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Chemopreventive and Antilipidperoxidative Potential of *Clerodendron inerme* (L) Gaertn in 7,12-dimethylbenz(a)anthracene Indcued Skin Carcinogenesis in Swiss Albino Mice

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Abstract: The present study has investigated the chemopreventive and antilipidperoxidative effects of the ethanolic extract of *Clerodendron inerme* leaves (CiELet) in DMBA induced skin carcinogenesis in Swiss albino mice. The skin squamous cell carcinoma was induced in the shaved back of mice, by painting with DMBA (25 µg 0.1 mL^{-1} acetone) twice weekly for 8 weeks. We have observed 100% tumor formation in the fifteenth week of experimental period. Elevated lipid peroxidation and decline in enzymatic and non-enzymatic antioxidants status was observed in tumor bearing mice. Oral administration of CiELet (300 mg kg^{-1} bw) for 25 weeks significantly prevented the tumor incidence, volume and burden of the tumor. The CiELet also showed potent antilipidperoxidative effect as well as enhanced the antioxidant defense mechanisms in DMBA painted mice. The present study thus demonstrated the chemopreventive and antilipidperoxidative efficacy of CiELet in DMBA induced mouse skin carcinogenesis.

Key words: Chemoprevention, skin cancer, *Clerodendron inerme*, lipidperoxidation, antioxidants

INTRODUCTION

Skin is the most common site of malignancy and represents 55% all human cancers. Three major forms of skin cancer are basal cell carcinoma, squamous cell carcinoma and malignant melanoma. The incidence of squamous cell carcinoma is increasing rapidly worldwide and ranks second place in the prevalence of skin tumors. It is most common in 5th and 6th decade of life and occurs mostly due to cumulative ultraviolet radiation. Skin squamous cell carcinoma accounts for 16% of all skin cancers (Jemal *et al.*, 2003; Einspahr *et al.*, 2002; Kyriazi *et al.*, 2006). 7,12-dimethylbenz(a)anthracene, a potent organ specific carcinogen, is present in the environment as a product of incomplete combustion of complex hydrocarbons. DMBA can act either as a complete carcinogen or as an initiator of mouse skin carcinogenesis (Shukla *et al.*, 1998). DMBA on metabolic activation produces diol-epoxide (ultimate carcinogen) which mediates carcinogenic process by inducing chronic inflammation, over production of reactive oxygen species (ROS) and oxidative DNA damage (Slaga *et al.*, 1979).

Over production of reactive oxygen species in the cell induces cellular oxidative damage through DNA strand breaks and lipidperoxidation. It has been suggested that exposure to various carcinogenic agents result in enormous production of reactive oxygen species which cause DNA damage and contributing to carcinogenesis (Mcord, 2000; Ray and Husain, 2002). The most important characteristics of free radicals *in vivo* are lipid peroxidation and free radical induced lipid peroxidation has been implicated in the pathogenesis of several disorders including cancer (Mcord, 2000). Free radical induced lipid peroxidation also causes damage to membrane structure and function, increase in membrane fragility and reduction in cell fluidity (Kolanjiappan *et al.*, 2002). However, human body has an array of sophisticated antioxidant defense mechanism (enzymatic and non-enzymatic) to combat the deleterious effects of ROS mediated oxidative stress. Enzymatic antioxidant defense system includes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) whereas the non-enzymatic antioxidants include vitamin E, C and reduced glutathione (Gutteridge, 1995).

Chemoprevention, a novel approach in experimental oncology, deals with the prevention, inhibition, suppression and reversal of carcinogenesis by the use of natural products and synthetic agents. Any natural

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products or synthetic agents which possess antimutagenic, anticarcinogenic, inhibitory effects on cellular proliferation, free radical scavenging activity are considered as a good chemopreventive agent (F-guyer *et al.*, 2003). Experimental studies have demonstrated the chemopreventive potential of a large number of traditional medicinal plants and their possible mechanism of action (Kelloff, 2000; Hong and Sporn, 1997).

Liver plays a crucial role in the detoxification process and thus the measurement of the activities of detoxification agents such as Glutathione-S-Transferase (GST), Glutathione Reductase (GR) and reduced glutathione help to assess the chemopreventive potential of the test compound (Wilkinson and Clapper, 1997). Medicinal plants which induce the activity of detoxification agents such as GST and GR are considered to have chemopreventive potential (Coles and Ketterer, 1990; Hu and Singh, 1997). GST detoxifies carcinogens either by destroying their reactive centers or facilitating their excretion by conjugation process. Glutathione Reductase (GR), an important enzyme required for reduced glutathione maintenance, catalyses NADPH dependent reduction of glutathione disulfide to reduced glutathione (Hu and Singh, 1997; Abraham and Singh, 1999). Altered level of reduced glutathione (GSH) in the liver indicates the insult by toxic foreign agents (McLellan and Wolf, 1999; Schwartz and Shklar, 1996).

