

Research Article

GC-MS Analysis and Cytotoxicity Evaluation of *Shammah* (Smokeless Tobacco) Samples of Jazan Region of Saudi Arabia as Promoter of Cancer Cell Proliferation

Asaad Khalid ¹, Hassan A. Alhazmi,^{1,2} Ashraf N. Abdalla,^{3,4} Waquar Ahsan ²,
Shahnaz Sultana,⁵ Mohammed Al Bratty ², Sadique A. Javed,² and Zia ur Rehman²

¹Substance Abuse Research Centre, Jazan University, P.O. Box 114, Jazan 45142, Saudi Arabia

²Department of Pharmaceutical Chemistry, College of Pharmacy, Jazan University, P.O. Box 114, Jazan 45142, Saudi Arabia

³Department of Pharmacology and Toxicology, Faculty of Pharmacy, Umm Al-Qura University, Makkah 21955, Saudi Arabia

⁴Medicinal and Aromatic Plants Research Institute, National Center for Research, P.O. Box: 2424, Khartoum 11111, Sudan

⁵Department of Pharmacognosy, College of Pharmacy, Jazan University, P.O. Box 114, Jazan 45142, Saudi Arabia

Correspondence should be addressed to Asaad Khalid; drasaad@gmail.com

Received 19 December 2018; Revised 17 February 2019; Accepted 4 March 2019; Published 15 April 2019

Academic Editor: Angelo Minucci

Copyright © 2019 Asaad Khalid et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Shammah is a locally manufactured form of smokeless tobacco (ST) which is traditionally used in Middle Eastern countries including Saudi Arabia, Sudan, and Yemen. Presence of a high concentration of nicotine, in addition to various other toxic and carcinogenic constituents, makes it a serious human health threat. It is an admixture containing powdered tobacco, along with several additives, such as lime, ash, black pepper, volatile oils, and flavoring agents. This study was conducted to investigate the constituents of eight different samples of widely used *shammah* varieties in the Jazan region of Saudi Arabia using GC-MS and to evaluate their cytotoxic effect against three cancer cell lines representing most of the top malignancies in the region including MCF-7, A2780, and HT29 cancer cells, in addition to MRC5 cells (normal human fetal lung fibroblast) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. GC-MS analysis showed the presence of nicotine or 3-(1-methyl-2-pyrrolidinyl)pyridine (2.1–91.9% of total constituents detected) in all ST samples, whereas its derivative (1s,2s)-nicotine-*N*-oxide (0.23–1.62%) was detected in four samples. In addition, several known carcinogenic constituents were also identified, and their carcinogenicity was confirmed by MTT results, in which, all the eight samples promoted the growth of MCF7, A2780, and HT29 cancer cells. The cytotoxic effects of samples against the normal cells MRC5 was proportional to the number of components detected by GC-MS. The ingestion of these constituents through saliva of *shammah* consumers could be the reason for many cancers including breast, ovary, and colon cancers. These results support the urgent local and international call to educate the users regarding the deleterious effects of *shammah* to avoid its use.

1. Introduction

Smokeless tobacco (ST) is used by over 300 million people worldwide, mostly from South Asian and Middle Eastern countries [1]. In Saudi Arabia, the use of ST is more prevalent in the southern region (Jazan Province) due to its proximity to Yemen, where its use and trade is legal. *Shammah* is a locally manufactured form of smokeless tobacco. It is an addictive substance, used by placing in the oral cavity, and saliva produced is ingested thereafter. Generally, *shammah* is produced

mainly from the tobacco leaves, including *N. tabacum*, *N. rustica*, *N. glauca*, or *N. nepalensis* [2, 3]. The tobacco leaves are further mixed with other materials such as carbonate of lime, ash, black pepper, volatile oils, and other coloring and flavoring agents to enhance its properties [4]. A number of harmful substances, including at least 28 potential carcinogens, have been identified in *shammah*; among these substances, nitrosamines are reported to be the most harmful [5].

Smoked tobacco have been reported to increase the risk of developing at least 14 types of cancer and is responsible

for about 87% of deaths from lung cancer and 25–30% of general cancer deaths [6]. On the contrary, the chewed ST has been responsible for about 400,000 oral cancer cases so far, which represents around 4% of all the cancer types [6]. In addition to that, the use of smokeless tobacco is also associated with a wide range of adverse health issues, including cardiovascular diseases, weight loss, increased blood urea, and creatinine levels which seem to cause serious degenerative alterations and lead to periportal fibrosis in liver and edematous and calcified changes in renal glomerulus [7]. Aqueous extracts of ST hinder growths of some oral flora, which affect their balance, causing oxidative stress via degradation of nicotine into hydroxynicotine and cotinine-*N*-oxide inside the bacterium [8, 9]. Furthermore, disruption of sperm-head morphology, decreased total sperm count, increased oxidative stress, and high genotoxic and germ cell toxic effects have been seen in animals treated with tobacco [10]. According to the Saudi Arabia cancer incidence report of 2014, the most common cancer in the Jazan region is colon cancer in males and breast cancer in females [11].

Despite the fact that nicotine itself is not carcinogenic, its derivatives such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosonornicotine might be responsible for the carcinogenic effect [12]. Moderate and reversible toxicity along with weight loss was found in the esophagus, stomach, liver, kidneys, and lungs [13]. The presence of ammonia, benzo[a]pyrene, cadmium, nickel, nicotine, nitrates, and tobacco-specific nitrosamines increase the risk of probabilistic cancer [14]. The use of tobacco is thought to cause systematic stress in humans with increased inflammation and RBC membrane damage [15]. Heavy metals such as arsenic and nickel have synergistic effects with risk factors associated with oral cancer [16, 17].

