

Original Article

EFFECT OF PIPERINE IN COMBINATION WITH 4-HYDROXYISOLEUCINE ENRICHED  
TRIGONELLA FOENUM-GRAECUM EXTRACT AND SILYMARIN ON PARACETAMOL INDUCED  
LIVER DAMAGE IN RATS

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ABSTRACT

**Objective:** The present study was undertaken to evaluate the hepatoprotective activity of 4-hydroxyisoleucine enriched extract of *Trigonella foenum-graecum* and silymarin in the presence of piperine, against experimentally induced liver injury.

**Methods:** Wistar albino rats (150-180g) of either sex (n=6) were employed in present study. Experimental hepatotoxicity was induced by the administration of paracetamol (3 g/kg, p. o.). All the test drugs were administered orally in the form of suspension to the animals. The combination of silymarin and piperine, 4-hydroxyisoleucine (28%) enriched *T. foenum graecum* extract and piperine were given to animals to compare with individual group as well as with toxicant.

**Results:** Administration of paracetamol caused significant increase in of SGOT, AST, ALP, bilirubin and total proteins characterizing experimental hepatotoxicity. 4-Hydroxyisoleucine enriched extract of *T. foenum-graecum* and silymarin in the presence of piperine, significantly attenuated the toxic effects of hepatotoxicants in liver. Histological study of liver was also carried out to estimate the extent of tissue injury.

**Conclusion:** The combination of piperine and silymarin has shown significant hepatoprotective activity against the injury induced by paracetamol. This was evident from significant reduction in serum enzyme AST, ALT, ALP and total bilirubin. These biochemical observations were also supplemented by histopathological studies of the liver.

**Keywords:** *Trigonella foenum-graecum*, Hepatoprotective, Silymarin, 4-hydroxyisoleucin, Paracetamol.

INTRODUCTION

In daily routine, liver continuously come in contact of xenobiotics, hepatotoxins and chemotherapeutic agents that lead to impairment of its functions by induction of lipid peroxidation and other oxidative damages. Toxic chemicals, excess consumption of alcohol, infections and autoimmune disorders etc. may cause liver diseases. Hepatotoxicity is one of the major causes that could give rise to severe metabolic disorders and even mortality [1]. Medicinal plants possess myriads of secondary metabolites that can protect the liver from such types of hepatotoxins. Silymarin is a standardized extract obtained from the seeds of *Silybum marianum* (L), family Asteraceae (Milk Thistle) which is largely used as a hepato protective agent against poisoning from chemical and environmental toxins. It can be used as a standard natural hepato protective agent for the hepato protective activity studies.

4-Hydroxyisoleucine (4-OH Ile) is a natural nonproteinogenic amino acid with absolute configuration as (2S, 3R, 4S)[2], [3], present in *Trigonella foenum-graecum* Linn., family Fabaceae (Fenugreek) seeds. It has been reported to be responsible for the insulinotropic activity of fenugreek seeds. It increases glucose-induced release of insulin which is strictly dependent on the glucose concentration [4]. This unique property helps in avoiding undesirable side effects such as hypoglycemia in the therapy of type II diabetes. Thus, 4-OH Ile seems a promising dietary supplement in the treatment and prevention of chronic diseases [5].

Piperine, an alkaloid present in the dried unripe fruits of *Piper nigrum* Linn, Family Piperaceae, has been well established bioenhancer if used in combination with other drugs. It reduces the drug dose, danger of drug resistance and toxicity of drugs. Piperine has been reported to bring about its bioenhancement effect by different mechanism including, DNA receptor binding [6], modulation of cell signal transduction [7], or by inhibition of drug efflux pump [8]. The major objective of this paper is to explore the bioenhancement effect of piperine on the hepatoprotective activity of 4-hydroxyisoleucine enriched fraction of fenugreek extract, in the experimental animals.

MATERIALS AND METHODS

Isolation and purification of TF4H (28%) Sugaheal®

Dried mature seeds of Fenugreek, *Trigonella foenum graecum*, family Fagaceae, were first subjected to screening for the presence of total amino acids and trigonelline using thin layer chromatography on pre-coated silica gel TLC plates using n-butanol: acetic acid: water in a ratio of 12:8:2 and initial scanning using UV at 254 nm for the presence of trigonelline. Ninhydrin reagent was used for colour development of total amino acids. Dried mature Fenugreek seeds in a quantity of 1 Kg were flaked in a flaker to expose the inner core, resulting in flakes of average 15 mm in size. The flakes were then subjected to hydro-alcohol extraction using 6 litres of isopropyl alcohol: water mixture in a ratio of 50:50 at 35°C for 12 hours. The resultant liquid (about 5500 ml) was concentrated to a final volume of 150 ml under vacuum at 45-50°C. This liquid was extracted with 3x50 ml of n-hexane to remove fats and lipids. The defatted concentrate was diluted with de-mineralized water to a final volume of 500 ml. This liquid was subjected to fine filtration through 200-mesh size to remove insoluble.

