

Original Article

Ames Mutagenicity Assessment of Flavored Water Pipe Tobacco Products :A Cross Sectional Study in Tehran

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Abstract

Waterpipe smoking has become a global youth trend especially in the Middle East countries and Iran . The aim of this study was to determine the mutagenic effects of three most popular flavored tobaccos by four different salmonella typhimurium strains and compare the possible mutagenic effects of the test samples. Ames mutagenicity assessment was conducted according to the OECD guideline using TA100, TA98 , YG1024 and YG1029 strains. Charcoal burned flavored tobaccos of three different flavors including Orange, Double Apple, and Lime Mint were filtered and exposed to all strains after strain identification tests and MIC ,MBC determinations. The Ames test results indicated significant mutagenic effects of tobacco samples in all four test strains when compared with negative control ($p \leq 0.0001$). The highest Mutagenic Factor (MF) was seen in Double Apple samples using TA 98 ($MF=11.5 \pm 3.3$) . In all experiments, TA strains showed higher sensitivity to the samples than YG strains which suggest these two strains for further regulatory toxicity tests ,policy making purposes and tobacco control programs . Present results represents an important step in understanding the genotoxic potentials of three most popular flavored tobaccos samples of a famous brand in the global markets .

Keywords: Waterpipe; Narghile, Shisha, Hookah, Flavored Tobacco; Mutagenicity, Ames; Salmonella typhimurium;

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Introduction

Nowadays widespread use of tobacco, along with its inducible diseases and subsequent deaths, has become as one of the biggest threats to public health worldwide (Mohammad-Alizadeh-Charandabi S et al., 2015). Tobacco use causes more than 5 million deaths per year globally, and current trends have indicated that tobacco use will cause more than 8 million deaths annually by 2030 (Berkowitz Z et al., 2016). According to the latest statistics published by the Ministry of Health and Medical Education of Iran, waterpipe consumption among Iranian adolescents has become a matter for concern because 15 percent of the young population aged between 13 and 15 are using waterpipe, and a total number of 35 hundred tons of tobacco are consumed per year (Roohafza H et al., 2015). Unfortunately the prevalence of waterpipe smoking (at least once in the previous 30 days) was estimated as 28.0%, according to a study in 2013 and the mentioned prevalence was significantly higher in males (34.8%) than females (21.4%). As a dramatic fact, total of 45.1% of adolescents have reported their lifetime uses of waterpipe and 34.2% had ever shared a waterpipe with others (Baheiraei A et al., 2013) moreover recreational use of waterpipe has been reported widespread among university students in Iran (Ghafouri N et al., 2011), (Sabahy AR et al., 2011), (Roohafza H et al., 2011), before the mentioned study has been published in adolescences.

Researches have showed that waterpipe smoke includes numerous harmful toxicants and carcinogens (Martinasek MP et al., 2011), (Shihadeh A et al., 2015) and waterpipe smoking (WPS) acutely leads to cardiovascular disorders including tachycardia and hypertension, impaired pulmonary function and carbon monoxide poisoning (El-Zaatari ZM et al., 2015). Chronic bronchitis, emphysema and lung oxidative stress are also serious complications of uses of waterpipe in animal models (Nemmar A et al., 2015) but dermatologic disorders (Wollina U et al., 2015), hematological abnormalities (Miri-Moghaddam E et al., 2014), reproductive toxicity (Ali BH et al., 2015), oral cancer as well as the other types of cancer (Al-Amad SH et al., 2014) are also associated with WPS in human studies.

The waterpipe tobacco epidemic has worsened with the growing list of flavors and additives

which have made waterpipe an appealing hobby to youth (WHO 2014). Studies on waterpipe tobaccos, that are usually flavored (CDC 2012), are suggesting that smoke from these preparations contain carbon monoxide, PAHs other toxic agents known to increase the risks for smoking-related cancers (Shihadeh A et al., 2012). All tobacco products, including flavored tobacco products are addictive and carry the same health risks as regular tobacco products (FDA 2011). Sweet-flavored tobaccos fall into the category of products likely to create an impression that the product is less harmful than other tobacco products (Manning KC et al., 2009). The aim of this study was to define if the nature of flavors used in waterpipe tobacco, may cause the mutagenic potency of the tobacco samples towards the strains TA100, TA98, YG1024 and YG1029 of *Salmonella typhimurium*. The second goal was also so to define, whether or not flavored tobacco have direct mutagenic effect on the strains mentioned above and compare the possible mutagenic effects of these samples.

