List of Publications:

1. Hepatoprotective effect of extracts from *Pergularia daemia* Forsk.
Journal of Ethnopharmacology 107, 2006, 164-168.
2. Hepatoprotective effect of extracts from *Pergularia daemia* Forsk. against carbon tetrachloride induced toxicity in rats.
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3. Protective effect of extracts from *Pergularia daemia* Forsk. against carbon tetrachloride-induced hepatotoxicity: An in vitro study.
Communicated to **Pharmacognosy Magazine (under revision)**
4. Hepatoprotective effect of *Pergularia daemia* ethanol extract and its fraction.
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5. Hepatoprotective activity of *Baliospermum montanum* (willd) Muell.-Arg. against paracetamol intoxication in rats.
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6. Protective Effect of Extracts from *Pergularia daemia* against Paracetamol and Thioacetamide Induced Hepatotoxicity
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Hepatoprotective effect of extracts from *Pergularia daemia* Forsk.

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Abstract

Pergularia daemia (Asclepiadaceae) is a perennial herb growing widely along the road sides of India. It has been used in folk medicine for the treatment of liver disorders. The aim of this work is to study the hepatoprotective effect of crude ethanolic and aqueous extracts from the aerial parts of *Pergularia daemia*. The aqueous and ethanolic extracts obtained from aerial parts of *Pergularia daemia* were evaluated for hepatoprotective activity in rats by inducing liver damage by carbon tetrachloride. The ethanolic extract at an oral dose of 200 mg/kg exhibited a significant ($P < 0.05$) protective effect by lowering serum levels of glutamic oxaloacetic transaminase, glutamic pyruvic transaminase, alkaline phosphatase, total bilirubin and total cholesterol and increasing the levels of total protein and albumin levels as compared to silymarin used as a positive control. These biochemical observations were supplemented by histopathological examination of liver sections. The activity may be a result of the presence of flavonoid compounds. Furthermore, the acute toxicity of the extracts showed no signs of toxicity up to a dose level of 2000 mg/kg. Thus it could be concluded that ethanolic extract of *Pergularia daemia* possesses significant hepatoprotective properties.
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Keywords: *Pergularia daemia*; Asclepiadaceae; Carbon tetrachloride; Ethanolic extract; Silymarin

1. Introduction

Pergularia daemia Forsk. Syn. *Daemia extensa* (Asclepiadaceae) commonly known with the name of "dustapu teega" in Telugu is a perennial twining herb, growing wildly along the road sides of Andhra Pradesh state in India. The plant is used to treat jaundice by the folklore people of Chittoor district, Andhra Pradesh state. The literature survey reveals that little work has been carried out on this plant. The plant is useful as anthelmintic, laxative, anti-pyretic and expectorant, and is also used in infantile diarrhoea. This drug was also strongly recommended for malarial intermittent fevers (Kirtikar and Basu, 1983). Phytochemically the plant has been investigated for cardenolides, alkaloids, triterpenes and saponins (Sathish et al., 1998). Sathish et al. (1998) reported the anti-inflammatory, anti-pyretic and analgesic activities of the plant. The plant was also found to possess anti-diabetic activity (Wahi et al., 2002). The plant was found to contain various triterpenes and steroidal compounds (Anjaneyulu et al., 1998). The present study was undertaken to scientifically prove the folklore use of the plant against liver disorders.

2. Materials and methods

2.1. Plant material

The aerial parts of the *Pergularia daemia* were collected from the foot hills of Tirumala, Andhra Pradesh state and their identity was conformed at The Botanical Survey of India, Southern circle, Coimbatore, India. The voucher specimen (BSI/SC/5/21/05-06/Tech: 1512) was also deposited at the Madras herbarium, The Botanical Survey of India, Coimbatore.

