

Chemoprotective Effect of *Tribulus terrestris* on DMBA/Croton Oil Mediated Carcinogenic Response

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Abstract: Chemoprevention is an important strategy to control the process of carcinogenesis. The potential of using medicinal herbs as cancer chemopreventive nutraceuticals and functional food is promising. There has been a growing awareness in recent years that dietary non-nutrient compounds can have important effects as chemopreventive agents and considerable work on the cancer chemopreventive effects of such compounds in animal models has been undertaken. *Tribulus terrestris* is a well known medicinal plant which has been used in Ayurvedic medicine as hepatoprotective, antiviral, antibacterial, analgesic. The present study was carried out to evaluate the anti-tumor activity of a hydro-alcoholic extract of the fruits on the two stage process of skin carcinogenesis. *Tribulus terrestris* fruit extracts against 7, 12 - dimethylbenz (a) anthracene (DMBA) induced papillomagenesis in *Swiss albino* mice was studied. The successively extracted fruit of *T. terrestris* with hydromethanolic and aqueous was analyzed for their toxicity and chemopreventive activity. In chemopreventive activity was evaluated by two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) two times in a week were employed. A significant reduction in tumor incidence, tumor burden and cumulative number of papillomas was observed, along with a significant increase in average latent period in mice treated topically with *Tribulus terrestris* fruit extract as compared to the control group treated with DMBA and croton oil alone. Significant elevation in the levels of catalase and reduced glutathione ($p < 0.05$) was noted in the group treated *Tribulus terrestris* fruit extracts in comparison with the negative control group. Conversely, lipid peroxidation levels were significantly decreased ($P < 0.05$). The results thus suggest that *Tribulus terrestris* extract exhibits significant anti-tumor activity, which supports the traditional medicinal utilization of this plant. Therefore, the present study is immensely important in future drug development programs for the cancer treatment.

Key words: *Tribulus Terrestris* Linn • Papillomagenesis • DMBA • Croton Oil • *In vivo* Antioxidant

INTRODUCTION

Cancer chemoprevention is currently one of the most urgent projects in public health. According to epidemiological surveys, the majority of human cancers are related to two factors; diet and smoking [1-2]. The study highlights the importance of environmental factors such as diet in cancer chemoprevention. Most cancer prevention research is based on the concept of multistage carcinogenesis and showed in (Figure: 1) : initiation → promotion → progression [3,4]. In contrast to both the initiation and progression stages, animal studies indicate that the promotion stage occurs over a long time

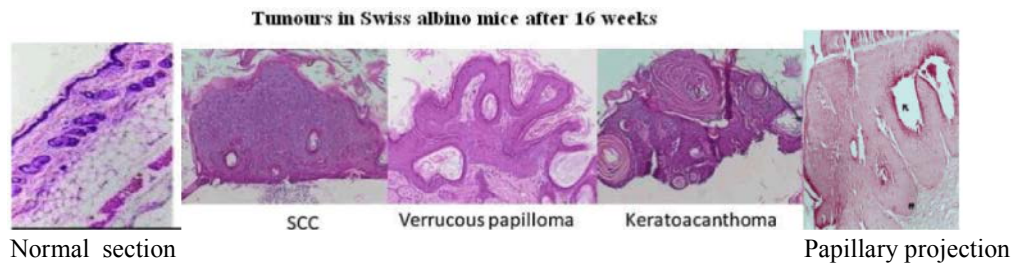
period and may be reversible, at least early on. Therefore, the inhibition of tumor promotion is expected to be an efficient approach to cancer control [5,6]. Cancer chemoprevention is defined as the use of specific natural and synthetic chemical agents to reverse or suppress carcinogenesis and prevent the development of invasive cancers. There has been a growing awareness in recent years that dietary non-nutrient compounds can have important effects as chemopreventive agents and considerable work on the cancer chemopreventive effects of such compounds in animal models has been undertaken. In the course of our research on potential antitumor promoters (cancer chemopreventive agents)

