Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Comparative Assessment of Physicochemical and Cytotoxic Characterisation, in Khaini and Filtered Khaini Smokeless Tobacco Products

Arun Kumar Sundaragopal
Madha Dental College and Hospital
Senthil Murugappan
Sri Balaji Vidyapeeth University
Sharath Asokan
KSR Institute of Dental Science and Research
Shyam Sivasamy
shyamsivyog@gmail.com

Meenakshi Ammal Dental College and Hospital
Divyarajprabhakar Subramani
Cancer Institute (W.I.A)
Ramya Sekar
Meenakshi Ammal Dental College and Hospital

Research Article

Keywords: Cool Lip, smokeless tobacco, TSNA, cytotoxicity, Indian SLT products

Posted Date: March 5th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8793685/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Comparative Assessment of Physicochemical and Cytotoxic Characterisation, in Khaini and Filtered Khaini Smokeless Tobacco Products

AUTHORS:

Arun Kumar Sundaragopal[1], Senthil Murugappan[2], Sharath Asokan[3], Shyam Sivasamy[4], Divyarajprabhakar Subramani[5], Ramya Sekar[6]

AFFILIATIONS:

1. Email: ak.s29@live.com
Institution: Madha Dental College and Hospital, Department of Public Health Dentistry, Chennai, India.
&
Phd Scholar, Sri Balaji Vidyapeeth, Pondicherry, India.

2. E mail: senthilm@igids.ac.in
Institution: Indira Gandhi Institute of Dental Sciences, Department of Public Health Dentistry, Sri Balaji Vidyapeeth, Pondicherry, India.

3. E mail: asokansharath@yahoo.com
Institution: KSR Institute of Dental Science and Research, Department of Paediatric and Preventive Dentistry, Tiruchengode, India.

4. E mail: shyamsivyog@gmail.com
Institution: Faculty of Dentistry - Meenakshi Academy of Higher Education and Research, Department of Public Health Dentistry, Chennai, India.

5. E Mail: divyarajprabhakar@gmail.com
Institution: Cancer Institute (WIA), Psycho Oncology, Chennai, India.

6. E Mail: drramyaopath@gmail.com
Institution: Faculty of Dentistry - Meenakshi Academy of Higher Education and Research, Department of Public Health Dentistry, Chennai, India.

Clinical trial number: Not applicable

Funding: Nil

Abstract

Objectives

Smokeless tobacco (SLT) products such as Cool Lip, marketed in India in filter-pouch form and resembling Swedish snus, are widely promoted as reduced-risk alternatives to traditional SLT such as Khaini(HANS). This study aims to systematically characterize the physicochemical, chemical, and biological toxicity profile of Cool Lip in comparison to a conventional SLT brand, Hans, to provide evidence on their health risks.

Study Design

Comprehensive analyses were performed to assess physical properties (pH, moisture, particle size using SEM imaging), chemical constituents (nicotine, TSNA, heavy metals, PAHs, alkalis, pesticides, and microplastic contamination), and microbiological load. Cytotoxicity was evaluated via MTT assay and apoptosis by AO staining in primary human gingival fibroblasts exposed to product extracts.

Results

Cool Lip exhibited a finer, more porous, and fragmented microstructure than Hans, which can accelerate chemical dissolution and absorption. Both products contained high levels of carcinogenic TSNAs and PAHs, and demonstrated higher concentrations of NNN, NNK, and nicotine, while Cool Lip had a substantially greater benzo[a]pyrene burden. Cytotoxicity and apoptosis were dose-dependent for both, with Hans consistently causing greater cellular toxicity.

Conclusions

Despite being marketed as a harm-reduced alternative, Cool Lip exhibits comparable or even

greater toxicological risks relative to established Indian SLT products. The findings emphasize the urgent need for regulatory scrutiny and consumer education to counter misleading safety perceptions.

Keywords

Cool Lip, smokeless tobacco, TSNA, cytotoxicity, Indian SLT products

1. Introduction

Smokeless tobacco (SLT) use remains a major public health concern in India, where its prevalence is among the highest globally, with millions consuming products such as khaini, gutkha, zarda, and mishri (1). Despite legal restrictions and state-wide bans in many regions, SLT products continue to be easily available due to systematic violations, low cost, and targeted marketing strategies, driving widespread use particularly among young people and those in lower socioeconomic strata (2).

