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CHEMOPREVENTIVE AND ANTIOXIDATIVE EFFECT OF FLAXSEED OIL AGAINST DMBA/CROTON OIL INDUCED TWO STAGES SKIN CARCINOGENESIS IN MICE

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Keywords:

7, 12-dimethyl benz(a)anthracene (DMBA), Skin carcinogenesis, Oxidative stress, Chemoprevention, Flaxseed oil, Antioxidative enzymes

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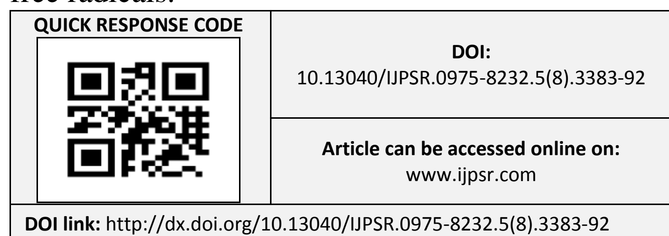
ABSTRACT: Cancer is one of the major threats to human health that cause considerable suffering and economic loss worldwide. The search for natural compounds which prevent cancer has upsurge with the mounting evidence that many types of cancer are caused or triggered by factors relating to our lifestyle. Chemopreventive agents include all those agents which can either prevent cancer or slow down the growth of malignant lesions. The present experiment was designed to explore the chemoprotective and antioxidative potential of Flaxseed oil (FSO) on 7, 12-dimethylbenz(a)anthracene (100 µg/100 µl of acetone) and croton oil (1% in acetone/three times a week) induced skin carcinogenesis in mammals. FSO administration to mice, by oral gavage at a dose of 50µl/animal/day for 17 weeks at peri-post initiation stage (i.e. started from 7 days before the experiment & continued till completion of experiment), reduced the tumor burden, tumor multiplicity, tumor yield, cumulative number of papillomas and percent tumor incidence while increased the average latent period when compared to control group. The lipid per oxidation levels in liver and skin were significantly ($P\leq0.001$) reduced along with the significant ($P\leq0.001$) elevation in enzymatic (superoxide dismutase and catalase) and non-enzymatic (reduced glutathione and vitamin C) antioxidants. Total protein content also increased significantly in both the tissues. These results demonstrate that the FSO treatment significantly reduces the chemical induced tumorogenesis and oxidative stress during skin carcinogenesis.

INTRODUCTION: Human body is constantly exposed to various exogenous (radiation, heavy metals, drugs etc.) and endogenous (cellular metabolism) factors responsible for generation of free radicals.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Oxidative stress is caused by the disturbance in the equilibrium between production of reactive oxygen species and their counterbalance mechanism i.e. anti-oxidative potential of our body. This increase in the free radicals results in injury to all the important cellular macromolecules and genetic material which can cause the cellular death [1]. Free radicals include ROS, RNS, superoxides, hydroxyl ions etc. Reactive oxygen species (ROS) are thought to underline the progress of numerous diseases including cancer [2, 3].

Cancer is one of the dreaded human diseases that cause considerable suffering and economic loss worldwide. Skin carcinogenesis is a result of genetic or subsequent epigenetic alterations that are crucial to different stages from initiation to promotion and progression of cancer development. Changes in lifestyle together with depletion in the atmospheric ozone layer during the last few decades have led to an increase in the skin cancer [4].

The two most frequent type of non-melanoma skin cancer comprises basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), are the commonly diagnosed cutaneous malignancies in the world and their incidences are exponentially increasing. Annual incidences of NMSC include 80% cases of BCC while SCC represents as about 16% occurrence [5]. The skin cancer incidence has increased so fast in the last decades that was considered as the silent epidemic of the 20th century [6].

Cancer chemoprevention involves the use of either natural or synthetic compounds to delay, inhibit or reverse the development of cancer in normal or pre-neoplastic conditions. The search for natural compounds which prevent cancer has upsurge with the mounting evidence that many types of cancer are caused or triggered by factors relating to lifestyle and nutrition. An effective chemopreventive agent should preferably intervene early in the process of carcinogenesis to eliminate pre-malignant cells before they acquire malignant character.[7,8] Many chemopreventive agents can retard the promotion and/or progression of premalignant or malignant cells by modulating cell proliferation, enhanced detoxification of the carcinogenic intermediates, selective promotion of apoptosis in cancerous cells, inhibition of angiogenesis and metastasis formation [9].

