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EVALUATION OF PROTECTIVE EFFECT OF *PHYLLANTHUS AMARUS* SEEDS ON PARACETAMOL INDUCED HEPATOTOXICITY AND NEPHROPROTECTIVE IN RATS

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ABSTRACT
To assess the hepatoprotective and nephroprotective effect of *Phyllanthus amarus* seeds extract on Paracetamol induced Hepatotoxicity and Nephrotoxicity. The antioxidant status of liver and kidneys were improved by substantiate protection of cellular damage induced by highly reactive toxic intermediates formed during biotransformation of paracetamol. In the present study test extract shown presence of polyphenolic compound and demonstrated antioxidant activity. The 70% methanolic extract 250 mg/kg of *Phyllanthus amarus* seeds have shown promising results as compared to aqueous extract of 300 mg/kg. Therefore the study concludes that the 70% methanolic extract of *Phyllanthus amarus* seeds have antioxidant and organ protective property. The results obtained in this study indicate that *Phyllanthus amarus* seeds possess hepatoprotective and nephroprotective activity. Methanolic extract effects were comparable to silymarin and cystone, and were highly significant, whereas aqueous extract were less potent compared standard drugs. Due to strong capability by reducing the enhanced levels of liver and kidney function blood parameters. Histopathological observation exhibited the improved hepatic and renal anatomy that is reversed the damage almost equal to standard drugs. Thus 70% metonolic extract of *Phyllanthus amarus* seeds possess a potent hepatoprotective and nephroprotective activity.

KEYWORDS: Hepatoprotective, Nephroprotective, Paracetamol, *Phyllanthus amarus.*

INTRODUCTION
The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver and kidney diseases by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization of herbal products and randomized placebo controlled clinical trials to support clinical efficacy.[1]

Liver diseases and kidney failure have become one of the major causes of morbidity and mortality in man and animals all over globe. Hepatotoxicity and nephrotoxicity due to drugs appear to be the most common contributing factors. Many investigators have turned to simpler experimental models for studying drug metabolism and response of the organ or liver cells to potentially toxic agents.[2] Conventional drugs used in the treatment of liver diseases are sometimes inadequate and can have serious side effects; therefore, it is necessary to search for alternative drugs for the treatment of live rand kidney diseases in order to replace currently used drugs of doubtful efficacy and safety.[3] Steroids vaccines and antiviral drugs that are employed as therapy for liver diseases have potential adverse effects especially when administered for long periods. There is worldwide trend for use of traditional herbal drugs for the treatment of liver and kidney diseases. Several plant sources have been found as potential hepato and nephroprotective agents with diverse chemical structures.

In the absence of a reliable liver and kidney protective drug in modern medicine, there are a number of medicinal preparations in Ayurveda recommended for the treatment of liver disorders. Due to severe undesirable side effects of synthetic agents, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the traditional herbal medicines that are claimed to possess hepatoprotective activity. A single drug cannot be effective for all types of severe liver diseases.[4] Drugs, diagnostic agents and chemicals are well known to be nephrotoxic. Nephrotoxic agents can produce damage

either by directly reacting with cellular macromolecules and membrane components or from metabolism with in the tubular cells to toxic products. These toxic metabolites mainly include free radicals.[5,6] Therefore an effective formulation has to be developed using indigenous medicinal plants, with proper pharmacological experiments and clinical trials.

The organ protective property was screened against paracetamol induced hepatotoxicity and induced nephrotoxicity model in rats, were used to assess the organ toxicity potential. The seeds of the plant were reported to possess lignans and related flavonoids which are known antioxidants, therefore, the *Phyllanthus amarus* Schum & Thonn (family: Euphorbiaceae) seeds was selected for assessing antioxidant and organ protective potential. However, till date no scientific evaluations are conducted on seeds for confirming its role as hepatoprotective and nephroprotective agent. Thus, the present study is designed to investigate the activities of *Phyllanthus amarus* seed extracts on hepatotoxicity and nephrotoxicity in rat models.

