INTRODUCTION

Cancer, one of the major causes of morbidity and mortality, continues to be an unresolved enigma and the quest to conquer the disease keeps eluding. This is partly due to the fact that there are a number of genetic and environmental factors affecting the causation of this disease. Among the environmental factors, there are physical, chemical and biological agents that significantly increase the frequency of malignant neoplasms in a population. These are termed as carcinogens. Human beings are exposed to various carcinogenic agents accidentally, occupationally or by lifestyle. When contact with such agents is habit associated, it is much easier to curtail the exposure by creating an awareness and changing the human perspective towards the habit. Such habit associated cancers fall in the category of 'self induced, preventable disease'.

Today, cancers of oral cavity present a major health problem in South-East Asia, with more than a hundred thousand new cases occurring every year, representing approximately thirty percent of all oral cancers worldwide (Stanley and Stjernsward, 1986). There is a striking variation in the patterns and incidence of these cancers in different countries. Oral cancer, in India, encompasses nearly one third of the total cancer cases (WHO, 1984) and is the commonest cancer among males and third commonest among females (Sankaranarayanan et al. 1989). In the United States, oral and pharyngeal cancers account for three percent of all cancers (Silverberg and Lubera, 1989). This reflects the differences in exposure to environmental risk factors and brings into sharp focus the alarming situation existing in our country. Epidemiologic studies have documented that the habit of chewing betel quid is a major risk factor in causation of oral cancers (IARC, 1985). A direct and dose-related effect of the habit has also been proposed (Hirayama, 1966; Wahi, 1968).

Betel quid is composed of betel leaf, areca nut, catechu and lime to which tobacco may or may not be added (IARC, 1985). Although the causative agents in betel quid have not been unequivocally identified (Hirono, 1985), tobacco and areca nut are the main suspected sources of carcinogens and/or their precursors. Tobacco is consumed in a variety of ways, and has been accounted to exhibit a close correlation between the manner of its consumption and the development of site specific cancers. Owing to this fact, the possible harmful effects of areca nut have been overshadowed and have not received due consideration. It is noteworthy here, that literary references to the habit of chewing betel quid (which always consists of areca nut) in India are atleast 2000 years old (Gode, 1961), and tobacco was introduced much later, around the sixteenth century (IARC, 1985). It also should be noted that especially elaborate descriptions of oral cancers have been given in 'Sushruta samhita', a treatise on Indian surgery, written in 600 B.C., indicating that oral cancers were common even in those days (Suraiya, 1973).

Areca nut, popularly known as 'betel nut', is almost symbolic to the culture of some of the oriental nations. To the Indians, Malayans or the Indonesians, areca nut chewing is as familiar as chewing gum to the Americans. There are about 250 million betel quid chewers worldwide (Dunn and Stich, 1986). Areca nut accounts for the major amount by weight, in betel quid, 'mava' (tobacco, areca nut and lime) or in 'pan masalas'. There has been some evidence that the risk of oral cancer is higher in people who chew betel quids even without tobacco, compared to those who do not chew betel quids at all, however, the relative risk associated with each ingredient used in the quids have not been clearly established (WHO, 1984; Gupta et al., 1982). A few short term assays and animal experiments have given indications about the possible carcinogenic potentials of areca nut (IARC, 1985). Recently, areca nut specific nitrosamines have been detected in the saliva of those chewing betel quid with or without tobacco (Wenke

et al., 1984b; Nair et al., 1985; 1987; Prokopczyk et al., 1987b). The micronucleus frequency has also been found to increase in the exfoliated cells of buccal mucosa of the betel quid chewers (Stich and co-workers, 1982-1986).

*Considering the magnitude of oral cancers in India and the increasing number of people practising areca nut chewing in a variety of combinations, the monitoring of individuals exercising this habit needs to be attended more seriously. However, faced with a limited data on areca nut, the International Agency for Research on Cancer has inferred that there is '**inadequate**' evidence that the habit of chewing betel quid without tobacco is carcinogenic to humans, and there is '**limited**' evidence that areca nut with and without tobacco is carcinogenic to experimental animals (IARC, 1985).*

Thus, the lack of hard evidence has allowed areca nut to be considered safe for human consumption. This paucity of comprehensive and conclusive data on areca nut, strengthens the need of estimate the carcinogenic potentials of this widely consumed and one of the oldest known masticatories.

ORIGIN AND OCCURRENCE:

Areca nut, (Areca catechu.Linn.), is a fruit of Areca catechu, a species of genus Areca Linn which belongs to the family 'Palmaceae'. The derivation of the name areca nut is not definitely known, however, it can be traced to the Kanarese 'adeke' or the Malayalam 'adakka' (Raghavan and Baruah, 1958). In vernacular (Hindi), it is called 'supari'. Although the actual source of origin of areca palms is still a matter of speculation, according to most of the reports, it seems to have originated from Malaya. It is cultivated in most tropical countries such as India, Bangladesh, Sri Lanka, Burma, Malaysia, Phillipines, Japan, Madagascar and East African countries. In India it is grown mainly in Maharashtra, Tamil Nadu, Karnataka, Assam and West Bengal.

PHYSICAL AND CHEMICAL PROPERTIES:

Areca catechu is a handsome palm with tall, slender, graceful stem, crowned by a tuft of large, elegant leaves. The fruit grows in large bunches at the base of the leaves. The areca palm requires 10 to 12 years before fruiting and each tree yields two-three bunches of 200-250 nuts per year.

A considerable variation has been found in the size, shape, hardness and astringency of the nuts. The fruit is orange yellow in colour when ripe and is of the shape and size of a small egg. The areca nut essentially consists of a hard fibrous outer covering commonly called the husk, enclosing within it the endosperm, which is the edible nut. The husk is separated from the endosperm, and it is then used fresh or after sundrying or curing.

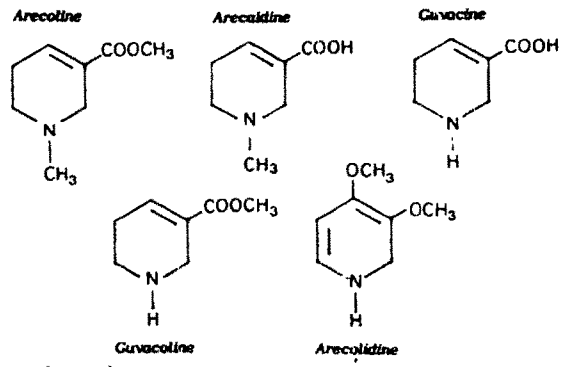
The 'refining' or 'curing' of the nuts is mainly carried out in south western provinces of India. The process involves shelling of the fruits, slicing the kernal and then boiling it in water together with the remnants of previous year's curing (choguru). The curing is practised to improve the colour and quality, reduce the tannin content and make the nuts palatable (CSIR, 1948). In India, varieties of areca nuts are available in the market under different trade names like Batlu, Churu, Lavanga chooru, Deshavar, Ramsupari, Ramchandrapur, Gotu, Shrivardhani, etc. The major chemical ingredients in areca nut, before curing are listed as follows: (Raghavan and Baruah, 1958).

