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Neferine suppresses diethylnitrosamine-induced lung carcinogenesis in Wistar rats

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ABSTRACT

Neferine is a bisbenzylisoquinoline alkaloid isolated from the embryos of lotus which has attracted attention for its anti-inflammatory and anti-cancer activities. The aim of this study was to evaluate the anti-cancer effect of neferine against diethylnitrosamine (DEN)-induced lung carcinogenesis in Wistar rats and to explore the underlying molecular mechanism. DEN-induced oxidative stress is mediated by alterations in the levels of pulmonary reactive-oxygen species, lipid peroxidation, protein carbonyl content and antioxidant status. Thus, treatment with neferine restored cellular normalcy, highlighting the antioxidant potential of neferine in mitigating the oxidative stress-mediated damage produced during DEN-induced lung carcinogenesis. Histopathological analysis showed disorganized alveolar structure, thickened alveolar wall, infiltration of inflammatory cells in DEN-induced rats, the damage was significantly reduced upon neferine treatment. DEN-induced rats exhibited increased gene expression of NF-κB, COX-2, CYP2E1, VEGF, Bcl-2, PI3K/AKT/mTOR and significantly decreased the gene expression of p53, Bax, caspase-9 and caspase-3. Neferine treatment restored the DEN- induced alteration of these gene expression levels. Further, blotting analysis also revealed increased expression of NF-κB, COX-2, Bcl-2 and decreased expression of Bax, caspase-9 and caspase-3 proteins in DEN-induced rats. Neferine treatment restored the expression of these proteins in DEN- induced lung carcinogenesis.

1. Introduction

Humans are exposed to various exogenous and endogenous environmental pollutants, which include emissions from industrial source or vehicle exhaust and constitute various chemical compounds that can potentially act as carcinogens (Hennig et al., 2012). Cancer arises principally as a consequence of exposure of individuals to carcinogenic agents, from air, water or food, tobacco use, occupational exposure and biological factors (Silva et al., 2007). Recent studies by Tomasetti and Vogelstein, 2015 have shown that unavoidable random mutations, as non-inherited factors are responsible for two-thirds of the mutations in human cancers which they described as 'bad luck'. However, somatic mutations are generated by the influences of genetic polymorphisms in the genome which suggests the contributory role of genetic constitution in cancer development. Thus carcinogenesis is a complex process which involves the genetic as well as environmental factors. Previous studies also reported that diethylnitrosamine-induced lung tumor in mice was accompanied with mutagenesis of the K-ras protooncogene. It induces acquired mutations in ras (point mutations at codon 16) and β-Catenin in liver cancer, which causes genome instability and eventually results in the transformation of pre-neoplastic or neoplastic cells.

Carcinogenesis is a multi-step process induced by one or several genotoxic or carcinogenic agents and involves several sequential steps from the time of administration to the progression of visible pre-neoplastic lesions. Nitrosamines are considered as an important class of environmental carcinogens owing to their carcinogenic and mutagenic properties. The sources of human exposure to nitrosamines include agricultural chemicals, tobacco products, pharmaceutical preparations,

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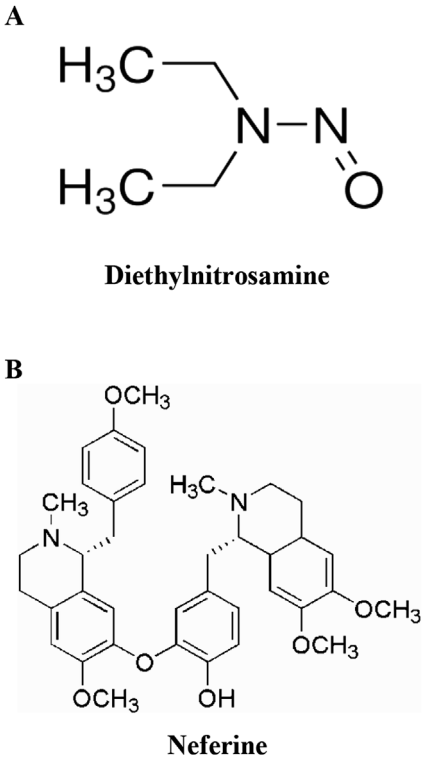

Fig. 1. Chemical structure of diethylnitrosamine and neferine.

cosmetics and food preservatives (Ciemniak, 2006). Nitrosamines are found in tobacco smoke at a concentration ranging from 1 to 2 ng/cigarette and in baby bottle nipples at a level of 10 ppb (IARC, 1972). Among various potent environmental carcinogens, diethylnitrosamine (DEN) is well known for its genotoxic, carcinogenic and mutagenic potential.

DEN causes tumors in the gastrointestinal tract, liver, lung, skin and other organs (Fig. 1A) (Verna et al., 1996).

DEN undergoes metabolic activation by cytochrome P450 enzymes leading to the generation of ROS which in turns to cause oxidative stress-mediated cytotoxicity, mutagenicity and carcinogenicity (Archer, 1989). Upon repeated administration DEN causes perturbations in the nuclear enzymes involved in DNA repair mechanism resulting in cell damage and tumor development (Priya et al., 2018). Several studies reported DEN-induced free radical generation, increased lipid peroxidation, depletion of endogenous antioxidants, cytotoxicity and carcinogenesis (McKillop and Schrum, 2009).

Lung cancer is a complex disease that develops through the progressive accumulation of both genetic and epigenetic alterations (Blanco et al., 2007). Therapeutic success of the lung cancer depends on the comprehensive understanding of the genetic events involved in the initiation and progression of human lung cancer. The recent studies involving genome-wide screening for the genes involved in lung cancer development and progression resulted in the identification of molecular targets with potential therapeutic relevance (Shao et al., 2016). Animal models of chemical carcinogenesis are considered as a suitable option for analysis of risk assessment from exposure to environmental agents as well as evaluation of therapeutic efficacy of anticancer agents (Hussein et al., 2015).

Recent research has demonstrated the efficacy of a number of bioactive components for their ability to prevent cancer and other chronic diseases (Cascao et al., 2017). Such promising research provides an impetus for the development of natural compounds as chemopreventive agents. The chemopreventive effects elicited by these natural compounds can be attributed to their ability to induce cellular defense systems which include upregulation of detoxification and antioxidant systems as well as inhibition of cell proliferation and inflammatory responses, leading to cell death by cell cycle arrest and apoptosis. In the present study, we evaluated the anticancer potential of neferine against DEN-induced lung cancer.

Neferine is a major bisbenzylisoquinoline alkaloid derived from the green plumule in the ripe seed of lotus *Nelumbo nucifera* Gaertn (Fig. 1B). Previous reports revealed the various biological and pharmacological activities of neferine which include: anticancer, antioxidant, anti-inflammatory, anti-fibrosis and anti-multidrug resistance (Zhang et al., 2012; Poornima et al., 2014b; Kadioglu et al., 2017). Neferine inhibited the proliferation of human osteosarcoma cells by promoting p38 MAPK-mediated p21 stabilization (Zhang et al., 2012) and endoplasmic reticulum (ER) stress-induced apoptosis in Hep3B cells (Yoon et al., 2013). Deng et al. (2017) recently reported the antitumor activities of neferine on cell invasion and oxaliplatin sensitivity regulated by EMT *via* Snail signaling in hepatocellular carcinoma. Yang et al. (2016) reported neferine increases anticancer effect of dehydroepiandrosterone on MCF-7 cells. Neferine enhances the responsiveness of tumor cells to anticancer drugs, acting as a natural sensitizer in chemotherapy (Zhao et al., 2010). Chemosensitization of anticancer drugs by neferine was reported earlier in MCF-7 cells (Tang and Cao, 2001) and A549 cells (Poornima et al., 2014a). Recently our research group has reported that neferine potentiates the antitumor effect of cisplatin *via* mitochondria-mediated apoptosis pathway (Kalai Selvi et al., 2017a) and ROS- mediated non-canonical autophagy (Kalai Selvi et al., 2017b) in lung cancer cells. Our present study is an extension of our previous *in vitro* finding which suggests that neferine inhibits human lung cancer cell growth and induce apoptosis of lung cancer cells. Hence, the objective of this study is to evaluate the chemotherapeutic efficacy of neferine in DEN induced carcinogenesis. Alkaloids have chemopreventive effects in cancer through the induction of enzymes affecting carcinogen metabolism and inhibiting various activities of tumor promoters which are involved in the process of carcinogenesis. In this investigation we evaluated the efficacy of neferine as a chemopreventive agent through investigation of the COX-2 and NF-κB signaling pathway, PI3K/AKT/mTOR pathway, angiogenic factor (VEGF), tumor suppressor gene p53, expression of CYP450, apoptotic and anti-apoptotic marker genes Bcl-2, Bax, caspase-9, caspase-3.