Photograph showing the skin tumour induced by DMBA + Croton oil for 16 weeks



MBA+TTFE+ Croton oil DMBA+Croton oil

Histopathology Study



Photomicrographs (40xH&E) of Skin tumour of Swiss albino mice showing various histopathological changes after termination of Experiments, with or without T.terrestris fruit extracts treatment (PP - Papillary Projection, PL - Papillary Lesion, SSC-squamous cell carcinoma)

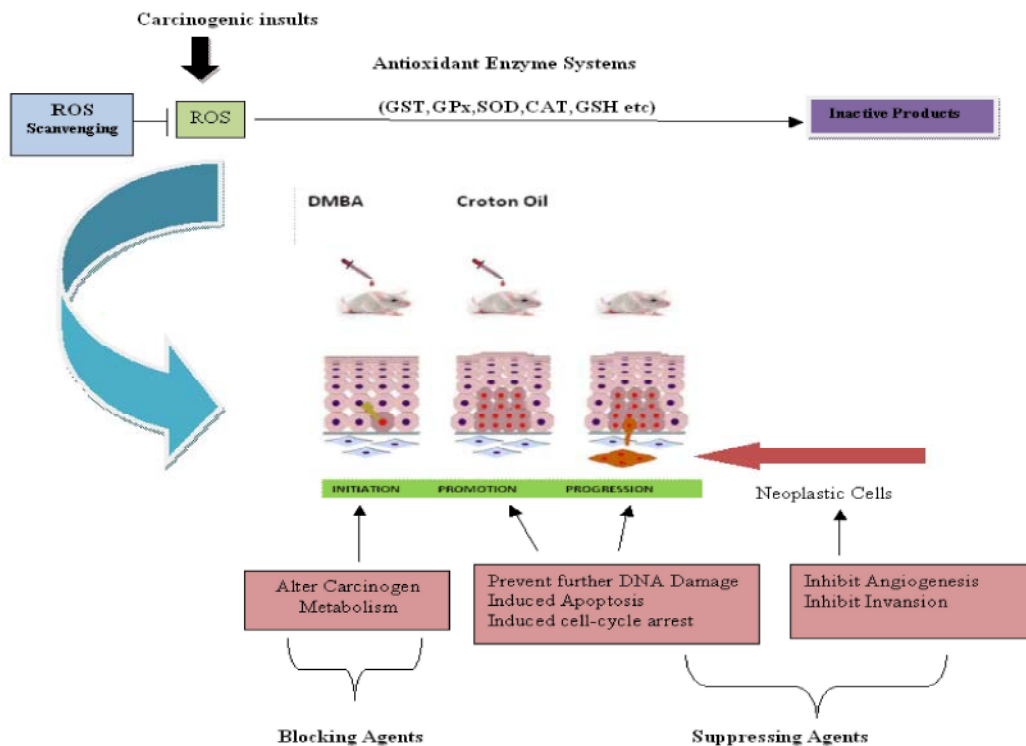


Fig. 1: Mechanism of cancer chemoprevention (Initiation, Promotion and Progression, Neoplasm)

from edible plants and fungi and from crude drugs, we have found that various triterpene alcohols and sterols and their oxygenated derivatives showed inhibitory effects on mouse ear inflammation induced by 12-Otetradecanoylphorbol- 13-acetate (TPA). We have recently reviewed the chemopreventive activities of naturally occurring terpenoids [7-9]. Primary prevention of cancer aims to avoid the development of cancer.

Thus, it is important to inhibit the initiation and/or promotion of carcinogenesis. However, the adult population bears tumor cells that cannot revert to normal cells and thus effective strategies to prevent cancer include avoiding continuous contact between these cells and promoters and/or aggressively inhibiting the tumor promoter effects. Therefore, to prevent cancer, it is essential to find effective compounds (anti-tumor promoters) that delay, inhibit or block tumor promotion, which is a reversible and long-term process. Active research is now being conducted using animal carcinogenesis models on cancer preventing substances contained in plants and vegetables. The chemopreventive activity of natural sources, foods, supplements, crude drugs and Kampo medicines (traditional Japanese herbal prescriptions).

