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Anti-tumor and Anti-oxidative Activity of R*osmarinus officinalis* in 7, 12 Dimethyl Benz(a) Anthracene Induced Skin Carcinogenesis in Mice

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Abstract

The present investigation was undertaken to explore the antitumor-promoting activity of *R.officinalis* on 2-stage skin carcinogenesis, induced by a single topical application of 7,12-dimethylbenz(a)anthracene and promoted by repeated treatment of croton oil for 16 weeks in Swiss albino mice. Oral administration of *R.officinalis* leaves extract, during the peri-and post-initiation stage induced by 7,12-dimethylbenz[a]anthracene (DMBA), reduced the tumor burden to 2.6 (positive control value: 5.16); cumulative number of papillomas to 13 (positive control value: 62) and percent incidence of mice bearing papillomas to 41.66 %, (positive control value: 100%). A significant ($p<0.001$) decreased was observed in Lipid peroxidation level by the administration of ROE. Reduced glutathione (GSH) and total proteins was found to be significantly elevated in liver and skin ($p < 0.001$) of mice in ROE treated group. Skin and liver of *R.officinalis* treated group animals showed a significant enhancement in antioxidant enzymes like superoxide dismutase (SOD) and Catalase, when compared with the carcinogen treated control. These studies indicate that *R.officinalis* could reduce the chemical induced tumor and oxidative stress during skin carcinogenesis.

Keywords: 2-stage skin carcinogenesis, Glutathione (GSH), SOD, papilloma, Anti-oxidants.

1. Introduction

Non-communicable diseases including cancer are emerging as major public health problems in India. These diseases are lifestyle related, have a long latent period and needs specialized infrastructure and human resource for treatment. Cancer is a serious global health problem. In

developed countries, cancer at present is the second most common cause of death and a similar trend is happening in developing countries as well. Substantial epidemiological data on populations indicate an association between many human cancers and lifestyle/ diet, moreover detailed studies of mutational events in human cancers have provided evidence for a direct action of environmental carcinogens in the development of certain cancers [1].

There are striking variations in the risk of different cancers by geographic area. Most of the international variation is due to exposure to known or suspected risk factors related to life style or environment, and provides a clear challenge to its prevention.

Carcinogenesis in the skin can be induced by the sequential application of a sub threshold dose of a carcinogen (initiation phase) followed by repetitive treatment with a non carcinogenic tumor promoter. The initiation phase requires only a single application of either a direct-acting carcinogen or a pro carcinogen (which has to be metabolized before being active) and is essentially irreversible; the promotion phase is initially reversible but later becomes irreversible. This system not only has provided an important model for studying carcinogenesis and for bio-assaying carcinogenic agents, but it is also one of the best systems for investigating the effects of inhibitors of chemical carcinogenesis [2].

Chemoprevention is regarded as one of the efficient strategies to prevent cancer. It can be defined as prevention by administration of chemical entities, either as individual drugs or as naturally occurring constituents of the diet.

Laboratory studies and epidemiological evidence lead credence to chemoprevention strategy in attenuating the risk of developing cancer in human beings [3, 4]. Many nutrient and non-nutrient dietary constituents of plant origin have evidence of chemoprevention by inhibiting and/or suppressing or reversing cancer incidence trend evoked by multitude of factors including environmental chemical carcinogens.

Ayurveda, the Indian traditional system of medicine, which dates back many centuries, uses many herbal extracts to cure a variety of diseases including cancer. One such popularly used plant is *Rosmarinus officinalis* that belongs to family Lamiaceae. Rosemary (*Rosmarinus officinalis* L.) is a common household plant grown in many parts of the world. It is used for flavoring food, as a beverage, and in cosmetics [5-7]. In folk medicine, it is used as an antispasmodic in renal colic and dysmenorrhea, in relieving respiratory disorders, and to stimulate growth of hair [8]. Extract of rosemary relaxes smooth muscles of the trachea and intestine, and it has choleretic, hepatoprotective, and antitumorogenic activities [9].

