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Research Article

QUENCHING OF N-NITROSOPYRROLIDINE INDUCED HEPATOCELLULAR CARCINOMA ON POST TREATMENT WITH THE *HELICTERES ISORA*

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ABSTRACT

Objective: The present study was aimed at probing the protective potential of *Helicteres isora* hydroethanolic stem bark extract (HIHSBE) against N-nitrosopyrrolidine (NPYR) induced hepatocellular carcinoma (HCC) in Swiss albino male mice.

Method: Mice were divided into six groups of six mice in each. Hepatocellular carcinoma (HCC) was induced by single intraperitoneal injection of the carcinogen nitrosopyrrolidine (NPYR). Followed by the subcutaneous injection of carbon tetrachloride (CCl_4). Carcinogen treated mice were then orally administered with *Helicteres isora* hydroethanolic stem bark extract (HIHSBE) at a dose of 100 and 200 mg/kg once daily for 4 weeks followed by investigation of liver injury markers like alanine transaminase (ALT), aspartate transaminase (AST), alanine phosphatase (ALP), gamma glutamyl transferase (GGT), Lactate dehydrogenase (LDH). Tumor markers alpha fetoprotein and carcinoembryonic antigen were determined in serum. Level of catalase (CAT), reduced glutathione (GSH), glutathione-s- transferase (GST) and lipid peroxidation were also estimated.

Results: The level of liver injury markers and antioxidant enzymes decreased in the liver tissue of NPYR treated mice compared to normal control mice. However, HIHSBE post treatment increased the level of these enzymes compared to only carcinogen treated mice. HIHSBE also lowered the level of tumor markers and lipid peroxidation in serum and liver tissue of mice bearing HCC respectively. Histological studies also supported biochemical investigations.

Conclusion: The chemopreventive effect of HIHSBE is well supported in our study as it hinders the development of HCC by interacting with ROS during carcinogenesis and thus counterbalancing the antioxidant defense system as analyzed.

Keywords: *Helicteres isora*, Hepatocellular carcinoma, Liver enzymes, N-nitrosopyrrolidine, Oxidative stress.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the frequent deadliest cancer across the globe. Several risk factors have been associated in the development of HCC such as chemical carcinogens, cirrhosis, hepatitis B and C virus infection, and other liver diseases like non-alcoholic fatty liver diseases. Among these factors chemical carcinogens play a key role in precipitating hepatocarcinogenesis. Nitrosamines are focused due to their increasing appreciation as potential human carcinogens [1,2]. Among some known carcinogens a significant presence of N-nitrosopyrrolidine (NPYR) has been reported as preservatives used in meat products, milk, alcohol, tobacco [3]. NPYR when administered with the drinking water it resulted in, HCC in rats [4].

During metabolic biotransformation of NPYR, there is perceptible generation of free radicals leading to oxidative stress inside the cells. As reported earlier oxidative stress is responsible for DNA lipid damage and may even cause alteration in gene expression by forming DNA adducts like 4-oxobutanediaohydroxideand related intermediates to precipitate cancer [5]. Signal transduction pathways are known to get activated by reactive oxygen species (ROS). When ROS are not neutralized by endogenous antioxidant system of the organism, the oxidative stress may induce DNA protein or lipid damage which can result in genetic changes, instability of chromosomes and variation in cell growth leading to cancer. DNA damage can develop single strand or double strand breakage, base modification, deoxyribose modification, and DNA cross linking [6-9].

Continuous efforts are being made to develop efficacious anticancer drugs with minimum side effects. Several drugs from plant origin are already in use for chemoprevention. An appreciable interest has evoked among researchers to identify the bioactive molecules found in medicinal plants to inhibit the formation of DNA adducts, tumorigenesis using different animal models [10,11]. In past ethnomedicinal studies have displayed an influential correlation between consumption of medicinal herbs and their chemopreventive response against carcinogens. Phytoconstituents in plants underline the mechanism of cancer prevention by stimulating the release of enzymes, inhibiting the formation of DNA adducts, and by activating tumor suppressor genes [12-15]. The above affirmations very well support the extensive use of potential anticancer drugs derived from natural products [16-18].

