ORIGINAL ARTICLE

Protective effect of extract of *Boerhaavia diffusa* and *Silybum marianum* in combination against fructose induced non-alcoholic fatty liver in rats

Sachin Jain, Kamna S. Tripathi[1], Gaurav Parihar, Praveen Sharma, Aakash Singh Panwar
Departments of Pharmacognosy, College of Pharmacy, IPS Academy, Indore, Madhya Pradesh, [1]Institute of Pharmaceutical Education and Research, Borgaon Meghe, Wardha, Maharashtra, India

Aim: The present study was undertaken with a view to validate the traditional use of *Boerhaavia diffusa* (BEE) root and *Silybum marianum* (SME) seeds in combination as a hepatoprotective agent against non-alcoholic fatty liver disease. **Materials and Methods:** The alcoholic extracts of BEE roots (150 mg/kg, p.o.) and SME (150 mg/kg, p.o.) seeds were administered to the experimental rats individually and in combination (75 mg/kg + 75 mg/kg), p.o. by dispersing it in 1% tween 80, were given, of different groups respectively. After intoxication with high fructose diet (HFD) fructose solution to the animals orally for 6 weeks serum levels of various enzymes were recorded. Serum levels of aspartate transaminase (AST), alanine amino transaminase (ALT), alkaline phosphatase (ALP), total bilirubin (TB), total protein and cholesterol (CHO) level were assessed. **Results:** BEE roots and SME seeds extracts exhibited a significant hepatoprotective effect as evident from the decreases of serum AST, ALT, ALP, TB and CHO and increases in levels of TP compared with control group ($P < 0.01$ or $P < 0.05$). The effect of combination of both the extract exerts more hepatoprotective as revealed by more level of significance. **Conclusions:** The present finding suggests that the hepatoprotective effect of BEE roots and SME seeds extract.

Key words: *Boerhaavia diffusa*, fructose, hepatoprotective, non-alcoholic fatty liver, *silybum marianum*

INTRODUCTION

The liver is an organ of utmost importance; liver plays an important role in the metabolism of materials and compounds viz. foreign particles entering into the body. Human beings are exposed to these compounds through environmental exposure, consumption of contaminated food or during exposure to chemical substances in the occupational environment. All these compounds produce a variety of toxic manifestations.[1]

Non-alcoholic fatty liver disease (NAFLD) has now emerged as a global health challenge. In 1980, Ludwig *et al*., has discovered fatty components in the liver biopsy of non-alcoholic patients.[2] NAFLD was also observed in diabetes mellitus (DM) type II and obesity also hepatic fibrosis.[3,4] DM and obesity create risk factor for liver-related deaths in NAFLD[5] accompanied by insulin deregulation of glucose and lipid metabolism. Potentially hepatotoxic fatty acids in hyperinsulinemic, hepatic steatosis.[6] Cirrhosis and/or hepatic carcinoma can be terminal complications.[7]

In our country, maximum death is predicted with diabetes only. Metabolic syndrome has NAFLD as a component in addition to visceral obesity, hypertension, dysglycemia, and dyslipidemia, insulin resistance, oxidative stress, and proinflammatory cytokines are implicated as causes of both steatohepatosis and steatohepatitis.[8,9] The spectrum of NAFL and its complications being wide, management of the disease is challenging. Weight reduction, ursodeoxycholic acid, clofibrate, gemfibrozil, vitamin E, metformin and betaine have been recommended. However, none of these is conclusively effective, and a well-controlled clinical trials are called for.[10] The effect on hepatic morphology of treatment of obesity with fasting, reducing diets, and small bowel bypass has been studied.[11,12]

Boerhaavia diffusa (BEE) is an herbaceous member of the family Nyctaginaceae. It is widely distributed in the tropics and subtropics.[12] It has a long history of uses by indigenous and tribal people and in Ayurvedic or natural herbal medicines.[13] The major active principle present in the roots is alkaloidal and is known as punarnavine. The world-wide use of BEE roots to treat liver disorders was validated when researchers demonstrated, in 1980 and 1991, that its root extract had antihepatotoxic properties.[14,15] Pharmacological

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Address for correspondence: Prof. Sachin Jain, College of Pharmacy, IPS Academy, Rajendra Nagar, Indore, Madhya Pradesh, India.
E-mail: sachin225819@rediffmail.com
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studies have demonstrated that BEE possesses diuretic,[16] anti-inflammatory,[17] antifibrinolytic,[18] anticonvulsant,[19] and antibacterial properties,[20] which makes it a very useful medicinal plant. All these properties have made this plant very interesting, and the plant has played an important role in the treatment of human and plant diseases.

