Indian J Microbiol (July–Sept 2011) 51(3):338–344
DOI 10.1007/s12088-011-0096-3

ORIGINAL ARTICLE

Effect of Mangrove Tea Extract from *Ceriops decandra* (Griff.) Ding Hou. on Salivary Bacterial Flora of DMBA Induced Hamster Buccal Pouch Carcinoma

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Received: 22 May 2009 / Accepted: 20 September 2009 / Published online: 27 February 2011

Abstract The objective of this study was to investigate the effects of mangrove tea on salivary bacterial flora in DMBA induced hamster buccal pouch carcinoma. Tea from mangrove plant *Ceriops decandra* was administered against DMBA induced buccal pouch carcinoma in hamster rats. The chemical constitutions and quality of mangrove tea is similar with the commercial tea *Camellia sinensis*. The Hamster rats were painted thrice a week with DMBA in their right buccal pouch, and also administrated orally with 1.25% of Ceriops tea extract, on alternate days of the DMBA treatment. Appropriate control animals were maintained. After 14 weeks of treatment, bacterial species in saliva were enumerated, tumor incidences were analyzed using histopathological section and tumor volume in the animals was quantified using water-displaced method. The decreased counts of beneficial bacteria and increased counts of harmful bacteria were associated with increased volume of tumors. The present study concluded that the tea extract from *C. decandra* prevents the oral cancer incidences and maintain the good health conditions of the animals.

Keywords Beneficial bacteria · Harmful bacteria · Oral cancer · *Ceriops decandra* · Mangrove tea · DMBA

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Introduction

Oral cancer is the sixth most common malignancy worldwide [1] and is particularly prevalent in developing countries, such as in Southeast Asia, where up to 40% of all malignancies are located within the oral cavity [2]. More than 90% of cancers in mouth are squamous cell carcinomas (SCCs), originating from the oral mucosa [3]. With an average of all stage, 5-year survival rate for oral cancer of less than 50%, the annual mortality figures are comparable to those of carcinoma of the cervix and malignant melanoma [4, 5]. Bacteria are known to associate with cancer tissues. Nagy et al. [6] have demonstrated a difference in the microflora associated with the surface of tumors in comparison to control sites. Patients with oral cancer tend to possess significantly low concentration of beneficial bacteria in their saliva. This is of particular interest because of its potential application as a diagnostic tool to predict oral cancer [7]. Tea that contains many antioxidants is a pleasant and safe drink that is enjoyed by people across the globe. Tea leaves are manufactured as black, green, or oolong. Black tea represents ~78% of total consumed tea in the world, whereas green tea accounts for ~20% of tea consumed. The concept of "use of tea for promotion of human health and prevention and cure of diseases" has become a subject of intense research in the last decade. The health benefits of tea are ranging from a lower risk of certain cancers to weight loss, and protection against infections, like bacterial and viral, to chronic debilitating diseases, including cancer, coronary heart disease, stroke, and osteoporosis. [8]. the mangrove plant *Ceriops decandra* has traditionally [9, 10] and scientifically rich in medicinal values [11–13]. Our research team attempted to extract black tea from a mangrove species *Ceriops decandra* which contains large amount of theaflavin (giving

flavour) and theambrugin (neurostimulant) [13]. These compounds are produced when polyphenols (present in cytoplasms) are allowed to ferment by polyphenol oxidase (present in cell vacuoles). The tea had no toxicity in mice, and had a better quality than commercial teas, as evident by sensory evaluation tests performed with our centre people. The present study was to determine the effect of mangrove tea on the bacterial flora which associated with saliva of DMBA induced hamster buccal pouch carcinoma.

Materials and Methods

Collection and Preparation of Plant Material

Leaves of the mangrove plant species, *Ceriops decandra* were collected from the forest of Pichavaram (11° 27′ N; 79° 47′ E) situated in south east coast of India. The specimen was identified and its holotype (No. R90) has been deposited in the herbarium of the Centre of Advanced Study in Marine Biology, Annamalai University, Parangipettai, Tamil Nadu, India. The leaf sample was washed with tap water to remove epiphytes and other external matter.

