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RESEARCH ARTICLE

Hepatoprotective impact of combined *Emblica urinaria* (L.), *Saussurea costus* and *Rheum webbianum* extracts against diethylnitrosamine (DEN) induced hepatic injury in Wistar rats

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ABSTRACT

Liver diseases have become one of the major causes of morbidity and mortality in men and animals all over the globe and hepatotoxicity due to drugs appears to be the most common contributing factor. A single drug cannot be effective against all types of severe liver diseases. The different plants exhibit different modes of action and in combination, that activity can be enhanced as compared to the single plant extract. The present study was planned to observe the *in vitro* cytotoxicity of 70% aqua ethanolic extracts of the plants namely *Emblica urinaria* (L.) (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) on human oral cancer KB cells, human lung cancer A549 cells and human cervical carcinoma SiHA cells, respectively followed by *in vivo* experimental study in Albino Wistar rats. In the *in-vivo* experimental protocol, a total of 42 rats were randomly divided into 6 groups, where, group I served as plain control and group II was provided with N-diethylnitrosamine (DEN) alone. Group III received DEN with silymarin and groups IV, V and VI were administered with a combination of *Emblica urinaria* (L.), *Saussureacostus* and *Rheum webbianum* extracts in 3 doses in the ratio of 1:2:2 to study growth response, mortality pattern, clinical signs, biochemical and pathological changes against DEN-induced hepatic damage in rats. *Emblica urinaria* (L.), *Saussureacostus* and *Rheum webbianum* extracts have shown maximal % cytotoxicity at their highest concentration i.e. 200 µg/ml. The minimal alterations in the values of liver enzymes (ALT, AST), total protein and creatinine levels, gross and histopathological changes in the ameliorative groups depicted the protective efficacy of these plant extracts in combination as compared with the group II treated with DEN alone. The present study warrants the hepatoprotective potential of the plant mentioned above combinations and signifies the need to use these medicinal plants as a therapeutic regimen in future.

Keywords: Hepatoprotection, liver, medicinal plants, pathology

INTRODUCTION

The liver is one of the most important vital organs concerned with the metabolism of toxins and drugs[1]. Hepatocytes play an important role in our body such as protein synthesis and storage, carbohydrate and lipid metabolism, glycogen storage, cholesterol, bile salts and hormone production, metabolism and detoxification of exogenous (drugs/insecticides) and endogenous (steroids) substances[2]. Several pharmacological agents, while essential for the therapeutic management of various ailments are frequently implicated in hepatocellular damge owing to their biotransformation primarily in the liver and to lesser extent, the kidneys with hepatotoxicity representing a prevalent dose and duration-dependent adrvese effect[3]. In addition, many pollutants are known to be toxic to the liver, such as organic toxicants and heavy metals. Furthermore, liver injuries can develop into fatty liver, hepatitis, fibrosis, cirrhosis, liver failure or even cancers. Hepatocellular carcinoma is one of the most common types of malignant tumours, which represents the third leading cause of death due to cancer and the fifth most prevalent malignancy worldwide[4].

N-diethylnitrosamine (DEN), is a nitrosamine compound used in the experimental research in the laboratory animals to induce hepatic damage and hepatocellular carcinoma. It is found in very trace amounts as a contaminant in some industrial processes, processed meats, smoke or alcoholic beverages, especially those involving nitrites/nitrates and high-temperature processing. Therefore, the search for compounds or drugs to be used as a complementary therapy alongside DEN administration helps reduce DEN-induced

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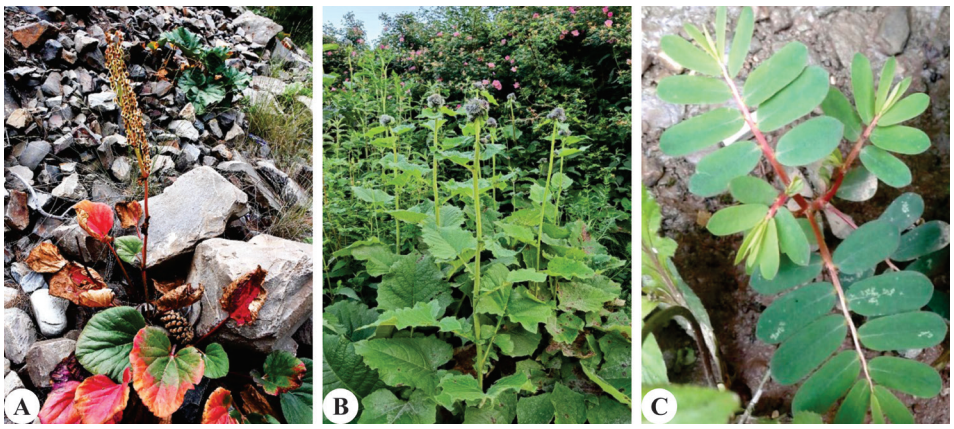

Fig. 1A. *Rheum webbianum*. **B.** *Saussurea costus*. **C.** *Emblica urinaria* (L.).

hepato-toxicity[5]. DEN is a perilous chemical which develops liver cirrhosis and is generally used to initiate hepatocarcinogenesis in rats[6]. DEN is one of the most important hepatocarcinogens and DEN-induced hepatocarcinogenesisis similar to those of human hepatocellular carcinoma (HCC)[7].

