INHIBITORY POTENTIAL OF *ROSMARINUS OFFICINALIS* AGAINST DMBA-INDUCED SKIN PAPILLOMAGENESIS AND MODULATION OF ANTI- OXIDANT DEFENCE SYSTEM IN MICE

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Summary

In the present study, we tested the inhibitory potential of *Rosmarinus officinalis* leaves extract (ROE) on 7, 12-dimethylbenz (a) anthracene induced skin papillomagenesis in Swiss albino mice. A significant reduction in tumor incidence, tumor burden, tumor multiplicity and cumulative number of papillomas along with the significant increase in the average latent period was recorded in mice treated orally with ROE at peri - and post initiational phases of carcinogenesis as compared to positive control treated with DMBA and croton oil alone. Furthermore, a significant increase in the activity of superoxide dismutase (SOD), reduced glutathione (GSH), catalase and total proteins but a decrease in lipid peroxidation (LPO) was observed in ROE treated groups as compared to carcinogen treated control. These results suggest the anti- tumor and anti-oxidative potential of *Rosmarinus officinalis* leaves extract.

Key Words: Carcinogenesis, Chemoprevention, Skin papilloma, Rosemarinus *officinalis*, Anti-oxidant defense system, Lipid peroxidation (LPO).

Introduction

Cancer is one of the major human diseases and causes considerable suffering and economic loss worldwide. Cancer can occur in all living cells in the body and different cancer types have different natural history. The induction of cancer depends on inherited and acquired susceptibility factors, on exposure to initiation factors (exogenous & endogenous carcinogens), and on promotion and progression factors[1]. Epidemiological studies have shown that 70- 90% of all cancers are environmental.

Chemoprevention, namely inhibition or reversal of carcinogenesis, may be conducted at variety of time points in this process to reduce occurrence of *in situ* or invasive cancers or cancer morbidity and/or mortality[1].
The goal of cancer prevention is to delay or block the processes of initiation and progression from pre-cancerous cells into cancer. Cancer chemoprevention, which targets normal and high risk populations, involves the use of drugs or other chemical agents to inhibit, delay, or reverse cancer development[2, 3].
Lifestyle related factors are the most important and preventable among the environmental exposures. At present, a major focus of research in chemoprevention of cancer includes the identification, characterization, and development of a new and safe cancer chemopreventive agent[4].

Dietary habits have been regarded as one of the important etiologic factors that lead to the wide variations in the risk and incidence of cancer. Natural products, such as grape seed, green tea, and certain herbs have demonstrated anti-cancer effects[5, 6, 7]. It has been shown through epidemiological studies that consumption of fiber rich diet with low lipid content and yellow-green vegetables is associated with the reduced risk of cancer[8, 9, 10].

To find a natural product that can be used in chemoprevention of cancer, we tested a medicinal plant, *Rosmarinus officinalis* L. (family: Lamiaceae). It is a small evergreen which grows wild in most Mediterranean countries[11], reaching a height of 1.5 m. Plant essential oils are obtained through steam distillation of herbs and medicinal plants[12]. These oils have been used traditionally as medicines in many countries, and ancient peoples were also aware of their pesticidal properties; however, only in recent years these oils have been commercialized as pest control products[13]. Most of these oils are environmentally non-persistent and non-toxic to humans[14, 15, 16] while being Rosemary (*Rosmarinus officinalis* L.) oil has been traditionally used as a medicine for colic, nervous disorders and painful menstruation. Recent studies revealed that rosemary oil is an effective antibacterial agent which can control many food micro-organisms[17]. It can also inhibit the activity of food spoilage bacteria and yeast strains[18].

Thus, the aim of the present study is to investigate the morphological and biochemical alterations during carcinogenesis induced by 7, 12-Dimethyl Benz (a) anthracene (DMBA)/ croton oil and their possible amelioration by *Rosmarinus officinalis* leaves extract (ROE).

Materials and Methods

The animal care and handling was approved by our institution and was done according to guidelines set by the World Health Organization, Geneva, Switzerland, and the Indian National Science Academy, New Delhi, India. The inhibition of tumor incidence by *Rosmarinus officinalis* leaf extract was evaluated on 2-stage skin carcinogenesis, induced by a single application of DMBA (initiator) and 2 weeks later promoted by repeated application of croton oil (promoter) thrice per week following the protocol for 16 weeks.

