# Dietary administration of Nexrutine inhibits rat liver tumorigenesis and induces apoptotic cell death in human hepatocellular carcinoma cells

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## A B S T R A C T

Epidemiological studies suggested that plant-based dietary supplements can reduce the risk of liver cancer. Nexrutine (NX), an herbal extract from *Phellodendronamurense*, has been shown to have anti-inflammatory, anti-microbial and anti-tumor activities. In the present study, we have shown the anti-tumor potential of NX against Solt-Farber model with elimination of PH, rat liver tumor induced by diethylnitrosoamine (DEN) as carcinogen and 2-acetylaminofluorene (2-AAF) as co-carcinogen. The elucidation of mechanistic pathways was explored in human liver cancer cells. Dietary intake of NX significantly decreased the cell proliferation and inflammation, as well as increased apoptosis in the liver sections of DEN/2-AAF-treated rats. Moreover, NX (2.5–10 μg/ml) exposure significantly decreased the viability of liver cancer cells and modulated the levels of Bax and Bcl-2 proteins levels. NX treatment resulted in increased cytochrome-*c* release and cleavage of caspases 3 and 9. In addition, NX decreased the expression of CDK2, CDK4 and associated cyclins E1 and D1, while up-regulated the expression of p21, p27 and p53 expression. NX also enhanced phosphorylation of the mitogen-activated protein kinases (MAPKs) ERK1/2, p38 and JNK1/2. Collectively, these findings suggested that NX-mediated protection against DEN/2-AAF-induced liver tumorigenesis involves decrease in cell proliferation and enhancement in apoptotic cell death of liver cancer cells.

## 1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world. The incidence of HCC is dramatically increasing and is the third-leading cause of cancer death in worldwide (Ferlay et al. [50]). The major risk factors for hepatic cancer include chronic infection with hepatitis B and C (accounting for 54% and 31% of cases worldwide respectively), the consumption of food grains contaminated with mycotoxins (produced by fungi during storage in tropical or sub-tropical climatic countries) and last, but not the least, heavy alcohol consumption [1–3]. Hepatocarcinogenesis involves initial genotoxic insult (initiation), clonal expansion from premalignant to malignant lesions (promotion) and finally tumor progression by means of further clonal expansion [4]. To date, surgery remains the best choice of treatment that could prolong HCC patients' survival. However, poor prognosis at times after surgery along with side effects of various chemotherapeutic drugs are also being seen as causes of relapse [5].

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In addition to surgery, chemoprevention is another key approach to control HCC, where one or more nontoxic, naturally occurring or synthetic agents are administered to prevent, improve or reverse the occurrence of disease substantially. Thus, chemopreventive intervention may serve as a feasible alternative strategy for prevention of liver tumorigenesis.

In recent years, considerable efforts have been made to search naturally occurring substances for the intervention of carcinogenesis [6,7]. Nexrutine® (NX), a commercially available herbal extract from *Phellodendronamurense*, widely used for the treatment of inflammation, gastroenteritis, abdominal pain and diarrhea, has shown to exhibit minimal toxicity to normal tissues [8]. Active components of NX are isoquinoline alkaloids, phenolic compounds and flavone glycosides. A recent study revealed that NX inhibited the proliferation of prostate and lung cancer cells through the modulation of Akt and CREB-mediated signaling pathways, and that its anti-proliferative effects are comparable to that of berberine, a well-known chemopreventive agent [9–11]. Other findings also established NX to be effective against early-stage prostate tumor development as well as tumor progression in the transgenic adenocarcinoma of mouse prostate (TRAMP) model [8,12]. In addition, recently our group showed that NX inhibited the promotion of skin tumorigenesis in the two-stage mouse skin tumorigenesis model [13]. Although NX has proven to be a potent anti-cancer agent for prostate, skin and lung cancer, no study so far has reported the anti-tumor effects of NX on liver cancer.

Therefore, in this study, anti-inflammatory and anti-tumor promoting potential of NX was demonstrated in partially modified Solt-Farber rat liver tumorigenesis model. We found NX's anti-tumor mechanisms involved inhibition of cell proliferation and induction of apoptosis, mediated by modulation of p53 and cyclin-dependent kinase (CDK) inhibitor levels, alternations in mitogen-activated protein kinases (MAPKs) signaling and activation of caspases 3 and 9.

## 2. Materials and methods

### 2.1. Preparation of NX

Nexrutine® (NX) was obtained from Next Pharmaceuticals (Irvine, CA). Stock solution of NX was prepared by dissolving NX in dimethylsulfoxide (DMSO) at a concentration of 1.0 mg/ml. The stock solution was further diluted either in milli Q water or culture medium to obtain various working concentrations.

### 2.2. Antibodies and chemicals

Antibodies specific for cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were procured from Cayman Chemical Company (Ann Arbor, MI). Antibodies specific for ERK1/2, p38, JNK, CDK2, CDK4, p27, p53, p21, cytochrome *c*, cyclin E1, cyclin D1 and β-Actin-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), while p-ERK1/2, p-p38, p-JNK, Bax, Bcl-2, cleaved-caspase 3, cleaved-caspase 9, and proliferating

cell nuclear antigen (PCNA) were purchased from cell signaling (Beverly, MA). 2-Acetylaminofluorene (2-AAF), 2-β mercaptoethanol (BME), bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), diethylnitrosamine (DEN), dithiothreitol (DTT), Dulbecco's Modified Eagle's Medium (DMEM), Fetal bovine serum (FBS), streptomycin, penicillin, ethylenediaminetetraacetic acid (EDTA) disodium salt, trypsin/EDTA solution, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), phenylmethyl-sulphonyl fluoride (PMSF), propidium iodide (PI), RNase A, protease inhibitor cocktail set-I, Tris buffer, Triton X-100 and Tween-20 were from Sigma Chemicals Co. (St. Louis, MO). All other chemicals and reagents used were of highest purity commercially available.

### 2.3. Animals

Four to six week old male Wistar rats (160–180 g), were obtained from the animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, acclimatized under standard laboratory conditions and given a commercial pellet diet (Provimi Animal Nutrition India Pvt Limited, India) and water ad libitum. Animals were housed in plastic cages on rice husk as bedding and maintained in controlled atmosphere of 12 h dark/light cycle, $22 \pm 2\,^\circ$C temperature and 50–60% humidity as per rules laid down by Animal Welfare Committee of CSIR-IITR, Lucknow. All the experiments involving animals were approved by the Institutional Animal Ethics Committee (IAEC), CSIR-IITR, Lucknow. Animals were sacrificed by cervical dislocation with minimal suffering as per CSIR-IITR guidelines.

