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EVALUATION OF CARCINOGENIC/CO-CARCINOGENIC ACTIVITY OF A COMMON CHEWING PRODUCT, PAN MASALA, IN MOUSE SKIN, STOMACH AND ESOPHAGUS

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Pan masala, a dry powdered mixture of areca nut, catechu, lime, unspecified spices and flavoring agents, has gained widespread popularity as a chewing substitute in India. In this study, the carcinogenic and tumor-promoting potential of an ethanolic pan masala extract (EPME) was determined using skin of S/RVCri-ba mice and forestomach and esophagus of ICRC mice as the target tissues. Carcinogenic activity of pan masala was tested by painting the mouse skin for 40 weeks with EPME or by gavage feeding for 6 months. Following initiation with 9,10-dimethylbenz(a)anthracene (DMBA), carcinogenesis of mouse skin was promoted with different doses of EPME, while gastric- and esophageal-tumor-promoting activity was determined by administering EPME by gavage to animals initiated with diethylnitrosamine (DEN). The ability of EPME to effect progression of skin papilloma to carcinoma and cutaneous alterations after a single or multiple EPME treatment were also evaluated. EPME at 25 mg per dose promoted skin-papilloma formation between 30 and 40 weeks of treatment and enhanced the rate of conversion of papilloma to carcinoma. Induction of mild epidermal hyperplasia, dermal edema, increase in epidermal mitotic activity and the rate of epidermal and dermal DNA synthesis by EPME correlated well with its skin-tumor-promoting potential. In ICRC mice, EPME was inactive as a complete carcinogen, but effectively promoted the development of forestomach and esophageal papilloma and carcinoma in a concentration-dependent manner. The tumor incidence at 25 mg EPME per dose was comparable with that obtained in the 12-0-tetradecanoylphorbol-13 acetate(TPA)-treated group. The findings indicate that habitual pan-masala use may exert carcinogenic and co-carcinogenic influence. *Int. J. Cancer* 75:225–232, 1998.
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Habitual chewing of betel quid, locally called ''pan,'' is widely prevalent in India since ancient times. A pan is prepared by smearing the betel leaf with lime and catechu; pieces of areca nut and desirable spices such as cardamom and cloves, are placed on the leaf, which is folded into an ornate shape. In the last two decades, the Indian market has been flooded with a powdered chewing mixture containing the main dry ingredients of betel quid, menthol, unspecified spices and flavoring agents. This chewing product, termed ''pan masala,'' has gained widespread popularity and social acceptance in several Asian countries, particularly among women and children who generally refrain from using tobacco in any form.

Areca nut, the major constituent of pan masala, has been found to exhibit clastogenic, genotoxic and carcinogenic potential in different experimental systems (Suri *et al.,* 1971; Ranadive *et al.,* 1976; IARC, 1985; Panigrahi and Rao, 1986; Sundqvist *et al.,* 1989; Dave *et al.,* 1992). Catechu was shown to cause dominant lethal mutation and chromosomal damage in mouse bone-marrow cells (Giri *et al.,* 1987), while lime is reported to induce irritation as well as hyperplasia of the oral mucosa (Dunham *et al.,* 1966; Sirsat and Kandarkar, 1968) and produce reactive oxygen species in combination with catechin fraction of areca nut *in vitro* (Nair *et al.,* 1992). Epidemiological studies have shown an association between the habit of chewing betel nut and high risk for oral cancer in the Indian population in South Africa and Malaysia (Schonland and Bradshaw, 1969; Chin and Lee, 1970).

In keeping with the genotoxic properties of the individual constituents, an extract of pan masala was found to increase the frequencies of sister chromatid exchanges and chromosomal aberrations in mouse bone-marrow cells (Mukherjee and Giri, 1991) and Chinese hamster ovary cells (Adhvaryu *et al.,* 1989). Mutagenic potential of ethanolic (Bagwe *et al.,* 1990) and aqueous extracts (Polasa *et al.,* 1993) of different brands of pan masala has also been reported. However, no information exists regarding the neoplastic potential of pan masala. In the present study, we investigated the carcinogenic and tumor-promoting activity of pan masala in 2 mouse models, using skin, esophagus and forestomach as the target tissues. The effect of pan masala on cutaneous alterations including epidermal hyperplasia, cell proliferation and inflammation considered to be crucial for mouse skin-tumor promotion (Argyris, 1985) was also evaluated.

MATERIAL AND METHODS

Chemicals

DMBA, DEN and TPA were purchased from Sigma (St. Louis, MO (^{3}H) methyl thymidine (specific activity 18000 mCi /mmol) was obtained from BRIT (Mumbai, India). The choice of the pan masala brand was based on the results of a market survey conducted at different locations in Mumbai, India. The brand with the highest sale, which we employed in the present study, was identical to the one used by us in mutagenicity experiments earlier (Bagwe *et al.,* 1990). In order to avoid possible batch-to-batch variation in chemical constituents, bulk quantities of the same batch were purchased and stored at −20°C until use. All other chemicals were of analytical grade.