The medicinal herb *Tribulus terrestris* Linn. (Family: Zygophyllaceae) is native to the mediterranean, tropics, subtropics and temperate regions of the world has been subjected to long term clinical trials in "AYURVEDA". *Tribulus terrestris* has long been used as a tonic and aphrodisiac and a diuretic in Unani system of medicine. The diuretic effect was attributed to the presence of potassium salts in high concentration. So many studies have been done on pharmacological activities of *T. terrestris*. The major constituents of these plants are steroidal saponins namely: terrestrosins A, B, C, D and E, desgalactotigonis, F-gitonis, desglucolanatigoneis, gitnin etc. The biological activity exhibited by saponins include: piscicidal, antimicrobial, molluscicidal, haemolytic, antiviral, cytotoxic, antihepatotoxic, spermicidal, insecticidal, antioedematous, antiulcer analgesic, immunomodulatory and sedative effects. It has been reported that *Tribulus terrestris* contains saponins, quercetin, kaempferol and rutin which are known to have antioxidant and anticancer properties [10]. Two new steroid saponins named terrestrinins A(1) and B(2), along with furostanol, gigenin, hecogenin, ruscogenin, gitogenin and tigonin have been isolated [11-12] and anticancer properties of *Tribulus terrestris* have been reported on various cell lines i.e. mouse sarcoma 180 (ASC), Bcap-37 breast cancer

cell line, BEL- 7402 liver cancer cell line, SK- MEL, KB, BT- 549 and SK- OV-3 [13-16]. The purpose of cancer prevention is to cause delay in onset of cancer, progression from precancerous lesion or recurrence after treatment, as an alternative to treatment of cancer cases after clinical symptoms have appeared [17]. In the present investigation, the chemopreventive potential of *Tribulus terrestris* against 7, 12 - dimethyl benz (a) anthracene (DMBA) induced skin carcinogenesis in Swiss albino mice has been evaluated.

MATERIALS AND METHODS

Chemicals: 7, 12 - Dimethylbenz (a) anthracene (DMBA), croton oil, reduced glutathione (GSH), 5,5/ - dithio-bis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA), TCA were obtained from Sigma Chemicals Co. (St. Louis, MO. USA). The other chemicals were obtained from local firms and were of the highest purity. DMBA was dissolved in acetone at a concentration of 104ug/100ul and croton oil was diluted in acetone to give a 1% dilution.

Animals: Random bred male Swiss albino mice (7- 8 weeks old), weighing 24 ± 2 gm were used for the experiments. These animals were housed in polypropylene cages in the animal house at temperatures of $24 \pm 30^\circ\text{C}$. The animals were provided with standard mice feed (from Hindustan Lever Ltd. India) and tap water ad libitum. The study protocol is approved by the Departmental Animal Ethical Committee and confirms to the guidelines set by World Health Organization, Geneva, Switzerland and Indian National Science Academy (INSA), New Delhi (India) No. 43, Ref. No. 672/200.IAEC/2010).

Preparation of *Tribulus Terrestris* Extract: Plant material (*Tribulus terrestris* Linn.) was collected locally and identified and the specimen was placed at Herbarium, Department of Botany, Safia college, Bhopal(MP), India. The voucher number is SPTT/010/2010. Mature fruits were washed, air dried, powdered and extracted separately, with double distilled water (DDW) and by refluxing for 36 hr (12×3) at 40°C . Both of the extracts thus obtained were vacuum evaporated to make it in powder form. These extracts were dissolved in DDW just before topically application.

Experimental Design for Skin Carcinogenesis: The dorsal skin on the back area of the animals was shaven 3 days before the commencement of the experiment and

only those animals in the resting phase of the hair cycle were chosen for the study. For induction of tumors a two stage protocol consisting of initiation with a single topical application of a carcinogen (7, 12 - dimethylbenz (a) anthracene (DMBA) followed by a promoter (croton oil) two times in a week were employed as per our previous modified method of Berenblum [18] reported elsewhere [19]. The animals were randomly allocated into 8 groups comprising six mice each. The treatment was provided topically on shaved area

Treatment Groups:

Group 1 (Untreated Control): No treatment

Group 2 (Vehicle Control): 100 µl acetone 2 times /week up to 16 weeks

Group 3 (DMBA Alone): - 104 µg DMBA was dissolved in 100 µl acetone and single application was given.

