

Evaluation of anticarcinogenic effects of *Clerodendron inerme* on 7,12-dimethylbenz(a) anthracene-induced hamster buccal pouch carcinogenesis

Manoharan S, Kavitha K, Senthil N, Renju G L

ABSTRACT

Introduction: Oral cancer is the fifth most frequent cancer worldwide and India has recorded the highest incidence (40-50 percent) of oral malignancy. *Clerodendron inerme* is used by Indian traditional practitioners for the treatment of various ailments, including cancer. Our aim was to investigate the chemopreventive potential of the aqueous leaf extract of *Clerodendron inerme* (CiAet) in 7,12-dimethylbenz(a) anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.

Methods: We developed oral squamous cell carcinoma in the buccal pouch of male Syrian golden hamsters by painting them with 0.5% DMBA in liquid paraffin thrice a week for 14 weeks. The tumour incidence, tumour volume and tumour burden that were formed in the hamster buccal pouches were determined.

Results: Oral administration of CiAet at a dose of 500 mg/kg body weight to DMBA-painted animals on days alternate to DMBA painting for 14 weeks significantly prevented the tumour incidence, tumour volume and tumour burden. CiAet also exerts potent antilipidperoxidative effect and improved the antioxidant defence system in DMBA-painted animals. The chemopreventive efficacy of CiAet was evident by inhibition of tumour formation (80%) in DMBA-painted animals.

Conclusion: The chemopreventive potential of CiAet is probably due to its antilipidperoxidative effect or the presence of some potent bioactive chemopreventive principles in the leaves of *Clerodendron inerme*.

Keywords: antioxidants, chemoprevention, *Clerodendron inerme*, hamster buccal pouch, lipid peroxidation, oral carcinogenesis

INTRODUCTION

Cancer of the oral cavity is one of the most common malignant diseases worldwide, and the highest incidence (40-50%) of oral cancer is observed in India⁽¹⁾. Epidemiological studies have shown that chewing of betel quid with tobacco is the major aetiological factor of oral carcinogenesis in India⁽²⁾. 7,12-dimethylbenz(a) anthracene (DMBA), a potent carcinogen, can initiate and promote the development of oral carcinoma of the buccal mucosa and DMBA-induced experimental oral cancer is the most widely-accepted experimental model, since it has many morphological and histological similarities with human oral carcinoma⁽³⁾.

Oxidative stress due to high flux of oxidants has been implicated in the pathogenesis of several cancers, including oral cancer⁽⁴⁾. Over-production of reactive oxygen species (ROS) has been well documented in betel quid and tobacco chewers⁽⁵⁾. ROS-mediated oxidative damage results in deoxyribonucleic acid (DNA) damage, and thereby contributes to mutagenesis and carcinogenesis⁽⁶⁾. It has been suggested that DMBA, on metabolic activation, induces cancer through an oxidative mediated genotoxicity by incorporating diol epoxide and other ROS into DNA⁽⁷⁾. The Human body has an array of defence mechanisms (enzymatic antioxidants – superoxide dismutase [SOD], catalase [CAT] and glutathione peroxidase [GPx] and non-enzymatic antioxidants – vitamin C, vitamin E and reduced glutathione [GSH]) to combat the deleterious effects of free radical-mediated oxidative damage⁽⁸⁾. Previous reports from our laboratory have documented the status of lipid peroxidation and antioxidants in both human and experimental oral carcinogenesis.

A wide number of traditionally important medicinal plants are still used by Indian traditional practitioners for the treatment of cancer. *Clerodendron inerme* (L.) Gaertn. (Pinarichangu) is one such medicinal plant popularly known as “Sangkupi” in Hindi and “Peechangu” in Tamil. Different parts of

Department of
Biochemistry
Faculty of Science
Annamalai University
Annamalai Nagar
608002
Tamil Nadu
India

