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Research Article

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

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ABSTRACT

Chrysin (5,7-dihydroxy flavone) possesses diverse pharmacological effects including anticancer potential. Aim of the present investigation was to assess the chemopreventive potential of chrysin in 7, 12-dimethylbenz(a)anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice. Skin tumors in mice were developed by painting with DMBA two times a week for 8 weeks. Hundred percent tumors was noticed in mice treated with DMBA alone after 14 weeks. Though oral administration of chrysin at a dose of 250 mg/kg bw to mice treated with DMBA significantly (86.6%) prevented tumor formation, severe hyperplasia, hyperkeratosis and dysplasia was noticed. The present results suggest that chrysin might have delayed rather than inhibiting the tumor formation during DMBA-induced skin carcinogenesis.

Keywords: Chemoprevention; DMBA; Skin cancer; Chrysin

INTRODUCTION

Skin, the largest organ covering the entire surface of the body, is the first line of defense against toxic external stimuli such as ultraviolet light, pro-oxidant, chemical compounds, infections and ionizing radiations (Schroder, 2010; Shindo & Hashimoto, 1995). Skin also prevents the loss of too much water and other fluids from the body. Skin cancer, abnormal mass of tissues in the skin, mainly appears on the face, hand, or neck, where they cause disfigurement and drastic alterations in the biochemical and molecular events. Skin cancers spread into other parts of the tissues and organs if not diagnosed and treated promptly (Senel, 2011; Erb, et al., 2008). Skin cancer is widely prevalent throughout the world and its incidence is increasing rapidly during the past two to three decades (Almahroos & Kurban, 2004). In USA, more than 1 million Americans are newly diagnosed with skin cancer every year (Criscione & Weinstock, 2010). Skin cancer affects 60,000 people every year in England (Kroll, et al., 2011). Australia has recorded the highest incidence of skin cancer than any other countries worldwide. More than 3,80,000 Australians are treated for skin cancer each year and the annual incidence is nearly four times the rates of Canada, US and UK (Mar, et al., 2011). Skin cancer accounts for 1-2 % of all cancers in India (Deo, et al., 2005).

7, 12-dimethylbenz(a)anthracene (DMBA), a potent site-specific carcinogen is commonly employed as both initiator as well as promoter to induce skin cancer in mice. DMBA mediates carcinogenesis by producing chronic inflammation, generating excess reactive oxygen species (ROS) and modulating the effect of phase I and II detoxification cascade. DMBA induced skin carcinogenesis is the preferred experimental model to study the chemopreventive potential of natural products and synthetic agents (Vellaichamy, et al., 2009).

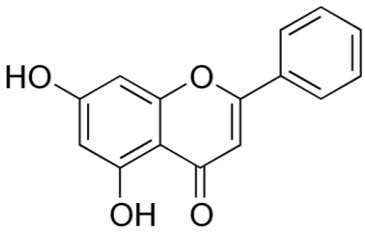

Figure 1: Chemical structure of chrysin

Chrysin (5,7-dihydroxy flavone. fig. 1) is abundantly present in *Passiflora caerulea* (passion flower), honeycomb and Indian trumpet flower (*Oroxylum indicum*). Chrysin, a biologically active flavone, possesses diverse pharmacological effects including anti-inflammatory, anticancer and antioxidant properties (Weng, et al., 2005). Extensive studies demonstrated the hepatoprotective effect of chrysin against galactosamine-induced liver toxicity (Pushpavalli, et al., 2010). Chrysin showed anti-inflammatory effect by inhibiting COX-2 expression and via IL-6 signaling (Lin, et al., 2010). Chrysin