Preparation of ethanolic pan-masala extract (EPME)

Finely powdered pan masala was mixed with 5 vol of redistilled ethanol and the contents were extracted by agitation on a rotary shaker for 24 hr at room temperature. The supernatant collected after centrifugation at 12050 g was evaporated to dryness by flash evaporation. The dry residue was dissolved in a minimal amount of redistilled ethanol and further in 100 µl of acetone or distilled water (DW) to obtain EPME doses equivalent to 12.5, 25 and 50 mg of original pan masala powder.

Animals and treatments

An inbred hairless mouse strain obtained from the Animal house of the Cancer Research Institute, Mumbai, India, designated S/RVCri-ba or ''Bare'' was used for skin tumorigenesis experiments. Female Bare mice 6- to 7-week-old were randomly divided into 11 groups of 15 each for carcinogenicity studies (Table I) and 8 groups of 5 each for histomorphometric analysis (Table II). For determining gastric and esophageal tumorigenesis, 6- to 8-week-old inbred male and female mice (30 per group, 15 of each sex) of

Abbreviations: EPME, ethanolic pan masala extract; DMBA, 9,10-dimethylbenz(a)anthracene; DEN, diethylnitrosamine; TPA, 12-O-tetradecanoylphorbol-13-acetate; DW, distilled water.

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TABLE I – EXPERIMENTAL DESIGN OF SKIN CARCINOGENESIS IN FEMALE S/RV Cri-ba MICE[1]

Group	Number of mice	Initiation	Promotion	Progression	Purpose
1	15	0.1 ml acetone	0.1 ml acetone	—	Solvent control for complete carcinogenesis
2	15	25 mg EPME	25 mg EPME	—	Complete carcinogenic activity of EPME
3	15	50 mg EPME	50 mg EPME	—	Complete carcinogenic activity of EPME
4	15	0.1 ml acetone	1.8 nmol TPA	—	Solvent control for tumor initiation
5	15	25 mg EPME	1.8 nmol TPA	—	Tumor-initiating activity of EPME
6	15	20 nmol DMBA	0.1 ml acetone	—	Solvent control for tumor promotion
7	15	20 nmol DMBA	12.5 mg EPME	—	Tumor-promoting activity of EPME
8	15	20 nmol DMBA	25 mg EPME	—	Tumor-promoting activity of EPME
9	15	20 nmol DMBA	50 mg EPME	—	Tumor-promoting activity of EPME
10	15	20 nmol DMBA	5 nmol TPA	0.1 ml acetone	Solvent control for tumor progression
11	15	20 nmol DMBA	5 nmol TPA	25 mg EPME	Tumor progression with EPME

[1]For details of treatment, see ''Material and Methods''.

ICRC/HiCri (ICRC) strain (Randelia *et al.,* 1988) were used. Animals of the same sex were housed 5 to a cage in an air-conditioned animal house (temperature 21 ± 1°C; humidity 55-60%; constant 12-hr light/dark cycle) and were provided with pelleted mouse feed and water *ad libitum.* Different doses of EPME and TPA or 20 nmol DMBA were dissolved in 100 µl acetone and applied topically using a micropipette to the back skin of Bare mice, while 100 µl aqueous solutions of EPME and TPA were administered by gavage to ICRC mice.

Complete carcinogenesis. The back skin of uninitiated S/RVCri-ba mice was treated twice a week with acetone or 25 and 50 mg EPME in acetone for 40 weeks. Groups of ICRC mice received by gavage the same doses of EPME in DW or DW only, 5 times a week for 6 months.

Tumor initiation. S/RVCri-ba mice were initiated with a single topical application of 25 mg EPME or acetone followed one week later by twice-weekly treatment with 1.8 nmol TPA in 100 µl of acetone for a period of 40 weeks. However, the ability of EPME to initiate gastric or esophageal tumors was not evaluated.

Tumor promotion and progression. Skin-tumor-promoting activity of EPME was determined in Bare mice initiated with 20 nmol of DMBA in 100 µl of acetone. One week after initiation, mice received twice-weekly applications of 12.5, 25 or 50 mg of EPME for 40 weeks. In experiments on the progression of skin papilloma to carcinoma, papillomas were induced by the standard DMBA-TPA protocol (Boutwell, 1974). After a treatment-free period of 6 weeks, the back skin was treated with acetone or 25 mg EPME for 14 weeks. To evaluate the tumor-promoting activity of EPME in the forestomach and esophagus, ICRC mice were initiated with a cumulative DEN dose of 16 mg/kg body weight administered in drinking water for 4 days. This dose of DEN was chosen on the basis of preliminary experiments wherein cumulative DEN doses ranging from 16 mg to 225 mg/kg body weight were given in drinking water for a period of 4 days to 8 weeks to determine the initiating dose of DEN (data not shown). Since no gross tumors were observed either in the esophagus or in the forestomach of ICRC mice killed 6 months after 4 days treatment with a cumulative dose of 16 mg/kg body weight, this dose of DEN was preferred for tumor initiation. One week later, mice were gavage-fed DW or 12.5-, 25- and 50-mg doses of EPME, 5 days a week for 3 and 6 months. TPA (5 nmol) dissolved in DW was administered by gavage as a positive control for 3 months.