Manoharan S, MSc,
MPhil, PhD
Senior Lecturer

Kavitha K, MSc, MPhil
Research Scholar

Senthil N, MSc, MPhil
Research Scholar

Renju G L, MSc
Research Scholar

Correspondence to:
Dr S Manoharan
Tel: (91) 4144 238 343
Fax: (91) 4144 238 145
Email: manshisak@
yahoo.com

the *Clerodendron inerme* (*C. inerme*) plant products are used in the Ayurvedic medicine for the treatment of rheumatism, skin disease, venereal infections, beri-beri and tumours^(9,10). To the best of our knowledge, there is no scientific data on chemopreventive efficacy and antilipidperoxidative potential of *C. inerme* aqueous leaf extract (CiAet) in DMBA-induced hamster buccal pouch carcinogenesis, except in the treatment of Ayurvedic and Siddha medicine. The present study was thus designed to provide the scientific evidence for the effectiveness of CiAet in modifying the carcinogenic process, as well as oxidative alterations induced during DMBA-induced oral carcinogenesis.

METHODS

The carcinogen, DMBA, was obtained from Sigma-Aldrich Chemical Pte Ltd, Bangalore India. All other chemicals used were of analytical grade. Male golden Syrian hamsters 8-10 weeks old weighing 80-120 g were purchased from National Institute of Nutrition, Hyderabad, India and were maintained in Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed five in a polypropylene cage and provided standard pellet diet and water ad libitum. The animals were maintained under controlled conditions of temperature and humidity with a 12 h light/dark cycle.

C. inerme was collected in and around Chidambaram and Cuddalore, Tamil Nadu, India. Dr R Panneer Selvam, Botanist, Department of Botany, Annamalai University verified the identity of the plant and a voucher specimen was also deposited in the Department of Botany, Annamalai University. 100 g of dried fine powder of *C. inerme* leaves was suspended in 250 ml of water for two hours and then heated at 60-65°C for 30 minutes. The extract was preserved and the process was repeated three times with the residual powder, each time collecting the extract. The collected extract was pooled and passed through fine cotton cloth. The filtrate, upon evaporation at 40°C, yielded 14% semisolid extract. This was stored at 0-4°C until use. A known volume of the residual extract is suspended in distilled water and was orally administered to the animals by gastric intubation using a force-feeding needle during the experimental period.

The local institutional animal ethics committee (Register number 160/1999/ CPCSEA), Annamalai University, Annamalai Nagar, India, approved the experimental design (Proposal No. 278: dated. 01-07-2005). The animals were maintained as per the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with Indian National Law on animal care and use.

A total of 40 hamsters were randomised into four groups of ten animals. Group I animals served as control and were painted with liquid paraffin thrice a week for 14 weeks on their left buccal pouches. Groups II and III animals were painted with 0.5% DMBA in liquid paraffin thrice a week for 14 weeks on their left buccal pouches. Group II animals received no other treatment. Group III animals were orally given CiAet at a dose of 500 mg/kg body weight, starting one week before exposure to the carcinogen and continued on days alternate to DMBA painting, until the scarification of the animals. Group IV animals received oral administration of CiAet alone throughout the experimental period. The experiment was terminated at the end of 14 weeks 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 mucosal tissues were fixed in 10% formalin and routinely processed and embedded with paraffin, 2-3µm sections were cut in a rotary microtome and stained with haematoxylin and eosin.

The plasma was separated by centrifugation at 3,000 rpm for 15 minutes. 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 minutes at 20°C. The erythrocyte membrane was prepared by the method of Dodge et al⁽¹¹⁾ modified by Quist⁽¹²⁾. Thiobarbituric acid reactive substances (TBARS) were assayed in plasma, erythrocytes, and buccal mucosa according to the methods of Yagi⁽¹³⁾, Donnan⁽¹⁴⁾ and Okhawa et al⁽¹⁵⁾, respectively. GSH was determined by the method of Beutler and Kelley⁽¹⁶⁾. Vitamins C and E were measured according to the methods of Omaye et al⁽¹⁷⁾ and Desai⁽¹⁸⁾, respectively. The activities of enzymatic antioxidants, SOD, CAT and GPx were estimated by the methods of Kakkar et al⁽¹⁹⁾, Sinha⁽²⁰⁾ and Rotruck et al⁽²¹⁾, respectively.

