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A synthetic coumarin (4-Methyl-7 hydroxy coumarin) has anti-cancer potentials against DMBA-induced skin cancer in mice

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ABSTRACT

Scopoletin, an alkaloid separated from ethanolic extract of the medicinal plant, *Gelsemium sempervirens* (Fam: Loganiaceae) has been reported to have anti-cancer potentials. The synthetic coumarin (4-Methyl-7 hydroxy coumarin) derived from resorcinol and ethyl aceto-acetate in presence of concentrated sulphuric acid is structurally close to scopoletin, being a coumarin derivative. Whether this synthetic compound also has anti-cancer potentials has been evaluated *in vivo* on DMBA (7,12-Dimethylbenz[a]anthracene) induced skin cancer in mice by analyzing results of several cytogenetic endpoints, Comet assay, and fluorescence activated cell sorting (FACS). Further, expressions of signal proteins like Aryl hydrocarbon receptor , p53, PCNA, Akt, Bcl-2, Bcl-xL, Bad, Bax, NF-kB Apaf, IL-6, Cytochrome-c, Caspase-3 and Caspase-9 were studied by immunoblot analysis along with histology of skin and immuno-histochemical localization of Aryl hydrocarbon receptor and PCNA in DMBA treated mice vis-a-vis carcinogen treated synthetic coumarin fed mice. Feeding of this synthetic coumarin induced positive modulations in expression of all biomarkers in DMBA administered mice, giving clues on its possible signaling pathway(s) - primarily through down-regulation of Aryl hydrocarbon receptor and PCNA and up-regulation of apoptotic proteins like Bax, Bad, Cytochrome c, Apaf, Caspase-3 and Caspase-9, resulting in an appreciable reduction in growth of papilloma in mice. Therefore, this synthetic coumarin shows promise for use in cancer therapy, particularly in skin cancer.

1. Introduction

7,12 Dimethylbenz[a]anthracine (DMBA) is a prototypical polycyclic aromatic hydrocarbon, a major class of environmental carcinogen that has been used to promote tumors in laboratory animals (Medina, 1974). Mammary and skin tumors (Das et al., 2005) can be produced in mice following administration of DMBA that causes up-regulation of the cellular cytosolic receptor, the Aryl hydrocarbon receptor. Upon ligand activation, the Aryl hydrocarbon receptor translocates into the nucleus and associates with the co-factor Aryl nuclear translation protein (Denison and Nagy 2003) to induce CYP1A1, CYP1A2 and CYP1B1 genes There is growing evidence that Aryl hydrocarbon receptor also regulates various down-stream proteins including proliferating cell nuclear antigen (PCNA) (Abdelrahim et al., 2003).

Further, other mechanisms like generation of reactive oxygen species and nuclear factor –kB (NF-kB) are also important controlling factors in proliferation and branching (Brantley et al., 2001). Thus because of the central role of Aryl hydrocarbon receptor and PCNA in many xenobiotics related toxicity and carcinogenesis, the search for chemical inhibitors of Aryl hydrocarbon receptor and PCNA has assumed importance in chemoprevention.

Several pathways are known to be mediated through p53-induced apoptosis and one of these involves the Bcl-2 and Bax proteins. The Bcl-2 family consists of both pro-apoptotic and anti-apoptotic members that elicit opposing effect on mitochondria. Bax promotes release of Cytochrome c into the cytosol from mitochondria that activates Caspase-3, which executes apoptosis (Donovan and Cotter, 2004) via the activation of Caspase-9. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL, which are transcriptionally suppressed by p53, preserve the integrity of mitochondria (Ryan et al., 2001) that blocks the release of Cytochrome c preventing apoptosis.

We (Bhattacharyya et al., 2008) demonstrated earlier anti-cancer potential of scopoletin, chemically a coumarin analogue, isolated from the ethanolic extract of plant, *Gelsemium sempervirens* (Family Loganiaceae) on an *in-vitro* human cancer cell line.

Thus, the hypothesis to be tested was whether a synthetic coumarin (4-Methyl-7-hydroxycoumarin) can also exhibit anti-cancerous properties against skin cancer induced *in vivo* by application of DMBA in

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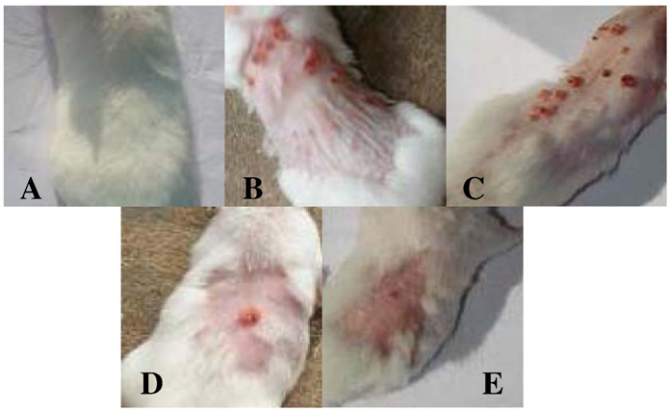


Fig. 1. External morphology of mice skin: A) Normal B) DMBA + Croton oil C) DMBA + Croton oil + alcohol D) DMBA + Croton oil + Synthetic coumarin (50 mg/kg bw) E) DMBA + Croton oil + Synthetic coumarin (100 mg/kg bw).

mice by employing such endpoints as: a) study of chromosome aberrations, micronulei, sperm head anomaly; b) DNA damage-through Comet assay; c) cell cycle analysis-through fluorescence activated cell sorting (FACS) and d) cell signaling responses-through expressions of Aryl hydrocarbon receptor, p53, Bcl-2, Bcl-xL, Akt, Bax, Bad, Apaf, NF- kB , IL-6, PCNA, Cytochrome c and Caspase-3 and -9 in mice subjected to DMBA/croton oil treatment vis-a vis mice fed synthetic coumarin alongside.

2. Materials and methods

2.1. Materials

Swiss albino mice (*Mus musculus*) of both sexes were inbred and reared in the animal house under standard hygienic conditions and food. The experiments were performed with clearance from and as per the guidelines of the Ethical Committee, University of Kalyani. Healthy mice weighing between 22–26 g were selected randomly for use in the experiment. The selected mice were subdivided into 4 groups, each comprising six mice:

Group 1 — negative control: mice fed normal diet and water *ad libitum* without any treatment on skin

Group 2 — carcinogen administered: mice were gently rubbed regularly on their skin of back with 100 µg DMBA (once a week) and with 1% Croton oil (twice a week) for 24 weeks.