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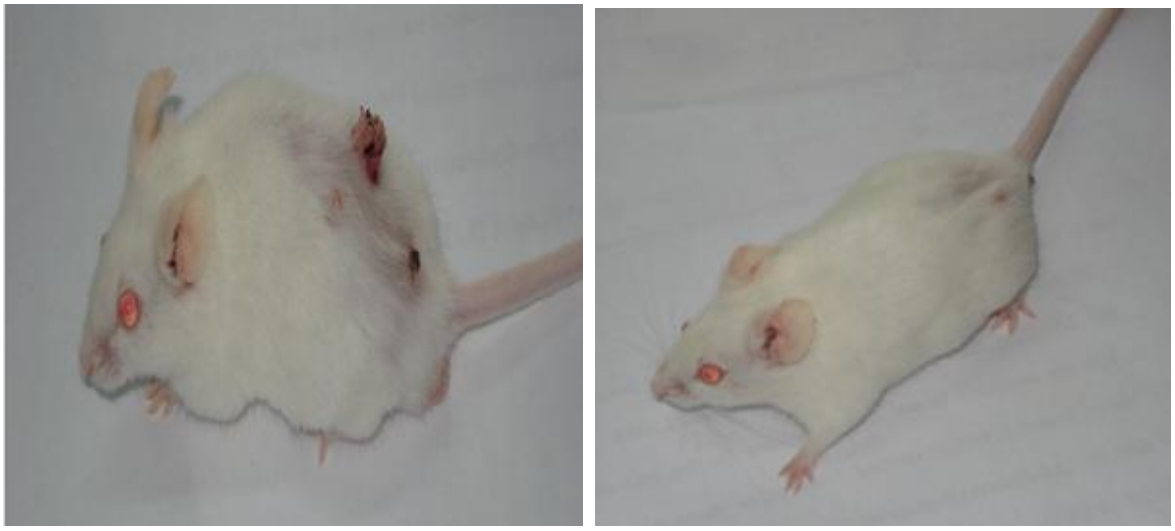
Table 1: Effect of chrysin on tumor incidence, tumor volume and tumor burden in DMBA treated mice

Groups	Tumor incidence	Total number of tumors	Tumor volume (mm^3)	Tumor burden (mm^3)
DMBA alone	100% (6/6)	19 / (6)	661.6 ± 59.92[a]	2095.1 ± 189.6[a]
DMBA + chrysin	16.66% (1/6)	3 / (1)	104.5 ± 9.76[b]	313.5 ± 22.83[b]

Values are expressed as mean ±SD (n=6). Tumor volume was measured using the formula $v = \frac{4}{3}\pi\left[\frac{D_1}{2}\right]\left[\frac{D_2}{2}\right]\left[\frac{D_3}{2}\right]$ where D_1, D_2 and D_3 are the three diameters (mm^3) 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. Values that are not sharing a common superscript in the same column differ significantly at $p<0.05$.



DMBA alone treated **DMBA + chrysin treated**

Figure 2: The gross appearance of skin tumors in DMBA alone and DMBA + chrysin treated mice

also exerted anticancer effect under in vivo and in vitro conditions (Miyamoto, et al., 2006). However, the mechanism of anticancer effect of chrysin is not well understood. There are however no scientific studies on the chemopreventive potential of chrysin in DMBA induced skin carcinogenesis. The present study is therefore designed to focus the chemopreventive potential of chrysin in DMBA induced skin carcinogenesis.

MATERIALS AND METHODS

Chemicals

7,12-dimethylbenz(a)anthracene (DMBA), chrysin and other biochemicals such as reduced glutathione, reduced nicotinamide adenine dinucleotide, 1,1',3,3'-tetramethoxypropane, were obtained from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, India. Heparin, thiobarbituric acid (TBA), trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), nitroblue tetrazolium (NBT) and phenazine methosulphate (PMS) were purchased from Hi-media Laboratories Mumbai, India. All other chemicals and solvents used were of analar grade.

Animals

Male, Swiss Albino mice 4-6 weeks old, weighing 15-20g 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 and water ad libitum and maintained under controlled conditions of temperature and humidity, with a 12 h light/ dark cycle. The local institutional animal ethics committee (Register number 160/1999/ CPCSEA), Annamalai University, Annamalai Nagar, India, approved the experimental design (Proposal No. 811: dated. 20-04-2011). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use.

