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Research Article

Chemopreventive effect of *Eulophia Herbaceae* on DMBA/Croton Oil-induced two stage mouse skin carcinogenesis

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ABSTRACT

Eulophia Herbaceae (EH) is a widely distributed plant in India and is found mainly on terrestrial and hill slopes as forest undergrowth in the area of Himalaya, Bengal and Eastern part of India. The tubers of EH are traditionally used for treatment of various diseases such as tumors of scrofulous glands of neck, treatment on worms, rheumatism and is also used for ailment of pimples. Decoction of tuber is used on spermatorrhoea, urinary complaints and menses. Tubers are source of salep, pseudobulbs and are used as tonic. In this study, the chemopreventive effect of triterpenoid fraction of tubers from EH was investigated. The triterpenoid fraction of EH (TFEH) showed a strong chemopreventive activity against DMBA/Croton oil induced two stage mouse skin carcinogenesis. The mechanistic pathway for the chemopreventive potential of TFEH was evaluated by analyzing the status of enzymatic (CAT, SOD) and non enzymatic (reduced glutathione) detoxification agents and lipid peroxidation during DMBA-induced skin carcinogenesis. The results obtained showed significant reduction of the incidence and number of skin papillomas, tumor burden, tumor volume along with significant elevation of phase II detoxifying enzymes (CAT and SOD) and inhibition of lipid peroxidation in liver. The present study thus demonstrates that TFEH has significant suppressing effect on cell proliferation during DMBA-induced mouse skin carcinogenesis. The chemopreventive potential of TFEH is probably due to its modulating effect on the status of lipid peroxidation, antioxidants and detoxification agents during DMBA-induced skin carcinogenesis.

Keywords: Lipid peroxidation; Phase II enzymes; Reduced glutathione; Triterpenoids.

INTRODUCTION

The human population is exposed to a number of chemical mutagens and carcinogens, accidentally, occupationally or by life style habits. The skin is a major environmental interface for the body and, as a consequence of its direct exposure to variety of xenobiotics, is at a uniquely high risk of developing cancer. Skin cancer is one of the most common cancers worldwide and accounts for 30% of all newly diagnosed cancers. More than 1million skin cancers are diagnosed each year in the United States (Diepgen et al., 2002).

7,12-Dimethylbenz[a]anthracene (DMBA) is commonly employed to induce skin carcinogenesis in mice. DMBA is metabolized to dihydrodiol-epoxide, the ultimate carcinogen, which mediates carcinogenic process by inducing chronic inflammation, over production of reactive oxygen species (ROS) and oxidative DNA damage (Das et al., 2006).

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Chemoprevention is a novel and promising approach to control, inhibit or suppress the tumor formation by using natural or synthetic entities. A large number of phytochemicals ingested in human diet, exhibit anticarcinogenic and antimutagenic effects. Triterpenoids are natural, biologically active compounds extracted from many plants. They possess cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglycemic, anti-inflammatory, and antiparasitic and antioxidant properties (Cipak et al., 2006; Roman et al., 2007). Triterpenes such as glycyrrhizin, oleanolic acid, ursolic acid, b-boswellic acid, have been reported to possess a variety of biological effects including anti-inflammation, hepatoprotection and Immunomodulation (Jung-ae et al., 2009). There is a growing interest in natural triterpenoids, also known as phytosterols, due to their wide spectrum of biological activities. Triterpenes are a wide-spread group of natural compounds with considerable practical significance. Triterpenes are also natural components of human diets (Mohammad S., 2009).

Recent studies have shown that diets rich in phytochemicals can significantly reduce cancer risk by as much as 20%. Epidemiological data suggest that the phytosterols content of the diet is associated with a reduction in common cancers including cancers of the colon, breast, and prostate (Bradford et al., 2007;

Sporn et al., 2007). Data emanating from molecular studies with various tumorigenic models suggest that triterpenoids modulate host systems potentially enabling more robust antitumor responses such as enhancing immune recognition of tumor cells, altering hormone-dependent growth of endocrine tumors and modulating sterol biosynthesis (Bradford et al., 2007).

A number of triterpenoids have shown promise as antineoplastic agents and exhibit antiproliferative activity when tested against various cancer cell lines. These triterpenoids include members belong to the cycloartane, lupane, friedelane, dammarane, ursane, oleanane, limonoid and cucurbitacin family (Hattori et al., 2002). Recent reports showed that triterpenes directly inhibit tumor growth, cell cycle progression, and induce the apoptosis of tumor cells under in vitro and in vivo situations (Bradford et al., 2007).