2. Materials and methods

2.1. Chemicals

Diethylnitrosamine and CelLytic™ MT Cell Lysis Reagent were purchased from Sigma Aldrich (St. Louis, MO, USA). Neferine was extracted from the embryo of the lotus seeds as previously described (Wu et al., 2004). The purity of the extracted neferine was calculated as 95.9% with reference to the neferine standard. All primary antibodies and HRP-conjugated secondary antibodies used for the study were obtained from Cell signaling technology, USA and Santa Cruz Biotechnology (Santa Cruz, CA). Western blot membranes were obtained from Whatman, USA and the enhanced chemiluminescence (ECL) reagent was obtained from Abcam, USA. DCF was obtained from Calbiochem (Merck, Darmstadt, USA). All other chemicals used were of analytical grade.

2.2. Experimental animals

Albino male Wistar rats were purchased from small animal breeding station, college of veterinary and animal sciences, Mannuthy, Kerala. Adult Wistar rats weighing 160 ± 20 g were used for this study. Animals were caged in groups and maintained at a temperature of 26 ± 2 °C with a normal 12-hr light/dark cycle. The animals were fed with commercially available pelleted rat chow (Sai Durga private limited, Bangalore) and water *ad libitum*. The experiment was carried out according to the guidelines of the Committee for the Purpose of Control

and Supervision of Experiments on Animals (Reg.No.688/2/C-CPCSEA).

2.3. Induction of lung carcinogenesis and neferine treatment schedule

An animal model of DEN-induced lung carcinogenesis is developed as previously described (Chuang et al., 2000). Animals were given DEN at a dose of 150 mg/kg body weight i.p. once a week for four weeks. After four weeks animals were divided into six groups (n = 6) and were treated with neferine (10 mg/kg, 15 mg/kg, 20mg/kg-dissolved in ethanol, final concentration not exceeding more than 0.1%), for 20 alternative days by oral intubation method.

2.4. Experimental design

After the induction of lung cancer, the experimental animals were randomly divided into six groups (comprising six rats in each group) as follows,

Group I - Normal control rats
Group II - Diethylnitrosamine-induced rats (150 mg/kg)
Group III - Diethylnitrosamine-induced rats were treated with Neferine 10 mg/kg body weight, 20 alternate days by oral intubation method.
Group IV - Diethylnitrosamine-induced rats were treated with Neferine 15 mg/kg body weight, 20 alternate days by oral intubation method
Group V - Diethylnitrosamine-induced rats were treated with Neferine 20 mg/kg body weight, 20 alternate days by oral intubation method
Group VI - Normal rats treated with neferine 20 mg/kg body weight, 20 alternate days by oral intubation method

24 h after the last treatment, animals were deprived of food overnight and all rats were anesthetized. Blood was collected from the jugular vein and serum was separated and used for biochemical investigations. The lung tissue was dissected out, washed in ice-cold saline, patted dry and weighed. A portion of the tissue was fixed in 10% formaldehyde for histopathological analysis. From the remaining tissue about 100 mg of tissue was weighed and homogenized with chilled 0.1 M Tris-HCl buffer in Potter- Elvehjem homogenizer for biochemical measurements. The residual portion of lung tissue was stored at −80 °C for further analysis.

2.5. Histopathological examination

The lung tissue from the control and experimental groups were fixed in 10% buffered formalin for analysis of histological alterations. The tissues were then processed for dehydration and clearing of fixative and embedded in paraffin wax. Tissue sections (3–5 mm thickness) were placed on slides and stained with hematoxylin and eosin to study the lung histology of rat under the light microscope. Histopathological changes were analyzed quantitatively by using Image J software.

2.6. Estimation of ROS levels

Generation of ROS levels in lung tissue homogenates were quantified using DCFH-DA fluorescent probe as described previously (Lawler et al., 2003).

2.7. Lipid peroxidation and protein carbonyl content

Lipid peroxidation (LPO) was assayed in lung tissue homogenate by estimating the formation of thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). Protein carbonyl content in lung tissue homogenates was examined by the method of Levine et al. (1990).

2.8. Cellular antioxidant enzymes

The activities of antioxidant enzymes- SOD (Marklund and Marklund, 1974), Catalase (Takahara et al., 1960), GSH (Moron et al., 1979), GPx (Rotruck et al., 1973) and GST (Habig et al., 1974) were determined in tissue homogenate.

2.9. Extraction and estimation of glycoproteins in serum

Glycoprotein extraction was performed using 0.1 ml of serum. To 0.1 ml of serum, 5 ml of methanol was added and centrifuged at 3,000 rpm for 10 min. The supernatant was removed and washed with 5 ml of 95% ethanol and centrifuged at 3,000 rpm for 10 min. Then the supernatant was discarded and glycoproteins were precipitated. Further, hexose (DuBois et al., 1956), hexosamine (William, 1979), fucose (Dische and Shettles, 1948) and sialic acid (Warren, 1959) were estimated from the precipitation respectively.

2.10. Extraction and estimation of glycoproteins in tissue

100 mg of the lung tissue was homogenized in 7 ml of methanol. The contents were filtered and homogenized with 14 ml of chloroform. It was filtered, the residue was subsequently homogenized in chloroform-methanol (2:1 v/v), and each time the extract was filtered. The residue (defatted tissues) was suspended in 3 ml of 2 N HCl and heated at 90 °C for 4 h. The sample was cooled and neutralized with 3 ml of 2 N NaOH. Aliquotes were used to estimate the hexose, hexosamine, fucose, and sialic acid by using the methods of previously described in serum glycoproteins.

2.11. Assay of membrane-bound ATPases

Activities of the membrane-bound ATPases were determined: Na^+K^+ATPase (Bonting et al., 1963), $Ca2^+$ ATPase (Hjerten and Pan, 1983) and $Mg2^+$ATPase (Ohnishi et al., 1982) in the tissue homogenate.

2.12. Real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from lung tissues using Trizol reagent (Invitrogen, USA) and the purity of the RNA was determined by measuring absorbance in Genova nano (Jenway, UK). cDNA was synthesized using high capacity cDNA reverse transcription kit (Applied Biosystems, USA). First strand cDNA was synthesized from 1.5 μg each of total RNA samples using a random primer and reverse transcriptase enzyme according to the manufacturer's instruction.

The mRNA expression of Bcl-2, Bax, p53, caspase-9, caspase-3, PI3K, AKT, mTOR, NF-κB, COX-2, CYP2E1 and VEGF were quantified by SYBR green method using Mx-3000P spectrofluorometric thermocycler (Stratagene, USA). β-actin was used as an endogenous control. The reactions were performed in a 25 μl volume of Power SYBR green master mix (Applied Biosystem, USA) with 10 pM of each primer. PCR was performed with specific primers of the genes as mentioned in Table 1.

The protocol for the PCR reaction used was as follows: Initial denaturation at 95 °C for 2 min, followed by 40 cycles of cyclic denaturation at 94 °C for 15s, annealing at 59 °C for 1min and extension at 72 °C for 15s.

2.13. Immunoblot analysis

Lung tissue was homogenized in lysis buffer (CelLytic™ MT Cell Lysis Reagent-Sigma) with protease inhibitor cocktail (PIC) on ice. The homogenates were centrifuged at 13,000 rpm, at 4 °C for 15 min. Proteins were resolved on polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to PVDF membranes (NENTM Life

Table 1
Sequences of primers used for real-time PCR analysis.

Gene	Forward primer	Reverse primer
Bax	CCACATGGCAGACAGTGACC	ACAGTCCAAGGCAGCAGGAA
Bcl-2	GGTGGTGGAGGAACTCTTCA	ATCTCCCTGTTGACGCTCTC
Cyt-C	TTTGGATCCAATGGGTGATGTTGAG	TTTGAATTCCTCATTAGTAGCTTTTTTGAG
Caspase-9	CCACGGACACATGAGGTTGT	TGGTTGGCAGAACTCAGGAG
Caspase-3	AGTGGTGGACATGACGACAG	GCTGCTGCATAATCGCTCTT
P53	CAGCACAGGAACCTGGAACT	GGAAGCCATAGTTGCCTTGG
PI3K	GGTAGACGATGACGAGGA	TACAGAGCAGGCATAGCA
AKT	CTGGAGGACAACGACTATGG	CTCGAACAGCTTCTCATGGT
mTOR	CCAACTACCTTCGGAACCTC	ACTCCACATACTCAGCAGTG
COX-2	GAAGTTGATAATCGGGTAGTCT	GATGTCACTGTAGCTTGGTT
VEGF	CAACTTCTGGGCTCTTCTCTC	CCTCTCCTCTTCCTTCTCTTTC
NF-KB	ATGGTGGAGTTTGGGAAGGA	CACGGAAGCTGGCTTTGTAA
CYP2E1	CGGTTCTTGGCATCACCATT	GGATGGGAAGAGGGAAAGGT
β-actin	AGGGTGTGATGGTGGGTATGGG	TTCACGGTTGGCCTTAGGGTTC

Table 2
Effect of neferine on body weight, lung weight, lung weight to body weight ratio in DEN induced lung carcinogenesis in rats.