The data are expressed as mean ± standard deviation (SD). Statistical comparisons were performed by One way analysis of variance (ANOVA), followed by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if the p-values were less than 0.05.

RESULTS

The tumour incidence, tumour volume and tumour burden of control and experimental animals in each group are shown in Table I. We have observed 100% tumour formation with mean tumour volume

Table I. Incidence of oral neoplasm in control and experimental animals in each group (n=10).

Parameter	Group I control	Group II DMBA	Group III DMBA + CiAet	Group IV CiAet alone
Tumour incidence (Squamous cell carcinoma)	0	100%	20%	0
Total number of tumours/animal	0 ^a	4.2 ± 0.6 ^b	0.2 ± 0.4 ^a	0 ^a
Volume (mm ³)/tumour	0 ^a	454.13 ± 21.52 ^b	74.43 ± 4.07 ^c	0 ^a
Tumour burden (mm ³)/animal	0 ^a	1906.8 ± 72.19 ^b	74.43 ± 4.07 ^c	0 ^a

Tumour 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 D_1 , D_2 and D_3 are the three diameters (mm) of the tumour. Tumour burden was calculated by multiplying tumour volume and the number of tumours/animal

Values are given as mean ± SD. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)

CiAet: *Clerodendron inerme* aqueous leaf extract

Table II. Histopathological changes in the buccal pouch of hamsters in control and experimental animals in each group (n=10).

Parameter	Group I control	Group II DMBA	Group III DMBA + CiAet	Group IV CiAet alone
Keratosis	Absent	Severe	Moderate (++)	Absent
Hyperplasia	Absent	Severe	Mild (+)	Absent
Dysplasia	Absent	Severe	Mild (+)	Absent
Squamous cell carcinoma	Absent	Moderately differentiated (10)	Well differentiated (2)	Absent

CiAet: *Clerodendron inerme* aqueous leaf extract

Numbers in parentheses indicate total number of animals bearing tumours

Table III. Status of plasma TBARS and antioxidants in control and experimental animals in each group (n=10).

Parameter	Group I control	Group II DMBA	Group III DMBA + CiAet	Group IV CiAet
TBARS (nmoles/ml)	2.82 ± 0.22 ^a	4.75 ± 0.37 ^b	3.32 ± 0.25 ^c	2.73 ± 0.12 ^a
GSH (mg/dL)	29.02 ± 2.3 ^a	18.47 ± 1.28 ^b	24.38 ± 2.02 ^c	30.52 ± 2.7 ^a
Vitamin C (mg/dL)	1.46 ± 0.12 ^a	0.82 ± 0.07 ^b	1.32 ± 0.09 ^c	1.52 ± 0.12 ^a
Vitamin E (mg/dL)	1.26 ± 0.08 ^a	0.68 ± 0.05 ^b	1.06 ± 0.10 ^c	1.30 ± 0.09 ^a
SOD (U*/ml)	2.85 ± 0.23 ^a	1.72 ± 0.13 ^b	2.52 ± 0.18 ^c	2.92 ± 0.25 ^a
CAT (U**/ml)	0.44 ± 0.03 ^a	0.25 ± 0.02 ^b	0.33 ± 0.03 ^c	0.47 ± 0.04 ^a
GPx (U***L)	134.2 ± 11.3 ^a	96.8 ± 8.4 ^b	128.58 ± 9.04 ^a	135.7 ± 12.4 ^a

* Values are given as mean ± SD. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT)

** The amount of enzyme required to inhibit 50% nitroblue-tetrazolium (NBT) reduction

*** Micromoles of H₂O₂ utilised/s

**** Micromoles of glutathione utilised/min

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(454 mm³) and tumour burden (1,906.8 mm³) in DMBA alone-painted animals (Group II). Oral administration of CiAet at a dose of 500 mg/kg body weight significantly prevented the tumour incidence (80%), tumour volume and tumour burden in DMBA-painted hamsters (Group III). No tumour was observed in control animals painted with liquid paraffin alone (Group I) as well as CiAet alone-administered animals (Group IV).

