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# The neem limonoids azadirachtin and nimbolide inhibit hamster cheek pouch carcinogenesis by modulating xenobiotic-metabolizing enzymes, DNA damage, antioxidants, invasion and angiogenesis

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**Abstract**
The neem tree has attracted considerable research attention as a rich source of limonoids that have potent antioxidant and anti-cancer properties. The present study was designed to evaluate the chemopreventive potential of the neem limonoids azadirachtin and nimbolide based on *in vitro* antioxidant assays and *in vivo* inhibitory effects on 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis. Both azadirachtin and nimbolide exhibited concentration-dependent anti-radical scavenging activity and reductive potential in the order: nimbolide > azadirachtin > ascorbate. Administration of both azadirachtin and nimbolide inhibited the development of DMBA-induced HBP carcinomas by influencing multiple mechanisms including prevention of procarcinogen activation and oxidative DNA damage, upregulation of antioxidant and carcinogen detoxification enzymes and inhibition of tumour invasion and angiogenesis. On a comparative basis, nimbolide was found to be a more potent antioxidant and chemopreventive agent and offers promise as a candidate agent in multitargeted prevention and treatment of cancer.

**Keywords:** *Angiogenesis, antioxidants, azadirachtin, DMBA, invasion, neem, nimbolide, xenobiotic-metabolizing enzymes*

## Introduction

Exposure to polycyclic aromatic hydrocarbons (PAHs) is associated with increased risk of cancer [1]. 7,12-Dimethylbenz[a]anthracene (DMBA), a prototype PAH and a potent carcinogen, is metabolized by the combined action of xenobiotic metabolizing enzymes (XME) to form electrophilic intermediates that bind to DNA, forming adducts [2]. Excessive proliferation of carcinogen-altered cells coupled with insufficient apoptosis can result in genomic instability and neoplastic transformation [3].

Although cell proliferation is an essential prerequisite for the development of a malignant tumour, tissue invasion and angiogenesis are major causes of cancer morbidity and mortality. Tumour invasion requires efficient degradation of the extracellular matrix (ECM) by urokinase plasminogen activator (uPA) and matrix metalloproteinases (MMPs) that are regulated by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs) [4]. Vascular endothelial growth factor (VEGF), released during ECM processing, triggers a network of signalling pathways that promote angiogenesis. Hypoxia-inducible factor-1$\alpha$ (HIF-1$\alpha$) and placental growth factor (PlGF) enhance VEGF signalling [5,6]. RECK (reversion-inducing cysteine-rich protein with Kazal motifs), a recently characterized membrane-bound protein that suppresses key components in the metastatic cascade, is in turn repressed by histone deacetylase [7]. Identification of agents that target these molecules is

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likely to be of significance in cancer chemoprevention. Research over the last several decades has identified several antioxidant phytochemicals capable of multi-targeted cancer prevention and therapy [8].

Of late, limonoids, modified triterpenes formed as secondary metabolites by plants in the *Meliaceae* and *Rutaceae* families, have attracted considerable research attention as promising candidates for chemoprevention [9]. The neem tree (*Azadirachta indica* A. Juss), widely distributed in Asia, Africa and other tropical parts of the world, is one of the richest sources of limonoids [10]. Neem leaf preparations have been demonstrated to inhibit the development of experimental carcinogenesis by targeting multiple signal transduction pathways [11–13]. Azadirachtin, isolated from seed kernels, and nimbolide present in leaves and flowers, are the two most important neem limonoids that exhibit antiproliferative properties [14,15]. Figure 1 represents the chemical structures of nimbolide and azadirachtin. Recently, we identified nimbolide as one of the major constituents in neem leaf fractions by HPLC and demonstrated its cytotoxic effects against human choriocarcinoma cells *in vitro* [12,16]. Although both azadirachtin and nimbolide have been demonstrated to exert cytotoxic effects against a panel of human cancer cell lines, they have not been tested for chemopreventive potential in animal tumour models.

The present study was designed to evaluate the relative antioxidant potential of azadirachtin and nimbolide *in vitro* and dose-dependent inhibitory effects on DMBA-induced hamster buccal pouch (HBP) carcinogenesis *in vivo*. The activities of phase I (total cytochrome P450 monooxygenases (CYP) as well as its isoforms CYP1A1, 1A2 and 2B and cytochrome $b_5$) and phase II ( glutathione-S-transferase (GST) and quinone reductase (QR) XMEs, the extent of oxidative DNA damage (8-hydroxy 2-deoxyguanosine; 8-OHdG) and the status of antioxidant defence systems were used to monitor chemoprevention. In addition, the ability of the neem limonoids to modulate markers of carcinogen detoxification (NQO1), antioxidant defences (Mn-SOD, catalase), invasion (MMP-2, MMP-9, TIMP-2 and RECK) and angiogenesis (PlGF, VEGF, VEGF receptor 1; VEGFR1, VEGF receptor 2; VEGFR2) as well as histone deacetylase-1 (HDAC-1) was also evaluated.

## Materials and methods

### *Chemicals*

Both nimbolide and azadirachtin (≥98% purity) were purchased from SPIC Science Foundation (Tuticorin, India). All other reagents used were obtained from Sigma Chemical Company (USA).

### In vitro *free radical scavenging assays*

The free radical scavenging capacity was evaluated by the DPPH assay described by Blois [17]. The total antioxidant potential was measured by 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay [18]. Hydroxyl radical and superoxide anion scavenging activity were determined by the methods of Halliwell et al. [19] and Nishimiki et al. [20], respectively. The nitric oxide radical inhibition activity was measured by the method of Sreejayan and Rao [21] and the reductive potential was determined according to the method of Oyaizu [22].

### *Animals and diet*

The experiment was carried out with male Syrian hamsters aged 8–10 weeks weighing 100–110 g obtained from the Central Animal House, Annamalai University, India. The animals, housed five to a polypropylene cage, were provided with standard pellet diet (Mysore Snack Feed Ltd, Mysore, India) and water *ad libitum* and maintained under controlled conditions of temperature and humidity with an alternating light/dark cycle in accordance with the guidelines of the Indian Council of Medical Research and approved by the ethical committee, Annamalai University.

