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Review Article

CISSUS QUADRANGULARIS INHIBITS TUMOR FORMATION DURING 7, 12-DIMETHYLBENZ (A) ANTHRACENE INDUCED ORAL CARCINOGENESIS

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ABSTRACT

The Indian system of traditional medicine has documented diverse pharmacological effects of *Cissus quadrangularis*, including anti-inflammatory and anticancer properties. The present study is indented to evaluate and explore the tumor preventive potential of the ethanolic extract of *Cissus quadrangularis* leaves (CqELet) in 7,12-dimethylbenza(a)nthracene (DMBA) induced hamster buccal pouch carcinogenesis. The tumor preventive efficacy of CqELet was determined by analysing the status of biomarkers (TBARS, Antioxidants, phase I and Phase II detoxification agents) and using histopathological studies, in addition to the observation of the tumor incidence during the experimental period. Although CqELet attenuated the tumor formation and restored the biochemical variables in the pre-initiation phase, tumor formation in the buccal pouch was noticed in 33% of the animals in the post- initiation phase. The results of the present study thus warrant for extending the experimental period to confirm the tumor preventive ability of CqELet in the pre-initiation phase.

INTRODUCTION

Cancer arises due to a spectrum of pathological diseases and is characterized by rapid and abnormal cell proliferation, spreading into adjacent tissues and to distant organs. Cancer usually occurs as a result of accumulation of multiple mutations in the genetic material, DNA. The abnormal mass of tissues seen in the lips, cheeks, tongue and floor of the mouth is in general termed as oral carcinoma, the cancer of the oral cavity. Though oral cancer is life threatening, it can be treated if diagnosed early. The risk factors that are strongly linked to oral carcinogenesis include high consumption of tobacco, either in the form of smoking or chewing, and alcoholic beverages. The incidence of oral carcinoma throughout the world is steadily increasing every year, especially in developing countries, including India (Castellsagué *et al,* 2004; Vigneswaran and Williams, 2014).

7,12-dimethylbenz(a)anthracene is a well known chemical carcinogen and is used to develop tumors in a wide variety of animal organs, including mouth, skin and breast. This site specific carcinogen mediates cancer through inducing chronic inflammation, causing extensive DNA damage and by generating excessive reactive oxygen species (ROS). DMBA induced experimental oral carcinogenesis imitates human oral carcinogenesis at histological, molecular and biochemical aspects and thus preferred to investigate the tumor preventive potential of natural products and or synthetic constituents (Miyata *et al,* 1999; Manimaran *et al,* 2017).

Oxidative stress has been documented as one of the major risk factors in the pathogenesis of several diseases, including cancer and diabetes (Rahman *et al,* 2012). Oxidative stress mediated chronic inflammation has been suggested as the major mechanism in the pathogenesis of these chronic diseases. Over production of ROS in the cell can cause extensive damage to cell structure and function, which can in turn lead to neoplastic transformation. On the other hand, host cells are endowed with enzymatic and non-enzymatic antioxidants to battle against the deleterious effects of ROS. An imbalance in the role of oxidants (reactive oxygen species) and antioxidants [vitamin E, glutathione (GSH), glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT)] lead to oxidative stress, which in turn cause oxidative damage to various biomolecules such as lipids, proteins and nucleic acids, there by leading to carcinogenesis (Reuter *et al,* 2010; Birben *et al,* 2012). Profound studies have also implicated the significance of phase I and II detoxification agents in carcinogenesis. Extensive

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studies on carcinogenesis reported an imbalance in the activities of phase I and phase II detoxification agents (Karthikeyan *et al,* 2013).

Cissus quadrangularis, popularly known as bone setter, is used in the traditional medicine, especially to treat several feminine disorders and for joint and bone health. This plant extract has been recommended for pain, peptic ulcer, hemorrhoids, obesity, diabetes, scurvy and cancer. Extensive in vitro studies documented its cytotoxic potential against skin cancer cell lines and Daltonls ascitic lymphoma cell lines (Ayesha *et al,* 2017; Bhujade *et al,* 2013). *Cissus quadrangularis* explored the anti-inflammatory and gastroprotective effect in the experimental animal model as well (Netaji *et al,* 2015; Jainu *et al,* 2006). The present study, for the first time, reported the tumor preventive efficacy of *Cissus quadrangularis* leaves in DMBA induced oral carcinogenesis.

