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Anti-tumor activity of rosmarinic acid in 7,12-dimethylbenz(a)anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice

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Aim of the present study was to evaluate the anti-tumor effect of orally administered rosmarinic acid in 7,12-dimethylbenz(a)anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice. Phase I and II detoxication agents, lipid peroxidation byproducts, antioxidants and apoptotic biomarkers were used to assess chemopreventive efficacy of rosmarinic acid in DMBA induced skin carcinogenesis. Skin squamous cell carcinoma was induced at the shaved back of mice by applying DMBA (20 µg in 0.1 mL acetone) twice weekly for 8 weeks. Tumor formation (100%) was observed within 15 weeks of treatment in DMBA alone. Marked alterations in the status of above mentioned biomarkers were observed in tumor bearing mice. Oral administration of rosmarinic acid completely prevented the formation of skin tumors during DMBA-induced mouse skin carcinogenesis. Also, oral administration of rosmarinic acid brought back the status of phase I and phase II detoxication agents, lipid peroxidation byproducts, antioxidants and apoptotic markers (p53, Bcl-2, caspase-3 and caspase-9) in DMBA treated mice. Results of the present study suggested that rosmarinic acid had potent anti-cancer, anti-lipid peroxidative and apoptotic effect in DMBA-induced skin carcinogenesis.

Keywords: Antioxidants, Apoptosis, Detoxication agents, Lipid peroxidation, Rosmarinic acid, Skin cancer

Skin, the largest organ of human body, contains three kinds of cells, squamous cells, basal cells and melanocytes. Skin cancer develops in the epidermis[1] and commonly of three types, basal cell carcinoma, squamous cell carcinoma, and melanoma. Oxidative stress may be the cause of skin cancer leading to DNA damage in the skin that includes DNA base damage, DNA single and double strand breaks and cross-linking between DNA and proteins[2]. Uncontrolled release of reactive oxygen species is involved in pathogenesis of a number of human skin disorders including cutaneous neoplasia[3]. Patients with actinic keratoses and basal cell carcinoma, have lowered activities of antioxidant enzymes in plasma or serum[4]. Superoxide dismutase (SOD) activity decreases in human non-melanoma skin cancers[5]. Development of novel strategies to prevent skin cancer is a desirable goal to reduce the incidence of skin cancer. Rosmarinic acid, a polyphenol, has been isolated from the leaves of *Rosmarinus officinalis*[6] and also occurs in other species of Lamiaceae and Boraginaceae[7]. Chemically, rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid[8]. Rosmarinic acid has been reported to protect biomembranes against peroxidative damage[9]. In the present study the chemopreventive efficacy of rosmarinic acid in DMBA induced skin carcinogenesis in Swiss albino mice has been reported.

Materials and Methods

Chemicals—7,12-Dimethylbenz(a)anthracene (DMBA), rosmarinic acid and other biochemicals such as reduced glutathione, reduced nicotinamide adenine dinucleotide, 1,1',3,3'-tetramethoxypropane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Heparin, thiobarbituric acid (TBA), trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), nitro blue tetrazolium (NBT) and phenazine methosulphate (PMS) were purchased from Hi-media Laboratories Mumbai, India. Caspase-3 and caspase-9 colorimetric assay kit were purchased from BioVision research products, USA. All other chemicals and solvents used were of analytical grade.

Animals—Male Swiss albino mice (No. 24) 4-6 weeks old, weighing 15-20 g were purchased

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from National Institute of Nutrition, Hyderabad, India and maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. Animals were housed in groups of 4 or 5 in polypropylene cages and provided standard pellet diet and water *ad libitum* and maintained at 22° ± 2°C under 12 h light/ dark cycle.

The Institutional Animal Ethics Committee (Register number 160/1999/ CPCSEA), Annamalai University, Annamalai Nagar, India, approved the experimental design (Proposal No. 698: dated. 11-01-2010). Animals were maintained as per the principles and guidelines of the Ethical Committee for Animal Care of Annamalai University.