Animals

The study was conducted on random-breed male Swiss albino mice (7-8 weeks old), weighing 24 ± 2 g. These animals were housed in polypropylene cages in the animal house under controlled conditions of temperature (25°C ± 2°C) and light (14 light:10 dark). The animals were fed a standard mouse feed procured from Aashirwad Industries, Chandigarh (India), and water *ad libitum*. Four animals were housed in 1 polypropylene plastic cage containing saw dust (procured locally) as bedding material. As a precaution against infections, tetracycline hydrochloride water was given to these animals once each fortnight. Three days before the commencement of the experiment, hair on the interscapular region of the mice was clipped. Only those animals in the resting phase of the hair cycle, showing no hair growth, were used for the study.

Chemicals

The initiator DMBA and croton oil (used as promoter) were procured from Sigma chemicals Co., St. Louis, USA. DMBA was dissolved at a concentration of 100 µg/100 µL in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

Preparation of *Rosmarinus officinals* Extract (ROE)

The identification of the plant *Rosmarinus officinalis* L. (Voucher Specimen No: DDC/2000/DEPTBT) belongs to Family Lamiaceae was done by a botanist of Department of Botany, Danielson College, Chhindwara, Madhya Pradesh (India). The non-infected leaves of the plant were carefully cleaned, shade dried and powdered. The plant material was then extracted with double distilled water (DDW) by refluxing for 36 hrs (12 x 3 hrs). Pellets of the drug were obtained by evaporation of its liquid contents in an incubator. The required dose for treatment was prepared by dissolving the drug pellets in double distilled water at dose levels of 750 mg/ kg body weight.

Experimental Design

The animals were randomized into control or experimental groups and divided into 5 groups of twelve mice each. Three days before the commencement of the experiment, hair on the interscapular region of the mice were clipped. Only the mice in the resting phase of the hair cycle were considered for the study. Body weights of the animals were recorded weekly.

Group I: Vehicle treated (Negative Control)

Animals of this group received topical application of acetone (100 µl/ mouse) on the shaven dorsal skin and double distilled water equivalent to ROE (100 µl/ mouse/ day) by oral gavage for 16 weeks.

Group-II: ROE treated (Control)

Animals of this group received Rosemary extract (ROE) orally at a dose of 750 mg/ kg of body wt./ mouse/ day, throughout the experimental period, i.e., 16 weeks.

Group-III: Carcinogen treated (Positive Control)

Mice of this group were applied topically a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin. Two weeks later, croton oil (1% w/v in acetone) was applied three times per week until the end of experiment. This group received double distilled water equivalent to ROE (100 µl/ mouse) by oral gavage for 16 weeks.

Group –IV: ROE treated (Experimental$_1$)

These experimental animals received the same treatment as in Group-III and also received ROE at a dose of 750 mg/ kg body wt. / animal/ day, orally for 7 days before and 7 days after DMBA application.

Group –V: ROE treated (Experimental$_2$)

Animals of this group received the same treatment as in Group-III and were administered ROE (750 mg/kg b. wt. / animal/ day) by oral gavage, starting from the time of croton oil treatment till the end of experiment (i.e., 16 weeks).

The following were studied:

I. Morphological Parameters

(i) Tumor incidence: The number of mice carrying at least one tumor expressed as a pe rcentage incidence.
(ii) Tumor yield: The average number of papillomas per mouse.
(iii) Tumor burden: The average number of tumors per tumor bearing mouse.
(iv) Diameter: The diameter of each tumor was measured.
(v) Weight: The weight of the tumors of each animal at the termination of each experiment was measured.
(vi) Body weight: The weight of the mice was measured weekly.
(vii) Average latent period: The lag between the application of the promoting agent and the appearance of 50% of tumors was determined. The average latent period was calculated by multiplying the number of tumors appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by total number of tumors.

$$\textbf{Average Latent Period} = \frac{\Sigma FX}{n}$$

Where F is the number of tumors appearing each week, X is the numbers of weeks, and n is the total number of tumors.

(viii) Inhibition of tumor multiplicity =

$$\frac{\text{(Total no. of papillomas in carcinogen control) – (total no. papillomas in treated) X 100}}{\text{Total no of papillomas in carcinogen control}}$$

II. Biochemical Parameters

Biochemical alterations were studied in animals of all groups at the time of the termination of the experiment (i.e., the 16th week). At the end of the 16th week, the animals were killed by cervical dislocation. The dorsal skin affected by tumors was quickly excised and washed thoroughly with chilled saline (pH 7.4). It was then weighed and blotted dry. A 10% tissue homogenate was prepared from part of the sample (skin) in 0.15 M Tris-KCl (pH 7.4), and the homogenate was then centrifuged at 2000 rpm for 10 minutes. The supernatant thus obtained was taken for estimation of lipid peroxidation (LPO) and reduced glutathione (GSH).
The following biochemical parameters were estimated in the skin of mice.

