Cytotoxic Effects of Smokeless Tobacco Extract on Fibroblast Cell Line

Muhammad Khairuzafeq Mohd Jamri
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
khairuzafeq@gmail.com

*** Siti Aishah Zainal, PhD**
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
sitizainal@usm.my

Azlina Ahmad
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
azlinakb@usm.my

Noor Khairiena Mohamad
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
khairiena@usm.my

Masitah Hayati Harun
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
masitahh@usm.my

Siti Fadilah Abdullah
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
sfadilah@usm.my

Badr Abdullah Al-Tayar
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
bader2221@windowslive.com

Sarah Fatimah Mohd Ikhsan
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
sarahikhsan@student.usm.my

Mohamad Ezany Yusoff
School of Dental Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia
ezany@usm.my

***Corresponding author:** Siti Aishah Zainal, sitizainal@usm.my

Article History:
Received on April 15, 2021
Accepted on November 8, 2021
Published on January 1, 2022

Abstract:

Introduction: The use of smokeless tobacco is prevalent among Asians. Smokeless tobacco products have long been determined to be associated with oral cancer. Studies on the effect of smokeless tobacco extract, in Malaysia, is limited. This study aimed to investigate the cytotoxic effects of locally source crude smokeless tobacco aqueous extract on the mouse fibroblast cell line, L929 as well as to observe the cell morphological changes. **Methods:** Tobacco purchased from a local market was blended with deionised water (1:2; w: v) followed by freeze-drying. The freeze-dried powder was dissolved in Dulbecco's Modified Eagle's Medium (DMEM), followed by dilution in seven concentrations of aqueous tobacco extract (100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%). L929, was incubated with the different concentrations of prepared aqueous tobacco extract. Cytotoxicity of the treated cell line was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay, and the half-maximal inhibitory concentration (IC_{50}) was determined after 24-, 48- and 72-hours incubation. Data was analysed by one-way ANOVA. **Results:** Increasing concentrations

of aqueous tobacco extract (1.56%, 3.125%, 6.25%, 12.5%, 25%, 50% and 100%) resulted in decreasing viability of mouse fibroblast cell L929 for all incubation times compared to the control and showed altered morphology. The IC_{50} value of areca nut extract on L929 was 52%, 34% and 28%, at 24, 48 and 72 hours, respectively. **Conclusion:** This study revealed that tobacco is cytotoxic to L929 with increasing concentrations and was also found to promote cell morphology changes.

Keywords: smokeless tobacco, mouse fibroblast cell line, cytotoxicity, MTT assay, oral cancer

Introduction:

Smokeless tobacco is a non-smoking type of tobacco that can be chewed (whole leaf, plug or twist tobacco) or inhaled (finely ground or cut tobacco) (IARC, 2012). Asian people, including those in Malaysia, India and Pakistan, have a history of consuming smokeless tobacco (NHMS, 2015; Suliankatchi et al., 2017). In Malaysia, smokeless tobacco is usually placed in the oral vestibule or kept in the mouth for long periods of time. Other form of smokeless tobacco use is in folded betel quid which is composed of betel leaf, areca nut, and lime (Zain et al., 1999). In 2015, the prevalence of smokeless tobacco use was 10.9% (NHMS, 2015). In 2019, the prevalence reduced to 6.5% among Malaysians above 14 years of age (NIH, 2019). Usually, smokeless tobacco users chew the tobacco and spit out the juice that forms. Thus, absorption of tobacco constituents by the oral mucosa is to be expected (Niaz et al., 2017).

Smokeless tobacco has been determined as carcinogenic to humans by the World Health Organization (IARC, 2012). Its use has been associated with periodontal diseases (Kamath et al., 2014), oral cancer (Asthana et al., 2018), and also cardiovascular disease (Asplund, 2003; Regezi et al., 2016; Rostron et al., 2018). Asthana and colleagues (2018) reported a significant risk of oral cancer with smokeless tobacco products by 95% for the Southeast Asia and Eastern Mediterranean regions. The major groups of carcinogens in smokeless tobacco include the non-volatile alkaloid-derived tobacco-specific *N*-nitrosamines and *N*-nitrosoamino acids (IARC, 2007), with the most prominent component being tobacco-specific *N*-nitrosamines (Banerjee et al., 2014; IARC, 2007). Other carcinogenic agents include cadmium, polonium-210, formaldehyde, benzo(a)pyrene, lead, urethane, metals, and uranium-235 and -238 (IARC, 2007; Janbaz et al., 2014).

