly ($P < 0.001$) increased the level of GPx and GST in a dose dependent manner.

Extensive vascular degenerative changes and centrilobular necrosis in hepatocytes was produced by acetaminophen. Treatment with different doses of methanolic extract of leaves of *P. polyphyllus* produced only mild degenerative changes and absence of centrilobular necrosis, indicating its hepatoprotective efficiency.

Free radical mediated process has been implicated in pathogenesis of most of the diseases. The protective effect of *P. polyphyllus* on acetaminophen induced hepatotoxicity in rats appears to be related to inhibition of lipid peroxidation and enhancement of antioxidant enzyme levels in addition to free radicals scavenging

action. Preliminary phytochemical studies reveal the presence of flavonoids in methanolic extract of *P. polyphyllus*. Flavonoids are hepatoprotectives (Seevola et al., 1984; Wegner and Fintelmann, 1999). The observed antioxidant and hepatoprotective activity of *P. polyphyllus* may be due to the presence of flavonoids. Further studies to characterise the active principles and to elucidate the mechanism are in progress.

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