PART III

STUDIES ON GENOTOXIC POTENTIALS OF PAN MASALA

In recent years, especially in India, a new product 'Pan masala' has captured the market gaining rapid popularity. There has been an irrefutable evidence that the use of tobacco is a major health hazard and public opinion against its use has heightened. However, with no counter evidence chasing its ever increasing popularity, and under the pretext of being 'safe', pan masalas are consumed abundantly even by those who generally refrain from chewing habits, including women and children. The manner in which it is advertised on all possible media has led to its profound social acceptance.

Pan masalas, as stated on their packings, consist of areca nut, catechu, lime, cardamom and other flavouring agents (unspecified). Much has been discussed in the previous chapters regarding the genotoxic potentials of areca nut, which, as assessed by us forms 70-80% of the product by weight for the brand studied. Our own results on areca nut and those of other investigators cited earlier, were sufficient enough to draw our attention to this new emergence. Apart from this, it is to be noted that despite all the claims of the manufacturers of using high standard substances, when areca nuts are used in a complex mixture such as this, where its identity is being camouflaged with other constituents, the quality of areca nut used may be questionable. When the nuts are infected with Asperguillus flavus or Asperguillus niger (Mahdihassan, 1981; Mahdihassan and Rabia, 1988; Borle and Gupta, 1987) they are sure to contain aflatoxins, which have been known as potential carcinogens (IARC, 1976; 1982).

Catechu, another constituent of pan masala, is the residue of a hot water extraction of the heart wood of Acacia catechu Linn. The main constituents are tannins and polyphenols; 25-35% catechunic acid, 2-10% catechin, catechu red and quercitin (IARC, 1983). Giri et al. (1987) have reported the genotoxic effects of

catechu. Catechin is a flavonoid and inhibits the mutagenicity of several environmental mutagens including tobacco (Nagabhushan and Bhide, 1988). It has also been reported that flavonoids are genetically active compounds (Brown, 1980). The mode of antimutagenic action of catechin has been studied (Nagabhushan et al., 1988).

Lime, prepared from either limestone or shells, has also been considered to play an important role in the genesis of oral cancer (Davis, 1915; Mendelson and Ellis, 1924; Orr, 1933; Hirayama, 1966; Dunham et al., 1966; Mori et al., 1979; Tanaka et al., 1983; Agrawal et al., 1986). It brings about the liberation of the arecoline from areca nut and the alkaline pH promotes the hydrolysis of arecoline to arecaidine (Nieschultz and Schmersahl, 1968; Boyland and Nery, 1969).

Although, the flavouring agents used in pan masala are not specified, the occasional use of synthetic flavours like musk ambrette and musk xylene to improve the taste and flavour of tobacco (zarda) is well-known. Musk ambrette and musk xylene have been detected in the saliva of chewers of betel quid with tobacco (Nair et al., 1985). Both the musks were found to be mutagenic in Salmonella/mammalian microsome test (Nair et al., 1986). Pan masalas with zarda (a kind of processed tobacco), are also available in the market, however, they are not being considered in the present investigation.

When all such suspected substances are combined in a complex mixture such as pan masala, the possibility exists that either the genotoxic potentials of the constituents may get nullified or synergized, resulting in more severe genotoxicity.

Unlike tobacco chewing, where the chewers spit out the saliva (juice) periodically, pan masalas are consumed in toto, and hence, together with the effects on oral mucosa, it is likely to produce systemic effects as well. It has been suggested

that high risk of oesophageal cancer in India is due to swallowing betel quid without tobacco (Jussawalla, 1981). The in vitro experiments and endogenous formation of nitrosamines, strongly imply that the nitrosation of areca nut specific alkaloids occur in mouth leading to an increased exposure of buccal mucosa and oesophageal lining to areca nut specific nitrosamines. Chewers who swallow the quid, a situation comparable to pan masala consumption, may form even higher amounts of these nitrosamines in their stomach due to nitrosation of the alkaloids (Nair et al., 1985).

Taking all these into consideration, and with a view to assess the potential hazards associated with this seemingly harmless product, we have attempted to determine its genotoxic potentials. As outlined earlier, short term assays can detect the events leading to the initiation of carcinogenesis i.e. the ability of a substance to induce DNA lesions and animal bioassays or human studies would help in making the report more comprehensive. Therefore, together with the in vitro studies, a parallel effort was made towards analysing peripheral blood lymphocytes and exfoliated buccal mucosa cells of individuals regularly consuming pan masalas.

MATERIALS AND METHODS

The present in vitro study was carried out on CHO cells. The cell line was supplied by NFATCC, Pune, India.

SELECTION OF SUBJECTS:

In comparison to the deep rooted custom of chewing areca nut and tobacco, pan masala consumption is a relatively new habit. In the present study, we have attempted to select only those individuals who consumed ONLY pan masala (without tobacco) the same brand that was utilized for our in vitro studies, for more

than one year and had no other past or concurrent habit of tobacco/areca nut consumption. Since these criteria were strictly adhered to, we had difficulty in searching the subjects. The control subjects were selected according to the criteria detailed in the previous chapter.

CHEMICALS:

MEM with NEAA, NCS, CMF-PBS, trypsin, colchicine, Hoechst 33258, Ilford L_4 autoradiography emulsion, Kodak D19B developer, Heparin, PHA-M and MMC were the same as specified in the preceding chapters.

CULTURE PROCEDURES:

CHO cells were maintained in GM, i.e. MEM containing 10-20% NCS and antibiotics. Experiments were initiated in exponentially growing cells after subcultivation and the culture conditions were maintained at 37°C.

The peripheral blood, collected in heparinized vials under asceptic condition was cultured as described in Part II. Briefly, one ml of whole blood was added to 7 ml GM containing 20% NCS, 0.3 ml PHA-M, 2 µg/ml BrdU and the antibiotics. Following colchicine treatment during the last three hours of incubation at 37°C, the cultures were harvested by routine hypotonic treatment with 0.56% KCl and fixed with 1:3 aceto-methanol. Air dried slides were then prepared.

The exfoliated buccal mucosa cells from the pan masala consuming individuals were collected, fixed and stained as described earlier in Part II.

PREPARATION OF AQUEOUS EXTRACT OF PAN MASALA (PM ext.):

Pan masala without tobacco (zarda) ('Pan parag', since it is most popular among the consumers) was purchased from the local market and an aqueous extract

was prepared. In order to allow comparison with the aqueous extract of areca nut, the method of extract preparation of pan masalas remained precisely the same as detailed for AN ext. preparation. 25 grams of finely ground pan masala powder was added to 225 ml distilled water (75 ml added every day, for three days consecutively). This was then centrifuged and the supernatant was filter sterilized with 0.22 µm porosity filter and stored in small aliquotes at -70°C. The extract contained 1.21 gms% water soluble materials.

