

RESEARCH COMMUNICATION

Chemopreventive Potential of *Tribulus terrestris* against 7,12-Dimethylbenz (a) anthracene Induced Skin Papillomagenesis in Mice

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Abstract

In the present investigation, the chemopreventive potential of aqueous extracts of the root and fruit of *Tribulus terrestris* (an Ayurvedic medicinal plant) on 7, 12 - dimethylbenz (a) anthracene (DMBA) induced papillomagenesis in male Swiss albino mice was studied. 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 orally with *Tribulus terrestris* suspension continuously at pre, peri and post-initiation stages of papillomagenesis as compared to the control group treated with DMBA and croton oil alone. Treatment with *Tribulus terrestris* suspension by oral gavage for 7 days resulted in a significant increase in the reduced glutathione content in the liver ($P < 0.001$ for both root and fruit extracts). Conversely, lipid peroxidation levels were significantly decreased ($P < 0.001$).

Key Words: Chemoprevention - DMBA- croton oil - *Tribulus terrestris* - reduced glutathione- lipid peroxidation

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Introduction

Epidemiological studies have indicated that the risk of cancer may be modified by changes in dietary habits (Prochaska, 1997). Humans ingest a large number of naturally occurring antimutagens and anticarcinogens in food, which may inhibit one or more stages of the carcinogenesis (Hocman, 1989). Several studies have indicated that compounds with antioxidant or anti-inflammatory properties as well as certain phytochemicals can inhibit tumor initiation, promotion and progression in experimental animal models (Perchellet and Perchellet, 1989; Chesson and Collins, 1997).

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 trails in "AYURVEDA". The fruits are credited with aphrodisiac, diuretic, cooling, demulcent and tonic properties, which are used to treat kidney stones, painful urination, genito-urinary disorders, diabetes, piles, rheumatism, dropsy, breathing difficulties, heart disease and impotence. The root is considered to have tonic properties, and is a constituent of the Ayurvedic preparation Dasamula (CSIR, 1948-1992; Sivarajan and Balachandran, 1994). It has been reported that *Tribulus terrestris* contains saponins, quercetin, kaempferol and rutin which are known to have antioxidant and anticancer

properties (Ross, 2001). Two new steroid saponins named terrestrinins A(1) and B(2), along with furostanol, gigenin, hecogenin, ruscogenin, gitogenin and tigonin have been isolated (Huang et al., 2003; De Combarien et al., 2003) 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 (Itokowa, 1998; Bedir et al., 2002; Sun et al., 2003; Sun et al., 2004). 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 (Tsuda et al., 2004).

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

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.

Chemicals

7, 12 - Dimethylbenz (a) anthracene (DMBA), croton oil, reduced glutathione (GSH), 5.5/ - dithio-bis-2-nitrobenzoic acid (DTNB) and thiobarbituric acid (TBA) 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 100 g/50 l and croton oil was diluted in acetone to give a 1% dilution.

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, University of Rajasthan, Jaipur. The voucher number is RUBL - 19900. Mature fruits and roots were washed, air dried, powdered and extracted separately, with double distilled water (DDW) by refluxing for 36 hr (12 x 3) at 400C. Both of the extracts thus obtained were vacuum evaporated to make it in powder form. These extracts were redissolved in DDW just before oral administration.

Experimental design

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) three times in a week were employed as per our previous modified method of Berenblum (1941), reported elsewhere (Prashar et al., 1994; Qiblawi and Kumar 1999, Panwar et al., 2005).

Group I. A group of 10 animals was treated with DMBA (100 g/50 l acetone per animal) on day 0 and two weeks later, 0.1 ml croton oil (1% in 100 l acetone) was applied topically on the shaven area. This treatment was continued thrice weekly till the termination of the experiment.

Group II. A group of 10 animals was administered root extract of *Tribulus terrestris* dissolved in double distilled water (DDW) (800 mg/kg body weight) from 7 days before the application of DMBA (100µg/50µl acetone per animal) followed by application of croton oil (thrice weekly) and throughout the experiment.

Group III. A group of 10 animals was treated with fruit extract of *Tribulus terrestris* dissolved in DDW (800 mg/kg body weight) from 7 days before the treatment of DMBA (100µg/50µl acetone per animal) followed by croton oil (weekly thrice) and till the termination of the experiment.

During the 16 weeks of experiments, mice were observed weekly and weighed. The mice were carefully examined once a week for the presence of skin papillomas and the number of papillomas on each affected mice were recorded. Papillomas were defined as a lesion with a diameter greater than 1 mm that persisted for at least two consecutive observations.

