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Protective Effects of Linalool on Diethynitrosamine and AAF-induced Hepatocellular Carcinoma in Wistar Rats

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***Abstract*:- Objective: To investigate the chemopreventive potential of Linalool (LIN) on diethynitrosamine and AAF-induced hepatocarcinogenesis in Wistar rats. Methods: The experimental animals were divided into six groups (n=6). Hepatocellular carcinoma was induced by single intraperitoneal injection of diethynitrosamine (DENA) in normal saline at a dose of 200 mg/kg b.w followed weekly once for three weeks subcutaneous injections of AAF (0.02g/kg b.w/week) for 3 weeks, as the promoter of carcinogenic effect. After administration of the carcinogen, 25, 50 and 100 mg/kg of LIN were administered orally once a day throughout the study. At the end of 12 weeks, the body weight, liver weight and relative liver weight were measured. The percentage of nodule incidence and liver cancer markers such as γ-glutamyl transferase (γ-GT), total bilirubin level (TBL) and estimated along with histopathological investigation in experimental groups of rats. Results: Obtained results demonstrated that the cotreatment with LIN significantly prevented the decrease of the body weight and also increased in relative liver weight caused by DENA+AAF. The treatment with LIN significantly reduced the nodule incidence and nodule multiplicity in the rats after DENA+AAF administration. The levels of liver cancer markers such as γ-glutamyl transferase, TBL and were substantially increased by DENA+AAF treatment. However, LIN treatment significantly reduced the liver injury and restored the entire liver cancer markers. Histological observations of liver tissues too correlated with the biochemical observations.**

Conclusions: These finding powerfully supports that Linalool exert chemopreventive effect by suppressing the tumor burden and restoring the activities of hepatic cancer marker enzymes on DENA and AAF-induced hepatocarcinogenesis in Wistar rats.

INTRODUCTION

Hepatocellular carcinoma (HCC) or liver cancer is the second most common cancer and the third leading cause of cancer mortality in the world [1]. The burden of cancer is increasing in economically developing countries as a result of population aging and growth as well as, increasingly, an adoption of cancer-associated lifestyle choices including smoking, physical inactivity, and "westernized" diets. Liver cancer in men/women is the second/fifth most frequently diagnosed cancer worldwide but the third most frequent cause of cancer death. An estimated 748 300 new liver cancer cases and 695 900 cancer deaths occurred worldwide in 2008[2]. Hepatitis viral infection, food additives, alcohol, fungal toxins (aflatoxins), toxic industrial chemicals, air and water pollutants are the major risk factors of liver cancer[3]. Human liver is the major site in the body that metabolizes ingested material. It has pore to carcinogenic insult. Moreover, due to the high tolerance of liver, HCC is seldom detected at the earlystage and once detected treatment faces a poor prognosis in most cases [4]. Diethynitrosamine (DENA) is a potent hepatocarcinogenic nitrosamine present in tobacco smoke, water, cheddar cheese, cured and fried meals, occupational settings, cosmetics, agricultural chemicals and pharmaceutical agents [5].

DENA is widely accepted for induction of preneoplastic lesions and hepatic tumors in rats and it is initiated by perturbations of nuclear enzymes involved in DNA repair or replication [6]. Investigations have provided evidence that DENA causes a wide range of tumors in all animal species and such compounds are hazardous to human health [7]. DENA+AAF-induced lesions as well as tumors in rodents show marked biochemical, histological and molecular similarity to the progression of HCC in humans [8]. Many pharmaceutical agents have been discovered by screening natural products from plants, animals, marine organisms and microorganisms. Vincristine, vinblastine, irinotecan, etoposide and paclitaxel are examples of plant-derived compounds that are being employed in cancer treatment as well as several chemicals are also known to possess chemopreventive properties against a broad spectrum of cancer [9]. Recently, identification of bioactive ingredients from medicinal plants to inhibit tumorigenesis in a variety of animal models of carcinogenesis, involving organ sites, such as the skin, lungs, oral cavity, esophagus, stomach, liver, pancreas, small intestine, colon, and

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prostate is gaining considerable attention[10]. Moreover, in recent years, naturally occurring plant products have getting increased attention for the intervention of malignant invasive progression in the late stage of neoplastic diseases [11].