EXPERIMENTAL PROTOCOL:

The experimental protocol and the concentrations used during the course of study, closely corresponded to those followed for in vitro studies of AN ext. A brief account of the concentrations and the duration of treatment is as follows:

Experiment 1:

With a view to assess the probable cytotoxic effects of pan masala on CHO cells, three concentrations, viz. 10 µl, 20 µl and 50 µl of PM ext. per ml GM were added for a treatment duration of 24 hours, and in another set of experiment for 48 hours. Each set contained a control (untreated) sample. At the end of the treatment percent viable cells were counted.

Experiment 2-3-4:

For analysing CA and SCE frequencies and cellular kinetics in CHO cells, in individual set of experiments, the cultures were subjected to four different concentrations of the PM ext., viz. 2.5 µl, 5 µl, 10 µl and 20 µl per ml GM containing 10 µg BrdU per ml GM. The experiments contained untreated and positive control cultures. The extract was present in the medium until the cells were harvested. In recovery experiments, where the cells were treated with the extract for just

3 hours, the concentrations used were 10 µl, 20 µl and 50 µl per ml GM. Following 3 hours' treatment, the cells were washed with CMF-PBS and supplemented with a fresh extract free GM. For CA assay, the cells were allowed to undergo one cell cycle and for SCE assay as well as for cellular kinetics, they were allowed to propagate for two cell cycles in extract free GM. During all these steps they were protected from direct light. Harvesting was performed following routine protocols.

Experiment 5:

Coverslip cultures of CHO cells were treated with 2.5 µl, 5 µl, 10 µl and 20 µl of PM ext. per ml GM. The treatment lasted for 12 hours in one set of cultures and 24 hours in the other. Autoradiographic studies following a 20 minute pulse of ^{3}H-thymidine, were performed to gather information regarding the possible effects of pan masalas on the synthesis of DNA.

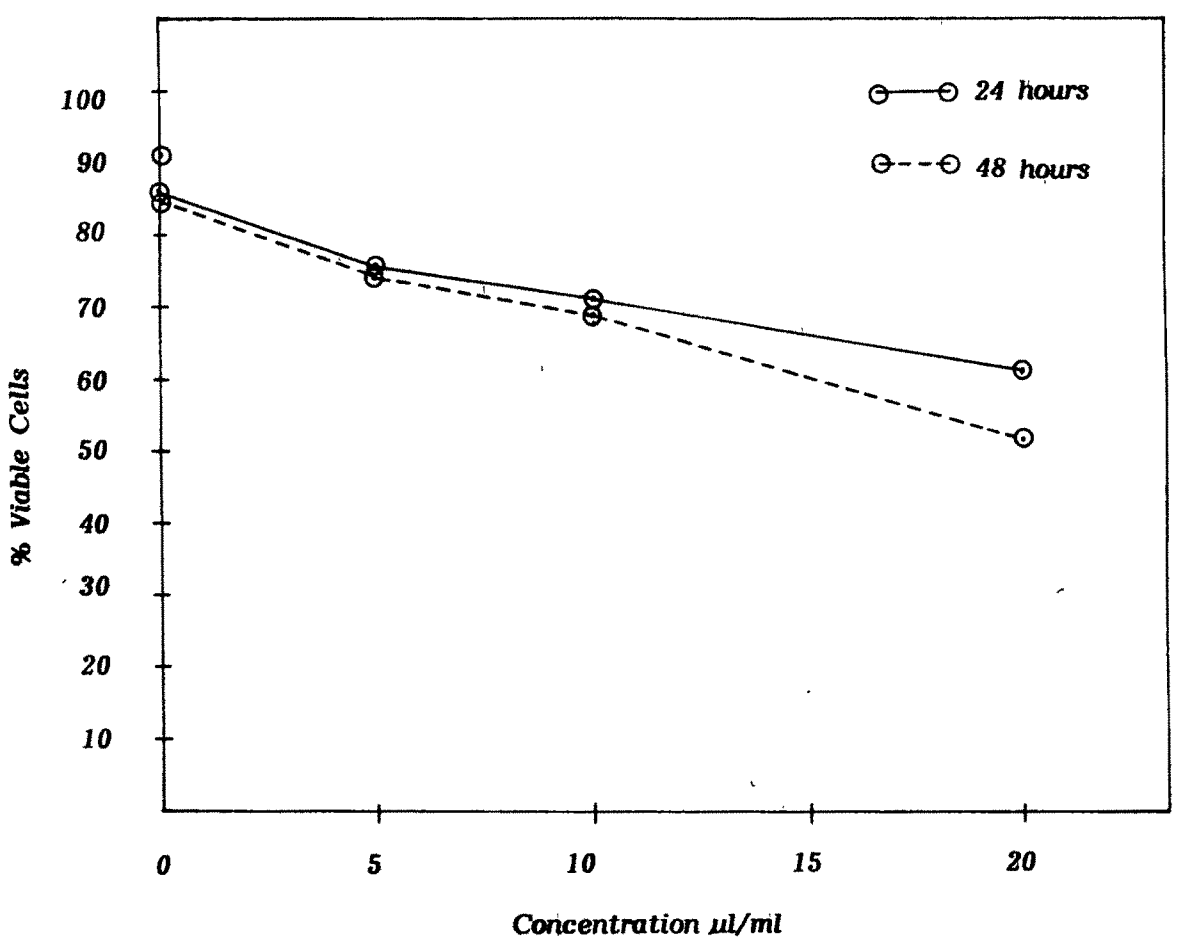
The staining and scoring methods for all the experiments in CHO cells and PBLs of individuals and the particulars of statistical analysis were essentially the same as accounted earlier in Part I and Part II.

RESULTS

1. The manner in which different concentrations of PM ext. affected the percentage viability of the cells, has been graphically presented in Fig. 20. At 0 hour, 90.11% of the cells were found to be viable and after 24 hours the viable cells were 85.92% in untreated cultures. In cultures treated with 5 µl, 10 µl and 20 µl of PM ext. per ml GM the percent viability of the cells decreased to 75.62%, 71.01% and 60.91%, respectively. After 48 hours, when the control cultures showed 85.00% cells to be viable, the treated cultures exhibited 75.00%,

Fig. 20

Cytotoxic effects of PM ext. on CHO cells



69.03% and 52.11% viable cells, respectively. Thus, a dose and duration dependent cell kill was observed when CHO cells were treated with PM ext.

2. The details of the number and types of CAs induced by different concentrations of the extract in CHO cells are presented in Table-37. When the cells were treated for just 3 hours with 10 µl, 20 µl and 50 µl of PM ext. per ml GM, and recovered for 24 hours in extract free GM, the number of aberrant metaphases were 11%, 13% and 18%, respectively, whereas, the untreated cultures and the positive controls respectively showed 7% and 21% aberrant metaphases. Almost all types of aberrations increased with the increasing concentration of the extract, however, only the highest concentration used, i.e. 50 µl per ml GM, provided a statistically significant elevation in the CA per cell value when compared to that of the controls ($p<0.02$).