To the best of our knowledge, no data are available on the constituents and cytotoxicity of ST/*shammah* locally manufactured in Saudi Arabia. Therefore, this could be the first study to explore the constituents of *shammah* samples from the Jazan region using GC-MS and their carcinogenic properties on the breast, ovary, and colon cancer cells, by MTT cytotoxicity assays. We assume that this study will effectively highlight the carcinogenic potential of locally used *shammah* and could strengthen the social and governmental efforts to minimize the use of these substances among the local inhabitants and stimulate the awareness in the society about the adverse health effects associated with the use of *shammah*.

2. Materials and Methods

2.1. Shammah Material. *Shammah* powder was collected from different producers in Jazan, Abu Arish, Sabiya and Ahad al Masariyah cities of the Jazan region. A total of eight *shammah* samples were collected, which include Arishi *shammah* from Jazan city (S1), Arishi *shammah* from Sabiya (S2), Arishi *shammah* from Ahad al Masariyah (S3), Adani cold *shammah* from Jazan city (S4), Adani cold *shammah* from Abu Arish (S5), Adani cold *shammah* from Sabiya (S6), special *shammah* from Sabiya (S7), and Khadrah *shammah* from Ahad al Masariyah (S8). These *shammah*

varieties also differ in their color as the Arishi *shammah* is yellow in color and is also called as yellow *shammah*, whereas other types are brown, white, black, and grey *shammah* depending upon the additives used as the coloring agent. As discussed earlier, *shammah* is manufactured locally by mixing powdered tobacco leaves with several other constituents that are added externally to increase its acceptability and potency. These ingredients include several known substances such as lime, ash, black pepper, volatile oils, coloring agents, and flavoring agents and several unknown substances that are added by the manufacturer to impart unique properties to the product. Many of these substances are harmful to human health and can cause cancer if exposed for long time.

2.2. Preparation of Different Extracts. Each of the eight *shammah* samples (0.2 g) was extracted and homogenated at 5000 R/sec with different polar (water and ethanol) and nonpolar solvents (chloroform and petroleum ether). After 24 h, the extracts were filtered, concentrated by distillation, and dried under reduced pressure to obtain a dark-colored mass. The residues were stored at 4°C in the dark for GC-MS analysis. The crude ethanolic extract of *shammah* samples was prepared for MTT cytotoxic investigations according to method described by Harborne [18]. In brief, 50 g of *shammah* powder was macerated in 300 mL of 80% (v/v) aqueous ethanol for 72 h at room temperature with shaking. The supernatant was filtered through 0.45 µm filter paper and dried.

2.3. GC-MS Analysis of the Extracts. GC-MS analyses were carried out on a Shimadzu Gas Chromatograph instrument fitted with a capillary column TR-5MS (30 m × 0.25 mm) and film thickness 0.25 µm. The carrier gas He was used at a flow rate of 1.2 mL/min. The initial temperature was 70°C, which was increased at a rate of 15°C per min to 290°C and held for 30 min. The chromatograph was coupled to Shimadzu QP2010 Ultra MS detector 70 eV.

2.4. Identification of Constituents. Constituents were identified by GC-MS by comparing their retention indices with those of authentic standards available in the laboratory or with the retention indices, which were in close agreement with reference. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using the NIST08 and Wiley 9 built-in libraries.

2.5. Cell Culture. Three cancer cell lines MCF7 (human breast adenocarcinoma), A2780 (human ovary adenocarcinoma), and HT29 (human colon adenocarcinoma) and one normal cell line MRC5 (normal human fetal lung fibroblast) were used in this study. All cells were obtained from the American Type Culture Collection (ATCC). The three cancer cells were subcultured in RPMI-1640 media (10% FBS), while MRC5 was maintained in the Eagles minimum essential medium (EMEM, 10% FBS), all incubated at 37°C, 5% CO₂, and 100% relative humidity.

2.6. Cytotoxicity Assay. The cytotoxicity of the eight *shammah* samples was evaluated by the MTT assay as per the method described in the literature [19]. The three cell lines and one normal fibroblasts were separately cultured in 96 well (3×10^3 /well) and incubated at 37°C overnight. Final compound concentrations 0, 6.25, 12.5, 25, 50, and 100 $\mu\text{g}/\text{mL}$ (DMSO 0.1%; $n = 3$) were used in the study. Plates were incubated for 72 h, followed by addition of MTT to each well. Plates were further incubated for 3 h, the supernatant were aspirated, and DMSO was added to each well. Absorbance was read on multiplate reader (Bio-Rad PR4100). Optical density of the purple formazan A_{550} is proportional to the number of viable cells. Compound concentration causing 50% inhibition (IC_{50}) compared to control cell growth (100%) was determined. GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA was used for analysis.

3. Results

3.1. GC-MS Analysis. Using GC-MS analysis, we were able to separate and identify various constituents of *shammah* samples efficiently. Representative chromatograms of three *shammah* samples are shown in Figure 1. All components identified in *shammah* samples along with their retention times are summarized in Table S1, whereas the major toxic/carcinogenic components are listed in Table 1 along with their structures.