Experimental Design—Male Swiss albino mice (24) were divided into 4 groups of 6 each. Skin carcinogenesis was developed in Swiss albino mice according to the method of Azuine and Bhide[10]. Depilatory cream was applied to remove hair from the back of each mouse and the mice were left untreated for two days. Mice having no hair growth after two days were selected for the study.

In Group I acetone (0.1 ml/mouse) was applied on the depilated back of the mice twice a week for 8 weeks (vehicle treated control). In Groups II and III, mice were applied with DMBA (25 µg in 0.1 ml acetone/mouse) twice weekly for 8 weeks. While Group II mice received no other treatment, Group III mice were orally administered with rosmarinic acid (100 mg/kg body wt in 1ml distilled water) by gastric gavage, starting 1 week before the exposure to the carcinogen and continued for 25 weeks (3 times/week on alternate days) thereafter. Group IV mice were orally administered with rosmarinic acid alone by gastric gavage throughout the experimental period. At the end of experimental period all animals were sacrificed by cervical dislocation. Tumor volume was measured using the formula

$$V=\left(\frac{4}{3}\right)\pi\left[\frac{D_1}{2}\right]\left[\frac{D_2}{2}\right]\left[\frac{D_3}{2}\right]$$

where D1, D2 and D3 are the 3 diameters (mm) of the tumors. Tumor burden was calculated by multiplying tumor volume and number of tumors/animal.

Histopathology—For histopathological studies, tumor tissues and normal skin tissues were fixed in formalin (10%), embedded in paraffin, sections (2-3 µm) were cut using a rotary microtome and stained with hematoxylin and eosin.

Biochemical estimations—Biochemical estimations were carried out in skin tissues and liver of control and experimental animals in each group. Biochemical parameters related to carcinogenic process were analyzed. Skin tissues from mice (DMBA treated, acetone treated and untreated area) were dissected, blotted dry, weighed and homogenized using appropriate buffer in a homogenizer with Teflon glass pestle. Skin tissue homogenate was centrifuged at 1000 × *g* for 5 min and the supernatant was used for assay of lipid peroxidation (TBARS)[11], superoxide dismutase (SOD)[12], catalase (CAT)[13] and glutathione peroxidase (GPx)[14] and reduced glutathione (GSH) [15]. Liver tissue homogenate was used for estimation of phase I and phase II detoxification agents.

Caspase-3 and caspase-9 activities in skin tissues were measured using caspase-3 colorimetric assay kit and caspase-9 colorimetric assay kit respectively according to manufacturer's instructions (Biovision Research Products, 980 Linda Vista Avenue, Mountain View, CA 94306, USA. www.biovision.com). Reduced glutathione level in skin tissues and liver was determined by the method of Beutler and Kelley[15]. Activities of liver glutathione-s-transferase (GST) and glutathione reductase (GR) were assayed by the method of Habig[16] and Carlberg and Mannervik[17] respectively. Cytochrome P_{450} and b_5 status in liver was estimated by the method of Omura and Sato[18].

Immunohistochemistry—Skin tissues from control and experimental animals in each group were immediately fixed in buffered neutral formalin solution (10%), embedded in paraffin, cut sections (2-3 µm) using rotary microtome, placed on clean glass slides, dried at 37°C and used for immunohistochemical studies. Paraffin embedded tissue sections were dewaxed and rehydrated through graded ethanol to distilled water. Endogenous peroxidase was blocked by incubation with hydrogen peroxide (3%) in methanol for 10 min. Antigen retrieval was achieved by microwave in citrate buffer solution (*p*H 6.0) for 10 min, followed by washing step with Tris-buffered saline (*p*H 7.6). Tissue sections were then incubated with power Block™ reagent (BioGenex, San Ramon, CA, USA), universal proteinaceous blocking reagent, for 15 min at room temperature to block non-specific binding. Tissue sections were then incubated with the respective primary antibody (DAKO p53 –DO-7 and DAKO Bcl-2/100) for overnight at 4°C. The bound primary antibody was