In the present communication, leaves extract of *R.officinalis has* been tested to explore its antitumor action during chemical induced carcinogenesis.

2. Materials and Methods

The animal care and handling were approved by our institution and was done according to the guidelines set by the World Health Organization, Geneva, Switzerland, and the Indian National Science Academy, New Delhi, India. The inhibition of tumor incidence by *R.officinalis* leaves extract was evaluated on two-stage skin carcinogenesis, induced by a single application of DMBA (initiator), and two weeks later, promoted by repeated application of croton oil (promoter) thrice per week, following the protocol for 16 weeks [10].

2.1 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℃ ± 2℃) 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 one 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.

2.2 Chemicals

7, 12-Dimethyl Benz (a) anthracene (DMBA) and croton oil was procured from Sigma Chemical Co., USA. DMBA was dissolved at a concentration of 100 µg/ 100µl in acetone. Croton oil was mixed in acetone to give a solution of 1% dilution.

2.3 Preparation of *Rosmarinus officinals* Extract (ROE)

The identification of the plant *Rosmarinus officinalis* L. (Voucher Specimen No: DDC/2000/DEPTBT), 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 the dose level of 750 mg/ kg. b. wt.

2.4 Experimental design

The dorsal skin (2 cm diameter) of Swiss albino mice were clipped off three days before the application of the chemicals or modulator, and animals in the resting phase of hair growth cycle was selected for the experiment. The animals were assorted into the following groups:

Group-I Normal (n = 12): Animals were put on a normal diet and double distilled water was given by oral gavage daily for 16 weeks; these animals served as a negative control.

Group-II ROE Alone (n=12): Animals belonging to this group received *R. officinalis* (750 mg/k.g. b.wt./animal/day) leaves extract by oral gavage throughout the experimental period i.e., 16 weeks and acted as drug treated control.

Group III Carcinogen treated Control (n=12): Animals of this group were topically treated with a carcinogenic dose of DMBA (100µg/100 µl acetone). After 2 weeks of the carcinogen treatment, application of croton oil (1% croton oil in acetone) was given three times a week for 16 weeks to serve as positive control group.

Group IV ROE treated Experimental (n=12): Animals were orally administered with *Rosmarinus officinalis* leaves extract (ROE) with the dose of 750 mg/ k.g. b.wt/ animal/day throughout the experimental period including 1 week before and 2 weeks after DMBA application followed by the application of croton oil in the promotion stage.

The following parameters were studied:

I. Morphological

(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 time 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.

$$\text{Average Latent Period} = \frac{\sum FX}{n}$$

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

II. Biochemical

Biochemical alterations were measured in animals of all the 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 necropsied. The dorsal skin affected by tumors and liver were 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 the part of the sample (skin & liver) 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 liver and 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)[11]. 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 on systronics UV-Vis spectrophotometer, and the contents were expressed as nmol/mg of tissue.

(ii) Glutathione (GSH): The level of GSH was estimated as total non protein sulphahydryl group by the method of Moron *et al*. (1979)[12]. The homogenate prepared 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 (CAT): The catalase activity was assayed by the method of Aebi[13]. 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*.,[14] using bovine serum albumin as a standard and the level was expressed as mg/ gm.

(v) Superoxide dismutase (SOD): SOD level was determined according to the method of Marklund and Marklund [15] by quantification of pyrogallol auto oxidation inhibition and the results expressed as units/mg protein. One unit of enzyme activity was defined as the amount of enzyme necessary for inhibiting the reaction by 50%. Auto oxidation of pyrogallol in Tris-HCL buffer (50 mM, pH-7.5) was measured by increase in absorbance at 420 nm.