### 2.4. Animal experimental protocol

To study the protective effect of NX slightly modified experimental schedule of Solt and Farber liver tumorigenesis protocol was followed [14,15]. This modified experimental protocol eliminates partial hepatectomy (PH). Because, PH is desirable to increase the sensitivity with weak agents and PH requires extensive surgical procedure that causes a lot of pain and mortality of animals. In the classical Solt-Farber model, along with 2-AAF, PH was done for vigorous liver cell proliferation and in this protocol growth can be grossly visible within a period of 1 week. While literature suggest that alone 2-AAF is sufficient to induce tumorigenesis in rats by stimulation of cell proliferation [16]. Therefore, in the present study, we have used a combination of DEN + 2-AAF to develop hepatotumorigenesis in Wistar rats. Here, thirty male Wistar rats were randomly allocated into five groups of six rat each. Animals of Group I received only saline intraperitoneally and kept on normal basal diet. Group II animals were initiated by single intraperitoneal injection of 200 mg/kg body weight of DEN in saline followed by 2-AAF (0.02% (w/w) in diet from day 14 until 8 weeks after initiation). Groups III and IV were served as prevention groups, where in addition to carcinogen treatment as in Group II, animals received dietary administration of NX at doses of 300 and 600 ppm respectively, along with 2-AAF. Group V served as a negative control and received only NX treatments in the diet

for 8 weeks. Eight weeks after initiation period, animals in all the groups were observed for any apparent signs of toxicity as well as mortality, were fasted overnight and sacrified. Livers were excised, part of which was used for whole cell lysate preparation and part fixed in 10% formalin for histopathologycal and immunohistopathological analysis.

### 2.5. Histopathological evaluation

The formalin-fixed tissue samples were processed conventionally to prepare paraffin blocks followed by tissue sectioning at 5 μm and hematoxylin-eosin staining. Stained slides were observed under light microscope of Leica (Heerbrugg, Switzerland) and photographed.

### 2.6. Immunohistochemical analysis

Immunohistochemical analysis of COX-2, iNOS and PCNA were performed in liver sections using Super Sensitive Polymer-HRP Detection System from BioGenex (San Ramon, CA) as per the manufacturer's instructions.

### 2.7. In situ cell death detection using TUNEL assay

In situ apoptosis analysis was performed in the paraffin-embedded liver sections by the TUNEL method using in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to manufacturer's protocol.

### 2.8. Cell culture

The liver cancer cell line (HepG2) cells, were obtained from National Centre for Cell Science, Pune, India. Cells were cultured in DMEM supplemented with heat inactivated FBS (10%), penicillin (100 U/ml) and streptomycin (100 U/ml) at 37 °C in humid air containing 5% $CO_2$.

### 2.9. Cell growth and viability assay

The liver cancer cells were plated at 5000 cells/cm$^2$ in 48-well plate as described above. At 60–70% confluency, the cells were fed with fresh medium and treated either with DMSO alone or different concentrations (1.0, 2.5, 5.0, 10.0 and 25 μg/ml) of NX in DMSO for 24 and 48 h. Viability of the HepG2 cells were determined by MTT assay as described previously [17]. The effect of NX on cell viability is presented as the relative cell viability compared with vehicle-treated control cells, which were arbitrarily assigned 100% viability.

### 2.10. Analysis of apoptotic cell death and cell cycle distribution by flow cytometer

Liver cancer cells (60–70% confluent) were seeded in 6-well cell culture plate at a concentration of $5 \times 10^5$ cells/ml and treated with NX at concentrations of 2.5, 5.0 and 10.0 μg/ml for 48 h, and both adherent and floating cells were collected, washed twice with ice-cold phosphate-buffered saline and $5.0 \times 10^5$ cells were used for apoptosis analysis using Annexin V: FITC Apoptosis Detection Kit (BD Pharmingen, San Jose, CA) as per the manufacturer's

instructions using FACS Canto$^{TM}$ II (Becton Dickinson, Franklin Lakes, NJ) flow cytometer. For cell cycle analysis, $5.0 \times 10^5$ cells were fixed in 70% ethanol for 1 h at $-20$ °C and subsequently incubated with PI (20 μg/ml) and RNase A (200 μg/ml) for another 30 min at 37 °C and a minimum of 10,000 events per sample were acquired in flow cytometer and DNA histograms were analyzed by FACS Diva software (Becton Dickinson, Franklin Lakes, NJ).

### 2.11. Protein extraction and western blot analysis

In another set of experiment, liver cancer cells (60–70% confluent) were treated with either DMSO or NX (0, 2.5, 5.0 and 10.0 μg/ml) and after 48 h, cells were harvested, washed with cold phosphate-buffered saline, and lysed with ice-cold RIPA (Radio-immunoprecipitation Assay) buffer supplemented with protease inhibitors. Proteins (50 μg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and incubated with specific primary antibodies at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody (Sigma, St. Louis, MO). Bound antibody was detected by enhanced chemiluminescence using Luminata Forte Western HRP substrate following the manufacturer's instructions (Millipore, Billerica, MA). All the blots were stripped and reprobed for either total of respective protein or β-actin to ensure equal loading of protein.

### 2.12. Statistical analysis

The results were expressed as the mean ± S.E. The statistical significance of difference between the values of control and treatment groups was determined using two-tailed Student's t test. A p value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. General observation

During the entire period of our study no difference in food or water consumption was observed among the various groups of animals. All the animals had a steady body weight during the treatment. The administration of DEN/2-AAF alone or along with NX (300 or 600 ppm) did not affect the growth of the rats measured at weekly interval.

