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Research Article

Dose dependent study of phloretin against 7,12-dimethylbenz(a)anthracene (DMBA) induced hamster buccal pouch carcinogenesis.

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Abstract

The aim of the present study was aimed to investigate the chemoprevetive effects of phloretin against 7,12-dimethylbenz(a)anthracene (DMBA) induced experimental oral carcinogenesis in male golden Syrian hamster in a dose dependent manner. Forty eight male golden Syrian hamsters were divided into six groups with eight animals in each group. Group I animals served as untreated control. The left side of buccal pouches of the hamster rats in group II were painted with 0.5% solution of DMBA, three times a week for 16 weeks. The same pouches of group III-V were subjected to the same DMBA painting; but at the same time, the animals received 20, 40 and 80 mg/kg b.wt of phloretin were orally administrated for the same period for alternative days. Group VI hamsters were orally administrated with phloretin alone and served as the drug control. The experiment was terminated at the end of 14^{th} week. The experimental animal's tumours were subjected into morphological examination and consequently monitored the status of lipid peroxidation; activities of enzymic, nonenzymic antioxidants and detoxification enzyme status were measured in plasma, erythrocyte, buccal mucosa and liver of both control and experimental groups. Our primary findings reveal that increased level of lipid peroxidation, altered status of antioxidant enzyme status were observed in plasma, erythrocyte and buccal tissue of DMBA alone treated animals. Phase I & II detoxification enzyme activities were markedly drastically altered in DMBA alone treated animals. Oral administration of phloretin significantly restored back the altered status of lipid peroxidation, antioxidants and detoxifying enzymes thus achieving complete detoxification of the carcinogen. Out of threes the three different doses, phloretin at a dose of 40 mg/kg b.wt had shown very potential chemopreventive activity. These results suggest that oral administration of phloretin beneficial in the control of DMBA induced oral carcinogenesis by noticeable antioxidant and detoxifying properties.

Keywords: Antioxidants, DMBA, free radical, reactive oxygen species, phloretin.

Introduction

Oral cancer is one of the most serious problems in oncology. It is a leading cause of death among men in many countries. The American Cancer Society estimates that in 2009, approximately 192,370 women are newly diagnosed with this disease, and estimated death is 40,170. Significant geographic variation is noted in the incidence of oral cancer, with high rates reported for the Indian subcontinent and parts of Asia (male incidence rates in excess of 10 per 100,000 per annum). In India, cancer of the oral cavity is one of the five leading sites of cancer in either sex.

An alternative approach to cancer avoidance could be to increase the intake of chemopreventive compounds, which might be reasonably expected to interfere with multi stage of carcinogenesis (Ramakrishnan *et al.*, 2007). Bioactive compounds from plant origin have the potential to subside the biochemical imbalance

induced by various toxins associated with free radicals. They provide protection without causing any side effects, and therefore, development of drugs from plant products is desired (Mittal *et al.,* 2001). Phloretin is one of the phytochemicals of apple; several studies showed that phloretin was an effective pharmacological effects especially in relevant for cancers treatment (Hung *et al.,* 2013). Recent study reported that phloretin had potent inhibitory action on human oral cancer cells with selective cytotoxicity. It has potential cancer preventive effects with the antioxidant capacity and apoptotic activities in many cancers cell line (Zhu *et al.,* 2013).

Chemopreventive potential of a plant derived compound on chemically induced carcinogenesis is very often related to its ability to induce the activities of the antioxidant enzymes, which are indeed considered as the markers for carcinogenesis (Monari *et al.,* 2006). Cells are equipped with various antioxidants such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) etc, which can counteract the deleterious action of ROS and thereby protect the cell from cellular and molecular damage (Blessy *et al.,* 2009). Apart from the enzymic antioxidants, non-enzymic antioxidants, such as GSH, vitamins C and E, also play an important role in protecting the cells from oxidative stress. Several studies have been reported that detoxification enzymes provide a sensitive indicator of a distant neoplasm in rats (Linden *et al.,* 1989).

