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Chemopreventive potential of geraniol in 7,12-dimethylbenz(a) anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice

Author Details

S. Manoharan (*Corresponding author*)
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar - 608 002, India
e-mail: sakshiman@rediffmail.com

M. Vasantha Selvan
Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Annamalainagar - 608 002, India

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Abstract

The present study investigated the chemopreventive potential of geraniol, an acyclic monoterpene alcohol, by monitoring the tumor incidence and analyzing the status of phase II detoxification agents, lipid peroxidation by products and antioxidants in 7,12-dimethylbenz(a)anthracene (DMBA) induced mouse skin carcinogenesis. Skin tumor was developed by painting DMBA (25 µg in 0.1 ml acetone mouse^{-1}) in the shaved back of the mice, twice weekly for 8 weeks. We noticed 100% skin tumor formation in mice treated with DMBA alone. The status of phase II detoxification agents and antioxidants were decreased where as lipid peroxidation by products were increased in tumor bearing mice. Oral administration of geraniol at a dose of 250 mg kg^{-1} body weight significantly prevented the tumor formation as well as brought back the status of phase II detoxification agents, lipid peroxidation by products and antioxidants to near normal range in DMBA treated mice. Present results suggest that geraniol might have inhibited abnormal cell proliferation occurring in skin carcinogenesis by modulating the activities of phase II detoxification agents and through its free radical scavenging potential.

Key words

Skin cancer, Geraniol, Antioxidants, Detoxification, Thiobarbituric acid reactive substances, 7, 12-dimethylbenz(a) anthracene

Introduction

Skin cancer, the most common form of human cancer, proceeds through three distinct phases, initiation, promotion and progression. It represents the most commonly diagnosed cancer, surpassing lung, breasts, colorectal and prostate cancer. They start as precancerous lesions and environmental toxins play a crucial role in the initiation of skin carcinogenesis (Krueger *et al*., 2008; Boukamp, 2005). It also represents a major and growing public health problem and of all new cancers diagnosed annually in the world, almost one-third originates in the skin (Greenlee *et al*., 2001). In USA, around 1.2 million new skin cancer cases are diagnosed each year (Stern, 2010). The highest incidence of skin cancers were reported every year in South Africa and Australia than any other countries throughout the world, due to the fact that populations of these countries receive high amounts of UV radiation (Kalicharran *et al*., 1993; Staples *et al*., 2006). In India, skin cancer accounts for 1-2% of all cancers (Deo *et al*., 2005).

7,12-dimethylbenz(a)anthracene (DMBA), a polycyclic aromatic hydrocarbon, is a procarcinogen and thus needs metabolic activation to become an ultimate carcinogen (Miyata *et al*., 2001). The active metabolite, dihydrodiol epoxide, generated during the metabolic activation of DMBA binds to and causes damage to DNA. Excessive reactive oxygen species are also generated during metabolic activation of DMBA.It is widely used as an initiator as well as promoter to induce skin carcinogenesis in Swiss albino mice (Das and Bhattacharya, 2004; Nigam *et al*., 2007). DMBA induces skin carcinogenesis and is therefore commonly employed to study the chemopreventive potential of natural and synthetic entities (Rastogi *et al*., 2007).

Oxidative stress occurs in the cells due to imbalance in oxidant and antioxidant status. Oxidative stress damages DNA, lipids and proteins as well impair the structure and function of biomembranes (Droge, 2002). Reactive oxygen species (ROS) mediated lipid peroxidation has been implicated in the pathogenesis of several cancers including skin cancer. Mammalian cells have, however, an array of sophisticated antioxidant defense mechanism

to compromise and combat the deleterious effects of ROS. Though skin antioxidants maintain the balance of cellular redox balance, premature ageing of skin and tumor initiation occurs if ROS are excessively generated in the skin (Lu *et al*., 2007; Ishii, 2007).

