



## Research article

## Particle size penetration rate and effects of smoke and smokeless tobacco products – An invitro analysis

J.Vini Mary Antony, Pratibha Ramani, Abilasha Ramasubramanian<sup>\*</sup>, Gheena Sukumaran

Department of Oral Pathology, Saveetha Dental College, Saveetha Institute of Medical and Technical Sciences 600077, Chennai, India

## ARTICLE INFO

## Keywords:

Tobacco  
Egg shell membrane  
Khaini  
Tobacco particle size penetration & proliferation  
Oral cancer  
potentially malignant disorders

## ABSTRACT

Utilization of tobacco products is a global epidemic that causes a health threat among the adolescents and adults of the world. If the current pattern perpetuates, it is estimated to cause 10 million deaths each year by 2020. According to the 2009–2010 survey by Global Adult Tobacco Survey, 53.5% of Indians use tobacco products (66.2% of men and 40% of women). Cancers of the lip and oral cavity are highly frequent in Southern Asia (eg: India and Sri Lanka) and it is also the leading cause of cancer death among men in India and Sri Lanka.

**Aim and objectives:** The aim of the study is to evaluate the particle size, and assess the penetration rate in egg shell membrane and proliferation rate using cell lines among 4 commercially available tobacco products.

**Materials and methods:** A total of 4 tobacco product samples namely Gutka, Khaini, Beedi and Cigarette was used to analyse the particle size using High resolution Scanning electron microscopy, the penetration rate on egg shell membrane using Spectrofluometry and proliferative rate using ELISA on cell lines respectively.

**Results:** Khaini revealed the smallest particle size and highest penetration and proliferation rate compared to Gutka, Beedi and Cigarette.

**Conclusion:** Current evidence shows that individuals who consume Khaini are at high risk to develop potentially malignant disorders and oral squamous cell carcinoma when compared to Gutka, Beedi and Cigarette (in the order of occurrence).

## 1. Introduction

Oral squamous cell carcinoma is one of the most prevalent non-communicable disease in the world with an estimated increase of 369,200 incipient cases per year [1], predominantly caused by tobacco habit. Utilization of tobacco products is a global epidemic and causes a preeminent health threat. According to the 2009–2010 survey by Global Adult Tobacco Survey, 53.5% of Indians use tobacco products (66.2% of men and 40% of women) and thereby there is rapid increase in the patients with OSCC [2]. It is well known that tobacco is one of the most important risk factors for premature death globally [3]. It is reported that there are more than 1.3 billion smokers worldwide. The World Health Organization (WHO) estimates that tobacco causes nearly 6.4 million deaths and hundreds of billions of dollars of economic damage worldwide each year. If current trends continue, by 2030 tobacco will kill more than 8 million people worldwide each year, most of which will occur in developing countries with lower incomes [4]. Tobacco is available as -smoked and smokeless tobacco products. In smoked tobacco products nicotine levels in the blood rise quickly after smoking, with arterial blood

levels exceeding venous levels within the first few minutes because nicotine is a weak base and is ionized at acidic pH, there is little absorption of nicotine through the membranes of the mouth [5]. The amount of nicotine in the blood depends on the tobacco particle size, the product's pH and other factors [6]. Nicotine absorption is also influenced by the pH at the buccal mucosa-product interface [7]. In an alkaline pH, nicotine is unionized and rapidly absorbed whereas in an acidic pH, nicotine is ionized and does not cross biological membranes. Absorption of nicotine across the buccal membrane appears to be related to the amount of nicotine present in the unionized “free base” form [8]. The size of the tobacco particle also plays an important role for penetration through the cell membrane. The smaller the particle size, the more is its penetration and effect on the cells. The term “smokeless tobacco” implies any unburned tobacco in the culminated product that can be consumed orally or nasally [9]. Various smokeless forms of tobacco include various processed forms of tobacco namely Gutka, Khaini, Mawa etc. Gutka, is a powdery, granular, light brownish to the white substance which is a coalescence of areca nut, slaked lime, paraffin, and catechu along with tobacco [10]. Khaini is a cumulation of Tobacco, Lime, Dihydrogen

<sup>\*</sup> Corresponding author.

E-mail address: [abilasha.ramasubramanian@gmail.com](mailto:abilasha.ramasubramanian@gmail.com) (A. Ramasubramanian).

<https://doi.org/10.1016/j.heliyon.2021.e06455>

Received 4 October 2020; Received in revised form 17 January 2021; Accepted 4 March 2021

2405-8440/© 2021 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

monoxide Menthol, Oil, spices contains integrated flavour. Consumption of Gutka and Khaini are more deleterious than any other form of tobacco because they are masticated and enter into circulation directly [11]. Smoked tobacco products which are potentially harmful includes various forms such as beedis, cigarette, e-cigarette, hookah, kreteks and pipes. Cigarettes are the only legal consumer products containing tobacco in the world [12]. Cigarette is an accumulation of remedied and finely cut tobacco and other additives rolled or stuffed into a paper wrapped cylinder [13]. Beedi (derived from "beeda") is a thin cigarette or mini-cigar and consist of tobacco wrapped in a tendu or temburni leaf [14] and is secured with a colourful string at one or both ends.

Smokeless tobacco consist of about 48% of the tobacco products consumed in India, 38% of the population use beedis and only 14% uses cigarettes [15]. All these tobacco products are manufactured predominantly with varying particle size and studies relating to their penetration, effects and its possible implications is very sparse. This in-vitro study is the first of its kind to explore and assess the particle size of the tobacco products namely Gutka, Khaini, Beedi and Cigarette in egg shell membrane using High Resolution Scanning Electron Microscope along with the proliferation rate determination using ELISA and the penetration rate using Spectroflurometry in order to quantify the residual amount of tobacco in the cell lines and egg shell membrane which will throw light on the etiopathogenesis and role of tobacco in development and progression of OPMDs and OSCC.

## 2. Materials and methods

A total of 4 tobacco product samples namely Gutka, Khaini, Beedi and Cigarette was used to analyse the particle size using High resolution Scanning electron microscopy, the penetration rate using Spectroflurometry and proliferative rate using ELISA on egg shell membrane and cell lines. The study was approved by the Saveetha Institutional Review Board (SRB).

### 2.1. Isolation of lyophilized extract

20g of the four tobacco samples (the average amount of tobacco in a commercially available product) namely Sample 1 (Beedi), Sample 2 (Gutka), Sample 3 (Khaini) and Sample 4 (Cigarette) were taken in four 200 mL conical flask and 100 mL of solvent such as water and ethanol was added to each beaker. The sample was kept in a reciprocating shaker for 24 h for continuous agitation at 150 rev/min for thorough mixing. Then, extract was filtered by using Whatman no 1 filter paper and finally filtered by using vacuum and pressure pump (AP-9925 Auto Science). The solvent from the extract was removed by using rotary vacuum evaporator RE52 with the water bath temperature of 50 °C. Finally, the residues were collected and used for the experiment. Lyophilisation of the aqueous extract samples: The samples first underwent Freezing (Thermal Treatment) at atmospheric pressure followed by Primary Drying (Sublimation) and Secondary Drying (Desorption) under vacuum. Isolation of egg shell membrane was done by the standard method and was cut into sheets of 1 cm and then used.