One of the toxicology tests to determine material cytotoxicity is the 3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide (MTT) assay (Aslantürk, 2017; Bahuguna et al., 2017). The assay measures cell metabolic activity by assessing the mitochondrial enzymes activity in viable cells. In active mitochondria, the tetrazolium ring is cleaved where the reaction occurs exclusively in living cells (Mosmann, 1983). Thus, the MTT assay results could showcase the effect of the smokeless tobacco extract on L929. Das et al. (2013) demonstrated the cytotoxic effect of crude smokeless tobacco extract on lung epithelial cell (A549), hepatic epithelial cells (HepG2) and mouse squamous epithelial cells (HCC7). However, following treatment with the substance, instead of undergoing apoptosis, cancer cells' (MCF-7, A2780 and HT29) growth increased, indicating smokeless tobacco's carcinogenicity (Khalid et al., 2019).

The most common type of oral cancer, squamous cell carcinoma, is defined as an invasive epithelial neoplasm, with variable degrees of squamous differentiation (Suciu et al., 2014). In addition to its delayed clinical detection and poor prognosis, there are no specific biomarkers for the disease and therapeutic alternatives are costly (Rivera, 2015). In 2018, Asia had the highest estimation of oral cancer prevalence, death and new cases, based on GLOBOCAN. Therefore, a study on smokeless tobacco as a risk factor for oral cancer is still relevant.

In the current study, we aimed to investigate the effects of crude aqueous smokeless tobacco extract exerted on mouse fibroblast cell line, L929, based on the cytotoxicity and cell morphological changes. The idea is to determine the smokeless tobacco extract's suitable value to be used in our subsequent experiments. Another of our study utilizes betel quid, a combination of multiple constituents, including tobacco, based on local Kelantanese consumption. In this experiment, we focused on a single crude component which is tobacco.

Materials and Methods:

Preparation of crude aqueous smokeless tobacco extract

Tobacco was purchased from a local market in Kota Bharu, Kelantan, to represent a local form of consumption (Figure 1 (a)). The tobacco was weighed, then blended with deionised water with a ratio of 1:2 (w:v) for 15 minutes using an electric blender followed

by soaking for 6 hours at ambient temperature. After soaking, the tobacco was centrifuged at 12,000 rpm (Biofuge primo, Heraeus, Germany) for 5 minutes, and the supernatant was then filtered using 200µm filter paper to remove sediments (Whatman, USA). The filtered solutions were kept in the refrigerator (-80° C) for 24 hours before freeze-drying for 4 days (Figure 1 (b)) (modified based on Sazwi et al., 2013).

Figure 1: Images of smokeless tobacco. The smokeless tobacco in (a) a raw form and (b) freeze dried, powdered crude aqueous tobacco extract.

Preparation of filtered extract from powdered tobacco crude extract

100 mg of the freeze-dried powder of tobacco was incubated in 10 ml of Dulbecco's Modified Eagle Medium (DMEM). The solution was left in a shaking incubator for 24 hours, followed by centrifugation at 2,500 rpm (Universal 32 R, Hettich Zentrifugen, Germany) for 5 minutes to remove large particles. Then, it was filtered using 0.2µm membrane filters. The extract was resuspended in DMEM: 100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%, based on previous findings from another experiment (unpublished).

Cytotoxicity of smokeless tobacco extract on L929 cells

L929 was purchased from American Type Culture Collection (no. CCL1, NCTC clone 929). This cell was selected based on ISO standard (ISO 10993-5, 2009) for cytotoxicity studies. The cell line was sub-cultured upon arrival, resuspended and kept at -80°C. Before experimental use, the stored cells were sub-cultured three times. The L929 cell (3×10^3) were seeded into each well of a 24-well plate and incubated at 37°C, 5% CO_2 for 24 hours. After the incubation, following culture media removal, the cells were treated with the filtered aqueous smokeless tobacco extract (100%, 50%, 25%, 12.5%, 6.25%, 3.125% and 1.56%) for 24, 48 and 72 hours. Untreated L929 was used as the control. MTT was then added to the treated cells and further incubated for 4 hours. This was followed with removal of MTT-containing media. In order to dissolve the formed formazan crystal, DMSO was added. The optical density of dissolved formazan was measured at 570 nm using an ELISA plate reader (Tecan, Japan). The L929 cell viability percentage was plotted against smokeless tobacco extract concentrations, and subsequently, the half-maximal inhibitory concentration (IC_{50}) was determined using GraphPad (Prism). The IC_{50} value indicates the smokeless tobacco extract concentration which inhibited cell growth by half (Aykul & Martinez-Hackert, 2016). The experiment was conducted in duplicates with eight technical repeats for each.

Cell morphological of L929 cell

Cell morphological alterations of L929 were observed at 24, 48 and 72 hours after treatment with the different concentrations of aqueous smokeless tobacco extract using an inverted microscope (Carl Zeiss, Germany) and photographed using a camera (Nikon, Japan).