Tumor Study

Cumulative number of papillomas. The cumulative

number of papillomas appeared till the termination of the experiment.

Tumor incidence. The number of mice carrying atleast one tumor expressed as percent incidence.

Tumor burden. The average number of tumors per tumor bearing mouse was measured.

Average latent period. The time lag between the application of the promoting agent and the appearance of 50% tumors was determined (Prashar et al., 1994). It 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 the total number of tumors.

$$\text{Average latent period} = \frac{\text{SFX}}{n}$$

Where, F is the number of tumors appearing in each week, X is the number of weeks and n is the total number of tumors.

Biochemical Study

Experiment was designed to study the reduced glutathione content and lipid peroxidation level in liver of Swiss albino mice. Animals were assorted randomly into the following groups :

Group I (n=10) : Animals were fed a normal diet and sham - treated with 100 l distilled water by oral gavage daily, for 7 days; this group of animals served as control.

Group II (n = 10) : Animals were fed a normal diet and treated with 100 l of root extract of *Tribulus terrestris* per animal per day by oral gavage daily, for 7 days.

Group III (n = 10) : Animals were fed a normal diet and treated with 100 l of fruit extract of *Tribulus terrestris* per animal per day by oral gavage daily, for 7 days.

Preparation of Homogenate for Biochemical Studies

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.

Reduced glutathione

Reduced glutathione was estimated as total nonprotein sulphhydryl group by the method as described by Moron et al. (1979). Homogenates were immediately precipitated with 0.1 ml of 25% trichloroacetic acid and the precipitate was removed after centrifugation. Free SH groups were assayed in a total 3 ml volume by adding 2 ml of 0.6 mM DTNB prepared in 0.2 M Sodium phosphate buffer (pH8.0), to 0.1 ml of the supernatant and absorbance was read at 412 nm using a UV-VIS Systronics spectrophotometer. GSH was used as a standard to calculate n mole of - SH content / gm tissue.

Lipid peroxidation

Lipid peroxidation in the liver was estimated

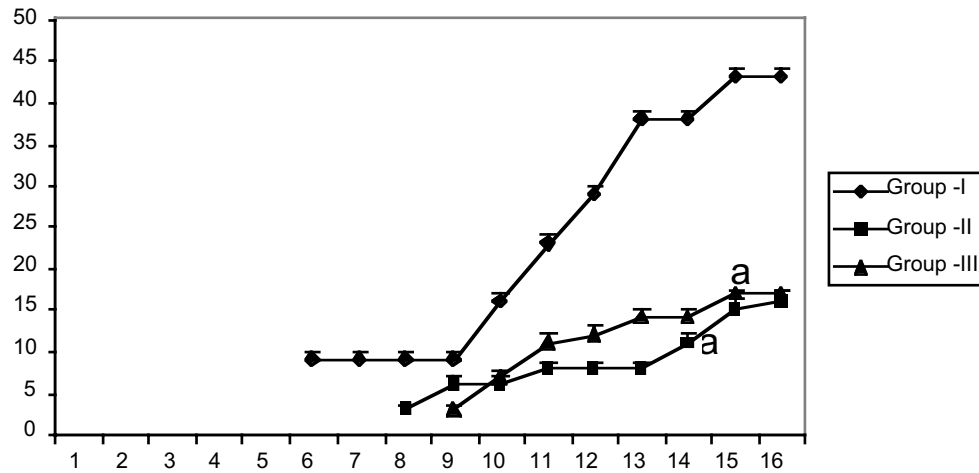


Figure 1. Cumulative number of papillomas recorded after initiation by DMBA followed 2 weeks later by croton oil treatment (thrice a week) for 14 weeks with and without *Tribulus terrestris* extract treatment

spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkawa et al(1979) and is expressed in terms of malondialdehyde (MDA) formed per mg tissue. In brief, 0.8 ml of homogenates was mixed with 0.2 ml of 8.1% Sodium dodesylsulphate (SDS) to which 1.5 ml of 20% acetic acid was added. Then 1.5 ml of 0.6% TBA was added and placed in a water bath for 1 hr at 80°C, cooled in ice and mixed with 5 ml mixture of n-butanol and pyridine (15:1). Then centrifuged at room temperature for 10 min at 3,000 rpm. The absorbance of the clear supernatant was measured against blank of distilled water at 532 nm.