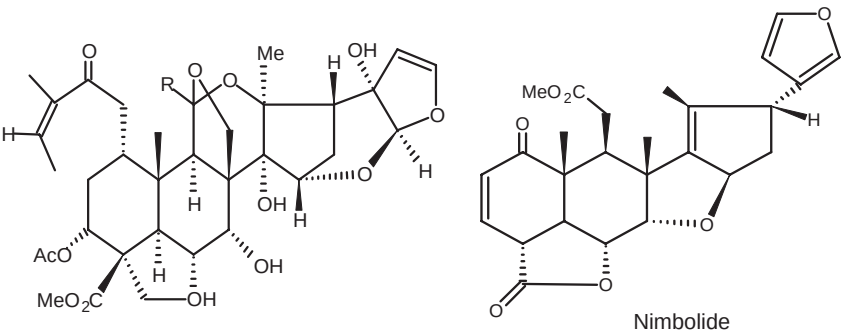



Figure 1. Chemical structures of azadirachtin and nimbolide.

*Experimental design*

The animals were randomized into experimental and control groups and divided into eight groups of 10 animals each. In group 1, the right buccal pouches of hamsters were painted with a 0.5% solution of DMBA in liquid paraffin three times per week for 14 weeks [23]. Hamsters in group 1 received no further treatment. Animals in groups 2–5, painted with DMBA as in group 1, received in addition, intragastric administration of azadirachtin and nimbolide at a dose of 10 and 100 μg/kg bw, respectively, three times per week on days alternate to the DMBA application. The doses of azadirachtin and nimbolide administered in the present study are based on those used for halichondrin B, an anti-tumour agent isolated from a marine sponge that is structurally similar to the neem limonoids [24]. Animals in groups 6 and 7 were administered azadirachtin and nimbolide alone, respectively, at a concentration of 100 μg/kg bw. Group 8 animals received basal diet and served as control. The experiment was terminated at the end of 14 weeks and all animals were sacrificed by cervical dislocation after an overnight fast.

The mean tumour burden was determined by multiplying the number of tumours in each group by the mean tumour volume in millimetres. Tumour volume was calculated using $4/3\ \pi r^3$, where r represents ½ tumour diameter in mm.

Tissues for histopathological examination were immediately fixed in 10% neutral buffered formalin, embedded in paraffin, processed by means of routine histological techniques and stained with haematoxylin and eosin. Tissue samples for biochemical analyses were weighed and homogenized using appropriate buffers in an all glass homogenizer with Teflon pestle. The S9 fractions were prepared at 4°C, as described by Ames et al. [25]. All biochemical estimations were carried out immediately. Since the lower dose (10 μg/kg bw) of azadirachtin and nimbolide administered in the present study showed significant modulatory effects on xenobiotic-metabolizing enzymes and antioxidants, we used only these groups to evaluate the effects on molecular markers.

*Biochemical estimations*

Cytochrome P450 and cytochrome $b_5$ content were assayed by the method of Omura and Sato [26]. The activities of ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD) and pentoxyresorufin *O*-dealkylase (PROD), indicative of CYP1A1, 1A2 and 2B1, were determined spectrofluorimetrically according to the method of Burke et al., [27]. The activity of GST and QR were assayed by the methods of Habig et al. [28] and Ernster [29], respectively.

Total superoxide dismutase (SOD) and Mn-SOD activities were assayed as described by Oberley and Spitz [30]. Cu-ZnSOD activity was calculated by deducting the activity of Mn-SOD from total SOD activity. The activity of catalase was assayed by the method of Sinha [31] and total GSH content by the method of Anderson [32]. Selenium-dependent glutathione peroxidase (GPx) and Se-independent GPx activity were assayed by the method of Rotruck et al. [33] and Lawrence and Burk [34], respectively. The activities of gamma-glutamyl transpeptidase (GGT) and GR were assayed by the methods of Fiala et al. [35] and Carlberg and Mannervick [36], respectively. The protein content was estimated by the method of Lowry et al. [37].

*Immunohistochemistry*

The antibodies used in the present study were as follows: mouse monoclonal antibodies for CYP1A1 (gifted by J. J. Stegeman (WHOI) and H. V. Gelboin (NCI), CYP1B1 (Santa Cruz), 8-OHdG (JaICA, Japan) and rabbit polyclonal antibody for RECK (Santa Cruz). Immunohistochemistry was performed as described previously [38]. The immunohistochemical data are expressed as the number of cells with positive staining per 400 counted cells in a random high power field. The scoring was conducted independently by RV and PM, who were blinded to treatment sequence.

*SDS-PAGE and Western blot analysis*

Proteins separated by SDS-PAGE were electrophoretically transferred to polyvinylidene difluoride membranes. The blots were incubated in 1X PBS containing 5% non-fat dry milk for 2 h to block non-specific binding sites. The blot was incubated with primary antibody (diluted according to the manufacturer's instructions) overnight at 4°C. After washing, the blots were incubated with 1:1000 dilution of horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) for 45 min at room temperature. After extensive washes with high and low salt buffers, the immunoreactive proteins were visualized using enhanced chemiluminescence detection reagents (Sigma). Densitometry was performed on IISP scanner and quantitated with Total Lab 1.11 software.

*Reverse transcriptase (RT) reaction: cDNA synthesis*

Reverse-transcription of isolated RNA (1 μg) to cDNA and further PCR amplification was done as described previously [38]. Table I provides details of primer sequences and thermocycling conditions for PCR reactions. Amplification products were analysed by electrophoresis in a 2% agarose gel containing ethidium bromide with 100 bp DNA ladder. The PCR

Table I. Oligonucleotide primers and thermocycling conditions for RT-PCR.