A continuous treatment of 48 hours with the extract, i.e. 2.5 µl, 5 µl, 10 µl and 20 µl per ml GM produced a variety of aberrations as detailed in Table-38. Although an increase in the number of aberrant metaphases was observed in the treated cultures, i.e. 15%, 15%, 20% and 21%, respectively, compared to the control values of 7% aberrant metaphases, a statistically significant elevation in CA per cell frequency was observed for the last two concentrations used ($p<0.05$).

3. Fig. 21 accounts for the SCE frequency evidenced after 3 hours' treatment and recovery for two cell cycles. When the control cultures presented a mean SCE per cell frequency of 6.76, the treated cells yielded a statistically significant increase in the SCE frequency ($p<0.001$). As witnessed in the figure, more number of cells with higher SCE frequency caused the elevated mean SCE per cell values. The mean values were 9.64, 11.00 and 14.16 SCE per cell for 10 µl, 20 µl and

Table-37

Types and frequency of CAs observed in CHO cells after 3 hours' treatment with PM ext. followed by recovery in fresh growth medium

Conc/ml	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	7	4	-	-	2	1	-	-	-	-	0.07±0.026
10 µl	11	6	-	-	2	2	1	-	-	-	0.11±0.031
20 µl	13	9	-	-	1	4	-	-	-	-	0.14±0.038
50 µl	18	8	2	-	3	4	2	-	1	1	^{b}0.21±0.048*
MMC 0.03 µg	21	9	1	1	4	3	1	2	-	2	^{a}0.23±0.047**

** $p<0.01$ * $p<0.02$

a - $p<0.01$ b - $p<0.05$ - excluding gaps

Table-38

Details of CAs visualized in CHO cells treated with PM ext.

Conc/ml	%Aberrant Metaphase	Chromatid				Chromosome					CA/Cell±S.E.
		G	B	I	Af	GI	BI	DM	R	Dic	
Control	09	7	1	-	2	-	-	-	-	-	0.10±0.033
2.5 µl	15	10	1	1	2	1	1	-	-	-	0.16±0.039
5 µl	15	6	2	1	1	3	1	-	1	1	0.16±0.039
10 µl	20	11	3	-	5	1	1	-	-	2	^{b}0.23±0.049*
20 µl	21	10	6	2	2	2	1	-	1	3	^{a}0.27±0.068*
MMC 0.007 µg	19	9	2	1	2	3	2	-	2	1	^{b}0.22±0.043*

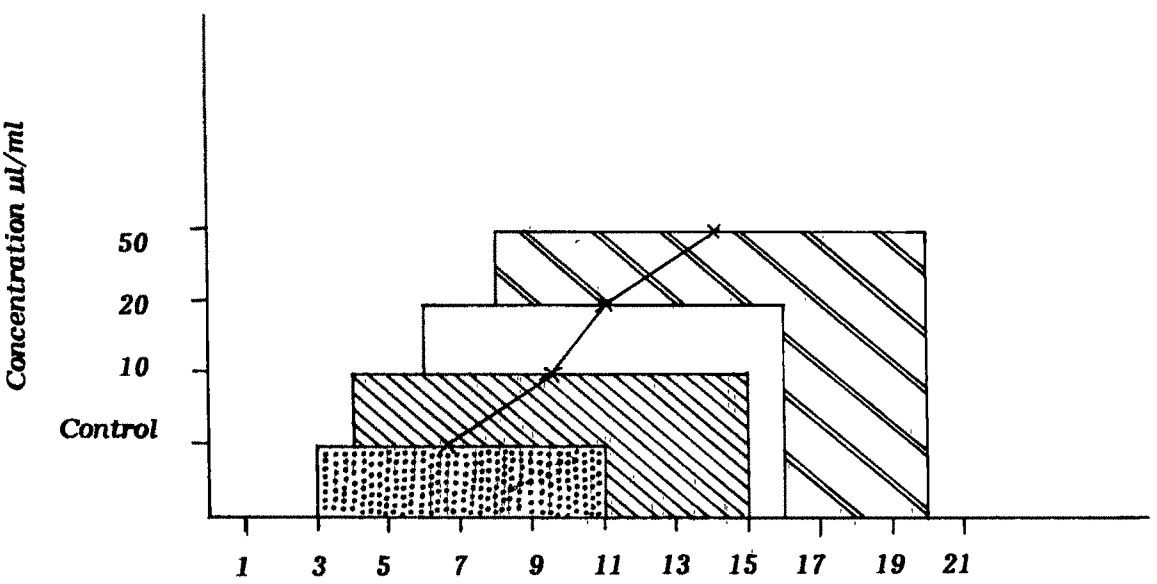
* $p<0.05$

b - $p<0.05$ | excluding gaps
a - $p<0.02$ |

G - Gap, B - Break, I - Interchange, Af - Acentric fragment,
GI - Gap isochromatid, BI - Break isochromatid, DM - Double minutes,
R - Ring, Dic - Dicentric

Fig. 21

Effect of 3 hours' treatment of PM ext. on SCE induction in CHO cells (Mean values with range)



p<0.001 for all concentrations when compared with control

50 µl of the extract, respectively. The positive controls provided a mean value of 21.81 SCE per cell.

All the four concentrations of the extract utilized for a continuous treatment of 48 hours, also induced a dose dependent rise in the SCE frequency. At 2.5 µl, 5 µl, 10 µl and 20 µl of the extract concentration per ml GM, the corresponding mean SCE per cell values were 11.16, 12.72, 14.36 and 15.56, while the control mean was 7.16 SCE per cell. The SCE inductions by all the four concentrations were statistically significant ($p<0.001$) (Fig. 22).

4. The AGT, determined by scoring one hundred metaphases and identifying them into MI, MII and MIII, was found to increase considerably even by three hours' treatment with PM ext. While the control cultures had 2% metaphases in MI, the treated cultures had 3%, 8% and 28% MI metaphases for 10 µl, 20 µl and 50 µl extract per ml GM, respectively. There was a substantial retardation in the proliferation rate and the AGT increased from 21.24 hours in controls to 27.75 hours in cultures treated with 50 µl of the extract, i.e. an increase of 6 hours (30%) by just three hours' treatment with PM extract (Table-39).

The cellular kinetics were also changed after a continuous treatment with the extract. The AGT increased from 23.88 hours in the controls to 31.58 hours with the highest concentration used, a prolongation of almost 8 hours (Table-40).

5. Fig. 23 depicts the observations of the effect of the PM ext. on the synthesis of DNA as judged by the incorporation of ^{3}H-thymidine in the S-phase nuclei. After 12 hours' treatment with the extract, the labelling index, which expresses the percentage of cells synthesizing DNA at the time of ^{3}H-thymidine pulse, was reduced from a control level of 68.0% to 55.6%, 49.0%, 42.4% and 30.5%

Fig. 22

SCE induction in CHO cells treated with different concentrations of PM ext.

