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Analysis and Toxicity of Plain (PMP) and Blended (PMT) Indian Pan Masala (PM)

Düz (PMP) ve Harmanlanmış (PMT) Hint Pan Masala (PM) Analizi ve Toksisitesi

Suresh Kumar Nigam[1], H. Venkatakrishna-Bhatt[2]
[1]Emeritus Scientist's Laboratory and Department of Neurobehavioural Toxicology, National Institute of Occupational Health(NIOH), Meghani Nagar, Gujarat, India
[2]Department of Neurobehavioural Toxicology, NIOH, Meghani Nagar, Gujarat, India

Abstract

Objective: Betel leaf combined with areca nut is known as betel quid pan masala (PM), and tobacco with areca nut, catechu and lime is pan masala (PMT) blended with gulkhand. These narcotics are popular among young and old individuals. A *prima facia* chemical analysis and a toxicity assessment of PM in mice were conducted to study the relationship between longtime consumption of PM and health hazards.

Materials and Methods: Chemical analysis of different types of *PM* was done employing HPLC , GLC, AAS, ES, TLC, GCMS and sequential extraction for PAH, pesticides, metals and minerals, electrolytes, drugs and xenobiotics. Ethanolic *PM* extracts were tested by IP and PO routes in inbred Swiss mice.

Results: *PAH*, which are known xenobiotics for pre-cancerous lesions, were significantly high ($p<0.01$) in *Rajaniganda and Pan Parag Zarda*. Isomers of DDT and BHC, which principally act on nerves and muscles, were also high ($p<0.01$) in PM. The enhanced metal and mineral content of *PM* results in massive oral fibrosis. There is a high level of narcotics in *PM*, especially nicotine, a potentially cancerous agent in the gastrointestinal tract.

Conclusion: Experimental studies with different extracts of plain and blended *PM* in mice fed for 16 and 90 days revealed no effect on blood and organ weights (kidney, heart, spleen and liver), but we did observe attenuated testis. However, in the bone marrow of the mice, chromosomes were most affected in the mice fed *PM-Zarda* blend for 3 months. The chromosomal abnormalities included ploidy, loss, breaks, gaps, deletions and exchanges in ring chromosomes. The *PM* caused sperm head anomalies (narrow, blunt, triangular and banana shapes), and the sperm were irregular, amorphous, tailless and rudimentary, with the maximum effect among the groups fed *PM* for 3 months. Significantly higher levels ($p<0.01$) of testis glycogen, cholesterol and protein were found. The group fed for 16 days showed no change in red blood corpuscles (*RBC*), white blood corpuscles (*WBC*), hemoglobin and erythrocyte sedimentation counts.

Key Words: Indian Pan Masala, Narcotics, Public health, Toxicity

Özet

Amaç: Areka fındık ile kombine betel yaprağı pan masala (PM) ve areka fındık ile tütün, pekiştirici ve kireç gulkhand ile harmanlanmış pan masala (PMT) olarak bilinir. Bunlar genç ve yaşlı bireyler arasında popüler uyuşturucu maddelerdir. PM bir toksisite değerlendirilmesi *prima facia* kimyasal analiz ve farelerde uzun zamandır PM tüketimi ve sağlık riskleri arasındaki ilişkiyi incelemek için gerçekleştirilmiştir.

Gereç ve Yöntem: Farklı kimyasal analizler; PM HPLC, GLC, AAS, ES, TLC, GCMS ve PAH, pestisitler, metaller ve mineraller, elektrolitler, uyuşturucu ve ksenobiyotiklere için sıralı ekstraksiyon ile gerçekleştirilmiştir. Etanolik PM özleri inbred Swiss farelerde IP ve PO yollar ile test edilmiştir.

Bulgular: Kanser öncesi lezyonlar için ksenobiyotikler olarak bilinen PAH, *Rajaniganda ve Pan Parag Zarda*'da ($p<0.01$) anlamlı olarak yüksek bulunmuştur. Başta sinir ve kasların hareket DDT ve BHC, izomerleri PM ($p<0.01$) yüksek bulunmuştur. Masif oral fibrozis PM sonuçlarının gelişmiş metal ve mineral içeriği, gastrointestinal sistemin potansiyel kanser ajanı, özellikle nikotin PM uyuşturucuda yüksek düzeyde bulunmaktadır.

Sonuç: Deneysel çalışmalarda 16 ve 90 gün boyunca beslenen farelerde düz ve karışımlı PM farklı özlerinin kan ve organ ağırlıkları (böbrek, kalp, dalak ve karaciğer) üzerinde hiçbir etkisi yoktur, ancak ufalmış testis gözlemlemek mümkün olmuştur. Ancak, *PM-Zarda* karışımı ile 3 ay boyunca beslenen farelerin kemik iliğinde, kromozomlar büyük oranda etkilenmiştir. Kromozom anomalilerine ploidi, kayıp, boşluk, silinme ve halka kromozomlar içerisinde değişim dahildir. PM sperm baş anomalileri (dar, künt, üçgen ve muz şekiller) ve 3 ay boyunca PM ile beslenen gruplar arasındaki maksimum etkisi ile sperm düzensiz amorf, kuyruksuz ve ilkel yapısı şeklinde gözlemlenmiştir. Testis glikojen, kolesterol ve protein düzeyleri arasında anlamlı ($p<0.01$) ilişki bulunmuştur. PM ile 16 gün boyunca beslenen grupta alyuvar (RBC), akyuvar (WBC), hemoglobin ve eritrosit sedimentasyon sayımlarında hiçbir değişiklik gözlenmemiştir.