A monthly record of animal weight and survival rate was maintained for all the mice. In the Bare mouse strain, the number of tumors (≥1 mm diameter) was recorded every week. Necropsy was performed on all moribund animals and at the end of the experimental period. Skin and tumor tissues from Bare mice and lung, liver, stomach, esophagus, kidney, spleen, intestine and urinary bladder of ICRC mice were fixed in Bouin's fluid and processed for routine histopathology. The esophagus was dissected completely open on a filter paper so that it remained flat, while the stomach was opened across the greater curvature. The number of tumors was recorded in the organs and confirmed histopathologically. The percentage of tumor bearers and the average number of tumors per mouse in experimental and control groups were compared using Chi-squared distribution and Student's *t*-test respectively.

Histomorphometric analysis

DMBA-initiated Bare mice received a single application or multiple applications (twice weekly for 40 weeks) of acetone or 12.5-, 25- and 50-mg doses of EPME. Mice were killed 1 hr after i.p. injection of 10 µCi (^{3}H) methyl thymidine in 0.1 ml of PBS and 48 hr after the last promoter treatment. Skin tissues were excised, fixed in Bouin's fluid and processed for routine histopathology. Additional sections were processed for autoradiography, and histomorphometric analysis was carried out as described (Bagwe *et al.,* 1994). Data were analyzed by Cochran-Fisher modification for analysis of variance and by Student's *t*-test for the comparison of means in control and experimental groups.

RESULTS

Skin tumorigenesis

In all the groups, EPME treatment did not induce overt signs of toxicity as judged by visual observation of the skin and change in body weight relative to controls. Multiple applications of 25 or 50 mg EPME to uninitiated Bare mouse skin or a single application of 25 mg EPME as an initiator followed by TPA promotion for 40 weeks failed to induce skin tumors. As compared with controls, a 2-fold increase in the rate of conversion of papilloma to carcinoma (27% *vs.* 13%) was observed when 25 mg EPME was applied in the progression phase. However, the increase was statistically not significant.

As shown in Table III, the time required for the appearance of the first tumor was similar in DMBA-initiated mice treated with acetone or 3 different doses of EPME. The time for tumor development in 100% of the animals was 32 weeks in the control group, while it was 39, 29 or 26 weeks in mice treated with 12.5, 25

TABLE II – HISTOMORPHOMETRIC CHANGES INDUCED BY SINGLE OR MULTIPLE EPME TREATMENT IN DMBA-INITIATED S/RVCri-ba MICE[1]

Promoter	Thickness (μm)		Number of nucleated epidermal cell layers	Number of basal cells	Epidermal mitoses			Labelled nuclei				
								Epidermis			Dermis	
	Epidermis	Dermis			Basal	Supra-basal	Total	Basal	Supra-basal	Total		
Single application												
Acetone	21.7 ± 1.6	103 ± 2.9	2.63 ± 0.02	531 ± 4.0	4.1 ± 0.3	0	4.1 ± 0.3	6.1 ± 1.5	0	6.1 ± 1.5	2.3 ± 0.4	
EPME, 12.5 mg	25.9 ± 0.7	176 ± 2.7	2.85 ± 0.04	526 ± 3.6	5.3 ± 0.3	1.0 ± 0.02	6.3 ± 0.3**	6.4 ± 0.3	1.1 ± 0.1	7.5 ± 0.3	3.5 ± 0.4	
EPME, 25 mg	31.9 ± 0.9**	204 ± 10.6**	3.83 ± 0.03**	514 ± 4.0	5.8 ± 0.7	1.4 ± 0.2	7.2 ± 0.8**	7.8 ± 1.5	2.7 ± 0.3	10.5 ± 1.8*	4.8 ± 0.7*	
EPME, 50 mg	24.0 ± 0.5	201 ± 6.2**	2.96 ± 0.04	540 ± 4.4	5.8 ± 0.4	0.4 ± 0.1	6.2 ± 0.5**	6.1 ± 0.5	0	6.1 ± 0.5	2.5 ± 0.2	
Multiple applications												
Acetone	21.5 ± 1.7	133 ± 20.1	2.8 ± 0.1	573 ± 8.0	5.2 ± 0.4	1.0 ± 0.1	6.2 ± 0.3	7.1 ± 1.8	0	7.1 ± 1.8	2.2 ± 0.6	
EPME, 12.5 mg	26.0 ± 1.4	190 ± 12.6*	3.2 ± 0.03	551 ± 1.5	5.7 ± 0.3	1.2 ± 0.1	6.9 ± 0.3	6.8 ± 0.5	1.5 ± 0.3	8.3 ± 0.6	3.9 ± 0.3*	
EPME, 25 mg	33.2 ± 0.9**	209 ± 6.5*	4.0 ± 0.1**	531 ± 6.4**	7.6 ± 0.5	2.5 ± 0.2	10.1 ± 0.6***	7.5 ± 0.5	2.8 ± 0.5	10.3 ± 0.8	4.5 ± 0.6*	
EPME, 50 mg	26.1 ± 0.5	197 ± 3.6*	3.0 ± 0.1	583 ± 10.5	6.0 ± 0.7	1.5 ± 0.3	7.5 ± 0.9	6.0 ± 0.5	1.2 ± 0.5	7.2 ± 0.6	3.4 ± 0.4	

