Research Paper

Determination of Trace Metals, Moisture, pH and Assessment of Potential Toxicity of Selected Smokeless Tobacco Products

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The characterization and classification of smokeless tobacco products has been a continuously evolving process. This is based on a number of different parameters like nicotine content, moisture content, amount of heavy metals, pH, and *in vitro* cytotoxicity assays. Their contexts often vary between countries, research institutions, and legal requirements. The categorisation of these products is quite challenging due to the diffused sample sizes, diverse array of branded products on offer, and the absence of a centralized manufacturing facility. This study aims at a systematic classification of 10 smokeless tobacco product samples from the retail market based on their potential toxicity upon long-term use. The estimation of potential toxicity follows a well-established method that employs the concentration of toxic metals in the different samples. The potential toxicity as well as heavy metal concentrations of the smokeless tobacco products analysed was found to be much higher than acceptable limits. For instance, the levels of lead, cadmium, copper and zinc of 2.5, 1, 4 and 23 ppm, respectively, are well above their recommended limits. The results from the study indicate that chronic use of smokeless tobacco products is a significant health risk, especially in the vulnerable population. Further studies of this nature will help establish a toxicological fingerprint on the diverse class of products that floods the market now.

Key words: Smokeless tobacco products, tobacco pH, chewing tobacco, potential toxicity, trace metals

Tobacco and related products exemplify a liberally used class of recreational substances. Their use remains an integral part of the sociocultural life of Indians. While rural men sharing a casual hookah supposedly symbolises solidarity and brotherhood, the act of smoking has started to represent modernity among the urban women. Although *bidi* and cigarette smokers who constitute 60% of the tobacco using population make smoking the preferred route of tobacco intake, smokeless tobacco product (STP) users who account for the remaining 40% of the share still represent a significant proportion[1]. Some of the common STP variants like Manipuri tobacco, *khaini, snus,* and *mawa* that are popular in the North Indian states comprise differing compositions of areca nut, slaked lime, camphor, and cloves besides tobacco[2].

A STP prevalence survey conducted by the International Institute of Population Sciences at the turn of the millennium suggested that 9.4% of the population in the 15-19 year age group and 37.6% of the 60-plus year olds used chewing tobacco in India. With 0.8-0.9 million deaths annually, India accounted for about one-fifth of the global tobacco related mortality[1]. Apart from the large death toll, tobacco use in India has resulted in 3.9 million cases of chronic obstructive pulmonary disorder, 0.16 million cases of cancer as well as 4.5 million cases of heart diseases. It is also reported that their is a cummulative 1.7 million cases of disability-adjusted life years in 1990 due to tabaco use[3,4]. It has been confirmed that metals are preferentially accumulated into the leaves and other parts of the tobacco plant during its cultivation and growth[5]. Tobacco products are therefore a potential source of toxic heavy metals. International Agency for Research on Cancer (IARC) has identified and listed about 4000 chemical agents as potential carcinogens present in tobacco products[6]. This list includes toxic substances such as tobacco specific nitrosamines (TSNAs), inorganic ions like nitrate and nitrite as well as heavy metals like cadmium, lead, beryllium, chromium, and nickel[6,7].

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Chemically a basic alkaloid, nicotine remains unionised at an alkaline pH. Hence, pH of the product is an important determinant of the bioavailable fraction of free nicotine. Moisture is another major factor influencing nicotine permeation and moist snuff preparations are typically absorbed better than the dry products[8]. This study, carried out using STPs available in a South Indian market, was aimed to estimate the moisture content and pH of these select products as well as to quantify the different heavy metals present in them which, in turn, was used to assess their carcinogenic potential with the help of well-established international standards. We hope this study will help the society to reach a consensus about the toxicity of the STPs sold in the retail market and stimulate the authorities to take the required regulatory measures to control the use and sale of these products.

