RESEARCH COMMUNICATION

Modulatory Influence of *Rosemarinus officinalis* on DMBA-induced Mouse Skin Tumorigenesis

Garima Sancheti, PK Goyal

Abstract

The present investigation was undertaken to explore the anti-tumor promoting activity of *Rosemarinus officinalis* on two-stage skin carcinogenesis, induced by a single topical application of 7, 12-dimethylbenz(a)anthracene and promoted by treatment of croton oil for 15 weeks in Swiss albino mice. Oral administration of Rosemary leaf extract at a dose of 1000 mg/ kg b. wt. / day at pre, peri and post-initiational phases, was found to be effective in decreasing the tumor incidence (50, 41.7, 58.3%, respectively) in comparison to the control (100%). Furthermore, the cumulative number of papillomas, tumor yield and tumor burden were also found to be reduced in *R. officinalis*-treated animals. This was associated with significant alteration in liver lipid peroxidation and glutathione (GSH) levels.

Key Words: Chemoprevention - glutathione - lipid peroxidation - *Rosemarinus officinalis* - mouse skin

Asian Pacific J Cancer Prev, **7**, 331-335

Introduction

Of the many diseases that affect people these days, cancer is among the most feared. Results from various epidemiological studies reveal that 80- 90 % of all cancers that are caused by environmental factors and chemicals, either naturally or synthetically, are the predominant environmental carcinogens (Higginson and Muir, 1973). These chemical carcinogens which are related to lifestyles include those in the tobacco smoke (De Flora et al, 2003), air, water, food, some drugs and cosmetics (Kupradinun et al, 2002).

In multistage carcinogenesis process, the inhibition of tumor promotion is regarded as an effective strategy for cancer chemoprevention, because tumor promotion occurs by repetitive exposure to tumor promoters in long term (Murakami et al., 1999). To be most effective, a chemopreventive agent should be able to inhibit several stages in multistage carcinogenesis (McCormick et al, 1998). Keeping this in view, recently medicinal plants and their active principles have received growing attention as potential chemopreventive agents as depicted by various studies (Khan and Sultana, 2002; Iqbal et al, 2003; Lee and Park, 2003). An abundance of data indicates that diets high in fruits and vegetables are effective in protection against cancer. In vitro data have been collected to support tumor- inhibitory properties of phytohormones, apoptosis-inducing compounds, and substances that induce detoxification of potential mutagens (Dixon et al, 1999; Smith et al, 2004).

The evergreen shrub, rosemary (*Rosemarinus officinalis*) belonging to the Labiatae family, is indigenous to Southern Europe, particularly on the dry rocky hills of the Mediterranean region. A well-known culinary and aromatic herb, rosemary has a long history of medicinal use. The plant has been used as a tonic and stimulant, analgesic, antireumatic, carminative, diuretic, expectorant, anti-epileptic, anti-spasmodic in renal colic, dysmenorrhoea, relieving respiratory disorders effects and for effects on human fertility (Hussain, 1978; Al-Serati et al., 1999). Rosemary extract is experimentally found to be antimutagenic in the Ames tester strain TA102 (Minnunni, 1992). Wide acceptability, common usage and diverse anti-oxidative and pharmacological properties of *R. officinalis* aroused our interest to obtain insight into the chemopreventive effect of the plant against DMBA-induced skin tumorigenesis in mice.

Materials and Methods

Animal care and handling

Animal care and handling was performed according to the guidelines set by World Health Organization, Geneva, Switzerland and INSA (Indian National Science Academy, New Delhi, India). The study was conducted on random bred, 6-7 week old and 24- 28 gm body weight bearing, male Swiss albino mice. Animals were maintained under controlled

Radiation & Cancer Biology Lab, Dept Zoology, University of Rajasthan,Jaipur – 302 004 (India) E-mail: garimasancheti@rediffmailcom

conditions of temperature and light (Light: dark, 10 hrs: 14 hrs.). Four mice were housed in a polypropylene cage containing sterile paddy husk (procured locally) as bedding throughout the experiment. They were provided standard mice feed (procured from Hindustan Levers Ltd., India) and water ad libitum. Tetracycline water once a fortnight was given as preventive measures against infections. The Departmental Animal Ethical Committee approved this study.

Chemicals

The initiator, 7, 12-dimethylbenz(a)anthrecene (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 the plant extract

Identification of the plant *Rosemarinus officinalis* (family: Labiatae) was by a competent botanist from the Herbarium, Department of Botany, University of Rajasthan, Jaipur (India). Non-infected leaves of the plant were carefully cleaned, shade dried and powdered. The plant material was then extracted with double distilled water by refluxing for 36 hrs. at 800 C. Pellets 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 a dose level of 1000 mg/ kg body weight.

