Effect of *Rosmarinus officinalis* on DMBA-induced mouse skin tumorigenesis: A preliminary study

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Summary

The present investigation envisages anti-tumor promoting activity of *Rosmarinus officinalis* on two-stage skin tumorigenesis in Swiss albino mice. Oral administration of *R officinalis* leaves extract at a dose of 800 mg/ kg body wt. / day at pre, peri and post-initiational phases, was found to be effective in decreasing the rate of tumor incidence in comparison to the control. Furthermore, cumulative number of papillomas, tumor yield and tumor burden were also found to be reduced. The level of lipid peroxidation was significantly reduced (near to normal) in blood serum and liver. In addition, depleted levels of glutathione were restored in rosemary-treated groups. The study has revealed the chemopreventive role of *Rosmarinus officinalis* against DMBA-induced skin tumorigenesis in mice.

Key Words: Chemoprevention, *Rosmarinus officinalis*, Glutathione, Lipid peroxidation, Tumor incidence.

Introduction

The areas of dietary modification and chemoprevention show considerable promise as effective approach and are a focus of research efforts in the field of cancer prevention. Dietary epidemiological studies have provided initial leads for the identification of numerous naturally occurring candidate chemopreventive agents (1) and laboratories studies have identified many potential agents that suppress carcinogenesis in animal models. Plants and plant products have been a source of medicinal agents since time immemorial. Various plants like *Withania somnifera, Hemidesmus indicus, Emblica officinalis, Hippophae rhamnoides, Alstonia scholaris, Swertia chirata* (2-7) etc., have been tested in mammalian systems and were found to be quite effective at optimum dose levels.

Oxidative damage has been reported to be involved in the pathogenesis of major diseases such as cancer, atherosclerosis (8) and certain neurological disorders (9). Also, the relevance of free radicals/reactive oxygen species (ROS) in tissue damage and carcinogenesis has been studied (10). Inactivation and removal of ROS depend on reactions involving the antioxidative defense system. Hence, it is important to explore natural antioxidative agents present in plants and plant derived compounds.

The dew of sea, rosemary (*Rosmarinus officinalis*) belonging to the Lamiaceae family, is indigenous to Southern Europe, particularly on the dry rocky hills of the Mediterranean region. Rosemary has been used as an analgesic, antirheumatic, tonic and stimulant, carminative, diuretic, expectorant, anti-epileptic, anti-spasmodic in renal colic, dysmenorrhoea, relieving respiratory disorders effects and for effects on human fertility (11). del Bano *et al.* (12) studied the antioxidant activity of six rosemary extracts with different polyphenolic composition and proposed that they are excellent antioxidants in both aqueous and lipid systems. Known for their antioxidant activity, leaves of *R. officinalis* are not only used in food industry but also shown to be safe and anti-toxic in animal tests (13). The present study is undertaken to obtain insight into the possible anti-cancer activity of *R. officinalis* against DMBA-induced skin tumorigenesis in mice.

Materials and Methods

Animals

The study was conducted on random bred, 6-7 weeks old and 24- 28 gm body weight bearing, male Swiss albino mice (*Mus musculus*; Fig. 1). Animals were maintained under controlled conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). They were provided standard mice feed (procured from Hindustan Levers Ltd., India) and water *ad libitum*. The study protocol is approved by the Departmental Animal Ethical Committee and confirms to the guidelines set by World Health Organization, Geneva, Switzerland and Indian National Science Academy (INSA), New Delhi (India).

Chemicals

The chemicals, 7, 12-dimethylbenz(a)anthracene (DMBA) and croton oil 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 the Rosemary Extract (RE)

The identification of the plant *Rosmarinus officinalis* (family: Lamiaceae) was done by a botanist (Voucher Specimen No: DDC/2001/DEPTBT/ACHARYA2430), Department of Botany, Danielson College, Chhindwara, Madhya Pradesh (India). The non-infected leaves of the plant were extracted with double distilled water (DDW) by refluxing for 36 hrs. at 50-60^0 C. Pellets of the drug were obtained and the required dose for treatment was prepared by dissolving the pellets in double DDW at a dose level of 800 mg/ kg body weight.

Experimental protocol

Three days before the commencement of the experiment, hair on the interscapular region of the mice were shaved. Only the mice showing no hair growth were selected for the study. The animals were randomly allocated into 5 groups comprising twelve mice each.

