Chemomodulatory Potential of Flaxseed Oil Against DMBA/Croton Oil–Induced Skin Carcinogenesis in Mice

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Abstract
The present study was conducted to evaluate the potential of flaxseed oil to prevent chemically induced skin cancer in mice. Cancer was induced on 2-stage skin carcinogenesis model by single topical application of 7,12 dimethylbenz [a] anthracene (DMBA), as, initiator, and two weeks later it was promoted by croton oil treatment thrice a week on the dorsal surface of mice for 16 weeks. Flaxseed oil (FSO; 100µL/animal/d) was orally administered 1 week before and 1 week after DMBA application (Peri-initiation stage). The animals of the FSO-administered group showed a significant reduction in tumor incidence (76.67%), cumulative number of tumors (37), tumor yield (3.7), and tumor burden (4.81) when compared with the carcinogen-treated control animals. Biochemical parameters in skin and liver tissue such as LPO and phase I enzymes were significantly (P < .01) reduced in the FSO-treated experimental group, whereas the phase II enzymes (GST, DT-diaphorase) and antioxidant parameters (GSH, GPx, SOD, catalase, and vitamin C) exhibited a significant (P < .01) elevation when compared with the animals of the carcinogen-treated control group. Histopathological alterations in the carcinogen-treated control animals were also observed in the form of epidermal hyperplasia, keratinized pearl formation, and acanthosis in skin and tumors, whereas these were found to be reduced after FSO administration. The results of the present study demonstrate that the oral administration of FSO has the potential to modulate the levels of LPO, antioxidants, and detoxification enzymes in the DMBA-croton oil–induced skin carcinogenesis in mice.

Keywords
skin carcinogenesis, chemoprevention, antioxidants, phase I and phase II enzymes, lipid peroxidation, α-linolenic acid

Introduction
Cancer is one of the noncommunicable diseases having a high global burden. Cases of cancer are increasing rapidly these days because of the changes in environment and lifestyle. All cancers develop by genetic damage, which is the result of either internal factors such as spontaneous mutations, hormones, and nutrient metabolism in the body or external factors such as excessive tobacco or alcohol consumption, exposure to chemicals, and radiations.

Cancer is the second leading disease in developed countries, accounting for nearly 1 in every 4 deaths. In the United States, about 1 658 370 new cancer cases are estimated to have been diagnosed in 2014, and of these patients, 589 430 are expected to die of this disease.¹ Skin carcinogenesis is the most prevalent of all cancer types, and its incidence is expected to rise substantially. There are 2 types of skin cancer: malignant melanoma of the skin and nonmelanoma skin cancer. Malignant melanoma is the most serious type of skin cancer. Epidemiological data and experimental evidence suggest that UV-B radiation has the potential to cause both squamous cell carcinoma and basal cell carcinoma because of its characteristic wavelength range.²

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vitamins, and minerals that inhibit, delay, or reverse the multistep process of carcinogenesis, including initiation, promotion, and progression of malignant cells. The chemopreventive potential of various natural products has been investigated during the recent past. Our laboratory has also evaluated the anticancer potential of various medicinal plants—Phyllanthus niruri, Aegle marmelos, Syzygium cumini, Aveorrha carambola.

Flaxseed oil (FSO) is obtained from Linum usitatissimum belonging to the family Linaceae. Such oil is consumed in various food formulations, and it is the subject of extensive research because of its potential health benefits. The physiological benefits of flax oil are attributed primarily to the high α-linoleic acid content, chlorophyll pigments, γ-tocopherol, plastochromanol-8, phenolic acids, and flavonoids, which may play significant and/or synergistic roles in the pharmacological quality of the oil.

Basch et al reviewed the medicinal properties of flaxseed and concluded that it had been studied on humans for the prevention and treatment of 13 diseases, including constipation (as a laxative), attention-deficit hyperactivity disorder, hyperlipidemia, atherosclerosis/coronary artery disease, menopausal symptoms, cyclic mastalgia (breast pain), breast cancer, prostate cancer, acquired immune deficiency syndrome (AIDS), and hyperglycemia/diabetes. The scientific literature also provides evidence of the radioprotective and photoprotective properties of FSO in animal models.