### 2.2. Determination of particle size

Exposure of egg shell membrane to tobacco products: This was used to assess the degree of diffusion and particle size of the various forms of tobacco in a previous study by the authors. The egg shell membrane, which is composed of ground substance and keratin, was used to simulate the buccal mucosa. The egg shell membrane was obtained from fresh chicken egg, by opening the egg shell at pole opposite to the air chamber, and then emptying the albumen and yolk followed by a thorough rinse with distilled water. After emptying the contents, the external egg shell was removed by dipping the shell in 0.1N hydrochloric acid for about 1 h followed by manual removal of the shell. Thereafter, the underlying membrane was isolated, taking care to maintain the integrity of the

membrane and it was washed and stored in distilled water at 4 °C until use. The membranes were first rinsed with ringer solution to provide a uniform environment for the measurement of diffusion. About 0.5 g of the lyophilised tobacco products were measured and placed on the membrane for about 5 days for every 4 h. The membrane along with tobacco products were subjected to 5 ml of artificial saliva at equal intervals for 5 days. During this period, a constant grinding force was applied on the membrane to simulate the oral environment. Egg shell membrane not subjected to any product served as control.

The eggs shell membrane which was exposed to various tobacco products was prepared for examination under the HR-SEM. The under-surface of the specimen was viewed with a JEOL JSM- 6360 LV SEM, at an accelerating voltage of 3–30 kV. The HR-SEM images of the four samples were examined under Image analysis software (Image J) for determination of the particle size.

After exposure of the lyophilized tobacco products to egg shell membrane, the membrane was washed in PBS and 0.5 ml of 0.1 M HCl at room temperature. After 30–45 s, 2ml Acridine Orange staining solution was added and first observed under fluorescence microscope with an appropriate filter set. Also cells were analysed alternatively by flow cytometry.

### 2.3. Preparation of KB cell suspension (cell lines)

A subculture of KB (taken from mouth, MEM(E) with NEAA + FCS medium, procured from National Centre for Cell Science, Pune, India) in Dulbecco's Modified Eagle's Medium (DMEM) was trypsinized

separately, after discarding the culture medium and to the disaggregated cells in the flask 25 mL of DMEM with 10% FCS was added. The cells were suspended in the medium by gentle passage with the pipette and the cells were homogenized.

### 2.4. Determination of penetration rate on cell lines

Exposure of the lyophilized tobacco products to cell lines: About 0.5 g of the lyophilised tobacco products was measured and placed on the cell lines every 4 h for about 5 days and was stored in incubator after use. Cell lines without any sample product served as control. The cells were fixed by transferring the cell suspension in 9 ml 1% paraformaldehyde in PBS, and incubated on ice. It was centrifuged at 200 g for 5 min and the cell pellet was resuspended in 5 ml PBS, centrifuge. The cell pellet obtained was suspended in 1 ml PBS and transferred in 9 ml of 70% ethanol, on ice and incubated for 4 h. It was then centrifuged at 200 g for 5 min and resuspended in 1 ml PBS. Then 0.5 ml of 0.1 M HCl at room temperature and after 30–45 s 2 ml AO staining solution was added. The cells were then observed similarly.

### 2.5. Determination of proliferation rate

Protocol for cell proliferation rate was followed, according to the recommended method in the ATCC instruction guide for MTT Cell Proliferation Assay [16].

### 2.6. Determination of tobacco products contents gas chromatography-mass spectroscopy (GCMS)

Shimadzu GC-2010 Plus gas chromatograph was used. GC/MS metabolomics database was used for the similarity search with retention index. Determination of particle size was performed using High Resolution Scanning Electron Microscope and image analysis software.

## 3. Results

### 3.1. Particle size determination

High Resolution Scanning Electron Microscope analysis of beedi, gutka, khaini and cigarette on egg shell membrane at 300 and 1200

magnification showed aggregates of tobacco particles scattered among the meshwork. At higher magnification ( $\times 5000$ ) the exposed to egg shell membrane showed clumps of tobacco particles adherent to the meshwork along with disruption of the collagen meshwork (Figures 1.1,1.2,1.3,1.4,1.5,1.6,1.7,1.8,1.9,1.10,1.11,1.12).The particle size of all the samples in terms of length and width is depicted in (Tables 1A and 1B).

### 3.2. Statistical analysis

The Normality test, Shapiro-Wilks test result reveals that the variable (Particle size) follows Normal distribution. Therefore, to analyse the data parametric methods were applied. To compare the mean values between groups one way ANOVA was used. To analyse the data SPSS software was used.

The mean square for particle size in terms of length was 0.162 between the groups and 6.677 within the groups. The net total between the groups are statistically not significant ( $P = 0.976$ ). The mean square for

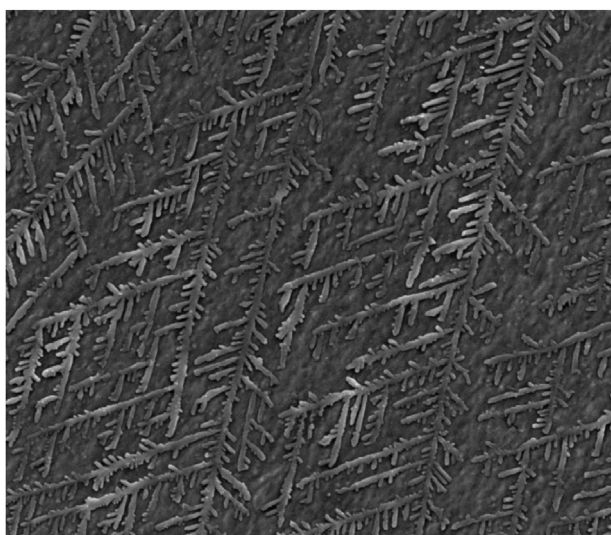


Figure 1.1. Lower magnification ( $\times 300$ ) of Beedi exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.

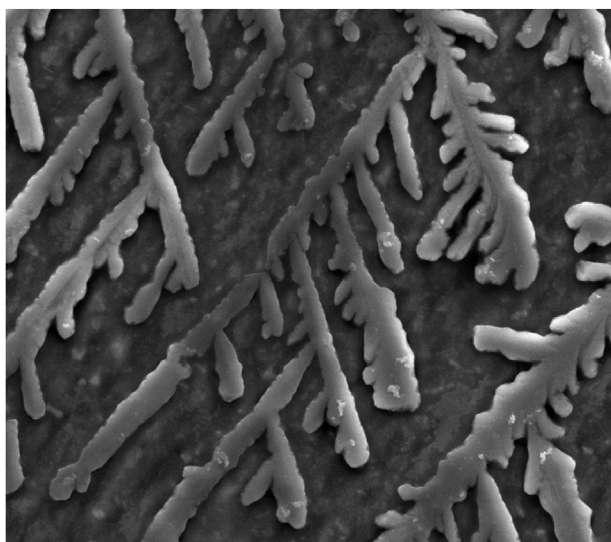


Figure 1.2. At 1200 x -Beedi exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.