A brand of SLT-Cool Lip that has gained popularity in recent years is often advertised as a safer alternative to other tobacco products, such as Khaini, Gutka, and Cigarettes (3). This product come in a teabag-like filter pouch that resemble Swedish snus. In India, these safer claims are often misapplied to another similar product - Chaini Khaini, which closely aligns compositionally with khaini, rather than snus (4). Swedish snus is internationally recognized for its relatively low levels of tobacco-specific nitrosamines (TSNAs) and has, in specific contexts, even received Modified Risk Tobacco Product (MRTP) authorization from regulatory bodies such as the US FDA, allowing claims of reduced risk compared with cigarettes (5). Notably, Swedish snus manufacturing practices minimize TSNA formation, which is strongly linked to smokeless tobacco-associated cancers (6).

The misleading association with Swedish snus exploits public perceptions of harm reduction, despite a lack of evidence supporting the safety of Indian products labeled as snus. Despite the increasing popularity and aggressive marketing of filter-pouch SLT products like Cool Lip, there is a critical lack of scientific characterization of their physicochemical, chemical, and cytotoxic properties (7). No previous study has systematically studied this product, leaving its true safety profile unknown and raising concerns about unsubstantiated harm reduction claims.

Hitherto, literature has extensively studied the physical and chemical properties of a traditional Khaini product- Hans. Thus, the present study was designed to address this gap by conducting a comprehensive comparative analysis of Cool Lip and Hans. The specific objectives were to assess their physical properties (pH, moisture content, and particle size). To determine their chemical composition (nicotine content, heavy metal concentrations, levels of tobacco-specific nitrosamines, calcium and magnesium carbonate, and calcium hydroxide). To investigate the presence of microplastics and pesticide residues, with particular emphasis on Cool Lip (filter khaini). To evaluate cytotoxic effects and cellular morphological changes using in vitro assays.

By providing a robust scientific baseline, this study seeks to clarify health risks and inform both clinicians and policymakers about the actual toxicological profiles of these widely available Indian SLT products, ultimately contributing to evidence-based public health strategies.

2. Materials and Methods

2.1 Sample procurement

The present cross-sectional laboratory study was conducted to measure the levels of chemical constituents such as nicotine, TSNA, heavy metals, PAHs, alkalis, pesticides, and microplastic contamination from the two most popular SLT, that is, FK and khaini, and to physical properties such pH, moisture content, and particle size . One pouches each of two top brands (KHAINI 1 pack and FK 1 pack) were randomly procured from three different retail stores in Chennai, Tamil Nadu, India. Individual pouches of the FK were emptied. The samples were separately powdered using a clean ceramic mortar and pestle, and stored in labelled polypropylene containers until analysis. For each category, one commercially popular brand of filtered khaini and one brand of non-filtered khaini were selected to minimize inter-brand variability and enable a controlled comparison between product types.

2.2 Determination of Physical and Chemical Properties

2.2.1Particle Size

Particle size distribution was determined using scanning electron microscopy (SEM). Images were captured at magnifications from 100x to 10,000x, and the average particle sizes were calculated using image analysis software.

2.2.2pH Measurement

Tobacco samples (1 g) were suspended in 10 mL of deionized water. After equilibration, pH was measured with a calibrated pH meter.

2.2.3Moisture Content

Moisture content was evaluated by weighing 1 g of the sample before and after drying at 105°C until constant weight. The difference was taken as moisture loss and expressed as a percentage of the initial weight.

2.2.4Analytical Chemistry

2.2.4.1 Nicotine Quantification

Nicotine content was assessed using high-performance liquid chromatography (HPLC) equipped with a C18 analytical column and UV detection at 259 nm. Powdered tobacco (0.5 g) was extracted in methanol:water (1:1), sonicated, filtered (0.45 μm), and injected. Calibration curves were prepared from certified nicotine standards, and sample concentrations were calculated accordingly.

2.2.4.2 Tobacco-Specific Nitrosamines (TSNAs)

TSNA analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). One gram of powdered sample was extracted with methanol:water (80:20), sonicated, centrifuged, and filtered (0.22 μm). Quantification was based on external calibration with certified reference standards for NNN, NNK, NDMA, NDEA, NDIPA, NDBA, NDPA, and others.

2.2.4.3 Polycyclic Aromatic Hydrocarbons (PAHs)

For PAH determination, samples were extracted with an organic solvent and analyzed by gas chromatography-mass spectrometry (GC-MS) as per validated methods. Quantitative detection targeted benzo[a]pyrene and related markers.

