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Assessment of Cytotoxicity of Smokeless Tobacco (Shammah) In Hepg2 and WRL68 Cells Line

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ABSTRACT

Shammah is a traditional form of chewing tobacco [Smokeless tobacco, (ST)] that is commonly used in the Middle east specially Saudi Arabia (KSA), Yemen and Sudan. The cytotoxicity of Sudanese and Yemenis ST hexane and methanol extracts was evaluated using MTT assay. Annexin-V assay has been used to detect the induction of apoptosis. Luminescence based assay also been conducted to check the level of caspases enzyme. The involvement of cell cycle check point arrest has been performed using flow cytometry analysis. The current study found that ST has the capacity to induce cell toxicity in human liver cells. The inhibitory capacity of ST in HepG2 and WRL 68 has been found to be 151 ± 2.5 and 305 ± 11.5 μg/ml for 24 h. An early apoptosis induction in HepG2 cells was observed by annexin V assay, which clearly exhibited significantly increased early and late apoptosis phases both at 24 and 48 h. Both the caspases-8 and-9 level was found to be increased by the introduction of ST to HepG2 cells significantly ($p<0.05$). Moreover the ST extract was able to arrest the cell cycle check point at G2/M phase. A significantly increasing pattern of hypodiploid phases of cells also been observed, which confirm the apoptosis induction again. Collectively, results presented in this study demonstrated that the ST, which is used as a euphoritic substance of abuse also, has significant level of toxicity in human cells. Moreover the mode of cell death was found to be though programmed cell death which is closely associated with cell cycle arrest.

Key words: Hepatotoxicity, *In vitro* models, Saudi Arabia, Smokeless tobacco, Substance Absue Research Centre, Shammah.

INTRODUCTION

Smokeless tobacco is an unburned tobacco products that chewed in the oral cavity.[1,2] The unwanted harmful effects associated with the chewing of ST include cancerous and leukoplakian lesions of the oral cavity, periodontal disorders, and nicotine addiction. Immune-dysregulation of immune cells and their components may play a significant role in the progression of some unfavorable health effects associated with ST use. Some evidences also suggesting reproductive outcomes also exist in conjunction with ST use. Cardiovascular endothelia and blood pressure may also be impacted by ST use. Nitrosamines as tumorigenic products were also detected in ST products within allowed levels of concentrations.[3-8] These carcinogens have been extracted and chemically identified from ST samples. On the other hand, mutagenesis of ST have well been established using *in vivo* and *in vitro* models,[9,10] Cytotoxicity of ST on oral cell lines was observed *in vitro* to include inhibition of cellular proliferation, apoptotic cell death and involvement of inflammation pathways.[11]

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Aqueous extract of ST at a single dose was previously found to cause oxidative stress in rat's liver causing increased levels of malondialdehyde. An earlier research showed treatment of HOK-16B cells with ST extract caused cell death. These findings provide an established scientific explanation to the ulceration and inflammation observed in people using ST at a new site within 2 days.[12-17]

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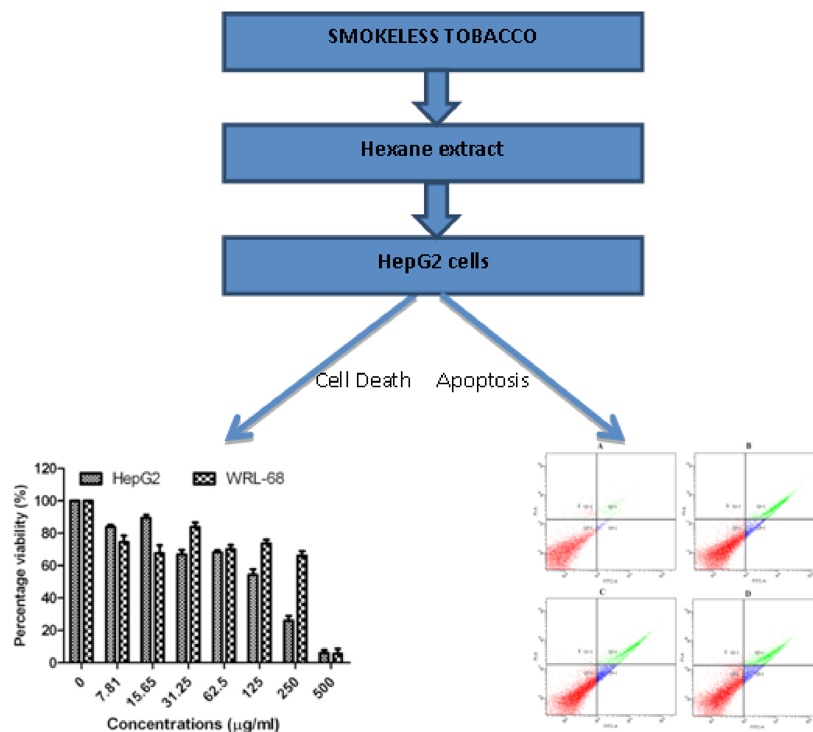