Statistical analysis

Statistical analysis for the experimental data was performed using SPSS Statistics 20 statistical software (IBM Software Group, Chicago, USA). The cytotoxic effects of smokeless tobacco extract on L929 was compared between control and treated cells using One-way Analysis of Variance (ANOVA) and Mann-Whitney. Statistically significant was considered at *p*-value less than 0.05 ($p<0.05$

Result:

Cytotoxic effect of aqueous smokeless tobacco extract on L929

Viability of L929 cells decreased after treatment with increased concentrations of aqueous smokeless tobacco extract. There was a significant decrease of cell viability when exposed to aqueous smokeless tobacco extract with dilution of 100%, 50% and 25% at 24 hours (Figure 2 (a), $p<0.05$) and with dilution 100%, 50%, 25%, 12.5%, 6.25% and 3.125% at 48 hours (Figure 2 (b), ($p<0.05$)). However, there was a slight increase of cell viability found at the 12.5% dilution. Additionally, the cell viability of treated L929 at 72 hours incubation also decreased as the concentration of the extract went higher and significantly decreased at 100%, 50%, 25%, 12.5% and 6.25% compared to the control (Figure 2 (c), ($p<0.05$)).

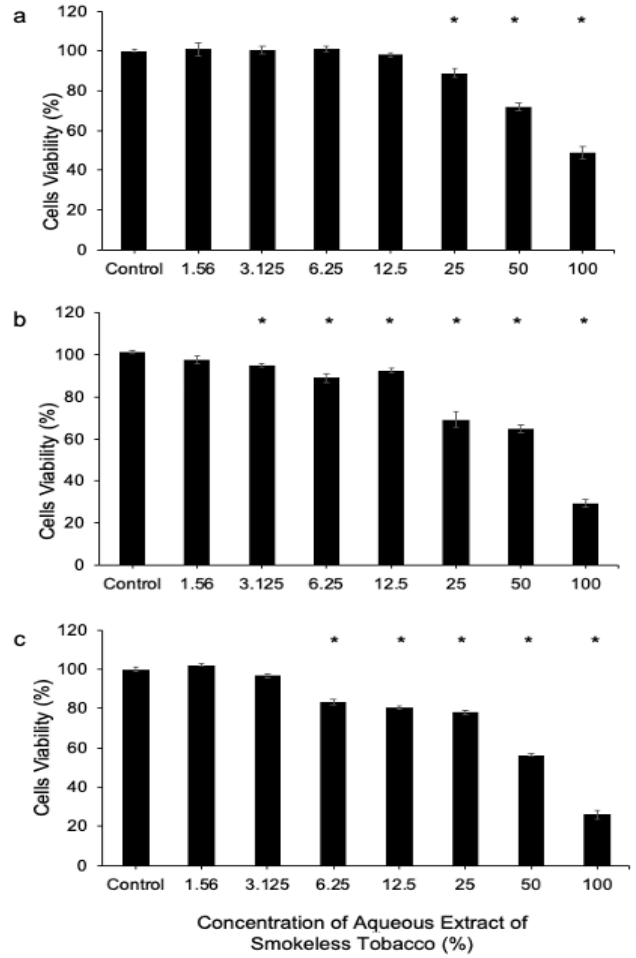

Figure 2: Effect of cytotoxicity of aqueous smokeless tobacco extract on L929. Viability of L929 cell when cultured with aqueous smokeless tobacco extract at (a) 24, (b) 48 and (c) 72 hours. Cell viability decreased with increasing concentrations of aqueous smokeless tobacco extract. * $p<0.05$. Data are presented as mean ± S.E.

The IC_{50} values of tobacco aqueous extract were 52%, 34% and 28% for 24, 48 and 72 hours, respectively (Figure 3). Decreased IC_{50} values was observed as the incubation time of L929 with aqueous extract of smokeless tobacco increased.

