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Cytotoxicity and Morphological Effects of Aqueous Areca Nut Crude Extract on L929 Fibroblast Cell Line

Noor Fadilatul Akmal Mat Salleh, Ahmad Azlina, Masitah Hayati Harun, Badr Abdullah Al-Tayar, Siti Nurnasihah Md Hashim, Mohamad Ezany Yusoff, Noor Khairiena Mohamad, Siti Fadilah Abdullah, Siti Aishah Zainal*

School of Dental Sciences, Universiti Sains Malaysia, Kampus Kesihatan, 16150 Kubang Kerian, Kelantan, Malaysia

*sitizainal@usm.my

Abstract

Betel quid chewing is a detrimental recreational habit amongst Asians and a risk factor for oral cancer. Arecoline, a component of areca nut (a major constituent of betel quid) is a known carcinogen. However, the effect of areca nut crude extract is not much studied. To evaluate the cytotoxicity and morphologic effects of areca nut aqueous extract on mouse fibroblast cell line (L929). Dried raw areca nut obtained from a local market in Kota Bharu, Kelantan was prepared and suspended in DMEM (Dulbecco's Modified Eagle's medium), prior to serial dilution of 1.56, 0.781, 0.39, 0.195, 0.0976, 0.0488, and 0.0244 mg/ml. The L929 was then exposed to each of the aqueous areca nut extract dilutions and incubated at 37 °C for 24, 48 and 72 hours. Following incubation, the cytotoxicity level of treated L929 was measured using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] assay. Five highest concentrations of areca nut extract showed significantly decreased L929 cell viability (1.56, 0.781, 0.39, 0.195, 0.0976 mg/ml) for all incubation periods compared to untreated cells ($p < 0.05$). The IC_{50} values of aqueous areca nut extract on L929 were 0.1516, 0.1040, and 0.09136 mg/ml at 24, 48 and 72 hours, respectively. The L929 cell showed altered morphology when cultured in the extract for 24 hours. Higher concentrations of the areca nut aqueous extract is cytotoxic to L929. Prolonged exposure to the extract reduced the IC_{50} value. Investigation on the role of areca nut in cell proliferation could be further undertaken to assess its association with oral cancer.

Keywords: areca nut, L929, mouse fibroblast cell line, cytotoxicity, MTT assay

*Author for Correspondence

Received (February 15th 2020), Accepted (April 5th 2020) & Published (April 30th 2020)

Cite as: Mat Salleh, N.F.A., Azlina, A., Harun, M.H., Al-Tayar, B.A., Md Hashim, S.N., Yusoff, M.E., Mohamad, N.K., Abdullah, S.F., Zainal, S.A. (2020). Cytotoxicity and Morphological Effects of Aqueous Areca Nut Crude Extract on L929 Fibroblast Cell Line. *Asian Journal of Medicine and Biomedicine*, 4 (2), 34–41.

DOI: <https://doi.org/10.37231/ajmb.2020.4.1.328>

Introduction

Betel quid (BQ) chewing is a popular recreational oral habit in Southeast Asia and Asia Pacific regions such as Malaysia, Indonesia, India, Yemen, and Taiwan.¹ The habit is a known risk factor for oral squamous carcinoma (OSCC).^{2,3} In Malaysia, about 7% adults chewed BQ⁴ with the highest prevalence observed among Indians.^{5,6}