Group 3 — carcinogen administered 2% alcohol (placebo) fed: DMBA and Croton oil administered mice fed for 24 weeks 100 µl of 2% alcohol (placebo) (vehicle of synthetic coumarin) once daily.

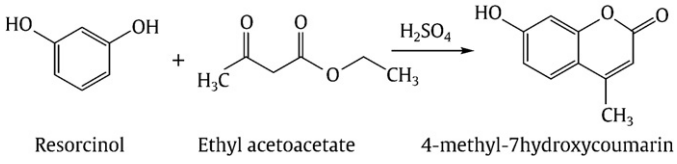
Group 4 — carcinogen administered synthetic coumarin fed: DMBA and Croton oil administered mice fed synthetic coumarin in two doses, 50 and 100 mg per kg body weight, respectively, once daily for 24 weeks.

All chemicals used were of Analytical grade and procured from Sigma, USA.

2.2. Preparation of 4-Methyl-7-hydroxy coumarin

The preparation of 4-Methyl-7-hydroxy coumarin was done by following the method of Kiskhan and Yagci (2007) as shown below. Concentrated H_2SO_4 (100 ml) was added to a 250 ml round flask and cooled in an ice bath at 0 °C. A solution of resorcinol (10 g, 90.8 mM) in ethyl aceto-acetate (12 ml, 95.0 mM) was added drop-wise to the flask with constant stirring, and the temperature was kept below 10 °C. The contents were stirred overnight at room temperature. Then, the reaction mixture was poured onto crashed ice with vigorous stirring. A solid product that formed instantly was filtered under suction and washed with copious amounts of cold water. The raw material was re-crystallized from ethanol to obtain pure 4-Methyl-7-hydroxycoumarin with an approximately 70% yield.

While scopoletin has the chemical formula $C_{10}H_8O_4$, with a molecular weight 192.7, the scopoletin derivative tested in the present study (i.e., 4-Methyl-7 hydroxy coumarin; henceforth called "synthetic coumarin") has the chemical structure of $C_{10}H_8O_3$ with a molecular weight 176.



2.3. Incidence of skin tumors

The onset of tumors (>2 mm) commenced at 8 weeks in DMBA + Croton oil treated mice and reached 100% at 24 weeks. The number of tumor formation in DMBA/Croton oil treated groups and also the carcinogen + synthetic coumarin fed groups were recorded weekly.

2.4. Cytogenetic assay

The standard cytogenetic protocols like assays of chromosome aberrations, micronuclei , mitotic index and sperm head anomaly have been adopted in the present study (Biswas et al., 2004). Mice were intra-peritoneally injected with 0.025% colchicine at the rate of 1 ml/ 100 mg body weight 1 h and 30 min before sacrifice. Marrow of the femur was flushed in 1% sodium citrate solution at 37 °C and fixed in acetic acid/ ethanol (1:3). Slides were prepared by the conventional flame drying technique followed by Giemsa staining for scoring bone marrow chromosome aberrations. Chromosome aberrations of various natures have been pooled into two categories: the "major type" comprising aberrations like breaks, rings, etc and the "minor types" comprising less significant aberrations like gap, stretching, etc. A total of 300 bone marrow cells were observed. For micronucleus preparation, a part of the suspension of bone marrow cells in 1% sodium citrate was smeared on clean grease free slides, briefly fixed in methanol and subsequently stained with May-Grunwald followed by Giemsa. Approximately 3000 bone marrow cells, comprising both polychromatic erythrocytes and normochromatic erythrocytes were scored. The mitotic index was determined from the same slide that was scanned for micronuclei, and a total of 5000 cells were examined from each series. The non-dividing and dividing cells were recorded and their ratios ascertained. For sperm head anomaly, the technique of

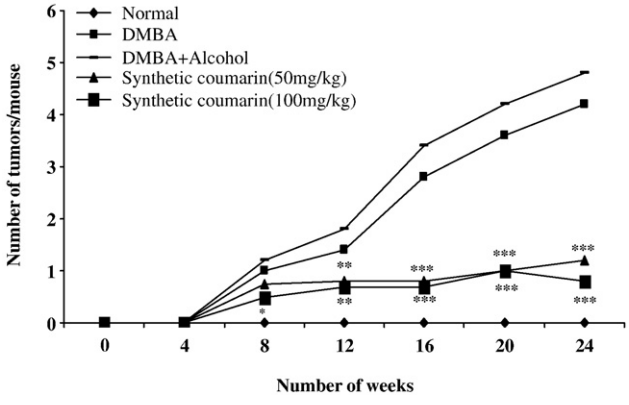


Fig. 2. Number of tumors/mouse plotted as a function of weeks on test. Value represents mean number of tumor per mouse. * = $P<0.05$, ** = $P<0.01$, *** = $P<0.001$.

Wyrobek (Wyrobek et al., 1984) was adopted. 5000 sperm were examined in each series. The epididymis of testis of control and treated series was dissected out separately and its inner content squeezed out into different vials containing 10 ml of 0.87% normal saline. The material was thoroughly shaken, filtered through a silken cloth and smeared on clean grease-free slides. The slides were allowed to air dry and then stained with dilute Giemsa (1 ml Giemsa in 10 ml distilled water). For estimation of DNA damage, Comet assay was performed with single cell gel electrophoresis, as per protocol described elsewhere (Wojewodza et al., 1998). Briefly, peripheral blood nuclear cells (PBMC) (1×10^4) were suspended in 0.6% low melting agarose and layered over a frosted microscopic slide previously coated with a layer of 0.75% normal melting agarose to ensure firm gripping. The slides were then kept at 4 °C for solidification. Subsequently slides were immersed in a lysis buffer of pH 10 and kept overnight for lysis of cell and nuclear membranes. The following day slides were transferred into a horizontal electrophoresis chamber containing electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA; ph 13.0) and presoaked for 20 min in order to unwind DNA. Electrophoresis was then carried out for 20 min (300 mA, 20 V). Slides were then washed thoroughly with neutralizing buffer (Tris 0.4 M, pH 7.5), stained with ethidium bromide (final concentration of 40 µg/ml) and examined under a Lyca fluorescence microscope. Comets were scored with computer aided image analysis system (Motic Image, China). % of DNA damage was determined from analysis of tail length.