Experimental Design

A total number of 24 male Swiss albino mice were divided into four groups of 6 in each. Skin carcinogenesis was developed in Swiss albino mice according to the method of Azuine and Bhide (Azuine & Bhide, 1992). Depilatory cream was applied to remove hair from the back of each mouse and the mice were left untreated

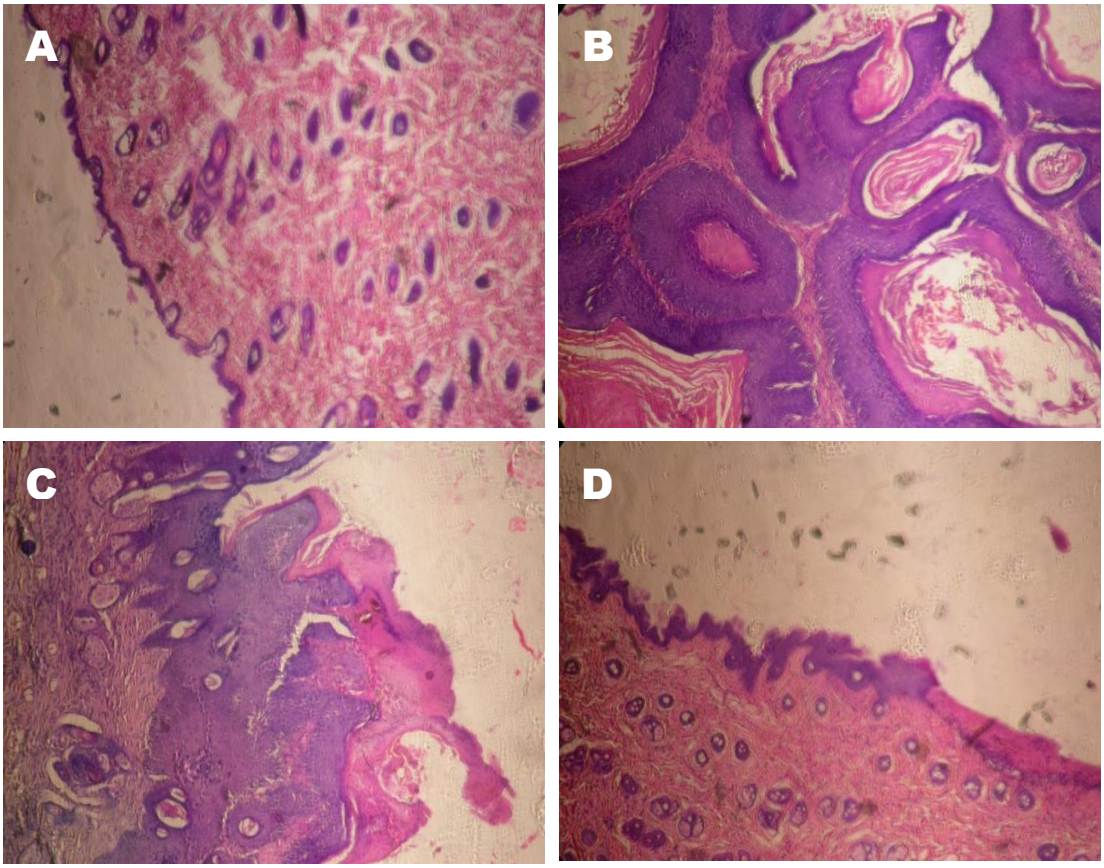


Figure 3: Histopathological changes observed in control and experimental mice in each group

A & D: Microphotographs of skin tissues from control and chrysin alone treated mice respectively, showing well-defined subcutaneous tissues and intact epithelial layer (40×)

B: Microphotograph of skin tissues from DMBA alone treated mice showing well-differentiated squamous cell carcinoma with dysplastic epithelium (H & E, 40X).

C: Microphotograph of skin tissues from DMBA + chrysin treated mice showing hyperplastic and mild dysplastic epithelium (H & E, 40X).

