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Tannin Screening, Phenolic Compounds Analysis, and Antiproliferative Activity of Areca Nut Extract by Decreasing Ki-67 Protein in Oral Squamous Carcinoma Cell Lines

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ABSTRACT

The target of cancer therapy based on chemoradiotherapy or herbal medicine is to inhibit the cancer cell proliferation. Due to the current interest in the potential activity of areca nut extract, we investigated its ability to affect cell-cycle arrest and Ki-67 activity in Oral Squamous Carcinoma (OSC) cell lines. The tannin content was estimated by titrimetric analysis. The phenolic composition was analyzed using Liquid Chromatography-Mass Spectrometry (LC-MS). Flow cytometry was performed for analyzing cell-cycle arrest and Ki-67 protein as an indicator of the proliferative activity in HSC-2 and HSC-3 cell lines. The areca nut extract showed 0.007% tannin, catechin, and quercetin. The cell-cycle arrest occurred in HSC-3 cells. The reduction of Ki-67 activities occurred in both cells after 24 hours. The areca nut extract has antiproliferative activity in OSC cell lines.

Keywords: Tannin, Catechin, Quercetin, Oral squamous carcinoma, Cell-cycle.

Introduction

Oral Squamous Carcinoma (OSC) is one of the most frequent malignant neoplasms in the oral cavity and about 8% of all malignancies of head and neck cancer worldwide.[1,2] This condition represents more than 90% of the malignancies that occur in the oral cavity.[3] Oral squamous carcinoma shows a relatively small safety rate; 35-50%, even though it has been combined with radiation, surgery, and chemotherapy.[4] Combined chemotherapy treatment with surgery has been shown to significantly improve cure rates.[5] However, the side effects of this high dose treatment due to exposure a large area of the skin, maxilla, mandibula, and salivary glands cannot be avoided during treatment, this leads to the onset of mucositis, xerostomia, and osteoradionecrosis.[6,7] The studies to find drugs that have anticancer activity and have minimal side effects are still being developed.

Anticancer activity not only induces cell apoptosis but also inhibits proliferation by cell-cycle arrest.[8] The cell-cycle is the most fundamental function leading to the accurate duplication of large amounts of Deoxyribose Nucleic Acid (DNA) in the chromosomes which subsequent chromosome replication once during the synthesis (S) phase and the identical chromosomal copies are distributed equally to two daughter cells during mitotic (M) phase.[9] Two gaps (G) phases separate S and M phase, known as G1 and G2.[10] During these periods, the effort of the cells is geared towards integrating growth signals, organizing replicated genomes, and also preparing for the chromosome segregation.[11] The cell has a quiescent phase which means that the cell is in the resting phase (G0 phase). One of the hallmarks of cancer is cell-cycle dysregulation. Genomic integrity depends on the ability of normal cells to undergo cell-cycle arrest after damage to DNA. The progression of the cell-cycle is controlled by checkpoints which occurred in metaphase to anaphase, G2/M, and G1/S transitions.[11]

In normal cells, if the damage is detected in the checkpoint, the cell will pause and subscribe to the checkpoint to make repairs.[12] If the damage can be repaired then the cell continues DNA complete replication, but if not, the cell may undergo apoptosis. This doesn't occur in cancer cells that will continue to replicate despite cell damage. The apoptotic cells can be identified by the presence of internucleosomal DNA fragmentation. The low molecular weight DNA fragments that leak out from permeabilized cells due to the influence of 70% ethanol or an aqueous solution in cell staining techniques will show the subG1 population identified on DNA content frequency histogram.[13] The defective checkpoint function results in genetic modifications that contribute to carcinogenesis.[14]

Proliferation is a fundamental biological process in the development and continuity of tissue homeostasis.[15] Uncontrolled cell proliferation is one of the biological mechanisms associated with carcinogenesis.[16] One of the most important proteins which act as a proliferation marker is Ki-67.[17,18] The Ki-67 protein is a non-histone nuclear protein expressed by the cell in G1, S, G2, and M phases but not observed in cells that are not dividing (G0).[19,20] This protein is also used to calculate the growth fraction of normal tissue and malignant tumors, making Ki-67 a prognostic marker of OSC and oral premalignant lesions.[21-23] The well-differentiated OSC expresses Ki-67 as a proliferation marker.[24]

Some attentions have been focused on identifying new chemotherapy agents with fewer side effects from natural plants. Indonesia is one of the countries whose tropical climate is rich with many herbal plants. Areca nut (*Areca catechu* L.) is a herbal plant that is one of the most widely used by the inland region of Indonesian people especially in

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Aceh province as a chewing material to increase stamina, but apparently, areca nut also has the risk to cause OSC. This fact raises a controversy as to whether areca nut can be used as a therapy in oral cancer or not. Studies which explained the association between areca nut extract and its ability to inhibit cancer cell function have not been widely conducted. Our previous research showed that areca nut contains phytochemical content such as alkaloids, flavonoids, and phenolics that contribute to the high antioxidant activity. Tannin is one of the active polyphenolic compounds in areca nut that has efficacy as a biological antioxidant, which means that this natural antioxidant can inhibit the free radical-induced carcinogenic, cardiovascular diseases, and aging in humans. The current study is aimed at exploring tannin content, analysis of phenolic compounds, and the effect of areca nut extract on the proliferation of OSC cells by assessing the cell-cycle arrest and analysis of Ki-67 activity in HSC-2 and HSC-3 cells after 24 and 48 hours treatment.