The histopathological features observed in the buccal mucosa of hamsters in control and experimental

animals in each group are depicted in Table II. The buccal pouches from DMBA-treated hamsters revealed severe keratosis, hyperplasia, dysplasia and well-differentiated squamous cell carcinoma (Group II). A mild to moderate preneoplastic lesions (hyperplasia [+], keratosis [++] and dysplasia [+]) were noticed in Group III animals (DMBA + CiAet). The severity of pathological changes was done by Dr CR Ramachandran, Dean, Faculty of Dentistry, Rajah Muthaiah Medical College and Hospital, Annamalai University, Annamalai Nagar, when

Table IV. TBARS and antioxidant status in erythrocytes of control and experimental animals in each group (n=10).

Parameter	Group I control	Group II DMBA	Group III DMBA + CiAet	Group IV CiAet alone
Erythrocyte TBARS (pmoles/mg Hb)	1.84 ± 0.12 ^a	2.68 ± 0.21 ^b	2.18 ± 0.16 ^c	1.81 ± 0.14 ^a
Erythrocyte membrane TBARS (nmoles/mg protein)	0.35 ± 0.03 ^a	1.1 ± 0.09 ^b	0.52 ± 0.04 ^c	0.33 ± 0.03 ^a
Vitamin E (µg/mg protein)	2.38 ± 0.14 ^a	1.39 ± 0.10 ^b	2.18 ± 0.18 ^c	2.41 ± 0.17 ^a
Erythrocytes GSH (mg/dL)	43.21 ± 3.6 ^a	28.52 ± 2.3 ^b	38.08 ± 3.2 ^c	44.32 ± 3.1 ^a
Erythrocyte lysate SOD (U*/mg Hb)	2.13 ± 0.19 ^a	1.42 ± 0.11 ^b	1.98 ± 0.17 ^c	2.26 ± 0.20 ^a
CAT (U**/mg Hb)	1.26 ± 0.10 ^a	0.81 ± 0.08 ^b	1.18 ± 0.09 ^a	1.31 ± 0.12 ^a
GPx (U***/g Hb)	14.58 ± 1.24 ^a	7.44 ± 0.61 ^b	11.35 ± 1.07 ^c	14.82 ± 1.13 ^a

* Values are given as mean ± SD. Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

* The amount of enzyme required to inhibit 50% NBT reduction

** Micromoles of H₂O₂ utilised/s

*** Micromoles of glutathione utilised/min

CiAet: *Clerodendron inerme* aqueous leaf extract

Table V. Buccal mucosa TBARS and antioxidant status in control and experimental animals in each group (n=10).

Parameter	Group I control	Group II DMBA	Group III DMBA + CiAet	Group IV CiAet alone
TBARS (nmoles/100 mg protein)	77.89 ± 6.23 ^a	48.3 ± 3.86 ^b	72.32 ± 6.84 ^a	78.45 ± 6.42 ^a
GSH (mg/100 mg tissues)	6.48 ± 0.41 ^a	13.16 ± 0.98 ^b	7.28 ± 0.42 ^c	6.42 ± 0.39 ^a
Vitamin E (mg/100 mg tissues)	1.89 ± 0.15 ^a	2.92 ± 0.16 ^b	2.13 ± 0.21 ^c	1.82 ± 0.14 ^a
SOD (U*/mg protein)	5.32 ± 0.29 ^a	3.58 ± 0.25 ^b	4.97 ± 0.36 ^a	5.36 ± 0.28 ^a
CAT (U**/mg protein)	38.85 ± 2.86 ^a	22.16 ± 1.8 ^b	35.24 ± 2.28 ^c	39.21 ± 2.51 ^a
GPx (U***/g protein)	7.12 ± 0.61 ^a	10.32 ± 0.72 ^b	7.51 ± 0.56 ^a	7.03 ± 0.53 ^a

* Values are given as mean ± SD. Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT)

* The amount of enzyme required to inhibit 50% NBT reduction

** Micromoles of H₂O₂ utilised/s

*** Micromoles of glutathione utilised/min

CiAet: *Clerodendron inerme* aqueous leaf extract

examining the histopathological slides under the microscope.