MATERIAL AND METHOD

Chemicals

DMBA, croton oil, hydroxylamine hydrochloride, triton X-100, ethylene diamine tetra acetic acid (EDTA), nitro blue tetrazolium (NBT) was obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide 30% (H_2O_2) was obtained from Tekay Products (Thane, India). 5, 5-dithio bis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA) was obtained from Loba Chemie (Mumbai, India).

Animals

Healthy adult male albino mice of Swiss strain, weighing between 20 and 25 g were purchased from breeding centre of R.C Patel Institute of Pharmaceutical Education and Research, Shirpur. The animals were acclimatized under laboratory condition for a fortnight before starting experiments. The animals were maintained on a standard diet and water ad libitum. They were housed in polypropylene cages and exposed to 10–12 h of daylight under standard conditions of temperature (25°C) and humidity (30%). The study was approved by Institutional Animal Ethical Committee registered with Committee for the Purpose of Control and Supervision of Experiments on Animals, India (Registration No. 651/02/C/CPCSEA).

Plant material

The plant of *Eulophia Herbaceae* was collected from hilly areas of Toranmal (Nandurbar, Maharashtra State, India) in the month of July and August. The plant specimen was authenticated by Mr. T. Chakraborty (Voucher No. BANDEUH1) at Botanical Survey of India (BSI), Pune.

Extraction and isolation of triterpenoid fraction

The air dried powdered tubers of *Eulophia Herbaceae* was extracted with methanol at room temperature to yield crude extract. After extraction with methanol all the crude extract was filtered & concentrated separately under reduced pressure in rotary vacuum evaporator. The dried extract was then suspended in water and partitioned with portions of n-hexane in a separating funnel. The combined n-hexane extract was then dried. Various preliminary phytochemical tests revealed the presence of triterpenoids in the hexane extract which was further confirmed by using sophisticated analytical techniques such as GC-MS which showed the presence of triterpenoids such as Stigmasterol and β-sitosterol. The triterpenoid fraction of Eulophia Herbaceae (TFEH) thus obtained was used for experimental purpose.

EXPERIMENTAL DESIGN

Male Swiss Albino mice were divided into five groups containing eight animals in each. The back of the animals of all groups were shaved 2 days prior to the start of the experiment. Animals of control group (Group I) received topical application of acetone (100 µl /mouse) on the shaved area of skin and water by oral gavage for 12 weeks and served as vehicle controls. DMBA group (Group II) received two topical application of 7, 12-dimethylbenz[a]anthracene at an interval of 72 h, at a dose of 0.05 g/kg b.w. in acetone (100 ml/mouse), followed by croton oil (1% w/v) in acetone (100 µl/mouse), twice in a week up to 9th week starting from day 8 of 1st 7, 12-dimethylbenz[a]anthracene application and served as carcinogen control. The remaining three groups (Group III, IV, V) received DMBA treatment same as Group II along with TFEH at the doses 50, 100 & 200 mg/kg, p.o. throughout the experimental period respectively.

Mice of group I, II, III, IV and V were sacrificed following the guidelines as mentioned by the ethical committee after 12 weeks of 1st DMBA application and the parameters described below were studied.

Detection of Papillomas

The experimental animals were carefully examined weekly up to 12th week for counting and recording the incidence of papilloma and the number of papilloma per papilloma bearing mouse. Skin papillomas with a diameter greater than 1mm that persists for at least two consecutive observations were used for counting. The papillomas, which regressed after one observation, were not considered for counting. Two different experts who were not concerned with information regarding the experimental groups performed the measurement of papillomas (Das et al., 2005).

Visual Parameters

1). Body weights of all the animals were recorded at an interval of 7 days till sacrifice.

2). Tumor burden (Number of papilloma per papilloma bearing mouse) is defined as the total number of tumors per tumor bearing mouse. Papillomas appearing on the shaven area were recorded at weekly intervals and papillomas >1 mm in diameter was included in

data analysis only if they persisted for 2 weeks or more. Animals were sacrificed 20 weeks after commencement of the treatments (Abraham et al., 2008).