detected by incubation with the secondary antibody conjugated with horse radish peroxidase (Bio Genex, San Ramon, CA, USA) for 30 min at room temperature. After rinsing with Tris-buffered saline, antigen-antibody complex was detected using 3,3'-diaminobenzidine (Sigma, USA), the substrate of horse radish peroxidase. When acceptable color intensity was reached, slides were washed, counter stained with haemotoxylin and covered with a mounting medium.

Statistical analysis—Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT). Values are represented as mean ± SD and $P< 0.05$ was considered statistically significant.

Results

Body and liver weight were significantly decreased in DMBA treated animals compared to control animals (Table 1). Oral administration of rosmarinic acid 3 times per week for 25 weeks significantly increased the body and liver weight in DMBA treated animals. Oral administration of rosmarinic acid alone to mice showed no significant difference in body and liver weight compared to control animals.

In DMBA treated mice, tumor formation (100%) with mean tumor volume (592.6 mm^3) and tumor burden (1679.1 mm^3) was observed. Skin tumors in DMBA treated mice were observed (Fig. 1a) that disappeared in DMBA + rosmarinic acid treated mice (Fig. 1b). Oral administration of rosmarinic acid completely prevented tumor incidence, tumor volume and burden that appeared in DMBA applied mice.

Histopathological evaluation showed that skin tissues from vehicle treated control mice (Fig. 2a) and rosmarinic acid alone treated mice (Fig. 2d) exhibited well defined subcutaneous tissue and intact epithelial layer. Severe hyperplasia, hyperkeratosis, dysplasia and well-differentiated squamous cell carcinoma were observed in all DMBA treated mice (Fig. 2b). Although, hyperplasia and mild dysplasia was observed in DMBA + rosmarinic acid treated mice (Fig. 2c), no tumor formation was observed.

Status of phase I (cytochrome P_{450} and cytochrome b_5) and phase II detoxication agents (GST, GR and

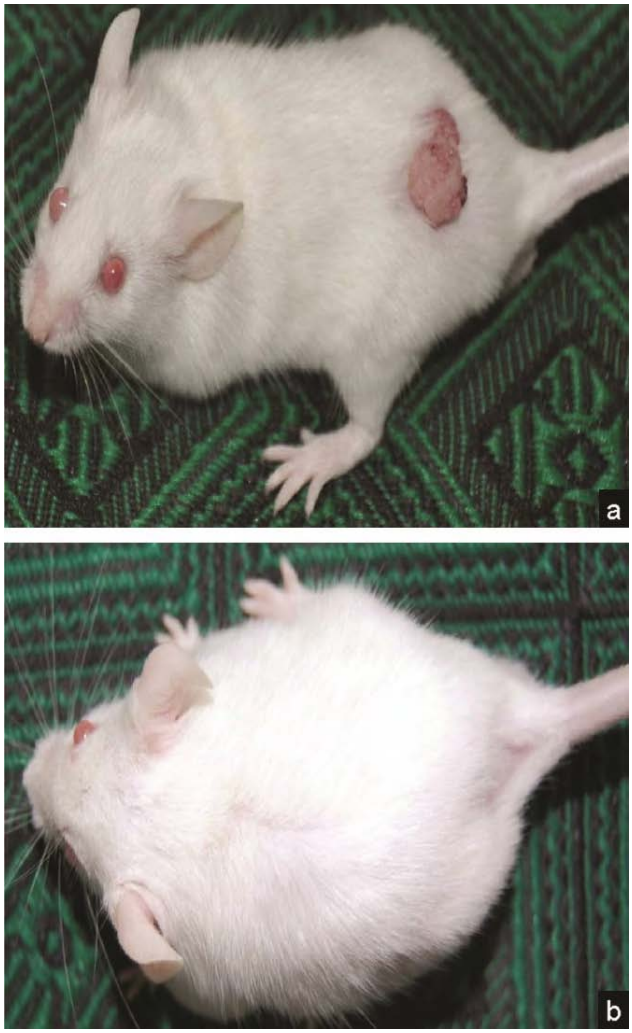

Fig. 1—Gross appearance of skin tumors in DMBA alone and DMBA + rosmarinic acid treated mice. [(A)-DMBA alone treated mice; (B)- DMBA + rosmarinic acid treated mice].