Experimental groups	Initial body weight (g)	Final body weight (g)	Lung weight (g)	Lung weight to body weight ratio (g)
Group I (Control)	160 ± 5.82	198 ± 4.18	1.48 ± 0.08	0.75 ± 0.04
Group II (DEN)	175 ± 2.92	147 ± 3.39[a]	1.96 ± 0.33[a]	1.33 ± 0.25[a]
Group III (DEN + 10 mg/kg NEF)	170 ± 3.74	164 ± 10.29[b]	1.67 ± 0.1[b]	1.01 ± 0.11[b]
Group IV (DEN + 15 mg/kg NEF)	173 ± 2.92	170 ± 15.2[c]	1.58 ± 0.07[c]	0.93 ± 0.08[c]
Group V (DEN + 20 mg/kg NEF)	175 ± 5.38	179 ± 20.0[d]	1.51 ± 0.16[d]	0.84 ± 0.16[d]
Group VI (20 mg/kg NEF alone)	165 ± 3.63	193 ± 9.40[NS]	1.42 ± 0.20[NS]	0.73 ± 0.03[NS]

Data are expressed as mean ± SD (n = 6).
Lung/body weight ratio = [Lung weight (gm)/body weight (g)] x 100%.
[a] P < 0.05 DEN- induced vs. control; [b,c,d] P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

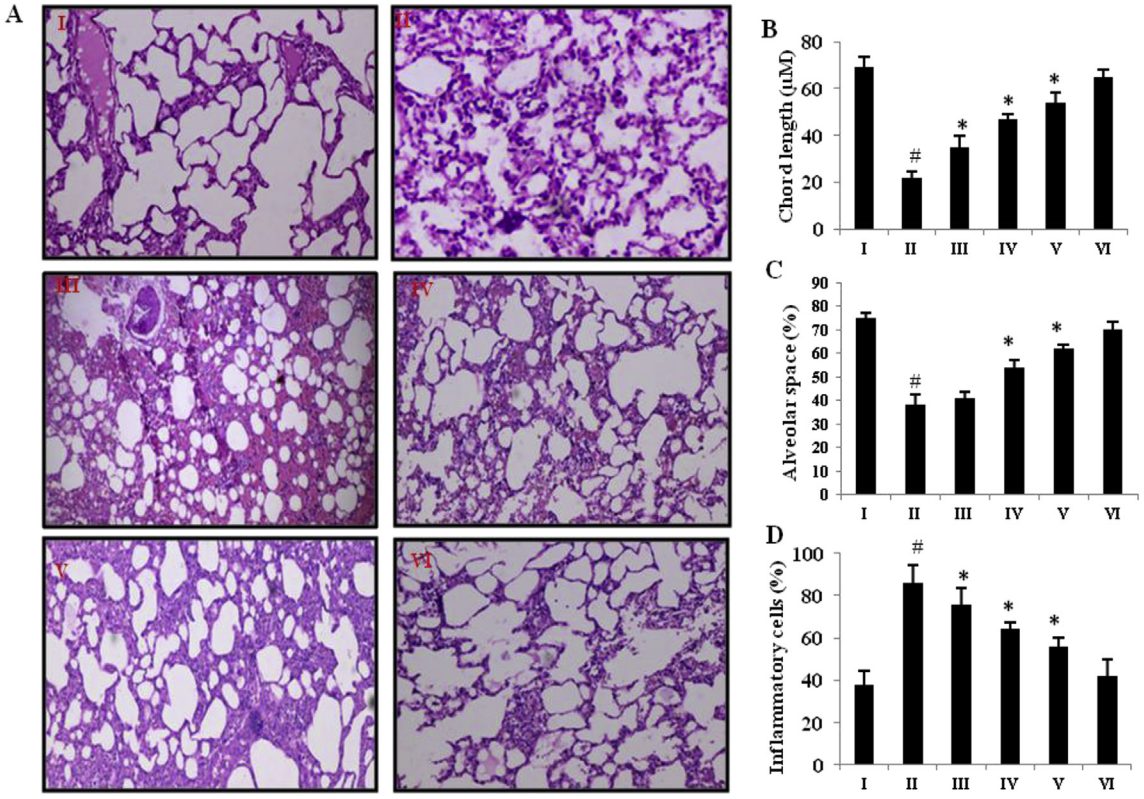

Fig. 2. Histopathological examination of lung. (A) Hematoxylin and Eosin staining of control rat showed normal lung histology. DEN-induced animals showed disorganized alveolar structure, thickened alveolar wall, red blood cells scattered in multiple alveolar cavities. Original magnification, ×100. **(B)** Chord length; **(C)** Lung alveolar space**; (D)** Inflammatory cells. Statistical significance at #P < 0.05 DEN- induced vs. control; *P < 0.05 DEN + Neferine vs. DEN-induced group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

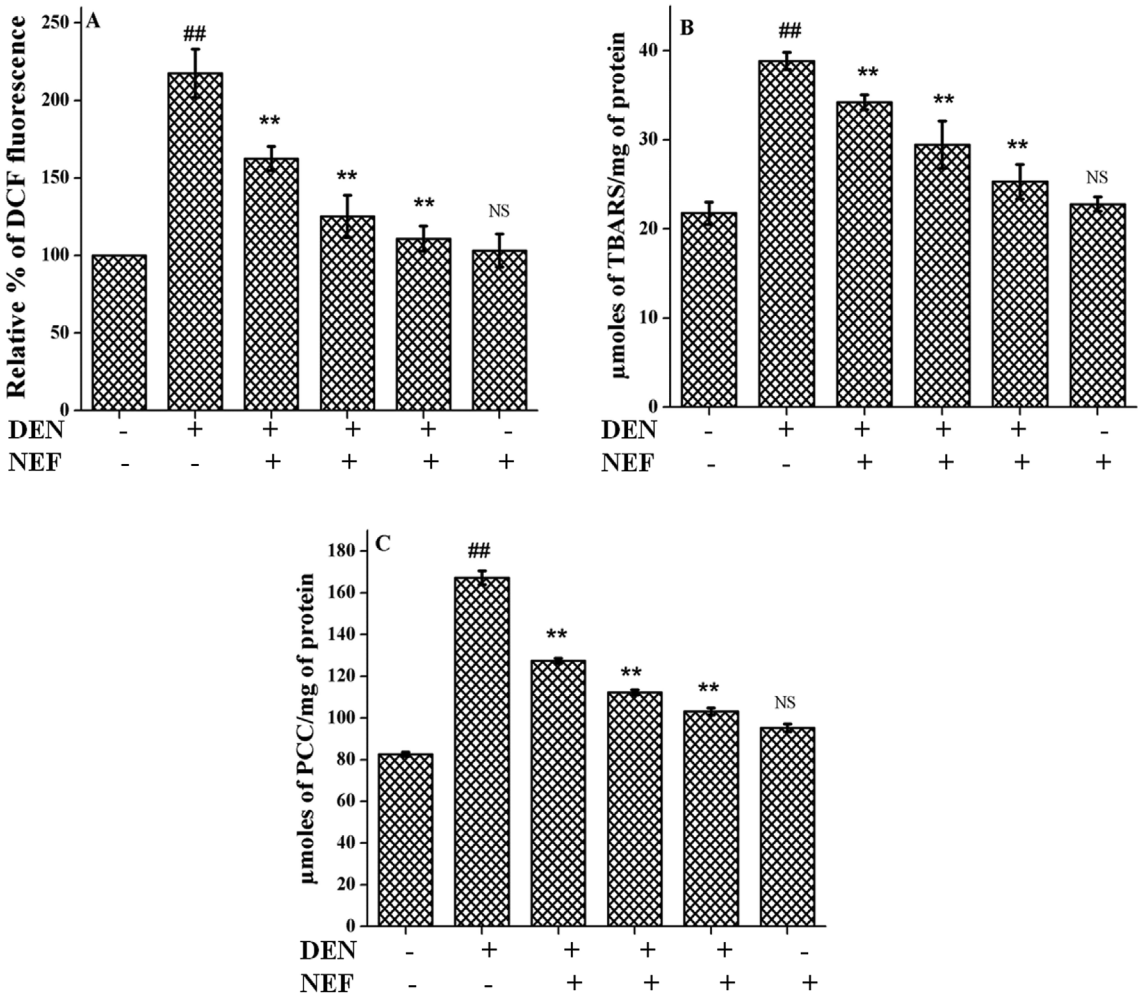

Fig. 3. Neferine attenuates DEN-induced (A) ROS generation (B) LPO (C) PCC levels. Data are expressed as mean ± SD (n = 6). Units are expressed as follows: LPO - µmoles of TBA reactants/mg of protein and PCC- µmoles of pcc/mg of protein. Statistical significance at ##P < 0.01, DEN-induced vs. control; **P < 0.01, DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

Table 3
Effect of neferine on the antioxidant status in DEN-induced lung carcinogenesis in Wistar rat.