| Gene Product | Primer sequences | Fragment size (bp) | Initial denaturation | Denaturation | Annealing | Extension | Final extension | No. of cycles |
|---|---|---|---|---|---|---|---|---|
| NQO1 | Sense 5′-ATTGTACTGGGCCATTCAGA-3′<br>Antisense 5′-GGCCATTGTTTACTTTGAG-3′ | 498 | 94°C, 5 min | 94°C, 1 min | 56°C, 1 min | 72°C, 2 min | 72°C, 7 min | 30 |
| Mn-SOD | Sense 5′-CCTGAACGTCACCCGAGGAGAA-3′<br>Antisense 5′-CTGCAGTACTCTATACCACTACA-3′ | 512 | 94°C, 5 min | 94°C, 1 min | 68°C, 1 min | 72°C, 2 min | 72°C, 10 min | 32 |
| Catalase | Sense 5′-GGTGAGATCGAATGGAT-3′<br>Antisense 5′-GGCGATGGCATTGAA-3′ | 486 | 94°C, 5 min | 94°C, 30 s | 49°C, 30 s | 72°C, 2 min | 72°C, 7 min | 29 |
| PlGF | Sense 5′- CATGGACTTTGACCACTGC -3′<br>Antisense 5′- CAAGAGAATCTGGCTTGGC -3′ | 150 | 95°C, 5 min | 95°C, 20 s | 58°C, 20 s | 72°C, 20 s | 72°C, 7 min | 45 |
| VEGF | Sense 5′-ATGAACTTTCTGCTGTCTTGG-3′<br>Antisense 5′-TCACCGCCTCGGCTTGTCACA-3′ | 444 and 576* | 94°C, 10 min | 95°C, 30 s | 51°C, 30 s | 72°C, 1 min | 72°C, 7 min | 40 |
| VEGFR1 | Sense 5′- AGGAGAGGACCTGAAACTGTCTT -3′<br>Antisense 5′- ATTCCTGGGCTCTGCAGGCATAG -3′ | 230 | 95°C, 5 min | 95°C, 30 s | 65°C, 30 s | 72°C, 1 min | 72°C, 6 min | 28 |
| VEGFR2 | Sense 5′- GTGATTGCCATGTTCTTCTGGC -3′<br>Antisense 5′- TCAGACATGAGAGCTCGATGCT -3′ | 268 | 95°C, 5 min | 95°C, 30 s | 60°C, 30 s | 72°C, 1 min | 72°C, 7 min | 30 |
| MMP-2 | Sense 5′-GGCCCTGTCACTCCTGAGAT-3′<br>Antisense 5′-GGCATCCAGGTTATCGGGGA-3′ | 249 | 94°C, 5 min | 94°C, 1 min | 64°C, 1 min | 72°C, 1 min | 72°C, 7 min | 32 |
| MMP-9 | Sense 5′-AGTTTGGTGTCGCGGAGCAC-3′<br>Antisense 5′-TACATGAGCGCTTCCGGCAC-3′ | 753 | 95°C, 5 min | 95°C, 30 s | 60°C, 30 s | 72°C, 1 min | 72°C, 7 min | 30 |
| TIMP-2 | Sense 5′- GTTTTGCAATGCAGACGTAG -3′<br>Antisense 5′- ATGTCAAGAAACTCCTGCTT-3′ | 539 | 94°C, 1 min | 94°C, 1 min | 60°C, 1 min 20 s | 72°C, 1 min | 72°C, 7 min | 30 |
| β-actin | Sense 5′ AACCGCGAGAAGATGACCCAGAT-CATGTTT-3′<br>Antisense 5′-AGCAGCCGTGGCCATC TCTTGCTCGAAGTC-3′ | 350 | 94°C, 5 min | 95°C, 1 min | 55°C, 1 min | 72°C, 1 min | 72°C, 7 min | 30 |

* The primers for VEGF detect two of four different molecular species produced by alternative splicing of mRNA-VEGFI21 and VEGFI65, with expected fragment sizes of 444 bp and 576 bp, respectively.

products were visualized as bands with a UV-transilluminator and photographs were taken using a gel documentation system (GelDocMega™, UK).

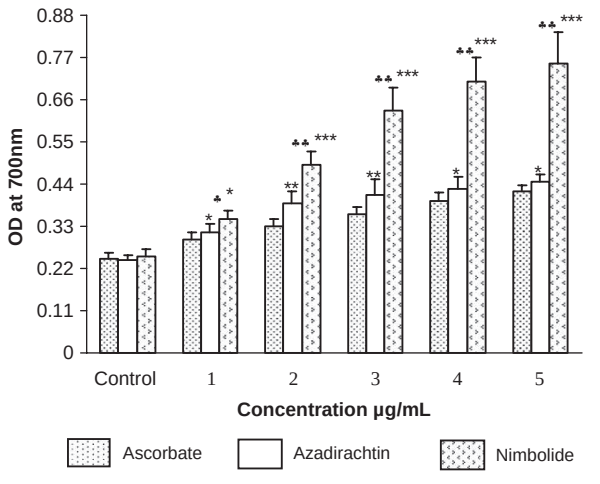
### *Statistical analysis*

The data are expressed as mean ± SD. The $IC_{50}$ for *in vitro* antioxidant potential was calculated using linear regression analysis. The reduction potential and changes in body weight were statistically compared by Student's *t*-test. Statistical analysis on the data for tumour incidence was carried out using Fischer's probability test. The data for biochemical assays and densitometric analysis were analysed using analysis of variance (ANOVA) and the group means were compared by the least significant difference test (LSD). The results were considered statistically significant if $p < 0.05$.

## Results

### In vitro *antioxidant assays*

Table II shows the $IC_{50}$ values of ascorbate, azadirachtin and nimbolide against various free radicals. Analysis of the free radical scavenging activities of azadirachtin and nimbolide revealed a concentration-dependent anti-radical activity resulting from reduction of $DPPH^{\bullet}$, $ABTS^{\bullet+}$, superoxide ($O^{\bullet-}$), hydroxyl ($OH^{\bullet}$) and nitric oxide (NO) radicals to non-radical form. The scavenging activity of azadirachtin and nimbolide was higher compared to ascorbic acid, a known antioxidant used as positive control and the scavenging potential was in the order: nimbolide > azadirachtin > ascorbate. Figure 2 presents the reduction potential of ascorbate, azadirachtin and nimbolide. The reducing power of ascorbic acid, azadirachtin and nimbolide increased gradually with increasing concentration. The order of the reduction potential was nimbolide > azadirachtin > ascorbate.




* Significantly different from ascorbate (p<0.05) by Student's *t* test.
** Significantly different from ascorbate (p<0.01)
*** Significantly different from ascorbate (p<0.001)
♣ Significantly different from azadirachtin (p<0.05).
♣♣ Significantly different from azadirachtin (p<0.001).

Figure 2. Reducing potential of azadirachtin and nimbolide *in vitro* (mean ± SD; $n = 6$).

Table II. $IC_{50}$ values of ascorbate, azadirachtin and nimbolide against various free radicals.

| | $IC_{50}$ values | | |
|---|---|---|---|
| Antioxidant activity | Ascorbate | Azadirachtin | Nimbolide |
| DPPH scavenging (µg/ml) | 4.94 | 3.53 | 2.23 |
| ABTS scavenging (µg/ml) | 5.31 | 3.28 | 2.90 |
| $OH^{\bullet}$ scavenging (µg/ml) | 4.18 | 4.54 | 2.11 |
| $O_2^{\bullet-}$ scavenging (µg/ml) | 5.34 | 4.08 | 2.82 |
| NO scavenging (µg/ml) | 4.51 | 3.48 | 2.59 |

$IC_{50}$ values were determined by plotting dose response curves of radical scavenging activities vs concentration of azadirachtin and nimbolide using GraphPad Prism version 4.00 for Windows, (GraphPadTM Software Inc., San Diego, CA).