A large number of traditionally valuable medicinal plants are still used in Indian traditional medicine for the treatment of cancer. *Clerodendron inerme* (L.) Gaertn is one such important medicinal plant which is commonly known as Seaside Clerodendron in English, Sankupi in Hindi and Peechangu in Tamil (Kiritikar and Bau, 1975). In Ayurvedic traditional medicine, different parts of *Clerodendron inerme* plant products are considered beneficial for the treatment of rheumatism, skin disease, veneral infections, beri-beri and tumors (Rehman *et al.*, 1997). No scientific studies were however available for its chemopreventive and antilipidperoxidative efficacy in DMBA induced skin carcinogenesis. The present study was therefore designed to provide scientific evidence for the chemopreventive and antilipidperoxidative efficacy of *Clerodendron inerme* in DMBA induced skin carcinogenesis.

MATERIALS AND METHODS

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

Animals: Male Swiss Albino mice 4-6 weeks old, weighing 15-20 g were purchased from National Institute of Nutrition, Hyderabad, India and maintained in the Central Animal House, Rajah Muthaiah Medical College and Hospital, Annamalai University. The animals were housed in groups of four or five in polypropylene cages and provided standard pellet diet and water *ad libitum* and maintained under controlled conditions of temperature and humidity, with a 12 h light/dark cycle.

Plant material: *Clerodendron inerme* (L.) Gaertn 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.

Preparation of plant extract
Ethanolic extract of *Clerodendron inerme* leaves: The ethanolic extract of *Clerodendron inerme* leaves was prepared according to the method of Hossain *et al.* (1992). Five hundard gram of fresh leaves of *Clerodendron inerme* leaves were dried, powdered and soaked in 1500 mL of 95% ethanol over night. After filtration, the residue obtained was again resuspended in equal volume of 95% ethanol for 48 h and filtered again. The above two filtrates were mixed and the solvents was evaporated in a rotavapour at 40-50°C, under reduced pressure. A 14% semisolid light greenish yellow material obtained was stored at 0-4°C until used. A known volume of the residual extract is suspended in distilled water and was orally administered to the animals during the experimental period.

Experimental design: A total number of 40 male Swiss albino mice were divided into four groups of 10 each. Skin carcinogenesis was developed in Swiss albino mice according to the method of Azuine and Bhide (1992). Depilatory cream was applied to remove hair from the back of each mouse and the mice were left untreated for two days. Mice having no hair growth after two days were selected for the experimental study.

The depilated back of group I mice was painted with acetone (0.1 mL/mouse) twice weekly for 8 weeks (vehicle treated control). The depilated back of groups II and III mice were painted with DMBA (25 μg in 0.1 mL acetone/mouse) twice weekly for 8 weeks. Group II mice received no other treatment. Group III mice were administered CiELet (300 mg kg^{-1} bw in 1 mL distilled

water) orally starting 1 week before the exposure to the carcinogen and continued for 24 weeks (3 times/week on alternate days) thereafter. Group IV animals were received CiELet (300 mg kg^{-1} bw in 1 mL distilled water) alone 3 times per week throughout the experimental period. At the end of experimental period all the animals were sacrificed by cervical dislocation. Biochemical estimations were carried out in plasma, erythrocytes, liver and skin tissues of control and experimental animals in each group. For histopathological examination, skin tissues were fixed in 10% formalin and embedded with paraffin, 2-3 μm sections were cut in a rotary microtome and stained with hematoxylin and eosin.

Biochemical analysis: Blood samples were collected into heparinised tubes and plasma was separated by centrifugation at 3000 rpm for 15 min. The erythrocyte membrane was prepared by the method of Dodge *et al.* (1968) modified by Quist (1980). Thiobarbituric Acid Reactive Substance (TBARS) was assayed in plasma, erythrocyte membrane, skin tissue, according to the methods of Yagi (1978), Donnan (1950) and Okhawa *et al.* (1979), respectively. Lipid hydroperoxides was assayed by the method of Jiang *et al.* (1992). Reduced glutathione was determined by the method of Beutler and Kelley (1963). The activities of enzymatic antioxidants, were estimated by the methods of Kakkar *et al.* (1984) (SOD), Sinha (1972) (CAT) Rotruck *et al.* (1973) (GPx), respectively. The phase II detoxification enzymes activities were estimated by the methods of Habig *et al.* (1974) (GST) and Carlberg and Mannervik (1985) (GR), respectively.