Graphical Abstract

and Sudan. In Sudan, about 40% of males and 10% of females use toombak (local name of ST).[18,19] Today, the trade of certain types of ST, such as moist snuff, is also illegal in several countries, e.g., in Saudi Arabia, Japan, Hong Kong, Singapore, Australia and New Zealand.[20] The There is various botanical taxonomy for tobacco plants. Therefore, the toxicity of these tobacco species and their health impacts may be reasonably different. To the best of our knowledge no studies were conducted to explore the hepatotoxicity of polar and non-polar extracts of ST using well-established *in vitro* models of liver toxicity. Therefore, the cytotoxicity of Sudanese and Yemenis ST hexane and methanol extracts was evaluated using established *in vitro* models of liver toxicity.

MATERIAL AND METHODS

Smokeless Tobacco Samples

Samples from Sudan and Yemen were obtained from exclusive shops in Khartoum (Sudan) and Jazan (Saudi Arabia), respectively.

Extraction procedure

Both Sudanese and Yemenis ST powder were added with enough quantity of hexane and macerated for three days with occasional shaking. The extracts were then filtered through filter paper Whatman® No. 41 and the residue were exposed to methanol for the polar extracts. The extraction procedure was same as done with hexane to obtain non-polar extracts. The filtrate was evaporated to dryness using (Buchi, R-210, Postfach, Switzerland) and store in refrigerator (-20ºC) until used.

Determination of cytotoxicity

Both HepG2 and WRL cells lines were purchased from ATCC, USA. Cells were plated and incubated until it reaches attachment and confluency. The medium was aspirated off and replaced with fresh medium (200 µl) containing ST extract of different concentrations in 96-well microplate except the last row which was left as a control. The plates were incubated at 37°C, 5% CO_2, for 24, 48 and 72 h. MTT containing medium was removed and replaced with 200 µl DMSO per well. The plate was mixed until the formazan crystals were dissolved and the plates were read on microtiter plate reader at 570 nm. The IC_{50} is generated from the dose-response curves for each cell lines. Three replicates in 96-well plates were analyzed for each cell type, concentration, and exposure period.

Cell cycle analysis by flow cytometry

The cell cycle phase arrest was studied using flow cytometry. HepG2 cells were plated and treated with ST. After 24 h, the cells were harvested in centrifugal tubes

and centrifuged at 3000 rpm for 5 min and fixed with 90% ethanol after washing with phosphate buffered saline (PBS) and refrigerated at -30°C. On the following day, the fixed cells were centrifuged at 3000 rpm for 5 min and the system was resuspended with 1 ml PBS. This washing process was repeated twice. One hundred microliter of 200 μg/ml DNAse free RNAseA enzyme was added to the cells and incorporated at 37°C for 30 min. The cells then were stained with 100 μl of 1mg/ml of propidium iodide and left at room temperature for 10 min. The cells were immediately analyzed using the FACS Canto II Becton-Dickinson Flowcytometryby analyzing at least 10,000 cells per sample. The percentage of cells in G1, S and G2 phases were analyzed by Mod Fit LT software (Verity Software House, Topsham, ME).

Caspase-8 and 9 activity assay

Caspase-8 and -9 activities were measured using luminescence-based assay, Caspase-Glo™ 8 Assay, and Caspase-Glo™ 9 Assay (Promega, US). Cells were cultured in 96-well culture plates in 50 μl of RPMI1640 supplemented with 10% Fetal Bovine Serum (FBS) and incubated for 24 h. Cells were then treated with different concentrations of ST extracts. At the end of incubation, 100 μl of assay reagent was added to be incubated for 1 h at room temperature. Luminescence was measured using a microplate reader (Tecan Infinite M 200 PRO, Männedorf, Switzerland).