2.2. Preparation of extracts

The shade dried aerial parts of about 500 g were subjected for size reduction to coarse powder. The powder was defatted with petroleum ether (60–80 °C) and then extracted with 5 l of 95% ethyl alcohol using soxhlet apparatus till exhaustion for about 32 h. The total aqueous extract was also prepared by percolation method using 2.5 l of chloroform water till the percolate

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is colourless for about 30 h. Both the ethanolic and aqueous extracts were concentrated under vacuum to get the residues. The percentage yields of ethanolic extract and aqueous extract were found to be 3.9% (w/w) and 4.23% (w/w), respectively. The ethanolic extract was found to contain cardenolides, triterpenes and flavonoids (Wagner and Blatt, 1996). Silymarin was used as a positive control at an oral dose of 200 mg/kg (Morazzoni and Bombardelli, 1995). All the test suspensions are prepared in vehicle, i.e., Tween-80.

2.3. Animals

Wistar albino rats of either sex, weighing 200–250 g maintained under standard husbandry conditions (temperature 23 ± 2 °C, relative humidity 55 ± 10% and 12-h light:12-h dark cycle) were used for all experiments. Animals were allowed to take standard laboratory feed and tap water. The experiments were performed after the experimental protocols approved by the institutional animal ethics committee, M.S. University of Baroda, Vadodara, Gujarat.

2.4. Toxicity studies

Acute toxicity study was performed for ethanolic and aqueous extracts according to the acute toxic classic method (as per OECD guidelines). Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 300 mg/kg and observed for 14 days. If mortality was observed in two out of three animals, then the dose administered was assigned as toxic dose. If the mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose, i.e., 2000 mg/kg. One-tenth of the maximum dose of the extract tested for acute toxicity was selected for evaluation of hepatoprotective activity, i.e., 200 mg/kg (Handa and Anupama, 1990).

2.5. Carbon tetrachloride-induced hepatotoxicity in rats

Rats were divided into five groups of six each, control, hepatotoxin, positive control and two test groups. The control group received oral vehicle treatment at 0, 24 and 48 h. The animals in hepatotoxin-treated group received vehicle at 0 h and at 24 h vehicle followed by carbon tetrachloride diluted in liquid paraffin (1:1, i.p.) at a dose of 1.25 ml/kg, while at 48 h these animals received only vehicle. The test groups have received the first dose of extracts at 0 h, second dose of extracts at 24 h, which was followed by a dose of carbon tetrachloride and at 48 h the third dose of extracts (Kurma and Mishra, 1997; Sureshkumar and Mishra, 2005). The positive control group has received the first dose of silymarin (200 mg/kg) (Morazzoni and Bombardelli, 1995) at 0 h, at 24 h the second dose of silymarin followed by a dose of carbon tetrachloride and at 48 h the third dose of silymarin. After 72 h blood was collected from all the groups, and allowed to clot for the separation of serum. The serum was used for estimation of biochemical parameters. Glutamic oxaloacetic transaminase (SGOT) and glutamic pyruvic transaminase (SGPT) are estimated by Reitman and Frankel Method (1957), alkaline phosphatase (ALKP) by PNPP method (Mac Comb and Bowers, 1972), total bilirubin (TBL) by Jendrassik and Grof method (1938), total cholesterol (CHL) by CHOD-PAP Method (Richmond, 1973), total protein (TPTN) by colour complexation with copper ions in an alkali solution (Peters, 1968) and albumin (ALB) was estimated by Bromo Cresol Green Method (Webster, 1974). All the determinations were carried out using standard kits by an autoanalyser of Merck make (300 TX, E. Merck-Micro Labs, Mumbai).

2.6. Histopathological studies

One animal from each of the treated groups showing maximum activity as indicated by improved biochemical parameters was used for this purpose. The animals were sacrificed and the abdomen was cut open to remove the liver. The liver was fixed in Bouin's solution (mixture of 75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5 ml of glacial acetic acid) for 12 h, then embedded in paraffin using conventional methods (Galighor and Kozloff, 1976) and cut into 5 μm thick sections and stained using haematoxylin–eosin dye and finally mounted in di-phenyl xylene. Then the sections were observed under microscope for histopathological changes in liver architecture and their photomicrographs were taken.