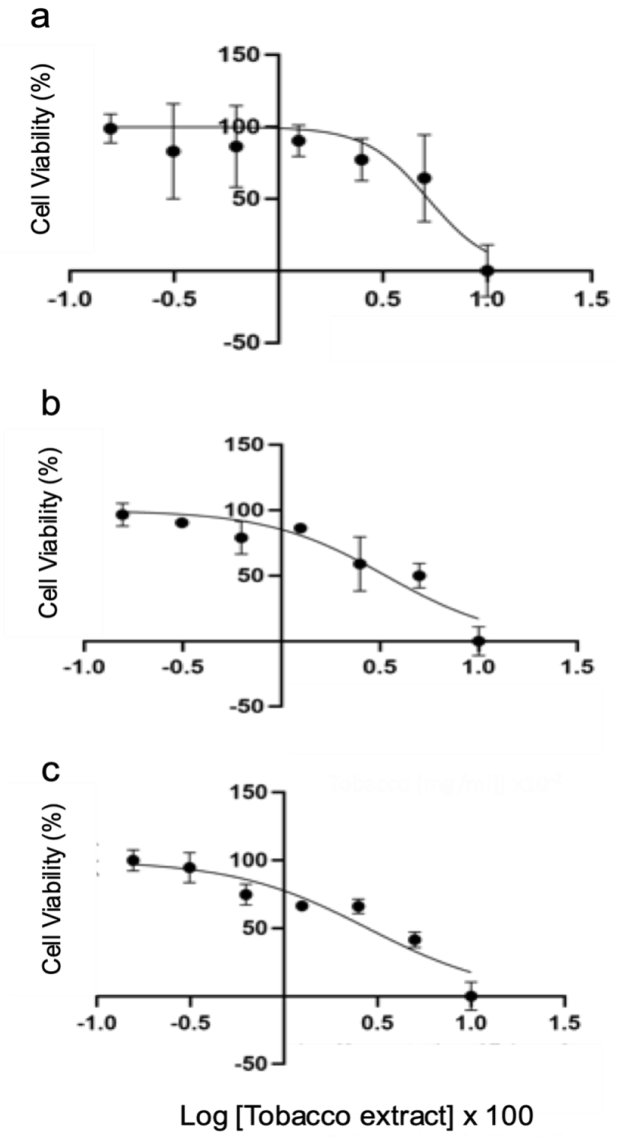

Figure 3: Half-maximal (IC_{50}) of cytotoxicity effect of aqueous smokeless tobacco extract. IC_{50} values are shown for (a) 24 hours; Log IC_{50} = 52%, (b) 48 hours; Log IC_{50} = 34% and (c) 72 hours; Log IC_{50} = 28%.

Cell morphology of smokeless tobacco extract treated L929

After incubation with the extract, there were morphological changes observed in treated L929 compared to the untreated control cells. The control cells at all incubation time exhibited elongated shape and were well-attached on the plastic surface (Figure 4 (a), 5 (a) and 6 (a)).

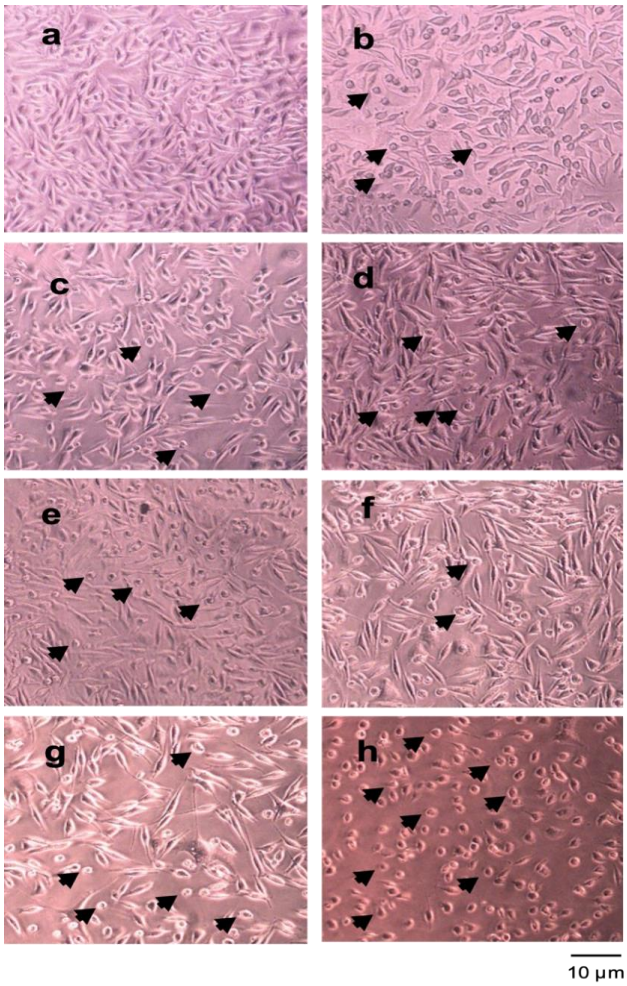

Figure 4: Changes of cell morphology of L929 treated with aqueous smokeless tobacco extract after 24 hours of incubation. The micrographs refer to (a) the untreated L929 and the others are the L929 treated with (b) 1.56%, (c) 3.125%, (d) 6.25%, (e) 12.5%, (f) 25%, (g) 50% and (h) 100% of aqueous extract of smokeless tobacco. (Arrow: rounded-shaped cells).