BQ, usually comprising tobacco, areca nut (AN) and lime wrapped in betel leaf, is consumed by chewing and kept in the mouth where it remains in contact with the mucosa.⁷ AN, also sometimes known as betel nut, is harvested from Areca palm tree (*Areca catechu*).⁸ BQ chewers in Malaysia typically consume combinations of betel leaf, AN, tobacco, lime and gambir.^{5,9} According to the International Agency for Research on Cancer, BQ, with or without tobacco, and AN are classified as Group 1 agents, and carcinogenic to humans.³ Since BQ contains AN, we were interested in studying the cytological effects of AN. Generally, AN contains alkaloids such as arecoline and arecaidine. Both compounds are carcinogenic and genotoxic agents which can cause oral cancer.^{10,11} AN-specific nitrosamines are released by nitrosation of areca alkaloids, and capable of inducing tumours in experimental animals.¹² Many previous studies focused on single chemical elements of AN, for example arecoline bromide¹³⁻¹⁵, with scarce available information on the effects of crude AN extract. A combination of chemical compounds may exert different effects on biological systems in comparison to single components.^{16,17} Thus, there is a need to investigate the underlying biological mechanisms of crude AN extract effect on cells which is still not fully established.

Oral mucosa lining is an epithelium that covers the oral cavity. More than 90% of oral cancers are OSCCs, arising from the squamous epithelial lining of the oral mucosa.¹⁸ The development of OSCC involves a number of biochemical activities which include initiation, promotion and progression. Direct mucosal contact with AN could result in a reactive response, and OSCC is the effect of the progressive accumulation of genetic changes after long-term exposure.¹⁹ The most critical and decisive event of chemical carcinogenesis is the interaction between presumed carcinogens (AN crude extract) and cellular macromolecules such as DNA, proteins, and lipids.¹⁹ As a country with high numbers of BQ chewers, the number of studies on local AN effect on cells is scarcely available. Our long-term aim is to investigate the mechanism of AN crude extract effect on the biological system, which we hypothesise will eventually lead to carcinoma. The concluding data could hopefully assist in the selection of appropriate treatment options for OSCC patients. In this current article, our short-term aim is to investigate the cytotoxic effect of aqueous AN extract at a series of low concentrations *in vitro* using mouse fibroblast cell line (L929) as a model, for 72 hours. Our conjecture is that during AN consumption, the extract is in direct contact with epithelium at low concentrations due to the presence of saliva. In the current study, the crude extract was prepared using AN flake acquired from a local market for relevancy with the local community. As expected, cytotoxicity was observed, even at low concentrations.

Materials and Methods

Extraction of aqueous AN

Areca nut was purchased from a local market in Kota Bharu, Kelantan, Malaysia in the form of dried flakes, ready for consumption. The AN was washed with deionised water, cut into small pieces and weighed. The AN pieces were blended with deionised water with a ratio of 1:2 for 3 minutes using an electrical blender and left to soak for 6 hours at room temperature. The mixture was then centrifuged at 2500 rpm for 5 minutes to separate insoluble material. The supernatants were filtered using no.1 filter paper (Whatman, USA), concentrated using a rotary evaporator. The concentrated supernatant was then frozen at -80 °C before being freeze-dried. The freeze-dried AN extract, in powder form, were then kept at -20 °C. The powder was weighed and resuspended with Dulbecco's Modified Eagle's Medium (DMEM) to a serial dilution of 1.56, 0.781, 0.39, 0.195, 0.0976, 0.0488, and 0.0244 mg/ml prior to use.

Cytotoxicity of AN on L929 cells

Mouse fibroblast cell line, L929, was purchased from American Type Culture Collection (no. CCL1, NCTC clone 929). The cells were subcultured in DMEM, propagated and stored in -80 °C. For the experiment, the stored cells were propagated and subcultured 3 times, prior to use. L929 cells (3×10^3) were seeded into each well of a 24-well plate and incubated for 24 hours at 37°C, 5% CO₂. After incubation, the culture media was removed, and the cells were treated with the serial dilutions of AN for 24, 48 and 72 hours. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] solution was then added to the treated cells which were further incubated. After 4 hours, the MTT-containing media was removed, and DMSO added to dissolve formazan crystals that have formed. The optical density dissolved formazan was measured at 570 nm using ELISA plate reader (Tecan, Japan). The percentage of L929 cell viability was plotted against AN concentration, and subsequently, the half-maximal inhibitory concentration (IC₅₀) was determined using GraphPad (Prism). IC₅₀ value indicates the concentration of AN extract which inhibited cell growth by half.²⁰

Cell morphology analysis

Morphological alterations of L929 were observed at 24 hours after treatment with 1.56, 0.781 and 0.39 mg/ml of AN extracts using a light microscope (Carl Zeiss, Germany).