The purpose of the present study is to evaluate the cancer preventive effect of phloretin against the 7,12 dimethyl benz(a)anthracene (DMBA) induced oral carcinogenesis. Therefore, the aim of our study was to assess the status of lipid peroxidation and antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH), vitamin C and vitamin E and liver marker enzymes such GST, GR, cyto p450, cyt b5 in oral cancer bearing animals and compare with phloretin and control animals.

Materials and Methods

Chemicals

7,12 Dimethyl benz(a)anthracene, phloretin, reduced glutathione, dinitrophenyl hydrazine were purchased from Sigma Chemical Company, USA. All the other chemicals used were of analytical grade.

Animals

Male golden Syrian hamster aged between 50 and 55 days was purchased from National Institute of Nutrition, Hyderabad and was housed in polypropylene cages. The recommendations of Annamalai University "Institutional Animal Ethics Committee" (Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA Regn. no.160 /1999/CPCSEA), India) for the care and use of laboratory animals were strictly followed throughout the study. The animals were maintained under controlled environmental condition on alternative 12-h dark/light cycle. Commercial pelleted feed by M/s Kamdhenu Ltd., Bangalaru, and water ad libitum were given to animals.

Experimental design

A total number of 48 hamsters were divided into 6 groups of 8 animals each. Group I animals were served as untreated control. Animals in groups II -V were induced oral carcinogenesis by painting with DMBA in liquid paraffin three times a week for 14 weeks. Group II received no other treatment. Group III- V hamsters were orally administered with phloretin, at a dose of 20, 40 & 80mg/kg b.wt respectively, starting 1 week before the exposure to the carcinogen and continued until 1 week after the final exposure to the carcinogen. Group VI animals were orally administered with phloretin alone at a concentration of 80mg/kg b.wt throughout the experimental period. The experiment was terminated at the end of 16^{th} week to find the effective dose of phloretin. Biochemical studies were conducted on plasma, erythrocyte, liver and buccal mucosa of control and experimental animals in each group.

Tumor study

Tumor size was estimated according to the method of Geren et al., (1972). The resultant solid tumor was considered to be prelate ellipsoid with one long axis and two short axis. The two short axes were measured with vernier caliper. Tumor volume was calculated by the formula $v = 4/3\ (\pi) \times (D_1/2) \times (D_2/2) \times D_3/2)$, where D_1, D_2, and D_3 are the three diameters (mm) of the tumors.

Biochemical determination

Estimation of lipid peroxidation

Lipid peroxidation was estimated as evidence by the formation of thiobarbituric acid reactive substances (TBARS). The activity was assayed by the method of Yagi (1987). The reaction mixture consists of 0.083N sulphuric acid, 10% phosphotungstic acid, and thiobarbituric acid (TBA). The changes in the absorbance were recorded at 530 nm and TBARS activity was expressed as nmoles/ml.

Estimation of lipid hydroperoxides

Lipid hydroperoxides were estimated by the method of Jiang *et al.,* (1992). Oxidation of ferrous ion (Fe^{2+}) under acidic conditions in the presence of xylenol orange leads to the formation of a chromophore with an absorbance maximum at 560nm. Lipid hydroperoxides were expressed as mmol/dL of plasma or mmol/100 g of tissues.

Estimation of conjugated dienes (CD)

Conjugated dienes were assayed by the method of Rao and Recknagel (1968). Lipid peroxidation is associated with rearrangement of the double bonds in the polyunsaturated fatty acids leading to the formation of conjugated dienes. The concentration of CD was recorded at 233 nm and the values were expressed as mmol/dL plasma or mmol/100 mg tissue or nmol/mg of protein for erythrocytes.

Estimation of enzymatic antioxidants

Superoxide dismutase activity (SOD)

Superoxide dismutase activity in plasma and buccal mucosa was assayed by the method of Kakkar, (1984). The assay is based on the inhibition of the formation of NADH-phenazine metho sulphate-nitroblue tetrazolium formazan. The optical density was measured at 560 nm.

