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Protective effect of *Eleteria cardamomum* (L.) Maton against Pan masala induced damage in lung of male Swiss mice

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ABSTRACT

Objective: To study the potential ameliorating properties of cardamom *Elettaria cardamomum* (*E. cardamomum*) L. Maton against pan masala induced damage in lung of male Swiss mice. **Methods:** The experimental animals were divided into 3 groups (control, pan masala treated group and pan masala with cardamom treated group) to evaluate pan masala toxicity. The observations were substantiated with profound changes in the lung tissue as revealed in the histologic and transmission electron microscopic examinations. **Results:** Lung of pan masala treated group showed adenocarcinoma, edema, and inflammation with increased activity of acid phosphatase, alkaline phosphatase, and lactate dehydrogenase. The deleterious effects were seen to be less in cardamom treated group and the enzymatic activity also decreased significantly ($P<0.05$) in the ameliorating group. **Conclusions:** Thus, the present experiment exciting results are observed when cardamom is supplemented with pan masala, or when given alone.

1. Introduction

Tobacco chewing has been a tradition in South-East Asia and the South Pacific and among people of Indian origin who have migrated elsewhere. The pattern of use varies across the globe, with relatively higher prevalence in the South Asian region (India, Pakistan, Bangladesh, China and Thailand), United States of America and Brazil[1]. Aggressive marketing and high profile advertising of pan masala developed inclination and addiction even among school children, youth and labors and in various occupations just to elevate their mood and psychoenlightenment[2]. Available studies demonstrate that the habit of chewing pan masala by students and adolesescents are on the increase, which may lead to deterioration of oral health and other organ systems[3]. Pan masala is a dry mixture of various ingredients like areca nut, catechu, lime, permitted spices, unspecified flavoring agents and tobacco, *etc*. 70%-80% of the mixture is composed of Areca nut which is reported to possess cytotoxic, mutagenic and genotoxic properties as well cause cancer of various tissues[4]. Catechu and slaked lime, another constituent of pan masala, is a highly abused agent with carcinogenic properties[5]. Areca nut/quid chewing is also a habit that is commonly practiced in the Indian subcontinent and this age-old social habit is still being practiced by the Indians in South Africa which is said to be associated with the development of oral submucous fibrosis (OSF), a premalignant lesion, oral leukoplakia and oral cancer[6]. Reactive oxygen species (ROS), implicated in multistage carcinogenesis, are generated in substantial amounts in the oral cavity during chewing and its formation is favoured by alkaline condition build up by $Ca(OH)_2$ present in slaked lime[7]. According to Archana et al[8], exposure to pan masala poses serious embryotoxic effects, along with postimplantation loss and utero and lactational fetotoxic effects. Pan masala also induced testicular damage, decreased spermatid count, sperm count and abnormal morphology of sperm head shape[9]. In other studies, pan masala was reported to impair liver function in mice with significant increase in serum alkaline phosphatase (ALP), glutamic oxalo acetic (GOT) and glutamic pyruvic (GPT) transferases[10]. Increased frequency of micronuclei in polychromatic erythrocytes (MNPCE) and normochromatic erythrocyte (MNNCE) was reported in the

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bone marrow cells, indicating the genotoxic potential of pan masala along with a dose-dependent significant increase in chromosome aberration[11]. Pan masala intake results in enhanced production of superoxide anion. The oxidative stress by reactive oxygen species thus plays a significant role in the cytotoxicity[5]. Various other works have been undertaken to elucidate the carcinogenic potential of pan masala; however, there are no data reported for amelioration toxicity induced by pan masala in pulmonary tissue.

Cardamom has been shown to have chemoprotective potential against chemically induced skin carcinogenesis in swiss mice and a significant decrease in the lipid peroxidation level of the liver was observed[12,13]. Cardamom extracts significantly enhance the cytotoxic activity of natural killer cells, indicating their potential anti-cancer effects[14]. Cardamom flavored gum was found to be effective in lessening the nicotine withdrawal symptoms in individuals[15]. It is, thus, reasonable to hypothesize that either supplementation of cardamom or pan masala with cardamom may at least partially protect the consumers from immediate health hazard by effectively reducing the deleterious effects produced after its consumption. In the present paper, lung was selected, among other tissues, to find out the degree of damage caused by this smokeless tobacco and possible reasons were suggested for the deleterious effects that it caused. At the end of the treatment period the test animals were subjected to amelioration with cardamom.