Helicteres isora (HI) commonly named as East Indian screw tree in English and also known as Avartani in Ayurveda, belongs to the family Sterculiaceae [19]. In different parts of India HI has been used by a large number of tribal people/ethnic groups for the treatment of various ailments such as gastrointestinal disorder, diabetes, scabies, eczema, sore ear, and snake bite [20-26]. Studies have been conducted on toxic effects of NPYR in hyper-cholesterolemic condition in rats, but there is no finding which particularize the invigorating response of HI stem bark on HCC induced by hepatocarcinogen NPYR using an *in vivo* model of Swiss albino male mice.

METHODS

Chemicals and reagents

NPYR, Silymarin were obtained from Sigma Aldrich Chemicals, USA, all other chemicals were procured from Hi-Media and Central Drug House, Mumbai.

Experimental animals and medicinal plant

Experimental animals were obtained from Haryana Agricultural University, Hissar (Haryana, India). The study was executed upon approval by Institutional Animal Ethical Committee (IAEC) of Banasthali

University. The experimental studies were carried out in accordance with the guidelines given by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA, Reg. No: IAEC BT/67 date. May 2, 2012). A total of 36 male Swiss albino mice weighing 23-28 g were selected. Animals were maintained on commercial rodent pellet diet. Stem bark of HI was collected in the month of February, from Shiwalik hills of Western Uttar Pradesh, India. It was identified and authenticated by a botanist at Homoeopathic Pharmacopeia Laboratory, Ghaziabad, India. Dose used in experiment was decided on the basis of previously published studies [27]. Soxhlet apparatus was used for preparing successive (non-polar to polar solvents) hydro-ethanolic extract (70%) of shade dried and powdered stem bark of HI.

Induction of HCC and post treatment of animals with hydroethanolic stem bark extract (HIHSBE)

Swiss albino male mice were divided into six groups of six animals each. The groups were classified as following:

Group I: Normal control mice (0.5% carboxy methyl cellulose [CMC]).
Group II: NPYR (120 mg/kg b.w) + carbon tetrachloride (CCl_4; 3 ml/kg b.w) + 0.5% CMC treated.
Group III: NPYR + CCl_4+ HISBE (100 mg/kg b.w).
Group IV: NPYR + CCl_4+HISBE (200 mg/kg b.w).
Group V: NPYR + CCl_4 + Silymarin (200 mg/kg b.w).
Group VI: HISBE alone (200 mg/kg b.w).

In Group I all the mice were given 0.5% CMC throughout the experiment. Group II, III, IV, and V were given hepatocellular carcinogen NPYR through single intra-peritoneal injection for initiating the process of carcinogenesis. After 2 weeks of injecting NPYR all the Groups (II-V) were given subcutaneous injections of CCl_4, a known promoter for carcinogenesis once in a week up to 6 weeks [28, 29]. On completion of 6 weeks, Group II was orally administered with 0.5% CMC. Group III and IV were orally administered with a low and high dose of HIHSBE respectively for 4 weeks. Animals in Group V were treated with a standard drug Silymarin, a known anti-HCC compound [30, 31]. Group VI served HIHSBE treated, mice in this group were administered only HIHSBE. All the doses of HIHSBE and Silymarin were given in the form of a suspension made in 0.5% CMC. After 12 weeks of study, body weight of each animal was noted and sacrificed by cervical dislocation after overnight fasting. Liver of all the mice were excised out immediately, washed with phosphate buffered saline, dried, and their weight was taken. A portion of tissue was kept for histopathological studies while rest of the tissue was used for preparing homogenate for further biochemical investigations.

Biochemical assays

Estimation of hepatic injury markers

The activities of alanine transaminase (ALT), aspartate transaminase (AST), and alanine phosphatase (ALP) were estimated using span diagnostic kits [32]. Gamma-glutamyl transferase (GGT) was assayed according to Orlowski and Meister and modified by Rosalki and Rau [33,34]. Briefly, 0.013% of glycylglycine (2.2 ml), 1 ml of Tris-HCL buffer (0.1 M, pH 8.5), and 0.5 ml of 3 mg/ml γ-glutamyl-p-nitroanilide were taken in a test tube. To the mixture, was then added 200 µl of tissue homogenate and volume of the mixture was made up to 4 ml by adding distilled water followed by incubation at 37°C for 30 minutes. Control tubes and standard of p-nitroaniline were prepared in similar way.