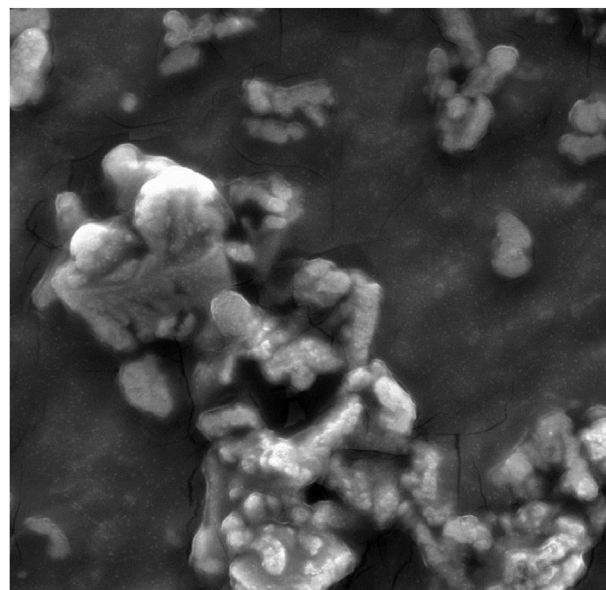


Figure 1.3. Higher magnification ( $\times 5000$ ) of beedi exposed egg shell membrane showing clumps of tobacco particles adherent to the meshwork along with disruption of the collagen meshwork.

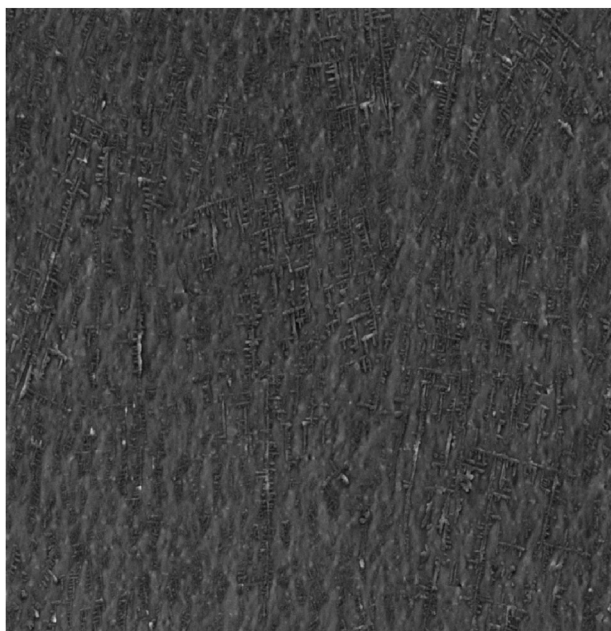


Figure 1.4. Lower magnification ( $\times 300$ ) of Gutka exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.

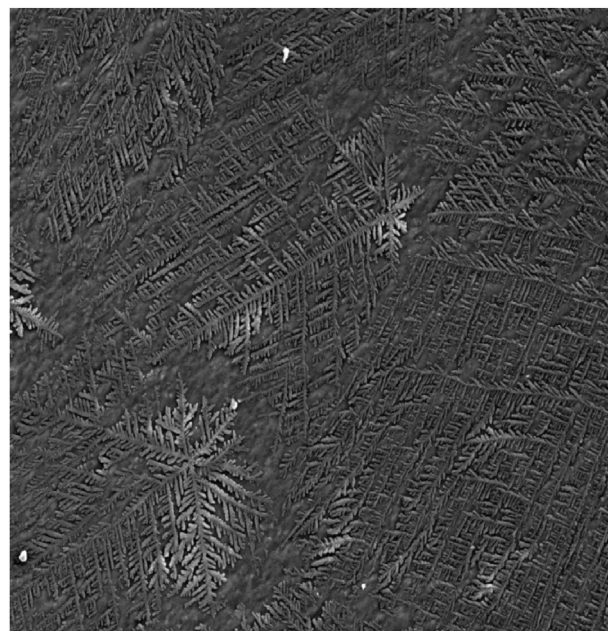
particle size in terms of width was 0.179 between the groups and 6.014 within the groups. The net total between the groups are statistically not significant ( $P = 0.971$ ).The above results showed that the particle size in terms of length and width was clinically significant and statistically not significant.

### 3.3. Determination of penetration rate

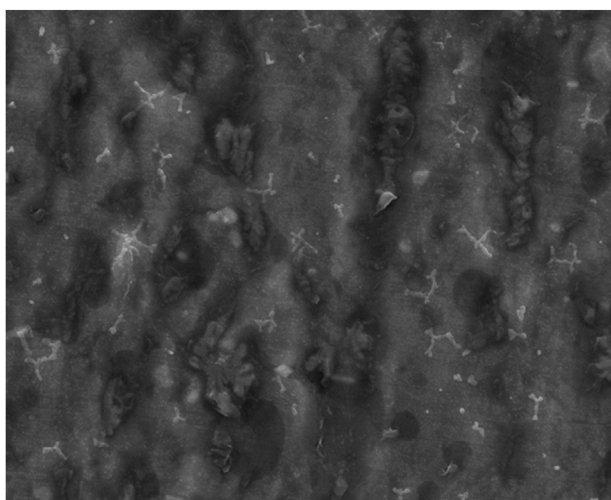
The residual amount of all the tobacco products on egg shell membrane was measured using Fluorescence emission spectrum ( $E_m = 500-8000$  nm), with acridine orange and when exposed to cell lines on Fluorescence emission spectrum ( $E_m = 500-8000$  nm), with acridine orange to determine the penetration rate (Table 2).



**Figure 1.5.** At 1200 x -Gutka exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.



**Figure 1.7.** Lower magnification ( $\times 300$ ) of Khaini exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.



**Figure 1.6.** Higher magnification ( $\times 5000$ ) of Gutka exposed to egg shell membrane showing clumps of tobacco particles adherent to the meshwork along with disruption of the collagen meshwork.