Catalase activity

Catalase activity in plasma and buccal mucosa was assayed by the method of Sinha, (1972). In this assay system, dichromate in acetic acid was reduced to chromic acetate when heated in the presence of hydrogen peroxide (H_2O_2) with the formation of perchloric acid as unstable intermediate and chromic acetate thus formed was measured spectrophotometrically at 570 nm. The results were expressed in terms of micromoles H_2O_2 liberated per minute per milligram protein.

Glutathione peroxidase activity (GPx)

Activity of glutathione peroxidase in plasma and buccal mucosa was determined by the method of Rotruck *et al.,* (1975) with modifications, which was based on the reaction of glutathione (GSH) with 5,5′-dithiobis-(2-nitrobenzoic acid to give a compound that absorbs at 412 nm. The values were expressed as micrograms of glutathione utilized per minute per milligram protein at 37°C.

Estimation of Non-enzymatic antioxidants

Estimation of Reduced Glutathione (GSH)

Reduced glutathione level was determined by the method of Beutler and Kelley (1963). This method was based on development of yellow color when 5, 5'-dithio-bis-2-nitrobenzoic acid (DTNB) is added to compound containing sulphydryl groups. The color developed was read at 412 nm. The values were expressed as mg/dl for plasma and mg/100 mg tissue for buccal mucosa.

Estimation of Vitamin C

Level of vitamin C was determined by the method of Omaye *et al.,* (1979). Ascorbic acid is oxidized by copper to form dehydro ascorbic acid and diketoglutaric acid. These products when treated with 2,4-dinitrophenyl hydrazine (DNPH) form the derivatives bis-2,4-dinitrophenylhydrazone which undergoes rearrangement to form a product with an absorption maximum at 520nm. The values were expressed as mg/dl for plasma and mg/100 mg tissue for buccal mucosa.

Estimation of Vitamin E

Level of Vitamin E was estimated by the method of Palan *et al.,* (1991). This method involves reduction of ferric ions to ferrous ions by the tocopherol and the formation of a pink coloured complex with batho phenanthroline orthophosphoric acid. Absorbance of the stable chromophore is measured at 536nm.

The values were expressed as mg/dl for plasma and mg/100 mg tissue for buccal mucosa.

Estimation of drug metabolizing enzymes
Assay of Cytochrome p450

Cytochrome p450 activity was measured by the method of Omura and Sato (1964). The reaction mixture consists of 0.1M phosphate buffer, Sodium dithionate and Carbon monoxide. The changes in the absorbance were recorded at 400-500nm and was expressed as µmol/mg protein.

Assay of cytochrome b5

Activity of cytochrome b5 was measured by the method of Omura and Sato (1964). The reaction mixture containing in phosphate buffer and NADH was added. The changes in the absorbance were recorded at 429-409nm and was expressed as µmol/mg protein.

Assay for glutathione reductase activity

Glutathione reductase (GR) activity was determined by method of Carlberg and Mannervik (1975). The reaction mixture consist of 1.65 ml phosphate buffer, EDTA, oxidized glutathione, NADPH, and 10% PMS. Enzyme activity was quantitated at 25ºC by measuring disappearance of NADPH at 340nm and was calculated as micromoles of NADPH oxidized per minute.

Assay for glutathione S-transferase activity (GST)

Glutathione S-transferase (GST) activity was assayed by the method of Habig et al. (1974). The reaction mixture consisted of phosphate buffer, reduced glutathione, CDNB, and PMS. The changes in the absorbance were recorded at 540nm and the specific activity of GST was expressed as micromoles of CDNB-GSH conjugate formed per minute per milligram Hb or protein.

Assay for G-glutamyl transpeptidase (GGT)

G-glutamyl transpeptidase (GGT) activity was determined by the method of Fiala et al. (1972), using g-glutamyl-p-nitroanilide as substrate. The reaction mixture consists of g-glutamyl-p-nitroanilide, glycylglycine, TCA, and $MgCl_2$ in tris-HCl buffer. The changes in the absorbance were recorded at 410nm and were expressed as micromoles of p-nitroaniline formed per gram of tissue per hour.