Experimental protocol

The inhibition of tumor incidence by leaf extract of *R. officinalis* was evaluated on two-stage process of skin tumorigenesis using the following protocol: A total of 72 animals were randomized into control and experimental groups and divided into 6 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 showing no hair growth were considered for the study. Body weights of the animals were recorded weekly.

Group-I: Vehicle Controls - received topical application of acetone (100 µl/ mouse) on the shaven dorsal skin and double distilled water at 100 µl/ mouse by oral gavage for 15 weeks.

Group-II: RE Controls - administered rosemary extract orally at a dose of 1000 mg/ kg b. wt. / animal, once a day for the 15 week study period.

Group-III: Carcinogen Controls - applied topically with a single dose of DMBA (100 µg/ 100 µl of acetone) over the shaven area of the skin of the mice. Two weeks later, croton oil (1% w/v in acetone) was applied three times per week until the end of experiment. Double distilled water equivalent to rosemary drug (100 µl/ mouse) was also given by oral gavage.

Group-IV: RE Experimental 1 - the same treatment as in group-II and also rosemary extract at a dose of 1000 mg/ kg b. wt./ animal, orally for 15 days, before and during DMBA application.

Group-V: RE Experimental 2 - the same treatment as for group-II and were administered rosemary drug (1000 mg/ kg b. wt./ animal) by oral gavage, starting from the time of croton oil treatment till the end of experiment (i.e. 15 weeks).

Group-VI: RE Experimental 3 - as for group-II plus the RE extract (1000 mg/ kg b. wt./ animal) throughout the experimental period, i.e., before and after DMBA application and also at the promotional stage.

Detection of papillomas

Papillomas appearing on the shaven area of the skin were examined and recorded at weekly intervals in all the above groups. Only those papillomas which persisted for two weeks or more, with a diameter greater than 1 mm, have been taken into consideration for final evaluation of the data. Skin papillomas which regressed after one observation were not considered for counting.

Biochemical parameters

Biochemical alterations were studied in animals of all the groups at the time of termination of the experiment (i.e., at 15th week). The hepatic level of glutathione (GSH) was determined by the method of Moron et al (1979). 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 (1963). The absorbance was read at 412 nm using a UV-VIS systronics spectrophotometer. 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. (1979). The absorbance was read at 532 nm.

Statistical 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 findings of the present study are furnished in Tables 1 and 2 and Figure 1. Animals of group-I (vehicle control) showed no incidence of papillomas during the entire experimental period. Administration of R. officinalis leaf extract had no adverse effects on mice of RE control (Group-II) in terms of sickness or mortality, urination and defecation pattern.

Topical application of DMBA followed by croton oil produced skin papillomas which started appearing from the 6th week onwards. The incidence in DMBA-croton oil treated mice (carcinogen control) reached 100% by the termination of the experiment (i.e., 15 weeks). The cumulative number of papillomas in these animals was

Table 1. Chemomodulatory Effects of *R. officinalis* Extract on DMBA-induced Tumorigenesis in Mice

Groups (number of mice)	Cumulative Number of Tumours	Tumor Incidence (%)	Tumor Burden	Tumor Yield	Average Latent Period (weeks)
Vehicle control (12)	-	-	-	-	-
RE control (12)	-	-	-	-	-
Carcinogen control (12)	63	100	5.25 ± 0.28	5.25 ± 0.28	9.7
RE experimental 1 (12)	19	50.0	3.16 ± 0.12*	1.58 ± 0.48*	11.4
RE experimental 2 (12)	29	58.3	4.14 ± 0.20*	2.42 ± 0.63*	11.3
RE experimental 3 (12)	14	41.7	2.80 ± 0.24*	1.16 ± 0.44*	12.3

*Significant difference from the carcinogen control group at $p < 0.05$

Table 2. Lipid Peroxidation (LPx) and Glutathione (GSH) Levels in Serum/Blood and Liver of Mice Given Rosemary Extract

