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Evaluation of Antioxidant and Hepatoprotective Activity of *Phyllanthus Virgatus* Leaves.

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ABSTRACT

Evaluate Antioxidant and hepatoprotective activity of the plant was “CCL₄ and Paracetamol Induced Hepatotoxicity” and the following steps were carried out. Acute toxicity study. Hepatoprotective activity. Estimation of biochemical parameters. Histopathological studies. This study aimed on evaluating antioxidant and hepatoprotective activity of *Phyllanthus virgatus*. It showed marked decrease in hepatotoxic effect of CCL₄ and Paracetamol which was evident by study of biochemical parameters. It is observed that methanolic extract of bark of *Phyllanthus virgatus* possesses hepatoprotective property which was evident by physical and biochemical parameters and histopathological reports.

Keywords: *Phyllanthus virgatus*, Silymarin, Antioxidant activity, Hepatoprotective activity.

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INTRODUCTION

Liver diseases are among the important diseases affecting mankind. According to WHO about 18,000 people die every year due to liver diseases. The common ailments of liver are cirrhosis, cholestasis, hepatitis, portal hypertension, hepatic encephalopathy, fulminant hepatic failure and certain tumors like hepatoma. It is estimated that two billion people around the world are infected with hepatitis B. About 350 million of these have the chronic form of the disease. This alarming statistics with perplexing report, warrant the immediate necessity of studies of any level to either ensure the effectiveness of available formulations or exploration of the new herbal therapies to reduce the morbidity and mortality rate due to hepatic complications.

In modern medicine cortico steroids and immunosuppressants are commonly used to treat liver disease in allopathic form of medicine. But, these drugs are associated with adverse effects such as immunosuppression and bone marrow depression. Further, the success rate of treating liver diseases is disappointing. Attempts are being made globally to get scientific evidences for this traditionally reported herbal drugs [1]. In view of severe undesirable side effects of synthetic agents and absence of reliable liver protective drugs in the modern medicine, there is growing focus to follow systematic research methodology and to evaluate scientific basis for the use of traditional herbal medicines which are claimed to possess hepatoprotective activity. About 70-80% of the world populations rely on the use of traditional medicine, which is predominantly based on plant materials. The traditional medicine refers to a broad range of natural health care practices including Ayurveda, Siddha, Homeopathy and Unani. About 600 commercial preparations with claimed liver protecting activity are available all over the world. About 100 Indian medicinal plants belonging to 40 families are used for herbal formulation. A few reports on the hepatoprotective activity are cited here, e.g. *Apium graveolens* Linn. (Umbelliferae), *Boerhaavia diffusa* Linn. (Nyctagina ceae), *Euphorbia antisyphilitica* (Euphorbiaceae), *Rubia cordifolia* (Rubiaceae), *Solanum lyratum* (Solanaceae), *Tylophora indica* (asclepiadaceae) [2].

MATERIAL AND METHODS

Extraction [3]:

The authenticated whole of *Phyllanthus virgatus* leaves were dried in shade and powdered coarsely. Extraction was done according to standard procedure using analytical grade solvents. The coarse powder of the leaves was Soxhlet extracted with the solvents with increasing order of polarity i.e. petroleum ether (60-80°C), chloro form (59.5-61.5°C), ethanol (64.5-65.5°C), and distilled water. After defating with petroleum ether, Methanolic extract was also prepared. The extracts so obtained were concentrated under reduced pressure.

In addition the shade-dried powder was extracted directly with Methanol (hydro-alcoholic) extract which was used for pharmacological investigations after subjecting it to preliminary qualitative photochemical studies. The extracts were concentrated under reduced pressure and stored in desiccators until further use and the percentage yield of corresponding extracts were calculated.

Animals

Albino wistar rats of either sex weighing between 150 to 200 gms and albino mice of either sex weighing between 20 to 25 gms. Animals were housed under standard conditions of temperature (25 ± 2°C) and relative humidity (30-70%) with a 12:12 light-dark cycle. The animals were fed with standard pellet diet and water *ad libitum*. Approved at the Institutional Animal Ethics Committee (IAEC) was taken for conducting antioxidant and hepatoprotective activities.

Acute Toxicity Studies

Acute toxicity studies for, Methanolic extracts of *Phyllanthus viragatus* leaves belonging to the family 'Euphorbiaceae' were conducted as per OECD using Albino Swiss mice. Each animal was administered Methanolic extracts solution of the extract by oral route. The test procedure minimizes the number of animals required to estimate the oral acute toxicity of a chemical and in addition estimation of LD₅₀, confidence intervals. The test also allows the observation of signs of toxicity and can also be used to identify chemicals that are likely to have low toxicity.

Experimental Schedule

Antioxidant activity

In-vivo Antioxidant Activity

An attempt is made to assess the influence of pre-treatment with 70% ethanolic extract of *Begonia malabarica* leaves on the levels of Glutathione and lipid peroxidation *in-vivo* in both CCl₄ and paracetamol induced hepatotoxicity.

- a) Glutathione (GSH) and lipid peroxidation estimation in CCl₄ induced hepatotoxicity in albino rats of either sex.
- b) Glutathione (GSH) and lipid peroxidation estimation in paracetamol induced hepatotoxicity in albino rats of either sex.