The first variety of tested *shammah* was Arishi *shammah*, the samples of which were purchased from Jazan (S1), Sabiya (S2), and Ahad al Masariyah (S3) cities of Jazan Province. The GC-MS analysis of all the samples revealed the presence of similar groups of constituents including alkanes, siloxanes, pyridine derivatives, fatty acid esters, and amides (Table S1). Nicotine was extracted mainly in the nonpolar solvents, petroleum ether, and chloroform. On the basis area%, proportion of nicotine in different samples was found to be 12.96–89.45% in S1, 4.52–72.86% in S2, and 18.96–66.78% of the total extract in S3. Siloxane, another important constituent, was found to be distributed among the samples in different types and quantities. Dodecamethyl cyclohexasiloxane (S1: 1.54–3.52%; S2: 1.34–2.01%; S3: 0.86–1.68%), 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane (S2: 0.77–1.77%; S3: 0.68–1.88%), and hexamethyl cyclo-trisiloxane (S1: 0.67–8.52%; S2: 0.5–18.19%) were detected in different sample extracts. Among alkanes, undecane (S1: 0.41–0.86%; S3: 1.83–1.89%), tridecane (S2: 0.93–4.19%; S3: 0.73–1.22%), and tricosane (S1: 4.82%; S3: 0.82–1.18%) were the important ones detected mainly in the nonpolar solvents. Fatty acids and esters were found to be present in significantly high concentrations in almost all the Arishi samples. The major fatty acid esters include methyl hexadecanoate (S1: 19.78–23.24%; S2: 1.7–13.69%; S3: 3.85–34.1%), methyl linoleate (S1: 3.32–3.84%; S2: 0.65–1.41%; S3: 1.69–2.28%), methyl 7-octadecenoate (S3: 1.87–13.45%), and methyl oleate (S1: 20.17–20.81%; S2: 0.67–12.6%; S3: 0.41–18.23%). Oleic acid, a fatty acid, was detected only in the *shammah* sample collected from Ahad al Masariyah (S3) at a proportion of 1.41–3.07%. Dimethyl 2-hydroxy-4-[(tetrahydropyran-2-yl)

oxymethyl]benzene-1,3-dicarboxylate was the only aromatic ester detected in all extracts of S2, except aqueous. A nitrogenous heterocyclic compound, 1-tert-butyl-3,3,4-trimethyl-3,4-dihydro-1H-benzopyran-1-ol, and an aromatic compound, 1-phenyl-5,5-dimethyl-4,6-dioxo-5-sila-8-nitrooct-1-ene, were present in S2 in different concentrations ranging from 2.87% to 7.33%. The *shammah* sample from Jazan (S1) was also characterized by the presence of butyl 2-amino-5-methylbenzoate, an aromatic ester, in varying concentrations from 0.93% to 1.0%.

Adani Baarid (cold) *shammah* collected from Jazan city (S4), Abu Arish (S5), and Sabiya (S6) was also characterized by GC-MS. Two pyridine derivatives, nicotine and nicotine-*N*-oxide, were detected. Nicotine-*N*-oxide was not present in S4 and S5 but present in minor quantities (0.29–1.05%) in S6. Nicotine was the major constituent detected in all the tested samples with concentrations ranging between 55.04 and 87.56% in S4, 66.1–89.2% in S5, and 0.03–90.03% in S6. Sample S6 was characterized by only two alkanes tridecane and tricosane in trace quantities (1.5–2.26%), while several alkanes, including undecane, tridecane, pentadecane, tricosane, pentatriacontane, nonacosane, tetradecane, and tetracontane, were detected in S4. The sample collected from Abu Arish (S5) was found to contain similar alkanes with slightly higher area% (0.34–7.26%) in different extracts. Among siloxanes, dodecamethyl cyclohexasiloxane and 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane were the major contributors in all the samples in minor quantities. Several fatty acids and their esters including methyl palmitate (S4–S6) and methyl linoleate (S4) were also observed in these samples in small to moderate concentrations. The Adani Baarid *shammah* from Sabiya (S6) showed the presence of some compounds that was unique and not detected in other samples. Hexanedioic acid bis(2-ethylhexyl)ester was the aliphatic ester present in aqueous extract in 3.84% of the total components detected. Oleanitrile was present in an area% of 5% in aqueous extract. 1,2-Benzenedicarboxylic acid dibutyl ester, 1,2-benzenedicarboxylic acid bis(2-methylpropyl)ester, 1,2-benzenedicarboxylic acid bis(2-methoxyethyl)ester, and 1,2-benzenedicarboxylic acid bis(2-ethylhexyl) ester were derivatives of aromatic esters present in aqueous extract at concentrations ranging from 1.85 to 45.29% of the total components. S4 showed the presence of an amide, 13-docosenamide (0.15–3.76%), whereas, interestingly, caffeine was identified in S4 (0.09–0.5%) and S5 (0.68–1.41%) samples which may be due to the presence of other additives such as tea leaves that would have been added to *shammah* to enhance its organoleptic properties.

The GC-MS analysis showed that the petroleum ether, chloroform, ethanol, and aqueous extracts of Khususi (special) *shammah* from Sabiya (S7) possessed various types of chemical constituents including alkanes, siloxanes, fatty acid ester, and pyridine derivatives (Table S1). Among pyridine derivatives, nicotine or 3-(1-methyl-2-pyrrolidinyl)pyridine was present in highest amount with area percentage ranging between 15.77 and 72.18%. Among siloxane derivatives, dodecamethyl cyclohexasiloxane was present in a concentration of 0.62–3.06%; moreover, 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane was found to be present in