Emblica urinaria (L.), one of the herbal plants belonging to the genus *Emblica* (Euphorbiaceae), is widely distributed in China, Southern India and Southern America. *P. Urinaria* is used for the treatment of diseases such as hepatitis and infectious diseases; the anti-tumour usage has also been described in "Chinese Materia Medica" and "Practical Anti-Tumor Herb Medicine"[8]. *Saussurea costus* (Falc.) Lipsch is a well-known medicinal plant growing in the Himalayan region between 2500 to 3000 m above sea level. It is cultivated in a few states of India, including Uttarakhand and Himachal Pradesh. The dried roots of *Saussurea costus* have been widely used in traditional systems of medicine in Asia, as treatments for abdominal pain, tenesmus, nausea and cancer[9]. Sesquiterpene lactones such as costunolide and dehydrocostus lactone, are major components of the roots of *Saussurea costus*, and have been reported to possess various biological activities such as antineoplastic and antihepatotoxic[10,11]. *Rheum Webbianum Royle* is an important medicinal plant belonging to the family Polygonaceae. It is commonly known as 'Himalayan Rhubarb' in English, 'Ravanchini' in Hindi, 'xu mi da huang' in Chinese, 'Chotal' in Pakistan and 'Lachhu' or 'Chu-rtsa' in Ladakh. It is native to Asia-Temperate to Asia-Tropical, from China to India, Nepal and Pakistan. In India, it is found in Himachal Pradesh, Jammu Kashmir and Uttar Pradesh. The extract from the roots of *R. webbianum* are diuretic, laxative, purgative and febrifuge used against indigestion, wounds, gastritis etc[12].

A single drug cannot be effective against all types of severe liver diseases. The different plants exhibit different modes of action and in combination, that activity can be enhanced as compared to the single plant extract. Therefore, effective formulations have to be developed using indigenous medicinal plants with proper pharmacological experiments and clinical trials[13]. Also, plants in combination may exhibit various pharmacological actions like synergism that can enhance the potential medicinal effects hence, further research is necessary to screen the plants in combination for their therapeutic effects. A new approach of combining allopathic and plant-based medicaments in combination is proposed and gaining popularity but the need to understand pharmacological interactions between the drugs is necessary for effective implementation. Moreover, many authors have argued that natural molecules from phyto extracts counter balance the side effects of pure therapeutic agents and therefore these extracts have better medicinal significance[14-16].

Therefore, the present research was proposed to study the hepatoprotective effect of medicinal plants namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) and in various combinations against DEN induced hepatotoxicity in rats.

MATERIAL AND METHODS

Identification and extraction

The plants namely *Emblica urinaria*, *Saussure acostus* and *Rheum webbianum* were collected from different regions of Himachal Pradesh and submitted to CSIR-Institute of Himalayan Bioresource Technology (CSIR-IHBT), Palampur, Himachal Pradesh, India for identification (Fig. 1). The powdered material from each of the plants namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) was weighed, macerated overnight with 70% ethanol and filtered using a double-layered muslin cloth. The filtrate was concentrated over the Rotary Evaporator (Model BUCHI Rotavapor R-210) at 40^{O}C and 175 mbar vacuum pressure until a thick slurry was obtained. The slurry was then subjected to lyophilisation for 24 hrs to obtain dried powdered plant extract. The percent yield

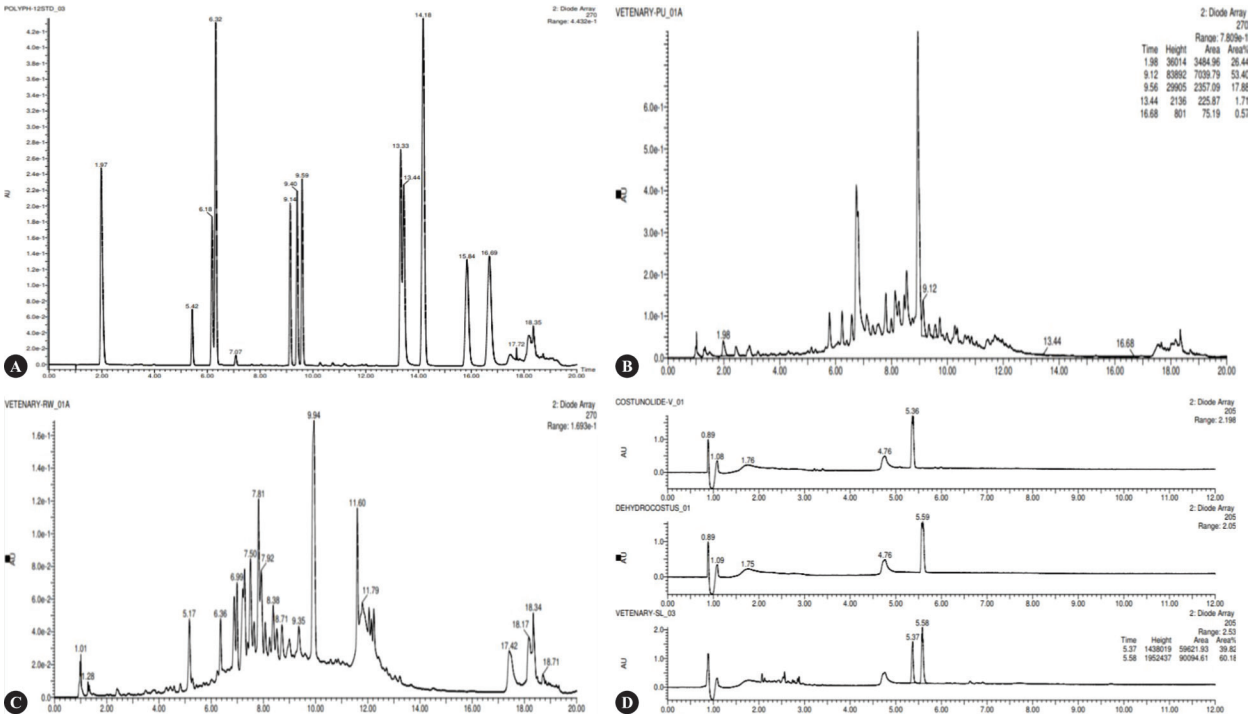

Fig. 2A. UPLC chromatogram of various standard polyphenols. **B.** UPLC chromatogram of *Emblica urinaria*. **C.** UPLC chromatogram of *Rheum webbianum*. **D.** UPLC chromatogram of standard costunolide and dehydrocostus lactone (1st and 2nd chromatogram) compared with *Saussurea costus* extract (3rd chromatogram).

was recorded. The extract was kept at 4°C temperature in the refrigerator until further use.