Statistical analysis: Values are expressed as mean±SD. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Student's t-test. The values were considered statistically significant, if p-value was less than 0.05.

RESULTS

We have observed 100% tumor formation with mean tumor volume (513.42 mm^3) and tumor burden (1488.91 mm^3) in DMBA alone-painted animals (Group II). Oral administration of CiELet at a dose of 300 mg kg^{-1} body weight significantly prevented the tumor incidence, tumor volume and tumor burden in DMBA painted Swiss albino mice (Group III). No tumor was observed in control animals painted with acetone alone (Group I) as well as CiELet alone administered animals (Group IV) (Table 1).

The skin tissues from DMBA treated mice revealed severe keratosis, hyperplasia, dysplasia and well differentiated squamous cell carcinoma Group II (Table 2). A mild to moderate preneoplastic lesions (keratosis [++], hyperplasia [++] and dysplasia [+] were noticed in group III animals (DMBA + CiELet). The severity of pathological changes was done by Dr.C.R.Ramachandran, Dean, Department of Oral Pathology, Faculty of Dentistry, Rajah Muthiah Dental College and Hospital, Annamalai University, Annamalainagar, when examining the histopathological slides under the microscope.

The concentrations of TBARS and lipid hydroperoxides were increased in tumor bearing animals (Group II), compared to control animals. Oral administration of CiELet to DMBA painted animals (Group III) significantly restored the status to normal concentrations of TBARS and lipid hydroperoxides. Mice treated with CiELet alone (Group IV) showed no significant different in TBARS and lipid hydroperoxides status as compared to control animals (Group I) (Table 3).

Table 1: Tumor incidence, tumor volume and tumor burden of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
Tumor incidence	0	100%	0	0
Total number of tumors/animals	0	29/10	0	0
Tumor volume (mm^3)	0	513.42±56.47	0	0
Tumor burden (mm^3)	0	1488.91±104.22	0	0

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 D$_1$, D$_2$ and D$_3$ are the three diameters (mm) of the tumors. Tumor burden was calculated by multiplying tumor volume and the number of tumors/animal; Values are expressed as mean±SD (n = 10 mice); CiELet-*Clerodendron inerme* ethanolic leaf extract

Table 2: Histopathological features of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
Keratosis	Absent	Severe	Moderate	Absent
Hyperplasia	Absent	Severe	Moderate	Absent
Dysplasia	Absent	Severe	Mild	Absent
Squamous cell carcinoma	Absent	Well differentiated	Absent	Absent

CiELet-*Clerodendron inerme* ethanolic leaf extract

Table 3: Levels of TBARS and Lipidhydroperoxides in plasma, erythrocyte membrane and skin tissue of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
TBARS				
Plasma (nmol mL^{-1})	3.42±0.26	6.1±0.94[a]	3.77±0.28[a*b]	3.40±0.22[a*]
Erythrocytemembrane (nmol mg^{-1} protein)	0.35±0.02	0.93±0.10[a]	0.41±0.05[a*b]	0.36±0.03[a*]
Skin tissue (mmol/100 g tissues)	12.39±0.75	22.08±2.88[a]	13.70±1.23[a*b]	12.20±0.62[a*]
Lipid hydroperoxides				
plasma (x10^{-5} mM dL^{-1})	4.20±0.34	7.2±1.09[a]	4.65±0.34[a*b]	4.13±0.27[a*]
Erythrocyte membrane (nmol mg^{-1} protein)	3.23±0.20	5.49±0.75[a]	3.59±0.34[a*b]	3.19±0.16[a*]
Skin tissue (mmol/100 g tissues)	143.3±7.86	192.45±10.14[a]	153.92±8.52[a*b]	141.59±7.21[a*]

Values are expressed as mean±SD (n = 10 mice); a-significantly different from control animals (group I) [a]p<0.001; [a*]p<0.01. [a*]p-Not significant; b-significantly different from tumor bearing animals (group II), [b]p<0.001; CiELet-*Clerodendron inerme* ethanolic leaf extract