The extract of *P. amarus* seeds was subjected for the following studies

1. Acute toxicity studies
2. Evaluation of hepatoprotective activity (Standard Silymarin)

- Paracetamol-induced hepatotoxicity
- Liver biochemical markers:

SGOT, SGPT, ALP, Total and Direct bilirubin, cholesterol, triglyceroides

1. Evaluation of nephrotoprotective activity

- Paracetamol-induced nephrototoxicity (Standard Cystone)
- Body weight
- Kidney biochemical markers:
- Serum criatinine, Blood urea

MATERIALS AND METHODS

Plant material

The seeds of *Phyllanthus amarus* were collected from local gardens of Tirupati. The plant was identified and authenticated by Dr. Madhava Chetty, Department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh. A herbarium specimen is deposited in Nizam Institute of Pharmacy and Research Centre, Hyderabad, India.

Preparation of extracts

The crushed and dried seeds of *Phyllanthus amarus* were divided into two parts; one part was extracted successively with petroleum ether, benzene, chloroform and finally with methanol by soxhlet extraction and concentrated by rotary vacuum.[7] The other part is extracted by cold maceration process for aqueous extraction.[8] The obtained extracts were dried by evaporation. The yield 30% w/w and 15.6% w/w were stored in refrigerator and weighed quantities were suspended in tween 80 and 2% tragacanth solution respectively, for the experiment. The extracts were used for its hepatoprotective and nephroprotective activity.

Experimental Animals

The Wister rats, weighing 180-200g of either sex were used in this study. They were procured from Mahaveer Agencies, Hyderabad. The animals were allowed to acclimatize for 7 days under laboratory conditions, before the experiment.[9] They were housed in polypropylene cages and maintained at 25˚C ± 2˚C under 12 hours dark / light cycle. The rats had free access to standard pellet chow (Gold Mohar Lipton India Ltd.) and water *ad libitum* throughout the experiment. They were housed in a cage of six animals per cage and were renewed thrice a week to ensure hygiene and maximum comfort for animals. Ethical clearance for handling the animals was obtained from the Institutional Animals Ethical Committee (IAEC Registration No. 1330/ac/10/CPCSEA) prior to the beginning of the work.

Acute toxicity studies

Healthy adult Wister rats were subjected for oral toxicity study. The animals were overnight fasted and divided into two groups, each contains three animals. They were fed with 70% methanolic and aqueous extracts.[10] The animals were observed continuously for 2hours for behavioral, neurological and autonomic profiles and after 24 and 72 hours for any lethality.[11]

Experimental procedures

Table 1: Evaluation of hepatoprotective & nephroprotective Activity.

Groups	Administration	Hepatoprotective activity	Nephroprotective activity
Group I	Negative control	Tween 80 (1 ml/kg p.o.)	Tween 80 (1 ml/kg p.o.)
Group II	Positive control	Paracetamol 2 g/kg p.o.	Paracetamol 2 g/kg p.o.
Group III	Standard	Silymarin 100 mg/kg p.o.	Cystone 5 ml/kg p.o.
Group IV	Methanolic extarct	250 mg/kg p.o.	250 mg/kg p.o.
Group V	Aqueous extract	300 mg/kg p.o.	300 mg/kg p.o.

Hepatoprotective activity

Wister rats were randomly assigned into 5 groups of 6 individuals each the study has carried out for 9 days.[12]

Group-I: Animals (-ve control) were administered tween 80 (1 ml/kg p.o).

Group-II: Animals (+ve control) were administered Paracetamol 2 g/kg p.o.

Group-III: Animals (Standard) were administered with Silymarin 100 mg/kg p.o.

Group-IV: Animals were administered with methanolic extract 250 mg/kg p.o.
Group-V: Animals were administered with aqueous extract 300 mg/kg p.o.

