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Genotoxicity and Carcinogenicity of Pan Masala: A Review

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Chewing habits have prevailed in India for more than two millenia. At present, a multitude of chewing products that contain the main ingredients of betel quid, such as areca nut, chatechu and lime are available in the market. Chewing mixes that do not contain tobacco are termed as 'pan masala' and those containing tobacco as an additional ingredient are termed 'gutkha'. A connection between gutkha habituation and adverse health effects is easily appreciated by the public since there is considerable awareness that tobacco chewing and smoking habits are responsible for the development of cardio-vascular, and pulmonary diseases as well as cancer at various sites. On the other hand pan masala is considered safe for human consumption because it does not contain tobacco. Wide spread popularity of pan masala has raised concern regarding its adverse effects on human health. The concern is based on the existing data on mutagenic, genotoxic and carcinogenic properties of areca nut and on the finding that areca nut chewers are at high risk for development of a pre-cancerous lesion, oral submucous fibrosis. This article surveys available literature on biological hazards of pan masala ingredients and the mixture as a whole. The studies provide reproducible evidence regarding genotoxic potential of areca nut and pan masala. The demonstration of chromosomal damage in lymphocytes from pan masala habitues, the association between pan masala chewing and early onset of oral submucous fibrosis and unequivocal data on pan masala carcinogenicity in three different mouse models strongly indicate that pan masala should be categorized as a human carcinogen.

Key Words: Genotoxicity, Carcinogenicity, Pan masala, Areca nut

Habitual mastication of plant products has prevailed in the Indian subcontinent since antiquity. Chewing of areca nut is mentioned in Sushruta Samhita, believed to have been written around 600 BC, while ancient writings refer to the prevalance of betel quid chewing habit in Sri Lanka as far back as 504 BC. Botanical evidence indicates that Areca catechu and piper betel plants are native to West Malaysia. Betel quid chewing appears to have been introduced to the Indian sub-continent during the period of colonization of Malaysia and South East Asia by Indian emperors. However, the Portuguese traders brought tobacco to India in the late 16th or early 17th century. Tobacco was considered a luxury item and due to the prevalence of other masticator products was accepted easily and chewed by itself, with areca nut, lime or with betel quid. Even in the present times an overwhelmingly large section of the Indian populace indulges in these habits.

During the last three decades, rapid industrialization has resulted in a decline in the leisurely life style of common Indians. Manufacturers of chewing tobacco, scented and spiced areca nut preparations were quick to sense the changing social needs and attitudes and introduced in early seventies, readymade chewing products containing all the main ingredients of betel quid i.e. areca nut, catechu and lime with or without tobacco. "Mawa", a preparation containing thin shavings of areca

nut, tobacco and slaked lime was wrapped in cellophane paper and sold by vendors. Another product, which adorned the shelves of many shops, was termed "pan masala", clearly conveying to the users that the product comprises of the main ingredients of betel quid without the betel leaf. Pan masala is available in attractive, airtight hermetically sealed, sachets or in larger tins under different brand names. The tin containing 100g of pan masala was targeted for family consumption while the sachets served the need of individuals who were spared the time required for preparing a betel quid. Presently, two types of ready to chew betel quid substitutes are available in the market. Those that contain the main dry ingredients of betel quid but are devoid of tobacco are labeled as "pan masala" while the products containing pan masala and tobacco are called as "gutkha". Aggressive marketing strategies and mass media advertising by reputed film personalities with emphasis on absence of tobacco have helped popularize pan masala not only in Indian males but also in females and children in all social strata. Pan masala and gutkha are exported to several countries, however these products are banned in USA.

There is widespread belief that pan masala is safe for human consumption as it does not contain tobacco. Contrary to this belief, experimental and human studies conducted over the last few decades have shown that individual ingredients of pan masala are genotoxic and hazardous to human health. More recent studies have provided clear evidence regarding genotoxic and carcinogenic potential of commercially available pan masala in experimental models. Increased chromosomal damage is known to occur in the peripheral blood lymphocytes and oral mucosal cells of regular pan masala users. Moreover, the habit is related to early development of oral submucous fibrosis, a painful condition known to precede development of oral cancer. Unfortunately, biological and human health hazards of pan masala consumption have not been conveyed to the public at large. During this decade, the topic received scientific attention and several studies were initiated to elucidate the biological effects of this chewing product.