2-Acetyl aminofluorene (AAF) is a model compound, widely used to evaluate the metabolism and carcinogenesis of arylamides and amines. The metabolic activation of this compound involves a number of steps such as N-hydroxylation, sulfate transfer, N-O-acetyl transfer, deacetylation, or 1-electron oxidation step to finally form the ultimate reactive intermediate [12]. One of the metabolites of AAF, 2-Nitrosoflourine, has been reported to induce redox cycling leading to superoxide production which reacts with DNA, disrupting the cell membrane, integrity, and finally lipid peroxidation.

Linalool (LIN) is a naturally occurring mono terpenoid mainly present in coriander. Coriander is commonly used for seasoning foods [13], which contains LIN (Fig.1) as the main constituent of its essential oil [14]. Safety evaluation studies show that LIN is not irritating, phototoxic or sensitizing but shows mild acute toxicity. LIN is known to inhibit mitochondrial complex I enzyme activity (NADH UQ-reductase/NADH oxidase) and mitochondrial complex II enzyme activity (succinate dehydrogenase), resulting in decreased ATP level. LIN also possesses antioxidant [15], antiinflammatory, antiviral, antibacterial [16], antidiabetic [17], anticancer [18], and antileishmanial [19] properties. So far, there are no reports demonstrating the chemotherapeutic efficacy of LIN against experimental hepatocarcinogenesis. Our present study was developed to provide an insight into the potent regulatory effects of LIN against DENA/AAF induced progression of NAFLD to HCC by evaluating the liver marker enzymes, lipid peroxidation, fibrotic changes, antioxidant status, cell proliferation and inflammatory status.

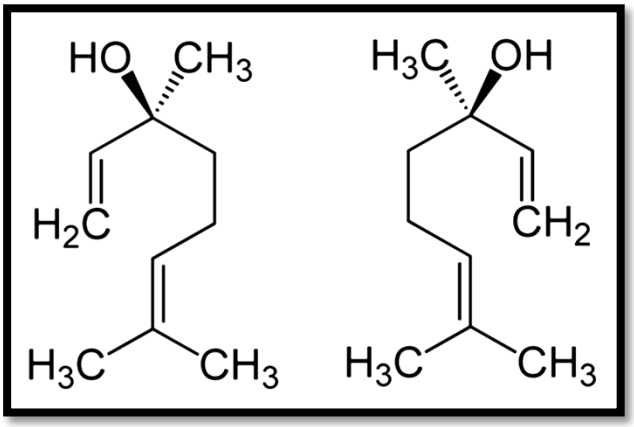

Figure.1. Molecular structure of LIN

MATERIALS AND METHODS

Animals

Eight-week-old male Albino Wistar rats weighing approximately 200 g were procured from the Biogen, Bangalore, India. The animals were kept in polypropylene cages (26 x 42cm) and housed in Rajah Muthaiah Medical College and Hospital, containing 6 groups of 6 animals in each group All the animals received humane care according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and as per the approval of Institutional Animal Ethics Committee (IAEC), Annamalai University (Reg No. 160/1999/CPCSEA and Proposal No.1138).

Chemicals and reagents

Diethylnitrosamine (DENA, N0258), 2-acetylaminofluorine (AAF) and linalool (LIN, L2602) were purchased from Sigma – Aldrich Chemical Company St. Louis, MO, USA. All other chemicals and reagents used were of analytical grade and obtained either from Sigma Aldrich or Himedia laboratories, Mumbai, India.

Linalool preparation

Linalool (LIN) was dissolved in corn oil and prepared just prior to use. Rats of various treatment groups (IV–VI) received a daily dose of 25, 50 and 100 mg/kg b.w. of LIN, respectively.

Experimental protocol

Thirty six rats of Albino Wistar strain were used in this study. Rats were randomly divided into six groups with 6 animals in each group.

Group I: (Normal control): animals were given standard pellet diet for 91 days. Group II: (LIN control): LIN at the dose of 100mg/kg b.w. p.o. every day was supplemented along with standard pellet rat diet throughout the experimental period. Group III (DENA control): Rats were administered DENA and AAF as described above (liver cancer induction). Group IV–VI (treatment group): Rats were supplemented with different doses of LIN (25 mg/kg b.w, 50 mg/kg b.w and 100 mg/kg b.w, respectively) for 12 weeks along with DENA and AAF as in group III. The quantity of food and water intake was assessed, and the body weights of all the rats were recorded once a week. Fresh food and water were

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provided every day and the husk for bedding were changed once a week.