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

Treatment Group	Body weight (gm) (mean ± S.E.)		Tumor size		Tumor weight (mg)
	Initial	Final	2-5 mm	6-9 mm	
I	25.55±1.31	37.00±1.23	-	-	-
II	22.00±1.22	35.50±1.11	-	-	-
III	23.33±0.99	30.00±1.09	22	40	71.56
IV	24.50±1.26	36.00±1.21	9	4	31.34

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

2.5 Statistical Analysis

Data from different experimental groups were analysed and expressed as mean ± SD. The significant level of difference between carcinogen treated control and ROE treated experimental groups were statistically analysed using Student's t-test.

3. Results

Table 1 depicts the findings of the present investigation. The gain in body weight is not affected by the application of *R. officinalis* extract. In the positive control group (Group III), in which a single topical application of DMBA was followed 2 weeks later by repeated application of croton oil, showed 100% tumor incidence and the average number of papillomas per mouse as well as the number of papillomas per papilloma bearing mouse was found to be 5.16.

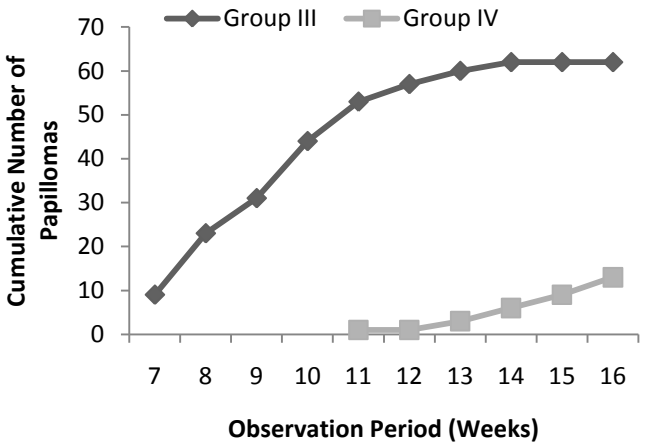

Figure 1. Variation in the cumulative number of papillomas during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Animals of Group IV, received the ROE treatment continuously at the peri-as well as at the post-initiational phase of papillomagenesis, showed 41.66% tumor incidence (Fig.2) and the average number of tumors per mouse and papillomas per papilloma bearing mouse were 1.083 and 2.6, respectively(Fig. 3,4). Interestingly, the extract of *R.officinalis* also delayed the onset of DMBA/Croton oil induced skin papilloma in mice, and the cumulative number of papillomas was recorded 62 in positive control group while the same was decreased to 13 in Group IV (Fig. 1).

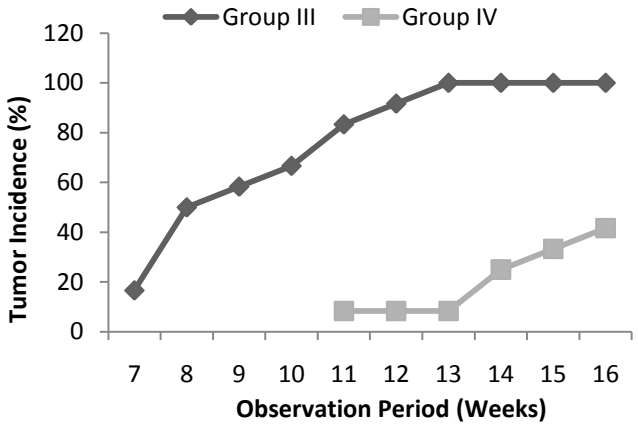

Figure 2. Variation in the Incidence of skin Papillomas during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

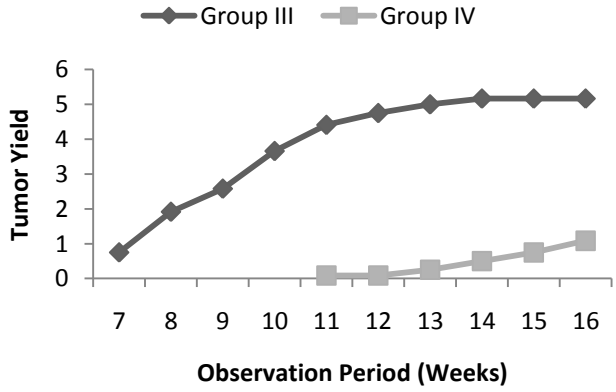

Figure 3. Variation in the Tumor Yield during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

The results indicated that the administration of *R.Officinalis* extract, could prolong the average latent period of tumor occurrence in Group IV (Group III- 7.53; Group IV-12.46) (Fig.5). No skin papillomas appeared in the animals treated orally, with ROE alone (Group II).