2.2.4.4 Alkali Compounds

Calcium Hydroxide, Calcium Carbonate, and Magnesium Carbonate quantification was achieved by titration and atomic absorption spectroscopy for cations following acid digestion of 0.5 g sample. Standards and procedural blanks ensured accuracy.

2.2.4.5 Pesticide Residue Analysis

Pesticide residues were isolated using the QuEChERS extraction method and analyzed using GC-MS/MS (for non-polar) and LC-MS/MS (for polar/thermolabile compounds). Quantification involved matrix-matched calibration and targeted classes included organochlorines, organophosphates, pyrethroids, and carbamates.

2.2.4.6 Microplastic Assessment

For microplastic analysis, 1 g of finely ground tobacco underwent digestion with 30% hydrogen peroxide at 60°C for 24-48 hours, followed by vacuum filtration through 0.45 μm

membrane filters. Retained particles were identified and counted using FTIR microscopy with reference polymer libraries.

2.3 Microbiology

Aerobic microbial and yeast counts were determined by inoculating 1 g of each smokeless tobacco sample (Cool Lip and Hans) into 9 mL sterile saline, followed by serial tenfold dilutions. 100 µL aliquots were spread onto nutrient agar for bacterial counts and Sabouraud dextrose agar for yeast isolation. Plates were incubated aerobically at 37°C for bacteria and 30°C for yeasts, for 48 hours. Colony-forming units (CFU) were enumerated and recorded as CFU per gram of sample. Distinct colony morphologies were further subcultured and identified using Gram staining (for bacteria) and standard mycological tests (for yeast).

2.4 Cell-Based Toxicity and Morphology

2.4.1 Chemicals and Reagents

All cell culture reagents, including DMEM F-12 media, antibiotics (streptomycin, penicillin), trypsin-EDTA, phosphate-buffered saline (PBS), and fetal bovine serum (FBS), were obtained from Gibco (Invitrogen, USA). The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemicals Pvt Ltd, USA. Additional analytical-grade chemicals were utilized as required throughout the experimental procedures.

2.4.2 Sample Preparation

Hans and Cool Lip samples served as test compounds in all cellular assays. Before experimentation, each sample was sterilized via ultraviolet (UV) irradiation within a laminar air flow cabinet. Sterilized samples (50 mg/mL) were then immersed in serum-free DMEM medium supplemented with 1X antibiotic/antimycotic solution and incubated at 37°C with 5% CO_2 in 10 mm Petri dishes for 24 hours. The resulting conditioned media were harvested and used for subsequent cell treatment protocols.

2.4.3 Cytotoxicity (MTT Assay)

Primary human gingival fibroblast cells (5×10^3/well, 96-well plate) were exposed to conditioned media (Hans, Cool Lip) at various concentrations 20 µL/mL for 24 hours. After treatment, cells were incubated with MTT solution, and formazan production was quantified at

570 nm. Cell viability (%) was calculated as (OD treated/OD control) × 100. All quantitative experiments were performed in triplicate.

2.4.4 Morphological Assessment

Optimal-dose treated cells (2×10^5/well, 6-well plate) were examined microscopically after 24 hours for morphological changes (shrinkage, rounding, detachment) using phase-contrast microscopy.

2.4.5 Apoptosis (Acridine Orange Staining)

Cells were exposed to various concentrations of conditioned sample media (20-120 µL/mL), then resuspended in AO stain (1 mg/mL) after washing. Apoptotic changes were identified under a fluorescence microscope by characteristic nuclear morphology and chromatin condensation.

2.5 Statistical Analysis

The MTT assay was performed in triplicate. Data were analysed using one-way ANOVA in SPSS version 27.0 software. Statistical significance was set at $p < 0.05$.

3. Results

3.1 Physio-Chemical Properties

Cool Lip demonstrated a finer particle size distribution with an average particle size of approximately 120 µm, whereas Hans showed larger particles averaging around 250 µm. Moisture content was slightly higher in Hans (9.54%) compared to Cool Lip (8.56%). The pH values were alkaline for both products: Hans exhibited a pH of 10.65, while Cool Lip measured a pH of 10.12, which may influence nicotine absorption (Table 1) (Figure 1).

