Chemomodulatory Effect of *Trigonella foenum graecum* (L.) Seed Extract on Two Stage Mouse Skin Carcinogenesis

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ABSTRACT

Cancer is not a single disease but a group of complex genetic diseases of aged cells. *Chemoprevention* of cancer is the attempt to use natural and synthetic compounds to intervene in the early stages of cancer, before invasive disease begins. Consuming a diet rich in plant foods can provide a milieu of phytochemicals and non-nutritive plant substances that possess health-protective effects. Some phytochemicals derived in spices and herbs as well as other plants possess substantial cancer preventive properties. Thus the cancer chemopreventive potential of naturally occurring phytochemicals is of great interest because of their preventive role and as they are not perceived as “medicine”. During the course of present study *Trigonella foenum graecum* (L.) seed- TFGS (commonly called fenugreek) extract was given at pre-initiational, post-initiational, promotional and throughout the experiment along with 7,12-dimethylbenz [a] anthracene DMBA and 12-O-tetradecanoylphorbol-13-acetate TPA treatment in Swiss albino mice. A significant reduction of papillomas in DMBA + TPA + TFGS (400 mg/kg. body wt.) treated group was found to be effective in decreasing the rate of tumor incidence in comparison to control. Furthermore, cumulative number of papillomas, tumor yield and tumor burden were also found to be reduced. The TFGS extract treatment before DMBA and TPA application (i.e. Pre initiation) were more effective than that of treatment during, and /or after DMBA treatment, however TFGS extract treatment was most effective when treated throughout all the stages of tumorigenesis. The TFGS treatment also showed a modulatory influence on mouse hepatic antioxidant defense system (GSH and LPO level).

Key words: Cancer, chemoprevention, DMBA, phytochemicals, TPA, *Trigonella foenum graecum*

INTRODUCTION

Research has demonstrated that cancer is a largely avoidable disease.[¹] It is estimated that more than two-thirds of cancer may be prevented through lifestyle modification.[²-⁴] *Chemoprevention* is the attempt to use natural and synthetic compounds to intervene in the early stages of cancer, before invasive disease begins.[⁵] Natural dietary agents including fruits, vegetables, and spices have drawn a great deal of attention from both the scientific community and the general public due to their various health promoting effects including suppression of cancers, many of them have been used as traditional medicines for thousands of years.[⁶,⁷] Some phytochemicals derived in spices and herbs as well as other plants possess substantial cancer preventive properties.[⁸-¹¹] Chemopreventive agents can be grouped into two major classes: blocking agents and suppressing agents. Blocking agents prevent carcinogenic compounds from reaching or reacting with critical target areas of the body, while suppressing agents inhibit the growth of existing tumors.

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sites by preventing the metabolic activation of carcinogens or tumor promoters by enhancing detoxification systems and by trapping reactive carcinogens. Suppressing agents prevent the evolution of the neoplastic process in cells that would otherwise become malignant.

There have been two major diet-related prevention strategies that have evolved to combat cancer, i.e., cancer chemoprevention and dietary cancer prevention, with appreciable overlap existing between them. Generally, cancer chemoprevention is recognized as the pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer. Dietary epidemiologic studies have provided initial leads for the identification of numerous naturally occurring chemopreventive agents and laboratory studies have identified many potential agents that suppress carcinogenesis in animal models. So, dietary prevention is considered as the change in food consumption patterns necessary to decrease cancer development. A diet rich in plant foods may provide protection against several chronic diseases including cancers. Differences among individuals, including inherited genetic susceptibility, could also contribute to inconsistent epidemiologic associations between dietary factors and specific cancers.

Trigonella foenum graecum (L.), commonly called fenugreek, is an aromatic leguminous plant native to many Asian, Middle Eastern, and European countries. Fenugreek belongs to the subfamily Papilionaceae of the family Leguminosae (bean family, Fabaceae). Research has shown that the seeds can inhibit cancer of the liver, lower blood cholesterol levels and also have an antidiabetic effect. The seed can be cooked or sprouted or even eaten raw. Fenugreek seeds are a good source of many essential elements such as iron, phosphorus and sulphur. These seeds and leaves have been used extensively in various medicinal preparations.

