CHEMOPREVENTIVE ACTIVITY OF TWO VARIETIES OF FREEZE-DRIED COCONUT WATER AGAINST CERVICAL CANCER CELLS, HELA.

Ansor NM[1], Ramlee SNL[1], Mail MH[1,2], Saifuddin SN[1], Murtey MD[3], Ahmad AMC[4], Singh R[5], Sapuan S[1], and Seeni A[1]

[1]Department of Toxicology, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Penang, Malaysia

[2]Malaysian Institute of Pharmaceuticals and Nutraceuticals, National Institute of Biotechnology Malaysia, Ministry of Science, Technology and Innovation, 11700 Penang, Malaysia

[3]Basic Sciences and Oral Biology Unit, School of Dental Sciences, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

[4]Department of Community Health, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Penang, Malaysia

[5]Faculty of Medicine, AIMST University, Kedah Malaysia

Correspondence:

Associate Professor Dr. Md Azman Bin PKM Seeni Mohamed,
Department of Toxicology,
Advanced Medical and Dental Institute,
Universiti Sains Malaysia, Bertam,
13200 Kepala Batas, Penang, Malaysia
Email: azmanseeni@usm.my

Abstract

Coconut water is considered to be nature's elixir. It is a refreshing beverage that can quench thirst, and it is consumed as a health tonic, especially by people living in the tropics. As cancer prevalence increases, novel anticancer therapies are urgently needed, and the chemoprevention approach using natural products is gaining research attention. The goal of this study was to evaluate the potential chemopreventive effects of two varieties of freeze dried coconut water (FDCW) against cervical cancer (HeLa) cells. Both the MATAG FDCW and the Aromatic Dwarf (AD) FDCW varieties exerted anti-proliferative activity against HeLa cells with a inhibitory concentration of 100 µg/ml. After 72 h of treatment, observation under an inverted microscope showed typical apoptotic morphological alteration in HeLa cells exposed to MATAG FDCW, and features of both apoptosis and autophagy were observed in HeLa cells treated with AD FDCW. Fluorescence microscopy revealed the presence of condensed chromatin and apoptotic bodies in HeLa cells from both treatments. To evaluate the anti-proliferative activity over a prolonged treatment period, HeLa cells treated with each type of FDCW were incubated for 8 d. MATAG FDCW was able to continuously suppress HeLa cell proliferation for the entire experiment, whereas the effect of AD FDCW was not stable and the suppression effect decreased over time. These results suggest that MATAG FDCW has a better anti-proliferative effect than AD FDCW. However, both FDCW varieties demonstrated positive chemopreventive activity and should be considered as potential novel anticancer therapies.

Keywords: Anti-Proliferative, Cervical Cancer (Hela cells), Health Tonic

Introduction

Cancer is a leading cause of death worldwide (1). In 2008, about 12.7 million people were diagnosed with cancer, and 7.6 million people died of cancer worldwide. Cancer cases expected to hit 20 million cases by 2030 unless there are effective and concentrated efforts (1, 2). Cervical cancer, albeit a preventable disease, still accounted as third most common cancer in women worldwide with 604,127 new cases and 341,831 deaths worldwide in the year 2020 (2).

In Malaysia, cervical cancer is the third most common cancer among women (3). Although there are reduction of cervical cancer mortality and morbidity through screening and treatment at earlier stages (4), the effectiveness of modern conventional therapies are not encouraging since the overall cancer burden is still high. Therefore, numerous researchers are trying to develop new therapeutic strategies to combat cancer, and chemoprevention is among the active areas of

investigation.

Natural phytochemicals and dietary supplements have gained increasing interest in the field of cancer therapy and prevention. Generally, prospect of having low toxicity in dietary phytochemicals means that these bioactive natural compounds have great potential as cancer chemopreventive agents (5, 6). Coconut water is a natural source of phytochemicals. In tropical countries, it is viewed as both a refreshing beverage and a health tonic. Hawaiians refer to coconut water as "the dew from the heavens" due to its enormous health benefits. They use it to treat food poisoning, dehydration, malnutrition, heatstroke, boils, kidney stones, fatigue, digestive disturbances, osteoporosis, urinary tract infections, constipation, diarrhea, and sterility (7, 8). In Malaysia, there are 13 varieties of coconut can be found and for this study, we only chose two popular varieties which were MATAG hybrid and Aromatic Dwarf (AD) as to evaluate their chemopreventive activity. They were selected based on the commercialization and market demand in this country. MATAG hybrid is bred from cross-pollination of Malayan Dwarf and Tagnanan Tall. This hybrid can grow up to 15 meters. Depending on the Malayan Dwarf parent, the daughter's (MATAG) fruits, leaves, and flowers are either pale green or orange in colour. The coconut fruit produced is large in size and suitable for various applications such as coconut water, coconut milk production, and also copra (coconut meat). On the contrary, AD was first introduced from Thailand in 1971. In Malaysia, it is known as "Kelapa Pandan". The uniqueness of this variety is the aromatic scent of its water and meat content which have the scent of "pandan" making it famous as a refreshing beverage. This coconut tree can reach a height of 6 meters. The fruits produced are small, round, notched three, and green in colour. The average fruits produced per bunches are approximately 10-15 fruits. Thus, with these features and quality, MATAG and AD are famous among domestic consumers in Malaysia (9).