At the end of the experimental period of 12 weeks, the rats were anesthetized using intra-peritoneal administration of ketamine hydrochloride, 30mg/kg body weight. Rats were sacrificed by cervical decapitation after an overnight fast and perfused with ice-cold saline.

Assessment of liver injury markers

γ-glutamyl transferase (γ -GT) were estimated by methods of King [20] and Szasz's [21], respectively. Total bilirubin level (TBL) was determined by modified dimethyl sulfoxide (DMSO) method [22] on the basis of sulfanilic acid reaction with sodium nitrite to produce deoxidized sulfanilic acid.

Histopathological assessment

For histologic studies, the liver and kidney sections were made immediately from the liver of different groups of rats, fixed in 10% formalin, dehydrated in gradual ethanol (50%-100%), cleared in xylene, and embedded in paraffin wax. Sections were cut at 4 µm thick (Automatic tissue processor, Lipshaw) in a rotary microtone and the pathological changes were observed microscopically after staining with hematoxylin and eosin (H&E).

Milligan's Trichrome staining for collagen

The paraffin-embedded liver sections were deparaffinized and hydrated with deionized water. The sections were subsequently incubated in Fouchet's reagent for 5 min, rinsed in distilled water, incubated in celestine blue for 2 min and again rinsed in distilled water. They were further incubated in hematoxylin for 3 min, rinsed in distilled water, stained with Sirius red solution for 10 min and again rinsed in distilled water. The sections were later mounted with DPX and allowed to dry. The presence of collagen deposition in the tissues was examined with light microscope (Axio scope A1, Carl Zeiss, Jena, Germany) by segmenting the red-stained collagen.

Statistical analysis

The data were represented as mean ± SEM. for six rats. Analysis of variance (ANOVA) test was followed by individual comparison by Newman-Keuls test using Prism Pad software (Version 3.0) for the determination of level of signifcance. The value of $P<0.05$ was considered statistically signifcant.

Results

Effect of LIN on the development of liver nodules Table 1 shows the total number of nodules, nodule incidence and average no of nodules per nodule bearing liver in the normal and experimental groups of animals. When rats were treated with DENA+AAF (group III), 100% developed nodules in the liver were observed, whereas administration of LIN 25 mg/kg (group IV), 50 mg/kg (group V) and 100 mg/kg (group VI) showed marked decrease in the number and multiplicity of the nodules as compared to group I rats. The incidence of nodule growth was reduced to 66.67% in group III and 33.33% in group V, respectively. There was a lesser incidence of nodule formation in LIN 50 mg/kg b.w treated group V compared to group VI rats that received LIN at 100mg/kg b.w. The occurrence of hepatic nodules was not observed in the normal group I and LIN alone treated group VI animals.

Table. 1.
Effect of LIN and DENA+AAF on the development of nodules in the liver of control and experimental rats.

Groups	Treatment	Nodules incidence [n (%)]	Total no of nodules	Average no of nodules/ nodules bearing rats
I	Control	0 (0.00)	0	0.0
II	Control + Linalool (100 mg/kg b.w)	6 (100.00)	43	7.3
III	DENA (200mg/kg) + AAF(0.02g/kg b.w)	4 (66.67)	27	6.8
IV	DENA+AAF + LIN (25mg/kg b.w)	3 (43.22)	14	5.4
V	DENA+AAF + LIN (50 mg/kg b.w)	2. (33.33)	9	4.5
VI	DENA+AAF + LIN (100 mg/kg b.w)	0 (0.00)	0	0.0

Values are given as mean SD of each group. Superscript letters (^{b}) is used to distinguish the values of the different groups.
Values not sharing a common superscript differ significantly at $p<0.05$ (DMRT).

Effect of LIN on liver injury

The effect of LIN on liver injury is shown in Table 2, DENA+AAF-treated group III rats showed increased serumγ-GT [(122.06 ± 9.12) U/L, $p<0.01$] and TBL [2.97 ± 0.44) U/L, $p<0.01$] compared to control group I rats [(29.43 ± 4.37) U/L, $p<0.01$] and TBL [0.62 ± 0.99) U/L, $p<0.01$], respectively. In contrast, the LIN-treated groups IV and VI rats at 25, 50 and 100 mg/kg b.w significantly decreased.