Parameters	Group I (Control)	Group II (DEN)	Group III (DEN + 10 mg/kg NEF)	Group IV (DEN + 15 mg/kg NEF)	Group V (DEN + 20 mg/kg NEF)	Group VI (20 mg/kg NEF alone)
GSH	143.24 ± 1.0	127.13 ± 1.4[a]	131.0 ± 1.5[b]	135.18 ± 1.3[c]	137.18 ± 0.7[d]	141.46 ± 0.9[NS]
SOD	657.34 ± 1.0	401.90 ± 6.3[a]	426.57 ± 7.6[b]	504.46 ± 5.0[c]	557.99 ± 3.6[d]	650.34 ± 1.8[NS]
CAT	118.54 ± 1.0	110.12 ± 0.6[a]	112.54 ± 0.7[b]	115.09 ± 0.6[c]	117.49 ± 0.2[d]	117.85 ± 0.6[NS]
GST	2530.53 ± 192.4	1113.22 ± 75.6[a]	1364.94 ± 111.7[b]	1837.94 ± 150.6[c]	2095.60 ± 228.4[d]	2530.66 ± 213.0[NS]
GPx	5798.97 ± 140.1	4113.68 ± 83.5[a]	4451.26 ± 155.6[b]	5148.74 ± 100.7[c]	5553.53 ± 38.5[d]	5727.96 ± 25.3[NS]

Data are expressed as mean ± SD (n = 6).
Units are expressed as follows: GSH- nmoles of glutathione released/mg of protein; SOD-U/mg of protein; CAT-nm of H_2O_2 consumed/min/mg of protein; GPx-µg of GSH utilized/min/mg of protein; GST-nm of CDNB conjugated/min/mg of protein.
[a]P < 0.05 DEN- induced vs. control.
[b,c,d] P < 0.05 DEN + Neferine vs. DEN-induced group.
NS- not significant compared to control.

Science Products, Boston, MA, USA). The blots were blocked with 5% non-fat milk in TBST saline (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) at RT for 1hr and incubated overnight with the appropriate primary antibody (NF-κ B, COX-2, VEGF, Bcl-2, Bax, caspase-9 and caspase-3) at 4 °C. After washing with TBST, the blots were incubated with peroxidase-conjugated secondary antibody for 1 h. Bands were monitored using enhanced chemiluminescence reagent. To ensure equal protein loading, GAPDH was used as an internal control. Densitometric analysis was carried out using the multi gauge image digitalizing software.

2.14. Statistical analysis

Gene expression was estimated using Ct values. The level of target genes relative to endogenous control was evaluated by $2^{-\Delta\Delta Ct}$ formulae and expressed in fold change. All Data were expressed as mean ± SEM. Multiple comparisons were done using one way ANOVA followed by Tukey's multiple comparison tests using SPSS 16 software. Differences

Table 4
Effect of neferine on serum glycoprotein levels in DEN-induced lung carcinogenesis in Wistar rats.

Glycoproteins	Group I (Control)	Group II (DEN)	Group III (DEN + 10 mg/kg NEF)	Group IV (DEN + 15 mg/kg NEF)	Group V (DEN + 20 mg/kg NEF)	Group VI (20 mg/kg NEF alone)
Hexose	79.55 ± 2.9	146.31 ± 5.5[a]	130.47 ± 3.4[b]	110.67 ± 4.0[c]	100.48 ± 5.9[d]	81.25 ± 4.07[NS]
Hexoseamine	65.26 ± 2.9	123.74 ± 1.9[a]	113.87 ± 2.5[b]	101.08 ± 3.8[c]	74.39 ± 2.2[d]	69.28 ± 4.4[NS]
Fucose	19.61 ± 3.2	69.10 ± 2.9[a]	51.72 ± 1.4[b]	36.96 ± 1.7[c]	32.05 ± 1.7[d]	23.63 ± 1.6[NS]
Sialic acid	110.85 ± 3.9	184.37 ± 3.2[a]	174.76 ± 2.3[b]	159.98 ± 4.8[c]	129.32 ± 3.5[d]	108.63 ± 4.8[NS]

Data are expressed as mean ± SD (n = 6).
Values of the glycoprotein components are expressed in mg/dL.
[a]P < 0.05 DEN- induced vs. control.
[b,c,d] P < 0.05 DEN + Neferine vs. DEN-induced group.
NS- not significant compared to control.

Table 5
Effect of neferine on tissue glycoprotein levels in DEN-induced lung carcinogenesis in Wistar rats.

Glycoproteins	Group I (Control)	Group II (DEN)	Group III (DEN + 10 mg/kg NEF)	Group IV (DEN + 15 mg/kg NEF)	Group V (DEN + 20 mg/kg NEF)	Group VI (20 mg/kg NEF alone)
Hexose	254.90 ± 4.4	140.01 ± 4.1[a]	163.28 ± 3.0[b]	200.61 ± 7.8[c]	218.06 ± 3.7[d]	243.27 ± 5.0[NS]
Hexoseamine	148.41 ± 4.0	86.11 ± 1.3[a]	99.97 ± 5.8[b]	121.93 ± 3.9[c]	134.13 ± 3.6[d]	142.70 ± 6.5[NS]
Fucose	41.10 ± 1.9	23.16 ± 1.0[a]	30.35 ± 2.9[b]	34.08 ± 1.8[c]	36.95 ± 3.2[d]	40.00 ± 1.6[NS]
Sialic acid	100.70 ± 3.8	31.48 ± 4.4[a]	45.50 ± 1.4[b]	57.81 ± 3.1[c]	85.31 ± 3.5[d]	96.91 ± 1.1[NS]

Data are expressed as mean ± SD (n = 6).
Values of the glycoprotein components are expressed expressed in mg/g of defatted lung tissues.
[a]P < 0.05 DEN- induced vs. control.
[b,c,d] P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

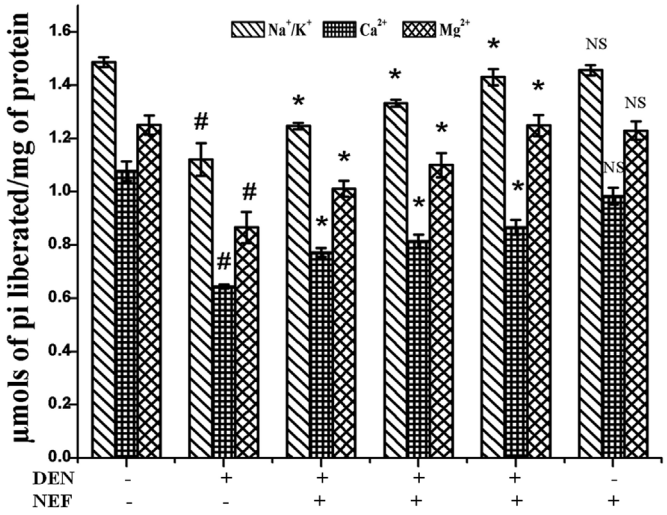

Fig. 4. Effect of neferine treatment on the activities of membrane bound ATPases. ATPase activities are expressed as µmoles of pi liberated/mg of protein in lung tissues. Data are expressed as mean ± SD (n = 6). Statistical significance at ##P < 0.0, DEN-induced vs. control; **P < 0.01, DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

were considered statistically significant when P < 0.01 or P < 0.05.

3. Results

3.1. Effects of neferine treatment on lung weight to body weight ratio in DEN-induced lung carcinogenesis in Wistar rats

Table 2 shows the body weight, lung weight and lung weight to body weight ratio of control and experimental groups. A significant decrease in the body weight was observed in DEN-induced rats compared to control rats. Administration of neferine to DEN-induced animals resulted in a significant increase in final body weight when compared to DEN-induced animals. Lung weight and lung weight to body weight ratio of DEN-induced animals were significantly increased compared to control. Whereas, administration of neferine to DEN-induced animals resulted in a significant decrease in lung weight and lung weight to body weight ratio dose-dependently when compared to DEN-induced rats. There was no significant difference in the lung weight and lung weight to body weight ratio in the neferine alone treated rats when compared to control group.

3.2. Histopathological examination of lung

In order to determine the pathological alterations in the lung, H&E staining were performed. The control group showed normal lung tissue architecture. The DEN-induced animals showed disorganized alveolar structure, thickened alveolar wall, interalveolar inflammatory cells, red blood cells scattered in multiple alveolar cavities (Fig. 2A). Histopathological features were analyzed quantitatively (chord length, alveolar space, number of inflammatory cells) (Fig. 2B, C & D). During the treatment with neferine these pathological damages were significantly reduced in a dose-dependent manner.