Group#	Lipid Peroxidation (LPx) level		Glutathione (GSH) level	
	Serum (n mole/ml)	Liver (n mole/mg)	Blood (μg/ml)	Liver (μmole/gm)
Vehicle control	1.29±0.21	2.73±0.23	3.43 ±0.11	63.17 ± 0.56
RE control	1.26±0.03	2.61±0.16	3.56 ± 0.12	63.86 ± 1.15
Carcinogen control	3.87±0.09	4.86±0.56	2.74 ± 0.07	56.22 ± 1.02
RE experimental 1	1.45±0.05*	3.02±0.02	3.19 ± 0.12*	61.86 ± 1.15*
RE experimental 2	2.01±0.08*	3.26±0.13*	3.05 ± 0.33	60.42 ± 0.13*
RE experimental 3	1.32±0.08*	2.90±0.10	3.32 ± 0.15*	62.83 ± 0.64*

12 mice in each group *Significant difference from the carcinogen control group at $p < 0.05$

recorded as 63. The average number of papillomas per mouse (tumor yield) as well as the papillomas per papilloma bearing mice (tumor burden) was found to be 5.25. These significantly were reduced in all experimental groups. Average latency period (i.e., time lag between the application of the promoter and the appearance of 50% of tumors) was also greater with rosemary extract treatment.

Significant lower glutathione peroxidase activity was noted in blood and liver in the carcinogen control mice as compared to RE experimental animals (Groups IV-VI), at the time of termination of the experiment (i.e., 15 weeks). Treatment of rosemary extract resulted in an enhanced level of this enzyme ($p<0.05$) in such groups. A considerable elevation in lipid peroxidation level was noted in blood serum and liver; whereas administration of rosemary significantly reduced the level ($p<0.05$) of LPx in all the RE experimental groups in comparison to the carcinogen controls.

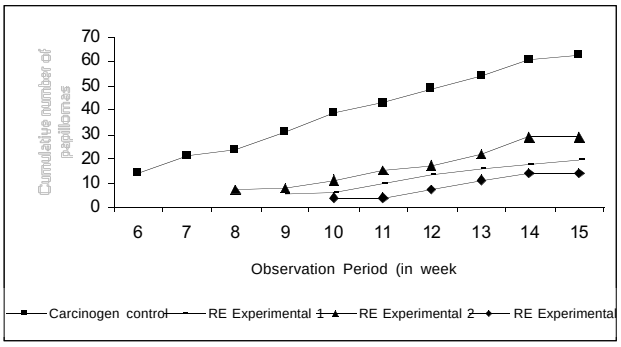

Figure 1. Cumulative Numbers of Papillomas in RE Treated mice Compared with Carcinogen Controls

Discussion

The most promising strategies for cancer control may be accomplished by pharmacological/ dietary factors as key modulators of carcinogenesis. Much progress in the understanding of functional relationships between nutritional factors has been provided by epidemiological and diet intervention studies (Byers and Guerroro, 1995), relevant laboratory animal model studies and investigations on mechanisms of tumor induction, progression and inhibition. This concept is based on the mechanism of action of the chemical compounds and/ or micronutrients present in food, their toxicity and the chemopreventive efficacy (Beecher and Khachik, 1989; Wattenberg, 1992).

The skin carcinogenesis model in experimental animals has been found to be a very useful system for investigating the influence of dietary chemopreventors both mechanistically and operationally (Morse and Stoner, 1993). In our previous study, we observed chemopreventive potential of *Emblica officinalis* (amla) against DMBA induced skin tumorigenesis in mice (Sancheti et al, 2005). The present study demonstrated similar findings with rosemary extract, the onset of papilloma development being delayed and the yield also reduced when *R. officinalis* was given in the initiation or post-initation stages. Greatest effects 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 or modulated ornithine decarboxylase protein kinase C activity or has decreased prostaglandin synthesis. The active constituents of rosemary, carnosol and urosolic acid, are lnpwn to inhibit TPA-induced ear inflammation, ornithine decarboxylase

activity and tumor promotion (Huang, 1994). Such depletion of tumorigenesis owing to similar factors in various plants has been reported by others (Prakash et al, 2002; Kausar et al, 2003; Sancheti et al, 2005).

It has been reported earlier that addition of rosemary extracts to the diet as a 1% supplement by weight may decrease the frequency of carcinogen-DNA adduct formation (Singletary et al, 1996). Carnosic acid and carnosol, the two major active ingredients of rosemary were found to exhibit anticarcinogenic activity against the action of oxygen radicals in animals (Minnunni et al, 1992). Natural polyphenols found in rosemary have not only potent antioxidant activities but also anticarcinogenic properties (Offord et al, 1997). Furthermore, it has been reported that extract of rosemary leaf has some superoxide dismutase like activity (Kim 1995).