**Figure 1.8.** At 1200 x -Khaini exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.

### 3.4. Determination of proliferation rate

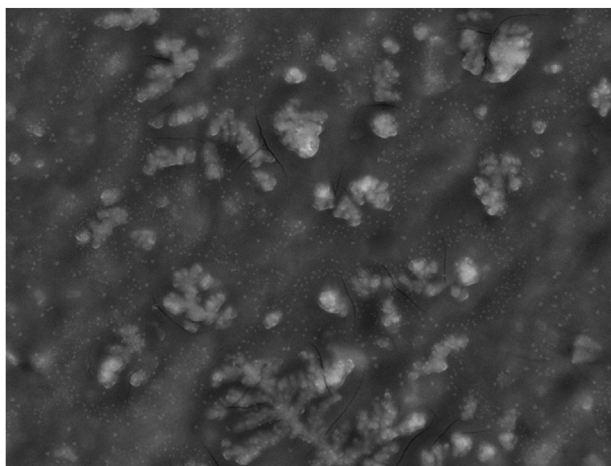
The proliferative rate was determined for both the egg shell membrane and cell lines. When the cell lines were subjected to tobacco products was determined using ELISA reader and Inverted Tissue culture microscope (Figures 2.1,2.2,2.3,2.4,2.5). The in-vitro proliferative activity proved that cancer cell lines were triggered significantly with the increasing sample concentration. The KB cell line when exposed to Khaini showed more proliferative rate followed by Cigarette, Gutka and Beedi (Figure 3). This effect is due to the tobacco products having more specific activity to cancer cell line.

### 3.5. Determination of components

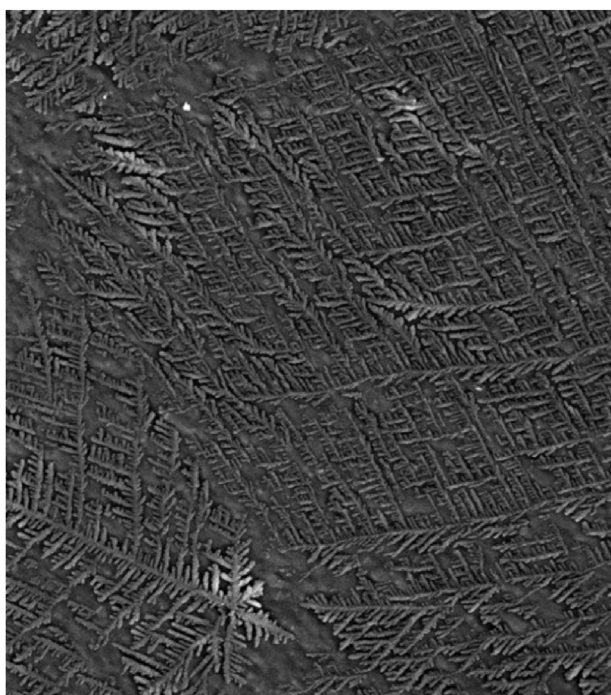
The GC-MS procedure was applied to determine the components in the tobacco samples. All the analyzed samples showed varied

components of tobacco like beedi, cigarette, khaini and ghutka are shown in (Table 3). The variation in the components may be due to environmental or climatic conditions based on location, variation in sample sourcing and the types of leaves used for the preparation of the samples.

Beedi contained more concentration of Nicotinamide which is 31.91 % and belongs to a group called Nitrosamines. The Polycyclic Aromatic Hydrocarbons(PAH) with two or more condensed aromatic and other cyclic rings of carbon and hydrogen atoms namely 3-Phenyl-2H-chromene, 2-Bromo-4,5-dimethoxycinnamic acid, 4-(4-methoxyphenyl)-2-methyl-3-oxo-1,4-dihydroquinoline-5-carboxylic acid and Carbonyl compounds such as N-Methyladrenaline, Ethyl aminomethylformimidate were detected using GC-MS in the sample. GCMS analysis of Cigarette sample showed Polycyclic Aromatic



**Figure 1.9.** Higher magnification ( $\times 5000$ ) of Khaini exposed egg shell membrane showing clumps of tobacco particles adherent to the meshwork along with disruption of the collagen meshwork.

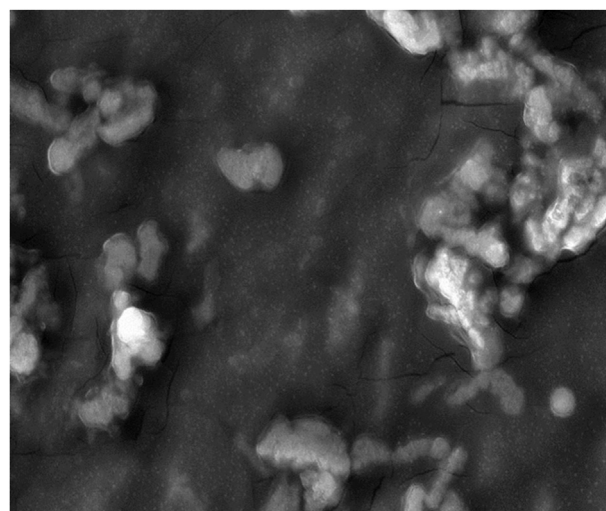


**Figure 1.10.** Lower magnification ( $\times 300$ ) of Cigarette exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.

Hydrocarbons(PAH) namely 1-Cysteine, N,S-bis(m-toluoyl)-, methyl ester, 3-Phenyl-2H-chromene 2H-1,3,4-Benzotriazepine-2-thione, 5-benzyl-1,3-dihydro 1,3,4-benzotriazepine-2-, Benzo[h]quinoline, 2,4-dimethyl- 2,4-Dimethylbenzene, Pyridine-3-carboxylic acid, and 1,4-dihydro-5-cyano-2-hydroxyl ester. Nitrosamines namely N,N-Dimethylformamide ethylene acetal, (3H)-Isobenzofuranone and 3-[(3,5-dimethylphenyl)amine and Volatile compounds namely Phosphonic acid, Cinnamic acid and Anthranilic acid was detected from the cigarette sample. Polycyclic Aromatic Hydrocarbons(PAH) namely Benzoyloxyacetaldehyde dimethyl aceta, 5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine, Acridine-9-carbaldehyde, Acridine-9-carbaldehyde, 1,2-Benzisothiazol-3-amine, Isophthalic acid, di(2-methoxyethyl) ester and 2-Ethylacridine -6. Nitrosamines namely Acetamide, 2-chloro-N and other Volatile compounds namely Morphinan, and Paredrine are



**Figure 1.11.** At 1200 x -Cigarette exposed to egg shell membrane showing aggregates of tobacco particles scattered among the meshwork.



**Figure 1.12.** Higher magnification ( $\times 5000$ ) of Cigarette exposed egg shell membrane showing clumps of tobacco particles adherent to the meshwork along with disruption of the collagen meshwork.

detected in Chaini samples. Nitrosamines namely N,N -Dimethyl -4-nitroso -3-(trimethylsilyl)aniline, Polycyclic Aromatic Hydrocarbons(PAH) namely Pyrrol-2(5H)-one, 4-acetyl-5-(2,4-dichlorophenyl)-1-(2-furfuryl), 1-Tripropylsilyloxy-3-phenylpropane, Benzo[h]quinoline, 2,4-dimethyl- 2,4-Dimethylbenzene, Esters namely Ethylphosphonic acid, bis(tert-butyl)dimethylester, diethyl bis(trimethylsilyl) ester and other Volatile compounds namely Homogentisic acid, Silicic acid are detected in the Gutka samples.

