RESEARCH COMMUNICATION

Anti-tumour Promoting Activity of Diphenylmethyl Selenocyanate Against Two-stage Mouse Skin Carcinogenesis

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Abstract

Epidemiological, clinical and experimental evidence collectively suggests that Se in different inorganic and organic forms provides a potential cancer chemopreventive agent, active against several types of cancer. It can exert preventive activity in all the three stages of cancer: initiation, promotion and progression. Literature reports revealed that organoselenocyanates have more potential as chemopreventive agents than inorganic forms due to their lower toxicity. In our previous report we showed chemopreventive efficacy of diphenylmethyl selenocyanate during the initiation and pre- plus post-initiation phases of skin and colon carcinogenesis process. The present study was undertaken to explore the anti-tumour promoting activity of diphenylmethyl selenocyanate in a 7,12-dimethylbenz (a) anthracene (DMBA)-croton oil two-stage skin carcinogenesis model. The results obtained showed significant ($p<0.01$) reduction of the incidence and number of skin papillomas, precancerous skin lesions, along with significant ($p<0.01$) elevation of phase II detoxifying enzymes (GST, Catalase and SOD) and inhibition of lipid peroxidation in liver and skin. Thus, the present data strongly suggest that diphenylmethyl selenocyanate also has the potential to act as anti-tumour promoter agent in a two-stage skin carcinogenesis mouse model, pointing to possible general efficacy.

Key Words: Antitumour promoter - diphenylmethyl selenocyanate - DMBA- croton oil - phase II enzymes - lipid peroxidation

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Introduction

In multistage carcinogenesis process the inhibition of tumour promotion (antitumour promoting) is regarded as an effective strategy for cancer chemoprevention, because the process of tumour promotion occurs through long-term, repetitive exposure to tumour promoters (Murakami et al., 1999; Murakami et al., 1996). To be most effective, chemopreventive agents should be able to inhibit several stages in multistage carcinogenesis (McCormick et al., 1998; McCormick et al., 1999). Experimental evidence suggests that the anticarcinogenic effects of Se may not be particularly stage specific. Se has been found to be anti-tumorigenic when treatment was after the initiation phase in several carcinogenesis models for liver cancer (Curphey et al., 1988), mammary cancer (Thomson et al., 1980; Thomson et al., 1981; Ip et al., 1981; Ip, 1985; Tempero et al., 1986) and colon cancer (Jacob, 1983). It was further reported that the antitumourigenic effect of selenium might vary according to the dose amount and form (Ip et al., 1985).

In our previous study, we showed chemopreventive activity of diphenylmethyl selenocyanate, a synthetic organoselenium compound, which effectively restricted the tumour burden of animals during DMBA - croton oil induced two-stage mouse skin carcinogenesis process by inhibiting lipid peroxidation and induction of phase II detoxifying enzymes in target organ skin (Das et al., 2004) and also in liver (Das et al., 2004) and at the same time by inducing apoptosis and inhibiting cell proliferation (Das et al., 2005) at the initiation and pre + post initiation of carcinogenesis process. The compound also showed inhibitory effects against azoxymethane induced colonic neoplasia (aberrant crypt formation) in rats in the same manner (Ghosh et al., 2005).

In continuation of our previous experiments, this present study was undertaken to evaluate the antitumour promoting activity of diphenylmethyl selenocyanate in the post initiation phase of carcinogenesis with use of a two-stage mouse skin cancer model induced by DMBA and promoted by phorbol ester type tumour promoter croton oil.

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Materials and Methods

Chemicals

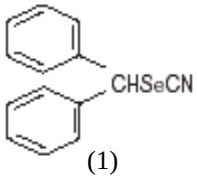
DMBA, 1-chloro-2, 4-dinitrobenzene (CDNB), ethylene diamine tetra acetic acid (EDTA), reduced glutathione (GSH), pyrogallol, sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA) and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). Hydrogen peroxide 30% (H_2O_2) was obtained from Merck Ltd., India.

