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Evaluation of Hepatoprotective Activity of *Passiflora foetida*

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Abstract

The present investigation encompasses phytochemical detection and its quantification using methanol as solvent from fresh leaves (FL) and shade dried leaves (SDL) of *P. foetida*. Furthermore, the work examines their hepatoprotective activity in murine system.

Keywords: Phytochemical screening, in vivo analysis, hepatoprotective, solvents extract.

Introduction

Passiflora foetida L., a perennial climbing herb of the family Rubiaceae is commonly known as skunk vine, stink vine, Chinese fever vine, gandhabhadulia (Bengali), gandhaprasarini (Hindi), prasarani (Sanskrit) and paduri lata (Assamese) (**Torkelson, 1999**). Different types of phytochemicals that are detected from *P. foetida* are iridoid glycosides namely paederoside, asperuloside and scandoside (**Shukla *et al.*, 1976**), alkaloids like paederin a and b and essential oil of which terpenoids are found to be present as major component (**Samy *et al.*, 2005**).

The plant species possesses wide range of therapeutic properties including antinociceptive (**Hossain *et al.*, 2006**), anti-diarrhoeal (**Afroz *et al.*, 2007**), antitussive anti-oxidative (**Osman *et al.*, 2009**), anti-helminthic, anti-ulcer (**Das *et al.*, 2013**), anti-hyperlipidemic (**Kumar *et al.*, 2014**), gastroprotective, anti-arthiritic, renoprotective and anti-inflammatory (**Kumar *et al.*, 2015**) among others. Present research work was done to evaluate hepatoprotective activity of ***Passiflora foetida.***

Materials and method

The plant species under study namely, *P. foetida* is collected from Experimental farm of Bidhan Chandra Krishi Viswavidyalaya, Kalyani, Nadia. The collections of the leaves are made during the months of August and October. The species were identified as *Passiflora foetida* L. (Voucher specimen no-KU/BOT/Pf/PBL-01)

Types of leaf samples used for experiments

Fresh leaves (FL)

The collected leaves of both the species were washed thoroughly in double distilled water (ddH2O) to remove adherent dust particles and blotted dry. The leaves were cut into small pieces (6×4 mm) with the help of a scissor and 20 gm of leaves were used for extraction process.

Shade dried leaves (SDL)

The fresh leaves were shade dried for 15 days at room temperature (RT; 30 ± 2 ºC). The leaves were ground to a fine powder using a mechanical grinder (Bajaj, India).

Extraction of phytochemicals

Extracts from FL and SDL

The leaf samples were extracted by cold maceration with four different solvents namely, methanol (90%, v/v; Spectrochem, India) at increasing and regular time intervals of 12, 24,

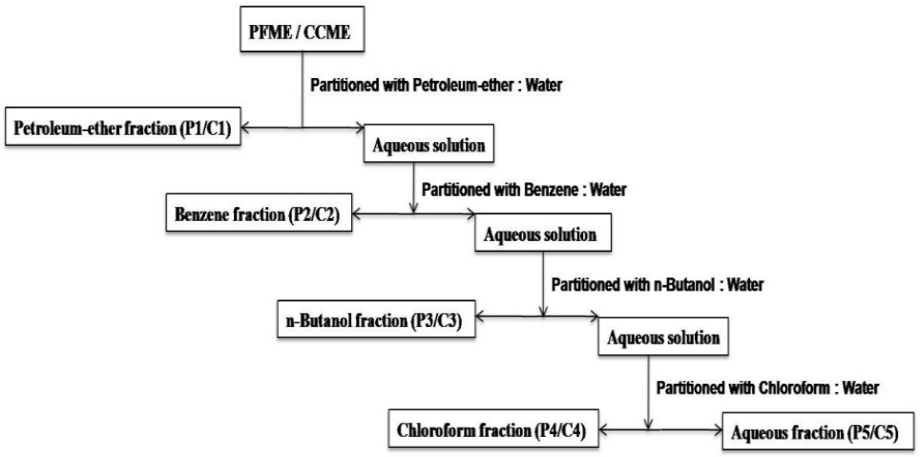
36, and 48h duration. The extractions were done at RT with gentle shaking at 45 rpm (Remi, RS-24 BL, India). The sample to solvent ratio (1:20; w/v) was kept constant during the experiment process. The solvent extracts were filtered through filter paper (Whatman No. 1) followed by centrifugation at 4000 rpm for 15 min at RT to remove the plant remains. The filtrates were concentrated under reduced pressure in a rotary vacuum evaporator (Büchi, Switzerland) as well as by lyophilisation (Heto, Germany) and the resultant concentrates (crude extracts) were weighed to determine the extract yield (EY). After drying, the crude extracts were kept at 4 ºC for further use.

