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**ORIGINAL RESEARCH ARTICLE**

# Dose Tolerance Study of Diosmin Against 7, 12-Dimethylbenz (a)Anthracene (DMBA) Induced Hamster Buccal Pouch Carcinogenesis

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## ABSTRACT

Naturally occurring phytochemicals consumed in foods that may prevent development of neoplastic transformation is of high prioritized manner. Diosmin is a flavonoid, most abundantly found in many citrus fruits. As a flavonoid, it possesses a multitude of biological activities including anti-hyperglycemic, anti-lipid peroxidative, anti-inflammatory, antioxidant, and anti-mutagenic properties. However, anticarcinogenic effects of diosmin not have yet to be fully explored. The objectives of the present study were to assess protective effect of diosmin against 7, 12-dimethylbenz (a)anthracene (DMBA) induced buccal pouch carcinogenesis in male golden Syrian hamsters.

**Methods:** Oral squamous cell carcinomas developed in the left buccal pouch of hamsters on painting with 0.5% of DMBA, three times in a week. To assess protective potential of Diosmin, it was orally administered to DMBA treated hamsters on alternate days from DMBA painting for 14 weeks.

**Results**: We observed 100% tumor formation with marked levels of neoplastic changes and altered biochemical and histological expressions were observed in the DMBA alone painted hamsters as compared to control hamsters. Oral administration of Diosmin at a dose of 100 mg/kg b.wt to DMBA treated animals on alternative days for 14 weeks significantly reduced the neoplastic changes and improved the status of biochemical and histological expressions. These observations confirmed that Diosmin acts as a tumor suppressing agent against DMBA induced oral carcinogenesis.

**Keywords:** Diosmin, DMBA, hamster, oral squamous cell carcinoma.

## INTRODUCTION

Oral cancer is a major contributor of cancer related deaths worldwide, estimated 263,000 new cases of oral cavity cancer arise worldwide, resulting in approximately 127,000 deaths [20]. Data from the National Cancer Registry Project of the Indian Council of Medical Research (ICMR) also confirm that oral cancer is common in India [41]. Oral squamous cell carcinoma is associated with a number of risk factors, of which tobacco and alcohol use present the greatest opportunity for intervention. Epidemiological research and the scientific literature have demonstrated that the use of tobacco and alcohol as well as human papiloma virus infection (HPV) are the predominant causative factors for oral carcinoma, which arises through a multistep process of cumulative genetic alterations leading to a loss of cell cycle regulation [26,37].

The golden Syrian Hamsters are the suitable animal model for OSCC, which is anatomically and physiologically similar with human population [25]. DMBA, one of the most potent carcinogen, has been extensively studied in view of the finding that it can induce not only squamous but also lung and mammary carcinomas [7, 35] in the human population. DMBA is metabolized to dihydro diol-epoxide, the ultimate carcinogen, which mediates carcinogenic process by inducing chronic inflammation, over production of reactive oxygen species (ROS) and oxidative DNA damage [39].

Flavonoids are a group of plant polyphenols that are generally found in vegetables, fruits, herbs, tea, and wine as secondary metabolites and have received much attention due to their anti-inflammatory and antioxidant activities (3).