Animals

Adult (5–6 weeks) Swiss albino female mice (22±2 g), bred in the animal colony of Chittaranjan National Cancer Institute, Kolkata, used for this study, were maintained at controlled temperature under alternating light and dark conditions. Standard food pellets and drinking water was provided ad libitum.

Synthesis and Structure of the Compound

Diphenylmethyl selenocyanate (1) was prepared following the procedure reported earlier (Das et. al., 2004). The compound is 99.9% pure. The purity was determined by using HPLC: Waters [μ-Bondapak C_{18} steel column; 30cm x 3.9mm; isocratic mobile phase acetonitrile-water (50:50) at a flow rate of 1.0 ml/min at ambient temperature; UV detection at 220nm; retention time 9.11min]. Purity of compound was checked at a regular interval during the experiment.



(1)

Drug Preparation

Synthetic organoselenium compound diphenylmethyl selenocyanate was used as a suspension in 5.5% propylene glycol in water, prepared each day of experiment, just before treatment.

Experimental Groups

All the experiment in this model was done in 2 different sets. Mice were divided into four groups (groups I-IV). Each group comprised 20 mice. The backs of the animals in each group were shaved 2 days prior to the start of the experiment. In each set all the experimental groups were categorized in accordance with parameter to be evaluated after the stipulated period of the treatment of carcinogen and treatment compound.

Vehicle Control (Gr. I):

Animals received topical application of acetone (100 μ l/mouse) on the shaved skin and propylene glycol (300 μl/mouse) by oral gavages for 12 weeks and served as vehicle controls.

Carcinogen Control (Gr. II):

Animals received two topical applications of DMBA at an interval of 72 h, at a dose of 0.05 gm./kg. b. w. in acetone (100 μl/mouse), followed by croton oil (1% w/v) in acetone (100 μl/mouse), twice a week for 9 weeks starting from day 8 of first DMBA application and served as carcinogen control.

Treatment Group (Gr. III):

Animals in this group received the same treatment as for group II and also received the selenium compound at a dose of 2mg./kg. b.w. from the day (on day 8th after the 1st DMBA application) of croton oil treatment.

Treatment Group (Gr. IV):

Animals in this group received the same treatment as for group II and also received the selenium compound at a dose of 3mg./kg. b.w. from the day (on day 8th after the 1st DMBA application) of croton oil treatment.

Mice of group I, II, III, IV were sacrificed following the guidelines as mentioned by the ethical committee of our Institute (Regn. no- IAEC-1.2/SD1/2001- 2005) after 12 weeks of 1st DMBA application and the parameters described below were studied.

Detection of Papillomas

The experimental animals were carefully examined weekly upto 12th week for counting and recording the incidence of papilloma and the number of papilloma per papilloma bearing mouse. Skin papillomas with a diameter greater than 1mm that persists for at least two consecutive observations were used for counting. The papillomas, which regressed after one observation, were not considered for counting. Two different experts who were not concerned with information regarding the experimental groups performed the measurement of papillomas.

Biochemical Estimation

Quantitative Estimation of Liver and Skin Lipid Peroxidation:

Lipid peroxidation was estimated in liver and skin microsomal fraction. The level of lipid peroxides formed was measured using thiobarbituric acid and expressed as nano mole of thiobarbituric acid reactive substances (TBARS) formed per mg of protein using extinction co-efficient of 1.56×10^5 M^{-1} cm^{-1} (Okhawa et al., 1974).

Estimation of Liver and Skin Glutathione - S - Transferase (GST) Activity:

Glutathione -S-Transferase (GST) activity was measured in the liver and skin cytosol (Mulder, 1995). The enzyme activity was determined from the increase in absorbance at 340nm with 1-chloro-2-4-dinitrobenzene (CDNB) as the substrate and specific activity of the enzyme expressed as formation of 1-chloro- 2-4-dinitrobenzene (CDNB)-GSH conjugate per minute per mg of protein. (Habig et al., 1974).

Estimation of Liver and Skin Superoxide Dismutase (SOD) Activity:

SOD activity in liver and skin cytosol was assayed by

the method of Marklund et al. (Marklund et al., 1974) and the partial extraction and purification of SOD was done as described by Mc Cord et al. (McCord et al., 1969). Superoxide dismutase (SOD) activity was determined by quantification of Pyrogallol auto oxidation inhibition and expressed as unit/mg of protein.