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
[1]Number of mice per group = 5.

and 50 mg EPME respectively (Fig. 1). Up to the 30th week of promotion, no significant changes in tumor yield or rate were observed between the control and experimental groups. At week 40, however, a dose-dependent increase in papilloma yield was observed in animals given 12.5- and 25-mg doses of EPME, while at the highest dose (50 mg) the papilloma yield was similar to that in animals treated with 12.5 mg EPME (Table III, Fig. 1). At the dose of 25 mg EPME, the increase in the average number of tumors per mouse was statistically significant (Table III, $p < 0.05$). Histopathologically, the papillomas were well-differentiated hyperplastic lesions with excessive keratinization and mild cellular atypia.

Histomorphometric alterations

The morphology of Bare mouse skin after a single application of acetone following DMBA initiation resembled that of the normal tissue. Similar treatment with 25 mg EPME resulted in mild epidermal hyperplasia and dermal edema, as evident from a significant increase in the epidermal and dermal thickness and the number of nucleated epidermal cell layers (Table II). Moreover, as compared with acetone control, a significant increase in epidermal mitotic activity was observed after a single application of EPME at all the 3 doses. The number of labelled nuclei per 5 mm surface length was significantly higher in EPME(25 mg)-treated epidermis and dermis.

Sustained epidermal hyperplasia and an increase in mitotic activity in the suprabasal layer, epidermal and dermal thickness, as well as increased number of labelled cells per 5 mm surface length in the epidermis and dermis, were also observed in mice treated with 12.5 or 25 mg EPME for 40 weeks. The increase in these parameters was statistically significant in the group receiving 25 mg EPME. The number of basal epidermal cells was significantly lower in this group as compared with the solvent control epidermis (Table II).

Gastric and esophageal tumorigenesis

Macroscopic observations. The survival rate of ICRC mice in the control and experimental groups ranged from 86 to 100%, since a few animals died due to lung infection. At the start of the experiment, the mean body weight of male and female mice was 34 g and 30 g respectively. Generally, the animals gained body weight during the first 3 months of treatment. However, weight loss of 2 to 3 g was observed in animals receiving EPME for 6 months. On gross observations, raised or nodular surfaces as well as papillomas were noted in the forestomach and esophagus of animals treated with EPME. The papillomas were generally located near the limiting ridge in the forestomach. No gross abnormalities were observed in lung, liver, spleen, kidney or intestine. The incidence and multiplicity of tumors in the forestomach and esophagus in males and females as well as cumulative rate and yield are summarized in Tables IV and V respectively.

Complete carcinogenic activity of EPME. Administration of EPME to uninitiated ICRC mice for 6 months did not induce forestomach or esophageal tumors.

Tumor promoting activity of EPME

Forestomach tumors. The overall rate and yield of forestomach tumors in the control animals treated with DW in the promotion phase for 3 or 6 months was similar (Table IV). Although tumors were not observed in male mice at 3 months, a yield of 1.5 papillomas was observed in 27% of females. However at 6 months, the tumor rate and yield was comparable in animals of both sexes.

The cumulative rate of tumor development and yield increased in a dose-dependent manner in animals given 12.5- or 25-mg doses of EPME for 3 or 6 months. At the 50-mg dose the tumor rate at 3 months was similar to that in animals receiving 25 mg EPME, while a significant reduction was noted in the 6-month-treatment group (34% *vs.* 67%, $p < 0.05$). In male mice treated for 3 months with different doses of EPME, the tumor rate and yield was higher than those in the control group. Moreover, a significant elevation in tumor incidence and yield (50% *vs.* 27%, 2.6 *vs.* 1.5 papillomas/tumor bearer) was recorded in females treated with the 25-mg dose

TABLE III – SKIN-TUMOR-PROMOTING ACTIVITY OF EPME IN DMBA-INITIATED S/RVCri-ba MICE

Promoter	Time when 1st tumor visible (weeks)	Tumor development at					
		Week 20		Week 30		Week 40	
		Yield	Rate	Yield	Rate	Yield	Rate
Acetone	18	0.1 ± 0.05	10	2.1 ± 0.5	80	4.2 ± 1.0	100
EPME							
12.5 mg	18	0.1 ± 0.05	7	3.1 ± 0.6	93	5.2 ± 1.2	100
25 mg	20	0.1 ± 0.1	13	3.4 ± 0.6	100	6.8 ± 0.7*	100
50 mg	18	0.9 ± 0.2	56	2.8 ± 0.3	100	5.7 ± 0.7	100

The yield is the average number of papillomas per mouse; the rate is the percentage of tumor bearers.
*$p < 0.05$.