### *Tumour incidence and histopathological observations*

Table III shows changes in the body weight, tumour incidence and histopathological changes in control and experimental animals. The mean final body weights were significantly decreased in group 1 compared to control (group 8). No significant differences in the body weights were observed in groups 2–8. In DMBA painted animals (group 1), the incidence of SCC was 100% with a tumour multiplicity of 1.7 per hamster. These tumours were large and exophytic with a mean tumour burden of 208.74 $mm^3$. In group 5, three of 10 animals developed SCC, while others exhibited moderate-to-severe dysplasia without infiltration. Although no tumours were observed in groups 2–4, histopathological examination of pouches revealed varying degrees of hyperplasia, hyperkeratosis and dysplasia. While administration of azadirachtin and nimbolide at 10 and 100 µg/kg bw decreased tumour incidence as well as pre-neoplastic lesions, the inhibitory effect was more pronounced at 10 µg/kg bw of azadirachtin and nimbolide, respectively. In groups 6–8, the epithelium was normal, intact and continuous. Representative photomicrographs of histopathological changes in the buccal pouch mucosa of control and experimental animals are shown in Figure 3.

### *Biochemical assays*

Figure 4 shows phase I and phase II xenobiotic-metabolizing enzyme activities in the S9 fraction of the buccal pouches of control and experimental animals. Administration of DMBA (group 1) increased the activities of phase I and II enzymes in the pouch compared to control (group 8). Intragastric administration of azadirachtin and nimbolide to DMBA painted animals significantly decreased phase I enzyme activities and elevated phase II enzyme

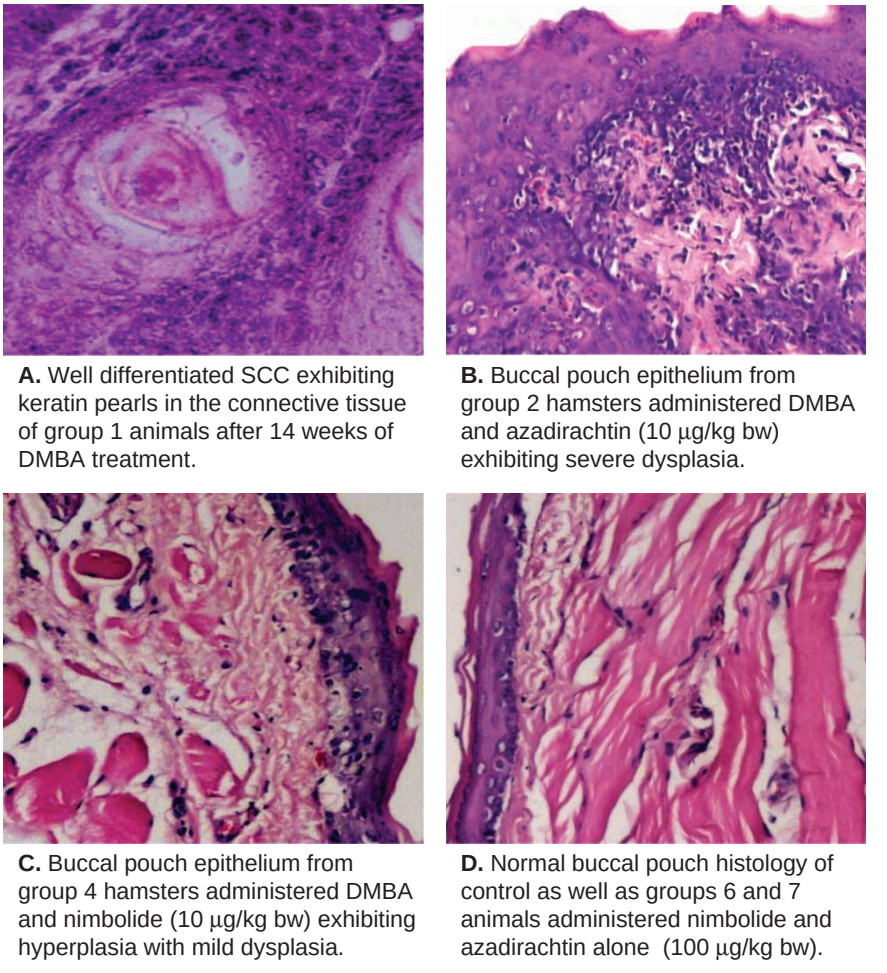
Table III. Body weight, tumour incidence and histopathological changes in control and experimental animals (mean ± SD; $n = 10$).

| Group | Treatment | Body weight (g) | *Keratosis* | Hyperplasia | *Dysplasia* | SCC (%) |
|---|---|---|---|---|---|---|
| 1. | DMBA | 129.18 ± 14.62* | +++ | +++ | +++ | 10/10 (100) |
| 2. | DMBA + Azadirachtin (10 μg/kg bw) | 132.65 ± 11.67 | ++ | +/++ | +++ | — |
| 3. | DMBA + Azadirachtin (100 μg/kg bw) | 129.56 ± 13.25 | +++ | +++ | ++/+++ | 3/10 (30)** |
| 4. | DMBA + Nimbolide (10 μg/kg bw) | 134.33 ± 13.98 | +++ | ++ | + | — |
| 5 | DMBA + Nimbolide (100 μg/kg bw) | 131.34 ± 14.23 | +++ | ++ | +/++ | — |
| 6 | Azadirachtin (100 μg/kg bw) | 137.12 ± 14.65 | — | — | — | — |
| 7 | Nimbolide (100 μg/kg bw) | 138.23 ± 14.97 | — | — | — | — |
| 8 | Control | 141.34 ± 15.47 | — | — | — | — |

+ = mild, ++ = moderate, +++ = severe, — = no change, SCC = Squamous cell carcinoma.
* Significantly different from group 8 by Student's *t*-test ($p < 0.01$).
** Significantly different from group 1 by Fischer's probability test ($p < 0.05$).

activities in the pouch compared to group 1. However, azadirachtin and nimbolide at 10 μg/kg bw (groups 2 and 4) showed greater modulatory effects on xenobiotic-metabolizing enzymes compared to groups 3 and 5. Although treatment with azadirachtin and nimbolide (100 μg/kg bw) alone did not induce any significant change in phase I enzymes (groups 6 and 7), the activities of phase II enzymes in the pouch were significantly increased compared with group 8 (control).