One unit of enzyme activity is 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) is measured by increase in absorbance at 420 nm. .

Estimation of Liver and Skin Catalase (CAT) Activity:

Activity of catalase (CAT) in liver and skin cytosol was determined spectrophotometrically at 250nm according to the method of Luck (Luck, 1963) and expressed as units per milligram of protein where the unit is the amount of enzyme that liberates half the peroxide oxygen from H_2O_2 in 100 sec at 25˚C.

Estimation of Protein:

Protein concentration in each sample was estimated spectrophotometrically (Lowry et al., 1951) with bovine serum albumin as standard.

Statistical Analysis:

The data obtained from each experiment were analyzed by ANOVA followed by Tukey's test (SYSTAT 9.0; SPSS Inc., Chicago, USA) to identify the differences between the means of different groups. The P-value of <0.05 was considered significant.

Results

Effect of Diphenylmethyl Selenocyanate on the Development of Papillomas:

The incidence of papillomas in DMBA-croton oil treated and DMBA-croton oil/selenium compound-treated groups at different weeks is shown in figure 1. The incidence in DMBA-croton oil treated mice (Gr. II) reached 96% after 12 weeks of experiment. Mice of Gr. III that received selenium compound treatment at the post-initiation phase of the experiment i.e. from the day of croton oil application at a dose of 2mg/kg body weight showed a tumour incidence of 46.66%, whereas animals of Gr. IV receiving the drug at a dose of 3mg/kg body weight showed 28.58% tumour incidence. Animals of Gr. I showed no incidence of papillomas.

The Numbers of Papillomas per Papilloma-bearing Mouse:

The cumulative numbers of papilloma per papilloma bearing mouse received the selenium compound (Gr. III & IV) from the day of croton oil application were significantly lower (P<0.01) than in the carcinogen alone control group (Gr. II). The mean numbers of papillomas per papilloma bearing mouse were 1.64±0.08 for Gr. III and 1.33±0.10 in Gr. IV whereas that in Gr. II was found to be 3.97±0.70 (Fig. 2).

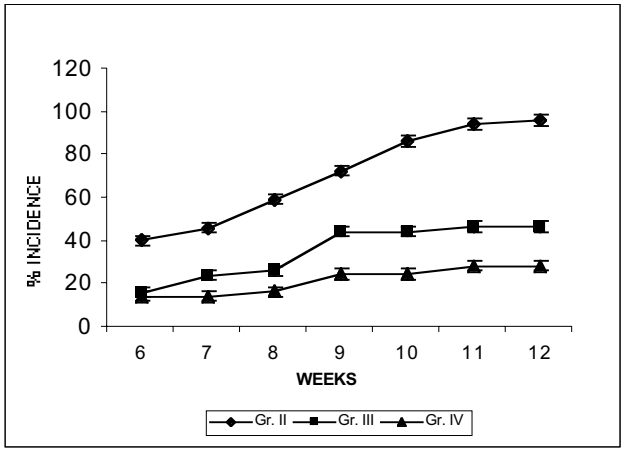


Figure 1. Percentage Incidence of Papilloma in Different Groups after 12 weeks of DMBA Application. Gr. II: carcinogen control; Gr. III and IV: Post initiation treatment groups. Incidence was significantly reduced (P<0.01) in different treatment groups with respect to the corresponding values obtained in the carcinogen control group in a dose dependent manner, with the highest level of inhibition in Gr. IV