3.2 Chemical Composition

3.2.1 Tobacco-Specific Nitrosamines (TSNAs)

LC-MS/MS analysis detected seven key TSNAs (NNN, NNK, NDMA, NDEA, NDIPA, NDBA, NDPA) in both products. Of which, IARC Group I carcinogens N-Nitrosonornicotine (NNN), and Nicotine-derived nitrosamine ketone (NNK), showed elevated concentrations of (1.45 mg/kg) and (0.97 mg/kg) in HANS compared to Cool Lip, which had lower but notable levels (NNN: 0.85 mg/kg; NNK: 0.52 mg/kg). Other carcinogens (Group IIA, IIB) were also detected. (Table 2).

3.2.2 Polycyclic aromatic hydrocarbons (PAHs); Alkalis and Nicotine:

Polycyclic aromatic hydrocarbons (PAHs) were detected in both Cool Lip and Hans, with total PAH concentrations of 30.96 µg/kg and 33.45 µg/kg, respectively; benzo[a]pyrene, a potent carcinogen, was much higher in Cool Lip (8.89 µg/kg) than Hans (1.26 µg/kg). Alkali analysis showed Calcium hydroxide $Ca(OH)_2$, Calcium carbonate $CaCO_3$, and Magnesium carbonate $MgCO_3$, were present in both samples, with $Ca(OH)_2$ notably higher in Hans (1.89% vs. 1.4%). HPLC quantification revealed nicotine levels of 2.5% w/w in Cool Lip and significantly higher levels in Hans at 3.15%w/w. This suggests comparatively greater nicotine exposure potential with Hans' use (Table 3). The presence of carcinogenic PAHs, alkalis, and nicotine in these products poses health risks, including potential oral and systemic toxicity.

3.2.3 Microplastics and Pesticides in Cool Lip

FTIR microscopy quantified microplastic particles in Cool Lip and Hans and detected no polyethylene and polypropylene fragments under 500 µm in both the products. Pesticide residue analysis via GC-MS/MS and LC-MS/MS did not detect any of the Organochlorine pesticides (OCPs), Organophosphorus Pesticides (OPPs), Pyrethroids, and Carbamate (supplementary).

3.3 Microbial Analysis

Aerobic microbial counts for Cool Lip and Hans revealed the presence of numerous bacterial and yeast colonies, with CFU levels indicative of microbial contamination within both products. Cool Lip displayed a slightly higher total aerobic bacterial count and total yeast and mold count, respectively (2.3×10^3 CFU/g; 1.5×10^3 CFU/g), while Hans demonstrated a comparatively lower diversity and number of yeast colonies per gram (2.15×10^3 CFU/g; 1.15×10^3 CFU/g). (Table 4)

3.4 Cytotoxicity and Cellular Effects

3.4.1 MTT Cell Viability Assay

Dose-dependent cytotoxicity was observed in gingival fibroblasts treated with both conditioned media. Hans' exposure reduced viability to 56.764% at 100 µL/mL, significantly lower than Cool Lip at the same concentration (65.235%) (Figure 2).

3.4.2 Morphological Changes

Phase-contrast images showed both treatments caused cell shrinkage, reduced cell density, and detachment. These changes were evident in cool lip conditioned media treated cells as comparable to Hans at 80 and 100 μL/mL, indicating higher cytotoxic stress (Figure 3).

3.4.3 Acridine Orange Staining

Fluorescence microscopy revealed hallmark apoptotic features chromatin condensation, and nuclear fragmentation in cells treated with both Cool Lip and Hans media. Apoptotic indices were higher in both groups, supporting their greater cytotoxic potential (Figure 4).

The comparative physicochemical and biological toxicity profiles of Cool Lip and Hans highlight higher toxicity and carcinogenic potential in both products.

4. Discussion

There is a critical gap in scientific knowledge regarding the physicochemical and toxicological properties of Cool Lip, a popular filter-pouch SLT product in India. Despite its rapid uptake and marketing as a safer alternative, no prior study has systematically characterized its composition and assessed its health risks in comparison to established products (7,8). This study addresses these urgent evidence gaps to inform clinicians, policymakers, and public health strategies of Cool Lip, comparing it with Hans, a well-established SLT brand. The study found that Cool Lip's finer structure, as revealed by SEM, may enhance the release and absorption of harmful constituents, including nicotine and heavy metals. Previous studies confirm that higher surface area and smaller particle size in SLT products correlate with more rapid nicotine release and potentially higher addiction risk [8]. Chemically, both products contained significant levels of carcinogenic tobacco-specific nitrosamines (TSNAs) and polycyclic aromatic hydrocarbons (PAHs); however, Hans consistently exhibited higher TSNA and nicotine content, while Cool Lip showed an alarming concentration of benzo[a]pyrene. Both products induced dose-dependent cytotoxicity in oral fibroblasts, with greater cytotoxic and apoptotic effects. Cool Lip is aggressively marketed in teabag-like pouches mimicking Swedish snus, yet it closely aligns with khaini(Hans) in formulation. Their packaging leverages international harm-reduction perceptions associated with Swedish snus and MRTP (Modified Risk Tobacco Product) regulatory authorizations abroad. However, scientific scrutiny reveals that these pouches contain TSNA and nicotine levels comparable to traditional khaini (Hans)