Preparation of Black Tea Extract

Black tea was extracted from the leaves of *Ceriops decandra*, adopting the method of [14]. Fresh leaves were spread on a trough and allowed to dry using a warming blender. A known weight of the macerated sample was placed in a piece of cloth and distilled water was continuously trickled over it for 1 h. The fermented sample was dried in a hot air oven at 95°C for 30 min. The tea extract was freshly prepared every day using 1.25 g of *Ceriops* tea powder in 100 ml boiled water.

Chemicals Used for the Study

Carcinogen Dimethylbenz[a]anthracene (DMBA) was purchased from Sigma Chemical Company, USA and used for this study. All other reagents used were of analytical grade.

Experimental Animals Used

The Syrian hamster cheek pouch epithelium has been a valuable model in studies of chemical carcinogens interactions with oral tissues [15, 16]. Therefore, male Syrian hamsters were used as experimental animals with an age of 8–10 weeks weighing 85–90 g obtained from the National Institute of Nutrition Hyderabad, India. The animals were housed at six per polypropylene cage and provided with standard pellet diet (Mysore Snack Feed Ltd., Mysore, India) and water ad libitum. The animals were maintained at a temperature of 28 ± 2°C with an alternating 12-h light/12-h dark cycle. The animals were maintained as per the norms provided by the Bioethic Committee of Annamalai University (IAEC/CPCSEA 270 Dated 01.07.2005).

Experimental Design

The animals were randomized into experimental, control groups, and divided into four groups of six animals each. Animals in group I was treated as untreated control. The animals in group II were painted with a 0.5% solution of DMBA in liquid paraffin on the right buccal pouch using a no. 4 brush three times a week for 14 weeks. Each application treated approximately 0.4 mg of DMBA. Group III animals were painted with DMBA as in group II; In addition, the animals were administered with 1.25% of freshly prepared mangrove tea extract twice per day. Group IV animals received the same dose of mangrove tea extract alone. The experiment was terminated at the end of 14th week and animals were sacrificed by cervical dislocation after an over night fast and the fresh tissue were used for estimations.

Isolation and Identification of Bacteria from Saliva

Saliva samples were collected from the buccal region of the animal using sterile Whatman filter paper discs (with a diameter of 5 mm). The sample was serially diluted and plated on culture media deMan Rogosa Sharpe (MRS), streptococcus selection agar (SSA) and modified milk agar and incubated at 37°C for 36 h. All the determinations were carried out in triplicate. The counts are expressed as colony forming unit (CFU) per ml of the sample. Identification was done by following the keys of Bergy's manual of determinative bacteriology [17, 18].

Histopathological Observations

The specimens were maintained in 10% formalin solution for processing. Embedded in paraffin, the specimens were sectioned and examined under a microscope at 40× magnification, after staining with hematoxylin and eosin.

Statistical Analysis

One-way ANOVA and Duncan multiple range tests were used to compare mean values at 0.05 probabilities.

Results

Cancer Incidences

Tea from mangrove plant *Ceriops decandra* effectively prevented the DMBA induced carcinogenesis. The DMBA treated animals showed 100% squamous cell carcinoma where as the tea extract treated animals showed hyperplasia alone. We did not observed any cancer incidences in both untreated control and tea extract alone treated animals.