Annexin V assay

Cells (1×10^5 cells/ml) were exposed to ST for 24, 48 and 72 h and an Annexin V assay was done according to BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit (APO Alert Annexin V, Clon Tech, California, USA) instructions. In short, cells were centrifuged at 200 x g for 10 min. Supernatant was removed and cells were rinsed with 1 x binding buffer and re-suspended in 200 μl of binding buffer from the kit. Cells were stained with 5 μl of Annexin V and 10 μl of propidium iodide (Sigma, Saint Louis, Missouri, USA) and incubated in the dark for 15 min at room temperature. Using a FACS Canto II Becton-Dickinson flow cytometer, analysis was performed with at least 10,000 cells/sample. The reaction volume was brought to 500 μl for the flow cytometry analysis with the aid of binding buffer available from the kit. Cells treated with DMSO (0.1%, v/v) served as control.

RESULTS

Cytotoxicity on Liver Cells

The effect of ST samples on cell growth inhibition was expressed as an IC_{50} value. Only hexane extract of Yemenis sample showed toxicity on liver cells. Cellular proliferation following 24 h of exposure to ST samples showed considerable inhibition in ST-treated cells compared to non-treated cells (controls). The proliferation of ST-treated cells decreased as the ST concentration with IC_{50} of 151 ± 2.5 and 305 ± 11.5 μg/mL on HEPG2 and WRL-68, respectively for the hexane extract of Yemen shamma (Figure 1).

Annexin V

To determine the occurrence of apoptosis, annexin-v assay has been used. The HepG2 cells have been treated with the IC_{50} concentration of ST for 24, 48 and 72 h. The results had

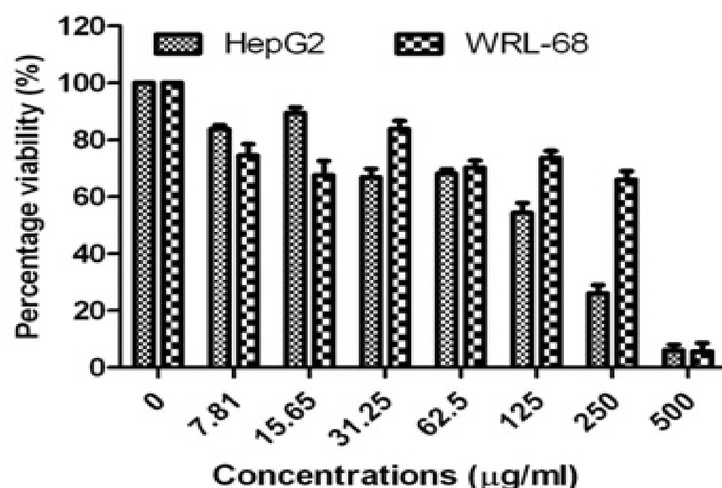

Figure 1: ST sample hexane extract was tested in HepG2 and WRL-68 cell lines. Cell viability was determined by MTT assay. The test agent induced cell cytotoxicity in a time-dependent manner. These dose titration curves allowed the IC_{50} of the test agent towards different cell lines to be determined. The proliferation of ST-treated cells decreased as the ST concentration with IC_{50} of 151 ± 2.5 and 305 ± 11.5 μg/mL on HEPG2 and WRL-68, respectively