Materials and Methods

Plant material

The roots, stems, leaves, and seeds of *Areca catechu* L. were collected from Aceh province, Indonesia in April 2019. The voucher specimen was identified by Dr. Sunaryo of the Herbarium unit of the Botanical Division of Biological Research Center in Cibinong, West Java, Indonesia. The voucher specimen (No. 735) has been deposited at the Botanical Division of Biological Research Center.

Extraction of the sample

The sample used was two kilograms (2 kg) of ripe areca nuts (gross weight). They were cleaned from dirt using running water and were subsequently dried. Areca nuts were dried in the open air and under sunlight. Further drying was conducted using a vacuum oven at 50ºC for 5 days. The dried sample was crushed into smooth powder form using a blender then strained with a 20-mesh sieve. The areca nut powder (1.5 kg) was macerated in 96% ethanol at room temperature for 7 days. The extract was filtered and the marc was dried and again extracted with 96% ethanol. The resulting extract was concentrated using a rotary evaporator at 30–40°C, and further concentrated over a water bath until it produced solid areca nut dry powder. The extract was stored at 36°C until further use.

Tannin assay

The areca nut extract (3 g) was mixed with distilled deionized water (dd H_2O) into a 250 mL volumetric flask for 4 hours at room temperature and then the sample was filtered. The analysis of tannin content was performed according to the International Pharmacopoeia and AOAC method with some modifications.[25,26] The infusion (25 mL) is measured into 1 L conical flask, the 25 mL of indigo solution, and 750 mL distilled deionized water (dd H_2O) are added. Then 0.1 N aqueous solution of $KMnO_4$ is used for titration until the blue coloured solution changes to green colour. Then a few drops of $KMnO_4$ were added little at a time until the solution becomes golden yellow (endpoint). The standard solution of Indigo carmine is prepared as follows: 6 g Indigo carmine is dissolved in 500 mL of distilled deionized water (dd H_2O) by heating, after cooling, 50 mL of 95-97% H_2SO_4 was added, the solution was diluted to 1 L and then filtered. The blank tests was done by titrating a mixture of 25 mL Indigo carmine solution and 750 mL dd H_2O with the 0.1 N $KMnO_4$. All samples were analyzed in duplicate. The tannins content (T %) in the sample was calculated as follows:

$$Tannin\ (\%) = \frac{(V - V0) \times 0.004157 \times 250 \times 100 \times}{g \times 25}$$

V = Volume (mL) of 0.1 N of an aqueous solution of $KMnO_4$ for the titration of the sample; V0 = Volume (mL) of 0.1 N of an aqueous solution of $KMnO_4$ for the titration of the blank sample; 0.004157 = tannins equivalent in 1 mL of 0.1 N aqueous solution of $KMnO_4$; g = mass (g) of the sample taken for the analysis; 250 = Volume (mL) of the volumetric flask; 100 = percent (%).

Analysis of phenolic composition

Liquid Chromatography-Mass Spectrometric (LC-MS) analysis was performed using 0.8 mg sample dissolved in 10 mL 95% methanol and injected into the LC-MS (Waters Alliance 2695 HPLC pump system) with a flow rate of 0.2 mL/min.[27] The standards were catechin, cinnamic acid, gallic acid, and quercetin. The mobile phase consists of solvent (A) 10% methanol; (B) Water + (C) 0.3% formic acid: (D) Acetonitrile. The gradient was programmed at 0, 1, 4, 6, 10, 12, and 13 minutes at an average rate of 2 mL/min. The reverse-phase has a nonpolar stationary phase (column). The column size was 150 mm x 4.6 mm ID Enduro C18G with a column temperature of 20ºC.[28]

Sample preparation for flow cytometry

The 10 mg of dry areca nut extract powder was dissolved in 150 µL of Dimethyl Sulfoxide (DMSO) (276855**,** Sigma-Aldrich) and diluted with complete culture medium to reach the desired dilution to make two different concentrations.