and are not subject to the rigorous processing that reduces carcinogen formation in Swedish snus (7,5).

Usage prevalence of Cool Lip, remains very high in India: over 20% of adults are current users, and prevalence is disproportionately high among rural males, informal workers, and low socioeconomic strata. Cool Lip's packaging and ease of discreet use enhance its acceptability among young and first-time users, compounding public health concerns (7,8,9).

Both Cool Lip and Hans contain substantial amounts of TSNAs, particularly NNN and NNK, class I IARC carcinogens extensively linked to oral and upper aerodigestive cancers. PAHs, especially benzo[a]pyrene (substantially higher in Cool Lip), add to the carcinogenic burden. Levels of free/unprotonated nicotine in these products, driven by their high pH, enhance both bioavailability and dependence potential (8,10).

On the contrary, this study reveals microbial counts for both Cool Lip and Hans, which are well within the USFDA guidelines (11). The in vitro cytotoxicity findings provide further cause for concern: both Cool Lip and Hans caused dose-dependent reductions in oral fibroblast viability, pronounced apoptotic features, and marked cellular morphological changes. These data echo established literature identifying Indian SLT products as potent inducers of DNA damage, oxidative stress, and cytogenetic alterations, contributing to the high oral cancer burden in South Asia. Thus, urging the addition of Cool Lip to the harmful SLT products in India (12,13). The findings underscore the urgent need for regulation of filter-pouch SLT products such as Cool Lip, which are falsely marketed as safer alternatives due to snus-like packaging and unsubstantiated harm-reduction messaging (14). The lack of scientific characterization leaves clinicians and policymakers unable to critically appraise the risks unique to these products (15). Current Indian regulatory frameworks lag behind those in Europe or the US, where snus is subject to stringent quality and safety standards and MRTP authorizations govern allowable "reduced risk" claims.

5. Conclusion

This first comparative study of Cool Lip filter-pouch SLT reveals significant physicochemical, toxicological, and cellular hazards, comparable to or exceeding established SLT brands. Marketing Cool Lip as a "safer snus alternative" is scientifically unsupported and potentially harmful. There is a pressing need for evidence-based policy intervention, consumer education, and product regulation to counteract misleading marketing and reduce the burden of oral cancer and other SLT-related diseases in India. The comparative physicochemical and biological

toxicity profiles of Cool Lip and Hans highlight higher toxicity and carcinogenic potential in both products.

Declarations

Ethical approval and Accordance

Not applicable. This study did not involve human participants, human data, or live vertebrate animals.

Consent to participate

Not applicable. This study did not involve human participants.

Consent to publish

Not applicable. This manuscript does not contain any person's data.

Availability of Data and Materials

The datasets generated and/or analysed during the current study are not publicly available due to institutional data-storage policies, but are available from the corresponding author on reasonable request.

References

1. Niaz K, Maqbool F, Khan F, Bahadar H, Hassan FI, Abdollahi M. Smokeless tobacco (paan and gutkha) consumption, prevalence, and contribution to oral cancer. Epidemiology and health. 2017 Mar 9;39:e2017009.
2. Vidhubala E, Pisinger C, Basumallik B, Prabhakar DS. The ban on smokeless tobacco products is systematically violated in Chennai, India. Indian journal of cancer. 2016 Apr 1;53(2):325-30.
3. https://extranet.who.int/fctcapps/fctcapps/fctc/kh/slt/news/smokeless-tobacco-slt-products
4. Stepanov I, Gupta PC, Dhumal G, Yershova K, Toscano W, Hatsukami D, Parascandola M. High levels of tobacco-specific N-nitrosamines and nicotine in Chaini Khaini, a product marketed as snus. Tobacco control. 2015 Dec 1;24(e4):e271-4.