On 9th day, after 30 min of vehicle, 100 mg/kg Silymarin, 250 mg/kg 70% methanolic extract and 300 mg/kg aqueous extract of *Phyllanthus amarus* seeds administration to Group-II, III, IV and V respectively, received Paracetamol 2 g/kg p.o., which was prepared in tween 80 (2% solution). Food was withdrawn 12 hours before paracetamol administration to enhance the acute liver damage in animals of group II, III, IV and V. The rats were sacrificed under mild ether anesthesia.

Blood samples were collected for evaluating the serum biochemical parameters and liver was dissected out, blotted off blood, washed with saline and stored in 10% formalin and preceded for histopathology to evaluate the details of hepatic architecture in each group microscopically. The blood so collected was centrifuged immediately to get clear serum and was subjected to various biochemical studies.

Nephroprotective activity

Five groups of six rats in each were used in this model. They were weighed individually on first and eighth day of treatment.[13]
Group-I: Animals (-ve control) were administered 1ml tween 80 p.o.
Group-II: Animals (+ve control) were administered with Paracetamol 2 g/kg p.o.
Group-III: Animals (Standard) were administered with cystone 5 ml/kg p.o.
Group-IV: Animals were administered with methanolic extract 250 mg/kg p.o.
Group-V: Animals were administered with aqueous extract 300 mg/kg p.o.

Group I received only normal saline throughout the course of the experiment was used as control. Group II animals received daily Paracetamol 2 g/kg p.o., for eight days. This dose has already been shown to produce nephrotoxicity. The animals of group III received Paracetamol 2 g/kg p.o., with cystone 5 ml/kg p.o., nearly for eight days. The animals of group IV received Paracetamol 2 g/kg p.o., nearly for eight days in addition to this they also received 250 mg/kg of *Phyllanthus amarus* seeds methanolic extract orally. Group V animals were given Paracetamol 2 g/kg p.o., for eight days in addition to this they also received 300 mg/kg of *Phyllanthus amarus* seeds aqueous extract orally.

This was started three days prior to the Paracetamol 2 g/kg p.o., and continues with eight days paracetamol treatment. At the end, animals were sacrificed under mild ether anaesthesia and blood samples were collected and assessed.

Blood sample collection

Blood samples of the animals were collected from retro orbital plexus from the inner canthus of the eye under mild ether anesthesia, using capillary tubes before the animals were sacrificed. The plasma was separated in a T8 electric centrifuge at 2000 rpm for 2 minutes assessment and then analysed for hepatoprotective and nephroprotective biochemical parameters[14] using the standard procedures prescribed in the enzymatic kits (MEDES LIMITED, U. K.)

Statistical analysis

Results were expressed as mean ± SEM, (n=6). Statistical analysis was performed with one way analysis of variance (ANOVA), followed by Turkey-Kramer multiple comparisons test by using Graph Pad Instat software. P value less than 0.05 was considered to be statistically significant. *p<0.05, **<0.01 and ***<0.001, when compared with control and toxicant group as applicable.

RESULTS AND DISCUSSION

There was increased level of SGPT observed in paracetamol treated group (299.58 U/L). Which was restored to 101.56 U/L by 250 mg/kg 70% methanolic extract of the seeds as that of 100mg/kg silymarin i.e., 98.33 U/L. SGOT levels increased significantly in paracetamol treated group i.e., 403.35 U/L. 250 mg/kg 70% methanolic extract of the seeds reduced to 108.56 U/L, which was very near to 100 mg/kg silymarin effect of 106.13 U/L. In case of the total and direct bilirubin, 250 mg/kg 70% methanolic extract reduced the elevated levels of total and direct bilirubin levels by i.e. from 2.38 mg/dl and 0.61 mg/dl to 1.03 mg/dl and 0.26 mg/dl respectively. The effect of 250 mg/kg 70% methanolic extract had better than100 mg/kg standard silymarin. There was no significant rise in total cholesterol and triglyceride levels in paracetamol treated group. 250 mg/kg 70% methanolic extract was almost same as that of standard 100 mg/kg silymarin. There was increase in ALP level observed in paracetamol treated group (468.73 IU/L). The level was restored to 149.70 IU/L by 250 mg/kg 70% mthanolic extract of the seeds which was near to effect of 100 mg/kg silymarin i.e., 145.45 IU/L. Increased biochemical parameters were restored significantly by 250 mg/kg 70% mthanolic extract which shows that the seeds have hepatoprotective activity.