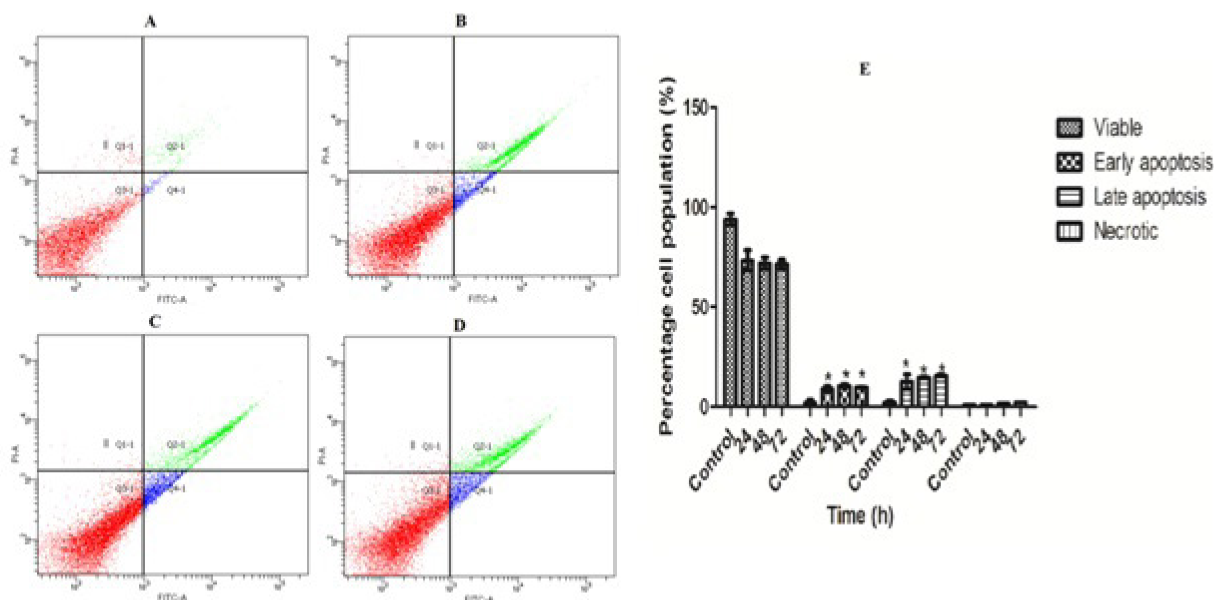

Figure 2: Annexin-V-FITC staining HepG2 cells were treated IC_{50} concentration of ST for 24, 48 and 72 h. After staining with FITC-conjugated Annexin V and PI, cells were analyzed by flow cytometry. Control cells received no drug treatments (A). (B–D) The effects of 24, 48 and 72 hr exposure (respectively) of HepG2 cells. The results showed statistically significant (p<0.05) (E)

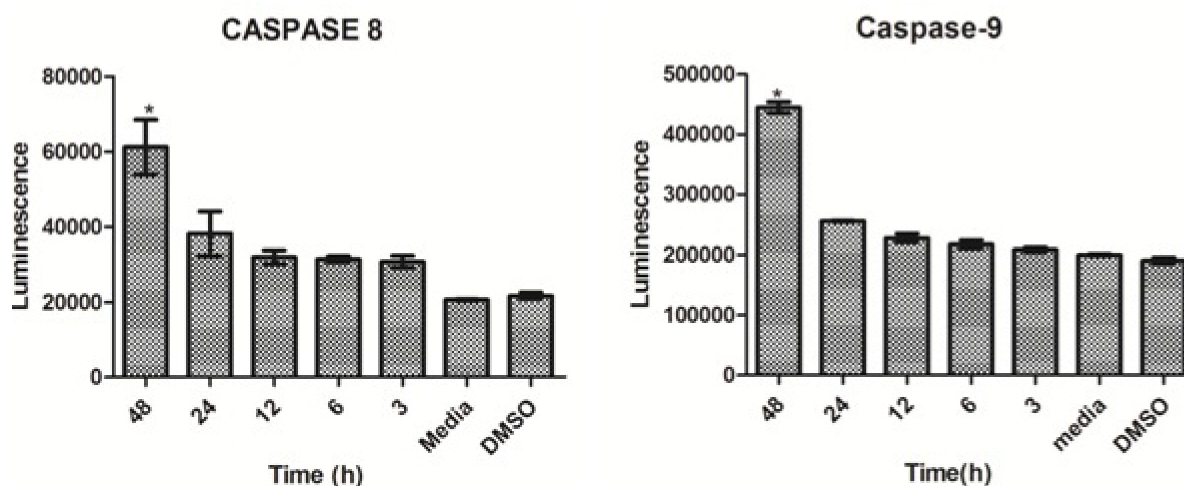

Figure 3: The luminescence-based assay of caspase-8 and caspase-9 in HepG2 cells treated and untreated with ST hexane extract at different time points. Cells were cultured in RPMI 1640 media maintained at 37°C and 5% CO_2