The filtered liquid was then passed through a glass column of 500 mm length x 25 mm diameter containing strong acid cation exchange resin in H<sup>+</sup> form freshly regenerated with 600 ml of 3% HCl in water, followed by washing to neutral pH. After passing the liquid, the column was washed with de-mineralized water to neutral pH. The loaded amino acid and trigonelline were eluted with 200 ml of 0.5 (N) ammonia solution. The ammonia liquid was circulated in the column until it attained a stable pH of 8.0.

The resultant solution was then passed through a glass column of 800 mm length x 25 mm diameter containing 200 ml of freshly regenerated weak acid cation resin in gel form. The eluent from this column was a colourless, neutral liquid having only compounds such as amino acids and trigonelline present in the ratio as in the mother seed. The product was spray dried with the conditions of co-current air flow, inlet temperature, 165°C, outlet temperature 85°C with automizer revolutions of 30,000 rpm. The resultant granules from

the spray drying process was found to be free flowing and suitable for formulation. The resultant powder referred to as Sugaheal® was further screened by HPLC for amino acids by derivatization using working standard of (99%) of 4-hydroxyisoleucine as a dinitrofluorobenzene derivative (347 nm), and trigonelline using UV. HPLC analysis of Sugaheal® on JASCO HPLC/LC 2000 with UV-2075 detector and reverse phase C-18 column L1 as defined in USP30/NF25 with 5µ particle size (diameter 250 mm X 4.6 mm). Results of the HPLC studies have indicated the content of Sugaheal® as 4-hydroxy isoleucine (28.44%), trigonelline (31.91%) and remainder as Galactomannan. Retention time and area under curve (AUC) were calculated to determine purity of sample [9].

#### Isolation of piperine

Black pepper (100g) was collected from local market of Bilaspur, coarsely powdered and extracted in Soxhlet extractor with ethyl alcohol (95%). Total ethanolic extract was concentrated under reduced pressure. The concentrated black pepper extract was added to 10% potassium hydroxide solution in 95% ethanol. Resulting solution was heated and water was added dropwise to yield yellow precipitate. The solution was filtered and the filtrate was further washed with diethyl ether. The crude filtrate afforded fine crystals of piperine after recrystallization in ethyl alcohol [10].

#### Silymarin

Silymarin which is used as a standard hepato protective agent in these studied was procured from Micro Labs Ltd, Hosur as a gift

sample. It was in the form of brownish yellow fine powder slightly soluble in ethyl acetate and hot methanol. The gift sample contained 70.32% of silymarin with content of silybin and isosilybin not less than 30%.

#### Experimental animals

For experimental protocol, Wistar albino rats (150-180g) of either sex and approximately of same age were procured from the animal house of the Institute of the Pharmaceutical sciences. The animals were housed in the polypropylene cages and fed with standard pellet diet and water *ad libitum*.

The animals were kept under alternate cycle of 12 hours of darkness and light in laboratory. The animals were fasted for at least 24 hours before the onset of the activity. The experimental protocols were approved by Institutional animal ethics committee (IAEC No. 994/a/GO/06CPCSEA, reference no. 31/IAEC/Pharmacy/2013) after scrutinization.

#### Hepatic damage

Hepatic damage was induced by the suspension of PCM (2g/kg) in the tragacanth (2%, 1 ml/kg, orally). Hepatic damage was induced within 24 hrs [11].

#### Experimental design

In the experiment, total 36 animals were taken in 6 groups. Each group had animals. Drugs were given by the oral route through oral gavage.

**Table 1: table shows experimental design: for determining the effect of piperine in combination with silymarin and 4-hydroxyisoleucine enriched *Trigonella foenum-graecum* extract**

Group I (Normal control)	Vehicle (2% Tragacanth, 1 ml/kg) for 10 days.
Group II (Toxicant)	PCM (2g/kg) daily for 03 days, vehicle (1 ml/kg) from 4th-10th day.
Group III	PCM (2g/kg) daily for 03 days, silymarin (100mg/kg) from 4th-10th day.
Group IV	PCM (2g/kg) daily for 03 days, silymarin (100mg/kg) + piperine (30mg/kg) from 4th-10th day.
Group V	PCM (2g/kg) daily for 03 days, 28% of 4-OH Ile (400mg/kg) from 4th-10th day.
Group VI	PCM (2g/kg) daily for 03 days, 28% of 4-OH Ile (400mg/kg) + Piperine (30mg/kg) from 4th-10th day.