Statistical analysis

Values are expressed as mean ± SD. Statistical comparisons were 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

Table 1 shows the tumor incidence; tumor volume and histological changes in the buccal pouches of control and DMBA treated animals. We have observed 100% tumor formation with increased levels of mean tumor volume in DMBA alone treated animals (Group II). Oral administration of phloretin significantly reduced the tumor incidence, tumor volume and tumor burden in DMBA painted animals (Group III-V). No tumors were observed in control animals (Group I) and phloretin alone administrated animals (Group VI). Histopathological examination of the pouches in DMBA+ phloretin treated animals revealed varying degrees of preneoplastic and neoplastic lesion. The examination of histopathological studies revealed mild to moderate hyperplasia. In groups I and VI animals, the epithelium was normal, intact, and continuous.

Table 2 shows the levels of TBARS, LOOH and CD levels in plasma, erythrocyte and buccal pouches of control and experimental animals in each group. The increased TBARS, LOOH and CD levels in plasma and erythrocyte and decreased same parameter in buccal tissue in DMBA treated animals when compared to control animals. Oral administration of varying doses of phloretin (20, 40 and 80mg/kg b.wt) on DMBA painted animals were significantly modulate near normal levels of TBARS, LOOH and CD when compared to control animals. Phloretin at a dose of 40 mg/kg b.wt was found to be more effective than other doses. However, animals treated with phloretin alone (80mg/kg b.wt) showed no significant differences when compared to that of control animals.

Table 3 shows the decreased levels of SOD, CAT and GPx in plasma, erythrocyte and buccal mucosa, however GPx were significantly increased in buccal tissue of DMBA treated animals.

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

Parameters	Control	DMBA	DMBA+ Phloretin (20 mg/kg b.w.)	DMBA + Phloretin (40 mg/kg b.w.)	DMBA + Phloretin (80 mg/kg b.w.)	Phloretin alone (80 mg/kg b.w.)
Tumor incidence	-	100	80	20	70	-
Total number of tumors /animals	-	14/(6)	10/(6)	4/(6)	12/(6)	-
Tumor volume (mm3)	-	133.74±11.13	110.13±10.19	76.98±6.61	109.18±2.99	-
Keratosis	No change	Severe	Moderate	Mild	Moderate	No Change
Hyperplasia	No change	Severe	Moderate	Mild	Moderate	No Change
Dysplasia	No change	Severe	Moderate	Mild	Moderate	No Change
Squamous cell carcinoma	No change	Moderate	Mild	Well	Mild	No Change

Data are expressed as the mean ± SD for 8 hamsters in each group. Values not sharing a common superscript letter in the same row differ significantly at $p<0.05$ (DMRT). Tumor burden was calculated by multiplying tumor volume and the number of tumor/animals. () indicates total number of animals bearing tumors.

Table 2 The levels of TBARS, LOOH and CD in plasma, erythrocyte and buccal mucosa of control and experimental animals in each group.

