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Evaluation of Chemopreventive Potential of *Zingiber Officinale Roscoe* Ethanolic Root Extract on 7, 12-dimethyl Benz[a]anthracene Induced Oral Carcinogenesis

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Abstract: The present study has investigated the chemopreventive and ant ilipidperoxidative efficacy of *Zingiber oficinale Roscoe* ethanolic root extract on 7,12-dimethyl benz[a]anthracene (DMBA) induced hamster buccal pouch carcin ogenesis. Oral squamous cell carcinoma was induced in hamster buccal pouches by painting with 0.5% 7,12 -dimethyl benz[a]anthracene(DMBA) three times per week for 14 weeks. We observed 100% tumor formation in DMBA painted hamsters. Oral administration of *Zingiber oficinale* ethanolic -1root extract at a dose of 300 mg kgbody weight prevented the tumor formation as well as decreased the levels of lipid peroxidation by products and enhanced the antioxidants defense mechanism in DMBA painted hamsters. Our results suggest that *zingiber oficinale* ethanolic root extract exert their anticarcinogenic effect by modulating the status of lipid peroxidation. and antioxidants in DMBA painted hamsters

Key words: DMBA, Oral cancer, *Zingiber oficinale,* lipid peroxidatio n, antioxidants

INTRODUCTION

Cancer of the oral cavity the disfiguring disease of human ,populations, morbidity and mortality worldwide. While oral squamous cell carcinoma accounts for - 3 5% of all cancers in Western industrialized countries, i t accounts for 40 -50% of all malignancies in developing countries including India [1]. Squamous cell carcinoma is the most common malignant neoplasm of the head and neck. It constitutes at least 75% of head and neck cancer in which patients show a high incidence of immunologic deficiencies and inflammatory symptoms [2]. India has recorded the highest incidence of oral cancer where the habits of excessive tobacco chewing with or without betel quid, smoking and alcohol consumption are attributed to the highes t incidence of oral cancer [3]. Betel quid chewing with tobacco has been identified as the most important risk factor for high oral cancer incidence in India [4]. DMBA induced hamster buccal pouch carcinogenesis is to therefore use d as an ideal model for evaluating chemoprevention of oral cancer [5]. Squamous cell carcinomas induced by the application of 7, 12, Dimethyl benz(a) anthracene (DMBA) to the buccal pouch s are morphologically, physiologically and histiopathologically similar to human carcinoma [6].

Free radicals are uncoupled electrons and are extremely active and unstable. Among the most important free radicals in the reactive oxygen species (ROS) are singlet oxygen ($^1O2$), super oxide anions (O 2Â), hydrogen peroxide (H_{2O2}), and hydroxyl radical (ÂOH) in the etiology of cancer [7]. Uncontrolled production of ROS results in the destruction macromolecules such as DNA, lipids and proteins [8]. Antioxidants act as a major defense against ROS mediated toxicity by protecting membrane and cytosolic compounds [9]. Free radicals are involved in both initiations as well as promotion stage of tumourigenesis [10]. Oxidative stress induced when an imbalance between free radical genera tion and scavenging capacity of antioxidants results in cancer [11].

Antioxidants are the chemical substances that reduce or prevent oxidation and have the ability to counteract the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart diseases, and several other diseases.Human body is equipped with various enzymatic and non enzymatic antioxidants viz, superoxide dismutase (SOD), glutathione pero xidase (GPx), Catalase (CAT), Glutathione (GSH), Ascorbic acid (Vitamin C), α -tocopherol (vitamin E), etc. which can counteract the deleterious action of ROS and protect from cellular and molecular damage [12]. Previous studies from our laboratory hence showed enhanced lipid peroxidation and disturbed antioxidants defense mechanism in experimental buccal pouch

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carcinogenesis [13].

Ginger *(Zingiber oficinale Roscoe, Zingiberaceae)* is widely used as a dietary species throughout the world. Besides its extensive utilization as a spice, the rhizome of ginger has been used traditional medicine to ameliorate such symptoms as inflammation, rheumatic disorders and gastrointestinal discomforts [14]. Ginger is used extensively in traditional Chinese me dicine to treat headaches, nausea and colds and in Ayurvedic and western herbal medicinal practice for the treatment of arthritis, rheumatoid disorders and muscular discomforts. [15]. Ginger is often used for the treatment of stomachache, and cardiovascul ar and motor diseases. It also possesses anti -inflammatory activity and regulates bacterial growth, as well as providing protection for immune-depressed patients, such as individuals who are HIV positive [16]. This species contains biologically active con stituents including the main pungent active principles, the gingerols and sh ogaols [17]. However, no scientific reports were available on the literature for its chemopreventive and antilipid. peroxidative effects in DMBA induced buccal pouch carcinogenesis.