Table 1—Effect of rosmarinic acid on body weight, liver weight and relative organ weight of experimental animals in each group. [Values are mean ± SD of 6 animals]

Groups	Body wt. (g)		Liver wt. (g)	Relative organ weight (liver wt./body wt.)
	Initial	Final		
Control (Vehicle Treated)	24.83 ± 0.61[a]	29.85 ± 0.56[a]	1.25 ± 0.08[a]	0.041 ± 0.004[a]
DMBA alone	25.12 ± 0.86[a]	24.53 ± 0.89[b]	0.90 ± 0.12[b]	0.036 ± 0.003[b]
DMBA + rosmarinic acid	24.46 ± 0.95[a]	29.11 ± 0.68[a]	1.18 ± 0.08[a]	0.040 ± 0.005[a]
Rosmarinic acid alone	24.97 ± 0.84[a]	30.04 ± 0.97[a]	1.27 ± 0.09[a]	0.042 ± 0.004[a]

Values that are not sharing common superscript in the same column differ significantly at $P < 0.05$

GSH) in liver of control and experimental animals in each group are shown in Table 2. Levels of GSH, GST and GR were significantly decreased whereas the status of cytochrome P_{450} and cytochrome b_5 was increased in the liver of tumor bearing animals compared to control animals. Oral administration of rosmarinic acid to DMBA treated animals significantly improved the status of phase I and phase II detoxication agents.

Levels of TBARS and levels of enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidant (GSH) in skin tissues of control and experimental animals in each group are shown in Table 3. Level of TBARS was significantly increased whereas, the activities of SOD, CAT, GPx and GSH level were significantly decreased in skin tissues of tumor bearing animals compared to control animals. Oral administration of rosmarinic acid to DMBA

