ANTILIPIDPEROXIDATIVE AND ANTIOXIDANT EFFECTS OF ZINGIBER OFFICINALE ROSCOE ROOT EXTRACT IN 7, 12-DIMETHYL BENZ[A] ANTHRACENE INDUCED ORAL CARCINOGENESIS

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Summary

The present study has investigated the chemopreventive and antilipidperoxidative efficacy of Zingiber officinale Roscoe aqueous root extract in 7,12-Dimethylbenz[a]anthracene (DMBA) induced hamster buccal pouch carcinogenesis. Oral squamous cell carcinoma was induced in hamster buccal pouches by painting with 0.5% 7, 12-Dimethylbenz[a]anthracene (DMBA) three times per week for 14 weeks. 100% tumor formation, altered levels of lipid peroxidation and antioxidants status were observed in DMBA painted hamsters. Oral administration of Zingiber officinale Roscoe aqueous root extract significantly prevented the tumor formation as well as decreased the levels of lipid peroxidation by products and enhanced the antioxidants defense mechanism in DMBA treated hamsters.

Key words: DMBA, Oral cancer, Zingiber officinale, Lipid peroxidation, Antioxidants.

Introduction

Oral squamous cell carcinoma (OSCC) of the oral cavity, the fifth most common cancer worldwide, is the leading cause of morbidity and mortality in the Indian subcontinent (1). In India, where the habits of chewing tobacco with betel nut, reverse smoking and heavy alcohol usage are common risk factors, there is a striking incidence of oral cancer which accounts for as many as 30-40% of all cancers (2). 7,12-Dimethylbenz(a)anthracene (DMBA) is commonly used potent organ and site-specific carcinogen to induce buccal pouch carcinogenesis in hamsters, The hamster oral cancer model has relevant similarities to human oral cancer development (3).
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 (4).

The most important free radicals in the reactive oxygen species (ROS) are singlet oxygen (O₂), super oxide anions (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (·OH) in the etiology of cancer (5). Free radicals induced oxidative stress has been implicated in the pathogenesis of several diseases including cancer. Free radicals can damage proteins, lipids, carbohydrates, and nucleic acids. The most important function of free radicals in vivo or in vitro is lipid peroxidation resulting in deleterious effects on membrane system and damages the cells (6).

Antioxidants are intimately involved in the prevention of cellular damage is the common pathway for cancer, aging and a variety of diseases (7). However, the body has developed several endogenous antioxidants defense systems (non enzymatic and enzymatic) to deal with the production of reactive oxygen intermediates. The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (Gpx) and the non-enzymatic antioxidants include vitamin E, vitamin C and reduced glutathione (GSH) (8).

Ginger (Zingiber officinale Roscoe, Zingiberaceae) is widely used as a dietary condiment throughout the world. Besides its extensive utilization as a spice, the rhizome of ginger has been used traditional oriental medicine to ameliorate such symptoms as inflammation, rheumatic disorders and gastrointestinal discomforts (9). Ginger is used extensively in traditional Chinese medicine to treat headaches, nausea and colds and in Ayurvedic and western herbal medicinal practice for the treatment of arthritis, rheumatoid disorders and muscular discomforts (10). Ginger is often used for the treatment of stomachache, and cardiovascular 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 (11). This species contains biologically active constituents including the main pungent principles, the gingerols and shogaols (12).

However, no scientific reports were available on the literature for its chemopreventive and anti-lipidperoxidative effects in DMBA induced buccal pouch carcinogenesis. In the present study, the chemopreventive and anti-lipidperoxidative effect of ginger was examined 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. All 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 Nutrition, 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 *add libitum*. The animals were maintained under controlled conditions of temperature and humidity with a 12h light dark cycle.

Plant material
*Zingiber officinale* roots were purchased from fresh market in Chidambaram, Tamil nadu, India and authenticated by the Botanist, Dr.K.Sivakumar, Department of Botany, and Annamalai University. A voucher specimen (AU04219) was also deposited.