Characterization of phytoconstituents

Qualitative and quantitative phytochemical screening of different plant extracts was done for the presence of polyphenols and their respective bioactive compounds. UPLC analysis was done to quantify the specific bioactive compounds in the extracts. Gallic acid, syringic acid, quercetin, catechin, caffeic acid, rutin, luteolin, apigenin, cinnamic acid, kaempferol, hyperoside and isoquercetin were employed as reference standards for both qualitative and quantitative analysis using Ultra-Performnace Liquid Chromatography with Diode Array Detector (UPLC-DAD) analysis. Identification of these standards on UPLC was done based on UV wavelength and retention time while the quantification was based on UV by comparing the area under the peak. UPLC columns used were Acquity UPLC-BEH-C18, 1.7 µ column of dimension 2.1x100 mm. UPLC-DAD analysis was carried out with a flow rate of 0.24 ml/min and run time was 20 minutes.

***In-vitro* studies**

The activity of the plant extracts (*Emblica urinaria*, *Saussurea costus* and *Rheum webbianum*) was subjected to *in-vitro* studies using KB (oral carcinoma), human lung and SiHa (cervical carcinoma) cells for measuring the percent cytotoxicity. For routine cell maintenance, RPMI-2640 medium containing 10% foetal bovine serum (FBS), sodium bicarbonate (2 g/L), penicillin (10,000 units/100 ml) and streptomycin (10 mg/100 ml) was used. Cells were kept at 37°C in a humidified 5% CO_2 incubator. The culture media was changed frequently. Cell confluence was

Table 1. Effect of various combinations of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts on the serum biochemical parameters of rats.

Groups	ALT	AST	TP	Creatinine
I	41.90 ± 0.52[a]	106.20 ± 2.35[a]	6.995 ± 0.09[a]	0.70 ± 0.00[a]
II	221.77 ± 7.50[b]	312.14 ± 10.17[b]	3.80 ± 0.21[b]	0.79 ± 0.00[b]
III	117.85 ± 2.41[c]	150.29 ± 7.13[c]	6.28 ± 0.14[a]	0.70 ± 0.01[a]
IV	184.14 ± 9.34[d]	222.21 ± 5.30[b]	5.85 ± 0.15[c]	0.75 ± 0.01[a]
V	121.476 ± 3.60[c]	163.65 ± 9.06[c]	6.31 ± 0.24[a]	0.74 ± 0.01[a]
VI	147.05 ± 12.10[c]	181.72 ± 7.41[c]	6.23 ± 0.44[a]	0.72 ± 0.01[b]

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

Table 2. Effect of various combinations of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts on the relative liver weight of rats.

Parameter	Groups					
	I	II	III	IV	V	VI
Relative liver weight (g)	2.28 ± 0.12[a]	3.42 ± 0.13[b]	2.77 ± 0.12[a]	3.25 ± 0.15[ab]	2.66 ± 0.02[a]	2.85 ± 0.10[a]

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

examined using an inverted microscope. The cells were sub-cultured when 60-70% confluency had been attained. Cells were counted using a haemocytometer under an inverted microscope for seeding and the leftover cells were sub-cultured. *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts were tested at four concentrations i.e. 20, 50, 100 and 200 µg/ml. Sulforhodamine B (SRB) assay was used to determine cytotoxicity. Optical density was measured at 540 nm through microplate reader.

Animals

The present study was conducted on 48 weaned Wistar rats of both sexes (weighing approximately 150-200 g body weight) procured from CSIR-IHBT, Palampur. Out of these 48 rats, 6 were used for the acute toxicity study, whereas 42 were used for the main experimental study. The rats were housed under strict hygienic conditions at the Laboratory Animal House facility of Dr G.C. Negi College of Veterinary and Animal Sciences, CSK HPKV, Palampur (HP) for 17 weeks. Animals were kept in polypropylene cages and rendered under standard laboratory conditions of 12 hours of light/dark cycles. The feed was autoclaved at 15 lbs pressure for 15 minutes prior to feeding to the rats. Water provided to rats was first boiled and subsequently cooled before giving to rats. Both feed and water were given *ad libitum*. During the entire period of the experiment, no medication was given to the rats. The experimental protocol was duly approved by the Committee for Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India.