Enumeration of Bacteria in Saliva

Bacterial counts in the hamsters induced with oral carcinoma are given in the Table 1. Animals in control group exhibited high counts of lactobacilli ($42 \pm 11 \times 10^3$ CFU ml^{-1}) followed by streptococci ($29 \pm 17 \times 10^3$ CFU ml^{-1}) and bifidobacteria ($22 \pm 17 \times 10^3$ CFU ml^{-1}). The tea extract alone treated animals were also rich in lactobacilli and bifidobacterial counts ($53 \pm 18 \times 10^3$, $31 \pm 15 \times 10^3$ CFU ml^{-1} respectively) and reduced counts of streptococci ($25 \pm 13 \times 10^3$ CFU ml^{-1}). The DMBA treated groups of animals exhibited low counts of lactobacilli ($25 \pm 10 \times 10^3$ CFU ml^{-1}) and bifidobacteria ($07 \pm 1.0 \times 10^3$ CFU ml^{-1}) however, the animals showed high counts of streptococci ($47 \pm 17 \times 10^3$ CFU ml^{-1}). Whereas the tea extract in the DMBA-treated animals showed increased counts of lactobacilli and bifidobacteria ($37 \pm 10 \times 10^3$; $17 \pm 1.1 \times 10^3$ CFU ml^{-1} respectively) and decreased counts of streptococci ($32 \pm 15 \times 10^3$ CFU ml^{-1}). Counts of lactobacilli and bifidobacteria were negatively correlated with tumor size, whereas streptococcus was positively correlated to tumor size (Figs. 1, 2, 3). The ratio between beneficial and harmful bacteria of 0.68 coincided with the incidence of tumor, whereas there was no tumor incidence when the ratio was equal or greater than 1.6 (Table 5).

Bacterial Species in Saliva

Bacterial species were identified in saliva based on morphological and biochemical characteristics as shown in Table 2. There were five species of lactobacilli (*Lactobacillus acidophilus, L. lactis, L. jensenii, L. casei* and *L. brevis*), two species of bifidobacteria (*Bifidobcterium bifidium* and *B. longum*) and two species of streptococci (*Streptococcus mutan* and *S. mitis*) identified. The characteristics of the bacterial species are given in Tables 2, 3, 4.

Histological Observations

The tumour formation is evident by histological observations. There was well developed squamous cell carcinoma, along with well-defined epithelial and keratin pearls in the connective tissue with cellular pleomorphism (Fig. 4). However, the animals treated with DMBA + mangrove tea extract, exhibited only hyperplasia. The histological studies proved the anti-cancer effect of the mangrove tea extract at the dose of 1.25% twice per day. No pathological observations were noted either in control animals or the animals treated with mangrove tea extract alone. Fig. 4a, d show the histological structure of mucosa extracted from control and mangrove tea extract alone treated animals which are showing normal and intact epithelium. Fig. 4b exhibits the abnormal epithelium intruded into the connective tissue leading to the formation of spherical pearl' like structures in the mucosa extracted from DMBA–treated animals. Fig. 4c exhibits only multi layer of epithelial cells (hyperplasia) in the mucosa of animal treated with both DMBA + mangrove tea extract. Here there is no abnormal intrusion of epithelium and formation of pearl like structures.

Discussions

The results of present study indicated that the tea extract from *Ceriops decandra* effectively prevented the DMBA induced carcinogenesis. The available salivary bacterial species of the test animals clearly indicated the health status of the host animals. The predominant types of bacteria isolated from the saliva, tongue, dorsum and buccal mucosa of rates were *Streptococcus* spp., *Lactobacillus*

Table 1 Effect of tea extract of *C. decandra* on salivary bacterial counts and tumour volume of experimental animals

Treatment	Lactobacillus ($\times 10^3$ CFU ml^{-1})	Bifidobacteria ($\times 10^3$ CFU ml^{-1})	Streptococci ($\times 10^3$ CFUml^{-1})	Tumour volume (ml)
Control	42 ± 11^a	22 ± 05^a	29 ± 17^a	0.00^a
DMBA	25 ± 10^b	07 ± 1.0^b	47 ± 17^b	2.45 ± 0.2^b
DMBA+ Tea extract	36 ± 9^c	16 ± 1.1^c	29 ± 11^a	0.00^a
Tea extracts alone (1.25%)	53 ± 18^d	31 ± 18^d	25 ± 13^a	0.00^a

Values are mean ± standard error from three replicates in each group of animals maintained with six each

