

BIOASSAY GUIDED HEPATOPROTECTIVE ACTIVITY OF *POLYGONATUM CIRRHIFOLIUM* AGAINST ISONIAZID AND RIFAMPICIN INDUCED HEPATOTOXICITY IN RATS

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ABSTRACT

The present investigation was performed to examine the hepatoprotective effect of the aqueous ethanolic extract of *Polygonatum cirrhifolium* in antitubercular drug-induced liver damage. *P. cirrhifolium* rhizomes were crushed, dissolved in various solvents (in order of polarity), and then tested for phytochemicals. Based on their findings, mass extraction utilizing the ethanol-water mixture (50: 50) was carried out using the Soxhlet method. The doses for animal research were established through acute toxicity tests. The hepatoprotective potential of aqueous ethanolic extract (50:50) of rhizomes was determined in Wistar rats at doses of 200 mg kg⁻¹ and 400 mg kg⁻¹ p.o. per day. Blood samples were examined for the biochemical markers SGOT, SGPT, ALP, total bilirubin, and albumin. Histopathology of the liver was also conducted followed by *in vitro* anti-oxidant studies. Simultaneously, the extract was subjected to LCMS characterization. *P. cirrhifolium* extract at both the doses 200 mg kg⁻¹ and 400 mg kg⁻¹ has shown significant hepatoprotective activity against hepatotoxicity induced by INH+ RIF in a dose-dependent manner, as depicted by the significant changes in the values of blood biomarkers and *in vitro* anti-oxidant studies. Histopathological studies showed that the treatment with 200 mg kg⁻¹ and 400 mg kg⁻¹ of *P. cirrhifolium* exhibited regeneration of liver architecture and portal system by reducing the haemorrhage and inflammatory infiltrate. LC-MS characterization showed serpentine, 5-hydroxy methylfurfural and cephalotaxine as active constituents. It can be inferred that hydroethanolic extract of *P. cirrhifolium* protects the liver from anti-TB induced toxicity and this protection could be due to the active phytoconstituents.

Keywords: *Polygonatum cirrhifolium*, hepatoprotective, hepatotoxicity, rifampicin, isoniazid, silymarin

INTRODUCTION

The body's largest gland and most crucial organ is the liver. It is involved in protein secretion and storage of fat-soluble vitamins and other useful substances. It also removes toxic substances from the body¹. But due to oxidative stress, lipid peroxidation occurs and the condition gets aggravated as a result of infections, immune disorders and high intake of alcohol. The plants which have the ability to reduce oxidative damage are considered to be hepatoprotective². Many adverse effects of anti-tubercular agents have been reported, and one of the foremost toxicities is hepatotoxicity. Anti-TB drugs

like isoniazid and rifampicin are the first-line standard drugs that have been reported to cause hepatotoxicity and are known to induce considerable mortality and morbidity mediated through oxidative stress. Isoniazid and rifampicin affect hepatocytes, liver vasculature and biliary epithelial cells^{3,4}.

P. cirrhifolium is a member of the genus *Polygonatum* of about 57 species belonging to the family Liliaceae or Convallariaceae. *P. cirrhifolium*, also known as Mahameda, Tridanti, Vasucchidra, and Devamani, consists of roots and rhizomes. It is widely distributed in China and Japan covering East Asia, Pakistan and India⁵. The presence of steroidal saponins and polysaccharides has been discovered in phytochemical investigations on this plant. Other species of the *Polygonatum* predominantly

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contain phytohormones, glycosides, polysaccharides, alkaloids, lignins, flavonoids, homoisoflavanones, phenethyl cinnamide, lectins, and saponins (steroidal and triterpenoid)⁶. The rhizomes have diuretic, adaptogenic, cardiogenic, demulcent, hypoglycaemic, and energizer properties and their tonics are used to treat pulmonary problems including tuberculosis⁷. Thus, the current study was planned to examine the hepatoprotective effect of this plant and to isolate the phytoconstituents present in it.