OSC cell lines

The HSC-2 and HSC-3 cell lines were provided by the Integrated Cytogenetic and Cell Culture Laboratory, Cipto Mangunkusumo Hospital. The HSC-3 and HSC-2 cell lines were derived from the tongue with a *p53* gene mutation, 4bp insertion (TAAG insertion in codon 305–306, exon 8) (JCRB0623), and p53 intron 6 splice mutation (JCRB0622), respectively.[29] All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) (D6429, Sigma-Aldrich) supplemented with 10% Fetal Bovine Serum (FBS), pyruvate, glutamine, nonessential amino acids, and vitamins at 37°C with 5% CO_2/95% air in a humidified CO_2 incubator. The supplements for the media were 100 units/mL of penicillin and 100 mg/mL of streptomycin (15070063, Thermo Fisher Scientific).

Cell culture

The cell lines in cryophilic liquid N_2 were moved into a 15 mL tube, then PBS (10010031, ThermoFisher Scientific) was added up to 10 mL. The cell lines were centrifuged by using Laboratory benchtop centrifuge (Liston C2201) for 10 min at 300 × g at room temperature. The supernatant was disposed of and to the cell pellets were added 2–3 mL complete DMEM medium and pipetted into a culture plate containing 7–10 mL DMEM medium. It was incubated at 37°C with a 5% CO_2 in a humidified CO_2 incubator. If the cells achieved 80% confluence, then it was ready to be harvested. The medium was disposed and rinsed with PBS Ca^{2+} and Mg^{2+}, then 1 mL Trypsin EDTA (59418C, Sigma-Aldrich) was added. After the addition of complete DMEM (2 – 3 mL) and centrifuging at 500 rpm for 10 minutes, the supernatant was discarded. The cells were resuspended in the culture medium and ready to be used for the experiment. Cell counting was done with a hemocytometer. The cell viability assay was performed previously to evaluate the percentage cytotoxicity and IC_{50} of areca nut extract after treating the HSC-2 cells for 72 hours is 629.50 µg/mL while in HSC-3 cells is 164.06 µg/mL.[30] The protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Indonesia (no. 501/H2.F1/Etik/2014) and conducted in compliance with the International biosafety guidelines (WHO laboratory biosafety manual, 2004). The flow cytometry assay was conducted in the Clinical Pathology Laboratory, Cipto Mangunkusumo Hospital.

Treatment with the areca nut extract

The HSC-2 and HSC-3 cells were plated at 1×10^5 cells/well in 60 mm dishes with DMEM. The cell cycle analysis uses the IC_{50} doses of areca nut extract (629.50 µg/mL and 164.06 µg/mL for HSC-2 and HSC-3 cell lines, respectively). This study examined the effect of areca nut extract on both cells after 24 and 48 h of treatment. The controls were HSC-2 and HSC-3 cells that were incubated at the same time and condition without areca nut extract treatment.

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Flow cytometry analysis for determining the distribution of cells per stage of cell-cycle

The principle of cell-cycle profile staining procedure is to dissolve the fat in the cell membranes using a non-ionic detergent, to eliminate cytoskeletons and nuclear proteins using trypsin, as well as to digest RNA using an enzyme and to stabilize nuclear chromatin using spermine. The final result of the flow cytometry was analyzed to detect the presence of an abnormal DNA (aneuploidy). This procedure used concentrated cells of $1x10^5$ cells/mL. The prepared cell suspension was centrifuged for 5 minutes at 500 rpm, afterward, the supernatant was discarded, and 200 µL of solution A, which contained trypsin in spermine tetrahydrochloride detergent buffer (340242, Becton Dickinson Cycletest™ Plus) was added to each tube and tapped with a finger, without a vortex. Solution A functioned to disaggregate solid tissue fragments enzymatically as well as to dissolve cell membranes and cytoskeletons. Solution A was allowed to react for ten minutes in a dark room at room temperature. Then, two hundred µL of solution B was added, which contained RNase A and trypsin inhibitor in spermine buffer (340242, BD Cycletest™ Plus), into each tube and each tube was tapped with a finger, without a vortex. Afterward, the tubes were placed in a dark room for ten minutes. Solution B functioned to inhibit trypsin activity and dissolve RNA. Two hundred microliter (200 µL) of solution C was added, which contained propidium iodide (PI) stain and spermine tetrahydrochloride in citrate stabilizing buffer (340242, BD Cycletest™ Plus), into each tube, following by incubation for ten minutes in a refrigerator at a temperature between 2ºC and 8ºC without discarding solution A and B. Solution C functioned to bind DNA. The samples were analyzed with the flow cytometry (BD FACS Calibur Flow cytometry System type E 34297502328, San Jose, California, USA) after at least three hours of completion of the staining procedure. The percentage of cells at each phase of the cell-cycle was calculated using Modfit LT Software version 4 (Verity Software House, Topsham, USA).