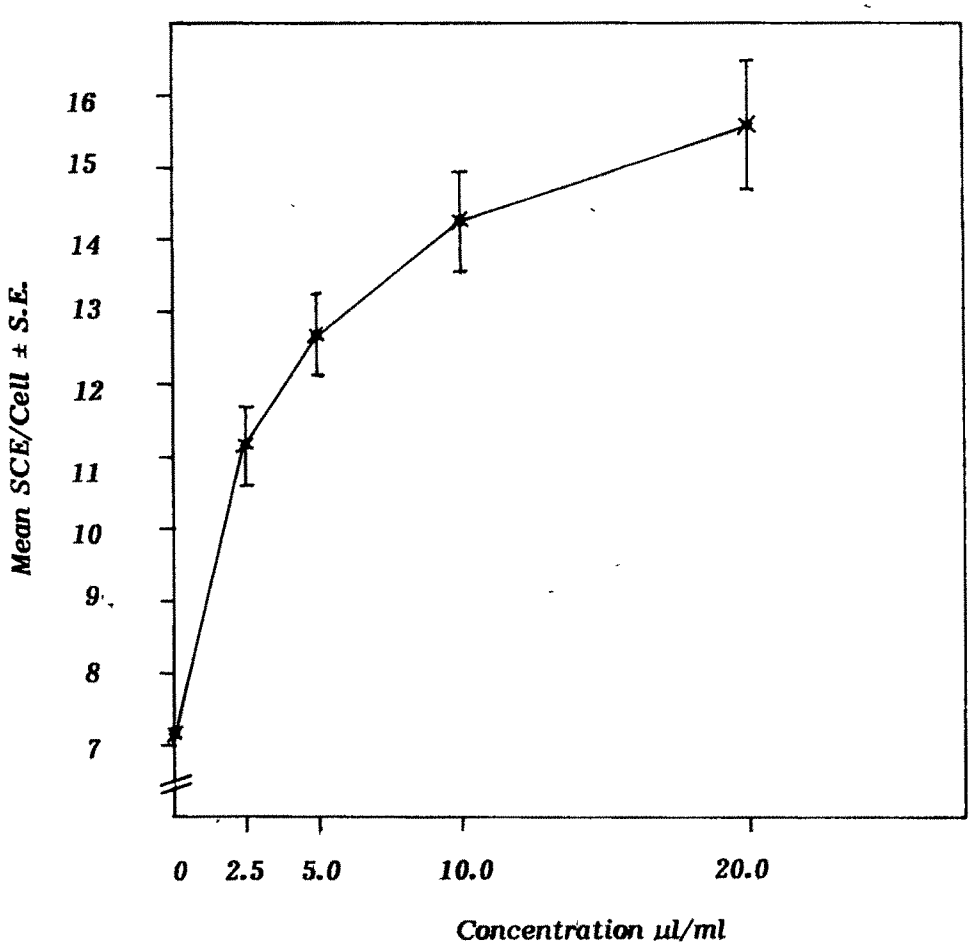


Table-39

Cellular kinetics in CHO following a 3 hour treatment with PM ext. and recovery in fresh growth medium

Conc/ml	MI	MII	MIII	PRI	AGT (hr.)
Control	02	70	28	2.26	21.24
10 µl	03	71	26	2.23	21.52
20 µl	08	85	07	1.99	24.12
50 µl	28	71	01	1.73	27.75
MMC 0.03 µg	22	78	00	1.78	26.97

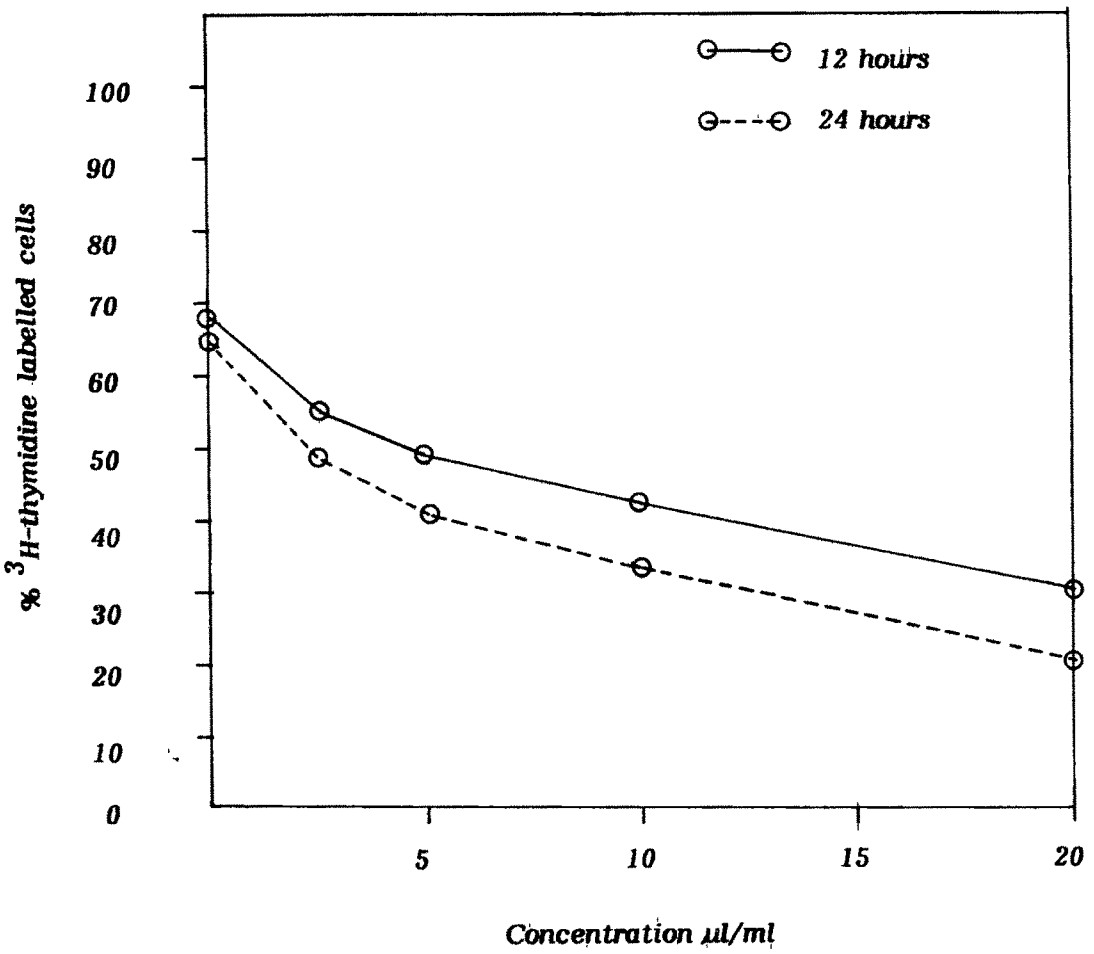
Table-40

CHO cell propagation on treatment with PM ext.