Silybum marianum (SME) is the well-researched plant in the treatment of liver diseases. *Silymarin* have been isolated, shown to have significant anti-inflammatory effects on hepatic tissue. Several studies have demonstrated a variety of anti-inflammatory effects, including mast cell stabilisation, inhibition of neutrophil migration, and Kupffer cell inhibition.[21-25] SME was used as in combination with BEE root extract in the present study. Significant effort was put into creating an animal model of NAFLD.

MATERIALS AND METHODS

Chemicals

Fructose was procured from LOBA Chemie, Pvt. Ltd. Biochemical kits for cholesterol (CHO), alanine aminotransferase (ALT), aspartate transaminase (AST) alkaline phosphatase (ALP), total bilirubin (TB) and total protein (TP) for assays, and liver tests used for the study were purchased from Merck, India. Merck Microlab-300, Semi Auto analyser was used for the estimation of all biochemical parameters. All the other chemicals and reagents used for experimental purpose were of laboratory grade.

Plant Material

The BEE roots and SME seeds were collected from Nagpur District, Maharashtra, India in the month of September 2009. The plant was identified and authenticated by Dr. N. M. Dongarwar "Post graduate Teaching, Department of Botany, Rashtrasant Tukadoji Maharaj, Nagpur University, Nagpur." A voucher specimen (No. 9471/9474) was deposited with the, "Post graduate Teaching Department of Botany, Nagpur University, Nagpur."

Preparation of Plant Extract

After proper cleaning, the BEE root and SME seeds were subjected to soxhlet extraction by the ethyl alcohol. Each extraction was done thrice for 4 h each. The total filtrate of the extract was put up for distillation. The filtrate was finally spray-dried to yield a powder form. The yield of the extract was 17.3% and 10.1% w/w. This alcoholic extract were then stored in a vacuum desiccators. During the experiment, an appropriate aliquot of the crude extract was diluted with tween 80 before administration to the animals.

Experimental Animals

Male Wistar rats, inbred at Institute of Pharmaceutical Education and Research, Wardha India, were procured after obtaining clearance for the experiment from the Institutional Animal Ethics Committee (Registration No. 535/02/a/CPCSEA/Jan2002) of the Institute. Animals weighing 200-250 g were reared on a balanced laboratory diet and given clean water *ad libitum*. They were kept at 25 ± 2°C, 65% to 70% humidity, and day/night cycle (12 h/12 h).

Preliminary Phytochemical Screening

Preliminary phytochemical analysis of BEE root and SME seeds extracts was performed to identify the nature of phytoconstituents [Table 1].[26]

Acute toxicity studies: Healthy adult male albino mice (18-22) were used for acute toxicity studies as per Organization for Economic Cooperation and development guidelines (Guideline 423: Acute toxic Category Method). Based on these studies, the doses of 150 mg/kg extracts per os (p.o.) were selected for *in-vivo* experiments.[27]

Preparation of High Fructose Diet

For 100 ml of liquid diet we dissolve 6 g of fructose with 125 ml of tween 80 in distilled water to have 50 ml of a solution noted A; we dissolve 0.4 g of CHO in lard to have 50 ml of oily solution noted B. The two solutions A and B were mixed to have an emulsion, which is administered to a dose of 10 mL/kg.[28]

Experimental Design

For the effect of BEE root and SME seeds extract on NAFLD model a protocol of 6 weeks was used. The animals were then randomized into five groups (n = 6/group).

Group 1 control

The animals were fed *ad libitum* for 2 weeks. After 2 weeks, 0.5 ml tween 80, the vehicle was given orally along the same diet for 4 weeks.

Group 2

The animals were fed on HFD orally for 6 weeks.

Table 1: Results of preliminary phytochemical analysis of *Boerhaavia diffusa* roots and *Silybum marianum* seeds extract

Phytoconstituents	BEE	SME
Sterols	+	+
Fats	–	–
Phenols	+	+
Tannins	–	+
Saponin glycoside	+	–
Flavonoids	+	+
Cardiac glycoside	–	–
Alkaloids	+	–
Sugars	+	+
Proteins	+	+

BEE – *Boerhaavia diffusa*; SME – *Silybum marianum*; + – Presence of constituents; – – Absence of constituents

Group 3-5

The animals were fed on HFD for 2 weeks. After 2 weeks, along with HFD, *Silymarin* methanol extract treated (150 mg/kg) (Group 3), BEE ethanol extract treated (150 mg/kg) (Group 4), and *Boerhaavia* ethanol + silymarin methanol (75 mg/kg + 75 mg/kg). (Group 5) were given p.o. for 4 weeks.