The liquid chromatography tandem mass spectrometry (LCMS) technique is used to perform analytical determination as it provides improved sensitivity and specificity and needs a matrix in a very small amount. It is capable to choose specific ion fragments for the investigative molecule. Based on molecular weight and patterns of fragmentation of thermally stable samples, this technique allows the identification and depiction of chemical compounds. Liquid Chromatography: Reverse-phase (RP) chromatography is generally used with successive MS analysis as it provides comparatively more resolution due to the absence of salts in buffers. Generally, stationary phases for LCMS consist of SiO₂ having a diameter of micrometer range and consist of C₈ or C₁₈ carbon chain compounds⁸.

MATERIALS AND METHODS

Plant material

The *P. cirrhifolium* plant was gathered from the Ponta Sahib, Dehradun, India in the month of August and was approved by Dr. Sunita Garg, Biological Scientist, Council of Scientific & Industrial Research, NISCAIR, Delhi with the voucher specimen No. Ref.no: NISCAIR / RHMD/ Consult / 2018/3282-83.

Phytochemical screening

P. cirrhifolium was dissolved in different solvents and then screened to detect the presence of phytoconstituents. Phytochemical screening was performed using conventional tests and gave information about the presence of plant constituents like alkaloids, glycosides, terpenoids, flavonoids, carbohydrates, lipids, steroids, volatile oils, and tannins⁹. Dragendorff and Mayer's reagents were used for the detection of alkaloids. A froth test was conducted for identification of saponins. Keller Kiliani test and Liebermann's test were used for the determination of glycosides. For testing terpenoids, chloroform was added to the extract followed by concentrated sulphuric acid. Flavonoids were tested by the addition of sodium hydroxide. Molisch test was conducted

Table I: Summary of preliminary phytochemical analysis of the constituents present in the rhizomes extract of *P. cirrhifolium*

Solvent	Alkaloids	Glycosides	Terpenoids	Flavonoids	Saponins	Carbohydrates	Lipids	Volatile oils	Steroids	Tannins
DMSO (Dimethyl-sulphoxide)	+	-	-	-	-	-	+	+	-	-
n-Hexane	-	-	-	-	-	-	+	+	-	-
Petroleum ether	-	-	-	-	-	-	+	++	-	-
Chloroform	+	-	-	+	+	-	+	+	-	+
Dichloromethane	-	+	-	-	-	-	+	++	-	-
Acetone	-	+	-	-	-	-	+	+	-	-
Ethyl acetate	-	+	-	-	-	-	+	+	-	-
Methanol	-	-	+	-	-	-	+	+	-	-
Ethanol: water	+	++	-	+	-	-	+	+	-	-
Water	+	+	-	-	-	-	+	+	-	-
Methanol: water (7:3)	+	+	-	-	-	-	+	+	-	-
BAW (n-butanol:acetic acid: water) (4:1:5)	+	+	-	-	-	-	+	+	-	-

for the detection of carbohydrates. An acrolein test was performed to detect lipids in the extract. The Salkowski test was conducted for the detection of steroids. For identification of tannins, ferric chloride was used. The extract was treated with Sudan III for the detection of volatile oils.

Preparation of mass extraction

In the laboratory, plant materials were dried in the open air at room temperature for 3 days and coarsely powdered and further stored in a tightly closed container. Approximately 250 g of the plant sample was extracted using a Soxhlet extractor with 50% ethanol and 50% water. It was filtered with filter paper (Whatman) and the solvents were then evaporated using a Rota-Vapor evaporator under low pressure and the product was obtained as a viscous semi-solid mass.

