Tumor suppressing potential of *Cajanus cajan* in 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis

Kuselar Hemalatha¹, Shanmugam Manoharan*², Mustafa Shabana Begum³ and Mani Neelakandan²  
¹Research and Development Centre, Bharathiar University, Coimbatore, India  
²Department of Biochemistry & Biotechnology, Annamalai University, Annamalainagar, India  
³Department of Biochemistry, Muthayammal College of Arts and Science, Rapisuram, India

ABSTRACT

Traditional medicine has explored the diverse pharmacological efficacy of *Cajanus cajan* leaves including anti-inflammatory and antioxidant potential. To scientifically validate the tumor inhibitory potential of *Cajanus cajan* leaves, the present study utilized the 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis as an experimental oral cancer model. The ethanolic extract of *Cajanus cajan* leaves (CcELet) suppressed the tumor formation in the pre-initiation phase and reduced the formation of tumors and tumor size as well in the post-initiation phase. Also, the *Cajanus cajan* leaves modulated the status of lipid peroxidation, antioxidants, and phase I and phase II detoxification agents towards tumor inhibition in the hamsters treated with DMBA. The results of the study thus explore the anti-tumor potential of *Cajanus cajan* leaves during DMBA induced hamster buccal pouch carcinogenesis.

Keywords: *Cajanus cajan*; oral cancer; lipid peroxidation; antioxidants; detoxification agents.

INTRODUCTION

Cancer, a detrimental group of diseases, is characterized by abnormal cell proliferation, invasion (spreading tendency into local adjacent tissues) and metastasis (spreading into other parts of the body). The incidence of cancer has profoundly increased year by year worldwide, especially in developing countries. Though cancer is treated by conventional modalities, the recurrence and multiple side effects warrants a search for new chemotherapeutic drugs with non-toxicity and fewer side effects (Campbell, 2017; Gao et al., 2017). Oral cancer, the cancer that arises in the lip, buccal mucosa, tongue and palate, is responsible for major morbidity and mortality worldwide. Though several forms of oral cancers are categorized histologically, oral squamous cell carcinoma accounts for 90% of all these cancers. The major risk factors responsible to elicit tumorigenesis in the oral cavity include excessive tobacco and alcohol use. In addition, viruses, UV radiation, immunodeficiency and nutritional deficiency account for oral carcinogenesis in a considerable proportion. The incidence and prevalence of oral cancer are sharply increasing in several parts of the world, especially in India, Pakistan, Bangladesh and Sri Lanka. In India, this form of cancer accounts for 40-50% of all cancers. Despite advancement and improvement in treatment modalities for oral cancer, late diagnosis and lack of awareness could still account for poor 5-year survival outcome of oral cancer patients (Manimaran and Manoharan, 2017; Manoharan et al., 2013).

The chemical carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA), is employed commonly to elicit tumors in the buccal pouch of golden Syrian hamsters. This carcinogen mediates oral carcinomas in the buccal pouch by inducing severe inflammation, generating excess reactive oxygen species (ROS) and causing multiple mutations in the DNA. The researchers commonly prefer DMBA induced hamster buccal pouch carcinogenesis model due to its close histological similarities with human oral carcinoma (Baskaran et al., 2017; Manimaran et al., 2017).

Natural products play a prominent and vital role in the prevention and management of several illnesses including cancer. Indian Siddha and Ayurvedic medicines explored enormous medicinal plants that have anticancer properties. Medicinal plants have been used by humans to treat various illnesses. Plants are the major source of bioactive secondary metabolites that fight against cancer. *Cajanus cajan* (L) Millsp. belongs to the family Fabaceae. It is commonly known as Pigeon pea in English, Arhar in Hindi, and Thuvarai in Tamil. India contributes 90% of the total world production of pigeon pea. This legume crop is rich in proteins and widely eaten as a dhal. In traditional Chinese medicine, it is used for pain...
relief and as a sedative. The leaf pastes are used for the treatment of inflammations and oral ulcers. Leaves are used to cure gingivitis and stomatitis as well. This plant has a more beneficial effect against diabetes, sores, skin irritations, hepatitis, measles, jaundice, dysentery and many other illnesses. It has been used in expelling bladder stones and stabilizing menstrual period. Phytochemical analysis revealed the presence of flavonoids and stilbenes (Zu et al., 2010; Pal et al., 2008). Experimental studies explored the potent anti diabetic, antimicrobial, antioxidant, hepatoprotective, antitumor and anti-inflammatory properties of Cajanus cajan leaves (Pal et al., 2008; Okigbo and Omodamiro, 2008, Nahar et al., 2014; Ezike et al., 2010). However, there are no experimental studies to scientifically validate the chemopreventive potential of Cajanus cajan leaves in DMBA induced oral carcinogenesis. The present study has therefore taken an effort to scientifically validate the chemopreventive potential of Cajanus cajan leaves by analyzing the status of biochemical markers and by carrying out histopathological studies in DMBA induced oral carcinogenesis.

