

The in vivo antineoplastic and therapeutic efficacy of troxerutin on rat preneoplastic liver: biochemical, histological and cellular aspects

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Abstract

Purpose Troxerutin (TXER), a trihydroxyethylated derivative of the natural bioflavonoid rutin, abundantly found in tea, various fruits and vegetables, is known to exhibit ample pharmacological properties. In the present investigation, we examined the antineoplastic, therapeutic efficacy and furthermore the possible mechanisms of action of TXER against NAFLD/NASH progression to hepatocarcinogenesis.

Methods The effect of TXER (12.5, 25 or 50 mg/kg b.w/day) was evaluated on the nitrosodiethylamine (NDEA) model of hepatocarcinogenesis in rats, after 16 weeks of oral treatment, with special focus on liver specific enzymes, xenobiotic metabolizing enzymes, antioxidant status, lipid peroxidation profile, DNA damage, fibrosis, cell proliferation and inflammatory status.

Results Administration of TXER to hepatocellular carcinoma-bearing rats restored the enzyme activities and the hepatic architecture. Furthermore, TXER significantly curtailed NDEA-induced DNA damage, cell proliferation, inflammation, fibrosis and hepatic hyperplasia.

Conclusion This study provides the evidence that troxerutin exerts a significant therapeutic effect against liver cancer by modulating liver function enzymes, xenobiotic enzymes, oxidative damage, inhibiting cell proliferation, suppressing inflammatory response and induction of apoptosis.

Keywords Troxerutin · Xenobiotic metabolism · Oxidative stress · Fibrosis · DNA damage · Cell proliferation

Introduction

Hepatocellular carcinoma (HCC), the primary form of liver cancer, remains the fifth most common malignancy and the third most common cause of cancer mortality worldwide [1, 2]. The risk factors associated with HCC include hepatitis viruses, consumption of alcohol, tobacco, aflatoxin-contaminated food stuff, exposure to vinyl chloride, environmental and industrial toxins, non-alcoholic fatty liver and hemochromatosis [3, 4]. Among these risk factors, recent epidemiological studies have pointed out NAFLD as a major cause of HCC. NAFLD is known as the most common liver disease that encompasses various disease conditions ranging from fatty liver non-alcoholic steatohepatitis (NASH), inflammation, fibrosis, cirrhosis and progression to HCC. At present, the ratio of NAFLD patients at the risk of developing HCC is shown to be much higher than the estimated survey of 9–32 % of the general Indian population [5, 6].

N-nitrosodiethylamine (NDEA) is a well-known carcinogenic dialkyl nitrosoamine used to induce hepatocellular carcinoma in rodent models that mimic human liver cancer [7, 8]. NDEA is also known to cause adverse effects in humans by exposure to tobacco products, cosmetics, pharmaceutical products, agricultural chemicals and by consumption of preserved meats, curd and fried meals [9]. Metabolism of NDEA in the liver results in the formation of reactive oxygen species (ROS), which induces oxidative stress resulting in DNA damage. Studies on experimental models have revealed that NDEA administration triggers hepatocyte proliferation, thus leading to the development of

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altered hepatic foci (AHF) and preneoplastic nodules that eventually results in HCC [10].

The treatment modalities for HCC have exponentially improved during the past few decades. Even so, the effective treatment regimens for HCC are still limited and the survival rate remains poor. This makes new treatment strategies essential. Phytochemicals especially flavonoids with diverse biological and pharmacological properties are nowadays gaining much attention for the treatment and prevention of various diseases. Flavonoids are one of the major components in the human diet that possess antioxidant and anti-inflammatory properties due to the presence of variable number of hydroxyl/phenolic groups in their structure. Flavonoids like quercetin, rutin, hesperidin, nobiletin and naringin have shown to exhibit beneficial effects over human health by interacting with various antioxidants and scavenging free radicals and thereby imparting anti-inflammatory and anticancer properties [11–13]. So currently, research is focused on the discovery of novel chemotherapeutic flavonoids as these bioactive plant components have high potential in cancer chemoprevention at the same time exhibits minimal or no side effects.

Troloxerutin (TXER) is a trihydroxyethylated derivative of the bioflavonoid rutin, abundantly found in tea, various fruits and vegetables. TXER has been used for a number of therapeutic purposes such as for the treatment of chronic venous insufficiency (CVI) and for improving capillary function. TXER also possesses antithrombotic, fibrinolytic, antioxidant, anti-inflammatory, anti-diabetic, anticancer, edema-protective, neuroprotective, radioprotective and rheological activities. In addition, its safety and effectiveness has been successfully evaluated in both elderly patients and pregnant women [14, 15]. Till date, there are no studies demonstrating the chemotherapeutic efficacy of TXER against hepatocarcinogenesis. Our present study provides an insight into the scientific knowledge about the effective modulatory effects of TXER against NDEA-induced NAFLD progression to HCC. To attain this goal, liver marker enzymes, lipid profile, antioxidant status, DNA damage, extent of cell proliferation, inflammatory status and fibrosis were evaluated.

Materials and methods

Animals

Five-week-old male albino Wistar rats weighing approximately 200 g were obtained from the Central Animal House, Raja Muthiah Medical College and Hospital (RMMCH), Tamil Nadu, India. 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).

Chemicals and reagents

N-nitrosodiethylamine [N0756] and troloxerutin [91950] 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.

Liver cancer induction

Liver cancer was induced using NDEA according to the method of Khan et al. [16]. Five-week-old male albino Wistar rats weighing 200 g were injected intraperitoneally (i.p) with NDEA (200 mg/kg b.w) dissolved in sterile distilled water. NDEA was injected thrice at a time interval of 15 days in between starting at second week of the experimental period and subsequently continued during the fourth and sixth week.

Compound preparation

TXER was dissolved in sterile distilled water and prepared just prior to use. Rats of various treatment groups (IV–VI) received a daily dose of 12.5, 25 or 50 mg/kg b.w of TXER, respectively [15].

Treatment regimen

The experimental design is as depicted in Fig. 1. Rats were randomly divided into six groups with six animals each. Group I (control): Rats were provided with standard pellet diet and water throughout the experimental period of 16 weeks. Group II (treatment control): Rats were administered 50 mg/kg b.w of TXER orally along with standard pellet diet and water throughout the experimental period. Group III (NDEA control): Rats were administered NDEA as described above (liver cancer induction). Group IV–VI (treatment group): Rats were supplemented with different doses of TXER (12.5, 25 or 50 mg/kg b.w, respectively) for 16 weeks along with NDEA as in group III.

At the end of the experimental period, after an overnight fast, the rats were killed by cervical dislocation and blood was collected from the jugular vein. Liver was dissected out, weighed and washed in ice-cold saline.

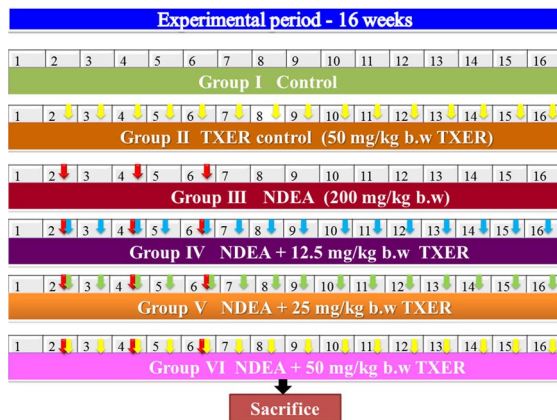


Fig. 1 Schematic representation of the experimental design

Processing of blood samples

The collected blood samples were centrifuged at 2000 rpm after 20 min at 4 °C. The supernatant (serum) was used for evaluating the activities of various liver specific enzymes.