2. Materials and methods

2.1. Test substance and diet preparation

A market survey on the rate of purchase of several brands of pan masala was conducted in different parts of Ranchi, India. The brand with the highest sale was then used for experimental study. Pan masala (2% of the feed) in a powdered form was mixed in the diet after grinding properly in an electric mixer[16]. The diet consisted of cracked wheat (68%), cracked Bengal gram (20%), fish meal (5%), yeast powder (4%), and groundnut oil (1%) in the form of dry mash. The amelioration group was given the same diet along with 0.2% of cardamom and the percentage of the feed adjusted vice-versa[17].

2.2. Animal's maintenance and experimental design

A total of 80 male Swiss mice [(22±5) g], obtained from B. N. Ghosh and Company, CIT Road, Kolkata) with an average age of four weeks were used in the experiment. The investigation was cleared by the Ethics Committee, Ranchi University, Ranchi, for conducting research on swiss mice and other strains of albino mice. Feeding of animals was done ad libitum, along with drinking water and maintained at natural day/night cycle. The animals were randomly divided into three groups (of 25 mice each), which were designated control (fed on formulated diet), PMT (pan masala treated, 2% of the feed) and PMCT (pan masala and cardamom treated, 2% and 0.2% of the feed respectively). A group of 5 mice was treated as a sham control (wild type) in order to provide baseline measurements for the experimental protocol. Food was given once daily, and the residual was measured next day. After the end of 9 months, mice of the group PMT and PMCT were continued with cardamom along with the food for a further 3 months to check the protecting effect of cardamom and to assess the ameliorating effect of the same. The process of amelioration was checked in two ways: simultaneously feeding the mice with pan masala and cardamom for nine months and a further three months the remaining mice were exposed only to cardamom along the diet. Meanwhile, 5 animals from each group were sacrificed after 3, 6, 9 and 12 months respectively and their tissues were collected for histological and biochemical analyses. During the observation stage, all animals were regularly weighed, and toxicological signs and symptoms were recorded.

2.3. Histopathology

Animals were sacrificed after 3, 6, 9 and 12 months by cervical dislocation under anesthesia, and lungs were excised. A part of the excised lung was fixed in Bouin's fixative (mixture of 75 mL saturated picric acid, 25 mL of 40% formaldehyde and 5 mL of glacial acetic acid). The tissues were dehydrated through an ethanol series, then treated with xylene and embedded in paraffin wax[18]. About 6 μm thick sections of tissues were stained with Crossman stain and observed under the light microscope. Another portion was fixed with a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 8-12 h at 4 ℃ and its ultrastructure was observed by Philips CM-10 transmission electron microscope (Netherland).

2.4. Biochemical assays

Tissues were homogenized (0.15 g tissue/mice) in ice cold 0.25 M sucrose (1:10, w/v)[19] after infusion with formalin and the homogenates were centrifuged at 10 000 g at 4 ℃ for 10 min to obtain a clear supernatant for biochemical estimation. Alkaline phosphatase (ALP, EC 3.1.3.1), Acid phosphatase (ACP, EC 3.1.3.2), and Lactate dehydrogenase (LDH, EC 1.1.1.27) activities were determined by the method of pNPP kinetic method, α - napthylphosphate kinetic method and IFCC method respectively. The assays were done using Acid phosphatase kit, Alkaline phosphatase kit (DEA) and LDH (P-L) kit of Crest Biosystems. The enzymatic activity was also determined in sham control mice and expressed in U/L.

2.5. Statistical analysis

Data were analyzed via one-way ANOVA, using graph pad Prism 5. O software. The results were presented as individual values or mean±SD. A P-value<0.05 was considered significant.

3. Results

The mortality and survival rates of animals after 12 months are presented in Table 1. There was no significant difference between the survival rates of the animals in the control and experimental groups. Signs of pan masala intoxication such as loss of fur, ruffled skin, loss in weight, and dermal lesions were observed in animals having 9 months of exposure. Ameliorated mice showed clear signs of improvement in weight and skin structure. Data on the body weight for the control and experimental groups are presented in Figure 1. The mean body weight of animals fed pan masala individually and in combination with cardamom was consistently lower than that of the controls, and the difference was particularly marked from month 6 onwards. Intake of pan masala caused a decline in food consumption of the treated group as compared to control resulting in their weight loss.