5. Liber AC, Seidenberg AB, Pesko MF. MRTP claim authorisation and General Snus sales in the USA: evidence from a difference-in-differences model. Tobacco control. 2024 Nov 1;33(6):825-8.
6. Valen H, Becher R, Vist GE, Holme JA, Mdala I, Elvsaas IK, Alexander J, Underland V, Brinchmann BC, Grimsrud TK. A systematic review of cancer risk among users of smokeless tobacco (Swedish snus) exclusively, compared with no use of tobacco. International Journal of Cancer. 2023 Dec 15;153(12):1942-53.
7. Sharma P, Cheah NP, Kaur J, Sathiya Kumar S, Rao V, Morsed FA, Choo MY, Murthy P. Physical and chemical characterization of smokeless tobacco products in India. Scientific Reports. 2023 Jun 1;13(1):8901.
8. Bhisey RA. Chemistry and toxicology of smokeless tobacco. Indian journal of cancer. 2012 Oct 1;49(4):364-72.
9. Bharati B, Sahu KS, Pati S. Prevalence of smokeless tobacco use in India and its association with various occupations: a LASI study. Frontiers in public health. 2023 Feb 27;11:1005103.
10. Nasrin S, Chen G, Watson CJ, Lazarus P. Comparison of tobacco-specific nitrosamine levels in smokeless tobacco products: High levels in products from Bangladesh. PLoS One. 2020 May 26;15(5):e0233111.
11. Han J, Sanad YM, Deck J, Sutherland JB, Li Z, Walters MJ, Duran N, Holman MR, Foley SL. Bacterial populations associated with smokeless tobacco products. Applied and environmental microbiology. 2016 Oct 15;82(20):6273-83.
12. Khan Z, Tönnies J, Müller S. Smokeless tobacco and oral cancer in South Asia: a systematic review with meta-analysis. Journal of cancer epidemiology. 2014;2014(1):394696.
13. Khan Z, Khan S, Christianson L, Rehman S, Ekwunife O, Samkange-Zeeb F. Smokeless tobacco and oral potentially malignant disorders in South Asia: a protocol for a systematic review. Systematic reviews. 2016 Aug 24;5(1):142.
14. Patwardhan S, Fagerström K. The new nicotine pouch category: a tobacco harm reduction tool?. Nicotine & Tobacco Research. 2022 Mar 1;24(4):623-5.
15. Travis N, Warner KE, Goniewicz ML, Oh H, Ranganathan R, Meza R, Hartmann-Boyce J, Levy DT. The potential impact of oral nicotine pouches on public health: a scoping review. Nicotine & Tobacco Research. 2025 Mar 24;27(4):598-610.

Tables

Table 1: Physical properties of Cool lip and Hans

Properties	**Cool Lip**	**Hans**
Moisture Content (%)	8.56%	9.54%
pH	10.12	10.65
Particle Size (µm)	20-200 µm	80-400 µm

Table 2: Tobacco Specific Nitrosamines content in Cool Lip and Hans using LC-MS/MS

TSNAs	**Cool Lip (mg/Kg)**	**Hans(mg/Kg)**
N-Nitrosonornicotine (NNN)	0.85	1.45
Nicotine-derived nitrosamine ketone (NNK)	0.52	0.97
N-nitrosodimethylamine (NDMA)	0.014	0.121
N-nitrosodiethylamine (NDEA)	0.008	0.02
N-nitroso-4-methyl-4-aminobutyricacid (NMBA)	ND	ND
N-nitrosodiisopropylamine (NDIPA)	0.006	0.05
N-nitrosodibutylamine (NDBA)	0.011	0.02
N-nitrosodipropylamine (NDPA)	ND	ND
N-nitrosomethylphenylamine (NMPA)	0.003	0.008

Table 3: Assessment of Polycyclic aromatic hydrocarbons (PAHs); Alkalis and Nicotine using GC-MS; Titrimetric method; and HPLC respectively.