3). Incidence of papilloma: The number of mice carrying at least one tumor expressed as percent incidence (Das et al., 2005).

4). Total number of papilloma was calculated at the end of the study.

5). Relative liver weight was measured at the end of the study for all groups.

Biochemical Parameter

Preparation of Liver samples for Biochemical Estimation:

Animals were sacrificed by cervical dislocation. The liver was quickly excised and washed thoroughly with chilled phosphate buffered saline (pH 7.4). A 10% tissue homogenate (w/v) was prepared from part of the sample (liver) in 0.15 M Tris–HCl (pH 7.4) and the homogenate was then centrifuged at 12 000 *g* for 15 min. The supernatant thus obtained was taken for estimation of Catalase (CAT), Superoxide Dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA).

1) Catalase (CAT) Assay:

This was assayed by the method of Aebi (Aebi, 1984). 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 of skin and liver tissues (of 10% tissue homogenate obtained as described above) in 50 mM phosphate buffer (pH 7). The activity of the enzyme is expressed as % of control.

2) Superoxide Dismutase (SOD) assay:

Superoxide dismutase was assayed in liver by utilizing the method of Kono (Kono, 1978). Wherein reduction of nitroblue tetrazolium (NBT) mediated by superoxide anions generated by photooxidation of hydroxylamine hydrochloride to blue formazon was measured at 560 nm. The activity of superoxide dismutase was expressed as percentage of control.

3). Reduced glutathione (GSH) Content:

Reduced glutathione was estimated as total non-protein sulphydryl groups by the method described by Moron (Moron et al., 1979). Homogenates were immediately precipitated with 0.75 ml of sulfosalicylic acid and precipitates were removed after centrifugation at 1500 *g* for 10 min. Free- SH groups were assayed in a total 3ml volume by adding 2 ml of 0.6 mM DTNB prepared in 0.2M sodium phosphate buffer (pH 8.0), to 0.1 ml of the supernatant and absorbance was read at 412 nm using a double beam spectrophotometer.

4). Lipid peroxide estimation (MDA)

Levels of lipid peroxides were estimated using the method of Ohkawa (Okhawa et al., 1979). Briefly, thiobarbituric acid (0.8%), sodium dodecyl sulphate (0.1%) and acetic acid (20%) were added to 100 ml of the tissue homogenate (10%) prepared as described above. This mixture was heated for 30 min, cooled, extracted with *N*-butanol-pyridine, and the OD of MDA recorded at 532 nm.

Statistical Analysis

Data obtained for all other experiments was expressed in MEAN ± SEM. All the groups were compared and analyzed by one way ANOVA followed by Dunnett's multiple comparison tests. The values were considered statistically significant, if the *p*-value was less than 0.05.

RESULTS

Effect of TFEH on body weight

The body weight of control and experimental animals in each group is shown in Figure 1. The body weight was significantly decreased in DMBA treated animals as compared to control animals. Oral administration of TFEH for 12 weeks significantly increased the body weight in mice painted with DMBA.

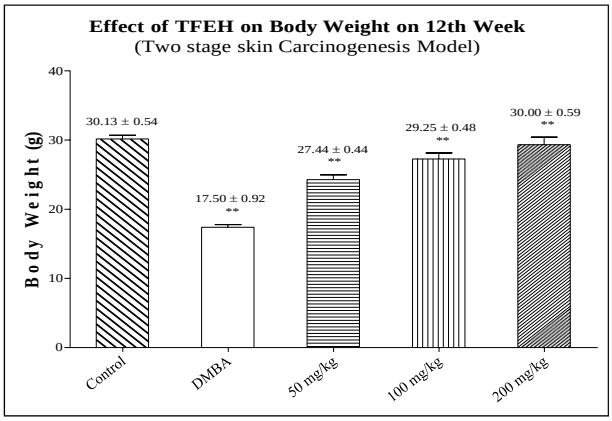

Figure 1: Effect of TFEH on body weight

Effect of TFEH on Tumor Burden

The tumor burden in control and experimental is shown in Figure 2. Tumor burden was found to be decreased in TFEH treatment groups when compared with DMBA alone treated group. Tumor burden in DMBA alone group was found to be 3.88 ± 0.67 tumors whereas there is dose dependent decrease in tumor burden in TFEH treatment groups. Highest level of inhibition was found to be in 200 mg/kg TFEH treatment group with significant reduction ($p<0.05$) 1.50±0.65 tumors.