Collection of Blood Samples

The treatments were continued for 6 weeks and all animals were retro-orbital bleeding was conducted under light ether anaesthesia at the end of the 6th week after overnight fasting. Blood collected without the use of anticoagulant for serum preparation and allowed to stand for 10 min before being centrifuged at 2,000 rpm for 10 min and the serum was collected using rubber micropipette. The levels for the biochemical tests, ALT, AST, ALP,[29] TP, TB[30] and CHO.[31]

Statistical Analysis

Statistical analysis was carried out using one way analysis of variance test followed by Tukey's multiple comparison test to assess the statistical significance between control, Fructose and intervention groups using Instant Software (version 5.0). The difference between the mean ± standard error of the biochemical values was tested for significance. The minimum level of significance was set at $P < 0.05$.

RESULTS

Preliminary Phytochemical Screening

On preliminary phytochemical analysis, the BEE root and SME seeds extracts showed the presence of certain phytochemicals respectively viz. flavonoids, glycosides, phenolic compounds, proteins, saponins and phytosterols.

Acute Toxicity Studies

In acute oral toxicity study, the BEE root and SME seeds extracts did not show any sign and symptoms of toxicity or mortality up to 2000 mg/kg body weight, which could be considered relatively safe.

Liver Function Marker Tests

Serum CHO levels in all groups are shown in Figure 1. There was an age-related progressive increase in serum CHO in the vehicle control group as shown. In the HFD group, the age-related increase in serum CHO was further amplified. Compared to normal control value, there was a significant increase in the CHO values after 6th weeks in model control group (40.68 ± 1.23, 95.75 ± 3.79 mg/dl, respectively). In the SME and BEE group, there was a significant increase in CHO levels w.r.t. normal control group (62.50 ± 3.15, 64.03 ± 10.41 mg/dl respectively). However, by the 6th week combination of both the extract rise in serum CHO levels had been attenuated (64.66 ± 12.22 mg/dl) compared to those in the HFD group.

After 6 week induction period, a marked rise in ALT levels were observed in the HFD group (73.03 ± 3.93), compared to the vehicle control (38.86 ± 5.60 U/L), whereas in the SME and BEE group the level was reduced (50.64 ± 4.07, 57.78 ± 3.32 respectively U/L). The combination of both extracts showed moderate level of ALT enzyme decrease (52.98 ± 3.01 U/L) [Figure 2].

Similar observations were found in the case of AST a marked rise in enzyme compared to vehicle control (110.61 ± 6.08); SME and BEE extract has reduced the increased (SGOT) Serum Glutamic Oxaloacetic Transaminase levels from (262.05 ± 28.00 IU/L Model control) to (161.35 ± 11.92, SME 181.43 ± 4.01 IU/L, BEE). The combination of both the extract showed moderate level of AST enzyme decrease (168.75 ± 5.90 IU/L) [Figure 3].

The ALP levels in HFD, SME and BEE and in combination groups were significantly lower in the 6th week (189.45 ± 16.82 U/L, 164.53 ± 24.78 U/L, 161.25 ± 17.32 U/L, respectively) compared to the vehicle control group (128.71 ± 2.19 U/L;

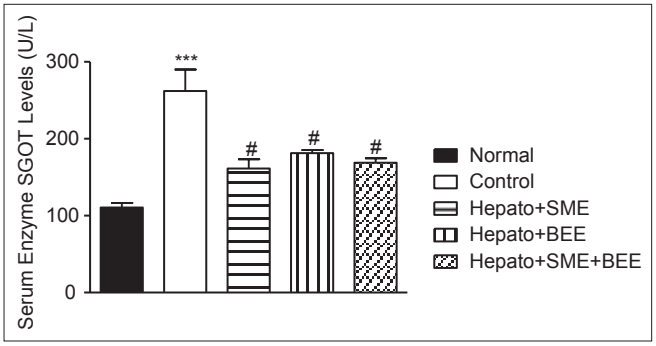

Figure 1: Effect of *Boerhaavia diffusa* root and *Silybum marianum* seeds extracts on SGOT of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean (n = 6) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. control group #$P < 0.05$, vs. HFD model control group