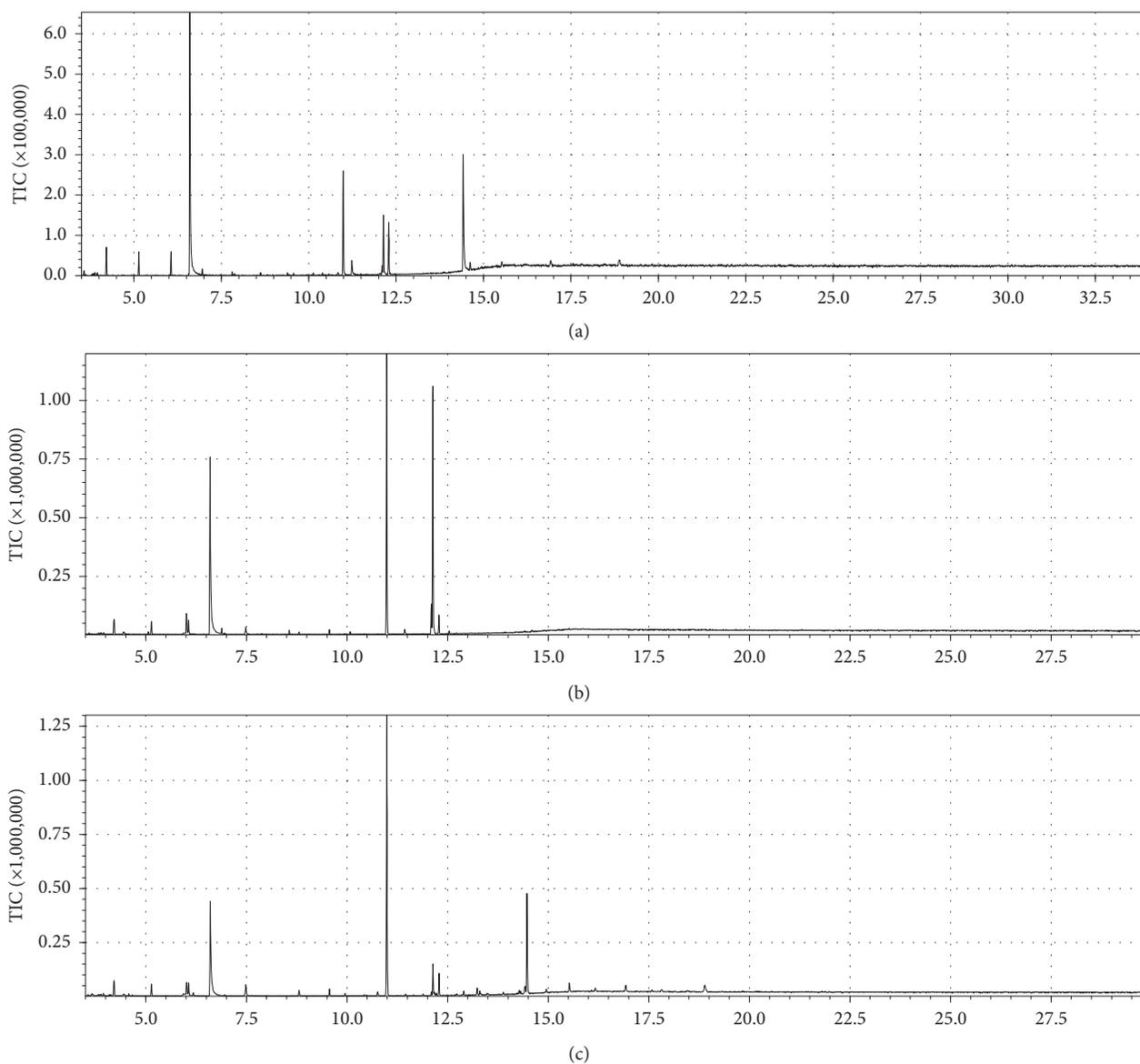


FIGURE 1: Representative typical GC-MS total ion current (TIC) chromatograms of three shamamah samples. (a) **S1** (pet. ether); (b) **S2** ethanol; (c) **S8** (chloroform).

S7. Several fatty acids and their esters were also found to be present in low to higher proportion with methyl linoleate (5.64%) and methyl oleate (35.26%) being highest in the aqueous extract of the samples. Alkanes, such as undecane, tridecane, dodecane, 2,6,10,14-tetramethyl heptadecane, pentadecane, and tricosane, were also detected.

Last sample, the Khadrah (green color) *shamamah* procured from Ahad al Masarihah (**S8**) also characterized and found to possess various types of chemical constituents including alkanes, siloxanes, pyridine derivatives, and fatty acid esters. Nicotine was present in all the extracts ranging from 10.12 to 87.91%, with the maximum amount again observed in petroleum ether extract. The other pyridine derivative, (1*s*,2*s*)-nicotine-*N*-oxide, was also present in **S8** in the non-polar extracts. Among siloxanes, dodecamethyl cyclohexasiloxane and 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane were the most important

constituents in all the extracts present in smaller quantities. Several fatty acids and their esters were detected, and these include methyl hexadecanoate (9.76–17.08%), methyl oleate (1.43–1.92%), methyl linoleate, methyl stearate, and methyl 9,10-dichloro-octadecenoate in different extracts, majorly in nonpolar solvents. **S8** was found to contain another constituent, 1-*tert*-butyl-3,3,4-trimethyl-3,4-dihydro-1*H*-benzopyran-1-ol, present in extremely low concentrations in petroleum ether (0.71%) and ethanolic extract (2.11%). Alkanes, on the other hand, were detected in low to good quantities, among which undecane (1.14–3%) and pentadecane (0.68–1.61%) were the major ones.

3.2. Cytotoxicity Assay. The MTT assay revealed that all the eight samples promoted the growth of MCF7, A2780, and HT29 cancer cells, as all the IC_{50} values (except samples no.

TABLE 1: The chemical formula/structure of toxic/carcinogenic components identified in various *shammah* types using GC-MS.