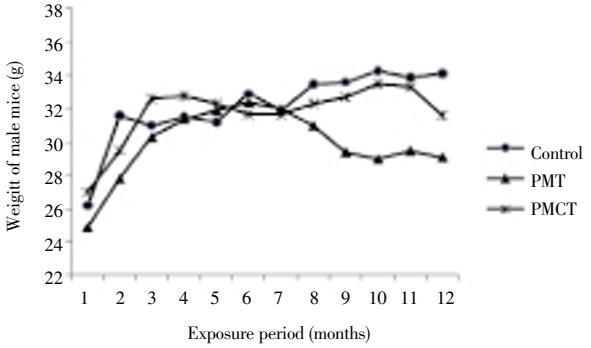

Figure 1. Data on the body weight for the control and experimental groups.

3.1. Histopathological alteration

Under the light microscope, the lung of mice in the control and sham control group showed a typical alveolar architecture, open intra-alveolar spaces with normal blood vessel as shown in Figure 2. Examination of the PMT group (pan masala treated group) exhibited marked lung histopathological abnormalities, characterized by fusion of alveoli and adenocarcinoma with compressive and destructive growth as evident from Figure 3. The pulmonary parenchyma is associated with extensive areas of hemorrhage and coagulative necrosis around the bronchovascular bundle enveloped by necrotic debris. There was total loss of alveolar structure with presence of vacuoles in the bronchiolar region, disruption of alveolar and bronchiolar epithelial cells

Table 1
Number of survived animals after 12 months.

Group	No. of animals at the beginning of experiment	No. of survivors											
		3 Months			6 Months			9 Months			12 Months		
		Sacrificed	Animals died before sacrifice	After Sacrifice	Sacrificed	Animals died before sacrifice	After Sacrifice	Sacrificed	Animals died before sacrifice	After Sacrifice	Sacrificed	Animals died before sacrifice	After Sacrifice
CONTR	25	5	NIL	20	5	NIL	15	5	NIL	10	5	1	4
PMT	25	5	NIL	20	5	1	14	5	1	8	5	1	2
PMCT	25	5	NIL	20	5	NIL	15	5	1	9	5	NIL	4

Table 2
Effects of co-administration of pan masala and cardamom along with individual treatment with pan masala and cardamom on the activities of lung acid phosphatases, alkaline phosphatases and lactate dehydrogenases in Swiss mice.

Treatment period (months)	ACP (U/L)			ALP (U/L)		
	Control	PMT	PMCT	Control	PMT	PMCT
0-3	59.00±5.09	112.13±4.10[a]	67.13±5.09[b,c]	67.47±5.71	116.13±10.65[a]	83.08±6.10[b,c]
0-6	62.75±3.04	145.25±6.05[a]	84.63±3.57[b,c]	76.19±11.94	146.42±18.44[a]	95.01±10.26[b,c]
0-9	64.88±1.70	175.00±3.01[a]	109.00±10.11[b,c]	86.29±10.26	215.73±18.18[a]	133.11±7.14[b,c]
10-12	66.00±1.63	109.50±6.79	71.75±2.23	90.88±10.52	136.78±3.76	93.64±13.37

Treatment period(months)	LDH (U/L)		
	Control	PMT	PMCT
0-3	245.11±10.91	323.47±14.00[a]	275.47±32.85[b,c]
0-6	260.20±6.33	335.47±12.52[a]	286.58±16.14[b,c]
0-9	278.19±16.25	395.90±20.57[a]	294.19±39.86[b,c]
10-12	279.81±15.88	321.97±19.67	292.13±38.21

Each value is a mean of 5 determinations ± SD. PMT, pan masala treated group; PMCT, group with co-administration of pan masala and cardamom; [a]P<0.05 compared to control group; [b]P>0.05 compared to control group; [c]P<0.05 compared to PMT (pan masala treated group).

as shown in Figure 4. There were perivascular/peribronchial acute inflammation and neutrophil infiltration. Extensive fibrosis in peribronchial region and increased thickness of bronchial smooth muscle due to accumulation of collagen was seen. The interalveolar septum showed focal thickness and fibrosis with a bronchiole (BR) showing mild, chronic peribronchitis (Figure 4).