Preparation of the plant extracts
Five hundred grams of dried and finely powdered *Zingiber officinale* root were suspended in 250 ml of water for 2h and then heated at 60-65°C for 30 min. the extracts was preserved and the process was repeated for three times with the residual powder, each time collecting the extract was pooled and passed through the fine cotton cloth. The filtrates upon evaporation at 40°C yielded 16% semisolid extract. This was stored at 0-4°C until used.

A known volume of 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 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 with *Zingiber officinale* aqueous root extracts (ZoAREt) (500 mg kg-1 B.wt) 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 ZoAREt (500 mg kg-1 B.wt) alone throughout the experimental period. The experiment was terminated at the end of 14th 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 histopathological examination, buccal mucosa tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3μm 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 haemolysate was separated by centrifugation at 10,000 rpm for 15 min at 20˚C. The erythrocyte membrane was prepared by the method of Dodge et al (13). Modified by Quis(14). Thiobarbituric acid reactive substances (TBARS) were assayed in plasma, erythrocytes, and buccal mucosa according to the methods of Yagi, Donnan and Okhawa etal (15, 16, 17) respectively. Reduced glutathione (GSH) was determined by the method of Beutler and Kelley (18). Vitamin C and E were measured according to the methods of Omaye et al (19) and Desai (20), respectively. The activities of enzymatic antioxidants, SOD, CAT and Gpx were estimated by the methods of Kakkar et al (21) Sinha (22) and Rotruck et al (23) 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

Table 1 shows the effect of *Zingiber officinale* Roscoe root extracts on tumor incidence, tumor volume, and tumor burden and histopathological features in DMBA induced hamster buccal pouch carcinogenesis. We observed 100% tumor formation with mean tumor volume (70.37±55.9 mm3) and tumor burden (140.64±7.88 mm3) in DMBA alone painted hamsters (Group II). Oral administration of ZoARet at a dose of 500 mg kg-1 body weight significantly prevented the tumor incidence, tumor volume and tumor burden in DMBA painted hamsters (groups III). No tumors were observed in control animals (Group I) and ZoARet 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 (group II). A mild to moderate preneoplastic lesions (hyperplasia, keratosis and dysplasia) were noticed in group III animals.

Table 2 shows the status of plasma, erythrocytes, erythrocyte membrane and buccal mucosa TBARS in control and experimental animals in each group. The levels 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 aqueous root extract of *Zingiber officinale* at a dose of 500mg/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 aqueous root extracts of *Zingiber officinale* alone showed no significant difference in TBARS as compared to control animals.
Tables 3 and 4 show the levels of circulatory, (plasma and erythrocytes) and buccal mucosa enzymatic and non-enzymatic antioxidants respectively in control and experimental animals in each group. The levels of non enzymatic antioxidants and activities of enzymatic antioxidants 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 mucosa of cancer animals as compared to control animals. Oral administration of Zingiber officinale Roscoe root extracts of Zingiber officinale at a dose of 500mg/kg-1 b.w normalized the status of antioxidants in circulation and buccal mucosal tissues. Hamsters treated with root extracts of Zingiber officinale alone showed no significant difference in antioxidants status as compared to control animals.
Table 1  Effect of *Zingiber officinale* root extract on squamous cell carcinoma in 0.5% DMBA painted golden Syrian hamsters.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>DMBA</th>
<th>DMBA+ZoERet (500mg/kg b.wt)</th>
<th>ZoERet alone (500mg/kg b.wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence (oral Squamous cell carcinoma)</td>
<td>0</td>
<td>100% (6)</td>
<td>33% 2/ (6)</td>
<td>0</td>
</tr>
<tr>
<td>Total number of tumors/animals</td>
<td>0</td>
<td>20(6)</td>
<td>4/ (2)</td>
<td>0</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>0</td>
<td>70.37±55.9</td>
<td>8.07±0.49</td>
<td>0</td>
</tr>
<tr>
<td>Tumor burden</td>
<td>0</td>
<td>140.64±7.88</td>
<td>32.48±2.00</td>
<td>0</td>
</tr>
<tr>
<td>Keratosis</td>
<td>_</td>
<td>Severe</td>
<td>Mild</td>
<td>_</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>_</td>
<td>Severe</td>
<td>Mild</td>
<td>_</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>_</td>
<td>Severe</td>
<td>Mild</td>
<td>_</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>_</td>
<td>Well differentiated squamous cell carcinoma</td>
<td>_</td>
<td>_</td>
</tr>
</tbody>
</table>