2.7. Statistical analysis

The mean values ± S.E.M. are calculated for each parameter. For determining the significant inter-group difference each parameter was analysed separately and one-way analysis of variance (ANOVA) (Gennaro, 1995) was carried out and the individual comparisons of the group mean values were done using Dunnet's Procedure (1964).

3. Results

The ethanolic and aqueous extracts did not cause any mortality up to 2000 mg/kg and were considered as safe (OECD, 1996). The rats which have received ethanolic extract at the dose of 2000 mg/mg exhibited ptosis.

Carbon tetrachloride (CCl_4) intoxication in normal rats elevated the levels of SGOT, SGPT, ALKP, TBL and CHL, whereas decrease in the levels of TPTN and ALB were observed significantly indicating acute hepato cellular damage and biliary obstruction. The rats treated with ethanolic extract and also silymarin, showed a significant decrease in all the elevated SGOT, SGPT, ALKP, TBL and CHL levels and significant increase in TPTN and ALB levels (Table 1). The rats treated with aqueous extract have shown significant decrease in the levels of SGOT and CHL and increase in the levels of ALB.

Histopathological examination of liver sections of control group showed normal cellular architecture with distinct hepatic cells, sinusoidal spaces and a central vein (Fig. 1). Disarrangement of normal hepatic cells with intense centrilobular necrosis

Table 1
Effect of *Pergularia daemia* on CCl_4-induced toxicity in rats

GROUP	SGOT (IU/l)	SGPT (IU/l)	ALKP (IU/l)	TBL (mg/dl)	CHL (mg/dl)	TPTN (mg/dl)	ALB (g/dl)
Control	141.83 ± 29.46	117.30 ± 14.44	332.50 ± 21.59	1.23 ± 0.19	92.85 ± 7.86	5.91 ± 0.51	3.90 ± 0.26
CCl_4	345.33 ± 34.36	249.02 ± 37.36	455.0 ± 19.66	2.37 ± 1.16	19.45 ± 25.82	3.21 ± 0.24	1.98 ± 0.17
Silymarin	140.33 ± 28.03*	115.50 ± 19.98*	352.50 ± 24.95*	1.07 ± 0.16*	63.36 ± 6.26*	5.27 ± 0.55**	3.26 ± 0.18**
Aqueous extract	173.83 ± 30.56*	217.16 ± 31.47	396.17 ± 27.36	1.53 ± 0.20	71.27 ± 8.92*	4.27 ± 0.54	2.33 ± 0.21**
Ethanolic extract	169.67 ± 29.73*	127.0 ± 17.54*	358.33 ± 19.90*	1.16 ± 0.30*	88.70 ± 13.38*	5.25 ± 0.56**	3.40 ± 0.33**
$F_{calculated}$	7.98	6.07	4.50	2.99	7.0	4.69	10.99
Dunnet's value	112.11	94.54	84.12	1.04	35.70	1.82	0.30

Values are mean ± S.E.M.; $F_{theoretical} = 2.79$ ($P < 0.05$).
* Significant reduction compared to hepatotoxin ($P < 0.05$).
** Significant increase compared to hepatotoxin ($P < 0.05$).

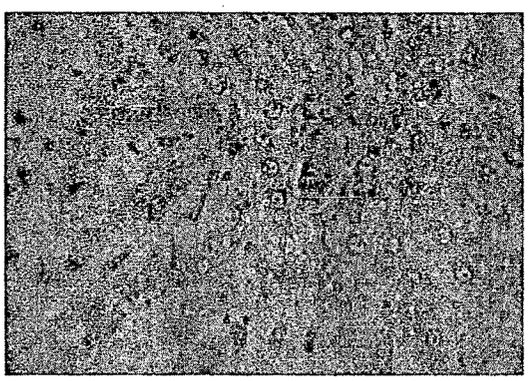

Fig. 1. Normal rat liver section, 400×, haematoxylin–eosin stain. Liver section of the rat showing normal cellular architecture with distinct hepatic cells, sinusoidal spaces and central vein. cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

and vacuolization of periportal vein are observed in CCl_4-intoxicated liver (Fig. 2). The liver sections of the rat treated with ethanolic extract and intoxicated with CCl_4 (Fig. 3), showed less vacuole formation and absence of necrosis and overall no