MATERIALS AND METHODS
Preparation of the plant extract
Cajanus cajan leaves (500 g) were dried, finely powdered and soaked in 1500 ml of 95% ethanol overnight. The residue and filtrate were collected separately and the residue was again soaked in equal volume of 95% ethanol for further 48 h and filtered again. The two filtrates were then mixed, and the solvents were evaporated in a rotavapor at 40-50°C under reduced pressure. The obtained semisolid material (9%) was stored at 4°C until further use. For the experimental study, residual extract at a dose of 250mg/kg body weight was suspended in distilled water and was orally administered to the animals by gastric intubation using force feeding tube.

Experimental protocol
Thirty golden Syrian hamsters were categorized into five groups and were maintained in the Animal House, Muthayammal college of Arts and Science, Rasiapuram as per ethical committee principles. Tumors were developed in the buccal pouch using the site and organ specific carcinogen, DMBA. Topical application of 0.5% DMBA in the buccal pouches, three times a week for 14 weeks, developed exophytic oral tumors. All the animals were allowed to take food and water ad libitum. The experimental design employed for the study was as follows.

Group I hamsters received topical application of liquid paraffin alone on their left buccal pouches, three times a week for 14 weeks.

Group II hamsters received topical application of 0.5% DMBA in liquid paraffin on their left buccal pouches, three times a week for 14 weeks.

Group III hamsters received topical application of 0.5% DMBA in liquid paraffin on their left buccal pouches, three times a week for 14 weeks and received the ethanolic extract of Cajanus cajan leaves (250mg/kg bw) orally three times a week for 14 weeks as well on alternate days of DMBA treatment.

Group IV hamsters received topical application of 0.5% DMBA in liquid paraffin on their left buccal pouches, three times a week for 10 weeks and thereafter received the ethanolic extract of Cajanus cajan leaves (250mg/kg bw) orally three times a week up to 16 weeks.

Group V hamsters received the ethanolic extract of Cajanus cajan leaves (250mg/kg bw) alone orally three times a week and continued up to the end of the experimental period.

The hamsters were sacrificed by cervical dislocation and the collected blood and tissues were subjected to biochemical and histopathological analysis.

Biochemical estimations
The specific and sensitive colorimetric procedures were used to analyze the biochemical parameters. Plasma and tissue lipid peroxidation by-products (TBARS) were analyzed using the method of Yagi (1987) and Ohkawa et al., (1979) respectively. Reduced glutathione level was determined by the method of Beutler and Kelley (1963). While vitamin E level in plasma was determined colorimetrically by the method of Desai, (1984), the tissue vitamin E was measured by the fluorimetric method of Palan et al., (1991). Superoxide dismutase, catalase, and glutathione peroxidase (GPx) activities were assayed by the method of Kakkar et al., (1984); Sinha (1972) and Rotruck et al., (1973) respectively. The activity of glutathione-S-transferase (GST) in the buccal mucosa and liver tissue homogenate was assayed by the method of Habig et al., (1974). Glutathione reductase activity in the buccal mucosa and liver tissue homogenate was assayed by the method of Carlberg and Mannervik (1985). The status of cytochrome P450 and b5 in liver and the buccal mucosa was determined according to the method of Omura and Sato (1964).

Statistical analysis
The statistical significance between the groups was analysed using one way of analysis of variance followed by Dancans multiple range Test. The two groups are considered statistically significant if the P values were found to be less than 0.05.

RESULTS
The tumor incidence and histopathological changes noticed in the control and experimental hamsters are shown in table 1. While the present study noticed tumors with severe histopathological abnormalities in the buccal pouch of all the hamsters treated with DMBA alone, moderate to severe precancerous lesions without any tumor formation were observed in the hamsters treated with DMBA + Cajanus cajan leaves in the
Table 1: Macroscopic and microscopic pathological observations in control and experimental hamsters (n=6)