Chemoprevention, a novel and appealing strategy, deals with the inhibition, reversal or suppression of carcinogenesis by the use of natural or synthetic agents (F'guyer *et al*., 2003). The possible mechanism so far reported for the chemopreventive potential of natural products include carcinogen detoxification, suppression of genetic mutation, suppression of cell proliferation, induction of apoptosis and modulation of the immune system (Dorai and Aggarwal, 2004). Agents that possess antimutagenic and antioxidant potential have the ability to exert striking inhibitory effects on diverse cellular events associated with multistage carcinogenesis (Crowell, 2005). Geraniol, a colourless liquid, is an acyclic terpene alcohol with a flowery rose-like odour. It is found widely as a chief constituent in essential oils including orange flower oil, lemon grass oil and lavender oil. Experimental studies demonstrated several pharmacological activities including antioxidant and anticancer potential of geraniol (Tiwari and Kakkar, 2009). Geraniol exerted anti-tumor activity against various cancer cells both *in vitro* and *in vivo* (Yu *et al*., 1995; Duncan *et al*., 2004; Carnesecchi *et al*., 2004). It has also been reported that geraniol exhibited potent insecticidal, antimicrobial and anti-inflammatory effects (Chen and Viljoen, 2010). Crowell (1999) reported that dietary geraniol suppressed hepatic HMG CoA reductase activity and lowered the levels of serum cholesterol in experimental animals. To the best of our knowledge, we found no studies on chemopreventive potential of geraniol in DMBA induced skin carcinogenesis. The present study is therefore designed to evaluate the same effect in DMBA induced skin carcinogenesis.

Materials and Methods

Experimental design: Male Swiss albino mice, 4-6 weeks old, weighing 15-20 g were purchased from National Institute of Nutrition, Hyderabad, India and maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages and provided standard pellet diet (Agro Corporation Private Limited, Bangalore, India) and water *ad libitum*. The mice were maintained under controlled conditions of temperature (22±2°C) and humidity (55±5%) with a 12 hr light dark^{-1} cycle.

The experiments were undertaken after IAEC approval of Annamalai University. Mice were divided into 4 groups (I–IV) of 6 each. Skin carcinogenesis was developed in Swiss albino mice according to the method of Azuine and Bhide (1992). Depilatory cream was applied to remove hair from the back of each mouse and the mice were left untreated for 2 days. Mice having no hair growth after 2 days were selected for the experimental study.

The depilated back of group I mice was painted with acetone (0.1 ml mouse^{-1}) two times weekly for 8 weeks (vehicle-treated control). The depilated back of groups II and III mice were painted with DMBA (25 µg in 0.1 ml acetone mouse^{-1}) two times weekly for 8 weeks. Group II mice received no other treatment. Group III mice received oral administration of geraniol (250 mg kg^{-1} b.wt. in 0.5 ml corn oil) by gastric gavage starting 1 week before the exposure to the carcinogen and continued for 25 weeks (three times week^{-1} on alternate days) thereafter (at morning 9 am). Group IV animals received oral administration of geraniol alone by gastric gavage throughout the experimental period (at morning 9 am) and were not treated with acetone. At the end of experimental period, all the animals were sacrificed by cervical dislocation. Blood was processed for plasma and erythrocytes and further for biochemical assays. Liver was blotted dry, weighed and processed for biochemical assays. Skin tissues were processed for histological evaluation and biochemical assays.

Tumor volume was measured using the formula

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

where D_1, D_2 and D_3 are the three diameters (mm) of the tumors. Tumor burden was calculated by multiplying tumor volume and the number of tumors / animal. Number in parenthesis indicated total number of animals bearing tumors.

Histological evaluation: The skin tissues were routinely processed and embedded with paraffin wax, sectioned at 2-3 µm in a rotary microtome and stained with hematoxylin and eosin. Single sections of each specimen were evaluated by Nikon Eclipse E-200 Photomicrographic System.