(i) Lipid Peroxidation (LPO)

The level of LPO was estimated spectrophotometrecally by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkhawa *et al.* (1979)[19]. Briefly, thiobarbituric acid (0.6%), sodium dodecyl sulphate (0.1%), and trichloroacetic acid (20%) were added to 200 µl of the tissue homogenate (10%) prepared as described above. This mixture was heated for 60 minutes, cooled, and extracted with N butanol- pyridine (15:1), the optical density (OD) was recorded at 532 nm and the contents were expressed as nmol/mg of tissue.

(ii) Reduced Glutathione (GSH)

The level of GSH was estimated as total nonprotein sulphahydryl group by the method of Moron *et al*. (1979)[20]. The homogenate was immediately precipitated with 100 µl of 25% trichloroacetic acid (TCA) and the precipitate was removed after centrifugation. Free endogenous-SH was assayed in a total volume of 3 ml by the addition of 200 µl of 0.6 mM 5, 5′ dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8.0) to 100 µL of the supernatant and the absorbance was recorded at 412 nm using a UV-VIS Systronics spectrophotometer. Reduced GSH was used as a standard. The levels of GSH were expressed as µmol/gm of tissue.

(iii) Catalase

The catalase activity was assayed by the method of Aebi[21]. The change in absorbance was followed spectrophotometrically at 240 nm after the addition of H_2O_2 (30 mM) to 100 µL of the supernatant (10% of skin homogenate prepared in 50 mM phosphate buffer and centrifuged for 10 min.) in 50 mM phosphate buffer (pH 7).The activity of the enzyme is expressed as U/mg of tissue, where U is µmol of H_2O_2 disappearance/min.

(iv) Proteins

Total Proteins were estimated by the method of Lowery *et al*., [22] using bovine serum albumin as a standard and the level was expressed as mg/ ml.

(v) Superoxide dismutase

Superoxide dismutase (SOD) level in skin was estimated by the method of Marklund and Marklund[23] and its activity was expressed as units/min/mg protein.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean. The data from biochemical determinants were analyzed using the Student's *t* test.

Results

The results from the present study show the effect of *R. officinalis* on DMBA induced skin papillomagenesis in mice. Animals from group III (Carcinogen treated control), in which a single topical application of DMBA was followed by croton oil, produced skin papillomas, which started appearing from the seventh week onward. The tumor incidence in the DMBA/croton oil treated mice was noted as 100% by the end of the experiment (i.e., 16 weeks). The cumulative number of papillomas in these mice was recorded as 62 while such number reduced significantly in experimental groups (Group IV: 20, Group V: 27). The average number of papillomas per mouse (tumor yield) as well as the papillomas per papilloma-bearing mice (tumor burden) were found to be 6.2 in carcinogen treated control (Group III), whereas Group IV and Group V showed reduction in tumor yield (1.66, 2.25) and tumor burden (2.85, 3.375). The average latent period was also found to be greater (Group IV: 11.80, Group V: 11.03) with ROE administration as compared to carcinogen treated control i.e., 7.53 (Fig. 1- 6). There was significant alteration in the body weight of animals were seen during experimental periods (Table 1). At the end of the experiment (i.e. at 16 weeks), even the tumor volume in ROE treated animals was much lower than tumor volume of Carcinogen treated animals (Table 1). No tumor formation was seen in the vehicle-treated control animals.

The level of MDA in the skin of mice treated with DMBA/TPA increased by 35.32% ($p < 0.001$); whereas in group IV and group V, such increase was only 16.47% ($p < 0.01$) and 19.53% ($p < 0.05$) respectively as compared to vehicle treated control (Fig. 7).

The levels of GSH in skin of mice treated with DMBA/TPA was decreased by 89.09% ($p < 0.001$) while the same was depleted by 48.42% ($p < 0.05$) and 51.15% ($p < 0.05$) in the skin of animals of Group IV and Group V respectively as compared with vehicle-treated control animals (Group I) (Fig. 8).