3.3. Neferine exerts dose-dependent protective effects during lung carcinogenesis

To explore the effects of neferine treatment on DEN-induced oxidative stress, we measured the levels of ROS, LPO and protein carbonyl content in all experimental groups. Neferine dose-dependently inhibited oxidative stress-induced cellular damage during DEN-induced lung carcinogenesis as evidenced by its ability to prevent ROS generation and lipid peroxidation in DEN-induced animals. A statistically significant decrease in ROS and lipid peroxidation was observed with neferine treatment to DEN-induced animals at a dose of 15 and 20 mg/kg (Fig. 3A and B). As shown in Fig. 3 C, DEN administration produced a significant increase in protein carbonyl content in the lung tissue compared to normal control animals. Treatment with neferine resulted in a dose dependent decrease of protein carbonyl content in DEN-induced animals. Interestingly, the level of protein carbonyl content in

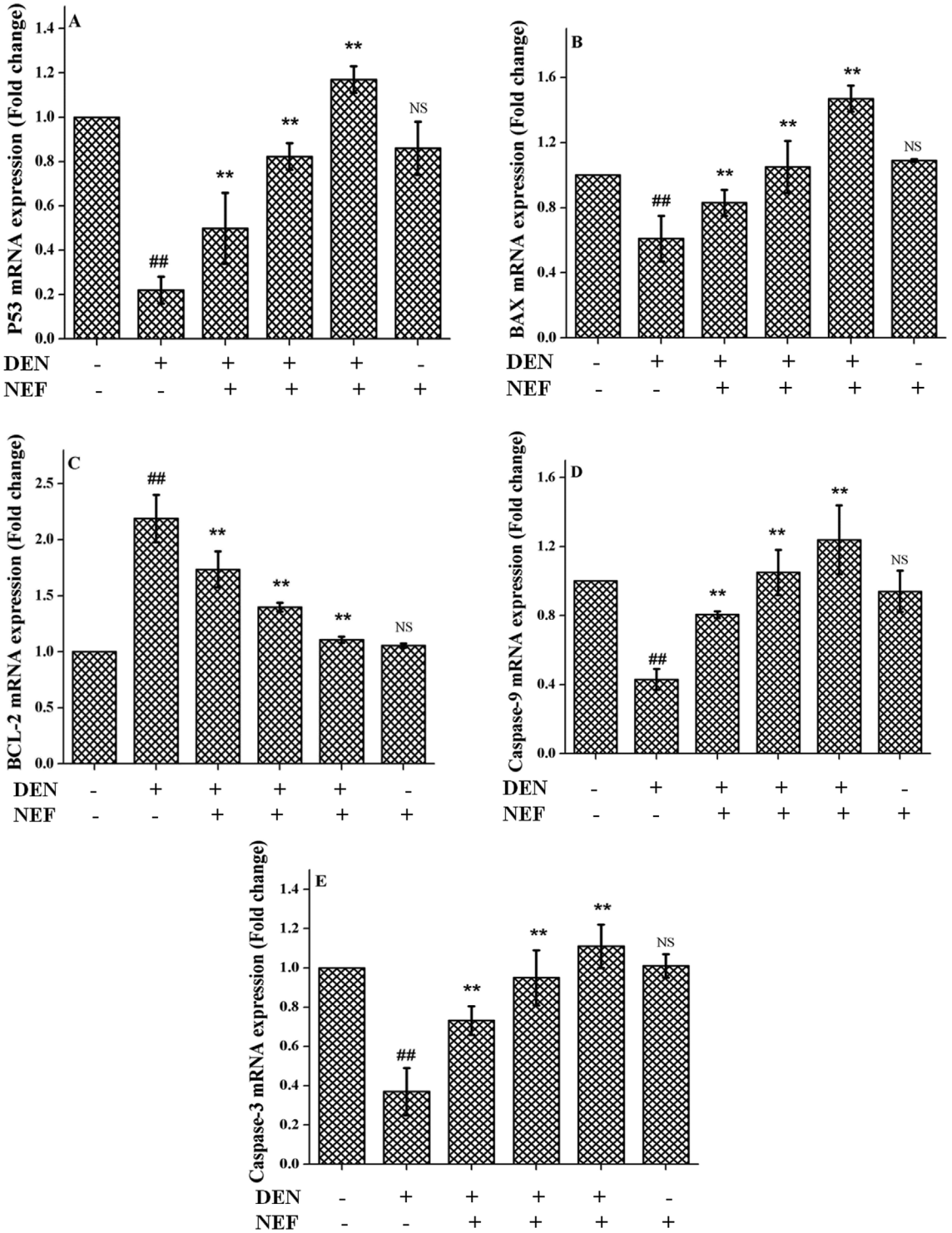

Fig. 5. Effect of neferine on mitochondrial mediated apoptosis. The gene expression levels of p53, Bax, Bcl-2 and caspases were analyzed in all experimental groups (A, B, C, D and E). Data are expressed as mean ± SD (n = 6). Statistical significance at ##P < 0.01 and #P < 0.05 DEN- induced vs. control; **P < 0.01 and *P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

the animals treated with neferine alone (20 mg/kg) was found to be slightly less than that of the normal control group.

Table 3 showed diethylnitrosamine significantly decreased the activities of various antioxidant enzymes namely- GSH, SOD, GPx, GST and CAT in lung tissues, indicating that ROS induced by DEN plays an important role in lung carcinogenesis. Neferine treatment to DEN-induced animals significantly increased the activities of these antioxidant enzymes compared to DEN-induced animals.

3.4. Effects of neferine treatment on the levels of glycoprotein components in the serum and lung tissue of DEN-induced lung carcinogenesis in Wistar rats

A significant increase in the levels of glycoprotein such as hexose, hexosamine, fucose and sialic acid were observed in the serum of DEN-induced animals with a concomitant decrease in the lung tissue when compared to control. Neferine treatment to DEN-induced animals resulted in significant decrease in the levels of glycoprotein components

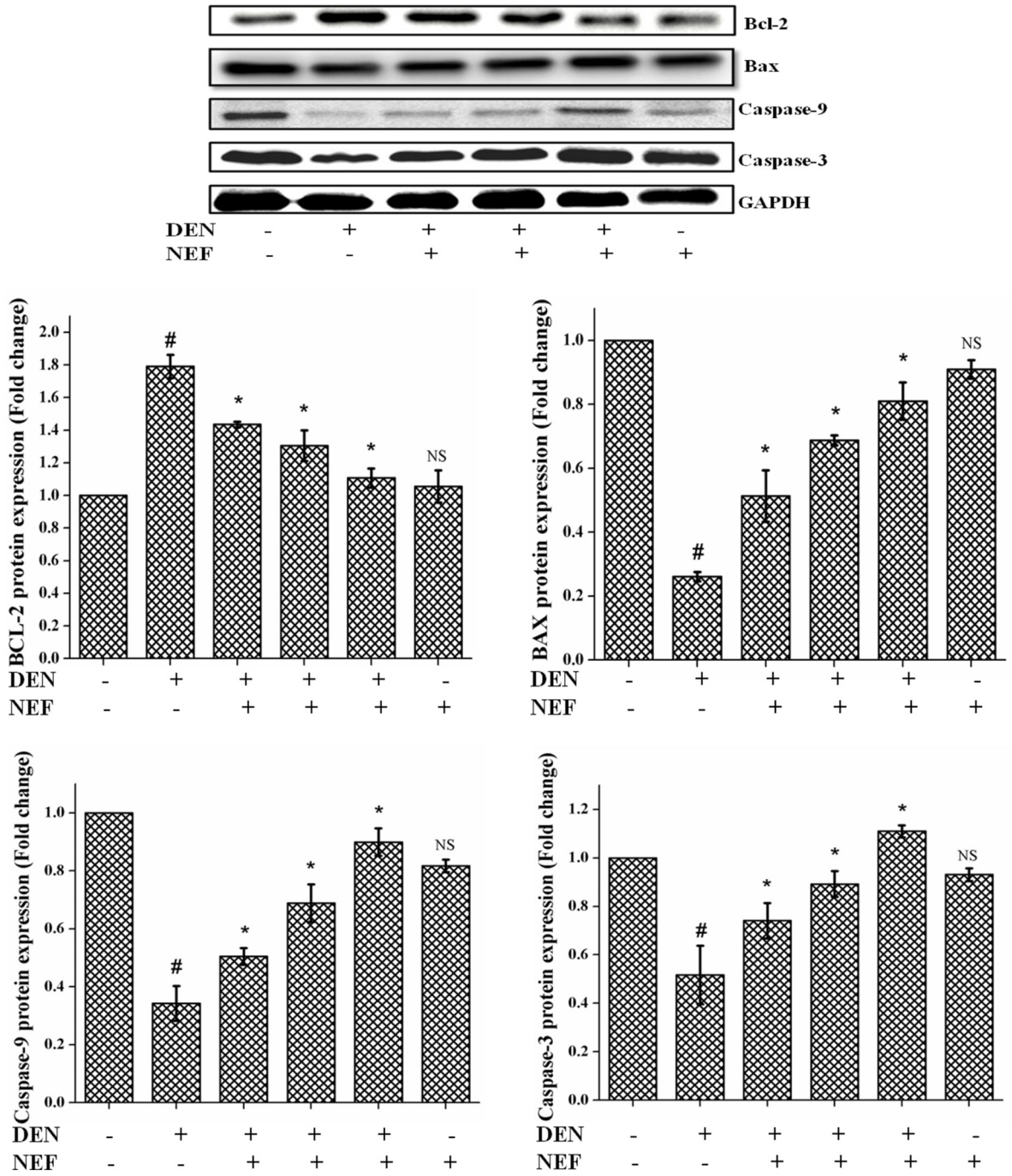

Fig. 6. Immunobloting analysis of Bcl-2, Bax, caspase-9 and caspase-3. Protein values are expressed as relative intensity arbitrary unit. Statistical significance at #P < 0.05 DEN- induced vs. control; *P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

in serum with a concomitant increase in the lung tissue in a dose-dependent manner when compared to DEN-induced animals. No significant difference was observed in the neferine alone treated rats when compared to control Table 4 and 5.