Acute toxicity study

An acute toxicity study was performed for a combination of 3 different plant extracts (*Emblica urinaria*, *Saussurea costus* and *Rheum webbianum*). Acute toxicity of extract in combination was studied in 6 Albino Wistar rats. The extract was administered orally thrice on alternate days during the first six days at the dose rate of 2 g/kg bw containing 400 mg of *Emblica urinaria*, 800 mg

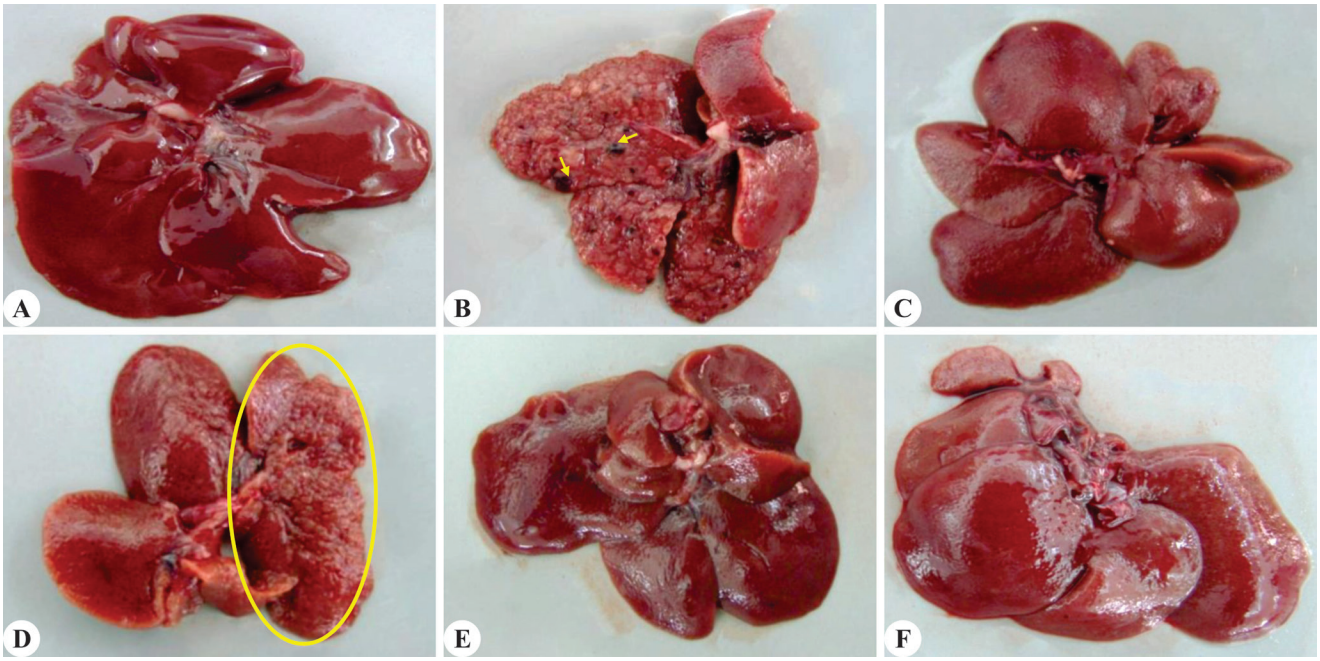

Fig. 3A. Gross pathology of normal liver (Group I). **B.** Severe multifocal to coalescing greyish-black nodules on the liver (yellow arrows) (Group II). **C.** Mild lesions present on the liver as compared to group II (Group III). **D.** Mild to moderate multifocal to coalescing greyish-black nodules on the liver (yellow circle) (Group V). **E & F.** Mild lesion present on the liver as compared to group II (Group VI).

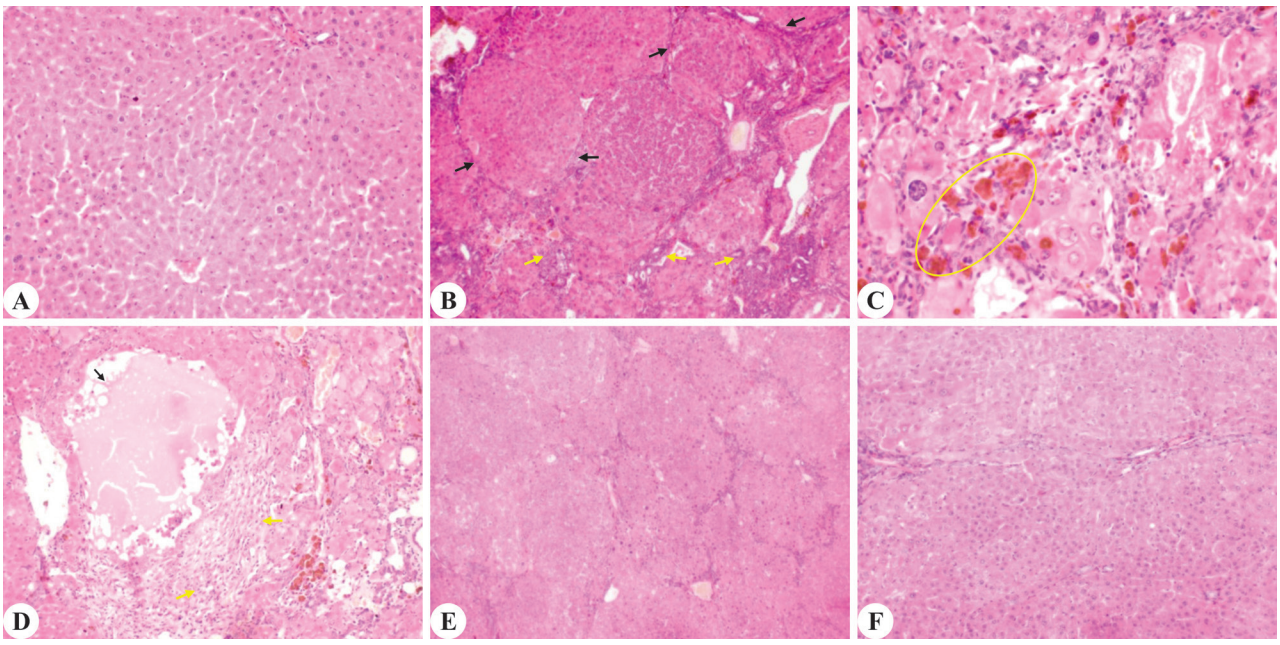

Fig. 4A. Photographs of liver showing: Normal hepatocytes and hepatic cord structure (H&E x10). **B.** Cirrhosis forming hepatocytic nodules (black arrows) and bile duct proliferation (yellow arrows) in and around the portal area (H&E x10). **C.** Apoptotic bodies, brown coloured bile accumulation in the bile duct (yellow circle) and presence of eosinophilic inclusion in the cytoplasm of the cell (H&E x20). **D.** Extensive fibrosis (yellow arrows) and bile cyst (black arrows) (H&E x10). **E.** Cirrhotic changes and presence of regenerating areas (H&E x40). **F.** Individualization of hepatocytes with maintained architecture (H&E x10).