The activity of catalase (CAT) in skin of mice treated with DMBA/TPA (Group III) exhibited 65.53% ($p < 0.001$) decrease from vehicle treated control, whereas only 33.55% ($p < 0.01$) and 35.32% ($p < 0.05$) decrease in CAT was measured in Group IV and Group V respectively (Fig. 9).

A significant decrease (51.96%; $p < 0.001$) in the level of protein was observed in the skin of DMBA/TPA-treated animals (Group III), whereas animals in Group IV and Group V exhibited only 13.98% ($p < 0.01$) and 16.05% ($p < 0.05$) decrease as compared with vehicle treated control. There was an increase in protein level in the skin of animals treated with DMBA/ROE/TPA as compared with the level in DMBA/TPA-treated animals (Fig. 10).

On application of DMBA/TPA, the SOD activity in skin showed 61.11% decrease ($p < 0.001$) whereas only 34.80% ($p < 0.01$) decrease in group IV and 29.17% ($p < 0.05$) in Group V was observed as compared with vehicle treated control (Fig. 11).

Table 1: Inhibitory potential of *R. officinalis* extract (ROE) against DMBA-induced skin papillomagenesis in mice*

Treatment Groups	Body weight (gm) (mean ± S.E.)		Tumor size		Tumor weight (mg)
	Initial	Final	2-5 mm	6-9 mm	
Group I	25.55±1.31	37.00±1.23	-	-	-
Group II	22.00±1.22	35.50±1.11	-	-	-
Group III	23.33±0.99	30.00±1.09	22	40	71.56
Group IV	25.50±1.36	35.00±1.13	12	8	38.7
Group V	23.50±1.55	34.50±1.25	17	10	49.59

*Treatment schedule of the groups is specified in materials and methods.

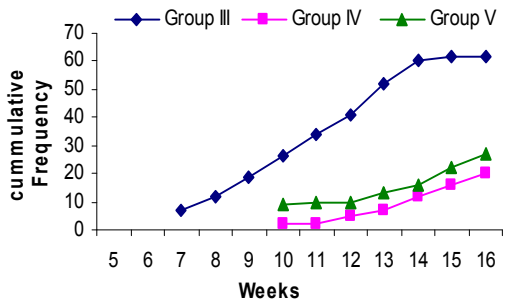

Figure -1 Variation in the cumulative frequency during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

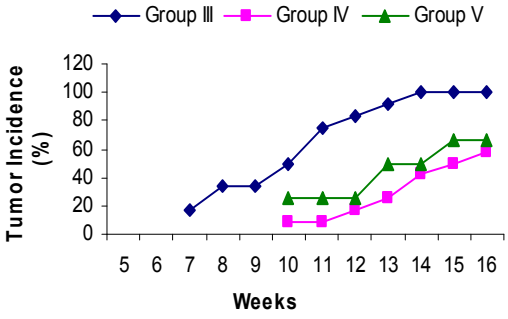

Figure -2 Variation in the tumor incidence during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

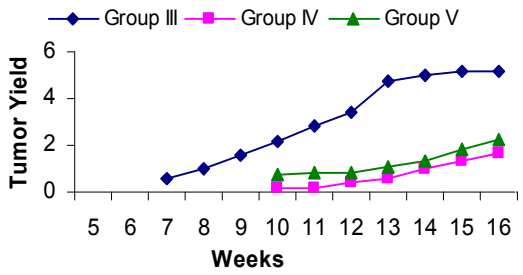

Figure -3 Variations in the tumor yield during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

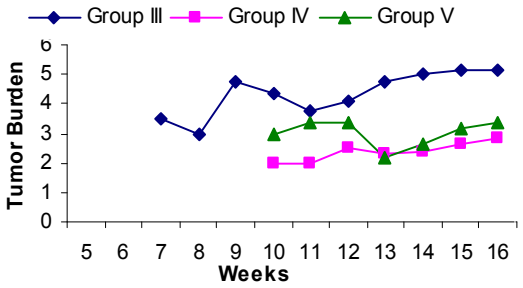

Figure- 4 Variations in the tumor burden during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

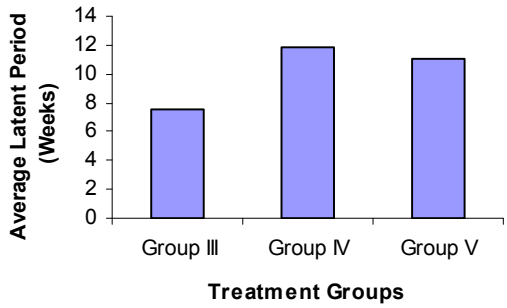

Figure - 5 Variations in the average latent period during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