'*' Indicates a significant difference from the control (p<0.05)

showed that the ST extract inhibited the proliferation of HepG2 cells significantly compared to control. As shown in (Figure 2), the apoptosis induction in the cells began after being treated for 24 h. For the untreated control, 89% ± 4.5 cells were viable and only 3 ± 0.22% cells were in the early apoptosis stage. But in 24 h the treated cells early viable cells phase reduced significantly to 73± 5.6% with a rise in early apoptosis cells to 10 ± 1.1% and 15 ± 2.0% late apoptosis cells. Even though the apoptosis phase was significant, there was no time depended increase in the apoptosis cells at 48 and 72 h compared to 24 h treatment. These results indicated that the ST allowed the translocation of PS to occur and hence induced early apoptotic induction in HepG2 cells.

Induction of caspases activation

Both the caspases under investigation were found to be induced while the treatment, and were found to be time-dependent. High levels of both caspase 8 and 9 were

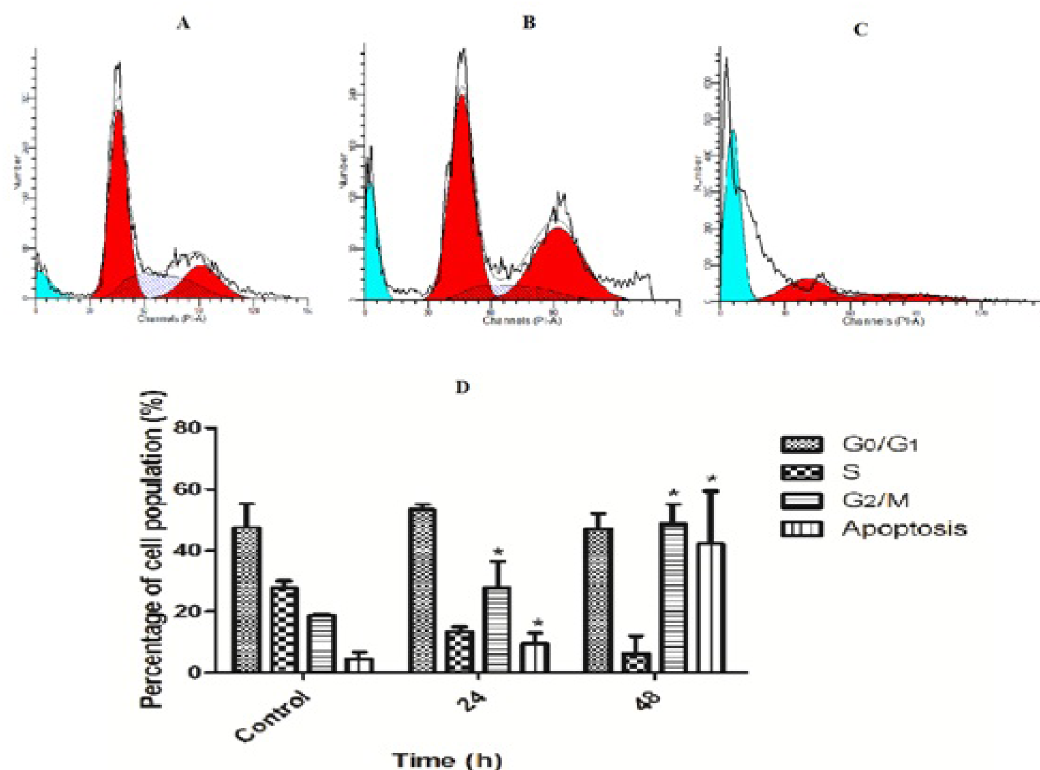

Figure 4: DNA analysis of ST hexane extract-treated HepG2 cells. Cells were exposed to IC50 concentrations at 24 h (B) and 48 (C) and control (A) were tested for DNA content of Sub-G0/G1, G0/G1, S and G2/M phase. Induction of G2/M arrest in the cell cycle progression of cells was observed (D).

'*' Indicates a significant difference ($p < 0.05$)

found at the highest time period of 48 h with a significance difference from control ($p<0.05$). But the activation of caspases was not significantly induced at early time periods. These results confirmed the activation of caspases was involved in the cell death induced by ST (Figure 3).