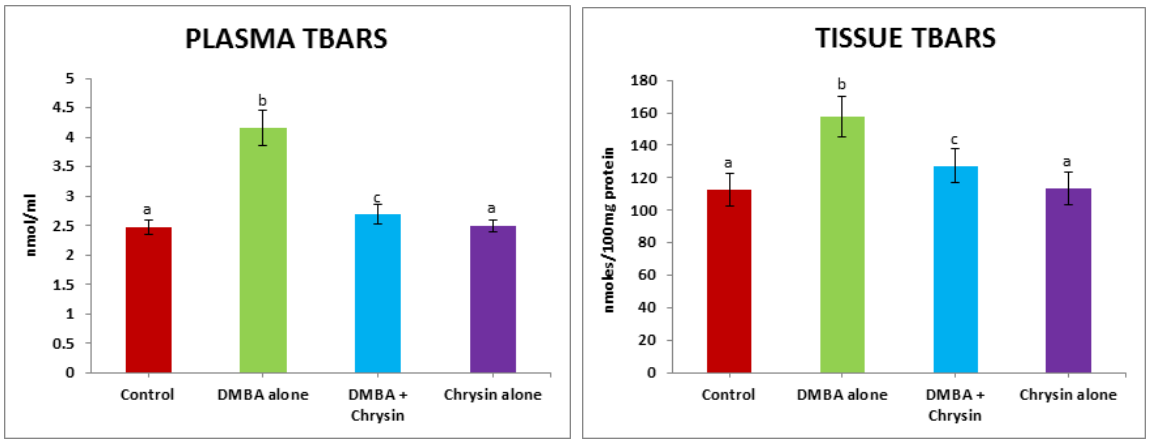


Figure 4: TBARS in plasma and skin tissues of control and experimental mice in each group

Values are expressed as mean ± SD (n=6). Values that are not sharing common superscript letter between groups differ significantly at p<0.05 (DMRT).

for two days. Mice having no hair growth after two days were selected for the experimental study.

The depilated back of group I mice was painted with acetone (0.1 ml/mouse) two times per week 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) two times per week for 8 weeks. Group II mice received no other treatment. Group III mice were orally administered with chrysin

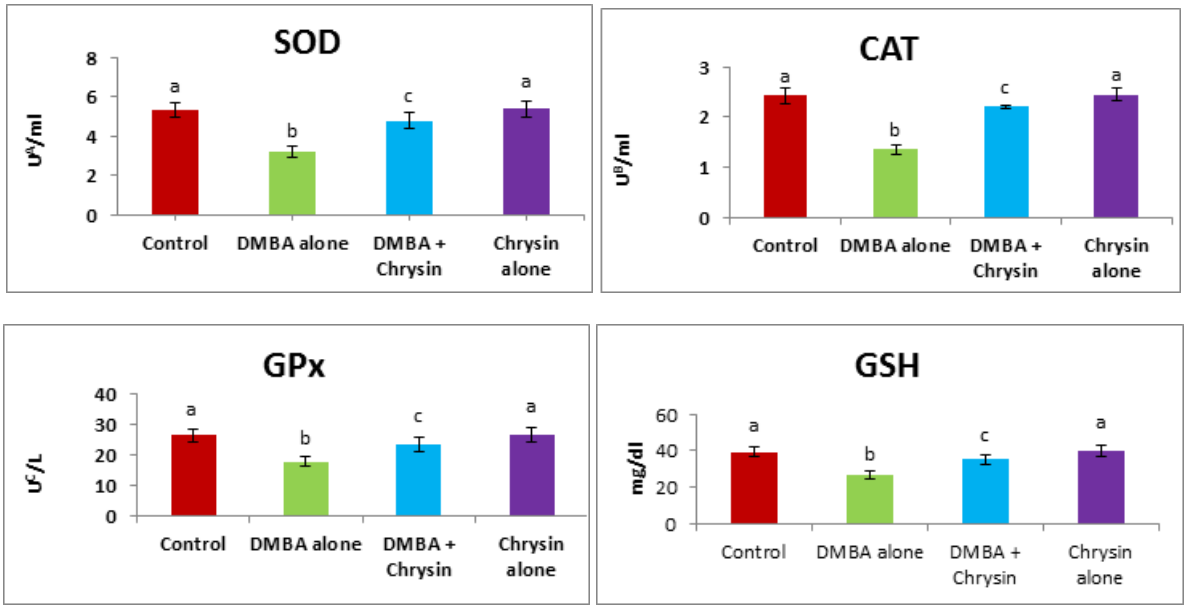


Figure 5: Status of enzymatic and non-enzymatic antioxidants in plasma of control and experimental mice in each group