MATERIALS AND METHODS

Preparation of the plant extract

The finely powdered *Cissus quadrangularis* leaves (0.5 Kg shade dried) was soaked in 1.5 L of 95% ethanol overnight. The filtrate obtained was separated and kept in a separate flask. The left over residue was again drenched in 1.5 L of 95% ethanol for 48 h. The filtrate was collected and mixed with the previous filtrate in the flask. The solvents were evaporated with a rotavapor at 40-50% under reduced pressure. The semisolid material (9%) was collected and stored at 4°C until further use.

Experimental protocol

The present study categorized 30 male golden Syrian hamsters (100-120g) into five groups of six each and the experiment was conducted as per the ethical guidelines of the Muthayammal College of Arts and Science, Rasipuram, Animal Ethical Committee. The experimental protocol is given in figure.1

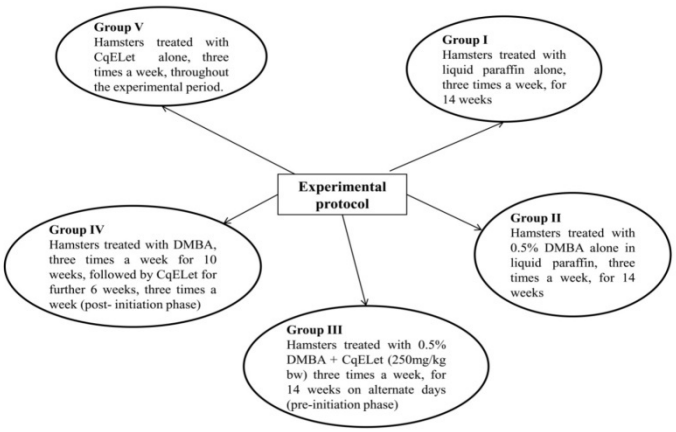

Figure 1. Experimental protocol of the present study

All the experimental hamsters are allowed to access the pellet diet and water freely and at the end of the experimental period, hamsters were sacrificed by cervical dislocation. The plasma, liver and buccal mucosa tissues were collected from the sacrificed animals and were subjected to biochemical analysis using specific and sensitive colorimetric method (table 1).

Table 1 Biochemical estimation carried out in the plasma and tissues

Biochemical parameters	References
Plasma TBARS	Yagi (1987)
Tissue TBARS	Ohkawa *et al,*(1979)
Plasma vitamin E	Desai (1984)
Tissue Vitamin E	Palan *et al,*(1991)
Reduced glutathione	Beutler and Kelley (1963)
Oxidized glutathione	Tietze (1969)
Superoxide dismutase	Kakkar and Viswanathan (1984)
Catalase	Sinha (1972)
Glutathione peroxidase	Rotruck *et al*, (1972)
Glutathione-S-transferase	Habig *et al,* (1974)
Glutathione reductase	Carlberg and Mannervik (1985)
DT-diaphorase	Ernster (1967)
Cytochrome P_{450} and b_5	Omura and Sato (1964)

RESULTS

Table 2 illustrates the tumor incidence and histopathological observations in the experimental animals. The exophytic tumor formation (well differentiated Squamous cell carcinoma) with severe hyperplasia and dysplasia was seen in all the hamsters treated with DMBA alone. The present study observed mild to moderate hyperplasia (group III) hyperkeratosis and dysplasia in the hamsters treated with DMBA + CqELet. However, no tumor formation was noticed in DMBA + CqELet treated hamsters. A significant decrease in tumor formation and tumor size with severe precancerous lesions was, however, observed in DMBA → CqELet treated hamsters (post-initiation phase).

Table 2 Tumor incidence and Histopathological observations of control and experimental hamsters (n=6)

	Control hamsters	DMBA treated hamsters	DMBA + CqELet treated hamsters	DMBA → CqELet treated hamsters	CqELet alone treated hamsters
Tumor incidence (%)	-	100	-	33.33%	-
Total number of tumors	-	16 (6 animals)	-	3 (2 animals)	-
Tumor volume (mm^3)	-	305.2±28.4	-	49.5±3.8	-
Tumor burden (mm^3)	-	814.2±83.6	-	75.1±6.4	-
Hyperkeratosis	-	+++	+ to ++	+++	-
Hyperplasia	-	+++	+ to ++	+++	-
Dysplasia	-	+++	+ to ++	++ to +++	-
Squamous cell carcinoma	-	Observed in all hamsters	-	Observed in two hamsters	-

+ − Mild, ++ − Moderate, +++ − Severe.