ST inhibits the HepG2 cell proliferation and arrest G2/M phase cell cycle

To check the involvement of cell cycle progression/arrest and apoptosis the cell cycle analysis has been performed. The results from the current study established that ST arrested the cell cycle at G2/M phase of the cell phase and induced significant apoptosis ($p<0.05$). The results shown in (Figure 4) indicated that the cell cycle arrest happened in time dependently, with a rise in G2/M phase to 30 ± 4.61and 48 ± 2.9% for 24 and 48 h respectively. Moreover the hypodiploid (sub G1/apoptosis), which is considered as the apoptosis phase has also rose up to 42 ± 6.7%.

DISCUSSION

The main toxic effects observed with the substance of abuse are carcinogenicity.[21] It is difficult to predict the particular agent causing this effect due to the fact that many a times their will be a mixture of substance, especially used by traditional practice. Shammah is one of the unburned tobacco products that chewed in the oral cavity. Even though officially banned in Saudi Arabia, it is chewing due to its psychoactive properties.[22] It has been found that chewing of Shammah has significant effect in mutagenicity and carcinogenicity. Previous studies had showed that the Shammah extract has the capacity to produce ROS in oral keratinocyte cells, capacity to increases in fragmentation of genomic DNA, and apoptotic mode of cell death.[23] But there is no report on the effect of Shammah in liver cells.

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death.[24,25] Inappropriate apoptosis (either too little or too much) is a factor in many human conditions including neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. Positive functions of apoptotic genes have been well established in a large number of biological contexts, including their role in eliminating damaged and

potentially cancerous cells. Evidence has suggested that proapoptotic proteins like caspases can induce proliferation of neighboring surviving cells to replace dying cells. This process is known as apoptosis-induced proliferation.[26] Whether the STE administration has the same kind of effect is unclear. In this study, the liver cells have been used to check it ability to undergo apoptosis. Out of HEPG2 and WRL-68 cells, HepG2 cells have been found to be more susceptible to toxicity produced by STE. It shows that the cytotoxicity produced by STE is cell specific and may be followed by specific cell signaling.

The Annexin V assay was used in the research as an initial attempt to investigate the probable mechanism of cell death responsible for the cytotoxic effect of the STE towards HepG2 cells. Phosphatidylserine (PS) externalization during apoptosis, a universal phenomenon during apoptosis is not limited in mammalian cells, but also occurring in both insect and plant cells.[27] Annexin V was found to bind specifically to PS, located at the outer membrane leaflet of cells in the presence of calcium. The present study demonstrated that treatment with STE is able to induce cell death via apoptosis. Even though the 24 h results had showed a significant amount of PS exposure there was no time dependent increase in the treatment. This may be due to cytostatic effect produced by the cells itself against the toxicity of STE.[28]

It is very apparent that cell cycle arrest happened concurrently with cell cycle arrest[29,30] In this study we have used flow cytometric measurements to assess apoptotic cell death using propidium iodide as the probe. At the time of cellular death, nuclei of the cells are stained with the fluorescent dye propidium iodide. The results clearly show that STE can induce time-dependent cell cycle arrest together with apoptosis in HepG2 cells. More over the increased hypo diploid cells observed in the cell cycle analysis further confirm the apoptosis induction.[31]

Caspases plays a vital role in initiating and executing apoptosis process.[32] Generally apoptosis happens though both intrinsic and extrinsic pathways. Both pathways have different caspases initiator. Caspase 8 and 9 plays the role of initiation in intrinsic and extrinsic pathways respectively.[33] At the downstream pathways Caspases are executioners of apoptosis that can dismantle a cell within hours. Hence the current study studied the role of caspases in STE in HepG2 cells. Both the caspases were found to be activated especially during the late time period of STE treatment.

In summary, we have established that the smokeless tobacco has the capacity to induce apoptosis and cell cycle arrest in the liver cells. The cell death was closely associated with caspases activation and typical apoptosis form of death. This suggests that Shammah has significant levels of toxicity with definite cell death mechanism. It needs further in-depth mechanism studies and animal based experiments.