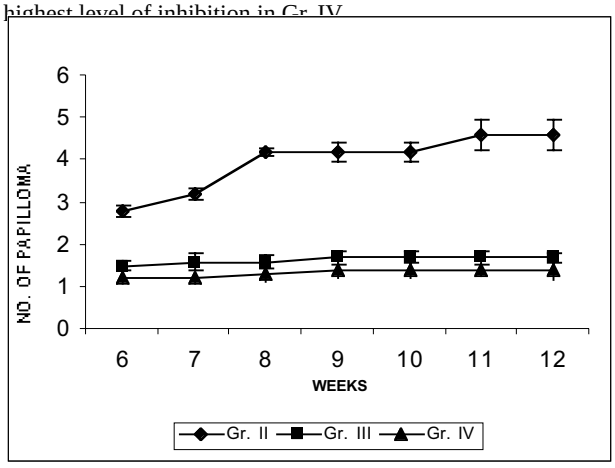


Figure 2.Cumulative Number of Papilloma per Papilloma-bearing Mouse after 12 weeks of DMBA Application. Gr. II: carcinogen control; Gr. III and IV: Post initiation treatment groups. Significant reduction (P<0.01) in the cumulative number of papilloma was observed in different Se-compound treated groups in comparison to that observed in the carcinogen control group.

Biochemical Studies in Mouse Liver and Skin

Hepatic Microsomal Lipid Peroxidation Level:

The level of lipid peroxidation in the liver microsomes of the animals treated with DMBA-croton oil (Gr. II) increased significantly (P<0.01) by 73.83% after after 12 weeks of treatment, compared with vehicle treated animals (Gr. I). The level of lipid peroxidation was decreased significantly (P<0.01) by 57.38% (Gr. III) and 63.29% (Gr. IV) after 12 weeks of treatment by the selenium compound as compared to the level in DMBA-croton oil treated animals (Gr. II) (Figure 3A).

Lipid Peroxidation Level in Skin Microsomes:

The level of lipid peroxidation in skin of the animals treated with DMBA- croton oil (Gr. II) increased significantly

(p<0.01) by 99.04% after 12 weeks of treatment compared to vehicle treated animal (Gr. I). The level of lipid peroxidation was decreased significantly (p<0.01) by 62.30% (Gr. II) and 75.39% (Gr. IV) after 12 weeks of treatment by the selenium compound as compared to the level in DMBA - croton oil treated animals (Gr. II) (Figure 3B).

Glutathione-S-transferase Activity in Liver:

The activity of GST in the liver of animals treated with DMBA-croton oil (Gr. II) showed a decrease of 73.74% after 12 weeks of DMBA treatment as compared with the vehicle treated animals (Gr. I). On treatment with diphenylmethyl selenocyanate, the GST activity rose sharply by 49.20% (Gr. III) and 73.75% (Gr. IV) when measured after 12 weeks treatment compared with the DMBA-croton oil control (Gr. II) (Figure 4A).

Glutathione -S-Transferase Activity in skin:

As compared with the vehicle treated animals (Gr. I), the activity of glutathione-S-transferase in the skin of animals treated with DMBA - croton oil (Gr. II) showed a decrease of 52.05% (p<0.01) after 12 weeks of DMBA treatment. On treatment with diphenylmethyl selenocyanate, the GST activity rose sharply by 63.15% (Gr. III) and 73.07% (Gr. IV) when measured after 12 weeks of DMBA treatment compared to the DMBA-croton oil control (Gr. II) (Figure 4B).

Superoxide Dismutase Activity in Liver:

A significant (P<0.01) decrease of 61.57% in the level of this enzyme activity was observed in DMBA- croton oil-treated animals (Gr. II) in comparison with the vehicle-treated mice (Gr. I) after 12 weeks of the experiment. This level was significantly (P<0.01) elevated by 52.70% (Gr. III) and 63.41% (Gr. IV) in the Se-compound treated groups after 12 weeks of treatment (Figure 5A).

Superoxide Dismutase Activity in skin:

A significant (p<0.01) decrease of 54.51% in the level of this enzyme activity was observed in DMBA - croton oil treated animals (Gr. II) in comparison to the vehicle treated mice (Gr. I) after 12 weeks of the experiment. This level were significantly (p<0.01) elevated by 70.89% (Gr. III) and 82.39% (Gr. IV) in the selenium compound treated group after 12 weeks of treatment (Figure 5B).