Group 4 (Croton Oil Alone): - 1 % Croton oil was applied on skin 2 times a week up to 16 week.

Group 5 (DMBA + Croton Oil): - 104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards 1 % Croton oil was applied on skin 2 times a week up to 16 week.

Group 6 (DMBA + *Tribulus terrestris* fruit Extract. (Ethanollic) + Croton Oil): 104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards the 100 µl dose of *Tribulus terrestris* fruit extract at the dose of 500 mg/kg b. wt. dose was given one hour before the each application of 1 % croton oil 2 times a week up to 16 weeks.

Group 7 (DMBA + *Tribulus terrestris* fruit Extract. (Aqueous) + Croton Oil): 104 µg DMBA was dissolved in 100 µl acetone and single application was given afterwards the 100 µl dose of *Tribulus terrestris* fruit extract at the dose of 500 mg/kg b. wt. dose was given one hour before the each application of 1 % croton oil 2 times a week up to 16 weeks.

Group 8 (*Tribulus terrestris* fruit Extract Alone): Was applied on skin 2 times a week up to 16 week.

Histopathology: At the end of the experiment, all mice were sacrificed by cervical dislocation. The skin from the treated area from each mouse was sampled and kept in

12% buffered formalin for at least 12 h. Skin tissues were trimmed and processed for histological evaluation based on Drury and Wallington [20]. All skin samples were dehydrated in a graded series of ethanol and water (from 70 to 100% ethanol), embedded in paraffin wax and cut into sections of 4 µm thick. Three adjoining sections of a tissue sample were placed on glass slides, stained with Hematoxylin-Eosin and observed under the light microscope. At least 10 fields from each slide and 10 slides from each group were examined [20-22].

Tumor study:

Body weight: Change in mean body weight was measured weekly.

Tumor Incidence: The number of mice carrying at least one tumor expressed as percent incidence.

Cumulative Number of Papillomas: Total number of tumors bearing mice.

Tumor Yield: The average number of papillomas per mouse.

Tumor Burden: The average number of tumors per tumor bearing mouse.

Average Latent Period: The lag between the application of the promoting agent and the appearance of 50% tumors was determined. The average latent period was calculated by multiplying the number of tumors appearing each week by the time in weeks after the application of the promoting agent and dividing the sum by total number of tumors

$$\text{Average latent period} = \sum fx/n$$

Where f is the number of tumors appearing in each weeks, x is the numbers of weeks and n is the total number of tumors.

Biochemical Study: Biochemical alterations were studied in all the groups at the time of termination of the experiment (i.e. at 16th week).

Preparation of Homogenates: Animals were killed by cervical dislocation and the entire liver was then perfused immediately with cold 0.9% NaCl and thereafter carefully removed, trimmed free of extraneous tissue. It was then weighed and blotted dry. For assaying reduced glutathione it was homogenized in ice-cold Tris- KCl

buffer (pH 7.4) to yield a 10% (w/v) homogenate. A 0.5 ml aliquot of this homogenate was used for assaying reduced glutathione. For assaying lipid peroxidation this tissue was homogenized in ice-cold 1.15% KCl to yield a 10% (w/v) homogenate. A 0.8 ml aliquot of this homogenate was used for assaying lipid peroxidation.

Determination of Reduced Glutathione (GSH): Hepatic level of reduced glutathione was determined by the method of Moron [23]. Reduced glutathione was used as a standard to calculate μ mole GSH/100 gm tissue.

Estimation of Lipid Peroxidation (LPO): The lipid peroxidation level was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa [24] and is expressed in terms of malondialdehyde (MDA) formed per mg protein.

Determination of Catalase (CAT) Activity: Catalase was estimated at 240 nm by monitoring the disappearance H_2O_2 as described by Sinha [25]. Catalase enzyme specific activity has been expressed as μ mole of H_2O_2 decomposed/min/mg protein.