**Table 2: Effect of piperine in combination with silymarin and 4-hydroxyisoleucine (28%) on paracetamol induced liver damage in rats**

Groups	Treatment	AST (SGOT)	ALT (SGPT)	ALP	Bilirubin	Total protein
I	Control	11.333 ± 0.843	2.666 ± 0.858	19.801 ± 3.189	0.166 ± 0.333	08.371 ± 0.124
II	Toxicant (PCM)	14.000 ± 1.265	3.110 ± 0.993 <sup>ns</sup>	15.539 ± 0.273 <sup>ns</sup>	0.125 ± 0.120 <sup>ns</sup>	09.747 ± 0.078 <sup>s</sup>
III	Silymarin	25.000 ± 1.673 <sup>**</sup>	4.000 ± 0.365 <sup>ns</sup>	05.964 ± 0.986 <sup>**</sup>	1.375 ± 0.111 <sup>**</sup>	10.853 ± 0.605 <sup>ns</sup>
IV	Silymarin + Piperine	03.333 ± 0.210 <sup>**##</sup>	9.354 ± 1.759 <sup>**##</sup>	18.684 ± 0.117 <sup>ns##</sup>	0.562 ± 0.027 <sup>ns##</sup>	07.264 ± 0.171 <sup>**##</sup>
V	28% 4-OH Ile	51.666 ± 0.666 <sup>**</sup>	4.143 ± 0.290 <sup>ns</sup>	03.660 ± 0.034 <sup>**</sup>	0.166 ± 0.039 <sup>ns</sup>	07.436 ± 0.118 <sup>**</sup>
VI	28% 4-OH Ile + Piperine	46.833 ± 0.654 <sup>**ns</sup>	0.122 ± 0.680 <sup>ns,#</sup>	11.736 ± 0.122 <sup>ns##</sup>	0.125 ± 0.009 <sup>ns,ns</sup>	06.705 ± 0.102 <sup>**ns</sup>

Values of <sup>s</sup>P<0.05 and <sup>\*\*</sup>P<0.001 were considered to be significant when control was compared with toxicant, <sup>P</sup><0.05 and <sup>\*\*</sup>P<0.001 were considered to be significant when all groups were compared with toxicant and <sup>#</sup>P<0.05 and <sup>##</sup>P<0.001 were considered to be significant when the group was compared with piperine containing group.

Blood was withdrawn through retro-orbital plexus of rats on 11<sup>th</sup> day. Blood was collected in the sample tubes and centrifuged at 2000 rpm for 25 min so as to obtain unhaemolysed serum. It was used for the estimation of serum AST, ALT [12], alkaline phosphatase [13], bilirubin [14] and total protein [14].

#### Statistical analysis

The results were presented as the mean ± S. E. M. One way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison.

#### Estimation of serum marker enzymes

Serum marker enzymes were estimated by the Liver Function Test kits (Span diagnostics Ltd, Surat). For assessing the bioenhancing effect of piperine [15] on hepato protective action of silymarin and 4-OH Ile (28%), paracetamol (PCM) was used as hepatotoxin (2g/kg).

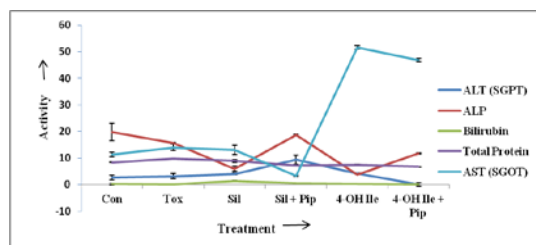
Assessment of liver function was done by estimating the activities of SGOT, AST, ALP, bilirubin and total proteins (Table-1, Graph-1). The degree of developed hepatotoxicity was determined by withdrawing

blood sample from retro orbital plexus and evaluated different parameters on 11<sup>th</sup> day. The elevated levels of SGOT, AST, ALP, bilirubin and suppressed level of total proteins was indicators of the hepatotoxicity. Liver damage and recovery from damage both were assessed on 11<sup>th</sup> day by measuring serum marker enzymes and biochemical changes in liver [16].