During the multistage carcinogenesis process, the activity of antioxidant enzymes tend to decrease which leads to a pro-oxidant state of the cell, facilitating tumor promotion and progression (Oberly et al, 1993). In the present study, it was noted that the treatment to the initiator and promoter alone in group III lowered the levels of glutathione (GSH) in blood and liver, while oral administration of RE did not influence the endogenous GSH level significantly either in liver or blood.

Treatment with rosemary extract in Groups IV, V and VI significantly elevated the level of GSH in the blood and liver. One of the mechanisms of RE protection against carcinogen can be an elevation in the glutathione level that is mediated through the modulation of cellular antioxidant level. This could have resulted in the reduction of lipid peroxidation level, thereby protecting against damage caused by the carcinogen in Group III. Rosmarinic acid, one of the active compounds of rosemary, has been experimentally found to have significant antioxidant role by free radical scavenging activity (Lamaison et al, 1991).

Lipid peroxides also cause damage to cellular macromolecules by generation of reactive species and are considered to promote carcinogenesis (Chung et al, 1996). The level of carcinogen-induced lipid peroxidation increased considerably in carcinogen-control animals whereas a decrease in the values was observed in the RE-treated groups. This study is supported by the anti-lipoperoxidant activities of the young sprouts of R. officinalis that have shown to reduce the formation of malondialdehyde significantly in rat hepatocytes (Joyeux et al, 1990). Sotelo-Felix et al. (2002) proposed that carnosol could scavenge free radicals induced by carbon tetrachloride, consequently avoiding the propagation of lipid peroxides in the liver of mice. Further studies by Haraguchi (1995) have revealed the inhibition of superoxide and lipid peroxidation by 4 diterpenoids from rosemary, i.e., carnosic acid, carnosol, rosmanol and epirosmanol. The exact mechanism of action of rosemary is not known; however, it may scavenge carcinogen-induced free radicals and thus reduces damage to the cellular DNA. Traditional use of spices and other plant parts as food ingredients may confer some protection from cancer. While it is generally accepted that a diet of large amounts of vegetables, fruits, and other plant products lowers cancer incidence, there is still a need to identify the most effective constituents of the diet as well as to elucidate their mechanisms of action. The present work demands additional study to explore the exact mechanism and clinical applicability of Rosemarinus officinalis as a chemopreventor.

Acknowledgements

The assistance in the form of chemicals from Special Assistance Program (SAP) running in Department is thankfully acknowledged.

References

Al-Sereiti MR, Abu-amer KM and Sen P (1999). Pharmacology of rosemary (Rosmarinus officinalis Linn.) and its therapeutic potentials. *Ind J Exp Biol*, **37**, 124-130.

Beecher GR and Khachik F (1989). Analysis of micronutrients in foods. In Nutrition and Cancer Prevention, T Moon and M Micozzi (Eds.). New York, Marcell Dekker, pp 103-58.

Beutler E, Duron O, Kellin BM (1963). Improved method for the determination of blood glutathione. *J Lab Clin Med*, **61**, 882-8.

Byers T, Guerrero N (1995). Epidemiologic evidence for vitamin C and vitamin E in cancer prevention. *Am J Clin Nutr*, **62**, 1385-92.

Chung Fl, Chen HJC, Nath RG (1996). Lipid peroxidation as a potential endogenous source for the formation of exocyclic DNA adducts. *Carcinogenesis*, **17**, 2105-11.

DeFlora S, Agostini D, Balansky RM, et al (2003). Modulation of cigarette smoke related end points in mutagenesis and carcinogenesis. *Mutat Res*, **523-524**, 237-52.

Dixon RA, Canovas P, Guo Z, et al (1999). Molecular controls for isoflavonoid biosynthesis in relation to plant and human health. *Recent Adv Phytochem*, **33**, 133-59.

Haraguchi H, Saito T, Okamura N, Yagi A (1995). Superoxide and lipid peroxidation were inhibited by 4 diterpenoids from rosemary: carnosic acid, carnosol, rosmanol and epirosmanol. *Planta Med*, **61**, 333-336.

Higginson J, Muir CS (1973). Cancer epidemiology. In 'Cancer Medicine' Eds Holland JF and Frei E. Lea and Febiger, Philadelphia. pp 241-306.

Huang MT, Ho CT, Wang ZY, et al (1994). Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Res*, **54**, 701-8.

Hussain FTK (1979). Medicinal plants, their cultivation and contents (Arabic Book Shop, Libya- Tunisia).