Values not sharing a common superscript are differ significantly at $P > 0.05$

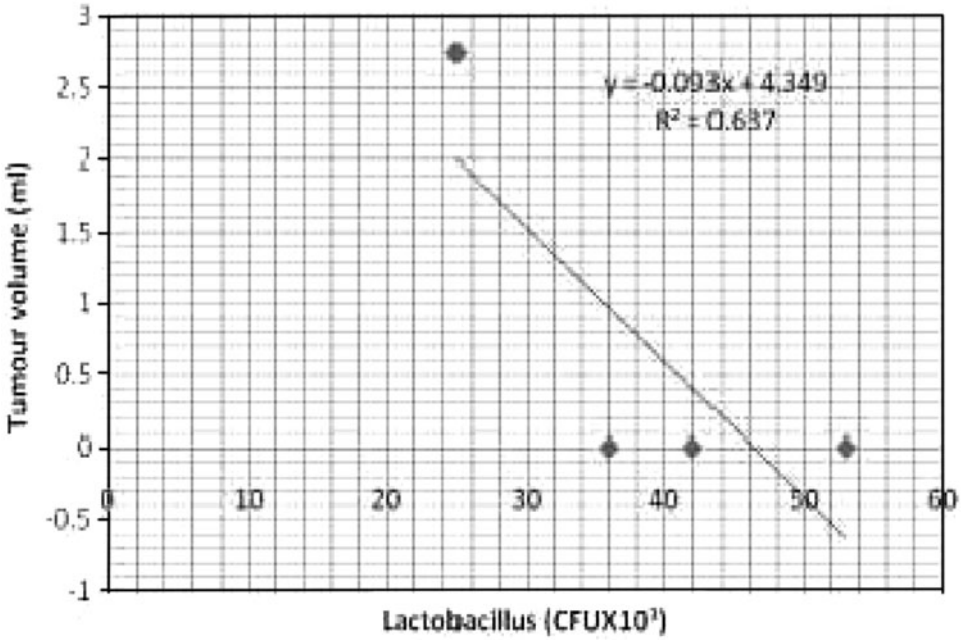

Fig. 1 Correlation between Lactobacilli (CFU × 10^3.ml^{-1}) count and tumour volume

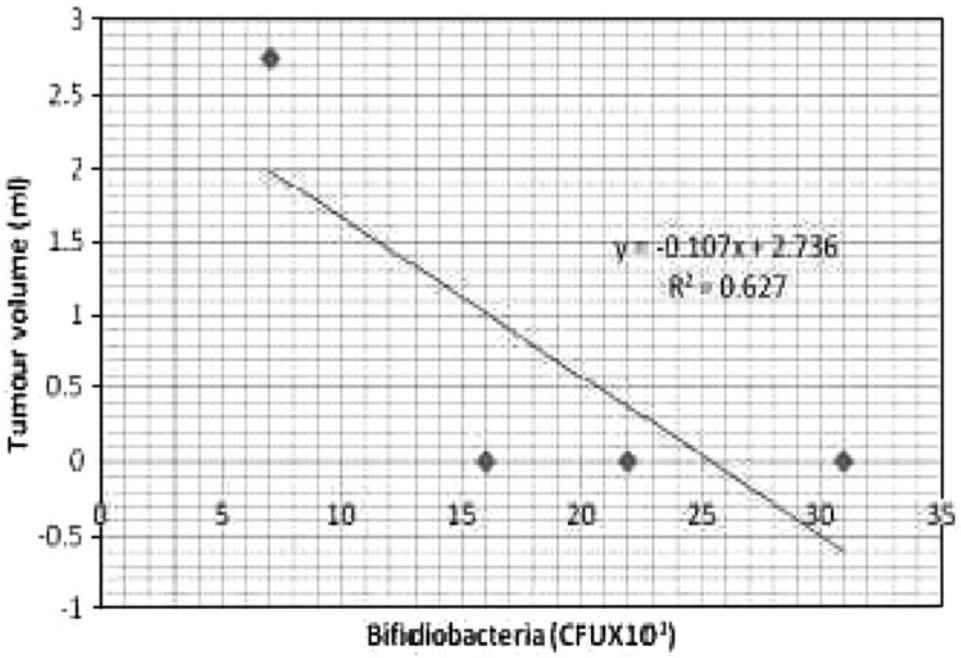

Fig. 2 Correlation between Bifidiobacteria (CFU × 10^3.ml^{-1}) count and tumour volume