#### RESULTS AND DISCUSSION

Thin layer chromatography (TLC) of 4-OH Ile (28%) containing extract of *T. foenum-graecum* revealed 3 spots (R<sub>f</sub>: 0.22, 0.64 and 0.85) in butanol: methanol: acetic acid: water (4:1.5:1:1) solvent system with iodine chamber.

Piperine was obtained as the pale yellow crystalline solid material in 4.5% yield. Piperine revealed a single spot (R<sub>f</sub> 0.52) on TLC plate in toluene: ethyl acetate (7:3) solvent system using Dragendroff's reagent. Silymarin is a standardized from the seeds of *Silybum marianum* (L) is a powerful hepatoprotective against poisoning from chemical and environmental toxins.



**Graph 1: Effect of piperine in combination with silymarin and 4-hydroxyisoleucine (28%)**

#### Effect of silymarin (100 mg/kg each) on serum markers

Administration of silymarin produced significant ( $p < 0.05$ ) decrease in the level of serum ALT when compared with the results of paracetamol treated rats. The AST, ALP and bilirubin levels were not found to be much affected as compared to normal untreated control rats. Level of AST was found to be significant when the results of toxicant (PCM) compared with silymarin ( $**P < 0.001$ ) as indicated in Table-1 and Graph-1.

#### Effect of silymarin and piperine in combination on serum markers

Administration of silymarin (100 mg/kg each) and piperine (30 mg/kg) produced significant ( $**P < 0.001$ ) increase in the level of serum ALT when compared with the results of paracetamol treated rats and as compared with the results of silymarin and 4-OH Ile ( $**P < 0.001$ ). Silymarin-piperine combination gave best results with significant values ( $**P < 0.001$ ) in case of all enzymes (AST, ALT, ALP), bilirubin and total proteins. Level of AST was found to be significant when the results of toxicant (PCM) compared with silymarin-piperine combination ( $**P < 0.001$ ). It decreased the level of AST and bilirubin to 86.66% and 59.12% respectively as compared to normal untreated control rats (Table-1, Graph-1).

#### Effect of 4-hydroxyisoleucine (28%) on serum markers

Results of 4-hydroxyisoleucine (28%) in the dose of 400 mg/kg has been quite insignificant. The serum ALT, AST, ALP and bilirubin levels were not found to be much affected when compared with normal untreated and paracetamol treated rats. Level of AST was found to be significant when the results of toxicant (PCM) compared with 28% 4-OH Ile ( $**P < 0.001$ ) as shown in Table-1 and Graph-1.

#### Effect of 4-hydroxyisoleucine (28%) and piperine in combination on serum markers

28% 4-OH Ile referred to as Sugaheal® in the dose of 400 mg/kg, in combination with piperine (30mg/kg) was found to be significant only in cases of ALT ( $**P < 0.05$ ) and ALP ( $**P < 0.001$ ). Level of AST was found to be significant when the results of toxicant (PCM) compared with 4-OH Ile (28%)- piperine combination ( $**P < 0.001$ ). It decreased the level of AST, ALT and bilirubin to 9.35%, 97.05% and 24.69% respectively (Table-1, Graph-1). Decrease in the elevated level of the above enzymes and increase in the suppressed level of protein indicates reversal of the induced toxicity of the liver. The overall pharmacological profile indicated that after hepatoprotective drug treatment, the levels of SGOT, AST, ALP and bilirubin were decreased while the levels of protein were increased. The use of piperine as bioenhancer showed good results with significant values.

#### CONCLUSION

Experimental data indicated that hepatoprotective effect of silymarin was most significant when used with piperine as compared to when used alone. Piperine has also indicated pronounced activity when used in combination with *T. foenum graecum* (28% 4-OH Ile). Increased hepatoprotective activity can be attributed to bioavailability enhancing effect of piperine by elevating the level of total protein and declining the liver enzymes

respectively. The use of piperine synergized the hepatoprotective action of silymarin and *T. foenum-graecum* (28% 4-OH Ile) possibly by inhibiting its metabolism or by increasing its absorption from the GIT and making it available to the blood plasma. In both these cases, recovery of damaged liver was observed in terms of various serum biochemical parameters after treatment with combination of drug and piperine. From all experimental results it can be concluded that silymarin as well as 4-hydroxyisoleucine (28%) enriched *T. foenum-graecum* extract could be formulated in combination of piperine as a bioenhancer for better protection of liver from the liver toxicants as well as for the treatment of the liver disorders.

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#### CONFLICT OF INTERESTS

Declared None

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