Estimation of Protein: Total plasma protein was estimated by the method of Lowry [26]. In this procedure, the final colour is a result of burette reaction of protein with copper ion in alkali and reduction of phosphomolybdic phosphotungstic reagents by the tyrosine and tryptophan present in the proteins. The absorbance was read at 540 nm using a UVVIS Systronic and results were expressed as mg/100 ml.

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

RESULTS

Effect of *Tribulus Terrestris* Fruit Extracts on Dmba Induced Skin Papillomagenesis: The findings of the present study are depicted in Tables 1 and Figure 2. Animals of Group- IV (control) in which a single topical application of DMBA, followed by croton oil produced skin papillomas, which started appearing from the 3th week onwards. The incidence in DMBA/croton oil treated mice (carcinogen control) reached 100% by the termination of the experiment (i.e. 16 weeks).

In the skin papilloma model, significant prevention of tumor incidences was observed in the *Tribulus terrestris* fruit both extract treated experimental groups (66.67% and 50 % in groups V & VI respectively) as compared to carcinogen control (100 %) group. The cumulative number of papillomas was also reduced in the *Tribulus terrestris* fruit both extract treated experimental groups (20 and 12 in groups V and VI respectively) as compared to carcinogen control (35) group. The tumor burden and tumor yield were significantly decreased (5.0 and 4.0 and 3.33 and 0.2) as compared to DMBA treated control (5.33 and 5.33) group.

Average latency period was significantly increased with *Tribulus terrestris* fruit both extract treatment (11.79 ± 2.88 and 12.83 ± 3.50 respectively) in compared to carcinogen control group (8.60 ± 1.78).

Antioxidant Enzymes: Significantly lower reduced glutathione (GSH), catalase, and protein activity was noted in the skin of carcinogen control mice (group IV) as compared with *Tribulus terrestris* fruit both extract treated experimental animals (groups V and VI) at the time of termination of the experiment (ie, 16 weeks). Treatment with *Tribulus terrestris* fruit both extracts resulted enhanced levels of GSH ($P < 0.05$), catalase ($P < 0.05$) and protein ($P < 0.05$) in these groups (Figures: 3, 4, 5).

Table 1: Chemopreventive Action of *Tribulus terrestris* fruit extract on DMBA induced Skin Carcinogenesis in Swiss albino Mice

Groups	Body Weight (gm)		Ist Appearance of papilloma (In Days)	Cumulative No. of total Papillomas	Tumour Incidence %	Tumour Burden	Tumour Yield	Average Latent Period (ALP, Weeks)	Inhibition Multiplication (%)
	Initial	Final							
Vehicle alone	25.83 \pm 1.27	27.31 \pm 1.46	-	-	-	-	-	-	-
DMBA alone	26.09 \pm 1.58	30.43 \pm 1.58	-	-	-	-	-	-	-
Croton oil alone	26.61 \pm 1.23	32.83 \pm 2.34	-	-	-	-	-	-	-
DMBA + Croton oil	27.20 \pm 1.32	32.18 \pm 2	21	35	(6/6) 100%	5.33	5.33	8.60 \pm 1.78	-
DMBA + TTEE + Croton oil	26.16 \pm 1.17	30.09 \pm 2.06	37	20	(4/6) 66.67%	5.00*	3.33*	11.79 \pm 2.88*	42.85*
DMBA + TTAE + Croton oil	26.42 \pm 1.3	31.09 \pm 2.19	52	12	(3/6) 50%	4.00*	2.00*	12.83 \pm 3.5*	65.71*