Acute toxicity studies

The acute toxicity studies of hydroethanolic extract (50:50) of *P. cirrhifolium* were performed in Wistar albino rats, as per the OECD Guideline No. 423¹⁰. The study protocol was approved by IAEC (Registration No: IAEC/KSOP/E/18/03). The doses of plant extract were given to various groups having 6 animals in each group. Before the experiment, animals were starved for the entire night. Dosages of 5, 50, 300, and 2000 mg kg⁻¹ were administered. All rats were carefully observed for any sign of toxicity within the first 4 h after extract administration and every day after that for 14 days.

Hepatoprotective activity

Selection of animals

Wistar rats of either sex, weighing between 150 and 250 g, were utilized. Rats were acclimatized to normal husbandry conditions for 10 days at 22 ± 2 °C and 50 ± 20% relative humidity with 12 h of dark and light cycle. Ethical clearances were acquired from the Institutional Animal Ethical Committee for performing animal experiments (IAEC Registration No./CPCSEA, IAEC/KSOP/E/18/03).

Study design

Five groups of Wistar rats were used comprising 6 animals per group.

Group I: Normal distilled water

Group II: Received isoniazid 50 mg kg⁻¹ day⁻¹, p.o. along with rifampicin 50 mg kg⁻¹ day⁻¹ p.o.

Group III: Received rifampicin and isoniazid followed by plant extract (200 mg kg⁻¹ day⁻¹ p.o.)

Group IV: Received rifampicin and isoniazid followed by plant extract (400 mg kg⁻¹ day⁻¹ p.o.)

Group V: Served as a standard group receiving silymarin (50 mg kg⁻¹ day⁻¹)

The whole experiment was conducted for a duration of 28 days¹¹.

Table II: Influence of extract of *P. cirrhifolium* on the biochemical and anti-oxidant parameters

Sr. No.	Parameter	Group-I	Group-II	Group- III	Group- IV	Group- V
1.	SGOT	50.06 ± 6.31	80.27 ± 6.04 ^a	41.39 ± 2.03 ^{***}	26.08 ± 3.59 ^{***}	24.21 ± 4.59 ^{***}
2.	SGPT	47.12 ± 4.87	70.58 ± 5.38 ^a	44.89 ± 2.52 ^{***}	26.63 ± 2.77 ^{***}	19.32 ± 1.95 ^{***}
3.	ALP	141.50 ± 4.02	284.30 ± 3.14 ^a	172.80 ± 3.33 ^{***}	149.80 ± 2.65 ^{***}	130.30 ± 1.45 ^{***}
4.	TBL	0.29 ± 0.01	0.67 ± 0.01 ^a	0.45 ± 0.02 ^{***}	0.37 ± 0.01 ^{***}	0.32 ± 0.02 ^{***}
5.	TPT	5.48 ± 0.45	2.21 ± 0.19 ^a	3.19 ± 0.17 ^{**}	4.13 ± 0.17 ^{***}	5.16 ± 0.24 ^{***}
6.	ALB	4.59 ± 0.27	2.45 ± 0.12 ^a	3.20 ± 0.06 [*]	4.04 ± 0.24 ^{***}	4.54 ± 0.20 ^{***}
7.	Glutathione	31.26 ± 3.15	12.78 ± 1.85 ^a	37.33 ± 2.16 ^{***}	41.90 ± 1.12 ^{***}	42.73 ± 2.18 ^{***}
8.	Catalase	23.64 ± 2.12	13.47 ± 2.39 ^a	21.55 ± 1.72 [*]	31.34 ± 2.20 ^{***}	34.40 ± 1.27 ^{***}
9.	TBARS	31.89 ± 3.01	71.48 ± 3.01 ^a	51.84 ± 2.62 ^{***}	25.45 ± 2.89 ^{***}	21.46 ± 1.98 ^{***}

^aP<0.05 vs Normal control *P<0.05 vs Toxic control **P<0.01 vs Toxic control, ***P<0.001 vs Toxic control (GII); SGOT, SGPT, ALP, TBL; TPT, ALB, TBARS