In the present study, the chemopreventive and antilipidperoxidative effect of Ginger was exam ined in DMBA induced experimental oral carcinogenesis.

MATERIALS AND METHODS

Chemicals: The carcinogen, 7, 12 -dimethylbenz (a) anthracene (DMBA), was obtained from Sigma - Aldrich Chemical Pvt. Ltd. Bangalore, India. A ll other chemicals used were of analytical grade.

Animals: Male golden Syrian hamsters 8-10 weeks old, weighing 80 -120g were purchased from National Institute of N utrition, Hyderabad, India and maintained in central animal house, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in poly propylene cages and provided standard pellet and water ad libitum .The animals were maintained under controlled conditions of temperature and humidity with a 1 2h light dark cycle.

Plant Material: *Zingiber oficinale* roots were purchased from fresh market in Chidambaram, Tamil nadu, India and authenticated by the Botanist, Dr.S.Si vakumar, Department of Botany, Annamal ai University. A voucher specimen (AU042 19) was also deposited.

Preparation of the Plant Extracts: Five hundred grams of dried and finely powdered *Zingiber oficinale* root were soaked in 1500 ml of 95% ethanol overnight. The residue obtained after filtration was again resuspended in equal volume of 95% ethanol for 48h and filtered again. The above two filterates were mixed and solvent were evaporated in a rotavapour at 40 - 50^{q}C under reduce d pressure. A dark semisolid material (8.6%) obtained was stored at 0 - 4qc until used.

A known volume of the residual extracts was suspended in distilled water and was orally administered to the animals by gastric intubation using a force-feeding needle during the experimental period.

Experimental Protocol: The local Institutional animal ethics committee, Annamalai University, Annamalai Nagar, India, has approved the experimental design. A total number of 24 golden Syrian Hamsters were. randomized into 6 animals in each group Group I animals were served as untreated control. Groups II animals were painted with 0.5% DMBA in liquid paraffin three times per week for 14 weeks on the left buccal pouches. Group III orally administered ZoERet (300 mg kg^{-1}bw) starting 1 week before the exposure to the carcinogen and continued on days alternate to DMBA painting, until the scarification of the animals. Group IV received ZoERet (300 mg kg^{-1}bw) alone throughout the exper imental period. The th experiment was terminated at the end of 15 week and all animals were sacrificed by cervical dislocation. Biochemical studies were conducted on blood and buccal mucosa of control and experimental animals in each group. For histopatholo gical examination, buccal mucosa tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2 - 3 μm sections were cut in a rotary microtome stained with haematoxylin and eosin.

Biochemical Analysis: After plasma separation, the buffy coat was removed and the packed cells were washed thrice with physiological saline. A known volume of erythrocytes was lysed with hypotonic buffer at pH 7.4. The heamolysate was separated by centrifugation at 10,000 rpm for 15 min at 20 Üc. The erythrocyte membrane was prepared by the method of modified by Thiobarbituric acid reactive substances were assayed in plasma, erythrocytes, and buccal mucosa according to the methods of and respectively. Reduced glutathione (GSH) was determined by the method of Vitamin C and E were measured according to the methods of and Desai, respectively. The activities of enzymatic antioxidants, SOD, CAT and Gpx were estimated by the methods of sinha and Rotruck, respectively.

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 AND DISCUSSION

Table 1 shows the effect of *Zingiber oficinale ethanolic* root extracts on tumor incidence, tumor volume, and tumor burden and histopathological features in DMBA induced hamster buccal pouch carcinogenesis. We have noticed 100% tumor formation with tumormm^3) meanvolume (340.37 ±28.89and tumor burden (1133.43 ±88.74mm^3) in DMBA alone painted hamsters (Group II). Oral administration of ZoERet at a dose of 300 mg^{-1}kg body weight significantly prevented the tumor incidence tumor volume and ,tumor burden in DMBA painted hamsters (groups II I). No tumors were observed in control animals (Group I) and ZoER et alone administered animals (Groups IV). We have observed severe Keratosis, hyperplasia, dysplasia and squamous cell carcinoma in the buccal mucosal tissues of hamsters painted with DMBA alone (gr oup II). A mild to moderate prene oplastic lesions (hyperplasia, keratosis and dysplasia) were noticed in groups III animals.