Biochemical assays: Blood samples from mice were collected into heparinized tubes. The plasma was separated by centrifugation at 3000 rpm for 15 min. The erythrocyte membrane was prepared according to the method of Dodge *et al*. (1963) modified by Quist (1980). Thiobarbituric acid reactive substances (TBARS) in plasma, erythrocyte membrane and skin were determined by the methods of Yagi (1987), Donnan (1950) and Ohkawa *et al*. (1979), respectively. The reduced glutathione (GSH) level in erythrocytes, liver and skin tissues was determined by the method of Beutler and Kelly (1963). Superoxide dismutase (SOD) activity in erythrocytes and skin tissues was assayed by the method of Kakkar *et al*. (1984). The activity of catalase (CAT) in erythrocytes and skin tissues was assayed by the method of Sinha (1972). The activity of gluthathione peroxidase (GPx) in erythrocytes and skin tissues was determined using the method of Rotruck *et al*. (1973). The activity of glutathione S-transferase (GST) in liver homogenate was assayed by the method of Habig *et al*. (1974). Glutathione reductase (GR) activity in liver homogenate was assayed by the method of Carlberg and Mannervik (1985). All the biochemical parameters were estimated colorimetrically in Elico SL 164 double beam UV-Visible Spectrophotometer.

Statistical analysis: Values are expressed as mean ± standard deviation (SD). Statistical analysis was analyzed with SPSS 9.0 for windows using one - way analysis of variance (ANOVA), followed

by Duncan's multiple range test (DMRT). The values were considered statistically significant, if the p-value was less than 0.05.

Results and Discussion

The body and liver weight of control and experimental animals in each group are shown in Table 1. The body and liver weight significantly decreased in DMBA treated animals as compared to control animals. Oral administration of geraniol three times week^{-1} for 25 weeks significantly increased the body and liver weight in DMBA treated animals. Oral administration of geraniol alone to mice (Group IV) showed no significant difference in body and liver weight as compared to control animals (Group I).

Table - 1: Effect of geraniol on body and liver weight, tumor incidence, tumor volume and tumor burden of experimental animals in each group

Parameters	I Control (vehicle treated)	II DMBA alone	III DMBA + Geraniol	IV Geraniol alone
Body weight (g)				
Initial	20.78 ± 0.55^a	21.16 ± 0.13^a	21.27 ± 0.23^a	20.95 ± 0.70^a
Final	26.12 ± 0.42^a	20.29 ± 0.46^b	25.47 ± 0.53^c	26.41 ± 0.57^a
Liver weight (g)	1.07 ± 0.05^a	0.79 ± 0.07^b	0.98 ± 0.06^c	1.09 ± 0.06^a
Tumor incidence	0	100% (6/6)	16.66 % (1/6)	0
Total number of tumors	0	17 / (6)	2 / (1)	0
Tumor volume (mm^3)	0	592.6 ± 53.72^a	69.8 ± 7.14^b	0
Tumor burden (mm^3)	0	1679.1 ± 146.2^a	139.6 ± 14.2^b	0

Values are mean of six replicates ±SD. Values that are not sharing a common superscript in the same column differ significantly at p<0.05. DMBA (25 µg in 0.1 ml acetone mouse^{-1}) two times weekly for 8 weeks and geraniol (250 mg kg^{-1} b.wt. in 0.5 ml corn oil) by gastric gavage three times week^{-1} on alternate days of DMBA treatment for 25 weeks

Table - 2: Effect of geraniol on TBARS in plasma, enzymatic and non-enzymatic antioxidants in erythrocytes and skin tissues of experimental animals in each group

