

Histological study of hepatoprotective aspect of indigo and its isolated isothiocyanate compound against *N*-Nitrosopyrrolidine in toxicated mice

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ABSTRACT

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In the present study, hepatoprotective role of hydroethanol extract of *Indigofera tinctoria* (HEIT) and its isolated isothiocyanate compound was assessed against hepatocellular Carcinoma (HCC) in mice at histopathological level. For the study, an isothiocyanate compound 'ITC-1' from HEIT was isolated, purified and characterized by using various chromatographic and spectroscopic techniques, and its possible structure was identified as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane. HCC was induced in mice by administration of Nitrosopyrrolidine (NPYR) intraperitoneally @120 mg/kg body wt., followed by weekly subcutaneous dose of CCl₄ (3 ml/kg body wt.) for 50 days. Crude HEIT at 100 and 300 mg/kg body wt. and 'ITC-1' (15 mg/kg body wt.) were given orally and intra-peritoneally, respectively to NPYR intoxicated mice for 21 days. Silymarin was also administered (25 mg/kg body wt.) to compare the results from plant and ITC-1. Histopathological analysis of liver sections of NPYR treated mice showed obliterated morphological architecture with abnormal hepatocytes and portal triad in comparison to control. Results significantly evidenced that both *I. tinctoria* and its isolated 'ITC-1' superiorly restored cellular architecture than Silymarin. The study concluded that both isolated compound ITC-1 and novel HEIT extract may have future prospects in treatment of liver cancer.

Keywords: Hepatocellular carcinoma, histopathology, *Indigofera tinctoria*, isothiocyanate, *N*-Nitrosopyrrolidine, silymarin

INTRODUCTION

N-Nitrosopyrrolidine (NPYR) is a potent hepatocarcinogenic nitrosamine that mainly found in side stream of cigarette smoke, fried bacon and other cured meats and also in drinking water¹⁻³. High mortality rate due to hepatocellular carcinoma (HCC) is a major health problem throughout the world⁴. Allopathic treatment of HCC causes various types of side effects in patient's body like damage of healthy tissues along with cancerous one and also this treatment is much expensive to afford. Since ancient time, herbal or ayurvedic treatment is considered as a safe and effective way to cure many health problems. Hence, HCC treatment by the use of isolated and pure herbal phytoconstituents would be an effective way.

Indigofera tinctoria L. (Nili or True Indigo; Family: Fabaceae) is cultivated preferably in Southern part of India⁵. Traditional importance in the treatment of several health problems made it as highly valuable medicinal plant⁶. Anti-lung carcinogenic⁷ and anti-proliferative⁸ activities of *I. tinctoria* were also studied. In our lab, potent antioxidant activity of novel hydroethanolic extract (80 ml ethanol + 20 ml distilled water) of *I. tinctoria* was investigated⁹ which was followed by the isolation of an isothiocyanate compound 'ITC-1' (1-[1,2-

Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane; C₁₆H₂₂N₄OS₅) from this¹⁰. Isothiocyanate is one of the most active phytochemical category of organosulfur compounds that possess potent anticarcinogenic activity¹¹.

Histology characterizes the carcinogenicity or protective activity of compound by analyzing cellular or subcellular morphological determinants and indicates the correlation between cellular structure and physiological function, the present study was designed to assess the anti-hepatocarcinogenic activity of crude hydroethanolic extract and isolated Isothiocyanate compound 'ITC-1' of *I. tinctoria* against NPYR-CCl₄ intoxicated HCC bearing male mice at tissue level.

MATERIALS AND METHODS

Plant collection and authentication

Aerial parts of shade dried *Indigofera tinctoria* plant sample was purchased from S.M. Heena Industries, Jaipur, Rajasthan (Order no. and date: 01/19-06-2012; Byers Invoice no./Batch no. 26). They had collected it from Tindivanam (latitude 12°15'0"N; longitude 79°39'0"E), District: Villupuram, Tamilnadu, India. The plant sample was identified, authenticated and submitted at Herbarium division of Department of Bioscience and Biotechnology, Banasthali University (BURI-13515).

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Extract preparation

Plant sample was minced in a grinder (Model:Unova, Mfd. by Vishva Enterprises, Mumbai), sieved (Sethi Standard Test Sieve, Yamberzal International, Jaipur) and extracted with Soxhlet using 80% ethanol after defatted with petroleum ether (non-polar solvent) to remove fatty acids. Then, extract was filtered and dried under reduced pressure in a rotary evaporator (HeidolphInKarp Instruments Pvt. Ltd., Germany) to afford 25.825 g crude hydroethanolic extract/100 g *I. tinctoria* powder.