#### 4. Discussion

The World Health Organization predicts that tobacco deaths in India may exceed 1.5 million annually by 2020 and this could be attributed to the use of smoked and smokeless tobacco, in various forms, which has

**Table 1A.** Particle size analysis of tobacco products using high resolution scanning electron microscope.

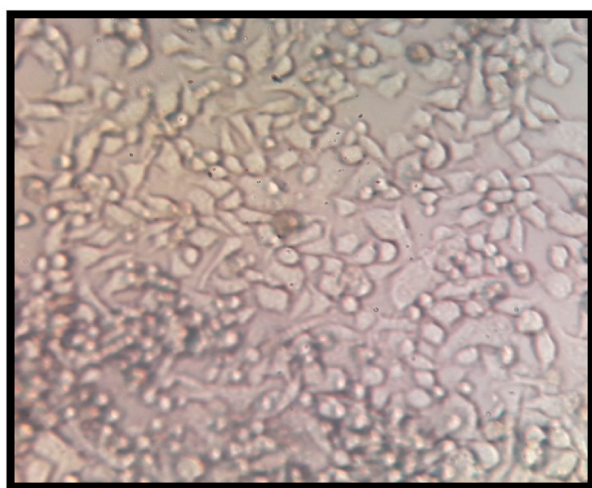
SAMPLES		Mean(mm)	SD(mm)
BEEDI	Length	10.51	0.44
	Width	9.36	0.57
GUTKA	Length	6.89	0.57
	Width	5.77	0.33
KHAINI	Length	4.85	0.53
	Width	3.80	0.28
CIGARETTE	Length	9.35	0.74
	Width	7.86	0.32

**Table 1B.** Comparison of means of particle size (length and width) of tobacco particles between groups using ANOVA.

Particle-size	Sum of Squares	Df	Mean Square	F-Value	p-Value	
Particle-size: Length	Between Groups	.323	2	0.162	0.024	0.976
	Within Groups	60.097	9	6.677		
	Total	60.420	11			
	Between Groups	.359	2	0.179	0.030	0.971

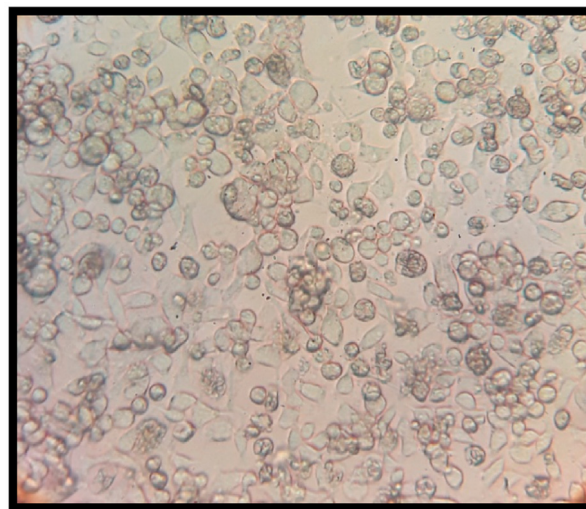
**Table 2.** Determination of penetration rate of the tobacco products on egg shell membrane using spectrofluorometer.

S.NO	SAMPLE NAME	Penetration rate on egg shell membrane ACRIDINE ORANGE UNITS (×10-3/ML)	Penetration rate on cell line ACRIDINE ORANGE UNITS (×10-3/ml)
1.	ESM 1-Beedi	23.05	2.187
2.	ESM-2-Gutka	25.86	2.388
3.	ESM 3-Khaini	27.45	2.534
4.	ESM 4-Cigarette	17.32	1.364
5.	ESM C-Control	0.69	0.058

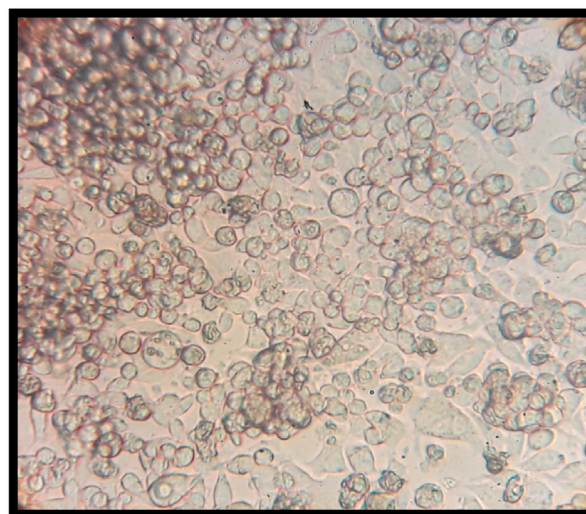


**Figure 2.1.** Control cell lines.

been shown to be strongly associated with Oral Squamous Cell Carcinoma and other potentially malignant disorders. Oral Squamous Cell Carcinoma (OSCC) affects as many as 369,200 people worldwide annually, and the frequency is often indicative of the patterns of tobacco use



**Figure 2.2.** At 300 µg/mL of Beedi concentration the cells showed the above proliferative activity.



**Figure 2.3.** At 300 µg/mL of Gutka concentration the cells showed the above proliferative activity.

[1]. This disparity in OSCC prevalence may be attributable to well-known risk habits of tobacco and alcohol abuse [17]. Most of the Indians (almost 75%) consume smokeless forms of tobacco that includes gutka and khaini [15]. Though various factors have been identified to evaluate the association of tobacco with OSCC and PMDs, this study was first of its kind to assess the particle size and penetration rate of various tobacco products.

There is a rise in the incidence of oral squamous cell carcinoma and potentially malignant disorders in recent years and the role of tobacco products in oral squamous cell carcinoma is of utmost concern. Though various factors have been identified to evaluate the association of tobacco with oral squamous cell carcinoma and potentially malignant disorders, the direct association of particle size on these disorders has not been studied. Therefore, this study was first of its kind to assess the particle size of various tobacco products.