of *Saussurea costus* and 800 mg of *Rheum webbianum*. After 14 days animals were sacrificed and examined grossly and microscopically for any toxicity.

Experimental protocol

In the present study, a total of 42 Wistar rats of either sex were randomly divided into six groups *viz.* group I served as normal control and received vehicle (DMSO) only; group II rats were treated with DEN@0.001% upto 10 weeks and thereafter the dose rate was increased to 0.01% for another 7 weeks; group III rats were treated with silymarin@25 mg/kg bw; group IV rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@100 mg/kg bw (1:2:2); group V rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@250 mg/kg bw (1:2:2) and group VI rats were treated with a combination of *Emblica urinaria*, *Saussurea costus*, *Rheum webbianum* extracts@500 mg/kg bw (1:2:2). After 10 weeks, six animals from group II were sacrificed to study the extent of liver damage on histopathological examination and the dose of DEN for the subsequent study was increased to 0.01% in groups II, III, IV, V and VI upto the end of the experiment. The doses were administered @ 0.5 ml/100g bw to the rats. The dosing of animals was done according to their daily-recorded body weights. The control animals received an equal volume of DMSO similar to those treated with the extracts.

Clinical signs, mortality pattern and growth response

The experimental animals of all the groups were observed closely throughout the experimental period thrice a day, for the development of any clinical signs and mortality. All the animals were carefully checked for the symptoms of dullness or depressed activity, reduced body weight, hair loss, skin condition and gastrointestinal symptoms. The food and water intake were monitored during the entire experiment. The body weight of all the animals was recorded every week for the calculation of dose accordingly.

Serum biochemical estimation

For serum biochemical estimation, the serum samples were collected from the animals of all the groups at the end of the experiment. Approximately 3 ml of blood was collected separately in a dry, clean and sterilized tube and allowed to clot. The sera were separated by centrifugation and further preserved at -20^{O}C till the estimations were carried out. The serum samples were analyzed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, serum creatinine and serum total protein levels using commercially available kits (Agapee Diagnostic Ltd., India) by using Semi-automatic Biochemistry Analyzer (AGAPPE MISPA-Neo, India).

Gross pathology

At the end of the experiment, all the rats from different groups were sacrificed using CO_2 euthanasia in a closed gas chamber. Detailed necropsy examination was carried out and gross changes in the internal organs of the rats were recorded, systematically. The liver and

Table 3. Microscopic lesion scores of liver in rats from various treatment groups.

Microscopic lesions	Groups					
	I	II	III	IV	V	VI
Bile duct hyperplasia	0.00 ± 0.00^{a}	2.83 ± 0.16^{b}	1.50 ± 0.22^{ab}	2.50 ± 0.22^{b}	1.83 ± 0.30^{ab}	2.50 ± 0.22^{b}
Enhanced lobular pattern	0.00 ± 0.00^{a}	3.00 ± 0.00^{b}	0.83 ± 0.30^{ab}	1.66 ± 0.33^{b}	0.33 ± 0.21^{a}	0.83 ± 0.30^{ab}
Pleomorphism	0.00 ± 0.00^{a}	2.50 ± 0.22^{b}	1.50 ± 0.22^{ab}	2.00 ± 0.25^{b}	2.00 ± 0.25^{b}	1.83 ± 0.16^{b}
Hyperchromasia	0.00 ± 0.00^{a}	3.00 ± 0.00^{b}	1.50 ± 0.22^{a}	2.30 ± 0.21^{b}	2.00 ± 0.25^{ab}	1.66 ± 0.21^{ab}
Cirrhosis	0.00 ± 0.00^{a}	2.83 ± 0.16^{b}	1.00 ± 0.00^{a}	1.50 ± 0.22^{ab}	1.00 ± 0.00^{a}	1.00 ± 0.00^{a}
Bile stasis	0.00 ± 0.00^{a}	2.50 ± 0.22^{b}	1.16 ± 0.16^{ab}	2.00 ± 0.00^{b}	1.66 ± 0.33^{ab}	1.83 ± 0.30^{b}
Lipid droplets	0.00 ± 0.00^{a}	2.16 ± 0.16^{b}	1.16 ± 0.16^{ab}	1.50 ± 0.22^{b}	1.00 ± 0.00^{ab}	1.00 ± 0.00^{ab}

*Data represent Mean ± SE (n=6); Group I: Control group (DMSO); Group II: DEN alone; Group III: DEN + Silymarin @ 25 mg/kg bw; Group IV: DEN + plant extract combination (20 mg/kg bw *Emblica urinaria* whole plant extract + 40 mg/kg bw *Saussurea costus* root extract + 40 mg/kg bw *Rheum webbianum* root extract); Group V: DEN + plant extract combination (50 mg/kg bw *Emblica urinaria* whole plant extract + 100 mg/kg bw *Saussurea costus* root extract + 100 mg/kg bw *Rheum webbianum* root extract); Group VI: DEN + plant extract combination (100 mg/kg bw *Emblica urinaria* whole plant extract + 200 mg/kg bw *Saussurea costus* root extract + 200 mg/kg bw *Rheum webbianum* root extract); ($p \leq 0.05$)

spleen were weighed and the relative organ weight (weight of organ as a proportion of the total weight of each rat) was calculated.