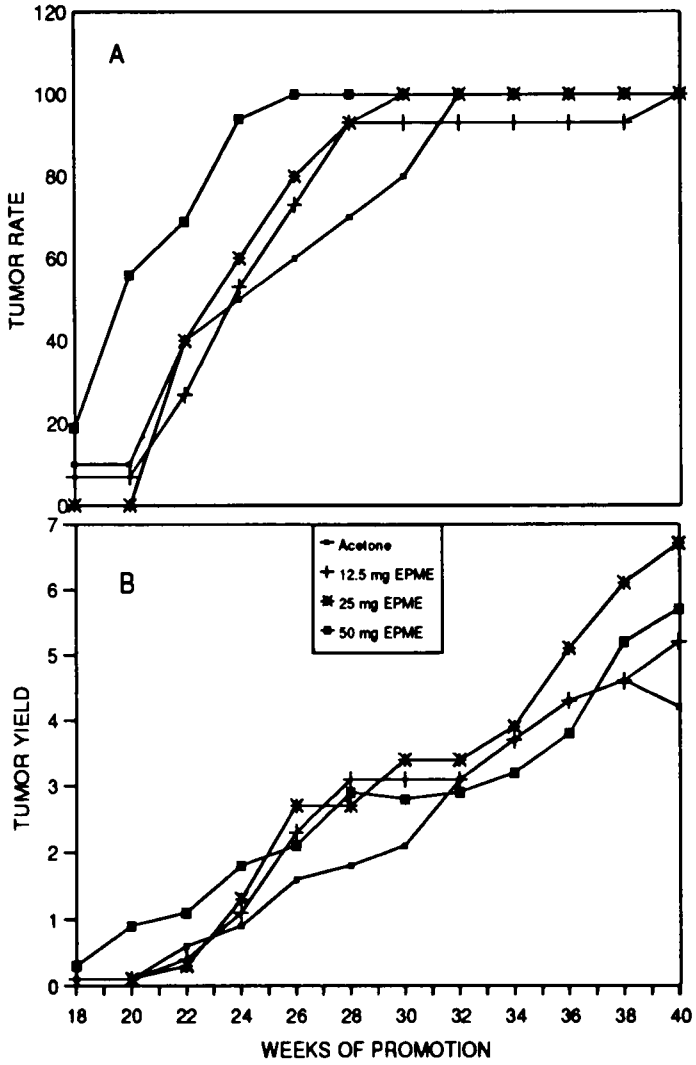

FIGURE 1 – Dose-response relationship in female S/RVCri-ba mice initiated with 20 nmol DMBA and treated with 12.5, 25 and 50 mg EPME for 40 weeks. *(a)* Tumor rate, the percentage of tumor bearers. *(b)* Tumor yield, the average number of papillomas per mouse.

(Table IV). As compared with controls, promotion with 12.5- and 25-mg doses of EPME for 6 months increased the tumor rate and yield in animals of both sexes, with maximum tumor incidence and rate (82%, 2.8 papillomas/tumor bearer) occurring in females treated with 25 mg EPME. At the 50-mg dose, although the tumor rate declined, the number of papillomas per tumor bearer was similar to that observed in animals receiving the 2 lower doses of EPME.

Esophageal tumors. As shown in Table V, administration of DW following DEN initiation for 3 or 6 months to control mice resulted in overall tumor development in 4 and 11% of the animals, the tumor yield being 1.0 and 1.7 respectively. Male mice treated with DW for 3 months did not develop any tumors, while a single tumor was observed in one female. At 6 months, although the tumor incidence was similar in mice of both sexes, the tumor yield was higher in males.

The total tumor rate and yield increased in a dose-dependent manner in animals treated with different doses of EPME for 3 or 6 months. The increase was statistically significant in mice treated

TABLE IV – INCIDENCE AND MULTIPLICITY OF TUMORS IN THE FORESTOMACH OF DEN-INITIATED ICRC MICE TREATED WITH EPME

Promoter	Sex	Duration					
		3 months			6 months		
		Number of effective animals	Tumor bearers (%)	Mean number of tumors/tumor bearer ± S.E.	Number of effective animals	Tumor bearers (%)	Mean number of tumors/tumor bearer ± S.E.
DW	M	14	0	0	14	14	1.5 ± 0.5
	F	15	27	1.5 ± 0.3	13	24	1.3 ± 0.3
	Total	29	14	1.5 ± 0.3	27	19	1.4 ± 0.3
EPME							
12.5 mg	M	15	26*	1.4 ± 0.3	15	34	1.7 ± 0.2
	F	15	15	2.7 ± 0.3**	15	37	2.5 ± 0.2
	Total	30	21	2.0 ± 0.3	30	35	2.1 ± 0.2
25 mg	M	13	23	2.0 ± 0.6	13	53*	1.7 ± 0.3
	F	14	50*	2.6 ± 0.2**	13	82**	2.8 ± 0.3*
	Total	27	39*	2.2 ± 0.2	26	67***	2.4 ± 0.3*
50 mg	M	15	53**	1.2 ± 0.1	15	13	2.0 ± 0.0
	F	15	27	1.5 ± 0.3	14	57	2.6 ± 0.3*
	Total	30	40*	1.3 ± 0.1	29	34	2.5 ± 0.2*
TPA	M	14	43**	2.7 ± 0.2			
5 nmol	F	14	43	2.7 ± 0.3			
	Total	28	43**	2.7 ± 0.2**			