The levels of TBARS and antioxidants (vitamins E and C, GSH, SOD, CAT and GPx) in plasma and erythrocytes, respectively, of control and experimental animals in each group are shown in Tables III and IV. The concentration of TBARS was increased, whereas the status of antioxidants was significantly decreased in tumour-bearing animals (Group II), compared to control animals. Oral administrations of CiAet to DMBA-painted animals (Group III) significantly reverted the status to normal concentrations of TBARS and antioxidants. Hamsters treated with CiAet alone (Group IV) showed no significant difference in TBARS and antioxidants status, compared to control animals (Group I).

The status of TBARS and antioxidants in the buccal mucosa of control and experimental animals in each group is given in Table V. Decrease in TBARS

level and disturbances in antioxidant status (vitamin E, GSH and GPx were increased; SOD and CAT were decreased) were noticed in oral cancer animals (Group II), compared to control animals (Group I). Oral administration of CiAet to DMBA-painted animals (Group III) reverted the concentration of TBARS and antioxidants to near normal range. Hamsters treated with CiAet alone (Group IV) showed no significant difference in TBARS and antioxidants status, compared to control animals (Group I).

DISCUSSION

Oral cancer, a disfiguring disease, has multifactorial aetiologies and occurs predominantly during the sixth to eighth decades of life⁽²²⁾. Oral carcinogenesis is a multifocal disease preceded by distinct premalignant lesions. DMBA-induced precancerous and cancerous lesions in hamsters resemble human oral precancerous and cancerous lesions.

Cancer chemoprevention deals with the prevention, inhibition and delay or reversal of carcinogenic process by using natural and synthetic agents⁽²³⁾. Several experimental and epidemiological studies documented the chemopreventive activities of many herbal plants and their bioactive constituents^(24,25). Medicinal plants may exert the carcinogenic potential by modulating carcinogen detoxification, inhibiting lipid peroxidation, or by improving in vivo antioxidants defence mechanism⁽²⁶⁾. In the present study, the chemopreventive potential of CiAet in DMBA-painted animals was monitored by observing the status of tumour burden, percentage of tumour-bearing animals, and by determining the status of lipid peroxidation and antioxidants in blood and buccal mucosal tissues.

We have observed hyperplasia, dysplasia and severe keratosis at the eighth to tenth week of carcinogen treatment and well-differentiated squamous cell carcinoma at 14th week in DMBA-painted animals. Although mild precancerous lesions were observed in all animals, tumour formation was seen only in two DMBA-painted animals treated with CiAet (Group III). Oral administration of CiAet at a dose of 500 mg/kg body weight to DMBA-painted animals on days alternate to DMBA painting for 14 weeks significantly reduced the tumour incidence, tumour volume and tumour burden.

Free radical-induced lipid peroxidation is regarded as one of the basic mechanism of cellular damage and therefore, the extent of tissue damage can be monitored by measuring the concentration of plasma or serum lipid peroxides⁽²⁷⁾. Increased plasma lipid peroxidation has been reported in several types of cancer patients⁽²⁸⁾. Erythrocytes are constantly exposed to oxidative stress, and susceptibility of erythrocytes to oxidative stress has been reported in several pathological conditions, including oral cancer⁽²⁹⁾. Elevated lipid peroxidation in cancer patients may also be correlated to their poor antioxidant system⁽³⁰⁾. Thus, the observed increase in plasma lipid peroxides in DMBA-painted animals is due to overproduction and diffusion from the damaged erythrocytes, erythrocyte membranes, and some other host tissues such as the liver.