Lactate dehydrogenase (LDH) was determined according to the method of King [35]. Briefly, in the test sample tubes, 100 µl of tissue homogenate was mixed with 1 ml of buffered substrate. Lactate was used as a substrate in the assay. In control tubes, 200 µl of distilled water was added in place of tissue homogenate. Further, both the test tubes were kept for incubation at 37°C for 5 minutes. To the mixture was added coenzyme nicotinamide adenine dinucleotide solution and again incubated for next 15 minutes. To arrest the reaction in tubes 0.002% of 2, 4- dinitrophenylhydrazine prepared in 1 N HCL was poured. Tissue homogenate was added to control tubes followed by incubation for further 15 minutes at 37°C. NaOH (0.4N, 500 µl) was added in all the tubes; the intensity of the color developed was measured spectrophotometrically at 420 nm. Different concentrations of the standard sodium pyruvate were also treated in similar way.

Estimation of hepatic tumor markers

Alpha feto protein (AFP) and carcinoembryonic antigen (CEA) were measured in serum using chemiluminescent immunoassay (Abbott, fully automatic immunoanalyzer, U.S.A).

Determination of liver antioxidant activities

Catalase (CAT) was determined using the procedure as given by Sinha [36]. Hydrogen peroxide (2 M, 400 µl) was mixed with of 1 ml phosphate buffer (0.01 M, pH 7) and 100 µl of homogenate sample. Control tubes were prepared in the absence of hydrogen peroxide. To this reaction mixture, 2 ml of dichromate acetic acid reagent (5% potassium dichromate and glacial acetic acid mixed in 1:3) was added. The rate of decomposition of H_2O_2 was measured at 620 nm. The enzyme activity was expressed as U/mg of protein.

Reduced glutathione (GSH) was estimated according to the method of Ellman [37]. Absorbance of the samples was noted at 412 nm within 2-3 minutes against reagent blank. A standard was also run in similar way using its different concentrations. DTNB (5, 5-dithiobis-(2-nitrobenzoic acid) reduced by thiol groups was expressed as nmoles of GSH oxidized per minute per milligram of protein.

The activity of GSH-S-transferase (GST) was measured spectrophotometrically according to the procedure of Habig *et al*. [38]. Homogenate (100 µl) of liver, 1 ml of phosphate buffer (100 mmol/l, pH 6.5), 100 µl 1-chloro-2, 4 dinitrobenzene (30 mmol/l), 1.7 ml distilled water were taken together and kept under incubation for 15 minutes at 37°C. Then 100 µl of GSH (30mM) was added. Intensity of the conjugate GSH-CDNB was noted at 340 nm up to 3 minutes. The activity of GST was expressed as U/mg of protein.

Measurement of lipid peroxidation (LPO) in liver tissue (thiobarbituric acid reactive substances [TBARS])

LPO results in the formation of malondialdehyde (MDA) and other aldehyde intermediates. Homogenate samples on mixing with thiobarbituric acid reagent gives pink colored species called TBARS. The absorbance of these resulting substances is measured at 532 nm. The MDA equivalents of the sample were calculated using an extinction coefficient of 1.56×10^5/M/cm and expressed as U/mg tissue [39].

Histopathological analysis

Liver portions fixed in 10% formalin were embedded in paraffin wax. Sections were prepared using a microtome and were stained with hematoxylin-eosin. Microscopically, the pathological alterations were observed and distinguished between slides of all the groups. An appropriate photograph of each slide was taken at ×40 magnification.

Statistical analysis

The results are represented as mean±standard error of mean for six mice. Statistical analyses of the results were performed using one-way ANOVA, followed by Tukey's *post-hoc* test for multiple comparisons with statistical significance of $p<0.01$.

RESULTS

Tumor incidence in hepatic tissue

All the animals were observed for NPYR induced tumor/nodule in the hepatic tissue. Group I which was treated as a control group and Group VI administered only HIHSBE at a dose of 200 mg/kg did not exhibited the presence of any tumor, whereas, Group II injected with NPYR and CCl_4 developed 100% tumors in hepatic tissue. Group III and IV post treated with HIHSBE at a dose of 100, 200 mg/kg lowered the tumor incidence from 50% to 0%, respectively. Similarly, Group V administered with reference drug silymarin did not reveal any development of tumor (Table 1).

Effects of HIHSBE on body and liver body weight ratio

The final body weight of control group showed significant difference with HCC induced group of mice. Similarly with that Group II showed a significant difference (p<0.01) with HIHSBE and silymarin treated groups (200 mg/kg). There was no significant difference of absolute and relative liver weights between NPYR and HIHSBE treated mice (Table 2).