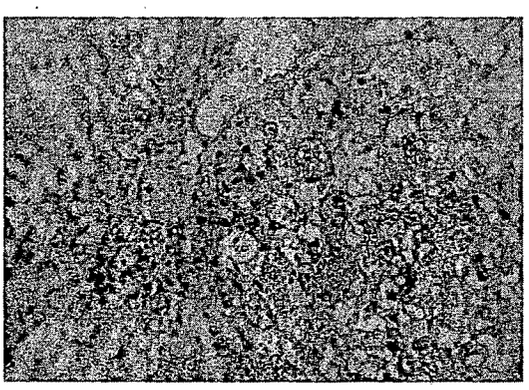

Fig. 2. Liver section of rat intoxicated with CCl_4, 400×, haematoxylin–eosin stain. Liver section of the rat showing disarrangement and degeneration of normal hepatic cells with centrilobular necrosis extending to midzone and sinusoidal haemorrhages and dilation. cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

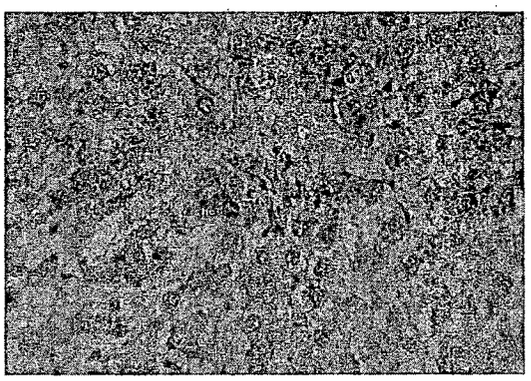

Fig. 3. Liver section of rat treated with ethanolic extract and intoxicated with CCl_4. 400×, haematoxylin–eosin stain. Liver section of the rat shows less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes. cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

visible changes observed as compared to silymarin (Fig. 4), supplementing the protective effect of the extract. Though the less visible changes are observed (Fig. 5) in the sections of the rats treated with aqueous extract and intoxicated with CCl_4, their

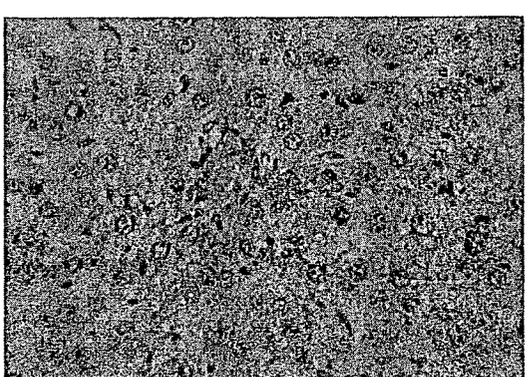

Fig. 4. Liver section of rat treated with silymarin and intoxicated with CCl_4, 400×, haematoxylin–eosin stain. Liver section of the rat shows less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes. cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

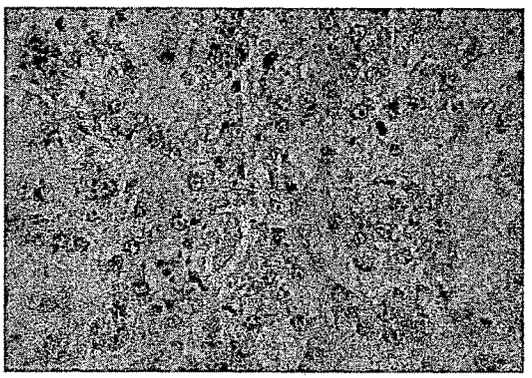

Fig. 5. Liver section of rat treated with aqueous extract and intoxicated with CCl_4, 400×, haematoxylin–eosin stain. Liver section of the rat shows less vacuole formation, reduced sinusoidal dilation, less disarrangement and degeneration of hepatocytes. cv: central vein; hc: hepatocyte; ss: sinusoidal space; vc: vacuole.

intensity was less compared to ethanolic extract-treated rat sections.