Parameters	I Control (vehicle treated)	II DMBA alone	III DMBA + Geraniol	IV Geraniol alone
Plasma TBARS (n mol ml^{-1})	1.85 ± 0.20^a	4.10 ± 0.33^b	2.20 ± 0.28^c	1.75 ± 0.22^a
Erythrocytes membrane TBARS (n mol mg^{-1} protein)	0.35 ± 0.07^a	1.10 ± 0.14^b	0.51± 0.15^c	0.32 ± 0.05^a
Skin tissues TBARS (m mol 100 g^{-1} tissue)	98.75 ± 6.73^a	131.25 ± 5.72^b	106.25 ± 2.79^c	100.02 ± 5.59^a
Erythrocytes				
SOD (U^A mg^{-1} Hb)	6.04 ± 0.27^a	3.45 ± 0.69^b	5.42 ± 0.55^c	6.16 ± 0.34^a
CAT (U^B mg^{-1} Hb)	2.73 ± 0.16^a	1.33 ± 0.36^b	2.44 ± 0.22^c	2.77 ± 0.17^a
GPx (U^C g^{-1} Hb)	25.31 ± 1.33^a	13.32 ± 2.17^b	22.20 ± 2.39^c	25.53 ± 1.19^a
GSH (mg dl^{-1})	39.39 ± 1.22^a	21.33 ± 1.45^b	36.49 ± 2.15^c	39.69 ± 1.32^a
Skin tissues				
SOD (U^A mg^{-1} Hb)	7.64 ± 0.55^a	4.22 ± 0.36^b	6.48 ± 1.13^c	7.89 ± 0.69^a
CAT (U^B mg^{-1} Hb)	42.55 ± 7.62^a	16.65 ± 5.55^b	34.09 ± 5.23^c	43.50 ± 6.74^a
GPx (U^C g^{-1} Hb)	42.20 ± 1.83^a	26.66 ± 1.53^b	39.62 ± 1.33^c	42.62 ± 2.17^a
GSH (mg dl^{-1})	35.55 ± 2.51^a	18.07 ± 1.22^b	32.39 ± 2.18^c	36.14 ± 2.43^a

Values are mean of six replicates ±SD, Values that are not sharing common superscript in the same column differ significantly at p<0.05. a= The amount of enzyme required to inhibit 50% NBT reduction, b= µmole of H_2O_2 utilized sec^{-1}, c= µmole of glutathione utilized min^{-1}. DMBA (25 µg in 0.1 ml acetone mouse^{-1}) two times weekly for 8 weeks and geraniol (250 mg kg^{-1} b.wt. in 0.5 ml corn oil) by gastric gavage three times week^{-1} on alternate days of DMBA treatment for 25 weeks

Table - 3: Effect of geraniol on phase II detoxification enzymes and reduced glutathione in liver of experimental animals in each group

Parameters/Groups	I Control (vehicle treated)	II DMBA alone	III DMBA + Geraniol	IV Geraniol alone
Reduced glutathione (mg g^{-1} tissue)	2.18 ± 0.31^a	1.06 ± 0.20^b	1.85 ± 0.18^c	2.24 ± 0.26^a
Glutathione S-transferase (U^A mg^{-1} protein)	156.96 ± 6.56^a	115.52 ± 8.88^b	142.16 ± 10.48^c	158.92 ± 10.24^a
Glutathione reductase (U^B mg^{-1} protein)	38.51 ± 1.74^a	20.36 ± 1.53^b	34.07 ± 2.65^c	38.88 ± 1.81^a

Values are mean of six replicates ±SD, Values that are not sharing common superscript in the same column differ significantly at p<0.05. a = µmole of CDNB-GSH conjugate formed hr^{-1}, b= µmole of NADPH oxidized hr^{-1}. DMBA (25 µg in 0.1 ml acetone mouse^{-1}) two times weekly for 8 weeks and geraniol (250 mg kg^{-1} b.wt. in 0.5 ml corn oil) by gastric gavage three times week^{-1} on alternate days of DMBA treatment for 25 weeks