Effect of TFEH on total number of papilloma

The total number of papilloma in control and experimental is shown Figure 5. Total number of papilloma in DMBA-Croton oil group was found to be 31 papillomas in after 12 weeks promotion period. In 50 mg/kg TFEH,

100 mg/kg TFEH and 200 mg/kg TFEH it was found to be 24, 15, 12 papillomas respectively.

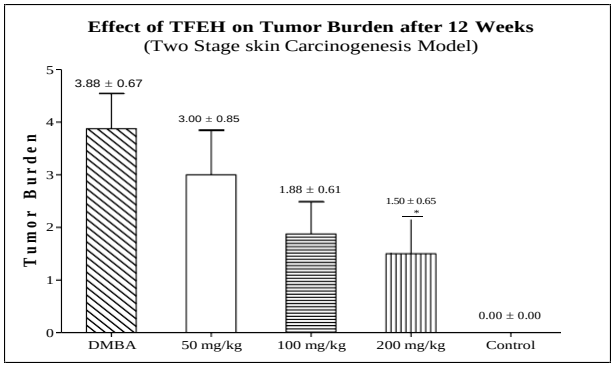

Figure 2: Effect of TFEH on tumor burden

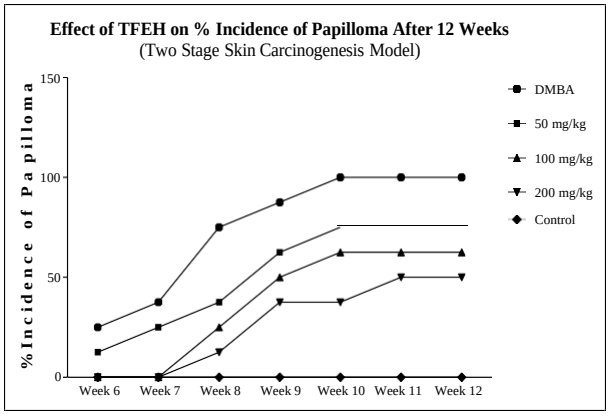

Figure 3: Effect of TFEH on percent incidence of papilloma

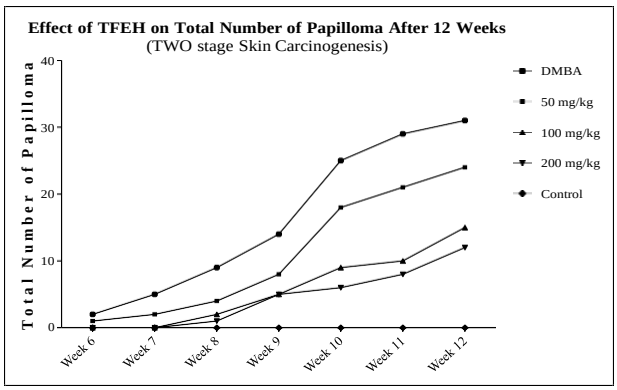

Figure 4: Effect of TFEH on total number of papilloma

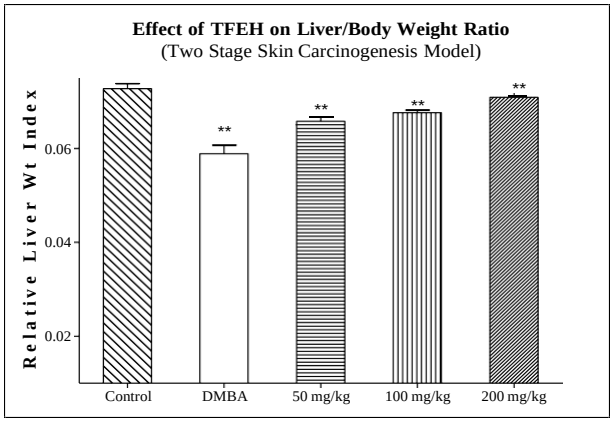

Figure 5: Effect of TFEH on Relative liver weight

Effect of TFEH on Relative liver weight

Effect of TFEH on relative liver weight in control and experimental animals is shown in Figure 5. Relative liver weight of control group after 12 weeks study was found to be 0.07 ± 0.001 g which is significantly increased ($p<0.001$) when compared with in DMBA-Croton oil group. There is dose dependent increase in relative liver weight in TFEH treatment groups with highest level of increase in 200 mg/kg TFEH 0.07 ± 0.0009 g which is significantly increased ($p<0.001$) when compared with DMBA group.