Materials and methods

Maassel Selection

Maassel is a preparation of shredded tobacco glycerol which contains many other unknown additives, and sold in a broad range of flavors that mimic the odors and tastes of various fruits, candy and beverages. The most popular Maassels which have been recognized at the center of the global waterpipe use in the past decade were considered for sample selection of present study. According to a preliminary survey on young population in Tehran's coffee shops and public areas, three types of most popular types of flavored tobacco (Maassel) including orange flavored, two apple flavored and lemon mint flavored were selected and considered for this work. Tobacco products were imported from neighboring countries through nongovernmental routes and obtained at retail from domestic markets in July 2014.

Sample preparation

Maassel is incapable of burning on its own and it should be smoked using charcoal as a heat source. Before sample preparations, the charcoal was placed on top of the maassel, separating by a thin, perforated sheet of aluminum foil which is well known as Ghelyan. The waterpipe was prepared by filling the head

with 10 g of “Nakhla” brand tobacco mixture known as “Two Apples”, “Orange” and “lemon Mint”, covering it with aluminum foil and perforating the foil to allow air passage. A charcoal, “Three Kings” brand quick-light briquette was ignited and placed on the top of the head at the beginning of the smoking session. Water in the water bowl was changed at the beginning of every smoking session for sample preparation. The condensate was collected using Pall Type A/E glass fiber filters as described before (Rammah M et al., 2013). Filters were stored in airtight containers and at -20°C . The content of filter was dispersed in DMSO (1:9, w/v) using a vortex mixer (9000 rpm, 15 minutes). The dispersed solution was then filtered with a syringe filter ($0.22\ \mu\text{m}$). The extract was stored in -4°C prior to assay.

Characteristics of Salmonella Strains

The Salmonella Typhimurium strains used in this study were TA100, TA98, YG1029, an O-acetyltransferase-overproducing derivative of TA100 (Sato G et al., 2000) and YG1024, an O-acetyltransferase-overproducing derivative of TA98 (Einisto P et al., 1990). YG1024 and YG1029 were cloned and their activity was first described and established by Prof. T Nohmi and colleagues (Watanabe M et al., 1994), who had provided and gifted all strains, from Biological Safety Research Center Co., Ltd. (Tokyo). All revertants colonies from 4 used strains were identified as colonies that grow in low levels of histidine or tryptophan. Frameshift and base-pair substitution defects were represented to identify of both. The DNA repair mutation (*uvrA/B*) eliminates excision repair, a repair pathway for DNA damage from UV light and certain mutagens. The presence of the *uvrA/B* mutation makes the strains more sensitive to the test articles that induce damage in this manner. The *uvrA/B* mutation is part of a deletion mutation extending into a gene for biotin synthesis; therefore, the biotin requirement is a result of the deletion of this region. The *uvrA/B* mutation is indicated by sensitivity to UV light. The *rfa* mutation changes the properties of the bacterial cell wall and results in the partial loss of the lipopolysaccharide (LPS) barrier increasing permeability of cells to certain types of chemicals. The *rfa* mutation is indicated by sensitivity to crystal violet. Additional genetic markers serve to make the strains more sensitive to certain types of mutagens.

Bacterial Reverse Mutation Assay

The Ames assays were performed according to the internationally accepted protocols (OECD 1997). Before starting the main experiments, the mentioned strains were checked for their genetic integrity by a series of tests: biotin-histidine dependence, histidine dependence, biotin dependence, *rfa* marker (crystal violet) and the presence of plasmid pKM101 (Ampicillin resistance) tests (Kumar A et al., 2000).

During all these preliminary experiments, the strains were grown overnight in nutrient broth for 16-18h in incubator at 37°C with a density of $1-2 \times 10^8$ (CFU)/ml in presence of $25\ \mu\text{g/ml}$ Ampicillin for TA98, TA100 and $10\ \mu\text{g/ml}$ Tetracycline for YG1029 and YG1024. The top agar was supplemented with biotin-histidine and prepared by dissolving 0.6g of agar-agar and 0.6g NaCl in 100ml distilled water. A sterilized aqueous solution of L-histidine and D-biotin (0.5mM/L) was added to top agar medium immediately before applications (Kumar A et al., 2000). In the next step, $100\ \mu\text{l}$ of overnight cultured bacteria with concentration of $1-2 \times 10^8$ CFU/ml were incubated at 37°C for 45 min in a sterile glass tube containing $500\ \mu\text{lit}$ sodium phosphate buffer ($0.1\ \text{M}$, pH 7.4) with the different flavors of the tobacco samples. After incubation, 2ml of Top agar supplemented with biotin-histidine (kept in 45°C water bath) and added to the mixture and mixed for 3 seconds using a vortex mixer, then poured on a plate of minimal glucose agar media (Mortelmans K and Zeiger E., 2000).