This review documents available information on the chemical and biological properties of individual ingredients of pan masala. Data on the influence of these chemicals in experimental systems and on human health hazards with particular reference to oral precancer and cancer are described. Publications on the biological activity of different brands of pan masala began to appear in this decade. Contributions demonstrating clastogenic, mutagenic and carcinogenic potential of this chewing mixture are also presented.

Chemical Analysis and Bioactivity of Pan Masala Ingredients

Catechu: Catechu is the residue of the hot water extraction of the heart-wood of Acacia catechu. Its main constituents are tannin and polyphenols, 25-35% catechu tannic acid, and 2-10% catechin. Catechu red, quercetin and gum have also been detected (IARC 1983, Windholz 1983). Polyphenols in catechu comprise of kaempferol, dihydroxykaempherol, taxifolin, isorhamnetin, (+)afzelchin and dimeric procyanidine and (-)epicatechins (Deshpande & Patil 1981). Epidemiological studies have shown an association between consumption of foods rich in tannins and high incidence of esophageal cancer (Morton 1972) in certain parts of the world. It was therefore felt that tannin rich catechu may exhibit genotoxic activity. Induction of sister chromatid exchanges (SCE) and dominant lethal mutations were studied in Swiss male mice injected various doses of an aqueous extract of catechu or gavage feeding of catechu to animals. A dose related increase in SCE frequency was observed in animals treated with 1.5 - 100 mg/kg bw of the aqueous extract of catechu. Acute or chronic exposure of male mice to the catechu extract also increased post-implantation loss of embryos (Giri et al. 1987). However, Nagabhushan et al. (1988) reported that an extract of catechu and pure catechin are non-mutagenic in the Ames assay in the absence

or presence of metabolic activation with Arochlor 1254 induced rat liver S9. They also found that oral administration of these compounds inhibited cytochrome P450 activity and increased glutathione level in liver tissues of Wistar rats.

Slaked lime : Two different types of lime are used in India. Lime is either prepared from sea shells or from limestones. Calcarious covering of marine invertebrates harvested along the coastal line of India are an important source of shell lime. Lime is prepared by roasting the shells on fire and mixing with water to form a paste. Stone lime or $CaCO_3$ is also quarried in central India. The *p*H of both types of lime is alkaline (Bhonsale et al. 1992). Whether one of the two types of lime or both the types of lime are added to pan masala is not known as the source is not mentioned on the container. In studies on biological activity of lime, repeated treatment with calcium hydroxide was found to induce epithelial atypia in hamster cheek pouches (Dunham et al.1966). In another study, slaked lime was painted on the palate and buccal mucosa of Wistar rats for varying time period ranging from 2-12 months. Histological examination revealed the presence of moderate to severe epithelial hyperplasia in all the tissues while hyperkerotosis, cytoplasmic vaccuolation and invagination of rete pegs into the papillary layer was observed in a large number of tissues (Sirsat & Kandarkar 1968).

Areca nut : Areca nut, the most abundant ingredient of pan masala is composed of tannins, alkaloids, fats, free fatty acids, polysaccharides, fibers and minerals (Majumdar et al. 1979). Atleast five alkaloids are present in areca nut--these are arecoline, arecaidine, arecolidine, guavacine and guvacoline (Arjungi 1976). *In vitro* experiments have shown that in the presence of nitrite, the major areca nut alkaloid arecoline can give rise to N-nitrosamines such as N-nitrosoguvacoline, 3 (methylnitrosamino) propionitrile (MNPN), 3-(methylnitrosamino) propionaldehyde (MNPA), (Wenke & Hoffmann 1983) and N-nitrosoguvacine (Nair et al. 1985). The latter has been detected in the saliva of betel quid chewers since the quid contains areca nut. Besides the alkaloids, areca nut contains appreciable amounts of polyphenols, generally termed as tannins, which include catechins, leucoanthocyanidins and polymerised compounds (Govindarajan & Mathew 1963). It is known that alkaloid content increases with the maturation of the areca nut (Mathew et al. 1964) and that a wide variation can occur in the chemical composition of the processed areca nut (Shivashankar et al. 1969).