In this study we have utilized egg shell membrane, a natural substrate to simulate the buccal mucosa and to determine the particle size and penetration of the tobacco products. Most of the Indians (almost 75%) consume smokeless forms of tobacco that includes gutka, khaini, mawa, paan and pan masala [15] and smoked tobacco products mostly beedi

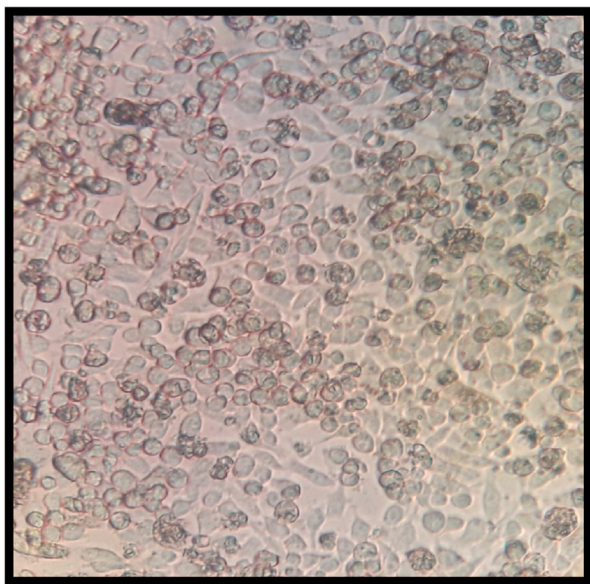


Figure 2.4. At 300 µg/mL of Khaini concentration the cells showed the above proliferative activity.

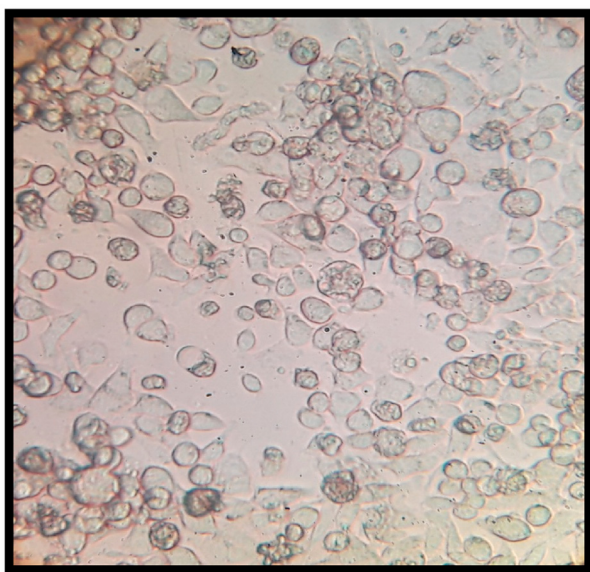


Figure 2.5. At 300 µg/mL of Cigarette concentration the cells showed the above proliferative activity.

and cigarette. So in this study we have used two most commonly available tobacco (smoking) products namely Beedi and Cigarette and smokeless tobacco products namely Gutka and Khaini.

Among the samples the particle size of Khaini was about  $0.53 \times 0.28$  mm and was the smallest when compared to the other products and it belongs to fine particle size. Under  $\times 500$  magnification, the egg shell membrane exposed to khaini showed maximum particle distribution which were scattered amidst the collagen meshwork of the egg shell which correlated with the literature. The particle size of Gutka was  $0.57 \times 0.33$  mm, which are also fine particles and was scattered among the collagen meshwork. The mean particle size of Beedi was  $0.44 \times 0.57$  mm and Cigarette was  $0.74 \times 0.32$  mm. The results of our study can be correlated to several studies wherein the prevalence of potentially malignant disorders and oral squamous cell carcinoma were assessed in

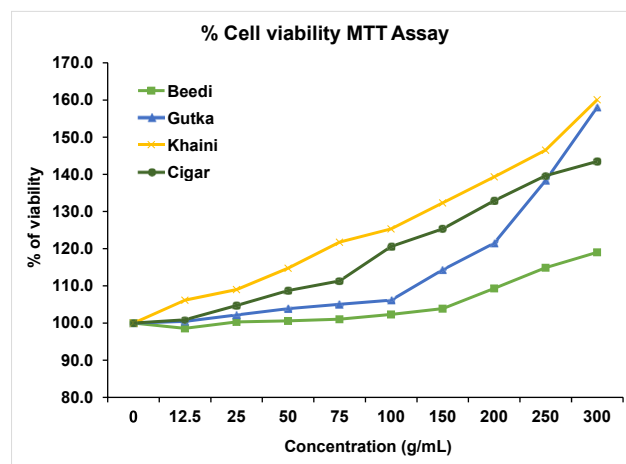


Figure 3. Depicts the overall proliferative activity of the tobacco samples with Khaini showing an increased rate of proliferative activity among all the tobacco products.

smokeless tobacco users [18]. A review of literature on various smokeless tobacco products in potentially malignant and malignant disorders demonstrated a greater prevalence of OSMF in gutka and mawa users and leukoplakia, erythroplakia and malignancies in tobacco leaf chewers [19] This could be due to the release of nitrosamines and other carcinogens from the tobacco leaf which could have a synergistic action on the particle size of the tobacco products [20]. Though khaini, having the smallest particle size has greatest association with OSMF compared with other forms, the number of studies done with khaini is comparatively less probably due to the late emergence of these products in 1990's. However, khaini and gutka which had the smallest dimension have shown a strong correlation with oral squamous cell carcinoma due to the differential release and absorption of toxic components of tobacco which are placed for a much longer time by the users when compared to the smoked tobacco products. The size of the tobacco particle thus plays an important role for penetration through the cells. The smaller the particle size, the more is its penetration into the cells and has increased effect. The size of the tobacco (finesness) influences buccal nicotine absorption [8]. More finely cut smokeless tobacco (smaller particle size) provides more surface area and a greater wetted surface which lead to the rapid absorption of nicotine [21].

GC-MS analysis was also done in our study to determine the components present in the four tobacco samples. Among them Khaini had more nitrosamines such as NNK (4-methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (N-nitrosornicotine) along with the phenolic components, followed by Gutka, Cigarette and beedi.

A number of studies have been conducted to elucidate the molecular mechanisms involved in tobacco mediated cell proliferation and has revealed the involvement of oncogenic derivatives 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosornicotine (NNN) in tobacco smoke has the capability of activating nicotinic acetylcholine receptors (nAChRs), which in turn stimulates multiple cancer-promoting signaling cascades including MAPK cascade. This is known to induce cell proliferation in a wide variety of tumours. Apart from the activation of MAPK pathway it also activates Rb-E2F pathway and JAK-STAT pathway which causes an increase in DNA synthesis and proliferation. Nicotine and NNK facilitate cell-cycle progression and upregulates cyclin-D1, CDK4 expression and downregulates cell cycle inhibitors (tumour suppressors) like p53, p21, and p27. NNK stimulation also increases the expression of the oncogene C-myc [22].

Tobacco contains numerous carcinogens such as polycyclic aromatic hydrocarbons, tobacco specific nitrosamines, and aldehydes which causes mutations of crucial genes such as KRAS, p53, and Rb (22). Nicotine, may be unable to initiate tumorigenesis in humans but promotes tumor

Table 3. Constituent analysis of cigarette, beedi, khaini and gutka using GC-MS.