Anahtar Kelimeler: Hint Pan Masala, Narkotikler, Halk sağlığı, Toksisite

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Correspondence to: H. Venkatakrishna-Bhatt, Flat No.13, Block No.2, Suramya Apartments, Sola Road PO PO, Mirambika Street, Near Naranpura Telephone Exchange, Ahmedabad 380063, Gujarat, India. Phone: +91 79-9724992949: 79-7874923334 e-mail: hvkbhatt@yahoo.co.in
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Introduction

The chewing of betel quid without tobacco is a widespread habit in the Orient. It began long ago when Portuguese people blended betel with tobacco in 1600 and called it betel quid, which was later made with areca nut, betel leaf, catechu, lime and tobacco. High consumption of betel quid is linked with oral cancer [1-6]. For two decades, a powdered chewing mixture, sold commercially as *pan masala (PM),* has been in abundant use in India among non-smokers as well as smokers who are attempting to refrain from smoking and tobacco use. Areca nut, a major portion of *PM,* is clastogenic, genotoxic and carcinogenic in animals [2, 7-8]. Catechu, an ingredient of *PM,* causes hepatic irritation, hyperplasia of oral mucosa [9], dominant lethal mutations and chromosomal damage in the bone marrow cells of mice [10]. Cancer incidence in southeast Asian countries is due not only to smoking but also to chewing betel quid and PM [5]. Betel quid is a mixture with different proportions of catechu, lime, areca nut, tobacco with spices, coconut, sugar and *gulkhand* (rose leaves and sugar). The different types of betel quid with certain addictive commercial products are sold in convenient one-dose sachets with familiar, popular brand names. *PM* is widely consumed by young and old individuals as a stimulant to remain anorectic and to compensate for a heavy extra workload; it is a mood elevator that provides relief from misery and grief. India and its neighboring countries are now in focus. Assam has the highest rates of cancers of the lip, buccal mucosa and pharyngeal areas due to the use of pan, betel nut and tobacco in betel quid or *PM* [11-12]. The tribal practice of reverse smoking with smokeless tobacco causes precancerous lesions [13], predominantly at the oral sites involved in squeezing and sucking the tobacco [14]. The proportions of the ingredients in *PM* differ by region. Assamese people incubate betel nuts with fibrous pericarp pits for 4 months and then strip the pericarp and use the endosperm (*tamul*). Gujarathi people make *mava* with dried betel nuts and lime, with or without tobacco. Raw kennel betel nuts, lime and tobacco are known as KWMP in Meghalaya. South Indians use betel leaves, nuts, lime and tobacco. Currently, northern Indians and people in the rest of the country consume *PM*. Worldwide, over 200 million people chew betel quid [15]. Juveniles may use as many as 30-40 betel quid per day, which may lead to the development of buccal cancer, stomatitis and pre-cancerous lesions. Many areas, such as the tongue, alveolus, buccal mucosa, hard palate, tonsils, oropharynx, larynx and esophagus are affected by chewing *PM* and smoking tobacco, which may act synergistically to cause leukoplakia [16], submucous fibrosis and cancers of the oral cavity, oropharynx, larynx and esophagus. The habit of chewing *PM* is relatively new and occurs all over the world, but it is most prevalent on the Indian sub-continent. The use of *PM* is gaining social acceptance, and consumption is also increasing, with an increase in the sales of *PM* products every day. The habit of *PM* chewing is also slowly gaining a foothold in Western countries.

A preliminary study of Indian *PM* showed 13 polycyclic aromatic hydrocarbons (*PAHs*), *DDT* (2,2,bis-*p*-chlorophenyl-l,l,l-trichloroethane) and *BHC* (benzene hexachloride) isomers. In addition, there were nitrosamine, toxic metals (lead, cadmium, nickel) and pesticide residues in *PM* reported to be unsafe. The genotoxic potential of *PM* constituents is reflected by an increase in the rate of sister chromatid exchange (*SCE*) and the chromosomal aberrations in mouse bone marrow cells from the Chinese hamster ovary (*CHO*) cell line [17]. Aqueous [18] and ethanol [19] extracts of different *PM* brands showed mutagenic potential. Thus, a preliminary report indicates that *PM* has carcinogenic, tumorigenic, teratogenic and mutagenic potential [20]. Therefore, we have analyzed different brands of *PM* and the toxicology of *PM* effects in pure inbred Swiss mice.

Materials and Methods

The study design includes two parts: A) the chemical portion and B) the experimental portion.

Chemical Studies

Polycyclic aromatic hydrocarbons (PAHs)

Different brands of *PM* were dissolved in various solvents. Standard samples of different *PAHs* and relevant standards were procured for the high-pressure liquid chromatography *(HPLC)* analysis. A comparison of the PAH levels (in *ug/g*) in *PM* to standard values was made by performing a concomitant test using *HPLC*.

Isomers of DDT and BHC

Various *PM* samples were extracted and analyzed using conventional methods of gas-liquid chromatography (*GLC*) in comparison with standards. The results are expressed in parts per million (*PPM*) or ug/gm of *PM*.

Atomic Absorption Spectrophotometer Analysis (*AAS*)

Different brands of *PM* (0.5 gm) were placed in flasks; 5 mL of double distilled water (DDW) and 15 mL of concentrated nitric acid (HNO_3) + 0.5 mL of concentrated sulfuric acid (H_2SO_4) were added and held overnight at room temperature. Next, the samples were dried in a sand bath until they became a black cherry color. They were processed by adding perchloric acid and nitric acid at a 2:1 ratio until the samples turned yellow, and a 10 mL sample was used for analysis.

Emission Spectroscopy (*ES*) for metal analysis

An ultraviolet (*UV*) spectrograph Q24 instrument with a slit attachment including condenser Q was used. The slit was

correctly set on the plate holder. The exposure wavelength scale was adjusted. The spectrograph image was adjusted from time to time, and the illumination of the camera lens was conducted according to the manufacturer's instructions. With the proper condenser distance, *UV* spectra were obtained to measure the elements by using a Fun Ken spectrum control plate.

Analysis of Narcotic Substances

Conventional *TLC* (thin-layer chromatography) was used. The spots obtained using *TLC* were compared with a standard sample for evaluation.

Gas Chromatography and Mass Spectroscopy (*GCMS*)

Five organic solvents were used: petroleum ether (60.80 grades), ethyl acetate and petroleum ether in the ratio of 20:1 on toluene, chloroform, acetone and ethanol 95%.