For cell cycle analysis, skin cells were taken out from rubbed area of mice and transferred to a dish with antibiotic/antimycotic solution (1:100 in PBS) and incubated for about 5 min at 37 °C. Then the skin cells were spread on a plastic surface and 2 ml of trypsin solution (0.025% trypsin in PBS/EDTA) was added. After storing them in refrigerator for over night, the cells were washed several times and single cell suspensions were made in DMEM medium. Cellular DNA was stained with propidium iodide and quantified by flow cytometry according to Nicoletti's procedure (Nicoletti et al., 1991). Briefly, cells were fixed with 70% (v/v) cold aqueous ethanol(−20 °C) and stored at 4 °C for at least 24 h. The cells were washed in phosphate buffer saline, after cell centrifugation, cell pellets were stained with propidium iodide staining solution containing 10 µg/ml propidium iodide, 5 Kunits of RNase, and 0.1%(v/v) Triton X-100. The cell suspension was incubated in the dark at room temperature for 30 min. By using Cell Quest software program, the distribution of nuclear DNA in different cell cycle phases was determined with the help of a fluorescence-activated cell sorter (Becton Dickinson, Mountain View, CA) equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale). A total of 40,000 events were acquired and MOD-FIT LT software (Becton Dickinson) was used.

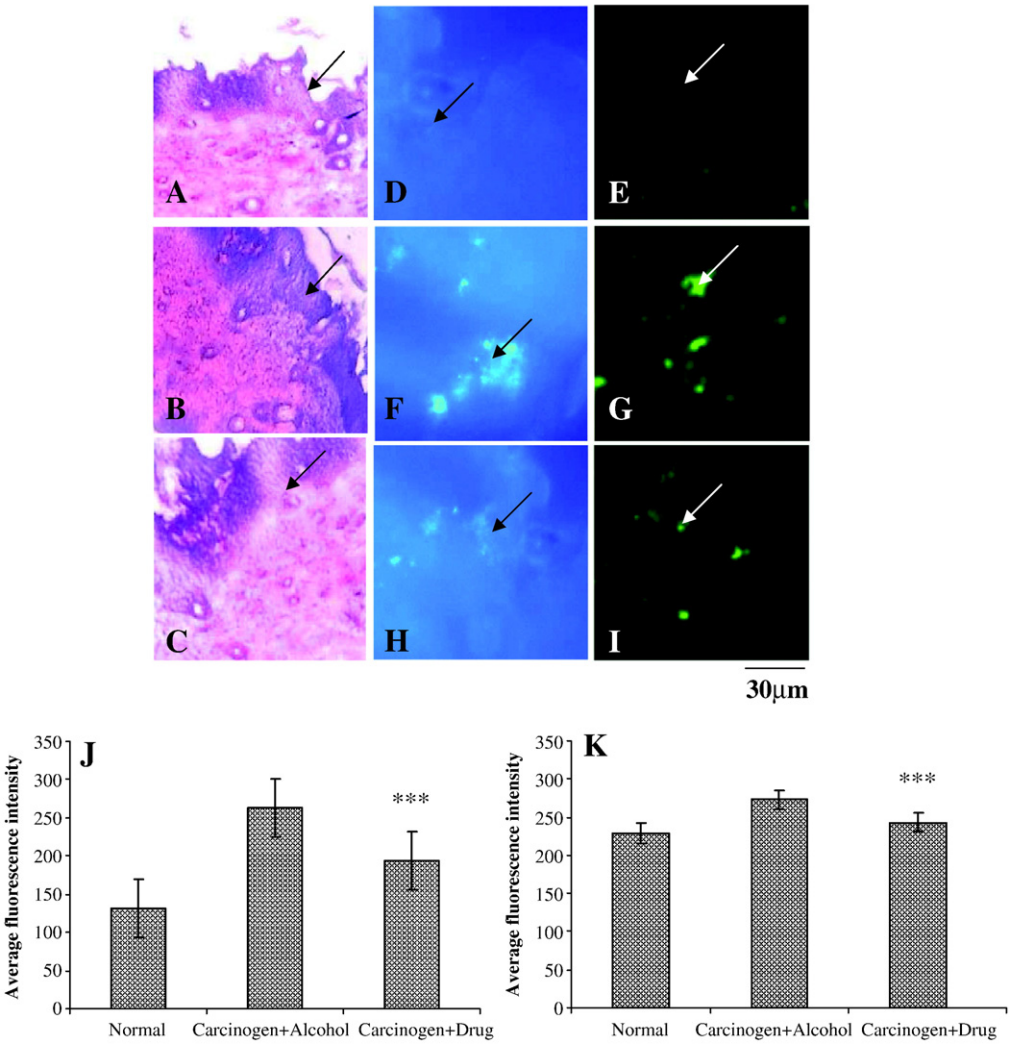


Fig. 3. Histology of skin by Haematoxylin/Eosin (A = normal, B = DMBA + Croton oil, C = Synthetic coumarin) and imuno-fluorescence of Aryl hydrocarbon receptor and PCNA proteins after 24th week of DMBA-Croton oil treatment.(D–E = normal, F–G = DMBA + Croton oil, H–I = Synthetic coumarin). Cells were visualized under light and fluorescence microscope respectively. (Black and white arrow represents changes in tissues compared to normal ones.) Bar indicates 30 µm. J–K represents proportion of average fluroscence intensity of Aryl hydrocarbon receptor and PCNA protein in different group. *** = $P<0.001$.

2.5. Preparation of nuclear extract

For analysis of expression of NF-kB, nuclear extract was used. Skin tissues were washed twice in 10 ml cold PBS and re-suspended in 500 µl of buffer (10 mM HEPES pH 7.9, 1.5 mM $Mgcl_2$, 10 mM Kcl, 0.5 mM DTT, including protease inhibitors 100 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1 µg/ml Pepstatin, 0.5 mM PMSF). NP-40 was added to a final concentration of 0.5% and the cells were vortexed for 10 s. The nuclei pellets were collected at the bottom of a centrifuge tube by spinning at 6500 rpm for 20 min; then 150 µl of buffer (20 mM HEPES pH 7.9, 1.5 mM $Mgcl_2$, 420 mM Nacl, 0.2 mM EDTA, 25% v/v Glycerol, including protease inhibitors 100 µg/ml Aprotinin, 5 µg/ml Leupeptin, 1 µg/ml Pepstatin, 0.5 mM PMSF) was added to re-suspend the nuclear pellet. The nuclear pellet was kept on ice with vigorous agitation. Nuclear extracts (supernatants) were recovered after centrifugation for 10 min at 12000 rpm at 4 °C.

2.6. Preparation of cytosolic extract

For analysis of all other signal proteins, cytosolic extract was used. Briefly, mouse skin tissues were homogenized in lysis buffer containing 50 Mm Tris–HCl, pH 8.0, 1% Nonidet P-40,125 mM NaCl, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 Mm Na_3VO_4 and 10 mM sodium pyrophosphate (Currier et al., 2005). The homogenized material was centrifuged at 13000 ×g for 15 min at 4 °C. The supernatant was analyzed for Western blot.