Plates were incubated for 48-72h at 37°C and the revertant colonies were counted. Three equal plates were used for each sample and each experiment was repeated three times to get maximum accuracy and reproducibility for this sensitive method (Li, et al. 2012).

Data analysis

For the Ames mutagenicity assay, positive responses required an increase in the number of revertant colonies/plate. For each of strains, sodium azide at $5\ \mu\text{g/ml}$ concentration (Sigma Aldrich, cat number: 438456) was used as positive control. Negative response was defined as no increase in the number of revertant colonies and DMSO was used as negative control in this study. A result is considered to be positive when there is a significant increase, where the number of revertant colonies is twice the spontaneous background (Chen, et al. 2008).

The Mutant Frequency (M.F.) was described for each assay and calculated as the ratio between number of histidine revertants induced per plate of the test sample and spontaneous revertants of the negative control (Lupi S et al., 2009).

Statistical analysis was conducted using IBM SPSS statistics software version 20, applying one-way ANOVA (LSD) to compare the colony counts of each plate in different groups. Multifactorial analysis was carried out considering the flavor as the main factor and p-values ≤ 0.01 was considered as statistically significant changes.

Results

Genetic integrities of strains

The genetic integrities of all strains were confirmed by a series of tests: biotin-histidine dependence, histidine dependence, biotin dependence, rfa marker (crystal violet) and plasmid pKM101 (Ampicillin resistance) tests. Lack of bacterial growth in rfa test confirmed the necessary mutations in all test strains (Fig 1).

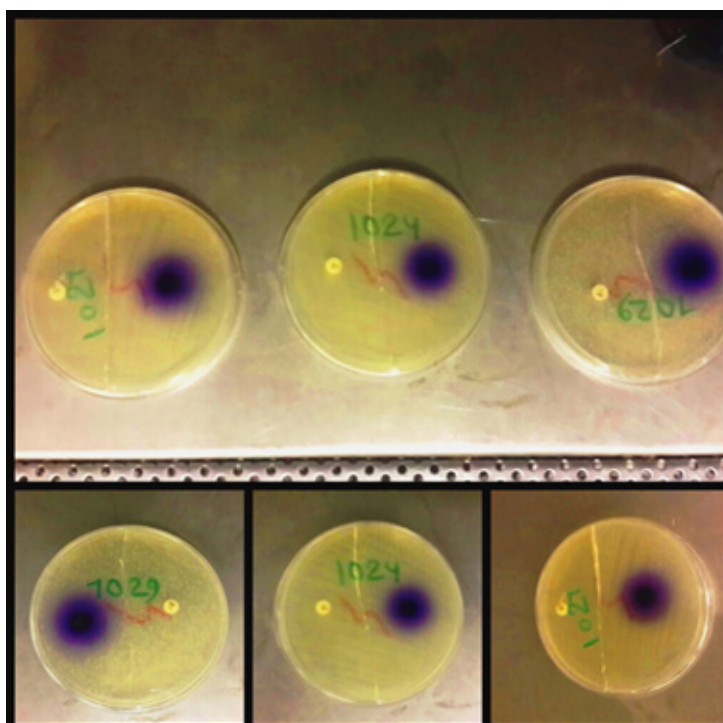


Figure 1: Confirmation of genetic integrity of strains by rfa marker (crystal violet) test and plasmid pKM101 (Ampicillin resistance) tests

MIC and MBC determination by broth serial macrodilution method

After confirming the activity of the strains by crystal violet, the inhibitory effects of 12 different concentrations of each test sample on four *Salmonella* strains were examined. No Bacteriostatic and bactericidal activity was detected in any dilution of each sample that means all experiments showed clearly that MIC and MBC levels were equal in all dilutions of all three samples (Fig 1b).