In either of the species, 20 g of fresh leaves (FL) were considered for extraction process; while for SDL extraction approximately 10 g (P. foetida: 10.20 g) of leaf samples were taken. Upon drying, 20 g of FL of P. foetida gave rise to weight of 10.20 g SDL respectively.

The methanolic extracts from SDL at 48 h from P. foetida was designated as PFME and the terminologies are used in the present study.

Solvent fractions of PFME

Crude extracts (1g) of PFME was subjected to liquid-liquid fractionation by solvent partioning using two immiscible liquids. The partioning was done using polar (water) and with four non polar (petroleum- ether, benzene, n-butanol and chloroform) solvents. The solvent fractionations were done in a separating funnel using 40 ml of each solvent in 1:1 ratio in each step. The scheme of the solvent fractionation is depicted in next page.



The obtained fractions were concentrated under reduced pressure using rotary vacuum evaporator, labelled properly, weighed and kept at -20 ºC for further experiments. Petroleum-ether, benzene, n-butanol, chloroform and aqueous fractions of P. foetida were termed as P1, P2, P3, P4 and P5 respectively whereas C. crista fractions were labelled as C1, C2, C3, C4 and C5 respectively. Stock solution (1 mg/ml) were prepared in methanol (P1- P4 and C1- C4) as well as in ddH2O (P5 and C5).

Experimental design and treatments with plant extracts

Hepatoprotective effect of PFME

The rats were divided into ten groups, each consisting of 6 animals, to include different doses of PFME in setup. Experiments involving PFME consisted of seven groups namely, normal control (Group - 1; receives normal food and water), hepatotoxic control (Group - 2; treated with CCl4 only), PFME treatment groups (3a, 4a, and 5a; fed with 100 mg/kg

body weight (b.w), 150 mg/kg b.w and 200 mg/kg b.w PFME respectively before CCl4 treatment) and two positive controls (Group - 6, receives silymarin 50 mg/kg b.w; Group - 7, receives Liv.52© 200 µl/ per rat before CCl4 treatment).

The normal control (Group 1) received no treatment. The extract treatment groups (PFME - 3a, 4a, and 5a) received different doses (100 mg/kg, 150 mg/kg and 200 mg/kg b.w) of crude extracts (suspended in ddH2O, 100-200 µl) for 7 days which were administered by oral gavage.

The positive control treatment groups received silymarin (Group -6; 50 mg/kg b.w, suspended in 1% carboxymethylcellulose sodium salt solution in water) and Liv.52© (Group -7; 200 µl / animal) also for 7 days and were administered orally. Before oral administration, the food was withdrawn for 12 h (overnight) to apply the samples in empty stomach of the animals. Irrespective of treatment, free access of water was allowed but food was given after 2 h of treatment. After 1h of the last administration on 7th day, acute hepatotoxicity was induced in all the groups, excepting group 1 (normal control), by intraperitoneal (IP) injection of CCl_4 (1 ml/kg b.w; prepared in olive oil, 1:1).

Collection of blood samples and preparation of samples from liver tissue

Rats were sacrificed under anesthetization with ketamine (50 mg/kg b.w) at 48 h after CCl4 administration. Blood samples were collected by cardiac puncture, kept at 16 ºC for 20 min, allowed to clot and centrifuged at 6000 rpm for 15 min at 4 ºC. The straw coloured serum samples were separated from the clot and either used immediately or stored at -80 ºC for future uses.

The liver was excised carefully, washed with ice cold normal saline (0.9% NaCl) to remove the adherent blood and blotted dry. The weight of the liver was recorded and then divided into tissue samples. The liver homogenate was prepared by crushing the liver tissue (0.2 g) with 10 volumes ice cold 0.1 M phosphate buffer (pH 7.4) containing 1mM phenylmethylsulfonyl fluoride (PMSF) in ethanol in a hand operated Potter-Elvehjem homogenizer. The homogenate was then centrifuged at 10,000 rpm for 20 min in 4 ºC and the supernatant was collected carefully and stored immediately at -80 ºC for enzyme assays. For lipid peroxidation assays, liver homogenate was prepared using ice-cold 1.5% KCl solution. Protein contents of various homogenates were estimated by the method of Lowry et al., (1951) using BSA as standard. The tissue samples were fixed in 10% neutral buffer formalin (NBF) for 48 h for histo-pathological studies.