Isolation and characterization of 'ITC-1': A natural Isothiocyanate derivative from *I. tinctoria*¹⁰

Positive phytochemical analysis of hydroethanolic extract of *I. tinctoria* (HEIT) for organosulfur compounds¹² was followed by the elution of compounds fractions through adsorption column chromatography via gradient elution of hexane, chloroform and methanol. Positive fraction was eluted with solvent system chloroform: methanol (90:10) with the yield of 192 mg/5 g of crude HEIT. It was green in color. HPLC of this fraction showed the presence of various compounds which was further separated by HPLC followed the conformation of single sulphur containing compound (ITC-1). HPLC (Dionex-Ultimate 3000 HPLC) was configured as quaternary gradient pump, variable wavelength UV detector (Ultimate 3000 RS), Genetix G Chrom Chromatography, Ultra P silica RP C18 HS, Standard column, size 5 µm, 250 mm × 4.6 mm. Gradient elution system was (95% water at 0 min; 80% at 3 min; 50% at 10 min; 50% at 20 min; 80% at 25 min; 95% at 30 min; 95% at 35 min) with Water:Acetonitrile (ACN). Isolated compound was then lyophilized (Henil Science Industrial Pvt. Ltd, Korea; Model: Clean Vac 8 Vacuum Freeze dryer) to get the pure compound. The yield of purified 'ITC-1' was 22 mg/5 g HEIT with 97% purity. Structural elucidation of ITC-1 was done by using various spectral techniques, i.e., Liquid Chromatography- Mass Spectrometry (LC-MS) (Agilent Technologies, California; Protein Digest method, Q-TOF B.05.00 (B5042.0) version, 6200 series TOF/6500 series), Proton Nuclear Magnetic Resonance (1H-NMR; BRUKER Advance III 500MHz) and FT-IR (Fourier transform-Infrared spectroscopy; Bruker, OPUS, Germany). The isolated compound that obtained after purification, was light pale yellow in color, liquid in nature, stable at room temperature and completely soluble in water. The

structure of ITC-1 was elucidated as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane (C₁₆H₂₂N₄OS₅; *m/z* = 446.17) (Fig. 1). Presence of "N=C=S group in the isolated compound indicates it as a natural isothiocyanate compound.

Chemicals and reagents

N-Nitrosopyrrolidine and CCl₄ were purchased from Sigma-Aldrich, U.S.A. and Emparta, ACS, Merck-Millipore, India respectively. Olive oil was purchased from Central Drug House (C.D.H.), Jaipur, Rajasthan, India. Commercial Silymarin drug "Silybon-140" was procured from "Micro Labs Limited, Himachal Pradesh, India. Staining dyes, i.e., Haematoxylin and Eosin were procured from Merck-Millipore, India. All chemicals were of highest purity and analytical grade (AR). Other unmentioned chemicals were also procured from reliable firms of India.

Animal care and monitoring

Male, Swiss albino mice weighing about 25-30 g were purchased from "Hissar Agriculture University", Hissar, Haryana, India. Ethical approval (BT/67-2.5.2012) was obtained from Institutional Animal Ethical Committee (IAEC). All animals were housed properly in polypropylene cages under well maintained environmental conditions (temperature regulated at 25±3°C, 12 h alternating light and dark cycle and relative humidity 50±5%) and fed with nutritious commercial pelleted diet (Ashirvad India Pvt. Ltd) and drinking water *ad libitum*.

Experimental regimen

Total of 36 mice aged 5 weeks were equally divided into 6 groups. The whole duration of experiments was 70 days. NPYR was administered on 1st day of experiment once whereas first dose of CCl₄ was administered on 14th day and then continued for next 5 doses (total 6 doses) once in a week. HEIT, Silymarin and ITC-1 were administered in to mice for 21 days regularly and were started on 50th day of experiment after 2 h of administration of last CCl₄ dose. The experimental regime is presented in table 1. On day 71th, all animals were sacrificed and necropsied, then liver lobules were collected in pre-chilled normal saline (0.9%) to avoid any osmotic disruption of cells. NPYR-CCl₄ doses were decided on the basis of published report¹³ and also on the basis of laboratory observations. Plant doses were