Recent findings support a growing body of evidence that flaxseeds, or its extracted oil exert anti-carcinogenic effects in some *in vitro* and *in vivo* experiments, and that flaxseeds oil and related extracts also play an important dietary role in various biological activities in the body [10, 11].

Flaxseed oil is obtained from *Linum usitatissimum* belonging to family linaceae and commonly known as linseed.

Flaxseed is an economically important oilseed crop grown around the world for its oil and fibers. The physiological benefits of flax oil are attributed primarily to the high α-linoleic acid content, chlorophyll pigments, tocopherol, plastochromanol-8, phenolic acids and flavanoids which may play significant and/or synergistic role in the pharmacological quality of the oil.[12,13] Therefore, the present study has been undertaken to evaluate the anticancer activity of Flaxseed oil on DMBA and croton oil induced skin carcinogenesis in Swiss albino 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 (USA). 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. Flaxseed oil was procured from Prano flax India Private Ltd.

Animals: The animal care and handling was approved by ethical committee of our institution and was 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 female Swiss albino mice (7-8 weeks old weighing 24 ± 2 g.), selected from a random breed inbreed 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 protocol: Animals for this study were divided into following groups:

1. **Group I: Vehicle treated control (Normal, n=10):** Animals of this group received topical application of acetone (100µl/ mouse) on the shaven dorsal skin and double distilled water (equivalent to FSO i.e.50µl /mouse) by oral gavage for a period of 16 weeks.

2. **Group II: FSO treated control (Drug control, n=10):** Animals belonging to this group received Flaxseed oil (50µl /mouse/day) by oral gavage throughout the experimental period i.e. 16 weeks and acted as drug treated control.

3. **Group III: Carcinogen treated control (Positive control, n=10):** Mice of this group were treated with single topical application of 100µl DMBA (100µg/100µl acetone) over the shaven area of skin. Two weeks later, croton oil (100µl as 1% w/v in acetone) was applied topically three times in a week until the end of experimental period.

4. **Group IV: Peri-and post treated experimental (FSO treated, n=10):** Animals of this group were administered FSO (50µl/animal/day) orally starting from 7 days before DMBA application and continued throughout experimental duration (i.e. 16 weeks).

Induction of tumor: For the induction of skin tumors, dorsal hairs between the cervical and caudal portions of the animals of Group III & IV were removed using a surgical clipper, 2 days prior to the initiation of the experiment, and 100µl DMBA (100 µg /100 µL acetone) was topically applied. After 14 days, the tumor initiation by DMBA was promoted with the topical application of 100µl croton oil (1% v/v in acetone), thrice a week for the next 14 weeks.

Appearance of tumors on the shaven dorsal surface of the skin was recorded at weekly intervals. For evaluation of the data only those papillomas were taken into the account that persists for 2 weeks or more (diameter≤2mm). Papillomas that regressed after one observation were eliminated from the study.

Morphological Parameters:

1. **Tumor incidence:** The number of mice carrying at least one tumor expressed as a percentage incidence.

2. **Tumor yield:** The average number of papillomas per mouse.

3. **Tumor burden:** The average number of tumors each tumor was measured.

4. **Weight:** The weight of the each tumor appeared in animals at the termination of each experiment was measured.

5. **Body weight:** The weight of the mice was measured weekly throughout the experimental period.

6. **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 total number of tumors.

$$\sum \frac{FX}{n}$$

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

7. **Inhibition of tumor multiplicity**:

$$\frac{(\text{Total no. of tumors in carcinogen control}) - (\text{Total no. tumors in treated})}{\text{Total no of tumors in carcinogen control}} \times 100$$

Biochemical Parameters: The animals from respective groups were sacrificed by cervical dislocation 16 week after the commencement of treatments, and their liver as well as dorsal skin affected by tumors were quickly excised and washed thoroughly with chilled 0.9% NaCl (pH 7.4). Both the tissues (liver & skin) were then weighed and blotted dry. A 10% tissue homogenate was prepared from the part of the tissue sample in 0.15 M Tris-KCL (pH 7.4) to estimate the reduced glutathione and lipid per oxidation.