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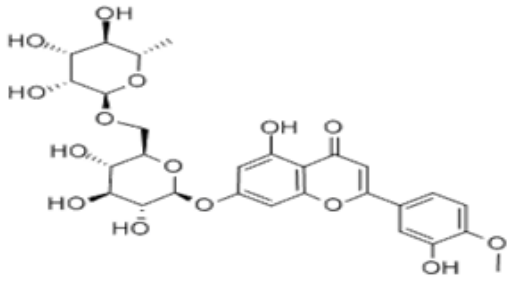
Diosmin ((3′,5,7-trihydroxy-4′-methoxyflavone-7ramnoglucoside) (**Fig 1**) is a flavonoid; most abundantly found in citrus fruits. As a flavonoid, it possesses a multitude of biological activities including anti-hyperglycemic [37], anti-lipid peroxidation [5] anti-inflammatory, antioxidant, and anti-mutagenic properties [8]. Diosmin exhibits attractive chemopreventive agent in urinary-bladder [46] colon carcinogenesis [42] as well exhibits an anti-inflammatory effect, and regulation of TNF-α, NF-kB and iNOS activation [40]. Furthermore diosmin prevented oxidative stress by restoring the levels of antioxidant enzymes, decreases DNA disintegration, and modulation of Bax and p53 gene expression [32]. However, there is no scientific literature were available to validate the chemopreventive potential of diosmin in DMBA induced oral carcinogenesis, for that reason the present study was designed to investigate the dose-tolerance effect of diosmin in the DMBA induced oral carcinogenesis by measuring the LPO, SOD, GPx, CAT, glutathione (GSH) and vitamin E& C as biochemical end point of chemoprevention.



**Structure of Diosmin**

## MATERIALS AND METHODS

### Chemicals

7, 12-dimethylbenz (a)anthracene, Diosmin and analytical grade methanol, were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). All other chemicals used in this study were of highest analytical grade obtained from Sisco Research Laboratories and Himedia, Mumbai, India.

### Animals

Male Syrian hamsters (80 ± 120 g), were obtained from National Institute of Nutrition, Hyderabad, India. Animals were housed in groups at constant temperature (23 ± 2˚C), and a light/dark (12 h/12 h) cycle. The animals were allowed to free access to food (VRK Nutritional Solutions, Maharashtra, India) and water (ad libitum) throughout the experimental period. The experiments were designed and conducted in accordance with the institutional ethical guidelines (Register number 1(6)0/1999/CPCSEA).

### Experiential design

The male golden Syrian hamsters were divided into 8 groups of 6 each. Group I, serve as an untreated control. The Group II treated with DMBA alone (0.5%) Group III to Group VII were painted with 0.5% DMBA in liquid paraffin three times a week for 14 weeks on the left buccal pouches using (No. 4 brush) to induce the buccal pouch carcinogenesis. Group III-VII animals were orally administered with Diosmin (80, 90, 100, 110 and 120mg/kg b.wt; dissolved in 0.5% DMSO) starting one week before the exposure to the carcinogen and continuing on alternate days of the DMBA painting until the animals were sacrificed. However, Group VIII animals were orally administrated with Diosmin alone (120mg/kg b.wt) to exclude any toxic effects.

### Sample collections

At the end of the experimental period, the hamsters were sacrificed by cervical decapitation under anesthetic conditions (Xylazine 30 mg/kg, i.p.). The control and experimental animal buccal tissues were immediately removed, washed using ice-cold phosphate buffer solution (pH 7.4), and then the buccal tissues were used for assessment of histological studies.

### Biochemical assays

After plasma separation, the buffy coat was removed and the packed cells were washed three times with physiological saline. A known volume of erythrocytes was lysed with hypotonic buffer at pH 7.4. The hemolysate was separated by centrifugation at 10,000 rpm for 15 min at 20°C. The erythrocyte membrane was prepared using the method (13) as modified by [31]. Thiobarbituric acid reactive substances (TBARS) were assayed in the plasma and erythrocytes according to the methods of [45,14] respectively. Reduced glutathione (GSH) was determined using the method of [6] Vitamins C and Vit E were measured according to the methods of [29,12] respectively. Activities of the enzymatic antioxidants SOD, CAT and GPx were estimated using the methods of [21,33,34] respectively. GST activity was assayed using the method of [19] Glutathione reductase activity was assayed according to the method of [9].

### Statistical Analysis

Values are expressed as mean ±SD. Statistical analysis was performed by One-way analysis of variance (ANOVA) followed by Duncan's

Multiple Range Test (DMRT). The Values were considered statistically significant if the p-value was less than 0.05.