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Table.2.
Effect of LIN and DENA+AAF on the activities of enzyme in the serum of control and DENA+AAF induced HCC in experimental rats.

Groups	Treatment	γ-GT(mg/dL)	TBL (mg/dL)
I	Control	29.43 ± 4.37	0.62± 0.99
II	Control + Linalool (100 mg/kg b.w)	49 ± 5.13	1.48 ± 0.16
III	DENA(200mg/kg + AAF (0.02g/kg b.w)	122.06 ± 9.12[b]	2.97 ± 0.44[b]
IV	DENA+ AAF +LIN (25 mg/kg b.w).	42.09 ± 7.60	0.94 ± 0.10
V	DENA+ AAF +LIN (50 mg/kg b.w).	28.62 ± 5.12	0.76 ± 0.08
VI	DENA+ AAF +LIN (100 mg/kg b.w).	20.99 ± 4.13	0.66 ± 0.05

Values are given as mean ±SD of each group. Superscript letters ([b]) is used todistinguish the values of the different groups. Values not sharing a common superscript differ significantly at $p<0.05$ (DMRT).

Histopathological observations

The histpathological examinations basically support the results obtained from serum enzyme assay. Figure 3. A and B) Control and LIN control the normal architecture (group I) and (group II) hepatic cells with granulated cytoplasm, small uniform nuclei and nucleolus. (C) Group III DENA+AAF-treated rats showed loss of architecture and neoplastic cells arranged in lobules separated by fibrous septa with inflammatory collection and small bile duct proliferation. Neoplastic cells were larger than normal cells with granular cytoplasm and larger hyperchromatic nuclei and hyaline globules (arrow) that represent proteins produced by the tumor cells. Group IV-VI rats architecture of liver sections of LIN treated (25 mg/kg b.w) group IV rats showed normal architecture with some hepatocytes and minimal inflammatory cell infiltration around the portal triads with few malignant hepatocytes. Whereas LIN-treated (50 mg/kg b.w) group V rats showed normal architecture with few preneoplastically transformed cells and hepatocytes maintaining near normal architecture which was compared to the control group I rats. (1000 mg/kg b.w) group V rats showed normal architecture. Administration of Linalool (50 mg/kg b.w) alone exhibited normal architecture of hepatocytes with granulated cytoplasm.