Conc/ml	MI	MII	MIII	PRI	AGT (hr.)
Control	05	89	06	2.01	23.88
2.5 µl	07	91	02	1.95	24.62
5 µl	15	84	01	1.86	25.81
10 µl	23	77	00	1.77	27.12
20 µl	48	52	00	1.52	31.58
MMC 0.007 µg	12	88	00	1.88	25.53

Fig. 23

CHO cells synthesizing DNA after treatment with different doses of PM ext.



for 2.5 µl, 5 µl, 10 µl, and 20 µl PM ext. per ml GM, respectively. At 24 hours, when the control level of labelling index was 65.15, the respective values for all the four concentrations were 49.1, 40.9, 33.6 and 20.08 in the increasing order of the extract concentration. Here too, a dose and duration related retardation in the number of cells synthesizing DNA was witnessed.

Human Studies: The peripheral blood lymphocytes of individuals consuming pan masalas showed a statistically significant increase in SCE as well as CA frequencies when compared to that of healthy controls, not consuming tobacco or areca nut in any form. The details of the mean SCE, CA and MNC frequencies in pan masala chewers are listed in Table-41. The number and types of CAs observed among pan masala chewers have been detailed in Table-42. The variations in SCE frequencies have been described in Fig. 24.

The group mean SCE per cell fequency in controls was 6.185±0.088, whereas,the mean value found in pan masala consumers was 7.592±0.112 SCE per cell. Similarly the CA frequencies also were found to increase greatly. The average CA per cell in controls was 0.050±0.004, which was elevated to 0.122±0.010, i.e. more than double the frequency of CA in PBLs of controls. The mean SCE and CA frequencies were marginally higher than areca nut chewers also. The frequency of MNC in the exfoliated buccal mucosa cells of the individuals consuming pan masalas was significantly higher when compared to that of the control individuals, the values being 0.70±0.051 in pan masala chewers and 0.193±0.022 in controls. A comparative chart (Table-43) of all the three cytogenetic parameters analysed in individuals consuming pan masalas clearly depicts increase in the DNA damage in the target as well as non-target tissues, compared to the controls.

Table-41

Details of different cytogenetic markers among pan masala chewers

No.	Age / Sex	Mean SCE/cell±S.E.	Mean CA/cell±S.E.	Frequency of % MNC	AGT(hr.)
1	22 M	7.40±0.388	0.10±0.0332	0.9	25.81
2	26 M	7.36±0.466	0.08±0.0271	0.5	33.33
3	28 M	7.82±0.477	0.15±0.0357	0.8	40.00
4	31 F	7.16±0.405	0.17±0.0506	0.8	41.38
5	32 M	8.00±0.449	0.10±0.0300	0.8	29.27
6	34 M	7.80±0.431	0.13±0.0391	0.6	25.40
7	36 F	7.93±0.480	0.16±0.0367	0.4	39.67
8	38 F	7.89±0.501	0.09±0.0320	0.6	34.78
9	39 M	7.68±0.407	0.14±0.0375	0.7	28.57
10	55 F	6.88±0.617	0.10±0.0360	0.9	41.03
Group Mean		7.592±0.112	0.122±0.010	0.700±0.051	33.92±1.913

Fig. 24

Distribution of SCE frequency among controls and pan masala chewers

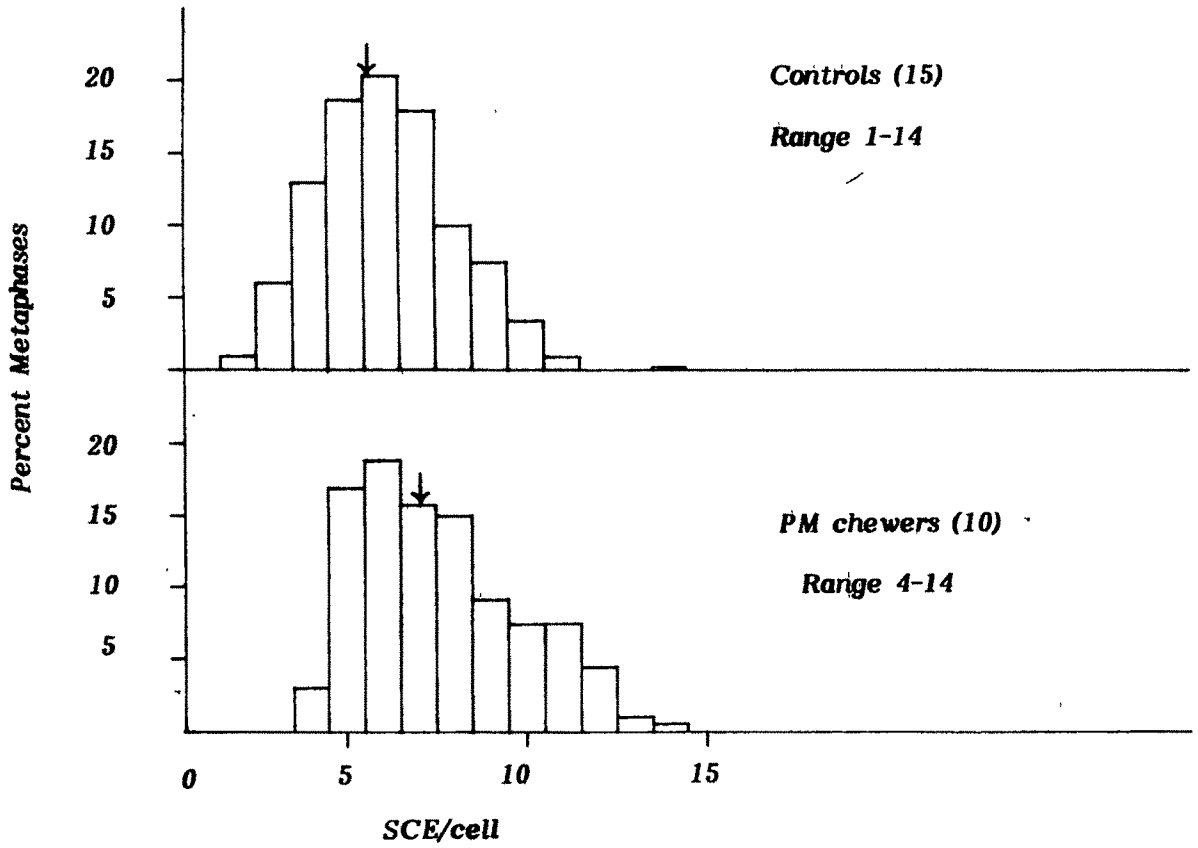


Table-42

Particulars of types of CAs(%) observed among Controls and Pan masala chewers

Group	%Aberrant Metaphases	Chromatid				Chromosome				
		G	B	I	Af	GI	BI	DM	Dic	R
Controls	4.80	3.7	0.6	-	0.3	0.3	0.1	-	-	-
Pan masala chewers	11.44	4.7	1.9	0.2	2.1	1.2	0.9	-	-	-

G - Gap, B - Break, I - Interchange, Af - Acentric fragment, GI - Gap Isochromatid, BI - Break Isochromatid, DM - Double Minutes, Dic - Dicentric, R - Ring.