Estimation of Glutathione and Lipid peroxidation

Glutathione is present in all types of living cells. Tissue such as mammalian liver normally contains high levels of reduced Glutathione. It has been suggested that GSH protects thiol groups in protein from oxidation, functions as an intracellular redox buffer and serves as a reservoir of cysteine [4]. The role of GSH in determining the extent of liver damage has been demonstrated in experiments where the hepatic concentration of GSH is altered by toxin treatments. Depletion of GSH contents has been reported to potentiate hepatic necrosis and covalent binding of toxic metabolites to cellular macromolecules [5]. Lipid peroxidation is accepted to be one of the principal causes of CCl₄ induced liver injury and is mediated by the production of free radical derivatives of CCl₄ [6].

Estimation of GSH

Tissue samples were homogenized in ice cold Trichloroacetic acid (1 gm tissue plus 10 ml 10% TCA) in an ultra turrax homogenizer. Glutathione measurement was performed using a modification of the **Ellman procedure (Aykae, et. al.) [7]**. Briefly, after centrifugation at 3000 rpm for 10 minutes, 0.5 ml supernatant was added to 2 ml of 0.3 M disodium hydrogen phosphate solution. A 0.2 ml solution of dithiobis nitrobenzoate (0.4 mg/ml in 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. Percentage increase in OD is directly proportional to the increase in the levels of Glutathione. Hence, % increase in OD is calculated.

Estimation of lipid peroxidation

The degree of lipid peroxide formation was assayed by monitoring thiobarbituric reactive substances formation. Stock solution of TCA-TBA-HCl reagent: 15% w/v trichloroacetic acid; 0.375% thiobarbituric acid; 0.25 N hydrochloric acid. This solution may be mildly heated to assist in the dissolution of the thiobarbituric acid.

Combine 1 ml of biological sample (0.1-0.2 mg of membrane protein) with 2 ml of TCA-TBA-HCL and mix thoroughly. The solution is heated for 15 min in a boiling waer bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the sample is determined at 535 nm against a blank that contains all the reagents minus the lipid [8].

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Hepato-Protective Activities

Evaluation of hepato-protective activity in CCl₄ inducedhepatotoxicity.

The method of **Suja SR. and et al.** was followed [9]

In the dose response experiment, albino rats were randomly assigned into 5groups of 6 animals in each groups.S

- Group-I Animals (-ve Control) were administered 1ml distilled water p.o, for 5 days.
- Group-II Animals (+ve Control) were administered 1ml distilled water p.o, for 5 days.
- Group-III Animals were administered with silymarin 100mg/kg p.o. or 5 days.
- Group-IV Animals were administered with M ethanolic extract 200 mg/kg p.o, for 5 days.
- Group-V Animals were administered with methanolic extract 00 mg/kg p.o, for 5 days.

Evaluation of Hepato-protective activity in paracetamol- induced Hepatotoxicity

The method of R.R. Chattopadhyay was followed.

In the dose response experiment, albino rats were randomly assigned into 5 groups of 6 animals in each group.

- Group-I Animals (-ve Control) were administered normal saline 1ml/kg p.o, for 7 days.
- Group-II Animals (+ve Control) were administered normal saline 1ml/kg p.o, for 7 days.
- Group-III Animals were administered with silymarin 100mg/kg p.o., for 7 days.
- Group-IV Animals were administered with Methanolic extract 100 mg/kg p.o, for 7 days
- Group-V Animals were administered with Methanolic extract 200 mg/kg p.o, for 7 days.

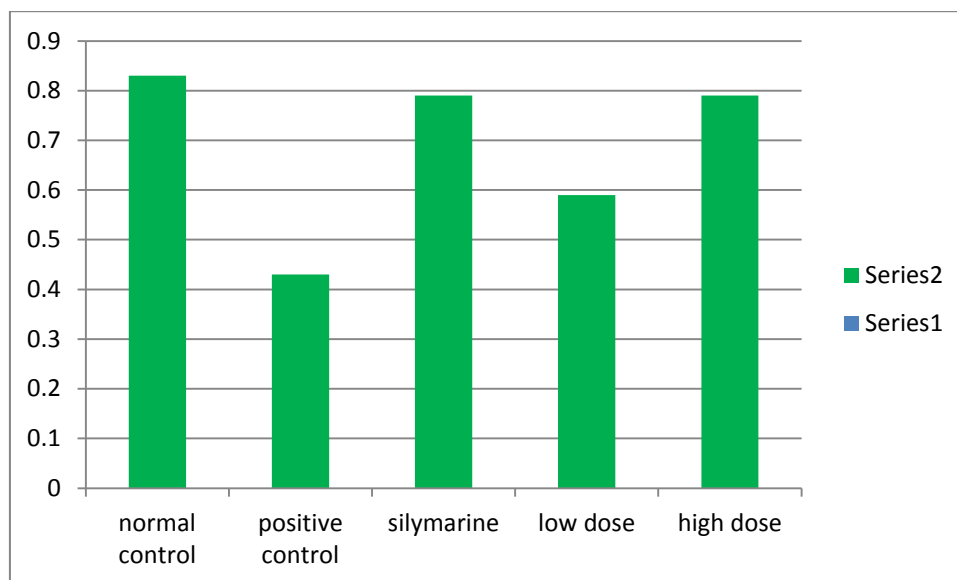
RESULTS

Table 1: Effect of 70% Methanolic extract of *Phyllanthus viragatus* leaves on tissue GSH levels in CCl₄ induced hepatotoxicity

Treatment	Absorbance Mean ± SEM	% Increase
Normal Control (1ml vehicle)	0.832 ± 0.02	
CCl ₄ (2ml/kg s.c)	0.436 ± 0.009	48.56
CCl ₄ + Silymarin (2ml/kg s.c. + 100 mg/kg p.o)	0.799 ± 0.03 ^{***}	82.60
CCl ₄ + Methanolic extract (2ml/kg s.c. + 100 mg/kg p.o)	0.591 ± 0.01 ^{**}	36.40
CCl ₄ + Methanolic extract (2ml/kg s.c. + 200 mg/kg p.o)	0.798 ± 0.02 ^{***}	83.98

Values are the mean ± S.E.M. of six rats/treatment.
Significance ***P< 0.001, **P< 0.01, *< 0.05, compared to CCl₄ treatment.