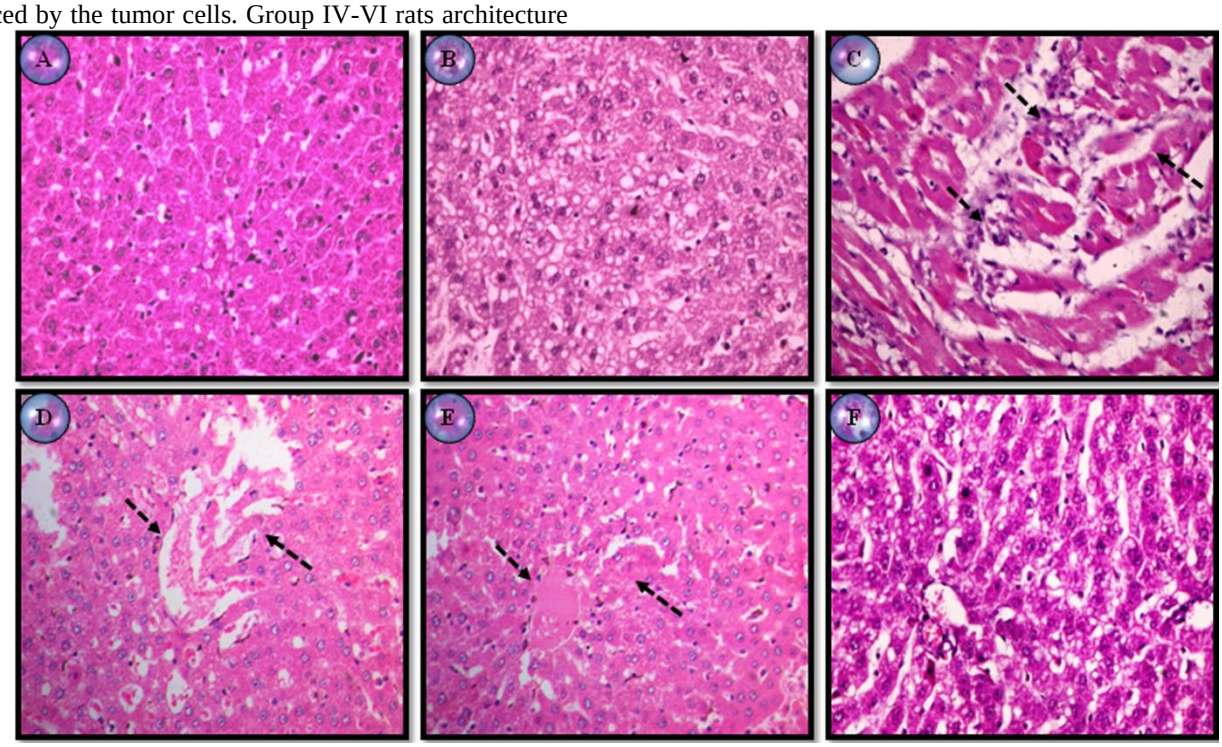

Fig. 3A. Histological changes in the liver of control and experimental groups (×40) [H and E staining]. A and B. Liver sections of control and treatment control rats showing normal architecture with central portal vein. C. Liver section of DENA + AAF -induced rat showing fibrosis with inflammation and nodule formation. D. Liver section of DENA + AAF + 25 mg/kg b.w LIN-treated rat showing inflammatory cells. E. Liver section of DENA + AAF + 50 mg/kg b.w LIN-treated rat showing degeneration of hepatocytes and mild periportal inflammation. F. Liver section of DENA + AAF + 100 mg/kg b.w LIN-treated rat showing moderate periportal inflammation. Each picture was taken at a magnification of 40X, with scale bars of 100 µm. Data are presented as the mean ± SD of six rats in each group (n = 6). Values not sharing a common superscript letter (A–F) versus Control, LIN differ significantly at $P < 0.05$ (DMRT).
Figure.3B Kidney of the control rat and LIN alone treated rat showed normal appearance of renal parenchymal cells, tubules, and glomeruli, whereas the DENA+AAF induced rat kidney shows congestion of blood vessels, swelling of tubules, scattered inflammatory cell infiltration, and damaged glomeruli. However, on supplementation with LIN, the changes induced by DENA+AAF were reversed as evident by the normal appearing glomeruli and regeneration of renal cells. A more pronounced effect of LIN treatment was observed in the groups of rats treated with 50 mg/kg b.w. (Group V).

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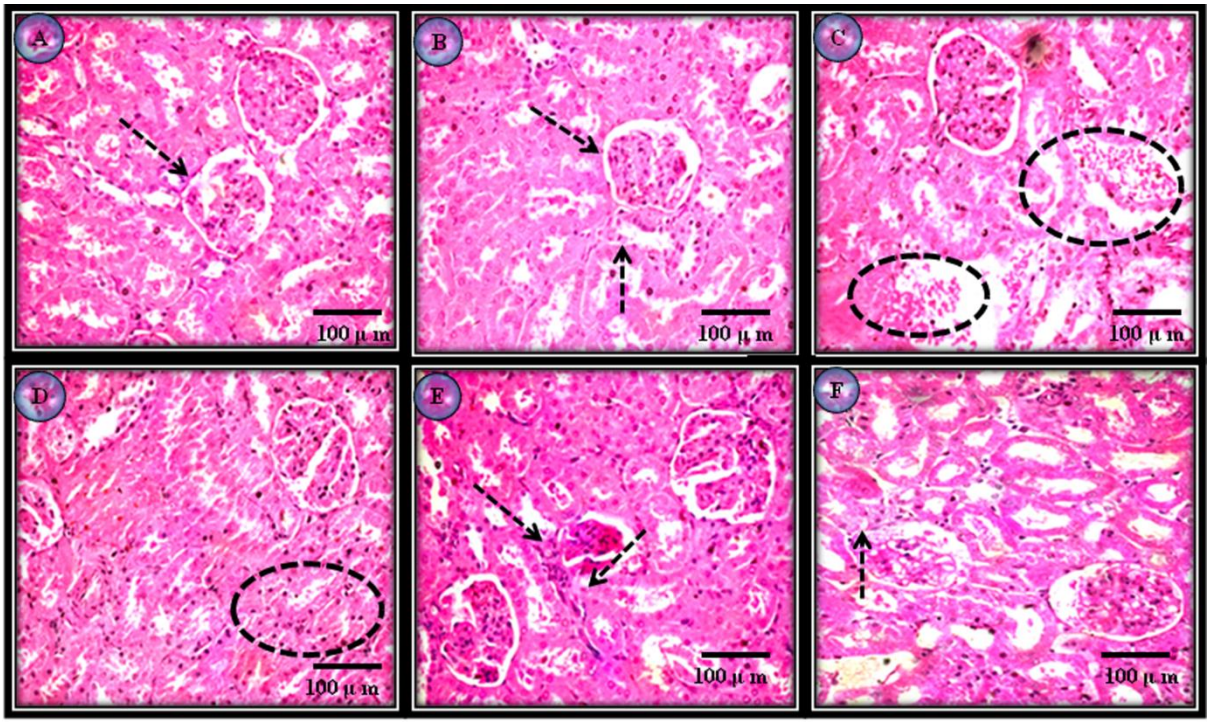