Histopathology

After a thorough gross examination, small representative samples of approximately 5 mm thickness (liver, kidneys, spleen and intestines) were collected in 10% neutral buffered formal saline solution. After 3-4 days of fixation, the tissues were subjected to dehydration in ascending grades of ethyl alcohol, clearing in benzene, embedding with paraffin and sectioning to obtain 3-4 μ thick sections. The sections were then stained with haematoxylin and eosin (H&E) stain as per the standard protocol[17]. The bile duct hyperplasia and other degenerative changes were graded with scores as Score 1: occasional/in few (mild), Score 2: fairly common/ multifocal areas (moderate) and Score 3: diffuse (severe)[18].

Statistical analysis

The data generated during the study was analysed using One-way Analysis of Variance (ANOVA) and the results were compared by Tukey's post hoc test. All analysis was performed with Graph Pad In-Stat software (San Diago, USA). All the statements of significance were based on a probability level of P≤0.05.

RESULTS

Percent recovery of the extracts

The final yield of raw plant materials namely *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) was 5.49%, 15% and 12%, respectively.

Phytochemical screening of 70% ethanolic extracts

Various polyphenols namely gallic acid, syringic acid, quercetin, catechin, caffeic acid, rutin, luteolin, apigenin, cinnamic acid, kaempferol, hyperoside and isoquercetin were detected in *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) plant extracts (Fig. 2A). UPLC chromatograms of various standard polyphenols were obtained which were then compared with the UPLC chromatograms of *Emblica urinaria* and *Rheum webbianum* (Fig. 2B & C). Quantification of bioactive compounds of *Saussurea costus* revealed the presence of costunolide and dehydrocostus lactone at the concentrations of 3.57% and 3.68%, respectively (Fig. 2D).

Percentage (%) cytotoxicity

The % cytotoxicity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) in human lung cancer A549 cells was 18.30, 45.82 and 9.98, respectively. The % anti-proliferative activity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) in oral cancer KB cells was 16.94, 23.83 and 5.59, respectively. On cervical carcinoma SiHA cells % cytotoxicity of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) at the highest dose (200 μg/ml, 72 h after incubation) was found to 2.54, 6.67 and 5.73, respectively. Cytotoxicity evaluation was carried out at four concentrations (20, 50, 100 and 200 μg/ml) as described in the Materials and Methods. The concentrations of 20, 50 and 100 μg/ml produced negligible effects. Accordingly, only the data at 200 μg/ml are presented and discussed.

Clinical signs, mortality and body weight gain

The rats used in the acute toxicity study showed no clinical signs. All the rats were healthy and no weight loss was observed. Upon sacrificing no significant changes were observed grossly and histologically.

The animals in the control group i.e. group I were completely healthy with normal behaviour throughout the experiment. The rats in group II treated with DEN only showed a decrease in body weight after 12 weeks,

no level of significance was observed. The rats in group II exhibited hair loss, anorexia, lethargy behaviour and hunched posture. The severity of clinical symptoms was reduced in the ameliorative groups as compared with group II treated with DEN only. The symptoms in ameliorative groups III, IV, V and VI included roughed hair coats and were almost as normal as those of the negative control group. No mortality was recorded in any of the experimental groups, but there was a drastic decline in the body weight of animals treated with DEN only (group II) when compared to the control group (group I).

Serum biochemistry

The rats in group II treated with DEN alone showed a significant ($p \leq 0.05$) increase in the levels of ALT in comparison to the control group. Mean serum ALT activity was observed to be significantly ($p \leq 0.05$) highest in group II treated with DEN only as compared to other ameliorative groups IV, V and VI treated with different combinations of *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts. However, the values of serum ALT in the other treatment groups (III, IV, V, VI) were significantly ($p \leq 0.05$) elevated in comparison to the control group. The rats treated with DEN only (group II) showed a significant ($p \leq 0.05$) elevation in the mean values of serum AST levels in comparison to group I. The mean serum AST activity was observed to be significantly increased ($p \leq 0.05$) in group II treated with DEN alone as compared to the treatment groups III (DEN+silymarin), V (50 mg *Emblica urinaria* whole plant extract + 100 mg *Saussurea costus* root extract + 100 mg *Rheum webbianum* root extract) and VI (100 mg *Emblica urinaria* whole plant extract + 200 mg *Saussurea costus* root extract + 200 mg *Rheum webbianum* root extract). The groups III, IV, V and VI significantly ($p \leq 0.05$) restored the level of AST to the control group. The group treated with DEN only exhibited a significant ($p \leq 0.05$) reduction in the levels of total proteins as compared with the control group. The various combinations of plant extracts were found to be significantly ($p \leq 0.05$) effective in attenuating the level of serum total protein in comparison with the DEN-treated group. The values of serum creatinine were found to be significantly ($p \leq 0.05$) increased in group II treated with DEN only as compared to the control group i.e. group I and other treatment groups i.e. groups III, IV, V and VI (Table 1).

Effect on relative liver weight

The relative liver weight of rats treated with DEN only increased significantly ($p \leq 0.05$) as compared with the control group I. The rats in group III and ameliorative groups (V and VI) treated with different combinations of *Emblica urinaria* (whole plant) *Saussurea costus* (roots) and *Rheum webbianum* (roots) extracts exhibited significant ($p \leq 0.05$) reduction in liver weight in comparison to DEN control (Table 2).