The evidence on production of tremors in rats treated with large doses of arecoline (Holmstedt & Lundgren 1967) indicated that arecoline affects the central nervous system presumably through its influence on muscarinic receptor (McKinney & Richelson 1984). Inhibition of both humoral and cell-mediated immune responses were noted in the laboratory animals treated with arecoline (Shahabuddin et al. 1980, Selvan et al. 1989, 1991, 1993). In an early study, aqueous and ethanolic extracts of areca nut were found to exhibit antibacterial and antifungal activity (Lalithakumari et al. 1965). Experimental studies were carried out by several groups of workers to determine the genotoxic potential of areca nut or its alkaloids. Arecaidine, a small fraction of the areca nut alkaloids was found to be mutagenic in the Ames assay (Shirname et al. 1983). These workers also reported that an aqueous extract of betel nut, arecoline and arecaidine increased significantly, the frequency of polychromatic erythrocytes in mouse bone marrow cells, and mutations in V-79 Chinese Hamster cells (Shirname et al. 1984). Stich and Tsang (1989) examined the effects of an aqueous extract of betel nut on the frequency of transformed foci in C3H 10 T½ cells transfected with bovine papilloma virus DNA. A ten fold increase in the number of transformed foci was observed in cultures treated with the areca nut extract. Exposure of human buccal epithelial cells to an aqueous extract of areca nut was found to decrease their viability and cause a significant increase in the formation of DNA single strand breaks and DNA protein cross links. A comparison of the areca nut related N-nitroso compounds and their precursor alkaloids

revealed that MNPA is the most potent inducer of DNA single strand breaks in human buccal epithelial cells in culture (Sundquist et al. 1991). A study of Hep2 cells derived from human larynx carcinoma showed induction of unscheduled DNA synthesis in cultures exposed to different concentrations of a betel nut extract (Sharan & Wary 1992).

Cytogenetic end points have also been employed to examine genotoxic influence of areca nut and it's alkaloids. Induction of chromosomal aberrations by arecoline was reported in CHO cells (Stich et al. 1981). An aqueous extract of areca nut was also found to be clastogenic in CHO cells (Stich & Tsang 1989). Dave et al. (1992) investigated the effects of an aqueous extract of areca nut and arecoline on the frequencies of SCE and chromosomal aberrations (CA) in CHO cells. They reported that the areca nut extract and arecoline induced a dose dependent increase in the frequencies of SCE and CA in continuous and pulse treatment protocols. The frequency of SCE and CA was higher in cultures treated with areca nut extract than in those treated with arecoline implying that substances other than arecoline are responsible for increased genotoxicity of the areca nut extract. Panigrahi and Rao (1982) showed that arecoline is clastogenic in murine bone marrow cells while arecoline as well as arecaidine were found to increase SCE frequencies in mouse bone marrow cells (Panigrahi & Rao 1984). Further studies revealed that intraperitoneal administration of an aqueous extract of areca nut and areca nut tannins to mice also increased the frequency of SCE in mouse bone marrow cells. Moreover at equivalent dosage, the aqueous extract was found to be a more potent inducer of SCE than tannins (Panigrahi & Rao 1986). On the other hand Umezawa et al. (1981) found that the ethyl acetate extract of areca nut did not induce SCE in virally transformed human lymphocytes. However, in another model system, viz the hepatocyte DNA repair test, MNPN, an arecoline-derived nitrosamine was found to be genotoxic (Williams & Laspia 1979). The effects of an extract of baked areca nuts on buccal mucosal fibroblasts derived from healthy individuals who did not indulge in the areca nut habit was examined by van Wyk et al. (1996). No discernable alterations were observed in fibroblasts from five individuals while growth of cells from one healthy individual was inhibited presumably due to individual differences in gene-environment interactions.