Effects of HIHSBE on hepatic injury and tumor markers

Group II (NPYR + CCl_4 treated) showed eloquent subsidence in the level of ALT (45.8±2.2 U/L), AST (86.5±1.7 U/L), ALP (178.2±14.1 U/L), GGT (3.0±0.5 U/mg protein), and LDH (7.4±1.1 U/mg protein) compared to Group I (78.9±5.1, 198±2.7, 481.8±10.5, 8.3±1.1, and 14.6±0.9), respectively. Whereas, the oral administration of HIHSBE to NPYR treated Group III (100 mg/kg) and IV (200 mg/kg) exhibited appreciable increase in ALT, AST, ALP, GGT, and LDH levels (Table 3).

In the serum samples of carcinogen treated mice (Group II), the prominent elevation in the levels of α-fetoprotein and CEA were monitored (18.76±0.69, 4.44±0.29 ng/ml), respectively. Though HIHSBE post-treated groups revealed convincingly lowered levels of AFP and CEA in serum samples (Figs. 1 and 2).

Effect of HIHSBE on antioxidant enzymes and LPO in HCC

Diminution of CAT, GSH, and GST (6.55±1.41, 5.44± .855, and 7.94±1.11 U/mg protein) were observed in NPYR treated mice of Group II as compared to Group I (Figs. 3-5). Groups III and IV post-treated with HIHSBE at a dose of 100 and 200 mg/kg, respectively exhibited restoration of these antioxidant enzymes. Whereas, the *per se* Group VI showed no significant variation in the enzymatic levels

Table 1: Effect of NPYR and HIHSBE on tumor development in Swiss albino mice liver

S. no.	Groups	Percentage of tumor incidence	Number of tumor-bearing mice
1	Control	0	0
2	NPYR+CCl_4 treated	100	6
3	NPYR+CCl_4+HIHSBE (100 mg/kg)	66.6	4
4	NPYR+CCl_4+HIHSBE (200 mg/kg)	33.3	2
5	NPYR+CCl_4+Silymarin (200 mg/kg)	33.3	2
6	HIHSBE alone (200 mg/kg)	0	0

HIHSBE: *Helicteres isora* hydroethanolic stem bark extract, NPYR: N-nitrosopyrrolidine, CCl_4: Carbon tetrachloride

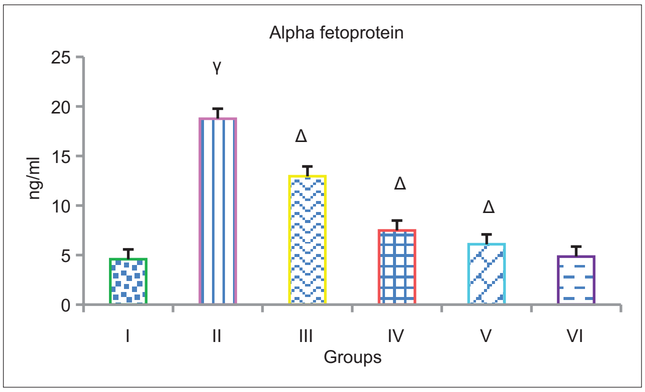

Fig. 1: Effects of hydroethanolic stem bark extract on alpha-fetoprotein in control and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue. Values are presented as mean±standard error of the mean of six mice in each group. $^{\gamma}$p<0.0001, as compared with Group I. $^{\Delta}$p<0.0001 as compared with Group II

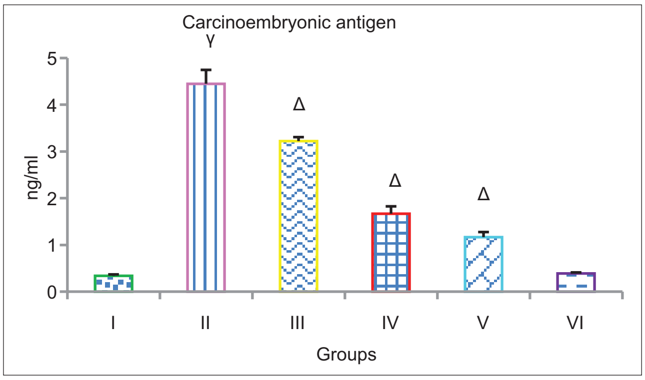

Fig. 2: Effects of *Helicteres isora* hydroethanolic stem bark extract on carcinoembryonic antigen in control, and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue. Values are presented as mean±standard error of mean of six mice in each group. $^{\gamma}$p<0.0001, as compared with Group I. $^{\Delta}$p<0.0001 as compared with Group II