## RESULTS

The tumor incidence, tumor volume, tumor burden, and histopathological changes in control and experimental groups are shown in (**Table 1**). In DMBA alone painted animals, the tumor incidence was 100%. These tumors were large and exophytic with a mean tumor burden of 97.01 mm3, histologically identified as well defined squamous cell carcinoma. Diosmin administration effectively suppressed the development of HBP carcinomas. In group 5 animals, Diosmin (100mg/kg) mean tumor burden of 21.70 mm3. (**Fig 1**) shows Mild to moderate preneoplastic lesions (hyperplasia, keratosis and dysplasia) were observed in DMBA and Diosmin-treated animals. And the effective dose 100mg/kg shows moderate dysplasia and hyperplasia. Control animals showed a well defined, intact epithelial layer whereas animal treated with DMBA only revealed hyperkeratosis, hyperplasia, dysplasia and moderately differentiated squamous cell carcinoma. A thick, rough and reddish oral mucosa was seen in DMBA only-treated hamsters after 14 weeks. Dysplasia with minimal tumor growth and well-developed oral squamous cell carcinoma were seen in the mucosa of the DMBA only-treated animals after 10 and 14 weeks, respectively.

Oral administration of Diosmin (80, 90, 100, 110, 120mg/kg bw) have been tested on DMBA painted animals. The 16 weeks DMBA application in hamster buccal pouch ensued well-developed oral squamous cell carcinoma (OSCC) with very mean tumor burden linked with severe hyperplasia, hyperkeratosis, and dysplasia. However, effective dose of diosmin (100 mg/kg bw) showed significant antioxidant activity and crucial tumor inhibitory effects than remaining high and low doses, which was studied by following parameters.

(**Fig 2**) shows the levels of TBARS and enzymatic antioxidants (SOD, CAT and GPx) in plasma, erythrocyte and buccal tissue of control and experimental animals. Increased the levels of thiobarbituric acid reactive substances (TBARS) in plasma and erythrocyte whereas decreased in buccal tissues. However, Oral administration of diosmin (80-120mg/kg bw) to DMBA painted animals significantly restored the levels of TBARS when compared to that of control animals. Among the tested doses the 100 mg/kg b.wt of diosmin effectively restored the the levels of TBARS and antioxidants status. Hamsters treated with diosmin (120mg/kg bw) alone did not show any significant difference.

(**Fig 3**) revealed levels of non-enzymatic antioxidants (Glutathione reductase (GSH), Vitamin-C and Vitamin-E) in plasma, erythrocyte and buccal tissue of control and experimental animals. The reduced levels of non-enzymatic antioxidants such as glutathione reductase (GSH), Vitamin-C and Vitamin-E in were observed in plasma and erythrocyte whereas the levels were significantly increased in buccal tissue of DMBA painted animals when compared to control animals. The animals treated with the effective dose of diosmin (100mg/kg bw) were able to retrieve the GSH, Vit-C and Vit-E levels to a significant level i.e, normal against DMBA induced toxicity when compared to control animals. Animals treated with diosmin alone (120mg/kg bw) showed no significant differences were observed when compared to control animals.

**Table 1: Incidence of oral neoplasm and histological changes in the control and experimental animals in each group**

| Parameters | Control | DMBA | DMBA+Diosmin (80 mg/kg b.wt) | DMBA +Diosmin (90 mg/kg b.wt) | DMBA +Diosmin (100 mg/kg b.wt) | DMBA +Diosmin (110 mg/kg b.wt) | DMBA +Diosmin (120 mg/kg b.wt) | Diosmin alone (120 mg/kg b.wt) |
|---|---|---|---|---|---|---|---|---|
| Tumor incidence | - | 100 | 80 | 20 | 70 | 70 | 70 | - |
| Total number of tumors/animals | - | 10/6 | 7/6 | 8/6 | 3/6 | 6/6 | 7/6 | - |
| Tumor volume (mm3) | - | 97.01±9.63 | 84.76±8.49 | 72.41±1.08 | 21.70±2.10 | 54.70±2.10 | 65.70±2.10 | - |
| Keratosis | No change | Severe | Mild | Mild | Moderate | Moderate | Moderate | No Change |
| Hyperplasia | No change | Severe | Severe | Mild | Moderate | Moderate | Severe | No Change |
| Dysplasia | No change | Severe | Severe | Mild | Moderate | Moderate | Severe | No Change |
| Squamous cell carcinoma | No change | Moderate | Well | Well | Mild | Well | Well | No Change |