Fig. 1: Effects of Methanolic extract of *Phyllanthus viragatus* leaves on in vivo GSH in CCl₄ induced hepatotoxicity.



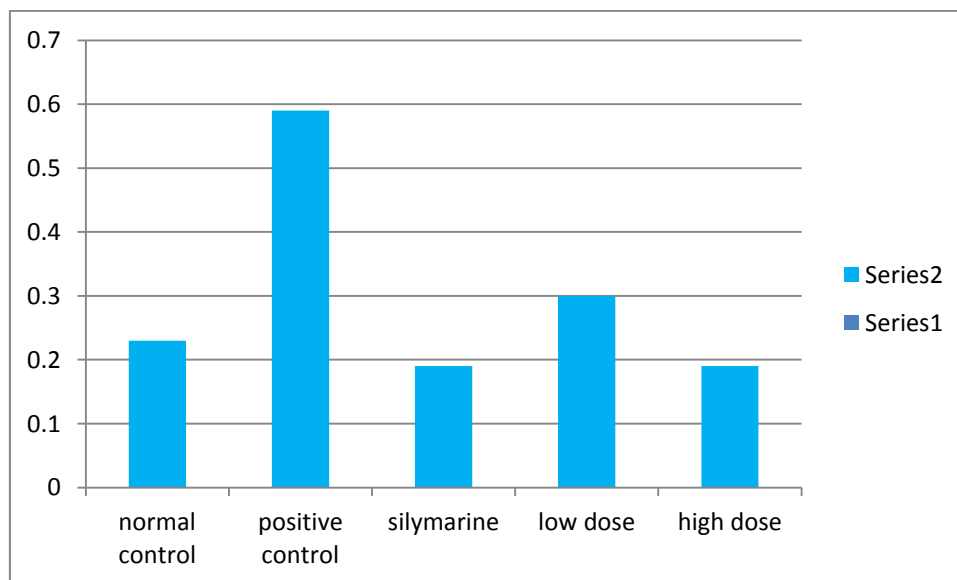
A: Normal Control (1 ml vehicle)
 B: CCl₄ (2 ml/kg s.c.)
 C: CCl₄ + Silymarin (2 ml/kg s.c. + 100 mg/kg p.o)
 D: CCl₄ + 70% Methanolic extract (2 ml/kg s.c. + 100 (mg/kg p.o)
 E: CCl₄ + 70% Methanolic extract (2 ml/kg s.c. + 200 (mg/kg p.o)

Table 2: Effect of Methanolic extract of *Phyllanthus viragatus* leaves on tissue lipid peroxidation levels in CCl₄ induced hepatotoxicity.

Treatment	Absorbance Mean ± SEM	% Increase
Normal Control (1ml vehicle)	0.232 ± 0.13	
CCl ₄ (2ml/kg s.c)	0.593 ± 0.10	
CCl ₄ + Silymarin (2ml/kg s.c. + 100 mg/kg p.o)	0.199 ± 0.007 ^{***}	66.44
CCl ₄ + methanolic extract (2ml/kg s.c. + 100 mg/kg p.o)	0.300 ± 0.006 [*]	49.40
CCl ₄ + methanolic extract (2ml/kg s.c. + 200 mg/kg p.o)	0.194 ± 0.005 ^{***}	67.28

Values are the mean ± S.E.M. of six rats/treatment.
Significance ***P < 0.001, **P < 0.01, * < 0.05, compared to CCl₄ treatment

Fig. 2 : Effects of Methanolic extract of *Phyllanthus viragatus* leaves on in vivo lipid peroxidation in CCl₄ induced hepatotoxicity



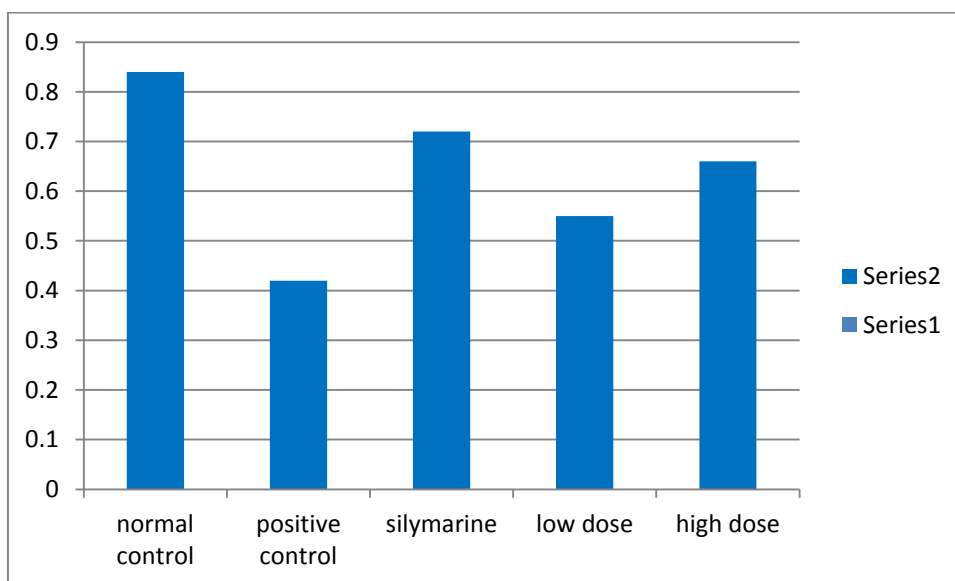
A: Normal Control (1 ml vehicle)
B: CCl₄ (2 ml/kg s.c.)
C: CCl₄ + Silymarin (2 ml/kg s.c. + 100 mg/kg p.o)
D: CCl₄ + Methanolic extract (2 ml/kg s.c. + 100 mg/kg p.o)
E: CCl₄ + Methanolic extract (2 ml/kg s.c. + 200 mg/kg p.o)