Treatment groups

Animals of group-I *(Vehicle Control)* received topical application of acetone (100 µl/ mouse) on the shaven skin and DDW equivalent to drug (100 µl/ mouse) orally for 15 weeks. Mice of group-II (*Carcinogen Control*) were applied topically a single dose of DMBA (100 µg/ 100 µl of acetone), two weeks later, followed by croton oil (1% w/v in acetone) application (thrice a week) until the end of experiment. This group received DDW equivalent to RE (100 µl/ mouse) orally for 15 weeks.

The animals of group- III *(RE Experimental-1)* were given the same treatment as in group-II and also received rosemary extract orally at a dose of 800 mg/ kg b. wt./ animal for 15 days (i.e., 7 days before and 7 days after DMBA application). Animals in group-IV (*RE Experimental-2)* received the same treatment as for group-II and were administered RE (800 mg/ kg b. wt./ animal) by oral gavage, starting from the time of croton oil treatment till the end of experiment. The treatment to mice of group-V (*RE Experimental-3)* was similar as for group-II. Also, these animals received the RE extract (800 mg/ kg b. wt. / animal) throughout the experimental period, i.e., for 15 weeks.

Detection of papillomas

Papillomas appearing on the dorsal skin of mice were recorded at weekly intervals. For final evaluation of the data, only those papillomas which persisted for two weeks or more (diameter < 1 mm) were taken into consideration.

Biochemical study

Biochemical alterations were studied in all the groups at the time of termination of the experiment (i.e., at 15^{th} week). The hepatic level of glutathione (GSH) was determined by the method of Moron *et al.* (14). The GSH content in blood was measured spectrophotometrically using Ellman's reagent with 5-5, dithiobis-2-nitrobenzoic acid (DTNB) as a coloring reagent, according to the method of Beutler *et al. (15).* The lipid peroxidation level in liver and blood was measured in terms of Thiobarbituric acid reactive substances (TBARS) by the method of Ohkhawa *et al.* (16).

Data analysis

The differences in the incidence of tumors among different groups were considered to be significant at 5% significance level ($p<0.05$) when evaluated by Student's 't' test.

Results

The results of the present investigation have been summarized in Tables 1 and 2. Vehicle control group did not show any tumor incidences. Topical application of DMBA followed by croton oil produced skin papillomas which started appearing from 6th week onwards. In group-II, the incidence of tumors reached 100% by the 15th weeks. Cumulative number of papillomas in these animals was recorded as 63. The average number of papillomas per mouse (tumor yield) as well as the total number of papillomas scored/ papillomas bearing mice (tumor multiplicity) was found to be 5.25 (Fig. 2).

In the RE experimental groups (III-V), mice showed a significant ($p< 0.05$) decrease in the number and incidence of tumor appearance as compared with that of the carcinogen control group. When rosemary extract was orally administered to animals of group-III, in addition to the initiator (DMBA) and promoter (croton oil) for 15 days (i.e., 7 days before and 7 days after DMBA application), the tumor incidence was found to be 50%. At the end of the experiment, the values of cumulative number of papillomas, tumor multiplicity and tumor yield were recorded 21, 3.50 and 1.75 respectively, and these were observed to be significant ($p< 0.05$) lower than group-II (Fig. 3).

Animals of Group-IV, administered RE for 13 weeks from the time of croton oil application, showed 58.33% incidence of tumor occurrence. Cumulative number of papillomas, tumor multiplicity and tumor yield were recorded as 30, 4.28 and 2.50 respectively, that was significantly ($p<0.05$) lower to that noted in the DMBA-croton oil alone treated group. Animals of Group-V, given rosemary extract throughout the experimental period (i.e., 7 day prior to DMBA application till the end of 15th week), showed 41.66% incidence of papillomas. Cumulative number of papillomas, tumor multiplicity and tumor yield were recorded as 16, 3.20 and 1.33 respectively, which was found to be significantly lower than carcinogen control group II.