### 3.2. Effect of Nexrutine in DEN/2-AAF-induced histopathological changes

Rats treated with DEN/2-AAF showed abnormal hepatocyte shape (Fig. 1B). These cells were small with large hyperchromatic nuclei compared to liver cells from control rats (Fig. 1A) and showed cytoplasmic granulation and intracytoplasmic violet-colored material. Treatment of animals with 300 pm NX along with DEN/2-AAF showed slightly enhanced hepatocellular architecture (Fig. 1C), while the liver architecture of rats those that received 600 ppm NX (Fig. 1D) were comparable to that of the
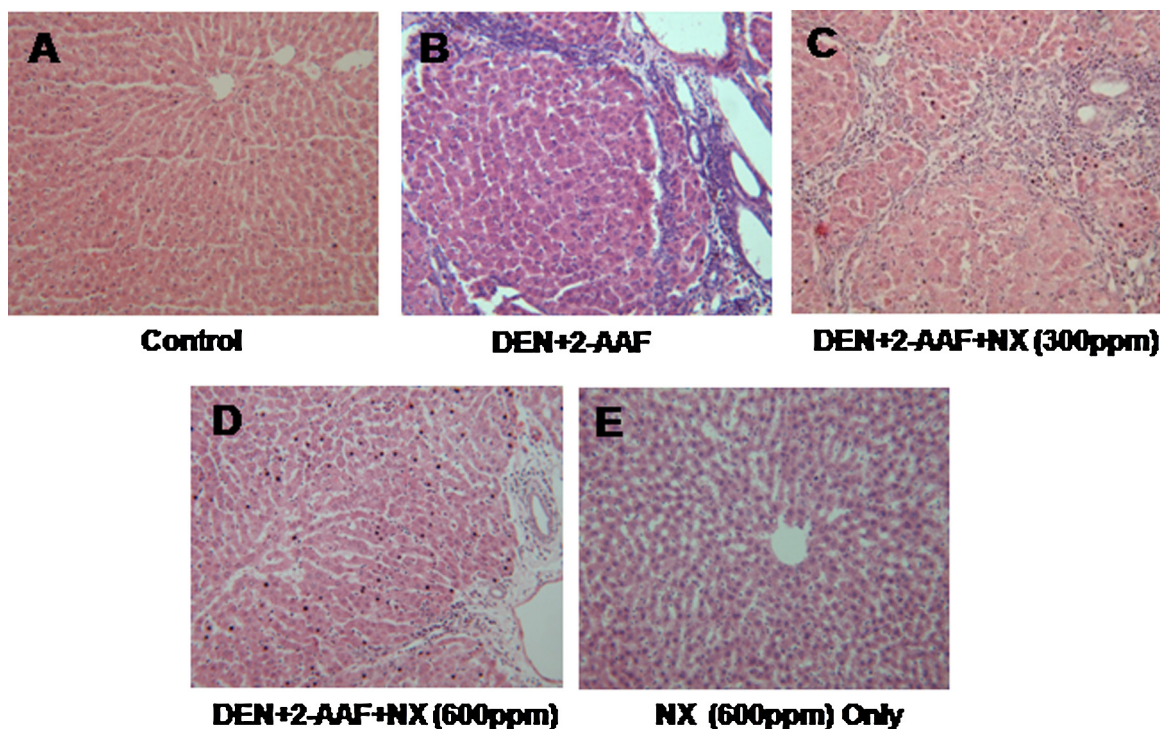
**Fig. 1.** Effect of Nexrutine on histopathology of DEN/2-AAF-treated rat liver. Control group rat liver (A) showed normal cellular architecture while DEN/2-AAF-treated rat liver (B) showed areas with cytoplasmic granulation, enlarged hyperchromatic nuclei and intracytoplasmic violaceous material. The group treated with DEN/2-AAF along with NX (300 ppm and 600 ppm; C and D, respectively) showed marginal to moderate improvement of hepatic histology over the DEN/2-AAF-treated livers. NX (600 ppm)-treated rat liver section (E) shows near-normal architecture.

normal rat (Fig. 1A). The size of the nuclei of mononuclear cells in the liver of NX-treated group was essentially uniform and fewer binucleated cells were seen in these rats compared to the DEN/2-AAF treated group (Fig. 1B).

### 3.3. Inhibitory effect of Nexrutine on DEN/2-AAF-induced COX-2 and iNOS protein expression

COX-2 and iNOS are well-established molecular biomarkers of inflammation and tumor promotion and thus could be promising molecular targets for designing of drugs targeting cancer prevention as well as therapy [18,19]. In the present study, we observed that both COX-2 and iNOS protein expression were elevated in DEN/2-AAF-treated rat liver (Figs 2B and 3B) respectively. Interestingly, dietary exposure of NX (300 and 600 ppm) resulted in substantial decrease in COX-2 and iNOS expression in DEN/2-AAF-treated rat liver (Figs 2C–D and 3C–D) respectively. These results suggest that NX suppresses DEN/2-AAF-induced inflammation by down regulating COX-2 and iNOS expression in the rat liver.

### 3.4. Inhibitory effect of dietary Nexrutine on PCNA labeling index

PCNA is an auxiliary protein of DNA polymerase-delta and higher level of its expression is correlated with cell proliferation, suggesting PCNA is an excellent marker of

cellular proliferation [20]. In our study, the PCNA antigen was not expressed in liver sections of control rats (Fig. 4A). However, liver sections from DEN/2-AAF-treated rats were positive for the PCNA staining, indicative of active cell proliferation in liver tissue (Fig. 4B). We observed lower PCNA expression (Fig. 4C–D) in the treatment groups of NX with DEN/2-AAF suggesting NX has an anti-proliferative effect on DEN/2-AAF-induced liver tumorigenesis in rats.

### 3.5. Nexrutine induced apoptosis in liver tissue treated with DEN/2-AAF animals

An apoptotic response of NX in the liver tissue of DEN/2-AAF-induced rats was investigated using TUNEL staining. Representative photographs for TUNEL-positive cells in DEN/2-AAF-treated alone or NX with DEN/2-AAF-treated animals are shown in Fig. 5. There was an increase in the number of TUNEL positive cells in the livers of NX +DEN/2-AAF treated rats (Fig. 5C–D) compared to DEN/2-AAF-treated rats (Fig. 5B). However, the apoptotic induction by NX was more pronounced in the group where 600 ppm of NX was given along with DEN/2-AAF (Fig. 5D).