4. Discussion

In Indian system of medicine certain herbs are claimed to provide relief against liver disorders. The claimed therapeutic reputation has to be verified in a scientific manner. In the present study one such drug *Pergularia daemia* was taken for the study. The ethanolic extract of *Pergularia daemia* possesses significant ($P < 0.05$) hepatoprotective effect in the CCl_4 model of intoxication in rats. Our investigation on the extracts showed the presence of triterpenoids and flavonoids in the ethanolic extract. According to these results, it may be hypothesized that flavonoids, which are present in the ethanolic extract, could be considered responsible for the hepatoprotective activity.

The hepatotoxicity of CCl_4 has been reported to be due to the formation of the highly reactive trichloro free radical, which attacks polyunsaturated fatty acids. It produces hepatotoxicity by altering liver microsomal membranes in experimental animals (Ashok et al., 2001). The effect of CCl_4 is generally observed after 24 h of its administration. Hence the withdrawal of the blood for biochemical parameters should be carried out only after 24 h of CCl_4 intoxication. From Table 1 it is evident that the ethanolic extract was able to reduce all the elevated biochemical parameters due to the hepatotoxin intoxication. The levels of total proteins and albumin were reduced due to the hepatotoxin intoxication. The reduction is attributed to the damage produced and localised in the endoplasmic reticulum which results in the loss of P_{450} leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides. Intoxication with CCl_4 also resulted in inhibition of synthesis of the bile acids from cholesterol which is synthesized in liver or derived from plasma lipids, leading to increase in cholesterol levels. Suppression of cholesterol levels suggests the inhibition of the synthesis of bile acids from cholesterol is reversed by the extract. Reduction in the levels of SGOT and SGPT towards the normal value is an indication of stabilisation of plasma membrane as well as repair of hepatic tissue damages caused by CCl_4. Reduction of ALKP levels with concurrent depletion of raised bilirubin level suggests the stability of the biliary function during injury with CCl_4. The raise in protein and albumin levels suggests the stabilization of endoplasmic reticulum leading to protein synthesis. The protective effect exhibited by the ethanolic extract is similar to silymarin treatment. The aqueous extract was not able to reduce the elevated parameters caused by CCl_4 intoxication except the CHL. Similarly, an increase in the levels of ALB was observed with aqueous extract.

Histological examination of the liver sections reveals that the normal liver architecture was disturbed by hepatotoxin intoxication. In the sections obtained from the rats treated with ethanolic extract and intoxicated with hepatotoxin, the normal cellular architecture was retained as compared to silymarin, there by confirming the protective effect of the extract. Although the less visible changes are observed in the sections of the rats treated with aqueous extract and intoxicated with CCl_4, the intensity was less compared to ethanolic extract-treated rat sections.

It can be concluded from this investigation that, among the aqueous and ethanolic extracts tested, the ethanolic extract of the aerial parts of *Pergularia daemia* possess hepatoprotective activity against CCl_4 intoxication in rats. Our further detailed studies may, however, confirm the utility profile of this drug.

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Hepatoprotective activity of extracts from *Pergularia daemia* Forsk. against carbon tetrachloride-induced toxicity in rats

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ABSTRACT - *Pergularia daemia* Forsk. (Asclepiadaceae) is a perennial herb grows along the road sides in India. Studies on the hepatoprotective effect of acetone and ethanolic sub fractions of ethanolic fraction obtained from total alcoholic extract was carried out using carbon tetrachloride- induced liver damage in wistar albino rats. Acetone sub fraction showed significant ($P<0.05$) protective effect by lowering serum levels of various biochemical parameters in the selected model. These biochemical observations were supplemented by histopathological examination of liver sections. Silymarin was used as positive control. The presence of flavonoid compounds in the ethanolic sub fraction of alcohol extract of *Pergularia daemia* may be responsible for significant hepatoprotective properties. The results justify use of *Pergularia daemia* as a hepatoprotective agent.