Preparation of cytosolic and microsomal fractions

Cytosolic and microsomal fractions were collected by the method of Schladt et al. [17]. Briefly, the liver samples were homogenized in 10 mM Tris–HCl buffer (pH 7.4) containing 0.25 M sucrose, centrifuged at $9000\times g$ for 20 min and the supernatant was collected. This supernatant was further centrifuged at $100,000\times g$ for 20 min, and the clear cytosolic fractions (supernatant) were collected for measuring phase II xenobiotic enzymes. The pellets from the above centrifugation were further resuspended in ice-cold 0.15 M Tris–KCl buffer (pH 7.4) and centrifuged for 30 min at $100,000\times g$. The pellets (microsomal fraction) were resuspended in equal volumes of homogenization buffer and used for the analysis of phase I xenobiotic enzymes.

Preparation of tissue homogenate for the determination of lipid peroxidation and antioxidant status

Liver tissues were dissected and further homogenized with an appropriate buffer using Potter-Elvehjem homogenizer with a Teflon pestle. The homogenate was centrifuged at 1000 rpm for 10 min at 4 °C to separate the cell debris. The supernatant was collected for the estimation of lipid peroxidation. Half the supernatant was again centrifuged at 2000 rpm for 10 min at 4 °C to obtain post mitochondrial supernatant (PMS) which was used for the estimation of antioxidants.

Biochemical analysis

Assay of liver marker enzymes

The extent of liver function was estimated spectrophotometrically (SL 159, UV–Vis spectrophotometer, ELICO, India) by assessing the activities of liver-specific enzymes such as aspartate transaminase [AST, EC 2.6.1.1], alanine transaminase [ALT, EC 2.6.1.2], alkaline phosphatase [ALP, EC.3.1.3.1] using a diagnostic kit based on the method of Reitman and Frankel [18] and gamma-glutamyl transpeptidase [GGT, EC 2.3.2.2] using the method of Patrick and Alan [19].

Estimation of activities of xenobiotic metabolizing enzymes

The activities of phase I enzymes in the liver such as cytochrome P450 [EC.1.14.14.1], cytochrome b5, cytochrome P4502E1 [EC.1.14.13.N7], NADPH-cytochrome P450 reductase [EC.1.6.2.4] and NADPH-cytochrome b5 reductase [EC. 1.6.2.2] were measured by the methods of Omura and Sato [20], Watt et al. [21], Mihara and Sato [22] and Omura and Takesue [23], respectively.

The activities of phase II enzymes in the liver such as glutathione-S-transferase [GST, EC.2.5.1.18], DT-diaphorase [DTD, EC.1.6.99.2] and UDP-glucuronyl transferase [UDP-GT, EC.2.4.1.17] were assessed by the method of Habig et al. [24], Ernster et al. [25] and Isselbacher et al. [26], respectively.

Estimation of lipid peroxidation status

Tissue lipid peroxidation was assessed spectrophotometrically (SL 159, UV–Vis spectrophotometer, ELICO, India) by measuring the levels of thiobarbituric acid reactive substances (TBARS) and lipid hydroperoxides (LOOH) by the method of Ohkawa et al. [27]. and Jiang et al. [28], respectively.

Determination of the antioxidants

The activity of superoxide dismutase (SOD) [EC. 1.15.1.1] was assayed by the method of Kakkar et al. [29]. The activity was determined on the basis of 50 % inhibition of the formation of NADH–phenazine methanosulphate–nitroblue tetrazolium (NBT) formazan at 520 nm. Activity of catalase [CAT, EC.1.11.1.6] was assayed by the method of Sinha [30], and the values are expressed as micro-moles of H_2O_2 utilized/min/mg protein. Glutathione (GSH) was determined by the method of Ellman [31] as an acid soluble non-protein sulphhydryl group, and values are denoted as mmol/mg tissue.

Histological characterization and quantification

For histological characterization, formalin-fixed samples were embedded in paraffin, sectioned into slices of 5 μm thickness, processed, stained and analyzed as described below.

Hematoxylin and Eosin staining

The paraffin-embedded liver sections were hydrated, dipped in Mayer's hematoxylin and agitate for 30 s. The sections were then rinsed in distilled water for 1 min and stained in 1 % eosin Y solution for 10–30 s with agitation. Further, the sections were dehydrated with two changes of 95 % alcohol and two changes of 100 % alcohol for 30 s each. The alcohol was extracted with two changes of xylene. The sections were subsequently mounted with DPX and allowed to dry. The slides were digitally scanned using a light microscope (Carl Zeiss). Multifractal analysis was performed with the software Fraclac V2.5. The parameter used was the generalized dimension spectra, denoted as D_q versus q calculated by setting an arbitrary range for $q = 210$ to $+10.75$ with 0.25 increments. The images were converted to binary images and outlined for improved quantification. Images can be seen in Fig. 4.

Argyrophilic nucleolar organizer regions (AgNORs) staining

AgNORs staining was performed according to the method of Treré [32]. Briefly, the paraffin-embedded sections were deparaffinized in xylene and rehydrated by placing in decreasing concentrations of ethanol to deionized water. The sections were then stained in freshly prepared silver colloid solution and kept in the dark for 45 min at room temperature. After incubation, the sections were rinsed in deionized water and dehydrated through different concentrations of ethanol to xylene. The sections were mounted with DPX and allowed to dry overnight. The number of AgNORs per nucleus was scanned using a light microscope (Carl Zeiss) and quantified by Image-based Tool for Counting Nuclei (ITCN).

Mast cell staining

Histological analysis of mast cell number was determined by the method of Ramakrishnan et al. [33]. Briefly, the liver sections were dewaxed, rehydrated and stained with toluidine blue for 2 min, and then rinsed in deionized water. The sections were further dehydrated in increasing

concentrations of ethanol and xylene. The slides were subsequently dried and mounted with the DPX mounting solution. The total number of mast cells in each section was observed and photographed using a light microscope (Carl Zeiss). The number of intact mast cells was quantitatively analyzed using ImageJ software (1.50 g) by inverting the image, thresholding and measuring using cell counter plugin.

Sirius red staining for collagen

The paraffin-embedded liver sections were deparaffinized and hydrated with deionized water. The sections were then incubated in Fouchet's reagent for 5 min, rinsed in distilled water, incubated in celestine blue for 2 min and rinsed in distilled water, further incubated in hematoxylin for 3 min, rinsed in distilled water, stained with Sirius red solution for 10 min and rinsed in distilled water. The sections were subsequently mounted with DPX and allowed to dry. The presence of collagen deposition in the tissues was observed using a light microscope (Carl Zeiss) and subsequently quantitatively analyzed using ImageJ software (1.50 g) by segmenting the red-stained collagen using thresholding and measuring the intensity of red staining in each experimental group. The 3D visualization of collagen deposition is presented as volumes and surfaces of each image stacks using the 3D Color Inspector module.

Deoxyribonucleic acid (DNA) extraction and fragmentation

DNA was extracted using GenPro™ 3-in-1 isolation kit. In brief, 10 mg of tissue was homogenized by adding lysis buffer provided in the kit. The tissue homogenate was then centrifuged for 1 min at 12,000 rpm at room temperature. Then, genomic DNA (gDNA) wash buffer I was added to the pellet and centrifuged at 12,000 rpm for 1 min. To the pellet, gDNA wash buffer II was added and centrifuged at 12,000 rpm for 2 min at room temperature and the flow through was discarded. To this, DNA elution buffer was added and stored at $-20\text{ }^{\circ}\text{C}$.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), and significant differences among various groups were evaluated by Duncan's multiple range test (DMRT) using SPSS 15.0 software package (SPSS, Tokyo, Japan). The results were considered statistically significant at $p < 0.05$. The values are given as mean \pm SD.