CONSTITUENT	*QUANTITY*
Tannins	*11.4 - 26.0%*
Gallotannic acid	*18.03%*
Gallic acid	-
D-Catechol	*3 gm/800 gm (0.4%)*
Phlobatannin	-
Alkaloids	*0.15 - 0.67%*
Arecoline	*0.07 - 0.50%*
Arecaidine	*small quantity*
Guvacine	*small quantity*
Isoguvacine	*trace quantity*
Arecolidine	*minute quantity*

Fats	*1.3 - 17.0%*
Sitosterol	*trace quantity*
Carbohydrates (saccharose, reducing sugars, galactan, mannan)	*47.2 - 84.5%*
Protein	*4.9- 9.3%*
Non-protein nitrogen	*0.22 - 1.6%*
Saponins	-
Gums	-
Carotene	*5 International Vit. A units/100 g.*
Mineral matter	
Calcium	*0.018 - 0.05%*
Phosphorus	*0.13 - 2.35%*
Iron	*1.5 - 11.6 mg/100 g (0.002 - 0.01%)*

It should be noted that wide variations can occur in the composition of 'cured' areca nut and the content of polyphenols, alkaloids, fat, crude fiber and total polysaccharides may vary in different varieties (Shivshankar et al., 1969). Among the polyphenols identified in areca nut are leucocyanidins, catechin, 3-4 flavandiols and hexahydroxyflavan (Arjungi, 1976). The chemical structures of the major areca nut alkaloids are as follows: (Source: Majumdar et al., 1982).

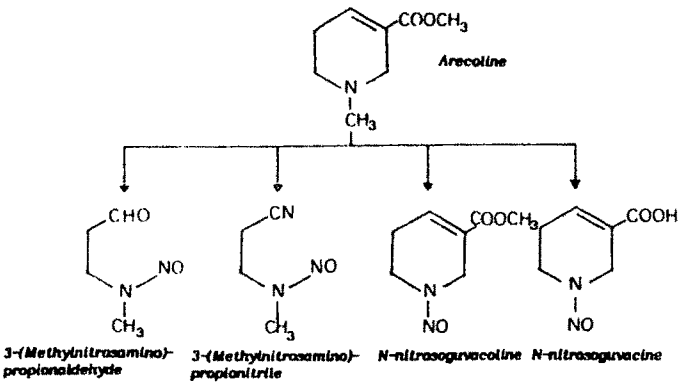
Fig: 1



The reported content of arecoline is 0.07-0.50% of dry weight, thus contributing as the major portion of the alkaloid content of areca nut (Arjungi, 1976). Arecoline is a colourless oily liquid (B. P. 230°C) and forms crystalline salts with acids

(Raghavan and Baruah, 1958). The in vitro experiments have revealed that the nitrosation of arecoline can give rise to atleast four N-nitrosamines namely, N-nitrosoguvacoline (NGCO), 3(methylnitrosamino) propionitrile (MNPN), 3-(methyl-nitrosamino)propionaldehyde (MNPA), (Wenke and Hoffmann, 1983) and N-nitroso-guvacine (NGCI) (Nair et al., 1985). They are reasonably stable in aqueous media (Wenke and Hoffmann, 1983).

Fig: 2 *(Source: IARC, 1985) Nitrosation of Arecoline.*



GEOGRAPHIC VARIATIONS AND DIFFERENT MODES OF CONSUMPTION:

Areca nut is chewed in a variety of forms, ripe or unripe, dried or wet, and processed or unprocessed. Different populations or even different individuals in a given population may be in a habit of chewing different preparations of areca nut. It is chewed either alone or as a component of betel quid. Pindborg et al. (1967), in a survey found no less than twenty two different ways of chewing 'betel' and/or tobacco, with or without other types of tobacco usage. Although the chewing of betel quid is practised in several different ways, the major compo-nents of the quid essentially remain constant. A brief description of the major components, other than areca nut, seems essential here (from IARC, 1985):

Betel Leaf: *Mature green leaves of Piper betle Linn (Piperaceae) mainly contain volatile oils, nitrate and small quantities of sugar, starch and tannin.*

Lime: *known colloquially in India as 'chuna', is prepared either from the calcareous or silicious covering of marine vertebrates (sea shells) or from quarried stone.*

Catechu: *is a resinous extract from the matrix of Acacia tree, usually Acacia catechu or Acacia suma. It mainly consists of tannins and polyphenols. It is known in India as 'kattha'.*

Tobacco: *It is often added to a betel quid. Chewing tobacco in India is made from Nicotiana rustica and Nicotiana tabacum and is usually consumed raw. Depending on the socio-economic level and individual preference, other spicy ingredients are also frequently added in betel quid (Hirono, 1981). In India, betel quid consists of sliced dry areca nut with or without flakes of sundried tobacco wrapped in a betel leaf, smeared with catechu and slaked lime. Spices such as cardamom, cloves or aniseed (WHO, 1984), grated coconut (Schonland and Bradshaw, 1969), ginger (Arjungi, 1976) and sugar (Millot, 1965) may be added as additional flavours. This may vary from region to region in India itself:*

Calcutta:	*Dried areca nut pieces, tobacco and spices wrapped in betel leaf smeared with catechu and lime (usually stonelime).*
Delhi:	*Basically the same quids as in Calcutta, with more spices.*
Agra:	*Betel leaf, catechu, lime and areca nut pieces (dried), usually tobacco is not added.*
Mainpuri: (Uttar Pradesh)	*A mixture of tobacco, areca nut, lime, camphor, cloves and other spices. Betel leaf is not used.*
Bombay:	*Betel leaf, dried areca nut pieces, catechu and lime. Tobacco may or may not be added. In absence of tobacco, various spices are added in greater amount.*
Gujarat:	*Besides the regular betel quid chewing, with or without tobacco, a relatively new (about 20 years) combination of tobacco, areca nut and lime is being chewed. This combination is called 'masala' in Ahmedabad city and the surrounding areas, 'mava' in Bhavnagar and 'faqui' in Porbandar district.*
Shillong:	*Raw and unprocessed areca nut (locally known as 'Tamol' or 'kwai'), is chewed with betel leaf and a little slaked lime.*
Sourthern Provinces:	*Areca nut (processed or 'cured') pieces with vaddakkan tobacco wrapped in betel leaf smeared with shell lime.*