Table 3: Effect of Methanolic extract of *Phyllanthus viragatus* leaves on tissue GSH levels in Paracetamol induced hepatotoxicity

Treatment	Absorbance Mean \pm SEM	Percentage Increase
Normal Control (1ml vehicle)	0.849 \pm 0.01	-
Paracetamol Treated (2ml/kg s.c)	0.421 \pm 0.007	-
Paracetamol + Silymarin (2ml/kg s.c. + 100 mg/kg p.o)	0.723 \pm 0.005 ***	71.73
Paracetamol + Methanolic extract (2ml/kg s.c. + 100 mg/kg p.o)	0.552 \pm 0.005 **	26.36
Paracetamol + Methanolic extract (2ml/kg s.c. + 200 mg/kg p.o)	0.666 \pm 0.004 ***	58.19

Values are the mean \pm S.E.M. of six rats/treatment.
Significance ***P < 0.001, **P < 0.01, * < 0.05, compared to Paracetamol treatment.

Fig.3 : Effects of Methanolic extract of *Phyllanthus viragatus* leaves on in vivo GSH levels in paracetamol induced hepatotoxicity



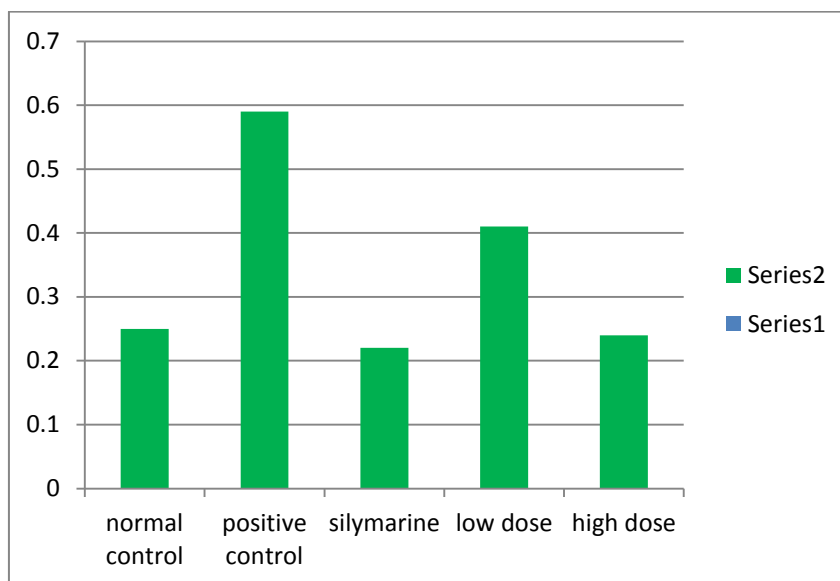
A: Normal Control (1 ml vehicle)
 B: Paracetamol treated (2 gm/kg p.o.)
 C: Paracetamol + Silymarin (2 gm/kg p.o. + 100mg/kg p.o.)
 D: Paracetamol + Methanolic extract (2 gm/kg p.o. + 100mg/kg p.o.)
 E: Paracetamol + Methanolic extract (2 gm/kg p.o. + 200mg/kg p.o.)

Table.4: Effect of Methanolic extract of *Phyllanthus viragatus* leaves on tissue lipid peroxidation levels in Paracetamol induced hepatotoxicity;

Treatment	Absorbance Mean \pm SEM	Percentage Increase
Normal Control (1ml vehicle)	0.240 \pm 0.029	-
Paracetamol Treated (2ml/kg s.c)	0.594 \pm 0.026	-
Paracetamol + Silymarin (2ml/kg s.c. + 100 mg/kg p.o)	0.225 \pm 0.010 ^{***}	62.12
Paracetamol + Methanolic extract (2ml/kg s.c. + 100 mg/kg p.o)	0.414 \pm 0.012 ^{**}	30.30
Paracetamol + Methanolic extract (2ml/kg s.c. + 200 mg/kg p.o)	0.245 \pm 0.024 ^{***}	58.75

Values are the mean \pm S.E.M. of six rats/treatment.
 Significance ***P< 0.001, **P< 0.01, *< 0.05, compared to Paracetamol treatment.

Fig. 4: Effects of Methanolic extract of *Phyllanthus viragatus* leaves on lipid peroxidation levels in paracetamol induced hepatotoxicity



A: Normal Control (1 ml vehicle)
 B: Paracetamol treated (2 gm/kg p.o.)
 C: Paracetamol + Silymarin (2 gm/kg p.o. + 100mg/kg p.o.)
 D: Paracetamol + Methanolic extract (2 gm/kg p.o. + 100mg/kg p.o.)
 E: Paracetamol + Methanolic extract (2 gm/kg p.o. + 200mg/kg p.o.)