S.NO	CIGARETTE	CONCENTRATION	KHAINI	CONCENTRATION	GUTKA	CONCENTRATION	BEEDI	CONCENTRATION
1.	N,N-Dimethylformamide ethylene acetal	0.03	Benzoyloxyacetaldehyde dimethyl acetal	10.02	Pyrrrol-2(5H)-one, 4-acetyl-5-(2,4-dichlorophenyl)-1-(2-furfuryl)	0.26	Ethyl aminomethylformimidate	0.63
2.	L-Cysteine, N,S-bis(m-toluoyl)-, methyl ester	0.16	Paredrine	0.09	3,5-Dimethoxycinnamic acid, tert-butyl dimethylsilyl ester	0.36	N-Methyladrenaline	1.2
3.	(3H)-Isobenzofuran one, 3-[(3,5-dimethylphenyl)amine	0.41	Dimethyl hydrastate	8.22	1-Tripropylsilyloxy-3-phenylpropane	1.06	Epinephrine	1.66
4.	Cinnamic acid	0.47	o-Methylisourea hydrogen sulfate	6.45	N,N-Dimethyl-4-nitroso-3-(trimethylsilyl)aniline	0.81	Methylmandelic acid	1.56
5.	Pyridine-3-carboxylic acid, 1,4-dihydro-5-cyano-2-hydroxy-4-(4-isopropylphenyl)phenol	1.17	Acetamide, 2-chloro-N	11.6	Homogentisic acid	0.67	Chloroaniline-5-sulfonic acid	1.21
6.	(3H)-Isobenzofuran one, 3-[(3,5-dimethylphenyl)amino]phenol	0.43	Acridine-9-carbaldehyde	0.35	Hexestrol di-TMS	0.86	3-Phenyl-2H-chromene	20.96
7.	Anthranilic acid	1.37	5-(p-Aminophenyl)-4-(p-tolyl)-2-thiazolamine	5.44	Benzo[h]quinoline, 2,4-dimethyl-2,4-Dimethylbenzene<	20.07	N-Methyl nicotinimide	31.91
8.	Phosphonic acid	1.98	Morphinan,	0.59	trans-4'-Ethyl-4-(methylthio)chalcone	9.43	2-Bromo-4,5-dimethoxycinnamic acid	13.17
9.	Phosphonic acid 2-	2.49	3-Butoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)hexane	0.73	Silicic acid	13.33		
10.	4-(4-methoxyphenyl)-2-methyl-2H-chromene	27.53						
11.	3-Phenyl-2H-chromene	22.09	Acridine-9-carbaldehyde	0.67				
12.	2',4'-Dihydroxyacetophenone, bis(trimethylsilyl) ether	11.03	Benzo[f][1,4]oxazepin-3-one,	0.05				
13.	2H-1,3,4-Benzotriazepine-2-thione,							
14.	5-benzyl-1,3-dihydro							
15.	1,3,4-benzotriazepine-2-	15.34						
16.	1,2-Benzisothiazol-3-amine	21.21	Ethylphosphonic acid, bis(tert-butyl dimethylsilyl) ester	37.64	Cyclobarbital	0.09		
17.	4-Quinolinecarboxylic acid	6.22	Isophthalic acid, di(2-methoxyethyl) ester		1.01	diethyl bis(trimethylsilyl) ester	0.34	3-Amino-4-chlorobenzenesulfonic acid



growth and metastasis by inducing cell cycle progression, epithelial-to-mesenchymal transition (EMT), migration, invasion, angiogenesis, and evasion of apoptosis. In addition, nicotine has been shown to induce secretion of growth factors and cytokines altering the physiology of multiple organ systems [23]. Further, while acetylcholine (ACh) is the physiological ligand for nAChRs, nicotine, NNK and NNN can bind these receptors with greater affinity than ACh and can displace ACh, thus altering their normal function and causing cellular proliferation [24]. These observations suggest that nicotine likely contributes to the progression and metastasis of tumors that are initiated by tobacco carcinogens.

The total particulate matter (TPM) retained by the tobacco products was determined using Spectrofluometry which is a simple, quantitative, expedient, inexpensive and reproducible assay [25] and will prove useful in evaluating the total particulate matter on cell lines and egg shell membrane. The assay is based on the known linear association of tobacco products with fluorescence. Different components in tobacco contribute to the fluorescence. Polycyclic aromatic hydrocarbons (PAH) present in the tobacco products namely fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[a]pyrene (BaP), benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[g,h,i]perylene and dibenzo[a,h]anthracene are known to fluoresce. It is envisioned that significant differences were observed in the TPM retained cell lines and egg shell membrane when exposed to smoked and smokeless tobacco products, which showed that the significant difference may be due to the tobacco composition and packing, menthol and other flavourings, nicotine, carbon/charcoal filtration and particle size.

Among the samples, the residual amount of Khaini when exposed to egg shell membrane and cell lines on Fluorescence emission spectrum ( $E_m = 500\text{--}8000\text{ nm}$ ), with acridine orange standard was  $27.45 \times 10^{-3}/\text{ml}$  and  $2.534 \times 10^{-3}/\text{ml}$  respectively. This may be due to the smallest particle size of Khaini which has caused more penetration when compared to the other tobacco products. All the particles showed penetration in cell lines and egg shell membrane. Among the samples, cigarette showed minimal penetration rate which may be due to the large particle size and Khaini showed more penetration rate due to the smaller particle size. This has proved that smaller the particle size more is the penetration rate and thus establishing an inverse relationship which correlated with the literature.

This study is the first of kind to demonstrate tobacco products inducing proliferation in oral cell lines. Based on previous literature review, tobacco is known to cause epigenetic alteration of oral epithelial cells, inhibit multiple systemic immune functions of the host, and through its toxic metabolites cause oxidative stress on tissues to induce oral potentially malignant disorders [26] which may transform into oral squamous cell carcinoma [27]. The results from our in-vitro study showed that cell lines exposed to the cell lines when exposed to Khaini at 300 g/ml concentration showed proliferation rate of 160.09%. The cell lines when exposed to beedi at 300 g/ml concentration showed proliferative rate of 119.03%, Gutka was 158.08% and Cigarette was 143.49%. The proliferation on cell lines was seen using Inverted Tissue Microscope and was calculated using an ELISA reader. Khaini showed more proliferation when compared to the other tobacco products which may be due to the Nitrosamines and others components which could induce proliferation and metastasis by inducing cell cycle progression, epithelial-to-mesenchymal transition (EMT), migration, invasion, angiogenesis and evasion of apoptosis. From our samples beedi showed more proliferative rate followed by Khaini which may be due to the more concentration of Nitrosamines which was detected from our GC-MS study. Gutka and cigarettes also contains numerous carcinogens which has a proliferative effect on the cell lines and is proved from our results. All the samples used in our study have carcinogens and all the products showed increased proliferative rate when compared to the controls. In vitro studies on lung cell lines when exposed to nicotine showed increase in size and number of tumors forming in the lung and enhanced metastasis [28].