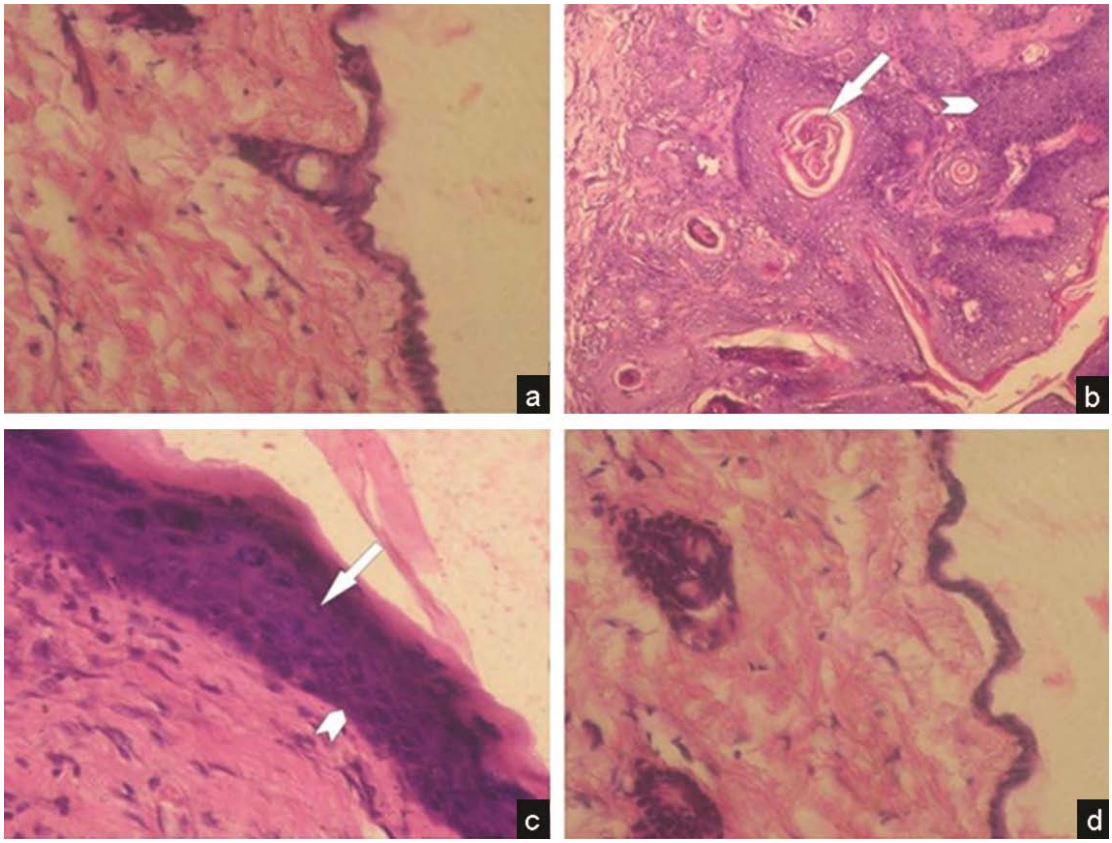

Fig. 2—Histological evaluations in skin tissues of control and experimental animals in each group. [Microphotographs of skin tissues of (a)-control; (b)-DMBA alone treated animals showing well-differentiated squamous cell carcinoma with dysplastic epithelium (arrow head) and keratin pearls (arrow; 40×); (c)-DMBA + rosmarinic acid treated animals showing hyperplastic (block arrow) and mild dysplastic (block arrow head) epithelium; (d)-rosmarinic acid alone treated animals, respectively showing well-defined subcutaneous tissues and intact epithelial layer (10×)].

Table 2—Levels of phase I and II detoxication agents in liver of control and experimental animals in each group.
[Values are mean ± SD of 6 animals]

Groups	Cyt P_{450} [A]	Cyt b_5 [B]	GST[C]	GR[D]	GSH[E]
Control (Vehicle Treated)	0.38 ± 0.05 [a]	0.53 ± 0.15 [a]	131.84 ± 8.31 [a]	43.98 ± 3.83 [a]	3.25 ± 0.18 [a]
DMBA alone	0.98 ± 0.17 [b]	0.95 ± 0.09 [b]	91.66 ± 6.81 [b]	22.58 ± 4.33 [b]	2.48 ± 0.14 [b]
DMBA + rosmarinic acid	0.45 ± 0.04[c]	0.69 ± 0.04 [c]	120.69 ± 5.75 [c]	37.05 ± 4.04 [c]	2.99 ± 0.21 [c]
Rosmarinic acid alone	0.40 ± 0.03 [a]	0.55 ± 0.14 [a]	134.57 ± 10.92 [a]	44.5 ± 5.99 [a]	3.29 ± 0.25 [a]

Values that are not sharing common superscript in the same column differ significantly at $P < 0.05$
Values are represented as [A]µm of cytochrome P_{450}/g of tissue/mg protein; [B]µm of cytochrome b_5/g of tissue/mg protein; [C]µm of CDNB-GSH conjugate formed/h/mg protein; [D]µm of NADPH oxidized/h/mg protein; and [E]mg/g tissue

Table 3—Levels of TBARS and enzymatic and non-enzymatic antioxidants in skin tissues of control and experimental animals in each group.
[Values are mean ± SD of 6 animals]