Flow cytometric analysis for Ki-67 activity

After the cells were centrifuged and the supernatant had been discarded, the solution was rinsed with 100 µL of cold PBS (10010031, ThermoFisher Scientific). One millilitre (1 mL) of 70% cold ethanol was added, followed by incubation in the refrigerator for thirty minutes, subsequently, the solution was rinsed again with PBS. Five micrlitre (5 µL) of Alexa Fluor® 647 anti-human Ki-67 antibody solution was added (562622, BD Pharmingen™) into each tube. Incubation was performed in a dark room for twenty minutes. Following the addition of 200 µL of PI (340242, BD Cycletest™ Plus) into each tube, 200 µL of solution B was added. Incubation was performed in a dark room for ten minutes. Two hundred µL of cooled solution C was added to each tube. Following the incubation for ten minutes in the refrigerator, the tubes were ready to be analyzed with the flow cytometer (BD FACS Calibur Flow cytometry System type E 34297502328, San Jose, California, USA).

Statistical analysis

All data of the flow cytometry were presented as the mean ± standard deviation of triplicate parallel measurements. Statistical analysis was done using SPSS 10.0 and the data were analyzed with the paired t-test using a significance level of $p < 0.01$.

Results and Discussion

The majority of phytochemical ingredients in areca nut extract are phenolic compounds such as flavonoids, tannins, and alkaloids.[30] The areca nut extract from Aceh has a low level of tannin (0.007%). The tannins have a potential role in increasing antioxidant activity in plants. It was established that a high quantity of tannins has been found in dark dry fruits such as tea. Although the areca nut is classified as a dark fruit, our result showed that the areca nut extract contained a small amount of tannin compared to areca nut from various districts in Karnataka, India (1.13–3.39% tannin).[31] The contents of tannin are different in areca nut found in different areas or regions. This differences could be caused by climate conditions (temperature, weather, and rainfall) and environmental factors (soil type and fertility, the height of growing, and plant maintenance) where it grows. The LC-MS analysis indicated the presence of catechin and quercetin in areca nut (Table 1). The major compound was found to be catechin. Gallate and cinnamic acid were not detected as part of the polyphenolic compounds in areca nut. Other studies have reported that areca nut contained procyanidin trimer and dimers (B1 and B2), catechin, and isorhamnetin 3-*O*-rutinoside.[28] This difference may be due to the types of the standard used. The term catechins are commonly used to refer to the family of flavonoids and the subgroup flavan-3-ols or simply, flavanol.[32] Catechins promote anticancer effects by modulation of multiple processes, including inhibition of carcinogen activity, tumorigenesis, proliferation, apoptosis induction, cell-cycle arrest, metastasis, and angiogenesis.[33-37] Although the quercetin level is not much, it has also a strong antioxidant activity that has the potential in inhibiting the development of cancer cells through the induction of apoptosis, and inhibition of proliferation in gastric, breast, esophageal, and ovarian cancer.[38] The limitation of the compound identification depends on the type and amount of the standards that we used in the analysis.

The result of the cell-cycle arrest in flow cytometry shows that twenty-four hours of areca nut extract treatment could cause a decrease in the percentage of HSC-2 cell population in the G1 phase (Figure 1). The decrease was also observed in the S phase, in which significant differences were found when comparing the control and treatment with areca nut extract, 65.45 ± 19.39% and 21.37 ± 4.16%, respectively ($p < 0.01$, unpaired t-test). The G2 phase also showed a significant difference between treated and control cells, which was 11-fold lower in the population of treated cells compared to the control cells ($p < 0.01$, unpaired t-test). There was a significant increase in the cell population in the SubG1 phase, which was three times higher compared to the control cells. The low cell population in all phases of the cell-cycle after 24 hours areca nut extract treatment indicated the presence of a disturbance in the synthesis and replication of RNA, DNA, cytosol organelles, as well as mitosis, causing cells unable to proliferate. A Significant increase was seen only in the subG1 phase, which was three times higher compared to the control cells. These results indicated that abundant apoptotic cells with DNA content had been split or cells that had undergone loss of DNA chromatin and subsequently formed apoptotic bodies. The flow cytometry result and the cell-cycle profile are presented in Figure 1.

The cell-cycle profile in HSC-2 cells after 48 hours of areca nut extract treatment could not be identified because there was no longer a population that still underwent cell-cycle as shown in Figure 2A.d. However, in the subG1 phase, which is a marker for apoptosis, a statistically significant difference was observed ($p < 0.01$, unpaired t-test) between the control cells (12.08 ± 36.06%) and those with the treated cells (27.06 ± 21.22%). The loss of cell populations in G1, S, and G2 phases was thought to be due to apoptosis or necrosis of almost all HSC-2 cells in the absence of a preparatory phase for mitosis or proliferation after the 48-hour exposure of areca nut extract. There were debris and aggregation due to plenty of DNA and cytosol organelle fractions, in the absence of cell-cycle patterns such as that observed in the control cells histogram.