Extraction of *PM*

Sequential extraction was carried out with various organic solvents according to their polarity so that the compounds present in the material were extracted completely. The following steps are involved in sequential extraction:

1. The material was dried completely at 40-50°C in a hot air oven before extraction.
2. The material was ground to a 40 mesh-powder.
3. The material was weighed (10 g of powder) and transferred into a conical flask (500-1000mL).
4. The weighed material was macerated for 20-24 hr in various organic solvents at a 1:10 ratio in a constant sequence that was based on the polarity of the solvents. The following sequence of solvents was used: a) Petroleum ether (60-80°C) b) Benzene/Toluene, c) Chloroform, iv) Acetone and v) Ethanol (95%).
5. After 20-24 hr, the macerated material was filtered with Whitman filter paper #1. The extract was collected by complete vacuum evaporation in a Rota vaporizer.
6. The residue was collected from the filter paper again and macerated in the same solvent for 20-25 hours. Processing was repeated three times with each solvent. The three extracts of one solvent were pooled in the same evaporating dish.
7. The solvent was changed after the material was fully dried.
8. The dried extract of each solvent was stored in an airtight bottle for analysis by GCMAS.

Determination of the volatile oil content of PM

1. Volatile oil extraction was performed by distillation using Clevenger's apparatus.
2. Fifty grams of PM in 600 mL distilled water with a few pieces of porcelain in a distillation flask were connected to a Clevenger's apparatus and kept on a heating mantle.
3. The graduated receiver was filled with water with no air bubbles.
4. Distillation was performed for 4-5 hrs at a rate that kept the lower end of the condenser cool.
5. The distillate was collected in the graduated receiver. Volatile oil, because it is light, remains in the upper layer.
6. The volatile oil was measured in the graduated receiver tube and % v/w was calculated on a dry weight basis.

The oil was collected in a small vial, and sodium sulfate was added to adsorb any water and moisture so that the pure oil remained for storage in a refrigerator (From 50 g *PM*, 0.6 ml volatile oil was obtained (1.2% v/w)).

Results

Levels of Polycyclic aromatic hydrocarbons

PAH was estimated with Manik Chand Sada and with Zarda, and the values were compared to common brands of PM (Table 1).The highest quantities of PAH were observed in Rajnigandha, Pan Parag and Zarda, suggesting that they can produce ill effects in individuals who regularly consume *PM*. Furthermore, some of the PAHs are known to be potent carcinogens, and regular consumption of them can even lead to the development of cancer in the mouth, esophagus, stomach or other parts of the body.

Levels of HCH and DDT

The most common brands of *PM* were evaluated for the presence of HCH, DDT and their isomers. The data suggest the presence of different isomers of DDT and HCH, and the total quantity was higher than permissible limits (Tables 2a and 2b). Some of the samples, such as Santoor, contained a very high concentration of HCH. Both these pesticides are persistent in nature and remain for a long period in human bodies, and they can have adverse effects on health, including neurotoxic symptoms in exposed persons. The new samples we analyzed contained levels of pesticides similar to those seen in our earlier studies.

Proximate analysis of *PM* samples with Atomic absorption spectrophotometer

Five Sada samples and 5 Zarda samples of common brands of PM were analyzed. All of the samples were free of cadmium and lead. In our earlier studies, cadmium, lead and nickel were found in traces, but the most interesting finding of the analysis was the presence of copper, zinc and magnesium in excessive amounts in all the samples. Magnesium, in particular, was found in excessive amounts (Table 3). Copper and magnesium are suspected to be the metals that most strongly stimulate the production of fibroblasts, leading to fibrotic changes in mucosal membranes. Cases of submucosal fibrosis in human beings are

Table 1. Levels of Polycylic Aromatic Hydrocarbons (PAH) in pan masala (ppm or µg/gm)

Compound	a	b	c	d	e	f	g	h	i
1 Napththalene	0.75	0.59	0.39	0.33	0.99	13.99	0.66	1.04	1.07
2 Acenaphthylene	0.06	0.41	0.07	0.38	0.06	12.06	0.19	0.07	0.06
3 Acenaphthene	0.05	ND	0.05	0.13	0.05	0.05	ND	0.05	0.05
4 Fluorene	0.06	ND	0.20	0.48	0.06	ND	ND	0.13	0.13
5 Phenanthrene	0.06	0.13	0.07	0.08	0.06	0.15	0.03	0.05	0.04
6 Anthracene	0.01	0.01	0.01	0.11	0.01	0.11	0.04	0.01	0.01
7 Fluoranthene	0.09	0.07	0.07	2.21	0.07	1.61	0.17	0.04	0.04
8 Pyrene	1.02	0.38	0.60	11.32	2.01	13.50	2.89	0.61	0.58
9 Chrysene	0.08	0.13	0.07	0.06	0.08	ND	0.13	0.08	0.08
10 Benz (Q) pyrene+perylene	0.03	0.02	0.03	0.04	0.03	0.04	0.08	0.04	0.04
11 Benz(b) Fluoranthene	0.04	0.04	0.05	0.03	0.04	0.07	0.08	0.06	0.06
12 Benz(k) Fluoranthene	0.09	0.06	0.11	0.09	0.07	0.07	0.10	0.11	0.12
13 Benzo(a)pyrene	0.03	0.04	0.02	0.07	0.04	0.10	0.07	0.04	0.04
14 PAH Total	2.37	1.81	1.77	15.39	3.51	41.75	4.71	2.33	2.29

a: Pan Parag Sada, b: Santoor Sada, c: Nawabi Masala , d: Rajnigandha Sada, e: Manikchand Sada, f: Pan Parag Zarda, g: Santoor Zarda, h: Pan King Zarda, i: Manik Chand Zarda

Table 2. (a) Pesticide concentration (pm or µg/gm) in pan masal (sada)

Compound	a	b	c	d	e	f	g
HCH							
Alpha HCH	0.061	2.546	0.047	0.090	0.52	0.56	0.070
Gamma HCH	0.075	11.544	1.075	0.045	0.045	0.075	0.060
Beta HCH	0.055	13.997	0.319	0.077	0.077	0.092	0.080
Total HCH	0.191	28.087	1.441	0.211	0.211	0.223	0.210
DDT							
P,p'DDE	0.009	0.010	0.008	0.006	0.007	0.016	0.012
O,p'DDT	0.010	0.027	0.012	0.007	0.010	0.024	0.010
P,p'DDT	0.027	0.063	0.030	0.015	0.026	0.060	0.028
Total DDT	0.046	0.100	0.050	0.028	0.043	0.100	0.050

a: Pan Parag, b :Santoor, c: Nawabi Masala, d: Rajnigandha, e: Manikchan, f: Mahak, g: Chutk

likely due to excessive levels of these minerals. The body has the capacity to absorb or excrete excess amounts of these metals, but it is likely that repeated exposure to these minerals could have other adverse effects on the body.