Some variations observed in other Asian countries are as follows:

Sri Lanka:	*Similar to the southern provinces in India.*
Malaysia: Singapore	*Catechu is in a different form as 'gambier'.*

Phillipines:	*The quid is called 'buyo', tobacco is half dry and seasoned.*
Guam:	*Areca nut is chewed without slaked lime, betel leaf or tobacco.*
Taiwan:	*Fresh unripe fruit of areca nut in toto with lime (stone lime) paste, a slice of long pepper and glycyrrhiza glabra.*
Thailand:	*Fresh unripe areca nut in betel leaf with lime. Tobacco is used to wipe teeth.*
Indonesia:	*Tobacco is not added to the betel quid but used as a dentrifice after the quid chewing.*
Papua New Guinea:	*Areca nut is chewed when ripe but not cured, it is chewed first and slaked lime is applied repeatedly to the buccal mucosa, followed by chewing of the stem of betel bush. Tobacco is never included.*
Hainan: (China)	*Peeled and sliced areca nut in betel leaf smeared with lime. Tobacco is never added in the quid.*

(The data has been collected from IARC, 1985; WHO, 1984; Mori, 1988; Sinor, 1989; Wary and Sharan, 1988; Stich and Tsang, 1989).

HISTORICAL BACKGROUND AND EPIDEMIOLOGY (IN RELATION TO ORAL CANCER):

The 'Mahawamsa', a register of events in Sri Lanka, written in Pali, is said to have made the first mention of 'Betel' in 504 B.C. (Krenger, 1942). The chewing of areca nut is mentioned in Sushruta samhita written around 600 B.C. near Benaras (Varanasi), India (IARC, 1985). 'Tambula', the Sanskrit name for betel leaf, is also mentioned in Sanskrit literature in Lalitasahasranama, an ancient eulogy of goddess in Sanskrit (Jayant and Deo, 1986). The name 'tambula' persists in Persian and Arabic (Muir and Kirk, 1960) while in modern Hindi it has been known as 'tambuli' (Gode, 1961). The chewing of 'betel' prevailed in southern coast of Arabia, Yemen and Mecca. This was accounted in 916 A.D. by Masudi, a traveller from Baghdad (Krenger, 1942). The earliest historic reference, by a European, to the habit of betel chewing among the orientals occur in the travelogues of Marco Polo in 1298, followed by Vasco de Gama in 1498 (Raghavan and

Baruah, 1958). The import of betel leaves from India and Malacca (West Malaysia) was forbidden in 1703 (Muir and Kirk, 1960).

Regardless of the ingredients and their quantity in betel quid, the term 'Betel' continued to be used as a synonym for the quid. Although areca nut had always been a component of the quid, the introduction of tobacco as a component was done around the sixteenth century (IARC, 1985). The inclusion of tobacco in the quid was a matter of choice. More than a hundred years ago, in the year 1860, Tennet made the first reference of 'Betel chewer's cancer', designated so due to their peculiar characteristics. Since the beginning of the century, it appeared that the prevalence of the cultural practice of chewing 'Betel' in the Asian continent had a correlation with the excess of oral cancer in this part of the world. Some of the early observations were those of Niblock (1902), Balaram (1902), Boak (1906), Bentell (1908). However, Fells (1908), was the first to observe that cancer developed at the site where the quid was placed. He considered lime to be the causative factor. It was observed in Manila (Phillipines), that the patients with cancer of cheek, chewed 'buyo', which is a 'betel chew' (Davis, 1915). Mendelson and Ellis (1924) in Thailand observed that all oral cancer patients were chewing betel quid with tobacco. A systematic case control study was conducted in 1933 (Orr, 1933) where the harmful effect of lime and the relative role of the diet, specially vitamin A, were questioned. These, of course, were some early observations of the association of betel quid chewing and oral cancer, even before epidemiology was established as a separate scientific discipline. Statistical methods have now been adopted to estimate the risk of the disease in those exposed to specific risk factors.

Oral cancers include cancer of lip, tongue, salivary gland, gum, floor of mouth and buccal mucosa (ICD 140-145) (WHO, 1977). The habit of chewing betel quid

(with or without tobacco) contributes as the prime etiological factor for oral ill health, however, compared to extensive studies on smoking in the etiology of lung cancer, the studies on betel quid chewing are fragmentary. The present study deals mainly with the effects of areca nut, but there are very few reports limited solely to areca nut chewers (Jayant et al., 1977; Jayant and Deo, 1986). In India, the limitation was observed due to the fact that wherever detailed studies were conducted, there were very few people among those studied, who were exclusively areca nut chewers, these include those chewing areca nut alone or with ingredients other than tobacco (Khanolkar et al., 1959). The number in Uttar Pradesh and Andhra Pradesh was even smaller, while in Gujarat, among those studied, only 1.5% were only areca nut chewers (Mehta et al., 1971; 1972; Dayal et al., 1978).

Regardless of the ingredients that are blamed by the epidemiologist, we have tried to sort out the studies that have been dealt exclusively with areca nut chewing (these include those of betel quid chewing without tobacco). However, the studies from Papua New Guinea have been included, despite the concurrent smoking habit since tobacco is never added in the chew. The earliest report from the country was that of Eisen (1946) who concluded that betel chewing does not appear to cause oral cancers. Atkinson et al. (1964) found that 17.8% of all cancers studied in the span of five years were oral, and that the prevalence of leukoplakia ranged from 1 to 13.8%, the highest being in the areas where areca nut was chewed and the lowest among non-chewers. They repeated the study after 21 years (Atkinson et al., 1982) and found the results to be consistent. According to IARC (1985), the authors have missed to consider smoking, which is a common practice in the region.

Farago (1963a; 1963b) observed that the rate of oral cancer was 18.1% and of these 98-99% were areca nut chewers. Smoking was also reported to be common.