In the present study attempt has been made to study the chemomodulatory potential of Trigonella foenum graecum (L.) against 7, 12-dimethylbenz (a) anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced mouse skin papillogenesis. The intermediate biomarkers for the study are changes in lipid peroxidation (LPO), the status of the antioxidants such as reduced glutathione (GSH) in the liver.

**MATERIALS AND METHODS**

**Animals**

Random-bred male Swiss albino mice (8–9 weeks old) were obtained from the animal facility (JNU, New Delhi). The animals were maintained in the animal house at temperature of 24 ± 3°C and a light: dark exposure period of 12 hours: 12 hours. The animals were housed in polypropylene cages and fed with standard mice feed (Hindustan Lever Ltd., India). Tap water was provided *ad libitum.*

**Chemicals**

5,5-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH), thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), 1,1,3,3-tetramethoxy propane (TMP), n-butanol, pyridine, meta phosphoric acid (MPA) were obtained from Qualigens, Himedia Laboratories Ltd., India and Sigma Chemicals Co., USA. 7,12-dimethylbenz (a) anthracene (DMBA), 12-O-tetradecanoylphorbol-13-acetate (TPA), colchicine, fetal calf serum (FCS), methanol, acetic acid, saline, may-grunwald and giemsa stain powder were procured from Sigma Chemical Co., USA.

**Preparation of Trigonella foenum graecum (L.) seed extracts**

Trigonella foenum graecum (L.) seeds (TFGS) were collected locally and identified at Herbarium, Department of Botany, University of Rajasthan, Jaipur, India (RUBL 20658). Seeds were air-dried in shade without direct exposure to sun rays and powdered. The extract of seeds of Trigonella foenum graecum (L.) (TFGS) was prepared using Soxhlet apparatus with methanol for 36 hours at 70°C.

**Experimental design**

**Seed extract tolerance study**

Mice for tolerance study were divided into two groups, group I served as control and group II as treatment group, which was further subdivided into several subgroups with nine animals in each group. Animals of group II were given 25, 50, 100, 200, 400, 800 mg/kg body weight/day of TFGS in double distilled water by oral gavages for seven consecutive days. All these animals were observed regularly till seven days and no toxic effect were observed in terms of sickness, mortality, morbidity and behaviour in animals treated with different doses (25, 50, 100, 200, 400, 800 mg/kg. b.wt. / day) of TFGS extract. This suggests that extracts of TFGS can be tolerated by mice up to 800 mg/kg. b. wt./day.

Experiments were designed to test the modulatory influence of TFGS seed extracts on mouse hepatic lipid peroxidation (LPO) level and reduced glutathione (GSH) content, as follows.

Depending upon the increase of GSH level and decrease in LPO content 400mg/kg. b.wt. of TFGS was selected [Table 1].
Mouse skin papilloma model

Animals were assorted into control and experimental groups. The animals were marked and body weight was taken. The hair on the dorsal region of the body (back) was removed, three days before the commencement of the experiment and only those animals in the resting phase of the hair cycle were selected for the experiments. Mice were shaved before each treatment to allow a better distribution of the chemical.

Two stage skin carcinogenesis models were used as reported earlier in our lab. For the induction of tumors/papillomas, the two-stage protocol consisting of initiation with a single topical application of the carcinogen DMBA, followed by three times a week treatment with a promoter TPA, was standardized.

Experiments were designed to see the effect of TFGS extract on DMBA / TPA induced skin papillomagenesis. All the animals were divided into two groups and each group was given separate treatments, as follows [Figure 1].