Data are expressed as the mean ± SD for 6 hamsters in each group. Values not sharing a common superscript letter in the same row differ significantly at p<0.05 (DMRT). Tumor volume was calculated by the formula $v = \frac{4}{3}\pi\left(\frac{D1}{2}\right)\left(\frac{D2}{2}\right)\left(\frac{D3}{2}\right)$, where D1, D2, and D3 are the three diameters (mm) of the tumors.

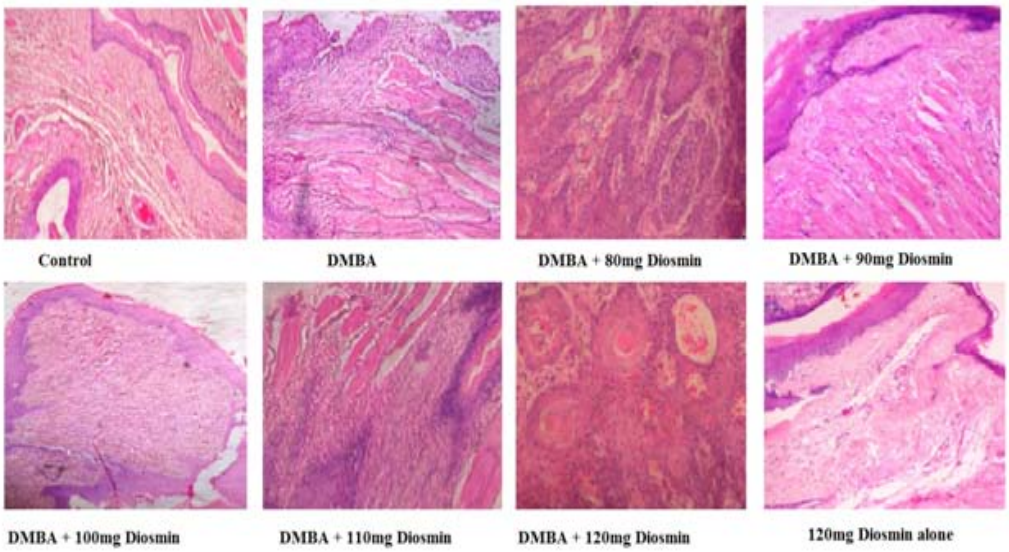



**Figure 1**: **Histopathological changes observed in the control and experimental animals in each group**

Histograph of untreated control animals, picturising normal epithelium in buccal mucosa. Microphotograph of DMBA alone treated animals, picturising well defined squamous cell carcinoma with hyper chromatic nuclei containing epithelial and keratin pearls.
Histograph of DMBA+ Diosmin(80 mg/kg b.wt) treated animals, picturising mild keratosis and severe dysplasia and hyperplasia.
Histograph of DMBA+ Diosmin(90 mg/kg b.wt) treated animals, picturising mild keratosis dysplasia and hyperplasia.
Histograph of DMBA+ Diosmin (100 mg/kg b.wt) treated animals, picturising moderate dysplasia and hyperplasia.
Histograph of DMBA+ Diosmin(110 mg/kg b.wt) treated animals, picturising moderate keratosis dysplasia and hyperplasia
Histograph of DMBA+ Diosmin(120 mg/kg b.wt) treated animals, picturising moderate keratosis but severa dysplasia and hyperplasia
Histograph of Diosmin alone treated animals, picturising normal epithelium in buccal mucosa. (H and E 10 X).