MATERIALS AND METHODS

Ten different brands of STPs were collected from the local retail markets of Cochin (Kerala) from the months of August to November 2011. The samples were selected on the basis of popularity among the people especially the teenage community, representing a large and uniform sample pool. They were personally collected from the shops due to the absence of a national level manufacturer. These were then labeled with unique identification codes and stored in airtight polythene bags under refrigeration. The samples were thoroughly ground and passed through 5 mesh size before being taken for analysis. For ease of use, the samples have been identified with numbering as samples 1-10: Sample 1 - DS Madras Snuff, Sample 2 - Shambhu, Sample 3 - Minar, Sample 4 - Madhu, Sample 5 - Cool Lip, Sample 6 - Hans, Sample 7 - Parag 9000, Sample 8 - Chaini Khaini, Sample 9 - Bombay 1000, Sample 10 - Rajanigandha.

Trace metal analysis:

The sample preparation procedures adopted to analyse trace metals are based on Health Canada, Centres for Disease Control and the United States Federal Registers and modified in-house techniques based on the most up to date information from published literature[9-11]. All the reagents used were of analytical grade unless specified otherwise. All the glassware were washed in distilled deionised water and dried in oven prior to use.

Three grams of the accurately weighed samples were digested using a 3:1 mixture of concentrated nitric acid and hydrochloric acid over a laboratory grade furnace. The samples were then filtered; the filtrate washed with distilled deionised water and made up to 100 ml in a standard flask. These samples were subsequently analysed by an atomic absorption spectrometer (GBC – Avanta, 2-0-2 version from GBC Scientific Equipments Pvt. Ltd., Victoria, Australia). A mixture of air-acetylene was used as fuel for all the elements except chromium and cadmium for which nitrous oxide-acetylene mixture was used. The air flow was maintained at 10 l/min while the fuel flow was adjusted to 2 l/min. The mean measuring duration was 3 s with an integrated measuring mode. The instrument parameters for the trace metal analysis are depicted in Table 1.

Analysis of pH:

This protocol revolved around the guidelines prescribed by the Federal Register[12] with slight in-house modifications. The freshly opened samples were extracted with 20 ml distilled water using a mechanical shaker and the clear supernatant was taken for analysis. The samples were analysed in triplicate using a laboratory grade pH meter (Digital pH meter M.K 6, Systronics, Ahmedabad) and the results expressed as mean pH±SD.

Moisture estimation:

The samples were prepared in accordance with Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA)-recommended method N°56[13] (for Karl Fischer method) and 57[14] (for Gas Chromatography-Thermal Conductivity Detector (GC-TCD) method) with slight in-house modifications in the techniques.

TABLE 1: INSTRUMENT PARAMETERS FOR TRACE METAL ANALYSIS

Metal	Lamp current (mA)	Wavelength (nm)	Slit width (nm)
Zinc	5	213.9	0.50
Magnesium	3	285.2	0.50
Nickel	4	232.0	0.20
Chromium	6	357.9	0.20
Iron	7	248.3	0.20
Cadmium	3	228.8	0.50
Lead	5	217.0	1.00
Copper	3	324.7	0.50

Variations in the lamp current, wavelength, and slit width for the different metals analysed

Moisture content was analysed by Karl Fischer titrator (Mettler Toledo model DL18 equipped with Mettler GA44 printer and Mettler AE200 balance) under conditions specified in CORESTA[13]. Five hundred milligram of the sample was extracted using Karl Fisher grade anhydrous carbinol and the clear supernatant was used for further analysis. The whole procedure was carried out in sealed flasks to avoid contamination with atmospheric moisture.

The moisture content was also determined by GC–TCD model HP5890 (Hewlett Packard). The GC was equipped with a porapack column of 25 m length with 0.53 mm internal diameter under conditions similar to those specified in CORESTA[14]. The same sample preparation procedure was adopted as that for the Karl Fischer method. Nitrogen at a flow rate of 30 psi was used as the carrier gas with the injection volume and oven temperature being maintained at 2 µl and 130º, respectively.