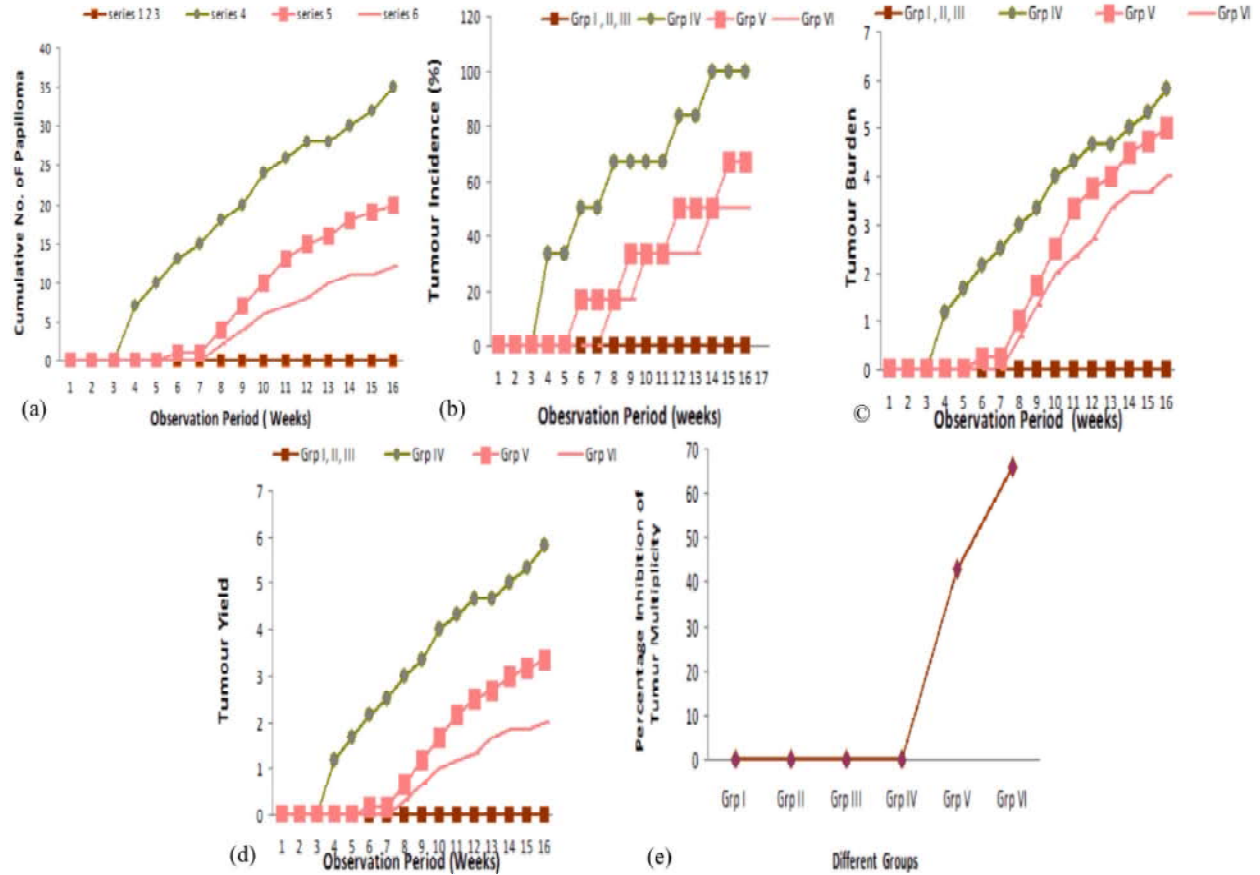


Fig. 2: DMBA-Induced Skin Papillomagenesis with or without Tribulus Terrestris Tumor Data over Time. a) Cumulative number of papillomas; b) Incidence; c) Burden; d) Yield; e) Tumor multiplicity