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

TABLE V – INCIDENCE AND MULTIPLICITY OF TUMORS IN THE ESOPHAGUS OF DEN-INITIATED ICRC MICE TREATED WITH EPME

Promoter	Sex	Duration					
		3 months			6 months		
		Number of effective animals	Tumor bearers (%)	Mean number of tumors/tumor bearer ± S.E.	Number of effective animals	Tumor bearers (%)	Mean number of tumors/tumor bearer ± S.E.
DW	M	14	0	0	14	14	2.0 ± 0.0
	F	15	7	1.0 ± 0.0	13	9	1.0 ± 0.0
	Total	29	4	1.0 ± 0.0	27	11	1.7 ± 0.3
EPME							
12.5 mg	M	15	0	0	15	29	1.4 ± 0.3
	F	15	7	1.0 ± 0.0	15	13	1.0 ± 0.0
	Total	30	3	1.0 ± 0.0	30	21	1.3 ± 0.2
25 mg	M	13	15	1.5 ± 0.5	13	47	2.3 ± 0.5
	F	14	23	1.3 ± 0.3	13	41	1.8 ± 0.3
	Total	27	19*	1.4 ± 0.2*	26	44**	2.1 ± 0.3
50 mg	M	15	13	1.0 ± 0.0	15	27	1.5 ± 0.3
	F	15	20	2.2 ± 0.7	14	43	1.5 ± 0.3
	Total	30	17	1.8 ± 0.5	29	34	1.5 ± 0.2
TPA	M	14	7	2.0 ± 0.0			
5 nmol	F	14	36*	1.4 ± 0.2			
	Total	28	21*	1.5 ± 0.2*			

*$p < 0.05$; **$p < 0.01$.

with 25 mg EPME (Table V). Esophageal tumors were not observed in male mice treated with 12.5 mg EPME for 3 months, while a solitary tumor was recorded in a female. The tumor rate and yield was similar in male and female mice treated with 25 or 50 mg EPME. Six months after promotion with different doses of EPME, a statistically insignificant increase in the percentage of tumor bearers was observed in males and females as compared to controls, while the tumor yield was similar in the control and the experimental groups.

Tumor promotion with TPA. In these experiments, 5 nmol TPA was administered by gavage as a positive control for 3 months. Due to retardation in body weight, promotion was not continued thereafter. Promotion with TPA significantly increased the cumulative yield and proportion of mice with forestomach and esophageal tumors (Tables IV, V). Moreover, the incidence of forestomach tumors was similar in male and female mice, while the percentage of animals with esophageal papillomas was significantly elevated in females (Table V).

Histopathological observations. Mild hyperplasia of the forestomach epithelium was observed in 20% and 35% of uninitiated or DEN-initiated mice receiving 25 mg of EPME respectively. Mild chronic inflammation indicative of esophagitis, as well as dyskeratoma, a warty lesion with a crateriform appearance characterized by keratinized and acantholytic cells (Fig. 2), were observed infrequently in mice treated with 50 mg EPME. Papilloma (Fig. 3) in both organs consisted of a localized exophytic excrescence lined by squamous epithelium of varying number of cell layers and increased keratinization, overlying a well-vascularized stroma. Carcinoma was not necessarily associated with a papilloma, but showed clear infiltration at the base of the lesion (Fig. 4). The maximum incidence of carcinoma in the forestomach (10%) and esophagus (7%) was observed at the 50-mg EPME dose. Other histopathological observations revealed fatty and dysplastic changes, and hemangioma in the liver in the experimental groups.

DISCUSSION

Pan masala is a complex mixture of various components whose mutagenic and genotoxic properties have been demonstrated in several short-term assays (IARC, 1985; Adhvaryu *et al.*, 1989;

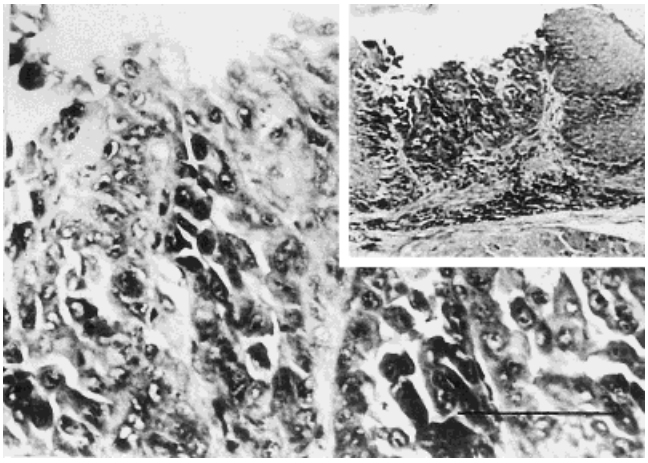

FIGURE 2 – Warty dyskeratoma of esophagus. The lesion shows a crateriform appearance with acantholytic papillae in the center (inset) and abnormal single-cell keratinization. Scale bar, 250 µm.