Assessment of potential toxicity:

The carcinogenic potential of the STPs was calculated using the method adopted by Ayo-Yusuf *et al.*[15] for which comparable carcinogenic potency data is available in the University of California carcinogenic potency database[16]. The following formulae were used:

Lifetime cancer risk=$ADE_{lifetime}$×CPF… (1), where $ADE_{lifetime}$= lifetime average daily oral exposure (mg/kg bodyweight/d) and CPF=cancer potency factor ((mg/kg bodyweight/d)$^{-1}$). Now, $ADE_{lifetime}$ = ADE × no. of years of snuffing/average lifetime… (2), where the ADE was calculated assuming the daily consumption of 10 g of STP by an individual for a period of 30 years out of an average lifespan of 70 years.

The CPF values for lead and cadmium to be used in formula 1, were obtained from Ayo-Yusuf *et al.*[15] as cadmium: 46.1 ((mg/kg bodyweight/d)$^{-1}$) and lead: 0.02 ((mg/kg bodyweight/d)$^{-1}$). This method of estimating potential toxicity is based on the assumption that 100% of the toxicant is potentially bioavailable in ideal conditions and can fully contribute to the overall risk of the product. Since some literature reports[17,18] justify the relevance of 6% bioavailability in the context of potential toxicity, this parameter has also been computed. However, all further calculations have been carried out under the assumption of an ideal 100% transfer.

The metals for which data was not available in the carcinogenic potency database were compared with their standard permissible daily intake levels in published literature[19-21]. These reference values for copper, nickel, iron, zinc, and chromium are 50, 20, 250, 300, and 25 µg/kg/day, respectively. The tolerable upper intake level of magnesium for 14-70 year old individuals as per the National Institutes of Health is 350 mg/day.

RESULTS

The recorded pH of the 10 samples was found to range from 7.18±0.00 to 10.21±0.01 with a mean pH of 9.18. Sample 5 had the highest value of 10.21 and sample 3 gave the lowest value of 7.18. Samples 2, 5, and 8 presented a pH around 10 (Table 2).

Moisture content of STPs is one of the key players influencing the nicotine delivery capacities of the product. In this study, moisture has been estimated using two methods viz. Karl Fischer titration method and gas chromatography method. A comparison of these results (Table 2) clearly indicated some variation in moisture content among the samples. The data from the Karl Fischer method was taken for further analytical interpretation due to its higher specificity compared to the GC method. The percentage moisture values obtained encompasses a range from 28.81% for sample 9 to 62.90% for sample 2 with a mean of 44.28%.

The heavy metal concentrations of the 10 brands of STPs expressed in ppm (i.e., µg/g) are represented in Table 3 as the mean concentration of triplicate readings along with the corresponding standard

TABLE 2: MOISTURE CONTENT AND pH OF STPS

Sample ID	Karl Fischer method (Percentage mean±SD)	GC-TCD method (Percentage mean±SD)	pH (Mean±SD)
1	33.68±0.04	35.45±0.21	9.27±0.03
2	62.90±0.05	67.50±0.14	10.02±0.03
3	36.33±0.16	47.90±0.00	7.18±0.00
4	60.07±0.04	68.00±0.14	9.20±0.03
5	38.68±0.04	47.44±0.19	10.21±0.01
6	48.18±0.00	55.64±0.22	9.18±0.03
7	31.16±0.22	33.65±0.21	8.93±0.15
8	53.71±0.11	64.73±0.09	9.93±0.02
9	28.81±0.05	33.80±0.14	8.68±0.00
10	49.31±0.02	43.24±0.21	9.19±0.00

GC-TCD=Gas chromatography-thermal conductivity detector, SD=Standard deviation, STPs=Smokeless tobacco products, Percentage moisture content from the two methods, SD is standard deviation for three observations, pH of the analysed samples expressed as the mean of three observations

deviation (SD). All analysis was performed under the instrument conditions specified in Table 1. Cadmium, chromium and nickel represent the group 1 carcinogenic metals analysed in this study. Cadmium levels varied between zero in samples 5, 7, 9, and 10 and 1.43±1.05 ppm in sample 1 with a mean concentration of 0.47±0.85 ppm. Nickel and chromium were not detected in any of the samples with the current experimental design. The IARC has categorized lead and cobalt as group 2 carcinogenic metals with potential threat to humans. The mean concentration of lead across the samples was 3.30±1.17 ppm with sample 6 exhibiting the highest concentration (5.06±0.53 ppm). Copper and zinc had mean concentrations of 4.17±1.48 and 23.33±1.36 ppm, respectively. The levels of iron and magnesium in the samples were significantly higher than all the other trace metals estimated, with iron displaying a mean concentration of 684.53±1.36 ppm and magnesium 6427.64±1.30 ppm.