The present study has been conducted to evaluate the chemomodulatory potential of FSO during DMBA and croton oil–induced skin carcinogenesis in mice.

Materials and Methods

Chemicals

The initiator, 7, 12-dimethylbenz [a] anthracene (DMBA) and croton oil (used as promoter) were procured from Sigma Chemicals Co, St Louis, MO. DMBA was dissolved in acetone at a concentration of 100 µg/100 µL. Croton oil was mixed in acetone to give a solution of 1% dilution. FSO was procured from Prano Flax India Private Ltd.

Animals

Animal care and handling were approved by the ethical committee of our institution (1678/GO/a/12/CPCSEA), and these were done according to guidelines set by the World Health Organization, Geneva, Switzerland, and the Indian National Science Academy, New Delhi, India. The present study was conducted on Swiss albino mice (7-8 weeks old weighing 24 ± 2 g), selected from a random breed inbred colony. These animals were housed in polypropylene cages in the animal house under controlled conditions of temperature (25°C ± 2°C) and light (14 light:10 dark). The animals were fed a standard mouse feed (procured from Aashirwad Industries, Chandigarh, India) and water ad libitum.

Experimental Design

Swiss albino mice in the resting phase of growth were selected from the inbred colony and divided into 5 groups. The dorsal hair between the cervical and caudal portions was clipped using a surgical clipper 2 weeks before application of the initiator.

Group I (vehicle-treated control; n = 10): animals of this group were treated with acetone (100 µL/animal) on the dorsal clipped portion, and double distilled water equivalent to FSO (100 µL/animal/d) was administered orally throughout the experimental period of 16 weeks.

Group II (FSO-treated control; n = 10): these animals were administered FSO alone at a dose of 100 µL/animal/d for the 16 weeks.

Group III (carcinogen-treated control; n = 10): a single dose of 100 µL DMBA (100 µg/100 µL acetone) was applied over the dorsal surface of mice in this group. Two weeks later, croton oil (100 µL as 1% w/v in acetone) was applied topically 3 times a week until the end of the experimental period.

Group IV (experimental; n = 10): In this peri-initiation treatment group, animals received FSO (100 µL/animal/d) from 7 days prior to 7 days after the DMBA application, whereas the croton oil was applied similarly to group III.

Animals of all the groups were monitored daily, and their weight was taken weekly. The mice were observed weekly for the detection of tumors on the dorsal surface, and tumors with diameter greater than 2 mm and whose persistence was more than 2 weeks were recorded. The parameters studied after the completion (ie, 16 weeks) of the experiment are discussed below.

Morphological

1. Tumor incidence: the number of mice carrying at least 1 tumor expressed as percentage incidence.
2. Tumor yield: the average numbers of tumors per mouse.
3. Tumor burden: the average number of tumors per tumor-bearing mouse.
4. Cumulative number of tumors: the total number of tumors carried by all mice.
5. Tumor diameter: the average diameter of tumors.
6. Tumor weight: the average weight of tumors measured at the end of the experiment.
7. Bodyweight: the average body weight of animals measured weekly.

8. Average latent period: the lag between the application of the promoting agent and the appearance of 50% of 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 the total number of tumors:

\[ \frac{\sum (F \times X)}{n} \]

where \( F \) is the number of tumors appearing each week, \( X \) the numbers of weeks, and \( n \) the total number of tumors.

9. Inhibition of tumor multiplicity:

\[ \frac{(\text{Total no. of tumors in carcinogen controls}) - (\text{Total no. tumors in treated group})}{\text{Total no. of tumors in carcinogen controls}} \times 100. \]

### Biochemical

The biochemical parameters measured in liver and skin at the termination of the experiment (ie, 16 weeks) are given below.

- **Lipid Peroxidation (LPO).** LPO was estimated in liver and skin spectrophotometrically by the thiobarbituric acid reactive substances method. \(^{11}\)

- **Total Proteins.** Proteins were determined in liver and skin by the method of Lowry et al. \(^{12}\) using bovine serum albumin as standard at 680 nm.