Results

Effect of TXER and NDEA on body weight and liver morphology

Initiation of hepatocarcinogenesis was perceptible within 2 months of NDEA administration. The amount of food intake by the HCC-bearing rats (group III) became much lesser as compared to the control and TXER-treated rats. The body weight changes of rats in each group are shown

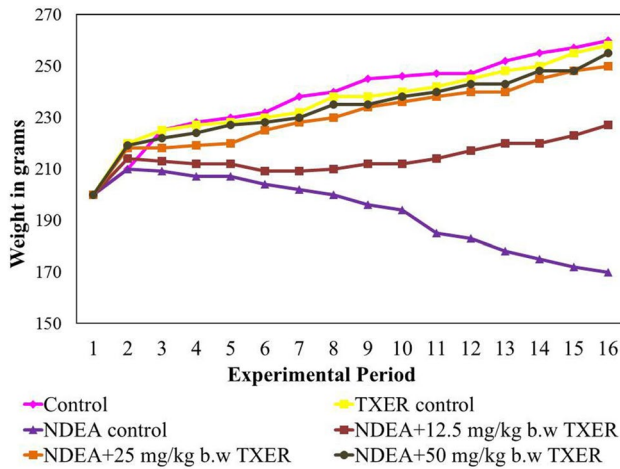


Fig. 2 Effect of TXER and NDEA on body weight changes

in Fig. 2. The mean body weights of NDEA-administered HCC rats (group III) were significantly reduced as compared to control and troxerutin control rats, whereas treatment with 25 and 50 mg/kg b.w TXER to the HCC-bearing rats (group V and VI) augmented the body weight gain and was comparable with those of the control rats. Moreover, TXER at the dose of 50 mg/kg b.w showed a more pronounced effect.

The morphology of liver specimens was analyzed after 16 weeks of the experimental period (Fig. 3). Administration of TXER to HCC-bearing rats (group IV–VI) showed significantly decreased hepatic nodules as compared to the NDEA alone treated rat (group III), especially the 25 and 50 mg/kg b.w TXER-treated rats (group V and VI) showed a marked suppression in tumor development, and no changes were observed in liver morphology. There were no significant changes between the liver morphology of control and TXER control rats.

Effect of TXER and NDEA on liver function enzymes

Table 1 summarizes the effects of TXER on NDEA-induced changes on the serum liver marker enzymes. Activities of hepatocyte marker enzymes such as AST, ALT, ALP and GGT were significantly increased in HCC-bearing rats (group III) as compared to the control rats (group I), indicating liver damage. TXER treatment to HCC-bearing rats showed a significant decrease in the activities of AST, ALT,

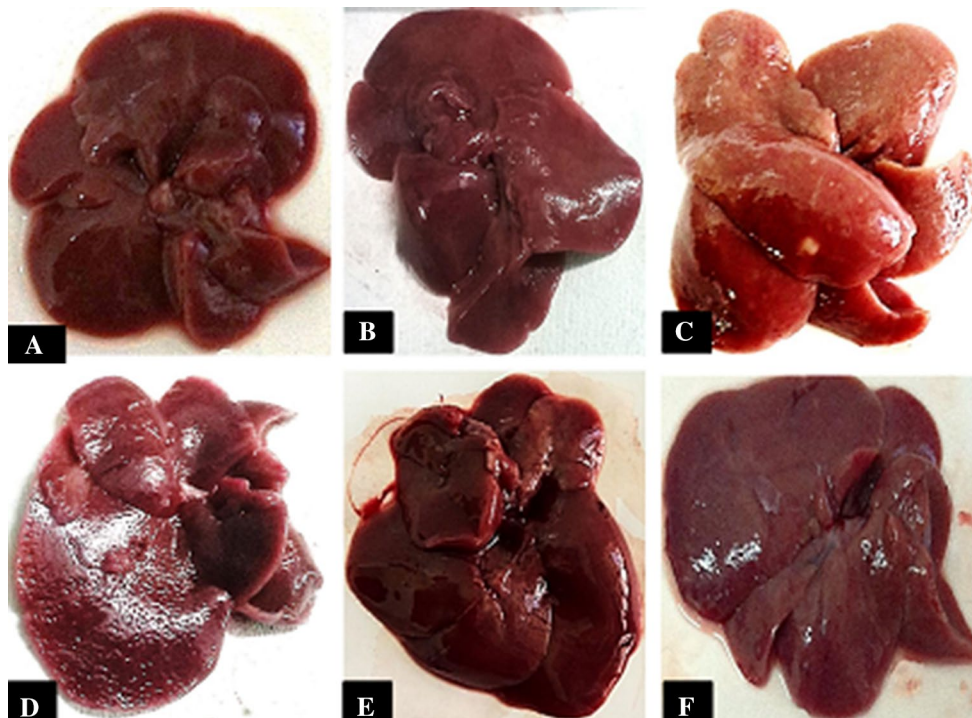


Fig. 3 Liver morphology of rats in each group (I–VI). **a** Control. **b** TXER control (50 mg/kg b.w). **c** NDEA control (200 mg/kg b.w). **d** NDEA + 12.5 mg/kg b.w TXER. **e** NDEA + 25 mg/kg b.w TXER. **f** NDEA + 50 mg/kg b.w TXER

Table 1 Effect of TXER and NDEA on the liver specific marker enzymes in the serum of control and experimental rats

| Parameters | Control | TXER control (50 mg/kg b.w) | NDEA control (200 mg/kg b.w) | NDEA + TXER (12.5 mg/kg b.w) | NDEA + TXER (25 mg/kg b.w) | NDEA + TXER (50 mg/kg b.w) |
|------------|---------------------------|--------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| AST (IU/L) | 52.24 ± 5.01 ^a | 53.71 ± 5.03 ^a | 81.40 ± 7.08 ^b | 75.46 ± 6.01 ^c | 55.13 ± 5.06 ^a | 50.15 ± 5.01 ^a |
| ALT (IU/L) | 61.98 ± 6.02 ^a | 60.20 ± 6.01 ^a | 77.03 ± 7.5 ^b | 70.82 ± 7.01 ^b | 68.89 ± 6.03 ^a | 62.44 ± 6.01 ^a |
| ALP (IU/L) | 87.93 ± 7.81 ^a | 87.01 ± 7.53 ^a | 120.35 ± 8.31 ^b | 115.6 ± 8.78 ^b | 90.80 ± 8.05 ^c | 90.93 ± 8.01 ^c |
| GGT(IU/L) | 20.84 ± 0.21 ^a | 20.95 ± 0.22 ^a | 60.54 ± 5.658 ^b | 43.23 ± 4.21 ^c | 22.49 ± 0.28 ^a | 22.98 ± 0.25 ^a |

All the values are expressed as the mean ± SD of six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT)

AST aspartate transaminase, ALT alanine transaminase, ALP alkaline phosphatase, GGT gamma-glutamyl transpeptidase

ALP and GGT as compared to the unsupplemented HCC-bearing rats (group III). Interestingly, administration of 25 and 50 mg/kg b.w TXER caused a pronounced decrease on the NDEA-induced augmentation of these enzyme activities. These results demonstrate that TXER depicts hepatoprotective effects against NDEA-induced hepatocarcinogenesis. There was no significant difference in the enzyme activities between the TXER control and control rats.

Effect of TXER and NDEA on xenobiotic metabolizing enzymes

Table 2 shows the influence of TXER supplementation on the activities of phase I and phase II enzymes in the liver. Administration of NDEA resulted in a dramatic increase in the activities of phase I enzymes followed by a decrease in

the activities of phase II enzymes (GST, DTD and UDP-GT) as compared to the control rats (group I). Interestingly, the adverse effects induced by NDEA were significantly suppressed on supplementation with TXER, a more pronounced effect being observed in the rats supplemented with 25 and 50 mg/kg b.w TXER (group V). However, there were no significant variations between the control (group I) and troxerutin alone treated rats (group II).