Statistical analysis

The experimental data was analysed using IBM SPSS Statistics 20 statistical software (IBM Software Group, Chicago, USA). Cytotoxicity effect was compared between the control and treated L929 cells by one-way analysis of variance (ANOVA). A p-value of less than 0.05 was considered as statistically significant.

Result

Cytotoxicity of AN aqueous extract on L929

Viability of L929 cells significantly decreased after exposure to increased concentrations of AN aqueous extract (1.56, 0.781, 0.39, 0.195, 0.0976, 0.0488, 0.0244 mg/ml) for all incubation times compared to the control (untreated) ($p < 0.05$) (Figure 1). The IC_{50} values of AN aqueous extract for 24, 48 and 72 hours were; 0.1516, 0.1040, and 0.09136 mg/ml, respectively (Figure 2).

Morphology of L929 treated by AN aqueous extract

Following 24-hour exposure to the extract, cytomorphological alterations were observed, as shown in Figure 3 (b, c and d). The control cells were well-dispersed onto the plate and exhibited bipolar to multipolar, elongated shapes (Figure 3 (a), arrow). Cells cultured with 0.39 mg/ml of AN extract however, formed dense aggregates, with a majority exhibiting irregular, rounded morphology. Occasional cells retaining the spindle-shaped morphology were also observed (Figure 3 (b), arrow). Treatment with 0.781 mg/ml AN resulted in similarly-shaped rounded cells as in Figure 3 (b), albeit in a more dispersed distribution (Figure 3 (c), arrow). Rounded, enlarged, unattached cells exhibiting intracellular vacuole-like structures were observed with exposure to 1.56 mg/ml AN extract (Figure 3 (d), arrow).

Discussion

Epidemiologically, BQ chewing habit is associated with the development of OSCC.^{2,8,21} Similarly, AN has been associated with a number of epithelial abnormalities ranging from oral epithelial hyperplasia to carcinoma. This may be attributed to the genotoxicity of AN components which alter the DNA structure, lipids and proteins.²² We are interested to investigate the effect of AN crude extract on the biological system, and the current study focuses on cytotoxicity analysis.

We began this study with a preliminary experiment to identify the suitable AN crude extract concentration on a cell biological model, L929. The AN extract concentration used was 5×10^6 mg/ml. After 24 hours incubation, all cells were found unattached and became senescent (unpublished data). The result suggested that the concentration of AN extract has a cytotoxic effect. The level of AN aqueous extract concentration was then reduced to assess the minimum concentration of AN extract that cytotoxic and affecting the cell morphology.

In the present study, AN aqueous extract concentration of 0.096 mg/ml and higher was cytotoxic to L929. Yeh *et al.*, (2014) reported a much higher concentration of AN (0.32 mg/ml) which led to the decrease in cell number of mouse C3H10T1/2, which implied cytotoxic effect.²³ The cytotoxic effect may be attributed to the carcinogenic compounds of AN such as arecoline.²⁴ Studies that have been conducted showed that arecoline is cytotoxic at concentrations of more than 0.05 mg/ml and was associated with delayed cell division.^{25,26} The cytotoxic effect of AN is not limited to normal cells as it also reportedly inhibited the growth of cancer cell lines.²⁷

L929 is a fibroblastic cell with spindle-shaped morphology. Based on the morphological changes observed in this study, we suggest that the cells do not adhere to the substrate upon exposure to the AN extracts.¹³ The present study only observed the cell morphology after 24-hour treatment with AN, and there is a possibility that prolonged incubation would show more extensive cell deformity.²⁸ In summary, the data supports the cytotoxicity findings.²⁴

In conclusion, AN crude extract is cytotoxic to L929 as it decreases the cell viability, and prolonged exposure increases the effect, hence reducing the IC_{50} . Cytotoxicity of AN extract may be associated with carcinogenesis by a disruption in cell proliferation.^{26,29} Further work to investigate the effect of aqueous AN extract on the regulators of the cell cycle will be conducted.