2.7. Western blot analysis

Western blot analysis was performed as described previously (Burnette, 1981). Equal amounts of protein as determined by Lowry assay were diluted with 5× sample loading buffer, boiled and loaded onto 12% polyacrylamide gels. After electrophoresis, the protein bands were transferred to a nitrocellulose membrane (Millipore Inc.) at 110 V for 1 h, and membranes were blocked in 5% milk in 1X TBS, 0.05% Tween, then the transferred bands of Aryl hydrocarbon receptor, PCNA, p53, Bcl-2, Bcl-xL, Bad, Bax, NF-kB (p65 sub-unit of nuclear extract), Cytochrome c, IL-6 and Caspase-9 were bound to their respective monoclonal antibodies purchased from Abcam, USA and Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Antibodies for Caspase 9, Akt, and Cytochrome-c were purchased from BD Bioscience, USA. The bound proteins were treated with the appropriate ALP-conjugated secondary antibodies (1:5000) (Sigma Chemicals, USA). The same membranes were also immuno-blotted against β-actin (house keeping enzyme) for data normalization. For quantitative analysis of each band, density was determined using Gel Doc System; Ultra Lum, USA.

2.8. Assessment of Caspase-3 activity

The activity of Caspase-3 was measured using the colorimetric substrate method from skin tissue employing an ELISA reader (Thermo Scientific, USA), following Das et al. (2005). Cytosolic extracts were prepared by homogenization of dorsal shaved area of skin tissues in extraction buffer containing 25 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.1%(w/v) CHAPS and 10 µg aprotinin. Subsequently, the homogenates were centrifuged at 13,000 ×g for 15 min at 4 °C. The supernatant fraction was used to determine Caspase-3 activity. In brief, Caspase-3 colorimetric substrates (Ac-DEVD-pNA), procured from Sigma, USA, were incubated with cell lysates for 1 h at 37 °C, then the amount of chromophore, p-nitroaniline (p-NA), released by Caspase-3 activity was quantified by measuring the optical density at 405 nm using a 96-well microtiter plate reader. Caspase-3 activity was expressed as µM p-NA released per hour per milligram cellular protein.

2.9. Histology and immunofluorescence

Back skin samples were dissected from the lesion and/or tumor regions of the mid-dorsal back skin and fixed in normal buffer formalin, followed by dehydration treatment,. After storing at room temperature for less than a week in 70% alcohol, the tissues were washed two times each for 30 min in xylene, and then transferred to the embedding bath. The paraffin blocks were cut into ribbon like pieces and placed on to slides after stretching into warm water. The paraffin was removed by xylene (two washes each for 10 min), then the xylene was removed by alcohols. Processed tissues were stained in haematoxylin for 2 min followed by washing in distilled and tap water, respectively. Then tissues were brought into alcohol medium (70%, 90% two washes each for 2 min), followed by staining with eosin (30 s) and dehydrated into 90% and absolute alcohol (two washes each for 2 min). Finally xylene treatment (two washes each for 2 min) was carried out followed by mounting into DPX. For immunofluorescence study, the technique of Arabzadeh et al. (2007) was adopted with a little modification. Briefly, cut tissue sections were de-paraffinized and incubated separately for 12 h with mouse monoclonal Aryl hydrocarbon receptor and PCNA antibodies, respectively, procured from Abcam International, USA. Then after blocking with 3% BSA, the tissue sections were incubated for 2 h with fluorescence isothiocyanite (FITC) conjugated secondary antibody purchased from Sigma, USA. The fluorescence intensity of FITC was measured under ZEISS fluorescence microscope, Germany.

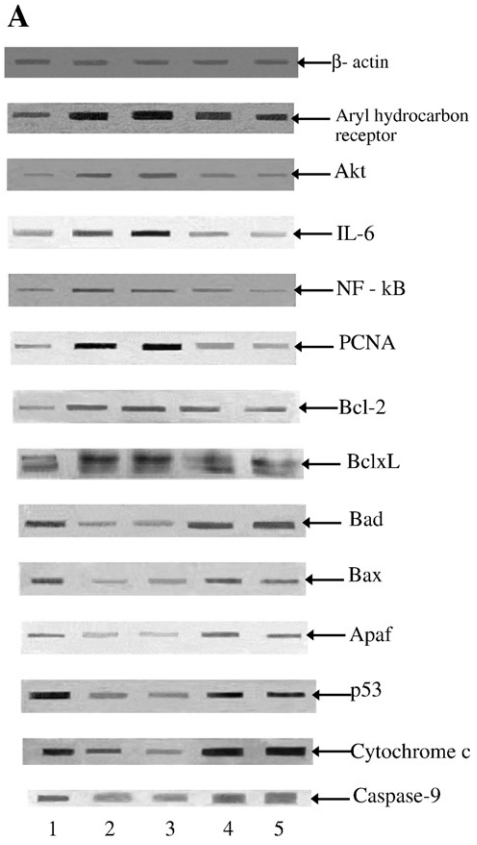


Fig. 4. A. Immunoblots of β-actin, Aryl hydrocarbon receptor, Akt, IL6, NF-kB, PCNA, Bcl-2, BclxL, Bad, Bax, Apaf, p53, Cytochrome c and Caspase-9. Ln1 — Normal; Ln2 — DMBA + Croton oil; Ln3 — DMBA + Croton oil + Alcohol; Ln4 — DMBA + Croton oil + Synthetic coumarin(50 mg/kg bw); Ln5 — DMBA + Croton oil + Synthetic coumarin (100 mg/kg bw). B–C. Band densities are expressed as a mean ± SE of the three independent experiments; ** = $P<0.01$, *** = $P<0.001$ of β-actin, Aryl hydrocarbon receptor, Akt, IL6, NF-kB, PCNA, Bcl-2, BclxL, Bad, Bax, Apaf, p53, Cytochrome c and Caspase-9. Each experiment is representative of three different sets of experiments. Ln1 — Normal; Ln2 — DMBA + Croton oil; Ln3 — DMBA + Croton oil + Alcohol; Ln4 — DMBA + Croton oil + Synthetic coumarin(50 mg/kg bw); Ln5 — DMBA + Croton oil + Synthetic coumarin (100 mg/kg bw).