Emission Spectroscopic analysis of *PM* samples

Nearly 40 samples of various brands of PM commonly used in Ahmedabad, Gujarat were analyzed using emission spectroscopy (Tables 4a and 4b). The results suggest that various metals are present (magnesium, copper, sodium, calcium, aluminum, potassium, manganese, silica, etc.) Continuous use of these brands of *PM* and the presence of these metals in excess may disturb the balance of these metals in the body, with diverse effects on both the individuals who use these brands regularly and those who use them in excess daily.

Qualitative estimation of narcotic substances and cannabis using the TLC method

The 10 most common varieties of *PM* were evaluated for charas, brown sugar, morphine and nicotine (Table 5).

Table 2. (b) Pesticide concentration (ppm or µg/gm) in pan masala (zarda)

Compound	a	b	c	d	e	f
HCH						
Alpha HCH	0.024	0.069	0.058	0.026	0.032	0.040
Gamma HCH	7.650	31.450	0.959	6.640	7.420	7.120
Beta HCH	0.037	19.632	1.165	0.037	0.046	0.036
Total HCH	7.711	51.151	2.182	6.703	7.798	7.196
DDT						
p.p' DDE	0.009	0.007	0.010	0.006	0.004	0.007
o.p' DDT	0.010	0.007	0.027	0.009	0.009	0.008
p.p' DDT	0.027	0.017	0.063	0.026	0.016	0.062
Total DDT	0.046	0.031	0.100	0.031	0.029	0.077

a: Pan Parag Zarda, b: Santoor Zarda, c: Panking Zarda, d: Manikchand Zarda, e: Mahak zarda, f: Chutki Zarda

Nicotine was present in excess in all of the samples, but no traces of narcotics or cannabis were present in any sample. However, the adverse effects of excessive nicotine in the body are known. It produces nausea in exposed persons, and it may also lead to the development of certain types of cancer in the gastrointestinal tract.

Experimental Studies

This experimental study was conducted with the approval of the local ethics committee. Pure inbred Swiss male mice were used for the experimental studies. The animals were exposed to PM mixed with food through *ip* and by oral intubation. In the short-term studies, primarily male Swiss mice were used. The aqueous and solvent (Dimethyl Sulfoxide; DMSO) extracts of the Manik Chand variety of *PM* with and without Zarda were prepared for exposure through the *ip* route.

Preparation of ethanol *PM* extract

Powdered Manik Chand brand of *PM* was mixed with 5 volumes of redistilled ethanol, and the contents were extracted by agitation using a rotary shaker overnight at room temperature. The supernatant collected after centrifugation at high speed in an ultracentrifuge was evaporated until dry. The dry residue was dissolved again in redistilled ethanol to obtain the desired dose of original *PM* powder.

Preparation of food with 2% *PM*

Manik Chand *PM* with or without *Zarda* was turned into powder using a mixer. A 100-g portion of this fine *PM* powder was mixed thoroughly with 4900 g of feed given to the mice routinely. Mice usually eat 5 g of feed per day; thus, 100 mg of *PM* was consumed by the experimental animals daily. Discrete biochemical changes in the testis were studied in mice by orally administering 3 different dose levels (i.e., 30, 40, 100 mg/kg b.w. of PMT extract) and a placebo (0.5 mL olive oil) for a period of 60 days using 4 sets of mice (n=10).

Animals and Treatments

Pure inbred Swiss male mice (and female mice in the chronic toxicity studies) 6 to 8 weeks old were used throughout the studies. Animals of the same sex were housed in a cage in an air-conditioned animal house (constant humidity

Table 3. Proximate (direct) analysis of pan masala samples

	Manik Chand		Pan Parag		Chutki	Rajni-gandh	Mahak		Tulsi	Vimol
	Tobacco	With-out	Tobacco	With-outN.T.[a]	N.T.[a]	N.T.[a]	Tobacco	N.T.[a]	Tobacco	Tobacco
Cadmium (µg/gm)	N.D.[b]	N.D.[b]	N.D.[b]	N.D.	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
Lead (µg/gm)	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	ND.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]
Cu (µg/gm)	21.6	17.60	21.44	19.68	5.92	19.36	14.56	13.28	9.60	15.68
Zn (µg/gm)	11.78	15.30	11.78	10.11	9.85	11.21	11.69	7.82	7.56	10.59
Mg (µg/gm)	7561.6	4345.89	3878.42	5321.42	19859.6	3642.12	11691.73	2688.64	7530.82	19501.0

[a]: Not Traceable, [b]: Not detected

Table 4. (a) Analysis of pan masala samples

Name of the sample	Mg	Cu	Na	Ca	Al	K
Vazin Gutkha (Tobacco)	++ (Excess)	+ (Present)	+	++	Traces	+
Kisan Gutkha (Tobacco)	++	+	+	++	-	+
Dawat Gutkha (Tobacco)	++	+	+	++	-	+
Zat Pat Gutkha (Tobacco)	++	+	+	++	-	+
Soni Gutkha (Tobacco)	++	+	+	++	-	-
Kuber Gutkha (Tobacco)	++	Traces	Traces	++	-	-
Mirad 2000 (Tobacco)	+	-	-	++	-	-
Budhlane (Tobacco) Raju Supari (Plain)	++	FT (Freely traceable)	+	++	-	FT
Raju Supari (Plain)	++	Traces	++	++	-	Traces
Dillagi Supari (Plain)	++	++	-	++	Traces	++
Plain Kacchi Supari						
Plain Kacchi Supari	+	FT	-	++	Traces	-
Saheli Sweet Supari (Plain)	++	-	-	++	Traces	-
Poonam Supari (Plain)	++	Traces	-	++	Traces	-
Simple Roasted Supari (Plain)	++	-	-	++	-	-
+ *Present but could not quantified*						
- *Nil*						