Parameters	Plasma			Erythrocyte			Buccal mucosa		
	TBARS (nmol/ml)	**LOOH** (mmol/ml)	**CD** (mg/dl)	**TBARS** (pmol/mg Hb)	**LOOH** (mmol/mg Hb)	**CD** (mg/g Hb)	**TBARS** (nmol/mg protein)	**LOOH** (mmol/mg protein)	**CD** (mg/g protein)
Control	3.14 ± 0.29^{a}	10.54 ± 0.58^{a}	8.12 ± 0.88^{a}	1.69 ± 0.13^{a}	4.97 ± 0.41^{b}	2.86 ± 0.28^{a}	15.24 ± 1.15^{a}	36.17 ± 3.16^{a}	30.57 ± 3.08^{a}
DMBA	5.98 ± 0.51^{b}	26.13 ± 2.12^{b}	21.17 ± 2.78^{b}	4.33 ± 0.17^{b}	19.13 ± 1.16^{b}	16.23 ± 1.32^{b}	9.71 ± 0.84^{b}	17.43 ± 1.68^{b}	17.19 ± 1.83^{b}
DMBA+ Phloretin (20 mg/kg b.w.)	5.13 ± 0.44^{d}	23.12 ± 2.15^{d}	19.42 ± 1.88^{d}	3.86 ± 0.37^{d}	17.47 ± 1.42^{d}	12.71 ± 0.93^{d}	9.12 ± 0.98^{d}	14.57 ± 2.56^{d}	22.14 ± 2.78^{d}
DMBA + Phloretin (40 mg/kg b.w.)	2.91 ± 0.20^{c}	17.43 ± 1.78^{c}	12.17 ± 0.11^{c}	2.18 ± 0.24^{c}	6.43 ± 0.33^{c}	7.54 ± 0.65^{c}	13.11 ± 1.37^{c}	29.48 ± 2.93^{c}	28.14 ± 2.41^{c}
DMBA + Phloretin (80 mg/kg b.w.)	4.87 ± 0.41^{d}	26.12 ± 2.34^{d}	18.41 ± 1.42^{d}	3.73 ± 0.31^{d}	14.15 ± 0.32^{d}	10.17 ± 0.07^{d}	8.74 ± 0.88^{d}	16.86 ± 1.77^{d}	23.17 ± 2.28^{d}
Phloretin alone (80 mg/kg b.w.)	3.19 ± 0.27^{a}	9.91 ± 0.88^{a}	8.89 ± 0.78^{a}	1.70 ± 0.14^{a}	4.99 ± 0.48^{a}	2.91 ± 0.19^{a}	15.41 ± 1.33^{a}	31.07 ± 3.14^{a}	29.14 ± 3.17^{a}

Data are expressed as the mean ± SD for 8 hamsters in each group. Values not sharing a common superscript letter in the same row differ significantly at $p<0.05$ (DMRT).

Table 3 The activities of enzymatic antioxidants in plasma, erythrocyte and buccal mucosa of control and experimental animals in each group.

Parameters	Plasma			Erythrocyte			Buccal mucosa		
	SOD (U^X/ml)	**CAT** (U^Y/ml)	**GP_X** (U^Z/l)	**SOD** (U^X /mg Hb)	**CAT** (U^Y/mg Hb)	**GP_X** (U^Z/g Hb)	**SOD** (U^X /mg protein)	**CAT** (U^Y/mg protein)	**GP_X** (U^Z/g protein)
Control	2.89±0.23[a]	0.81±0.04[a]	126.03±11.69[a]	2.16±0.21[a]	1.88±0.17[b]	20.64±1.07[a]	5.14±0.53[a]	39.13±3.62[a]	5.97±0.53[a]
DMBA	1.31±0.09[b]	0.38±0.01[b]	89.27±6.13[b]	1.11±0.13[b]	0.46±0.03[b]	11.13±1.43[b]	2.17±0.87[b]	19.25±1.01[b]	19.53±1.03[b]
DMBA+Phloretin (20 mg/kg b.w.)	1.97±0.19[d]	0.31±0.03[d]	74.31±6.12[d]	1.08±0.11[d]	0.64±0.05[d]	13.36±1.41[d]	2.68±0.22[d]	18.53±1.63[d]	15.13±1.57[d]
DMBA + Phloretin (40 mg/kg b.w.)	2.31±0.21[c]	0.69±0.04[c]	114.17±1.25[c]	1.17±0.21[c]	1.49±0.03[c]	17.14±1.89[c]	4.84±0.43[c]	33.19±3.04[c]	9.71±0.98[c]
DMBA + Phloretin (80 mg/kg b.w.)	1.86±0.18[d]	0.38±0.01[d]	77.47±7.03[d]	1.09±0.11[d]	0.71±0.04[d]	13.83±1.45[d]	2.76±0.21[d]	21.74±1.12[d]	15.14±1.53[d]
Phloretin alone (80 mg/kg b.w.)	2.65±0.23[a]	0.83±0.07[a]	127.14±2.08[a]	2.19±0.20[a]	1.89±0.06[a]	20.16±1.87[a]	5.17±0.51[a]	38.97±3.71[a]	5.91±0.51[a]

Data are expressed as the mean±SD for 8 hamsters in each group. Values not sharing a common superscript letter in the same row differ significantly at $p<0.05$ (DMRT).Units for SOD^X, CAT^Y and GPx^Z are expressed as the amount of enzyme required to inhibit 50% of NBT reduction, micromoles of H_2O_2 utilized/second, and micromoles of glutathione utilized/minute, respectively.