KEY WORDS- Carbon tetrachloride; Ethanolic extract; *Pergularia daemia* Forsk; Silymarin.

INTRODUCTION

Pergularia daemia Forsk. Syn. *Daemia extensa* R Br. (Asclepiadaceae) known as "Dustapu teega" in Telugu, "Uttaravaruni" in Sanskrit and "Utranajutuka" in Hindi is a perennial twining herb, grows wildly along the road sides throughout Andhra Pradesh state. The plant is used to treat jaundice in Chittoor district of Andhra Pradesh in India. The plant is described as anthelmintic, laxative, antipyretic and expectorant, also used to treat infantile diarrhoea and malarial intermittent fevers (1). Presence of triterpenes and saponins cardenolides and alkaloids were reported by Sathish et al. (2). Aanjaneyulu et al reported the presence of various triterpenes and steroidal compounds (3). Sathish et al investigated the anti inflammatory, anti pyretic and analgesic activities of the plant (2). The plant exhibited anti diabetic activity also (4). The present studies were performed to assess the hepatoprotective activity in rats against carbon tetrachloride as hepatotoxin to prove its claims in folklore practice against liver disorders.

MATERIALS AND METHODS

Plant material

The aerial parts of *Pergularia daemia* were procured from the foot hills of Tirumala, Andhra Pradesh, India. The identity of the plant was conformed at The Botanical Survey of India, Southern circle, Coimbatore, India. The voucher specimen (BSI/SC/5/21/05-06/Tech: 1512) was deposited at the Madras herbarium, The Botanical Survey of India, Coimbatore.

Preparation of extracts

About 43 g of the ethanolic fraction (EFTE) obtained by the fractionation of 60 g of total alcohol extract (TE) was adsorbed on to the 250 g of silica gel of 60-120 mesh size and fractionated with chloroform, acetone and 95% ethyl alcohol, resulting fractions concentrated in vacuum yielded 2.32 g, 11.57 g and 20.26 g solid mass respectively. Preliminary TLC studies of EFTE revealed the presence of flavonoids and cardenolides. The chloroform fraction (CFEFTE) showed cardenolides, acetone fraction (AFEFTE) showed flavonoids and cardenolides while ethanolic fraction (EFEFTE) showed flavonoids (5). The AFEFTE and EFEFTE were used for hepato protective activity in rats. Silymarin was used as positive control at an oral dose of 100 mg/kg (6). All the test substances were suspended in vehicle i.e. 5 % acacia mucilage. The extracts were tested for activity at doses of 50, 100 and 150 mg/kg p.o.

Animals

Wistar albino rats weighing 175-225 g of either sex, maintained under standard husbandry conditions (Temp 23 ± 2°C, relative humidity 55 ± 10% and 12 h light dark cycle) were used for all studies. Animals were allowed to take standard laboratory feed and tap water. The experiments were performed after the experimental protocols approved by the institutional animal ethics committee, M.S.University of Baroda, Vadodara, Gujarat. Groups consisted of 6 rats each unless otherwise noted.

Toxicity studies

Acute toxicity study was performed for AFEFTE and EFEFTE according to the acute toxic classic method as per OECD guidelines (7). Female albino rats were used for acute toxicity study. The animals were kept fasting for overnight providing only water, after which the extracts were administered orally at the dose of 300 mg/kg and observed for 14 days. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If the mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose i.e., 2000 mg/kg.