Catalase Activity in Liver:

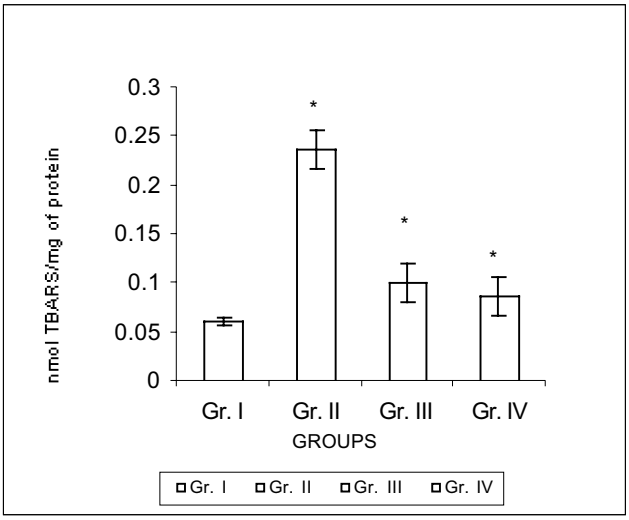
The activity of this enzyme significantly decreased in the carcinogen control group (Gr. II) compared with the vehicle-treated mice (Gr. I) after 12 weeks (76.81%) of the experiment (P<0.01). A significant (P<0.01) elevation of this enzyme activity was noted after 12 weeks (43.99% (Gr. III) and 70.18% (Gr. IV) after 12 weeks in different drug-treated groups as compared to the carcinogen control group (Gr. II) (Figure 6).

Catalase activity in skin:

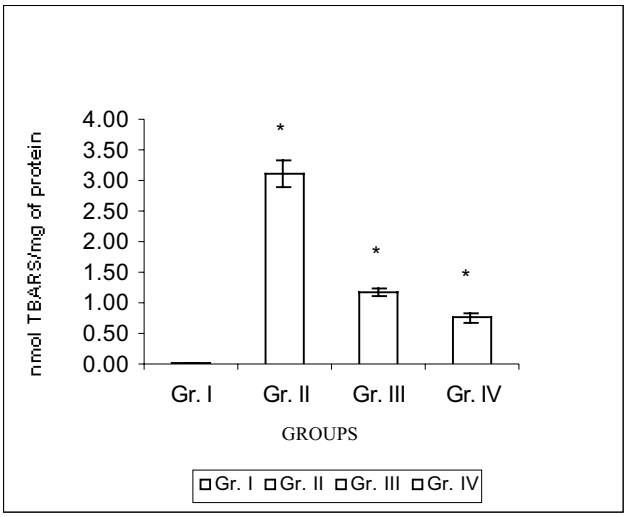
No significant alteration of catalase activity was observed in the skin cytosol after 12 weeks of experiments.