The changes in the levels of GSH and the activities of SOD (total SOD, Mn-SOD, Cu-Zn SOD), CAT and GSH-dependent enzymes in the buccal pouch of control and experimental animals are shown in Figure 5. A significant increase in the concentration of GSH and the activities of GPx (Se-dependent and



**A.** Well differentiated SCC exhibiting keratin pearls in the connective tissue of group 1 animals after 14 weeks of DMBA treatment.

**B.** Buccal pouch epithelium from group 2 hamsters administered DMBA and azadirachtin (10 μg/kg bw) exhibiting severe dysplasia.

**C.** Buccal pouch epithelium from group 4 hamsters administered DMBA and nimbolide (10 μg/kg bw) exhibiting hyperplasia with mild dysplasia.

**D.** Normal buccal pouch histology of control as well as groups 6 and 7 animals administered nimbolide and azadirachtin alone (100 μg/kg bw).

Figure 3. Photomicrographs of histopathological changes in the buccal pouch mucosa of control and experimental animals (Hematoxylin and eosin, ×10).

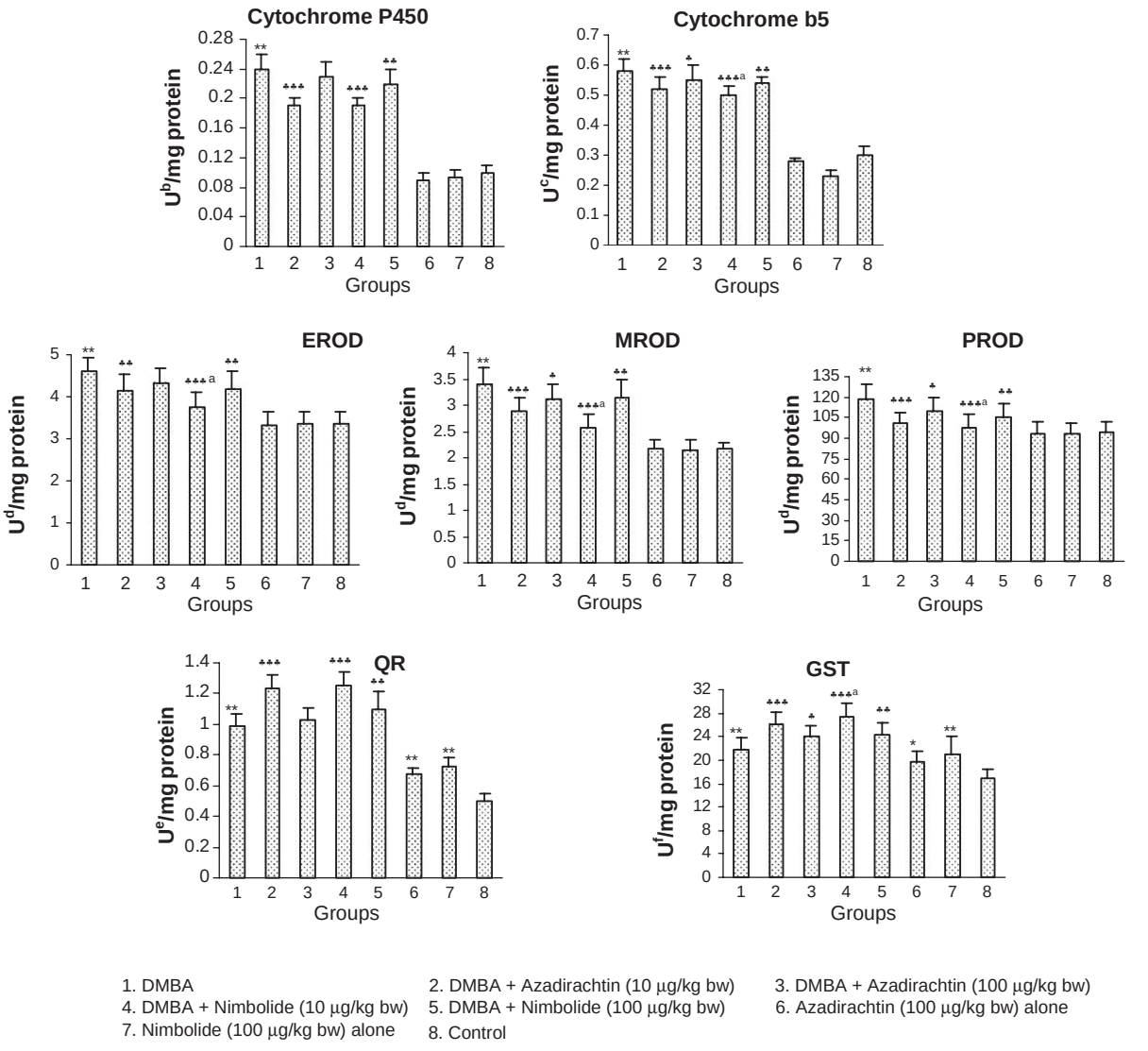
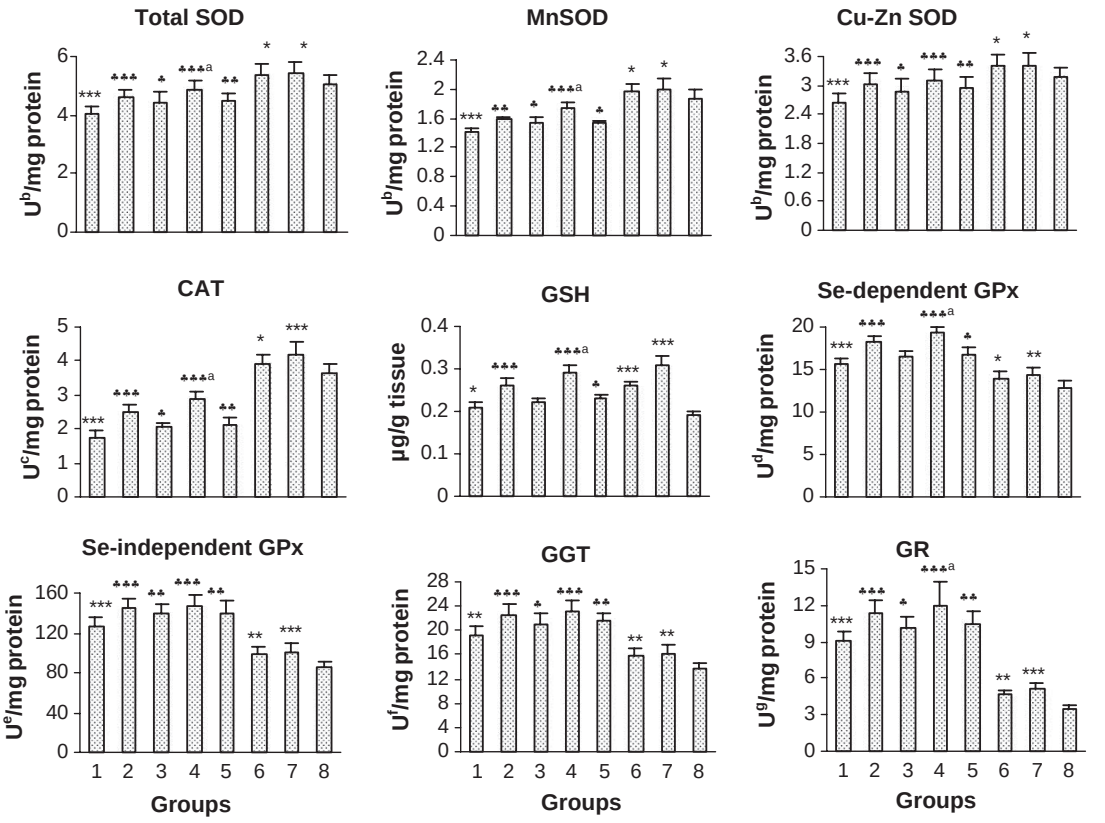