### 3.6. Nexrutine treatment resulted inhibition in cell growth of liver cancer cells

The inhibitory effect of NX (0.5–20.0 μg/ml) on the growth of liver cancer cells was assessed by MTT assay and
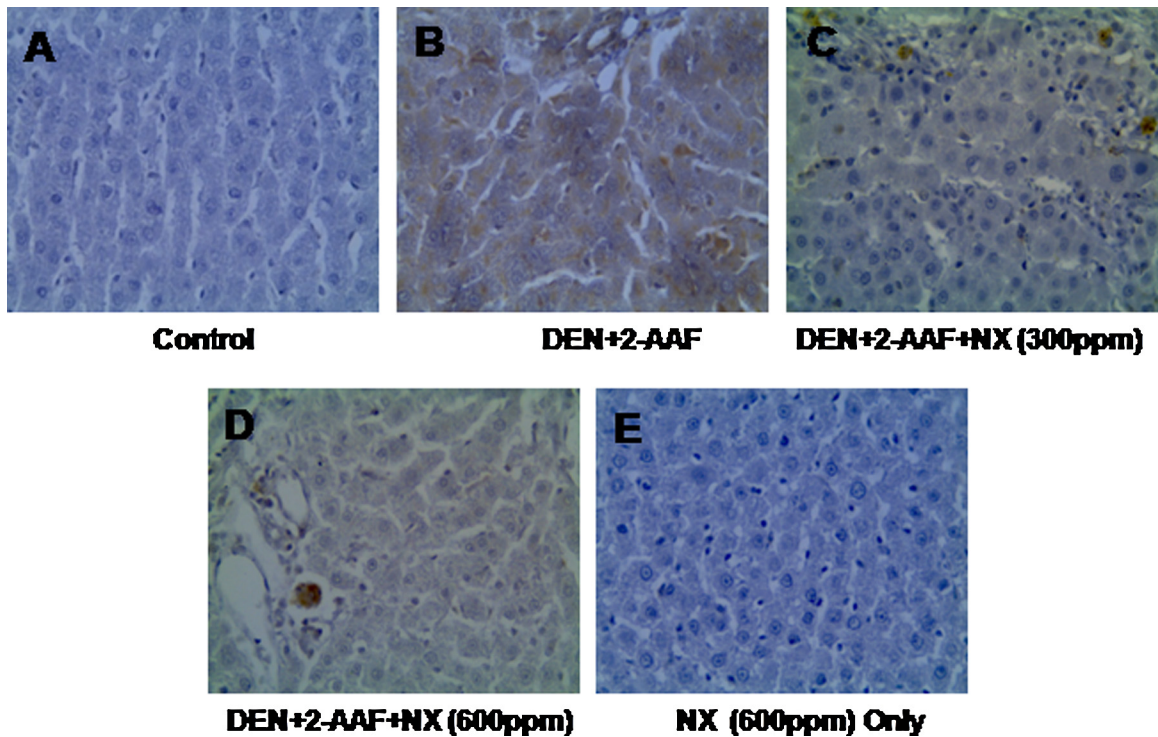
**Fig. 2.** Effect of Nexrutine on DEN/2-AAF-induced COX-2 expression in rat liver. Livers of DEN/2-AAF-treated rats (B) showed overexpression of COX-2 compared to control livers (A), while that of rats treated with DEN/2-AAF along with 300 ppm (C) and 600 ppm (D) NX showed marginal and significant reduction in COX-2 overexpression, respectively, compared to DEN/2-AAF treatment alone. NX (600 ppm)-treated rat liver section (E) shows no expression of COX-2.
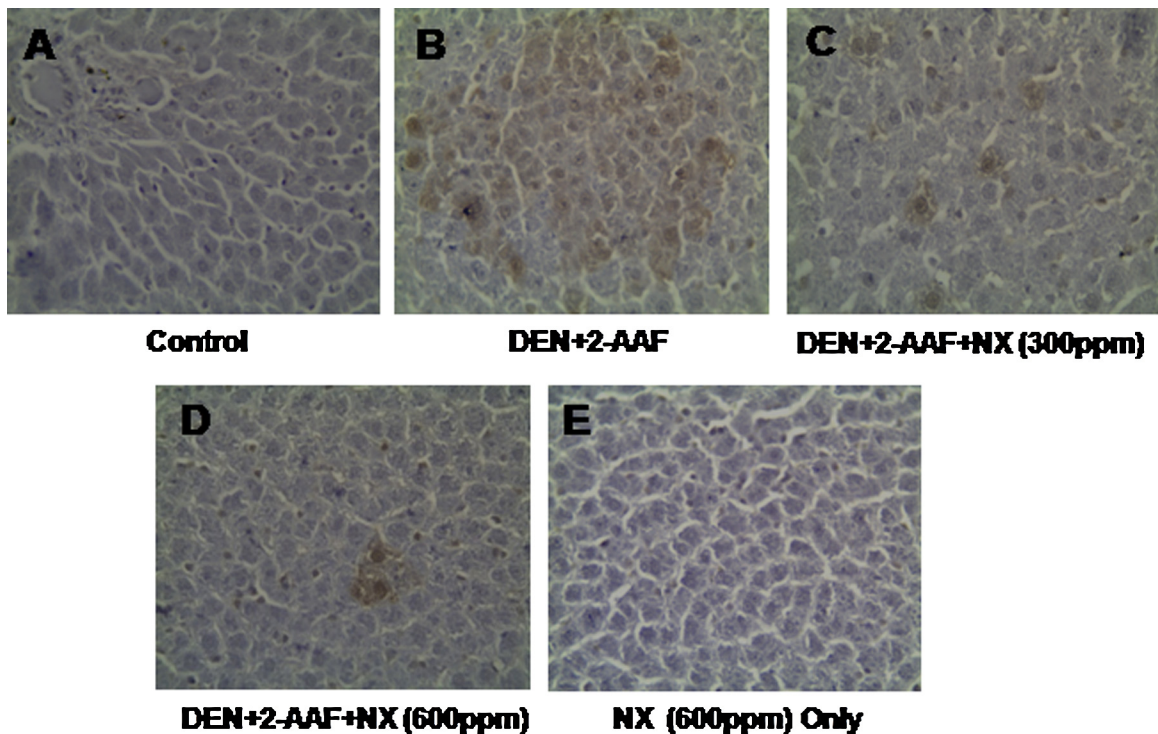