Table-43

Comparison of the mean values (±S.E.) of all the cytogenetic endpoints among controls, Areca nut chewers and Pan masala chewers

Groups	SCE	CA	MNC
Controls	6.185±0.088	0.050±0.004	0.193±0.022
Areca nut chewers (NAC)	7.218±0.288**	[b]0.105±0.006*	0.730±0.085*
Pan masala chewers	7.592±0.112*	[a]0.122±0.010*	0.700±0.051*

* $p<0.001$ ** $p<0.01$

a - $p<0.001$ b - $p<0.01$ (excluding gaps).

Illustrations 57-60 *Chromosomal damage observed in CHO cells after treatments with PM ext.*

57 *Arrow indicates chromatid gap.*

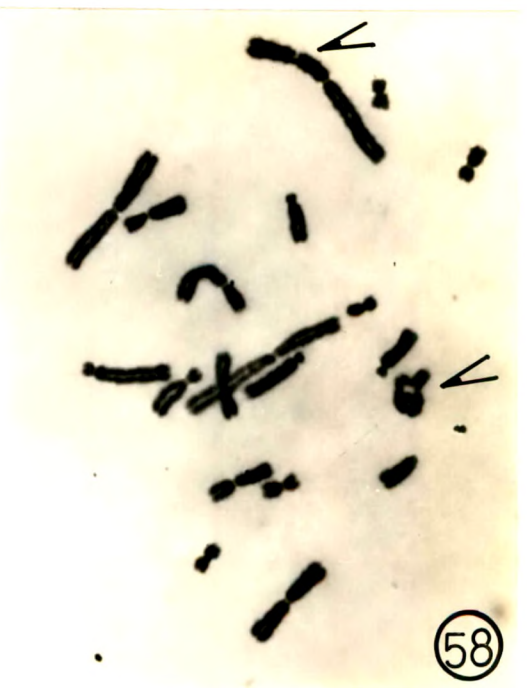
58 *A metaphase with chromatid gap and interchange.*

59 *A metaphase cell showing double minutes.*

60 *Isochromatid and chromatid breaks indicated by arrows.*

Magnification: *1400x.*

57



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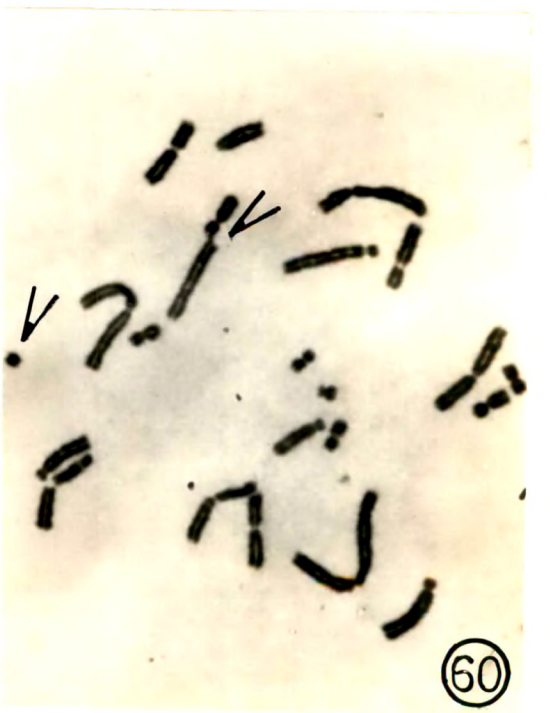

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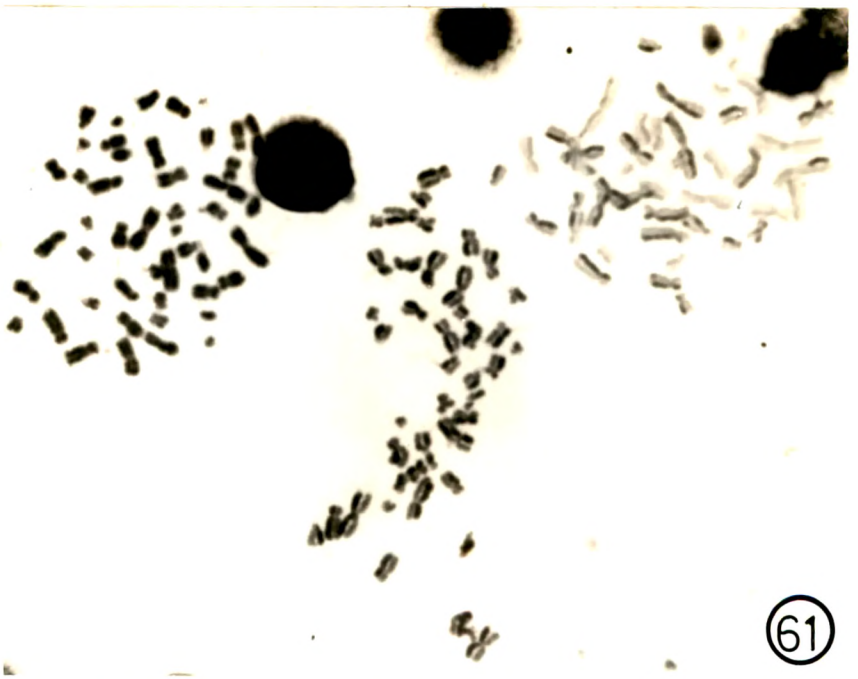
Illustration	*61*	*Human lymphocytes in MI, MII and MIII.*
	62	*Metaphase cell from a control subject showing 3 SCEs.*
	63	*Metaphase from the lymphocytes of a pan masala chewer showing 11 SCEs.*
Magnifications:	*61*	*1250x*
	62	*1400x*
	63	*1600x*



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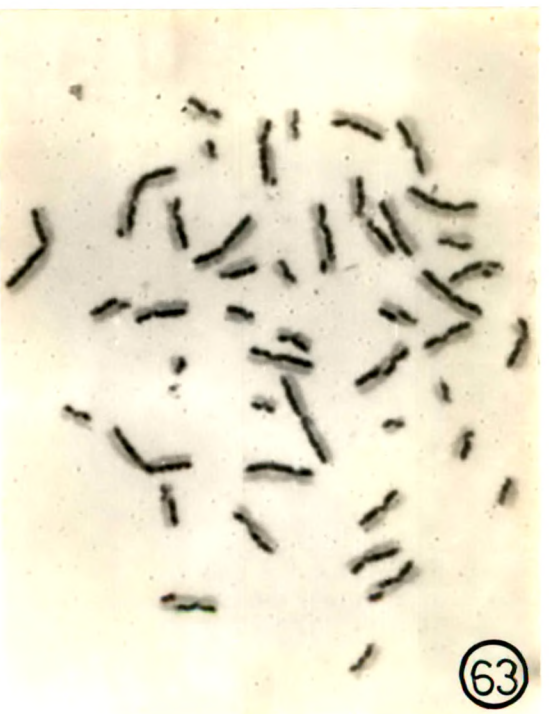