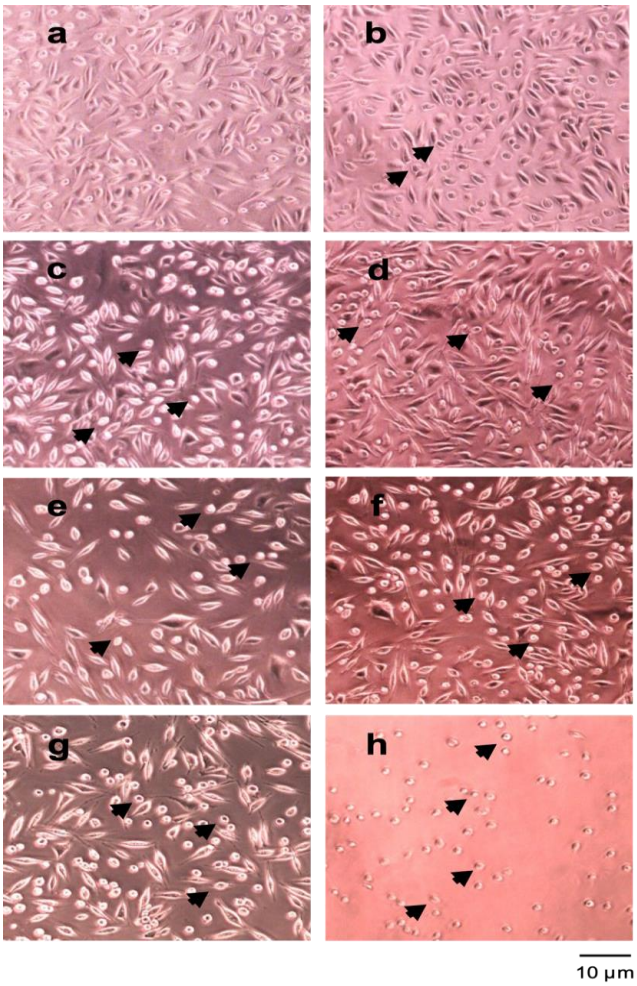

Figure 5: Changes of cell morphology of L929 treated with aqueous smokeless tobacco extract after 48 hours of incubation. The micrographs refer to (a) the untreated L929 and the others are the L929 treated with (b) 1.56%, (c) 3.125%, (d) 6.25%, (e) 12.5%, (f) 25%, (g) 50% and (h) 100% of aqueous extract of smokeless tobacco. (Arrow: rounded-shaped cells)

Treatment at 24 hours with the extract demonstrated that increasing concentrations resulted in more rounded-shaped cells (Figure 4 (b-h, arrow) with the highest concentration showing unattached and rounded cells. Similarly, at 48 hours, higher concentrations led to rounded-shaped cells (Figure 5 (b-g, arrow) whereby cells exposed to 100% smokeless tobacco extract (Figure 5 (h), arrow) were seen unattached and decreased in number. These conditions were also observed on cultures following 72 hours treatment (Figure 6 (b-h, arrow).

Discussion:

Smokeless tobacco use is often associated with oral cancer besides causing other health problems such as elevated blood pressure, physiologic dependence, and worsening periodontal disease (Asplund, 2003; Kamath et al., 2014; Regezi et al., 2016). Due to its high effect of toxicity, many studies have been conducted to substantiate the cytotoxic effects of smokeless tobacco using different concentrations and different type of cells. This present study assessed the cytotoxicity of smokeless tobacco using locally produced tobacco from Kota Bharu, Kelantan, Malaysia on L929 cell line.

The present study demonstrated that there was a dose-dependent cell death in L929 cells after exposure to the aqueous smokeless tobacco extract for 24, 48 and 72 hours. The MTT assay results revealed reduced cell viability as the concentration of the aqueous smokeless tobacco extract increased. A smokeless tobacco effect study on human squamous epithelial