Effect of TXER and NDEA on lipid peroxidation

Lipid peroxidation is pointed out as one of the basic mechanisms that cause tissue damage by liberating free radicals. Table 3 depicts the levels of TBARS and LOOH in the liver of control and experimental rats. Group III HCC-bearing rats exhibited significant ($p < 0.05$) increase in the levels of

Table 2 Effect of TXER and NDEA on the hepatic xenobiotic metabolizing enzymes of the control and experimental rats

| Parameters | Control | TXER control (50 mg/kg b.w) | NDEA control (200 mg/kg b.w) | NDEA + TXER (12.5 mg/kg b.w) | NDEA + TXER (25 mg/kg b.w) | NDEA + TXER (50 mg/kg b.w) |
|---|---------------------------|--------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| <i>Phase I enzymes</i> | | | | | | |
| Cytochrome P450 | 5.20 ± 0.52 ^a | 4.95 ± 0.49 ^a | 8.52 ± 0.85 ^b | 7.46 ± 0.74 ^c | 5.91 ± 0.58 ^a | 5.33 ± 0.53 ^a |
| Cytochrome P4502E1 | 4.64 ± 0.15 ^a | 4.79 ± 0.20 ^a | 7.32 ± 0.45 ^b | 6.82 ± 0.59 ^b | 5.10 ± 0.32 ^a | 5.03 ± 0.19 ^c |
| Cytochrome b ₅ | 6.12 ± 0.40 ^a | 5.81 ± 0.21 ^a | 12.95 ± 0.9 ^b | 10.33 ± 0.67 ^c | 6.04 ± 0.29 ^a | 7.48 ± 0.54 ^d |
| NADPH-cytochrome P450 reductase | 53.43 ± 5.15 ^a | 53.53 ± 5.16 ^a | 80.21 ± 7.56 ^b | 72.23 ± 6.84 ^c | 58.02 ± 5.23 ^a | 57.23 ± 5.20 ^a |
| NADPH-cytochrome b ₅ reductase | 15.25 ± 0.61 ^a | 15.05 ± 0.63 ^a | 30.15 ± 2.83 ^b | 26.89 ± 2.52 ^c | 16.59 ± 0.64 ^a | 16.01 ± 0.64 ^a |
| <i>Phase II enzymes</i> | | | | | | |
| GST | 0.40 ± 0.04 ^a | 0.42 ± 0.04 ^a | 0.22 ± 0.02 ^b | 0.28 ± 0.02 ^b | 0.39 ± 0.04 ^a | 0.45 ± 0.03 ^c |
| DTD | 1.62 ± 0.07 ^a | 1.72 ± 0.07 ^a | 0.43 ± 0.04 ^b | 0.88 ± 0.05 ^c | 1.58 ± 0.07 ^a | 1.89 ± 0.07 ^d |
| UDP-GT | 3.25 ± 0.28 ^a | 3.36 ± 0.28 ^a | 1.78 ± 0.15 ^b | 2.32 ± 0.20 ^c | 3.40 ± 0.28 ^a | 3.89 ± 0.38 ^d |

All the values are expressed as the mean ± SD of six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT)

Cytochrome P450- $\mu\text{mol}/\text{mg}$ protein; Cytochrome P4502E1-mmol of *p*-nitrocatechol liberated/min/mg protein; Cytochrome b₅- $\mu\text{mol}/\text{mg}$ protein; NADPH-cytochrome P450 reductase-One unit of enzyme activity is defined as that causing the oxidation of one mole of NADPH per minute; NADPH-cytochrome b₅ reductase-One unit of enzyme activity is defined as that causing the reduction of one mole of ferricyanide per minute; GST glutathione-S-transferase (μmol of 1-chloro-2,4-dinitrobenzene (CDNB)-GSH conjugate formed/min/mg protein); DTD-DT-diaphorase (μmol of 2,6-dichlorophenolindophenol reduced/min/mg protein); UDP-GT-UDP-glucuronyl transferase (nmol/min/mg protein)

Table 3 Effect of TXER and NDEA on hepatic lipid peroxidation byproducts and activities of the hepatic enzymic and non-enzymic antioxidants of the control and experimental rats

| Parameters | Control | TXER control (50 mg/kg b.w) | NDEA control (200 mg/kg b.w) | NDEA + TXER (12.5 mg/kg b.w) | NDEA + TXER (25 mg/kg b.w) | NDEA + TXER (50 mg/kg b.w) |
|---|---------------------------|--------------------------------|---------------------------------|---------------------------------|-------------------------------|-------------------------------|
| <i>Lipid peroxidation</i> | | | | | | |
| TBARS | 0.84 ± 0.08 ^a | 0.81 ± 0.08 ^a | 4.20 ± 0.32 ^b | 2.97 ± 0.31 ^c | 0.88 ± 0.08 ^a | 0.88 ± 0.08 ^a |
| LOOH | 48.89 ± 4.9 ^a | 45.20 ± 4.6 ^a | 91.51 ± 9.01 ^b | 80.74 ± 8.2 ^c | 43.70 ± 4.4 ^a | 45.89 ± 4.5 ^a |
| <i>Enzymic and non-enzymic antioxidants</i> | | | | | | |
| SOD | 6.57 ± 0.65 ^a | 6.77 ± 0.67 ^a | 3.38 ± 0.34 ^b | 4.89 ± 0.4 ^c | 6.13 ± 0.61 ^a | 6.32 ± 0.63 ^a |
| CAT | 31.83 ± 3.18 ^a | 31.66 ± 3.16 ^a | 9.56 ± 0.83 ^b | 10.86 ± 1.05 ^c | 28.26 ± 2.82 ^a | 29.59 ± 2.95 ^a |
| GSH | 80.53 ± 8.14 ^a | 81.89 ± 8.18 ^a | 30.97 ± 3.08 ^b | 44.22 ± 4.32 ^c | 78.35 ± 7.83 ^a | 79.13 ± 7.93 ^a |

Data are presented as the mean ± SD of six rats in each group. Values not sharing a common superscript letter (a–c) differ significantly at $p < 0.05$ (DMRT)

TBARS thiobarbituric acid reactive substances (mmol/mg tissue); LOOH lipid hydroperoxides (mmol/mg tissue); SOD Superoxide dismutase (50 % NBT reduction/min/mg protein); CAT Catalase (μmoles of H₂O₂ utilized/min/mg protein); GSH Glutathione (mmole/mg tissue protein)

TRARS and LOOH as compared to the control rats. On the other hand, administration of TXER to HCC-bearing rats restored the levels of lipid peroxidation by-products to near those of the control rats. The effect was more significant when the dose of TXER administration was 25 and 50 mg/kg b.w. There were no remarkable differences in the levels of the lipid peroxidation markers between the control and TXER control rats.

Effect of TXER and NDEA on the antioxidant status

The antioxidant property of TXER was determined by evaluating the enzymic and non-enzymic antioxidants in the liver of control and experimental rats (Table 3). In group III HCC-bearing rats, there was a significant decrease in the activities of the antioxidants such as SOD, CAT and GSH as compared to the control rats. On the other hand, in TXER-treated rats (group IV–VI), a significant increase in the activities of these antioxidants were observed as compared to HCC-bearing rats (group III). Group II TXER control rats showed no significant difference in the activities of these antioxidants when compared with group I control rats. Thus, supplementation with TXER to HCC-bearing rats appeared to stabilize the antioxidant status, and thereby, offering protection against NDEA-induced oxidative damages in the liver.

Effect of TXER and NDEA on liver histology

Histological changes in the liver of control and experimental rats are illustrated in Figs. 4, 5, 6 and 7. Histological examination revealed that administration of NDEA-induced fibrosis with inflammation, cell proliferation and nodule formation [Figs. 4(c, ci, cii); 5(c, ci, cii); 6(c, ci, cii, ciii); 7(c, ci, cii, ciii)], whereas treatment with TXER to NDEA-administered rats showed near normal appearing hepatocytes with reduced degeneration, less formation of nodules and normal

central veins [Fig. 4(e, ei, eii, f, fi, fii)]. It is noteworthy that TXER also reduced the accumulation of AgNORs, MCs and collagen deposition in the tissue [Figs. 5(e, ei, eii, f, fi, fii); 6(e, ei, eii, eiii, f, fi, fii, fiii); 7(e, ei, eii, eiii, f, fi, fii, fiii)], indicating the efficacy of TXER to suppress cell proliferation, inflammation and fibrosis. Moreover, beneficial effect of the supplementation was more noticeable in the rat administered with 25 mg/kg and 50 mg/kg b.w TXER. The statistical comparison between the various experimental group using Multifractal spectra [Fig. 4 g], ITCN [Fig. 5 g], Cell Counter [Fig. 6 g], and RGB Profiling [Fig. 7 g] are in supportive to the qualitative results.