Table 4. (b) Analysis of pan masala samples

Name of the sample	Si	Mn	Mg	Cu	Na	Ca
Manikchand Gutkha	-	-	+	FT (Freely treaceable)	FT	+
Tulsi Mix	-	-	+	FT	FT	+
Manikchand Plane	Traces	Traces	+	+	Traces	+
Rajnigandha Plain	+ (Present)	Traces	+	++	Traces	+
Pan Parag Tobacco	-	Traces	+	FT	Traces	+
Pan Parag Plain	-	-	+	FT	FT	+
Mahak Tobacco	-	-	+	FT	FT	+
Mahak Plain	Traces	-	+	FT	-	+
Moolchand with Tobacco	-	-	+	Traces	Traces	+
Tara Gutkha Tobacco	-	-	+	Traces	FT	+
Vimal Gutkha (Tobacco)	Traces	-	+	FT	FT	+
Pan King (Tobacco)	-	-	+	-	FT	+
Kuber Khaini (Tobacco)	-	-	+	-	Traces	+
Katha Paste	-	-	+	-	FT	
Janta Banarasi Brand	Traces	Traces	-	-	-	-

Table 5. Qualitative estimation of narcotic and cannabis substances in pan masala

Name of the sample	Charas	Brown Sugar	Diacetyl Morphine	Morphine	Papavarine	Opium	Nicotine
Manikchand Gutkha (Tobacco)	-	-	-	-	-	-	+ (Present)
Pan Parag utkha (Tobacco)	-	-	-	-	-	-	+
Dawat Gutkha (Tobacco)	-	-	-	-	-	-	+
Mahak Gutkha (Tobacco)	-	-	-	-	-	-	+
Tulsi Mix Gutkha (Tobacco)	-	-	-	-	-	-	+
Moolchand Gutkha (Tobacco)	-	-	-	-	-	-	+
Som Gutkha (Tobacco)	-	-	-	-	-	-	+
Vazir Gutkha (Tobacco)	-	-	-	-	-	-	+
Vimal Gutkha (Tobacco)	-	-	-	-	-	-	+
Zat Pat Gutkha (Tobacco)	-	-	-	-	-	-	+

55.6%; 12-hr light/dark cycle) and were provided with food prepared in the Institute. The composition of the food was 70% wheat, 20% cracked Bengal gram, 5% fishmeal, 4% yeast powder and 1% shark liver oil in the form of dry mesh, and water was given *ad libitum*. A group of animals were exposed by the ip route through oral intubations and *PM* mixed into their feed. Through oral intubations, the animals were given the scheduled dose 5 days each week, whereas *PM* mixed in feed was given daily. The institutional ethical committee for animals approved the experimental protocols and the maintenance of the animals and their usage during this study.

Toxicological evaluation through *ip* and *po*

The aqueous and solvent (DMSO) extracts of the *Manik Chand* brand of *PM* with and without *Zarda* were prepared. A preliminary experiment to evaluate the toxic dose level was initiated by exposing mice to doses of 0.2, 0.5, 1.0 and 2 g/kg bwt PM through the ip route. The animals could not tolerate doses of 0.5 g/kg b wt and above; therefore, the experimental animals were actually exposed to 8, 40 & 200 mg/kg bwt. One group (a) was exposed for two weeks *ip,* and another group was exposed through oral feeding for three months. Distilled water (0.1 mL) or DMSO (02 mL) was administered as a negative control, while benzo[a]pyrene or trichloroacetic acid was used as a positive control. After scheduled *ip* exposure, only histopathological and hematological evaluation could be done, whereas in the group exposed for 3 months, histopathological hematological cytogenetic and spermatological studies were performed. (b) Another group of animals received ethanol *PM* extract through oral intubations for ninety days. Special emphasis was placed on examining testicular tissue, and biochemical parameters, such as glycogen, cholesterol and protein, were estimated in testicular tissue. Histopathological alterations and the weights of different organs were also recorded. (c) For the chronic toxicity studies, *PM* with and without *Zarda* was mixed with feed and fed to the animals routinely.

This experiment offered a direct and effective way to identify the chemicals responsible for spermatogenic damage in rodents and men. The exposed animals were euthanized after two weeks or three months by cervical dislocation, and the cauda epididymis was removed. Suspensions were prepared from each cauda by mincing the cauda in 4 mL phosphate buffer saline (pH 6.8) and allowing the mixture to stand for 15 minutes at room temperature. Tissue fragments were removed, and an aliquot (0.5 ml) of the sample was mixed (10.1) with 1% aqueous eosin. Smears were made 30 minutes later from the stained sperm suspension. The slides were allowed to dry in air and mounted with per- mount. Ten slides were prepared from each mouse (5 slides per cauda), and the head shape morphology of 100-200 sperm (1000-2000

sperm per mouse) was examined under the light microscope at 400-fold magnification. Different organ weights were also recorded. The data were analyzed for statistical significance by applying Student's t test and the X^2 test.

Bone marrow metaphase assay

The animals were injected with colchicine solution 1.5 hr prior to bone marrow sampling (4 mg colchicine/kg b/wt). Bone marrow was flushed in HBSS, centrifuged for five minutes. The supernatant was discarded, and then hypotonic solution (1% sodium citrate) was slowly added to dispense the pellet. The mixture was centrifuged again after hypotonic treatment cells were fixed to the re-suspended pellet in a freshly prepared cold methanol/acetic acid mixture (3:1). The slides were stained with Giemsa 5% for 10 minutes and then examined for any chromosomal abnormalities.

Biochemical estimation

The estimation of protein was done according to a previously reported method [21]. A known amount of liver tissue was homogenized in 5 mL of distilled water. To 1 ml of homogenate (which was obtained after centrifugation), 4 mL of biuret reagent was added. Distilled water was added in place of the homogenate. The tubes were kept at room temperature for 30 minutes. The resultant color intensity was recorded at 540 nm.