Values are expressed as mean ±SD (n=6). Values that are not sharing common superscript letter between groups differ significantly at p<0.05 (DMRT). A-The amount of enzyme required to inhibit 50% NBT reduction; B-Micromoles of H_2O_2 utilized/ Sec; C-Micromoles of glutathione utilized/ min.

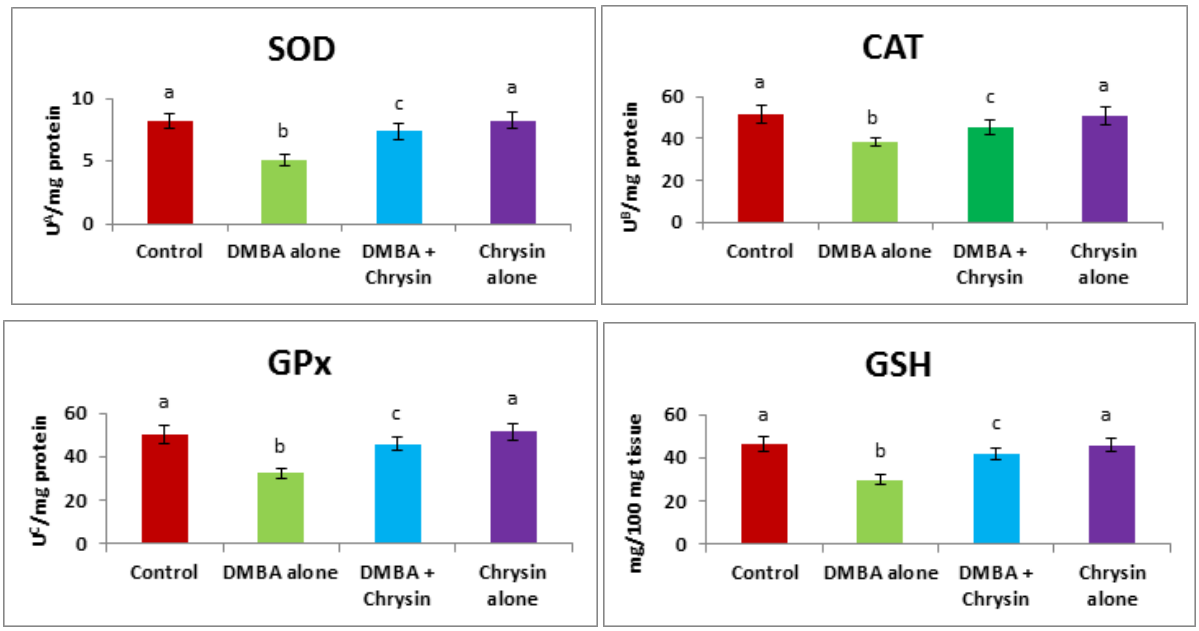


Figure 6: Status of enzymatic and non-enzymatic antioxidants in skin tissue of control and experimental mice in each group

Values are expressed as mean ±SD (n=6). Values that are not sharing common superscript letter between groups differ significantly at p<0.05 (DMRT). A-The amount of enzyme required to inhibit 50% NBT reduction. B-Micromoles of H_2O_2 utilized/ Sec. C-Micromoles of glutathione utilized/ min

(250 mg/kg body wt) by gastric gavage starting 1 week before the exposure to the carcinogen and continued for 25 weeks (3 times/week on alternate days of DMBA painting) thereafter. Group IV mice were orally administered with chrysin alone by gastric gavage throughout the experimental period. At the end of experimental period all the animals were sacrificed by cervical dislocation.