**Fig. 3.** Effect of Nexrutine on DEN/2-AAF-induced iNOS expression in rat liver. Livers of DEN/2-AAF-treated rats (B) showed overexpression of iNOS compared to control livers (A). Treatment groups which were administered NX (300 ppm and 600 ppm) along with DEN/2-AAF (C and D, respectively) showed reduction in iNOS overexpression compared to DEN/2-AAF alone.
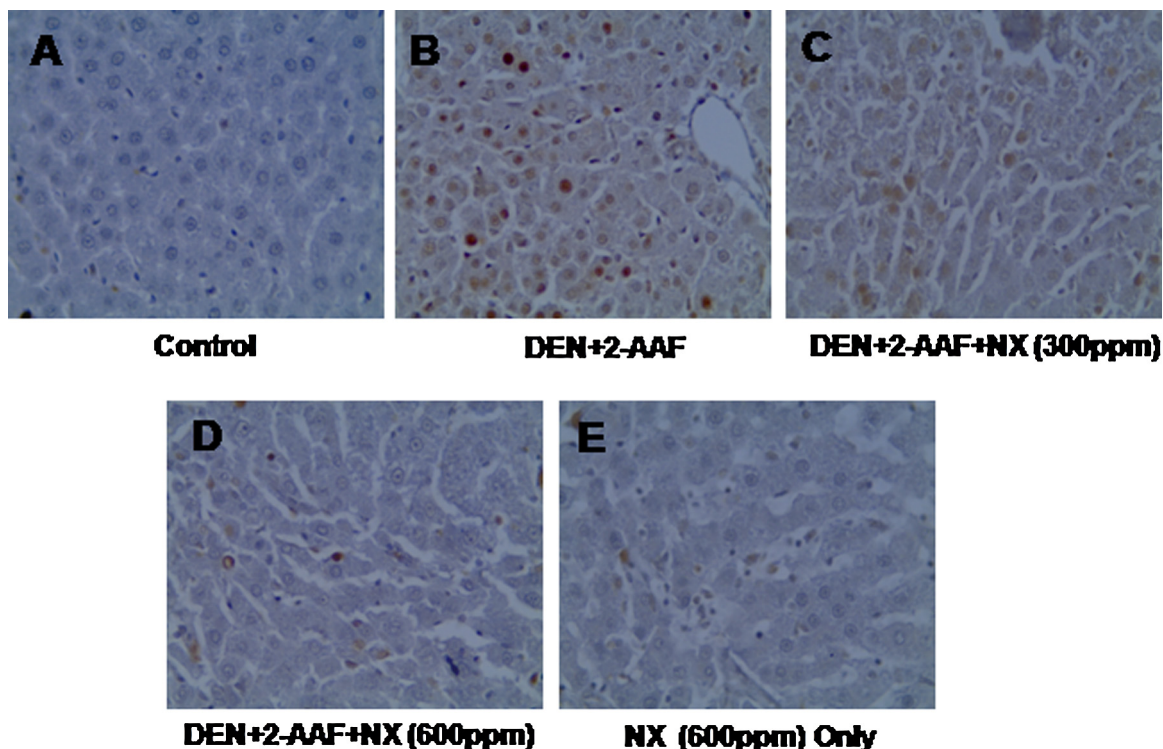
**Fig. 4.** Effect of Nexrutine on DEN/2-AAF-induced PCNA expression in rat liver. Livers of DEN/2-AAF-treated rats (B) showed overexpression of PCNA compared to control livers (A). Rats treated with DEN/2-AAF along with 300 ppm NX (C) showed marginal reduction in PCNA overexpression, while addition of 600 ppm NX (D) showed significant reduction in PCNA overexpression in the liver compared to DEN/2-AAF treatment alone. NX (600 ppm)-treatment alone induced no expression of PCNA in the liver (E).

is shown in Fig. 6A. Treatment with NX (0.5–20.0 μg/ml) for 24 h decreased the cell viability by 12–66%; while, at 48 h, the decrease in cell viability was even more pronounced (16–88%). Based on these findings, we selected NX doses of 2.5, 5.0 and 10.0 μg/ml and 48 h time point for further studies.

### 3.7. Nexrutine treatment induced apoptosis and cell cycle arrest in liver cancer cells

In view of above mentioned growth inhibitory effect, we were interested in determining whether NX also induces apoptosis in liver cancer cells. It was observed that treatment of liver cancer cells for 48 h with 2.5–10.0 μg/ml NX increases the number of apoptotic cells from 3.7 to 16.0%. The total percent of apoptotic cells was directly related to NX concentration increasing from 3.7% (control) to 16.0% (10 μg/ml), indicating that NX-induced apoptosis of liver cancer cell is dose-dependent (Fig. 6C). As the induction of apoptosis might also be mediated through the regulation of the cell cycle, we also examined the effect of NX treatment on cell cycle perturbations compared with the vehicle alone treatment. As shown in Fig. 6B, exposure of NX (2.5–10.0 μg/ml) to liver cancer cells for 48 h resulted in significant increase in the proportion of cells in the

G0/G1 phase (57–73%) with a concomitant decrease in the S (10–19%) and G2/M (17–24%) phases.