The protein concentration was determined using the following regression formula:

$$X = 0.077 + 18.70\ (y)q$$

Where X = Concentration of protein in mg

Y = O D of unknown sample

$$\text{Calculation: Amount of protein} = \frac{\text{Concentration of Mean}}{\text{Tissue weight}} \quad \frac{\text{OD Dilutrin X 100}}{\text{Aliquot volume}}$$

Histopathological examination: After the animals were euthanized, different organs were fixed in 10% formal saline. Histopathology is currently being conducted using conventional methods, and the results will be reported at a later date.

Statistical analysis

Student's *t* test was employed [22].

Chemicals

All the chemicals used were analytical grade products from Sigma-Aldrich Company with impurities ranging from 0.001% to 0.0001%.

Results

***IP* exposure to *PM* for 16 days**

Hematological changes, including RBC and WBC counts, differential hemoglobin and ESR, were analyzed in groups exposed to different doses of PM and compared with positive and negative control data (Table 6). The data suggest that there is no significant change in the hematological values of the experimental groups compared to the controls.

Oral Administration of *PM* for three months

After 3 months of *PM* administration, the animals' final weights were measured and compared to their initial weights. Blood was collected directly from the heart, and then the ani-

Table 6. Haematological findings intraperitoneal (*IP*) exposure to Manikchand pan masala in Swiss inbred mice

Groups	Units	R.B.C.[f] Million/mm^3	W.B.C.[g] -/mm^3	DC%[h] P	L	E	M	B	Hbi gm%	E.S.R.[j] mm
Negative control (NC)	0.1mL DMSO[d]	5.71±0.1	8682±140	N=6 47.5±8.81	N=6 51.83±8.94	N=6 0.83±0.17	-	-	13.42±0.54	9.25±1.19
Positive control (PC)	4 mg/kg b.wt BAP on 0.1mL DMSO[e]	5.09±0.2	8000±570.7	N=6 59.36±5.34	N=6 40.36±5.40	N=6 0.71±0.29	-	-	15.58±0.47	8.0±0.87
Pan Masala I (PM[a])	40 mg/kg b wt	5.18±0.6	7300±475.6	N=6 32.7±1.69	N=6 67.0±1.63	N=6 0.2±0.13	-	-	16.1 ± 0.39	12.2±1.38
Pan Masala II (PM[b])	200 mg/kg b wt	4.85±0.7	7400±507.2	N=4 63.0±2.08	N=4 37.2±2.08	N=4	-	-	13.33±0.37	5.58±0.30
Pan Masala III (PM[c])	8 mg/kg b wt	5.14±0.2	8180±202.4	N=6 42.5±4.29	N=6 57.0±4.18	N=6 0.5±0.29	N=6	-	16.25±0.25	5.39+0.39

NC, PC, PM[a] and PM[c] = Each group 16 days exposure, PM[b] = 8 days exposure, [de]Vehicle for NC and PC
(n=6 mice/group) Each 6 weeks old. P=Polymorphs, L=Leucocytes, E=Eosinophils, M=Monocytes, B=Basophiles, R.B.C.=Red Blood Corpuscles, W.B.C.=White Blood Corpuscles, DC=Differential Count, Hb=Hemoglobin, E.S.R.=Erythrocyte Sedimentation Rate

mals were euthanized. Organ weights were recorded (Tables 7a and 7b). There were apparently no changes in organ weights compared to the controls. No significant changes were seen in the hematological parameters in the *PM/sada* group or the *Zarda* group when compared with the control group (Tables 8a and 8b).

Bone marrow chromosomes

After 2 weeks of *ip* exposure and 3 months of continuous oral exposure to PM with and without *Zarda,* there was a pronounced effect on the bone marrow chromosomes of the experimental mice when compared with the controls. The effect appeared to be more pronounced in the *Zarda* group; when compared with the plain *PM* group, there was a significantly higher prevalence of chromosomal abnormalities (Table 9). The changes observed were ploidy loss, breaks, gaps, deletions and exchanges, including ring chromosomes.

Sperm head abnormalities: Compared to the controls, there were no significant differences in the organ weights of the kidneys, heart, spleen or liver. However, a slight reduction in testicular weight was observed. There was a slight (although non-significant) reduction in testicular tissue, but there were more pronounced changes in sperm head abnormalities, which included narrow, blunt, triangular, and banana shapes, irregular amorphous shapes, and tail-less and rudimentary sperm (Table 10). The effects were more pronounced in the group with 3 months of exposure.

Biochemical findings

When biochemical parameters, including glycogen, cholesterol and protein, were evaluated in testicular tissue, there were highly significant changes in glycogen, cholesterol level and protein content. This effect appeared to be more pronounced in the group that consumed more *PM* (Table 11).

Table 7. (a) Body and organ weights of pan masala extract treated pure inbred Swiss mice

Treatment	Body weight (gm)		Testes	Liver	Heart	Kidney	Spleen
	Initial	Final	(gm)	(gm)	(gm)	(gm)	(gm)
Group I (n=6)	21.5	22.5	0.150	1.51	0.120	0.401	0.043
Intact control or	22.0	23.4	0.164	2.07	0.140	0.430	0.097
vehicle treated	26.0	27.4	0.174	1.78	0.135	0.429	0.077
	20.5	22.4	0.168	1.62	0.129	0.400	0.052
	24.0	25.2	0.160	1.70	0.130	0.398	0.095
	25.0	26.1	0.196	1.58	0.120	0.500	0.072
Group II (n=6)	26.5	27.0	0.152	2.25	0.138	0.480	0.-066
Intact+Pan	23.5	24.6	0.146	1.78	0.129	0.420	0.043
Masala 20	27.0	28.2	0.164	1.90	0.150	0.410	0.102
Mg/kg b wt/day	27.5	28.0	0.160	1.80	0.110	0.385	0.056
for 60 days	24.0	25.8	0.164	1.65	0.145	0.400	0.074
	22.0	22.8	0.150	1.87	0.130	0.390	0.084
Group III (n=6)	28.0	29.0	0.146	2.31	0.132	0.407	0.191
Intact+Pan	24.5	25.4	0.164	2.18	0.119	0.415	0.096
Masala 40	24.0	25.0	0.150	1.90	0.105	0.400	0.069
Mg/kg b wt/day	25.0	25.8	0.152	2.07	0.126	0.368	0.085
for 60 days	22.0	23.2	0.160	1.87	0.136	0.368	0.138
	26.0	26.8	0.174	2.47	0.141	0.496	0.127
Group IV (n=6)	24.5	25.0	0.151	2.56	0.136	0.500	0.102
Intact+Pan	26.5	27.2	0.162	2.43	0.140	0.475	0.100
Masala 100	26.5	27.3	0.164	2.34	0.136	0.407	0.070
Mg/kg b wt/day	24.0	26.0	0.141	2.40	0.149	0.419	0.090
for 60 days	23.0	24.0	0.146	1.90	0.153	0.410	0.095
	22.0	23.4	0.140	1.78	0.120	0.429	-

