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Research Article

GC-MS and Cellular Toxicity Studies on Smokeless-Tobacco Show Alerting Cytotoxic effect on Human Gingiva and Lung Fibroblasts

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Smokeless tobacco (SLT) has been reported to have deleterious effects on the health of its users. This study aims to analyze the constituents of locally collected SLT sample extracts (S1–S11) from Tabuk region of Saudi Arabia using GC-MS and investigate their cytotoxic effect on human gingival fibroblasts (hGFs), normal human fibroblasts (MRC5), and two cancer cell lines (HT29 and HepG2) using MTT assay. GC-MS results showed that pyridine, 3-(1-methyl-1H-pyrrol-2-yl)-, tetracyclo[4.4.1.1(7,10).0(2,5)] dodec-3-en-11-ol, and cotinine were found in S1, while ethyl iso-allocholate was traced in S2. Compounds 9,12-octadecadienoic acid, ethyl ester, 7-methyl-Z-tetradecen-1-ol acetate, cis-10-heptadecenoic acid and octadecanoic acid, ethyl ester, and nicotine traces were found in S4, while compound 3,7,11,15-tetramethyl-2-hexadecen-1-ol, tetradecamethyl-hexasiloxane, and phytol in S5. Additionally, octadecamethyl cyclononasiloxane, oleic acid, and trimethylsilyl ester were found in S6 and S9, respectively. Interestingly, extracts S4, S10, and S6 were the most cytotoxic to the normal fibroblasts (hGF and MRC5, with low selectivity index: <1), compared with doxorubicin and with their effect on the cancerous cells (HT29 and HepG2). Various components detected in SLT samples were carcinogenic, including nicotine and its derivatives, hexadecanoic acid, 1,2-benzenedicarboxylic acid, and octadecanoic acid. The present study showed that the cytotoxic and possibly carcinogenic effects of the SLT samples on gingiva and lung cells are attributed to many compounds and not only nicotine derivatives, all of which could create health threats for SLT users and lead to various types of cancers, including oral, lung, colon, and liver cancers.

1. Introduction

Smokeless tobacco (SLT) and its chemical constituents have been linked to numerous physical and mental depressive disorders. In addition, it was also linked with 4% of all cancer types so far. During its cultivation, harvesting, or processing stages, more than two thousand compounds have been identified in tobaccos. The concentrations of these compounds may vary based on the method of use. For example, chewing or snuffing tobacco may contain compounds that exceed two orders of magnitude levels compared to compounds resulting from tobacco used by other methods [1].

The toxicity of SLT was the focus of several studies, as SLT exerts both local and systemic effects. The constituents found in SLT either originate from the leaves or as a result of additives during the production stage [2]. Investigating the cytotoxicity of SLT on normal cells could explain its possible toxicities. This is because cells from different body sites can be widely exposed to the tobacco material or its metabolites. Minimal to no toxicity is essential for the successful development of useable consumables [3].

The adverse effects of SLT in the human body are different; some are local, like leukoplakia, dental staining gingivitis, periodontitis, and carcinogenicity, while some are systemic like cardiovascular or psychosocial effects, including mitigating sleepiness and the social stigma [4–6]. In a Swedish study, SLT was proved to have distal effects, with a clear role in inflammatory bowel disease [7]. Moreover, SLT may affect cells by inducing mutations or cell transformation [1].

Worldwide, there are different forms of SLT with different names varying from country to country. Therefore, eleven SLT samples from Saudi Arabia were investigated using GC-MS in this study and were subjected to cytotoxicity assays to determine their effect on two normal cells (human gingival fibroblasts (hGF) and normal human fetal lung fibroblast (MRC5)), in addition to two cancer cells (human colon adenocarcinoma (HT29) and human hepatocellular carcinoma (HepG2)).

2. Materials and Methods

2.1. Collection and Preparation of SLT Extracts. Eleven SLT samples (S1–S11, Table 1) were purchased from different manufacturers in the Tabuk region, Saudi Arabia, in June 2019. Each of these samples (0.2 g) was macerated with 100 ml of ethanol. Each extract was concentrated with a rotary evaporator at 5000 r/sec. After 24 h, the extracts were separately filtered using Whatman filter paper (0.45 μ), concentrated by the Rota evaporator, and dried under reduced pressure to obtain dark-colored extracts, which were stored at 4°C in the dark.

2.2. GC-MS Analysis. Thermo Scientific GC-MS (US) equipped with the AS 3000 autosampler, Trace Ultra GC, and ISQ detector was used for gas chromatography-mass spectrometry analysis using a Thermo Scientific capillary column TR-5MS (30 m × 0.25 mm), film thickness 0.25 μm.

Table 1: Local names and physical nature of SLT samples of Tabuk region.

Sample	Local name	Physical nature
S1	Jeddah Shamma Barid-1	Dry
S2	Jeddah Shamma Barid-2	Dry
S3	Sudanese Tumbak-1	Wet
S4	Sudanese Tumbak-2	Dry
S5	Yellow Arishi Shamma	Dry
S6	Pakistani Niswar—green-1	Wet
S7	Pakistani Niswar—green-2	Dry
S8	Pakistani Niswar—brown-1	Dry
S9	Pakistani Niswar—brown-2	Wet
S10	Adani Shamma Barid—black	Wet
S11	Indian Shamma	Dry

Helium was the carrier gas at a 1.2 mL/min flow rate. The initial temperature was 70°C, which was increased at a 15°C/min rate to 290°C, and held for 30 min [8, 9]. Sample extracts (1 mg) were reconstituted in 1 ml ethanol and filtered through a 0.45 μ filter. Next, 2 μl was injected into the GC-MS system in the spitless mode.

2.3. Identification of Chemical Constituents. The GC-MS was used to identify the phytochemical constituents of SLT samples. The individual peaks identified the most volatile components by matching their retention indices with accurate values accessible in the library, closely resembling the reference samples. The mass spectra's shattering patterns achieved further identification matched with those deposited in the spectrometer catalog using the NIST08 and Wiley 9n/Adams MS library of the GC/MS records system and/or established with the aid of retention indices (RI) from available published sources. The relative percentage of separated compounds was calculated from FID chromatograms. According to the peak area integrated by the analysis program, the relative concentration of each compound was quantified [8, 9].