Oral administration of varying concentrations of phloretin (20, 40 and 80mg/kg b.wt) on DMBA painted animals were significantly restored the levels of SOD, CAT and GPx when compared to control animals. Among the various doses phloretin at a dose of 40mg/kg b.wt were found to be more effective than other doses. Animals were treated with phloretin alone (80mg/kg b.wt) showed no significant differences were observed compared to control animals.

Table 4 shows the decreased levels of GSH, Vit-C and Vit-E in plasma and erythrocyte and enhanced same parameter in buccal tissue of DMBA painted animals when compared to control animals. Oral administration of varying concentrations of phloretin on DMBA painted animals were more effectively modulate to normal levels of GSH, Vit-C and Vit-E, which was compared to that of control animals. However, among the various doses phloretin at a dose of 40mg/kg b.wt were found to be more effective than other doses. Animals treated with phloretin alone (80mg/kg b.wt) showed no significant differences were observed compared to control animals.

Table 5 shows the activities of phase I enzymes of liver and buccal mucosa (Cyt p^{450} and Cyt b_5) were significantly increased as well as phase II detoxifying enzymes (GST, GGT and GR) were significantly enhanced in buccal tissue and significantly diminished in liver tissue of DMBA painted animals when compared to control animals. Oral administration of phloretin (40mg/kg b.wt) on DMBA painted animals were more effective and significantly near normal levels of phase I and phase II enzymes when compared to control animals. Animals treated with phloretin alone showed no significant differences were observed compared to control animals.

Discussion

Comprehensive reviews provide strong evidence the DMBA-induced hamster buccal pouch carcinogenesis is an accepted experimental animal model to test the chemopreventive potential of natural products and synthetic agents (Crowell, 2003). In the present study, the chemopreventive potential of phloretin was assessed in DMBA-induced hamster buccal pouch carcinogenesis, by monitoring the percentage of tumor bearing hamsters and tumor size as well as by analyzing the status of detoxification agents, lipid peroxidation and antioxidants status in experimental animals.

In the present study, the metabolic activation of DMBA results in the generation of ROS and other toxic metabolites. These ROS can diffuse from the site of generation to other targets and produce deleterious effects by initiating lipid peroxidation directly or by acting as second messengers for the primary free radicals that initiate lipid peroxidation (Das, 2002). In recent years; there has been a growing interest in studying the role played by lipid peroxidation and antioxidant status. The enhanced levels of LPO in carcinoma of hamster could be attributed to the overproduction of ROS and also due to poor antioxidant defence system. The oral administration of phloretin could maintain membrane fluidity by preventing membrane lipid peroxidation, which in turn restores membrane, associated immunological activity.

Ebeed *et al.,* (2010) reported that the chemopreventive potential of natural products includes carcinogen detoxification, suppression of genetic mutation, suppression of cell proliferation, induction of apoptosis and modulation of the immune system (Ebeed *et al.,* 2010). Enzymatic antioxidants are the first line of defence system for ROS mediated oxidative stress. Endogenous antioxidant enzymes such as SOD, CAT, and GPx play a major role in the cellular defense mechanism against ROS generated carcinogenesis (Somigliana *et al.,* 2006). Lowered activities of SOD, CAT and GPx in plasma are probably due to exhaustion of these enzymes to scavenge excessively generated reactive oxygen species in the system (Suresh et al., 2010).The present study reveals that SOD levels are decreased in the DMBA treated animals, which may be due to altered antioxidant status caused by carcinogenesis. GPx catalyzes the oxidation of GSH to GSSG at the expense of H_2O_2, Decreased GPx activity was also observed in cancerous conditions (Bewick *et al.,* 1987). The findings of this study also shows that decreased level of CAT observed in cancer bearing animals may be due to the utilization of antioxidant enzymes in the removal of H_2O_2 by DMBA. Our results are in line with these findings. Oral administration of phloretin restored the status of antioxidants in the plasma and buccal mucosa of DMBA treated animals, which may be attributed to the free radical quenching activity of phloretin and also might be hydrogen donating capacity of the hydroxyl group.