Preparation of Tissue Homogenate

Tissue samples from mice were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer [appropriate buffer of concerned parameter (TBARS– 0.025 M Tris-HCl buffer, pH 7.5; GSH and GPx – 0.4 M phosphate buffer, pH – 7.0; SOD – 0.025 M sodium pyro-

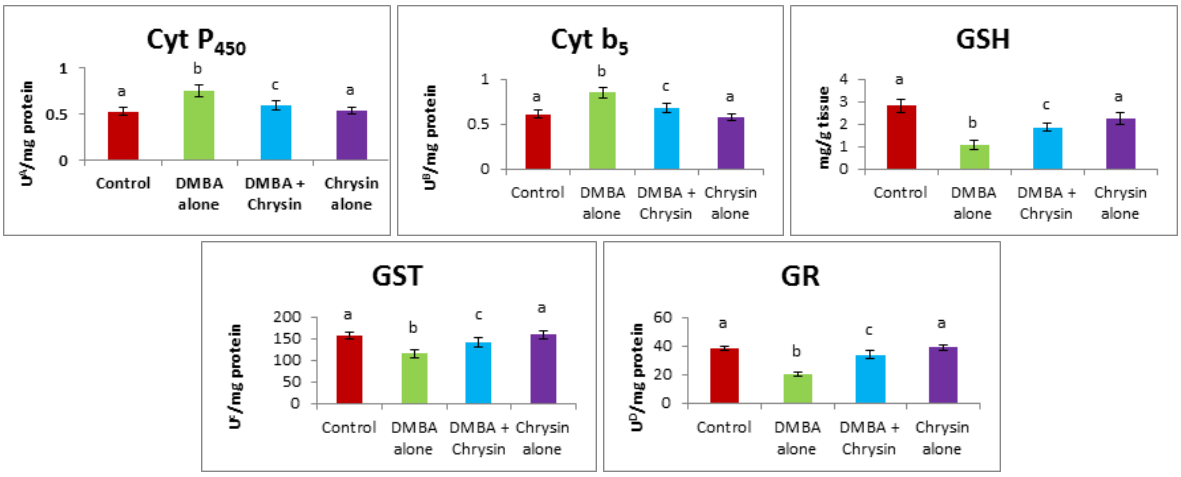


Figure 7: Status of phase I and phase II detoxication agents in the liver of experimental mice in each group

Values are expressed as mean ±SD (n=6). Values that are not sharing common superscript letter between groups differ significantly at p<0.05 (DMRT). A - µm of cytochrome P_{450}; B - µm of cytochrome b_5; C - µm of CDNB-GSH conjugate formed per hour; D - µm of NADPH oxidized per hour.

phosphate buffer, pH 8.3; CAT – 0.01 M phosphate buffer, pH 7.0)] in an all glass homogenizer with teflon pestle. The homogenate was centrifuged at 1000g for 5 minutes and the supernatant was then used for the biochemical estimations.

Histological evaluation

For histopathological studies, tumor tissues and normal skin tissues were fixed in 10 % formalin and were routinely processed and paraffin embedded, 2-3µm sections were cut in a rotary microtome and were stained with hematoxylin and eosin.

Biochemical assays

Biochemical estimations were carried out in blood and tissues of control and experimental mice in each group. Lipid peroxidation was estimated as evidenced by the formation of thiobarbituric acid reactive substances (TBARS). TBARS in plasma and skin was determined by the methods of Yagi (Yagi, 1987) and Ohkawa et al (Ohkawa, et al., 1979) respectively. The reduced glutathione level in liver and skin tissues was determined by the method of Beutler and Kelly (Beutler & Kelly, 1963). Superoxide dismutase, catalase and glutathione peroxidase activities in plasma and skin tissues was assayed by the method of Kakkar et al., Sinha and Rotruck et al (Kakkar et al., 1984; Sinha, 1972; Rotruck, et al., 1973) respectively. The levels of cytochromeP450 and b5 in liver tissue homogenate were determined according to the method of Omura and Sato (Omura & Sato, 1964). The activities of GST and GR in liver tissue homogenate was assayed by the method of Habig et al and Carlberg and Mannervik (Habig, et al., 1994; Carlberg & Mannervik , 1985) respectively.