2.4. Cell Culture

2.4.1. Ethical Clearance and Extraction of hGF. Human gingival fibroblasts (hGFs) were collected from the gingiva of a healthy adult male at the Dental Teaching Hospital (Umm Al Qura University, Makkah, Saudi Arabia, UQU) after obtaining signed informed consent from the subject and approval of the Ethical Committee of the Faculty of Dentistry, UQU, Makkah, Saudi Arabia (IRB: 190-20, on 5-10-2020). The gingival tissues were washed with phosphate-buffered saline (PBS) and incubated in dispase 1 mg/mL (Sigma, USA) overnight at 4°C to facilitate the removal of the connective tissue from the epithelial layer. The epithelial layer was removed, and the connective tissue was cut into small pieces, cultured in a complete cell growth medium in a 25 mL tissue culture flask, and incubated at 37°C in a humidified atmosphere of 5% CO_2. The complete growth medium contained Dulbecco' modified Eagle medium (DMEM, Gibco Thermo Scientific, USA) supplemented with 10% fetal bovine serum (HyClone Thermo Scientific, USA),

100 U/mL penicillin, 100 μg/mL streptomycin (Sigma, USA), and 2.5 μg/mL amphotericin B (Gibco Thermo Scientific, USA). Another normal cell line was used, MRC5 (human fetal lung fibroblast), in addition to two cancer cell lines, HT29 (human colon adenocarcinoma) and HepG2 (human hepatocellular carcinoma); all three cell lines sourced from the ATCC, USA. MRC5 cell line was maintained in Eagles minimum essential medium (10% FBS, 1% penicillin/streptomycin). In comparison, the two cancer cells were subcultured in RPMI-1640 media (10% FBS, 1% penicillin/streptomycin), all at 37°C, 5% CO_2, and 100% relative humidity, for a maximum of 5–10 passages.

2.4.2. Cytotoxicity and Selectivity Studies. The cytotoxicity of the eleven SLT extracts was evaluated by MTT assay, as previously reported [9, 10]. Each of the normal or cancerous cell lines were separately cultured in the 96-well ($3–5 \times 10^3$/well) and incubated with each of the extracts or doxorubicin (positive control) at final concentrations 0–100 μg/mL for 72 h at 37°C (DMSO 0.1%; $n = 3$, three independent experiments). Subsequently, MTT (0.5 mg/ml) was added for each well and incubated for 3 h at 37°C. The MTT solution was removed, and formazan granules were dissolved in dimethyl sulfoxide (DMSO). Absorbance was read on a multiplate reader (BioRad, PR 4100, Hercules, CA, USA). The number of viable cells is proportional to the optical density of the purple formazan (A_{550}). The IC_{50} (sample concentration causing 50% inhibition compared to 100% control cell growth) was determined using GraphPad Prism. The selectivity index (SI) for a given extract was calculated by dividing its IC_{50} against hGF or MRC5 cells by IC_{50} against either HT29 or HepG2 cells [11].

3. Results

3.1. GC-MS Analysis and Identification. Various phytochemical constituents were identified in the SLT samples using GC-MS and are given in Table 2 (representative chromatograms are shown in Figure 1). The components in Table 2 are arranged in order of their elution on the TR 5MS capillary column. S1 ethanolic extract was characterized by large amount nicotine derivatives: pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (64.91%) followed by pyridine, 3-(1-methyl-1H-pyrrol-2-yl)- (3.82%) and cotinine (1.61%), in addition to phthalic acid ester (8.17%) and tetracyclo [4.4.1.1(7,10).0(2,5)]dodec-3-en-11-ol (1.01%). The comparative GC-MS analysis showed that the maximum amount of nicotine was present in S1.

While, 27.15% of nicotine was identified in S2, along with (1′s,2′s)-nicotine-N′-oxide (3.04%). Six different derivatives of fatty acid esters were also determined in the same samples like hexadecanoic acid, methyl ester (1.52%), hexadecanoic acid, ethyl ester (3.62%), 9-octadecenoic acid (Z)-, methyl ester (2.35%), 9,12-octadecadienoic acid (Z,Z)-methyl ester (1.55%), 9,12-octadecadienoic acid, ethyl ester (1.55%), and ethyl oleate (4.21%). Two phytosterols, viz., stigmasterol (1.99%) and sitosterol (1.35%) and major traces of octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (11.79%) and two minor amounts of ethyl iso-allocholate (1.01%) and silane, and 1,6-heptadiyne-1,7-diylbis(trimethyl- (1.02%) were also present.

Eight different types of fatty acid esters (43.48%) were detected in S3, followed by pyridine derivative (8.94%), nicotine oxide (1.55%), and cotinine (0.94%). The other compounds found were two phytosterols (2.71%), and individually identified compounds were phthalic acid ester (6.78%), silane derivative (4.1%), and siloxane derivative (8.11%). In S4, eleven different derivatives of fatty acid esters (69.55%) were identified. However, cis-10-heptadecenoic acid (2.43%), siloxane derivative (4.22%), and 1,2-benzenedicarboxylic acid, diisooctyl ester (2.41%) were the only compounds present individually in the S4 sample. Surprisingly, nicotine and its derivatives were found in trace amounts (1.06–0.24%) in the sample.

Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (33.15%), with 0.19% of minor nicotine oxide and 1,2-benzenedicarboxylic acid and diisooctyl ester (5.22%) were identified in S5 sample. Each of two fatty acid esters (4.83%), silane derivatives (6.91%), and siloxane derivatives (19.33%) along with single 3,7,11,15-tetramethyl-2-hexadecen-1-ol (1.23%), phytol (1.01%), 7-hexyl-eicosane (1.18%), and sitosterol (1.29%) compounds were also detected in S5.

After two siloxane derivatives (33.68%), nicotine (12.17%), silane derivative (8.67%), and phthalic acid ester (7.83%) were found to be the most prominent compounds identified in S6 ethanolic extract. Nicotine oxide (0.77%) was the only important compound present in a minor amount. Hexadecanoic acid, methyl ester (1.09%), was the only fatty acid ester present in traces. Regarding S7, nicotine and its oxide were present in 42.86% of the sample. Three higher alkanes (6.1%), siloxane derivative (8.74%), silane derivative (4.73%), two phytosterols (5.1%), and only phthalic acid ester (3.83%) were the other components detected in the extract.