Acknowledgement

This study was jointly supported by grants from Research University Individual (RUI) (1001/PPSG/8012281) and Universiti Sains Malaysia Short Term Grant (304/PPSG/6315127). Authors declared no conflicts of interest.

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Table and Figure

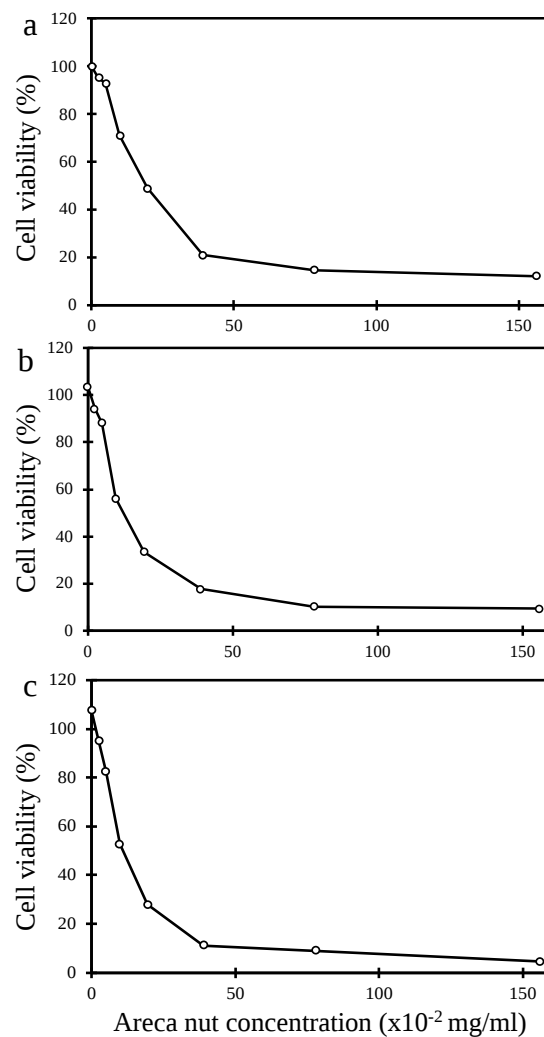


Figure 1: Cytotoxicity effect of aqueous areca nut (AN) crude extract on mouse fibroblast cell (L929) line. Cell viability of L929 when cultured with AN at (a) 24 (b) 48 and (c) 72 hours. Decrease of the cell viability was observed with increasing concentration of AN in all conditions.