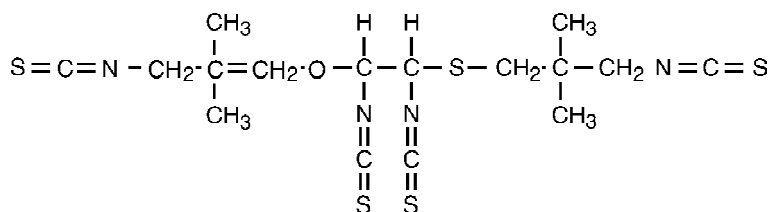


Fig.1. Possible proposed structure of ITC-1 on the basis of FT-IR, LC-MS and 1H-NMR having IUPAC name 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane.

also decided on the basis of previous published report¹⁴ and modified according to the experimental observations conducted in our own laboratory. The dose of Silymarin was also selected on the basis of literature¹⁵.

Tissue slide preparation

Histopathology was studied in Haematoxylin and Eosin stained micro level sections of liver tissues of all treated mice according to previously published method¹⁶. All slides were seen at both low and high magnification (40 and 100 X) with the help of Phase Contrast microscope (Magnus, Model-Metzer-M; Olympus Pvt. Ltd., Noida) equipped with high resolution Sony camera.

RESULTS

Histopathological observations of various treated groups are depicted in Fig. 2 to 7. In control or healthy liver (Group 1; Fig. 2), well-shaped portal triad was seen. Large hepatic portal vein (HPV) with rounded bile ductules (BD) and ovoid hepatic artery (HA) were clearly seen. Radially arranged hepatocytes plate (HCR) or hepatic chords (HC) were observed around the well-shaped central vein (CV). Hepatic lobule exhibited polyhedral shaped hepatocytes (PH) (Fig. 2g) with prominent nucleus (N) with well-preserved cytoplasm (Fig. 2c). Hepatic portal vein and central vein were well

lined by the flattened endothelial cells, a type of sinusoidal lining cells. Fig. 2b showed kupffer cells (KC) which is the major part of the monocyte-macrophage defense system.

Fig. 3 shows histological analysis of liver tissues of carcinogen (NPYR) intoxicated group (Group 2). NPYR induced HCC implies loss of architecture and thickening of lobule parts. Disrupted architecture of central vein (DCV) is clearly visible in Fig. 3a and 3d when compared with healthy one. Bile ductules (DBD) and hepatic arteries (DHA) became dilated whereas morphologically disrupted portal vein (DPV) was also observed. Infiltrated RBCs (DRBCs) in central vein with loss of membrane integrity that causes its distorted shape was clearly examined in Fig. 3e. Large inflammatory collection i.e. accumulation of kuffer cells (KC) and lymphocytes (IL) with thickening of portal tract is seen in Fig. 3g. Neoplastic cells with granular and dispersed cytoplasm were observed in swollen hepatocytes (SNH). Distorted hepatic chords (hepatocytes plates; DHC) with necrosis of hepatocytes pointed out the cancerous condition. Irregular shaped nucleus (N) and enlargement in sinusoids (ES) also represented the necrotic condition of liver after carcinogen exposure.

Sectioned liver of mice which received hydroethanolic extract of *I. tinctoria* low dose (HEIT-L; 100 mg/kg b.w.) and high dose (HEIT-H; 300 mg/kg b.w.) showed normal histological appearance (Fig. 4 and 5). Low