Paracetamol induced kidney damage shown very high decrease of body weight in paracetamol treated animals (-10.31%). However, there was equal decrease in body weights in animals treated with aqueous extract 250 mg/kg -7.17%. 70% methanolic extract showed decrease in body weight by -4.12%. When compared to standard cystone 5ml/kg i.e., -1.14%. Blood urea nitrogen level increased in paracetamol treated group i.e. 59.68 mg/dl. 250 mg/kg 70% methanolic and aqueous extracts of seeds restored the elevated level to 30.06 mg/dl and 47.16 mg/dl, respectively, with compared with the standard cystone 5 ml/kg i.e. 28.01 mg/dl. Serum

creatinine level also increased in paracetamol treated group to 1.74 mg/dl. However, 250 mg/kg 70% methanolic extract of seeds reduced the elevated level to 0.96 mg/dl with that of standard cystone 5 ml/kg to 0.95 mg/dl. Toxicant group have shown significant decrease in body weight, 70% methanolic extract of seeds was restored back to normal levels. Also the increased creatinine and urea was restored back to normal. Hence, the results show that the seeds possess hepatoprotective and nephroprotective activities.

Table 2: Effect of 70% methanolic extract of *Phyllanthus amarus* seeds on biochemical markers in Paracetamol induced hepatotoxicity.

Treatment	Biochemical parameters Mean ± SEx						
Groups	**SGPT U/L**	**SGOT U/L**	**Total Bilirubin mg/dl**	**Direct Bilirubin Mg/dl**	**Total Cholesterol Mg/dl**	**ALP U/L**	**Triglycerides mg/dl**
Negative control (1ml vehicle)	75.78 ±2.818	87.38 ± 6.121	0.93 ± 0.047	0.28 ± 0.023	112.74 ± 3.585	129.68± 5.013	176.94± 3.667
Positive control (Paracetamol) + Tween 80 (1:1) 2mg/kg, p.o.,	299.58 ± 10.722	403.35 ± 8.667	2.38 ± 0.116	0.61 ± 0.070	190.54 ± 11.128	469.35± 7.218	215.87± 10.264
Paracetamol + Std (Silymarin) 2mg/kg, p.o., + 100 mg/kg, p.o	98.33 ± 4.043***	106.13 ± 6.927***	1.05 ± 0.061***	0.29 ± 0.030***	125.28 ± 5.656***	146.62± 7.408*	174.37± 9.903**
Paracetamol + 70% meth. extract 2mg/kg, p.o., + 250 mg/kg, p.o	134.67 ± 1.069***	141.49 ± 1.198**	1.19 ± 0.060***	0.47 ± 0.013	156.08 ± 0.954**	188.78± 1.108**	191.63± 0.595
Paracetamol + aqueous extract 2mg/kg, p.o., + 300 mg/kg, p.o	101.53 ± 0.667***	108.56 ± 1.134**	1.03 ± 0.025***	0.26 ± 0.016***	129.31 ± 0.981**	149.70± 0.905**	177.49± 0.815*

Values are the x ± SEM of six rats / treatment
*Significance *p<0.05, **p<0.01 and ***p<0.001 compared to Paracetamol treatment*

Table 3: Effect of 70% methanolic extract of *Phyllanthus amarus* seeds on Paracetamol induced renal damage in rats.