Gross pathology

No significant gross pathological changes were evident in the liver of rats in the control group (group I) (Fig. 3A). Hepatomegaly characterized by marked enlargement of the liver with rounding of edges was a consistent gross observation in group II rats given with DEN alone. The liver surface in most of the rats in group II showed rough boundaries and shrinkage. Multiple grey to whitish coloured raised nodules ranging 1-5 mm in diameter were scattered over the entire surface of the liver (Fig. 3B). In several rats, multiple black-coloured, raised nodules were present. The rats in group III treated with silymarin exhibited a moderately rough appearance with a few coalescing nodules in comparison to DEN treated group i.e. group II (Fig. 3C). The overall appearance of the liver in group III was improved in comparison to the DEN-treated group. Groups IV, V and VI treated with different combinations of plant extracts have shown small greyish-white coloured, raised and poorly demarcated nodules over the liver surface with no evidence of black-coloured nodules (Figs. 3D & F). The sevrity of the liver damage was minimal in ameliorative groups as compared with group II rats treated with DEN only. The severity of lesions was reduced in various treatment groups and group VI was treated with *Emblica urinaria* (whole plant), *Saussurea costus* (roots) and *Rheum webbianum* (roots) in the combination of 100, 200 and 200 mg/kg bw, respectively, has shown maximal protection against DEN induced liver damage.

Histopathology

The liver sections in rats in the control group i.e. group 1 showed normal hepatic parenchyma with no distortion in its hepatic cords. Hepatocytes in group I showed uniform-sized nuclei throughout the hepatic parenchyma (Fig. 4A). The rats in group II treated with DEN only showed markedly distorted hepatic cords with lobulated hepatic parenchyma. The lobular structures in the liver were surrounded by a mild to moderate degree of fibrous connective tissue. The hepatocytes within the lobule revealed varying degrees of degenerative changes which were characterized by increased cellular swelling due to eosinophilic cytoplasmic granularity. The liver section of rats in group II showed severe bile duct proliferation along with hydropic or vacuolar changes. In many of the hepatocytes, cytoplasmic details were altogether lost and appeared to contain dark homogenous eosinophilic deposits. In some places, the fibrous tissue proliferation wandered into the hepatic parenchyma which resulted in the small islands of hepatic cells. Lymphocytic infiltration along with massive deposition of golden brown pigment was consistently present in the portal areas (Fig. 4B). Microscopic examination of liver tissues in various ameliorative groups was

studied, the changes were evident in all the groups but the extent of damage was limited as compared to the positive control group i.e. group II (Figs. 4C-E). Among all the combination groups, the rats in group VI (*Emblica urinaria*:*Saussurea costus*:*Rheum webbianum*, 100:200:200 mg/kg bw) showed significantly lower lesion intensity (Fig. 4F and Table 3). The mean microscopic lesion score of changes in the liver of rats in various treatment groups is depicted in Table 3.

Lesions in the spleen were characterized by mild congestion in group II treated with DEN only. Histopathological changes in the spleen sections in group II rats revealed an increase in lymphoid pulp. The lymphoid pulp area often coalesces to form a bigger area in the splenic parenchyma. In the white pulp splenic artery seems to be displaced towards the periphery. There was a slight increase in the red pulp. Golden brown hemosider in pigment was evident. Similar changes were noticed in the combination groups (groups IV, V and VI), but the changes were less severe as compared to the group II provided with DEN only.

The examination of kidney sections in rats kept in the DEN only treated group i.e. group II exhibited obliteration in the bowman's space, swelling and severe congestion, whereas, no histopathological changes were evident in the renal tissue of the rats kept in various treatment groups. The histopathological examination of intestinal tissue of rats in group II has shown lymphocytic infiltration in the villi, hyperplasia of enterocytes and lymphoid depletion in the Peyerpatches along with fragmented nuclei. Clubbing of the villi was also evident along with the goblet cell hyperplasia. In ameliorative groups (groups IV, V, VI), the histopathological changes in the intestine section were less severe as compared to group II treated with DEN only and were comparable to group III treated with silymarin.

DISCUSSION

The different plant extracts exhibit different modes of action for curing diseases and in mixture form may exhibit enhanced activity than that of individual plants, which is known as 'synergistic action'. A particular principle in its pure form may have only a fraction of the pharmacological activity that it has in its plant matrix. This highlights the importance of using the plant as a whole or a mixture of plants for treating a disease as done in the present experimental study[19,20]. The hepatoprotective effect of the ethanolic extract of *M. azedarach* (MAE) and *P. Longum* (PLE) and their combination (BHE) against hepatic damage in rats concluded that a combination of ethanolic extract *of M. Azederach* and *P. Longum* (BHE) exerts more hepatoprotective activity as compared to the group in which these were administered separately and may act as an adjuvant in clinical conditions associated with liver damage[14]. The potential use of "SAL," a standardized blend comprised of three extracts from *Schisandrachinensis*, *Artemisia capillaries* and *Aloe barbadensis*, in mitigating chemically induced acute liver toxicities. They concluded that the composition of SAL in the ratio of 4S:8A:3L could potentially be considered as a mitigating agent for chemical induced hepatotoxicity[21].