The most prominent constituents present in S8 were nicotine and its oxide (33.14%) and phthalic acid ester (9.15%). Siloxane derivative (17.21%), silane moieties (8.78%), fatty acid ester (1.77%), and phytosterol (1.65%) were other essential constituents characterized in the chromatogram. Significant groups of seven different types of fatty acid esters (42.63%) were identified in S9. Phthalic acid ester (2.98%) and higher alkanes (7.29%) were present in high amounts. Moreover, nicotine and its oxide were characterized in trace amounts (0.95%).

Two siloxane derivatives (38.35%), 1,2-benzenedicarboxylic acid, diisooctyl ester (13.33%), 1-monolinoleoylglycerol trimethylsilyl (7.9%), pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- (3.48%), 9-octadecenoic acid (Z)-, methyl ester (4.4%), sitosterol (2.37%), and nicotine oxide (0.82%) were other important compounds present in small amounts in S10. Similar to S10, the same types of siloxane derivatives (35.05%), silane derivatives (5.32%), and phthalic acid ester (14.76%) were present in S11 in different proportions. Benzaldehyde, 2-nitro-4-trimethylsilyl- (1.42%), and 9-octadecenoic acid (Z)-, methyl ester (1.58%) were also identified in the S11 sample. Finally, 3.22% of nicotine and its oxide were found in S11.

Table 2: GC-MS analysis of SLT samples.

No.	RT	Chemical constituents	Area%/peak area (in million)											MW	MF	Identification
			S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11			
1	7.23	Benzaldehyde, 2-nitro-4-trimethylsilyl-	—	0.16/18	—	—	0.13/09	0.1/05	—	—	—	—	1.42/51	223	$C_{10}H_{13}NO_3Si$	Aromatic aldehyde
2	11.56	2-(O-Trimethylsilyloxyphenyl)-1-	—	0.21/23	—	—	—	—	—	—	—	—	1.12/40	282	$C_{14}H_{26}O_2Si_2$	Silane derivative
3	16.06	Silane, 1,6-heptadiyne-1,7-diylbis [trimethyl-	—	1.02/143	—	—	2/145	—	—	3.85/194	—	—	—	236	$C_{13}H_{24}Si_2$	Silane derivative
4	16.36	Cyclohexasiloxane, dodecamethyl-	—	—	—	—	—	3.1/168	—	—	0.45/144	1.83/79	4.82/175	444	$C_{12}H_{36}O_6Si_6$	Siloxane derivative
5	18.15	3-(1-Methyl-2-pyrrolidinyl)-,(S) pyridine	64.91/31716	27.15/3034	8.94/1497	0.16/67	33.15/2412	12.17/659	38.29/3388	32.58/1766	0.65/209	3.48/152	2.6/94	162	$C_{10}H_{14}N_2$	Pyridine derivative
6	20.35	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl-	3.30/1072	11.79/1363	8.11/1687	4.22/1716	18.01/1309	31.14/1649	8.74/765	17.21/901	1.76/505	36.52/1590	30.23/1112	578	$C_{16}H_{50}O_7Si_8$	Siloxane derivative
7	21.49	(1′s,2′s)-Nicotine-N′-oxide	0.12/10	3.04/339	1.55/258	0.24/97	0.19/14	0.77/41	3.64/322	0.56/30	0.30/95	0.82/35	0.62/33	178	$C_{10}H_{14}N_2O$	Nicotine oxide, alkaloid
8	21.65	3-(1-Methyl-1H-pyrrol-2-yl)- pyridine	3.82/1868	—	—	—	—	—	—	—	—	—	—	158	$C_{10}H_{10}N_2$	Pyridine derivative
9	26.97	Cotinine	1.61/788	—	0.94/157		—	—	0.93/82	—	—	—	—	176	$C_{10}H_{12}N_2O$	Alkaloid, an analog of nicotine
10	27.55	Cyclononasiloxane, octadecamethyl-	—	—	—	—	—	2.54/137	—	—	—	—	—	666	$C_{18}H_{54}O_9Si_9$	Siloxane derivative
11	28.67	Tetracyclo[4.4.1.1(7,10).0(2,5)]dodec-3-en-11-ol	1.01/494	—	—	—	—	—	—	—	—	—	—	176	$C_{12}H_{16}O$	A tetracyclic alkene
12	29.27	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	—	0.25/28	0.17/28	—	1.23/89	—	0.35/31	0.51/27	0.60/193	—	—	296	$C_{20}H_{40}O$	Higher alkene
13	31.10	Hexadecanoic acid, methyl ester	0.23/110	1.52/63	9.06/1517	7.3/2993	1.67/121	1.09/59	0.1/04	0.12/05	16.48/534	0.61/26	0.1/04	270	$C_{17}H_{34}O_2$	Fatty acid ester
14	32.41	Hexadecanoic acid, ethyl ester (palmitic acid ester)	0.1/34	3.62/404	2.81/470	19.0/8024	0.11/05	0.4/02	0.11/10	0.14/09	2.18/705	0.85/36	0.29/10	284	$C_{18}H_{36}O_2$	Fatty acid ester
15	34.98	9-Octadecenoic acid (Z)-, methyl ester or 9,12-octadecadienoyl chloride, (Z,Z)-	0.12/54	2.35/45	15/2112	3.91/1605	0.63/45	0.56/30	0.77/67	0.1/04	0.22/71	4.40/159	1.58/57	296	$C_{19}H_{36}O_2$	Fatty acid ester
16	35.11	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	—	—	—	—	3.16/229	—	—	1.77/95	—	—	—	292	$C_{19}H_{32}O_2$	Fatty acid ester
17	35.32	Phytol	—	—	—	—	1.01/73	0.26/13	0.5/44	0.75/40	—	0.2/08	—	296	$C_{20}H_{40}O$	Diterpenol
18	35.76	Octadecanoic acid, methyl ester	0.24/119	0.61/68	1.35/225	1.3/533	—	0.32/17	—	—	2.78/899	0.26/11	—	298	$C_{19}H_{38}O_2$	Fatty acid ester
19	36.78	9,12-Octadecadienoic acid (Z,Z)-methyl ester	—	1.55/173	2.81/469	1.26/583	—	—	—	—	—	—	—	280	$C_{18}H_{32}O_2$	Fatty acid ester
20	36.85	9,12-Octadecadienoic acid, ethyl ester	—	1.55/173	—	3.65/1498	—	—	—	—	0.57/183	—	—	308	$C_{20}H_{36}O_2$	Fatty acid ester

Table 2: Continued.