Treatment	Change in b.w (%)	Blood urea (mg/dl)	Serum creatinine (mg/dl)
Negative control (1ml vehicle)	5.44 ± 0.583	27.348 ± 2.946	0.93± 0.065
Positive control Paracetamol + Tween 80 (1:1) 2mg/kg, p.o.,	-10.31 ± 1.03	59.68 ± 4.205	1.74 ± 0.082
Paracetamol + Standard (cystone) 2mg/kg, p.o., + 5 ml/kg, p.o	-1.14 ± 0.546***	28.01 ± 1.782**	0.95 ± 0.015**
Paracetamol + 70% meth. extract 2mg/kg, p.o., + 250 mg/kg, p.o	-4.12 ± 0.505***	30.06 ± 0.782*	0.96 ± 0.025*
Paracetamol + aqueous extract 2mg/kg, p.o.. + 300 mg/kg, p.o	-7.17 ± 1.964*	47.16 ± 0.884	1.18 ± 0.075

Values are x ± SEM of six rats / treatment
*Significance **P<0.01 and ***P<0.001 (vs. Control). b.w. – Body weight*

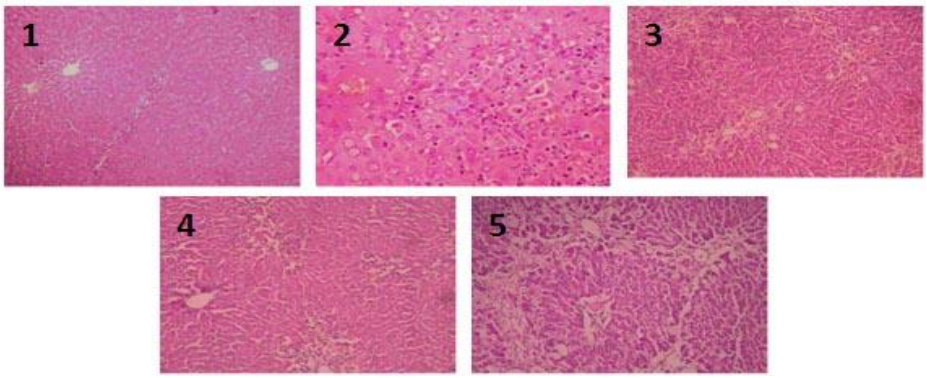

Figure 1: Histopathological studies in paracetamol induced hepatotoxicity.

Figure 1
1: Normal Group: Sections studied show structure of liver. Architecture is normal. Central vein, sinusoids and hepatocytes are normal.

2: Paracetamol Group: Sections showed marked necrosis and inflammatory cell infiltration in the centrizonal area, inflammatory cells were also observed in the portal triad. Development of fibrosis septae was observed between central veins and portal traid.

3: Silymarin Group: Sections studied shows structure of liver .The architecture is maintained. There is mild congestion and portal triaditis and inflammatory cells were observed in the centrizonal area.
4: Methonolic Group: Sections studied showed greater reduction of the necrosed area and sparse inflammatory cell infiltration around the central vein showing regeneration of hepatocytes.
5: Aqueous Group: Sections studied features of mild necrosis with mild congestion and reduced necrosis area around central veins).