Groups	TBARS[A]	SOD[B]	CAT[C]	GPxU[D]	GSHU[E]
Control (Vehicle Treated)	68.69 ± 5.36[a]	9.13 ± 1.02[a]	55.55 ± 4.17[a]	33.75 ± 3.32[a]	53.32 ± 5.44[a]
DMBA alone	110.08 ± 9.17[b]	4.69 ± 1.01[b]	42.44 ± 3.04[b]	14.21 ± 3.19[b]	38.32 ± 3.19[b]
DMBA + rosmarinic acid	79.12 ± 6.43[c]	7.53 ± 1.10[c]	50.61 ± 3.17[c]	28.81 ± 8.06[c]	47.58 ± 2.69[c]
Rosmarinic acid alone	69.42 ± 7.35[a]	9.05 ± 1.07[a]	55.49 ± 4.14[a]	32.67 ± 1.67[a]	52.76 ± 4.37[a]

Values that are not sharing common superscript in the same column differ significantly at $P < 0.05$
Values are represented as [A]m mol/100 g tissue; [B]The amount of enzyme required to inhibit 50% NBT reduction/mg protein; [C]μm of H_2O_2 utilized/sec/mg protein; [D]μm of glutathione utilized/min/mg protein; and [E]mg/100 mg tissues

Table 4—Levels of caspase-3 and caspase-9 activities in skin tissues of control and experimental animals in each group.
[Values are mean ± SD of 6 animals]

Groups	Caspase-3[A]	Caspase-9[A]
Control (Vehicle Treated)	0.162 ± 0.014[a]	0.105 ± 0.011[a]
DMBA alone	0.094 ± 0.008[b]	0.074 ± 0.006[b]
DMBA + rosmarinic acid	0.144 ± 0.012[c]	0.093 ± 0.007[c]
Rosmarinic acid alone	0.163 ± 0.013[a]	0.107 ± 0.009[a]

Values that are not sharing common superscript in the same column differ significantly at $P < 0.05$
Values are represented as [A]μm of p-NA liberated/mg protein/hr/mg protein

treated animals restored the level of TBARS and antioxidants to near normal range. Control mice treated with rosmarinic acid alone showed no significant difference in the skin tissue levels of TBARS and antioxidant status compared to control mice.

Activities of caspase-3 and caspase-9 in skin tissues of control and experimental animals in each group are shown in Table 4. Activities of caspase-3 and caspase-9 showed significant decrease in skin tissues of tumor bearing animals compared to control animals. Oral administration of rosmarinic acid to DMBA treated animals significantly restorerd the activities of caspase-3 and caspase-9.

p53 and bcl-2 immuno-expression pattern in skin tissues of control and experimental Swiss Albino mice is shown in Figs 3 and 4 respectively. Positive staining for p53 and bcl-2 were considered when more than 10% of the tumor cells showed strong nuclear and cytoplasmic staining respectively. A positive staining for p53 (60%) and bcl-2 (65%) was observed in skin tumor tissues, which was significant compared to normal tissues. Nuclear expression of p53 and cytoplasmic expression of bcl-2 increased in skin tissues of DMBA alone treated mice and observed negative staining for p53 in skin tissues of control animals. In normal epithelium, immunostaining for bcl-2 protein was identified in basal keratinocytes and dendritic cells adjacent to basement membrane. Oral administration of rosmarinic acid to DMBA treated mice significantly prevented up-regulation of p53 and bcl-2.

Discussion

With an aim to investigate the anti-tumor activity of rosmarinic acid on DMBA induced skin carcinogenesis in Swiss albino mice, tumor incidence, tumor volume and tumor burden and status of phase I and II detoxication agents, lipid peroxidation and antioxidants in DMBA treated mice were assessed. Oral administration of rosmarinic acid to DMBA treated mice completely prevented tumor formation, which suggested that rosmarinic acid inhibited the abnormal cell proliferation in DMBA induced skin carcinogenesis. It has been reported that activities of phase I and II enzymes were drastically altered during carcinogenesis including skin cancer[19,20]. Results of this study further confirm these findings. Oral administration of rosmarinic acid restored the status of detoxication enzymes in DMBA treated mice, which suggested that rosmarinic acid may have inhibited the metabolic activation of carcinogens or assisted in excretion of carcinogenic metabolite of DMBA.