spp., and Bifidobacteria [19]. Therefore these three types of bacteria were studied in the present investigation. Lactic acid bacteria and bifidobacteria are two well-known groups of beneficial bacteria which constitute an integral part of the health condition. They impart nutritional and therapeutic benefits to their host. The vitamins and enzymes produced by the lactic acid bacteria contribute to host metabolism. The antimicrobial substances produced by these bacteria control the proliferation of undesired pathogens. Lactic acid bacteria produce a soluble compound which may interact directly with oral tumor cells in culture and inhibit their growth [20]. Singh et al. [21] have observed that *Bifidobacterim longum* exerts a strong anti-tumor activity against colon cancer. Data from epidemiological and experimental studies indicate that ingestion of lactobacilli and bifidobacteria and their fermented products reduce the risk of certain types of cancer and inhibit tumor growth [22, 23]. In the present work, the counts of

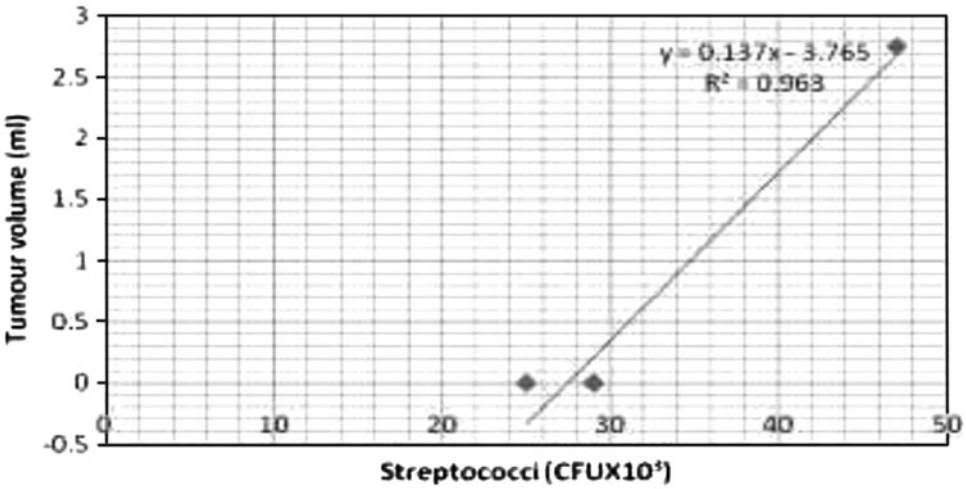

Fig. 3 Correlation between Streptococcus (CFU × 10^3.ml^{-1}) count and tumour volume.

Table 2 Biochemical reactions of the species of genus *Lactobacillus*

Biochemical test	Species of lactobacilli				
	L. acidophilus	*L. lactis*	*L. casei*	*L. jensenii*	*L. brevis*
Fructose	+	+	+	+	+
Galactose	+	+	+	+	+
Glucose (acid)	+	+	+	+	+
Glucose (gas)	−	−	−	−	+
Gluconate	−	−	+	−	+
Lactose	+	+	−	−	+
Maltose	+	−	d	+	+
Mannitol	−	−	+	−	+
Melizitose	−	−	+	−	−
Melibiose	−	−	−	−	+
Raffinose	−	−	−	−	−
Ribose	−	−	+	−	−
Xylose	−	−	−	−	d
Esculin	+	−	+	−	d
Oxidase	−	−	−	−	−
Catalase	−	−	−	−	−
Gelatin liquified	+	+	+	+	+
Nitrate reduction	−	−	−	−	−
Casein hydrolysis	−	−	−	−	−
Indole	−	−	−	−	−
H2S	−	−	−	−	−

+ Positive,− Negative, *d* Variable

lactobacilli and bifidobacteria were high in normal and tea extract treated animals when compared to streptococci which were low in number (Table 1).