Table.5: Effect of Methanolic extract of *Phyllanthus viragatus* leaves on biochemical markers in CCl₄ induced hepatotoxicity:

Treatment	SGPT U/L	SGOT	Total Bilirubine	Directbilirubine mg/DL	Total Cholesterol mg/DL	HDL MG/DL
Negative control	64.23± 23.98	78.89± 4.23	0.99±0.06	0.26±0.03	112.34± 3.59	7.87± 0.89
Positive control 2ml/kg s.c	328.18± 7.72	225.19± 6.67	4.54±0.28	1.49±0.16	168.38± 8.89	4.89± 0.32
Silymarine 100mg/kg	99.16± 7.56***	99.75± 12.67***	1.39±0.04***	0.41±0.03***	116.59± 4.59***	6.89± 0.19***
Low dose 100mg/kg	198.18± 6.76***	155.78± 9.45***	2.78±0.27***	0.88±0.05***	139.68± 7.16***	5.46± 0.22***
High dose 200mg/kg	119.67± 6.87***	110.12± 7.70***	1.49±0.19***	0.49±0.05***	117.16± 8.32***	6.55± 0.22***

Fig.5 ; Effect of Methanolic extract of *Phyllanthus viragatus* leaves on hepatic enzymes in CCl₄ induced hepatotoxicity