A. **Reduced Glutathione:** The level of GSH was estimated as total non-protein sulphahydryl group by the method of

Moron *et al*., 1979 [14]. The homogenate was immediately precipitated with 100 µl of 25% tri chloroacetic acid (TCA) and the precipitate was removed after centrifugation. Free endogenous-SH was assayed in a total volume of 3 ml by the addition of 200 µl of 0.6 mM 5, 5 dithio-bis (2-nitrobenzoic acid) dissolved in 0.2 M phosphate buffer (pH 8.0) to 100 µL of the supernatant and the absorbance was recorded at 412 nm using a UV-VIS Systronics spectrophotometer. Reduced GSH was used as a standard. The levels of GSH were expressed as µmol/gm of tissue.

B. **Lipid Peroxidation:** The LPO level was calculated spectrophotometrically by the thiobarbituric acid reactive substances (TBARS) method, as described by Ohkhawa *et al*., 1979 [15]. In this, thiobarbituric acid (0.6%), sodium dodecyl sulphate (0.1%) and trichloroacetic acid (20%) were added to 0.2 ml of the tissue homogenate (10%). This mixture was heated for 90 min, cooled, and extracted with N-butanol-pyridine (15:1), and centrifuged. The optical density (OD) of LPO was observed at 532 nm and the content of TBARS was expressed as n mole/mg of tissue.

C. **Superoxide dismutase:** SOD level was determined according to the method of Marklund and Marklund, 1974[16] by quantification of pyrogallol auto oxidation inhibition and the results expressed as units/mg protein. One unit of enzyme activity was defined as the amount of enzyme necessary for inhibiting the reaction by 50%. Auto oxidation of pyrogallol in Tris-HCL buffer (50 mM, pH-7.5) was measured by increase in absorbance at 420 nm.

D. **Catalase:** Catalase activity was measured by the method of Aebi, 1984[17] 50mM phosphate buffer is used for homogenate preparation and centrifuged for 10 min. The change in absorbance was observed spectrophotometrically at 240 nm. The activity of the enzyme is expressed as U/mg of tissue, where U is µmol of H_2O_2 disappearance/min.

E. **Vitamin C:** For this, tissue, the fresh organs were weighed, homogenized in acetate buffer (20 mg/ ml) extracted with cold 4 per cent trichloroacetic acid, centrifuged, and filtered. Ascorbic acid was determined by the method of Roe and Kuether, 1943 [18].

F. **Protein:** Total protein content of the skin and liver was estimated by the method of Lowry *et al*., 1951[19] preparing homogenate in distil water and absorbance was recorded at 670nm. Protein concentration was measured from a standard curve (BSA) and the level was expressed as mg/ gm.

(iii) Statistical Analysis

Data from different experimental groups were analyzed and expressed as mean ± SE. The significant levels of difference between carcinogen treated control and FSO treated experimental groups were statistically computed using Student's t-test.

RESULTS:

1) **Morphological analysis:** FSO administration significantly alters the different stages of skin carcinogenesis as depicted in Table 1. A gradual increase in the body weight was noted in all the animal groups which were found near to normal weight of the control animals.

   Topical application of DMBA followed by croton oil produced skin tumors which started appearing from week seventh onwards in positive control group and reached to 100% by the end of experiment (i.e. 16 weeks) whereas tumors were absent in both negative control and drug alone group.

   Cumulative number of tumors in group III was 53 (Fig.1). The average number of papillomas per mouse (tumor yield) as well as papillomas per tumor bearing mice (tumor burden) was found to be 5.3 (**Fig. 3-4**). The average latent period was 7.26 as shown in **figure 5**.