Table 4 The levels of non-enzymatic antioxidants in plasma, erythrocyte and buccal mucosa of control and experimental animals in each group.

Parameters	Plasma			Erythrocyte			Buccal mucosa		
	Vit-E (mg/dl)	**Vit-C** (mg/dl)	**GSH** (mg/dl)	**Vit-E** (µg /mg protein)	**Vit-C** (µg /mg protein)	**GSH** (µg /mg protein)	**Vit-E** (mg/100 mg tissues)	**Vit-C** (mg/100 mg tissues)	**GSH** (mg/100 mg tissues)
Control	2.06±0.19[a]	1.97±0.13[a]	33.11±32.09[a]	2.97±0.23[a]	2.87±0.21[b]	59.17±5.02[a]	1.94±0.19[a]	1.18±0.13[a]	9.17±0.80[a]
DMBA	0.93±0.07[b]	0.81±0.07[b]	19.14±1.18[b]	1.83±0.17[b]	1.77±0.11[b]	30.14±3.02[b]	3.19±0.31[b]	3.83±0.31[b]	16.83±1.53[b]
DMBA+ Phloretin (20 mg/kg b.w.)	0.99±0.19[d]	0.93±0.16[d]	17.23±1.08[d]	1.86±0.17[d]	1.31±0.13[d]	33.03±3.02[d]	3.21±0.27[d]	2.32±0.19[d]	14.74±1.24[d]
DMBA + Phloretin (40 mg/kg b.w.)	1.67±0.16[c]	1.52±0.04[c]	27.95±2.03[c]	2.43±0.24[c]	2.18±0.25[c]	46.95±4.05[c]	1.62±0.16[c]	1.57±0.15[c]	7.25±0.88[c]
DMBA + Phloretin (80 mg/kg b.w.)	1.09±0.08[d]	0.98±0.09[d]	19.94±1.82[d]	1.91±0.18[d]	1.57±0.13[d]	39.11±3.17[d]	3.82±0.31[d]	2.84±0.20[d]	16.29±1.06[d]
Phloretin alone (80 mg/kg b.w.)	2.08±013[a]	1.87±0.78[a]	32.31±3.06[a]	2.99±0.26[a]	2.88±0.21[a]	58.87±5.07[a]	1.91±0.14[a]	1.17±0.12[a]	9.03±0.97[a]

Data are expressed as the mean±SD for 8 hamsters in each group. Values not sharing a common superscript letter in the same row differ significantly at $p<0.05$ (DMRT).

Table 5 The levels of phase I and phase II detoxification enzyme status in buccal mucosa and liver homogenate of control and experimental animals in each group.

Parameters	Buccal mucosa					Liver homogenate				
	Cyt P^{450} (U^X /mg protein)	**Cyt b^5** (U^Y/mg protein)	**GST** (U^P/mg protein)	**GGT** (U^Q /mg protein)	**GR** (U^R/mg protein)	**Cyt P^{450}** (U^X /mg protein)	**Cyt b5** (U^Y/mg protein)	**GST** (U^P/mg protein)	**GGT** (U^Q /mg protein)	**GR** (U^R/mg protein)
Control	0.97±0.06[a]	0.53±0.02[a]	1.87±0.08[a]	12.93±1.26[a]	3.13±0.33[b]	1.86±0.08	1.96±0.18[a]	4.13±0.41[a]	21.13±1.23[a]	5.13±0.51[a]
DMBA	2.89±0.22[b]	0.91±0.02[b]	3.04±0.30[b]	21.15±1.08[b]	6.87±0.06[b]	3.87±0.03	2.23±0.21[b]	1.82±0.14[b]	8.45±0.81[b]	2.34±0.23[b]
DMBA+ Phloretin (20 mg/kg b.w.)	2.64±0.24[d]	0.89±0.03[d]	3.11±0.36[d]	16.73±1.48[d]	6.47±0.61[d]	3.76±0.04[d]	2.18±0.25[d]	2.56±0.21[d]	9.83±0.91[d]	3.73±0.34[d]
DMBA + Phloretin (40 mg/kg b.w.)	1.93±0.21[c]	0.61±0.08[c]	1.87±0.05[c]	12.19±1.33[c]	4.14±0.43[c]	1.83±0.09[c]	1.92±0.09[c]	3.38±0.32[c]	13.19±1.27[c]	4.24±0.41[c]
DMBA + Phloretin (80 mg/kg b.w.)	2.99±0.23[d]	0.84±0.02[d]	3.14±0.32[d]	17.13±1.03[d]	6.81±0.61[d]	3.73±0.23[d]	2.43±0.01[d]	2.71±0.25[d]	9.94±0.91[d]	3.81±0.35[d]
Phloretin alone (80 mg/kg b.w.)	0.91±.04[a]	0.56±0.04[a]	1.89±0.75[a]	12.19±1.28[a]	3.19±0.29[a]	1.84±0.09[a]	1.97±0.19[a]	4.18±0.39[a]	21.71±1.16[a]	5.62±0.57[a]