Carcinogenicity of Areca Nut and Its Derivatives

Suri et al. (1971) reported that multiple applications of a dimethylsulfoxide extract of areca nut to hamster cheek pouch results in the development of leukoplakia followed by squamous cell carcinoma. Subsequently, Dunham et al. (1974) reported that application of arecoline to the cheek pouch of Syrian hamsters results in the induction of squamous cell carcinoma. Proliferation and atypia of basal cells in the esophageal epithelium and esophageal papilloma were also observed. Ranadive et al. (1979) reported that topical application of areca nut extract, or its polyphenolic fraction to hamster cheek pouch as well as implantation of betel nut pieces in the cheek pouch, induced precancer and cancer in the cheek pouch and stomach of the treated animals. In another study using Syrian hamsters, animals were fed powdered areca nut in the diet, throughout their lifetime. Adenoma of the pancreatic islet cells, adrenal cortex and lacriminal gland, pheochromocytoma and papilloma of esophagus and forestomach were induced. The total tumor response in animals fed areca nut was significantly different from that in animals fed normal diet (Ernst et al. 1987). In another study, Rao and Das (1989) investigated the carcinogenic potential of areca nut given in diet for 12 months at concentrations ranging from 0.25% to 1% and oral feeding of areca nut paste to Swiss mice. Survival rate and body weight of the experimental animals was found to be slightly lower than that of the controls. In animals fed areca nut diet for 12 months, hyperplastic changes were observed in oral, esophageal and forestomach epithelium while esophageal carcinoma developed in a limited number of animals. These workers observed

that only unprocessed areca nuts have weak carcinogenic potency in mice.

Areca nut contains a number of phenolic compounds, which are considered to be responsible for the development of proliferative lesions. Bhide et al. (1979) observed that administration of areca nut extract and polyphenol fractions of areca nut to Swiss and C-17 mice by gastric intubation resulted in the development of gastrointestinal tract tumors in 50% of Swiss mice and in 25% of the mice of C-17 strain. Kapadia et al. (1978) found that subcutaneous injections of an aqueous extract of Areca Catechu plants, which are rich in tannins, produced malignant histiocytoma at the site of injection in 100% of NIH Black rats.To determine whether the phenolic compounds are responsible for the induction of these lesions, a comparative sutdy was carried out using 13 phenolic compounds. In that study, a number of phenolic compounds were found to induce fore-stomach hyperplasia (Hirose et al. 1986). Shivapurkar et al. (1980) injected Swiss mice with an aqueous extract of areca nut, areca nut polyphenolic fraction and arecoline for 13 weeks and observed the animals for their lifetime. Fibrosarcomas developed at the site of injection in 30% of mice injected with betel nut extract, in 100% of mice injected polyphenol fraction of areca nut while no tumors were observed in animals injected arecoline. Male and female Swiss mice were administered arecoline by gavage 5 days a week for the life span of the animals. In this study, 43% of the males developed tumors. There were 8 liver haemangiomas, 4 lung adenocarcinomas and 3 squamous cell carcinomas of the forestomach (Bhide et al. 1984). MNPN is formed *in vitro* under mild nitrosation conditions from the major areca nut alkaloid arecoline. Wenke et al. (1984) administered MNPN to F344 rats by multiple subcutaneous injections. All the rats developed tumors in 24 weeks. A majority of the tumors were papilloma in the esophagus and the nasal cavity; some tongue papillomas were also observed. However, the percentage of animals with carcinoma was low.

Human studies: In a study on genotoxic risk among oral users of Indian chewing products Stich et al (1982) reported an elevated micronucleated cell frequency in the buccal mucosal epithelium of individuals who chewed areca nut alone as compared to habit free controls. They also found that saliva of betel nut chewers contains chromosome damaging activity (Stich & Stich 1982). A study on peripheral blood lymphocytes from areca nut chewers comprising of healthy individuals, patients with oral submucous fibrosis, oral cancer, and healthy non-chewer controls, showed that the frequencies of SCE and CA were significantly higher in each group of chewers than in the controls (Adhvaryu et al. 1986). An interesting questionnaire based study was carried out in Papua New Guinea, where areca nut chewing is highly prevalent among the native population. The results showed that asthma was aggravated by betel nut chewing (Kiyingi 1991). In another study, betel nut chewing was found to affect cardiovascular function among chronic as well as recent chewers who showed a significant increase in heart rate lasting for an average of nearly 17 minutes (Chu 1993).