Administration of FSO by oral gavage in group IV significantly reduces the cumulative number of tumors up to 75.47% whiles the, tumor yield and tumor burden was lowered by 1.3 and 3.2 respectively. Tumor incidence (Fig. 2) in animals of FSO treated group was checked to 40% as well as average latent period was also increased by 12.30 when compared with positive control group.

2) **Biochemical analysis:** The levels of TBARS, total proteins and antioxidants (GSH, SOD and CAT), in liver and skin of animals of each group are shown in Figs. 6-11. The concentration of TBARS was significantly ($p \leq 0.001$) increased, whereas the status of antioxidants and the total proteins was significantly ($p \leq 0.001$) decreased in animals of carcinogen treated control (Group III), as compared to vehicle treated control (Group I).

   A Significant ($p \leq 0.001$) decrease in Lipid peroxidation was documented in Liver (49.19%) and Skin (49.39%) of peri-and post group animals when compared to positive control group. Intake of Flaxseed oil in group IV revert the values of TBARS towards normal i.e. negative control group (**Fig. 6**).

On the contrary antioxidant parameters (GSH, SOD, CAT, Vit. C) in liver and skin of flaxseed oil administered group i.e. Group IV showed significant ($p \leq 0.001$) elevation in comparison to Group III (Fig. 7-10).

Reduced glutathione level is elevated by 93.48% and 250.59% in liver and skin of FSO treated animals when compared with the animals without any treatment i.e. Group III (**Fig.7**) .

In the activity of Superoxide dismutase a decrease of 39.08% and 44.72% in liver and skin respectively of carcinogen treated control group as compared to vehicle treated group whereas after the ingestion of FSO the level showed significant ($p \leq 0.001$) rise of 46.17% in liver and 54.78% in skin (**Fig. 8**).

In Group III catalase level was observed with a significant ($p \leq 0.001$) fall of 39.69% (liver) and 47.63% (skin) with respect to negative control. There was an increase of 44.97% and 58.12% in both the tissues (liver & skin) of group IV when compared to Group III (**Fig. 9**).

As compared with the vehicle treated group the ascorbate levels exhibited 39.27% and 50.85% decrease in liver and skin of Group III, while the elevation of 50.67% and 75% was revealed in both tissues of pre-post treated experimental group (**Fig. 10**).

Total protein content in the animals of group III was lowered by 40.34% in liver and 35.92% in skin when compared with negative control group. After the oral administration of drug the protein content is increased by 48.16% and 39.88% in liver and skin in comparison of group III (**Fig. 11**).

TABLE 1: ANTICARCINOGENIC INFLUENCE OF FLAXSEED OIL (FSO) ON DMBA-INDUCED SKIN CARCINOGENESIS IN MICE*

Treatment Group	Body weight(gm) Mean± S.E.		Tumor Size (mm)		Tumor Weight (gm)
	Initial	Final	2-5	6-9	
Normal	25.56±1.73	32.67±0.45	-	-	-
Drug control	24.85±1.34	33.43±1.29	-	-	-
Positive control	25.63±1.95	28.67±1.23	38	15	1.53
Exp. Group I	25.46±1.43	37.36±1.45	8	5	0.49

*Treatment schedule of the groups is specified in materials and methods.