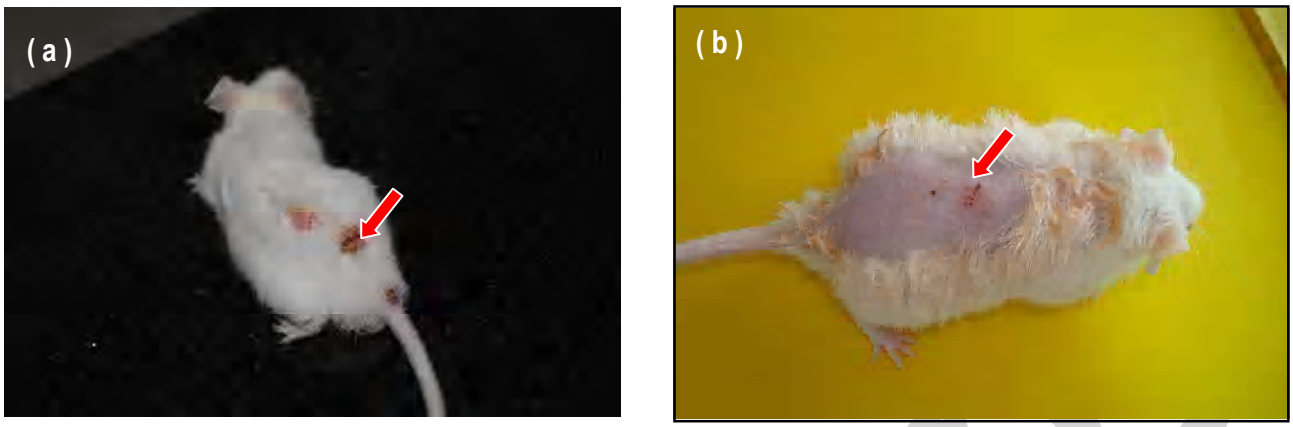

Fig. 1: The gross appearance of skin tumors in DMBA alone and DMBA + geraniol treated mice (a) DMBA alone treated mice with large skin tumor (b) DMBA + geraniol treated mice with small skin tumor