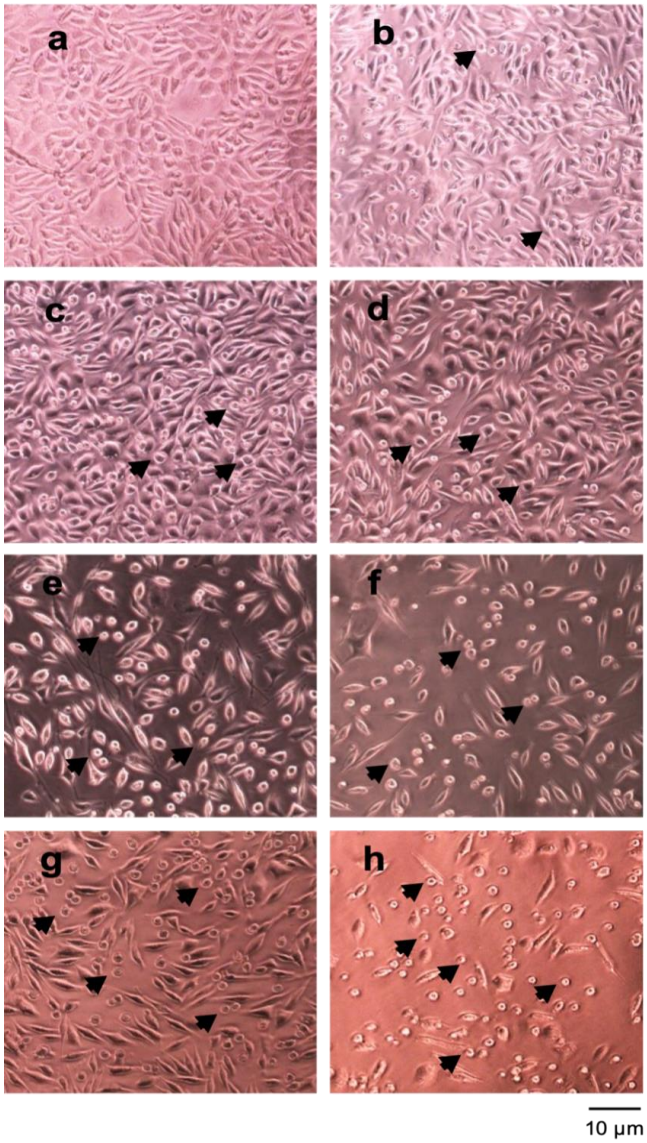

Figure 6: Changes of cell morphology of L929 treated with aqueous smokeless tobacco extract after 72 hours of incubation. The micrographs refer to (a) the untreated L929 and the others are the L929 treated with (b) 1.56%, (c) 3.125%, (d) 6.25%, (e) 12.5%, (f) 25%, (g) 50% and (h) 100% of aqueous extract of smokeless tobacco (Arrow: rounded-shaped cells).

cells, SCC-25, also observed cell viability reduction after treatment with the extract in a concentration-dependent manner (Ganguli et al., 2016). From the morphological observation of the treated cells under the inverted microscope, it was seen that there was a significant reduction of cells confluency and changes of the cell morphology from the elongated spindle shape to rounded, shrunken and detachment as the concentration of the aqueous smokeless tobacco increased. This finding was similar to a previous study by Das et al., (2013) using crude aqueous smokeless tobacco extract. The presence of smokeless tobacco extract altered the cellular morphology of HepG2 and A549 cells resulting in contraction and shrinkage in form accompanied by the loss of cellular integrity. The observation on cell migration revealed the inhibition of HepG2 cells migration in the presence of 400 µg/ml STE and inhibition of A549 cells migration in the presence of 300 µg/ml smokeless tobacco for 24 hours. The researchers also studied the dose-dependent fashion on non-cancerous human peripheral blood mononuclear cells (PBMC). Approximately 43% of PBMC were found to be apoptotic when treated with the smokeless tobacco extract (Das et al., 2013).

The reduced L929 viability in our study may possibly be attributed to apoptosis (Bagchi et al., 1999, 2001; Mangipudy & Vishwanatha, 1999). Smokeless tobacco extracts reportedly induced apoptosis by targeting the reactive cysteine residues of tubulin causing disruption and degradation of cellular microtubules (Das et al., 2013). The gradual decrease of cell viability describes apoptosis of the cultured cell. Additionally, Li and colleagues (2018) suggested that the extract increases the concentration of ROS which subsequently induces cell apoptosis (Li et al., 2018; Brodská & Holoubek, 2011). Cellular ROS surplus cause damage to proteins, nucleic acids, lipids, membranes and organelles, which can lead to activation of apoptosis (Redza-Dutordoir & Averill-Bates, 2016). Chronic use of smokeless tobacco induces the production of ROS and free radicals, damaging normal DNA and RNA (Niaz et al., 2017). Besides apoptosis, the occurrence of autophagy in cells treated with smokeless tobacco extract has also been reported (Ganguli et al., 2016). The presence of ROS may also lead the action of autophagy as a cell death machinery (Filomeni et al., 2015; Ganguli et al., 2016). However, the exact mechanism of cell death was not determined in this study.

Regular use of smokeless tobacco causes an increase in the incidence of buccal micronuclei (Proia et al., 2006), which is the sign of genotoxicity (Biswas et al., 2015). The chewing of smokeless tobacco can interrupt the cell cycle progression through p53/p21 signalling axis and induces the apoptosis through Bcl-2 and Bax disequilibrium which arrests the cell cycle (Biswas et al., 2015; Li et al., 2018).