Parameters studied for skin tumor model system

General parameters
- Tumor Rate / Tumor Incidence
  The number of mice carrying at least one tumor, expressed as percent incidence.
- Tumor yield
  Total number of tumors per group and the mean number of tumors per mouse.
- Diameter of each tumor
- Weight of tumors of each animal at the termination of the experiment.
- Tumor burden
  The average number of tumors per tumor bearing mouse.
- Average latent period

It is the time lag between the application of the promoting agent and the appearance of 50% of tumors. The average latent period was computed by multiplying the number of tumors appearing each week by the time in weeks after the

| Group | Treatment | Treatment schedule (days) |
|-------|-----------|---------------------------|
| I (Control) | Double Distl. Water (DDW) | 1 2 3 4 5 6 7 Sacrifice |
| II i) | TFGS extract (25 mg/kg b.wt./day) | TFGS Extract |
| II ii) | TFGS extract (50 mg/kg b.wt./day) | TFGS Extract |
| II iii) | TFGS extract (100 mg/kg b.wt./day) | TFGS Extract |
| II iv) | TFGS extract (200 mg/kg b.wt./day) | TFGS Extract |
| II v) | TFGS extract (400 mg/kg b.wt./day) | TFGS Extract |
| II vi) | TFGS extract (800 mg/kg b.wt./day) | TFGS Extract |

Figure 1: Dose application pattern in control and treatment group
application of the promoting agent and dividing the sum by the total number of tumors.

\[
\text{Average latent period} = \frac{\sum FX}{n}
\]

Where,

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

**Biochemical study**

- **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 (GSH) Assay**
  Reduced glutathione was estimated as total nonprotein sulphydryl group by the method as described by Moron et al.[27]. Homogenates were precipitated immediately 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 and 0.9 ml prepared 0.2 M Sodium phosphate buffer (pH 8.0), 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 μmole of - SH content / gm tissue.

- **Lipid Peroxidation (LPO) Assay**
  Lipid peroxidation in the liver was estimated spectrophotometrically by thiobarbituric acid reactive substances (TBARS) method, as described by Ohkhawa et al.[28] and is expressed in terms of malondialdehyde (MDA) formed per mg of tissue. In brief, 0.8 ml of homogenate 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). It is 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 difference between control and experimental groups was determined by student’s t-test and chi-square test.

**RESULTS**

Findings of present investigations are depicted in Tables 2-6. In the control group (Group I), in which a single topical application of DMBA was followed, two weeks later, by repeated application (three times in a week) of TPA, skin papillomas appeared in all the animals (100% tumor incidence). A significant reduction in tumor incidence (60 ± 1.13, 59.92 ± 1.06, 60.66 ± 11.06, 56.65 ± 2.41) at pre, post, promotional and throughout stages of treatment respectively were observed in animals of TFGS extracts treated groups as compared to control group where it is 100% [Table 4]. The cumulative number of papillomas during observation period of 16 weeks was significantly reduced in TFGS extract treated group (i.e. 17,20,19,15) at pre, post, promotional and throughout stages of treatment respectively as compared to control group where it is 48 [Table 3]. Whereas average latent period was significantly increased from 9.87 ± 0.15 weeks in control group to 11.58 ± 0.19, 10.44 ± 0.25, 11.11 ± 0.19, 11.96 ± 0.65 weeks in the treated groups [Table 4]. The average weight of tumors (in mg) was also reduced in pre, post, promotional and throughout stages of treatment with TFGS extracts [Table 5].

| Table 2: Modulatory influence of *Trigonella foenum graecum* (L.) (TFGS) extract on mouse hepatic antioxidant status |
|---|
| **Group** | **Treatment and dose** | **Duration (days)** | **GSH (μmole/gm tissue)** | **LPO (nmole MDA/mg of tissue)** |
| I | D.D.W. by oral gavage | 7 | 38.82 ± 1.21 | 9.36 ± 0.88 |
| II (i) | 25 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 39.72 ± 0.62 (ns) | 9.30 ± 0.07 (ns) |
| II (ii) | 50 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 39.76 ± 0.88 (ns) | 8.22 ± 0.95 (ns) |
| II (iii) | 100 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 46.62 ± 0.95*** | 7.22 ± 0.78** |
| II (iv) | 200 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 48.62 ± 0.95*** | 6.32 ± 0.08** |
| II (v) | 400 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 48.66 ± 1.00*** | 5.00 ± 1.00*** |
| II (vi) | 800 mg/kg b.w./day of TFGS extract by oral gavage | 7 | 45.34 ± 0.76*** | 6.39 ± 1.00* |