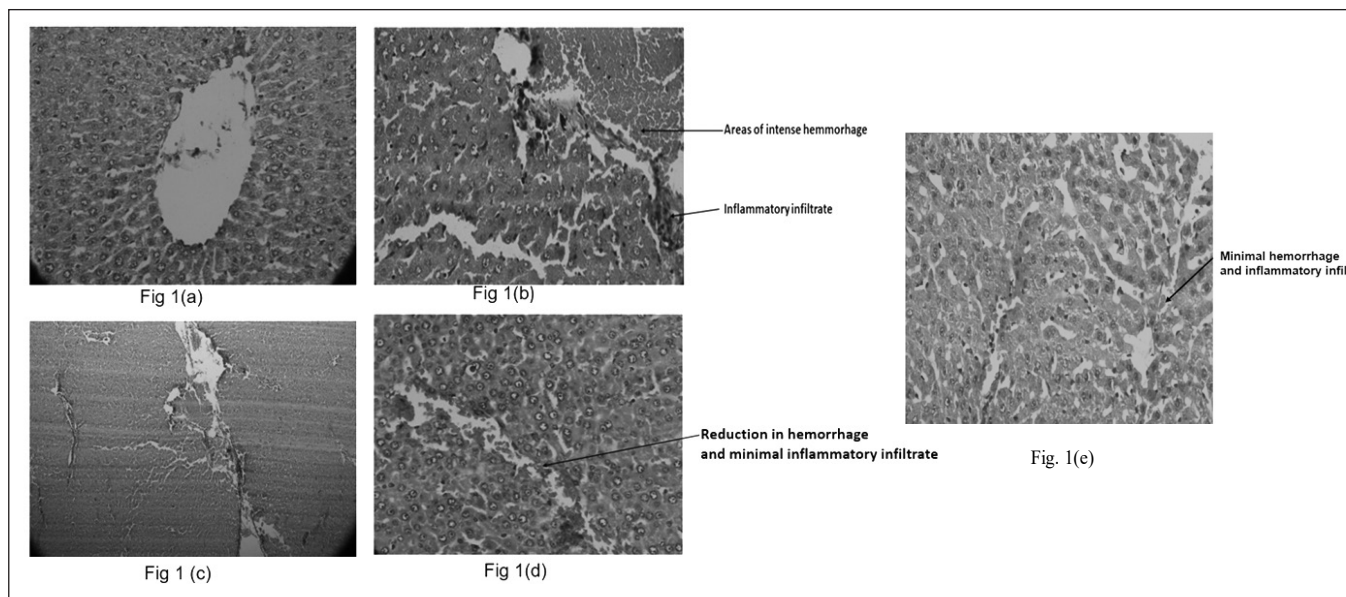


Fig. 1: a) Normal control (intact architecture); b) Anti-TB induced hepatotoxicity group (damaged liver architecture & portal system); c) low dose 200 mg kg⁻¹ group; d) high dose 400 mg kg⁻¹ group; e) standard drug group (silymarin treated) minimal hemorrhage and Inflammatory infiltrate

Collection of blood and assessment of liver function

On the 14th day and 28th day, the retro-orbital plexus was punctured to obtain blood samples from each group. The blood was collected in Eppendorf tubes and left to coagulate for 15-20 minutes at room temperature prior to centrifugation for 10 minutes at 2,500 rpm. The separated portion was utilized to perform biochemical measurements using kits for total bilirubin (TBL), alkaline phosphatase (ALP), serum glutamic oxaloacetate transaminase (SGOT), and serum glutamic pyruvate transaminase (SGPT).

Histopathological examination

All of the animals were sacrificed once the 28-day period of treatment was completed to get their liver tissue. Tissues of 4-5 μ m thickness were sliced and treated with haematoxylin-eosin after being maintained in a 10% formaldehyde solution. Finally, histological findings were determined with the help of a microscope.