Many human cancers result from exposure to chemicals derived from tobacco, diet and the environment. Oral cavity cancers are the commonest cancers among men and third commonest in women in India (Sankarnarayan et al. 1989). In recent years, areca nut chewing is recognized to play an etiological role in the development of oral submucous fibrosis, which is an insidious and chronic condition that precedes development of oral cancer. In a seminal report, Paymaster (1956) described the development of a slow growing squamous cell carcinoma in one third of all patients with oral submucous fibrosis. The condition has been observed repeatedly in association with frank oral malignancy and is reported to occur mainly in Indians and people of Indian origin who reside in other countries but chew areca nut regularly (Schonland & Bradshaw 1969, Chin & Lee 1970). Epidemiological studies have also indicated that the risk of oral cancer is higher in people who chew betel quid without tobacco than in those who do not chew betel quid, its ingredients or tobacco (Gupta et al. 1982,

WHO 1984). In a review of oral submucous fibrosis case series too, the range of frequency of areca nut chewing habit was found to be 34% to 100% (Sinor et al. 1990).

Pan Masala

Pan masala was introduced in the Indian market around 1975. As stated earlier it is a mixture of catechu, lime, areca nut and flavouring agents. Catechu forms about 10%, lime about 1%, areca nut pieces account for nearly 80% of the mixture and the remaining 9% include menthol and various spices such as cardamom (Dave et al. 1991). During this decade, this product has gained wide acceptance as a substitute for betel quid not only in India but also in several other Asian countries. Health hazards of pan masala were initially reported by dentists who noticed that oral submucous fibrosis occurs frequently in pan masala users. The observation raised concern regarding its safety and paved the way for scientific inquiry into the biological effects of pan masala.

Chemical analysis of five common brands of pan masala revealed the presence of polycyclic aromatic hydrocarbons (PAH), nitrosamines and toxic metals like lead, cadmium and nickel (Kashyap et al. 1989-90). However, actual levels of PAH and nitrosamines were not provided in the report. Volatile aldehydes including formaldehyde, acrolein, crotonaldehyde, propionaldehyde and isobutyraldehyde have been detected in studies on chemical analysis of pan masala in our laboratory (Pakhale S.S. personal communication). Some of the aldehydes are known to be carcinogenic and are of special interest as inhibitors of ciliary movement and lung clearance. Menthol is commonly added to pan masala to improve its flavor. Menthol concentration was estimated in 130 brands of pan masala and in 53 non-branded samples. The results showed that in 75% of the branded samples and in 92% of non-branded samples, menthol content was higher than 0.1 % (10 mg/g) the permissible limit prescribed by the Food Adulteration Act of India (Kannan et al. 1997).

Bagwe et al. (1990) analyzed the mutagenic activity of aqueous, ethanolic, aqueous- ethanolic and chloroform extracts of a popular brand of pan masala. In these studies, the ethanolic extract was found to produce a dose dependent increase in the number of TA98 revertants in the absence of S9. The maximum number of induced revertants was short by unity of the definition of mutagenicity ie. two-fold increase over spontaneous revertant number. Hence, the mutagenic potential of the extract was considered as borderline or weak. Subsequently Polasa et al. 1993 found that several brands of pan masala were mutagenic in the Ames assay.

Adhvaryu et al. (1989) conducted cytogenetic studies to examine genotoxic activity of pan masala and reported that an aqueous extract of pan masala increased the frequency of CA and SCE in CHO cells in culture. They also observed a dose dependent increase in the duration of the cell cycle of CHO cells. In further studies, Patel et al. (1994a) assessed the genotoxic potential of a dimethyl sulfoxide extract of pan masala in the same system. CHO cells were treated with various doses of the extract and the frequencies of CA, SCE and micronuclei in treated and control cultures were compared. As in the case of the aqueous extract, DMSO extract of pan masala was found to elicit a dose dependent increase in these parameters. In further studies ethanol was found to potentiate the clastogenic effect of pan masala. (Patel et al. 1994b). This group also carried out cytogenetic studies in regular pan masala consumers using PBL and exfoliated buccal epithelial cells as biological samples. A statistically significant increase in CA, SCE in PBL and micronucleated cell frequency in PBL and buccal mucosal cells with respect to controls (Dave et al. 1991) indicated that pan masala is genotoxic in humans as well. These findings were extended in a similar study wherein the effect of an aqueous extract of pan masala on genetic alterations in CHO cells was examined in the presence or absence of metabolic activation with rat liver S9 (Jaju et al. 1992). In these studies, a dose dependent increase was noted in the frequency of CA, SCE and micronuclei in cells cultured without rat liver S9. Presence of direct acting mutagens in the extract was implied by the observed suppression of chromosomal damage

in cells grown in culture medium containing rat liver S9 fraction. Cytogenetic analysis of meiotic metaphase I cells and abnormality of sperm head morphology was carried out by Mukherjee et al. (1991) who administered different doses of pan masala dispersed in polysorbate solution to Swiss mice. They observed a significant increase in the frequency of sperm head abnormalities at all the doses tested indicating that pan masala exerts clastogenic effect in the germinal cells. Mukherjee and Giri (1991) also administered an aqueous suspension to Swiss mice and studied SCE frequency in bone marrow cells. A significant dose related increase in SCE frequency was observed at low doses while higher doses induced a significant cell cycle delay.