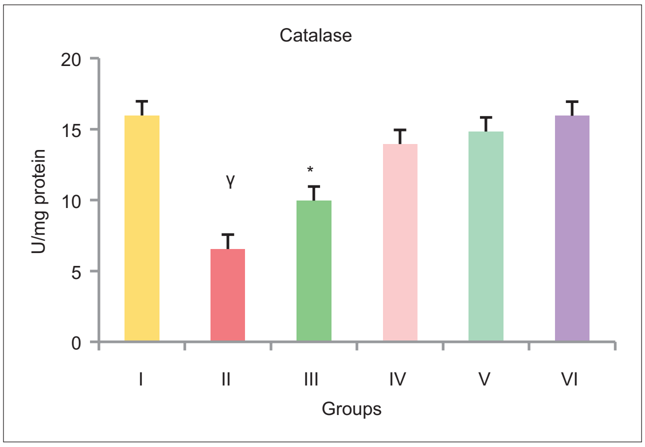

Fig. 3: Effect of *Helicteres isora* hydroethanolic stem bark extract on catalase (μmol H_2O_2 consumed/minutes/mg protein) in control, and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue homogenate of various groups of mice. Statistical significance $^{\gamma}$p<0.0001, as compared with Group I, *p<0.01 compared to Group II

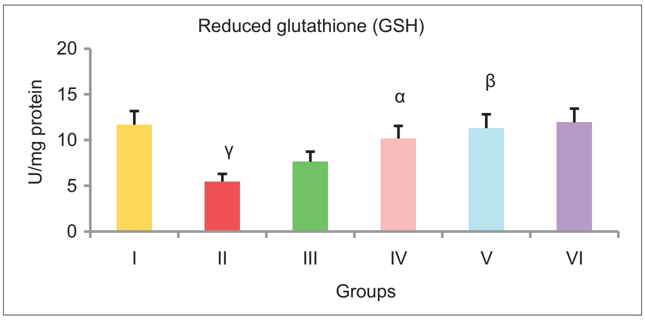

Fig. 4: Effect of *Helicteres isora* hydroethanolic stem bark extract on glutathione (μmol/minutes/mg protein) in control, and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue. Effect of *Helicteres isora* bark extract on the activity of GSH-S-transferase in tissue homogenate of various groups of mice. Statistical significance $^{\gamma}$p<0.0001 compared to Group I, $^{\alpha}$p<0.001, $^{\beta}$p<0.0001 as compared to Group II

as compared to control group. LPO levels (223.4±26.0 U/g tissue) in mice with NPYR induced HCC were found to be remarkably prominent as compared to Group I. However, post treatment with HIHSBE at 200 mg/kg lowered the formation of products such as MDA or other aldehyde products as indicated by decrease in LPO levels of Group II and IV (166.2±30.2; 136.8±25.7 U/g tissue), respectively (Fig. 6).

Effect of HIHSBE on hepatic histology

Histopathological analysis of liver supported our findings of biochemical estimations. The livers section from normal group of mice presented normal cells with granulated cytoplasm, central vein, and small uniform nuclei (Fig. 7a). Animals administered with NPYR and CCl_4 revealed loss of architecture, irregular shaped hepatocytes, development of neoplastic cells, and thick fibrous bands clearly demonstrated the condition of HCC (Fig. 7b). Treatment of mice with HIHSBE (100 mg/kg) reasonably recovered the effects of NPYR exhibiting normal architecture (Fig. 7c). Similarly in Group IV high dose of HIHSBE maintained near normal architecture of the tissue, showed ameliorated effects against HCC which seemed to be comparable to the liver sections of normal and silymarin treated group (Fig. 7d, and e). HIHSBE at the dose of 200 mg/kg exhibited no significant changes in the histological observations confirming its non-toxic nature (Fig. 7f).