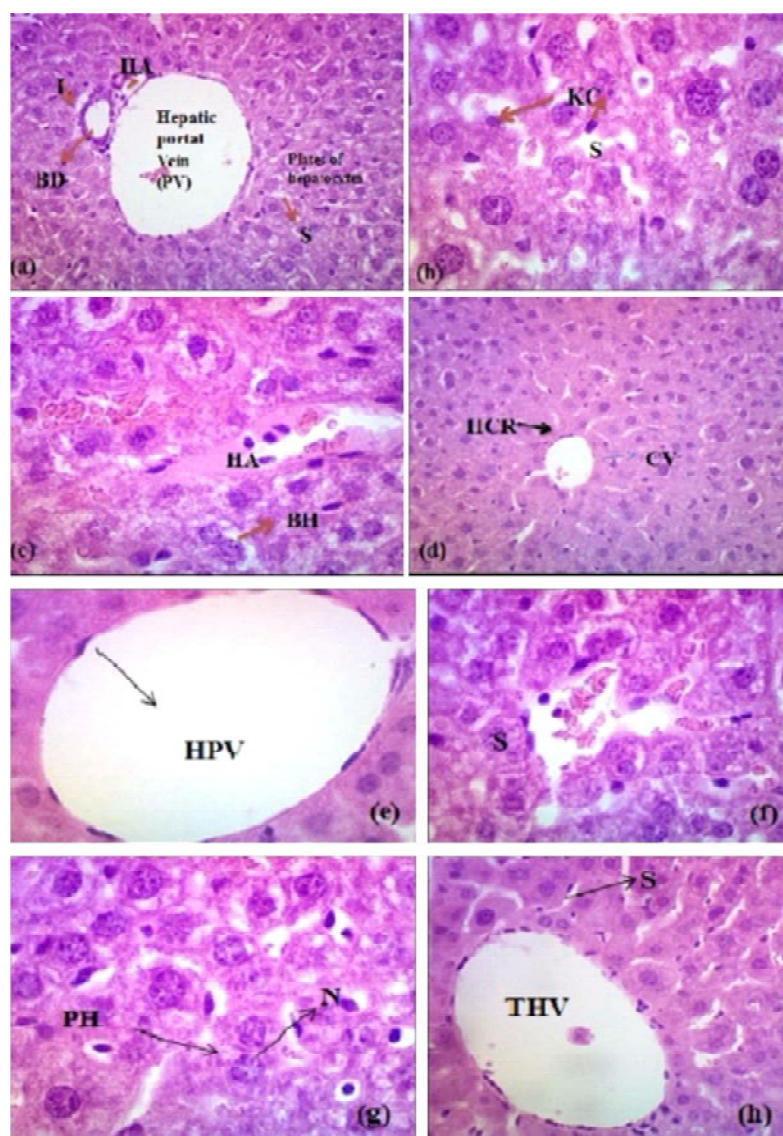


Fig. 2. Transverse section of Liver tissues of control group (healthy mice); H&E stain (a) Complete portal system of liver that include large Hepatic portal Vein (PV) with ovule shaped Bile ductules (BD), Lymphatics (L) and Hepatic artery (HA). Sinusoids (S) are present as large intracellular spaces. (x400); (b) Kupffer cells (KC) as sinusoidal lining cells are present (x1000); (c) Hepatic artery with RBCs and Binucleate hepatocyte (BH) are present (x1000); (d) Hepatic cell radiate (HCR) around round shaped small Central Vein (CV) (x400); (e) Hepatic Portal Vein (HPV) lined with flattened endothelial cells (x1000); (f) large sinusoidal space with RBCs (x1000); (g) Polyhedral shaped Hepatocytes (PH) with large single nucleus (N) (x1000); (h) Terminal hepatic venule (THV) with sinusoids (x400).

dose of HEIT showed the initiation process of recovery in cancerous liver cells. Several determinants were observed in microsectioned liver of HEIT-L group (Group 3) i.e. thin lined and arranged flattened epithelial cells with proper shaped central vein, less infiltrated RBCs (IRBCs), well-shaped portal vein, normal architecture

of bile ductules and regeneration of cytoplasm (Fig. 4) but this is the initiation process because some structures were still looked abnormal morphologically like nuclei shape (DN), dilated sinusoids, hyper vacuolation and swollen epithelial cells (SECs) around portal vein. Hepatic chords also started to rearrange. Proper morphological

Table 1. Grouping and treatment schedule of mice in experimental regimen.

Group	n=6 in each group	Treatments
Group 1	Control vehicle only	Olive oil
Group 2	N-Nitrosopyrrolidine (NPYR) + CCl ₄	120 mg/kg b.w. and 3 mL/kg b.w. respectively
Group 3	NPYR-CCl ₄ + HEIT-L	Hydroethanol extract of <i>I. tinctoria</i> ; low dose; 100 mg/kg b.w.
Group 4	NPYR-CCl ₄ + HEIT-H	Hydroethanol extract of <i>I. tinctoria</i> ; high dose; 300 mg/kg b.w.
Group 5	NPYR-CCl ₄ + Silymarin	Silymarin; 25 mg/kg b.w.
Group 6	NPYR-CCl ₄ + ITC-1	Isolated compound 'ITC-1'; 15 mg/kg b.w.