Effect of TXER and NDEA on DNA damage

DNA fragmentation assay was used to determine the breaks in the DNA due to oxidative stress. DNA laddering induced by NDEA and the protective effect of TXER are depicted in Fig. 8. NDEA administration caused a very significant DNA damage as indicated by the increased DNA laddering in group III HCC-bearing rat liver as compared to the control rat 12.5 mg/kg b.w TXER treatment to HCC-bearing rats did not show a significant decrease in the DNA laddering but supplementation with 25 and 50 mg/kg b.w TXER (group V–VI) showed a marked protection of the cellular DNA, as evident by the decreased DNA laddering. There was no significant difference in the hepatic DNA of the TXER control and the control rat.

Discussion

TXER is a bioflavonoid that has undergone many clinical trials in human beings, even with high doses and has shown excellent safety and tolerability profiles [34, 35]. The present study shows that administration of NDEA engendered

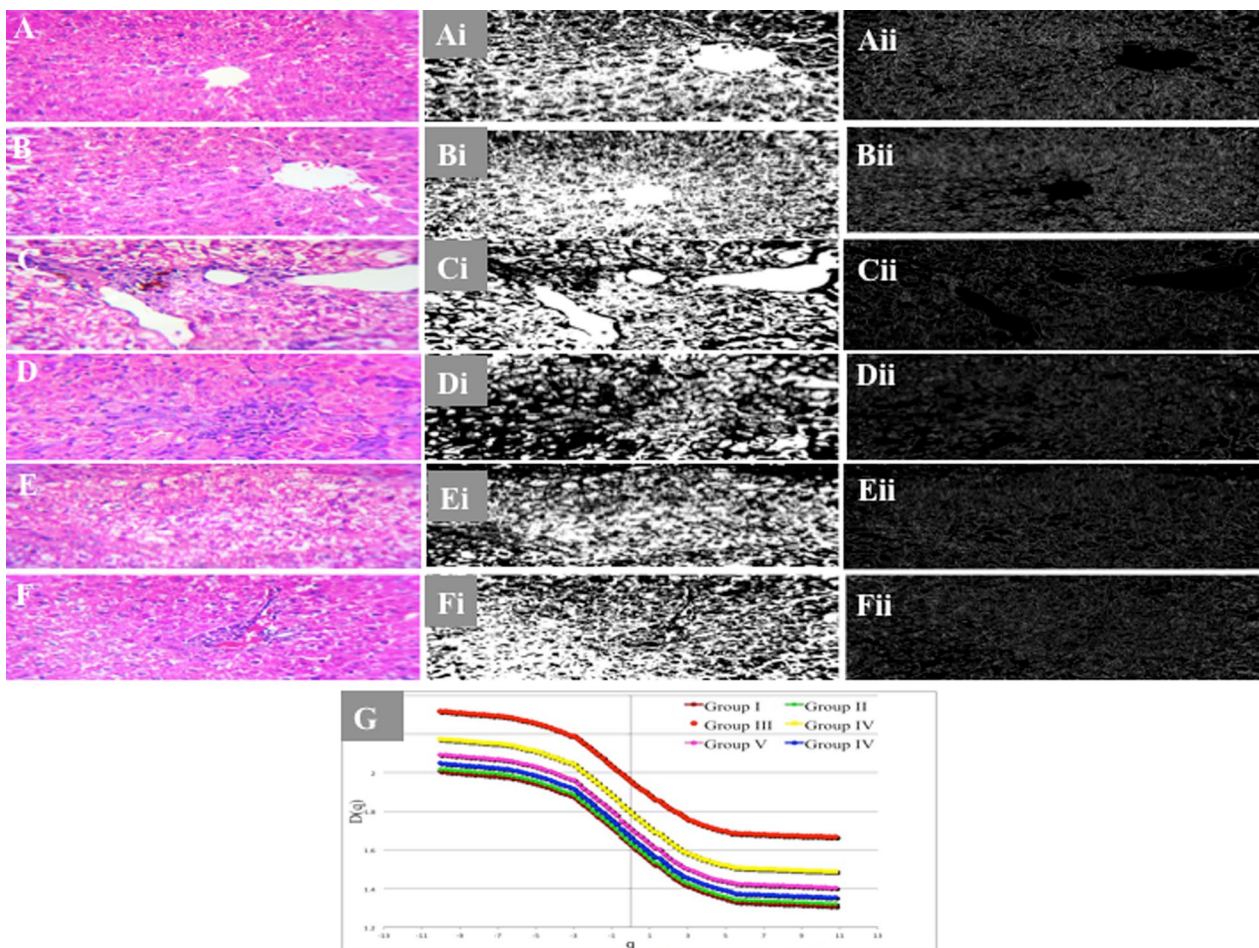


Fig. 4 Histological changes in the liver of control and experimental groups ($\times 100$) [H and E staining]. **a, b** Liver sections of control and treatment control rats showing normal architecture with central portal vein. **c** Liver section of NDEA-induced rat showing fibrosis with inflammation and nodule formation. **d** Liver section of NDEA + 12.5 mg/kg b.w TXER-treated rat showing inflammatory cells. **e** Liver section of NDEA + 25 mg/kg b.w TXER-treated

rat showing degeneration of hepatocytes and mild periportal inflammation. **f** Liver section of NDEA + 50 mg/kg b.w TXER-treated rat showing moderate periportal inflammation. **ai** and **fi** and **aii** and **fii** represent the binary and outlined image of **a–f** images showing a clear morphological difference, enlarging the boundaries of the regions of the foreground pixels. **g** Fractal analysis of histological image **a–f** illustrating Multifractal spectra

the development and/or progression of NAFLD to HCC and resulted in macroscopically and microscopically perceptible hepatic neoplasms and a significant decrease in the body weight as well demonstrating that TXER at the doses 25 and 50 mg/kg b.w has a potent therapeutic efficacy against NDEA-induced hepatocarcinogenesis. Oral administration of TXER to HCC-bearing rats showed not only an increase in the body weight and a drastic reduction in the occurrence of hepatic neoplasms but also the suppression of preneoplastic hepatic lesions.

Hepatic damage induced by NDEA disrupts the membrane permeability and metabolism of hepatocytes. This disruption leads to release of the liver-specific enzymes (ALT, AST, ALP and GGT) into the circulatory system resulting in the increased activities of these enzymes in the circulatory system [36]. Evaluating the activities of these

enzymes in the serum has been important in the diagnosis of liver damage [37]. Transaminases are the most sensitive markers for the diagnosis of functional membrane integrity and liver damage. Increased activities of AST and ALT in the serum indicates high incidence of HCC in patients. ALP, another key liver-specific enzyme in the serum, indicates pathological alterations in the bile flow. Rapidly, proliferating hepatocytes located in the bile canalicular plasma membrane sheds ALP in large amounts. GGT, an enzyme localized on the outer membrane of the hepatocytes, functions to maintain the homeostasis of cellular glutathione. During malignancy the concentration of GGT increases, indicating cholestasis and bile duct necrosis [38]. The observations of the present study correlate with the above findings, showing elevated activities of the liver-specific enzymes AST, ALT, ALP and GGT in