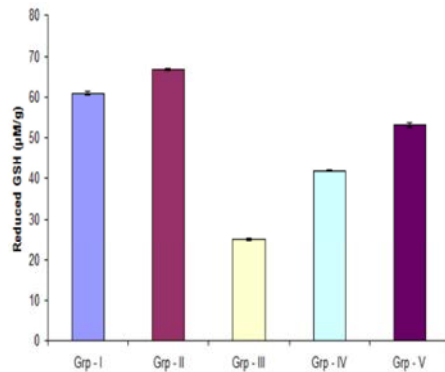


Fig. 3

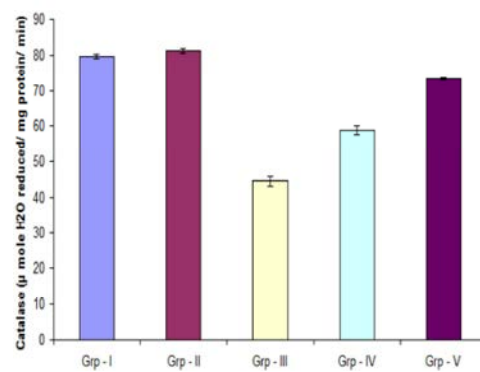


Fig. 4

Fig 3 and 4: Showing the variation in the GSH and Catalase level during DMBA-induced skin carcinogenesis with/without Tribulus terrestris extract treatment

A considerable elevation of LPO level was noted in the skin after DMBA and croton oil treatment, where as administration of *Tribulus terrestris* fruit both ($P < 0.05$)

significantly reduced the level of LPO in all the *Tribulus terrestris* treated experimental groups (V and VI) in comparison with the carcinogen control group (Figure 6).

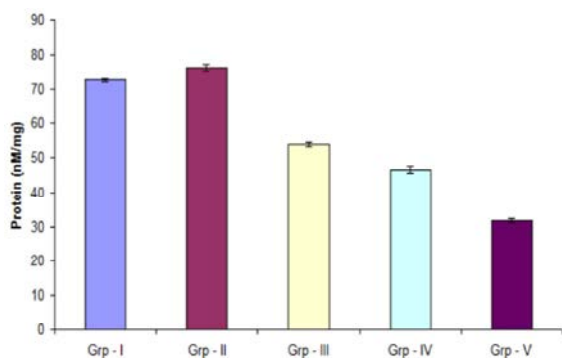


Fig. 5: Showing the variation in the Protein level during DMBA-induced skin carcinogenesis with/without *Tribulus terrestris* extract treatment

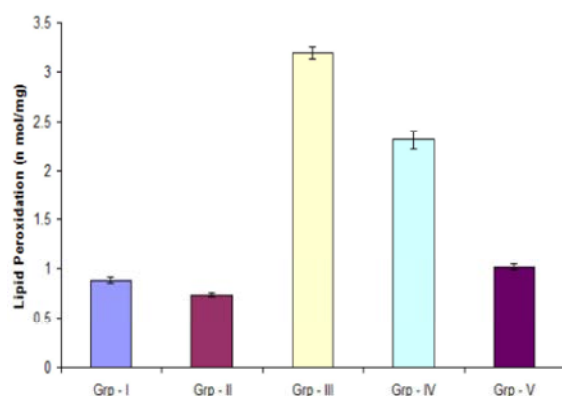


Fig. 6: Showing the variation in the Lipid Peroxidation level during DMBA-induced skin carcinogenesis with/without *Tribulus terrestris* extract treatment

DISCUSSION

The induction of cancer (carcinogenesis) is a multistage process and depends on inherited and acquired susceptibility factors, on exposure to initiation factors, i.e. exogenous and endogenous carcinogens and on promotion and progression factors.

Cancer chemoprevention can be defined as prevention by administration of chemical entities, either as individual drugs or as naturally occurring constituents of the diet. Chemoprevention has earned serious consideration as a mean of controlling cancer incidence, as it is no longer merely a theoretical strategy, but an approach yielding more impressive experimental and clinical results [4].

Tumor incidence, cumulative number of papillomas, tumor yield, tumor burden, tumor weight and tumor size were found to be decreased in all the experimental mice

(groups V & VI) but maximum reduction in all such parameters was evident in *Tribulus terrestris* extract treated mice (group V and VI). This fall may be due to factors such as inhibition of DMBA metabolism to its active form or delay in the promotion phase of tumorigenesis via down regulation in the production of ROS and inhibitory effects on tumor promoter-induced epidermal ODC activity. Because reactive oxygen species have been implicated in premature skin aging, carcinogenesis, DNA damage, activation of signal transduction pathways related to growth differentiation and cell death, it is assumed that antioxidants could act as potential anticarcinogens at multiple stages of skin carcinogenesis [27]. Treatment of *Tribulus* extracts increase reduced glutathione level and decrease malondialdehyde formation than sham treated animals of control group. However, fruit extract of *Tribulus* is more effective than root extract to increase reduced glutathione level. Whereas, root extract of *Tribulus* is more potent than fruit extract to decrease lipid peroxidation level.