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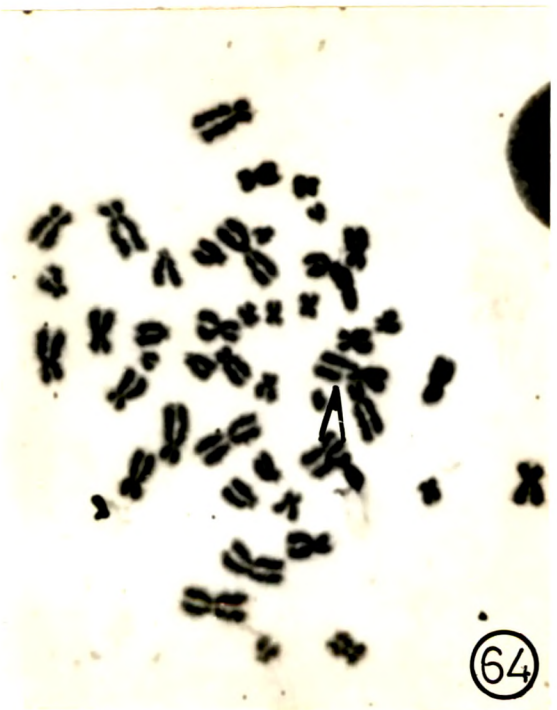
Illustrations 64-67 *Photomicrographs demonstrating chromosome aberrations in lymphocytes of pan masala chewers.*

64-65 *Chromatid gaps (arrows).*

66 *An incomplete interchange recorded in a metaphase.*

67 *A metaphase showing interchange and isochromatid break.*

Magnification : *1800x*



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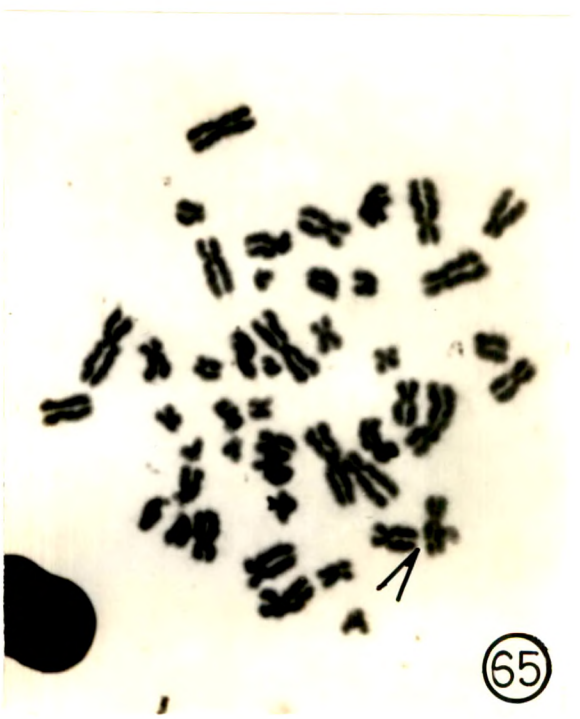

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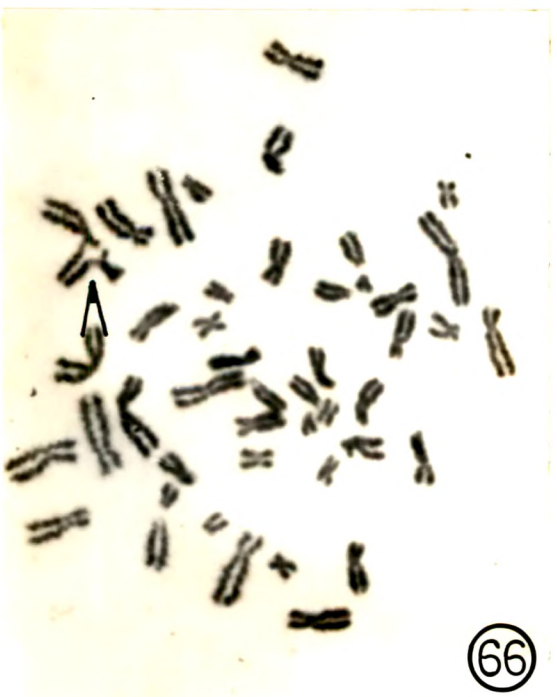

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DISCUSSION

The implications of the synergistic/antagonistic or additive/inhibitory interactions that may take place in a complex mixture, should be considered while interpreting the genotoxic potentials of pan masala. The carcinogenic and mutagenic activities of a substance may be modified by the presence of another compound in a variety of ways. Potentiating actions can occur between compounds that by themselves lack genotoxic activity. As outlined earlier, the major fraction (70-80%) of pan masala, is areca nut, the genotoxic properties of which have already been studied. Any change in the genotoxicity thereof, could be attributed to the presence of other components in the mixture. The ambiguity of the concentrations of other components limits our understanding, however, chemical fractionation and bioassay techniques could be of help.

According to the method employed in the present study, a 10 µl of aqueous extract contains the soluble constituents of 1.11 mg of pan masala. A small pouch of pan masala contains about 4.0 to 5.0 gms. of the material. By interviewing some pan masala consumers of either sex, we could work out an average human consumption in the range of 6.0 to 8.0 gms., i.e. 6000 to 8000 mgs/day. An average male weighs 60 kg. Thus, the estimate of consumption would be 100 mg/kg/day. Now, considering the amount of blood/kg body weight, (79 ml/kg - males, 65 ml/kg - females) and an average consumption of 6000 mgs/day, the estimate of consumption comes to 1.27 mg/day/ml of blood, which is very near to the lowest concentration of the extract used in the in vitro studies, i.e. 10 µl ≡ 1.11 mg pan masala.

Cell cultures and the dye exclusion test are widely used for studying cytotoxicity. Our previous experiments with areca nut extract have been carried out utilizing similar concentrations. The PM ext. elicited a dose and duration dependent cyto-

toxic effect on the CHO cells. An almost 40% cell kill was observed with 50 µl PM ext. per ml GM at 48 hours. The results, however, when compared to areca nut extract showed that the areca nut extract elicited a more severe effect with about 60% cell kill at comparable duration and concentration. An explanation to this may be that pan masala contain only 70-80% of areca nut which was found to be cytotoxic in our studies (Part I). Together with our observations, the lethal effects of areca nut have also been observed by Lalithakumari et al. (1965), Majumdar et al. (1982), Wary and Sharan (1988).