DISCUSSION

Nitrosamine compounds (NOCs) are known to cause numerous detrimental biological consequences along with induction of tumors. Two stage mechanism of carcinogenesis associating initiation-promotion has been studied extensively. NOC on metabolic biotransformation into reactive intermediates interferes with cellular macromolecules that may be one of the leading causes for developing cancer. One of the most provocative expressions of nitrosamines

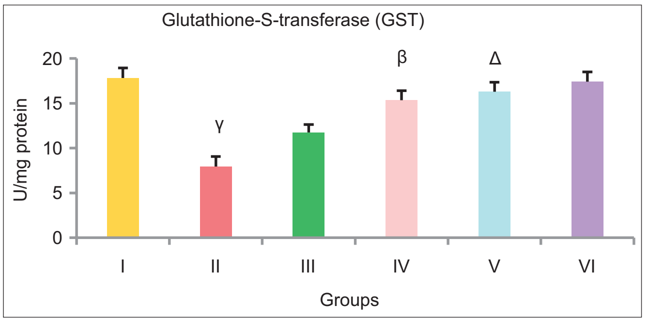

Fig. 5: Effects of *Helicteres isora* hydroethanolic stem bark extract on glutathione-S-transferase (GST) in control, and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue Effect of *Helicteres isora* bark extract on the activity of GST in tissue homogenate of various groups of mice. Statistical significance $^{\gamma}$p<0.0001, compared with Group I, $^{\beta}$p<0.0001. $^{\Delta}$p<0.0001 as compared with Group II

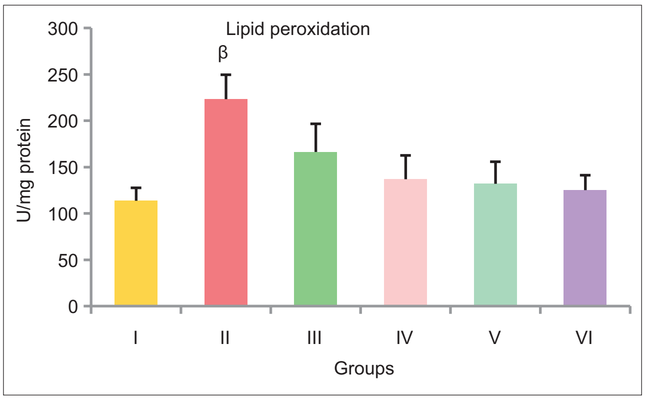

Fig. 6: Effects of *Helicteres isora* hydroethanolic stem bark extract on lipid peroxidation in control, and N-nitrosopyrrolidine induced hepatocellular carcinoma in liver tissue homogenate of various groups of mice. Statistical significance, $^{\beta}$p<0.01 as compared with Group I

Table 2: Effect of HIHSBE and NPYR on body and liver weight in mice

S. no	Groups	Final body weight (g)	Absolute weight of liver (g)	Relative weight liver (g/100 g b.w)
1	Control	30±0.38	1.56±0.109	5.21±0.33
2	NPYR+CCl_4 treated	20±0.63[a]	1.98±0.12	9.9±0.58
3	NPYR+CCl_4+HIHSBE (100 mg/kg)	26±0.68	1.88±0.13	7.22±0.44
4	NPYR+CCl_4+HIHSBE (200 mg/kg)	28±0.93[b]	1.68±0.11	6.04±0.46
5	NPYR+CCl_4+Silymarin (200 mg/kg)	28±1.06	1.64±0.11	5.97±0.54
6	HIHSBE alone (200 mg/kg)	29±0.68	1.54±0.12	5.30±0.36

Values are presented as mean±SEM of six mice in each group. Single intra-peritoneal injection was administered to mice in Group II, III, IV, and V followed by subcutaneous injection of CCl_4 for 42 days. After which Group III and IV were given HIHSBE at a dose of 100 and 200 mg/kg, respectively. [a]p<0.001 as compared with Group I, [b]p<0.01 as compared with Group II). HIHSBE: *Helicteres isora* hydroethanolic stem bark extract, NPYR: N-nitrosopyrrolidine, SEM: Standard error mean, CCl_4: Carbon tetrachloride

Table 3: Effect of HIHSBE and NPYR on biochemical markers of mice liver

S. no	Groups	ALT IU/L	AST IU/L	ALP IU/L	GGT	LDH U/mg
1	Control	78.92±5.1	198±2.7	481.8±10.5	8.37±1.1	14.6±0.9
2	NPYR+CCl_4 treated	45.82±2.2$^{\gamma}$	86.5±1.7$^{\gamma}$	178.2±14.1$^{\gamma}$	3.0±0.5$^{\alpha}$	7.4±1.1$^{\gamma}$
3	NPYR+CCl_4+HIHSBE (100 mg/kg)	55.57±3.7	131.1±1.0$^{\Delta}$	319.6±6.6$^{\Delta}$	4.43±0.6	9.25±0.9*
4	NPYR+CCl_4+HIHSBE (200 mg/kg)	65±4.3*	179.4±4.6$^{\Delta}$	467.3±11.3$^{\Delta}$	6.94±1.0*	11.67±1.1$^{\Delta}$
5	NPYR+CCl_4+Silymarin (200 mg/kg)	69.2±3.7**	188±3.0$^{\Delta}$	473.5±10.9	7.53±1.0	12.56±1.0
6	HIHSBE (200 mg/kg)	75.2±4.1	192.4±2.6	479±10.1	9.48±0.9	13.88±0.9