STATISTICAL ANALYSIS

Values are expressed as mean ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test (DMRT). The values were considered statistically significant, if p value was less than 0.05.

RESULTS

The tumor incidence, tumor volume and burden of mice treated with DMBA alone and DMBA+chrysin treated mice are shown in table 1. In mice treated with DMBA alone, 100% tumor formation with mean tumor volume (661.6 mm^3) and tumor burden (2095.1 mm^3) was observed. The gross appearance of skin tumors in mice treated with DMBA alone and DMBA+ chrysin treated mice is depicted in figure 2. Oral administration of chrysin significantly prevented the tumor incidence, tumor volume and burden in mice treated with DMBA.

The histopathological evaluation in skin tissues of control and experimental mice in each group is shown in figure 3 (A-D). Skin tissues from vehicle treated control mice (A) and chrysin alone treated mice (D) exhibited well defined subcutaneous tissue and intact epithelial layer. We observed severe hyperplasia, hyperkeratosis, dysplasia and well-differentiated squamous cell carcinoma in all the mice treated with DMBA alone (B). Although severe hyperplasia and dysplasia was noticed in all the DMBA + chrysin treated mice, a small size tumor was also observed in only one of the DMBA+chrysin treated mice (C).

The levels of TBARS in plasma and skin tissues of control and experimental mice in each group are shown in figure 4. The levels of TBARS were significantly increased in plasma and skin tissues of tumor bearing mice as compared to control mice. Oral administration of chrysin at a dose of 250mg/kg b.w three times per week for 25 weeks to DMBA treated mice significantly reduced the levels of TBARS. Control mice treated with chrysin alone showed no significant difference in the plasma and skin tissue TBARS as compared to control mice.

The activities of enzymatic antioxidants (SOD, CAT, GPx) and non-enzymatic antioxidant (GSH) level in the plasma and skin tissues of control and experimental mice in each group are shown in figure 5 and 6 respectively. The status of antioxidants was significantly decreased in the plasma and skin tissues of tumor bearing mice as compared to control mice. Oral administration of chrysin to DMBA treated mice significantly increased the activities of enzymatic antioxidants and non-enzymatic antioxidants levels. Control mice treated with chrysin alone showed no significant difference in plasma and skin tissue enzymatic antioxidants and non-enzymatic antioxidant status as compared to control mice.

The status of phase I (Cyt $P_{450,}$ Cyt b_5) and phase II detoxication agents (GST, GR and GSH) in the liver of control and experimental mice in each group are shown in figure 7. The status of Cyt $P_{450,}$ Cyt b_5 were significantly increased whereas the status of GSH, GST, and GR were significantly decreased in the liver of tumor bearing mice as compared to control mice. Oral administration of chrysin to DMBA treated mice significantly improved the status of phase I and phase II detoxification agents. Control mice treated with chrysin alone showed no significant difference in the activities of phase I and II detoxification enzymes and reduced glutathione level as compared to control mice.

DISCUSSION

In the present study, we have investigated the chemopreventive efficacy of chrysin in DMBA induced skin carcinogenesis in mice by monitoring the percentage of tumor bearing animals, tumor volume and burden as well as by analyzing the status of detoxification enzymes, lipid peroxidation and antioxidants status in the plasma and skin tissues of mice painted with DMBA. Mice treated with DMBA alone for 8 weeks developed 100% tumor formation and the tumor was histopathologically confirmed as well differentiated squamous cell carcinoma. We also noticed severe hyperkeratosis, hyperplasia and dysplasia in mice treated DMBA alone. The tumor volume and tumor burden was significantly increased in mice treated with DMBA alone. Oral administration of chrysin at a dose of 250 mg/kg b.w significantly inhibited (86%) the tumor formation in mice treated DMBA alone. We however observed severe hyperplasia and dysplasia in all DMBA + chrysin treated mice. Our results suggest that chrysin has considerable suppressing effect on abnormal cell proliferation occurring in DMBA-induced skin carcinogenesis.