The clastogenic potentials of PM ext. were tested in terms of its capacity to induce CAs. They have been extensively applied in genotoxicity studies since the mechanisms of their formation have been thoroughly elucidated. Most mutagenic carcinogens induce CAs. Areca nut has been found to be mutagenic in Salmonella tester strains and Chinese hamster V_{79} cells (Shirname et al., 1983; 1984). The mutagenic properties of catechu is an open question. Nagabhushan and Bhide (1988) have reported the antimutagenic property of catechin. Giri et al. (1987) have found catechu extract to be mutagenic. We have observed the chromosome damaging ability of areca nut extract. This has also been reported by Stich et al. (1983a). Under alkaline conditions, in absence of metabolic activation the aqueous extract of areca nut can induce mitotic gene conversion in Saccaromyces cerevesie (Rosin, 1984). The presence of lime in the mixture of pan masala might help in keeping the pH alkaline. Stich and Stich (1982), in an attempt to trace the source of clastogenic agents in the saliva of betel quid users, tested the effects of saliva of five such chewers on CHO cells. Each constituent of the quid was chewed separately. Pan Bahar was one such constituent (Pan Bahar is a pan masala containing areca nuts, catechu, cardamom, lime, copra, menthol and perfumes). Despite individual variations, the highest number

of average chromatid breaks were observed by treating the cells with saliva, after chewing Pan Bahar. The capacity of the clastogenic substances, present in pan masala to leach out in saliva, indicates the chromosome damaging capacity of the mixture and its ability to damage the cells of the oral mucosa. In our experiments the pan masala ext. was found to induce a dose dependent increase in CAs in CHO cells, either treated continuously or after recovering the cells following three hours' treatment. A statistically significant elevation in the number of CA per cell was observed with the final concentrations, i.e. 10 µl and 20 µl in 48 hours' treatment and 50 µl in three hours' treatment. Since the number and types of aberrations were quite comparable to those produced by AN ext., it seems reasonable to attribute the chromosome damaging capacity of the pan masala to areca nut present in the mixture.

SCE assay proved to be the most sensitive marker in the in vitro assessment. A statistically significant induction of SCEs was brought about by all the concentrations employed for brief as well as extended treatments ($p < 0.001$). It was noteworthy that the mean SCE frequency, observed with 20 µl of AN ext. treatment for three hours, was witnessed with 10 µl of PM ext.(Table-5 and Fig. 20), suggesting that PM ext. possesses a higher potency of inducing SCEs than the AN ext. This implies that pan masala contain substance(s) extractable in water, which add to DNA damage, as analysed by enumeration of SCEs. The clastogenic effects of areca nut, together with its alkaloid have been discussed in detail in the earlier chapters. Catechu was found to induce SCEs and dominant lethal mutations in mice and elicits strong clastogenic and mutagenic effects, as observed by Giri et al. (1987). The enhancement of the genotoxicity of the compounds in betel quid, when applied concurrently was evidenced in the experiments on CHO cells (Stich et al., 1981). The presence of lime and flavouring agents (unspeci-

fied), might also play a role in increasing the SCE frequencies rendering the PM ext. more genotoxic. Compounds that are non-genotoxic by themselves might potentiate the activity of each other or may add to the potentials of a genotoxic component. However, the ambiguity regarding the concentration of the constituents in this mixture constrains us in holding any one of them responsible for potentiating the genotoxic efficiency of areca nut.

A treatment with AN ext. caused the inhibition of DNA synthesis in CHO cells. This ultimately might result in the impaired cellular turn-over and hence, the decrease in cell proliferation. A similar picture emerged when the proliferation index and AGT were calculated after analysing the cells in different cycles, following the exposure of PM ext. Even a short treatment of just three hours could result in a dose related increase in the AGT. The preliminary data on the in vitro effects of PM ext. has been published (Adhvaryu et al., 1989. Reprint annexed).

Autoradiographic studies revealed that PM ext. affected the synthesis of DNA. This was observed as the decrease in the number of DNA synthesizing interphase nuclei. A 50% inhibition in the synthesis was found on treating the cells with 10 µl of the extract for 24 hours. Once again areca nut present in the extract appeared to be the major factor affecting the cells in S phase inhibiting the synthesis of DNA. Our observations, as well as those of other investigators (Yang et al., 1979; Shivapurkar and Bhide, 1979), have been discussed in detail in Part I.

The saliva of betel quid chewers without tobacco contains nitrite, thiocynate and arecoline (Shivapurkar et al., 1980a; Nair et al., 1985). Some areca nut specific nitrosamines as well as areca nut tannins have also been detected from the

saliva of individuals chewing betel quid without tobacco (Prokopczyk et al., 1987b; Wenke et al., 1984b; Nair et al., 1985; 1987; Stich and Anders, 1989). Saliva of pan bahar chewers was more clastogenic to CHO cells than other constituents of betel quid (Stich and Stich, 1982). A very high frequency of micronucleated cells were observed among Indians chewing areca nut, betel leaf and lime (Stich et al., 1986). A similar information could be gathered from our data, detailed in previous chapter. Given these, little remains to be explained about the increased micronucleus frequency in the buccal mucosa cells of pan masala consumers. It implies that even in this complex mixture, areca nut continues to act in a similar manner, as it does otherwise.

A significant increase in the SCE and CA frequencies in the PBLs of individuals consuming pan masala without tobacco compared to the controls, corroborate the findings of the in vitro genotoxic potentials of pan masala. The SCE and CA frequencies in the PBLs of pan masala chewers were marginally higher than that in the PBLs of areca nut chewers and it would not sound untimely to deduce that pan masala consumption can hardly be considered safe. Further studies, covering individuals consuming pan masalas with a concurrent tobacco/ areca nut chewing habit and also with those consuming pan masala with zarda, would prove to be more informative. Nevertheless, the results confirm that pan masala, per se, can cause DNA damage in the target as well as non-target tissues.

SUMMARY

It can be inferred from our results that even in the absence of metabolic activation, pan masala without tobacco

* *Is cytotoxic to CHO cells.*
* *Induces CAs and SCEs in CHO cells.*
* *Possesses the property to reduce the proliferation rate of CHO cells.*
* *Inhibits DNA synthesis in vitro.*
* *The human studies reveal that its consumption increases the frequency of MNC in exfoliated buccal mucosa cells and elevates the frequency of CAs and SCEs in PBLs of individuals consuming pan masalas. Hence, while scrutinizing the individuals during cytogenetic monitoring, pan masala consumption should also be considered.*

In case of oral diseases, it is of paramount importance to monitor habits of etiologic significance. The harmful effect of a habit, if not controlled, will be observed only after a decade, since a long latent period may exist between the damage and manifestation of the disease. The absence of warning labels and the active advertisement campaign, promotes the misconception that consuming pan masala is 'safe'.

Our data on in vitro system and in the individuals consuming pan masala, preliminary as they are, may help in making more reasoned decisions about the safety of this product. They may serve as indicators justifying further in depth studies and in restricting the indiscriminate use and sale of this widely consumed product, erroneously considered safe.