Values are presented as mean±SEM. of six mice in each group. Single intra-peritoneal injection was administered to mice in Group II, III, IV, and V followed by subcutaneous injection of CCl_4 for 42 days. After which Group III and IV were given HIHSBE at a dose of 100 and 200 mg/kg, respectively. $^{\gamma}$p<0.0001 and $^{\alpha}$p<0.001 as compared with Group I $^{\Delta}$p<0.0001 as compared with Group II (NPYR+CCL_4 treated), *p<0.01, **p<0.001 as compared with Group I. HIHSBE: *Helicteres isora* hydroethanolic stem bark extract, NPYR: N-nitrosopyrrolidine, ALT: Alanine transaminase, AST: Aspartate transaminase, ALP: Alanine phosphatase, GGT: Gamma-glutamyl transferase, LDH: Lactate dehydrogenase, CCl_4: Carbon tetrachloride

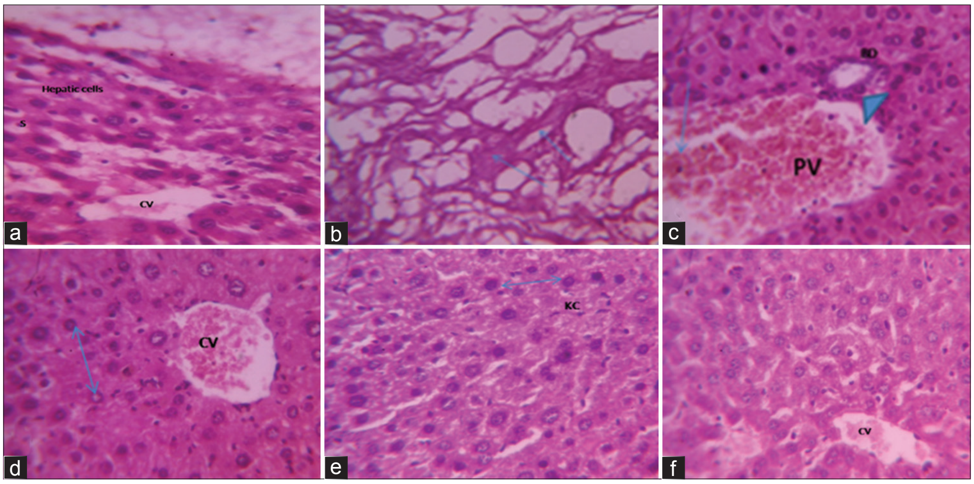

Fig. 7: Histological representation at (H and E stained) ×40, (a) Normal control mice liver (Group I) showing normal hepatocytes with maintained granulated cytoplasm and central vein (CV); (b) N-nitrosopyrrolidine and carbon tetrachloride treated Group II showing aberrant hepatocellular phenotype, irregular sinusoids, fibrous bands; (c) Group III post treated with Helicteres isora hydroethanolic stem bark extract (HIHSBE) at a dose of 100 mg/kg showed moderate improvement in hepatocytes histology; (d and e) HIHSBE at a dose of 200 mg/kg hepatocytes maintaining near normal architecture, CV, no fibrous bands, and neoplastic cells as observed in Group II; (f) HIHSBE alone, showing normal liver architecture similar to liver section of Group I. CV, Portal vein, Bile duct, Kupffer cell

toward carcinogenicity is their organ specificity. Smaller molecules of cyclic nitrosamines are more potent carcinogen than larger molecules [40]. Chemically-induced hepatocarcinogenesis, especially by using diethylnitrosamine (DENA) in the rodent model has gained huge attention in past few years, since rodent liver metabolizes DENA in much the same manner as does the human liver, also demonstrating identical morphology and gene expressions [41].

NPYR is a well-established hepatocarcinogen that is present in the diet, tobacco smoke, and may form endogenously in humans. Adduct formation between the metabolites of NPYR and the deoxyguanosines of DNA in tissues of rats treated with NPYR, is a major biomarker to assess the metabolic activation of NPYR [42, 43]. Our experiments focused on suppressing HCC induced by NPYR and CCl_4.