PAHs	**Cool Lip (µg/kg)**	**Hans (µg/kg)**
Benzo[a]pyrene	8.89	1.26
Benzo[a]anthracene	4.58	3.58
Benzo[b]fluoranthene	2.85	5.24
Benzo[k]fluoranthene	5.89	6.89
Chrysene	6.65	11.24
Dibenzo[a,h]anthracene	BQL	BQL
Indeno[1,2,3-cd]pyrene	2.1	5.24
Σ7 PAHs (sum)	**30.96**	**33.45**
Alkali		
$Ca(OH)_2$	1.4 (%w/w)	1.89 (%w/w)
$CaCO_3$	6.2 (%w/w)	5.89 (%w/w)
MgCO3	0.5 (%w/w)	0.78 (%w/w)
Nicotine		
Nicotine	2.5 (%w/w)	3.15 (%w/w)

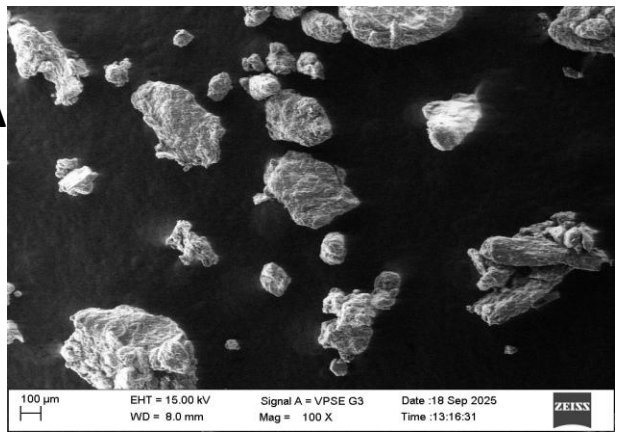
Table 4: Assessment of Total Aerobic Microbial Count and Total Yeast and Mold count in Cool Lip and Hans

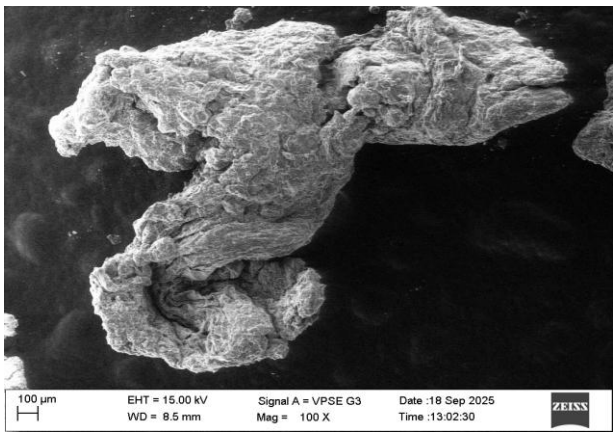
Microbiology Analysis	**Cool Lip (CFU/gm)**	**Hans (CFU/gm)**
Total Aerobic Microbial Count (TAMC)	2.3×10^3	2.15×10^3
Total Yeast and Mold Count (TYMC)	1.5×10^2	1.15×10^2
Escherichia coli	ND	ND
Salmonella spp.	ND	ND
Pseudomonas aeruginosa	ND	ND
Staphylococcus aureus	ND	ND

Figures:

Figure 1: A) Structural image of Cool Lip Particles; B) Structural image of Hans Particles