Statistical comparisons: Group I vs. Group II (i), II (ii), II (iii), II (iv), II (v) and II (vi) * = P < 0.05, ** = P < 0.01, *** = P < 0.001, ns = not significant
Table 3: Cumulative number of papillomas recorded after initiation by DMBA followed two weeks later by TPA treatment (three times a week) for 14 weeks with / without TFGS treatment

| Group | Treatment and dose | Weeks | Cumulative number of papillomas |
|-------|-------------------|-------|--------------------------------|
| I     | DMBA (100 µg/ 50 µl acetone) + TPA (2 µg in 200 µl of acetone) |       | 10 ± 0.76                      |
|       |                   | 2     | 10 ± 0.76                      |
|       |                   | 3     | 16 ± 0.94                      |
|       |                   | 4     | 23 ± 0.79                      |
|       |                   | 5     | 25 ± 1.22                      |
|       |                   | 6     | 28 ± 0.94                      |
|       |                   | 7     | 38 ± 0.94                      |
|       |                   | 8     | 39 ± 0.95                      |
|       |                   | 9     | 39 ± 0.95                      |
|       |                   | 10    | 48 ± 0.44                      |
|       |                   | 11    | 48 ± 0.44                      |
|       |                   | 12    | 48 ± 0.44                      |
|       |                   | 13    | 48 ± 0.44                      |
|       |                   | 14    | 48 ± 0.44                      |
|       |                   | 15    | 48 ± 0.44                      |
|       |                   | 16    | 48 ± 0.44                      |
| II    | DMBA (100 µg/ 50 µl acetone) + TPA (2 µg in 200 µl of acetone) + TFGS extract at preinitiation stage |       | 4 ± 1.26                      |
|       |                   | 2     | 8 ± 0.54                       |
|       |                   | 3     | 10 ± 0.74                      |
|       |                   | 4     | 10 ± 0.98                      |
|       |                   | 5     | 12 ± 0.84                      |
|       |                   | 6     | 15 ± 0.88                      |
|       |                   | 7     | 15 ± 0.98                      |
|       |                   | 8     | 20 ± 0.98                      |
|       |                   | 9     | 20 ± 0.76                      |
| II    | DMBA (100 µg/ 50 µl acetone) + TPA (2 µg in 200 µl of acetone) + TFGS extract at postinitiation stage |       | 9 ± 1.24                       |
|       |                   | 2     | 14 ± 1.4                       |
|       |                   | 3     | 12 ± 0.94                      |
|       |                   | 4     | 15 ± 1.44                      |
|       |                   | 5     | 18 ± 0.98                      |
|       |                   | 6     | 20 ± 0.98                      |
|       |                   | 7     | 20 ± 0.76                      |
| II    | DMBA (100 µg/ 50 µl acetone) + TPA (2 µg in 200 µl of acetone) + TFGS extract at promotion stage |       | 6 ± 1.41                       |
|       |                   | 2     | 14 ± 1.4                       |
|       |                   | 3     | 12 ± 1.46                      |
|       |                   | 4     | 15 ± 1.44                      |
|       |                   | 5     | 18 ± 1.03                      |
|       |                   | 6     | 19 ± 1.03***                   |
| II    | DMBA (100 µg/ 50 µl acetone) + TPA (2 µg in 200 µl of acetone) + TFGS extract at throughout stage |       | 3 ± 0.54                       |
|       |                   | 2     | 3 ± 0.54                       |
|       |                   | 3     | 10 ± 0.94                      |
|       |                   | 4     | 11 ± 0.94                      |
|       |                   | 5     | 11 ± 0.47                      |
|       |                   | 6     | 15 ± 0.98                      |
|       |                   | 7     | 15 ± 0.74***                   |

Statistical comparisons: Group I vs. Group II (a), II (b), II (c) and II (d) * = P < 0.05, ** = P < 0.01, *** = P < 0.001, ns = not significant