Among other reports from Papua New Guinea, are those of Cooke (1969) and Henderson and Aiken (1979) who found 2.1% and 14.4% prevalence of oral cancer respectively. The occurrence of leukoplakia was also found to be more frequent in areca nut chewers and still more among areca nut chewers with smoking habit (Forlen et al., 1965; Pindborg et al., 1968).

Among the studies carried out in Malaysia, the report of Ahluwalia and Duguid (1966) was specific. Of the total cancer cases examined in two years, it was found that among the chewers of betel quid without tobacco 9.5% cases were of oral cancer, while among those chewing betel quid with tobacco, 33.6% cases were oral cancers. This implied that oral cancers were more frequent among those chewing betel quid with tobacco. In a clinical study, 26% of the Indian areca nut chewers without tobacco, exhibited discernible changes in the oral mucosa (Ahluwalia and Ponnampalam, 1968). The incidence was greater when tobacco was added. The observations made by Lee and Chin (1970), and Chin and Lee (1970) were also comparable to this report.

The study of Jussawalla and Deshpande (1971) from India, was detailed with the relative risks of developing cancers at various sites among the individuals with the chewing and smoking habits. It reported that among the betel quid chewers (with or without tobacco), the relative risk of developing cancer of buccal cavity was observed to be the highest of all cancers, i.e. 7.7 times higher than the controls. Among the betel quid chewers without tobacco, the risk was found to be 3.0 times greater and among those chewing the quid with tobacco, it was 4.8 times more.

Gupta et al. (1982), reassessing the available data, found that the relative risk of developing oral cancer among the chewers of betel quid without tobacco is higher than the non-chewers, however, the risk was significantly greater among the chewers of betel quid with tobacco.

The IARC Working Group (IARC, 1985) could calculate the relative risks from the case control studies on oral cancers by Jafarey et al. (1977) in Pakistan, Hirayama (1966) in India and Sri Lanka, and Chandra (1962) in India who precisely described the chewing and smoking habits. It was found that the risk was lowest among those chewing betel quid without tobacco, however, it increased 3 to 6 times with concurrent tobacco chewing or smoking habits. Such increase due to tobacco habits, was also evidenced in the reports of Shanta and Krishnamurthy (1959; 1963).

A positive dose response relationship between oral cancer and betel quid chewing could be inferred from the reports of Orr (1933) and Hirayama (1966). Wahi (1968) examined the strength of such dose response association in many ways such as frequency of chewing, age at starting the practice, retention time per quid, period of total exposure per day, etc. and reported the association to be positive by every criterion. The prevalence was greatest among those retaining the quid in the mouth even during sleep.

On reviewing the existing epidemiological data pertaining to the effects of betel quid containing tobacco, a more serious picture emerged with regard to the causation of oral cancer. This was evidenced from the reports which clearly specified the chewing of the quid with tobacco, without intermingling the smoking habit (Khanolkar, 1944; Marsden, 1960; Muir, 1962; Ahluwalia and Duguid, 1966; Paymaster, 1956; Sidiq et al., 1964; Jayant et al., 1977; Gupta et al., 1982). There have been a number of reports which have not specified the presence of tobacco in the betel quid, in their studies, however, they indicated that the prevalence of oral cancer was higher among the chewers (Sanghvi et al., 1955; Sarma, 1958; Khanolkar, 1959; Balendra, 1949; 1965; Piyaratna, 1959; Huq, 1965; Kwan, 1976; Pindborg et al., 1984a).

After reviewing the epidemiological data, it seems reasonable to note that a

more systematic approach, precisely defining the mode of tobacco and areca nut consumption separately would be of much help and of a real value. A large scale study on only areca nut consumers is very much due.

Oral cancers are almost always preceded by a precancerous lesion/condition (WHO, 1984), one of which is oral submucous fibrosis (OSMF). It has been hypothesized that areca nut chewing plays an etiologic role in the development of OSMF (Sirsat and Khanolkar, 1962; Mehta et al., 1972; Shiau and Kwan, 1979; Gupta et al., 1980; Seedat and Wyk, 1988). It was first described as a precancerous condition by Paymaster (1956), who observed the development of carcinoma in one third of the OSMF cases. It has also been suggested by some that it mainly occurs among Indians (Evenson, 1983; Varghese et al., 1986). Pindborg (1980) estimated that no less than 2,50,000 cases of OSMF were present in India. The prevalence of OSMF has been studied in detail by Mehta et al. (1971) and Bhargava et al. (1975) and the malignant transformation has also been observed by many investigators (Pindborg and Zachariah, 1965; Gupta et al., 1980; McGurk and Craig, 1984; Pindborg et al., 1984b).

EFFECTS OF ARECA NUT

PHARMACOLOGICAL EFFECTS:

The pharmacological effects of areca nut have been extensively reviewed by Arjungi (1976), Mathew and Govindarajan (1964), Mathew et al. (1969) and Majumdar et al. (1979; 1982). It causes addiction, euphoria, excessive salivation and tremor. The alkaloids have been considered to be the active principles of areca nut.

In experimental system:

The aqueous extract of areca nut increased the glutathione content and decreased protein SH groups in liver, kidney and muscle in swiss mice (Shivapurkar and

Bhide, 1978). The aqueous extract as well as arecoline increased hepatic DNA and RNA content and stimulated DNA and RNA synthesis in liver (Shivapurkar et al., 1978; Shivapurkar and Bhide, 1979). The aqueous and ethanolic extracts of areca nut were found to be bactericidal (Lalithakumari et al., 1965; Majumdar et al., 1982). The ethanolic extracts of areca nut stimulated collagen synthesis in human fibroblasts in vitro (Caniff and Harvey, 1981). Tannins from chewed areca nuts reduced the susceptibility of collagen to degradation by collagenase in vitro (Meghji et al., 1982).

The ip LD_{50} of arecoline in rats was approximately 40 mg/kg body weight (Boyland and Nery, 1969). Arecoline was found to be cholinomimetic and to possess a pharmacological action similar to muscarine and pilocarpine (IARC, 1985). Arecoline selectively stimulated parasympathetic nervous system by binding to muscarine and nicotinic receptors (Euler and Domeji, 1945; Gilman et al., 1980). It produced tremors (Holmstedt and Ludgren, 1967) and showed effects on central nervous system (Leslie, 1965; Domino, 1967; Meltzar and Rosecrans, 1982). These effects on central nervous system could be prevented by atropine (McKinney and Richelson, 1984).