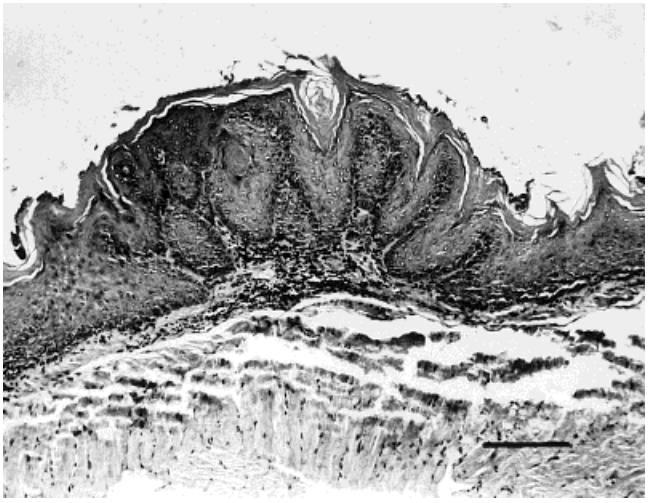

FIGURE 3 – Papilloma. The lesion consists of keratinizing exophytic processes of a squamous nature forming an elevated nodule in forestomach mucosa. Scale bar, 125 µm.

Bagwe *et al.,* 1990; Mukherjee and Giri, 1991). Increased cytogenetic damage has been reported in peripheral-blood lymphocytes and exfoliated buccal mucosa of pan-masala consumers (Dave *et al.,* 1991). However, the implications of habitual pan-masala use with reference to cancer risk have not been fully evaluated by epidemiological or experimental studies. Unlike tobacco chewers, who spit out the juice, pan-masala users often swallow the liquid extract. This increases the possibility of severe carcinogenic effects of pan masala at sites other than the oral cavity. In addition to the mouse-skin model of carcinogenesis, the forestomach and esophagus serve as model systems wherein papillomas appear in response to carcinogen initiation and promotion with croton oil or phorbol ester (Goerttler *et al.,* 1979). Hence in the present investigation, complete carcinogenic, tumor-initiating, tumor-promoting as well as progressor activities of pan masala were evaluated using Bare mouse skin and esophagus, and forestomach of ICRC mice, as the target organs. S/RVCri-ba or ''Bare'' mice are highly useful for mechanistic studies on skin carcinogenesis (Bhisey and Veturkar, 1990), while the ICRC mouse strain is known to be susceptible to the development of esophageal tumors, due to the presence of

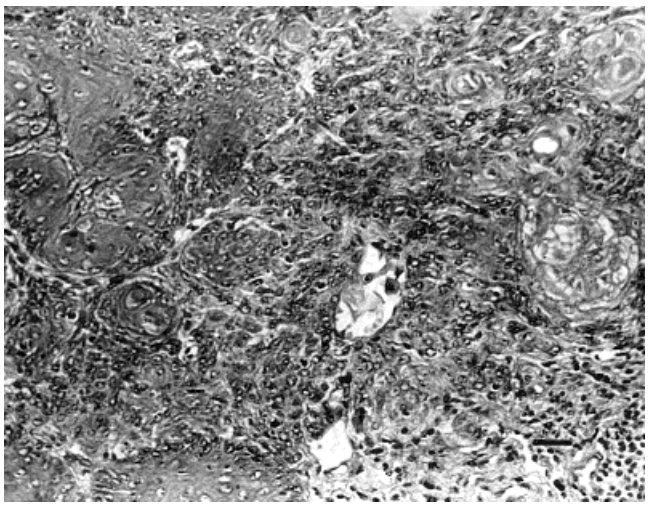

FIGURE 4 – Carcinoma. Sheets of focally keratinizing invasive squamous carcinoma are noted. Scale bar, 50 µm.

hyperplastic mega-esophagus and achalasia or constriction at the gastro-esophageal junction (Randelia *et al.,* 1988; Ghaisas *et al.,* 1989).