Increase in TBARS and decrease in antioxidants status was noticed in the skin tissues of DMBA treated mice. Results of this study confirm the imbalance in oxidant and antioxidant (oxidative stress) status in tumor bearing mice. Oral administration of rosmarinic acid restored the status of lipid peroxidation and antioxidant status in DMBA treated mice, which suggested free radical scavenging efficacy of rosmarinic acid. Antioxidant property of rosmarinic acid is probably due to its two ortho position hydroxyl groups in the two phenolic rings.

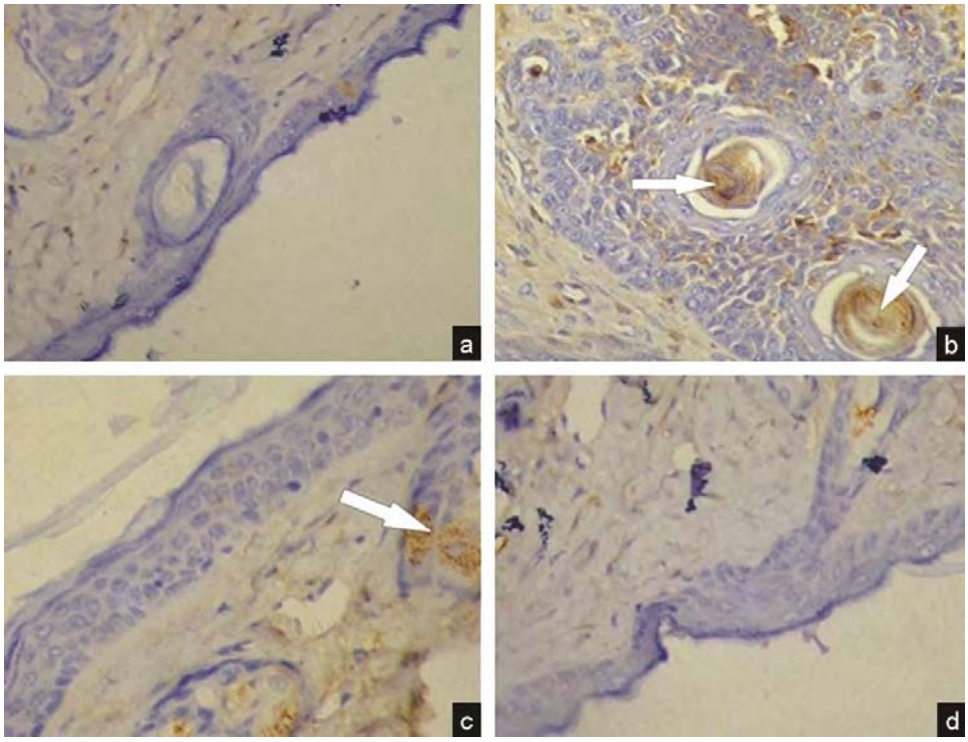

Fig. 3—Microphotographs shows the immunoexpression of p53 in the skin tissues of control and experimental Swiss albino mice (40×). [(a)-control (expression not detectable); (b)-DMBA treated (overexpressed); (c)-DMBA + rosmarinic acid treated (down regulated); and (d)-rosmarinic acid treated (expression not detectable)].