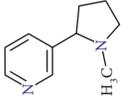
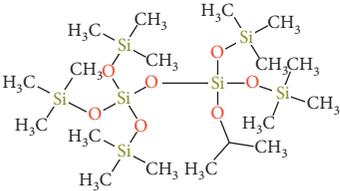
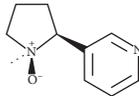
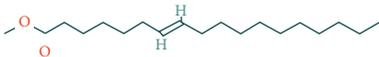
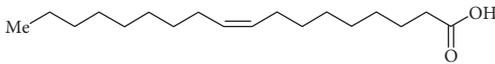
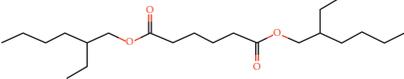
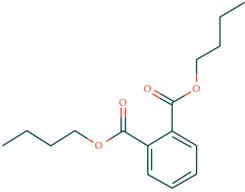
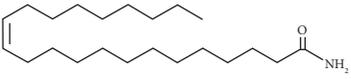
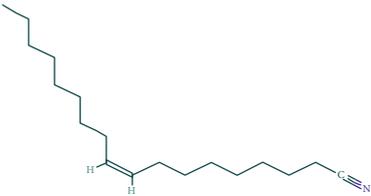
S. No.	Component
1.	$\text{CH}_3(\text{CH}_2)_{11}\text{CH}_3$ Tridecane
2.	$\text{CH}_3(\text{CH}_2)_{13}\text{CH}_3$ Pentadecane
3.	 3-(1-Methyl-2-pyrrolidinyl) pyridine
4.	 3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5- tris(trimethylsiloxy)tetrasiloxane
5.	 (1s,2s)-Nicotine-N-oxide
6.	 Methyl 7-octadecenoate
7.	$\text{CH}_3(\text{CH}_2)_{32}\text{CH}_3$ Tetratriacontane
8.	 Oleic acid (9-octadecenoic acid)
9.	$\text{CH}_3(\text{CH}_2)_{10}\text{CH}_3$ Dodecane
10.	$\text{CH}_3(\text{CH}_2)_{12}\text{CH}_3$ Tetradecane
11.	 Hexanedioic acid bis(2-ethylhexyl) ester

TABLE 1: Continued.

S. No.	Component
12.	 1,2-Benzenedicarboxylic acid dibutyl ester
13.	 13-Docosenamide
14.	$\text{CH}_3(\text{CH}_2)_{14}\text{CH}_2\text{C}(=\text{O})\text{OCH}_3$ Methyl heptadecanoate
15.	 Oleanitrile

S5 and S6 against A2780) were between 25 and 70 $\mu\text{g}/\text{mL}$. The samples S1–S5 and S7 were found to be more cytotoxic against MRC5 normal cells compared to the cytotoxicity of these samples on the three cancer cells (Table 2). On the other hand, samples S6 and S8 have exhibited less cytotoxic property against MRC5 (IC_{50} 48 and 54 $\mu\text{g}/\text{mL}$, respectively) compared to their effect on the three cancer cells.

4. Discussion

From different materials, additives, and flavors used in manufacturing *shammah*, the major ingredient tobacco contains at least 50 carcinogens [6]. The degree and intensity of exposure to carcinogens in terms of duration and quantity is crucial in developing cancer [20]. This may indicate the increased danger of extracted and swallowed components of smokeless tobacco over the components of smoking tobacco. Smokeless tobacco (*shammah*) is used by placing about 10 g into the mouth cavity, and it is placed between the gum and the lip, under the cheek or below the tongue on the floor of the mouth. It is then sucked slowly for a period of 15 minutes to few hours, which vary from

TABLE 2: Cytotoxic activity of the eight extracts against three cell lines and one normal fibroblast (MTT 72 h IC₅₀ ± SD µg/mL).

Sample no.	IC ₅₀ ± SD (µg/mL)			
	MCF7	A2780	HT29	MRC5
S1	34.43 ± 3.62	39.60 ± 0.79	43.07 ± 1.24	23.02 ± 3.09
S2	41.47 ± 0.15	70.04 ± 0.72	39.66 ± 0.48	9.55 ± 0.44
S3	54.84 ± 1.19	48.92 ± 2.42	55.39 ± 5.68	21.28 ± 1.27
S4	26.44 ± 1.70	52.75 ± 1.51	51.29 ± 1.91	15.34 ± 1.44
S5	64.52 ± 4.92	24.00 ± 1.98	57.03 ± 3.45	16.21 ± 0.18
S6	26.23 ± 1.50	17.27 ± 1.04	39.63 ± 2.24	48.17 ± 1.03
S7	25.34 ± 1.15	44.44 ± 2.10	48.54 ± 2.62	13.19 ± 1.58
S8	35.75 ± 5.15	47.62 ± 1.23	44.45 ± 2.07	54.86 ± 2.50

person to person. The saliva generated meanwhile is swallowed, resulting in significantly high concentration of *shammah* components ingested.