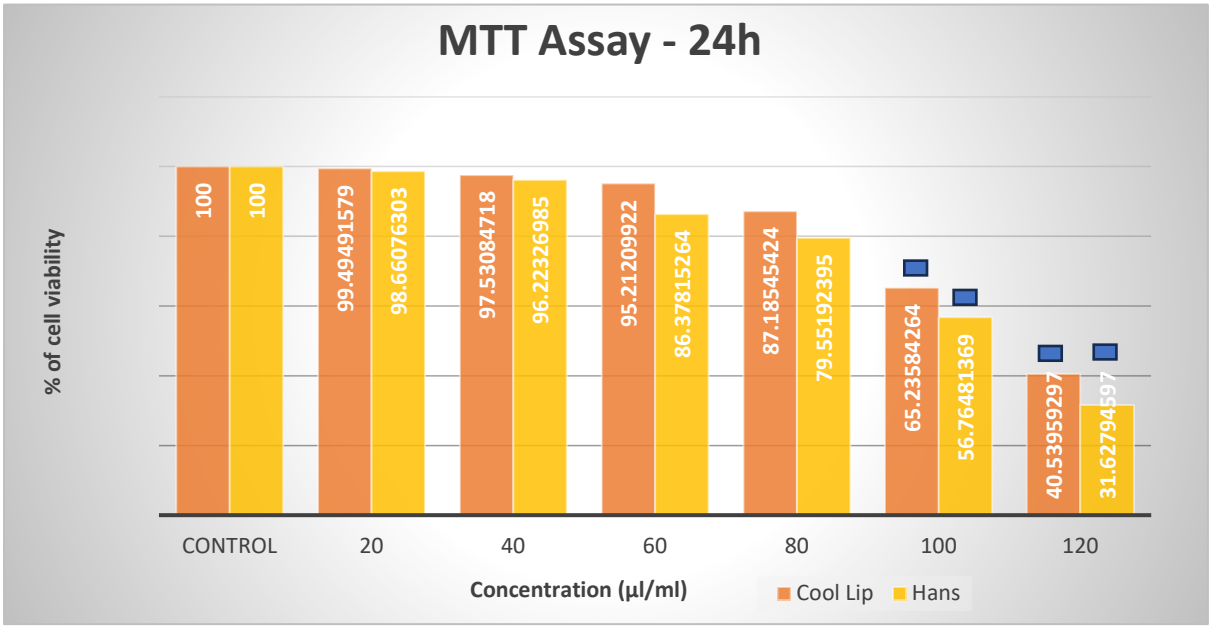
A





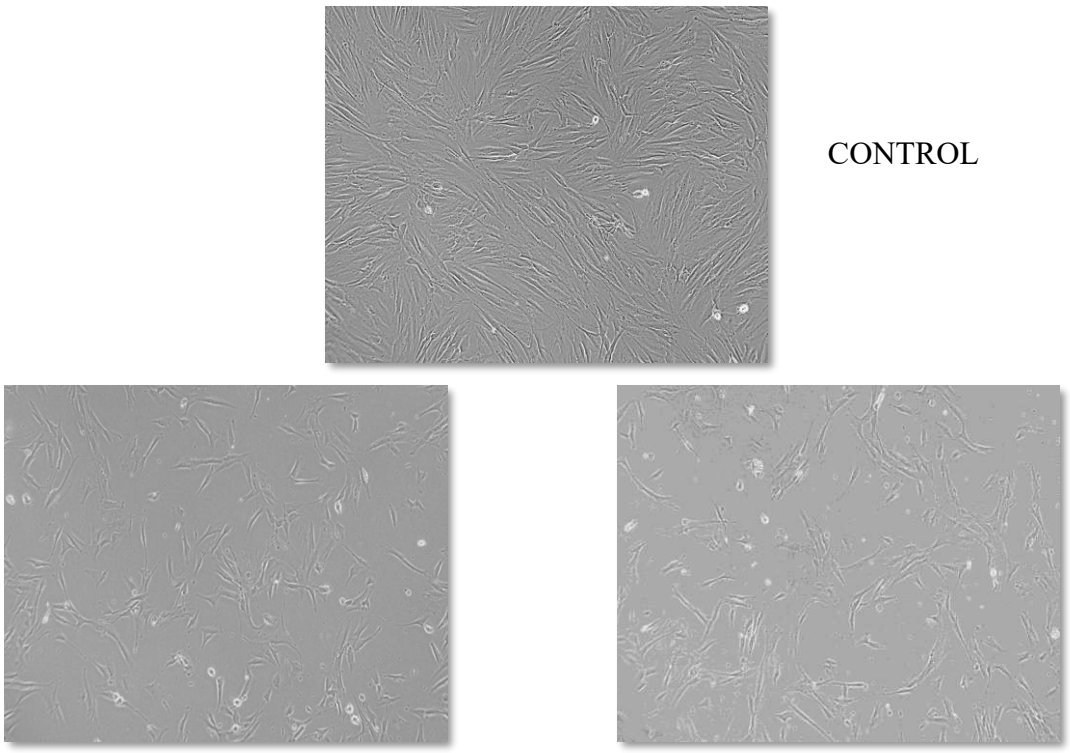
B

Figure 2: Bar diagram representing the depletion of cells on the introduction of increasing concentrations of Cool Lip and Hans in comparison to the control



- **ANOVA, <0.05(Statistically significant)**

Figure 3: Effect of Cool Lip and Hans on immersed condition media showing cell morphology of gingival fibroblast cells under Phase Contrast Microscope.



CONTROL

COOL LIP (100µl/ml)

HANS (100µl/ml)

Figure 4: Effect of Cool Lip and Hans on gingival fibroblast cells depicting apoptosis using Acridine Orange staining under inverted Phase Contrast Fluorescent Microscope.