Values are represented as mean ± SD. Tumor volume was measured using the formula, v=4/3π [D1/2] [D2/2] [D3/2] where D1, D2 and D3 are three diameters of the tumors. Tumor burden was calculated by multiplying the tumor volume and the number of tumors per animal.

The activities of phase I and II detoxification agents [Cytochrome P_{450} (CYT P_{450}), Cytochrome b_5 (CYT b_5), glutathione-S-transferase (GST), Glutathione reductase (GR), DT-Diaphore)] and the status of Thiobarbituric acid reactive substances (TBARS) and antioxidants [Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Vitamin E)] were illustrated in figures 2 to 5. The status of the above

said biochemical variables found to be significantly altered in hamsters treated with DMBA alone as compared to control hamsters. However, the status of these biomarkers was found to be reverted in DMBA + CqELet treated hamsters. A significant improvement in the status of the biochemical markers was also noticed in the DMBA → CqELet treated hamsters.

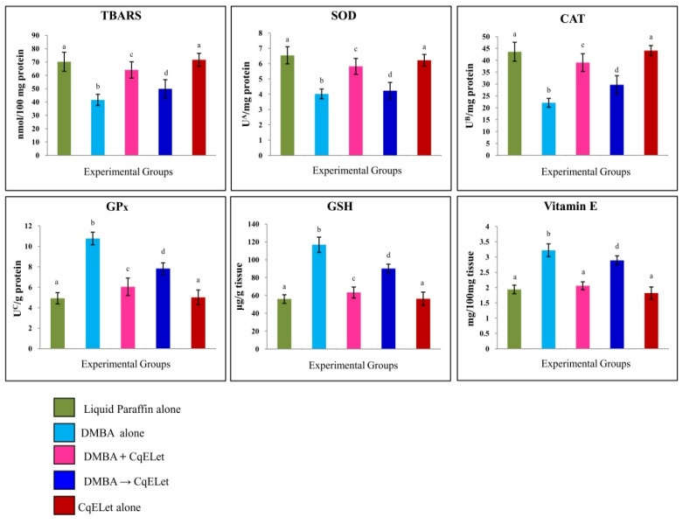

Figure 2 Buccal mucosa TBARS and antioxidants in control and experimental hamsters [n=6].

Values are expressed as mean ± standard deviation. Values that do not share a common superscript between two groups differ significantly at P<0.05 (DMRT). A- the amount of enzyme required to inhibit 50% NBT reduction; B-micromoles of hydrogen peroxide utilized/s; C- micromoles of glutathione utilized/min.

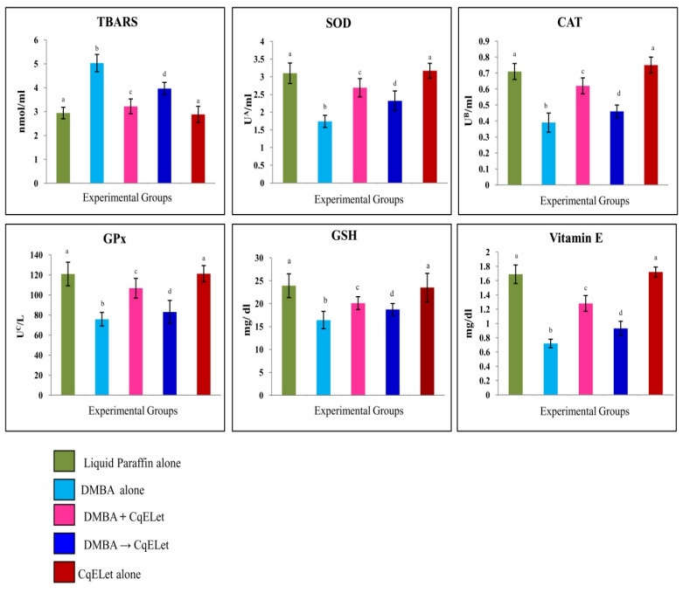

Figure 3 Plasma TBARS and antioxidants of control and experimental hamsters [n=6]

Values are expressed as mean ± standard deviation. Values that do not share a common superscript between two groups differ significantly at P<0.05 (DMRT). A-the amount of enzyme required to inhibit 50% NBT reduction; B-micromoles of hydrogen peroxide utilized/s; C-micromoles of glutathione utilized/min.