It has been well accepted that CCl_4 induces toxicity in liver cells. In one of the study carried on CYP 2E1, it was observed that bio-transformation of CCl_4 produces trichloromethyl free radicals and then reacts with molecular oxygen and undergoes cleavage to form new radicals [44]. These free radicals further invade lipids on the membrane of endoplasmic reticulum originating LPO and ultimately leading to the cell death. Currently, no experimental study has been carried out to uncover the carcinogenicity of NPYR in Swiss albino male mice. In the present study, metabolic activation of NPYR and CCl_4 resulting in hepatocarcinogenesis was examined by assessing some inescapable biomarkers such as ALP, ALT, AST, γ-GT, and LDH. GGT the most pronounced enzyme found in the liver has a significance of potential diagnostic marker studied in HCC. LDH also serves as a biomarker for tissue breakdown. LDH intends to initiate and metabolize the tumors. Hypoxia and angiogenesis are the likely mechanisms responsible for low and high LDH levels. The deliverance of these enzymes from liver cells into the blood during hepatic necrosis exhibits their impaired functioning in liver tissue. In concomitant with the above observations the outcomes of our experiments were well corroborated. We witnessed that enzymatic levels apparently lowered in the group of animals administered NPYR and CCl_4 as compared to the control group. However, post treatment of carcinogenesis induced mice with *Helicteres isora* bark extract (HIBE) rehabilitated the enzymatic levels in their liver cells by the interaction between carcinogens and liver components.

AFP is an abundant plasma protein synthesized by the liver cells during fetal development. According to the literature reported, fetal, and cancer cells reflect similar biochemical and antigenic features which was verified through *in vitro* and *in vivo* studies. *In vitro* studies provided the conformation about the uptake of AFP by various cancer cell lines. Whereas, *in vivo* studies in mice also exhibited prominent aggregation of AFP in carcinomas when compared to normal tissues. CEA is a set of glycol-proteins produced in gastrointestinal tissue during fetal development. It is reported CEA level raises in few types of cancer [45]. This correlates with the findings of our experiments illustrating augmentation in the levels of serum AFP and CEA in HCC induced animals (Group II) in contrast mice that were administered with HIHSBE for the treatment of HCC revealed a significant decline in their serum levels.

Oxidative stress embodied in animals treated with NPYR + CCl_4 was exhibited by elevated level of LPO. Since LPO is associated with enhanced formation of ROS; it constitutes a major marker for detecting HCC. It is noteworthy that plant extract treated groups showed significant decline in LPO level indicating its indispensable role in the development of HCC [46].

CAT enzyme is imperative for shielding the cell from oxidative damage by ROS by catalyzing the decomposition of hydrogen peroxide to water and oxygen. GSH is also a crucial antioxidant, which protects the cellular structure which is prone to damage by ROS. GST promotes conjugation of reduced form of GSH to xenobiotic substrates making it more water soluble and likable for the purpose of detoxification; thereby GST is hindering the involvement of toxic compounds with essential cellular proteins and nucleic acids. Reduction in the activities of these enzymes is presumably related with the steeping oxidative stress in tissues preceded by increase in generation of free radicals. In the present study, we observed that post treatment with HIHSBE influences all the antioxidant enzymes associated with normal liver functions responsible for neutralizing the oxidative stress. HIHSBE significantly sustained the CAT, GSH, and GST activity in hepatic tissue and level of these antioxidant enzymes were significantly reinforced in liver cell. Our current acquisitions are supported with the previous studies carried on HIHSBE, it exhibited considerable antioxidant efficacy by quenching the propagation of free radicals [47].

CONCLUSIONS

The findings of our experiments show that HIHSBE endeavors a quenching effect against NPYR and CCl_4 progenerated HCC in mice, by preserving the liver enzymes ALT, AST, ALP, LDH, and GGT. The chemopreventive effect of HIHSBE is well supported in our study as it hinders the development of HCC by interacting with ROS during carcinogenesis and thus counterbalancing the antioxidant defense system as analyzed. Histological findings also demonstrated that HIHSBE alleviated the carcinogenic effects of NPYR by maintaining the normal liver architecture, normal nuclei, and suppressing the formation of neoplastic cells and fibrous bands thus exhibiting its anticancer effects.

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