No.	RT	Chemical constituents	Area%/peak area (in million)											MW	MF	Identification
			S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11			
21	37.03	Ethyl oleate	—	4.21/470	2.77/464	18.52/7600	—	—	—	—	2.03/657	—	—	310	$C_{20}H_{38}O_2$	Fatty acid ester
22	37.89	Octadecanoic acid, ethyl ester	—	0.74/82	0.46/76	3.21/1315	—	—	—	—		—	—	312	$C_{20}H_{40}O_2$	Fatty acid ester
23	40.81	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	0.6/295	—	3.77/630	6.49/2664	—	—	—	—	6.97/8740	—	—	330	$C_{19}H_{38}O_4$	Fatty acid ester
24	42.53	7-Methyl-Z-tetradecen-1-ol acetate	—	—	—	3.66/1500	—	—	—	—		—	—	268	$C_{17}H_{32}O_2$	Fatty acid ester
25	43.68	cis-10-Heptadecenoic acid	—	—	—	2.43/995	—	—	—	—		—	—	268	$C_{17}H_{32}O_2$	Higher acid
26	45.73	1,2-Benzenedicarboxylic acid, diisooctyl ester	8.17/3992	16.1/2179	6.78/484	2.41/990	5.22/379	7.83/424	3.83/339	9.15/496	2.98/967	13.33/582	14.76/536	390	$C_{24}H_{38}O_4$	Phthalic acid ester
27	48.5	Oleic acid, trimethylsilyl ester	—	—	—	—	—	—	—	—	1.41/458	—	0.19/06	354	$C_{21}H_{42}O_2Si$	Fatty acid ester
28	48.69	9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester	—	—	5.91/756	1.35/145	—	—	—	—	10.78/3494	—	—	356	$C_{21}H_{40}O_4$	Fatty acid ester
29	51.02	7-Hexyl-eicosane	—	—	—	—	1.18/69	—	1.84/162	—	—	—	—	366	$C_{26}H_{54}$	Higher alkane
30	51.08	Hentriacontane	—	0.72/80	—	—	—	—	3.26/286	—	4.84/1566	—	—	437	$C_{31}H_{64}$	Higher alkane
31	52.69	Tetracosane, 11-decyl-	—	—	0.76/126	—	—	—	1/88	—	2.45/796	—	—	479	$C_{34}H_{70}$	Higher alkane
32	57.71	Ethyl iso-allocholate	—	1.01/112	—	—	—	0.54/29	0.83/73	—	0.24/77	—	0.25/09	436	$C_{26}H_{44}O_5$	Steroid
33	58.22	Stigmasterol	—	1.99/222	1.52/255	—	—	—	2.55/225	—	0.87/283	—	—	412	$C_{29}H_{48}O$	Phytosterol
34	59.47	Sitosterol	0.11/32	1.35/150	1.19/198	0.21/85	1.29/93	0.13/11	2.19/193	1.65/89	0.48/157	2.37/103	0.1/21	414	$C_{29}H_{50}O$	Phytosterol
35	66.26	1-Monolinoleoylglycerol trimethylsilyl ether	0.67/325	0.12/48	4.1/671	0.81/330	4.91/479	8.67/445	4.73/413	4.93/257	1.07/345	7.90/338	4.2/147	498	$C_{27}H_{54}O_4Si_2$	Silane derivative
36	70.32	Hexasiloxane, tetradecamethyl-	—	—	—	—	1.32/131	—	—	—	—	—	—	458	$C_{14}H_{42}O_5Si_6$	Siloxane derivative

RT, retention time; S1–S11, samples 1 to 11; MF, molecular formula; MW, molecular weight.