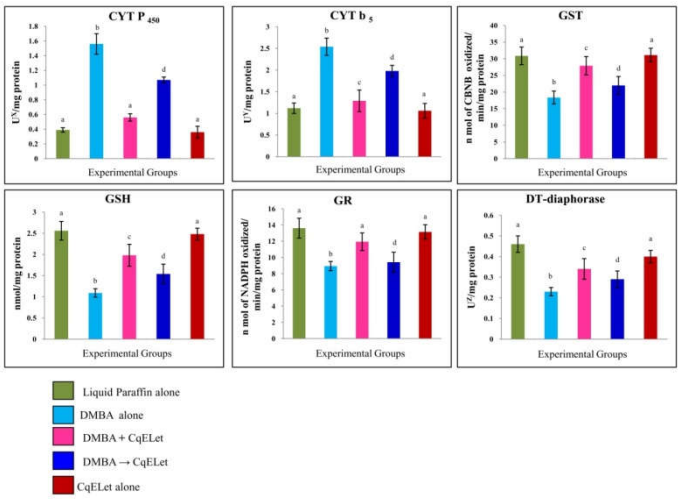

Figure 4 Liver phase I and phase II detoxification agents in control and experimental hamsters [n=6].

Values are expressed as mean ± Standard deviation values that do not share a common superscript between two groups differ significantly at P<0.05 (DMRT). X-micromoles of cytochrome P_{450}; Y - micromoles of cytochrome b_5; Z-micromoles of 2, 6-dichlorophenol reduced/min.

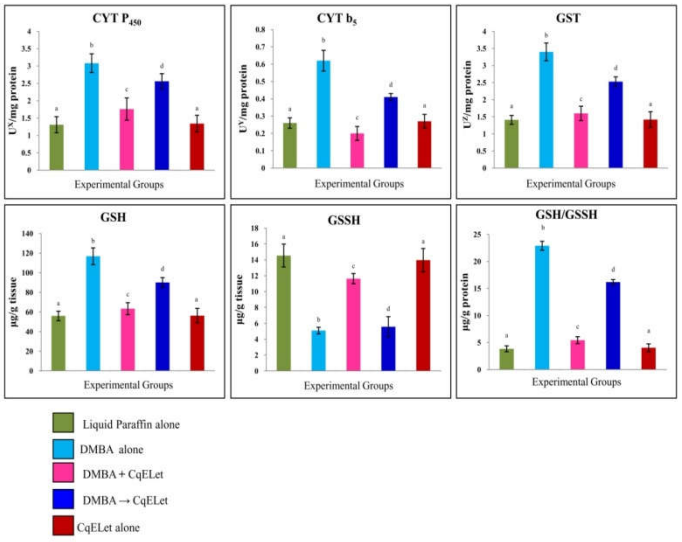

Figure 5 Buccal mucosa phase I and phase II detoxification agents of control and experimental hamsters group [n=6].

Values are expressed as mean ± standard deviation values that do not share a common superscript between two groups differ significantly at P<0.05 (DMRT). X- micromoles of cytochrome P_{450}; Y- micromoles of cytochrome b_5; Z-micromoles of 1-chloro 2, 4 dinitrobenzene (CDNB)/reduced glutathione conjugate formed/min.

DISCUSSION

The present study makes use of the status of detoxification agents, lipid peroxidation by-products (TBARS) and antioxidants to validate the tumor inhibiting potential of CqELet in experimental oral carcinogenesis. ROS explored its harmful effect in mediating tumor promotion and progression. ROS mediated cell signaling pathways play diverse roles in all the steps of carcinogenesis, including tumor cell survival. It has been reported that lowered activities of catalase and superoxide dismutase in the tumor tissues favor tumor cell proliferation

(Manoharan *et al,* 2005; Anbalagan *et al,* 2017). ROS such as superoxide radicals and hydrogen peroxides have accumulated in the tumor cells during carcinogenesis. Excessive generation of ROS persuades DNA damage and genetic instability as well (Liou *et al,* 2010; Choudhari *et al,* 2014). Profound studies reported over production of lipid peroxidation by-products in the circulation in cancerous conditions. Studies have documented decreased levels of TBARS in various tumor tissues (Manoharan *et al,* 2005; Anbalagan *et al,* 2017). Also, studies have shown compromised lipid peroxidation and antioxidant status in the tumor tissues of and circulation of solid tumors bearing animals (Manoharan *et al,* 2012; Kolanjiappan *et al,* 2003). It has been suggested that ROS exert a synergistic role with therapeutic antioxidants in the prevention of early carcinogenesis (Choudhari *et al,* 2014; Waris and Ahsan, 2006; Barrera, 2012).