Although the 70% ethanolic extracts of *Emblicaurinaria*, *Saussurea costus* and *Rheum webbianum* showed moderate *in vitro* activity and but their combined use exhibited quite promising hepatoprotection against DEN induced liver damge in rats. In the present study, *Emblicaurinaria*, *Saussurea costus* and *Rheum webbianum* exhibited measurable *in vitro* cytotoxic activity. Among them, *Saussurea costus* demonstrated comparatively higher effects (45.82% on A549 cells), whereas the responses of *Emblicaurinaria* and *Rheum webbianum* were modest, with particularly low cytotoxicity observed on SiHa cells (2.54% and 5.73% at 200 µg/ml, respectively). These plant extracts after *in vitro* study were used further for *in vivo* assessment of the protective effect in combination against DEN induced hepatic damage in rats. The hepatoprotective and antioxidant activity of Indian *Phyllanthus* species. The extracts were screened for hepatoprotective activity against *tert* butyl hydroperoxide (*t*-BH) induced toxicity in HepG2 cells[22]. Similarly, the induction of apoptosis by *Saussurea costus* and *Pharbitisnil* on AGS Gastric Cancer Cells. They analyzed the effects of these medicinal herbs on the proliferation and the expression of cell growth/apoptosis related molecules, by using AGS gastric cancer cells[10]. Moon and associates studied the anticancer activity of *Saussurea costus* extract by an apoptotic pathway in KB human oral cancer cells. They evaluated cell viability of KB cells by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay after treatment with 30 mg/ml of methanol extract from the dried roots of *S. costus*[23]. Another study concluded by Tabin observed that maximum aloe emodin, emodin and rhein were found in *Rheum webbianum* as compared to *Rheum spiciforme*[24]. Sun and Bu investigated the anticancer effect of emodin. They studied the effects of emodin on SGC-7901 human gastric carcinoma cell proliferation, apoptosis and regulation of phosphatase of regenerating liver-3 (PRL-3). The results showed that the proliferation of SGC-7901 cells was inhibited by emodin in a time and concentration dependent manner[25].

Diethylnitrosamine (nitrosamine) is a member of the N-nitroso compound and is considered the most important hepato toxin and carcinogen in the environment. Nitrosamine containing compounds are considered to be more effective hazards to human health as they are widely used in the industry[26]. In the liver, cytochrome P450 (CYP2E1) stimulates the N-diethylnitrosamine to form reactive oxygen species

and electrophiles. Another mechanism of nitrosamine to induce the HCC, CYP2E1 excite the Kupffer cells, which start the generation of reactive oxygen species[27]. In the present study, hair loss was evident almost in all the animals. However, the symptoms such as anorexia, hunched back posture and roughed hair coat were more pronounced in group II treated with DEN only as compared with the control and other ameliorative groups. There was a drastic decline in the body weight of animals treated with DEN only as compared to various treatment groups.

The serum ALT and AST activities were elevated, whereas, total serum protein concentration was lower in the DEN treated group as compared to the control group. The values of various serum biochemical parameters in the combination groups were generally lower as compared to group II given DEN alone, thereby suggesting the *in vivo* protective effect of the ethanolic extract of plants in combination (1:2:2) against hepatic damage. The hepatoprotective effect of *Phyllanthus Urinaria* L. on CCl_4 induced liver injury. They observed that *Phyllanthus Urinaria* treatment could reverse the increase in ALT, AST and ALP induced by CCl_4 and attenuate the pathological changes in rat liver[28]. The antihepatotoxic activity of aqueous methanolic extract of *Saussurea costus* Clarke root on D-galactosamine (D-GalN) and Lipopolysaccharide (LPS) induced hepatitis in mice. When D-GalN and LPS were co-administered resulted in higher plasma transaminase levels (ALT/AST) as compared to the control group[11].

A severe intensity of gross lesions was evident in the liver of the rats treated with DEN only. The lesions in group II rats treated with DEN only comprised hepatomegaly, liver paleness, presence of greyish-white nodules and black-coloured nodules (peliosishepatis). However, the gross lesions score intensity was lower in combination groups (groups IV, V and VI) as compared to the group administered with DEN alone (group II). Hau and co-researchers investigated that the mice, administered through an intraperitoneal route with a lethal dose of acetaminophen, followed by oral administration of *Phyllanthusurinaria* extract did not show any mortality[29]. Elsayed and associates tested the efficacy of *Saussurea costus* root extract on the reversion of already established liver fibrosis[30]. Akhtar and co-workers studied the hepatoprotective effects of *Rheumemodi* roots and their aqueous and methanolic extracts against liver damage induced by paracetamol in albino rats. The findings of our study are in connection with the studies mentioned above, where the plant extracts are found to have hepatoprotective activity[31].

Group VI (highest dose combination) showed a trend toward reduced lesion scores compared with the DEN only group; these reductions were not statistically significant for several histopathological parameters, including bile duct hyperplasia, enhanced lobular pattern, pleomorphism, hyperchromasia and bile stasis. A clear and significant hepatoprotective effect was observed only in cirrhosis, where Group VI recorded a distinctly lower score. In our study, the treatment groups exhibited marked regenerative changes and were concordant with a study conducted by[32]. The microscopic changes were also observed in the spleen, kidney and intestinal tissues of rats treated with DEN only indicating the systemic effect of DEN, although these changes were nil or minimal in other ameliorative groups given with 70% ethanolic extract of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* extracts in various combinations.

CONCLUSION

The clinical signs, mortality pattern, serum biochemistry and gross and histopathological lesions strongly suggest the promising hepatoprotective action of *Emblica urinaria*, *Saussurea costus* and *Rheum webbianum* (1:2:2) in a dose dependent manner in rats. The highest dose of the combination of extracts from all 3 plants i.e. 100:200:200 mg/kg bw showed a combined hepatoprotective action against DEN induced liver damage in rats. Therefore, the present investigation warrants incorporating a combination of these plants as a medication for therapeutic purposes and needs to explore its possible mechanisms in future.

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