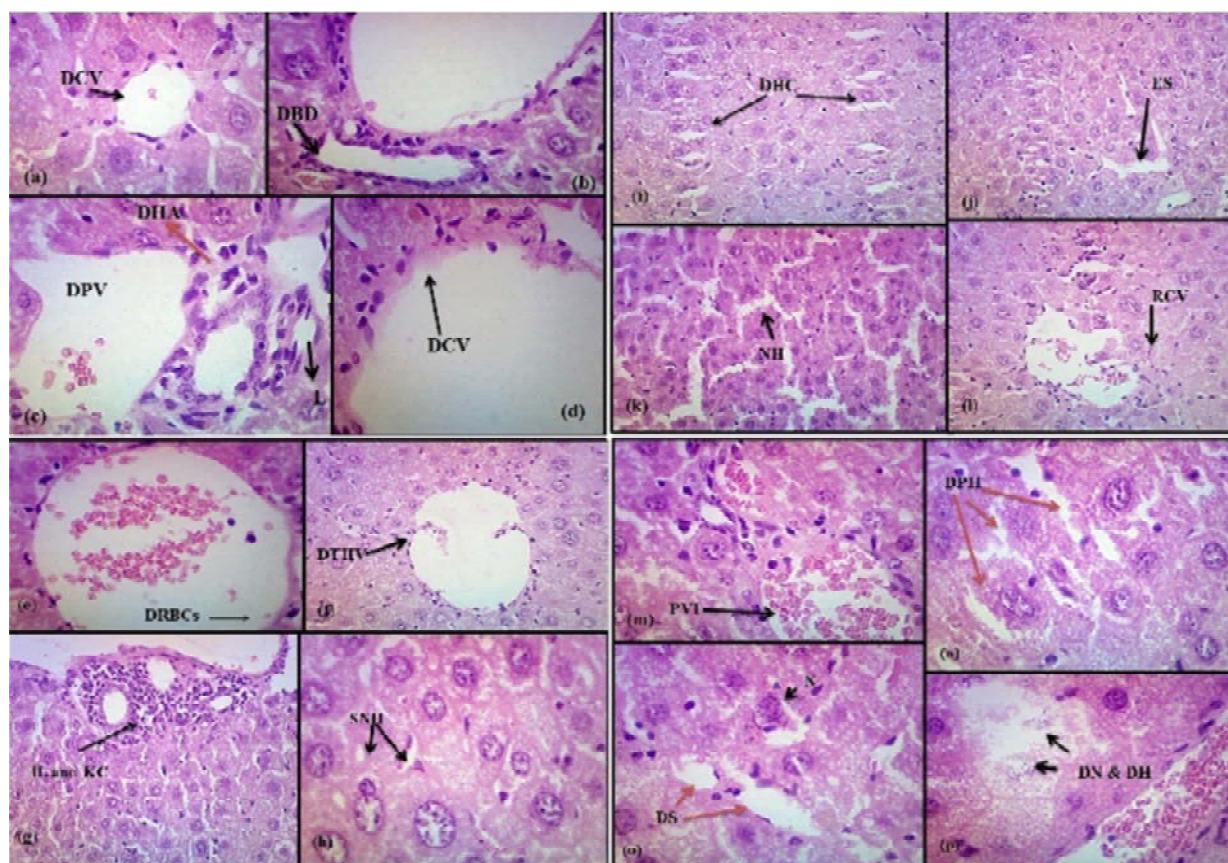


Fig. 3. Transverse section of Liver tissues of NPYR-CCl₄ treated group; H&E stain (a) distorted shape of central vein (DCV) (x1000); (b) dilated bile ductules (DBD) (x1000); (c) disruption of hepatic portal system, i.e., distorted portal vein (DPV) with infiltrated erythrocytes, damaged hepatic artery (DHA), congestion of Lymphatics (L) (x1000); (d) damaged lining of central vein (DCV) with disappearance and disarrangement of flattened endothelial cells (x1000); (e) distorted shape and cell lining of RBCs (DRBCs); (f) highly disturbed cell lining of terminal hepatic venule (DTHV) (x400); (g) broad infiltration of lymphocytes (IL) and accumulation of Kuffer cells (KC) around portal tract and thinking of bile ductules, hepatic artery and lymphatics (x400); (h) degenerated and dispersed cytoplasm in nuclei and swollen hepatocytes (SNH) (x1000); (i) clearly distorted hepatic chords (DHC) (x400); (j) enlargement of sinusoidal space (ES) (x400); (k) necrosis of hepatocytes (NH) and disarrangement of hepatic cells; hepatocytes lining and parenchyma degeneration (x400); (l) highly ruptured central vein (CV) with infiltrated RBCs (x400); (m) large amount of infiltrated RBCs in Portal vein (PVI) (x1000); (n) severally dilated and distorted sinusoids with damaged plating of hepatocytes (x1000); (o) Irregular shaped nucleus (N) with sinusoids (DS) (x1000); (p) severally necrosis of hepatic cells and nuclei (degeneration); DN & DH (x1000).