The potential toxicity of the 10 STP brands is listed in Tables 4 and 5. The average daily oral exposure (ADE) and the lifetime cancer risk for lead and cadmium were computed using the mathematical relationships described earlier. While the former was calculated on the basis of the individual consuming 10 g dry weight of the product, the estimation of the latter utilised the underlying assumption that 100% of this consumed product is bioavailable. For metals other than lead and cadmium, the potential toxicity was assessed by comparison with established permissible daily intake values in µg/kg/day.

The daily intake of magnesium from the consumption of 10 g of STP per day is represented in Table 6. It is pertinent to note here that the dietary intake of these metals is an important consideration in the context of potential toxicity of an individual metal. The contribution from occupational, environmental as well as geographical factors also needs to be quantified and subjected to in-depth analysis with respect to their toxic potential to humans.

DISCUSSION

The total nicotine content is the primary determinant of addiction potential and consumer attractiveness

TABLE 3: MINERAL AND HEAVY METAL COMPOSITION OF SELECTED STPS BY AAS

Sample ID	Lead (Pb)	Cadmium (Cd)	Iron (Fe)	Copper (Cu)	Zinc (Zn)	Magnesium (Mg)
1	2.73±0.23	1.43±1.05	1073.33±1.65	2.73±1.09	21.96±0.25	6926.81±0.25
2	2.61±2.00	0.66±0.28	493.52±0.28	28.91±0.24	26.52±1.35	5226.08±2.00
3	4.86±2.10	0.52±2.16	526.66±0.24	0.72±2.14	28.46±0.25	7588.00±0.27
4	2.73±1.10	0.76±1.36	1137.33±1.61	0.73±0.59	15.83±1.57	5250.14±2.21
5	4.33±0.26	BDL[a]	596.31±1.54	1.22±1.42	29.23±2.07	4818.24±1.64
6	5.06±0.53	0.92±1.65	624.51±1.24	0.53±2.09	23.03±1.68	7760.00±0.21
7	3.56±0.24	BDL[a]	770.66±2.04	1.13±1.67	10.06±2.07	6603.20±2.05
8	3.90±2.02	0.43±2.08	1123.66±1.68	0.02±2.08	41.21±2.01	6705.28±2.09
9	1.83±1.25	BDL[a]	380.11±1.64	3.06±1.89	27.26±1.26	10332.81±0.25
10	1.46±2.00	BDL[a]	119.21±1.65	2.66±1.57	9.76±1.09	3065.82±2.04

All concentrations are in ppm (µg/g)±standard deviation, [a]BDL=below detectable limits, Note: all the samples were analysed for nickel, chromium and were found to be BDL, standard deviation for three observations, STPs=smokeless tobacco products, AAS=atomic absorption specroscopy.

TABLE 4: ASSESSMENT OF POTENTIAL TOXICITY OF LEAD AND CADMIUM IN STPS

Sample ID	ADE life time Pb[a]	ADE life time Cd[a]	Lifetime cancer risk, Pb (100% transfer)[b]	Lifetime cancer risk, Cd (100% transfer)[b]	Lifetime cancer risk, Pb (6% transfer)	Lifetime cancer risk, Cd (6% transfer)	Total lifetime risk Pb+Cd
1	11.68	6.12	0.23	282.15	0.01	16.92	282.38
2	11.17	2.82	0.22	130.22	0.01	7.81	130.44
3	20.80	2.22	0.41	102.60	0.02	6.15	103.01
4	11.68	3.25	0.23	149.95	0.01	8.99	150.17
5	18.53	NM	0.37	NM	0.02	NM	NM
6	21.60	3.93	0.43	181.52	0.02	10.89	181.95
7	15.23	NM	0.30	NM	0.01	NM	NM
8	16.69	1.84	0.33	84.84	0.01	5.09	85.17
9	7.83	NM	0.15	NM	NM	NM	NM
10	6.12	NM	0.12	NM	NM	NM	NM

[a]According to equations 1 and 2 and assuming an average daily consumption of 10 g of the sample for 30 years and the lifespan to be 70 years, [b]According to equation 1 and the CPF values for lead and cadmium being 0.02 $(mg/kg\ body\ weight/d)^{-1}$ and 46.102 $(mg/kg\ body\ weight/d)^{-1}$, NM is not meaningful, STPS=Smokeless tobacco products.