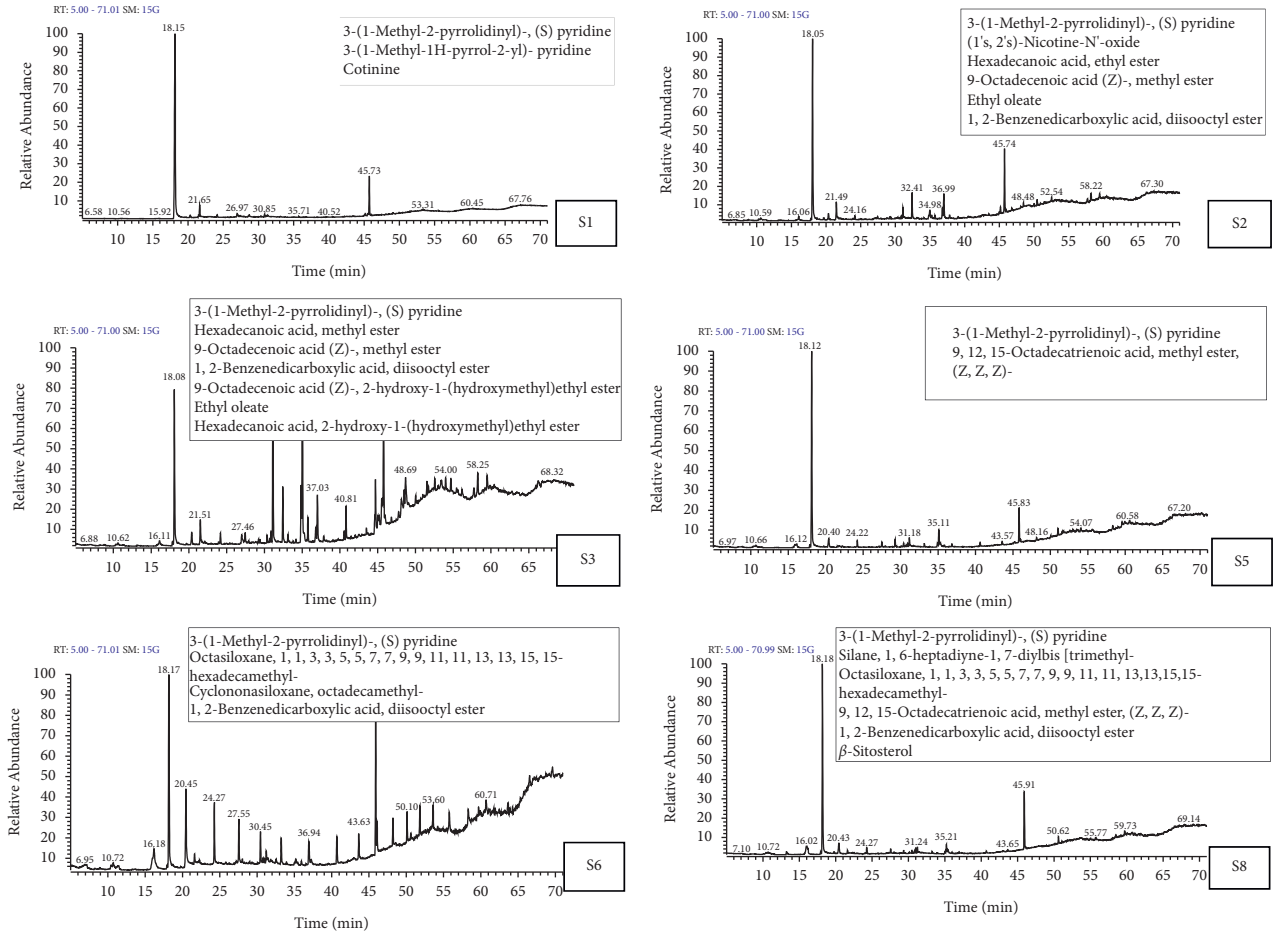


Figure 1: Total ion GC-MS chromatogram of some SLT samples showing (R, S)-nicotine (peak no. 1) with some major bioactive components (chromatograms of all samples are included in the supplementary file).

3-(1-Methyl-1H-pyrrol-2-yl)-pyridine (3.82%) and tetracyclo[4.4.1.1(7,10).0(2,5)]dodec-3-en-11-ol (1.01%) were only detected in S1, cis-10-heptadecenoic acid (2.43%) was identified in S4, hexasiloxane, tetradecamethyl- (1.32%) was limited to S5, and cyclononasiloxane, octadecamethyl- (2.54%) was unique compound present in S6.

3.2. Cytotoxicity and Selectivity Studies. The hGF and MRC5 cells were chosen for this study as they represent some of the main human tissue organs that could be affected by the SLT extracts following its consumption, i.e., the mouth gingiva and lungs, respectively. While, HT29 and HepG2 cancer cells were selected because they were derived from vital organs in the human gastrointestinal tract (GIT) that can be affected by SLT extracts and represent two of the most important GIT cancers worldwide. According to the cytotoxicity and selectivity index results (Tables 3 and 4), six out of the eleven samples showed a higher cytotoxic effect on normal cells when compared to doxorubicin. In contrast, all samples were cytotoxic against two cancer cells, but that toxicity was higher against two normal cells. All samples, except S1 against HepG2, showed low selectivity index (<1) towards normal cells, especially samples S4, S6, and S10 (Figures 2(c) and 2(d)), which were the most toxic to the normal fibroblasts (hGF and MRC5, average SI: 0.00, 0.03, and 0.00, respectively).

4. Discussion

4.1. GC-MS Analysis and Identified Cytotoxic Compounds. The GC-MS is one of the most effective techniques to study metabolomes with very high sensitivity and excellent separation capability. Despite its great chromatographic resolution, however, it can only be used for the identification of low-molecular-weight volatile compounds [12]. Overall, among the identified compounds, various types of chemical constituents including thirteen different fatty acid esters, four siloxane derivatives, four pyridine analogs, three types of higher alkanes, three silane derivatives, two different derivatives of phytosterols, and each of aromatic aldehyde, tetracyclic alkene, higher alkene, higher acid, diterpenol, phthalic acid ester, and steroid were all identified in different SLT samples. The major class of harmful compounds found in smokeless tobacco was a wide range of fatty acid esters, higher alkanes, steroids, siloxanes, and different pyridine derivatives.

Table 3: Cytotoxic effect of the eleven SLT extracts and doxorubicin against the human gingival fibroblasts, normal lung fibroblast, colon cancer, and liver cancer cells (MTT 72 h, $IC_{50} \pm SD$ μg/mL, $n = 3$).

Sample	hGF	MRC5	HT29	HepG2
S1	25.89 ± 3.56	2.13 ± 0.23	38.11 ± 0.21	22.52 ± 3.42
S2	12.67 ± 2.33	22.04 ± 5.22	36.89 ± 5.58	32.66 ± 4.93
S3	18.00 ± 2.91	4.14 ± 0.33	27.82 ± 2.24	52.48 ± 5.66
S4	0.14 ± 0.01	0.64 ± 0.10	29.07 ± 1.89	58.59 ± 1.61
S5	21.29 ± 3.22	2.83 ± 0.76	55.20 ± 3.77	56.48 ± 7.91
S6	1.08 ± 0.20	0.34 ± 0.17	11.03 ± 1.39	41.58 ± 2.37
S7	8.04 ± 1.22	13.97 ± 2.35	47.00 ± 5.96	50.32 ± 1.68
S8	4.89 ± 0.21	7.26 ± 1.88	9.90 ± 1.05	50.74 ± 4.58
S9	1.28 ± 0.21	35.14 ± 3.28	40.48 ± 8.05	46.19 ± 3.04
S10	0.11 ± 0.01	0.39 ± 0.01	22.66 ± 2.97	30.49 ± 1.63
S11	6.65 ± 1.10	10.41 ± 2.21	39.03 ± 5.89	47.63 ± 2.69
Doxorubicin	7.30 ± 0.66	5.91 ± 0.41	1.90 ± 0.15	2.03 ± 0.20

Table 4: Selectivity of the eleven SLT extracts and doxorubicin against HT29 and HepG2 cancer cells compared with hGF and MRC5 normal cells.