3.5. Effect of neferine on the activity of ATPase in DEN-induced carcinogenesis in the lung

Fig. 4 depicts the activities of membrane-bound ATPases in the lung tissue of control and experimental animals. The activities of Na^+/K^+ -ATPase, Ca^{2+} -ATPase and Mg^{2+} -ATPase were found to be significantly decreased in DEN-induced animals when compared with the control. DEN-induced animals treated with neferine showed a significant increase in the activities of these ATPases when compared with the DEN-induced animals. However, there was no significant difference in the activities of these ATPases in the control animals and the animals treated with neferine alone.

3.6. Effect of neferine on mitochondrial-mediated apoptosis in DEN-induced lung carcinogenesis

To illustrate the effect of neferine on mitochondrial-mediated apoptosis, the expression levels of p53, Bcl-2, Bax and caspases were analyzed in various experimental groups. The mRNA expression level of p53, Bcl-2, Bax, caspase-9 and caspase-3 is depicted in Fig. 5. DEN-induced animals showed significantly increased expression of Bcl-2

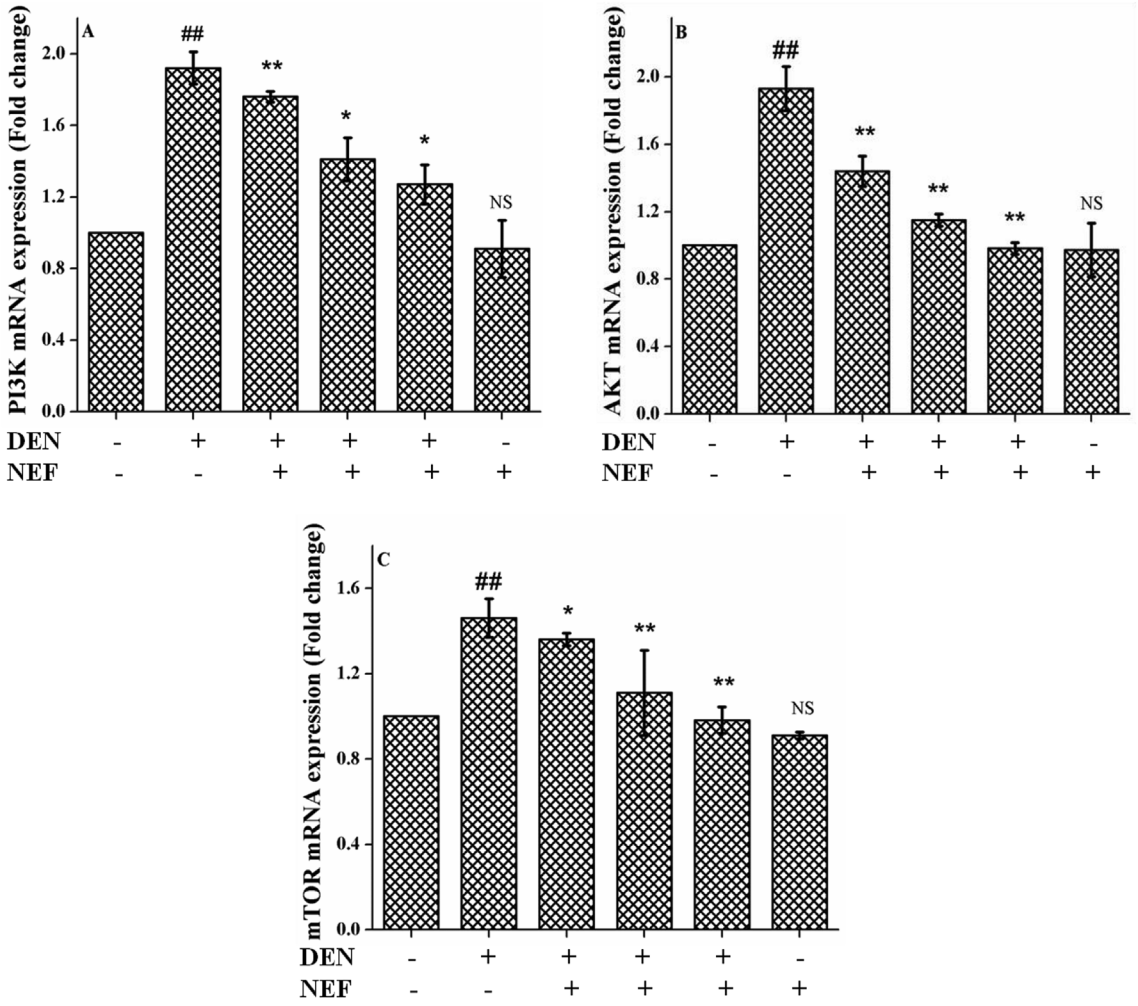

Fig. 7. Effect of neferine on gene expression of PI3K/AKT/mTOR signaling pathway (A, B and C). Data are expressed as mean ± SD (n = 6). Statistical significance at ##P < 0.01 and #P < 0.05 DEN- induced vs. control; **P < 0.01 and *P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

with a concomitant decrease in the expression of p53, Bax, caspase-9 and caspase-3 when compared with control animals. DEN-induced groups treated with neferine showed significantly increased expression of p53, Bax, caspase-9 and caspase-3 with a significant decrease in the level of anti-apoptotic gene Bcl-2, when compared with DEN-induced animals (Fig. 5A, B, C, D & E).

The Bcl-2 protein level was significantly increased in DEN-induced animals with a concomitant decrease in the protein expression of Bax, caspase-9 and caspase-3 when compared with control animals. DEN-induced group treated with neferine showed decreased expression of anti-apoptotic protein Bcl-2 with a concomitant increase in the expression of Bax, caspase-9 and caspase-3 in a dose-dependent manner compared with the DEN-treated group (Fig. 6).

3.7. Neferine suppresses the gene expression of PI3K/AKT/mTOR signaling pathway in DEN-induced lung carcinogenesis

The mRNA expression level of PI3K/AKT/mTOR is depicted in Fig. 7. DEN-induced animals exhibited higher expression levels of PI3K/AKT/mTOR when compared with control animals. DEN-induced group treated with neferine showed markedly lower gene expressions of PI3K/AKT/mTOR, when compared with DEN-induced animals (Fig. 7A, B & C).

3.8. Neferine regulates the expression of inflammatory genes and proteins

NF-κB, COX-2, CYP2E1 and VEGF mRNA and protein expression levels were determined in all the experimental groups to explore the possible molecular mechanism of the anticancer activity of the neferine. The mRNA expression levels of COX-2, NF-κB, CYP2E1 and VEGF were significantly increased in DEN-induced animals when compared with control. DEN-induced group treated with neferine resulted in decreased expression of COX-2, NF-κB, CYP2E1 and VEGF in a dose-dependent manner compared with the DEN-induced animals (Fig. 8A, B, C & D).

To determine the effect of the neferine on the DEN-induced inflammatory response, the protein expression levels of NF-κB, COX- 2 and VEGF were determined by western blotting analysis. DEN-induced animals showed a significant increase in the expression of COX-2, NF-κB and VEGF when compared with control. Administration of the neferine in DEN-induced animals significantly decreased the protein expression levels of COX-2, NF-κB and VEGF in a dose-dependent manner compared to DEN-induced animals (Fig. 9).

4. Discussion

Lung cancer is the most prevalent human cancer in the world (Siegel et al., 2014). As it poses a serious health threat to human race across the globe and the existing chemotherapeutics have serious side effects, several studies focused on finding new bioactive agents with