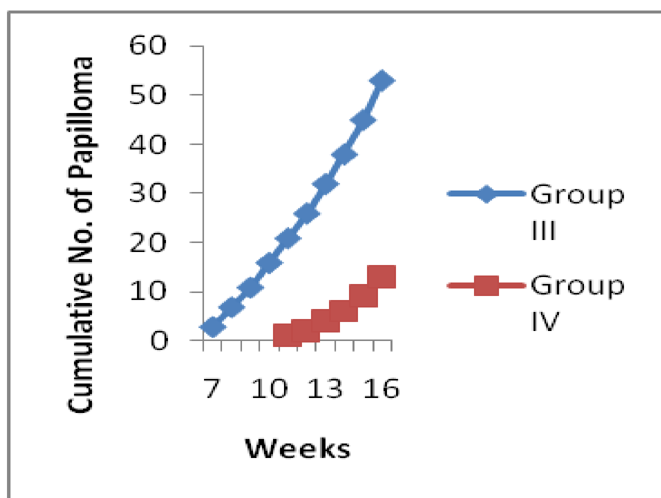

FIG. 1: EFFECT OF FLAXSEED OIL ON CUMULATIVE NO. OF PAPILLOMA DURING CHEMICAL INDUCED SKIN CARCINOGENESIS

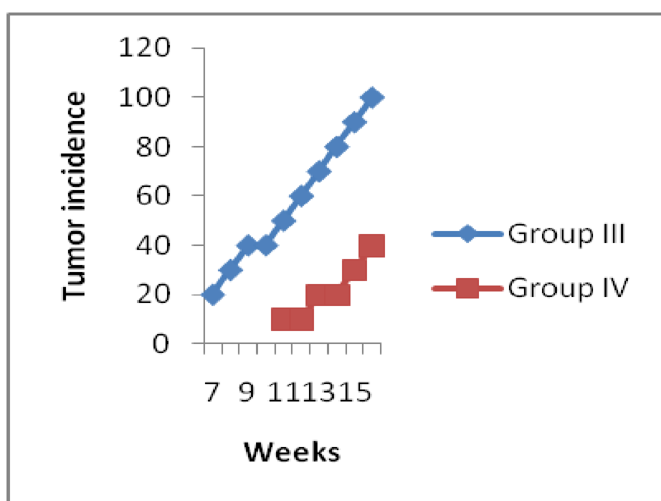

FIG. 2: EFFECT OF FLAXSEED OIL ON TUMOR INCIDENCE DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS

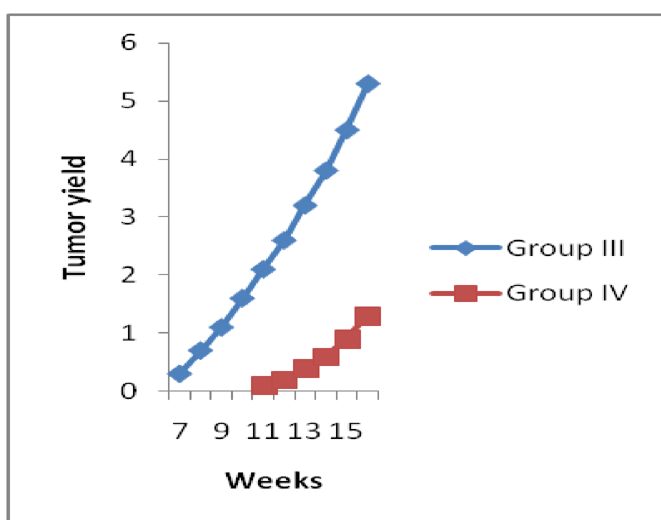

FIG. 3: EFFECT OF FLAXSEED OIL ON TUMOR YIELD DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS

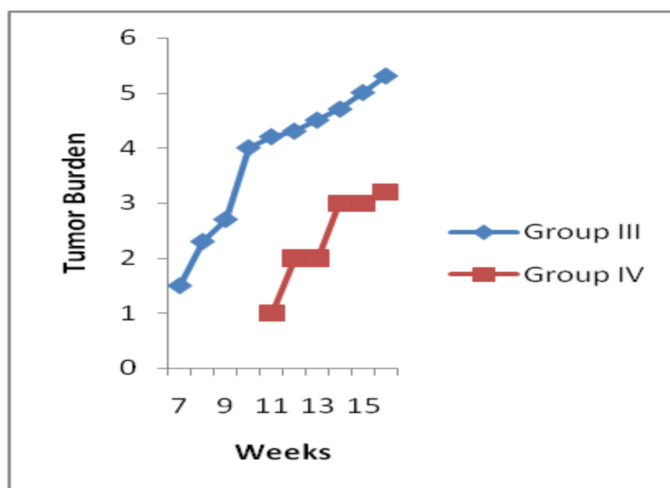

FIG. 4: EFFECT OF FLAXSEED OIL ON TUMOR BURDEN DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS

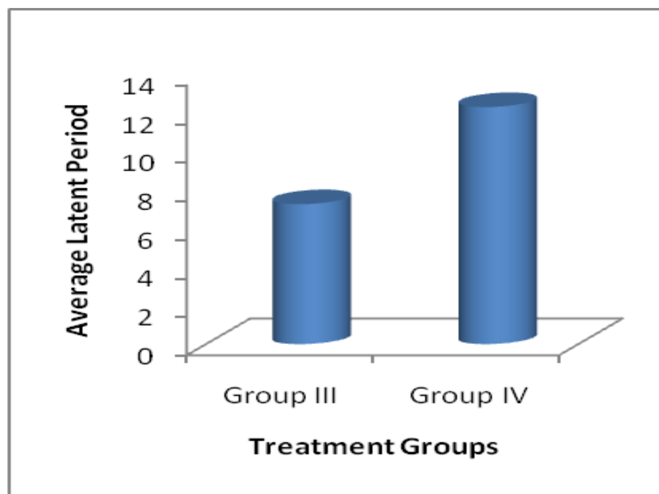