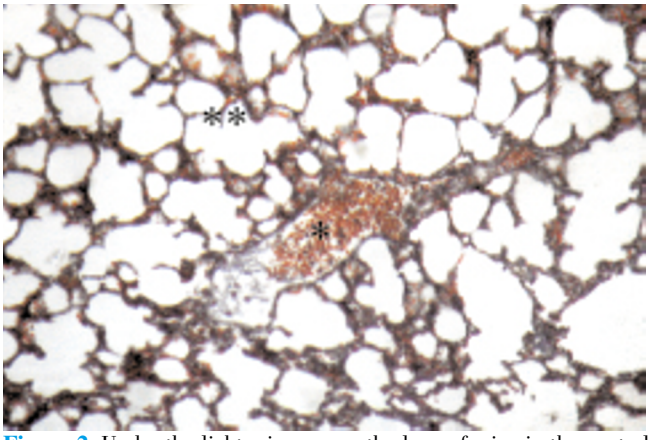

Figure 2. Under the light microscope, the lung of mice in the control and sham control group showed a typical alveolar architecture, open intra-alveolar spaces with normal blood vessel.

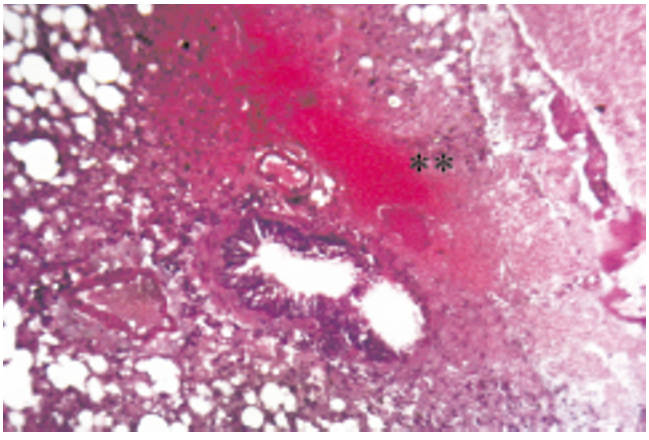

Figure 3. Examination of the PMT group (pan masala treated group) exhibited marked lung histopathological abnormalities, characterized by fusion of alveoli and adenocarcinoma with compressive and destructive growth as evident.

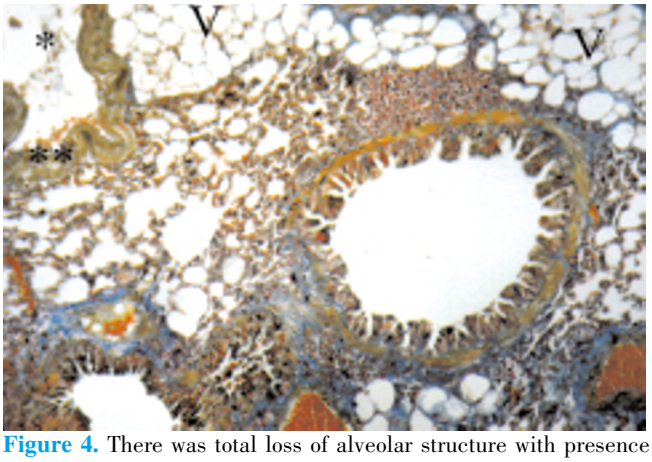

Figure 4. There was total loss of alveolar structure with presence of vacuoles in the bronchiolar region, disruption of alveolar and bronchiolar epithelial cells.

Microscopic images of PMCT group revealed focal emphysematous alterations in a form of emphysematous blebs with a few tissues lying in the alveolar lumen. However there was no oedamatous fluid and hemorrhage seen in the sections as compared to pan masala treated group (Figure 5). On cardamom treatment in the PMT and PMCT mice, congestion of lungs was mild with almost no medullary hemorrhage. There was no evident air space enlargement, lesions and membrane damage (Figure 6).