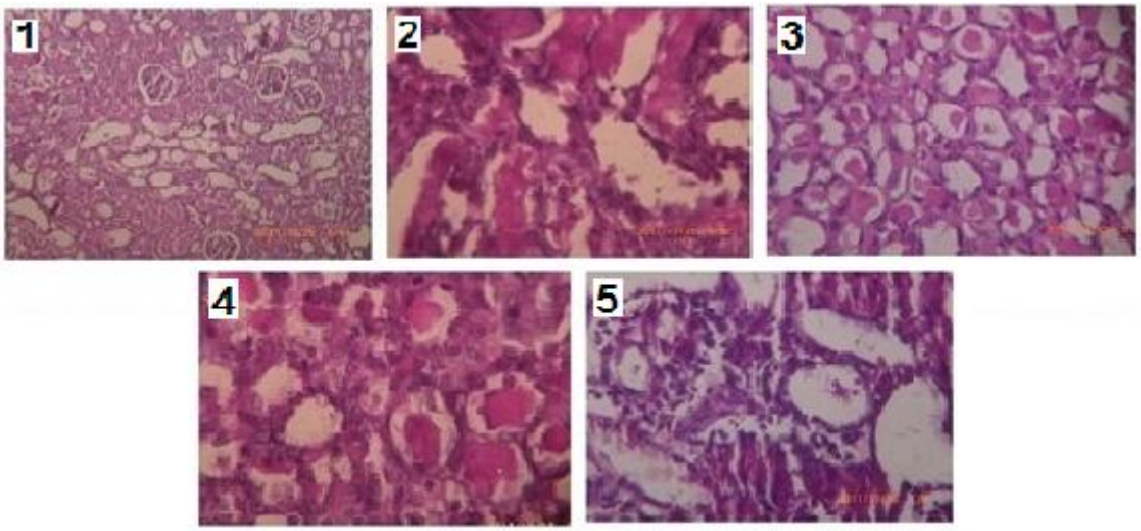

Figure 2: Histopathological studies in paracetamol induced nephrotoxicity.

Figure 2
1: Normal Group: Normal control group showed structure of kidney with normal glomeruli, proximal and distal tubules with normal interstitium and blood vessels.
2: Paracetamol Group: (Massive total necrosis). Paracetamol treated group showed structure of kidney with glomerular congestion. Interstitium showed scant lymphatic infiltration with inflammatory cells and interstitial total necrosis.
3: Cystane Group: (Interstitial Inflammation). In the case of 5 ml/kg cystone treated group showed structure of kidney with normal glomeruli, proximal and tubules with interstitium showing few lymphocytes.
4: Methonolic Group: (Residual Interstitial Nephritis). In the case of 250 mg/kg methanolic extract group showed structure of kidney with normal glomeruli, proximal and distal tubules with normal blood vessels with mild interstitial nephritis.
5: Aqueous Group: (Glomerular Congestion). In the case of 300 mg/kg aqueous extract group showed structure of kidney with glomerular congestion and mild tubular nephritis.

CONCLUSION

Many antioxidants have been used to protect the organs from these free radical challenges. Keeping in view of its antioxidant and organ protective activities, the present research work is made by use of Wister rats to assess the influence of pre-treatment with extracts of *Phyllanthus amarus* seeds. Liver and kidney functions were assessed by collecting the blood samples from each group and evaluating the biochemical parameters. Histopathological studies were done by isolating the liver and kidney of all the groups. Treatment with 70% ethanolic extract has brought back the elevated levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), bilirubin, protein, serum cholesterol and serum triglycerides in paracetamol induced hepatotoxicity in rats to near normal levels. Whereas 70% methanolic extract prevented the reduction in body weight and reduced the levels of total urea and serum creatinine in paracetamol induced nephrotoxicity.

Histopathological observation exhibited the improved hepatic and renal anatomy that is reversed the damage almost equal to standard drugs. Thus 70% methanolic extract of *Phyllanthus amarus* seeds possess hepatoprotective and nephroprotective activity.

In the present study test extract shown presence of polyphenolic compound and demonstrated antioxidant activity. The 70% methanolic extract 250 mg/kg of *Phyllanthus amarus* seeds have shown promising results as compared to other extracts. Therefore the study concludes that the 70% methanolic extract of *Phyllanthus amarus* seeds have antioxidant and organ protective property. The results obtained in this study indicate that *Phyllanthus amarus* seeds possess hepatoprotective and nephroprotective activity. Methanolic extract effects were comparable to silymarin and cystone, and were highly significant, whereas aqueous extract were less potent compared standard drugs.

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