<table>
<thead>
<tr>
<th></th>
<th>Control Hamsters</th>
<th>DMBA treated Hamsters</th>
<th>DMBA + CcELet treated Hamsters</th>
<th>DMBA → CcELet treated hamsters</th>
<th>CcELet alone treated Hamsters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor incidence (%)</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>50%</td>
<td>0</td>
</tr>
<tr>
<td>Total number of tumors</td>
<td>0</td>
<td>18 (6 animals)</td>
<td>0</td>
<td>4 (3 animals)</td>
<td>0</td>
</tr>
<tr>
<td>Tumor volume (mm³)</td>
<td>-</td>
<td>294.3±30.6</td>
<td>-</td>
<td>58.2±6.7</td>
<td>-</td>
</tr>
<tr>
<td>Tumor burden (mm³)</td>
<td>-</td>
<td>883.0±90.6</td>
<td>-</td>
<td>77.4±8.5</td>
<td>-</td>
</tr>
<tr>
<td>Hyperkeratosis</td>
<td>Absent</td>
<td>Severe</td>
<td>Mild to moderate</td>
<td>Severe</td>
<td>Absent</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>Absent</td>
<td>Severe</td>
<td>Mild to moderate</td>
<td>Severe</td>
<td>Absent</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>Absent</td>
<td>Severe</td>
<td>Mild to moderate</td>
<td>Moderate to severe</td>
<td>Absent</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>Absent</td>
<td>Seen in all animals</td>
<td>Absent</td>
<td>Seen in three animals</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Values are represented as mean ± SD. 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 three diameters of the tumors. Tumor burden was calculated by multiplying tumor volume and the number of tumors per animal.

Figure 1: 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 - amount of enzyme required to inhibit 50% NBT reduction; B - micromoles of hydrogen peroxide utilized/s; C - micromoles of glutathione utilized/min.
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 - amount of enzyme required to inhibit 50% NBT reduction; B - micromoles of hydrogen peroxide utilized/s; C - micromoles of glutathione utilized/min.

Figure 3: 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 P450; Y - micromoles of cytochrome b5; Z - micromoles of 2, 6-dichlorophenol reduced/min.
preinitiation phase. *Cajanus cajan* reduced the tumor formation and tumor size as well in the post-initiation phase.

The present study has analyzed the modulating effect of *Cajanus cajan* leaves on the status of lipid peroxidation, antioxidants and phase I and phase II detoxification agents in DMBA induced oral cancer in golden Syrian hamsters. The obtained results are shown in the figures 1 to 4. While TBARS was decreased in the tumor tissues, enhanced TBARS was noticed in the plasma of tumor bearing hamsters. Similarly, we noticed poor antioxidant defense in the plasma accompanied by disturbed buccal mucosa tumor tissue antioxidants in the tumor bearing hamsters. The present study noticed an increase in the activities of phase I and phase II detoxification agents in the buccal mucosa of DMBA alone treated hamsters. However, an inverse correlation was noticed between the activities of phase I and phase II detoxification agents in the liver. Oral administration of the ethanolic extract of *Cajanus cajan* leaves modulated the above biomarkers towards the inhibition of tumor formation, as evidenced by no tumor formation in DMBA + *Cajanus cajan* leaves extract treated hamsters. The *Cajanus cajan* leaves significantly inhibited the formation of tumors and improved the status of biomarkers in the post-initiation phase as well.

**DISCUSSION**

The present study has taken the effort to scientifically validate the tumor preventing potential of the ethanolic extract of *Cajanus cajan* leaves in DMBA induced oral carcinogenesis. Though the present study did not notice any tumor formation in the animals treated with DMBA + *Cajanus cajan* leaves extract, hyperkeratosis, hyperplasia and dysplasia were observed. The number of tumor formation and the tumor size was found to be decreased in the post-initiation phase (DMBA → *Cajanus cajan* leaves). The result thus suggests that the ethanolic extract of *Cajanus cajan* leaves delayed the formation of tumors rather than inhibiting the tumorigenesis. The extension of the experimental protocol may provide a clear picture about the tumor preventive role of *Cajanus cajan* leaves in DMBA induced buccal pouch carcinogenesis.
Excessive generation of reactive oxygen species (ROS) has been documented in almost all forms of carcinogenesis. Reactive oxygen species not only initiate the tumor formation, but also involved in the tumor promotion and progression, if they are excessively generated in the system. Host cells, however, have a compensatory antioxidant defense mechanism to scavenge the excess ROS (Chen et al., 2017; Liou and Storz, 2010). Mitochondrial dysfunction and defect in metabolism could lead to accumulation or excessive generation of ROS in the tumor cells. Accumulation of hydrogen peroxide and superoxide radicals were reported in solid tumors (Manoharan et al., 2010; Mittal et al., 2014; Mencalha et al., 2014). In the present study, we noticed an increase in the levels of lipid peroxidation by-products (TBARS) in the plasma and decreased TBARS in the tumor tissues. It has been pointed out that most of the cancer arises due to chronic irritation and inflammation at the site of tumor formation. Profound studies highlighted that DMBA mediates carcinogenesis at the site of buccal mucosa through chronic irritation and inflammation (Manoharan et al., 2009). Tumors arise in the buccal mucosa due to DMBA induced morphological and histological changes at the site of DMBA application. Tumor cells were reported to have disorganized membrane integrity, cellular fluidity and permeability (Manoharan et al., 2008). Manoharan et al (2005) have reported low levels of PUFA, a substrate for lipid peroxidation, in oral tumor tissues. It has been reported that tumor cells expressed high levels of antioxidant such as vitamin E, glutathione and glutathione peroxidase and lowered activities of catalase and superoxide dismutase (Manimaran and Manoharan, 2017). Low concentration of PUFA with disturbed antioxidants might be responsible for low TBARS in tumor tissues.