### 3.8. Nexrutine treatment results in activation of intrinsic pathway of apoptosis in liver cancer cells

Bax and Bcl-2 proteins play a central regulatory role in apoptotic cell death. Therefore, the expression levels of Bax and Bcl-2 following NX treatment were measured by western blot analyses. As shown in Fig. 7A, NX treatment (2.5–10.0 μg/ml) resulted a dose-dependent increase in the expression level of Bax and decrease in the expression level of Bcl-2. To further confirm whether modulation of Bax/Bcl-2 ratio is correlated with the release of cytochrome $c$ in cytosol, the levels of cytochrome $c$ in the cytosolic fraction were measured. We found the levels of cytochrome $c$ were significantly elevated in a dose-dependent manner following NX treatment as shown in Fig. 7A. It is well documented that the apoptotic process is executed by cysteinyl aspartate-specific proteases known as caspases, which demolish the cell in an orderly fashion by cleaving a large number of cellular protein substrates [21]. Therefore, activation of caspases 3 and 9 was assessed after NX treatment by western blot analyses. Results indicated that NX treatment resulted in increased levels of cleaved-caspases
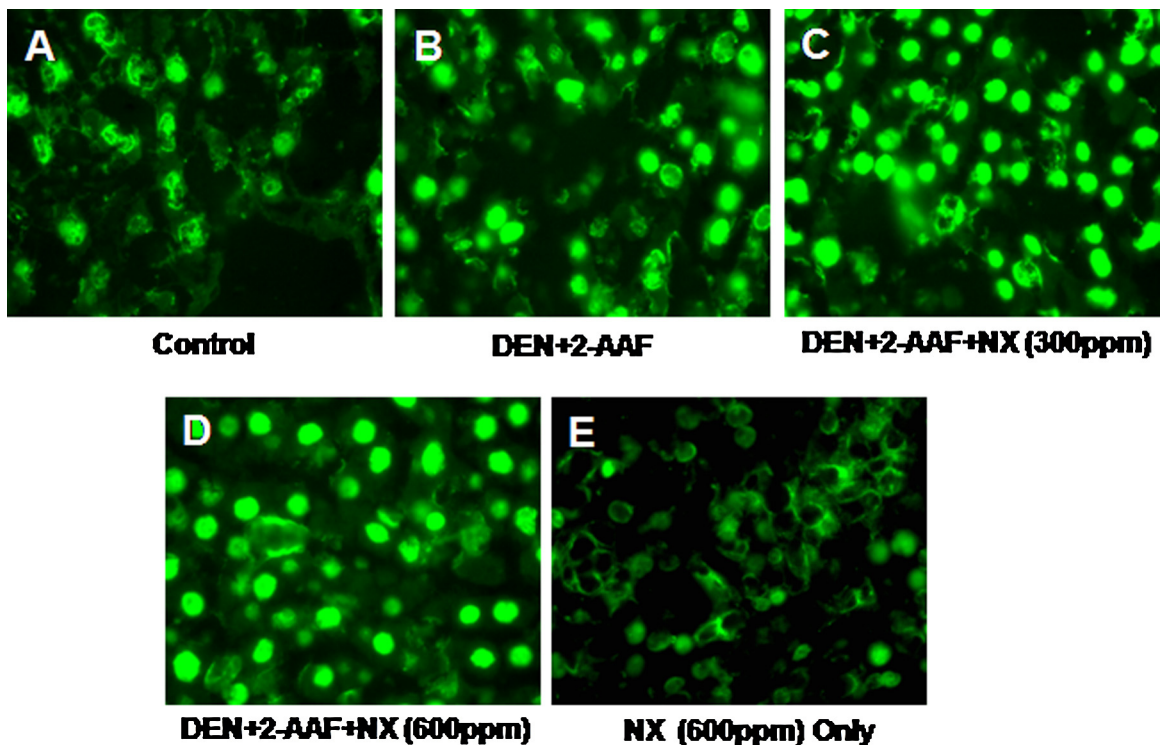
**Fig. 5.** Effect of Nexrutine on apoptosis in liver tissue of DEN/2-AAF animals. An apoptotic response to NX in the liver tissue of DEN/2-AAF-induced rats was investigated using TUNEL staining. An increase in the number of TUNEL-positive cells was seen in the group that was treated with NX + DEN/2-AAF (C and D) compared to DEN/2-AAF-treated rats (B). However, the apoptotic induction by NX was more pronounced in group where the rats are dosed with 600 ppm of NX along with DEN/2-AAF (D).

3 and 9 in a dose-dependent manner, while there was no change in expression level of caspase 8 (Fig. 7A).

### 3.9. Nexrutine modulate cell cycle regulators and phosphorylation of MAPKs

Altered expression of cell cycle regulatory protein such as CDKs and cyclins has been implicated in tumorigenesis [22,23]. As our results demonstrated inhibition of cell proliferation upon NX treatment, we further examined it's effect on the expression of cell regulatory proteins. As shown in Fig. 7B, NX exposure caused a decrease in cyclinE, cyclinD1, CDK2 and CKD4 levels in liver cancer cells. During cell cycle analysis we found that NX treatment caused G1 phase cell cycle arrest. We also found from immunoblot analysis that NX treatment caused significant induction of p21$^{WAF}$, a key regulator of G1-S phase transition, in a dose-dependent manner (Fig. 7B). Kip1/p27 is another important CDK inhibitor that regulates Cdk-cyclin activity at G1-S transition [24]. Protein levels of Kip1/p27 were also strongly upregulated after NX exposure. In addition, we found that NX treatment to liver cancer cells caused a dose-dependent increase expression of p53 (Fig. 7B). Further, we investigated the level of activated (phosphorylated) and total ERK1/2, JNK and p38 kinases in NX-treated HepG2 cells and found phosphorylation of ERK1/2, JNK, and p38 kinase levels were downregulated by NX without any change in their total protein levels (Fig. 7C)

### 4. Discussion

The present study we have shown that NX inhibited 2-AAF-mediated liver tumor promotion in DEN-initiated rats, which was correlated with a decrease in proliferation index together with inhibition of COX-2, iNOS and PCNA expression. Besides its anti-tumor promoting activity, we also observed that NX causes apoptotic cell death to human liver cancer cells.