Statistical Analysis

Statistical significance of differences between the groups was determined by Student's t-test.

risk for colorectal adenoma was performed. The author used the electronic search engine PubMed (www.pubmed.com) and Scopus (www.scopus.com) in searching for the literatures. The available reports were collected and extracted for the data about the pattern of VDR BsmI polymorphism. Those primary data were used for further metanalysis study.

Concerning the metanalysis study, the overall prevalence rate of each VDR BsmI polymorphism among overall cases

and controls was calculated. Also, the association between prevalence rate and nationality of the populations was assessed using Chi square test. The SPSS 11.0 for Windows was used for statistical analysis in this study.

Results

Findings of present investigations are depicted in Figures 1-3. In the control group (Group I), in which a single topical application of DMBA was followed, 2 weeks later, by repeated application (three times in a week) of croton oil, skin papillomas appeared in all the animals (100% tumor incidence). The cumulative number of papillomas as induced during the observation period of 16 weeks was 43.00 ± 0.94 (Figure 1). The tumor burden (mean number of tumors per effective mice) was recorded as 4.75 ± 0.10 . (Fig. 2B) The average latent period was observed as 10.81 ± 0.10 weeks in the control group (Figure 2A).

In the treatment groups (Groups II and III, where root and fruit extract of *Tribulus terrestris* was given orally), the animals showed a significant decrease in the cumulative number of papillomas, tumor burden and tumor incidence compared with control group (Group I). In animals of the treatment group, given a continuous treatment at pre, peri

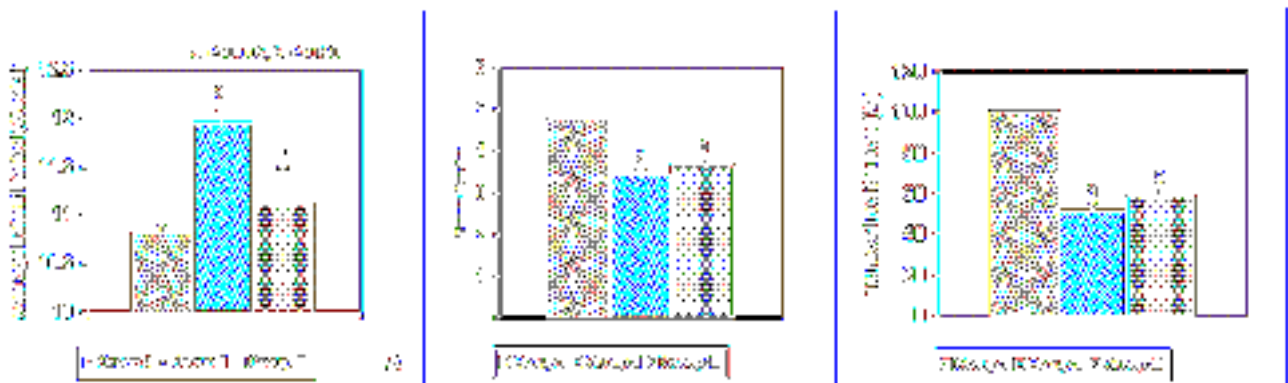


Figure 2. Average latent period, Tumor Burden and Tumor incidence Recorded after Initiation by DMBA Followed 2 Weeks Later by Croton Oil treatment for 14 weeks with and without *Tribulus terrestris* Extract

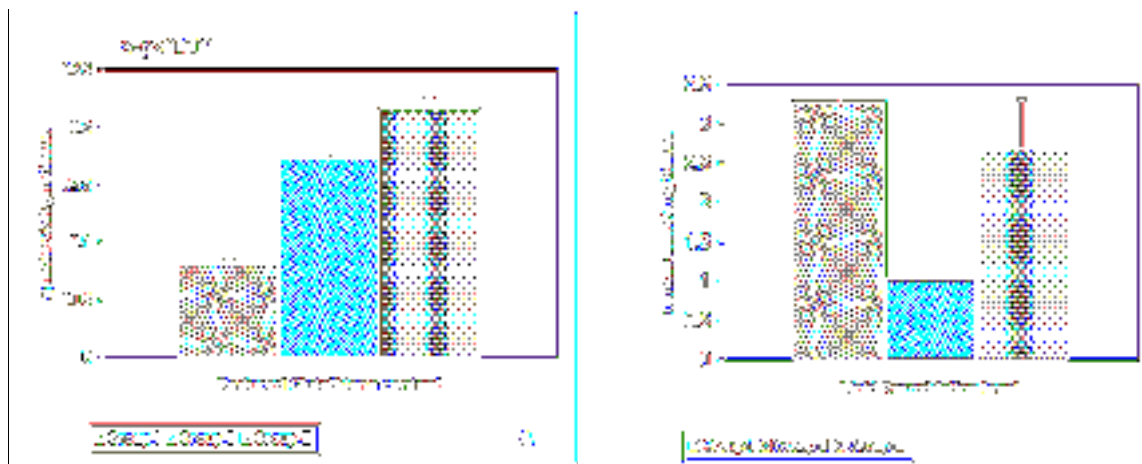


Figure 3. Modulatory Influence of Root and Fruit Extracts of Tribulus Terrestris on Reduced Glutathione (GSH) and Lipid Peroxidation (LPO) in Livers of Mice