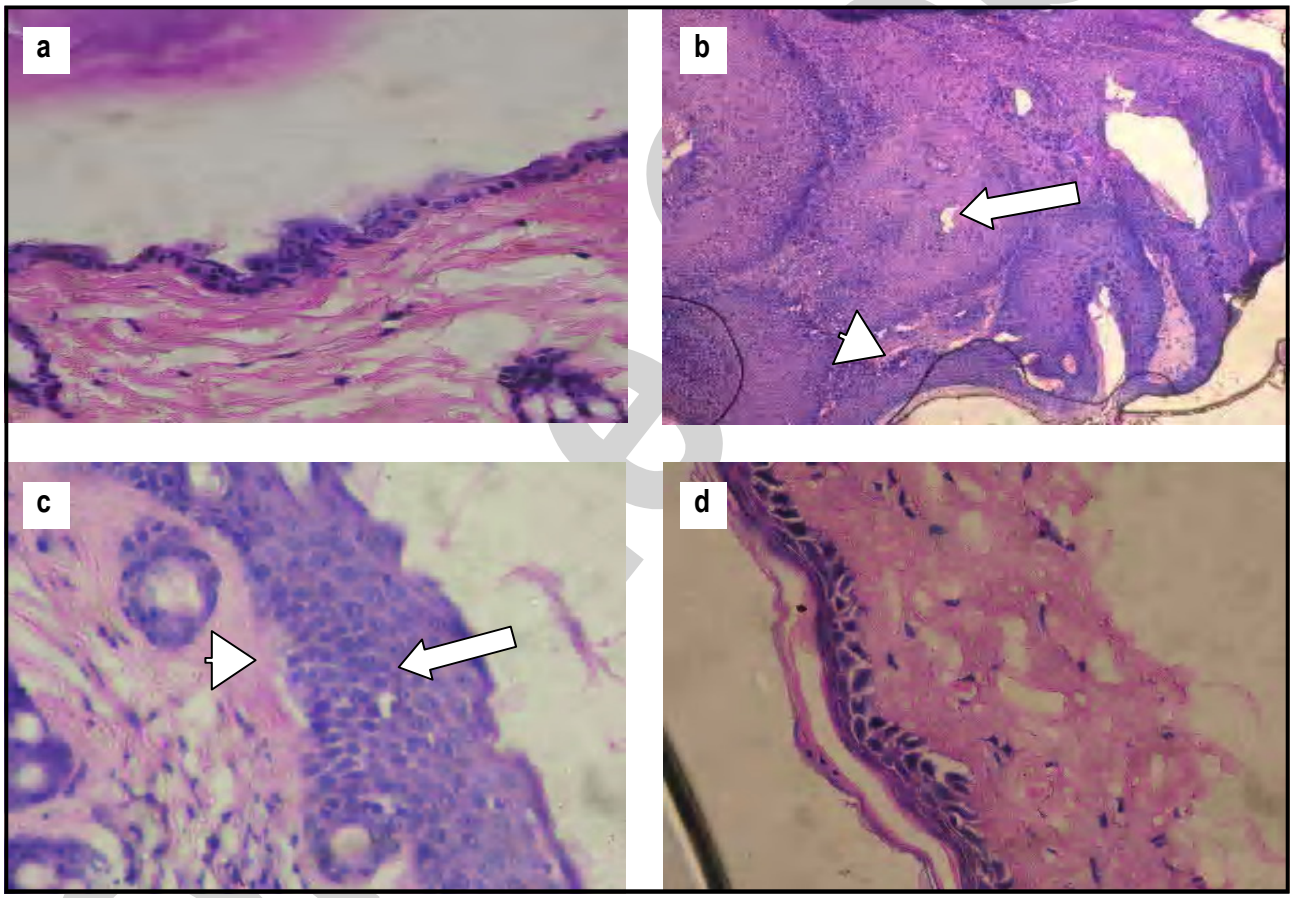

Fig. 2: The histological observation in skin tissues of control and experimental animals in each group (a) and (d) Microphotographs of skin tissues from control and geraniol alone treated animals respectively, showing well-defined subcutaneous tissues and intact epithelial layer (H and E x magnification) (b) Microphotograph of skin tissues from DMBA alone painted animals showing well-differentiated squamous cell carcinoma with dysplastic epithelium (△) and keratin pearls (⇨) (H and E x magnification); (c) Microphotograph of skin tissues from DMBA + geraniol treated animals showing hyperplastic (⇨) and dysplastic epithelium (△) (H and E x magnification)

The tumor incidence, tumor volume and burden of DMBA alone and DMBA + geraniol treated animals are shown in Table 1. In DMBA painted mice (group II), 100% tumor formation with mean tumor volume (592.6 mm^3) and tumor burden (1679.1 mm^3) was observed. The gross appearance of skin tumors in DMBA alone and DMBA + geraniol treated mice is depicted in Fig. 1a,b, respectively. Oral administration of geraniol significantly prevented tumor incidence, tumor volume and burden in DMBA painted mice.

The histopathological evaluation in skin tissues of control and experimental animals in each group is shown in Fig. 2 (a-d).

Skin tissues from vehicle treated control mice (2a) and geraniol alone treated mice (2d) exhibited well defined subcutaneous tissue and intact epithelial layer. We observed severe hyperplasia, hyperkeratosis, dysplasia and well-differentiated squamous cell carcinoma in all DMBA alone painted mice (2b). Although, we noticed hyperplasia and dysplasia (2c) in DMBA + geraniol treated mice, the squamous cell carcinoma was developed in one animal only.

The levels of TBARS in plasma, erythrocyte membrane and skin tissues of control and experimental animals in each group are shown in Table 2. The levels of TBARS were significantly increased in plasma, erythrocyte membranes and skin tissues of tumor bearing animals (group II) as compared to control animals. Oral administration of geraniol at a dose of 250 mg kg^{-1} b.wt. three times $week^{-1}$ for 25 weeks to DMBA painted animals significantly reduced the levels of TBARS. Control mice treated with geraniol alone (group IV) showed no significant difference in plasma, erythrocyte membrane and skin tissue TBARS as compared to control mice (group I).