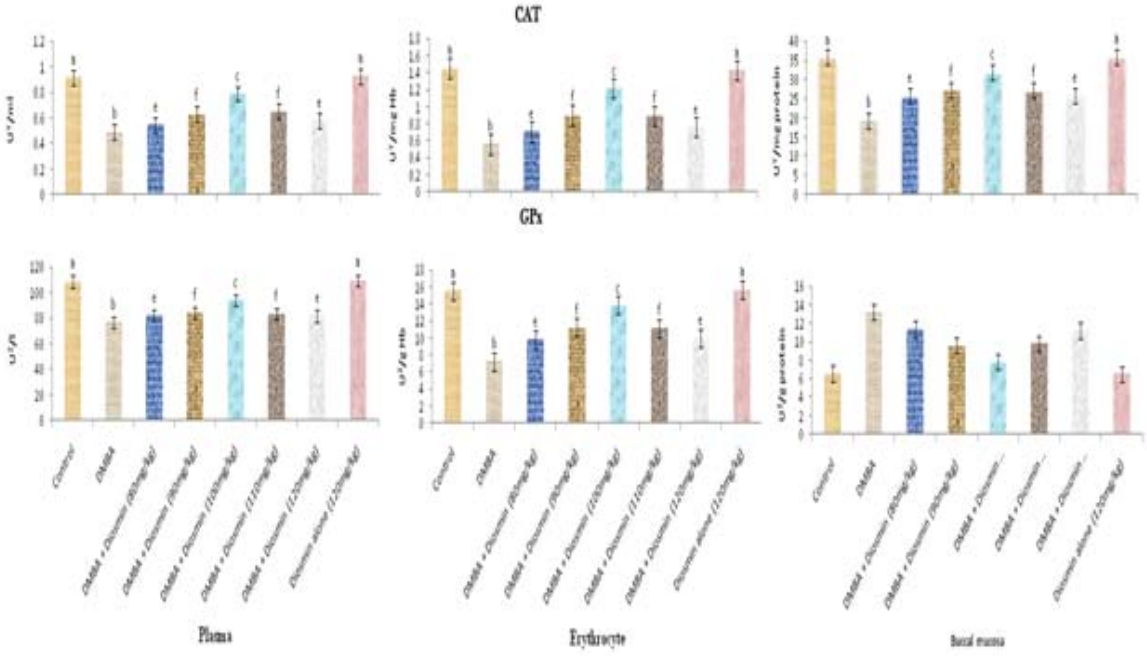



**Fig 3: Status of plasma, erythrocyte and buccal tissue for CAT and GPx assay in Control and Experimental animals in each group**

Data are expressed as Mean±SD for 6 hamsters in each group. Units for CAT** and GPx*** are expressed as enzyme required to inhibit 50% NBT reduction, micromoles of $H_2O_2$ utilized per second and micromoles of glutathione utilized per minute respectively. Values not sharing a common superscript letter in the same row differ significantly at $p<0.05$ (DMRT)

## DISCUSSION

Oral cancer is the most common neoplasm in India populations and has a tremendous impact on health and morbidity [17]. Though the incidence and mortality rates of oral cancer vary widely across the world, the highest rates reported every year from developing countries particularly from India [27]. Continued search for novel chemopreventive agents offers a promising new strategy for improving current cancer treatment [11]. Diosmin a naturally occurring flavones

glycoside readily obtained by dehydrogenation of hesperidin was act as particularly attractive chemopreventive agent [10]. Diosmin have strong HA22T cell viability inhibition in a dose dependent manner and significantly reduced the cell proliferative proteins as well as inducing cell cycle arrest in the G2/M phase through p53 activation and PI3K–Akt–MDM2 signaling pathway inhibition [15] .The synergistic antiproliferative effect shown by Diosmin and the lowest dose of IFN-alpha on metastatic pulmonary melanoma [1]. Chemopreventive potential for diosmin, naringin, naringenin and rutin towards CYP450 mediated mutagenesis of Heterocyclic amines [2]. Diosmin has been investigated in a number of animal models and human cancer cell lines, and has been found to be chemopreventive and antiproliferative agents [24,46] .

The polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)-anthracene (DMBA) serve as a potent organ and site specific carcinogen. It generates reactive oxygen species, such as peroxides, hydroxyl and superoxide anion radicals, which induce cellular oxidative damage through DNA strand breaks, lipid peroxidation and ultimately lead to carcinogenesis similar to human cancer [28]. Present study reveals Tumor incidence, tumor volume, tumor burden, and histopathological changes in control and experimental groups are shown in (Table 1). In DMBA alone painted animals, the tumor incidence was 100%. DMBA painting three times a week of 16 weeks caused OSCC with very mean tumor burden associated with severe hyperplasia, hyperkeratosis, and dysplasia selectively in the buccal mucosa of hamster. Oral administration of diosmin at various doses (80, 90, 100, 110 and 120mg/kg b.wt) possibly the effective dose (100mg/kg b.wt) significantly restored the malignant abnormalities.

Oxidative stress that arises due to an imbalance between generation of reactive oxygen species (ROS) and antioxidant defences has been implicated in malignant transformation and it produces deleterious effects by initiating lipid peroxidation directly or indirectly by acting as second messengers for the primary free radicals and the toxic metabolites of DMBA bind to adenine residues of DNA causing damage. Increased plasma and erythrocyte TBARS were decreased in buccal tissue could be due to over production of lipid peroxidation by product during DMBA metabolic activation as well as from the damaged host tissue [18]. In this study, we were observed an excessive generation of reactive oxygen species, as evidenced by increased formation of lipid peroxidation by product (TBARS) has been reported in DMBA induced hamster buccal pouch carcinogenesis. Previous report on the mechanisms and metabolic activity of diosmin significant dose (100 mg/kg bw) recover the concentration of TBARS in diabetic rat stated that antioxidant by scavenging free radicals properties of diosmin [38]. In the same line of attack oral administration of diosmin significant dose (100 mg/kg bw) to DMBA treated hamsters restored the TBARS levels in plasma and erythrocyte and buccal tissue, which suggests that diosmin has anti-lipid peroxidative potential during DMBA induced oral carcinogenesis.

Enzymatic and non-enzymatic antioxidants play crucial roles against ROS-mediated oxidative stress. Decreased levels of non-enzymatic antioxidants were reported in several types of carcinogenesis and enzymatic antioxidants such as SOD, CAT, GPx and GSH can protect cell and tissue damage from enhanced lipid peroxidation and over production of ROS [23]. DMBA application significantly reduces the SOD, CAT, GPx and GSH activities in plasma and erythrocytes and increase GPx and GSH levels in buccal tissue described in tumors is regarded as markers of malignant transformation [22]. The same lines of observation are also documented by [39]. Oral administration of diosmin significant dose (100 mg/kg bw) modulate these enzymatic antioxidants activities plus reflects a favorable balance between potentially harmful oxidants and protective antioxidants. Lipid soluble antioxidant Vitamin E present in plasma and erythrocyte membrane and vitamin C is an essential water-soluble antioxidant; both of which protect cell membranes from oxidative damage initiated by carcinogens [39]. Moreover, vitamin E reacts with lipid peroxy and alkoxy radicals and donates its labile hydrogen to the radicals and the reaction terminates. Decreased levels of non-enzymatic antioxidants in the plasma of tumor-bearing animals suggest that these antioxidants are utilized by malignant tumors to meet the nutrient demands of growing tumors or to combat the deleterious effects of ROS in the circulation system. Oral administration of diosmin restores Vit-C and Vit-E following reflects the regression of altered buccal tissue as a result of its oral protective role.

The present study clearly demonstrates the antioxidant and antitumor potential of Diosmin against DMBA induced oral carcinogenesis. Also 100 mg/kg of diosmin significantly up-regulate antioxidants and confer the protective effect of diosmin on DMBA induced oral cancer. Hence, this study also demonstrates that the chemopreventive potential of diosmin could be attributed to its modulatory effect on enzymatic and non-enzymatic antioxidants, lipid peroxidation, against DMBA induced hamster buccal pouch carcinogenesis.

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