The formation of malondialdehyde measured as index of lipid peroxidation, in liver (6.87 ±0.51) and skin (8.98 ±0.21), revealed a significant ($p<0.01$-$p<0.001$) decrease in ROE experimental Group IV animals in comparison to the carcinogen treated control (Group III). There was no statistical significant difference was evident in

ROE treated Group II as compared to Negative control (Group I) (Fig.6).

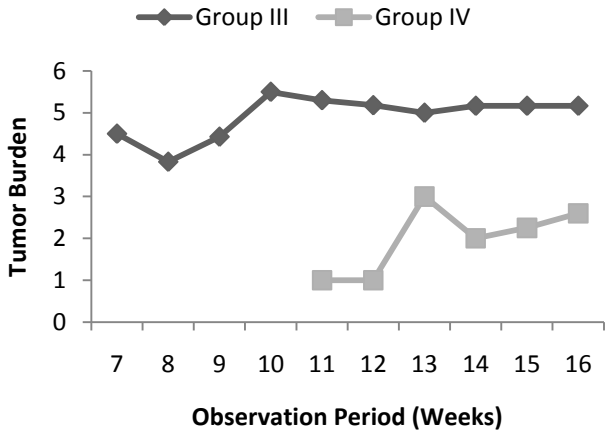

Figure 4. Variation in the Tumor Burden during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

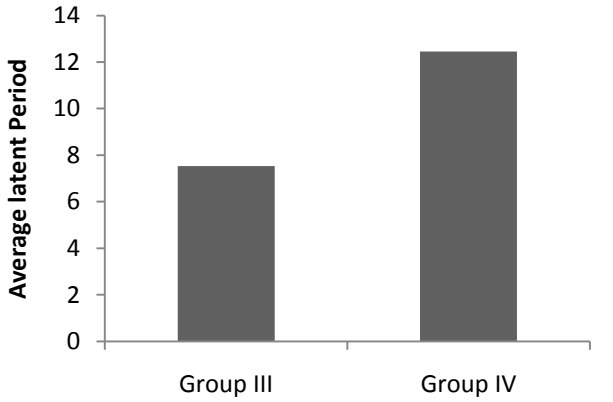

Figure 5. Variation in the Average latent period during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Specific activities of both the antioxidant enzymes studied, i.e., SOD (Fig.10) and catalase (Fig.8) have shown a significant increase ($p<0.001$- $p<0.01$) in liver (55.82 ± 2.69; 43.81 ± 4.66) and skin (62.53 ± 4.17; 40.88 ± 3.64) of ROE treated experimental animals as compared to the positive control group.

The treatment of *R.officinalis* could appreciably ($p < 0.001$) enhance the GSH (Fig.7) and total proteins content (Fig. 9) in liver (2.17 ± 0.41; 120.21 ± 8.56) & skin (3.17 ± 0.43; 115.76 ± 8.85) of mice of Group IV, when compared with positive control values (Group III).

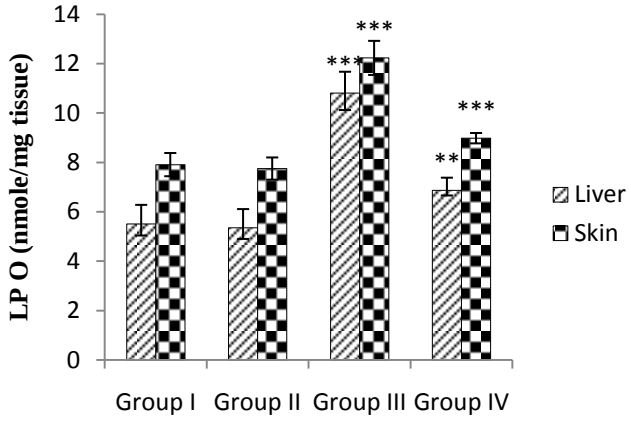

Figure 6. Variation in the LPO level in liver and skin of mice during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Statistical comparison:
Normal v/s Carcinogen treated Control; Carcinogen treated Control v/s ROE Experimental