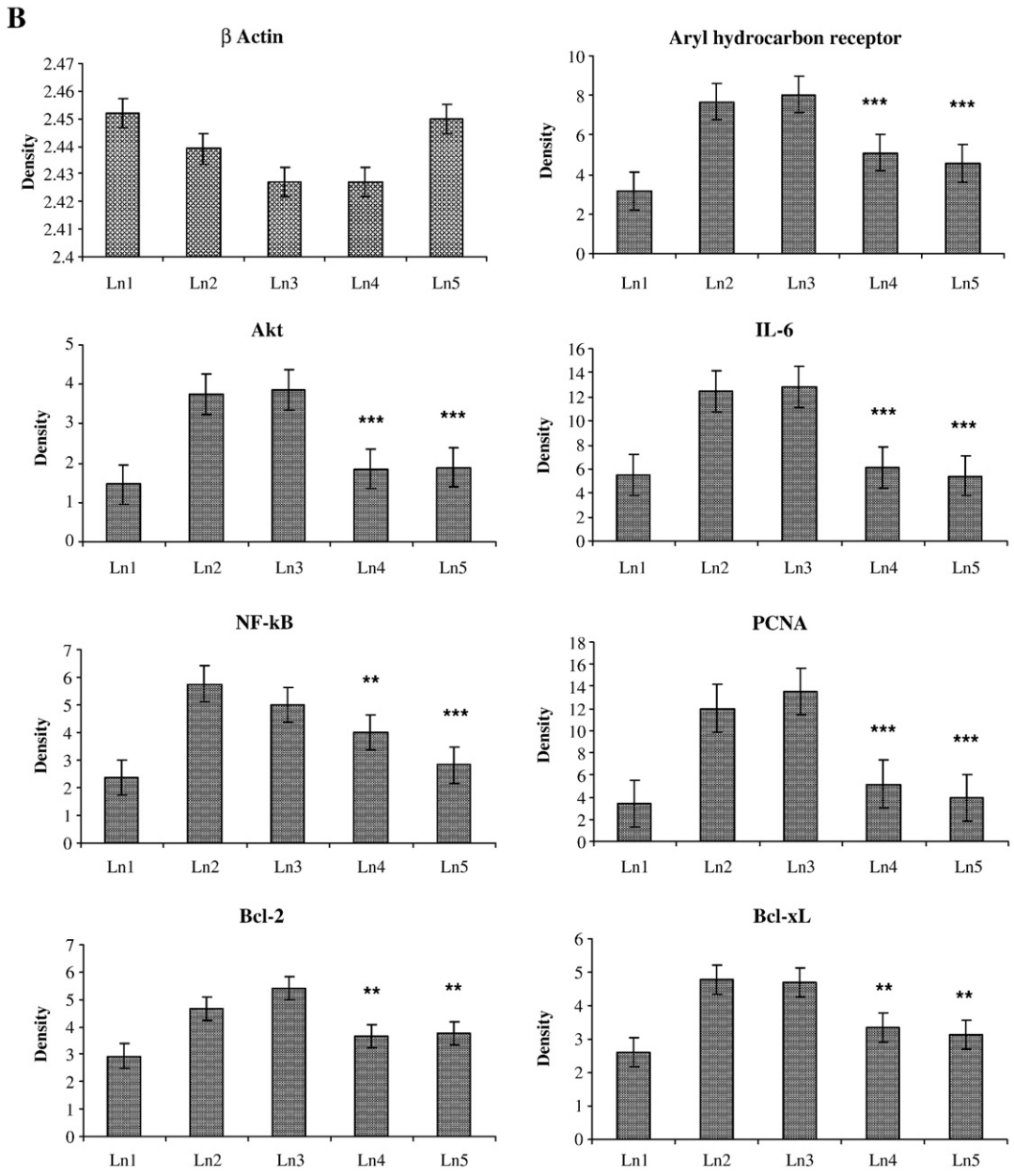


Fig. 4B.

2.10. Statistical analysis

The significances of differences between data of carcinogen plus 2% alcohol administered mice and carcinogen administered synthetic coumarin fed mice were analyzed by the Student's t test. For all quantitative analyses, data were obtained from representative experiments with triplicate and were expressed as mean ± Standard Error (SE). Homogeneity of the different series was further analyzed by LSD one-way ANOVA (analysis of variance) in Tables 1–3 (see Supplementary materials) using SPSS version-10.0; SPSS Inc., Chicago, III., USA. $P<0.05$ was considered significant.

3. Results

3.1. External morphology

The changes in external morphology of skin in mice subjected to i) no treatment (Fig. 1A; negative control — Gr-1), ii) carcinogen treatment (Fig. 1B; Gr-2), iii) carcinogen plus 2% alcohol treatment (Fig. 1C; Gr-3) and iv) carcinogen plus synthetic coumarin (50 mg and 100 mg/kg bw dissolved in 2% ethanol) treatment (Fig. 1D, E; Gr-4). While Gr-2 and Gr-3 mice showed distinct skin ulcerations and growth of papilloma, both the synthetic coumarin fed groups (i.e. 50 mg and 100 mg fed) showed much less growth of ulcer (the latter showing more pronounced effects) as well as loss of hair than their counterparts which did not receive synthetic coumarin treatment. The incidence of papilloma growth in different weeks in control and treated mice has been provided in Fig. 2. No tumor was found in any untreated mice. Animals receiving DMBA, croton oil and 2% alcohol for 24 weeks developed tumor at the rate of 4.8 ± 0.20 papillomas/mouse. The number of papillomas significantly decreased at 24 weeks in mice fed 50 mg and 100 mg of synthetic coumarin, showing papilloma at the rate of 1.2 ± 0.20 and 0.8 ± 0.20, respectively, as compared to that of control group. Papillomas observed in DMBA + Croton oil treated group were significantly bigger in size (70% papillomas of ≥2.5 mm in diameter) as that of synthetic coumarin fed series (8%–10%).