- **Phase I Enzymes**
  - **Cytochrome P450 and cytochrome b5.** Cytochrome P450 was determined using the carbon monoxide difference spectra. Both cytochrome P450 and cytochrome b5 content were assayed in the microsomal suspension by the method of Omura and Sato. \(^{13}\)

- **Phase II Enzymes**
  - **Glutathione S-transferase (GST).** GST activity was determined in liver and skin spectrophotometrically at 37°C, according to the procedure of Habig et al. \(^{14}\)

  - **DT diaphorase.** DT diaphorase activity was measured in the liver and skin, as described by Ernest et al. \(^{15}\)

- **Antioxidant Parameters**
  - **Reduced glutathione (GSH).** Reduced glutathione was estimated in liver and skin as total non-protein sulphydryl group by the method as described by Moron et al. \(^{16}\)

  - **Glutathione peroxidase (GPx).** GPx activity was measured in liver and skin by the coupled assay method, as described by Paglia and Valentine. \(^{17}\)

### Catalase (CAT)

CAT was estimated in liver and skin at 240 nm by monitoring the decrease of \( \text{H}_2\text{O}_2 \) as described by Aebi. \(^{18}\)

### Superoxide dismutase (SOD)

SOD was assayed utilizing the technique of Marklund and Marklund. \(^{19}\)

- **Vitamin C.** Skin and liver of animals were homogenized in acetate buffer (20 mg/mL), and 4% cold trichloroacetic acid was used for tissue extraction. The determination of ascorbic acid activity was done according to the protocol given by Roe and Kuether. \(^{20}\)

### Histopathological Analysis

After the 16th week, tumors and skin of sacrificed animals were removed and fixed in 10% formalin fixative for 24 hours. Dehydration of the tissue was done in ascending series of alcohol, embedded in paraffin wax, and 4-µm thick sections were prepared and studied using a light microscope.

### Statistical Analysis

All the above parameters were compared among different groups statistically by using Student’s \( t \) test.

### Results

#### Morphological

A gradual increase in body weight from the initiation of treatment was noted in all the groups, and such weight was found to be near normal at the end of the experimentation. Vehicle-treated (group I) as well as FSO-treated control animals (group II) did not show any tumor appearance throughout the experimentation (Table 1).

The incidence of tumors in DMBA/croton oil–treated animals was noted as 100%, and the average latent period of tumor appearance was recorded as 9.92 ± 0.02 weeks. The oral administration of FSO caused a 23.33% reduction in tumor incidence, and the cumulative number of tumors decreased to 37.00 ± 2.4 from 55.33 ± 2.04 in the experimental animals (group IV) when compared with the carcinogen-treated controls (group III). Furthermore, the average latent period was increased to 11.68 ± 0.13 weeks after FSO administration (Tables 1 and 2). Tumor yield and tumor burden in the animals were significantly reduced to 3.7 ± 0.24 and 4.81 ± 0.21, respectively, after treatment with FSO, and these were 5.53 ± 0.20 and 5.53 ± 0.20 in group III. Similarly, the inhibition of tumor multiplicity was calculated as 33.13% in the animals in group IV (Table 2; Figure 1).
Table 1. Variations in Tumor Size, Weight, and Body Weight During Chemical-Induced Skin Carcinogenesis in Mice With or Without FSO.

| Group                        | Treatment                          | Body Weight (g)     | No. of Tumors | Tumor Weight         |
|------------------------------|------------------------------------|---------------------|---------------|----------------------|
| Vehicle-treated control I    | Acetone (100 µL)                   | 25.56 ± 1.73        | 32.67 ± 0.45  | —                    |
| Flax seed oil–treated control II | 100 µL/animal/d                  | 27.48 ± 1.87        | 33.53 ± 1.32  | —                    |
| Carcinogen-treated control III | DMBA + Croton oil                  | 25.63 ± 1.95        | 23.67 ± 1.23** | 55.33 ± 2.03         | 1.33 ± 0.04 |
| FSO experimental IV          | FSO + (DMBA + Croton oil)         | 26.91 ± 1.44        | 34.83 ± 2.04  | 37.00 ± 2.40***      | 0.97 ± 0.06** |

Abbreviations: FSO, flaxseed oil; DMBA, 7,12 dimethylbenz[a]anthracene.