Human studies:

Only a few studies have been carried out on the effects of arecoline on human beings. It was found that very small doses, about 2-4 mgs., affect the human central nervous system in a way similar to that in animals (Sitaram and Weingartner, 1971; Christie et al., 1981). The salivary nitrite levels were found to increase without any change in the thiocynate levels in betel quid (without tobacco) chewers and the reverse, was observed among those chewing betel quid with tobacco (Shivapurkar et al., 1980a). Kutzung (1982) observed that the immediate effects of betel quid chewing appeared to be due to the stimulation of parasympathetic nervous system by arecoline. The secretion of gastric juices, sweat and tears were found to increase.

PHARMACOKINETICS (Absorption, distribution, metabolism and excretion)

Experimental systems:

The reaction of arecoline and arecaidine with thiol groups showed that they were alkylating agents (monofunctional), as arecoline was found to lose only one of its methyl groups during metabolism (Boyland and Nery, 1969). In rats, arecoline was de-esterified in liver and both arecoline and arecaidine were excreted as mercapturic acid. Arecoline hydrochloride, in rats, was metabolized by 1-oxide formation (Nery, 1971). Dosed with nitrite, when the total aqueous extracts and extracted tannins from areca nut were administered in rats, they either catalysed or inhibited endogenous nitrosation. This was measured by the amount of N-nitrosoproline excreted in urine (Stich et al., 1984a). Ernst et al. (1987) observed that N-nitrosonipecotic acid, a major urinary metabolite of areca nut derived nitrosamines, was detected in the urine of hamsters fed with nitrite and areca nut, indicating that areca nut alkaloids underwent in vivo nitrosation to form areca nut specific nitrosamines. Recently, this metabolite has been identified in the urine of rats who were given N-nitrosoguvacoline (NGCO) and N-nitrosoguvacine (NGCI) (Ohshima et al., 1989). The formation of areca nut specific N-nitrosamines has been observed in vitro (Wenke and Hoffmann, 1983; Nair et al., 1985, see Fig. 2).

Human system:

Since Wenke et al. (1984b) detected the presence of areca nut specific nitrosamine NGCO in the saliva of betel quid chewers, the search for other areca nut specific nitrosamines has been in progress. Nair et al. (1985) reported the presence of NGCI and Prokopczyk et al. (1987b) observed the occurrence of MNPN in the saliva of betel quid chewers without tobacco. Arecoline has also been found in the saliva of these chewers. In the saliva of chewers of betel quid with tobacco, besides these areca nut specific compounds, nicotine, cotinine and three of the

tobacco specific nitrosamines have also been detected (Wenke et al., 1984b; Nair et al., 1985; 1987; Sipahimalani et al., 1984). The studies of Nair et al. (1985; 1987) and Stich et al. (1983b), who carried out urine analysis, suggest that the endogenous nitrosation increases in individuals consuming areca nut with or without tobacco.

EXPERIMENTAL STUDIES: (CARCINOGENESIS):

SHORT TERM TESTS:

The various short term experiments performed to search the carcinogenic effects of areca nut have been detailed in Table-1. The source of the substance, methods of storage, preparation of samples and extraction procedures varied greatly, hence, the results may not necessarily be comparable. The results given here are as interpreted by the investigators under reference.

CARCINOGENICITY STUDIES IN ANIMALS:

There have been diverse approaches to study the carcinogenicity of areca nut, utilizing a variety of animal models, exposures and routes of administrations. The details of the studies have been summarized in Table-2.

HUMAN STUDIES:

The most extensive studies on humans are those of the exfoliated cells from buccal mucosa of areca nut/tobacco chewers and also of chewers of betel quid with or without tobacco. It was observed that the frequency of micronucleated cells increased among the chewers and it augmented with the addition of tobacco (Stich et al., 1982; Stich and Rosin, 1984; Stich et al., 1984c). The site specificity and dose response relationship with regard to micronucleus frequency was also observed (Stich et al.,

Table-1 **DETAILS OF SHORT TERM STUDIES**

Substance	Cells used	Assay	Results	Reference
ARECA NUT				
Aqueous ext.	Human lymphocytes Rat Mammary tumour cells	Inhibition of DNA synthesis	positive	Yang et al. (1979)
Areca nut powder	Drosophila Larvae	Autosomal translocation	negative	Abraham et al. (1979)
Areca nut powder	Drosophila Larvae	Sex cromo.lethal mut.	negative	Abraham et al. (1979)
Ethyl acetate ext.	Chinese hamster V79	Mutation	negative	Umezawa et al. (1981)
Ethyl acetate ext.	Human lymphoblastoid	SCE	negative	Umezawa et al. (1981)
Ethyl acetate ext.	Syrian hamster embryo	Cell transformation	negative	Umezawa et al. (1981)
Aqueous ext.	Chinese hamster ovary (CHO)	Chromosome Aberrations (CA)	positive	Stich et al (1983a)
Ethyl acetate ext.	CHO	CA	positive	Stich et al. (1983a)
n-butanol ext.	CHO	CA	positive	Stich et al. (1983a)
Aqueous ext.	S. typhimurium (TA1535, TA100)	Mutation (with and without S9)	positive	Shirname et al. (1983)
Aqueous ext.	Chinese hamster V79	Mutation (with and without S9)	positive	Shirname et al. (1984)
Aqueous ext.	Mouse bone marrow	Micronucleus (MN)	positive	Shirname et al. (1984)
Aqueous ext.	Saccharomyces cerevisie	Gene conversion (alkaline pH) (acidic pH)	positive negative	Rosin (1984)
Aqueous ext.	Mouse bone marrow	Sister Chromatid Exchange (SCE)	positive	Panigrahi and Rao (1986)
Aqueous ext.	Human epithelial cells	Colony forming	positive	Sundquist et al. (1987)
Aqueous ext.	Mouse kidney cells	DNA strand breaks	positive	Wary and Sharan (1988)
Aqueous ext.	CHO	CA	positive	Stich and Tsang (1989)
Aqueous ext.	C3H/10T 1/2	Transformation assay	positive	Stich and Tsang (1989)