Discussion

Selenium was identified more than four decades ago as



A

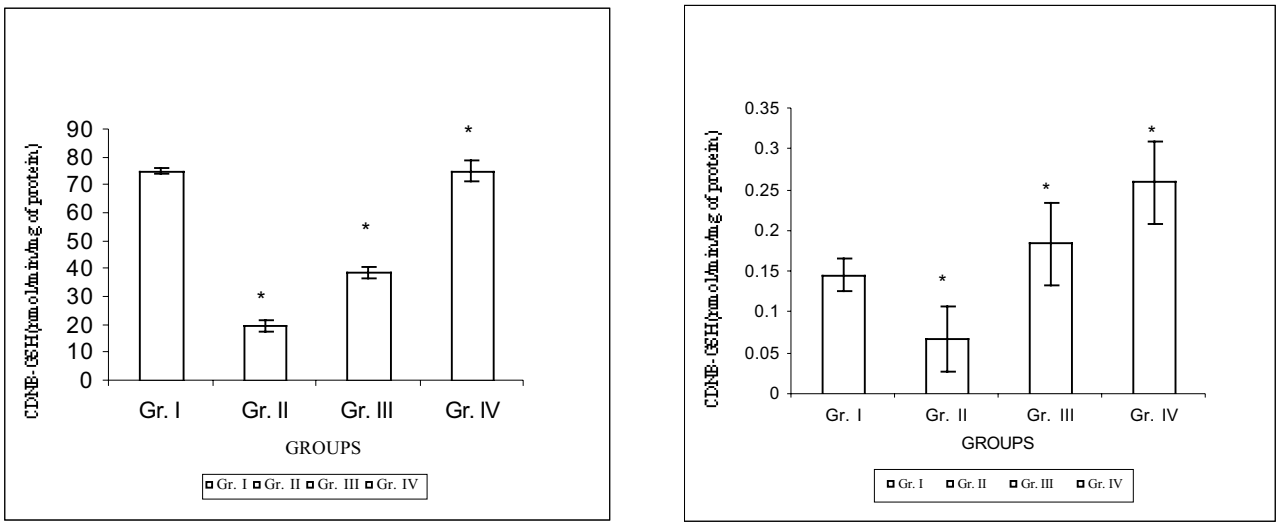


B

Figure 3A-B. Quantitative Estimation of Lipid Peroxidation in Liver and Skin Microsomes After 12 weeks

Data represent the Mean ±SE. * p<0.01. Comparisons are made in the text between

1. Gr. I (vehicle control) and Gr. II (DMBA+Croton oil treated carcinogen control group)
2. Gr. II (Carcinogen control) and Gr. III (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 12 weeks from the day of croton oil application)
3. Gr. II (Carcinogen control) and Gr. IV (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 12 weeks from the day of croton oil application)

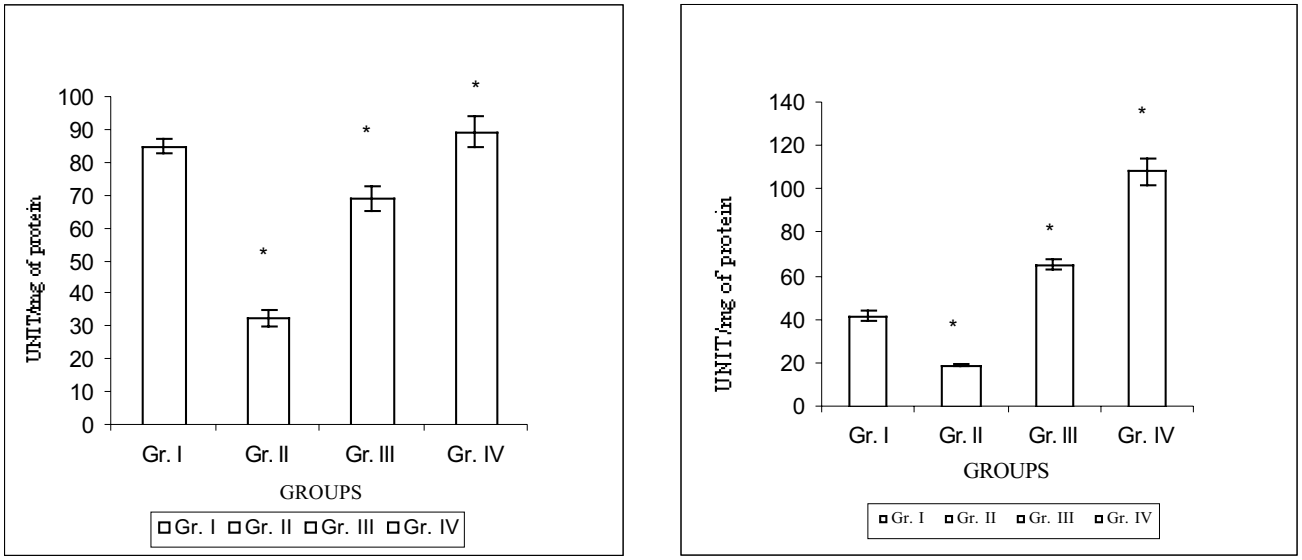


A B

Figure 4A-B. Estimation of GST Activity in Liver and Skin Cytosol after 12 Weeks

Data represent the Mean ±SE. * p<0.01. Comparisons are made in the text between

1. Gr. I (vehicle control) and Gr. II (DMBA+Croton oil treated carcinogen control group)
2. Gr. II (Carcinogen control) and Gr. III (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 12 weeks from the day of croton oil application)
3. Gr. II (Carcinogen control) and Gr. IV (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 12 weeks from the day of croton oil application)