Results were double checked and confirmed on plates (Fig 2). On the basis of these initial results, one optimized dilution was considered for each tobacco sample during the main tests. Each single dilution was assessed in 4 strains (TA100, TA98, YG1024, YG1029) and repeated three times.

Ames Mutagenicity Assay: As described in method and materials, bacterial mutagenicity was assessed in *S.typhimurium* tester strain

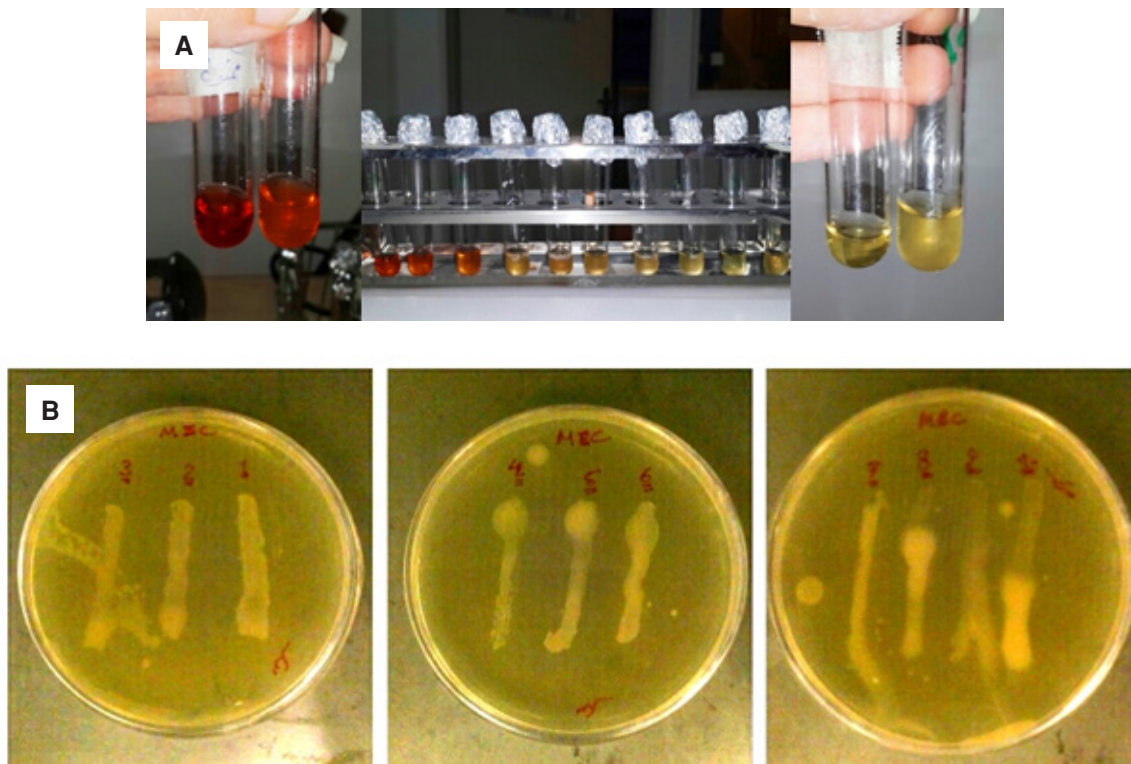


Figure 2: Lack of bacteriostatic and bactericidal effects in serial dilutions of test samples
A: broth serial macrodilution method B: plate tests

TA98 for detection of frame shift mutation, TA100 for measurement of base pair substitution, YG1024 and YG1029, with the enzymatic activity of O-acetyltransferase for their point mutation capacities via metabolic activation. Significant increase in the number of revertant colonies was detected in positive control plates and the number compared with negative control (<0.001). Details are described below for each tobacco sample:

Orange flavored tobacco: After comparing the revertant colonies of this sample to the negative control (untreated), significant mutagenicity of all samples were seen (≤ 0.0001). As described in Table 1, the highest MF level was detected in TA98 (11.4 ± 3.3) but TA 100 and YG1024 showed significant mutagenic potentials of orange flavored tobacco when compared with negative control ($P < 0.001$) (Fig 3).