Highly toxic/carcinogenic compounds identified in several *shammah* samples were higher *n*-alkanes such as tridecane, tetradecane, pentadecane, tetratriacontane, and dodecane. Tridecane, tetradecane, and pentadecane are asphyxiant (similar to the C6–C10 alkanes) when aspirated into the lungs. These alkanes can cause chemical pneumonitis and slow death [21]. Tetradecane is shown to be a carcinogen and tumor promoter in a two-stage experiment of benzo[a]pyrene carcinogenicity in mice [21]. It has also been found to enhance mitogenic response of murine spleen lymphocytes to the lectin phytohemagglutinin [21]. However, pentadecane is not a known toxic compound but may be harmful if taken in higher quantity by inhalation, ingestion, or skin absorption. Pentadecane, when aspirated into the lungs, has acted as an asphyxiant and was incubated *in vitro* with rabbit heart mitochondrial model [22]. On the contrary, dodecane can be irritating to mucous membranes [21]. It has also been shown to be a promoter of skin carcinogenesis for ultraviolet radiation [21]. Dodecane did not increase mutations when tested in the Ames *Salmonella typhimurium* assay with and without metabolic activation. However, it was able to enhance mutagenesis induced by methylazoxymethanol acetate at the ouabain resistance locus [21]. Tetratriacontane is similar to homologous series of *n*-alkanes ranging from *n*-C12-*n*-C31 and was found in all samples of the liver, heart, kidneys, muscle, and adipose bovine tissues [23]. Other potential carcinogenic components detected in *shammah* samples include fatty acid methyl 7-octadecenoate, which has been reported to promote skin tumor formation in mice [21]. Components with a lower degree of toxicity include 1,2-benzenedicarboxylic acid dibutyl ester (toxic for reproduction), and hexanedioic acid, which have produced kidney and testis damage when given to the experimental animals for a longer period of time [24].

Nicotine and nicotine derivative ((1*s*,2*s*)-nicotine-*N*-oxide) were also identified in *shammah* samples included in this study. Nicotine is a pale yellow to dark brown liquid present in 2–8% of the tobacco leaves dry mass. Cotinine is a predominant metabolite of nicotine, which have been studied for its antidepressant activity. 1,3-Dimethylurea is a colorless crystalline powder with low toxicity and is being used for synthesis of caffeine, pharmaceuticals, and

herbicides. Cis-13-docosenoamide/erucylamide is an amide of docosenoic acid that reduces mobility and awareness in rats, whereas 9-octadecenamide induces physiological sleep. The siloxane (organosilicone) compounds are probably added to the mixture as surfactants (wetting agents) or antifoaming agents. Cyclohexasiloxane and dodecamethyl are widely used as conditioning agents, emollients, in personal care products, lubricants, and defoaming and antimicrobial agents [25, 26]. Constituents like 3-(methylnitrosamino)-propionitrile, nitrosamines, and nicotine have been reported to induce the production of reactive oxygen species in ST, leading eventually to fibroblast, DNA, and RNA damage with carcinogenic effects in the mouth of tobacco consumers. The metabolic activation of nitrosamine in tobacco by cytochrome P450 enzymes may lead to the formation of *N*-nitrososonornicotine, a major carcinogen, and micronuclei, which are an indicator of genotoxicity. These effects further enhance DNA damage, which eventually lead to oral cancer [27].

Interestingly, 3-isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane which is one of the components in *khat* plant (*Catha edulis*) was also identified in the *shammah* sample in this experiment. This has indicated the possibility of mixing *khat* plant with *shammah* by some of the manufacturers. *Khat* is a well-known CNS stimulant with amphetamine-like properties [28].

All the *shammah* samples were subjected to the MTT cytotoxicity assay. The results of the MTT assay and the IC₅₀ values of the eight tested samples on MCF7, A2780, and HT29 cancer cells and the MRC5 noncancerous/normal cells have been shown in Table 2. The difference of the pattern of cytotoxicity produced by the tested samples against MRC5 is evident in sample S7 (14 detected component), as it has promoted HT29 cancer cell growth and was found to be cytotoxic for MRC5 cells too (Table S1, Figure 2(a)). The sample S5 (8 detected components) has also promoted HT29 cancer cell growth; however, it was less cytotoxic for MRC5 cells (Table S1, Figure 2(b)). It has indicated that if more components are present or mixed in the *shammah* samples, there are more possibility of both promotion of cancer cell growth and cytotoxicity of the normal cells. Samples, S6 and S8, were less cytotoxic against MRC5, which could be attributed to the fewer components detected in the GC-MS chromatogram, compared to samples S1–S5 and S7.

Studies regarding the constituents of *shammah* are very scarce and more emphasis has been given to the phytochemical analysis of smokeless tobacco. This study aimed to detect the chemicals added to smokeless tobacco during the manufacture of *shammah* and to evaluate their overall cytotoxicity. Earlier, 28 cancer-causing chemicals have been identified in ST and is regarded as known human carcinogen [29, 30]. This effect is mainly due to the presence of tobacco-specific nitrosamine (TSNA) which is a proven and potent carcinogen, in addition to polycyclic aromatic hydrocarbons (PAHs) in varying concentrations. Therefore, previous studies mainly aimed to detect the concentrations of nicotine, TSNA, and PAHs in various ST samples [31, 32].