A B

Figure 5A-B. Estimation of SOD Activity in Liver and Skin Cytosol after 12 Weeks

Data represent the Mean ±SE. * p<0.01. Comparisons are made in the text between

1. Gr. I (vehicle control) and Gr. II (DMBA+Croton oil treated carcinogen control group)
2. Gr. II (Carcinogen control) and Gr. III (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 12 weeks from the day of croton oil application)
3. Gr. II (Carcinogen control) and Gr. IV (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 12 weeks from the day of croton oil application)

an essential nutrient for animals and humans. It is being recognized as an essential component of a number of enzymes responsible for antioxidatve function. Different naturally and synthetic selenium compounds have been found till date to inhibit tumorigenesis in a variety of animal models. *In vitro* and *in vivo* studies also indicate that selenium intervention in the form of organoselenocyanate can delay, reverse or/and inhibit at all the stages of carcinogenesis process (Gerald et al., 1998; Schwarz et al., 1977). Furthermore, there are limited evidences that support the theory that dietary selenium can prevent the growth of transplanted tumours in animals (Medina et al., 1980; Ip C et al., 1981; Watrach et al., 1984). Studies also revealed that various selenocompounds, in a dose dependent manner,

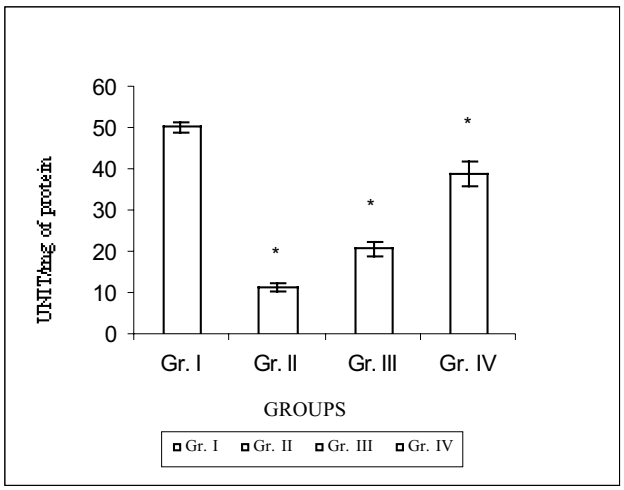


Figure 6. Estimation of Catalase Activity in Liver Cytosol after 12 Weeks

Data represent the Mean ±SE. * $p<0.01$. Comparisons are made in the text between

1. Gr. I (vehicle control) and Gr. II (DMBA+Croton oil treated carcinogen control group)
2. Gr. II (Carcinogen control) and Gr. III (DMBA-croton oil + drug treatment at a dose of 2mg /kg. b.w. for 12 weeks from the day of croton oil application)
3. Gr. II (Carcinogen control) and Gr. IV (DMBA-croton oil + drug treatment at a dose of 3 mg /kg. b.w. for 12 weeks from the day of croton oil application)

induced apoptosis of tumour cells in culture (Lu et al., 1994; Thompson et al., 1994; Wu et al., 1995). These growth-inhibiting and apoptosis inducing mechanism may be important in the promotion phase of tumour development, where there is a clonal expansion of preneoplastic cells that escaped the normal death machianry.

In the present study two-stage mouse skin carcinogenesis model was used to evaluate the antitumour promoting activity of diphenylmethyl selenocyanate, as in this model all the three stages (initiation-promotion-progression) of malignant transformation of normal keratinocytes of skin to squamous cell carcinoma is prominent (Senmez et al., 2003). Metabolization of DMBA by Cyp1A1 and Cyp1B1, the two enzymes of the Cyp-450 family (Kawajiri, 1999; Angus et al., 1999 and Buters et al., 1999), produces the ultimate carcinogen 1,2-epoxide-3, 4-diol DMBA, which form adducts with DNA (Cheng et al., 1988b and Cheng et al., 1988a). This adducts lead to mutations, which are a prerequisite for the development of mallignancy. The tumour promotion stage; clonal expansion of selective hyperplastic keratinocytes can be triggered by repeated application of croton oil, which is rich in phorbol esters, ultimately producing a squamous papilloma (Allen et al., 2003 and Slaga et al., 1996). The tumour progression is characterized by high level of genetic instability that leads to chromosomal alterations and malignant tumour formation (Warren et al., 1993).