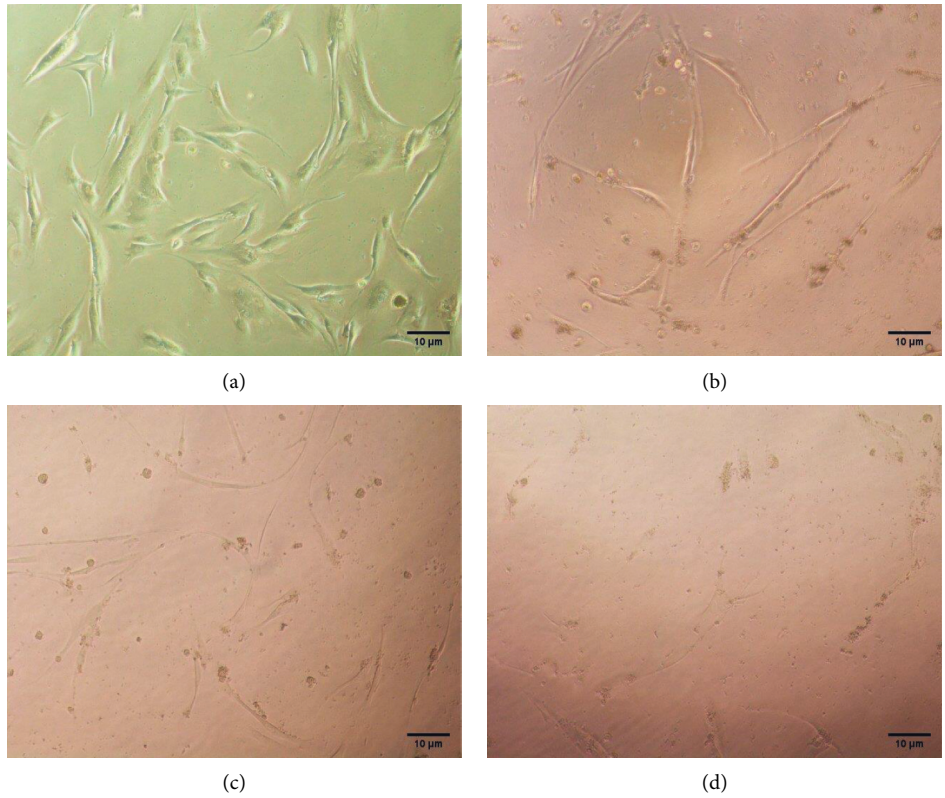
Sample	hGF		MRC5		Average SI
	HT29	HepG2	HT29	HepG2	
S1	0.67	1.14	0.05	0.09	0.48
S2	0.34	0.38	0.59	0.67	0.49
S3	0.64	0.34	0.14	0.07	0.29
S4	0.00	0.00	0.02	0.01	0.00
S5	0.38	0.37	0.05	0.05	0.21
S6	0.09	0.02	0.03	0.00	0.03
S7	0.17	0.15	0.29	0.27	0.22
S8	0.49	0.09	0.73	0.14	0.36
S9	0.03	0.02	0.86	0.76	0.41
S10	0.00	0.00	0.01	0.01	0.00
S11	0.17	0.13	0.26	0.21	0.19
Doxorubicin	3.84	3.59	3.11	2.91	3.36

<1, nonselective effect against the normal cell.

Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl- (1.76–36.52%), hexadecenoic acid, methyl ester (0.1–16.84%), hexadecanoic acid, ethyl ester (0.1–19%), 9-octadecenoic acid (Z)-, methyl ester (0.1–4.4%), 1,2-benzenedicarboxylic acid, diisooctyl ester (2.41–16.1%), sitosterol (0.11–2.37%), and 1-monolinoleoylglycerol trimethylsilyl ether (0.12–8.67%) were present in large amounts in all S1–S11 in different concentrations calculated on the bases of the peak area. Most of them were previously described as carcinogenic agents [1–4, 13–15].

7-Hexyl-eicosane, hentriacontane, and 11-decyl-tetracosane were the three higher alkanes found in S5, S7, and S9. These higher alkanes are asphyxiants that can slowly damage the lungs and skin when used in higher concentrations for a prolonged duration. Different degrees of carcinogenic and mutagenic properties are also reported [5, 6]. 3-(1-Methyl-2-pyrrolidinyl) pyridine (potent parasympathomimetic alkaloid) represented 0.16% and 64.91% of all chemicals determined in all SLT samples, while its derivative (1s,2s)-nicotine-N-oxide (0.12–3.64%) was identified in all samples with variable amounts, and cotinine (0.911173–1.61%) was found in S1, S3, S4, and S7 [16]. Nicotine and its derivatives are well-known triggers of various cancers, gene mutations, and malformations [8, 17, 18]. Interestingly, in different experimental models, cyclohexasiloxane and dodecamethyl were reported to have carcinogenicity, reproductive developmental toxicity, and neurotoxicity [17, 19]. Ethyl isoallocholate and stigmasterol were also known for their cytotoxic activity [20, 21]. Table 5 provides the major compounds present in more than one SLT sample, their structure, and cytotoxic effect against normal cells. Obviously, nicotine and its derivatives were reported to have major carcinogenic effects [17, 19]. They are also well-known triggers of gene mutations and malformations [8, 17, 18].

4.2. Cytotoxicity and Selectivity Studies. The MTT cytotoxicity assay showed that all SLT samples demonstrated a low selectivity index towards each of the normal fibroblasts: hGF and MRC5, which was worse than the selectivity caused by doxorubicin. Primarily samples S4, S10, and S6 showed zero selectivity. This could be attributed to their inclusion of toxic compounds previously discussed. In another study, which also used MTT assay, the hGF cells cultures were exposed once (5–15 min) for cigarette smoke, which caused the reduction of its growth [28]. Thus, it is convincing to anticipate that the more toxic effect of SLT samples used in the present study on the hGF cells, compared with the smoked cigarettes, could be correlated to the long and direct exposure time. The number of toxic compounds identified and the



(a) (b)

(c) (d)

Figure 2: Microscopic examination (×40, scale 10 μM) showing the cytotoxic effect of some samples on hGF normal cells treated (72 h) with (a) vehicle control, (b) doxorubicin (7.30 μg/mL), (c) S4 (0.14 μg/mL), and (d) S10 (0.11 μg/mL).