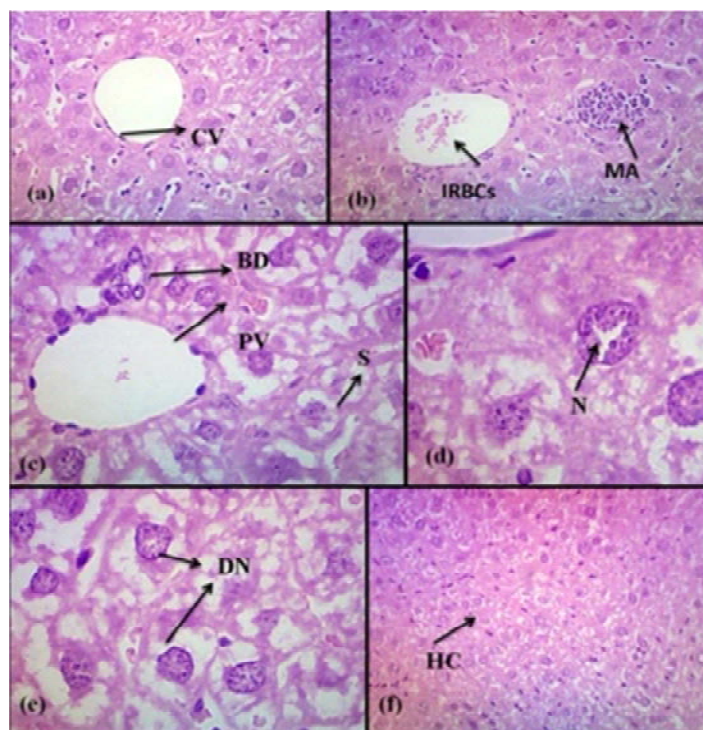


Fig. 4. Transverse section of Liver tissues of Low dose (100 mg/kg body weight) of hydroethanolic extract of *I. tinctoria* treated group; H&E stain (a) well lined central vein (CV) with properly arranged epithelial cells (x400); (b) less infiltration of erythrocytes (IRBCs) and monocyte cells aggregation (MA) (x400); (c) well organized portal vein (PV) lined with arranged epithelial cells and normal shaped bile ductules (BD); vacuolation and dilated sinusoids (S) are still present (x1000); (d) Nucleoplasm regeneration starts (N) in nucleus (x1000); (e) distorted shaped nuclei (DN) are still present (x1000); (f) rearrangement of hepatic chords (HC) starts (x400).

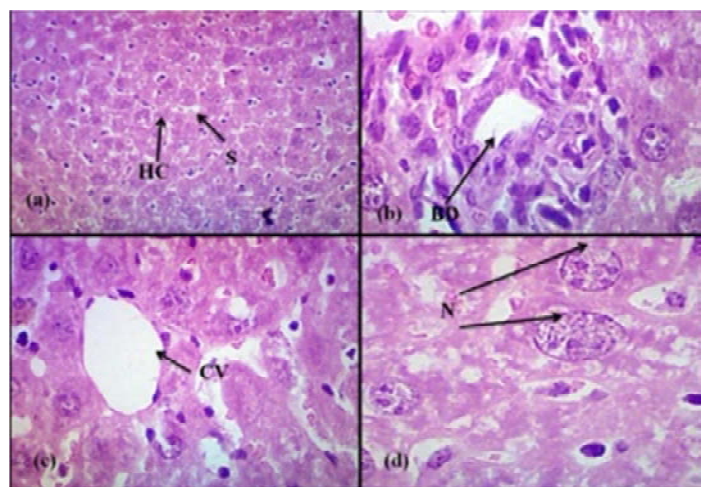


Fig.5. Transverse section of Liver tissues of High dose (300 mg/kg body weight) of hydroethanolic extract of *I. tinctoria* treated group; H&E stain (a) normal hepatic chords (HC); normal sinusoids with no vacuolation (S) (x400); (b) normal bile ductules (BD) (x1000); (c) normal central vein, i.e., round shaped and lined with properly arranged flattened epithelial cells (x1000); (d) normal round or ovoid shaped nucleus (N) (x1000).