*Each value represents mean ± standard error. Statistical comparison: normal versus control; control versus experimental. Significance levels: *P ≤ .05; **P ≤ .01; ***P ≤ .001.

Table 2. Morphological Alterations in Tumors During Chemical-Induced Skin Carcinogenesis, With or Without FSO.

| Group                        | Tumor Yield | Tumor Burden | Average Latent Period | Inhibition of Tumor Multiplicity (%) | Tumor Incidence (%) |
|------------------------------|-------------|--------------|-----------------------|--------------------------------------|---------------------|
| Vehicle-treated control I    | —           | —            | —                     | —                                    | —                   |
| Flax seed oil–treated control II | —          | —            | —                     | —                                    | —                   |
| Carcinogen-treated control III | 5.53 ± 0.20 | 5.53 ± 0.20  | 9.92 ± 0.02           | —                                    | 100                 |
| FSO experimental IV          | 3.7 ± 0.24*** | 4.81 ± 0.2*  | 11.68 ± 0.13***       | 33.13                                | 76.67               |

Abbreviation: FSO, flaxseed oil.

*Each value represents mean ± standard error. Statistical comparison: normal versus control; control versus experimental. Significance levels: *P ≤ .05; **P ≤ .01; ***P ≤ .001.

Figure 1. Morphological alterations in the chemical carcinogen–treated animal with or without flaxseed oil.

Biochemical

Lipid Peroxidation. Oral administration of FSO in the peri-initiation treatment group showed 1.17- (P < .01) and 1.42-fold (P < .01) reduction of the malondialdehyde levels in the liver and skin of animals when compared with the carcinogen-treated control group (Figure 2).

Total Protein. The total protein levels of the animals in group III were significantly reduced to 1.68- and 1.70-fold (P < .001) in the liver and skin, respectively, in comparison to the vehicle-treated control group. In contrast, there was significant (P < .01) elevation of total protein levels in the liver (0.77-fold) and skin (0.74-fold) in animals in group IV after treatment with FSO when compared with the DMBA/croton oil–treated group (Figure 3).

Phase I Enzymes. Cytochrome P450 in the carcinogen-treated control group showed a marked decrease: 0.81-fold (P < .001) in skin and 0.84-fold (P < .001) in liver as compared with group I. The levels were elevated to near normal as 1.13- and 1.07-fold (P < .01) in skin and liver, respectively, when FSO was administered (Figure 4).

Similarly, the activity of cytochrome b5 exhibited a significant (P < .001) elevation in the liver (1.29-fold) and skin (1.28-fold) of the carcinogen-treated control group in comparison to group I. Oral administration of FSO to the animals in group IV significantly decreased the levels of cytochrome b5 in both the tissues: 0.90-fold (P < .01) in
liver and 0.91-fold \( (P < .05) \) in skin when compared with animals in group III (Figure 5).

**Glutathione S-Transferase.** In the animals of the carcinogen-treated control group, the GST level exhibited a significant reduction of 0.45-fold \( (P < .001) \) in liver and 0.50-fold \( (P < .001) \) in skin when compared with the vehicle-treated control group (Figure 6).

After FSO treatment, a significant \( (P < .01) \) decrease in GST activity was registered in the liver and skin (1.64- and 1.72-fold, respectively) in experimental animals (Figure 6).

**DT-Diaphorase.** The activity of enzyme DT-diaphorase exhibited a significant \( (P < .001) \) reduction in liver (0.48-fold) and skin (0.47-fold) in group III animals when compared with the animals in group I. In contrast, FSO administration in group IV led to a significant elevation \( (P < .05) \) in DT-diaphorase level: 1.26-fold in the liver and 1.30-fold in the skin of animals in comparison with carcinogen-treated control group (Figure 7).

**Reduced Glutathione.** DMBA-croton oil treatment caused a significant \( (P < .001) \) reduction in GSH activity: 0.45-fold in liver and 0.19-fold in skin compared with group I. An increment in GSH levels of 1.50-fold and 1.55-fold, respectively, was noted in animals in group IV after FSO treatment in comparison with animals in group III (Figure 8).