Table 5: Major compounds present in SLT samples, structure, and cytotoxic effects against normal cells.

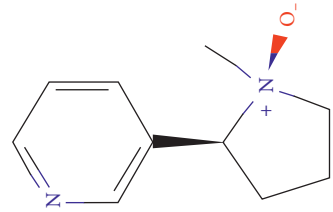
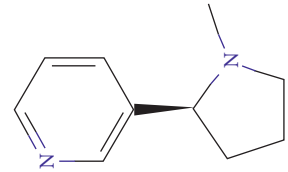
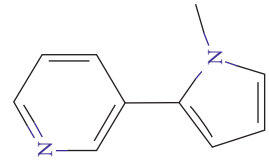
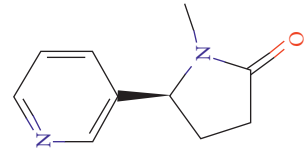
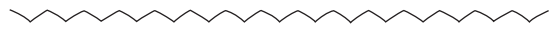
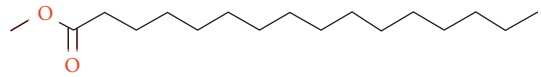
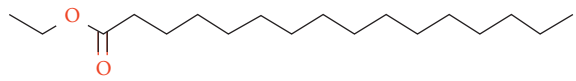
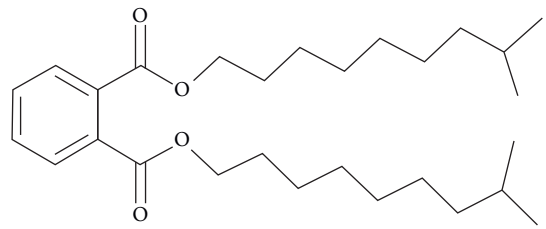
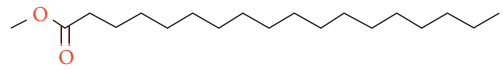
Compound		Structure	Effect	Reference
Nicotine derivatives	(1′s,2′s)-Nicotine-N′-oxide		Carcinogenic, promote tumor formation	[22, 23]
	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-		Carcinogenic, cytotoxic against normal fibroblasts (MRC5)	[8]
	Pyridine, 3-(1-methyl-1H-pyrrol-2-yl)-			
	Cotinine		Carcinogenic, genotoxicity, impaired osteogenesis	[24, 25]

Table 5: Continued.

Compound	Structure	Effect	Reference
Hentriacontane		Genotoxicity, impaired osteogenesis	[26, 27]
Hexadecanoic acid, methyl ester		Gingival recession, carcinogenic	[4]
Hexadecanoic acid, ethyl ester			
1,2-Benzenedicarboxylic acid, diisooctyl ester		Fatal toxicity, carcinogenic	[3, 8]
Octadecanoic acid, methyl ester		Carcinogenic	[23]

cytotoxic effect revealed in this study are very alarming, keeping in mind the long-term use of SLT by users and the potential role of saliva as important dissolving media which facilitate the ingestion of the SLT toxic materials inside the user's body through the digestion system.

5. Conclusions

The current study resulted in the identification of uncommon constituents noted along with the tobacco leaves. It also resulted in identifying toxic and carcinogenic components in several SLT local samples of the Tabuk region using GC-MS. The cytotoxicity investigations identified that samples are more toxic for the gingiva and lung cells compared to their effect on the colon and liver cancer cells. Samples S4, S10, and S6 were the most cytotoxic to the normal cells. S4 contained traces of nicotine derivatives, which shows that its toxicity is due to the synergistic effect of other components. Considerations for these constituents and their harmful effects are necessary. The correlation between the composition of these samples and their cytotoxic properties is evident in this study, as many compounds with previously reported cytotoxicity were identified. Thus, the saliva of persons consuming SLTs may contain carcinogenic and toxic compounds that may cause oral, lung, colon, liver, and other related cancers. These findings make good reasons to call for the manufacturers and users to abandon such harmful SLT products.

Data Availability

The data used to support this study are included within the article and the supplementary file.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

The supplementary file contains the GC-MS chromatograms of eleven SLT samples (S1–S11) showing major identified compounds (Figure S1) and a table showing all the samples identified by the GC-MS analysis with peak area% of 0.1 and higher (Table S1). (*Supplementary Materials*)

References

[1] IARC, "Monographs on the evaluation of the carcinogenic risk of chemicals to humans," *Tobacco Habits Other than Smoking; Betel Quid and Avoca Nut Chewing; and Some Related Nitrosamines*, Vol. 37, International Agency for Research on Cancer, Lyon, France, 1985.

[2] S. G. Cannella, S. S. Kagan, M. Kagan et al., "Mass spectrometric analysis of tobacco-specific nitrosamine hemoglobin adducts in snuff dippers, smokers and nonsmokers," *Cancer Research*, vol. 50, pp. 5438–5445, 1990.

[3] L. J. McGaw, E. Elgorashi, and J. N. Eloff, "Cytotoxicity of african medicinal plants against normal animal and human cells," *Toxicological Survey of African Medicinal Plants*, Elsevier, Amsterdam, Netherlands, pp. 181–233, 2014.

[4] USDHHS, *The Health Consequences of Using Smokeless Tobacco. A Report of the Advisory Committee to the Surgeon General*, US Department of Health and Human Service, Bethesda, MD, USA, 1986.

[5] E. A. Izzaddinn, S. A. Khalid, A. M. Mohammed, I. M. Yassin, O. M. Hyder, and K. Asaad, "Is smokeless tobacco used to mitigate the daytime sleepiness and depression? A case-control study in Tabuk city, Saudi Arabia," *Medical Science*, vol. 24, no. 105, pp. 2925–2931, 2020.