Phorbol ester type tumour promoter stimulates the production of reactive oxygen species by polymorphonuclear leukocytes, macrophages and non-phagocytic cells (Singh et al., 1985). Repeated topical application of the tumour promoter croton oil on mouse skin involves both oxidative burst as well as generation of nitric oxide (Das et al., 2004). Phorbol ester type tumour promoter also can activate Protein kinase C (PKC), which regulates tumour promotion and cell proliferation by inducing activation of transcription factor such as activator protein 1 (AP-1) and nuclear factor - kappa B (NF-kappa B) (Gopalakrishna et al., 1989; Gopalakrishna et al., 1991; Larsson et al., 1989), and by enhancing the expression of several growth regulatory key enzymes, such as ornithine decarboxylase (ODC) (Fischer et al., 1993; O' Brien et al., 1997), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Gopalakrishna et al., 2000). Results obtained from the present study demonstrated that, the incidence and number of skin papillomas formed by application of DMBA-croton oil can be significantly controlled by treatment with diphenylmethyl selenocyanate at the post initiation phase. Maximum inhibition was noted at the dose of 3mg/kg. b.w. (Gr. IV).

Selenium compounds are able to reduce hydrogen peroxide, lipid and phospholipid hydroperoxide, thereby restrict the propagation of lipid peroxidation by inhibiting the generation of reactive oxygen species leading to DNA damage (Matsumoto et al., 2001). The significant decrease in lipid peroxidation levels by post-treatment with the selenium compound indicates its role in reducing oxidative stress. Here also after 12 weeks of experiment maximum effect was observed at a dose of 3 mg/kg body weight (Gr. IV), which is in line with the observation made in the incidence and number of papilloma formation.

During the multistage carcinogenesis process the activity of antioxidant enzymes tend to decrease which leads to a pro-oxidant state of the cell, facilitating tumour promotion and progression (Oberly et al., 1993). It is noted from the present study that after repeated topical application of promoter, to the DMBA treated animals skin, lowered the level of antioxidant enzymes such as GST, catalase and SOD in metabolic organ liver as well as in target organ skin, suggesting involvement of these enzymes for the detoxification and inactivation of ultimate carcinogenic metabolites and reactive oxygen species, responsible for DNA damage. Treatment with diphenylmethyl selenocyanate from the day of promoter application elevated the levels of GST, catalase and SOD significantly.

Given the importance of selenium as an important inhibitor of tumour promoter, the molecular target has to be identified. PKC serves as a receptor for tumour promoters, including oxidants and lipid hydroperoxides, and activated by these agents. Selenometabolites acts on the same cellular target on which tumour promoters act, but they induce the inactivation of this kinase, which bring an efficient deregulatory mechanism to block the signal transduction pathway responsible for tumour promotion and progression by the tumour promoters (Kausar et al., 2003). Ornithine decarboxylase (ODC) an enzyme responsible for polyamine biosynthesis is upregulated by tumour promoter. Similarely, COX-2 protein, which is responsible for prostaglandin

biosynthesis is also induced by phorbol ester type tumour promoer. Selenium compounds were reported to inhibit ODC as well as COX-2 activity (El-Bayoumy et. al., 2001).

Thus, it seems clear at this point of investigation that the antitumour promoting activity of diphenylmethyl selenocyanate may be due to the combined effect of enhancing detoxification enzymes activity and downregulation of lipid peroxidation by controlling oxidative burst, that maintains the favorable shift in the intracellular oxidation/reduction balance. Whether, the compound has any effect on blocking the signal transduction pathway triggered by activated PKC which intern activates key enzymes related to cell proliferation and progression due to promoter insult or has the ability to downregulate ODC and COX-2 activity at the promotion stage are to be studied.

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