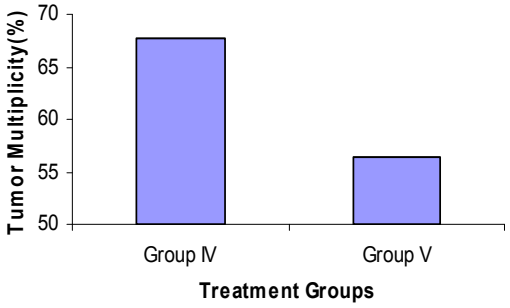

Figure - 6 Variations in the tumor multiplicity during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract

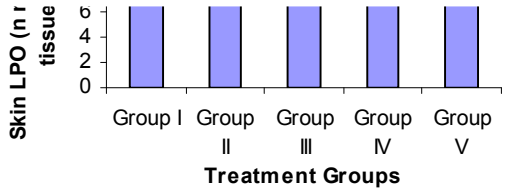

Figure- 7 Variations in the lipid peroxidation (LPO) level in skin during DMBA-induced skin papillomagenesis with or *R. officinalis* extract
[a]p < 0.05, [b]p < 0.01, [c]p < 0.001

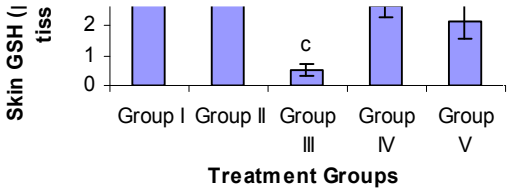

Figure-8 Variation in the glutathione (GSH) level in skin during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract
[b]p < 0.01, [c]p<0.001

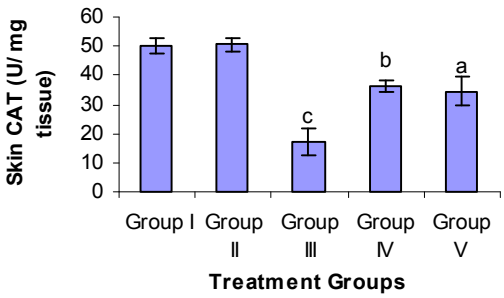

Figure -9 Variations in the catalase (CAT) level in skin during DMBA-induced skin papillomagenesis with or Without *R. officinalis* extract
$^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$

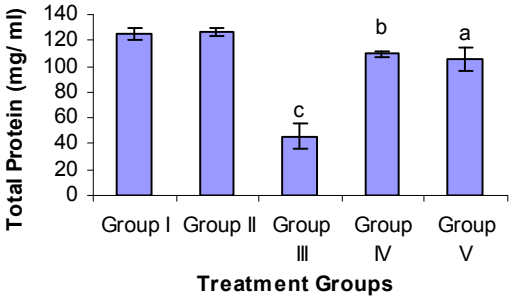

Figure-10 Variation in the protein level in skin during DMBA -induced skin papillomagenesis with or without *R. officinalis* extract
$^{a}p < 0.05$, $^{b}p < 0.01$, $^{c}p < 0.001$

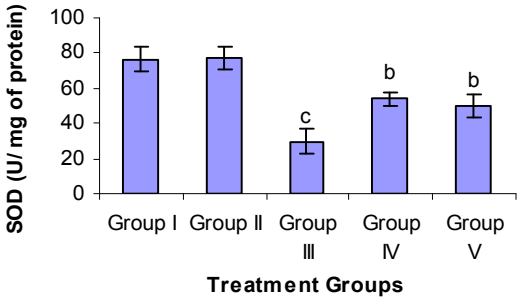

Figure-11 Variation in the protein level in skin during DMBA -induced skin papillomagenesis with or without *R. officinalis* extract administration
$^{b}p < 0.01$, $^{c}p < 0.001$

Discussion

Chemoprevention is an innovative area of cancer research that focuses on the prevention of cancer through pharmacologic, biologic, and nutritional interventions. Thus, there is a need for exploring drugs/agents which act as chemopreventive agents. In the present study, we have studied the chemopreventive effect of ROE against 7, 12-Dimethyl Benz (a) anthracene (DMBA)/ croton oil, using a mouse skin carcinogenesis model.

DMBA is a synthetic polycyclic aromatic hydrocarbon (PAH), which has been used extensively as a prototype carcinogen. The main target sites for the potent carcinogenicity of this agent in rodents are the skin and the mammary glands[24].