Figure.3B Kidney histology of control and experimental rats (H and E, staining). A and B. normal kidney histology. C. DENA+AAF fed rat kidney shows severe degenerative alterations in the tubules, congestion of blood vessels and diffused inflammatory cell infiltration. D. DENA + AAF + LIN (25 mg/kg body weight) treated rat kidney shows few tubules containing fat vacuoles. E. DENA + AAF + LIN (50 mg/kg body weight) treated rat kidney shows slight congestion of peritubular capillaries and normal glomeruli. F. DENA + AAF + LIN (100 mg/kg body weight) treated rat kidney shows normal histology. Each picture was taken at a magnification of 40X, with scale bars of 100 µm. Data are presented as the mean ± SD of six rats in each group (n = 6). Values not sharing a common superscript letter (A–F) versus Control, LIN differ significantly at $P < 0.05$ (DMRT).

Effect of LIN on liver collagen (Milligan's trichrome staining)

Figure. 4. Shows the liver sections stained with Milligan's trichrome for collagen. The liver sections of control (group I) and control rat supplemented with LIN (group II) showed normal collagen levels (stained blue). DENA+AAF induced rats (group III) showed massive deposition of collagen in the liver (stained blue). Supplementation with LIN to DENA+AAF induced rats (group IV) showed reduced collagen deposition in the liver sections.

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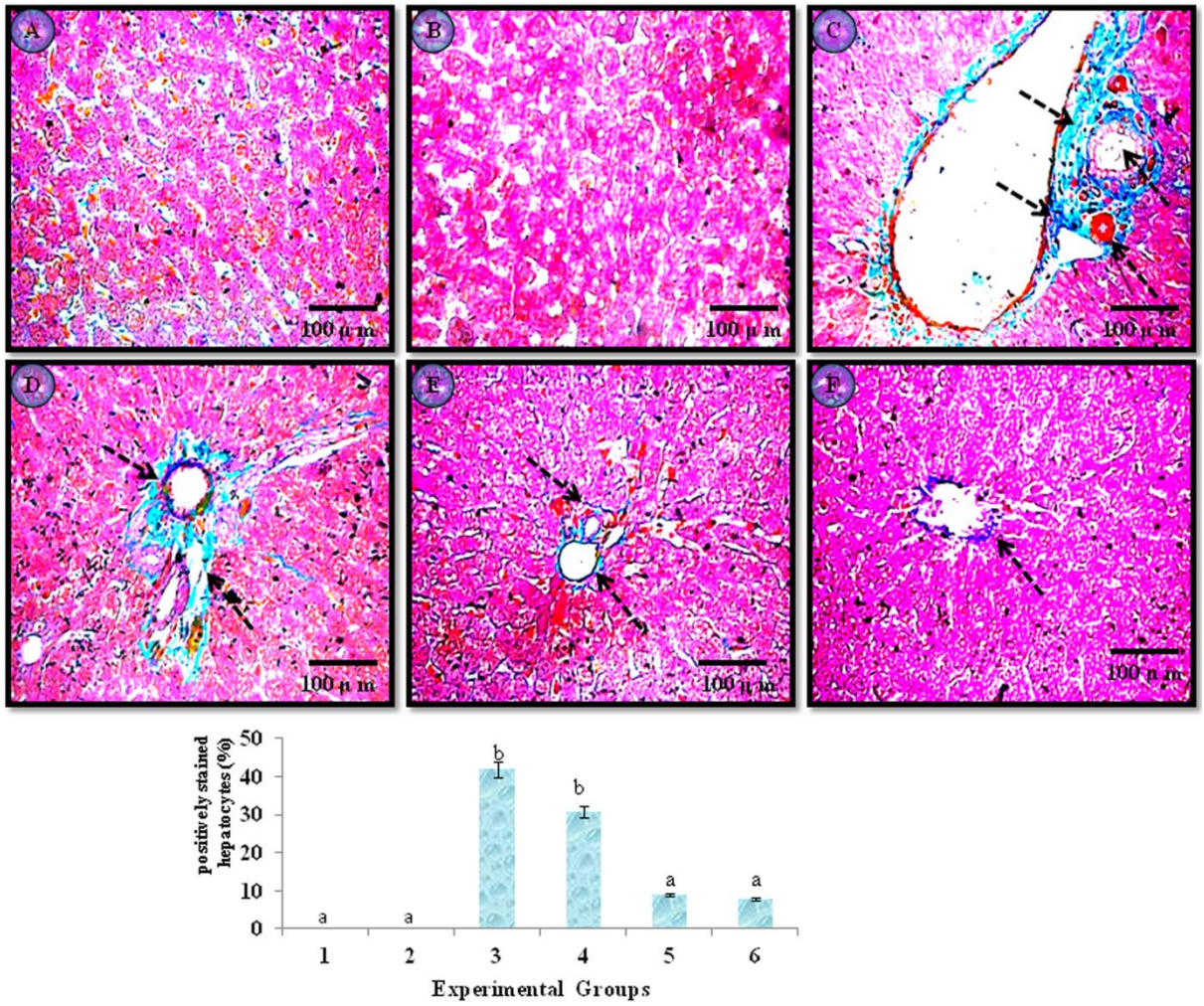