Figure 4. Activities of phase I and II enzymes in the S9 fractions of the buccal pouch of control and experimental animals (mean ± SD; $n = 10$).

independent), GR and GGT was associated with decreased activities of SODs and CAT in the buccal pouch of DMBA painted animals compared to control. Administration of azadirachtin and nimbolide significantly increased all the antioxidant enzymes in the buccal pouch of animals in groups 2–5. However, treatment with azadirachtin and nimbolide at 10 µg/kg bw (groups 2 and 4) was more effective. Treatment with azadirachtin and nimbolide (100 µg/kg bw) alone significantly enhanced GSH and the activities of all the antioxidant enzymes in groups 6 and 7 animals compared to control.

*Immunohistochemical analysis*

Figure 6 shows the effect of azadirachtin and nimbolide on CYP1A1, CYP1B1, 8-OHdG and RECK expression in the buccal mucosa of control and experimental animals. In DMBA-painted animals (group 1), the expression of CYP1A1, CYP1B1 and 8-OHdG was significantly higher and that of RECK was significantly lower compared to control animals (group 8). Administration of both azadirachtin and nimbolide at 10 µg/kg bw significantly decreased the expression of CYP1A1, CYP1B1 and 8-OHdG and up-regulated RECK expression compared to group 1 animals. The effects




* Significantly different from group 8 ($p<0.05$) ANOVA followed by LSD
** Significantly different from group 8 ($p<0.01$) ANOVA followed by LSD
*** Significantly different from group 8 ($p<0.001$) ANOVA followed by LSD
♣ Significantly different from group 1 ($p<0.05$)
♣♣ Significantly different from group 1 ($p<0.01$)
♣♣♣ Significantly different from group 1 ($p<0.001$)
a Significantly different from groups 2, 3 and 5 ($p<0.05$)
b Amount of enzyme required to give 50% inhibition of NBT reduction
c μmoles of $H_2O_2$ utilized per minute
d μmoles of GSH utilized per min
e μmoles of NADPH utilized per min
f μmoles of p-nitroaniline formed per h
g μmoles of NADPH oxidized per h

Figure 5. The levels of GSH and activities of SODs, CAT, Se-dependent and independent GPx, GGT and GR in the buccal pouch control and experimental animals (mean ± SD; $n = 10$).

were more pronounced in animals treated with nimbolide compared to azadirachtin treated animals.

*Western blot analysis*

Figure 7 shows the representative Western blot analysis of markers of carcinogen activation (CYP1A1 and CYP1B1), invasion (MMP-2, MMP-9, TIMP-2 and RECK) and angiogenesis (HIF-1α and VEGF) as well as HDAC-1 in the buccal pouch of control and experimental animals. Topical application of DMBA significantly increased the expression of CYP1A1, CYP1B1, MMP-2, MMP-9, VEGF, HIF-1α and HDAC-1 and decreased the expression of TIMP-2 and RECK compared to control. While administration of azadirachtin and nimbolide at a concentration of 10 μg/kg bw to DMBA painted animals (groups 2 and 3) significantly decreased the expression of CYP1A1, CYP1B1, MMP-2, MMP-9, VEGF, HIF-1α and HDAC-1

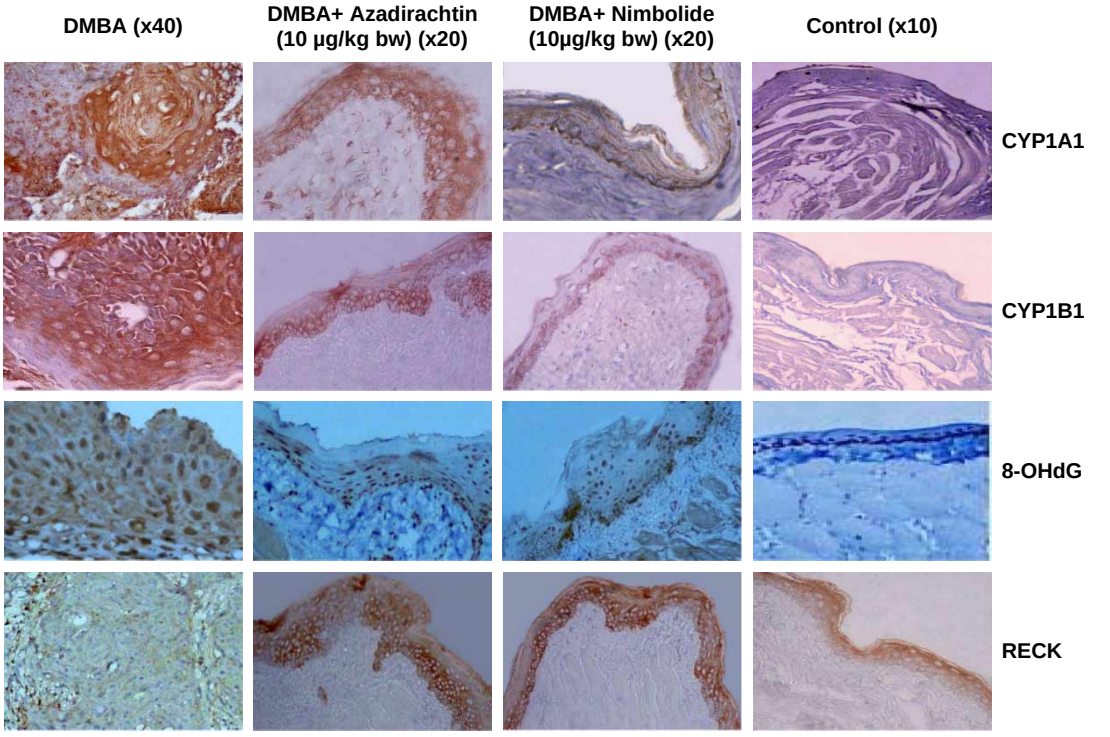



Figure 6. Photomicrographs of immunohistochemical staining of CYP1A1, CYP1B1, 8-OHdG and RECK in control and experimental animals (Avidin-biotin peroxidase method, Hematoxylin counterstain).



and increased the expression of TIMP-2 and RECK compared to group 1 animals, nimbolide was more effective in modulating the above markers. $\beta$-actin was used as a loading control.