Table 1: The composition of phenolic compounds in areca nut extract

Proposed compound	RT (min)	Observed mass	Calculated mass
Quercetin	11.20	301	15.263
Cinnamic acid	-	147	-
Gallate acid	-	169	-
Catechin	1.66	289.1	1863.327

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Furthermore, the investigation results on HSC-3 cells demonstrated that 24-hour exposure to areca nut extract could inhibit the HSC-3 cell-cycle in the G1 phase but no inhibition after 48-hour exposure. Statistically, a significant increase was observed in the G1 phase of HSC-3 cells, which was 7.3 times higher than that of the control cells ($p < 0.01$, unpaired t-test). The graphs and analysis results of the flow cytometry are presented in Figure 2. These results indicated that there was an increase in the number of cells that duplicated the organelle and cytosolic component as well as replicated the centrosome. However, in S and G2 phases, the number of the cell population was decreased. This decrease caused a decrease in the number of cells which underwent the DNA replication process in the S phase, as well as protein synthesis and complete chromosome replication in the G2 phase, and therefore the proliferation was inhibited. The flow cytometry on the HSC-3 cells after 48-hour exposure to areca nut extract did not show any significant changes in the cell population in G1, S, and G2 phases with or without the exposure of areca nut extract. Forty-eight hours of exposure to areca nut extract at a dose of 164.06 µg/mL did not affect the cell-cycle profile of HSC-3 cells.

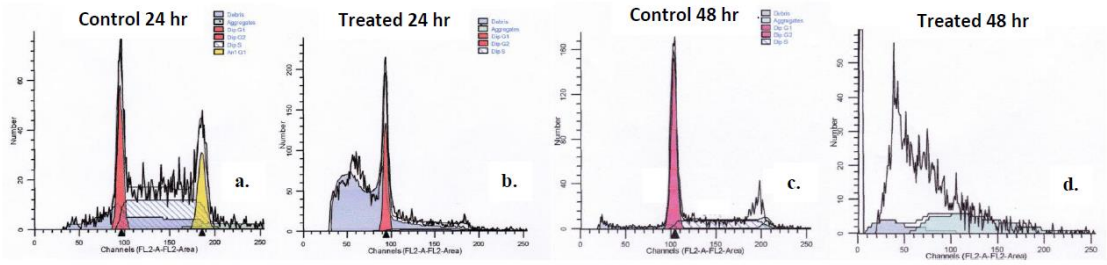
This study showed a significant decrease in Ki-67 activity following the 24-hour exposure to areca nut extract in HSC-2 cells ($p < 0.01$, unpaired t-test) (Figure 3). The 48-hour of areca nut extract exposure demonstrated a result consistent with the result of the HSC-2 cell-cycle analysis, which showed that the extract was unable to induce cell-cycle arrest. The M1 and M2 quadrant, which exhibited the percentage of cell population with and without Ki-67 protein, also showed a similar result in both the treatment and control groups ($p < 0.01$, unpaired t-test). The study results showed that areca nut extract does not affect Ki-67 activity after 48-hour exposure.

The Ki-67 activity of HSC-3 cells was similar to the HSC-2 cells. There was a significant decrease in a cell population with active Ki-67 compared to the control cells following 24-hour exposure, but not at 48-hour exposure ($p < 0.01$, unpaired t-test). The extract only inhibits HSC-3 cell proliferation after 24-hours treatment with areca nut extract, but not after 48-hour exposure. The flow cytometry analysis results and graphs are presented in Figure 4.

The areca nut extract arrests cell-cycle progression by significantly restricting cells in the G0/G1 phase only in HSC-3 cells after 24-hour exposure. The maximum effect of areca nut only happened 24 hours after treatment in HSC-3 cells, whereas in HSC-2 cells a decrease in the number of cells in the G1, G2, and S phases occurred at both times. This result indicated that these cells were unable to enter the next phases. This event might be because most of the cells have undergone apoptosis, resulting in the cell build-up in the subG1 phase. The increasing cell population in the subG1 phase is the characteristic of the amount of fractional DNA content and apoptotic bodies. This result has been confirmed by our previous research that there was a presence of apoptosis in HSC-2 cells after areca nut treatment.[39] Furthermore, the areca nut extract might have the ability to perturb the protein synthesis that is important to cell progression from G1 to S-phase. It is known that p53 and mdm2 proteins are important to the progression of the cell-cycle at G0/G1.[40] It may be possible that the extract plays a role in the disturbance of these proteins, but unfortunately, this aspect was not investigated in our study.