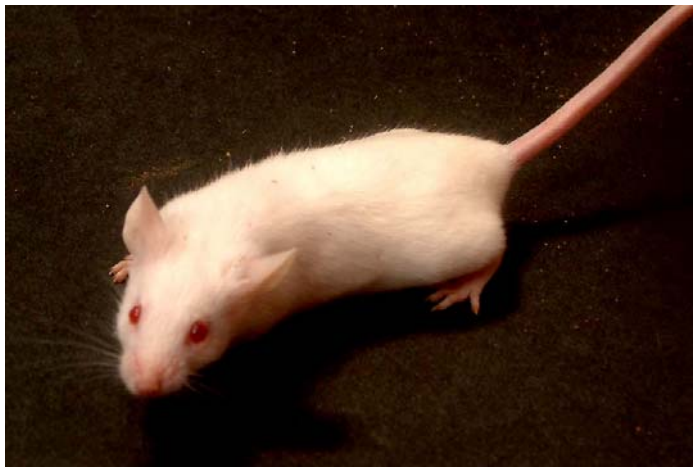

Fig 1. Normal Mouse (*Mus musculus*)

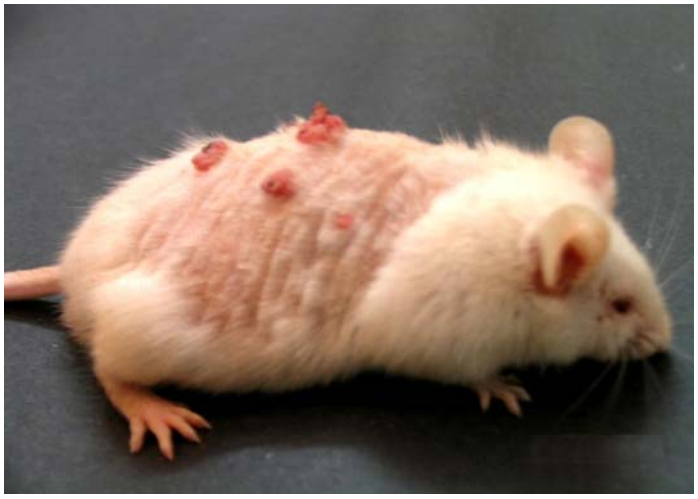

Fig 2. Mouse bearing papillomas (Without RE administration)

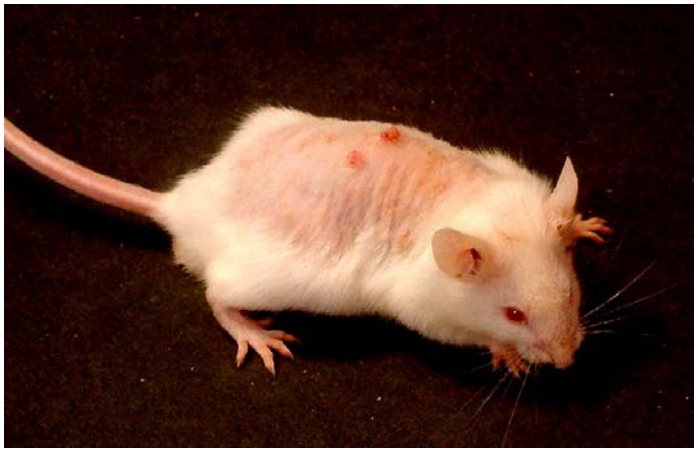

Fig 3. Mouse bearing papillomas (RE-administered group)

Result showed a prolonged average latent period (i.e., time lag between the application of the promoter and the appearance of 50% of tumors) in all the RE experimental groups. Such latent period was found to be 9.67 weeks in group II, whereas the same was significantly higher in the experimental groups III to V (11.14, 10.70 and 12.18 weeks respectively).

Table-1: Chemopreventive effect of *Rosmarinus officinalis* extract on DMBA-induced skin tumorigenesis in mice

Treatment Group	Average Latent Period (week)	Cumulative Frequency	Tumor Multiplicity	Tumor Yield	Tumor Incidence (%)
Vehicle control (Gr -I)	-	-	-	-	-
Carcinogen Control (Gr - II)	9.67±0.01	63.00±0.92	5.25±0.28	5.25±0.28	100.00
RE Experimental-1 (Gr- III)	11.14±0.02*	21.00±0.32*	3.50±0.26*	1.75±0.58*	50.00
RE Experimental-2 (Gr – IV)	10.70±0.04*	30.00±0.15*	4.28±0.46*	2.50±0.29*	58.33
RE Experimental-3 (Gr - V)	12.18±0.04*	16.00±0.28*	3.20±0.24*	1.33±0.64*	41.66

***** *Significance level among different groups at p< 0.05*

Carcinogen Control v/s RE experimental

A significant fall in glutathione (GSH) activity was noticed in blood and liver in the group-II animals as compared to RE experimental (groups III- V), at the time of termination of the experiment (i.e., 15 weeks). Treatment of RE resulted in an enhanced level of GSH ($p<0.05$) in such groups. A considerable elevation in lipid peroxidation level was noted in blood serum and liver; whereas administration of plant extract significantly reduced the level ($p<0.05$) of LPx in all the RE experimental groups in comparison to group-III (Table-2).