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

\[ V = \frac{4}{3} \pi \left( \frac{D_1}{2} \right) \left( \frac{D_2}{2} \right) \left( \frac{D_3}{2} \right) \]

where D1, D2, and D3 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. ZoARet - *Zingiber officinale* Aqueous Root extract.
Table 2. The levels of Thiobarbituric acid reactive substances (TBARS) in Plasma, Erythrocytes, Erythrocyte Membrane and Buccal tissue of control and experimental animals in each group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Plasma (nmol/ml)</th>
<th>Erythrocytes (pmol/mg Hb)</th>
<th>Erythrocyte Membrane (nmol/mg protein)</th>
<th>Buccal tissue (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>2.32 ± 0.30 a</td>
<td>1.90 ± 0.28 a</td>
<td>0.33 ± 0.10 a</td>
<td>69.30 ± 1.20 a</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>4.65 ± 0.30 b</td>
<td>2.41 ± 0.32 b</td>
<td>0.98 ± 0.08 b</td>
<td>41.90 ± 1.77 b</td>
</tr>
<tr>
<td>3</td>
<td>DMBA+ZoARet (500mg/kg b.wt)</td>
<td>3.20 ± 0.44 c</td>
<td>2.00 ± 0.23 c</td>
<td>0.47 ± 0.06 c</td>
<td>62.00 ± 1.00 c</td>
</tr>
<tr>
<td>4</td>
<td>ZoARet alone (500mg/kg b.wt)</td>
<td>2.00 ± 0.41 a</td>
<td>1.83 ± 0.10 a</td>
<td>0.32 ± 0.09 a</td>
<td>70.10 ± 1.50 a</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SD; n = 6. Values not sharing a common superscript significantly differ at P < 0.05. (DMRT)

ZoARet - *Zingiber officinale* Aqueous Root extract
Table 3. The levels of enzymatic antioxidants in plasma, erythrocytes and buccal tissue of control and experimental animals in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Plasma</th>
<th>Erythrocyte membrane Vitamin E</th>
<th>Erythrocyte GSH (mg/dl)</th>
<th>Buccal tissue Vitamin E (mg/100mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vitamin E (mg/dl)</td>
<td>Vitamin C (mg/dl)</td>
<td>GSH (mg/dl)</td>
<td>Vitamin E (µg/mg protein)</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>1.25 ± 0.10</td>
<td>1.32 ± 0.29</td>
<td>28.77 ± 2.63</td>
<td>2.30 ± 0.43</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>0.74 ± 0.06</td>
<td>0.78 ± 0.13</td>
<td>25.10 ± 2.42</td>
<td>1.47 ± 0.18</td>
</tr>
<tr>
<td>3</td>
<td>DMBA+ZoARet (500mg/kg b.wt)</td>
<td>0.91 ± 0.20</td>
<td>1.11 ± 0.28</td>
<td>28.10 ± 2.62</td>
<td>1.88 ± 0.32</td>
</tr>
<tr>
<td>4</td>
<td>ZoARet alone (500mg/kg b.wt)</td>
<td>1.26 ± 0.11</td>
<td>1.37 ± 0.27</td>
<td>29.10 ± 2.29</td>
<td>2.36 ± 0.11</td>
</tr>
</tbody>
</table>