Estimation of hepatic marker enzymes in serum samples

Assessment of alanine transaminase (ALT)

Alanine transaminase, also termed as serum glutamate - pyruvate transaminase (SGPT) was determined following the method described by Bergmeyer et al., (1986) using the kit (Liquid Gold ALT, Autospan, India).

The working ALT reagent was prepared fresh by mixing 10 ml of supplied buffer (0.1 M tris buffer, pH 7.5 containing 0.5 M L-alanine and 1200 U/L lactate dehydrogenase) with 2.5 ml of substrate solution (15 mM α-ketoglutarate and 0.18 mM reduced nicotinamide adenine dinucleotide). Serum samples (100 µl) were added to 1 ml of working ALT reagent and after gentle mixing, the kinetic assay was performed by recording the absorbance after every 30 sec up to 3 min at 340 nm and mean change in absorbance per min (ΔA/min) was deduced. ALT activity (IU/L) was calculated as: ΔA/min x K, where kinetic factor (K) = 1768.49.

Assessment of aspartate aminotransferase (AST)

AST, also known as serum glutamate-oxaloacetate transaminase (SGOT), was determined following the method described by Schumann et al., (2002) using the kit (Liquid Gold AST, Autospan, India).

The working AST reagent was prepared fresh by mixing 10 ml of supplied buffer (0.08 M tris buffer, pH 7.8 containing 0.24 M L-aspartate, 600 U/L malate dehydrogenase and 600 U/L lactate dehydrogenase) with 2.5 ml of substrate solution (12 mM α- ketoglutarate and 0.18 mM reduced nicotinamide adenine dinucleotide). Serum samples (100 µl) were added to 1 ml of working AST reagent and after gentle mixing, the kinetic assay was performed by recording the absorbance after every 30 sec up to 3 min at 340 nm and mean change in absorbance per min (ΔA/min) was deduced. AST activity (IU/L) was calculated as: ΔA/min x K, where kinetic factor (K) = 1768.49.

Assessment of alkaline phosphatase (ALP)

ALP activity from serum samples was determined based on the method of Copeland et al., (1985) using the kit (ALP; pNPP-AMP Kinetic assay, Autospan, India). The hydrolysis of colourless para- nitrophenyl phosphate (pNPP) into yellow coloured p-nitrophenol and

phosphate was done by ALP at alkaline pH 10.2.

The ALP reagent was prepared fresh by mixing 1.2 ml 2- amino-2-methyl-1propanol (AMP) buffer (300 mM AMP, 2mM magnesium acetate and 0.8 mM zinc sulphate) with 10 mM pNPP as provided by the kit. Serum samples (20 µl) were added to 1 ml of working ALP reagent and after gentle mixing, the kinetic assay was performed by recording the absorbance after every 30 sec up to 3 min at 405 nm and mean change in absorbance per min (ΔA/min) was deduced. ALP activity (IU/L) was calculated as: ΔA/min x K, where kinetic factor (K) = 2712.77.

Results and discussion

Hepatoprotective effect of PFME in CCl4 induced hepatotoxic rats

Evaluation of serum marker enzymes

The serum marker enzymes namely ALT (IU/L), AST (IU/L) and ALP (IU/L) are evaluated in different sets of experimental rats including control (untreated), CCl4 induced hepatotoxic rats (hepatotoxic control), different concentrations of PFME with or without CCl4 and positive controls (50 mg silymarin + CCl4; 200 µl Liv.52 + CCl4).

In relation to the hepatotoxic activity of CCl4, the serum liver marker enzymes in combination with different doses of PFME show significant ($P < 0.05$) reduction and such activities are nearly corroborating with positive controls like silymarin and Liv.52. Compared to control, the liver marker enzymes do not show any significant ($P > 0.05$) variation with different doses of treatments. It is worth mentioning that the estimates of liver marker enzymes are nearly same in untreated control rats as well as in PFME treated (different doses) rats.