and post-initiation phases, a significant reduction in the tumor incidence ($51.84 \pm 3.70\%$ and $58.33 \pm 4.16\%$ in Group II and III, respectively) compared with 100% tumor incidence in the control group was observed (Fig.2C). The cumulative number of papillomas during the observation period was 16 ± 1.15 and 17 ± 0.57 in Group II and III respectively (Fig.1). The tumor burden was recorded as 3.43 ± 0.12 and 3.66 ± 0.17 in these groups, respectively (Figure 2B). The average latent period in the treatment group was respectively, 11.95 ± 0.14 and 11.13 ± 0.37 was significantly higher than the control group (Figure 2A).

Both the investigated extracts of *Tribulus terrestris* significantly increased reduced glutathione level in the liver of mice over that of sham treated control animals. With root extract of *Tribulus terrestris* treatment, the basal reduced glutathione level was increased 2.15 fold ($p < 0.001$), whereas the fruit extract induced 2.67 ($p < 0.001$) fold increase in reduced glutathione concentration (Figure 3A). The formation of malondialdehyde measured as index of lipid peroxidation revealed a significant decrease. It was decreased 0.30 fold ($p < 0.001$) with root extract treatment, whereas the fruit extract induced 0.82 fold ($p < 0.001$) decrease in lipid peroxidation level (Figure 3B).

Discussion

The present study demonstrated that the oral administration of the *Tribulus terrestris* extract at pre, peri and post-initiation phases showed a significant reduction in tumor incidence, tumor burden and cumulative number of papillomas and a significant increase in the average latent period. However, it was observed that root extract of *Tribulus terrestris* has more chemopreventive potential than fruit extract at same concentration (800 mg/kg body weight), in skin papillomagenesis in Swiss albino mice. In root extract treated group, more reduction in tumor incidence, tumor burden and cumulative number of papillomas was observed than fruit extract treated group. The average latent period was significantly increased by root extract treated group.

Epidemiological evidence suggests that a diet high in fruits and vegetables is associated with a decrease incidence of cancer, cardio vascular disease and may be of other degenerative or age related diseases (Pool-Zobel et al., 1997). Chemical carcinogenesis in skin as well as in other organs is a multistep process comprising of initiation, promotion and progression, that requires both initiating and promoting substances for development of cancer (Mukhtar et al., 1989). Evidences have accumulated to suggest that reactive oxygen species (ROS) play an important role in tumor initiation by enhancing or facilitating the metabolic activation and/or initiating effects of carcinogens (Athar, 2002). Reactive oxygen and/or nitrogen oxide species-induced stress (RONOSS) and its downstream events are clearly important for carcinogenesis. RONOSS can be induced by exposure of animals and humans to a variety of carcinogenic xenobiotics and microorganisms (Kohen and Nyska, 2002; Hussain et al., 2003) and the various cellular alterations induced by RONOSS play crucial role in carcinogenesis (Olinski et al., 2002; Owuor and Kong, 2002; Ohshima, 2003). The antioxidants are expected to inhibit RONOSS, because of alteration in relevant enzyme profiles and quenching (Nakae et al., 2002; Ohshima, 2003).

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 (Gupta and Mukhtar, 2002). 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 (Perchellet and Perchellet, 1989). 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 (Gonzales, et al., 1984). 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 (Ketterer, 1998). 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 process (Seo et al., 2000).

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 (Thiele et al., 2001). The multiple physiological and metabolic function of GSH include 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 (Rana et al., 2002). 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 (Krishnaswamy and Raghuramulu, 1998). 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 (Safe et al., 1999 ; Bickers and Athar, 2000; Lamson and Brignall, 2001). The present investigation demonstrated the chemopreventive action in Swiss albino mice against 7,12-dimethylbenz (a) anthracene and croton oil may be due to the immunomodulatory and pharmacological properties of *Tribulus terrestris*.

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