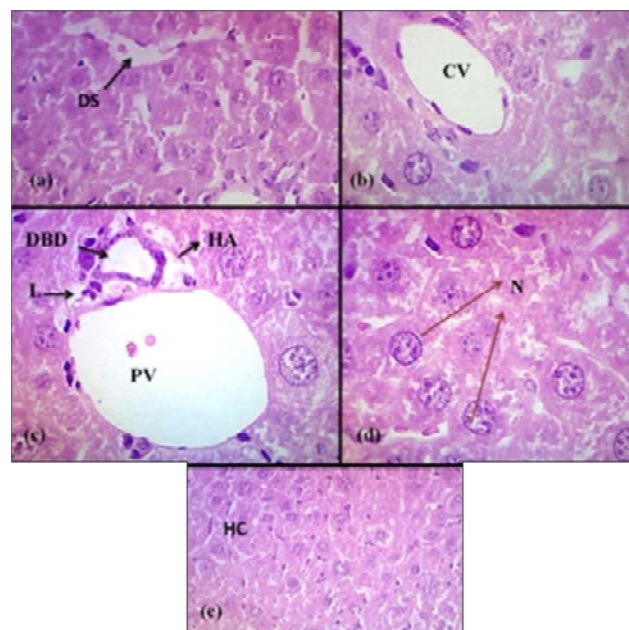


Fig.6. Transverse section of Liver tissues of Silymarin treated group; H&E stain (a) dilated sinusoids (DS) (x1000); (b) well lined central vein (CV) (x1000); (c) recovered hepatic portal system; well-lined portal vein (PV) with less infiltration of RBCs; less dilated bile ductules (DBD); no congestion of Lymphatics (L); dilated hepatic artery (HA) (x1000); (d) normal well shaped nucleus (N) (x1000); (e) regenerated hepatic chords (HC) (x400).

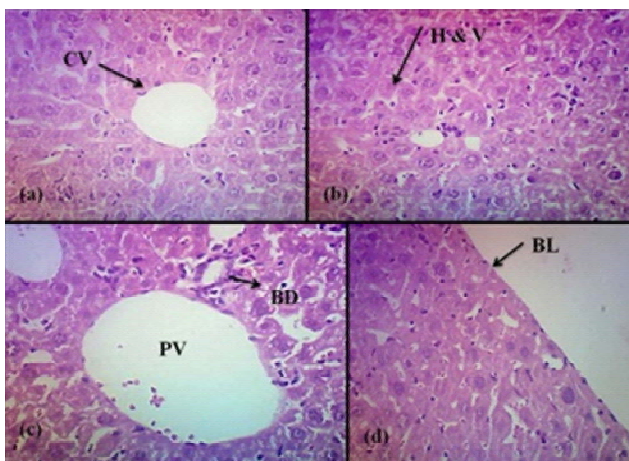


Fig. 7. Transverse section of Liver tissues of isolated compound (ITC"1) treated group (15 mg/kg body weight); H&E stain (a) well organized central vein (CV) and lined with flattened epithelial cells (x1000); (b) less hepatocytes (H) damage and vacuolation (V) (x400); (c) recovered hepatic portal system; well-lined portal vein (PV) with less infiltration of RBCs; less dilated bile ductules (BD) (x1000); (d) proper base line (BL) showed no damage at periphery of tissue (x400).

architecture of sectioned liver tissue is seen in Fig. 5 when high dose of HEIT was given to animals. Normal sinusoids and proper shaped nuclei (ovoid or round) were clearly visible and gave an indication that higher dose of HEIT gave better response in comparison of lower dose.

Silymarin, a well-known hepatoprotective drug, also gave evidence to its protective response (Fig. 6) against HCC but some structures were still not recovered like dilated bile ductules (DBD), sinusoids (DS) and hepatic artery (HA) in portal triad but portal vein showed non disrupted morphology.