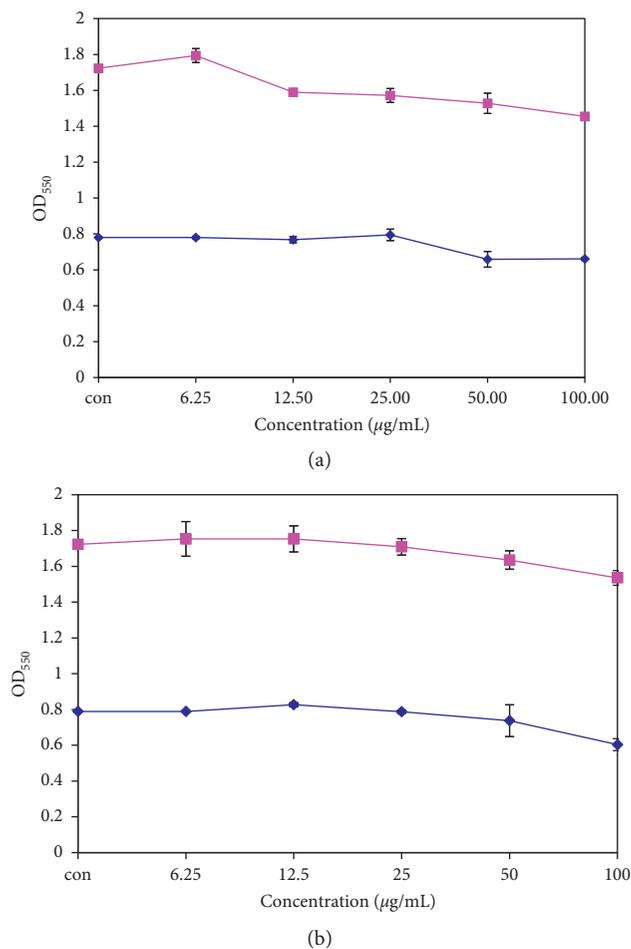


FIGURE 2: Cytotoxic effect of samples (a) S7 and (b) S8. Pink: MRC5 cells; blue: HT29 cells. X-axis: concentration $\mu\text{g/mL}$; Y-axis: optical density A_{550} ($n=3$). Experiments were repeated $\times 3$.

5. Conclusion

In conclusion, various toxic and carcinogenic components have been identified by GC-MS analysis of several *shammah* samples collected from the Jazan region. Several uncommon constituents have also been identified owing to the fact that *shammah* is locally manufactured and is an admixture of various additives along with tobacco leaves. These additives are added to enhance the organoleptic properties, taste, and mood-enhancing properties of *shammah*. However, these additives vary from one manufacturer to another and also among *shammah* samples collected from different places and may have serious adverse effects on human health. The knowledge of these constituents and their harmful effects are necessary. The cytotoxicity investigations showed that these samples have promoted the proliferation of cancer cells and killing the normal human cells within rich mixtures. Correlations are clear between the composition of these *shammah* samples and their carcinogenic properties. Thus, it is more likely that the human saliva containing digested *shammah* could be reason for many cancers including breast, ovary, and colon cancers.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was financially supported by a fund from Deanship of Scientific Research, Jazan University (Research Group no. RG-2-9). The authors are thankful to the Deanship of Scientific Research, Jazan University, for providing financial assistance (Research Group no. RG-2-9) to carry out this research work. The authors are also thankful to Mr. Hamad Hakami for his technical assistance in this study.

Supplementary Materials

Table S1: components identified in various *shammah* samples by GC-MS. (*Supplementary Materials*)

References

- [1] National Cancer Institute and Centers for Disease Control and Prevention, *Smokeless Tobacco and Public Health: A Global Perspective*, U.S. Department of Health and Human Services, Centers for Disease Control and Prevention and National Institutes of Health, National Cancer Institute, NIH Publication No. 14-7983, Bethesda, MD, USA, 2014.
- [2] J. C. Winter, *Tobacco Use by Native North Americans: Sacred Smoke and Silent Killer*, Vol. 236, University of Oklahoma Press, Norman, OK, USA, 2000.
- [3] D. L. C. Valdés, C. Gonzalez, R. Lara et al., "Genetic diversity of wild species of Nicotiana genus. I: characterization using biochemical markers," *Revista de Protección Vegetal*, vol. 25, no. 2, pp. 88–97, 2010.
- [4] R. M. Alsanosy, "Smokeless tobacco (shammah) in Saudi Arabia: a review of its pattern of use, prevalence, and potential role in oral cancer," *Asian Pacific Journal of Cancer Prevention*, vol. 15, no. 16, pp. 6477–6483, 2014.
- [5] IARC, "Smokeless tobacco and some tobacco-specific N-nitrosamines," in *IARC Monographs on the Evaluation of Carcinogenic Risks to Human*, IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, Ed., vol. 89, pp. 1–592, WHO Press, Geneva, Switzerland, 2007.
- [6] P. Anand, A. B. Kunnumakara, C. Sundaram et al., "Cancer is a preventable disease that requires major lifestyle changes," *Pharmaceutical Research*, vol. 25, no. 9, pp. 2097–2116, 2008.
- [7] N. Al-Mukhaini, T. Ba-Omar, E. Eltayeb et al., "Liver and kidney toxicity induced by Afzal smokeless tobacco product in Oman," *Tissue and Cell*, vol. 49, no. 2, pp. 307–314, 2017.
- [8] M. Liu, J. Jin, H. Pan et al., "Effect of smokeless tobacco products on human oral bacteria growth and viability," *Anaerobe*, vol. 42, pp. 152–161, 2016.
- [9] J. Sun, J. Jin, R. D. Beger, C. E. Cerniglia, M. Yang, and H. Chen, "Metabolomics evaluation of the impact of smokeless tobacco exposure on the oral bacterium *Capnocytophaga sputigena*," *Toxicology In Vitro*, vol. 36, pp. 133–141, 2016.