Conclusion:

We may conclude that smokeless tobacco is cytotoxic to mammalian cell lines (IC_{50}) with subsequent alteration on the cell morphology which may promote apoptosis.

Acknowledgment:

This study was supported by grants from the Research University Individual (RUI) (1001/PPSG/8012281) and the Universiti Sains Malaysia Short Term Grant (304/PPSG/6315127). No conflicts of interest have been declared by authors.

References:

Aslantürk, Ö. S. (2017). "*In Vitro* Cytotoxicity and Cell Viability Assays: Principles, Advantages, and Disadvantages" pp 4, Larramedy, M. L & Soloneski, S. Editor, Genotoxicity - A Predictable Risk to Our Actual World. IntechOpen.

Asplund, K. (2003). Smokeless tobacco and cardiovascular disease. *Progress in Cardiovascular Diseases*, 45(5), 383–394. DOI: 10.1053/pcad.2003.00102

Asthana, S., Labani, S., Kailash, U., Sinha, D. N., & Mehrotra, R. (2018). Association of smokeless tobacco use and oral cancer: A systematic global review and meta-analysis. *Nicotine & Tobacco Research*, 1–10. DOI: 10.1093/ntr/nty074

Aykul, S., & Martinez-Hackert, E. (2016). Determination of half-maximal inhibitory concentration using biosensor-based protein interaction analysis. *Analytical Biochemistry*, 508, 97–103. DOI: 10.1016/j.ab.2016.06.025

Bagchi, M., Balmoori, J., Bagchi, D., Ray, S. D., Kuszynski, C., & Stohs, S. J. (1999). Smokeless tobacco, oxidative stress, apoptosis, and antioxidants in human oral keratinocytes. *Free Radical Biology and Medicine*, 26(7), 991–1000.

Bagchi, M., Kuszynski, C. A., Balmoori, J., Joshi, S. S., Stohs, S. J., & Bagchi, D. (2001). Protective effects of antioxidants against smokeless tobacco-induced oxidative stress and modulation of Bcl-2 and p53 genes in human oral kerathnocytes. *Free Radical Research*, 35(2), 181–194.

Bahuguna, A., Khan, I., Bajpai, V. K., & Kang, S. C. (2017). MTT assay to evaluate the cytotoxic potential of a drug. *Bangladesh Journal of Pharmacology*, 12(2), 115–118. DOI: 10.3329/bjp.v12i2.30892

Banerjee, S. C., Ostroff, J. S., Bari, S., D'Agostino, T. A., Khera, M., Acharya, S., & Gany, F. (2014). Gutka and tambaku paan use among south Asian immigrants: A focus group study. *Journal of Immigrant and Minority Health*, 16(3), 531–539. DOI: 10.1007/s10903-013-9826-4

Biswas, S., Manna, K., Das, U., Khan, A., Pradhan, A., Sengupta, A., Bose, S., Ghosh, S., & Dey, S. (2015). Smokeless tobacco consumption impedes metabolic, cellular, apoptotic and systemic stress pattern: A study on Government employees in Kolkata, India. *Scientific Reports*, 5, 18284.

Brodská, B., & Holoubek, A. (2011). Generation of Reactive Oxygen Species during Apoptosis Induced by DNA-Damaging Agents and/or Histone Deacetylase Inhibitors. *Oxidative Medicine and Cellular Longevity*, 2011, 1-7. DOI: 10.1155/2011/253529

Das, A., Bhattacharya, A., Chakrabarty, S., Ganguli, A., & Chakrabarti, G. (2013). Smokeless Tobacco Extract (STE)-Induced Toxicity in Mammalian Cells is Mediated by the Disruption of Cellular Microtubule Network: A Key Mechanism of Cytotoxicity. *PLoS ONE*, 8(7). DOI: 10.1371/journal.pone.0068224

Filomeni, G., De Zio, D., & Cecconi, F. (2015). Oxidative stress and autophagy: The clash between damage and metabolic needs. *Cell Death and Differentiation*, 22(3), 377–388. DOI: 10.1038/cdd.2014.150

Ganguli, A., Das, A., Nag, D., Bhattacharya, S., & Chakrabarti, G. (2016). Potential role of autophagy in smokeless tobacco extract-induced cytotoxicity and in morin-induced protection in oral epithelial cells. *Food and Chemical Toxicology*, 90, 160–170.

IARC. (2007). Smokeless tobacco and some tobacco-specific N-nitrosamines. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* (Vol. 89). http://monographs.iarc.fr/ENG/Monographs/vol89/mono89.pdf

IARC. (2012). Personal Habits and Indoor Combustions. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, 100E, 46–167.