Isolated compound from HEIT, i.e., as 1-[1,2-Diisothiocyanato-2-(3-isothiocyanato-2,2-dimethyl-propylsulfanyl)-ethoxy]-3-isothiocyanato-2,2-dimethyl-propane or ITC¹ showed hepatoprotective activity against NPYR induced HCC through the appearance of well-lined central vein, portal vein and base line at periphery (Fig. 7). Slightly dilated bile ductule was observed in microsectioned hepatic tissues whereas no hepatocytes damage (H) and vacuolation (V) were observed that represent its chemo-preventive property. Proper base line (BL) showed no damage at periphery of hepatic tissue.

DISCUSSION

Liver develops embryologically as a glandular outgrowth of the primitive gut. It plays an important role in detoxification of various metabolic waste products from the body, synthesis and secretion of bile, plasma lipoproteins, and plasma proteins like clotting factors and also in metabolism like gluconeogenesis¹⁷. In general structure, liver is composed of several lobules which are roughly hexagonal in shape. Terminal branches of the hepatic artery, hepatic portal vein and a small bile duct are located at the angle of the boundaries of these lobules and are collectively known as portal tract or portal triad. Blood from portal tract converges upon central vein or terminal hepatic venule of each lobe through sinusoids (spaces) which pass between plates of hepatocytes. Liver is composed of parenchymal cells or hepatocytes which are arranged radially around the central vein and then spread throughout the liver lobules. Numbers of lymphatics were also present near the portal vein¹⁸⁻²⁰.

Hepatocellular carcinoma (HCC) is primarily associated with the severely damaged liver. Histopathological evaluation increase the chances of earlier detection of liver associated diseases that based on morphological changes in hepatocytes, deposition of some unusual structures, distorted nuclear structure, cytoplasmic disappearance etc. Numbers of changes were observed in liver sections after several treatments in mice.

Metabolism of various xenobiotics promotes the production of ROS and free radicals that further affects various cellular macromolecules like lipids, proteins, DNA etc. that lead to cell dysfunction via DNA damage, oxidative stress, DNA adducts formation²¹. The observed histopathological structures directly correlate with the

previous research reports²²⁻²⁴. NPYR has capability to produce various free radicals via liver microsomes which is the main reason of cell damage and responsible for HCC development²⁵. Various hepatic DNA adducts are formed through the metabolism of NPYR i.e. dThd, dAdo and dGuo that have potent mutagenic and carcinogenic potential thus responsible for liver cancer progression through cell damage²⁶. Several studies reported that gross changes in nitrosamine affected liver sections like severe hepatocytes damage/necrosis and infiltration of inflammatory cells around portal triad which are significantly associated with the observed changes in NPYR treated mice²⁷⁻²⁹.

HCC leads loss of architecture in liver cells as verified by observed morphological changes in NPYR treated group. It might be due to disruption in frame work of reticulin fibers. These fibers are spread throughout the hepatic chords and support the architecture of hepatocytes and sinusoid lining cells (SLCs). Disruption in reticulin fibers also might be responsible for morphological disruption of central vein and portal vein due to presence of thin lined flattened epithelial cells³⁰.

Results avowed that HEIT and its isolated compound ITC-1 possess good potential to restore the structural designing of liver components. HEIT possess good antioxidant and scavenging activity⁹ which is due to the presence of varieties of both non polar and polar phytoconstituents. Isothiocyanate compounds are reported to possess anticarcinogenic activity that is due to their capability to arrest G2/M phase of cell cycle and inhibition of production of HDAC (histone deacetylase) protein^{11,31}. There is quite possibility that HEIT and ITC-1 both have capability to regenerate of reticulin fibers.

Recently, progression in cancer cases every year lead to increase in consumption of allopathic medicines. A severe side effect of synthetic medicines during chemotherapy causes condition more vulnerable. So, now a days, it is very important to develop new and more effective herbal medicine which would be affordable to everyone and have less or no side effects. In our study, ITC-1 showed no side effect in experiment animals and results also favor its protective role against hepatocellular carcinoma (HCC).

Thus, on the basis of above study, we can concluded that both crude hydroethanol extract and isolated isothiocyanate compound 'ITC-1' have protective role against hepatocellular carcinoma that was validated by the histopathological examination of liver tissues. 'ITC-1' showed much better hepatoprotective role through normalizing the cellular architecture of liver tissues than silymarin, a commercially available hepatoprotective drug thus has future prospect in development of better hepatoprotective drug.

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