- [10] S. Das, P. Upadhaya, and S. Giri, "Arsenic and smokeless tobacco induce genotoxicity, sperm abnormality as well as oxidative stress in mice in vivo," *Genes and Environment*, vol. 38, p. 4, 2016.
- [11] Z. Al-Shahrani, A. Al-Rawaji, AN. Al-Madouj, and MS. Hayder, *Cancer Incidence Report, Saudi Arabia 2014*, Saudi Health Council, Saudi Cancer Registry, Riyadh, Saudi Arabia, 2014.
- [12] N. Moghbel, B. Ryu, P. J. Cabot, and K. J. Steadman, "In vitro cytotoxicity of *Nicotiana glauca* leaves, used in the Australian Aboriginal smokeless tobacco known as pituri or mingkulpa," *Toxicology Letters*, vol. 254, pp. 45–51, 2016.
- [13] C. Yu, Z. Zhang, Y. Liu et al., "Toxicity of smokeless tobacco extract after 184-day repeated oral administration in rats," *International Journal of Environmental Research and Public Health*, vol. 13, no. 3, 2016.
- [14] M.-A. Song, C. Marian, T. M. Brasky, S. Reisinger, M. Djordjevic, and P. G. Shields, "Chemical and toxicological characteristics of conventional and low-TSNA moist snuff tobacco products," *Toxicology Letters*, vol. 245, pp. 68–77, 2016.
- [15] S. Biswas, K. Manna, U. Das et al., "Smokeless tobacco consumption impedes metabolic, cellular, apoptotic and systemic stress pattern: a study on Government employees in Kolkata, India," *Science Reports*, vol. 5, p. 18284, 2015.
- [16] S. S. Arain, T. G. Kazi, H. I. Afridi et al., "Estimation of nickel in different smokeless tobacco products and their impact on human health of oral cancer patients," *Nutrition and Cancer*, vol. 67, no. 7, pp. 1063–1074, 2015.
- [17] P. Roy, A. Mukherjee, and S. Giri, "Evaluation of genetic damage in tobacco and arsenic exposed population of Southern Assam, India using buccal cytome assay and comet assay," *Ecotoxicology and Environmental Safety*, vol. 124, pp. 169–176, 2016.
- [18] J. Harborne, "Methods of plant analysis," in *Phytochemical Methods*, pp. 1–36, Springer, Berlin, Germany, 1984.
- [19] M. M. Bkhaitan, A. Z. Mirza, A. N. Abdalla et al., "Reprofiling of full-length phosphonated carbocyclic 2'-oxa-3'-azanucleosides toward antiproliferative agents: synthesis, antiproliferative activity, and molecular docking study," *Chemical Biology and Drug Design*, vol. 90, no. 5, pp. 679–689, 2017.
- [20] M. C. White, D. M. Holman, J. E. Boehm, L. A. Peipins, M. Grossman, and S. Jane Henley, "Age and cancer risk," *American Journal of Preventive Medicine*, vol. 46, no. 3, pp. S7–S15, 2014.
- [21] K. P. Coleman and W. A. Toscano, "Esters of mono- and alkenyl carboxylic acids and mono- polyalcohols," in *Patty's Toxicology*, E. Bingham, B. Cofrancesco, and C. H. Powel, Eds., p. 67, John Wiley & Sons, New York, NY, USA, 2001.
- [22] A. R. Borgatti, G. Trigari, V. Ventrella, and A. Pagliarani, "Interaction of n-alkanes with respiration and oxidative phosphorylation in rabbit heart mitochondria: n-dodecane, n-pentadecane and n-octadecane," *Bollettino Della Società Italiana di Biologia Sperimentale*, vol. 57, no. 15, pp. 1583–1589, 1981.
- [23] C. Lintas, A. M. Balducci, M. P. Bernardini, and A. Di Muccio, "Distribution of hydrocarbons in bovine tissues," *Lipids*, vol. 14, no. 3, pp. 298–303, 1979.
- [24] V. O. Sheftel, *Indirect Food Additives and Polymers: Migration and Toxicology*, Lewis Publishers, Boca Raton, FL, USA, 2000.
- [25] V. Gaddaguti, S. Jwala Mounika, K. Sowjanya et al., "GC-MS analysis and in silico molecular docking studies of mosquito repellent compounds from *Hyptis suaveolens* L," *International Journal of Bioassays*, vol. 1, no. 9, pp. 36–41, 2012.
- [26] K. A. Wagner, C. B. Huang, M. S. Melvin, R. Ballentine, N. K. Meruva, and J. W. Flora, "Gas chromatography-mass spectrometry method to quantify benzo[a]pyrene in tobacco products," *Journal of Chromatographic Science*, vol. 55, no. 7, pp. 677–682, 2017.
- [27] K. Niaz, F. Maqbool, F. Khan, H. Bahadar, F. Ismail Hassan, and M. Abdollahi, "Smokeless tobacco (paan and gutkha) consumption, prevalence, and contribution to oral cancer," *Epidemiology and Health*, vol. 39, article e2017009, 2017.
- [28] P. Kalix, "A constituent of khat leaves with amphetamine-like releasing properties," *European Journal of Pharmacology*, vol. 68, no. 2, pp. 213–215, 1980.
- [29] National Cancer Institute, *Smokeless Tobacco or Health: An International Perspective*, Smoking and Tobacco Control Monograph 2, National Cancer Institute, Bethesda, MD, USA, 1992.
- [30] National Cancer Institute (NCI), "Smokeless tobacco and cancer," September 2014, <http://www.cancer.gov/about-cancer/causes-prevention/risk/tobacco/smokeless-fact-sheet#r1>.
- [31] I. Stepanov, P. W. Villalta, A. Knezevich, J. Jensen, D. Hatsukami, and S. S. Hecht, "Analysis of 23 polycyclic aromatic hydrocarbons in smokeless tobacco by gas chromatography– mass spectrometry," *Chemical Research in Toxicology*, vol. 23, no. 1, pp. 66–73, 2009.
- [32] S. B. Stanfill, R. E. Croucher, P. C. Gupta et al., "Chemical characterization of smokeless tobacco products from South Asia: nicotine, unprotonated nicotine, tobacco-specific N'-Nitrosamines, and flavor compounds," *Food and Chemical Toxicology*, vol. 118, pp. 626–634, 2018.



Hindawi

Submit your manuscripts at
www.hindawi.com