Carbon tetrachloride-induced hepatotoxicity

Rats were divided into six groups of six each, control, carbon tetrachloride, silymarin and test groups. The rats of control group received three doses of 5% acacia mucilage (1 ml/kg, p.o.) at 12 h intervals (0 h, 12 h and 24 h). The rats of carbon tetrachloride group received three doses of vehicle at 12 h intervals and a single dose of carbon tetrachloride (1.25 ml/kg i.p.) diluted in liquid paraffin (1:1) 30 min after the administration of first dose of vehicle.

The animals in silymarin group received three doses of silymarin (100 mg/kg) at 0 h, 12 h and 24 h. Carbon tetrachloride (1.25 ml/kg i.p.) was administered 30 min after the first dose of silymarin while the test groups were given first dose of extract in acacia mucilage at 0 h which was followed by a dose of carbon tetrachloride (1.25 ml/kg i.p.) after 30 min, while at 12 h, and 24 h the second and third dose of respective extracts (50, 100 and 150 mg/kg p.o.) (8). After 36 h of administration of carbon tetrachloride, blood was collected and serum was separated and used for determination of biochemical parameters.

Assessment of liver function

Blood was collected from all the groups by puncturing the retro-orbital plexus and was allowed to clot at room temperature and serum was separated by centrifuging at 2500 rpm for 10 min. The serum was used for estimation of biochemical parameters to determine the functional state of the liver. Serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were estimated by a UV kinetic method based on the reference method of International Federation of Clinical Chemistry (9) in which both SGOT and SGPT were assayed based on enzyme-coupled system; where keto acid formed by the aminotransferase reacts in a system using NADH. The coenzyme is oxidised to NAD and the decrease in absorbance at 340 nm is measured. For SGOT malate dehydrogenase is used to reduce oxaloacetate to malate where as for SGPT the pyruvate formed in the reaction is converted to lactate by lactate dehydrogenase. Alkaline phosphatase (ALKP) was estimated by method described by Mac Comb and Bowers (10) involving hydrolysis of *p*-nitrophenylphosphate by alkaline phosphatase to give *p*-nitrophenol which gives strong yellow colour in alkaline solution. The increase in absorbance due to its formation is directly measured photometrically at 400 nm and is directly proportional to ALKP activity; while total bilirubin (TBL) by Jendrassik and Grof method (11) which involves the reaction of bilirubin with diazotized sulphanilic acid to form an azocompound, the color of which is measured at 546 nm. Total cholesterol (CHL) was determined by CHOD-PAP Method of Richmond (12) in which the free cholesterol is hydrolysed by cholesterol oxidase to cholestenone-4-en-3-one and hydrogen peroxide. Hydrogen peroxide by the action of peroxidase liberates oxygen which reacts with 4-amino antipyrine and phenol to form red coloured compound which is measured at 500 nm.

Total protein (TPTN) was estimated by Biuret method (13) where proteins produce a violet colour complex with copper ions in an alkali solution. The absorbance of the colour complex is directly proportional to the protein in the sample, while the albumin (ALB) was estimated by BCG (14) involving formation of blue-green complex with bromocresol green at slightly acidic pH which is measured photometrically. All the estimations were carried out using standard kits on auto analyser of Merck make (300 TX, E.Merck-Micro Labs, Mumbai).

Histopathological studies

Animals from control and treated groups were used for this purpose. The animals were sacrificed and the abdomen was cut open to remove the liver. The liver was fixed in Bouin's solution (mixture of 75 ml of saturated picric acid, 25 ml of 40% formaldehyde and 5ml of glacial acetic acid) for 12 h, then embedded in paraffin using conventional methods (15) and cut into 5 µm thick sections and stained using haematoxylin-eosin dye and finally mounted in di-phenyl xylene. The sections were then observed under microscope for histopathological changes in liver architecture and their photomicrographs were taken.

Statistical analysis

The mean values±SEM are calculated for each parameter. For determining the significant inter group difference each parameter was analysed separately

AFEFTE, could be considered responsible for the hepatoprotective activity. In conclusion this study underlines the therapeutic potential of *Pergularia daemia*.

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