Substance/Extraction	Cells used	Assay	Result	Reference
ARECOLINE				
Arecoline	BHK-21/C1 13	Cell transformation	positive	Ashby et al. (1979)
Arecoline	CHO	CA	positive	Stich et al. (1981)
Arecoline	Mouse bone marrow	CA	positive	Panigrahi and Rao (1982)
Arecoline	Mouse bone marrow	SCE	positive	Panigrahi and Rao (1983)
Arecoline	S. typhimurium TA100, TA98, TA1535, TA1538.	Mutation (with and without S9)	positive	Shirname et al. (1983)
Arecoline	Chinese hamster V79	Mutation (with and without S9)	positive	Shirname et al. (1984)
Arecoline	Mouse bone marrow	MN	positive	Shirname et al. (1984)
Arecoline	Mouse sperm cells	Morphological changes	positive	Sinha and Rao (1985a)
Arecoline	Mouse germ cells	Unscheduled DNA synthesis	positive	Sinha and Rao (1985b)
Arecoline	Mouse germ cells	Transplacental MN	positive	Sinha and Rao (1985b)
Arecoline	Mouse kidney cells	DNA strand breaks	positive	Wary and Sharan (1988)
BETEL QUID WITHOUT TOBACCO				
Aqueous	S. typhimurium (TA1535, TA100)	Mutation (with and without S9)	positive	Shirname et al. (1983)
Aqueous	Chinese hamster V79	Mutation (with and without S9)	negative	Shirname et al. (1984)
Aqueous	Mouse bone marrow	MN	negative	Shirname et al. (1984)

Table-2 *SUMMARY OF CARCINOGENICITY STUDIES IN ANIMALS*

Species	*Route*	*Extraction*	*Effect*	*Reference*
ARECA NUT				
Hamster	*Cheek pouch, beeswax pellet*	*None*	*No local malignant tumour*	*Dunham and Herrold (1962)*
Hamster	*Cheek pouch application*	*DMSO*	*Local squamous-cell carcinomas, 8/21*	*Suri et al. (1971)*
Hamster	*Cheek pouch application*	*DMSO*	*No malignant tumour*	*Ranadive et al. (1976)*
Mouse	*Subcutaneous*	*Aqueous*	*Local tumours, 24/40*	*Ranadive et al. (1976)*
Mouse	*Skin application*	*DMSO*	*No skin tumour*	*Ranadive et al. (1976)*
Rat	*Subcutaneous*	*Aqueous*	*Local tumours, 30/30*	*Kapadia et al. (1978)*
Mouse	*Oral intubation*	*Aqueous*	*Liver, lung and stomach tumours (polyphenolic fraction not active)*	*Bhide et al. (1979)*
Rat	*Mixed with diet (with & without lime)*	*None*	*No increased tumour incidence*	*Mori et al. (1979)*
Hamster	*Cheek pouch application*	*Aqueous*	*Cheek pouch carcinoma 1/15 Forestomach carcinoma 4/21*	*Ranadive et al. (1979)*
Hamster	*Cheek pouch, gelatin capsules*	*None*	*Cheek pouch carcinoma 4/19 Forestomach carcinoma 6/19*	*Ranadive et al. (1979)*
Mouse	*Subcutaneous*	*Aqueous*	*Local sarcomas, 2/12*	*Shivapurkar et al. (1980b)*
Mouse	*Subcutaneous*	*Polyphenolic fraction*	*Local sarcomas, 16/20*	*Shivapurkar et al. (1980b)*
Mouse	*Intraperitoneal*	*Aqueous*	*No tumour*	*Shivapurkar et al. (1980b)*
Mouse	*Intraperitoneal*	*Polyphenolic fraction*	*No tumour*	*Shivapurkar et al. (1980b)*
Mouse	*Mixed with diet (saccharin coated nuts)*	*None*	*No increased incidence of stomach tumours*	*Pai et al. (1981)*
Mouse	*Oral intubation*	*Aqueous*	*Reported increased incidence of lung tumours*	*Shirname et al. (1983)*

Species	Route	Extraction	Effect	Reference
Rat	Mixed with vitamin A-deficient & sufficient diet	None	No increased incidence of tumours	Tanaka et al. (1983)
Hamster	Cheek pouch application	None	Cheek pouch papillomas / carcinomas, 2/20	Rao (1984)
Mouse	Gavage, (pregnant dams) (Ripe, unprocessed and processed nuts)	Aqueous	Dose dependent increase in death,percentage frequency of resorption, as well as dead, macerated fetuses.	Sinha and Rao (1985c)
Rat	Mixed with diet (previous oral intubation of 4NQO)	None	Tongue papillomas and carcinomas, 13/17, carcinoma of buccal mucosa, 2/17, oesophageal carcinoma, 1/17, Total neoplasms 17/17	Tanaka et al. (1986)
Rat	Mixed with diet (with and without previous feeding with FAA)	None	Increased incidence of liver neoplastic nodules, 4/10 only in animals fed with FAA and areca nut. Altered liver foci in only areca nut	Tanaka et al. (1986)
Hamster	Mixed with diet (with and without Sodium nitrite)	None	Increased incidence of malignant tumours only in animals fed with areca nut with sodium nitrite. (12/30)	Ernst et al. (1987)
Mice	Mixed with diet Ripe, unprocessed, sun-dried (R-UP-SD), water soaked (WS) undried (UD), processed (P)	None	Oesophageal carcinoma/ papillomas (R-UP-SD) 3/47, (R-UP-SD-WS) 2/45, (R-UP-UD-WS) 3/44, No tumours due to processed variety of nuts.	Rao and Das (1989)

Species	Route	Extraction	Effect	References
Mice	Oral feeding of five above mentioned varieties of areca nuts	None	Oesophageal carcinomas, 4/37 (R-UP-SD), 2/34 (R-UP-SD-WS), 3/33 (R-UP-UD-WS). No tumour incidence by diet with processed nuts.	Rao and Das (1989)
ARECOLINE				
Hamster	Cheek pouch application	None	Papilloma of oesophagus	Dunham et al. (1974)
Hamster	Mixed with diet, with calcium hydroxide	None	Carcinoma of glandular stomach	Dunham et al. (1975)
Mouse	Subcutaneous	None	No local tumour	Shivapurkar et al. (1980b)
Mouse	Intraperitoneal	None	No tumour	Shivapurkar et al. (1980b)
Mouse	Oral intubation	None	Liver, lung and stomach tumours in males.	Bhide et al. (1984)
Mouse	Oral intubation with potassium nitrate alone or with lime	None	No increase in tumour incidence	Bhide et al. (1984)
BETEL QUID WITHOUT TOBACCO				
Hamster	Cheek pouch application	Aqueous	Forestomach carcinomas 5/20	Ranadive et al. (1979)
Hamster	Cheek pouch wax pellets	None	Cheek pouch carcinomas, 4/18 Forestomach carcinomas, 8/18	Ranadive et al. (1979)
Mouse	Subcutaneous	Aqueous	Local carcinomas, 7/20	Shivapurkar et al. (1980b)
Mouse	Oral intubation	Aqueous	Non-statistically significant increase in lung tumours.	Shirname et al. (1983)

1983c). A reduction in the micronucleated cells was found following vitamin A and β-carotene administrations (Stich et al., 1984b; 1984c). The chromosome damaging ability of the saliva of tobacco chewers and areca nut chewers was observed by Stich and Stich (1982) in the CHO cells.