Aerobic organisms utilise enzymatic and non-enzymatic antioxidants as the first line of defence against oxidative stress induced by ROS. Antioxidants exert their protective role either by suppressing the formation of free radicals or by scavenging them. In the multistage process of carcinogenesis (initiation and promotion), cell immortalisation and transformation have been shown to be inhibited by antioxidants⁽³¹⁾. Vitamin E, vitamin C and GSH can protect cells and tissues

by eliminating or quenching excessively generated ROS in the body⁽³²⁾.

It has been reported that tumour cells can sequester essential nutrients and antioxidants to meet the demands of the growing tumour⁽³³⁾. Thus the lowered concentration of plasma vitamin E, vitamin C and GSH are probably due to their utilisation by tumour tissues for their growth or to combat the deleterious effect of excessively-generated lipid peroxides in the circulation. Lowered activities of SOD, CAT and GPx in erythrocytes were reported in cancer by several authors⁽³⁴⁻³⁶⁾. Our results corroborate these observations.

Increased levels of lipid peroxidation byproducts play a role in the early phases of tumour growth⁽³⁷⁾. Substantial amounts of H₂O₂ and superoxide are produced in tumour cells⁽³⁸⁾. Tumour progression is associated with the low levels of malonaldehyde, one of the most extensively investigated products of lipid peroxidation⁽³⁹⁾. It has been reported that lipid peroxidation and the rate of cell proliferation in tumour tissues are inversely correlated⁽⁴⁰⁾. Low availability of peroxidisable substrates, such as polyunsaturated fatty acids, (PUFA), has been demonstrated in tumour tissues⁽⁴¹⁾. Thus, decreased susceptibility of tumour tissues to lipid peroxidation can be related to increased cell proliferation occurring in oral carcinogenesis or low availability of PUFA in tumour tissues. Activities of SOD and CAT were found to be decreased in oral cancer tumour tissues as compared to their normal counterparts. Lowered activities of SOD and CAT are probably due to exhaustion of these enzymes to scavenge excessively-generated superoxide and hydrogen peroxides, respectively, in tumour cells⁽⁴²⁾.

GSH, a cosubstrate of glutathione peroxidase, neutralises hydroxyl radicals and singlet oxygen. GSH in tissues keeps up the cellular levels of vitamins C and E in an active form. Recent studies have demonstrated that glutathione and GPx have regulatory effects on cell proliferation and are over expressed in various malignant tumours⁽⁴³⁾. Increased levels of glutathione and vitamin E in tumour tissues suggest that tumour tissues sequester these nutrients for their growth and enhancement of their antioxidant capacity⁽³³⁾. Low availability of peroxidisable substrates and enhanced antioxidant capacity of tumour tissues make them less susceptible to oxidative stress.

Oral administration of CiAet not only prevented the tumour formation but also significantly improved the status of lipid peroxidation and antioxidants in DMBA-painted animals, which clearly indicates its potent chemopreventive, antilipidperoxidative, and antioxidant potential in DMBA-induced hamster buccal pouch carcinogenesis. Oral administration of CiAet at a

dose of 500 mg/kg body weight significantly prevented tumour incidence, tumour volume, tumour burden and the number of tumours in DMBA-painted hamsters, which indicates that CiAet has a suppressive effect on cell proliferation in DMBA-induced hamster buccal pouch carcinogenesis. The phytochemical examination of *C. inermis* revealed the presence of flavanoids, sterols, flavones, triterpenes, diterpenes, quinone and neolignans⁽¹⁰⁾. The chemopreventive effect of CiAet is probably due to the presence of several bioactive chemopreventive principles and their synergistic effects.

Several authors have suggested that the chemopreventive properties of plant anticarcinogens are either due to antilipidperoxidative action (scavenging excess ROS), modulating carcinogens detoxification (elevation of carcinogen detoxifying enzymes) or improving the antioxidant defence mechanism^(44,46). Our results therefore indicate that *C. inermis* exerts its chemopreventive action by modulating lipid peroxidation and antioxidant defence mechanisms. Thus, the present investigation warrants further studies to isolate and characterise bioactive chemopreventive principles from the leaves of *Clerodendron inermis*.

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