Significance levels: $**p \leq 0.01$, $***p \leq 0.001$

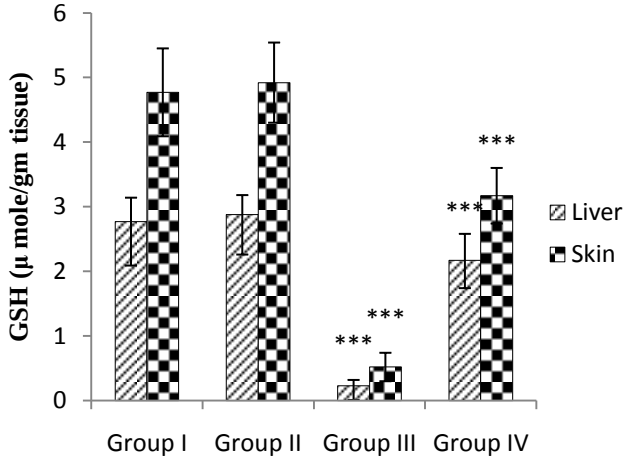

Figure 7. Variation in the GSH level in liver and skin of mice during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Statistical comparison:
Normal v/s Carcinogen treated Control; Carcinogen treated Control v/s ROE Experimental

Significance levels: $***p \leq 0.001$

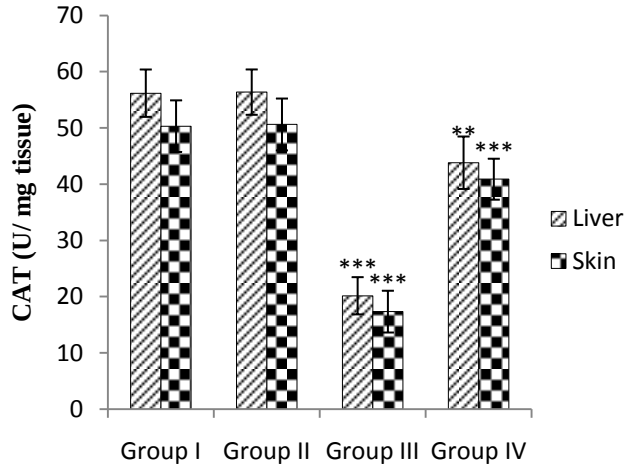

Figure 8. Variation in the Catalase activity in liver and skin of mice during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Statistical comparison:

Normal v/s Carcinogen treated Control; Carcinogen treated Control v/s ROE Experimental

Significance levels: **p ≤ 0.01, ***p ≤ 0.001

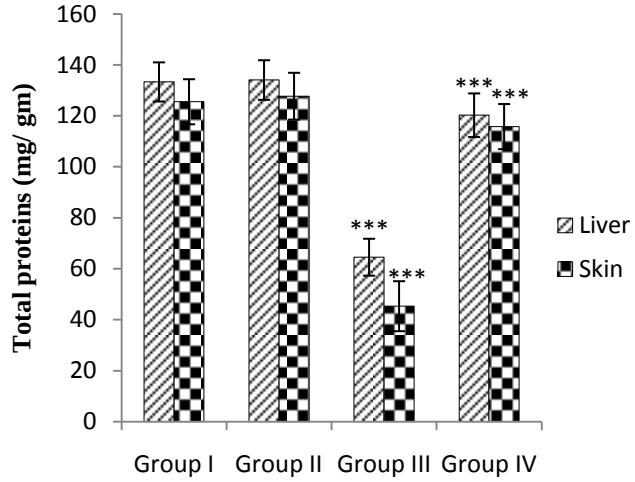

Figure 9. Variation in the Total protein level in liver and skin of mice during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Statistical comparison:

Normal v/s Carcinogen treated Control; Carcinogen treated Control v/s ROE Experimental

Significance levels: ***p ≤ 0.001

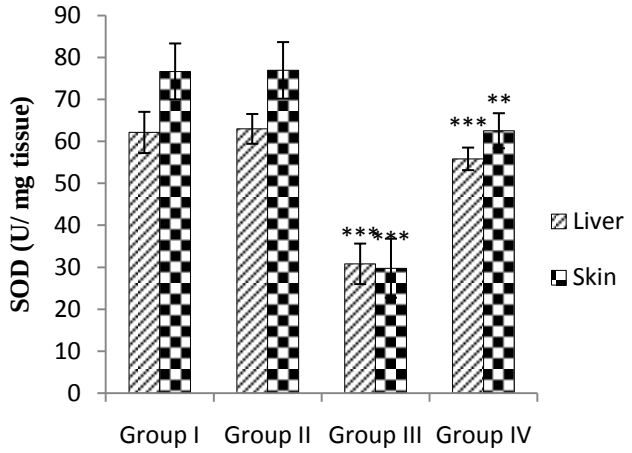

Figure 10. Variation in the SOD activity in liver and skin of mice during DMBA-induced skin papillomagenesis with or without *R. officinalis* extract (ROE) administration.

Statistical comparison:

Normal v/s Carcinogen treated Control; Carcinogen treated Control v/s ROE Experimental

Significance levels: **p ≤ 0.01, ***p ≤ 0.001

4. Discussion

In the present study, we have examined the effect of *R.officinalis* leaves extract on DMBA-initiated and croton oil promoted tumorigenesis in a two-stage skin carcinogenesis model. DMBA is present in the environment as a product of incomplete combustion of complex hydrocarbons. DMBA, being an indirect carcinogen, requires further metabolic activation to become an ultimate carcinogen. The thiol epoxide and other toxic reactive oxygen species formed during metabolic activation of DMBA can cause chromosomal damage by binding with adenine residues of DNA. It has been sugested that DMBA is highly mutagenic to lac I in mammary tissues and that adducts with both G:C and A:T base pairs participate in forming mutations in DMBA-treated Big Blue rats [16].

Carcinogenesis involves mainly three steps namely initiation, promotion and progression. The implication of free radicals in different steps of carcinogenesis is well documented [17, 18]. Generally, free radicals attack the nearest stable molecule, "stealing" its electron. When the "attacked" molecule loses its electron, it becomes

a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell. Normally, the body can handle free radicals, but if antioxidants are unavailable, or if the free-radical production becomes excessive, damage can occur. The generation of reactive oxygen species (ROS) and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal biochemical process, which further leads to the reduction of body weight[20,21].

Epidemiological data from more than 250 case control and cohort studies shows an inverse relationship between the risk of certain types of cancer and consumption of dietary phytochemicals and fibers [19]. Multiple mechanisms have been identified for the anti-neoplastic effects of plants, including antioxidant, anti-inflammatory and anti-proliferative activities, inhibition of bio- activating enzymes and induction of detoxifying enzymes [20].

Most human tumors have a long history of pathological development during which they pass through several preneoplastic and premalignant stages before becoming malignant. This situation offers the opportunity to interrupt or reverse tumor development at a still harmless stage, for instance by properly adjusting lifestyle (stop smoking, eat more vegetable diet, etc.) or by chemoprevention, i.e. by taking drugs acting on distinct molecular processes of tumorigenesis.

Anti-oxidants neutralize free radicals by donating one of their own electrons, ending the electron-"stealing" reaction. The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form they act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease.

In the present study, DMBA/ Croton oil-treated mice exhibited macroscopically detectable tumors and a significant inhibition of body growth. DMBA treatment increased the cumulative number of papillomas, tumor incidence, tumor burden and tumor yield in carcinogen treated control animals (Group III).