TABLE 5: ASSESSMENT OF POTENTIAL TOXICITY OF ZINC, NICKEL, IRON, AND COPPER

Sample ID	Zinc (Zn)	Iron (Fe)	Copper (Cu)	Nickel (Ni)
1	31.37	1533.32	3.90	3.90
2	37.88	705.02	41.3	3.72
3	40.65	752.37	1.02	6.94
4	22.61	1624.75	1.04	3.91
5	41.75	851.87	1.74	6.18
6	32.90	892.15	0.75	7.22
7	14.37	1100.94	1.61	5.08
8	58.87	1605.22	0.02	5.57
9	38.94	543.01	4.37	2.61
10	13.94	170.3	3.80	2.08

All values are expressed as µg/kg/day and calculated assuming the average body weight to be 70 kg and an average of 10 g of the sample was being used by the individual per day

TABLE 6: DAILY INTAKE OF MAGNESIUM FROM THE 10 SAMPLES STUDIED

Sample ID	Magnesium (Mg) (mg/day)
1	69.26
2	52.26
3	75.88
4	52.50
5	48.18
6	77.60
7	66.03
8	67.05
9	103.32
10	30.65

All values are calculated assuming an individual consumes 10 g of the sample per day

of STPs. The relevant component of this net nicotine content is the amount of biologically available unprotonated nicotine which depends on the product pH. A variety of compounds like ammonium bicarbonate, ammonium chloride, ammonium carbonate, sodium bicarbonate, sodium citrate, potassium bicarbonate, calcium bicarbonate, and slaked lime are deliberately added during the curing process of these products to modulate their acidity levels, increase their bioavailable nicotine content and consequently to create a higher addiction potential[22]. Unprotonated nicotine is quickly absorbed through the mucosal membranes of the oral cavity and transported to the central nervous system resulting in the instant stimulation that is desired by the habitual snuff dipper[23]. There is a weak correlation between the pH of snuff brands and the extent of oral lesions like leukoplakia, oral tumors, and overall tumor yields as well as the formation of TSNAs in the oral cavity[24]. Studies indicate that moist snuff shows the highest amount of nicotine while dry snuff, having the least percentage of moisture, also displays the lowest nicotine content[8]. Therefore, it can be safely concluded that the samples with higher moisture content will be more addictive in nature due to the presence of a larger amount of orally available free nicotine in them.

The region of tobacco cultivation, climatic conditions prevalent therein and soil chemistry as well as pH are all important in determining the extent and rate of metal accumulation in plants[25,26]. Many metal ions, in their capacity as components of metalloenzymes and metalloproteins, play a significant role in regulating the normal physiological functions of the body. The acceptable daily maximal levels of some of these metals are as follows: Iron 8-18 mg, manganese 1.8-2.3 mg, copper 0.9 mg, zinc 8-11 mg, and nickel 0.5 mg[27].

Lead has been known to increase the rate of hemolysis, suppress cognitive development, cause renal tumors, hypertension, cardiovascular diseases, and negatively impact the male reproductive system[28,29]. The Food and Agricultural Association/ World Health Organization expert panel on food additives has established a provisional tolerable daily intake value of 3.57 µg of lead per kg of body weight[30]. In this context, all the samples with the exception of samples 9 and 10 are found to contain a very high concentration of lead that ranges from about one to seven times the tolerable limit. This data clearly indicates a deleterious effect on the health of the users upon long-term intake of these products.