Pathogenic forms of oral cavity bacteria have recently been known to associate with cancer tissues. The development of illness such as cancer is associated with significant shifts in the number of gram negative bacteria detectable in oral samples [24]. Nagy et al. [6] have demonstrated a difference in the micro-flora associated with the surface of tumors in comparison to control. Patients with oral cancer tend to possess significantly high concentration of certain bacteria in their saliva. Many bacterial species have been similarly tested for their carcinogenic potential in monkeys, rats, hamsters, rodents and mice. The most carcinogenic bacteria are streptococci including *Streptococcus mutans, S. sobrinus*, *S. cricetus*, and *S. rattus;* and other carcinogenic bacterial species include *Actinomyces naeslundii*, (formerly *A. viscosus*), *S. salivarius*, *S. sanguis*, and *Entrococcus faecalis* [15]. The mutants of streptococcus (*S. cricetus*) are known to cause "caries" [25]. The present study recorded high counts of streptococci in oral system of tumor bearing Hamsters (Table 1). The contribution of probiotic bacteria, such as lactobacilli and bifidobacteria are mainly in the control of pathogenic microbes, that cause various diseases through production of antibacterial protein namely bacteriocin [26, 27] and anti-cancer substances [28]. The dietary supplements of lactobacilli are reportedly decreased the induction of experimental colon cancer [29]. They stimulate and modulate the mucosal immune system by reducing the production of pro-inflammatory cytokines through actions on NFκB pathways, increasing production of anti-inflammatory cytokines such as IL-10 and host defense peptides such as β-defensin 2, enhancing IgA defenses and influencing dendritic cell maturation. Modulation of cell proliferation and apoptosis through cell responses to, for example, microbially produced short chain fatty acids [30]. The present study expected the DMBA induced oral cancer could change the chemistry of the mouth, allowing the bacteria to flourish. It may be due to the cellular leakage of cancer tissues. The

Table 3 Carbohydrate reactions of the species of genus *Bifidobacterium*

Species	Sugars								
	Arabinose	Xylose	Ribose	Glucose	Cellulose	Lactose	Mannitol	Melibiose	Starch
Bifidobacterium bifidium	−	−	−	−	+	+	−	−	−
Bifidobacterium longum	+	+	+	−	−	+	−	+	−

Table 4 Some important tests used to differentiate *Streptococcus* species

Species	Growth					Hae	Raf	Suc	Inu
	10°C	45°C	2% NaCl	6.5% NaCl	pH 9.5				
S. mitis	−	+	+	−	−	D	+	+	+
S. mutans	−	−	+	−	−	D	−	−	−

Hae Haemolysis, *Raf* Raffinose, *Suc* Sucrose, *Inu* Inulin

types of bacteria present in the saliva are depending upon the conditions of the saliva. The alkaline condition of the saliva was observed in DMBA treated tumor bearing animals, whereas acetic and near neutral pH was observed in DMBA + tea treated animals. So the lactobacillus and bifidiobacteria cannot survive in the tumor bearing animals as they grow in acetic condition. It is inferred that the reduction in the beneficial bacterial counts increased the pathogenic bacterial counts in buccal pouch of Hamsters, and this situation made the animal weak and susceptible to incidence of tumor (Tables 1 and 5). The changes in bacterial flora are not the direct effects of DMBA treatment as proved by anti microbial method using disk diffusion method. DMBA showed no antimicrobial activity (data not shown). Therefore the DMBA effects on bacterial flora are indirect perhaps through metabolic changes induced in the test animals.