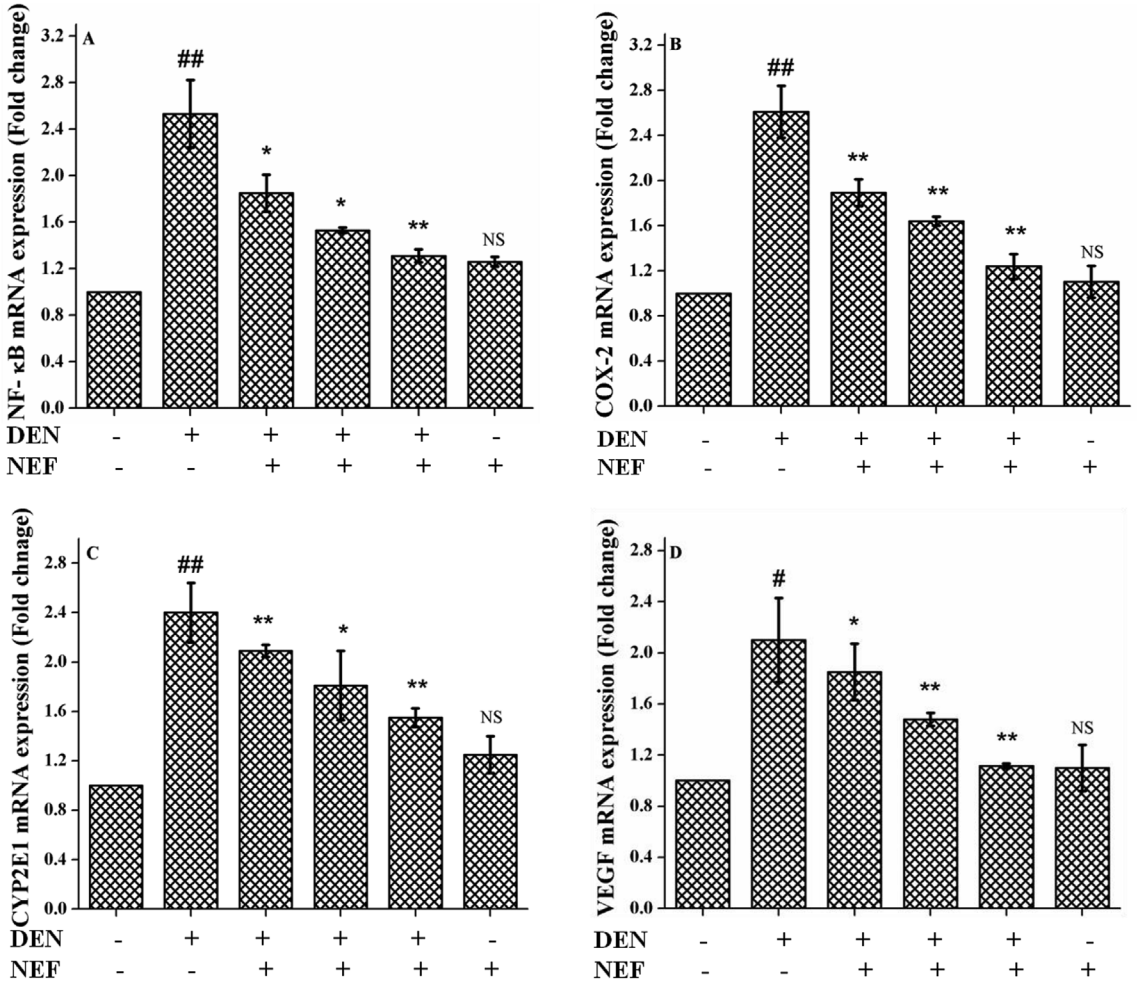

Fig. 8. Neferine regulates the expression of inflammatory genes NF-κB, COX-2, CYP2E1 and VEGF (A, B, C and D). Data are expressed as mean ± SD (n = 6).). Statistical significance at ##P < 0.01 and #P < 0.05 DEN- induced vs. control; **P < 0.01 and *P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

therapeutic potential. Since deregulated apoptosis leads to carcinogenesis, apoptosis has been considered an appropriate therapeutic target (Pfeffer and Singh, 2018). Preventing tumor incidence and delaying the onset of a tumor by natural bioactive compounds may be a valuable strategy in cancer chemoprevention. In the present study, we have elucidated the anticancer effect of neferine against DEN-induced lung cancer in Wistar rats.

Reactive-oxygen species (ROS) and oxidative stress play an important role in DEN-induced lung cancer. ROS and lipid peroxidation play a major role in initiation, promotion and progression of lung cancer through oxidation. Oxidative stress is a result of an imbalance between the production of ROS and detoxification of the reactive intermediates by the cellular antioxidant defence. Lipid peroxidation levles increased during carcinogenesis, hence, malondialdehyde (MDA) a product of lipid peroxidation can serve both as a potent mutagen and carcinogen (Barrera, 2012). In this study, treatment with DEN resulted in elevated levels of ROS and LPO which were considerably reduced in neferine-treated animals. Enzymatic and non-enzymatic antioxidants such as SOD, CAT, GPx, GST and GSH scavenge reactive oxygen species and prevent lipid peroxidation. Several investigators reported significantly reduced activities of SOD, CAT, GPx, GST and GSH in cancer-bearing animals with elevated free radicals and certain humoral factors (Sun, 1990; Willis et al., 2017). Reduced glutathione and associated enzymes present in the epithelial lining of the lower respiratory tract may be the first line of defense against lung damage. A persistent oxidative challenge to lungs depletes GSH and other antioxidants in lungs (Meister, 1994). The present study revealed significantly decreased activities of SOD, CAT, GP_x, GST and GSH in lung cancer bearing-animals. Administration of neferine restored the activities antioxidants in a dose-dependent manner. Continuous ROS generation and decreased antioxidant enzymes in lung tissues have long been reported in many chemically induced models of lung carcinoma. These results suggest that treatment with neferine alleviate oxidative stress and free radical scavenging action thereby modulating the levels of antioxidants against DEN-induced lung carcinogenesis.

Cell membrane constituents such as glycoproteins, play a prominent role in neoplastic diseases (Thakkar et al., 2014). They play a key role in mediating cell surface function, such as cell-cell recognition, cellular adhesion, binding and clearance of serum glycoproteins and metabolic transport among others. Elevated levels of glycoproteins serve as useful indicators of the carcinogenic process. Previous studies reported an increased level of serum glycoproteins in DEN administered animals (Rachesky et al., 1983). In our study glycoproteins such as hexose, hexosamine, fucose and sialic acid levels were found to be significantly elevated in serum and significantly reduced the lung tissue in cancer-bearing animals. Administration of neferine restored the levels of glycoproteins in a dose-dependent manner.

Several studies indicate that LPO affects cell membrane fluidity thereby cellular function. Free radical-induced change in membrane structure may have very specific effects on membrane proteins as documented by selective modification of different active sites in tissues. ATPases are intimately associated with the plasma membrane and

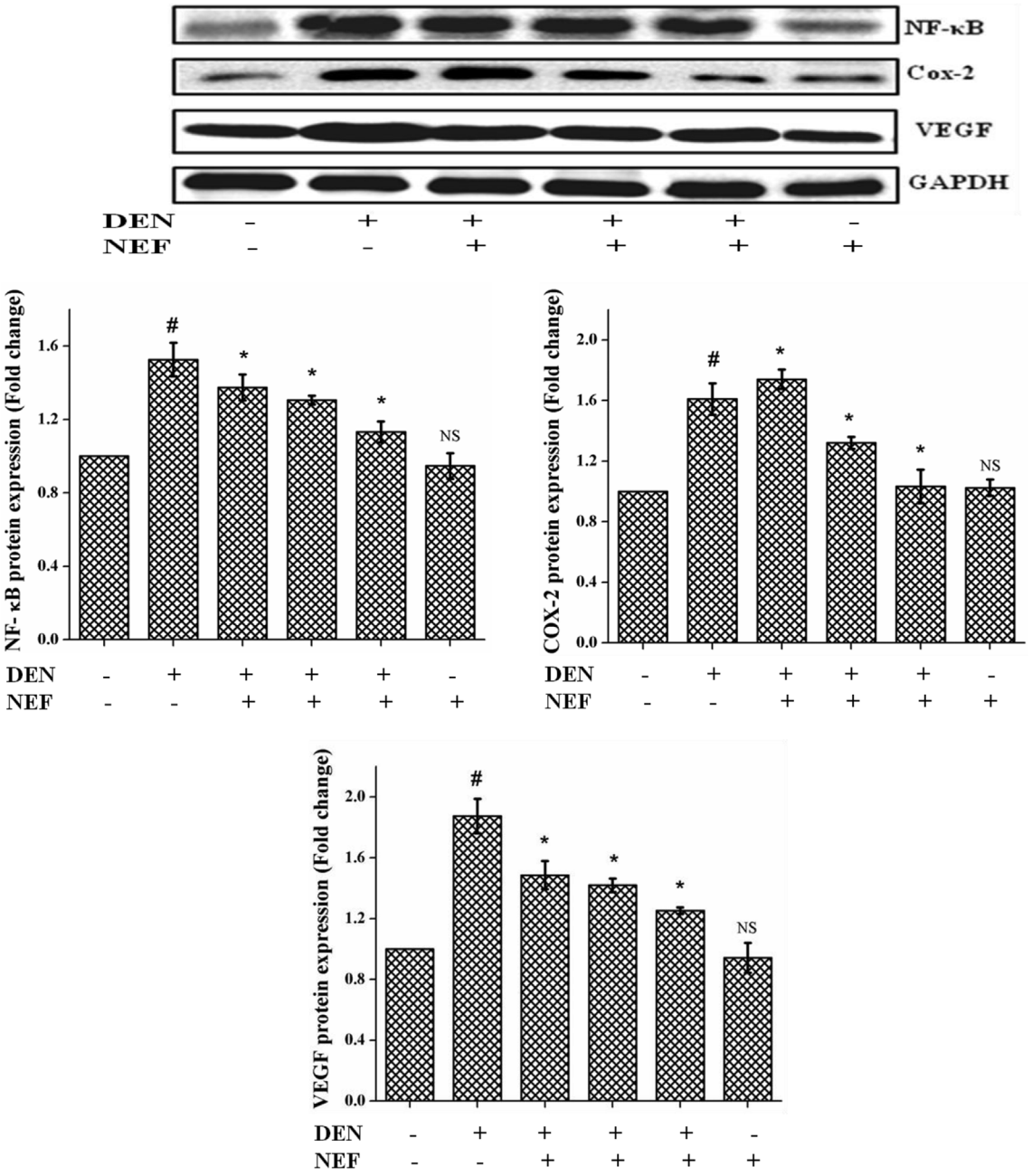

Fig. 9. Neferine regulates the expression of inflammatory proteins NF-κB, COX-2 and VEGF. Protein values are expressed as relative intensity arbitrary unit. Statistical significance at #P < 0.05 DEN- induced vs. control; *P < 0.05 DEN + Neferine vs. DEN-induced group. NS- not significant compared to control.