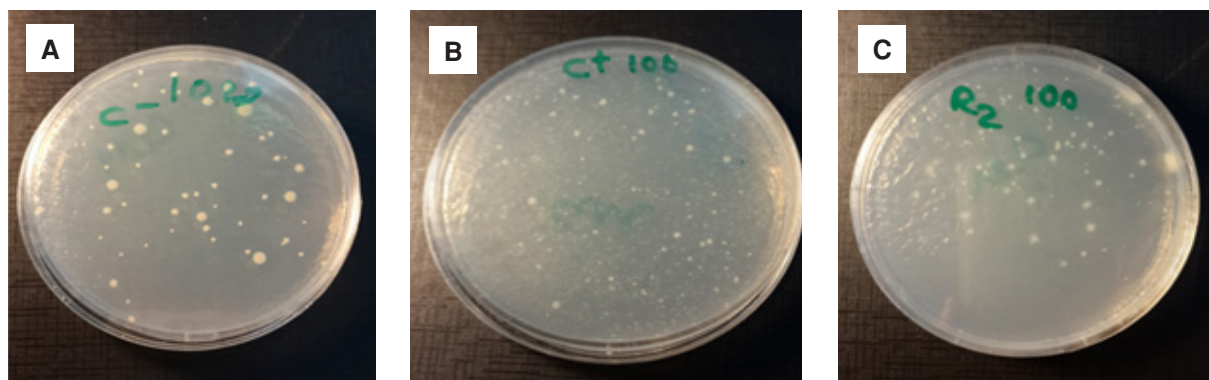


Figure 3-Revert colonies of TA100 in different levels: A:Negative control of TA100 B:Positive control of TA100 C:Mutagenic effects of Orange flavored tobacco in TA100(MF: 6.2±2.4)

Table 1: Mutagenic effects of orange, two apple and lemon-mint flavored tobacco samples on TA98,TA100,YG1024 and YG1029 strains in comparison to negative and positive controls

Strains	Revertant colonies per plate ¹ ±SEM	MF ² ±SEM	SEM± ³ Positive control
Orange flavored tobacco			
TA98	137.6±3.8	11.4±3.3*	158±3.1
TA100	111.6±2.2	6.2±2.4*	198±2.6
YG1024	26.3±2	2.6±1.6*	44±1.9
YG1029	27.3±2.7	1.5±1.2	81±1.8
Two Apple flavored tobacco			
TA98	208±3.8	11.5±3.3*	221±3.5
TA100	230.3±3.8	6.2±2.4*	241±2.8
YG1024	87.6±3	2.6±1.6*	129±2.7
YG1029	78.6±1.7	1.52±1.2	168±3.3
Lemon –Mint Flavored tobacco			
TA98	43.6±2.3	2±1.4	101±1.9
TA100	59.3±1.8	3.2±1.8	98±1.8
YG1024	13±2	1.8±1.3	56±1.3
YG1029	8±1.7	1.6±1.2	44±2

Two Apple flavored tobacco: After comparing the revertant colonies of this sample to the negative control (untreated), significant mutagenicity of all samples were seen (≤ 0.0001). As described in Table 1, the highest MF level was detected in TA98 (11.5±3.3) but TA 100 and YG1024 showed significant mutagenic potentials of two apple flavored tobacco when compared with negative control ($P < 0.001$).

Lemon-mint flavored tobacco: After comparing the revertant colonies of this sample to the negative control (untreated), significant mutagenicity of all samples were seen (≤ 0.0001). As described in Table 1, the highest MF level was detected in TA100 (3.2±1.8) but TA 98 showed significant mutagenic potentials of two apple flavored tobacco when compared with negative control ($P < 0.001$).

Comparison of mutagenicity of samples: Among the samples the highest mutagenicity was detectable in two apple flavored and the lowest one was seen in lime mint flavor. In all 3 samples TA strains showed higher sensitivities than YG strains. The difference was significant (≤ 0.0001) (Table 2).

Discussion

Although some studies have been under way since 40 years in the field of waterpipe smoking and its toxicity in comparison to cigarette smoking there is still insufficient knowledge on the real composition existing tobacco products in the market and the genotoxic potentials of the burned tobaccos, smoke inhaled and the resulting levels of exposure against particular hazardous ingredients. Unsurprisingly, studies of WTS have so far focused on identifying and quantifying chemicals that appear in the 'Hoffmann list' (Hoffmann D and Hoffman I; 1998), a list of tobacco or tobacco smoke constituents thought to be major or contributing causative agents in tobacco-related diseases. In this regard 82 toxicants have been quantified in WTS to date, including Polyaromatic Hydrocarbons (PAHs), heterocyclic amines, Tobacco Specific Nitrosamines (TSNAs), Volatile Organic Compounds (VOCs), and miscellaneous organic and inorganic compounds have been identified. Moreover there have been numerous reports over the years of the Ames assay being used to determine and compare the mutagenicity of tobacco smoke condensates