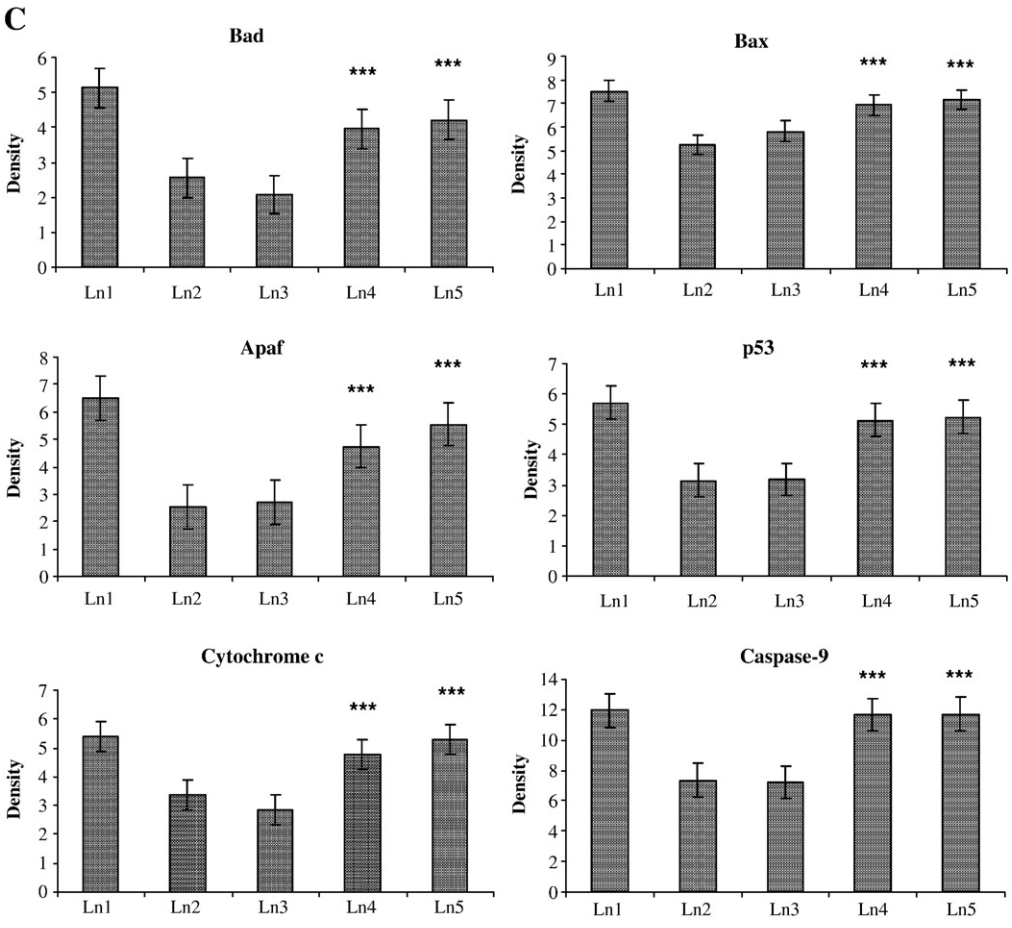


Fig. 4C.

3.2. Histology

As compared to the skin section with normal histological features (Fig. 3A), the histological features of DMBA + alcohol administered mice (Fig. 3B) consists of irregular distribution of different cell types and contains finger like projections (papilloma), indicative of cancerous growth. Histologically, the DMBA treated group exhibited varying degrees of structural and cytological divergence as compared to untreated group. Tumors of animal belonging to DMBA + Croton oil treated group were composed of focal proliferation of squamous cells, presence of some necrotic cells, keratinization and epithelial pearls. This clearly indicates the malignant nature of tumors that is well-differentiated squamous cell carcinoma. On the other hand, in the synthetic coumarin fed mice, these features were visible to a much lesser extent (Fig. 3C).

3.3. Immunofluorescence

The Aryl hydrocarbon receptor and PCNA specific immunochemical staining reveals lack of expression in normal skin (Fig. 3D, E) while in DMBA treated mice, Aryl hydrocarbon receptor and PCNA receptors were seen to take up higher level of green fluorescence in DMBA treated mice without synthetic coumarin feeding (Fig. 3F, G), indicating localization of Aryl hydrocarbon receptor and PCNA proteins in carcinogen treated mice, while in synthetic coumarin fed mice, the fluorescing intensity was much less (Fig. 3H, I). The proportion of average fluorescence intensity was quantified for Aryl hydrocarbon receptor (Fig. 3J) and PCNA (Fig. 3K) by image analyzer (Gel Doc System, Ultra Lum, USA).

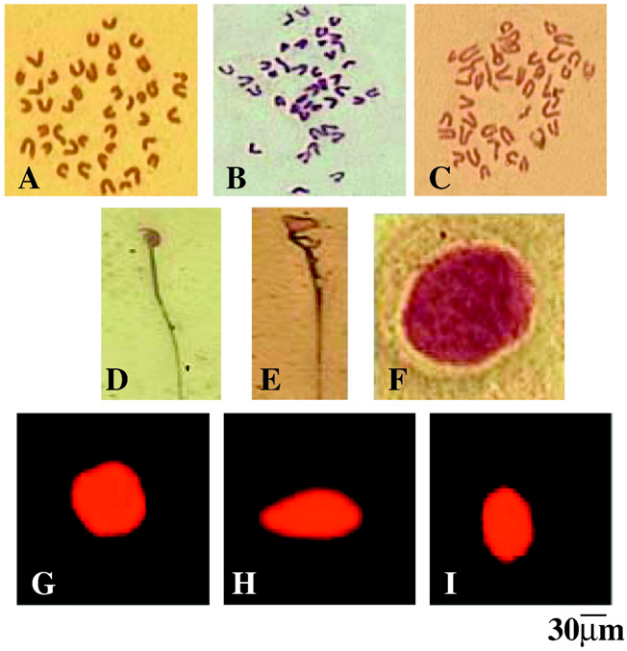


Fig. 5. Phomicrographic representation of chromosome spreads (A = normal, B and C = aberrated), sperm head (D = normal), abnormal sperm head (E = abnormal),micronucleus (F) DNA breakage by Comet assay (G = normal, H = carcinogen treated, I = Synthetic coumarin fed). Bar indicates 30 µm.

3.4. Immunoblots

The expression of different signal proteins in normal healthy mice (Gr 1) can be observed in the lane 1 of Fig. 4A. Of these, the expression levels of Aryl hydrocarbon receptor, Akt, IL-6, NF- kB, PCNA, Bcl-2 and Bcl-xL were low, and those in Bad, Bax, Apaf, p53, Cytochrome c and Caspase-9 were moderate. In the DMBA/Croton oil induced mice, the expression levels were increased in Aryl hydrocarbon receptor, Akt, IL-6, NF-kB, PCNA, Bcl-2 and Bcl-xl while in the carcinogen administered synthetic coumarin fed mice (Gr-4), there was suppression on the enhanced expression of these signal proteins. Correspondingly, there was decrease in expression of proteins like Bad, Bax, Apaf and p53 in the DMBA/Croton oil induced mice, which were increased in Bad, Bax, Apaf and p53 in the synthetic coumarin fed mice. The densitometric analysis of the immuno blots has been plotted in Fig. 4B, C, indicating the level of significance of difference between drug treated and alcohol controls. All the immunoblots were normalized by the expression of a house-keeping protein, β-actin.