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 H₂O₂ utilized /min; C - µ moles of GSH utilized / min; D - µ moles of H₂O₂ utilized /sec. ZoARet. – *Zingiber officinale* Aqueous Root extracts.
### Table 4. The levels of enzymatic antioxidants in plasma, erythrocytes and buccal tissue of control and experimental animals in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Plasma</th>
<th>Erythrocyte lysate</th>
<th>Buccal tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SOD (U^a/ml)</td>
<td>CAT (U^b/ml)</td>
<td>GPx (U^c/l)</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>2.45± 0.37^a</td>
<td>0.48 ± 0.03^a</td>
<td>119.70±8.50^a</td>
</tr>
<tr>
<td>2</td>
<td>DMBA</td>
<td>1.43± 0.50^b</td>
<td>0.25 ± 0.02^b</td>
<td>72.05± 9.00^b</td>
</tr>
<tr>
<td>3</td>
<td>DMBA+ZoARet (500mg/kg b.wt)</td>
<td>2.43± 0.32^c</td>
<td>0.43±0.04^c</td>
<td>92.90± 9.67^c</td>
</tr>
<tr>
<td>4</td>
<td>ZoARet alone (500mg/kg b.wt)</td>
<td>2.40± 0.33^a</td>
<td>0.51±0.06^a</td>
<td>121.63±10.03^a</td>
</tr>
</tbody>
</table>

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 H2O2 utilized/min; C - μ moles of GSH utilized /min; D - μ moles of H2O2 utilized /sec

ZoARet – *Zingiber officinale* Aqueous Root extract.
Discussion

Cancer chemoprevention is currently regarded as one of the most promising avenues for cancer control (24). Recent advances in our understanding at the cellular and molecular levels of carcinogenesis have led to the development of a new promising strategy for cancer prevention that is chemoprevention (25). Chemoprevention offers a novel approach to control the incidence of oral cancer, an important contributor of cancer morbidity and mortality in the Indian subcontinent. A wide variety of phenolic substances found in our diet have been shown to exert substantial chemopreventive effects against experimental carcinogenesis (26).

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 (27). Reactive oxygen species are able to produce chemical modifications and to damage proteins, lipids, carbohydrates and nucleotides in the tissues (28). Reactive free radicals may damage cells by initiation of lipid peroxidation that causes profound alteration in the structural integrity and functions of cell membranes. Free radical induced lipid peroxidation has been implicated in the pathogenesis of several pathological disorders including cancer (29).

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 (30). Lipid peroxides play an important role in the control of cell division. Low concentrations of oxygen free radicals have been reported to stimulate cell proliferation; whereas high levels induce cytotoxicity and cell death (31). An inverse relationship has been observed between lipid peroxidation and the rate of cell proliferation, with highly proliferating tumors showing low levels of lipid peroxidation (32). The decline of lipid peroxidation in DMBA-induced oral tumors was associated with enhanced levels of GSH, GPx and GST. GSH plays an important role in scavenging reactive oxygen species protecting cell against cytotoxic and carcinogenic chemicals (33). Enhanced lipid peroxidation associated with antioxidant depletion in circulation is a characteristic finding in malignant transformation (34).

Lowered activities of SOD and CAT enzymes were reported in patients with malignant and as well as carcinogen induced experimental carcinogenesis (34). The deficiency of ascorbic 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. A decrease in the activities of GPx, SOD and catalase, the major cellular detoxifying enzyme systems, has been reported in malignancies (35). 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 (36).
Oral administration of *Zingiber officinale* aqueous root extracts significantly prevented the formation of oral squamous cell carcinoma in the hamster buccal pouches, which indicates its potent chemopreventive role in DMBA induced oral carcinogenesis. Although the possible mechanism include induction of phase II detoxification enzymes and increase enzymatic degradation of DMBA by liver and or enhance antioxidant defense mechanism to degrade the toxic effects of reactive oxygen species, generated by DMBA. Ginger extract was found to have immense antioxidant potential to scavenge free radicals, possibly due to its bioactive constituents, with the ability to donate electron(s) and scavenging the free radicals, specifically superoxide anions. This characteristic of plant extract was found to have a positive correlation with its antilipid peroxidation potential. Thus, the present study demonstrates the antilipidperoxidative potential of *Zingiber officinale* aqueous 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 officinale*.

References