DISCUSSION

The present investigation demonstrates the chemopreventive action of *Trigonella foenum graecum* (L.) seed extract (TFGS) which is more effective when given from seven days before the DMBA treatment till the end of the experiment (the level of reduced glutathione (GSH) in this group is 35.29 ± 2.09 µmole/gm and that of LPO is 18.52 ± 1.49 nmole/mg of tissue) followed by the animals treated with TFGS in the preinitiation stage. The TFGS extract treatment before DMBA and TPA application (i.e. preinitiation) were more effective than that of treatment during, and/or after DMBA treatment, however TFGS extract treatment was most effective when treated throughout all the stages of tumorigenesis. TFGS administered orally at a dose of 400 mg/kg b.wt. showed a reduction in tumor rate/tumor incidence, tumor yield, cumulative number of papillomas, average weight of tumors remarkably compared to control, with a maximum reduction in the throughout treatment group. All these observation are reflection of chemopreventive activity of the methanolic extract of TFGS on DMBA induced skin papillomagenesis of Swiss albino mice.

The TFGS extract treatment before DMBA and TPA application (i.e. preinitiation) were more effective than that of treatment during, and/or after DMBA treatment. TFGS extract treatment was more effective when treated throughout the entire treatment group. However, TFGS extract treatment before DMBA and TPA application (i.e. preinitiation) were more effective than that of treatment during, and/or after DMBA treatment. This leads to the supposition that the inhibition of tumorgenesis by the seed extract might have been executed either by preventing the formation of active carcinogens from their precursors or by augmenting detoxification process, preventing promotional events in the mouse skin.
The chemopreventive activity of the methanolic extract of TFGS may be due to the rich chemical constituents (such as, saponins, flavonoids, alkaloids, galactomannans) that are present in the seed working synergistically at various stages of angiogenesis.

Diosgenin [(25R)-5-spirosten-3β-ol], a steroid sapogenin constituent of fenugreek seeds suppresses proliferation, osteoclastogenesis and inhibits invasion through inhibition of necrosis factor NF-κB-regulated gene expression and Tumor Necrotic Factor (TNF)–induced activation of AKT.[29,30]
Flavonoids, a group of about 4000 naturally occurring polyphenolic compounds were shown to inhibit various TPA3-induced phenomena, such as protein kinase C, and protein phosphorylation, all of which are believed to represent nonspecific markers of tumor promotion.

Seeds of fenugreek contain the alkaloid, trigonelline (0.38%, methyl betaine of nicotinic acid). It yields nicotinic acid on heating with hydrochloric acid at 260–270°C. Trigonelline has shown potential for use in cancer therapy.

Antioxidant potential of plants is known to be closely linked with their cancer chemoprevention properties. Many types of chemopreventors against cancer evoke large inductions of phase II enzymes of xenobiotic metabolism and increase glutathione levels in animal tissues.

In the present study the significant increase in GSH level and decrease in LPO in the group with TFGS extract treatment before DMBA and TPA application (preinitiation) satisfies the same.

Glutathione, often regarded as the first line of defence against oxidative stress, is the most important cellular thiol that acts as a substrate for several transferases, peroxidases and other enzymes that prevent the detrimental effects of oxygen free radicals.

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. 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.

Lipid peroxidation products modify the physical characteristics of biological membranes. Incorporation of LOOH, changes the physical structure of the membrane by decreasing the fluidity and increasing the permeability. Furthermore, the decreased lipid peroxidation which is measured by thiobarbituric acid reactive substances (TBARS) in the liver homogenate of TFGS 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.

The present investigation demonstrated the chemopreventive action in Swiss albino mice against DMBA and TPA, which may be due to the immunomodulatory, antiinflammatory, pharmacological properties of Trigonella foenum graecum (L.) and the mechanism underlying the antiinflammatory action of its seed may be due to the presence of steroidal compounds including two steroidal saponins which, on hydrolysis, give two steroidal sapogenins (diogenin and gitogenin), flavonoids, galactomannnan.

Hence, fenugreek is suggested as a promising protective medicinal herb for complementary therapy in cancer patients under chemotherapeutic interventions.

So it is well accepted that to reduce the occurrence of cancer, one promising approach is its prevention, especially by chemical intervention through minor nutritional dietary constituents.

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