Estimation of liver homogenate for total protein, TBARS, glutathione and catalase

All blood cells were removed from the excised livers by perfusing them in cold normal saline. They were then chopped into small pieces, soaked in phosphate buffer

(0.1 M, pH 7.4), and homogenized with the aid of a Remi homogenizer to reach 20% homogeneity, followed by centrifugation for a total of 15 minutes at 1000 g (3000 rpm), and the supernatant was taken in a special tube called Eppendorf tube. The final supernatant was used for the estimation of protein, TBARS, glutathione and catalase. The quantity of protein was determined using Lowry's method¹². Reduced glutathione was estimated using the Ellman method¹³. Superoxide dismutase was estimated using the Marklund method¹⁴. Catalase was assessed by using the Clairborne method¹⁵.

Statistical analysis

The values mean \pm SEM were calculated for each parameter. In order to determine the important intergroup distinction, liver functioning parameters were determined for each animal. Version 9.0 of Graph Pad Prism (Graph Pad Software, San Diego, California, USA) was employed to carry out a one-way ANOVA.

Isolation and characterization of phytoconstituents

The hydroalcoholic extract of rhizomes of *P. cirrhifolium* was subjected to LC-MS spectroscopic analysis and the various spectra obtained using this technique were analyzed to identify and characterize the natural compounds present in the plant¹⁶.