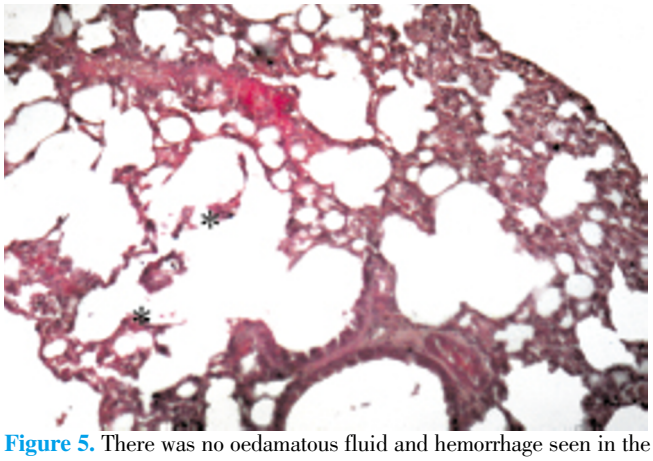

Figure 5. There was no oedamatous fluid and hemorrhage seen in the sections as compared to pan masala treated group.

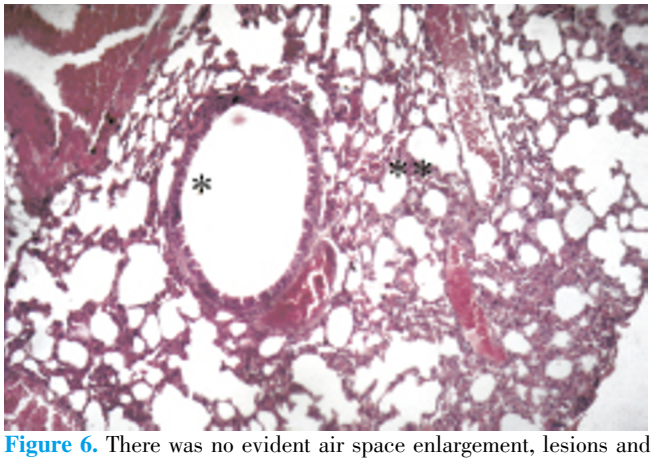

Figure 6. There was no evident air space enlargement, lesions and membrane damage.

At the ultrastructure level, images of the control group showed regular morphology with distinct nuclei and distinct, scattered mitochondria. Microvillus was distinct in the alveolar lumen, with no lesions seen in the membrane (Figure 7). Images of the pan masala treated group, showed swelling and focal fragmentation of the capillary endothelial cells and epithelial cells, leading to a denudation of the basal lamina. Huge accumulation of edema fluid was observed in various compartments of the lung: the peribronchovascular space, in the alveolar space and in the interstitium of the septum (Figure 8). Membrane blebbing was seen in the endothelial wall along with the accumulation of fibrin and cellular debris in the alveolar lumen. Extensive accumulation of lamellar bodies, erosion of epithelial lining, reduced microvilli, dilated mitochondrial cristae and disintegrating nuclei was observed in pan masala treated group. Microscopic images of the ameliorating group showed

great improvement in the histopathological picture, which nearly showed normal lung architecture, but there was mild inflammation of peribronchial and perialveolar tissues with moderate edema of interalveolar spaces and minimal fibrotic changes. Nuclei and epithelial membrane seems to be intact along with few lamellar bodies. Microvilli were prominent. However, little cellular debris was observed in the alveolar lumen (Figure 9).

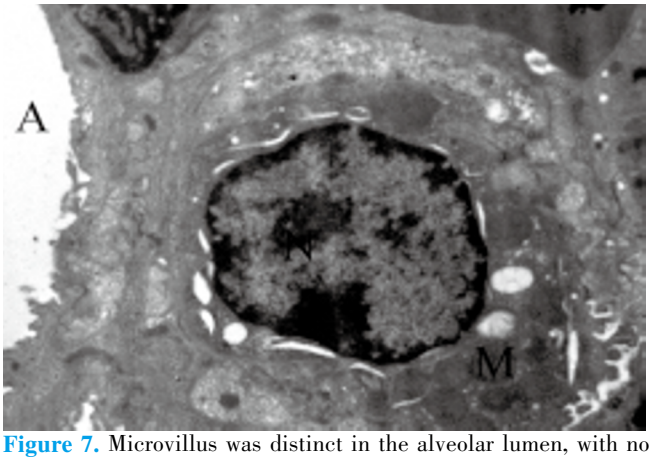

Figure 7. Microvillus was distinct in the alveolar lumen, with no lesions seen in the membrane.