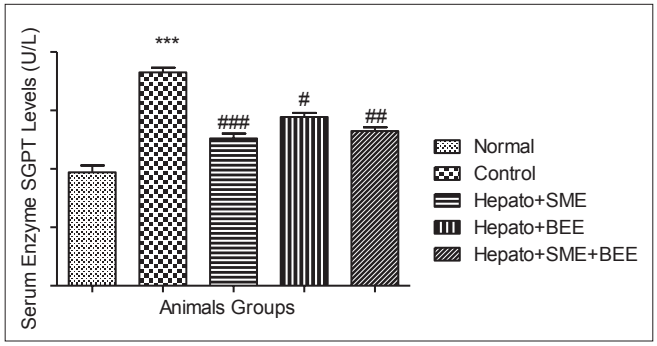

Figure 2: Effect of *Boerhaavia diffusa* root and *Silybum marianum* seeds extracts on SGPT of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean (n = 6) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. Control group #$P < 0.05$, vs. HFD model control group

$P < 0.001$). At the end of the study, the ALP values showed a marked rise in the HFD group compared to the model control (258.46 ± 7.17 U/L). A reduction in ALP levels was observed by the 6th week of treatment, the values had not normalized but significantly reduced [Figure 4].

The basal levels of TB and TP levels were 0.28 ± 0.07 mg/dl and 8.65 ± 0.27 mg/dl respectively. There was significant increase in TB (0.92 ± 0.04 mg/dl), accompanied by significant decrease in level of TP (4.24 ± 0.07 mg/dl) in model control group as compared to the control. There was significant decrease in TB (0.72 ± 0.03 mg/dl), accompanied by significant increase in level of TP (6.57 ± 0.18 mg/dl) in SME group as compared to the toxic control. There was significant decrease in TB (0.72 ± 0.04 mg/dl), accompanied by significant increase in level of TP (5.72 ± 0.18 mg/dl) in BEE as compared to the toxic control. In group which combination of both extract were given TB was decrease to more extent (0.643 ± 0.02 mg/dl and TP was increased to more extent i.e., 7.01 ± 0.83 [Figures 5 and 6].

DISCUSSION

From the phytochemical screening of the BEE roots it is revealed that it contains a large number of such compounds as flavonoids, alkaloids, steroids, triterpenoids, lipids, lignins, carbohydrates, proteins, and glycoproteins, Punarnavine, punarnavoside. The plant contained large quantities of potassium nitrate, besides punarnavine.[32] The herb and roots are rich in proteins and fats.

The seed of SME contain major phytoconstituents contain flavones lignans, collectively referred to as silymarin, tyramine fixed oil, linoleic acid, oleic acid and palmitic acid, protein, tocopherol, sterols, including CHO, potassium, protein, selenium, silandrin, silicon, silybin, silydianin, silyhermin, silymonin, sodium, stearic-acid, tin, zinc. Flowering heads are consumed by diabetics. Seeds are used for their demulcent, antispasmodic and anti-haemorrhagic properties. Though used for the treatment of jaundice and calculi of liver and gall-bladder.[33] So taking into consideration the traditional use and presence of these phytochemicals, SME seeds were evaluated for their *in vitro* antioxidant and *in vivo* hepatoprotective activity.

For assessment of hepatoprotective activity a normal level of different parameters were determined on first day. A repeated dose of a known hepatotoxic, Fructose was administered for six weeks to induce liver damage in experimental animals. The degree of hepatotoxicity developed was determined by withdrawing blood and evaluating different parameters after the protocol, the elevated levels of SGOT, Serum

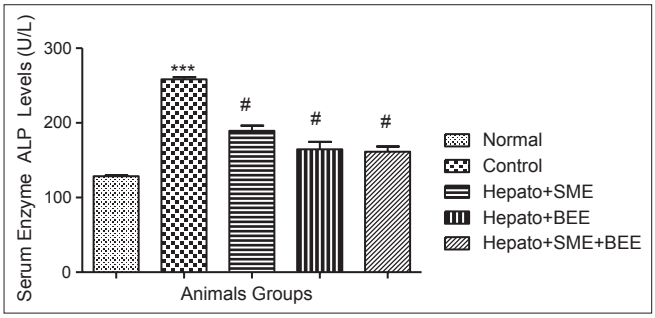

Figure 3: Effect of *Boerhaavia diffusa* root and *Silybum marianum* seeds extracts on alkaline phosphatase of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean ($n = 6$) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. control group #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. HFD model control group