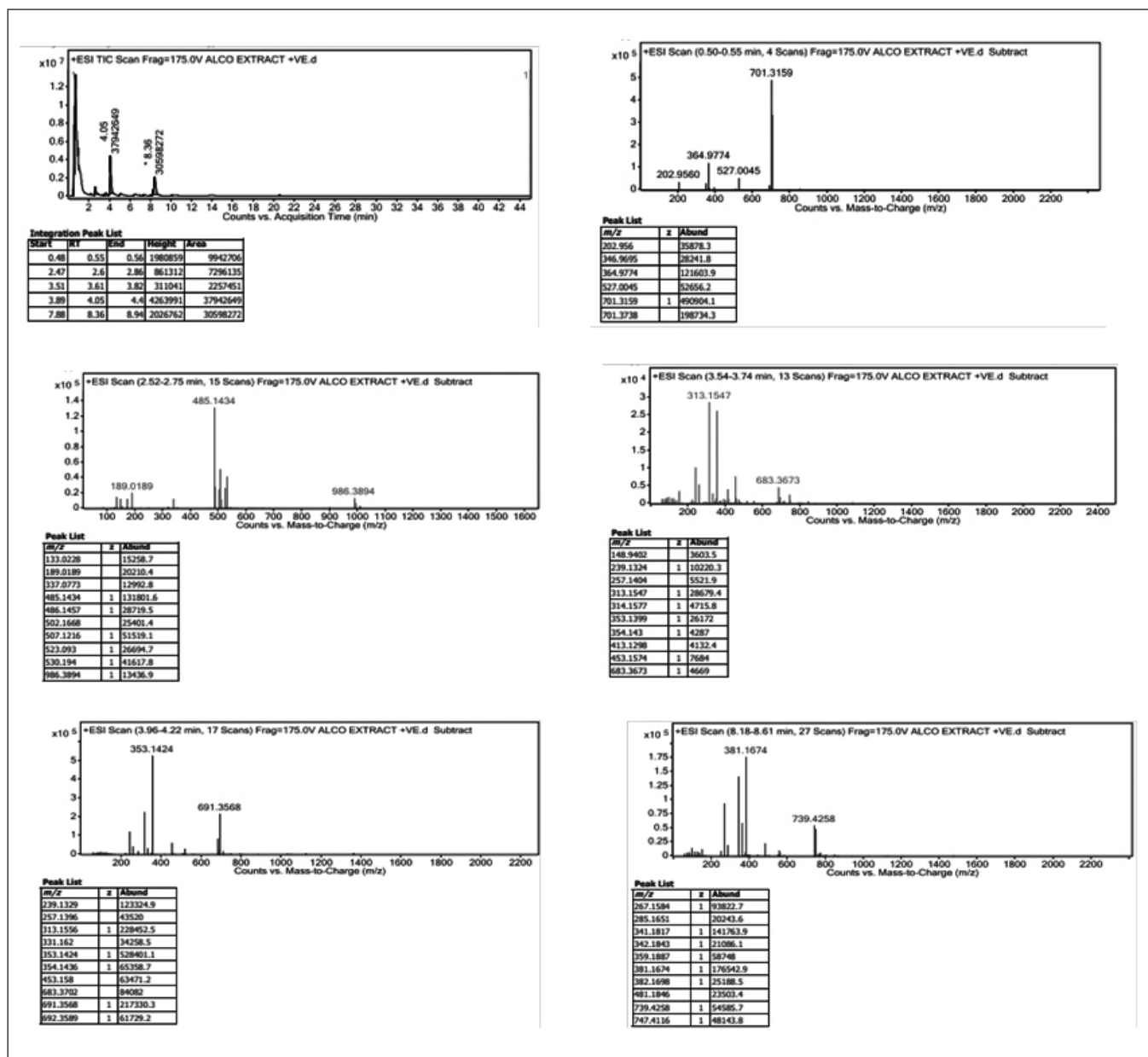


Fig. 2: LC-MS spectra of the *P. cirrhifolium* extract

RESULTS

Phytochemical screening

A preliminary phytochemical screening test for various ingredients was performed and this is summarized in Table I.

Impact of hydroethanolic extract of *P. cirrhifolium* on biochemical parameters

In either of the experimental animal groups, there was no mortality. When comparing the experimental animals' body weight and relative liver weights at the conclusion

of the investigation to the control group, there was no statistically significant change.

Table II displays the findings of hepatoprotective actions of a hydro-ethanolic extract of *P. cirrhifolium* on isoniazid and rifampicin intoxicated rats. On comparing with the healthy control group, the administration of INH and RIF at a dose of 50 mg kg⁻¹ b.w. significantly raised the levels of SGPT, SGOT, ALP and bilirubin. Treatment with *P. cirrhifolium* hydroethanolic extract at doses of 200 mg kg⁻¹ day⁻¹ and 400 mg kg⁻¹ day⁻¹ sixty minutes prior to the administration of INH and RIF lowered

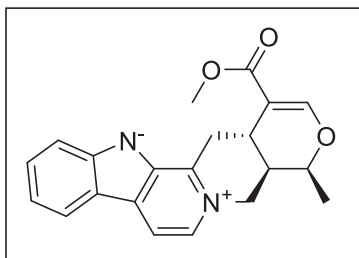


Fig. 3(a): Serpentine

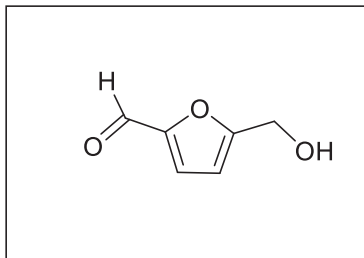


Fig. 3(b): 5-hydroxy methylfurfural

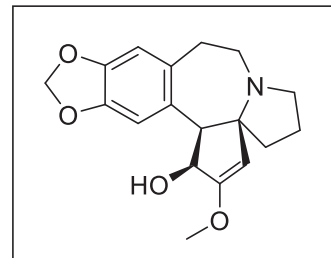


Fig. 3(c): Cephalotaxine

Table III: Characterization of the peak for identification of the first compound

Sr. No.	Mass	Ion	Product ion (PI) and components of neutral particle lost (NPL)	Sub-structure	Specific m/z ratio
1.	1	-	[M-1] ⁻	[M-1] ⁻ occur for moderately basic and acidic types	346.9

Table IV: Characterization of the peak for identification of the second compound

Sr. No.	Mass	Ion	PI and components of NPL	Sub-structure	Specific m/z ratio
1.	7	Li ⁺	[M+7] ⁺	Li adducts as final compound (5-hydroxy methylfuryl)	133