By the application of rosemary to mouse skin had a strong inhibitory effect on DMBA/TPA-induced tumor promotion in mice and the present investigation are based on an examination of the inducibility of anti-oxidant enzymes involved in detoxifying the free radicals. There were no adverse effects on normal animal health at the given dose levels of *R.officinalis*, as there were no decreases in body weight and body weight gain. An increase in the levels of protein in Group IV is indicative of induced protein synthesis and possibly that associated with endoplasmic reticulum, which could be responsible for the increase in mouse liver weight.

There was no significant difference in weight gain in experimental group throughout the duration of the experiment (i.e., 16 weeks). Thus, *R.officinalis* treatment did not influence the normal growth and development of the animals during the experimental period.

Interestingly the extract of *R.officinalis* also significantly delayed the onset of DMBA/Croton oil induced skin papilloma in mice of group IV. A significant inhibition in tumor burden as well as tumor incidence has been observed in the skin papillomagenesis studies. Literature suggest that naturally occurring substances have been known to cause inhibition of tumorigenesis either by preventing the formation of active carcinogen from their precursors or by suppressing the expression of neoplasia [21,22].

Rosmarinus officinalis leaves contains various antioxidants include carnosol, carnosic acid, rosmaridiphenol, rosmanol, isorosmanol, epirosmanol, and rosmariquinone [23], about 90% of the antioxidant activity of rosemary can be attributed to carnosol and camosic acid [24].

Rosemary crude extract and its constituents carnosol and carnosic acid have been reported to show chemopreventive benefits in *in vivo* anti-tumorigenesis studies, with the activation of phase I and phase II detoxifying enzymes being implicated as the mechanisms of action [25,26,27].

Antioxidants are reported to act as protective agents against cancer [28–31]. Antioxidants act as the primary line of defense against ROS and suggest their usefulness in eliminating the risk of oxidative damage induced during carcinogenesis. SOD and CAT acts as mutually supportive antioxidative enzymes, which provide protective

defense against reactive oxygen species [32]. The present study reveals that the activity of SOD is depleted in the cancer-bearing animals (Group III), which may be due to altered antioxidant status caused by carcinogenesis. A similar result was observed for CAT in group III, which may be due to the utilization of antioxidant enzymes in the removal of H_2O_2.

The liver is a versatile organ involved in drug metabolism and detoxification, hence the antioxidant enzymes and the other parameters were estimated in liver as well as in mouse skin. During oxidative stress, MDA and/or other aldehydes are formed in biological systems. These can react with amino acids and DNA and introduce cross linkages between proteins and nucleic acids, resulting in alterations in replication, transcription [33] and leading to tumor formation. Elevated levels of MDA were observed in liver as well as in skin of animals treated with DMBA/croton oil (Group III) suggesting oxidative stress in DMBA/croton oil-induced mouse skin carcinogenesis. Whilst, significant decrease in MDA levels by ROE treatment in Group IV indicates reduced oxidative stress, thus indicating its protective potential against skin carcinogenesis. This anti-oxidative effect of ROE as indicated by reduced lipid peroxides might be due to increase in non-enzymatic antioxidant molecule GSH and the antioxidant enzymes viz, CAT and SOD. ROE alone- treated animals showed no significant difference in TBARS and antioxidants status.

Glutathione, a biologically important tripeptide, is essential for maintaining cell integrity, due to its reducing properties and participation in the cell metabolism. We also observed decreased activities of GSH in positive control animals. ROE administration increased the GSH levels, which clearly suggest their antioxidant property. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also directly detoxifies reactive species [34].

The free radical hypothesis supported the fact that the antioxidants can effectively inhibit carcinogenesis and the observed properties may be attributed to the antioxidant principles present in the extract. It is likely that a combination of several components is responsible for the inhibitory effect of rosemary on DMBA induced skin carcinogenesis.

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