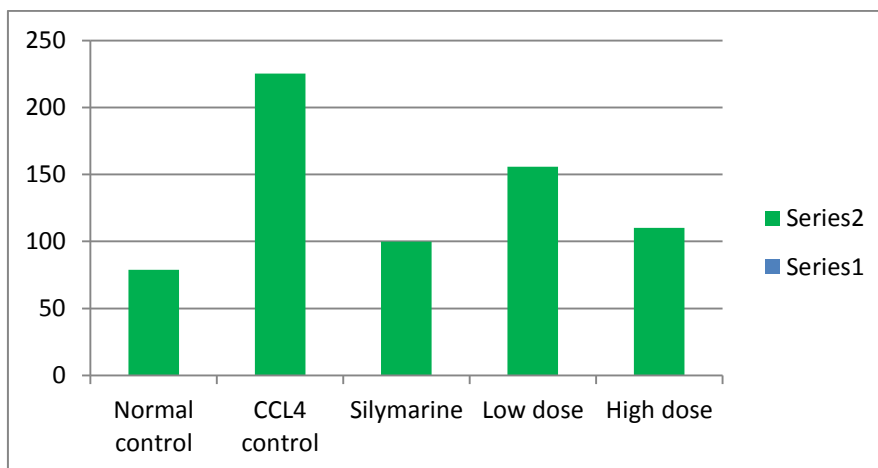


Fig. 6. Total Bilirubine Mg/Dl

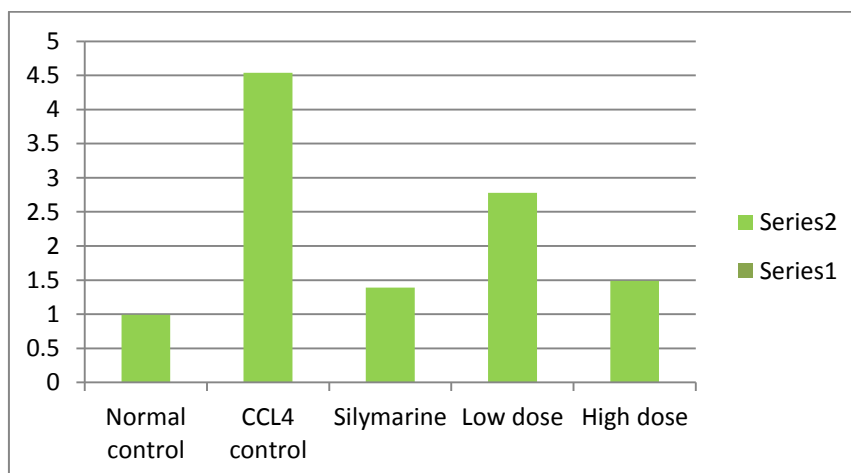


Fig.7. Direct Bilirubine Mg/Dl

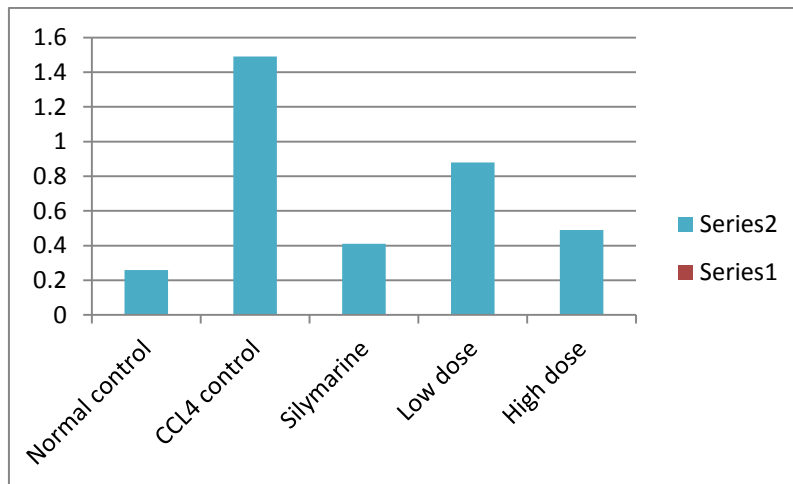


Fig.8 Total Cholesterol Mg/Dl

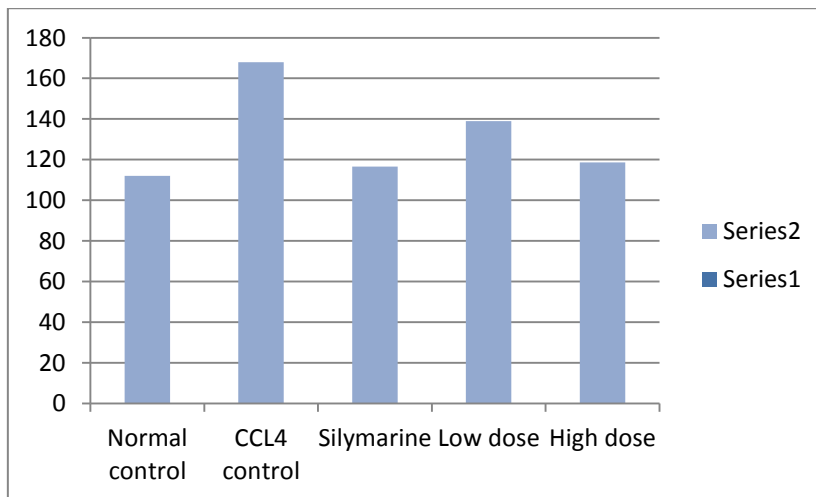


Fig.9. HDL

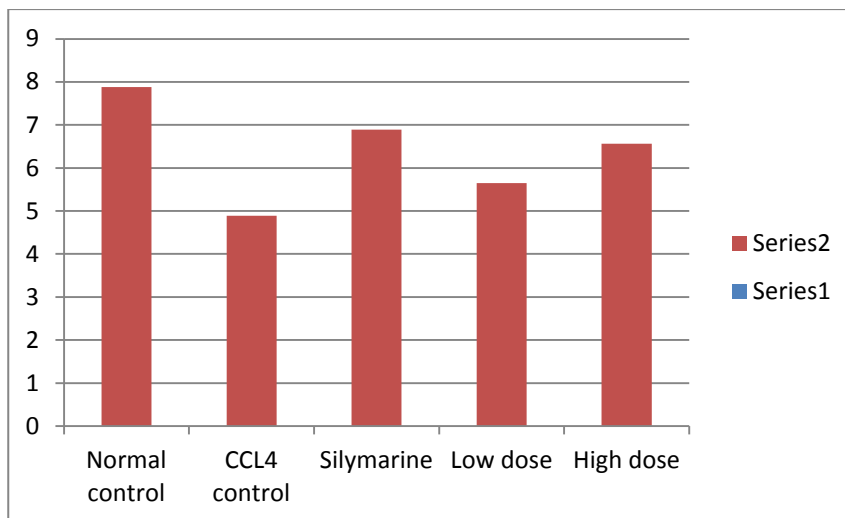


Table.6: Effects of Methanolic extract of *Phyllanthus viragatus* leaves on biochemical markers in Paracetamol induced hepatotoxicity:

Treatment						
	SGPT U/L	SGOT	Total Bilirubine	Directbilirubine mg/DL	Total Cholesteromg/DL	HDL MG/DL
Negative control	66.23± 7.16	75.09± 5.31	0.97±0.46	0.278±0.037	121.54± 3.19	8.37± 0.539
Positive control2ml/kg s.c	209.18± 6.59	261.9± 6.79	4.14±0.28	0.710±0.046	168.38± 8.89	4.39± 0.422
Silymarine100mg/kg	79.8.± 7.19***	111.75± 9.67***	1.39±0.04** *	0.32±0.023***	116.59± 2.95***	7.19± 0.479***
Low dose 100mg/kg	151.32.± 7.46***	185.78± 9.45***	2.78±0.27** *	0.51±0.059***	173.21± 1.89***	5.22± 0.342***
High dose 200mg/kg	89.6± 7.73***	119.13± 8.35***	1.39±0.12** *	0.36±0.005***	127.19± 2.82***	6.91± 0.612***

Fig.10 ; Effect of Methanolic extract of *Phyllanthus viragatus* leaves on hepatic enzymes in Paracetamol induced hepatotoxicity

Fig.10 SGPT(U/L)

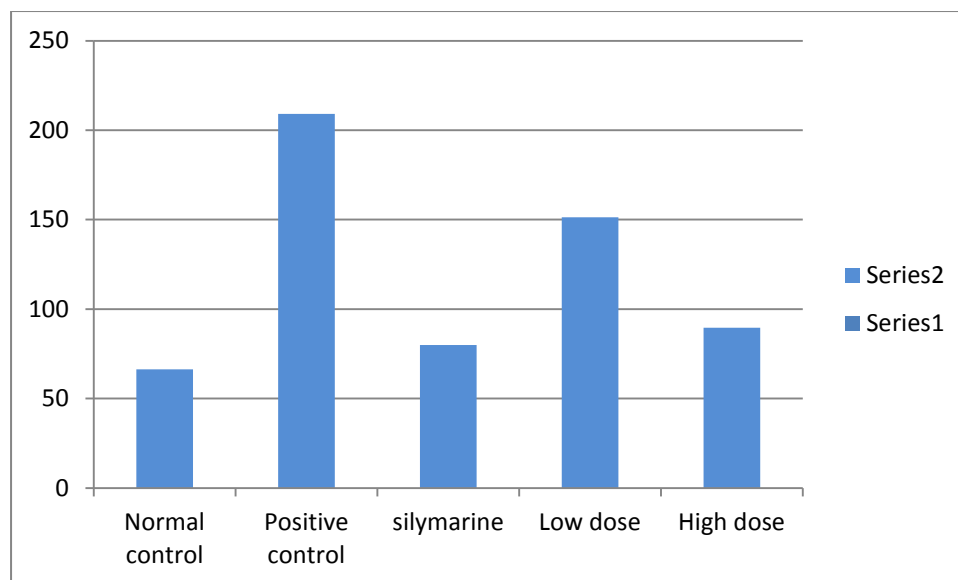


Fig.11 SGOT (U/L)