3.5. Cytogenetical assay

Representative photographs of chromosome plates, sperm head, micronuclei and comet assay of peripheral blood mononuclear cells etc. have been documented in Fig. 5A–I. While the % of chromosome

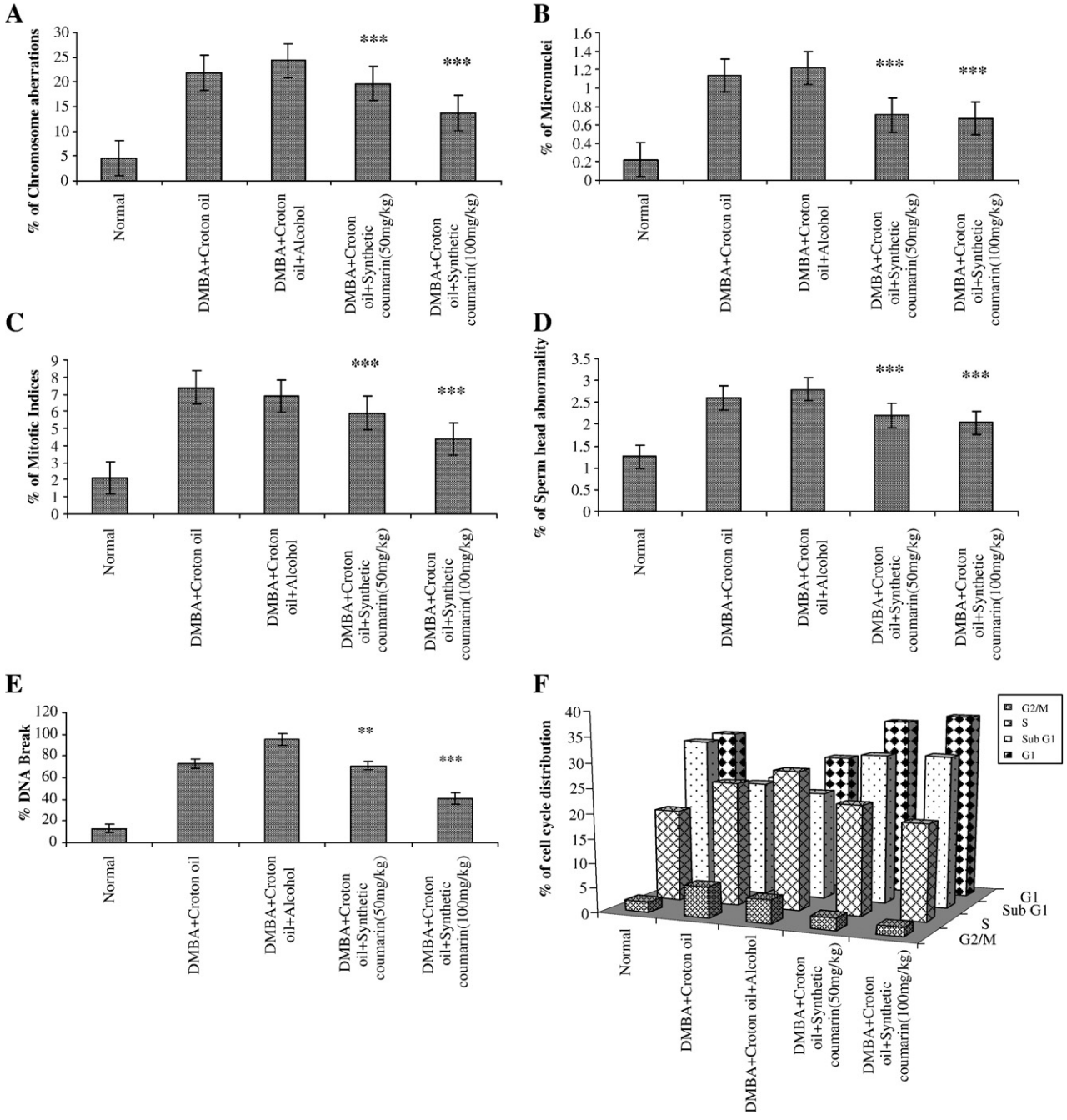


Fig. 6. Histogram showing % of chromosome aberration (A), micronucleated erythrocytes (B), mitotic indices (C), sperm head anomaly (D), DNA damage (E), cell cycle distribution obtained by FACS (F) in different series. Normal, DMBA + Croton oil, DMBA + Croton oil + alcohol, DMBA + Croton oil + Synthetic coumarin (50 mg/kg bw), DMBA + Croton oil + Synthetic coumarin (100 mg/kg bw). Value represents mean ± S.E ($n = 3$) and ** = $P < 0.01$, *** = $P < 0.001$ represent significant differences from alcohol control.

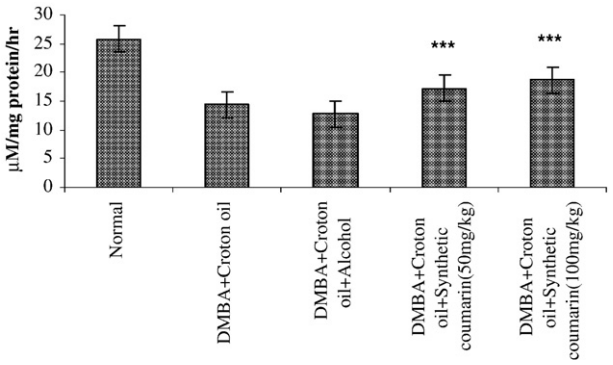


Fig. 7. Histogram showing quantitative data of Caspase-3 activity in different series. Value represents mean ± S.E ($n = 3$); *** = $P < 0.001$ represent highly significant differences from alcohol control.

aberrations, micronuclei, mitotic index and sperm head abnormality (Fig. 6A–D) were significantly elevated in the DMBA induced mice as compared to normal untreated control, the values were significantly decreased by the feeding of synthetic coumarin. The same was supported by the data of Comet assay (Fig. 6E) that indicated a much more amount of broken DNA in mice treated with DMBA/Croton oil, but much less in the synthetic coumain fed mice, slightly more pronounced in the 100 mg fed group than that of 50 mg fed group. The FACS analysis revealed that in the synthetic coumarin fed mice, the cell cycle was arrested more in the sub G_1 (30.45%,30.74% for 50 mg and 100 mg/kg bw) and G_1 (36.17% and 37.6% respectively) and S phase (22.18% and 19.31% respectively). From this finding it is very clear that synthetic coumarin in both doses block the cell cycle mostly in sub G_1 and G_1 and thus prevent the cell to enter into S phase or it induces more apoptosis. Further, DMBA showed higher % of S phase (28%) in contrast to the synthetic coumarin that showed 19.31% of cells in S phase (Fig. 6F).