In the present study, low levels of TBARS and inequity of the antioxidants was noticed in tumor tissues as compared to normal oral tissues. A large number of studies claimed low content of PUFA in the tumor tissues could be responsible for the decreased levels of TBARS. The abnormalities in the structural integrity and functions have been reported in the tumor tissues. Previous reports from our laboratory pointed out the unevenness in the antioxidant defense mechanism accompanied by a decrease in TBARS in the tumor tissues of the oral cavity (Manoharan *et al,*2012; Kolanjiappan *et al,* 2003). The present results are in line with our previous findings. Elevated glutathione peroxidase activity and its co-substrate reduced glutathione and increase in vitamin E content in the tumor tissues are partly responsible for the reduced oxidative stress in the tumor tissues. The exhaustion of SOD and CAT activities in the tumour tissues might be due to excessive generation of superoxide radicals and hydrogen peroxide in the tumour tissues. On the other hand, insufficient antioxidant defense mechanism, as evidenced by lowered activities of SOD, CAT and GPx and diminished content of reduced glutathione and vitamin E, could be attributed to enhanced TBARS levels in the plasma. The low content of plasma vitamin E and glutathione reflects their utilization by the oral tumor tissues to favor for their abnormal cell proliferation. Hamsters treated with DMBA +CqELet revealed a significant improvement in the antioxidant defense mechanism in the pre-initiation phase, which clearly focuses the antilipid peroxidative efficacy of CqELet during DMBA induced oral carcinogenesis. The results also imply the presence of antioxidant principles in the CqELet. A considerable improvement in the lipidperoxidation and antioxidant status was noticed in the DMBA→ CqELet treated hamsters as well.

Liver plays a putative and prominent role in the detoxification of xenobiotics and drugs via phase I and phase II detoxification agents. The harmful or toxic chemical substances are metabolically activated by phase I detoxification agents into less harmful or sometimes more harmful substance, depending on the amount of exposure to the liver. During this metabolic conversion, excessive ROS are generated as well, which further complicate the oxidative damage to lipids, proteins and DNA. Phase II detoxification agents are involved in the deactivation or detoxification of xenobiotic substances. They facilitate the excretion of these substances via urine or bile by catalyzing the conjugation reaction with glutathione or glucuronic acid. Any defect in the activation of either phase I or phase II detoxification mechanism could lead to accumulation of potent carcinogenic metabolites, thereby leading to neoplastic transformation (Gu *et al,* 2012; Manimaran and Manoharan, 2018; Rajasekaran *et al,* 2015). A large number of studies pointed out defective detoxification cascade in several cancers, including oral cancer (Palanimuthu *et al,* 2012; Bakaran *et al,* 2012). Repeated and frequent topical application of the carcinogen, DMBA, on the buccal pouch of the hamsters could account for the increased activities of phase I detoxification agents in the buccal mucosa and the liver of the hamsters treated with DMBA alone. The unevenness in the activities of phase II detoxification agents in the liver (decreased) and buccal mucosa (increased) clearly indicates the defect in the excretion of carcinogenic metabolites in the hamsters treated with DMBA alone. Oral administration of CqELet to DMBA treated hamsters maintained the status of phase I and II detoxification agents in the liver and buccal mucosa in the pre-initiation phase and significantly improved the activities in the post-initiation phase. The results of the present study thus explore the protective efficacy of CqELet in restoring the activities of detoxification agents to inhibit the formation of oral tumors in DMBA induced hamster buccal pouch carcinogenesis.

The results of the present study conclude that CqELet inhibited the formation of tumors in the buccal pouch of hamsters treated with DMBA alone through its antilipid peroxidative ability and by improving the detoxification mechanism to effectively excrete the carcinogenic metabolites. Though CqELet inhibited the formation of oral tumors in the buccal pouch of the DMBA treated hamsters, the observed precancerous lesions such as hyperkeratosis, hyperplasia and dysplasia in the pre-initiation phase and small size tumors in the post-initiation phase warrants further extension of the duration of the experimental period to confirm, whether CqELet has the tumor inhibiting potential (chemopreventive effect) or delaying the tumor formation during DMBA induced oral carcinogenesis.

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