CONCLUSION

In summary, we have established that the smokeless tobacco has the capacity to induce apoptosis and cell cycle arrest in the liver cells. The cell death was closely associated with caspases activation and typical apoptosis form of death. This suggests that Shammah has significant levels of toxicity with definite cell death mechanism. It needs further in-depth mechanism studies and animal based experiments.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this work.

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Highlights of Paper

- Shammah is a traditional form of chewing tobacco.
- Hexane extract of Shammah induced cell death and apoptosis in HepG2 cells.
- The cell death happened together with Caspases induction and cell cycle arrest.
- Cell cycle arrest has been happed at G2.M phase.

Author Profile

- **Dr.Siddig Ibrahim Abdelwahab** is Associate professor in Jazan University, Jazan, KSA. He graduated from Faculty of Veterinary Sciences- University of Khartoum in the field of Veterinary Medicine in the year 1999 and obtained his Master of Science and Doctor of Philosophy Degrees in Pharmacology from University Putra Malaysia, Malaysia in the years 2001 and 2008 respectively. He also had opportunity to join as Head of the Biomedical Research Unit, (Medical Research Centre, and Substance Abuse Research Centre) and Core Laboratory, Medical Research Centre,Jazan, Saudi Arabia. In his attempt to share his expertise, he acquired an overall H-index of 19 with his high number of research publications (130) and two patents filed. He is active member of four scientific communities and is been considered as potential reviewer for more than 10 biomedical journals. His excellence in research also extends to statistical analysis.