Table 4: Activities of enzymatic and non enzymatic antioxidants in erythrocytes of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
SOD (U^A mg^{-1} Hb)	6.14±0.48	3.84±0.34[a]	5.47±0.56[a*b]	6.17±0.35[a*]
CAT (U^B mg^{-1} Hb)	2.69±0.20	1.42±0.22[a]	2.40±0.27[a*b]	2.75±0.16[a*]
GPx (U^C g^{-1} Hb)	29.09±2.25	17.73±2.03[a]	26.06±2.41[a*b]	29.69±1.79[a*]
GSH (mg dL^{-1})	40.21±2.95	24.16±3.56[a]	35.98±3.52[a*b]	41.62±2.08[a*]

Values are expressed as mean±SD (n = 10 mice); a-significantly different from control animals (group I); [a]p<0.001.[a*]p<0.01.[a*]p-Not significant; b-significantly different from tumor bearing animals (group II), [b]p<0.001; ^{A}-The amount of enzymes required to inhibit 50% nitroblue-tetrazolium (NBT) reduction.^{B}-Micromoles of H_2O_2 utilised/s ^{C}-Micromoles of glutathione utilised min^{-1} CiELet-*Clerodendron inerme* ethanolic leaf extract

Table 5: Activities of enzymatic and non enzymatic antioxidants in skin tissues of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
SOD (U^A mg^{-1} protein)	6.61±0.52	3.84±0.48[a]	5.87±0.61[a*b]	6.70±0.46[a*]
CAT (U^B mg^{-1} protein)	39.27±3.14	22.39±2.37[a]	34.23±4.38[a*b]	40.35±3.15[a*]
GPx (U^C g^{-1} protein)	46.87±3.74	28.26±3.15[a]	41.93±3.78[a*b]	46.59±3.16[a*]
GSH (mg/100 mg tissues)	39.14±3.18	22.69±2.82[a]	34.43±3.87[a*b]	39.49±2.36[a*]

Values are expressed as mean±SD (n = 10 mice); a-significantly different from control animals (group I);[a]p<0.001. [a*]p< 0.01. [a*]p-Not significant; b-significantly different from tumor bearing animals (group II), [b]p<0.001; ^{A}-The amount of enzymes required to inhibit 50% nitroblue-tetrazolium (NBT) reduction; ^{B}-Micromoles of H_2O_2 utilised/s ^{C}-Micromoles of glutathione utilised min^{-1} CiELet-*Clerodendron inerme* ethanolic leaf extract

Table 6: Activities of phase II enzymes and reduced glutathione of control and experimental animals in each group

Parameters	Group I Control	Group II DMBA	Group III DMBA + CiELet	Group IV CiELet alone
Reduced glutathione (mg g^{-1} tissue)	2.79±0.25	1.54±0.21[a]	2.47±0.28[a*b]	2.85±0.25[a*]
Glutathione S-transferase (U^A mg^{-1} protein)	171.26±9.0	107.39±11.0[a]	157.39±1.76[a*b]	174.23±9.15[a*]
Glutathione reductase (U^B mg^{-1} protein)	40.21±3.21	25.64±1.76[a]	36.04±3.12[a*b]	39.96±2.59[a*]

Values are expressed as mean±SD (n = 10 mice); a-significantly different from control animals (group I); [a]p<0.001. [a*]p<0.01. [a*]p-Not significant; b-significantly different from tumor bearing animals (group II), [b]p<0.001; ^{A}-μ moles of CDNB-GSH conjugate formed per h; ^{B}-μ moles of NADPH oxidized per h; CiELet-*Clerodendron inerme* ethanolic leaf extract

The status of antioxidants was significantly decreased in tumor bearing animals (Group II) as compared to control animals. Oral administration of CiELet to DMBA painted animals (Group III) brought back the concentration of antioxidants to near normal range. Mice treated with CiELet alone (Group IV) showed no significant difference in antioxidant status as compared to control animals (Group I) (Table 4 and 5).

The status of detoxification enzymes was significantly decreased in tumor bearing animals (Group II) as compared to control animals. Oral administration of CiELet to DMBA painted animals (Group III) significantly revert back the status to near normal concentrations. Mice treated with CiELet alone (Group IV) showed no significant difference in phase II detoxification enzymes activities as compared to control animals (Group I) (Table 6).