Iqbal J, Minhajuddin M, Beg ZH (2003). Suppression of 7, 12-dimethylbenz(alpha)anthracene- induced carcinogenesis and hypercholesterolaemia in rats by tocotrienol-rich fraction isolated from rice bran oil. *Eur J Cancer Prev*, **12**, 447-53.

Joyeux M, Rolland A, Fleurentin J, Mortier F, Dorfman P (1990). Tert-butyl hydroperoxide-induced injury in isolated rat hepatocytes: A model for studing anti-hepatotoxic crude drugs. *Planta Med*, **56**, 171-4.

Kausar H, Bhasin G, Zargar MA and Athar M (2003). Palm oil alleviates 12-O tetradecanoyl-phorbol-13-acetate-induced tumor promotion response in murine skin. *Cancer Lett*, **192**, 151-60.

Khan N, Sultana S (2005). Inhibition of two stage renal

carcinogenesis, oxidative damage and hyperproliferative response by Nigella sativa. *Eur J Cancer Prev*, **14**, 159-68.
Kim SJ, Han D, Moon KD, Rhee JS (1995). Measurement of superoxide dismutase-like activity of natural antioxidants. *Biosci Biotechnol Biochem*, **59**, 822-6.
Kupradinun P, Reinkijakarn M, Tanyakaset M, Tepsuwan A, Kusamran WR (2002). Carcinogenicity testing of the cosmetic dye: D & C Red No. 36. *Asian Pacific J Cancer Prev*, **3**, 55-60.
Lamaison JL, Petitjean Freytet C, Carnat A (1991). Medicinal Lamiaceae with antioxidant properties, a potential source of rosmarinic acid. *Pharm Acta Helv*, **66**,185-8.
Lee BM and Park KK (2003). Beneficial and adverse effects of chemopreventive agents. *Mutat Res*, **523-524**, 265-78.
McCormick DL, Rao KVN, Steele,VE, et al (1999). Chemoprevention of rat prostrate carcinogenesis by 9-cis-retinoic acid. *Cancer Res*, **59**, 521-524.
Minnunni M, Wolleb U, Mueller O, Pfeifer A, Aeschbacher HU (1992). Natural antioxidants as inhibitors of oxygen species induced mutagenicity. *Mutat Res*, **269**, 193-200.
Moron MS, Depiere JW and Mannervik B (1979). Levels of GSH, GR and GST activities in rat lung and liver. *Biochem Biophys Acta*, **582**, 67-78.
Morse MA and Stoner GD (1993). Cancer chemoprevention principles and prospects. *Carcinogenesis*, **14**, 1737-1746.
Murakami A, Ohigashi H, Koshimizu K (1999). Chemoprevention: Insights into biological mechanisms and promising food factors. *Food Rev Int*, **15**, 335-395.
Oberley TD, Oberley LW (1993). Oxygen radicals and cancer. In 'Free Radicals in Aging' Ed Yu BP, CRC Press, Boca Raton, FL, pp 247-267.
Offord EA, Mace K, Avanti O, Pfeifer AM (1997). Mechanisms involved in the chemoprotective effects of rosemary extract studied in human liver and bronchial cells. *Cancer Lett*, **114**, 275-81.
Ohkhawa H, Ohishi N, Yogi K (1979). Assay for lipid peroxidation in animal tissue by thiobarbituric acid reaction. *Anal Biochem*, **95**, 351.
Prakash J, Gupta SK, Dinda, AK (2002). Withania somnifera root extract prevents DMBA-induced squamous cell carcinoma of skin in Swiss albino mice. *Nutr Cancer*, **42**, 91-97.
Sancheti G, Jindal A, Kumari R, Goyal PK (2005). Chemopreventive action of *Emblica officinalis* on skin carcinogenesis in mice. *Asian Pacific J Cancer Prev*, **6**, 197-201.
Singletary K, MacDonald C, Wallig M (1996). Inhibition by rosemary and carnosol of 7, 12-dimethyl-benz[a]anthracene (DMBA)-induced rat mammary tumorigenesis and in vivo DMBA-DNA adduct formation. *Cancer Lett* **104**, 43–8.
Smith SH, Tate PL, Huang G, et al (2004). Antimutagenic activity of berry extracts. *J Med Food*, **7**, 450-5.
Sotelo-Felix JI, Martinez-Fong D, Muriel De la Torre P (2002). Protective effect of carnosol on CCl (4)-induced acute liver damage in rats. *Eur J Gastroenterol Hepatol*, **14** , 1001-6.
Wattenberg LW (1992). Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res*, **52**, 2085-91.