The frequency of sister chromatid exchange (SCE) in peripheral blood lymphocytes (PBL) of betel quid chewers with or without tobacco was higher compared to the non-chewing controls (Ghosh and Ghosh, 1984). Similar results were observed among 'betel' chewing pregnant women (Ghosh and Ghosh, 1988). Elevated levels of SCEs in PBLs of tobacco/areca nut chewers have also been reported from our laboratory (Adhvaryu et al., 1986; 1988a).

Urine samples from some of the chewers of betel quid with tobacco when concentrated on XAD column, exhibited mutagenicity with Salmonella typhimurium TA1538 in presence of metabolic activation. None of the control samples showed such effect (Menon and Bhide, 1984). Urinary metabolites of arecoline were evaluated for their use as short-term indicators of the level of exposure to arecoline. It was shown that arecoline does bind to haemoglobin (Prokopczyk et al., 1988a).

The results of these studies in experimental systems and humans, however, have only provided 'inadequate' or 'limited' evidence regarding the carcinogenicity of areca nut (IARC, 1985), which means we still need to substantiate the existing data, and the present work is an attempt in this direction.

ABOUT THE PARAMETERS:

The experimental design adopted, was expected to provide us sufficient information and cover as many aspects as practicable. The preliminary data were generated from relatively simple, rapid and economical in vitro tests, utilizing established cell lines. The in vivo effects were examined

in humans, using some of the parameters that were employed for in vitro studies.

The in vitro effects were studied employing short term assays which offer many advantages in that they are sensitive, require small amount of the test substance, reliable and reproducible. A wide range of concentrations can be utilized, including near lethal dose. It has been suggested that in genotoxicity testing, the in vitro cytogenetic tests should take a central role in a test battery (Natarajan and Obe, 1982).

Cell cultures provide an ideal test system with homogenous cell population growing in chemically defined media and controlled conditions. The Chinese Hamster Ovary (CHO) cells were the cells of choice since they have a relatively short propagation time, can be synchronized easily, and possess large sized small number of chromosomes (Au and Hsu, 1982).

To estimate the genotoxic effects of areca nut and its major alkaloid arecoline, the cell cultures were subjected to various experimental protocols and the effects were studied on: (1) Cell viability, (2) Cell division, (3) Micronucleus (MN) formation, (4) Chromosome aberration (CA), (5) Sister chromatid exchange (SCE), (6) Cellular kinetics and (7) DNA synthesis.

(1) Cell Viability:

In estimating the carcinogenic/genotoxic potentials, the choice of a suitable dose range is governed by the toxicity of the compound. Studies should be undertaken at doses that cause 50% or less cytotoxicity. The cell death and viability can be classically elucidated by dye exclusion test, which is valuable in determining the possible lethal effects of the test substance on the cells. It is simple, rapid and does not require additional treatment which may affect the results (Tsukeda et al., 1978). It is widely used to

decide in vitro toxicity, the lethal and sublethal concentrations and thus help in the selection of appropriate doses for subsequent experiments.

(2) Cell division:

A group of chemicals, collectively called mitotic poisons, allegedly interfere with the mitotic apparatus, spindle proteins, centrioles, etc., but do not react with DNA. These substances may not be detected by mutation assay. Successful operation of cell division depends on properly sequenced completion of many macromolecule synthesizing events. Inhibition or alteration of anyone of these will be reflected as abnormal cell division. These may produce gross genetic imbalance leading to a more devastating effect on the cells than point mutations (Au and Hsu, 1982). In order to detect the effect of the test substance on mitotic activity and cell division, this assay was employed.

(3) Micronucleus assay:

Micronuclei originate from chromosomal regions lagging or irregularly migrating during anaphase. Their presence can be taken as an indication of previous existence of chromosomal aberration (Carrano and Natarajan, 1988). The detection of micronuclei is widely used as a short term mutagenicity test, both in cultured mammalian cells and in experimental animals. It offers a simple and rapid screening of chromosomal damage in cytological preparations (Heddle et al., 1978). Its sensitivity is comparable to that of scoring chromatid breaks and exchanges (Kliesch and Adler, 1980). Micronuclei resulting from spindle poisons are, as a rule, larger than those resulting from chromosome fragments (Schmid, 1976; Yamamoto and Kikuchi, 1980; Hogstedt and Karlsson, 1985). In recent years, the MN test has also been applied in the exfoliated cells of the individuals at elevated risk of developing organ specific cancers such as oral cavity (Stich and co-

workers, 1982-1986; Sarto et al., 1987), urinary bladder (Raafat et al., 1984), oesophagus (Zaridze et al., 1985) and in patients with upper digestive tract cancers (Mandard et al., 1987). Some investigators also used peripheral blood lymphocytes for their studies (Hogstedt et al., 1981; 1983a; 1983b; Stenstrand, 1985; Hogstedt and Karlsson, 1985). The profound understanding of the origin of micronucleus, and a reasonable relationship between the carcinogenicity and capacity of the chemicals to induce micronuclei and also the ease in scoring, stimulated the application of this test in our in vitro and human studies.

(4) Chromosome aberrations:

A number of different types of lesions are induced in DNA depending on the type of mutagen/carcinogen employed. These lesions are subjected to cellular repair, and the unrepaired or misrepaired lesions lead to chromosome aberrations (Natarajan, 1984). The changes in the chromosome structure offers morphological evidence of the damage to the genetic material and the type and persistence of lesions induced in DNA by various agents will determine the degree of cytogenetic damage. The principles and the mechanism underlying the formation of CA are clearly understood and this contributes significantly in the understanding of the carcinogenic effect of the substance. Most neoplasms exhibit chromosome changes in number and/or rearrangements (Atkin, 1976; Rowley, 1977; Mitelman and Levan, 1978). In several chemically induced tumours, chromosome changes were shown to be an initial observable event (Rees et al., 1970; Yunis, 1983). Elevated frequencies of CA may serve as an indicator of exposure to carcinogenic substances, a factor that increases the risk of cancer and genetic ill health (Carrano and Natarajan, 1988). Thus, the estimation of CA is considered to be an extremely sensitive indicator and one of the most

unambiguous techniques to determine chromosome breakage in vitro and in vivo (Evans and O'Riardon, 1975).