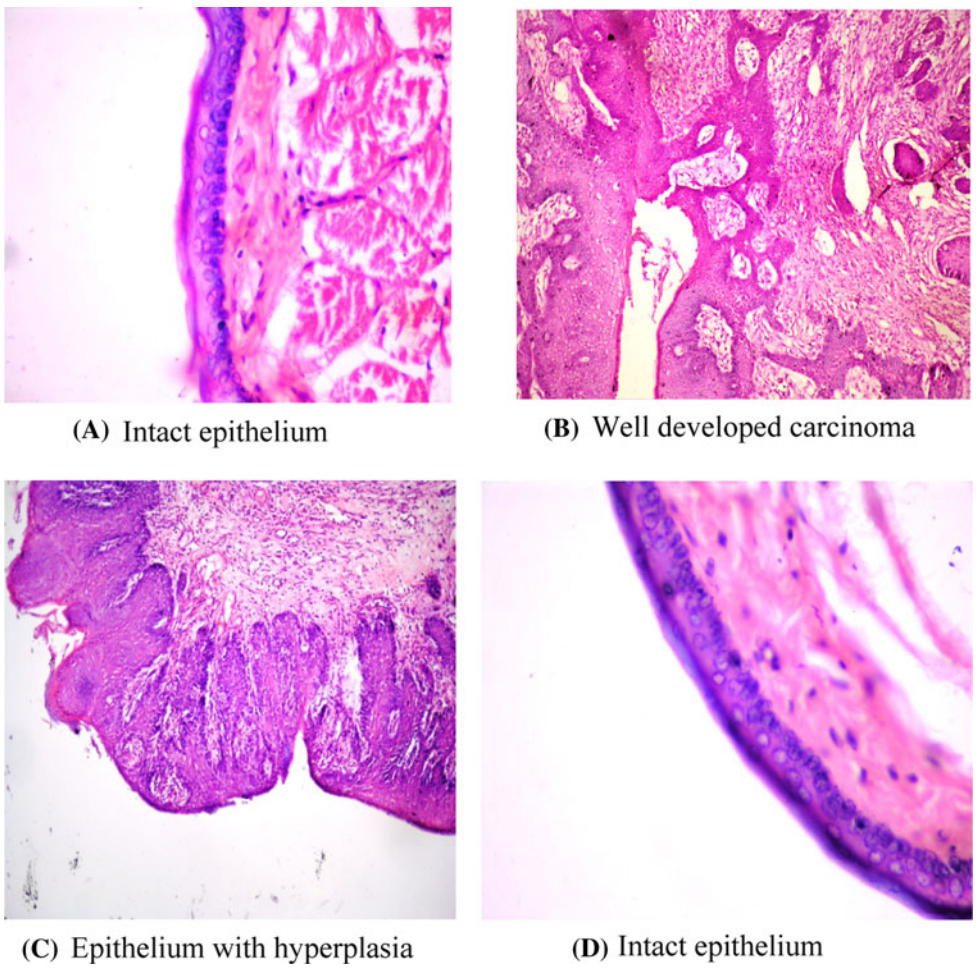

Fig. 4 Histological changes in the mucus tissue of Hamster buccal pouch. Untreated control (**A**), treated with DMBA (**B**) treated with DMBA + mangrove tea (**C**) and mangrove tea alone (**D**) (magnification 40×)

Table 5 Ratio between beneficial (lactobacilli, bifidobacteria) and harmful (streptococcus) bacteria and incidence of tumour volume in buccal pouch of Hamsters

Treatment	Ratio between beneficial and harmful bacteria(CFU $\times 103.ml^{-1}$)	Tumor volume (ml)
Control	1.93 ± 0.15^{a}	0.00^{a}
DMBA	0.68 ± 0.05^{b}	2.45 ± 0.2^{b}
DMBA+ Tea extract (1.25%)	1.60 ± 0.13^{a}	0.00^{a}
Tea extracts alone (1.25%)	3.14 ± 0.28^{a}	0.00^{a}

Values not sharing a common superscript are differ significantly at $P > 0.05$

Acknowledgments The authors are thankful to the authorities of Annamalai University for having provided with facilities.

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