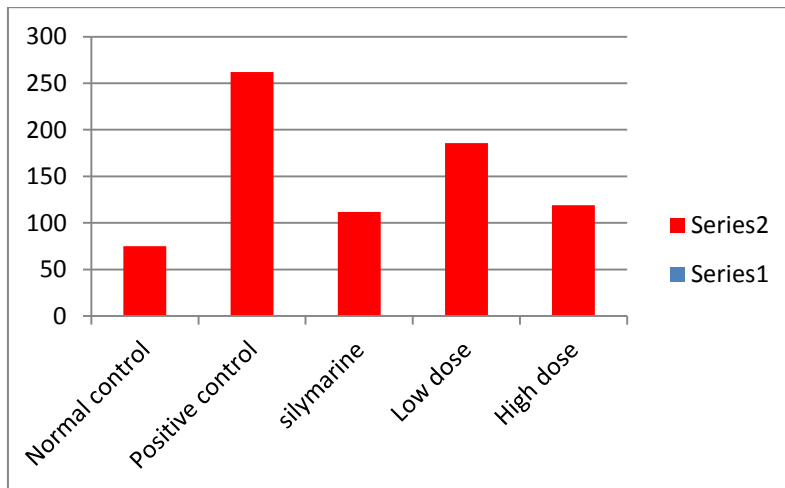


Fig.12 Total bilirubine(mg/dl)

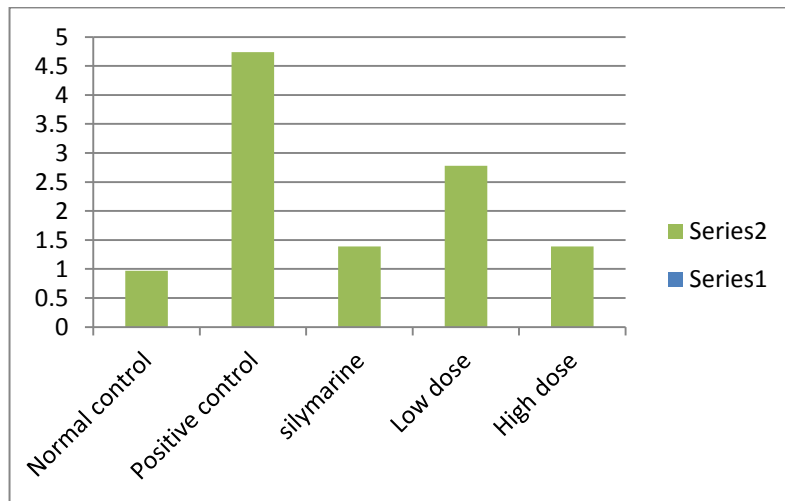


Fig.13 Direct bilirubine (mg/dl)

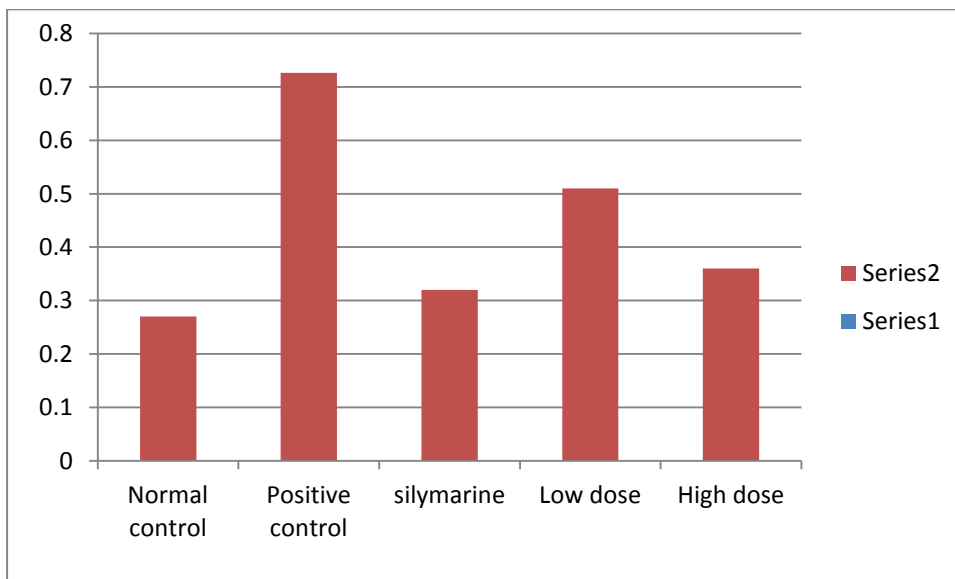


Fig.14 Total cholesterol(mg/dl)