3.6. Quantitative assay of Caspase 3 by ELISA

The Caspase-3 activity was also higher in synthetic coumarin fed mice as compared to the DMBA administered mice (Fig. 7).

4. Discussion

The results of the present study would unequivocally demonstrate that synthetic coumarin had anti-cancer activities not experimentally verified earlier in any *in vivo* or *in vitro* model. The topical application of DMBA/Croton oil produced ulceration followed by growth of papilloma. The present study provides ample evidence that synthetic coumarin could inhibit the DMBA-induced up-regulation of Aryl hydrocarbon receptor and over-production of PCNA that ultimately reduced the tumor formation in mice. The FACS data revealed that in the synthetic coumarin fed mice, the cell cycle was intervened more at sub-G1 and G phase, preventing cells to enter the S phase. PCNA is a proliferating cell nuclear antigen associated with the S phase of DNA replication. Both immuno-blot and immunofluorescence analyses showed that PCNA expression was less in the synthetic coumarin fed mice. The possible involvement of several signal proteins has been schematically presented in Fig. 6 for DMBA induced tumorigenesis. Briefly, DMBA induced signal proteins CYP1A1 and CYP1B1 through up-regulation of Aryl hydrocarbon receptor. These Cytochrome P450 enzymes were responsible for breaking down the pro-carcinogenic DMBA into active carcinogenic metabolites. These metabolites damaged DNA presumably by formation of adducts, which could be evidenced by the increase in Comet tail lengths and elevated frequencies of chromosomal aberrations in the carcinogen administered mice. The chromosome damage was less severe in the synthetic coumarin fed mice. The thiol epoxides and other toxic reactive oxygen species formed during metabolic activation of DMBA can cause chromosomal damage by binding with adenine residues of DNA. The bone marrow chromosome aberrations and peripheral blood micronucleus tests have therefore been used as a tool to evaluate the mutagenicity of environmental mutagens and carcinogens. The DNA damage, chromosome aberrations and consequently disorder in metabolic functioning, contributed to the initiation of the carcinogenetic process, through generation of reactive oxygen species. The generation of reactive oxygen species contributed further to the up-regulation of several down-stream signaling proteins like NF-kB, Akt and PCNA. While as an initial response to DNA damage there should normally have been an increase of p53 products, but because of over production of PCNA, the p53 eventually could not be up-regulated and the cells could neither be pushed into the apoptotic pathway. Up-regulation of NF-kB would induce the anti-apoptotic Bcl-2 and Bcl-xL genes through up-regulated Akt and thereby prevent the cells to undertake the apoptotic pathway via down-regulation of pro-apoptotic genes Bad, Bax and Apaf, Caspase-3 and -9.

Animals that received DMBA/Croton oil application and also fed two different doses of synthetic coumarin showed significant decrease in numbers of papilloma, particularly in mice fed the higher dose of synthetic coumarin. Inhibition of DMBA induced mammary tumors in mice has been reported earlier with some coumarin derived compounds by Wattenberg et al. (1979), but not with this particular synthetic coumarin, and not on skin tumor of mice. From the results of this study, the possible mechanism (s) and pathways through which synthetic coumarin acted can be somewhat speculated. Synthetic coumarin clearly inhibited Aryl hydrocarbon receptor and PCNA expressions, acting as an antagonist of Aryl hydrocarbon receptor and PCNA. Similar inhibitory role played by some other agents like Resveratol (Kundu and Young-Joon, 2005), di-phenylmethyl-selenocyanate (Das et al., 2005) and some alkaloids of plant extract and bioflavonoids (Quardri et al., 2000) have been attributed specific role of tumor inhibition in earlier studies.

Induction of apoptosis has been another avenue through which chemoprevention can be achieved (Chu et al., 2001). p53 plays an important role in mitochondrial membrane stability and promotes apoptosis (Rich et al., 2000). p53 is a tightly regulated transcription factor that induces cell cycle arrest or apoptosis in response to cellular stress such as DNA damage (Evans and Littlewood, 1998). Although p53 has a central role in driving cell to apoptosis, the mechanism of p53 mediated apoptosis after cellular stress still remains unclear. A number of phosphorylation sites on p53 are known to be altered after DNA damage and such phosphorylation events have been shown to result in alterations in p53 that make it more stable and more active (Vogelstein et al., 2000). Presently, the mode of action of p53-mediated apoptosis is believed to involve transactivation of target genes, which directs signaling events that may induce two sets of genes upon stress signal. One set, such as p21/waf-1 and GADD45, mainly function in cell growth control, and the other, such as Bax and Bcl-2, act on apoptosis (Agarwal et al., 1998). In the present study we observed down-regulation of p53, but up-regulation of PCNA in DMBA treated mice, but the expressions were reversed in the synthetic coumarin fed mice. The same was true for the other signaling proteins, consistent with the expressions that favored apoptosis and antagonized tumor formation. This contention was also supported by the findings of down regulation of Apaf and Caspase-3 which are directly involved in cell apoptosis. In fact Caspases are key proteins that modulate apoptotic response through regulation of Caspase-3 and Caspase-9. Caspase-3 is known to be a key executioner of apoptosis, which is activated by an initiator like Caspase-9. This activated Caspases cleave many cellular substrates, ultimately leading to the death of cells (Wang et al., 2006). The findings of up-regulation of Caspase-3 and 9 in synthetic coumarin fed mice would thus indicate that some of the cells with damaged unrepairable DNA were ultimately pushed to the apoptotic pathway.

TNF-α is also one of the known mediators of apoptosis (Yoshimura et al., 2006). TNF-α can function as effectors of chondrocyte apoptosis (Fernandes et al., 2002), presumably through production of high level of Nitric oxide (NO). Because of over-expression of the tumor necrosis factor the toxicity developed reflects also in the up-regulation of cytokine inflammatory signal, IL-6. There was down-regulation of both NF-kB and IL-6 in the synthetic coumarin fed mice which also supported that this derivative was not only anti-necrotic but also anti-inflammatory in action.

Thus, from the overall results of this study, it may be concluded that this synthetic coumarin compound has a great potential as a possible anti-cancer drug, particularly in polycyclic aromatic hydrocarbon induced skin cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2009.04.015.

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