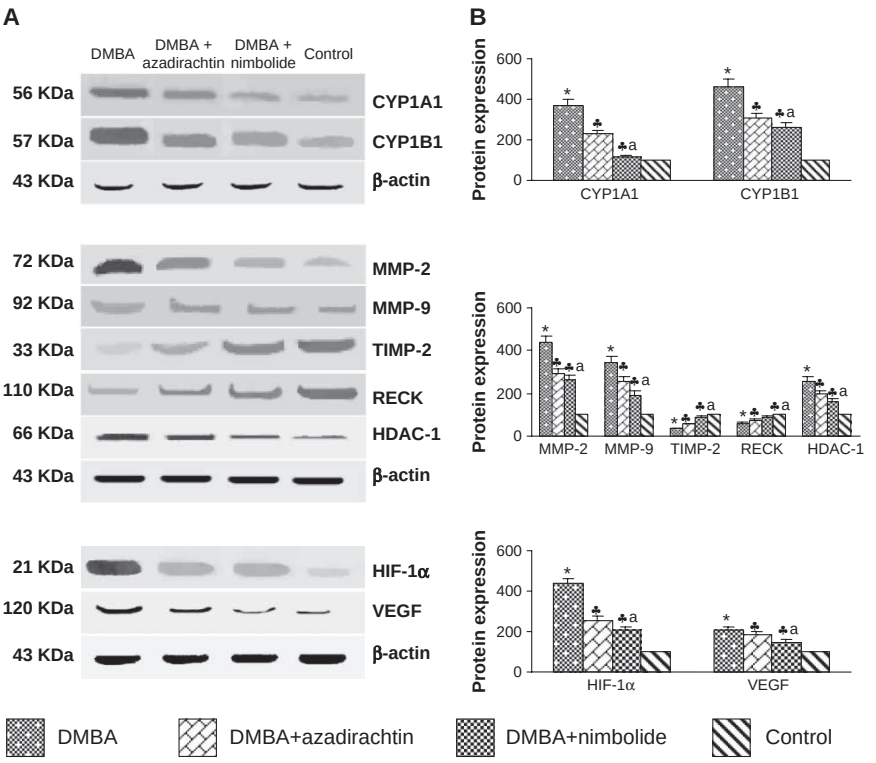
### *RT-PCR analysis*

Figure 8 shows the representative RT-PCR data for markers of antioxidant and xenobiotic-metabolism, tumour invasion and angiogenesis in the buccal pouch of control and experimental animals. Quantification of each band by densitometric scanning shows significant increase in the expression of PlGF, VEGF, VEGFR1, VEGFR2, MMP-2 and MMP-9, with decreased expression of NQO1, Mn-SOD, catalase and TIMP-2 in DMBA painted animals (group 1) compared to control. Although administration of both azadirachtin and nimbolide at 10 µg/kg bw significantly decreased PlGF, VEGF, VEGFR1, VEGFR2, MMP-2 and MMP-9 expression and increased the expression of NQO1, MnSOD, catalase and TIMP-2, the effects were more pronounced in animals treated with nimbolide compared to group 1. β-actin was used as an internal control.

## Discussion

Neem preparations as well as the neem limonoids azadirachtin and nimbolide have been documented to inhibit the growth of malignant cells *in vitro* [14–16,39,40]. Although neem extracts and fractions have shown chemopreventive potential in different animal tumour models, there are no reports on the *in vivo* protective effects of azadirachtin and nimbolide on tumorigenesis. We report for the first time inhibition of DMBA-induced HBP carcinogenesis by azadirachtin and nimbolide based on reduced incidence of pre-neoplastic and neoplastic lesions and modulation of XME, antioxidant status, 8-OHdG and markers of invasion and angiogenesis.

The decrease in CYP activity and expression of CYP isoforms and 8-OHdG by azadirachtin and nimbolide may be attributed to increased activities of phase II enzymes and antioxidants that can block the generation of toxic electrophiles and ROS and inhibit formation of DNA adducts. These findings are in line with studies by us as well as others that demonstrated a positive correlation between modulation of XME and up-regulation of antioxidants by neem leaf extracts in rodent models of carcinogenesis [12,41]. Dual-acting agents such as azadirachtin and nimbolide that inhibit phase I carcinogen activation enzymes and simultaneously induce phase II carcinogen detoxification enzymes are useful in chemoprevention, because they are capable of interfering with early stages of carcinogenesis. In particular, inhibitors of CYP1B1 have assumed significance in oral cancer prevention and therapy [42]. The potent *in vitro* ROS scavenging properties of azadirachtin and nimbolide




A Representative immunoblots of CYP1A1, CYP1B1, MMP-2, MMP-9, TIMP-2, RECK, HDAC-1, HIF-1α, and VEGF. Protein samples (50 μg/ lane) resolved on SDS-PAGE gels were probed with corresponding antibodies.

B Densitometric analysis. The mean protein expression from control lysates for ten determinations was designated as 100% in the graph. Each bar for other experimental groups represents the mean protein expression ± SD of ten determinations. β-actin was used as loading control.