There is a difference in extract effect on the cell-cycle distribution, where the percentage of HSC-2 cells undergoing apoptosis is higher than HSC-3 cells. This result might be possible because although both cells have gene mutations, there are different characteristics between HSC-3 cells and HSC-2 cells.[29,41,42] The HSC-3 cells can evade apoptosis higher than HSC-2 cells. On p53 activation, HSC-2 cells undergo predominantly apoptosis instead of cell-cycle arrest. However, this finding may vary by the study design and so much more data must be collected to better understand this phenomenon.

The cell-cycle test refers to a frequency histogram of cellular DNA content in each phase of the cell-cycle. The results of this analysis proved that cell-cycle arrest did not occur after 24 and 48-hour exposure of the areca nut extract in HSC-2 cells but a decrease in proliferation occurred after 24-hour exposure to the extract, indicated by a decrease in the cell population in G2/M phases, while after 48-hour exposure, a decrease in proliferation occurred due to the high number of cells undergoing apoptosis (increase in subG1 phase).



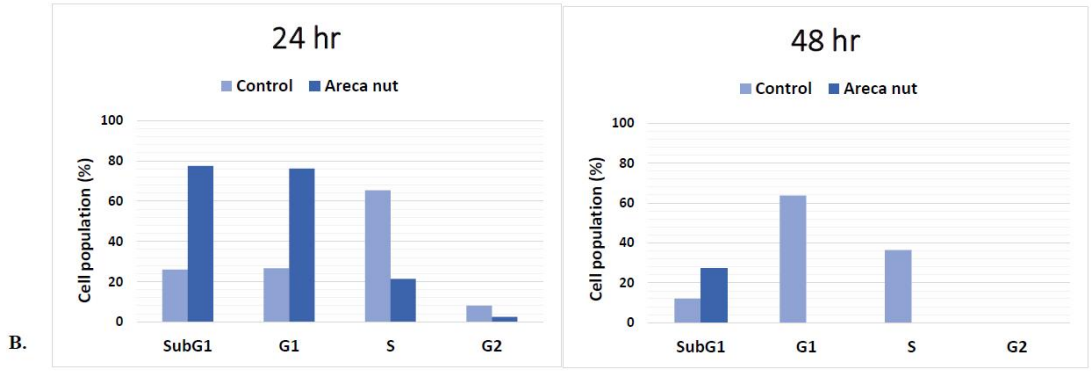


Figure 1: A. Cell-cycle distribution of areca nut extract on HSC-2 cells, (**a, c**) control in 24 and 48 hr; (**b, d**) areca nut extract treatment in 24 and 48 hr. **B**. Graph of comparison between HSC-3 cell percentage with and without areca nut extract after 24 and 48 hours

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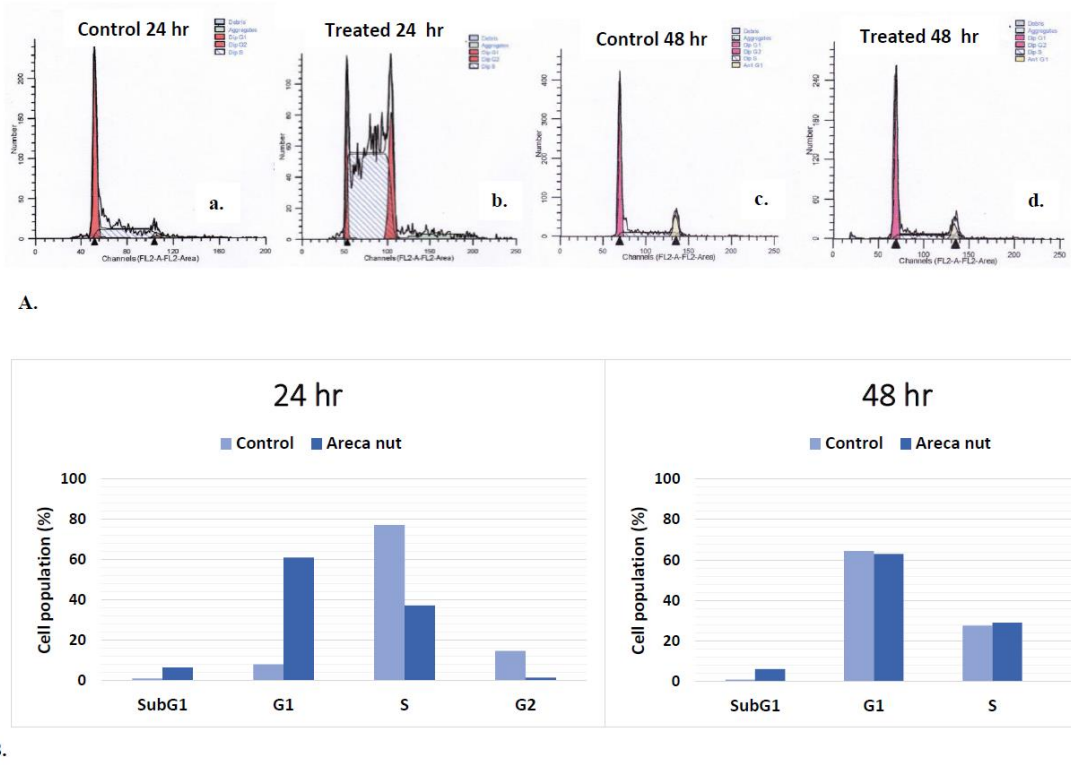