P- micromoles of CDNB conjugated with GSH/minute; Q- micromoles of p-nitroaniline formed/hr; R-Micromoles of NADPH oxidized/hr; X-Micromoles of cytochrome P^{450}; Y- Micromoles of cytochrome b_5

The non-enzymatic antioxidant defences include glutathione as well as vitamins C and E. They act in concert to reduce the oxidative damage by scavenging free radicals and by detoxifying the oxidants (McCall and Frei, 1999). GSH in conjunction with GST detoxifies reactive intermediate species generated during DMBA metabolism, thereby enhancing resistance against oxidative stress (Locigno and Castronovo, 2001). The depletion of vitamin C, vitamin E and glutathione in the circulation of tumor-bearing animals may be due to oxidative stress caused by DMBA. Several studies also documented that the decrease in the activities of non-enzymatic antioxidants especially GSH, the major cellular detoxifying enzyme systems, has been reported in malignancies (Chanda and Dave, 2009). Our results are in line with these findings. The depleted levels of non-enzymatic antioxidants status were restored to normal by phloretin treated animals. This might be due to release of antioxidants and chemoprotective role of phloretin.

Phase I (cytochrome p^{450} and b^{5}) and Phase II (GR, GST and GSH) biotransformation enzymes play pivotal role in the metabolic activation and excretion of carcinogens and their metabolites (Miller, 1988). Birkett *et al.,* (1993) reported the activities of phase I and II biotransformation enzymes in liver thus can be used as reliable biochemical markers to assess the chemopreventive potential of the plant derived photochemicals (Birkett *et al.,* 1993). Profound studies reported that the activities of phase I and II detoxification enzymes were significantly altered during DMBA induced oral carcinogenesis (Szaefer *et al.,* 2004). Our results are in line with these findings. Increased the levels of phase I enzymes and decreased the status of phase II enzymes in the liver of DMBA painted animals underlie ultimate carcinogenic metabolite of DMBA. Oral administration of phloretin to DMBA treated animals restored the status of phase I and phase II enzymes in the liver and buccal mucosa suggests that phloretin might have played crucial role in the detoxification of carcinogens, this may be attributed to the fact that phloretin is a bifunctional inducer, which influences expression of both Phase I and II biotransformation enzymes, which detoxify carcinogenic metabolites.

Animal studies were performed to investigate the effective dose of phloretin; various doses of phloretin (20, 40 and 80mg/kg b.wt) were assessed to find out the effective chemopreventive dose of phloretin in DMBA induced oral carcinogenesis. The effective dose of phloretin (40mg/kg b.wt) exhibited significant antioxidant enhancing and tumor inhibitory effects compare to the low dose and high dose of phloretin. Based on these above data, thus the present study shows that oral administration of phloretin at a concentration of 40mg/kg b.wt effectively inhibits the tumor incidence, stimulating the antioxidant and detoxification defense mechanism to neutralize the toxic effects generated by DMBA.

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