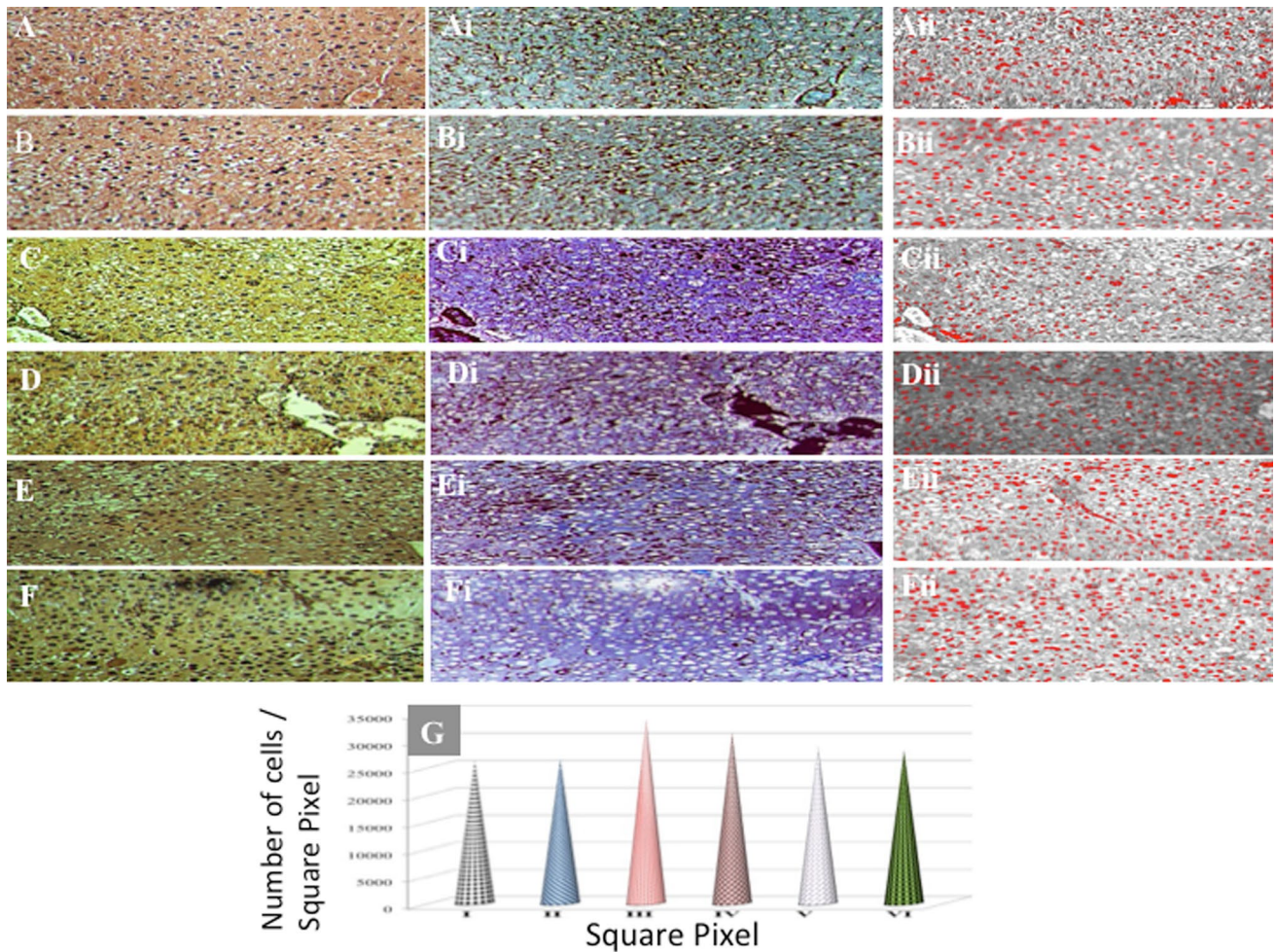


Fig. 5 Histological analysis of the liver sections with silver nitrate staining for AgNORs in the liver of control and experimental rats ($\times 40$). **a** AgNORs in the liver of control rat. **b** AgNORs in the liver of a TXER control rat. **c** AgNORs in the liver of a NDEA-administered rat, arrow shows nuclei with increased AgNORs. **d** AgNORs in the liver of a NDEA + 12.5 mg/kg b.w TXER-treated rat. **e** AgNORs in the liver of a NDEA + 25 mg/kg b.w TXER-treated rat, showing

nuclei with decreased number of AgNORs. **f** AgNORs in the liver of a NDEA + 50 mg/kg b.w TXER-treated rat. **ai–fi** Depicts the inverted image and **aii–fii** Depicts the threshold image that shows the NORs in the nuclei of proliferating cells. **g** Comparative graph illustrating extends of cell proliferation in each of the experimental group shown in **a–f**. $p < 0.05$ versus control

the circulation, indicating NDEA-induced hepatic injury and hepatocarcinogenesis. Supplementation with TXER to HCC-bearing rats significantly reduced the activities of these enzymes denoting the protective effects of TXER against HCC, which could be probably due to the inhibition of membrane damage caused by NDEA, thereby controlling the release of these enzymes into the circulation.

CYP450 enzymes, the heme-thiolate proteins located in the smooth endoplasmic reticulum of various tissues, are known to be responsible for the oxidation, peroxidation and reduction of various exogenous and endogenous substrates [39]. Previous reports have proved that increased activities of CYP450 and CYP2E1 are considered to be an important risk factor for cancer because they biotransform procarcinogens to electrophilic derivatives, that in turn alkylates DNA

and initiates chemical carcinogenesis. These enzymes are capable of inducing oxidative damage within the tissue by generating ROS [40, 41]. The procarcinogenic NDEA is hydroxylated by CYP450 through an alkylation mechanism to ethylacetoxylethyl nitrosamine and is oxidized to ethyl diazonium ions by CYP2E1 in the rat liver microsomes [42].

Phase II drug metabolizing enzymes (GST, DTDs and UDP-GT) biotransform endogenous and xenobiotic compounds into non-toxic and easily excretable form, playing an important role in the biological system. The decreased metabolizing capacity of the phase II enzymes leads to the overexpression of the toxic effects of the xenobiotics. The detoxification enzyme GST catalyzes the formation of thioether conjugates between the endogenous glutathione and the xenobiotic substances. GST acts as the defense