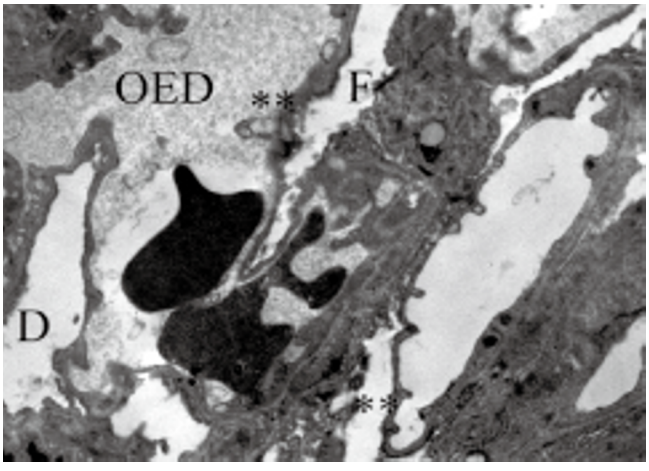

Figure 8. Huge accumulation of edema fluid was observed in various compartments of the lung: the peribronchovascular space, in the alveolar space and in the interstitium of the septum.

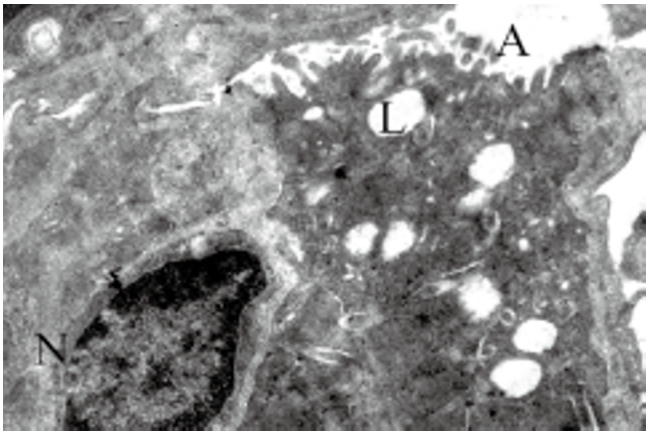

Figure 9. Little cellular debris was observed in the alveolar lumen.

3.2. Assay of biochemical parameters

The effect of treatment of pan masala and co-administration of pan masala with cardamom on acid phosphatase activity, alkaline phosphatase activity and lactate dehydrogenase activity in mice lungs are shown in Table 2. The sham control values were also evaluated to ascertain the actual changes in the level of enzyme activities that have taken place in treating mice. The enzymatic activity increased with the increase in the treatment period. It was less during 3 months of treatment period which gradually increased after 6 and 9 months of exposure showing severe damage in the exposed mice.

The enzymatic activity of the control group was (62.21±3.28) U/L, (76.65±9.30) U/L, (261.17±11.16) U/L for acid phosphatase, alkaline phosphatase and lactate dehydrogenase respectively, whereas the sham control values were (58.00±3.6) U/L, (72.06±12.46) U/L, (268.19±26.50) U/L indicating similar trends in sham control and control. Administration of pan masala alone increased (P<0.05) enzymatic activity in mice lung compared to sham control, control and PMCT groups (144.13±4.38) U/L, (159.43±15.75) U/L and (351.61±12.78) U/L for ACP, ALP and LDH respectively). On the other hand the enzymatic activities significantly decreased (P<0.05), during amelioration, when the treatment groups were exposed only to cardamom and the values reached almost near the control group as shown in Table 2.

4. Discussion

In Indian system of medicine, few herbs are claimed to provide relief against some of the disorders that occurred by the consumption of certain harmful products. Cardamom is one of them whose properties have been utilized in the present study to lower down the damaging effect in lung produced by pan masala whose consumption is on the rise due to the expanding global population, trade liberalization and extensive marketing strategies, not only in India and South East Asian countries but also in Commonwealth countries.