Cancer development is a sequential event which often involves chronic inflammation and hyperplasia. COX-2 is well known biomarker of cell proliferation and tumor promotion as it catalyzes the formation of prostaglandin E2, which is reported to be involved in cell proliferation, inflammation and angiogenesis [25]. Similarly, another major mediator in chronic inflammatory processes is nitric oxide (NO•), which is produced by liver parenchymal and non-parenchymal cells from L-arginine via nitric oxide synthase (NOS). NO• is considered to exert a hepatoprotective action against tissue injury and cytotoxic effects due to invading microorganisms, parasites and tumor cells. However, many situations that cause uncontrolled, prolonged and/or massive production of NO• by inducible NOS (iNOS) may result in liver damage, leading to inflammation and even tumor development [26]. iNOS produces much larger amounts of NO• and has been detected in many human tumors, such as breast cancer, melanoma, bladder cancer, and colorectal cancer [27–30]. A considerable amount of compelling evidence suggests that the inhibition of iNOS
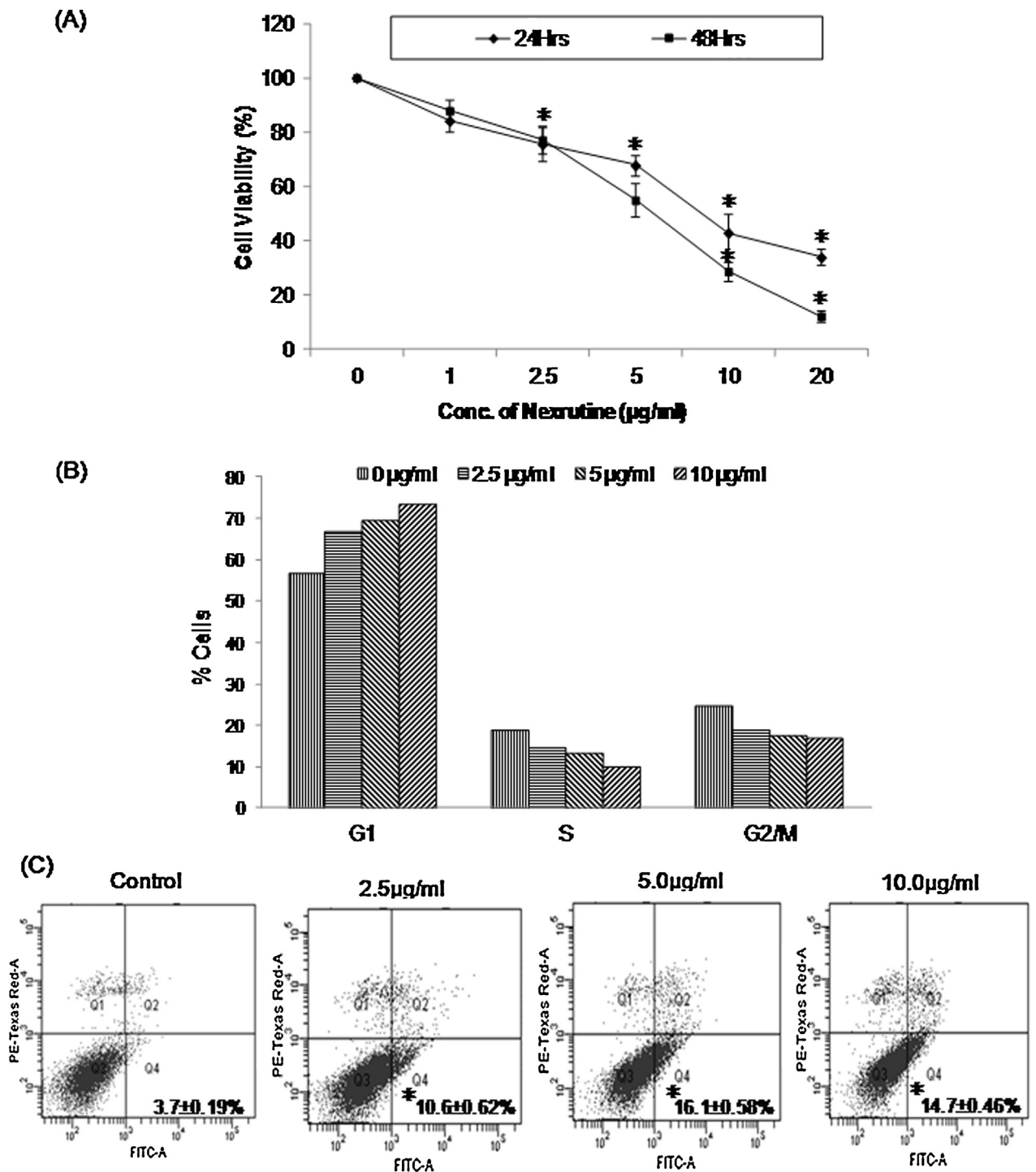
**Fig. 6.** Effect of Nexrutine on cell growth, cell cycle distribution and apoptosis. (A) Effect of NX on liver cancer cells (HepG2) growth. Liver cancer cells were treated with NX (0.5–20.0 μg/ml) for 24 and 48 h and the viability of cells was determined by the MTT assay. The data are shown as the relative cell viability compared to vehicle-treated control cells and represent the means ± S.E. of three experiments, in which each treatment was performed in multiple wells. (*$p < 0.05$ versus control). (B) Effect of NX on cell cycle phase distribution in liver cancer cells. The cells were treated with NX (2.5–10.0 μg/ml) for 48 h and harvested, stained with propidium iodide solution and data were acquired by flow cytometry as described in text. The data are shown as percentage of cells in each phase and represent the mean ± S.E. of three experiments in which each treatment was performed in multiple flasks. (C) Effect of Nexrutine on induction of apoptosis in liver cancer cells as assessed by Annexin-V FITC staining. The cells were treated with NX (2.5–10.0 μg/ml) for 48 h and collected, stained with Annexin-V FITC (FITC-A; x-axis) and propidium iodide (PE-Texas Red; y-axis). The data were acquired by flow cytometry as described in text and are shown as percentage of cells in Q2 quadrant (positive for both annexin V and PI) and tabulated as the mean ± S.E. of three experiments, in which each treatment was performed in multiple flasks (*$p < 0.05$ versus control).
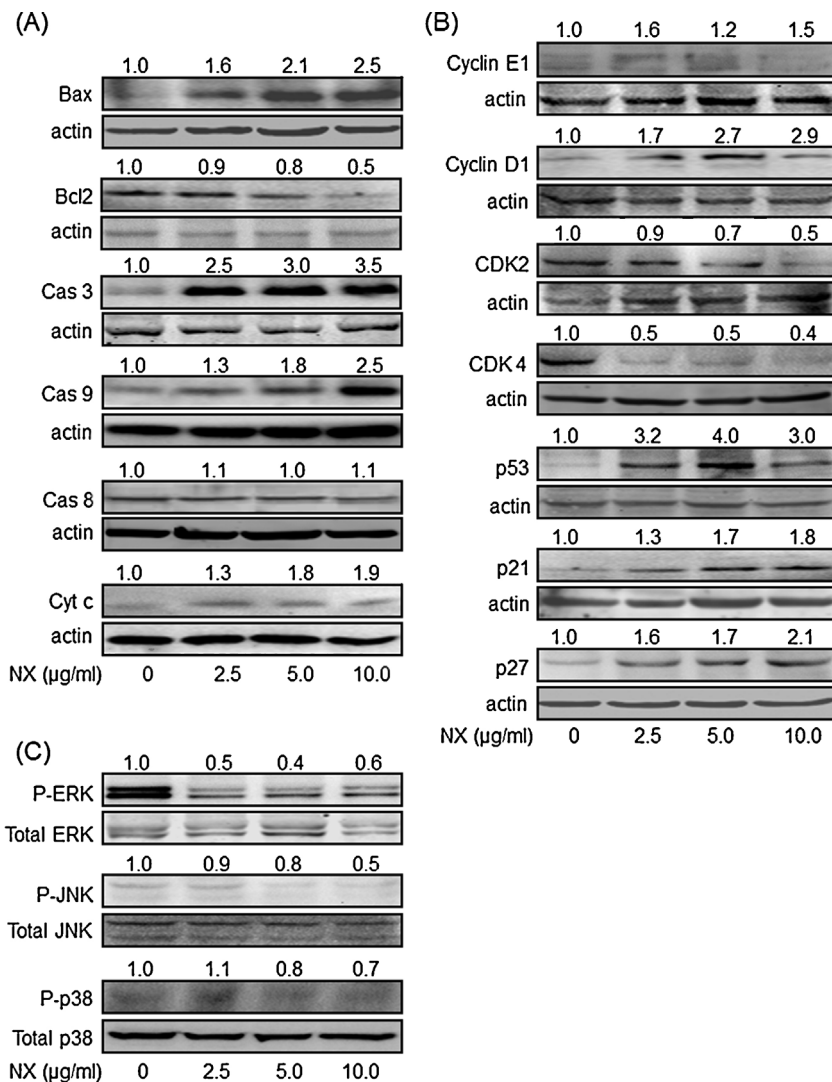
**Fig. 7.** Effect of Nexrutine on proteins involved in apoptosis, cell cycle regulators and MAPK activation cascades in liver cancer (HepG2) cells. As detailed in text, the cells were treated with NX (2.5–10.0 μg/ml) for 48 h and then harvested. Total cell lysates were prepared and 50 μg protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by western blot analyses. The membranes were probed for Bax, Bcl-2, caspases 3, 8, 9 and cytochrome c, cell cycle regulators and MAPKs, employing specific antibodies detailed in Section 2 followed by peroxidase-conjugated appropriate secondary antibody. Proteins were visualized with enhanced chemiluminescence detection system. Equal loading of protein was confirmed by stripping the blot and reprobing it for β-actin or for total MAPK expression. The values above the figures represent relative density in the term of fold change as compared to control after normalization with total protein or β-actin.