The effect of pan masala on liver function was studied by Sarma et al. (1992) in rats fed pan masala by gavage. Increased levels of serum, GOT, GPT and alkaline phosphatase indicated toxic influence of pan masala with resultant impairment of liver function. Khrime et al (1991) painted the oral cavity of albino rats with pan masala for a period of 6 months. Oral mucosa was biopsied at the beginning of the study and every two months thereafter. Histological observations revealed loss of cellular polarity, and increase in keratosis, inflammatory response and vascularity in the buccal mucosal tissues of pan masala treated rats as compared to controls. Alterations in connective tissue included thickening and condensation of collagen in the submucosa. These alterations resembled the changes seen in oral submuous fibrosis

Carcinogenic and co-carcinogenic activity of pan masala were examined by Ramchandani et al. (1998) using mouse skin, stomach and esophagus as the target tissues. Two different mouse models, hairless Swiss bare mice S/RV Cri-ba and ICRC mice with a mega-esophagus were used. The ethanolic extract of pan masala (EPME) found to be mutagenic in earlier studies was applied topically to the back skin of uninitiated mice or those initiated with 7,12-dimethylbenz (a)anthracene (DMBA) to evaluate complete carcinogenic, tumor promoting or progressor activity. Gastric and esophageal tumor promoting activity was determined in ICRC mice by administration of the extract by gavage to animals initiated with diethylnitrsaomine (DEN). Cutaneous alterations induced by a single or multiple EPME treatment were also recorded. EPME was found to increase epidermal mitotic activity and promoted development of papillomas in the skin, forestomach and esophagus. EPME also increased the rate of conversion of papilloma to carcinoma. In further long term studies, these workers fed pan masala in pelleted diet at two different doses to S/RVCri mice for 24 months. Benign and malignant neoplasms were observed in the liver, lung, stomach, skin, prostate and testes. Statistical analysis revealed a significant dose dependent increase in the incidence of lung adenocarcinoma in mice exposed for life time to pan masala indicating that lung is the major target for the carcinogenic action of pan masala (Bhisey et al. 1999). These results clearly indicate that pan masala should be considered as a potential human carcinogen.

Clinical investigations have shown prevalence of areca nut and pan masala chewing habits in patients suffering from oral submucous fibrosis (Anuradha & Devi 1993). A high frequency of oral cancer was reported in subjects who consumed tobacco, betel quid or pan masala. (Mehta et al. 1992). In another study on 50 patients, use of pan masala was found to be associated with an earlier age of onset of oral submucous fibrosis. Chewers of these products presented with oral submucous fibrosis 2.7 ± 0.6 years after commencement of the habit whereas the betel quid chewers presented with the condition after 8.6 ± 2.3 years of habituation (Babu et al. 1996). Subsequently, a larger study, using cases and controls, matched for age, sex and socio-economic conditions has revealed a direct relationship between chewing of areca nut/betel quid or pan masala and oral submucous fibrosis (Shah & Sharma 1998).

Thus a survey of relevant literature indicates that pan masala constituents, individually and in the mixture form induce DNA strand breaks, gene mutations, as well as chromosomal damage in short term assays and give rise to a variety of tumors in rodent models. The demonstration of increased genotoxic risk in users of pan masala

and its association with impaired liver function and oral submucous fibrosis indicates that pan masala habituation can cause significant health hazards in the Indian population. Epidemiological data suggest that oral submucous fibrosis is likely to reach an epidemic level in the next decade. This situation is expected to increase the incidence of oral cancer, the most frequent cancer type in India. The alarming scenario demands that federal regulatory and health agencies and non-governmental organizations should launch awareness programmes to inform and educate the public, particularly women and children, regarding the adverse health consequences and possible cancer risk associated with pan masala habituation.

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