[6] I. E. Ahmed and S. H. Rami, "Effects of smokeless tobacco on the blood pressure and pulse rate among Sudanese users: a

cohort study," *Medical Science*, vol. 25, no. 110, pp. 781–787, 2021.

[7] S. Mackey, *The Saudis: Inside the Desert Kingdom*, W. W. Norton and Company, New York, NY, USA, 2002.

[8] A. Khalid, H. A. Alhazmi, A. N. Abdalla et al., "GC-MS analysis and cytotoxicity evaluation of Shammah (smokeless tobacco) samples of Jazan region of Saudi Arabia as promoter of cancer cell proliferation," *Journal of Chemistry*, vol. 2019, Article ID 3254836, 8 pages, 2019.

[9] M. Al Bratty, H. A. Makeen, H. A. Alhazmi et al., "Phytochemical, cytotoxic, and antimicrobial evaluation of the fruits of Miswak plant, Salvadora persica L." *Journal of Chemistry*, vol. 2020, Article ID 4521951, 11 pages, 2020.

[10] A. N. Abdalla, U. Shaheen, Q. M. A. Abdallah et al., "Pro-apoptotic activity of Achillea membranacea essential oil and its major constituent 1,8-cineole against A2780 ovarian cancer cells," *Molecules*, vol. 25, no. 7, p. 1582, 2020.

[11] A. N. Abdalla, A. Qattan, W. H. Malki, I. Shahid, M. A. Hossain, and M. Ahmed, "Significance of targeting VEGFR-2 and cyclin D1 in luminal-A breast cancer," *Molecules*, vol. 25, no. 20, p. 4606, 2020.

[12] P.-G. Persson, G. Hellers, and A. Ahlbom, "Use of oral moist snuff and inflammatory bowel disease," *International Journal of Epidemiology*, vol. 22, no. 6, pp. 1101–1103, 1993.

[13] A. K. Singh and R. Chandra, "Pollutants released from the pulp paper industry: aquatic toxicity and their health hazards," *Aquatic Toxicology*, vol. 211, pp. 202–216, 2019.

[14] B. Atasever-Arslan, K. Yilancioglu, M. G. Bekaroglu, E. Taskin, E. Altinoz, and S. Cetiner, "Cytotoxic effect of extract from dunaliella salina against SH-SY5Y neuroblastoma cells," *General Physiology and Biophysics*, vol. 34, no. 2, pp. 201–207, 2015.

[15] F. Maiyo, R. Moodley, and M. Singh, "Phytochemistry, cytotoxicity and apoptosis studies of B-sitosterol-3-oglucoside and B -amyrin from Prunus africana," *Africa Journal of Traditional Complementary and Alternative Medicine*, vol. 13, no. 4, pp. 105–112, 2016.

[16] S. A. Grando, "Connections of nicotine to cancer," *Nature Reviews Cancer*, vol. 14, no. 6, pp. 419–429, 2014.

[17] S. Etteieb, S. Cherif, A. Kawachi et al., "Combining biological and chemical screenings to assess cytotoxicity of emerging contaminants in discharges into surface water," *Water, Air, & Soil Pollution*, vol. 227, no. 9, p. 341, 2016.

[18] A. Aziz, P. Agamuthu, and S. Fauziah, "Effective removal of p-tert-butylphenol and pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)- from landfill leachate using locust bean gum," *Waste Management & Research: The Journal for a Sustainable Circular Economy*, vol. 36, no. 12, pp. 1146–1156, 2018.

[19] H. El-Naggar and A. Hasaballah, "Acute larvicidal toxicity and repellency effect of Octopus cyanea crude extracts against the filariasis vector, Culex pipiens," *Journal of the Egyptian Society of Parasitology*, vol. 48, no. 3, pp. 721–728, 2018.

[20] R. S. Thakur and B. Ahirwar, "A steroidal derivative from Trigonella foenum graecum L. that induces apoptosis in vitro and in vivo," *Journal of Food and Drug Analysis*, vol. 27, no. 1, pp. 231–239, 2019.

[21] M. Ayaz, A. Sadiq, A. Wadood, M. Junaid, Z. K. Nadir, and N. Zaman Khan, "Cytotoxicity and molecular docking studies on phytosterols isolated from Polygonum hydropiper L." *Steroids*, vol. 141, pp. 30–35, 2019.

[22] E. J. LaVoie, A. Shigematsu, A. Rivenson, B. Mu, and D. J. Hoffmann, "Evaluation of the effects of cotinine and nicotine-N′-oxides on the development of tumors in rats initiated with N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide," *Journal of the National Cancer Institute*, vol. 75, no. 6, pp. 1075–1081, 1985.

[23] N. A. Habib, C. B. Wood, K. Apostolov et al., "Stearic acid and carcinogenesis," *British Journal of Cancer*, vol. 56, no. 4, pp. 455–458, 1987.

[24] D. Dalberto, C. C. Nicolau, A. L. H. Garcia, A. P. Nordin, I. Grivicich, and J. D. Silva, "Cytotoxic and genotoxic evaluation of cotinine using human neuroblastoma cells (SH-SY5Y)," *Genetics and Molecular Biology*, vol. 29, no. 2, p. 43, 2020.

[25] R. H. Aspera-Werz, S. Ehnert, D. Heid et al., "Nicotine and cotinine inhibit catalase and glutathione reductase activity contributing to the impaired osteogenesis of SCP-1 cells exposed to cigarette smoke," *Oxidative Medicine and Cellular Longevity*, vol. 2018, Article ID 3172480, 13 pages, 2018.

[26] I. El-Alam, A. Verdin, J. Fontaine et al., "Ecotoxicity evaluation and human risk assessment of an agricultural polluted soil," *Environmental Monitoring and Assessment*, vol. 190, no. 12, p. 738, 2018.

[27] E. Bingham, B. Cohrssen, and C. H. Powell, *Patty's Toxicology*, John Wiley & Sons, New York, NY, USA, 5th edition, 2001.

[28] A. Semlali, J. Chakir, and M. Rouabhia, "Effects of whole cigarette smoke on human gingival fibroblast adhesion, growth, and migration," *Journal of Toxicology and Environmental Health. Part A*, vol. 74, no. 13, pp. 848–862, 2011.