Apart from its carcinogenic potential vis-á-vis pancreatic cancer, animal studies have shown that cadmium can cause renal failure, placental necrosis, hypertension, anemia, hepatic damage, osteomalacia, testicular tumors, pulmonary edema, emphysema, and induce deficiencies of other essential minerals like iron, zinc, and copper[31]. The reference daily dose of cadmium for chronic oral ingestion leading to proteinuria is 0.5 µg/kg. Assuming that a 70 kg individual daily consumes 10 g of STP containing 1000 ng cadmium/g of the product, it can result in an overall exposure of 0.6 µg/kg bodyweight/d which is effectively higher than the reference dose for causing proteinuria[31]. The Food and Drug Administration (FDA) has set 55 µg as the tolerable daily intake of cadmium per individual;

however, none of the samples studied exceeded this limit.

Although iron and copper are essential minerals for humans, a chronic exposure to iron can cause iron oxide deposition in Parkinson patients while high levels of copper have been shown to cause liver damage[32,33]. In this study, none of the samples was found to exceed the recommended daily intake levels of copper and iron. However, when this data is coupled with accumulation from environmental, occupational and/or geographic sources, the net bioavailable concentration of these trace metals will be much above the permissible limits resulting in toxic effects.

Zinc is believed to cause adverse nutrient interactions with copper and other minerals. It also suppresses immune functions and reduces the high density lipoprotein (HDL) levels in the body[27]. A beneficial effect attributed to zinc is its ability to lower bouts of depression by about 15% for every gram consumed per day[34]. Zinc concentration in the STP samples analysed was also found to be below the stipulated daily upper intake levels. Consideration of the other accumulation factors mentioned above will, however, lead to greater toxicity, especially in high-risk populations.

Magnesium has been found to have beneficial roles in reducing hypertension as well as in improving the insulin sensitivity and lipid profile in patients at risk of cardiovascular diseases; however, a chronic exposure can cause hypermagnesemia and other related toxic effects[35,36]. The daily permissible upper levels of magnesium are 350 mg/day. Table 6 clearly indicates that none of the samples have exceeded the permissible limits of magnesium. Again it needs to be noted that STP consumption can increase the risk of magnesium overdose and toxicity when the contributions from other environmental and dietary sources are also considered.

The lead and cadmium based total lifetime cancer risk calculated as per the United States Environment Protection Agency (USEPA) guidelines ranges from 85.17 for sample 8 to 282.38 for sample 1 with a mean value of 93.31. This is about 18 lac (1.8 million) times higher than the minimum USEPA-stipulated target of 5.05E5 for potentially hazardous substances. It is also of significant concern that this risk is besides that posed by the ingestion of carcinogenic materials including other metals and TSNAs that has already been reported[37,38].

Metallic contamination of drinking water, soil, air, and food (ecosystem products like fish and grains) may be much higher in areas in the vicinities of mining sites, smelters, and hazardous waste sites[39]. Consequently, STP users originating from such high-risk geographical regions may be under an elevated threat of accumulated chronic metal toxicity. It has also been confirmed that natural sources including aquifers are a potentially major source of ground water contamination that again increases the risk of cumulative toxicity from STP use[40]. In the event of exposure to a metallic mixture, the expected clinical effects of metals in humans may be altered. Each component of the mixture may then affect a target organ or a particular system in an unpredictable manner that compounds the risk associated with such an exposure and may even lead to complete organ failure[39].

The results of this analysis point to toxic levels of lead and estimate a prolonged cancer risk associated with the STPs under consideration. This warrants an in-depth analysis of other brands of STPs that are manufactured and marketed in the South Indian states. The trace metal concentration can become a health risk when the additive toxicity potential from dietary as well as miscellaneous environmental exposure is taken into consideration. Considering all these facts the authors feel that the production of STPs should be coupled with thorough quality control protocols as per international guidelines and that their marketing be under stricter government control. Furthermore, marketing strategies like advocating STP as an alternative to cigarette smoking, including STPs in smoking cessation programs and promoting STPs in areas where smoking is prohibited[41] need to be critically reevaluated in the light of the present results. This study may also act as an impetus for further research with a much larger sample size representative of the Indian retail market as well as focussing more on the carcinogenic nitrosamines and other identified mutagens.

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