Reactive oxygen species (ROS) formed during DMBA metabolism can diffuse from the site of generation to other targets within the cells or even propagate the injury outside to intact cells. These ROS produce deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation[25].

During oxidative stress, malondialdehyde (MDA) and/or other aldehydes are formed in biological systems. These reactive oxygen species are responsible for initiating the multistage carcinogenesis process starting with DNA damage and accumulation of genetic events in one or few cell lines which leads to progressively dysplastic cellular appearance, deregulated cell growth, and finally carcinoma[26]. Hence, therapy using free-radical scavengers or antioxidants has potential to prevent, delay or ameliorate many of these disorders[27].

The polyunsaturated fatty acids present in cell membranes are easily oxidized both by enzymatic and autoxidative peroxidation via free radical chain reactions. Lipid peroxidation can be induced by free radicals (superoxide & hydroxyl) and by the singlet oxygen produced in biological systems. Thus, lipid peroxidation may be prevented by free radicals scavengers or by singlet oxygen quenchers. Elevated levels of MDA were observed in the present study in skin of animals treated with DMBA/TPA suggesting oxidative stress in DMBA/TPA-induced mouse skin carcinogenesis. Significant decrease in MDA levels by ROE treatment indicates reduced oxidative stress, thus indicating its protective potential against skin carcinogenesis. This protective effect of ROE as indicated by reduced lipid peroxidation could be due to increase in reduced glutathione (GSH) and the antioxidant enzymes CAT and SOD.

Superoxide dismutase is a ubiquitous chain breaking antioxidant and is found in all aerobic organisms. It is a metalloprotein widely distributed in all the cells and plays a protective role against ROS-induced oxidative damage. It converts superoxide radical into hydrogen peroxide whereas glutathione peroxidase (GPXs) and catalase (CATs) convert H_2O_2 into water[28]. SOD and CAT activity has been augmented significantly in the current study by the ROE administration.

Epidemiological and *in vitro* studies strongly suggest that food containing phytochemicals with antioxidants have potentially protective effects against many disorders including cancer, diabetes and cardiovascular diseases[29]. Consumption of fruits and vegetables has contributed to the prevention of degenerative processes caused by oxidative stress[30, 31]. An attractive hypothesis is that vegetables and fruits contain compounds that have a protective effect independent of that of known nutrients and micronutrients. This is supported by *in vitro* and *in vivo* studies which show that naturally occurring plant compounds may inhibit various stages in the cancer process[32].

Extracts of leaves of *R. officinalis* are widely used as antioxidant in the food industry and are shown to be safe by not producing any acute toxicity in animal tests[33-36]. Extracts of this plant have also been shown to exhibit an inhibitory activity against KB cells[37], an assay which is widely used to identify anticancer agents in natural products. The potentiality of rosemary extract to inhibit chemically-induced mammary tumorigenesis in female rats and to prevent carcinogen-DNA adduct formation in mammary epithelial cells was investigated[38]. Dietary supplement with 1% (w/w) rosemary extract in rats treated with 7, 12-dimethylbenz (a) anthracene (DMBA) significantly decreased the mammary tumorigenesis by 47% and inhibited total *in vivo* binding of DMBA to mammary epithelial cell DNA by an average of 42%, suggesting that the use of rosemary extract and its individual antioxidant constituents as chemopreventive agents for tumorigenesis warrant further investigation.

It has been reported that the constituents (caffeic acid, rosmarinic acid, carnosol or carnosic acid) of *R.officinalis* are antioxidants[39-41]. Presence of these compounds reduces the level of oxidation products during the autoxidation of the polyunsaturated fatty acid (linoleic acid). It reduces the risk of lipid peroxidation by elevation in the level of skin GSH and catalase, thereby reduced the tumor incidence, count of skin papillomas, tumor burden, tumor yield. The onset of papillomas development being delayed when Rosemary extract was administered at both the intervals. However, the greatest effects in the present study were achieved to the assumption that the plant extract may have either inhibited DMBA metabolism to its active form, or delayed the promotion phase of carcinogenesis or down regulated reactive oxygen species formation, by modulating ornithine decarboxylase, protein kinase C activity, or have decreased prostaglandin synthesis.

The results obtained from the present study concerning antioxidant activity and chemopreventive potential of the hydralcoholic extracts of *R. officinalis* suggest the possible use of such plant in the prevention of cancer, however such plant extract may also be trialed for other tumor models.

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