DISCUSSION

Skin cancer is the most common malignancy in White population with tremendous impact on health and morbidity (Jemal *et al.*, 2003; Kyriazi *et al.*, 2006). The mouse skin carcinogenesis model is commonly used to test the chemopreventive and antitumor promoting efficacy of medicinal plants and their constituents. In the present study, the chemopreventive efficacy of CiELet was assessed in DMBA induced skin carcinogenesis in mice by monitoring the percentage of tumor bearing animals, tumor volume and burden as well as by analyzing the status of detoxification enzymes, lipid peroxidation and antioxidants in DMBA painted animals. DMBA, a polycyclic aromatic hydrocarbon induces skin lesions when applied repeatedly on the mouse skin. Control animals treated with vehicle alone (acetone) showed well

defined skin and the presence of subcutaneous tissues. DMBA alone treated animals showed 100% tumor incidence and the tumor was histopathologically diagnosed as well differentiated squamous cell carcinoma. The tumor cells have pleiomorphic hyperchromatic nuclei with epithelial pearl formation. Oral administration of CiELet completely prevented the formation of well differentiated squamous cell carcinoma. We have however observed precancerous lesions such as hyperplasia and dysplasia. Our results thus indicate that CiELet has suppressing effect on cell proliferation. The chemopreventive efficacy may also be due to the presence of one or more bioactive principles and their cumulative effects.

In the present study the activities of glutathione-s-transferase (GST) and Glutathione Reductase (GR) and the level of reduced glutathione (GSH) were decreased in DMBA treated animals as compared to control animals. Several studies have pointed out that chemopreventive agents convert DNA damaging entities into excretable metabolites through the induction of detoxifying agents (Hu and Singh, 1997; Abraham and Singh, 1999). Oral administration of CiELet to DMBA treated mice restored the status of GST, GR and GSH, which indicates its role on carcinogen detoxification.

Oxidative stress, an imbalance in oxidant-antioxidant status, has been implicated in multistage carcinogenesis as well as in the genesis of other diseases (Mcord, 2000; Ray and Husain, 2002). Over production of ROS can induce strand breaks and can modify DNA bases, contributing to mutagenesis and carcinogenesis. In the present study, elevated levels of TBARS and lipid hydroperoxides were observed in plasma, erythrocyte membrane and skin tissues of DMBA painted animals.

Enormous production of Reactive Oxygen Species (ROS) has been well documented in carcinogenesis (Mccord, 2000; Ray and Husain, 2002). The major target of free radicals in vivo or in vitro is membrane lipid peroxidation which inturn cause alterations in the structural integrity and function of the membrane (Kolanjiappan *et al.*, 2002). In diseased condition, erythrocytes are more prone to oxidative stress (Kolanjiappan *et al.*, 2002; Manoharan *et al.*, 2005). Gutteridge (1995) has reported that over production of lipid peroxides serve as biomarkers of tissue damage. Several experimental and human studies have demonstrated increased levels of TBARS in carcinogenesis (Manoharan *et al.*, 2006; Kavitha and Manoharan, 2006). The observed plasma TBARS and lipid hydroperoxides are probably due to overproduction and diffusion from the damaged tissues (erythrocyte membrane, skin tissues and other host tissues) with subsequent leakage into plasma. The increase in skin tumor tissue TBARS is probably due to repeated carcinogenic (DMBA) insult on the skin.

Antioxidants acts synergistically to counteract the deleterious effects of free radical mediated lipid peroxidation. SOD, CAT and GPx, major antioxidant enzymes, protect cells by catalyzing the harmful reactive oxygen species such as O_2^- (SOD) and H_2O_2 (CAT and GPx). Lowered activities of these enzymes were reported in several cancerous conditions including skin carcinogenesis (Manoharan *et al.*, 2005; Dhawan *et al.*, 1999). Our results are in line with these findings.

Glutathione, an endogeneous antioxidant, can able to scavenge several potent ROS alone and together with glutathione dependent enzymes (McLellan and Wolf, 1999). The decreased levels of these non-enzymatic antioxidants are probably due to utilization by tumor tissues for their growth as well as to combat the harmful effects of excessively generated ROS. Lowered enzymatic and non enzymatic antioxidants observed in the present study confirm the state of oxidative stress in DMBA induced mouse skin carcinogenesis.

Oral administration of CiELet restored the status of lipid peroxides and antioxidants in DMBA painted animals. Our results thus indicate that CiELet has potent antilipidperaxidative and antioxidant functions. The antioxidant function of CiELet is also due to the presence of one or more antioxidant principles and their synergistic effects. The present study thus demonstrated the chemopreventive, antilipidperoxidative and antioxidant function of CiELet in DMBA induced skin carcinogenesis. Further studies are needed to isolate and characterize the bioactive constituents from the leaves of *Clerodendron inerme*.

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