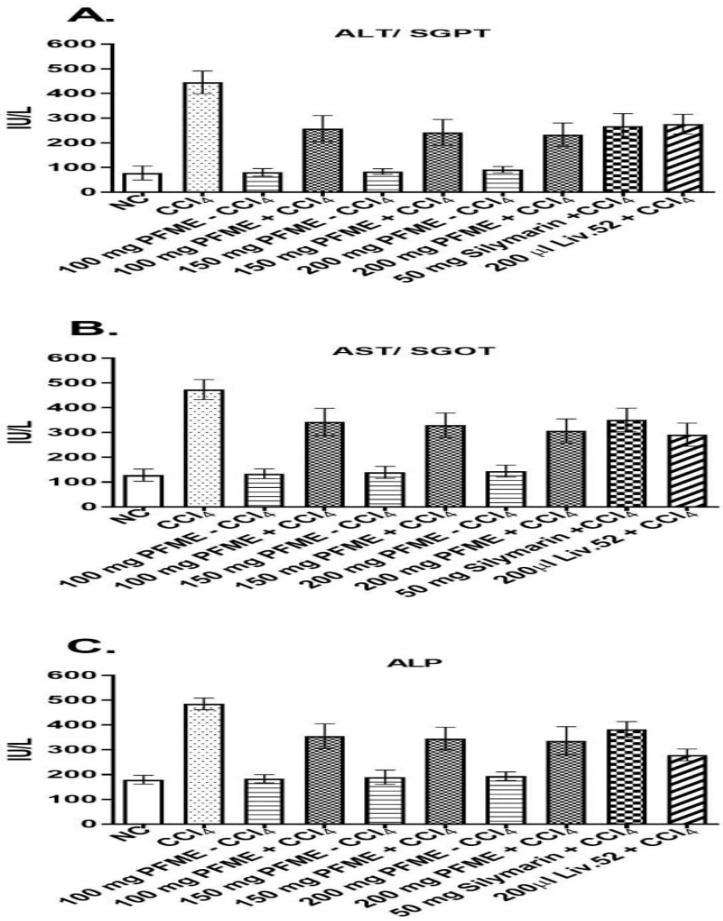


Figure (A-C): Bar diagram showing the effect of PFME on liver marker enzymes in the serum of CCl_4 induced hepatotoxic rats

Table 1: Effect of PFME on liver marker enzymes in the serum of CCl4 induced hepatotoxic rats

Experimental Groups		Liver marker enzymes		
		ALT/ SGPT (IU/L)	**AST/ SGOT (IU/L)**	**ALP (IU/L)**
Control		77.87 ± 28.56	128.30 ± 24.33	179.40 ± 17.62
CCl_4 only		445.20 ± 46.17	473.43 ± 40.36	485.17 ± 23.47
100 mg PFME	- CCl_4	80.61 ± 15.30	133.72 ± 19.57	183.21 ± 16.35
	+ CCl_4	257.90 ± 52.90	342.47 ± 55.00	355.43 ± 50.08
150 mg PFME	- CCl_4	83.59 ± 11.49	139.72 ± 24.26	189.45 ± 28.74
	+ CCl_4	242.33 ± 52.24	329.33 ± 49.80	345.17 ± 45.93
200 mg PFME	- CCl_4	91.64 ± 12.70	144.74 ± 24.18	193.68 ± 17.31
	+ CCl_4	233.03 ± 47.32	306.50 ± 48.23	336.27 ± 56.78
50 mg Silymarin* + CCl_4		266.77 ± 51.01	351.23 ± 47.05	382.33 ± 31.43
200 µl Liv.52* + CCl_4		276.03 ± 39.65	291.40 ± 46.81	279.43 ± 23.77
CD at 5% level		***35.74***	***35.92***	***32.34***

Serum marker enzymes ALT (IU/L), AST (IU/L) and ALP (IU/L) are evaluated in murine model considering sets like control (untreated), CCl4 induced hepatotoxic rats, treatment with different concentrations (100, 150 and 200 mg/kg b.w) of PFME and CCME before administration with or without CCl4 and positive controls (50 mg silymarin + CCl4; 200 µl Liv.52 + CCl4). In relation to hepatotoxic activity of CCl4, different doses of PFME show significant ($P< 0.05$) reduction in serum marker enzyme levels and such decrement are nearly corroborating with the positive controls like silymarin and Liv.52. Compared to control, the liver marker enzymes show non-significant ($P>0.05$) variations with different doses of extracts (PFME) treatments. Estimates of liver marker enzymes are nearly identical in untreated control rats compared to treatments with only PFME.

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