**Glutathione Peroxidase.** The activities of GPx significantly \( (P < .001) \) declined in skin (0.46-fold) and liver (0.48-fold) after treatment with carcinogen and promoter in group III as compared with group I. FSO administration resulted in a
significant ($P < .05$) increase in GPx activity: 1.38-fold in liver and 1.47-fold in skin (Figure 9).

**Catalase.** Relative to the vehicle-treated controls, the activity of CAT was significantly ($P < .001$) diminished in both liver (0.60-fold) and skin (0.52-fold) in animals belonging to group III. In contrast, the levels of CAT were significantly ($P < .01$) enhanced to 1.27- and 1.33-fold in liver and skin, respectively, in group IV as compared with the carcinogen-treated control group (Figure 10).

**Superoxide Dismutase.** When compared with group I, animals in the carcinogen-treated control group exhibited a significant ($P < .001$) inhibition of SOD activity in liver (0.61-fold) and skin (0.55-fold). Specific activity of this enzyme was significantly increased by 1.30-fold in the liver and 1.43-fold in the skin of FSO-treated animals with respect to group III (Figure 11).

**Vitamin C.** In contrast to vehicle-treated controls, ascorbate activity showed a significant decline in liver and skin—0.61-fold and 0.49-fold, respectively—whereas FSO administration resulted in a significant increment in vitamin C levels of 1.22-fold in the liver and 1.37-fold in skin when compared with group III (Figure 12).

**Histopathological Alterations**

The animals in the vehicle-treated group showed normal skin layers—that is, epidermis, dermis, and basal layer (Figure 13A). Carcinogen and promoter treatment indicated the occurrence of squamous cell carcinoma. The slides in
group III animals exhibited thickening of the epidermis (acanthosis), invasion of epidermal cells in the dermis, and formation of keratin pearls. The dermal region showed reduced dermal fibroblasts and fibroconnective tissue in carcinogen-treated control animals (Figure 13B). Sections of tumor revealed cells with atypical nuclei, dyskeratosis of keratin, and lymphocytes with reduced stroma accumulated at margins (Figure 13C).

Administration of the FSO during the peri-initiation stage demonstrated a lower degree of epidermal hyperplasia, acanthosis, and damage in sebaceous glands. A moderate number of keratinized pearls were observed with suprabasal hyperplasia in the FSO-treated experimental groups in comparison with carcinogen-treated control (Figures 13D-13E).

Discussion

FSO is a rich source of α-linolenic acid, which can be converted into long chain n-3 polyunsaturated fatty acid (PUFA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) via elongation and desaturation reactions.21 The present study was performed to evaluate the potential of FSO against DMBA-croton oil–induced skin carcinogenesis.

DMBA is an indirect-acting and organ-specific carcinogen, which is metabolized to the ultimate carcinogen 1,2-epoxide-3,4-diol DMBA by CYP1A1 and CYP1B1 enzymes of CYP450. The DMBA intermediate causes mutations in the genome by forming adducts with DNA and acts as initiator in chemical carcinogenesis.22 12-O-tetradecanoyl phorbol-13-acetate is the active constituent of croton oil that binds with protein kinase C and leads to transcription of early genes c-fos and c-jun, and stimulates the activating protein-1 and NF-κB transcription factors, which result in cellular proliferation and apoptosis.23

Reduction in the cumulative number of tumors, tumor yield, tumor burden, and tumor incidence by administering various plant extracts during chemical-induced carcinogenesis has been observed earlier.24,25 Similarly, a considerable decrease in skin tumors with the oral administration of FSO to mice has been reported in the present study. It might be possible that α-linolenic acid, flavanoids, phenolic acid, and tocopherols in FSO elevated the cellular antioxidants and reduced the oxidative damage caused by carcinogen and promoter.

LPO is produced during life processes in a chain reaction initiated by free radicals produced. Malondialdehyde is the lipid electrophile generated from peroxidation, and an increase in its levels is an alarming sign of various diseases, including cancer.26 Topical application of DMBA and croton...
oil to animals increases the production of ROS and ultimately leads to higher LPO levels in skin and liver, as observed in the present study. In contrast, FSO, rich in PUFA, protects the cellular membranes, and its other phytochemicals scavenge free radicals and inhibit LPO in experimental animals.