Intracellular levels of reactive oxygen species are constantly maintained by sophisticated enzymatic and non-enzymatic defense mechanism. These antioxidants scavenge or detoxify ROS and protect the cells from ROS mediated oxidative damage. Low levels of plasma antioxidants, both enzymatic and non-enzymatic, were reported in several cancers, including oral cancer (Barrera, 2012; Singh et al., 2013). Insufficient antioxidant defense mechanism, as evidenced by lowered levels/activities of antioxidants, observed in the plasma of tumor bearing animals could account for increased plasma TBARS. Oral administration of the ethanolic extract of Cajanus cajan leaves to hamsters treated with DMBA, brought back the status TBARS and antioxidants to near normal range. The present study thus explores the antilipid peroxidative potential of the plant extract, which could be due to the bioactive antioxidant constituents of the plant extract.

The human body has a wonderful detoxification mechanism to combat the deleterious effects of harmful toxins from food, environment and pharmaceutical drugs. Phase I and phase II detoxification systems are comprised of a battery of enzymes, which play a pivotal role in the detoxification of these xenobiotics. Phase I detoxification systems is involved in the metabolic activation of harmful xenobiotics including carcinogen. The ROS that are generated excessively during this phase I reaction can damage DNA, if not excreted by the phase II detoxification cascade. Phase II detoxification reactions excrete the harmful metabolites through urine or bile, involving the conjugation reactions such as glucuronidation and glutathione conjugation. Thus, impairment in the activities of phase I and phase II detoxification system could enhance the risk of several diseases including cancer (Manoharan et al., 2010).

Liver, the major metabolic organ of the human body, plays a crucial role in the metabolic activation and excretion of DMBA. In the present study, defect in phase I and phase II detoxification agents were noticed in both the liver and buccal mucosa of hamsters treated with DMBA alone. Increased activities of liver cytochrome P450 and b5, accompanied by decreased activities of glutathione reductase, glutathione S-transferase and glutathione content in tumor bearing hamsters, clearly pointing out the defect in the detoxification cascade in the tumor bearing hamsters. The defect in phase I and II detoxification system could lead to accumulation of ROS and dihydrodiol epoxide (the ultimate carcinogenic metabolite), which could in turn leads to carcinogenesis.

In contrast to liver, both phase I and phase II detoxification systems are enhanced in the buccal mucosa of tumor bearing hamsters. This is probably due to frequent and continuous topical application of the carcinogen, DMBA, on the hamster buccal pouch. The present study reveals that imperfection in the activities of phase I and phases II detoxification agents could partly be responsible for tumorigenesis in the hamsters treated with DMBA. Oral administration of the ethanolic extract of Cajanus cajan leaves to DMBA treated hamster improved the detoxification cascade in both liver and buccal mucosa towards the inhibition of tumorigenesis. The present study has thus explored the tumor preventive potential of the ethanolic extract of Cajanus cajan leaves during DMBA induced oral carcinogenesis in the buccal pouch of golden Syrian hamsters. The tumor preventive efficacy of the ethanolic extract of Cajanus cajan leaves could either be attributed partly to its antilipid peroxidative potential or due to its ability in enhancing the activities of enzymes involved in the phase I and phase II detoxification mechanism.

The present study, thus, makes the first attempt to explore the antitumor potential of Cajanus cajan leaves using DMBA induced hamster buccal pouch carcinogenesis model. Further studies are needed to confirm whether the plant extract has inhibited or delayed the tumor formation in the DMBA treated hamsters, as these animals’ buccal mucosa showed severe precancerous lesions in the pre-initiation phase. This could be confirmed by extending the experimental period for further 5 to 6 weeks duration of the pre-initiation phase.
ACKNOWLEDGEMENTS

The authors gratefully acknowledge the authorities of the Muthayammal College of Arts and Science for providing necessary animal house facilities to carry out the research work successfully.

REFERENCES