FIG. 5: EFFECT OF FLAXSEED OIL ON AVERAGE LATENT PERIOD DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS

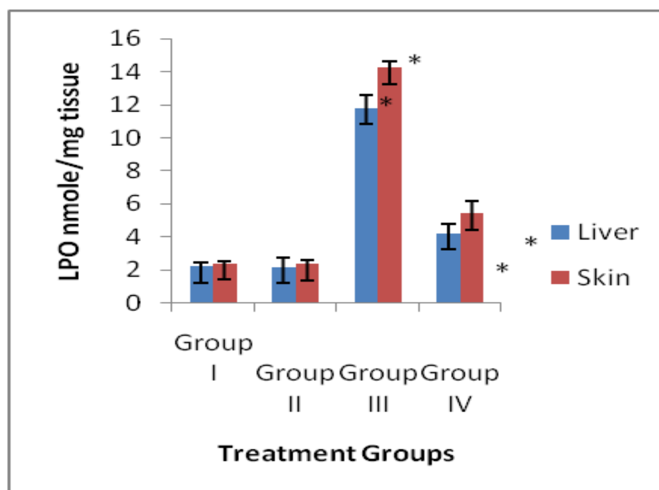

FIG. 6: EFFECT OF FLAXSEED OIL ON LPO LEVEL (mean±S.E) DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

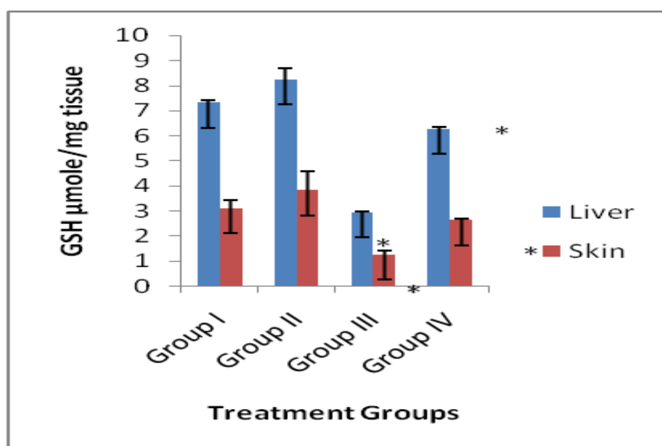

FIG. 7: EFFECT OF FLAXSEED OIL ON REDUCED GLUTATHION (mean±S.E) DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

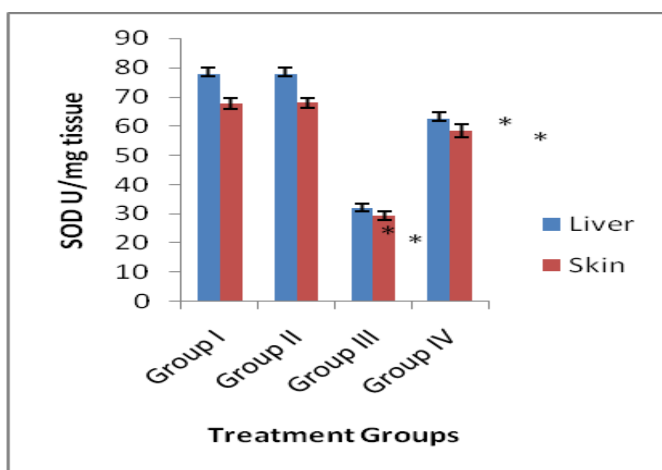

FIG. 8: EFFECT OF FLAXSEED OIL ON SUPEROXIDE DISMUTASE (mean±S.E) DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