Figure. 4. Effect of LIN and DENA + AAF on liver collagen, stained with picrosirius red (×40). A. and B. Liver sections of control and control rat supplemented with LIN shows normal collagen levels. C. DENA + AAF rats show massive deposition of collagen in the liver. D. Hepatic section of DENA + AAF + 25 mg/kg b.w LIN-treated rat shows reduced collagen deposition in the liver sections. E. Hepatic section of DENA + AAF + 50 mg/kg b.w LIN-treated rat shows marked reduction in the accumulation of collagen. F. Hepatic section of DENA + AAF + 100 mg/kg b.w. LIN-treated rat, shorts minimal accumulation of collagen. Each picture was taken at a magnification of 40X, with scale bars of 100 µm. Data are presented as the mean ± SD of six rats in each group (n = 6). Values not sharing a common superscript letter (A–F) versus Control, LIN differ significantly at $P < 0.05$ (DMRT).

EFFECT OF LIN TREATMENT ALONE

Surprisingly, when LIN (50 mg/kg b.w) was administered to the non-DENA+AAF-treated rats, no significant changes were observed in any of the enzyme and non-enzyme activities assayed when compared to normal control group I rats. At this dose level, there were no significant changes in the histological observations depicting the non-toxic nature of LIN. Figure 3. Histological study of liver tissue in control and experimental groups of rats. (A) Group I: Control animals revealed normal architecture. (B) Group II: DENA +AAF induced carcinoma bearing animal showing neoplastic hepatocyte with inflammatory collection and loss of architecture. Neoplastic cells were larger than normal cells with larger hyperchromatic nuclei and hyaline globules (arrow) that represent proteins produced by the tumor cells. (C) Group III: Administration of LIN (25 mg/kg) on HCC bearing animal hepatocyte showing minimal inflammatory cell with few malignant hepatocytes. (D) Group IV: Administration of LIN (50 mg/kg) on HCC bearing animals exhibited hepatocyte maintaining near normal architecture. (E) Group V: Administration of LIN (100 mg/kg) on HCC bearing animals shows the structure close to proximity of normal hepatocytes. (F) Group VI: Administration of LIN (50 mg/kg) alone exhibited normal architecture of hepatocytes indicating the non-toxic nature of the compound.