REFERENCES

1. Hoffmann D, Hecht S. Nicotine-derived N-nitrosamines and tobacco-related cancer: current status and future directions. Cancer research 1985; 45(3): 935-44.
2. Russo P, Nastrucci C, Alzetta G, Szalai C. Tobacco habit: historical, cultural, neurobiological, and genetic features of people's relationship with an addictive drug. Perspectives in Biology and Medicine 2011; 54(4): 557-77.
3. Walsh MM, Langer TJ, Kavanagh N, Mansell C, MacDougal W, Kavanagh C, *et al*. Smokeless tobacco cessation cluster randomized trial with rural high school males: Intervention interaction with baseline smoking. Nicotine and Tobacco Research 2010; 12(6): 543-50.
4. Klesges RC, Ebbert JO, Morgan GD, Sherrill-Mittleman D, Asfar T, Talcott WG, *et al*. Impact of differing definitions of dual tobacco use: implications for studying dual use and a call for operational definitions. Nicotine and Tobacco Research 2011; 13(7): 523-31.
5. Warnakulasuriya S, Dietrich T, Bornstein MM, Peidró EC, Preshaw PM, Walter C, *et al*. Oral health risks of tobacco use and effects of cessation. International dental journal 2010; 60(1): 7-30.
6. Ebbert J, Montori VM, Erwin PJ, Stead LF. Interventions for smokeless tobacco use cessation. Cochrane Database Syst Rev. 2011; 16(2).
7. Bhattacharyya N. Trends in the use of smokeless tobacco in United States, 2000–2010. The Laryngoscope 2012; 122(10): 2175-8.
8. Willis D, Popovech M, Gany F, Zelikoff J. Toxicology of Smokeless Tobacco: Implications for Immune, Reproductive, and Cardiovascular Systems. Journal of Toxicology and Environmental Health, Part B 2012; 15(5): 317-31.
9. Mognetti B, Di Carlo F, Berta G. Animal models in oral cancer research. Oral oncology 2006; 42(5): 448-60.
10. Hecht SS. Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nature Reviews Cancer. 2003; 3(10): 733-44.
11. Warnakulasuriya KAAS, Ralhan R. Clinical, pathological, cellular and molecular lesions caused by oral smokeless tobacco – a review. Journal of Oral Pathology and Medicine 2007; 36(2): 63-77.
12. Bagchi SSDBM. Toxicity of trace elements in tobacco smoke. Inhalation Toxicology 1997; 9(9): 867-90.
13. Bagchi M, Bagchi D, Hassoun E, Stohs S. Smokeless tobacco induced increases in hepatic lipid peroxidation, DNA damage and excretion of urinary lipid metabolites. International journal of experimental pathology 1994; 75(3): 197.
14. 1Chole RH, Patil RN, Basak A, Palandurkar K, Bhowate R. Estimation of serum malondialdehyde in oral cancer and precancer and its association with healthy individuals, gender, alcohol, and tobacco abuse. Journal of cancer research and therapeutics 2010; 6(4): 487.
15. Kurtul N, Gökpınar E. Salivary lipid peroxidation and total sialic acid levels in smokers and smokeless tobacco users as Maraş powder. Mediators of inflammation 2012; 2012: Article ID 619293, 8 pages.
16. Ramya R, Prakash S, Sudha S. Assessment of Serum Malondialdehyde in Oral Squamous Cell Carcinoma patients and its association with tobacco habits. JPBMS. 2011; 10(16): 1-3.
17. Mitchell C, Joyce AR, Piper JT, McKallip RJ, Fariss MW. Role of oxidative stress and MAPK signaling in reference moist smokeless tobacco-induced HOK-16B cell death. Toxicology letters 2010; 195(1): 23-30.
18. Idris A, Warnakulasuriya K, Ibrahim Y, Nielsen R, Cooper D, Johnson N. Toombak-associated oral mucosal lesions in Sudanese show a low prevalence of epithelial dysplasia. Journal of oral pathology and medicine 1996; 25(5): 239-44.
19. Idris AM, Ibrahim SO, Vasstrand EN, Johannessen AC, Lillehaug JR, Magnusson B, *et al*. The Swedish Snus and the Sudanese Toombak: are they different? Oral oncology 1998; 34(6): 558-66.
20. Klus H, Kunze M, Konig S, Poschl E. Smokeless Tobacco–An Overview. Beitrage zur Tabakforschung International/Contributions to Tobacco Research 2009; 23(5): 125-8.
21. Li J-H, Lin L-F. Genetic toxicology of abused drugs: a brief review. Mutagenesis 1998; 13(6): 557-65.
22. Allard WF, DeVol EB, Te OB. Smokeless tobacco (shamma) and oral cancer in Saudi Arabia. Community dentistry and oral epidemiology 1999; 27(6): 398-405.
23. Bagchi M, Balmoori J, Bagchi D, Ray SD, Kuszynski C, Stohs SJ. Smokeless tobacco, oxidative stress, apoptosis, and antioxidants in human oral keratinocytes. Free Radical Biology and Medicine 1999; 26(7): 992-1000.
24. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology 2007; 35(4): 495-516.
25. Ren B, Liu C, Wu L-H, Jin P, Li P, Liu Y-J, *et al*. Role of SMAD signaling in drug-induced apoptosis in colon cancer. Bangladesh Journal of Pharmacology 2014; 9(4): 539-44.
26. Ryoo HD, Bergmann A. The role of apoptosis-induced proliferation for regeneration and cancer. Cold Spring Harbor perspectives in biology 2012; 4(8): a008797.
27. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. Journal of immunological methods 1995; 184(1): 39-51.
28. Zhang J-J, Zheng T-T, Cheng F-F, Zhang J-R, Zhu J-J. Toward the early evaluation of therapeutic effects: An electrochemical platform for ultrasensitive detection of apoptotic cells. Analytical chemistry 2011; 83(20): 7902-9.
29. Li T, Kon N, Jiang L, Tan M, Ludwig T, Zhao Y, *et al*. Tumor suppression in the absence of p53-mediated cell-cycle arrest, apoptosis, and senescence. Cell 2012; 149(6): 1269-83.
30. Thakur VS, Gupta K, Gupta S. Green tea polyphenols causes cell cycle arrest and apoptosis in prostate cancer cells by suppressing class I histone deacetylases. Carcinogenesis 2012; 33(2): 377-84.
31. Santos RC, Salvador JA, Cortés R, Pachón G, Marín S, Cascante M. New betulinic acid derivatives induce potent and selective antiproliferative activity through cell cycle arrest at the S phase and caspase dependent apoptosis in human cancer cells. Biochimie 2011; 93(6): 1065-75.
32. Sui X, Sun Z, An W, Cheng K, Jiang S, Xianfeng G. 2, 4, 3′, 4′-tetramethoxy-biphenyl induces apoptosis in MGC-803 cells through a mitochondrial/caspase pathway. Bangladesh Journal of Pharmacology 2014; 9(2): 235-43.
33. Hyman BT, Yuan J. Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. Nature Reviews Neuroscience 2012; 13(6): 395-406.