Table 2 shows the status of plasma, erythrocytes, erythrocyte membrane and buccal mucosa TBARS in control and experimental animals in each group of experimental design. The concentration of TBARS were increased in plasma, erythrocytes and erythrocyte membrane and decreased in buccal mucosa of DMBA painted hamsters (Group II) as compared to control animals, Oral administration of *ethanolic* root extract of *Zingiber oficinale* at a dose of 300mg/kg^{-1} body weight significantly decreased the levels of TBARS in plasma, erythrocyte, erythrocyte membrane and significantly increased in buccal mucosa of DMBA painted hamsters (Group III). Hamsters treated with *ethanolic* root extracts of *Zingiber oficina el* alone (Zo ERet) showed no significant difference in TBARS as compared to control animals. (Group I).

Tables 3 and 4 shows the levels of circulat ory, (plasma and erythrocytes) and buccal mucosal enzymatic and non-enzymatic antioxidants respectively, in control and experimental animals in each group of experimental design. The concentration of non enzymatic antioxidants (GSH, Vitamin C and VitaminE) and activities of enzymatic antioxidants (SOD, CAT and GPx) were significantly decreased in plasma and erythrocytes whereas disturbances in antioxidants status (Vitamin E, GSH and GPx were increased; SOD and CAT were decreased) were noticed in buccal mu cosa of cancer animals as compared to control animals. Oral administration of *ethanolic* root extracts of *Zingiber oficinale* at a dose of 300mg/kg^{-1} b.wt normalized the status of antioxidants in circulation and buccal mucosal tissues. Hamsters treated with *ethanolic* root extracts of *Zingiber oficinale* alone showed no significant difference in antioxidants status as compared to control animals.

Discussion: Chemoprevention offers a novel approach to control the incidence of oral cancer, an important contributor of cancer morbidity and mortality in the Indian subcontinent. [18]. First focused the research for chemopreventive agents by examining the various dietary components. Dietary patterns may account for wide differences in the risk for leading cancers across the world. It was logical to propose that dietary factors in countries with populations at low risk for certain cancers could be identified and exploited for use in man as cancer inhibitors [19]. DMBA, a potent carcinogen used in the present study has been reported to produce toxic and highly diffusible reactive oxygen species, capable of producing deleterious effects at sites far from the tumor [20]. Free radical -induced lipid peroxidation is an oxidative process associat ed with membranes lipid destruction [21]. It causes profound alterations in the structural organization and functions of the cell membrane [22]. Generation of ROS and the peroxidation of membrane lipids are well associated with the initiation of carcinogenesis affecting the normal bio -chemical process, which further leads to the reduction of body weight [23].

Naturally, there is a dynamic balance between the amount of free radicals generated in the body and antioxidant defense system that quench or scavenge them and thereby protect the body against pathogenesis [24]. The *Zingiber oficinale* contain s number of anti -tumor compounds such as 6-paradol, 6 -gingerol, and 6 -shogaols. *Zingiber oficinale* ethanolic root extracts significantly prevent ed the tumor formation in the hamster buccal pouchs, which indicates its potent chemopreventive role in DMBA induced oral carcinogenesis. Although the exact mechanism include induction of phase II detoxification enzymes and increase enzymatic degradation of DMBA by li ver and or enhance antioxidant defense mechanism to degrade the toxic effects, of reactive oxygen species generated by DMBA.

Lipid peroxides play an important role in the control of cell division. Low concentration of oxygen free radicals have been reported to stimulate cell proliferation where as high levels induce cytotoxicity and cell death [25]. An inverse relationship has been observed between lipid peroxidation and the rate of cell proliferation, with highly proliferating tumors [26]. The decline in lipid peroxidation in DMBA-induced oral tumors was associated with enhanced levels of GSH, GPx and GST. GSH plays an important role in

Table 1: Eff ect of *Zin giber officinale* root extracts on squamous cell carcinoma in 0.5% DMBA painted golden Syrian hamsters.