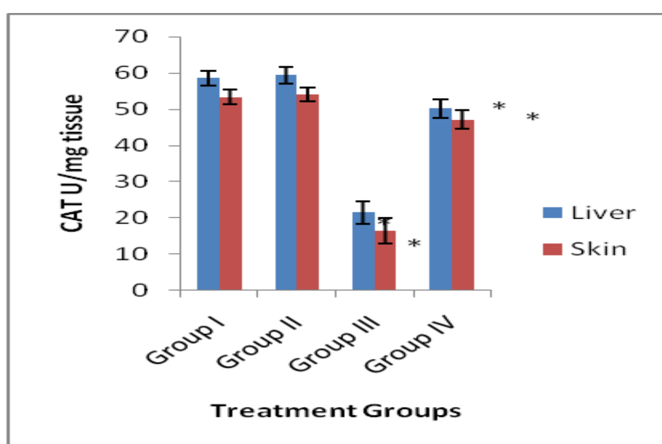

FIG. 9: EFFECT OF FLAXSEED OIL ON CATALASE (mean±S.E) DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

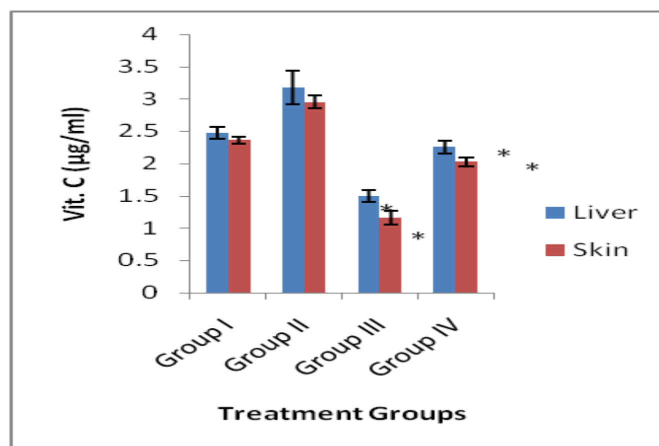

FIG. 10: EFFECT OF FLAXSEED OIL ON VITAMIN C (mean±S.E) DURING THE CHEMICAL INDUCED SKIN CARCINOGENESIS. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

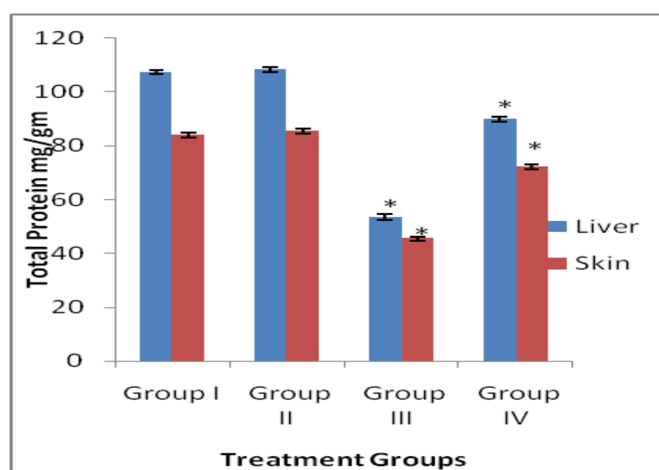

FIG. 11: EFFECT OF FLAXSEED OIL ON TOTAL PROTEIN (MEAN±S.E) DURING THE CHEMICAL INDUCED SKIN carcinogenesis. Statistical comparison: Normal v/s Control; Control v/s Experimental. Significance levels *P≤ 0.001

DISCUSSION: Polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants with high carcinogenic potential. DMBA is a potent polycyclic aromatic hydrocarbon which is metabolically activated by the P450 and converted into 3, 4-diol-1, 2-epoxide that is capable of binding covalently to DNA and causing gene mutation which ultimately leads skin carcinogenesis in Swiss albino mice [20].