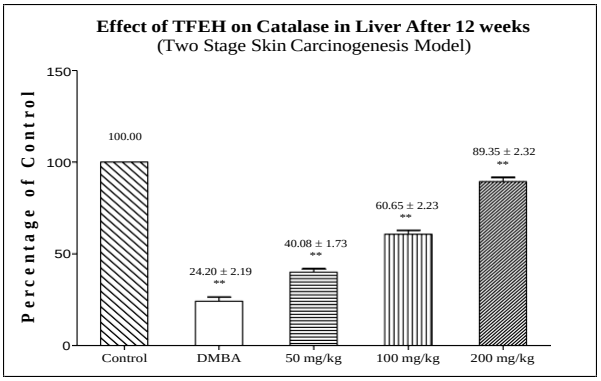

Figure 6: Effect of TFEH on CAT level

Effect of TFEH on Catalase level

Effect of TFEH on catalase level in control and experimental animals is shown in Figure 7. Activity of catalase in liver of DMBA-Croton oil group was found to be 24.20 ± 2.19 % when compared with control group after 12 weeks study. There was dose dependent increase in catalase activity in liver of treatment groups when compared with DMBA- Croton oil group. Significant increase in catalase activity was found in 50 mg/kg TFEH ($p<0.01$) up to 40.08 ± 1.73 %, 100 mg/kg TFEH ($p<0.01$) up to 60.65 ± 2.3 % and 200mg/kg TFEH ($p<0.01$) up to 89.35 ± 2.32 %. There was highest level of increase in catalase activity in 200 mg/kg TFEH group after 12 weeks study.

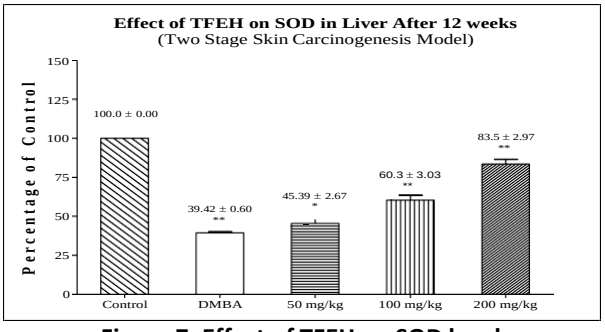

Figure 7: Effect of TFEH on SOD level

Effect of TFEH on SOD level in liver after 12 weeks study

Effect of TFEH on SOD level in control and experimental animals is shown in Figure 7. Activity of SOD in liver of DMBA-Croton oil group was found to be 39.42 ± 0.60 % when compared with control group after 12 weeks study. There was dose dependent increase in

SOD activity in liver of treatment groups when compared with DMBA- Croton oil group. Significant increase in SOD activity was found in 100 mg/kg TFEH ($p<0.01$) up to 60.30 ± 3.03 %, 200 mg/kg TFEH ($p<0.001$) up to 83.50 ± 2.97 % and 50 mg/kg TFEH ($p<0.05$) up to 45.39 ± 2.67 %. There was highest level of increase in SOD activity in 200 mg/kg TFEH group after 12 weeks study.