Figure 13. (A) Photomicrograph showing histological section of skin of normal mice (group I), ×100; (B) photomicrograph showing histological section of DMBA/croton oil–treated mice skin (group III), ×200; (C) photomicrograph showing histological section of DMBA/croton oil–treated skin in mice (group III), ×200; (D) photomicrograph showing histological section of DMBA/croton oil–treated skin in mice administered flaxseed oil (FSO; group IV), ×200; (E) photomicrograph showing histological section of DMBA/croton oil–treated skin tumor in mice administered FSO, ×200 (group IV).

Abbreviations: DMBA, 7,12 dimethylbenz[a]anthracene; E, epidermis; D, dermis; B, basal lamina; KP, keratinized pearls; EH, epidermal hyperplasia; DI, dermal invasion; DF, dermal fibroblast; SG, sebaceous gland; AC, acanthosis; AN, atypical nuclei; RS, reduced stroma with lymphocytes; TN, tumor nest

Cytochrome P450 and b5 are the phase I enzymes responsible for the biotransformation of DMBA into its active intermediate. The elevated levels of phase I enzymes act as a biomarker for cancer detection, and a related trend was noted in the carcinogen-treated control group.27 Modulatory
action of FSO reverted back the levels of both enzymes near to normal in the liver and skin of animals in group IV.

Mounting evidence reveals that phytochemicals enhance phase II enzyme levels in biological tissues. GSTs and DT-diaphorase facilitate the detoxification of carcinogens by conjugating the carcinogen with GSH and uridine diphosphoglucuronic acid, respectively, for its fast detoxification and excretion. In the present study, elevated phase II enzyme levels in skin and liver of experimental animals were recorded after FSO administration.

Treatment with FSO increased the levels of enzymatic (SOD, CAT, GPx) and nonenzymatic antioxidants (GSH) in both skin and liver, which were noted to be decreased in the carcinogen-treated control group. SOD accelerates the dismutation of superoxide anions to hydrogen peroxide and finally CAT, and GPx degrades $\text{H}_2\text{O}_2$ into water and oxygen. GPx is a selenium-containing enzyme, which works in association with GSH and needs different secondary enzymes (GR and G-6-PDH) and cofactors (NADPH and glucose-6-PO$_4$) for efficient action. Carcinogen treatment reduced the levels of SOD by generating ROS, whereas the free radical scavenging action of FSO restored the same toward normal in the experimental animals.

Tripeptide glutathione is a nonprotein thiol that plays a crucial role in scavenging free radicals. GSH can readily form adducts with ROS and protects the macromolecules in our body. The depletion in GSH content in the control animals was associated with tumor genesis, and administration of FSO reduced the oxidative stress and hence balanced the GSH levels.

Similarly, levels of vitamin C were observed to be reduced in carcinogen-treated controls because such vitamins are used in quenching free radicals in the aqueous phase to protect cellular membranes from degradation. FSO intake in mice conserves the vitamin C activity toward the normal range by scavenging the reactive species.

This study was limited to the mouse model. The literature shows the anticancer properties of FSO against breast, colon, prostate, and intestinal cancer and others, with the possible mechanism associated with its high ω-3 PUFA content, which reduces the formation of proinflammatory mediators PGE2 and LTB4 from arachidonic acid. Omega-3 fatty acids of FSO hinder the production of prostaglandins by acting on the cyclooxygenase pathway. Fatty acid metabolism is the same in all mammalian models, including mice and humans, although evidence from animal and human experimentation suggests that the products of omega-3 fatty acid (EPA and DHA) do have different potentials to alter gene expression and resulting metabolic and cellular functions.

Conclusion
The results of the present study suggest that FSO administration at the peri-initiation stage of carcinogenesis has chemopreventive potential by modulating the phase I and phase II enzymes and antioxidants in mammals. The anticancer and antioxidative effect of FSO might be a result of the synergistic action of various antioxidants present in such oil.

Declaration of Conflicting Interests
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