DISCUSSION

Natural products have long been used to prevent and treat many diseases, including cancer and thus they are good candidates for the development of anti-cancer drugs. The large population use ayurvedic medicine worldwide. Different in vivo and in vitro screening models are available for anticancer activity. The present investigation showed that oral administration of LIN counteracts the activities of tumor marker enzymes and prevents the development of HCC that is usually induced by DENA and AAF in experimental rats. On metabolic biotransformation

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of DENA+AAF produces promutagenic products, O6 -ethyldeoxyguanosine and O4 and O6 -ethyldeoxythymidine in liver which are responsible for their carcinogenic effects [31-35]. It is well established that AAF induces hepatotoxicity by metabolic activation and therefore selectively causes toxicity in liver cells maintaining a semi-normal metabolic function. AAF is bio-transformed by cytochrome P450 enzyme system in the endoplasmic reticulum to produce trichloromethyl free radicals. Trichloromethyl free radicals (AAF) then combine with cellular lipids and proteins in the presence of oxygen to form trichloromethyl peroxyl radical, which further attack lipids on the membrane of endoplasmic reticulum faster than trichloromethyl free radical. Thus, trichloromethyl peroxyl free radical leads to elicitation of lipid peroxidation and destruction of Ca2+ homeostasis, resulting in cell death [35].

The results of the present study seem to provide support for the chemopreventive effects of LIN against DENA+AAF-induced hepatocarcinogenesis in rats. There is an appreciable reduction in body weight and increase in liver weight observed in HCC bearing group III rats as compared to control group I rats. Decreased appetite and food intake contribute to the weight loss which could be an indirect indication of the declining hepatic function, an increase in the liver weight of the animals, which could be attributed to the formation of nodules and tumors in the liver following carcinogen exposure. Sreepriya and Bali [9] have also reported marked loss of body weight and increase in liver weights. The steadily increase in body weight and decrease in liver weight after LIN treatment (groups v and VI) indicate that, increased appetite and reduced tumor incidence shows its anticancer effectiveness. Administration of DENA+AAF is reported to produce neoplastic nodules in experimental animals [9]. Group III rats given DENA+AAF showed an incidence of 100% in the presence of nodules. Administration of LIN was able to reduce the percentage incidence of nodules as compared to group I. The effect of LIN to prevent the multiplicity of neoplastic nodules gives substantial support to the chemopreventive effects of plant extract. Hepatic damage caused by DENA and AAF generally reflects instability of liver cell metabolism which leads to distinctive changes in the serum enzyme activities [34]. Serum transaminases (TBL and γ-GT) are representative of liver function and their increased levels are sensitive indicators of hepatic injury [4]. Similarly, discharge of TBL reflects a nonspecific alteration in the plasma membrane integrity and/ or permeability [4]. γ-GT is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain and its liberation into serum indicates damage of the cells and thus injury to liver[5]. It is important to point out that serum γ-GT activity is considered to be one of the best indicators of liver damage [35]. In the present investigation, treatment with LIN significantly lowered the enhanced level of activities of these enzymes. It is suggested that LIN aids in parenchymal cell regeneration in liver and thereby protects membrane integrity by decreased enzyme leakage against carcinogenic effect of DENA+AAF. AFP is widely used as tumor marker for diagnosis of HCC, which is a unique immunomodulatory glycoprotein (65 kDa) normally made by the immature liver cells in the fetus [29]. Its detection during monitoring of HCC treatment is well accepted in patients with increased AFP levels prior to therapy, and is recommended by the European Association for the Study of the Liver. It has long been recognized that exposure of rats to certain carcinogens like DENA+AAF increases the circulating AFP levels. This corroborates the results showing significant rise in levels of AFP obtained in DENA+AAF-induced rats [5] and AFP levels were found to be significantly reduced in LIN treated (groups IV and VI) rats. To verify the anticancer activity of LIN, histopathological studies were carried out. In this investigation, marked changes were observed in the architecture of liver of cancer bearing animals. These indicate the presence of neoplastic conditions following DENA and AAF administration. In LIN treated animals, the DENA+AAF damage was recovered due to anticancer potency of coriander. From our study, it is concluded that the oral administration of LIN would exert regression of hepatocarcinogenesis induced by DENA and AAF may be due to restoring the activities of entire liver cancer marker enzymes and diminution in tumor incidence. Further investigations are required to understand the detailed mechanism of action which may lead to identification of potent molecules from coriander against DENA and AAF-induced HCC.

Conflict of interest statement The authors declare that there are no conflicts of interest.

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