Antioxidants such as GSH, cysteine and a tocopherol were shown to prevent the TPA- mediated decrease in the ratios of reduced to oxidized glutathione in mouse epidermal cells [28]. The increased glutathione reductase level plays a significant role in the reduction of oxidized glutathione to reduced glutathione at the expense of NADPH and regulates GSH-GSSG cycle in the cell [29]. The elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle and also detoxifies reactive oxygen species directly and/or neutralizes reactive intermediate species generated from exposure to xenobiotics including chemical carcinogens [30]. GSH has been endowed with an important function in maintaining the reduced state of cellular environment, in addition to its conjugating ability owing to nucleophilic center and its involvement in detoxification of xenobiotics that cause toxicity and carcinogenicity. Such a mechanism would decrease the level of reactive electrophiles available to bind DNA, reducing the likelihood of DNA damage and possible induction of carcinogenic [31]. Glutathione, often regarded as the first line of defense against oxidative stress, is the most important cellular thiol that acts as a substrate for several transferases, peroxidases and other enzymes that prevent the deleterious effects of oxygen free radicals [32]. The multiple physiological and metabolic function of GSH includes thiol transfer reactions that protect cell membranes and proteins. GSH participates in reactions

that destroy hydrogen peroxide, organic peroxides, free radicals and certain foreign compounds. The apoptotic processes in cells are often associated with decreased levels of GSH due to increased efflux of this antioxidant from the cells [33]. Furthermore, the decreased lipid peroxidation which is measured by thiobarbituric acid reactive substances (TBARS) in the liver homogenate of *Tribulus* treated mice, is correlated well with the induction of antioxidant enzymes above basal level. A wide range of plant products are source of antioxidants and act as modifiers of the carcinogenic process, appear to be the right approach for modifying cancer risk in the population [31]. The supplementation or topical application of synthetic agents viz. retinoids, vitamins, inhibitors of ornithine decarboxylase, cyclooxygenase, lipoxygenase and other antioxidant compounds including thiols and minerals have gained much attention on one hand while the use of natural agents like polyphenols, monoterpenes, flavonoids, organosulfides, indoles, etc. have shown promise for their development as chemopreventive agent against skin cancer [34-37].

CONCLUSION

Humans have used plants as foods and natural medicines since ancient times. Crude drugs, typically safer than synthetic drugs, have been used as both spices and supplements. Natural medicines have been used as anti-cancer agents by inhibiting the promotion process and it is important that these are consumed in small quantities for extended periods of time. The study of cancer prevention using plants is generating vast amounts of information regarding their benefits. This paper provides, an outline of studies focusing on plant extracts. Several active components have been isolated and their chemical structures have been and continue to be determined. In addition, structure-activity relationships, elucidation of physiological activities at the molecular level and development of strategies that allow for the production of sufficient supplies of these agents are issues for further investigation. The continued search for natural medicines is necessary for finding additional sources of active components that are suitable for clinical application. For this purpose, we will harness the strength of researchers from various fields with the goal for cancer prevention.

From the present study, it is evident that *Tribulus terrestris*, the Indian medicinal plant, is a source of many anti-carcinogenic agents and antioxidants, which may be

useful for the prevention of chemical induced skin cancer in mice. This work demands further study to evaluate the exact mechanism of chemoprevention offered by *Tribulus terrestris* constituents as well as its possible chemopreventive efficacy against other types of tumors in various models.

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Abbreviations: DMBA: 7, 12 - Dimethylbenz (a) anthracene, GSH: reduced glutathione, DTNB: 5.5/ - dithio-bis-2- nitrobenzoic acid, TBA: thiobarbituric acid, TCA: Trichloroacetic acid

Conflict of Interest Statement: I declare that I have no conflict of interest.

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