Histologic evaluation of pan masala exposed mice over 9 months demonstrated a temporal progression of pulmonary lesions that are characteristic of an acute lung injury followed by toxic insult. Early lesions were characterized by leakage of edema, fibrin, and erythrocytes from the pulmonary microvasculature into the alveolar spaces and interstitial tissues. There was also evidence of multifocal, mild epithelial degeneration, and necrosis in the terminal bronchioles and alveoli. This could reflect either inflammatory-mediated damage or a direct toxic effect of

pan masala on epithelial cells. According to other studies, mice fed with pan masala indicated subsequent development of tumours of lung, liver, stomach and testes[16,20]. The present study also suggested that pan masala plays a role in the development of adenocarcinoma as shown in Figure 3. In this context, the anti-cancerous property of cardamom have been utilized which showed clear signs of improvement in PMCT group with no cancerous like growth in the tissue. Also the repair and regeneration of epithelial damage that is focused on the terminal bronchioles, accompany the resolution of edema in PMCT group. Microscopic changes observed in lungs of pan masala treated mice also showed congestion of the lung and emphysematous changes around collections of macrophages. Administration of cardamom at a dose of 0.2% of the feed effectively reduced these pathological changes induced by pan masala with almost no emphysematous alteration, reduced alveolar damage and normal parenchyma. In an earlier study, green cardamom was used against congestion of the lungs, pulmonary tuberculosis, asthma, heart disease, inflammation of the eyelids and digestive disorders[21].

Alkaline phosphatase, acid phosphatase and lactate dehydrogenase are extremely used as marker enzymes. A direct relationship between the degree of protection by cardamom and the level of ACP, ALP and LDH was found. The effect of pan masala treatment included increased pulmonary capillary permeability, influx of different inflammatory cells and release of lysosomal enzymes raising its activity in the lung tissue. The present study indicated that pan masala caused a significant increase in the aforesaid enzymatic activities ($P<0.05$ compared to control and sham control). ACP and ALP are employed to assess the integrity of plasma membrane and endoplasmic reticulum while its increased activity can be correlated with cellular injury, inflammation, tissue damage and progression of fibrosis in chronic interstitial lung disorders[22]. Their increase in tissue homogenate indicates an enhanced Golgi activity and peroxidation in lysosomal membranes after exposure to pan masala causing membrane loss leading to enzyme leakage.

A significant increase in the activity of the LDH ($P<0.05$ compared to control and sham control) also depicted cellular injury that may be attributed to the loss of membrane integrity due to pan masala exposure. These changes closely mirror the early pan masala-induced pulmonary edema as observed in the exposed mice after treatment with pan masala for 9 months shown in Figure 7. The increase in LDH level may be associated with free radical production from areca nut, catechu and lime, the major ingredients of pan masala. Yamano and Morita[23] have also reported increased levels of LDH as a result of membrane damage caused by xenobiotic toxic agents. Lactate dehydrogenase is also recognized as a potential tumor marker in assessing the progression of the malignant cells due to its increased turnover[24]. In the present study, it was considered that extensive multiplication of cells and catabolism of their membranes may have lead to the elevation in alkaline phosphatase, acid phosphatase and lactate dehydrogenase levels in the lung tissue. This extensive proliferation of cell resulted into adenocarcinoma after 9 months of pan masala exposure which is shown in Figure 3.

However, a significant decrease in the enzyme activity was observed in the group co-administered with pan masala and cardamom. A similar result was observed when the animals of both the treated group were only exposed to cardamom after the end of 9 months treatment period till 12 months i.e. an additional exposure of 3 months. This suggests the protective effect of cardamom against loss of membrane integrity, high edema levels and emphysematous alteration in the treated group. Abdel-Waha and Aly[25] observed that treatment with clove and cardamom effectively decreased liver enzyme levels in the serum[26-30]. This can be also be attributed to the presence of antioxidant in clove and cardamom which contain phenolic compounds that can act by scavenging free radicals[17]. Limonene and cineole present in cardamom demonstrated promising effects against carcinogenesis[31]. Thus, the study suggests that the properties of cardamom can be utilized in protecting acute lung damage that results from the consumption of pan masala.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

The authors are thankful to the electron microscopy division AIIMS, New Delhi for providing facilities for TEM examination. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors. The idea of using cardamom as an ameliorating agent against pan masala toxicity is novel. It is possible that the measurement of these parameters may be ex-tended towards a useful application in human trauma or disease.

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