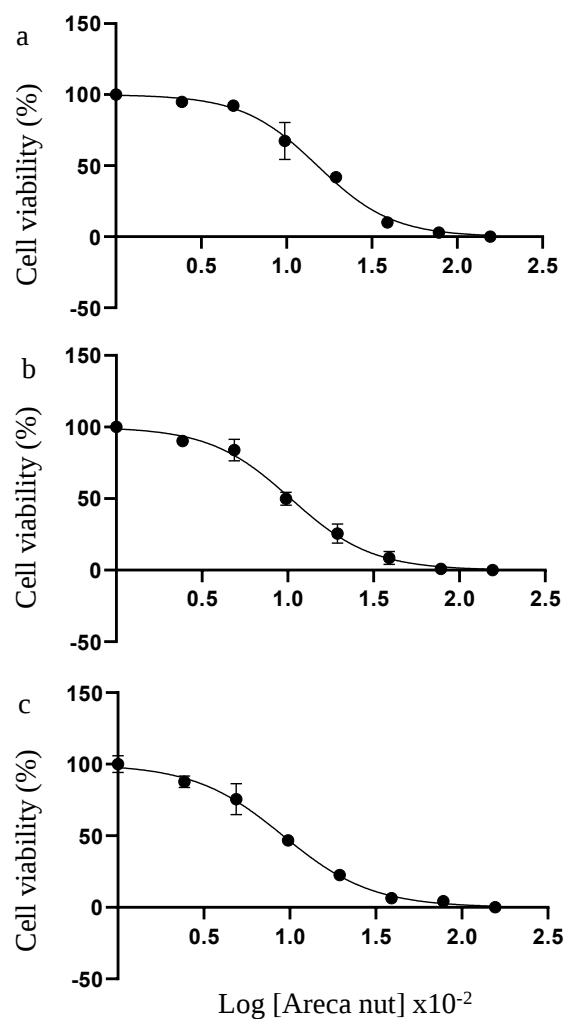


Figure 2: Half maximal of cytotoxicity effect of AN extract. IC₅₀ values are shown for (a) 24 hours; Log IC₅₀ = 0.001181 mg/ml, IC₅₀ = 0.1516 mg/ml and R² = 0.9834; (b) 48 hours; Log IC₅₀ = 0.01017 mg/ml, IC₅₀ = 0.1040 mg/ml and R² = 0.9857; (c) 72 hours; Log IC₅₀ = 0.009608 mg/ml, IC₅₀ = 0.09136 mg/ml and R² = 0.9850.

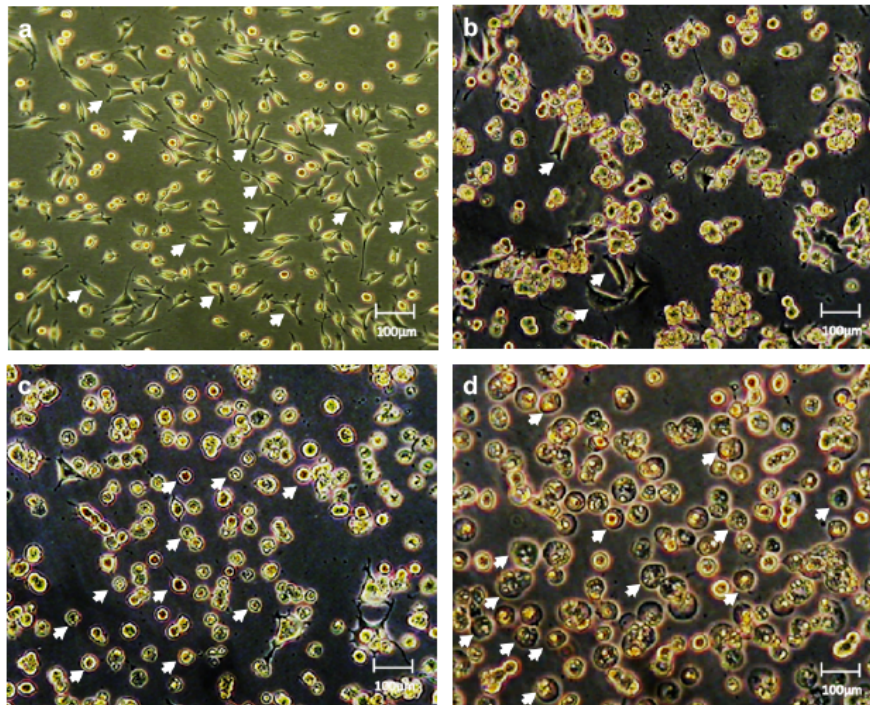


Figure 3: Cell morphological changes of L929 treated with AN for 24 hours. The micrographs are referring to (a) the untreated L929, (b) of cells exposed to 0.39 mg/ml, (c) to 0.781 mg/ml, and (d) 1.56 mg/ml of AN extract.