participate in the energy-requiring translocation of sodium, potassium, calcium and magnesium ions (Senthilnathan et al., 2006). Peroxidation of the membrane is accompanied by alteration of the structural and functional characteristics of membranes. ATPases are very sensitive to peroxidation reactions and abnormal lipoperoxides affect ATPase activities. The activities of Na+/K + -ATPase, Mg2+ -ATPase and Ca2+ -ATPase were found to be significantly decreased in the DEN induced animals when compared with the control group. Neferine treatment caused a significant increase in these enzyme activities in DEN induced animals when compared to DEN alone treated animals. Treatment with neferine could reverse the DEN induced alteration in membrane fluidity and thereby improved the cell membrane integrity and restored the activity of the membrane ATPases in animals which received DEN injection followed by neferine treatment.

Carcinogenesis is the result of a disturbed balance between cell division and growth on one hand, and programmed cell death (i.e., apoptosis), on the other. In recent years, altered PI3K/Akt signaling pathway has been reported in many human cancers (Khan et al., 2013). Previous findings suggest that PI3K/Akt signaling pathways play a major role in carcinogenesis as they promote cell survival. Apoptosis helps to establish a natural balance between cell death and cell renewal in mature animals by causing the destruction of damaged or abnormal cells, hence any disturbance in this sharp balance would direct the cells to carcinogenesis (Aggarwal et al., 2003). In this study, induction of carcinogenesis by DEN administration was accompanied by a strong upregulation of PI3K/Akt/mTOR mRNA expression which suggests its pro-survival role, an essential process in tumor development. Interestingly, neferine treatment for lung cancer-bearing rats significantly reduced the PI3K/Akt/mTOR expression levels, which correlate with reduced tumor burden in these animals.

p53 as a tumor suppressor is a powerful growth regulator of apoptosis, which regulates the expression of genes involved in apoptosis,

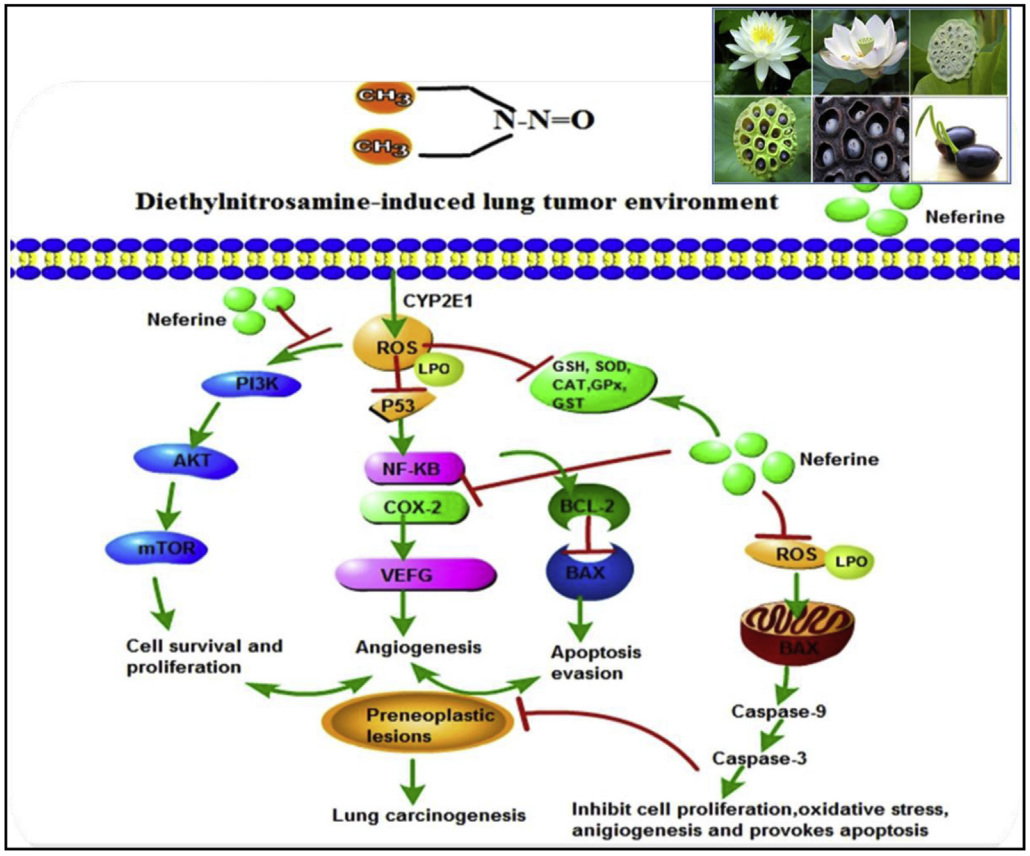

Fig. 10. Schematic representation of the mechanism of action of neferine against DEN-induced lung carcinogenesis in *in vivo* model.

such as Bax or Bcl-2 (Purvis et al., 2012; Ou et al., 2015). Bax/Bcl-2 regulates mitochondrial outer membrane permeabilization and the release of cytochrome C into the cytosol, which in turn induce caspase activation and apoptosis. Our studies revealed that neferine treatment up-regulated the expression of Bax, caspase 9 and 3 and down-regulated the expression of Bcl-2 in DEN-induced lung cancer-bearing rats. Thus the present investigation suggests that neferine induce mitochondrial mediated apoptosis through the activation of caspase cascade. These results are comparable with our previous findings in A549 cells (Poornima et al., 2014a).

NF-κB is the transcription factor involved in the regulation of inflammation, cell cycle, and cell survival. Under oxidative stress, NF-κB gets activated and translocated to nucleus where it is strongly associated with cancer initiation and progression (Hamza et al., 2018). Similarly, COX-2 overexpression which activates the prosurvival Akt-dependent cell proliferation pathway in cancer cells has been reported in several carcinomas (Ladu et al., 2008). COX-2, an inducible form of cyclooxygenase, serves as an interface between inflammation and cancer (Aggarwal et al., 2006). Aberrant induction of COX-2 has been implicated in the pathogenesis of various types of malignancies, including those arising in the lung (Hida et al., 1998; Yip-Schneider et al., 2000). Nuclear factor-κB (NF-κB) is an oncogenic transcription factor that serves as a regulator for COX-2 and VEGF expression in various types of cancer cells (Verstrepen and Beyaert, 2014). Previous studies showed that the inhibition of NF-κB signaling in human cancer cells decreases their tumorigenic and metastatic abilities by suppressing angiogenesis and inflammation through downregulation of VEGF and COX-2 activity (Araujo et al., 2015). Neferine treatment significantly down-regulated the gene and protein expression of NF-κB, VEGF and COX-2 in cancer-bearing rats. The gene expression levels of NF-κB, VEGF and COX-2 mRNA levels were paralleled with the protein expression. In lung cancer , neferine showed chemotherapeutic efficacy by down-regulating PI3K/Akt and NF-κB pathway and other regulated gene products, such as Bcl-2, COX-2 and VEGF. This finding was in the same line with the previous report demonstrating that suppression of PI3K/Akt/NF-κB pathway, which gives protection against diethylnitrosamine-induced oxidative stress injury, inflammation, tumor cancer stem cells proliferation in lung cancer (Man et al., 2017).

The chemical carcinogen DEN is primarily metabolized by phase I and II drug metabolizing enzymes and the metabolites further react with nucleophilic sites of DNA or RNA to generate adducts. All these serve as the initial step in the DEN-induced carcinogenesis (Liu et al., 2016). It has been well documented that DEN-induced lung carcinogenesis requires metabolic activation by some forms of CYP450s, especially CYP2E1. Hence, in the current study, we examined the mRNA expression level of phase I enzyme cytochrome P4502E1 (CYP2E1) and the results suggest that neferine could inhibit the expression of CYP2E1 which is evident from the decreased mRNA levels in cancer-bearing rats.

In conclusion, findings of the present study suggests that neferine inhibits DEN-induced lung carcinoma through suppression of oxidative stress, decreased expression of cell survival PI3K/Akt/mTOR and inflammatory genes NF-κB, CYP2E1, VEGF and COX-2 with a concomitant apoptosis induction. Fig. 10, depicts schematic representation of the mechanism of action of neferine against DEN-induced lung carcinogenesis in *in vivo* model.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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