ISO 10993-5. (2009). Biological evaluation of medical devices - Part 5: Tests for in vitro cytotoxicity. In *International Standard ISO 10993-5,* 1–34. DOI: 10.1016/j.ijbiomac.2017.02.080

Janbaz, K. H., Qadir, M. I., Basser, H. T., Bokhari, T. H., & Ahmad, B. (2014). Risk for oral cancer from smokeless tobacco. *Contemporary Oncology*, 18(3), 160.

Kamath, K. P., Mishra, S., & Anand, P. S. (2014). Smokeless tobacco use as a risk factor for periodontal disease. *Frontiers in Public Health*, 2(OCT), 1–4. DOI: 10.3389/fpubh.2014.00195

Khalid, A., Alhazmi, H. A., Abdalla, A. N., Ahsan, W., Sultana, S., Al Bratty, M., Javed, S. A., & Rehman, Z. U. (2019). 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*, 2019(April). DOI: 10.1155/2019/3254836

Li, L., Zhou, X., & Wang, Y. (2018). Smokeless tobacco

extract inhibits proliferation and promotes apoptosis in oral mucous fibroblasts. *Oncology Letters*, 16(4), 5066–5074.

Mangipudy, R. S., & Vishwanatha, J. K. (1999). Role of nitric oxide in the induction of apoptosis by smokeless tobacco extract. *Molecular and Cellular Biochemistry*, 200(1), 51–57.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, 65(1–2), 55–63. DOI: 10.1016/0022-1759(83)90303-4

NHMS. (2015). *National Health and Morbidity Survey 2015 – Report on Smoking Status among Malaysian Adults*.

Niaz, K, Maqbool, F., Khan, F., Bahadar, H., Hassan, F., & Abdollahi, M. (2017). Smokeless tobacco (paan and gutkha) consumption, prevalence, and contribution to oral cancer. *Epidemiology and Health*, 39, 1–11.

NIH. (2019). *Non-communicable diseases: Risk factors and other health problems* (Vol. 1).

Proia, N. K., Paszkiewicz, G. M., Nasca, M. A. S., Franke, G. E., & Pauly, J. L. (2006). Smoking and smokeless tobacco-associated human buccal cell mutations and their association with oral cancer—a review. *Cancer Epidemiology and Prevention Biomarkers*, 15(6), 1061–1077.

Redza-Dutordoir, M., & Averill-Bates, D. A. (2016). Activation of apoptosis signalling pathways by reactive oxygen species. *Biochimica et Biophysica Acta - Molecular Cell Research*, 1863(12), 2977–2992. DOI: 10.1016/j.bbamcr.2016.09.012

Regezi, J. A., Sciubba, J. J., & Jordan, R. C. K. (2016). "Clinical Overview" ppO12-O14, Editor, Oral Pathology: Clinical Pathologic Correlations (7th). Elsevier, United States of America.

Rivera, C. (2015). Essentials of oral cancer. *International Journal of Clinical and Experimental Medicine Pathology*, 8(9), 11884–11894.

Rostron, B. L., Chang, J. T., Anic, G. M., Tanwar, M., Chang, C. M., & Corey, C. G. (2018). Smokeless tobacco use and circulatory disease risk: A systematic review and meta-analysis. *Open Heart*, 5(2), 1–15. DOI: 10.1136/openhrt-2018-000846

Sazwi, N. N., Nalina, T., & Rahim, Z. H. R. (2013). Antioxidant and cytoprotective activities of Piper betle, Areca catechu, Uncaria gambir and betel quid with and without calcium hydroxide. *BMC Complementary and Alternative Medicine*, 13(351), 1–12.

Suciu, M., Morariu, S. H., Ormenisan, A., Grigoras, R. I., Bostan, R. H., Mocanu, S., Vartolomei, M. D., & Cotoi, O. S. (2014). Oral squamous cell carcinoma of the maxilla, a second malignancy after a right ethmoido-maxillary chondrosarcoma. *Rom J Morphol Embryol*, 55(3 Suppl), 1247–1251.

Suliankatchi, R. A., Sinha, D. N., Rath, R., Aryal, K. K., Zaman, M. M., Gupta, P. C., Karki, K. B., & Venugopal, D. (2017). Smokeless tobacco use is "replacing" the smoking epidemic in the South-East Asia region. *Nicotine & Tobacco Research*. 1-6.

Zain, R. B., Ikeda, N., Gupta, P. C., Warnakulasuriya, S., van Wyk, C. W., Shrestha, P., & Axéll, T. (1999). Oral mucosal lesions associated with betel quid, areca nut and tobacco chewing habits: consensus from a workshop held in Kuala Lumpur, Malaysia, November 25-27, 1996. *Journal of Oral Pathology & Medicine : Official Publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology*, 28(1), 1–4. DOI: 10.1111/j.1600-0714.1999.tb01985.x