Parameters	Control	DMBA 14th Week	DMBA+ZoERet (300mg/ b.w) 14th Week	ZoERet alone (300 mg/ b.w)
Tumor incidence (oral Squamous cell carcinoma)	0	100 %(6)	33% 2/ (6)	0
Total number of tumors/animals	0	20(6)	4/ (2)	0
Tumour volume (mm^3)	0	72.37±57.9	8.87±0.52	0
Tumour burden(mm^3)	0	1447±115.8	35.48±2.08	0
Keratosis	_	Severe	Mild	_
Hyperplasia	_	Severe	Mild	_
Dysplasia	_	Severe	Mild	_
Squamouscell carcinoma	_	Well differentiated squamous cell carcinoma	_	_

Values are expressed as ± SD for 6 animals in each group. Tumor volume was measuring using the

$$V = \frac{4}{3}\pi\left(\frac{D1}{2}\right)\left(\frac{D2}{2}\right)\left(\frac{D3}{2}\right)$$

where D_1, D^2, and D^3 are the three diameters (mm) of the tumor. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal indicates ( ) total number of animals bearing tumors.
ZoERet - *Zin giber officinale* Ethanolic Root extract

Table 2: The levels of Thiobarbituric acid reactive substances (TBARS) in control and experimental animals in each group of experimental design. (n=6)

Groups	Treatment	TBARS			
		Plasma (nmol/ml)	Erythrocytes (pmol/mg Hb)	Erythrocyte Membrane (nmol/mg protein)	Buccal tissue (nmol/mg protein)
1	Control	2.35 ± 0.35 a	1.90 ± 0.30 a	0.45 ± 0.16 a	70.75± 1.54 a
2	DMBA	4.70± 0.36 b	2.70 ±0.26 b	0.97 ± 0.12 b	42.92 ±1.17 b
3	DMBA+ZoERet (300mg/kg b.wt)	2.95± 0.44 c	2.33 ± 0.22 c	0.66± 0.19 c	65.75 ±1.12 c
4	ZoERet alone (300mg/kg b.wt)	2.30± 0.48 a	1.95 ±0.31 a	0.44 ± 0.15 a	71.62 ± 1.94 a

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P < 0.05. (DMRT)
ZoERet - *Zin giber officinale* Ethanolic Root extract

Table 3: The levels of enzymatic antioxidants in plasma, erythrocytes and buccal tissue of Control and experimental animals in each group of experimental design (n=6)

Groups	Treatment	Plasma			Erythrocyte membrane VitaminE (Pg/mg protein)	Erythrocyte GSH (mg/dl)	Buccal tissue Vitamin E (mg/100mg protein)
		VitaminE (mg/dl)	VitaminC (mg/dl)	GSH (mg/dl)			
1	Control	a 1.25 ± 0.11[a]	1.36± 0.19 [a]	28.77± 2.63 [a]	2.30± 0.43 [a]	37.77± 2.00 [a]	1.71± 0.41 [a]
2	DMBA	0.65 ± 0.19 [b]	0.80 ±0.17 [b]	25.10± 2.42[b]	1.47± 0.18 [b]	21.75±2.64 [b]	2.27 ±0.25 [b]
3	DMBA+ZoERet (300mg/kg b.wt)	0.91± 0.22 [c]	1.06 ± 0.10 [c]	19.10± 2.62	1.88± 0.32 [c]	32.54± 3.11 [c]	2.07± 0.20 [c]
4	ZoERet alone (300mg/kg b.wt)	1.26 ± 0.12 [a]	1.37± 0.15 [a]	2.29 2±9.10[a]	2.36± 0.11 [a]	37.32 ±2.38 [a]	1.77 ± 0.22 [a]

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P <- 0.05. (DMRT). A Amount of enzyme required to inhibit 50% Nitroblue tetrazolium- reduction/min; B µ moles of H_2O_2 utilized- /min; C-µ moles of GSH utilized / min; D - µ moles of H_2O_2 utilized /sec.
ZoERet . – *Zin giber officinale* Ethanolic Root extract

Table 4: The levels of enzymatic antioxidants in plasma, erythrocytes and buccal tissue of Control and experimental animals in each gro up of experimental design (n=6)

Groups	Treatment	Plasma			Erythrocyte lysate			Buccal tissue		
		SOD (U^A/ml)	CAT (U^B/ml)	Gpx (U^C/l)	SOD (U^A/mg protein)	CAT (U^D/mg Hb)	Gpx (U^C/g Hb) protein)	SOD (U^A/mg protein)	CAT (U^B/mg protein)	GPx (U^C/mg
1	Control	2.62± 0.39 a	0.50 ± 5.30 a	119.56±7.20 a	2.16 ± 0.19	1.29 ±0.12	14.63±0.94 a	4.55± 0.26 a	32.15± 2.78 a	6.00± 0.35 a
2	DMBA	1.43± 0.50 b	0.26 ± 3.09 b	72.39± 3.11 b	1.16± 0.26 b	0.81± 0.11 b	9.31± 1.40 b	2.75± 0.54 b	20.48 ±0.77 b	9.39± 1.04 b
3	DMBA+ZoERet (300mg /kg b.wt)	2.01± 0.35 c	0.43± 6.19 c	89.97± 9.67 c	1.75± 0.51 c	1.13± 0.30 c	12.93±1.70 c	3.82± 0.40 c	29.21± 2.77 c	7.37± 0.79 c
4	ZoERet alone (300mg/kg b.wt)	2.60± 0.33 a	0.51± 8.32 a	118.63± 9.64 a	2.14± 0.19 a	1.30±0.12 a	14.58±0.96 a	4.56± 0.22 a	32.45± 2.76 a	5.93± 0.50 a