Table 7. (b) Body and organ weights of pure inbred Swiss mice after oral admini-stration of Manikchand pan masala for 3 months

Treatment	Body weight (gm)		Testes	Liver	Heart	Kidney	Spleen	Lungs
	Initial	Final	(gm)	(gm)	(gm)	(gm)	(gm)	(gm)
Control	24	28	0.168	1.51	0.120	0.401	0.043	0.180
	28	29	0.164	2.07	0.140	0.430	0.097	0.210
	26	28	0.174	1.78	0.135	0.429	0.077	0.205
	24	26	0.183	1.62	0.129	0.400	0.052	0.190
Manikchand	26	28.2	0.132	1.70	0.130	0.398	0.956	0.200
Plain (8 mg)	28	29.8	0.150	1.58	0.120	0.500	0.072	0.195
	26	28.2	0.169	2.25	0.138	0.480	0.066	0.188
	20	23.1	0.172	-	0.129	0.420	0.043	0.210
Manikchand	24	26.4	0.142	1.78	0.15	0.410	0.102	0.15
Plain (40mg)	26	27.8	0.140	1.90	0.11	0.385	0.056	0.19
	26	27.8	0.165	1.80	0.145	0.400	0.074	0.17
	28	27.6	0.140	1.65	0.130	0.390	0.084	0.19
Manikchand	24	26.2	0.148	1.87	0.132	0.339	0.191	0.207
Plain (100 mg)	30	31.4	0.176	2.31	0.119	0.357	0.096	0.183
	26	27.8	0.132	2.18	0.105	0.315	0.069	0.179
	24	25.8	0.150	-	0.126	0.407	0.085	0.190
Manikchand with	25	26.6	0.148	2.08	0.136	0.419	0.080	0.194
Zarda	26	28.0	0.143	2.02	0.140	0.405	0.100	0.199
(8 mg)	24	26.2	0.150	2.12	0.136	0.400	0.050	0.205
	25	27.1	0.162	1.98	0.117	0.038	0.900	-
Manikchand with	26	27.6	0.148	2.43	0.149	0.486	0.075	0.180
Zarda (40 mg)	28	29.4	0.134	2.01	0.162	0.496	0.090	0.175
	28	29.6	0.142	1.98	0.153	0.500	0.095	0.221
	24	25.8	0.162	1.96	0.120	0.475	0.800	0.221
Manikchand	26	28.2	0.142	1.92	0.136	0.300	0.094	0.180
with Zarda	28	29.2	0.136	1.86	0.140	0.419	0.056	0.135
(100 mg)	26	28.1	0.155	2.22	0.136	0.486	0.061	0.140
	28	29.2	0.149	2.42	1.49	0.475	0.075	0.160

6 Animals in each group. (n=6)

After 60 days of PMT exposure, glycogen and cholesterol in the testis showed significant deterioration at all the dose levels compared with the controls, although the effect was not dose-dependent. Protein, although significantly higher in the 30-mg/kg group, was significantly lower at the 40- and 100-mg/kg dose levels compared with control (vehicle treated) mice. Discrete biochemical changes of the testis were studied in 4 sets of mice (n=10) that received orally administered PMT extract at 3 dose levels (i.e., 30, 40, 100 mg/kg b.w.) or a placebo (0.5 mL olive oil) for a period of 60 days.

Discussion

The study determined the chemical components of plain and blended *PM* as well as its sub-acute and biologic effects on weight, blood, bone marrow and sperm. The chemical composition of *PM* was found to be responsible for its biochemical actions and toxicity. Currently, *PM* usage is spreading widely among youth, workers and executives because it is a more effective mood elevator than smoking and may also act as a central nervous stimulant, offering temporary relief

Table 8. (a) Oral administration of pan masala to pure inbred Swiss male mice (Haematological findings-3 months)

Groups	Units	R.B.C. Million/mm^3	W.B.C. -/mm^3	DC% P	L	E	M	B	Hb Gm%	E.S.R. mm
Negative Control	0.4ml sterile water	5.71±0.1	8680±140	21.2±0.6	72.3±0.84	-	1.5±0.3	-	13.4±0.5	10.±0.7
Positive Control	25 mg/kg TCA b.wt	5±0.2	8000±460	19.7±3.2	81.3±0.99	0.33±0.2	1.2±0.3	-	15.5±0.5	11.3±1.9
Pan Masala With Sada I	8 mg/kg b.wt.	5.1±0.4	7300±475	26±1.17	71.3±1.87	0.17±0.1	2.5±0.4	-	13.6±0.4	7.7±1.6
Pan Masala With Sada II	40 mg/kg b.wt.	4.9±0.2	7100±407	27±1.13	70±1.24	0.33±0.2	2.7±0.3	-	13.3±0.3	9.4±0.48
Pan Masala With SadaIII	200mg/kg b.wt.	4.6±0.7	7050±202	26.4±3.6	70.67±3.6	1±3.6	1.3±0.4	-	12.2±0.2	8.5±0.43

(n=6 mice/group) Each 6 weeks old . P=Polymorphs, L=Leucocytes, E=Eosinophils, M=Monocytes, B=Basophiles, R.B.C.=Red Blood Corpuscles, W.B.C.=White Blood Corpuscles, DC=Differential Count, Hb=Hemoglobin, E.S.R.=Erythrocyte Sedimentation Rate

Table 8. (b) Oral administration of pan masala to pure inbred Swiss mice (Haematological findings-3 months)