The activities of enzymatic antioxidants (SOD, CAT and GPx) and non-enzymatic antioxidant (GSH) level in erythrocytes and skin tissues of control and experimental animals in each group are shown inTable 2. The activities of SOD, CAT, GPx and GSH level were significantly decreased in erythrocytes and skin tissues of tumor bearing animals (group II) as compared to control animals. Oral administration of geraniol to DMBA painted animals significantly increased the activities of enzymatic antioxidants and non-enzymatic antioxidants levels. Control mice treated with geraniol alone (group IV) showed no significant difference in erythrocytes and skin tissue enzymatic antioxidants and non-enzymatic antioxidant status as compared to control mice (group I).

The status of phase II detoxification agents (GST, GR and GSH) in the liver of control and experimental animals in each group are shown in Table 3. The status of GSH, GST and GR were significantly decreased in the liver of tumor bearing animals (group II) as compared to control animals. Oral administration of geraniol to DMBA painted animals significantly improved the status of phase II detoxification agents. Control mice treated with geraniol alone (group IV) showed no significant difference in the activities of phase II detoxification enzymes and reduced glutathione level as compared to control mice (group I).

Oral administration of geraniol to DMBA treated mice protected tumor formation in 83% of animals. Also, geraniol effectively reduced the histological abnormalities in the skin tissues of DMBA painted mice. Present results thus suggest that geraniol might have inhibited or suppressed abnormal cell proliferation in skin tissues during DMBA induced skin carcinogenesis.

It has been reported that glutathione-S-transferase detoxifies epoxides, the reactive metabolite produced during DMBA metabolism. Glutathione-S-transferase also plays crucial role the detoxification of xenobiotics and uses reduced glutathione to scavenge excessively generated potentially toxic compounds in the cells (Nakamura *et al*., 2000; Fiander and Schneider, 2000). Lowered activities of liver phase II detoxification were reported in skin cancer (Alias *et al*., 2009; Vellaichamy *et al*., 2009). Our results corroborate these observations. Oral administration of geraniol significantly increased the activities of phase II enzymes and glutathione content in DMBA painted mice. Present results suggest that geraniol stimulated the activities of phase II detoxification cascade to detoxify and excrete the active metabolite of DMBA, the dihydrodiol epoxide. The antitumor effect of geraniol is therefore partially associated with an induction of phase II detoxification enzymes and other antioxidant enzymes.

ROS causes severe damages to biomolecules and biomembranes as well as leads to several pathological diseases if excessive ROS are not promptly eliminated from the cells by host antioxidants defense mechanisms (Das and Saha, 2009; Dasgupta *et al*., 2003). Over production of ROS and insufficient antioxidant potential has been documented well in mice bearing skin tumors (Alias *et al*., 2009; Vellaichamy *et al*., 2009). Our results lend credence to these observations. Insufficient antioxidant potential in DMBA painted mice is probably due to exhaustion of these antioxidants to scavenge excessively generated reactive oxygen species or to combat the deleterious effects of ROS (Renju *et al*., 2007). Oral administration of geraniol at a dose of 250 mg kg^{-1} b. wt.significantly reduced the levels of TBARS and improved the status of antioxidant defense mechanism in DMBA treated mice. Present results suggest that geraniol has potent antioxidant function and free radical scavenging potential during DMBA induced skin carcinogenesis.

The present study thus demonstrated the chemopreventive potential of geraniol in DMBA induced skin carcinogenesis. We thus conclude that the chemopreventive potential of geraniol could be due to its anti-lipid peroxidative and antioxidant properties as well as modulatory effect on carcinogen detoxification process during DMBA induced skin carcinogenesis.

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