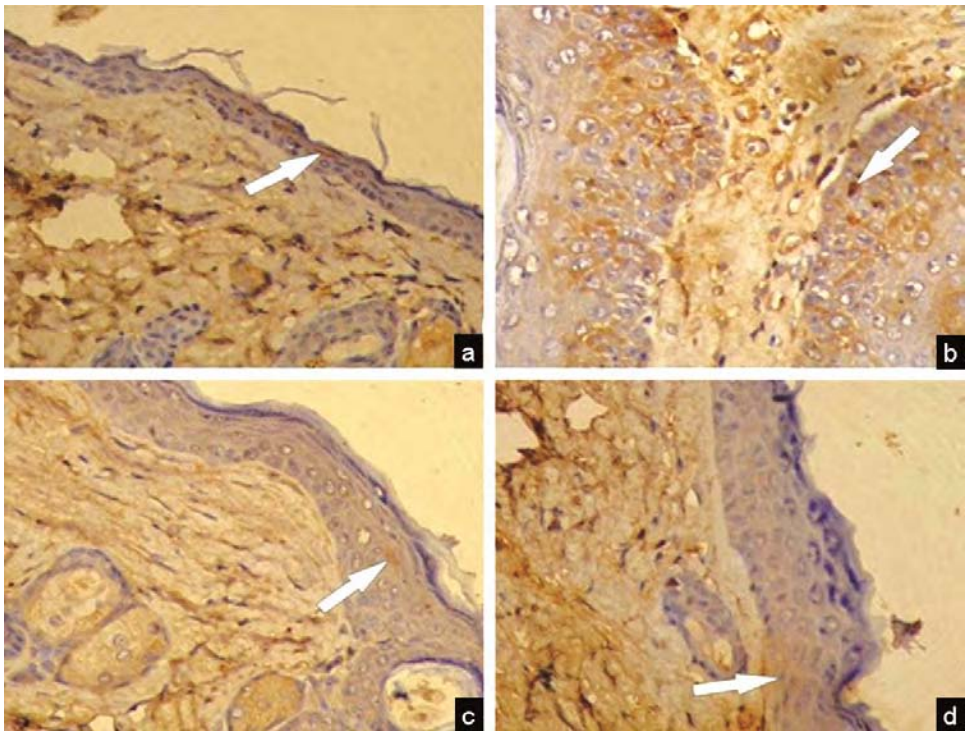

Fig. 4—Microphotographs shows the immunoexpression of Bcl-2 in the skin tissues of control and experimental Swiss albino mice (40×). [(a)-control (expression not detectable); (b)-DMBA treated (overexpressed); (c)-DMBA + rosmarinic acid treated (down regulated); and (d)-rosmarinic acid treated (mild expression detectable)].

Chemopreventive agents of natural origin act through the induction of apoptosis[21]. bcl-2 proteins execute their role in the regulation of apoptosis in conjunction with p53, bax and c-myc proteins[22]. Tumor-suppressor protein, p53, accumulates when DNA is damaged and any disruption to regulation of p53 will result in impaired apoptosis and the possible formation of tumors. Mutations in p53 gene have been detected in 50% of all human cancers and in almost all forms of skin carcinomas[23]. Over expression of p53 and bcl-2 has been documented well in skin carcinogenesis[24]. Results are in line with these findings. Over expression of p53 and bcl-2 observed in the skin tumor tissues of DMBA painted animals suggested abnormal cell proliferation and evasion of apoptosis[24]. Caspase-3 is a caspase protein that interacts with caspase-8 and caspase-9 in executing apoptosis. Caspase-3 and 9 are activated during programmed cell death[25]. Caspase-3 and 9 levels were significantly decreased in several cancers[26]. Results of the study showed that the apoptotic machinery was impaired in mice treated with DMBA alone. Oral administration of rosmarinic acid to DMBA treated mice restored the expression of p53, bcl-2, caspase-3 and 9, indicating that rosmarinic acid may have aided or stimulated the process of apoptosis during DMBA induced skin carcinogenesis.

The present study thus demonstrated the chemopreventive potential of rosmarinic acid in DMBA induced skin carcinogenesis. The chemopreventive potential of rosmarinic acid may be due to its anti-lipid peroxidative potential and modulating effect on detoxication cascade and expression pattern of p53, bcl-2, caspase-3 and 9 during DMBA induced skin carcinogenesis.

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