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P < 0.05. (DMRT). A Amount of enzyme required to inhibit 50% Nitroblue tetrazolium reduction/min; B- µ moles of H_2O_2 utilized /min; C - µ moles of GSH utilized / min; D - µ moles of H_2O_2 utilized /sec
ZoERet – *Zin giber officinale* Ethan olic Root extract

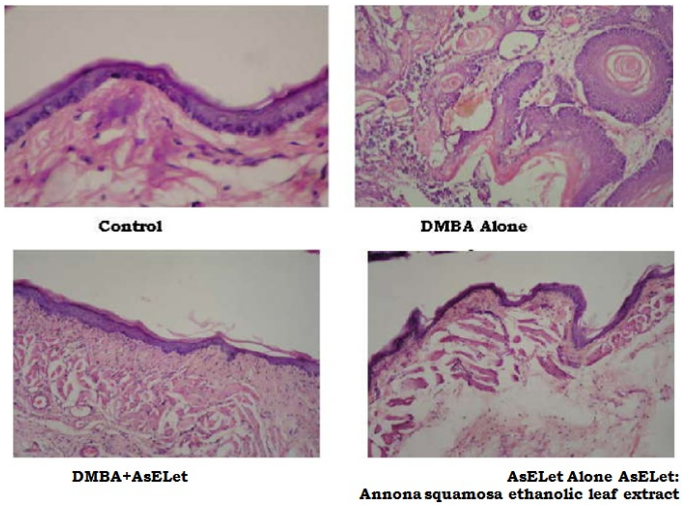

Fig. 1: Histological features observed in the buccal mucosa of control and experimental animals in each group

scavenging reactive oxygen speciesprote cting cell against cytotoxic and carcinogenic chemicals. [27]. Enhanced lipid peroxidation associated with antioxidant depletion in circulation is a characteristic finding in malignant transformation [28]. Lowered activities of SOD and CAT enzymes were rep orted in patients with malignant and as well as carcinogen induced experimental carcinogenensis. [29]. The deficiency of a scorbic acid, vitamin E and glutathione in the circulation of tumor bearing hamsters may be due to their increased utilization to scavenge the products of lipid peroxidation [30].

A decrease in the activities of GPx, SOD and catalase, the major cellular detoxifying enzyme systems, has been reported in malignancies [31]. Enzymatic and non enzymatic antioxidants from the first and second line of defense mechanism respectively against the deleterious effects of oxidative stress induced cell damage [32]. GSH and GPx play a crucial role in protecting membrane proteins and the thiol groups of Vitamin E, potent quenchers of free radicals and singlet oxygen, prevent the oxidation of glutathione. Cells depleted of glutathione are susceptible to membrane damage due to oxidative stress [33]. The deficiency of GSH, GPx, GST as well as ascorbic acid and vitamin E in the cir culation of tumors bearing animals may be due to increased utilization to scavenge lipid peroxides as well as sequestration by tumor cells. Glutathione helps to maintain membrane integrity, optimal transport of aminoacids, enzyme activity and also provides biological protection through the detoxification of xenobiotics and free radicals. Glutathione has been documented to have regulatory effects in cell proliferate activity [34].

Oral administration of *Zingiber oficinale* ethanolic root extracts to DMBA painted hamsters significantly protected the status of antioxidant and lipid peroxidation byproducts, which indicates their potent antilipidperoxidative potential during neoplastic transformation. The antilipidperoxidative property of the plant extrac ts suggests that presence of one or more bioactive principles in *Zingiber oficinale* ethanolic root extractThus, the present study demonstrates the antilipidperioxidative potential of *Zingiber oficinale* ethanolic root extracts in DMBA induced hamster buccal pouch carcinogenesis. Further studies are needed to isolate and characterize the bioactive antioxidants principles from the root of *Zingiber oficinale*

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