Most of the metabolic reactions including detoxification of carcinogenic substances takes place in the liver and thus measuring the status of detoxification agents may help to assess the chemopreventive potential of the natural products (Manoharan, et al., 2010a). The cytochrome P_{450} superfamily is a large and diverse group of enzymes, which metabolize lipophilic compounds to more polar products, which are then acted upon by the phase II enzymes, further increasing their polarity and assisting in their excretion (Muruganandan, & Sinal, 2008). Cytochrome b_5 is a ubiquitous, 15.2 K Da haemoprotein involved in a number of cellular processes such as fatty acid desaturation, drug metabolism, steroid hormone biosynthesis and methaemoglobin reduction (Finn, et al., 2011). Phase II enzymes are involved in the detoxification of carcinogens either by destroying their reactive centers or by conjugating them with glucuronic acid or reduced glutathione, facilitating their excretion (Manoharan, et al., 2010a). GSTs are a family of enzymes that catalyzes the conjugation of reactive chemicals with GSH and play a major role in protecting cells (Manoharan, et al., 2010b). Glutathione reductase catalyzes NADPH-dependant reduction of glutathione disulfide to glutathione, thus maintaining reduced glutathione levels in the cells (Vinothkumar & Manoharan, 2011).

Chemopreventive agents modulate the activities of phase I and phase II detoxification enzymes in favour of the excretion of carcinogenic metabolites (Silvan, et al., 2011). In the present study, the activities of phase I and phase II enzymes and reduced glutathione levels were drastically altered in the liver of mice treated with DMBA alone. Our results suggest that the liver of DMBA treated mice is highly exposed to carcinogenic insult. Oral administration of chrysin brought back the status of phase I and phase II detoxification agents to near normal range in the liver mice treated with DMBA. The present study thus suggest that chrysin modulated the activities of phase I and phase II detoxification agents to stimulate the excretion of the carcinogenic metabolite of DMBA, dihydrol diol epoxide.

Over production of reactive oxygen species in the cell cause damage to biomolecules that perform pivotal role in the regulation of cell cycle, thereby contributing to mutagenesis (Klaunig, et al., 2011). Excessive reactive oxygen species are generated during the metabolic activation of DMBA. Enzymatic and non-enzymatic antioxidants form the first line of defense against ROS mediated lipid-peroxidation. Measurement of plasma TBARS could help to monitor the extend of tissue damage. Increase in plasma TBARS in the tumor bearing mice could be related to over production of lipid peroxidation by-products and diffusion from damaged skin tissues and other host tissues with subsequent leakage into plasma (Renju, et al., 2007). Increase in skin tumor tissue TBARS is probably due to repeated carcinogenic (DMBA) insult on the skin (Alias, et al., 2009).

Lowered activities of enzymatic antioxidants and decreased content of GSH level in plasma and tumor tissues are due to exhaustion of these antioxidants to combat the deleterious effects of excessively generated ROS during carcinogenic process (Manoharan, et al., 2009). Lowered levels of antioxidants thus confirm the status of oxidative stress in mice treated with DMBA (Alias, et al., 2009). Oral administration of chrysin significantly improved the status of lipid peroxida-

tion and antioxidants in mice treated with DMBA, which suggests its free radical scavenging potential and stimulation of endogenous antioxidant function. The antioxidant potential is probably due to its two phenolic hydroxyl group in the chemical structure of chrysin.

The present study thus demonstrates the chemopreventive potential of chrysin in DMBA induced mouse skin carcinogenesis. The chemopreventive effect of chrysin is probably due to its anti-lipid peroxidative and antioxidant properties as well as modulatory effect on carcinogen detoxification process during DMBA induced skin carcinogenesis. Though chrysin significantly suppressed abnormal cell proliferation in mice treated with DMBA, it fails to completely prevent the skin tumor formation. The present study thus concludes that chrysin might have the ability to extend the survival time of tumor bearing mice by delaying the tumor formation.

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