Table 2: Comparison of sensitivity levels of three strains (TA 100,TA 98 and YG1024)

Strain 1	Strain 2	P values			
		Orange	Two Apple	Lemon mint	
TA100	TA98	0.001	0.005	0.039	p≤0.001**
	YG1024	0.0001	0.0001	0.0001	p≤0.001***
	YG1029	0.0001	0.0001	0.0001	p≤0.001***
TA98	YG1024	0.0001	0.0001	0.0001	p≤0.001***
		0.0001	0.0001	0.0001	p≤0.001***
YG1024	YG1029	0.891	0.223	0.494	

(DeMarini 2004), (Chen J, et al., 2008) however; there hasn't been a considerable review on the mutagenic effects of flavored tobacco "moassel" by the means of Ames test which makes present study as a novel work in this area of research. We showed and compared the mutagenic potentials of waterpipe flavored tobaccos by Ames mutagenicity assessment using 4 different Salmonella strains in this study.

To date there are no study on the constitutes of existing moassel products in the market of Tehran as well as the others in worldwide markets moreover there are not widely accepted methods for the toxicological testing of complex gaseous mixtures and aerosols of WPS although some modifications to the standard regulatory methods have been developed and used (Kilford J et al., 2014). This study has primarily focused on the mutagenic potentials of the solid particulate fraction of waterpipe flavored burned tobacco which was dissolved in DMSO in appropriate concentration. Although we were not able to assess the total inhaled smoke by this method and although this fraction may not accurately show the full toxicity and mutagenicity of the smoke aerosol as a whole, which contains semi-volatiles and short-lived products of combustion, but this works reflects exactly the genotoxic potencies of all evaluated favorite flavored tobacco products with a wide range of human health effects.

In this study we have used four strains of Salmonella typhimurium (TA98, TA100, YG1024 and YG1029) without necessity for metabolic activation (Watanabe M et al., 1994) and following exposure of strains to appropriate concentration of diluted mainstream

flavored tobaccos, highly significant and reproducible increases in the number of revert colonies were observed in all four Salmonella strains with MF > 2, but T strains showed clearly higher sensitivity in comparison to YG strain and suggest their possible more values for regulatory purposes in the field of tobacco control in the future worldwide.

We believe that the extrapolation of the findings of present toxicological assessment to human biological response is difficult, as in vitro test results do not necessarily correlate with results from in vivo tests but our result could strengthen the small but growing literature on maassel waterpipe smoke toxicants which has developed over the past decade. To address this global, rapidly growing tobacco use among young generation present findings are highly valuable because of limited reports on waterpipe toxicants (Rammah M et al., 2012). In this study researches used normal not flavored waterpipe smoke condensate (WSC), and assessed its mutagenicity using Ames test. Within the range of tested doses of WSC, WSC did not elicit sufficient response to be considered mutagenic in any of the strains tested (TA98, TA100, TA102, and TA97a) but were found to be toxic for strains TA97a and TA102 at the highest tested doses. This study showed for the first time the sensitivity of all these four strains to tobacco samples with special emphasis to existence of more hazardous substances in flavored ones with unknown chemical composition. Future studies should be done on the chemical compositions, biological effects through in vivo studies as well as the exposure biomarker studies in waterpipe users corroborate patterns in toxicant yields reported in studies of

waterpipe smoke.

In conclusion the data reported here represent the first in vitro demonstration of the effect of waterpipe smoke on salmonella strains providing evidence of the potential involvement of WPS in the mutagenesis in bacterial cells. While mutagenic frequencies (MF) vary widely across studies, all test strains have indicated that flavored waterpipe tobacco smoke generates mutagenicity and possible carcinogenicity in humans which should be confirmed by further studies but we observed 'A clear mutagenic response in Salmonella strains which is partially predictive for carcinogenic responses Despite the limitations of present work, these results may represent an important step in understanding the genotoxic potential of the most popular flavored tobaccos brands in the market of Tehran.

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Conflicts of Interest

None of the authors have any conflict of interest associated with this study.

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