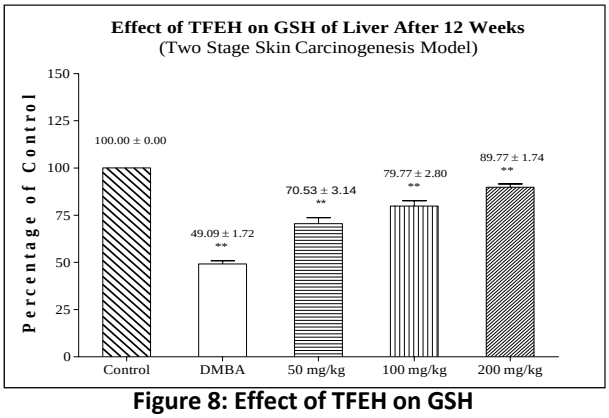

Figure 8: Effect of TFEH on GSH

Effect of TFEH on GSH level in liver after 12 weeks

Effect of TFEH on GSH level in control and experimental animals is shown in Figure 8. Reduced Glutathione content in liver of DMBA-Croton oil group was found to be 49.09 ± 1.72 % when compared with control group after 12 weeks study. There was dose dependent increase in Reduced Glutathione content in liver of treatment groups when compared with DMBA/Croton oil group. Significant increase in Reduced Glutathione content was found in 50 mg/kg TFEH ($p<0.01$) up to 70.53 ± 3.14 %, and 100 mg/kg TFEH ($p<0.01$) up to 79.77 ± 2.80 %. There was highest level of increase in reduced glutathione content ($p<0.01$) in 200 mg/kg TFEH group after 12 weeks study.

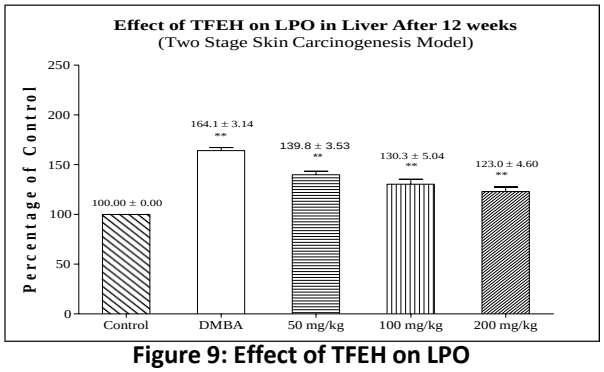

Figure 9: Effect of TFEH on LPO

Effect of TFEH on Lipid Peroxidation level in liver after 12 weeks

Effect of TFEH on lipid peroxidation level in control and experimental animals is shown in Figure 9. Lipid Peroxidation in liver of DMBA-Croton oil group was found to be 164.1 ± 3.14 % when compared with control group after 12 weeks study. There was dose dependent decrease in Lipid peroxidation in liver of treatment groups when compared with DMBA- Croton oil group. Significant decrease in lipid peroxidation was found in 50 mg/kg TFEH ($p<0.01$) up to 139.80 ± 3.53 %, 100 mg/kg TFEH ($p<0.01$) up to 130.30 ± 5.04 % and 200 mg/kg ($p<0.01$) up to 123.00 ± 4.60 %. There was highest level of inhibition of lipid peroxidation in 200 mg/kg TFEH group after 12 weeks study.

DISCUSSION

In recent years, the development of more effective and safer agents has been intensively required for chemoprevention of human cancer, and natural products from plants and their synthetic derivatives have been expected to play an important role in creating new and better chemopreventive agents (Tanaka et al., 2003). Triterpenoids occur widely in nature. They possess anti inflammatory, anti cancer, and antioxidant properties. Triterpenes such as glycyrrhizin, oleanolic acid, ursolic acid, b-boswellic acid, have been reported to possess a variety of biological effects including anti-inflammation, hepatoprotection and immunomodulation.

The mouse skin carcinogenesis model has become very useful in studying the genetic and biological changes involved in tumor promotion. Some of the genetic changes associated with the chemical initiation of benign papillomas and the transition to squamous cell carcinoma have been characterized in this system. The mouse model of multi-step carcinogenesis has taken researchers a long way towards understanding the molecular events that underlie the transition from a normal cell to a transformed cell and neoplasia. This model has been extended to transgenic mouse studies.

Reactive oxygen species (ROS) have been suggested as causative factors in mutagenesis, carcinogenesis and tumor promotion and have been implicated in the etiology and pathophysiology of many human diseases. The relevance of free radicals/ROS in tissue damage and carcinogenesis has been reported (Goyal et al., 2005; Oberly et al., 1979). They induce strand breaks in DNA and oxidative modification of DNA bases leading to mutagenic and carcinogenic effects. They also modulate gene expression by an epigenetic mechanism. Many tumor promoters have been shown to exert their action by overproduction of ROS and many compounds that possess antioxidant activity have been reported to inhibit tumor promotion. (Dhawan et al., 1999).

Inactivation and removal of ROS depends on reactions involving the antioxidative defense system. Chemoprevention aims to directly modulate specific steps in the carcinogenic process, i.e., block mutagenic carcinogens, prevent DNA damage by free radicals, suppress epithelial cell hyperproliferation and/or modulate epithelial cell differentiation and apoptosis (programmed cell death). Antioxidant enzymes may interfere with the initial mediation of apoptosis by ROS as well as later membrane lipid peroxidation (Goyal et al 2005; Salganik et al., 2001).