Figure 2: A. Cell-cycle distribution of areca nut extract on HSC-3 cells, (**a, c**) control in 24 and 48 hr; (**b, d**) areca nut extract treatment in 24 and 48 hr. **B**. Graph of comparison between HSC-3 cell percentage with and without areca nut extract after 24 and 48 hours

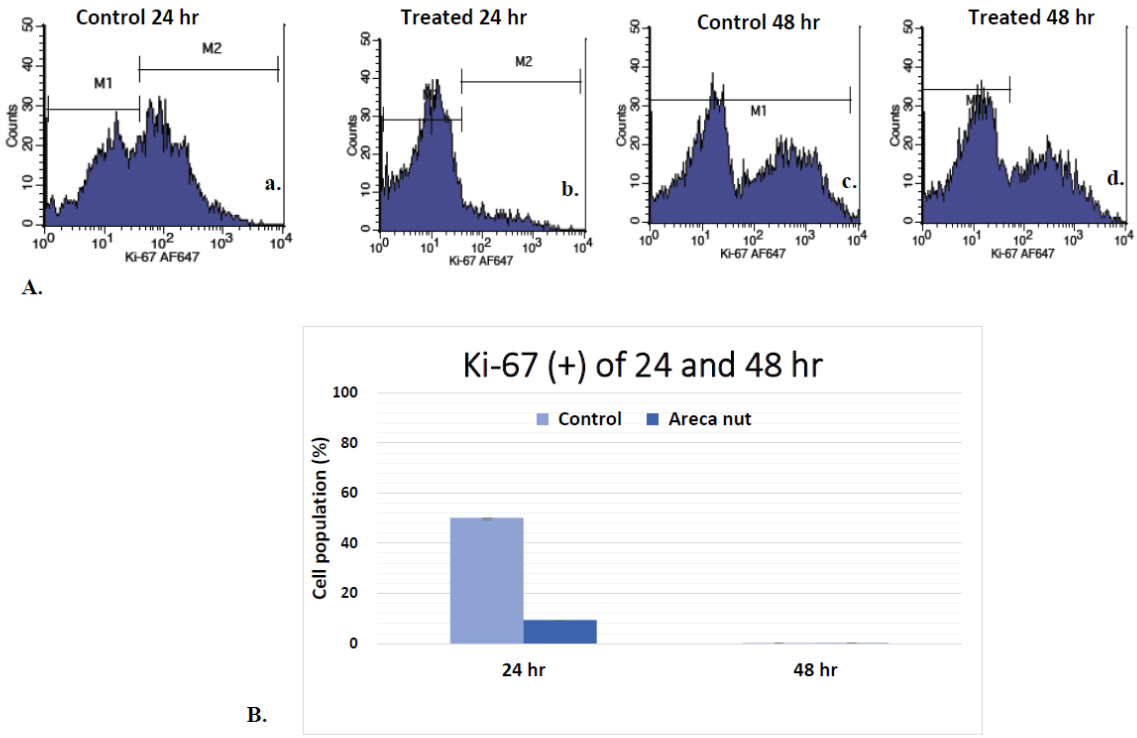

Figure 3: A. The Ki-67 activity on HSC-2 cells, (**a, c**) control in 24 and 48 hr; (**b, d**) areca nut extract treatment in 24 and 48 hr. **B**. Graph of comparison between the percentage of HSC-3 cells with active Ki-67 with and without areca nut extract after 24 and 48 hours