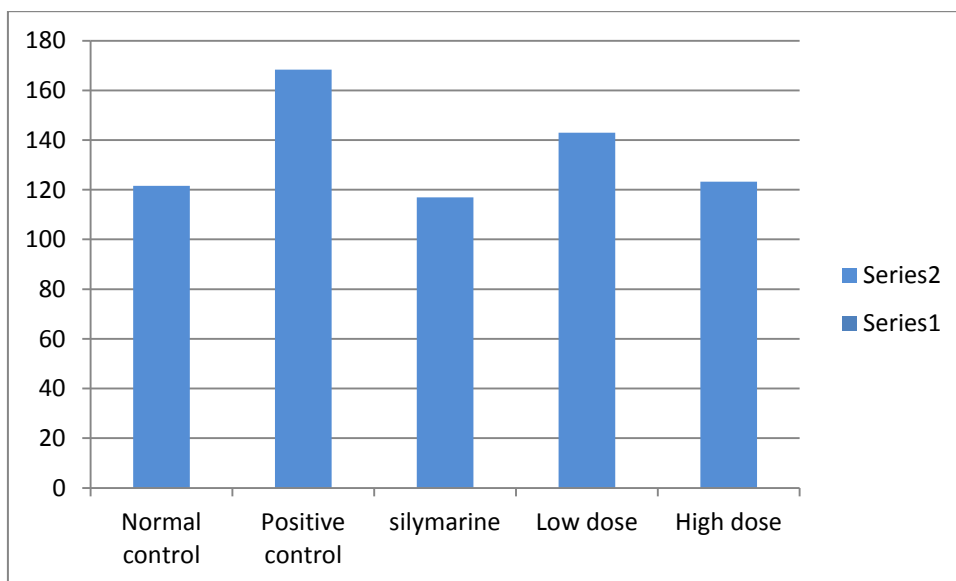
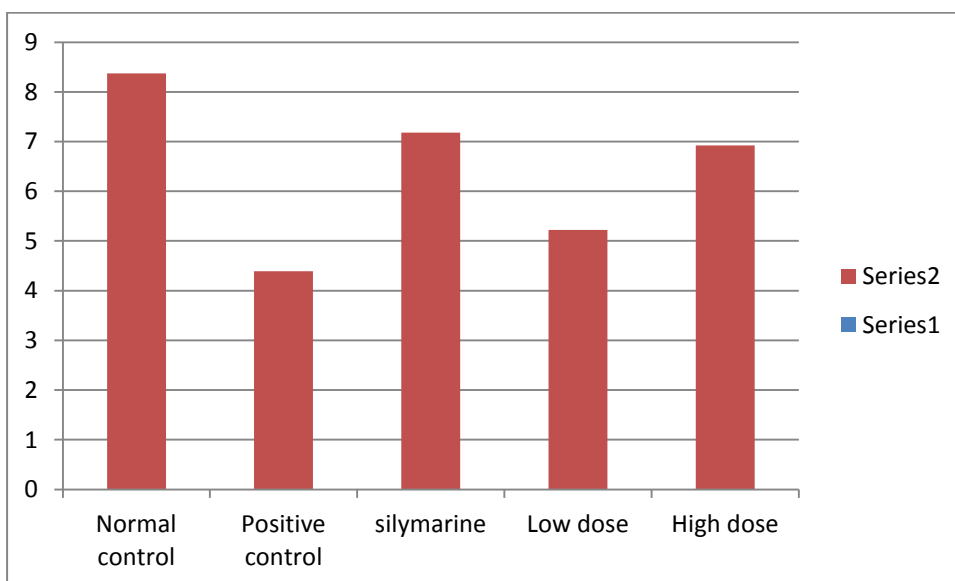


Fig.15.HDL (mg/dl)



DISCUSSION

This results into increase biochemical markers into the serum. Even it was observed that paracetamol decrease GSH and increased lipid per oxidation levels.It appears that the anti-oxidant property i.e. increased hydroxyl scavenging and super anion scavenging activities may be involved in the protective action of 70% methanolic extract of levels of *phyllanthus viragatus*. These results are indicating that the hepatoprotective activity of methanolic extract of whole of *phyllanthus viragatus* against paracetamol induced hepatotoxicity may be due to scavenging of NAPQI free radical and other free radicals. In addition increased GSH levels also play a role in this protective action. In both these models free radical generation by the hepatotoxins/conversion of hepatotoxin into its active radicals are involved in the hepatic destruction. It is evident from our study that the antioxidant property is involved in the hepatoprotective activity against both the toxins. There reports that flavonoids and catechol derivatives like 1-nor-adrenaline, dopamine and dopa are present in the leaves of *phyllanthus viragatus*

and are known to have very good antioxidant property. There is a report that the leaves contain glutathione (GSH), which is a known anti-oxidant and tissue protective component. In addition, the tissue GSH levels were raised due to treatment with test extract (this rise may be due to GSH content of the leaves or due to prevention of its depletion by exogenous challenges). Therefore, hepatoprotective activity of leaves of *Phyllanthus viragatus* may be attributed to these principles including GSH. However, further studies are needed to quantitatively assess the role of these principles in hepatoprotection.

CONCLUSION

The present investigation shows that the methanolic extract of the leaves of *Phyllanthus viragatus* has demonstrated dose-dependent increase in the depletion of tissues GSH and decrease in lipid peroxidation levels by both CCl₄ and paracetamol. Treatment with 70% ethanolic extract has brought back the elevated levels of SGPT, SGOT, ALP, Total and Direct Bilirubin, Cholesterol in both CCl₄ and paracetamol-induced hepatotoxicity in rats. Similarly, reduced HDL levels were increased. Further investigations are needed to be carried out with regard to the isolation of active principles responsible for hepatoprotective activity and also for the intoxication with other models such as iron, alcohol, etc. to prove its efficacy.

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