ROS such as superoxide radical, hydroxyl radical and hydrogen peroxides are frequently generated in the biological systems either by normal metabolic pathways or as a consequence of exposure to physical, chemical and biological agents. ROS attack biomembranes and lead to oxidative destruction of polyunsaturated fatty acids (PUFA) by a chain reaction known as lipid peroxidation. ROS interfere with the structure and function of the cells, making them weak and defenseless. Overproduction of ROS within tissues can damage DNA and contribute to mutagenesis and carcinogenesis. Human body has however an array of sophisticated antioxidant defense mechanism to combat the deleterious effects of ROS-mediated oxidative damage. This defense mechanism includes non-enzymatic antioxidants (Vitamin E, Vitamin C and glutathione) and enzymatic antioxidants (SOD, CAT) (Mc Cord, 2005).

Superoxide is inactivated by SOD, the only enzyme known to use a free radical as a substrate. However, the free radical scavenging activity of SOD is effective only when it is followed up by increase in the activity of CAT, since SOD generates hydrogen peroxide as a metabolite, which is more tissue toxic than oxygen radicals and has to be scavenged by CAT. CAT plays important roles in cellular defense as well as maintenance of cellular membranes from oxidative damage of free radicals by eliminating H_2O_2 (Sunde et al., 1986). Thus, a concomitant increase in CAT activity is essential if a beneficial effect from increase in SOD activity is to be expected.

The anti cancer effect of phytochemicals is based on their ability to quench ROS and thereby protecting critical cellular molecules from oxidative insult. Also, anti proliferative activity and induction of apoptosis in cancer cells are other important anti cancer mechanisms of phytochemicals (Mc Cord J, 2005).

In our study, Swiss Albino Mice weighing between 22-25 g were used. In Group I (Vehicle control) the weight of the animal increased from 23 g to 30.125 g. But in group II (DMBA control) the body weight of the animals reduced drastically showing toxic effects of DMBA. The weight loss was mostly due to swindling appetite, anorexia, declining functional status and cachexia. Our investigation shows that in TFEH treated groups (III, IV and V) the weight of the animal increased dose dependently showing protective effects against the toxicants.

The present investigation exhibited 100% tumor incidence as well as higher tumor burden, tumor volume and total number of tumor in Group-II (DMBA control) animals. This is perhaps due to the free radical oxidative stress resulting usually from deficient natural antioxidant defenses as well as lipid peroxidation. On the other hand, animals of Groups III, IV and V received similar treatment of DMBA and croton oil, when subjected to an oral administration TFEH, a significant reduction in tumor incidence, tumor yield, tumor volume and tumor burden were recorded. The maximum inhibition of papillomas was recorded in group V. The cumulative number of papilloma were also found to be reduced in the TFEH treated groups (III, IV & V) when compared to the Group-II (DMBA control) mice.

Liver is the primary site for biotransformation of xenobiotics (including carcinogens and anticancer drugs) and detoxification process. Relative liver weight was found to be reduced in DMBA group. There was significant reduction in liver weight was observed in DMBA group when compared with control group. Liver weight was significantly increased in TFEH treated group when compared with DMBA group.

In the present study, after repeated topical application of promoter, to the DMBA treated animals skin, caused decreased in the level of antioxidant enzymes such as GSH, CAT and SOD in metabolic organ liver, suggesting involvement of these enzymes for the detoxification and inactivation of ultimate carcinogenic metabolites and reactive oxygen species, responsible for DNA damage. Our results suggest that TFEH increases the activity of mainly phase II enzymes. Our data shows that TFEH increases the activity of antioxidant enzymes such as SOD, CAT and GSH. These effects could have a potential role in the detoxification and elimination of potential carcinogens from the body, leading to the cancer preventive efficacy of TFEH.

The property of TFEH as an antioxidant as well as an antioxidant enzyme system stimulant, lead to the supposition that it has played a role in augmenting the activities of antioxidant enzymes such as CAT, SOD and reduced glutathione GSH concomitant with reduction in lipid peroxidation.

From the investigation we can say that there is a protective role of TFEH against carcinogenic exposure by their action on the physiological detoxification processes.

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