Application of DMBA on the shaved dorsal surface causes initial mutation in stem cells mainly hair follicles and intra follicular epidermis compartment of skin epidermis [21].

In the present investigation topical application of DMBA and croton oil considerably increased the incidence, cumulative number of papillomas, tumor yield and tumor burden whereas it decreased average latent period in animals of carcinogen treated control (Group III) because DMBA, after metabolic activation, has been found to induce cancer through an oxidatively mediated genotoxicity by incorporating diolepoxide and other ROS into DNA [22, 23].

The classical skin tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) is an active ingredient of croton oil which has been responsible for the increase production of ROS and hydroperoxides in keratinocytes both *in vitro* and *in vivo* [24] and such organic peroxides have been shown to promote skin tumorogenesis [25].

Recent research on natural products of dietary origin, associated with high degree of safety margins, has been found to be beneficial as potent chemopreventive agents [26]. Comprehensive reviews provide strong evidence that high intake of vegetables and fruits are associated with the reduced cancer incidence [27, 28].

In the present experiment, carcinogenic potential of DMBA and croton oil was significantly overcome by flaxseed oil administration which is evident by reduction in cumulative tumor number, tumor burden, tumor incidences and simultaneously increase in the average latent period. The presence of α-linolenic acid and various plant lignans such as secoisolariciresinol diglucoside (SDG), matairesinol, pinoresinol may help in antitumorous activity [13].

Oxidative stress arising due to the overproduction of reactive oxygen species (ROS) induces various transcriptional factors like nuclear factor kappa B (NFκB) and AP-1 which leads to inflammation, cellular proliferation and apoptosis. ROS when coupled with deficiency of antioxidant defence mechanism has been instrumental in tumor promotion [29].

Lipid peroxidation is a chain reaction process characterized by the oxidative destruction of polyunsaturated fatty acids which is associated with pathological conditions [30].

Oxidative stress is a well-known mechanism of cellular injury that occurs with the increased lipoperoxidation of cell phospholipids and it has been implicated in various cellular dysfunctions [31]. Melonyldialdehyde is considered as important lipid peroxidation metabolite which exhibits high reactivity with biomolecules, such as proteins, DNA and phospholipids generating intra and intermolecular adducts. In the present study, elevated levels of MDA in Group III can be possible cause of cancer promotion and this condition is revert back to normal when FSO is administered in Group IV.

Reduced glutathione is the premier antioxidant which directly quenches reactive hydroxyl free radicals, superoxides and radical centers on DNA and other biomolecules. Elevated level of GSH protects cellular proteins against oxidation through glutathione redox cycle [32]. This experimental study also showed the increase in reduced glutathione in FSO treated experimental groups which aids in cancer prevention by checking the oxidative stress when compared with carcinogen control group.

SOD and CAT act as mutually supportive antioxidative enzymes, involve in protective defence against reactive oxygen species [33]. The present study reveals that the activity of SOD is depleted in the cancer-bearing animals (Group III), which may be due to altered antioxidant status induced during carcinogenesis. A similar result was observed for CAT in Group III, which may be due to the utilization of such antioxidant enzymes in the removal of H_2O_2.

Vitamin C is a key antioxidant which particularly protects lipids from per-oxidative damage by aqueous solution, thereby blocking the initiation of carcinogenesis [34]. Ascorbate level in this study was reduced because it is utilized in scavenging of free radicals generated by DMBA, and FSO restored the levels towards the normal by increasing the antioxidant levels.

CONCLUSION: The oxidative stress is mainly responsible for various life threatening diseases including cancer and the antioxidants can effectively inhibit carcinogenesis, and the same may be attributed to the presence of various phytochemicals like α-linolenic acid, γ-tocopheols,

lignans, p-coumeric acid and ferulic acid in the flaxseed oil. Therefore, it can be inferred that the flaxseed oil possess anti-carcinogenic and anti-oxidative potential against DMBA induced skin carcinogenesis in mammals.

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