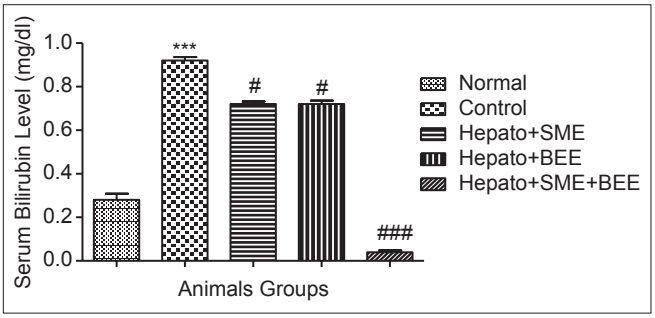

Figure 4: Effect of *Boerhaavia diffusa root* and *Silybum marianum* seeds extracts on bilirubin of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean ($n = 6$) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. control group #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. HFD model control group

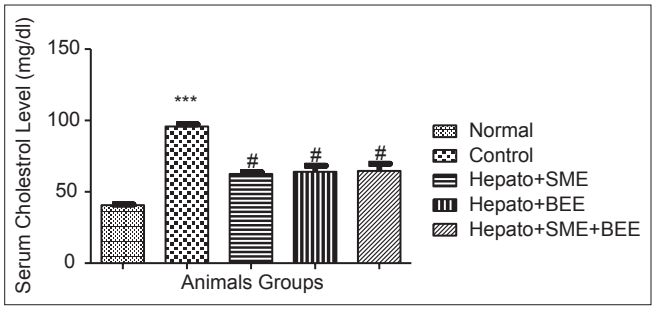

Figure 5: Effect of *Boerhaavia diffusa* root and *Silybum marianum* seeds extracts on serum cholesterol level of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean ($n = 6$) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. control group #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. HFD model control group

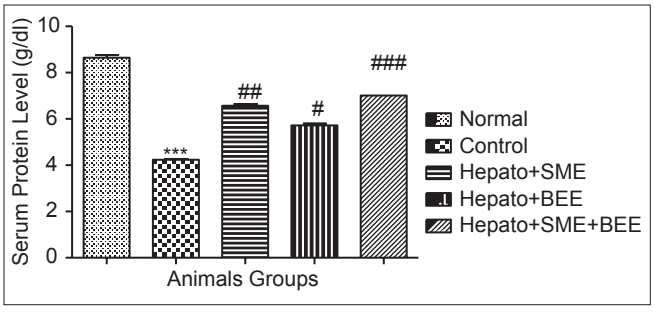

Figure 6: Effect of *Boerhaavia diffusa* root and *Silybum marianum* seeds extracts on protein level of high fructose diet induced hepatotoxicity in mice. Values are in Mean ± standard error of mean ($n = 6$) ANOVA followed by Tukey's Test. ***$P < 0.001$ vs. control group #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. HFD model control group

glutamic-pyruvic transaminase, ALP, CHO, Bilirubin and decreased level of Total protein indicates the hepatotoxicity. In present study fructose was used as hepatotoxin and it produced expected hepatotoxicity by elevating the liver enzyme levels. The mechanism of Fructose induced hepatic injury has interested by many investigators and the compound has become the reference substance for all hepatotoxic compounds.

The mechanism of fructose absorption in the small intestine is not completely understood. Some evidence suggests active transport, because fructose uptake has been shown to occur against a concentration gradient.[34]

Assessment of liver function was made by estimating the activities of SGOT, SGPT, ALP, CHO, Bilirubin and Total protein. SGPT and SGOT are the enzymes originally present in higher concentration in cytoplasm. When there is hepatic injury, these enzymes leak into the blood stream in conformity with the extent of liver damage.[35] The elevated levels of these marker enzymes in Fructose induced hepatic injury in rats in the present study corresponded to the extensive liver damage induced by the toxin.

ALP is a membrane bound enzyme and its elevation in plasma indicates membrane disruption in the organ. ALP though is not a liver specific enzyme the liver is the main source of this enzyme. The level of this enzyme increases in the hepatic injury.[36] ALP levels showed in better reduction in their high concentration induced by Fructose after treatment with extracts for rats.

Proteins are synthesized in liver, if liver injured by any hepatotoxin its cells are unable to perform their work and thus serum or plasma protein concentration decreases in liver. Decrease in the elevated level of the above biochemical's would indicate reversal of the induced toxicity of the liver. Both the extracts in combination had shown significant decrease in enzyme level of SGOT, SGPT, ALP, CHO, Bilirubin ($P < 0.05$) and significant increase in enzyme level of total protein ($P < 0.05$). Thus, hepatoprotective action of these extracts is likely to be due to its ability to induce microsomal enzymes.

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