* Significantly different from control ($p<0.001$) ANOVA followed by LSD

♣ Significantly different from DMBA ($p<0.01$) ANOVA followed by LSD

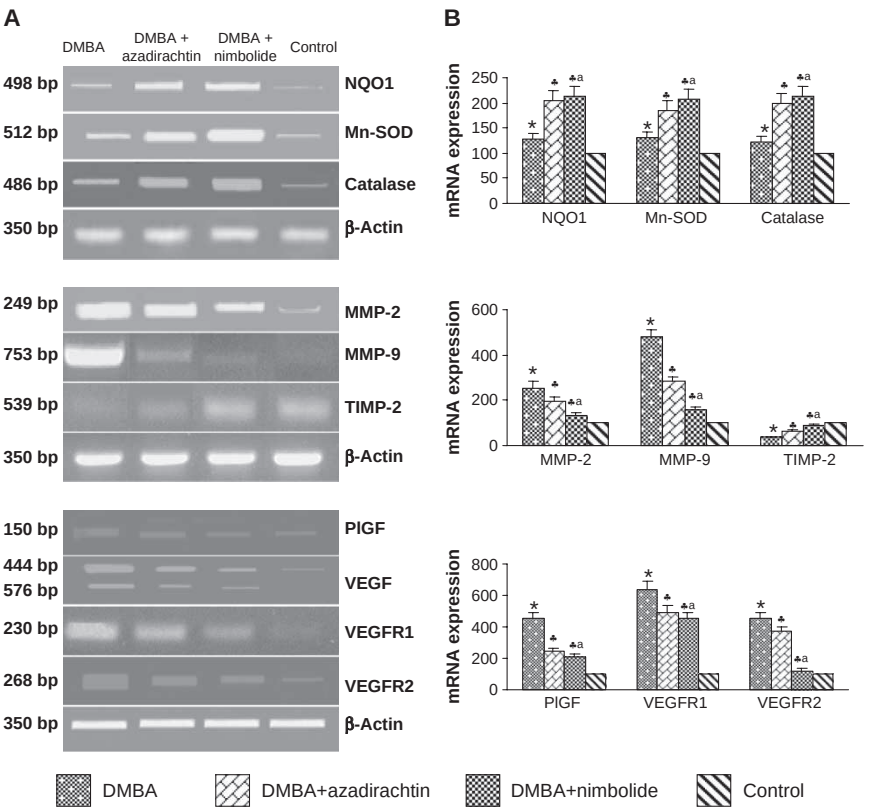
a Significantly different from DMBA + Azadirachtin (10 μg/kg bw) treated group ($p<0.05$)

Figure 7. Representative western blot analysis of markers of carcinogen activation (CYP1A1 and CYP1B1), invasion (MMP-2, MMP-9, TIMP-2 and RECK), HDAC-1 and angiogenesis (HIF-1$\alpha$ and VEGF) in the pouch of control and experimental animals (mean $\pm$ SD, $n=10$).

as well as their *in vivo* antioxidant enhancing effects observed by us in the present study lend credence to the tenet that antioxidants decrease carcinogen-induced oxidative DNA damage, a potentially critical event in neoplastic transformation [43]. In addition to mitigating oxidative DNA damage, antioxidant phytochemicals are recognized to influence invasion, metastasis and angiogenesis associated with tumour progression [44].

Alterations in the extent and distribution of markers of invasion and angiogenesis have been documented in the HBP model [44,38]. Increased expression of MMP-2 and -9 with downregulation of TIMP-2 and RECK observed in HBP tumours in the present study indicates ECM degradation and is in line with similar reports in human and experimental cancers [7,44,45]. RECKlessness seen in HBP carcinomas is a hallmark of cancer and may be correlated to increased expression of HDAC-1. While both RECK and TIMPs inhibit MMPs, RECK is membrane-anchored and acts in a localized manner at the cell surface, whereas TIMPs being diffusible can act in longer distances [7]. Overexpression of HIF-1$\alpha$ can trigger neovascularization in HBP carcinomas, as evidenced by enhanced expression of VEGF, PlGF and VEGFR1 and R2. Downregulation of proinvasive and angiogenic proteins with up-regulation of their inhibitors by azadirachtin and nimbolide observed in the present study is in line with the anti-invasive and anti-angiogenic potential of neem preparations [12,46]. Inhibition of HDAC-1 by these limonoids is of significance in the context of the growing interest in the involvement of epigenetic alterations in cancer in general and the potential anti-cancer effects of HDAC inhibitors in particular [47].

Among the multitude of molecules involved in invasion and angiogenesis, MMPs and VEGF have become major targets for therapeutic intervention and agents that inhibit these molecules have entered clinical trials [48,49]. Recent studies have shown




A Representative RT-PCR analyses of NQO1, Mn-SOD, catalase, MMP-2, MMP-9, TIMP-2, PlGF, VEGF, VEGFR1and VEGFR2 are indicated.
B Densitometric analysis
* Significantly different from control ($p<0.001$) ANOVA followed by LSD
♣ Significantly different from DMBA ($p<0.01$)
a Significantly different from DMBA + Azadirachtin (10 μg/kg bw) treated group ($p<0.05$)

Figure 8. The effect of azadirachtin and nimbolide on mRNA expression of NQO1, Mn-SOD, catalase, MMP-2, MMP-9, TIMP-2, PlGF, VEGF, VEGFR1, VEGFR2, and $\beta$-actin in the pouch of control and experimental animals (mean $\pm$ SD, $n=10$).

that an $\alpha$PlGF monoclonal antibody could serve as a safer anti-angiogenic agent than an $\alpha$VEGF monoclonal antibody and could perhaps substitute for or augment the effect of anti-VEGF therapy [50,51]. Despite the strong correlation between the extent of RECK expression and improved prognosis in multiple cancers, the therapeutic potential of this protein has remained largely unexplored [7,45]. However, a major obstacle in treatment targeted against individual molecules is the propensity for malignant tumours to switch to production of other pro-invasive/angiogenic molecules. Agents such as azadirachtin and nimbolide that target multiple molecules involved in invasion and angiogenesis are an attractive option for preventing tumour progression.

The results of the present study demonstrate that azadirachtin and nimbolide inhibit development of DMBA-induced HBP carcinomas, owing to their ability to modulate phase I and phase II xenobiotic-metabolizing enzyme activities, enhance antioxidant defence systems, reduce oxidative DNA damage, block tumour invasion and angiogenesis and inhibit HDAC. The chemopreventive efficacy of nimbolide was however more significant than azadirachtin and achievable even at a low dose of 10 μg/kg body weight. Studies have shown that nimbolide is the most important contributor to the cytotoxicity of neem extracts and its higher efficacy has been attributed to the $\alpha,\beta$-unsaturated ketone element [52]. Cohen et al. [53] found nimbolide to be the most potent of the six neem limonoids examined for cytotoxicity against a panel of cancer cell lines. Recently, we reported the anti-proliferative and pro-apoptotic effects of nimbolide in choriocarcinoma cells *in vitro* [16]. Taken together, these results suggest that nimbolide offers promise as a candidate agent for cancer prevention and therapy.

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