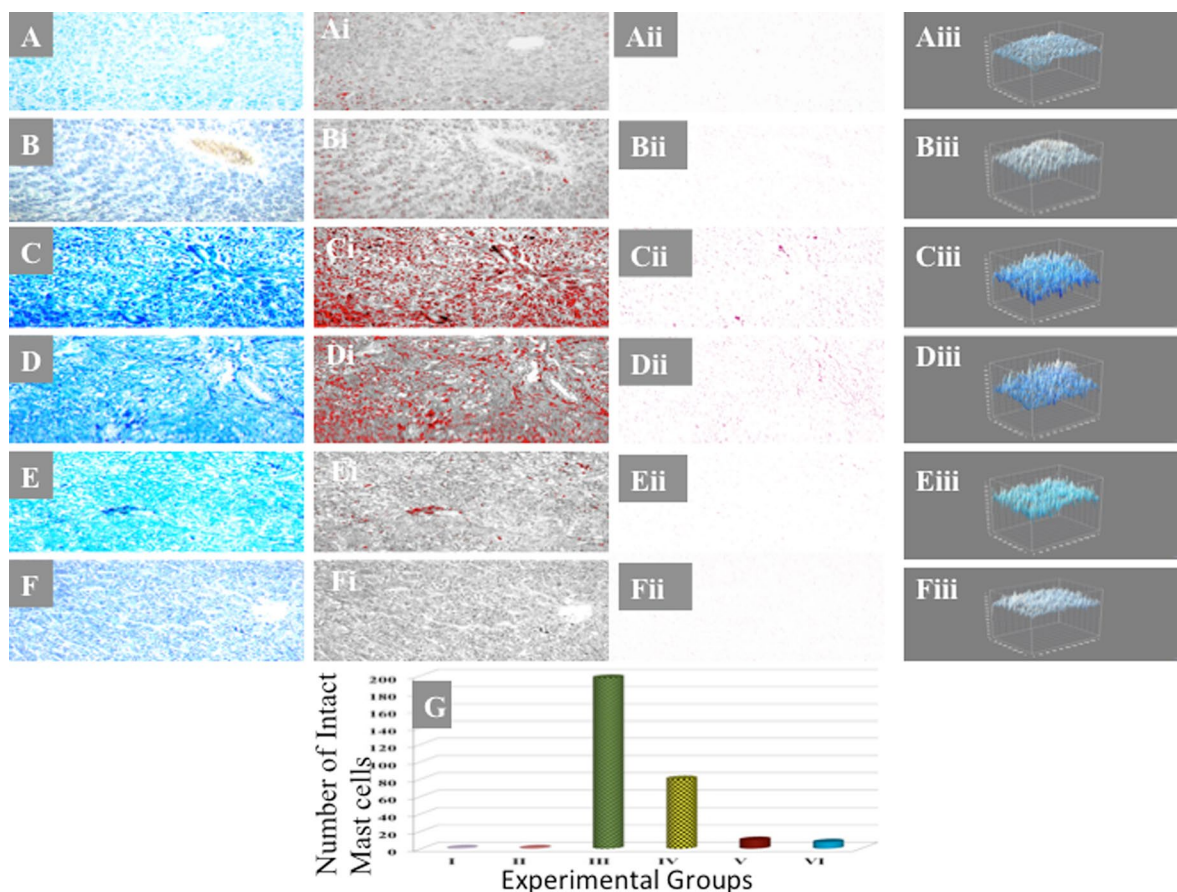


Fig. 6 Effect of TXER and NDEA on mast cell number, stained with toluidine blue ($\times 40$). **a, b** Hepatic section of control and TXER control rats shows no accumulation of mast cells. **c** Hepatic section of NDEA treated rat showing accumulation of mast cells (blue granule like structures) indicating the onset of pathological changes. **d** Hepatic section of NDEA + 12.5 mg/kg b.w TXER-treated rat, shows slightly reduced accumulation of mast cells. **e** Hepatic section of NDEA + 25 mg/kg b.w TXER-treated rat, shows reduced accumula-

tion of mast cells. **f** Hepatic section of NDEA + 50 mg/kg b.w TXER-treated rat, shows very few mast cells. **ai–fi** The red color shows the threshold section of the tissue. **aii–fii** Color deconvolution using new OD vector. **aiii–fiii** Colors of each section were also analyzed by the 3D Color Inspector module. **g** Comparative graph illustrating the presence of number of intact mast per square pixel in the histological image of each experimental group. $p < 0.05$ versus control

mechanism against the toxic and reactive electrophiles that are generated by cellular oxidative reactions catalyzed by CYP450. UDP-GT catalyzes the conjugation of glucuronides with many xenobiotics and endobiotics, thus eliminating the biotransformed toxins through renal and biliary excretion. This glucuronidation reaction catalyzed by UDP-GTs in liver is the most important drug detoxification pathway in humans [43]. DTD, a flavoprotein, catalyzes the reduction of quinones without producing ROS [44] and thereby protecting the cell against mutagenicity [45]. Thus, determining the activities of phase I and phase II drug metabolizing enzymes in chemically induced carcinogenesis is a key diagnostic factor. In our present study, the activities of phase I enzymes were significantly increased and the activities of phase II enzymes (GST, DTD and UDP-GT) were significantly reduced in NDEA alone treated rats, which could be due to the over

utilization of these enzymes to counteract NDEA-induced toxicity or due to the reduced metabolizing capacity of these enzymes. Supplementation with TXER to HCC-bearing rats significantly enhanced the activities of phase II xenobiotic metabolizing enzymes and suppressed the activities of phase I enzymes (Table 2). This underlines the protective effect of TXER where it detoxifies and inhibits the metabolic activation of NDEA in the liver.

Studies have reported that NDEA stimulates hepatic lipid peroxidation, an important causative factor of carcinogenesis, causing liver damage by generating free radicals and forming by-products like TBARS and LOOH which cross-links with macromolecules and promotes carcinogenesis [46]. The free radicals disrupt the cellular process and antioxidant status by altering the activities of the antioxidants such as SOD, CAT and GSH, leading to oxidative stress [47].

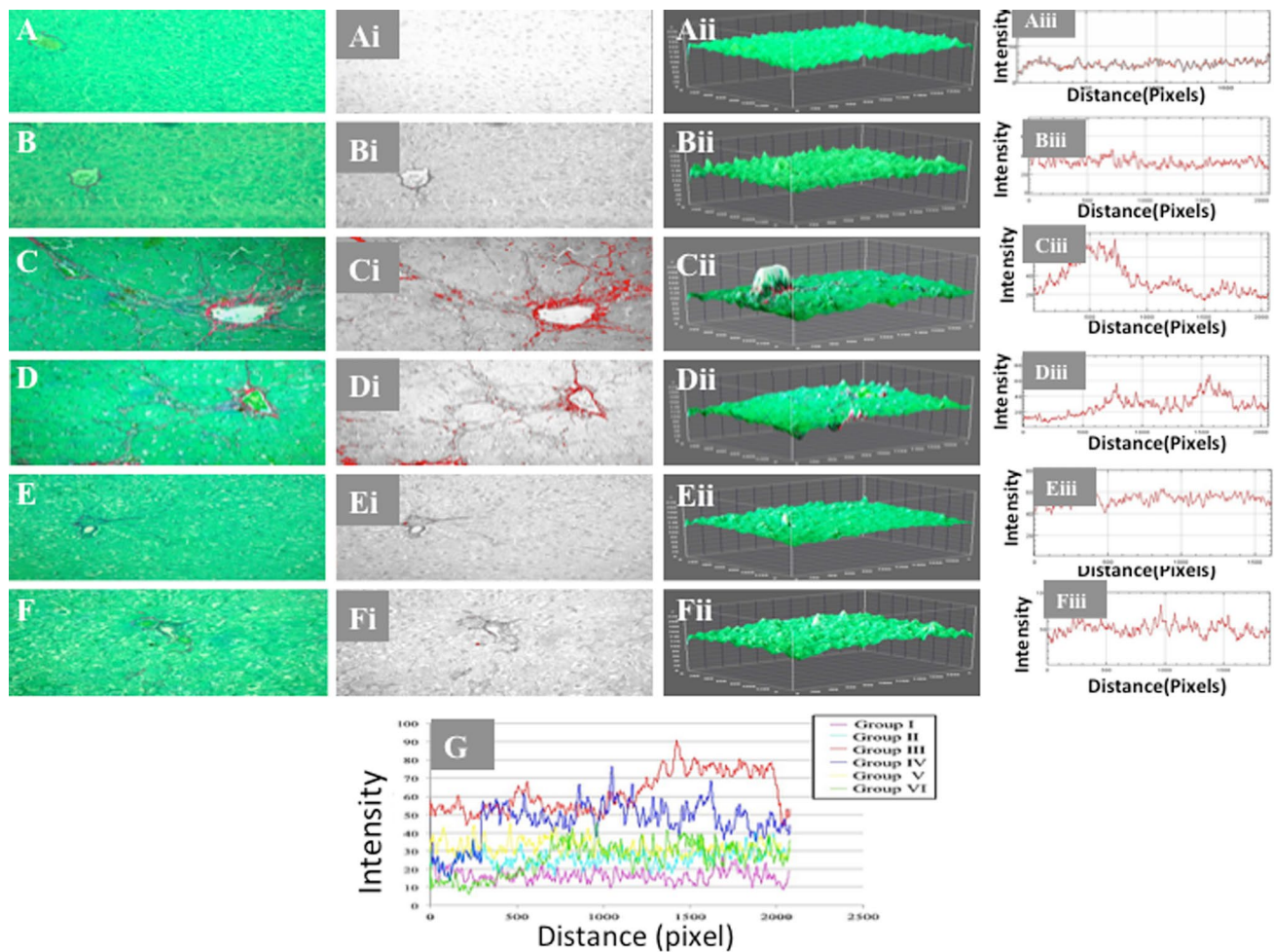


Fig. 7 Effect of TXER and NDEA on liver collagen, stained with sirius red ($\times 40$). **a, b** Hepatic sections of control and TXER control rats shows very minimal collagen deposition. **c** Hepatic section of NDEA treated rats shows marked accumulation of collagen. **d** Hepatic section of NDEA + 12.5 mg/kg b.w TXER-treated rat shows slightly reduced accumulation of collagen in the tissue. **e** Hepatic section of NDEA + 25 mg/kg b.w TXER-treated rat shows marked reduction in the accumulation of collagen. **f** Hepatic section of NDEA + 50 mg/

kg b.w TXER-treated rat, minimal accumulation of collagen. **ai–fi** The red color shows the threshold section of the tissue indicating collagen deposition. **aii–fii** Colors of each section were also analyzed by the 3D Color Inspector module. **aiii–fiii** RGB profiling image showing the intensity of collagen staining per square pixel. **g** Comparative graph representing the collagen intensity variation in each experimental group. $p < 0.05$ versus control

SOD and CAT are the two most important antioxidant enzymes that provide cellular protection against oxidative stress by directly eliminating the toxic-free radicals and electrophiles. NDEA-induced oxidative stress produces superoxide anions, which are dismutated into hydrogen peroxide by the catalytic activity of SOD. Further, CAT or GPx detoxifies the hydrogen peroxide into molecular oxygen and water. GSH, a non-protein thiol plays an important role in the innate antioxidant defense mechanism by detoxifying endogenous and exogenous substances. GSH also scavenges a variety of free radicals, thereby acting as an antioxidant [15, 38].