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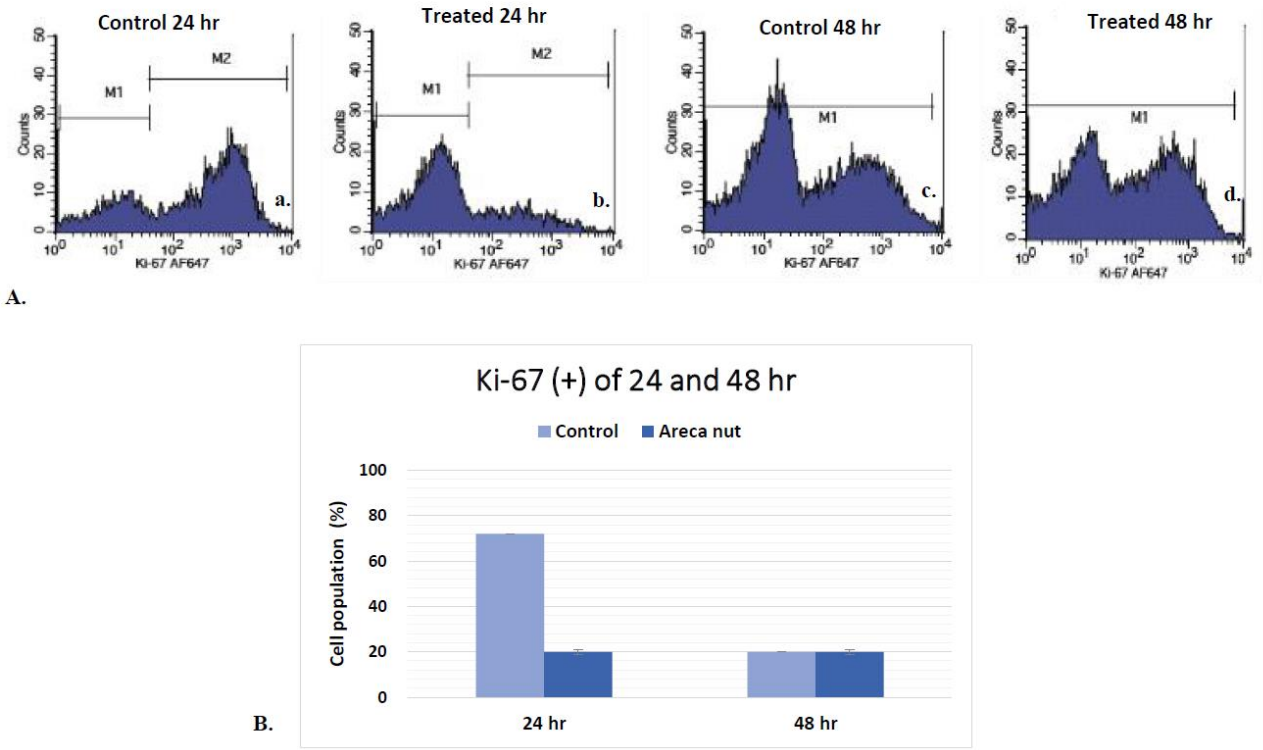

Figure 4: A. The Ki-67 activity on HSC-3 cells, (**a, c**) control in 24 and 48 hr; (**b, d**) areca nut extract treatment in 24 and 48 hr. **B**. Graph of comparison between the percentage of HSC-3 cells with active Ki-67 with and without areca nut extract after *2*4 and 48 hours

The drawback of flow cytometry in the Ki-67 assay is that the cell preparation or staining causes about 50% of the cells to experience apoptosis or necrosis without treatment and causes a much more decrease in the cell population in G2/M phases.

However, the areca nut extract was only able to inhibit proliferation after 24-hour exposure in HSC-3. This result was confirmed with the Ki-67 activity analysis, which indicated that a decrease in the proliferation activity due to areca nut extract only occurred after 24-hour exposure, whereas no significant difference was observed between the treated and the control groups after 48-hour exposure. Twenty-four-hour exposure of the extract caused an increase of the cell count in HSC-3 cells which were retained at the G1 phase. Inhibition of cell-cycle occurred due to the presence of a checkpoint at the transition of the G1/S phase, causing the cells which underwent DNA damage in the G1 phase not to be able to proceed to the S phase.[43] The p53 mutation in the HSC-3 and HSC-2 cells caused the cell-cycle not to involve the p53 pathway. This cell-cycle inhibition could occur from two pathways. The first pathway was the activation of Cyclin (Cyc) D1 by Anaphase Promoting Complex (APC), leading to the release of p21Cdk-Interacting Protein (CIP1) which bind with Cyclin-dependent kinase (Cdk)4/CycD1 and inhibited Cdk2/CycE.[43] The second pathway was through an increase in the phosphorylation of Cdk2 at the site of cell division control (cdc) 25A phosphatase, which task is to phosphorylate this site thus inhibiting the function of Cdk2/CycE. The involvement of the Checkpoint kinase (Chk) 2 pathway which induced p53 and later activated p21 to inhibit the phosphorylation of CycE/Cdk2 did not affect the HSC-3 cell-cycle inhibition.

Conclusion

The findings showed that the areca nut extract showed tannin, catechin, and quercetin. The areca nut caused the cell-cycle arrest in HSC-3 cells after 24 hours of extract treatment while the HSC-2 cells cannot be determined. The areca nut inhibited cell proliferation by the enhancement of Ki-67 after 24 hours of extract treatment in both cells.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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