Table V: Characterization of the peak for identification of the third compound

Sr. No.	Mass	Ion	PI and components of NPL	Sub-structure	Specific m/z ratio
1.	1	-	[M-1] ⁻	[M-1] and [M-2] occur for moderately basic and acidic compound type	314.15
			[M-2] ⁻		313.15

SGPT, SGOT, ALP, and bilirubin levels substantially when compared to the normal control group. A hydro-ethanolic extract of *P. cirrhifolium*, given at the dosages of 200 mg kg⁻¹ and 400 mg kg⁻¹ per day an hour before the administration of isoniazid and rifampicin administration, dramatically reduced the rise of transaminases and alkaline phosphatase activity to baseline levels. Treatment

with 200 mg kg⁻¹ and 400 mg kg⁻¹ doses of *P. cirrhifolium* significantly reduced serum bilirubin in comparison to hepatotoxic controlled rats. The effects of a 400 mg kg⁻¹ dose of *P. cirrhifolium* were shown to be comparable to those of a standard drug silymarin.

Impact of hydroethanolic extract of *P. cirrhifolium* on anti-oxidant parameters

A rise in liver TBARS levels in group II is indicative of increased oxidative stress brought on by INH + RIF therapy. Administration of the extract in two separate concentrations (200 and 400 mg kg⁻¹ b.w.) and the silymarin treated group considerably reduced increased liver TBARS levels. Reduced liver glutathione and catalase levels in Group II indicate increased oxidative stress as a result of INH + RIF therapy. The plant extract concentrations at 200 mg kg⁻¹, as well as 400 mg kg⁻¹, were observed to boost glutathione and catalase levels as depicted in Table II.

Effect of hydroethanolic extract (50:50) of *P. cirrhifolium* on histopathology of liver

Hepatocyte cords organized around the major vein were found in the livers of control rats, indicating normal histological architecture. Hepatic sinusoids with haphazardly dispersed Kupffer cells were found between the hepatic cords (Fig.1a). The histology of the liver was altered after exposure to the medication's isoniazid and rifampicin. There were multiple sites of Kupffer cells infiltration in the sinusoids. Lobular inflammation was caused by Kupffer cells and mononuclear cell hyperplasia (Fig.1 b). Treatment with the *P. cirrhifolium* extract at dose of 200 mg kg⁻¹ and subsequently 400 mg kg⁻¹ exhibited regeneration of liver architecture and portal system by reducing the haemorrhage and inflammatory infiltrate. This effect was more prominent at 400 mg kg⁻¹ dose (Fig.1c and d). Further, silymarin also showed regeneration of liver architecture and portal system by reducing the haemorrhage and inflammatory infiltrate, and no necrosis was observed with silymarin (Fig.1e).

LC-MS analysis of the *P. cirrhifolium* extract

LC-MS of the *P. cirrhifolium* extract was conducted at Indian Pharmacopoeia Commission, Ghaziabad, Uttar Pradesh, India by the instrument LC-MS-MS Agilent 6520 and their spectra are depicted in Fig. 2.

After obtaining the data and spectra from the LC-MS technique, a CD-Rom titled "Structure of Determination of Organic Compounds, Tables of Spectral Data" authored by Pretsch et. al. and published by Springer electronic media (ISBN 3-540-67815-8) was used in developing the data tables. Tables III, IV, and V further helped in the identification of three compounds.

These three compounds may be responsible for their various activities including hepatoprotective effects. The structures of the compounds are depicted in the Fig. 3(a), 3(b) and 3(c).

DISCUSSION

Due to the existence of active ingredients in various sections of the plant such as leaves, stems, fruits, and seeds, a vast variety of plants have beneficial characteristics. To explore the phytoconstituents, hydroalcoholic extract of *P. cirrhifolium* rhizomes was prepared using Soxhlet extraction¹⁷.