and COX-2 expression or activity is important not only for treatment of chronic inflammation, but also for the prevention of cancer [13,31,32]. Therefore, suppression of iNOS and COX-2 induction during cancer progression is recognized as an important and commonly accepted approach to effectively inhibit tumor promotion. These biomarkers were highly expressed in liver of DEN/2-AAF-treated animals. Treatment with NX remarkably suppressed COX-2 and iNOS in DEN/2-AAF-induced animals, suggesting a plausible anti-tumor promotion role of NX in vivo. These results agree with earlier studies that have been shown NX to inhibit prostate, lung and skin cancer cell proliferation by modulation of COX-2 and iNOS inhibition [8,12,13].

PCNA, is a 36 kDa nuclear protein and its expression in the nucleus is associated with the DNA synthesis phase of cell cycle, and serves as a biomarker of proliferation [20]. Earlier studies have reported that PCNA is highly associated with DEN/2-AAF-induced liver carcinogenesis, which could be detected immunohistochemically [33]. In our study, we found that NX reduced the hepatic PCNA expression in DEN/2-AAF treated rats.

Cell cycle regulation is one important mechanism of anti-proliferation in cancers [34]. In the present study, we investigated the cell cycle distribution after treatment with NX and found accumulation of liver cancer cells at G1 phase of cell cycle. Similarly, earlier reports with skin and prostate cancer cells showed NX treatment arrested cell

cycle progression at the G0/G1 phase [13]. Studies have also suggested that regulation of cyclin activity plays a key role in cell cycle progression at different phases, in which CDKs are negatively regulated by a group of functionally related proteins known as CDK inhibitors [24]. Cip/p21 binds and inhibits the cyclins E1, D1 and Adependent kinases, regulating the G1 to S phase transition of the cell cycle. Cip1/p21 is also known to influence the outcome of the p53 response to DNA damage and plays a protective role in survival signal against apoptosis. Kip1/p27 is up-regulated in response to anti-proliferative signals [35,36]. In accordance with these observations, our study also revealed an up-regulation of Kip1/p27 and Cip1/p21, and a decrease in the levels of CDK2, CDK4, cyclins E1 and D1 proteins. These results provide a mechanism by which NX induces cell cycle arrest that results in a decrease in cell proliferation of liver cancer cells.

MAPKs are important upstream regulators of transcription factor activation and their signaling is critical to transduction of a wide variety of extracellular stimuli into intracellular cascades, thereby controlling the cellular events such as proliferation, differentiation and apoptosis [37]. Our results demonstrated that NX treatment blocked the phosphorylation, and hence, activation of MAPKs, including ERK1/2 p38, and JNK in liver cancer cells. These findings are similar to previous studies where inhibition of ERK1/2, p38 and JNK by chemopreventive agents are capable of preventing skin carcinogenesis [38,39].

Apoptotic cell death represents a universal and exquisitely efficient suicidal pathway and an ideal way for elimination of unwanted cells; however, cancerous cells show dysregulation of this mechanism, which makes the cells virtually immortal and resistant to stress stimuli as well as therapeutic agents [40]. Therefore, the apoptotic pathway is widely studied as a potential target for cancer chemotherapy [41,42]. In our study, NX treatment to liver cancer cells resulted in a dose-dependent apoptotic cell death, which would contribute to NX-mediated cell growth inhibition. In support these findings, prior studies have shown that various chemotherapeutic phytochemicals possess the ability to induce apoptosis in cancer cells by arresting the cell cycle progression in various phases of cell division [43–45]. Furthermore, NX treatment to liver cancer cells results in significant decrease in the levels of Bcl-2 protein along with an increase in the levels of Bax protein, thus enhancing the Bax/Bcl-2 ratio, which favors apoptosis. Increase in Bax/Bcl-2 ratio acts as a proapoptotic signal resulting in the release of cytochrome *c* protein from mitochondria to cytoplasm, activating the apoptosome, which further leads to auto-activation of caspase 9 and cleavage of pro-caspase 3 to its activated form caspase 3, the executioner caspase [46–48]. Caspases are the mediators of execution mechanism of apoptosis, and their activation results in the cleavage of PARP protein, a DNA repair enzyme in the cell, and subsequent DNA degradation and apoptotic death [21]. Since, caspase 8 was not found to be activated after NX treatment in liver cancer cells, it can be deduced that NX-induced apoptosis is mediated via activation of the intrinsic pathway.

In summary, this study demonstrated that NX inhibited DEN/2-AAF-induced enhanced cell proliferation in liver. In addition, it also caused dose-as well as time-dependent cytotoxicity in liver cancer (HepG2) cells. NX induced accumulation of liver cancer cells at the G1 phase of cell cycle as well as apoptosis. Taken together, these in vivo and in vitro studies provide strong evidence that NX could be useful in the management (chemoprevention as well as chemotherapy) of liver cancer.

## Conflict of interest

None.

## Transparency document

The Transparency document associated with this article can be found in the online version.

## Acknowledgements

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