## 5. Conclusion

Thus from the present study it is concluded that all the products have carcinogens and are more prone to potentially malignant disorders and oral squamous cell carcinoma. Among the tobacco products used in our study Khaini has smallest particle size, more retained total particulate matter on cell lines and egg shell membrane and more proliferative rate when compared to the other tobacco products namely Gutka, Cigarette and Beedi. Khaini contains more concentration of nitrosamines and other carcinogens followed by Gutka, beedi and cigarette. According to the results obtained Khaini consumption proves to show higher risk to develop potentially malignant disorders and oral squamous cell carcinoma. The limitations of the study are that it is an in-vitro study based on a previously established study model which may not simulate the entire oral environment; hence in-vivo research and prospective studies on usage of various tobacco products should be conducted to prove the potential of these harmful products to develop carcinoma.

## Declarations

### Author contribution statement

Abilasha Ramasubramanian: Analyzed and interpreted the data; Wrote the paper.

Vini Mary Anthony: Performed the experiments; Wrote the paper.

Pratibha Ramani: Conceived and designed the experiments; Analyzed and interpreted the data.

Gheena Sukumaran: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

### Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

### Data availability statement

Data will be made available on request.

### Declaration of interests statement

The authors declare no conflict of interest.

### Additional information

No additional information is available for this paper.

## References

- [1] Y. Ghantous, I. Abu Elnaaj, Global incidence and risk factors of oral cancer, *Harefuah* 156 (10) (2017) 645–649.
- [2] Government of India, Ministry of Health & Family Welfare, Global Adult Tobacco Survey, India, 2010.
- [3] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, *CA Cancer J. Clin.* 68 (6) (2018 Nov) 394–424.
- [4] A.R. Jethwa, S.S. Khariwala, Tobacco-related carcinogenesis in head and neck cancer, *Canc. Metastasis Rev.* 36 (3) (2017) 411–423.
- [5] N.L. Benowitz, Nicotine and smokeless tobacco, *CA A Cancer J. Clin.* 38 (1988) 244–247.
- [6] M.J. Schroeder, A.C. Hoffman, Electronic cigarettes and nicotine clinical pharmacology, *Tobac. Contr.* (2014), 23 ii30–ii35.
- [7] V. Hegde, A. Nanukuttan, Comparison of nicotine concentration and pH of commercially available smokeless tobacco products, *J. Oral. Res. Rev.* 9 (2017) 21–24.
- [8] W.B. Pickworth, Z.R. Rosenberry, W. Gold, B. Koszowski, Nicotine absorption from smokeless tobacco modified to adjust pH, *J. Addiction Res. Ther.* 5 (3) (2014) 1000184.
- [9] T.C. Poulson, J.E. Lindenmuth, R.O. Greer Jr., A comparison of the use of smokeless tobacco in rural and urban teenagers, *CA Cancer J. Clin.* 34 (1984) 248–261.

- [10] G.A. Mishra, S.A. Pimple, S.S. Shastri, An overview of the tobacco problem in India, *Indian J. Med. Paediatr. Oncol.* 33 (3) (2012) 139–145.
- [11] R.A. Bhisey, Chemistry and toxicology of smokeless tobacco, *Indian J. Canc.* 49 (2012) 364–372.
- [12] V.U. Ekpu, A.K. Brown, The economic impact of smoking and of reducing smoking prevalence: review of evidence, *Tob. Use Insights* 8 (2015) 1–35.
- [13] J.S. Wigand, Additives, Cigarette Design and Tobacco Product Regulation. A Report to WHO, Tobacco Free Initiative and Tobacco Product Regulation Group, Kobe-Japan, 2006, pp. 12–13.
- [14] M. Rahman, J. Sakamoto, T. Fukui, Bidi smoking and oral cancer: a meta-analysis, *Int. J. Canc.* 106 (4) (2003) 600–604.
- [15] R. Chadda, S. Sengupta, Tobacco use by Indian adolescents, *Tob. Induc. Dis.* 1 (1) (2003) 8.
- [16] ATCC, ATCC instruction guide for MTT cell proliferation assay, 2011, pp. 1–6. <https://www.atcc.org/~media/DA5285A1F52C414E864C966FD78C9A79.ashx>.
- [17] K.R. Coelho, Challenges of the oral cancer burden in India, *J. Cancer Epidemiol.* 2012 (2012) 1–17.
- [18] G. Yardimci, Z. Kutlubay, B. Engin, Y. Tuzun, Precancerous lesions of oral mucosa, *World J. Clin. Cases* 2 (12) (2014) 866–872.
- [19] H. Mortazavi, M. Baharvand, M. Mehdipour, Oral potentially malignant disorders: an overview of more than 20 entities, *J. Dent. Res. Dent. Clin. Dent. Prospects* 8 (1) (2014) 6–14.
- [20] N. Jagannathan, A.R. manian, P. Ramani, P. Premkumar, A. Natesan, H.J. Sherlin, In vitro analysis of particle penetration of smokeless tobacco forms using egg shell membrane as a substrate, *J. Canc. Res. Therapeut.* 11 (2015) 204–210.
- [21] G. Connolly, Worldwide expansion of transnational tobacco industry, *J. Natl. Cancer Inst. Monogr.* 12 (1992) 29–35.
- [22] C. Schaal, S.P. Chellappan, Nicotine-mediated cell proliferation and tumor progression in smoking-related cancers, *Mol. Canc. Res. : MCR* 12 (1) (2014) 14–23.
- [23] K. Jensen, D. Nizamutdinov, M. Guerrier, S. Afroze, D. Dostal, S. Glaser, General mechanisms of nicotine-induced fibrogenesis, *Faseb. J.* 26 (12) (2012) 4778–4787.
- [24] N. Dang, X. Meng, H. Song, Nicotinic acetylcholine receptors and cancer, *Biomed. Rep.* 4 (5) (2016) 515–518.
- [25] G.M. Paszkiewicz, Pauly JL Spectrofluorometric method for measuring tobacco smoke particulate matter on cigarette filters and Cambridge pads, *Tobac. Contr.* 17 (2008) i53–i58.
- [26] X. Jiang, J. Wu, J. Wang, R. Huang, Tobacco and oral squamous cell carcinoma: a review of carcinogenic pathways, *Tob. Induc. Dis.* 17 (2019) 29.
- [27] A. Ramasubramanian, P. Ramani, H.J. Sherlin, P. Premkumar, A. Natesan, C. Thiruvengadam, Immunohistochemical evaluation of oral epithelial dysplasia using cyclin-D1, p27 and p63 expression as predictors of malignant transformation, *J. Nat. Sci. Biol. Med.* 4 (2) (2013) 349–358.
- [28] R. Davis, W. Rizwani, S. Banerjee, et al., Nicotine promotes tumor growth and metastasis in mouse models of lung cancer, *PLoS One* 4 (10) (2009), e7524.