In general, acute toxicity testing in experimental animals is used to conduct safety research on herbal remedies. In the present investigation, *P. cirrhifolium* was assessed for the liver protective impact against anti-TB drugs i.e., isoniazid and rifampicin-induced damages to the liver in Wistar rats. The main adverse impact of these first-line anti-TB drugs is hepatotoxicity. Previous studies have also reported hepatotoxicity induced by these first-line antitubercular drugs as exhibited in the form of inflammation of the liver architecture and its portal system. The metabolites of isoniazid like acetylhydrazine and acetylisoniazid are also injurious and have the capability to combine with cellular molecules¹⁸. Rifampicin, as an enzyme inducer, accelerates isoniazid metabolism, resulting in the production of isonicotinic acid and hydrazine, both of which cause hepatotoxicity¹⁹.

Besides the above mechanisms, hepatotoxicity can also be induced by the generation of free radicals, especially ROS, contributing to oxidative stress. The combination of these first-line drugs is known to increase TBARS levels in the hepatocytes^{20,21}.

The diagnostic enzymes like SGOT, SGPT, ALP, and bilirubin serve as biochemical parameters for liver injury. Due to liver damage, these enzymes leak

into the blood leading to a rise in serum levels. In the current study, at the dosage of 200 mg kg⁻¹ per day and at 400 mg kg⁻¹ per day, the hydroethanolic extract of *P. cirrhifolium* considerably ($P < 0.001$) reduced the values of liver enzymes, which were elevated by anti-TB medicines.

It's hypothesized that 50% hydro-ethanolic extract of *P. cirrhifolium* assisted in liver cell renewal, protecting the membrane structure, thus reducing enzyme leakage during antitubercular drug-induced hepatotoxicity. As per many research studies, serum bilirubin levels are significantly elevated with hepatic damage, so in the INH + RIF-induced toxicity group of rats, serum bilirubin increased dramatically, whereas albumin levels in the serum fell significantly^{22,23}. All examined parameters largely returned to normal conditions from the aberrant ones, as demonstrated in the standard drug-treated group and the high-dose extract group.

It has been proven that the human body possesses a defensive system that efficiently prevents and neutralizes free radical damage. The levels of superoxide dismutase and catalase were low in rats with hepatic damage treated with anti-TB medications, but the enzyme levels in the treated group (200 mg kg⁻¹ and 400 mg kg⁻¹) exhibited a considerable rise in the level because of the extract's capacity to scavenge reactive oxygen species. Lipid peroxides are an indicator of liver damage. Histopathological investigations supported the extract's overall hepatoprotective efficacy.

LCMS technique, also called as tandem mass spectrometry, is used to perform analytical determination as it provides improved sensitivity and specificity and needs a matrix in a very small amount. Characterization of the phytoconstituents by LC-MS yielded 3 components viz serpentine, 5-hydroxy methylfurfural, and cephalotaxine. Serpentine and cephalotaxine are alkaloids while 5-hydroxy methylfurfural belongs to the class of furans²⁴.

Further studies are required to be conducted on these 3 isolated cum characterized compounds. *P. cirrhifolium* hydroethanolic extract was found to have a protective action on liver to counter the hepatotoxicity caused by INH+ RIF. *P. cirrhifolium* extract at 400 mg kg⁻¹ was reported to lower the SGOT, SGPT, ALP, and total bilirubin levels in the experimental rats. The results with a dosage of 400 mg kg⁻¹ were found to be better than at the dosage of 200 mg kg⁻¹. Additionally, levels of antioxidants like glutathione and catalase significantly increased.

CONCLUSION

It can be concluded that *P. cirrhifolium* shows a significant effect as a hepatoprotective drug. It has comparable effects with silymarin which is used as a standard drug. Efforts are underway to extract and identify the active components responsible for this remarkable medicinal plant's hepatoprotective effectiveness.

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