Table 2: Variation in the lipid peroxidation and glutathione level during DMBA-induced skin tumorigenesis with/without rosemary extract treatment

Treatment Group	Lipid Peroxidation level		Glutathione level	
	Blood Serum (n mole/ml)	Liver (n mole/mg)	Blood (µg/ml)	Liver (µmole/gm)
Vehicle control (Gr- I)	1.29±0.21	2.73±0.23	3.43 ±0.11	63.17 ±0.56
Carcinogen Control (Gr- II)	3.87±0.09*	4.86±0.56*	2.74±0.07*	56.22±1.02*
RE Experimental-1 (Gr- III)	1.69±.48*	3.21±0.16*	3.08±0.12*	60.42±0.64*
RE Experimental-2 (Gr. IV)	2.12±0.13*	3.39±0.18*	2.83±0.04*	59.56±0.03*
RE Experimental-3 (Gr- V)	1.46±0.06*	3.14±0.51*	3.21±0.06*	61.94±0.04*

***** *Significance level among different groups at p< 0.05*

Carcinogen Control v/s Normal

Carcinogen Control v/s RE Experimental

Discussion

The present study demonstrates 100% tumor incidence in the carcinogen control group. Topical application of TPA (active constituent of croton oil) has been reported to increase production of free radicals (17). This is perhaps due to the free radical oxidative stress that has been implicated in the pathogenesis of a wide variety of clinical disorders (18).

In all the RE-treated experimental groups (III-V), the incidence and the number of skin papillomas decreased significantly. The present findings also show prolonged latent period in the RE experimental groups in comparison to the carcinogen control treated animals. The reduction in tumor counts may be due to inhibition of DMBA metabolism to its active form or delay in the promotion phase of tumorigenesis *via* down regulation in the production of ROS (19). A number of potent inhibitors of tumor initiation appear to be effective because they either prevent the formation of ultimate carcinogen or scavenge the reactive ultimate carcinogen (20).

Many antioxidants and anticarcinogenic compounds appear to have major effect on the detoxification of the carcinogens by the induction of Phase II detoxification enzymes since these enzymes divert carcinogens to react with critical cellular macromolecules (21). Singletary *et al*. (22) reported that the addition of rosemary extracts to the diet as a 1% supplement by weight may decrease the frequency of carcinogen-DNA adduct formation in rats. Huang *et al*. (23) reported that the topical application of carnosol and ursolic acid, isolated from rosemary, inhibited TPA-induced ear inflammation, ornithine decarboxylase activity, and tumor promotion against DMBA initiated and TPA promoted skin carcinogenesis in mice.

Glutathione is one of the antioxidant enzymes that act as the first line of defense against pro-oxidant stress. One of the mechanisms by which rosemary rendered protection against carcinogen can be an elevation in the glutathione level that could have been mediated through the modulation of cellular antioxidant level. Lipid peroxides are known to play an indirect role in the conversion of procarcinogens to the ultimate carcinogens (24). Malondialdehyde, a byproduct of LPx is said to be involved in DNA adduct formation which is believed to be responsible for carcinogenesis (25).

Studies conducted by Haraguchi *et al*. (26) reports an inhibition of superoxide and lipid peroxidation by 4 diterpenoids from rosemary, i.e., carnosic acid, carnosol, rosmanol and epirosmanol. The reduction of LPx and an increase in GSH in liver, the major site of carcinogen metabolism, is reflected in the reduction of incidence and count of skin papillomas.

The major proposal for action of rosemary leaves extract seems to be the effectiveness to intercept the free radicals and protect cellular molecules from oxidative damage. Further, it modulates glutathione level and is found to inhibit lipid peroxidation in liver and blood. The mechanism underlying the chemopreventive action of rosemary and its active principles is not clear; the beneficial effect of *R. officinalis* may be due to either individual or combined effects of its constituents.

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