(5) Sister chromatid exchange:

SCE is the cytological manifestation of DNA breakage and repair at apparently homologous sites on the two chromatids of a single chromosome. SCEs are efficiently induced by substances that form covalent adducts to the DNA or otherwise interfere with DNA precursor metabolism or repair (Perry and Evans, 1975; Wolff, 1977; Perry, 1980; Latt, 1981; Natarajan et al., 1981; Carrano and Thompson, 1982). Although the occurrence of SCE may reflect DNA damage and repair process that are closely correlated with mutational changes, the molecular mechanisms involved in the formation of SCE are not yet fully resolved, however, SCE and mutagenesis are closely related (Carrano et al., 1978). The SCE assay is a highly sensitive indicator of genetic injury, and has been studied in cell cultures, animals as well as humans exposed to known mutagens and carcinogens (Evans, 1988). SCEs have also been studied in relation to various types of cancers and in some precancerous conditions. Examination of SCE in lymphocytes of persons exposed to possible genotoxic agents is widely used for monitoring the exposure to possible mutagens/carcinogens (Carrano, 1982; Carrano and Natarajan, 1988). The study has been carried out in smokers (Lambert et al., 1978; Hopkin and Evans, 1980; Watanabe and Endo, 1984; Husum et al., 1986; Ghosh and Ghosh, 1987) and in tobacco and areca nut chewers (Adhvaryu et al., 1986; 1988a; Ghosh and Ghosh, 1984; 1988). The assay was employed for in vitro and human studies in the present work.

(6) Cellular kinetics:

The application of BrdU labelling technique in the analysis of cellular

kinetics in a logarithmically growing cell population has been validated in different areas of mutagenicity/carcinogenicity research (Craig-Holmes and Shaw, 1976; Tice et al., 1976; Crossen and Morgan, 1977; Tice and Ivett, 1985; Shah et al., 1986; 1989). The technique allows an unequivocal identification of the number of S phases completed during the exposure of the cell to 5-Bromo-2-deoxyuridine (BrdU), by using the differential staining pattern. It allows precise enumeration of the cells in first, second and third cycle of the cell division, and thus of the proliferation index and the average generation time (AGT). Impaired cellular immunity in patients with oral cancers has been evidenced by this technique (Bazopoulou-kyrkanidou et al., 1983). The analysis of cell kinetics may thus be used to estimate the delay in cell proliferation induced by exogenous agents and in unsynchronized cell cultures as in our study.

(7) DNA synthesis:

There is a direct relationship between the extent of DNA damage and the cell death induced by various physical and chemical agents. The ability of genotoxic agents to interfere with DNA replication has long been recognized (Roberts, 1984). DNA is duplicated during the S phase of the cell cycle. With the help of ^{3}H-thymidine incorporation in the interphase cells followed by autoradiographic analysis, we can determine the effect of any agent on the S phase events. Frequently, cells exhibit a progressive depression of the rate of synthesis of nascent DNA following short treatment with various agents (Roberts, 1984). The accumulation of chromosomal aberrations by DNA synthesis inhibitors, is a finding of relevance (Preston, 1982). The inhibition of repair may potentially contribute to increased number of DNA lesions that can interact to form chromosomal aberrations (Fram and Kufe, 1984). Thus, it would be of interest to learn about the

effects of areca nut on DNA synthesis. The assay was carried out in the present study utilizing CHO cells.

PLAN OF WORK IN NUTSHELL:

The first part of the thesis embodies the in vitro short term studies utilizing different parameters as has been already described. The aqueous extract of areca nut was tested for its genotoxic potentials. Together with this, arecoline, the major areca nut alkaloid in its pure form, was also subjected to various short term assays in order to pinpoint the substance responsible for the possible genotoxic effects of areca nut. Since areca nut is mostly, if not always, consumed together with tobacco, an analysis regarding the end effect on combining them was thought to provide a better insight. The in vitro studies were carried out taking the major alkaloids of both, areca nut and tobacco, i.e. arecoline and nicotine in combination. Much of the data were generated from relatively simple tests, availing established cell lines, however, it should be emphasized at the outset that the pharmacokinetic/metabolic factors may prevent the substance to elicit comparable response in humans. It has been suggested that the in vivo genotoxicity data would serve well to substantiate the in vitro data, when using it to predict genotoxic carcinogenicity (ICPEMC, 1988).

The second part, therefore, was designed to study the ability of areca nut to express its genotoxic potentials in vivo. The studies were carried out in humans. There are relatively few direct methods to measure the extent of genomic damage in human beings exposed to potential mutagens or carcinogens. The most extensively employed method to assess the genetic effects of such exposures has been the analysis of chromosomal aberrations (CA) and SCEs in phytohaemagglutinin (PHA) stimulated peripheral blood

lymphocytes (PBLs) of exposed persons. In the present study it was also possible to study the DNA damage in the target tissue with the help of micronucleated cell (MNC) estimation in exfoliated cells of buccal mucosa. These methods have been successfully employed as an index in various studies. Investigations were carried out considering two types of habits, (1) Chewing areca nut alone, (2) Chewing areca nut alongwith tobacco (with no other tobacco consuming habit, like smoking, snuff rubbing, etc.). A test battery of four assays was employed among individuals categorized in four groups; viz. Controls, Normal chewers, Chewers with oral submucous fibrosis (OSMF) and Chewers suffering from oral cancer.

The third part envisages the studies on 'pan masalas', a new product that has captured the market gaining rapid popularity. The active advertisement campaign has resulted in its escalating sales and profound social acceptance. 'Pan masalas', as stated on their packings, are a complex mixture of various ingredients which include areca nut (maximum amount by weight), catechu, lime, cardamom and unspecified flavouring agents. When all such substances combine to form a complex mixture, the genotoxic potentials of the individual constituents may either get nullified or they may act in a synergistic manner adding to the severity of the ultimate effect. Hence, it was thought worthwhile to study the effect of pan masala, which contains areca nut as the major constituent. An aqueous extract of the product was prepared and subjected to short term tests in CHO cells. The in vivo genotoxic effects were also studied in individuals (in PBLs and exfoliated buccal mucosa cells) who were regularly consuming pan masalas (without zarda) with no concurrent tobacco and/or areca nut habit.