Groups	Units	R.B.C. Million/mm^3	W.B.C. -/mm^3	DC% P	L	E	M	B	Hb Gm%	E.S.R. mm
Negative control	0.4ml sterile water	5.71±0.1	6.97±0.6	28±2.6	71±2.5	-	0.7±0.3	-	13.4±0.5	10.±0.7
Positive control	25 mg/kg TCA b.wt	5±0.2	5.3±0.34	35.8±2.9	62.6±3.2	0.38±0.3	1±0.2	-	15.5±0.5	11.3±1.9
Pan Masala With Zarda I	8 mg/kg b.wt.	4.1±0.2	5±0.4	35±2.3	62.8±2.2	0.4±0.4	1.8±0.2	-	13.1±0.4	8.8±1.6
Pan Masala With Zarda II	40 mg/kg b.wt.	4.12±0.6	5.51±0.4	32.8±2.2	64.1±2.1	0.92±0.3	2.2±0.2	-	12.9±0.2	9.4±0.48
Pan Masala With ZardaIII	200mg/kg b.wt.	4.1±0.7	5±0.58	35±2.9	61.6±3.1	1.45±0.2	1.5±0.3	-	12.2±0.2	8.6±0.43

(n=6 mice/group) Each 6 weeks old . P=Polymorphs, L=Leucocytes, E=Eosinophils, M=Monocytes, B=Basophiles, R.B.C.=Red Blood Corpuscles, W.B.C.=White Blood Corpuscles, DC=Differential Count, Hb=Hemoglobin, E.S.R.=Erythrocyte Sedimentation Rate

from grief and exhaustion due to manual and non-manual stress. Many reports on *PM* are available, but there are no long-term experimental studies about *PM* toxicity and the related health impairments. For example, 95% rats developed dysphasia and carcinogenesis over a period of time of dermal pasting *PM* [23]. Further effects, including leukoplakia, submucous fibrosis, loss of nuclear polarity, keratosis, parakeratosis, inflammatory cell infiltration, vascularity and decreased weight of the gonads and the brain [24], were found. Bone marrow and chromosomal deformities in mice fed *PM* were also reported [25]. Recently, carcinogenic effects were confirmed in rats fed *PM* for 56 weeks [20]. Our data addresses the composition of *PM* and its *po* and *ip* effects, over both short-term and long-term periods. According to our data, the toxicity of *PM* administered *po* and *ip* in mice is obvious, and the toxicity depends on the constituents and the dose administered. The areca nut has significant toxicity [26]. It contains many alkaloids, and its active component, arecoline, has been shown to induce cancer as a result of its reaction with cysteine *in vivo* and *in vitro*. The reaction between polyphenols and catechu and lime produces cysteine/3 alkylation addicts and superoxide ions and antioxidants [27].

In practice, many forms of *PM* consumption and the increasing number of components in *PM* betel quid cause a wide range of health impairments. For instance, catechu and lime are smeared on the betel leaf, and then pieces of areca nut and tobacco are added in addition to flavoring agents (such as cardamom, cloves, coconut, ginger, sugar and gulkhand (rose petals and sugar)) before betel quid consumption. Thus, clinical assessment is difficult and must include tracing the etiology of disease by recording the history and lifestyle of the patient. The tobacco in betel quid

Table 9. Effect of oral administration of Manikchand pan masala with and without zarda on the somatic (bone marrow) chromosomes of pure inbred Swiss mice after 2 weeks and 3 months exposure

Group	Dose (mg/kg b wt)	Chromosomal abnormalities (%)	
		2 weeks	3 months
Control (Distilled water)	0.2 mL	1.08	2.20
Manikchand Plain	8	1.82	1.74
Manikchand Plain	40	3.62	3.88
Manikchand Plain	100		
Manikchand with Zarda	8	4.20	5.64
Manikchand with Zarda	40	4.6	5.82
Manikchand with Zarda	100	5.8	6.60

6 Animals killed in each group (n=6)
bwt-body weight
p<0.01 (Contro verses test groups

Table 11. Biochemical changes in testicular tissue after administration of pan masala exctract

Treatment (n=6)	Glycogen (mg/gm)	Cholesterol (mg/gm)	Protein (mg/gm)
Group I: Vehicle treated group	3.46±0.04	8.62±0.2	182.2±2.5
Group II: 30 mg/kg bwt/day for 60 days	1.82±0.04*	5.22±0.2*	1130.32±1.4*
Group III: 40 mg/kg bwt/day for 60 days	1.92±0.02**	5.12±0.12*	108.07±0.2**
Group IV: 100 mg/kg bwt/day for 60 days	1.80±0.01**	3.75±0.2**	120.64±2.2*

Mean±SEM of animals
N.S.=Non-significan Group II, III & IV were compared with I.
*p<0.01-Significant
**p<0.001-Highly significant
bwt-body weight

(28) and catechu (tannin) in *PM* (IARC 1985) were found to cause cancer. Chewing areca nuts caused submucous fibrosis [29] but seldom ended in malignant cancer [30]. *PM, BQ* and areca nut chewing resulted in oral submucosis much earlier in a younger group that consumed only PM. Cytogenic anomalies, such as sister chromatid exchange, chromosomal aberrations and micronuclei were found in *PM* and tobacco consumers [8].

Conflict of interest statement: The authors declare that they have no conflict of interest to the publication of this article.

Table 10. Effect of oral administration of Manikchand Pan Masala on the sperm after 2 weeks and 3 months exposure

Group	Dose (mg/kg b wt)	Sperm abnormalities (%)	
		2 weeks	3 months
Control (Distilled water)	0.2 mL	1.42	1.48
Manikchand Plain	8	1.56 NS	1.82 p<0.05
Manikchand Plain	40	1.82 p<0.05	1.96 p<0.05
Manikchand Plain	100	2.26 p<0.01	3.64 p<0.01
Manikchand with Zarda	8	2.18 p<0.01	2.62 p<0.01
Manikchand with Zarda	40	2.62 p<0.01	2.88 p<0.01
Manikchand with Zarda	100	3.82 p<0.001	4.66 p<0.001

6 Animals in each group
p<0.01 Significant (Significance: Control versus test group)
p<0.001 Highly Significant
NS: Not Significant

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