Administration of pan masala to Swiss albino mice in the dose range of 0.5 to 2 g/kg body weight is reported to be toxic, while the lower dose range of 8 to 200 mg/kg body weight was found to cause cytogenetic damage in bone-marrow cells, as well as sperm-head abnormalities (Mukherjee *et al.,* 1991). In the present study, the dose range was selected to include the dose of 25 mg EPME shown to elicit mutagenicity in the Ames assay (Bagwe *et al.,* 1990). EPME at doses of 25 or 50 mg/mouse was inactive as a complete carcinogen or a tumor initiator in S/RVCri-ba mouse skin. However, EPME exhibited mild skin-tumor-promoting activity requiring 30 to 40 weeks of treatment for eliciting a significant increase in tumor yield. Experimental studies have shown that while few papillomas progress to carcinomas by TPA promotion, an increase in the appearance of carcinomas on treatment of papilloma-bearing mice with initiating carcinogens can be attributed to an additional mutational event(s) occurring in the target cell (Hennings *et al.,* 1983). Thus, a 2-fold increase in the rate of conversion of papilloma to carcinoma by application of EPME in the progression phase observed in this study may be related to its mutagenic potential (Bagwe *et al.,* 1990).

Induction of sustained epidermal hyperplasia, inflammation and increased cell proliferation are closely linked to the tumor-promoting activity of many compounds (Argyris, 1985). Mild to moderate loss of nuclear polarity, increase in keratoses and mitotic figures, inflammatory cell infiltration, and edema, were observed upon application of pan-masala paste to the oral mucosa of albino rats (Khrime *et al.,* 1991). An extract of betel-quid constituents which contains the main pan-masala ingredients was found to stimulate cell proliferation and induce DNA-strand-break formation in oral mucosal fibroblasts *in vitro* (Jeng *et al.,* 1994). A decrease in the number of basal cells and an increase in suprabasal mitotic cells observed in EPME-treated epidermis in this study were similar to that reported in TPA-treated mouse skin (Astrup and Iversen, 1981; Argyris, 1985). Moreover, an increased rate of epidermal and dermal DNA synthesis, mitotic activity and induction of mild but sustained epidermal hyperplasia and dermal edema by a single or multiple applications of EPME correlated well with its tumor-promoting potential.

Upper gastrointestinal lesions and oral submucous fibrosis are known to be associated with high risk for cancer development in those addicted to chewing of betel quid with or without tobacco (Jussawalla, 1981; Sinor *et al.,* 1990; Anuradha and Devi, 1993;

Ahmed *et al.,* 1993). Betel-quid ingredients, when administered separately or in various combinations to Syrian hamsters, have been shown to induce gastric and buccal-pouch tumors (Ranadive *et al.,* 1979). In the present study, administration of EPME to DEN-initiated ICRC mice significantly enhanced, in a concentration-dependent manner, the development of papillomas in the forestomach and esophagus for up to 25 mg EPME. The gastric- and esophageal-tumor-promoting activity exhibited by EPME at the 25 mg dose, was comparable with that observed with the potent tumor promoter, TPA, although EPME promoted papilloma development in the forestomach to a greater extent than in the esophagus. Additionally, the induction of squamous-cell carcinomas in the forestomach and esophagus of EPME-treated mice indicates that pan masala may influence the progression of malignant tumors. Generally, female mice appeared to be more susceptible than males to the development of papilloma and carcinoma in both organs. These observations are interesting in view of the fact that a high incidence of upper-alimentary-tract cancers was observed in Indian females in South Africa who habitually chewed betel quid (Oettle, 1967; Schonland and Bradshaw, 1969).

Although the precise nature of the carcinogenic/co-carcinogenic effects of pan masala in both the mouse models is not known, there are several possibilities. Chemical toxicological evaluation of pan masala has revealed the presence of polyaromatic hydrocarbons, nitrosamines, toxic metals such as lead, cadmium and nickel, and residual pesticides (Kashyap *et al.,* 1989). Areca nut, the main component of pan masala, contains several alkaloids which are converted to carcinogenic nitrosamines in mild nitrosation conditions (Nair *et al.,* 1985). Arecaidine and its methyl ester, arecoline have been suspected of exhibiting carcinogenic/mutagenic properties, since they are capable of reacting with cysteine *in vivo* and *in vitro* to produce cysteine/3-alkylation adducts (Boyland and Nery, 1969). In addition, reactive oxygen species, which are implicated in the multistep process of carcinogenesis, may be generated during the extraction of pan masala, due to auto-oxidation of polyphenols and interaction of catechin with lime (Nair *et al.,* 1992). The possibility of arecaidine being produced by hydrolysis of arecoline in mild alkaline conditions during the extraction procedure cannot be overlooked. Ethanol is reported to potentiate the clastogenicity of pan masala in Chinese hamster ovary cells (Patel *et al.,* 1994). However, whether the small amount of ethanol used in this study for solubilizing the dry extract of pan masala can contribute to its carcinogenicity needs to be investigated. Thus it is reasonable to assume that some of these compounds, as well as tannins in catechu (IARC, 1985), may be responsible for the carcinogenic and tumor-promoting properties of pan masala observed in the present study. More importantly, the findings on increased induction of benign and malignant tumors in all 3 target organs suggests that pan-masala use may exert carcinogenic as well as co-carcinogenic influence in habitual users of this product.

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