The present study reveals a significant increase in the levels of TBARS and LOOH and a significant decrease in the activities of SOD, CAT and GSH in the liver of NDEA

alone induced rats as compared to control. This could be due to that (1) ROS generated by lipid peroxidation can directly reduce the activities of these enzymes or (2) cancer tissues/hepatomas can sequester antioxidants from the circulation. Treatment with TXER to HCC-bearing rats significantly decreased the levels of lipid peroxidation by-products and significantly augmented the activities of the antioxidants. TXER being a flavonoid acts as an ideal scavenger of peroxy and oxygen-derived free radicals due to the presence of a 3-hydroxyl group in the B-ring that possess electron donating property. The above results prove that TXER exerts its hepatoprotective activity by scavenging free radicals and by enhancing the activities of the endogenous enzymic and non-enzymic antioxidants.

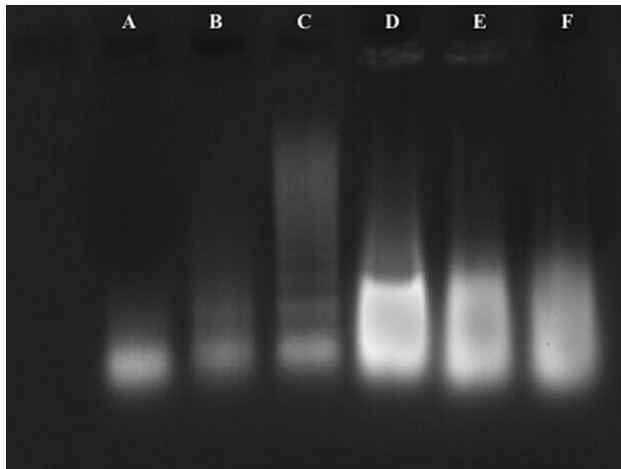


Fig. 8 Effect of NDEA and TXER on DNA fragmentation of control and experimental groups. **a, b** DNA of control and TXER control rat liver shows no DNA laddering. **c** DNA of NDEA control rat liver shows DNA laddering indicating DNA damage. **d** DNA of NDEA + 12.5 mg/kg b.w TXER-treated rat liver shows slightly reduced DNA ladder. **e** DNA of NDEA + 25 mg/kg b.w TXER-treated rat liver shows no significantly reduced DNA ladder. **f** DNA of NDEA + 50 mg/kg b.w TXER-treated rat liver shows no significantly reduced DNA ladder

The proliferative activities of hepatocytes are increased progressively in the non-cancerous lesions that are in the onset of HCC [48]. AgNORs, a set of nucleolar proteins that accumulates in the proliferating cells acts as indicators of the proliferative status of hepatomas and the number of cells in the S phase of the cell cycle. Our present study shows a marked reduction in the number of AgNORs in treatment groups as compared to HCC group, thereby indicating the anti-proliferative efficacy of TXER. Moreover, these effects of TXER could be due to its ability to modulate the cell cycle regulators and cell cycle processes.

MCs, a group of long-living cells which originate from hematopoietic stem cells in bone marrow, are known to be present in both the normal healthy and diseased livers. A dense amount of MCs are detected near the fibrotic septa and portal regions in the diseased liver. Accumulation of MCs accelerates tumor growth, invasion, neovascularity and modulates the host immune system by releasing factors such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF- β), metalloproteinase (MMPs), histamine, interleukin-10 (IL-10) and tumor necrosis factor alpha (TNF- α) [49–52]. In the present study, large number of MC's were found in the liver section of HCC-bearing rat indicating the first phase of inflammatory process, whereas treatment with TXER reduced the MC accumulation in the hepatocytes exhibiting its anti-inflammatory efficacy against HCC.

Hepatic fibrosis occurs as a dysfunctional wound healing response with excessive deposition of extracellular matrix

components which results in enhanced accumulation of collagen in the liver during chronic liver injury [53]. In the normal liver, collagen forms only a small portion of the total proteins of the liver, whereas in cirrhosis or cancerous liver, it is deposited in large amount leading to the structural disruption of the tissue [54]. In the present study, HCC-bearing rat expressed a continuous and progressive accumulation of collagen in the tissue which could be due to increased rate of collagen synthesis. This also indicates the extent of fibrosis in the liver. TXER supplementation to HCC-bearing rats showed less collagen deposition in the liver which could be by inhibiting or suppressing the activity of prolyl hydroxylase which synthesizes hydroxyproline of collagen, thereby decreasing the rate of collagen synthesis. Our present result correlates with the previous findings [54].

TXER is known to suppress inflammation by (1) inhibiting oxidative stress and (2) suppressing the expression patterns of the inflammatory markers such as COX-2 and iNOS through functional alterations in the transcription factor NF- κ B [55, 56]. Our present study also correlates with the above findings as evidenced by the marked inhibition of fibrosis and inflammation on TXER supplementation.

NDEA being a well-known mutagen exhibits its hepatocarcinogenic potential by interacting with and damaging DNA. DNA damage leads to bulky chemical adducts of the purine and pyrimidine bases that distorts the DNA double-stranded helix and interrupts DNA replication and transcription [57]. Our present results show an enhanced formation of DNA ladders in NDEA alone treated rat, whereas treatment with TXER showed a protective effect against NDEA-induced DNA damage. This protective effect of TXER could be by masking the binding site of DNA from being occupied by NDEA or by the ability of TXER to scavenge the reactive intermediates that approach the binding site on DNA.

Elevated liver marker enzymes, oxidative stress and generation of ROS are the hallmarks of NASH. Notably, the alterations in the activities and expressions of phase I drug metabolizing enzymes (especially CYP2E1) has proved to have an impact in the progressive stages of NAFLD to NASH and finally to HCC [58]. Clinicopathological studies have specified the role of increased inflammation and cirrhosis in the development of NAFLD to HCC [59]. In our present study, the biochemical, histological and cellular characterization of NDEA alone induced group is in correlation with the above findings, whereas interestingly our observation focused on the TXER-treated groups revealed that TXER effectively suppressed the progression of NAFLD to NASH and HCC by (1) enhancing the antioxidant defense mechanism (2) inhibiting lipid peroxidation and ROS generation (3) lowering the levels of CYP family enzymes (iv) suppressing cell proliferation and inflammation (v) reducing DNA damage, fibrosis and hepatic nodule formation.

The present study clearly concludes that the lowest dose 12.5 mg/kg b.w TXER was not observed to be much effective, which may be due to its insufficient concentrations to counter the adverse effects of NDEA. Even though 50 mg/kg b.w of TXER (the highest dose) showed beneficial effects, we conclude 25 mg/kg b.w TXER as the effective optimum dose for HCC. Further investigations are currently being done with this effective dose (25 mg/kg b.w), to unravel the mechanism of action of TXER particularly by targeting the specific signaling pathways and also by using different treatment regimens.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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