Therapeutically, coconut water content resembles intracellular fluid; it is sterile within its envelope and is used as intravenous hydration for patients in remote areas in cases of emergency. It was also used as a resuscitation fluid during World War II (8, 10). Furthermore, due to the variety of inorganic ions present in coconut water, it is able to replenish electrolytes in the human body. Electrolytes in coconut water create similar osmotic pressure with human body and show no interference with normal body homeostasis (10). Hence, coconut water is an effective rehydration drink (11).

Savalas and co-workers reported that coconut water contains cytokinins (e.g., kinetin, transZeatin), which are a class of phytohormones (9). Kinetin has shown significant anti-ageing effects, as it slows down the ageing process of endothelial cells and enhances cell proliferation and metabolic capacity. They concluded that coconut water through kinetin acts as an anti-carcinogen, anti-proliferative and apoptogenic by inhibiting cancer cell development. Plenty of research done to develop new therapeutic strategies in combating this terrifying disease with chemoprevention is among the active area of investigation with enormous potential. Natural phytochemicals or dietary supplements have gained an upsurge of interest in the field of cancer prevention and therapy. The numerous health benefits of coconut water that have already been discovered suggest that this beverage may exhibit positive chemoprevention activity against cancer cells. The goal of this study was to assess the chemopreventive activity of coconut water against two different varieties of coconut. These two types of coconut water were tested for their ability to inhibit or suppress the growth of cervical cancer (HeLa) cells. HeLa (ATCC®CCL-2™) cell line is cervix epithelial adenocarcinoma cells, an adherent cell. It is the first cell line that is successfully grown in laboratory condition, which it was known as "immortal cell line" later on, by a researcher named Otto Gey in John Hopkins University.

Materials and Methods

Plant materials

The samples used in this study were freeze dried coconut water (FDCW) made from two different coconut varieties (MATAG and Aromatic Dwarf (AD)). The coconut fruits (*Cocos nucifera*) were obtained from Agriculture Department, Penang.

Cell lines

Human cervical carcinoma cells (HeLa, ATCC# CCL-2) purchased from the American Type Culture Collection (Manassas, VA, USA) were used in this study.

Collection, preparation, and freeze drying of plant materials

Coconut water from six MATAG and six AD coconut fruits was filtered using a 1000 ml vacuum-driven filter, 0.22 µm PES hydrophilic to remove debris from the coconut husk. The filtered coconut water was then transferred into round-bottom flasks for freeze drying. The coconut water was poured into the flask until it was half- or three-quarters full to provide a large total surface area to speed up the freeze drying process, which took about 3 d to complete. The FDCW became concentrated, sticky, and smelled like normal coconut water; it was yellowish in colour for AD and pale-yellowish for MATAG coconuts. About 100 mg and 50 mg of FDCW was obtained from the six MATAG and six AD coconuts, respectively. The FDCW samples were transferred into 50 ml centrifuge tubes and stored at –20 ºC.

Determination of the inhibitory concentration of the FDCW samples

Upon reaching 80–90% cell confluence, HeLa cells were washed once with phosphate buffered saline (PBS). Immediately, cells were trypsinised by adding 3 ml of 0.25% trypsin/EDTA and incubating them for about 15 min at 37 ºC. Once the cells had detached from the flask surface, 4 ml of fresh complete medium were added to neutralize the trypsin reaction. Using a pipette, the cells were flushed a few times to ensure that all cells were completely detached, and the cell suspension was collected into a 15 ml centrifuge tube. The cell suspension was centrifuged at 200×g for 5 min. The supernatant was discarded and replaced with 5 ml of fresh complete medium to resuspend the cell pellet (12). For seeding, 2.5×10^4 cells were plated into each well of 6-well culture plates, which contained 3 ml of fresh complete medium. After 24 h, HeLa cells were treated with varying concentrations of both FDCW varieties separately by removing the old medium and replacing it with a mixture of 3 ml of fresh complete medium and the FDCW product. Screening was conducted from the lowest concentration to the highest concentration, which ranged from 0 to 180 µg/ml of FDCW product. The screening was conducted in triplicate, and the range was the same for both FDCW varieties. Control cells were exposed to a mixture of fresh complete medium and 10 µl of sterile ultrapure water without FDCW. Treated HeLa cells were incubated in a 37 ºC humidified CO_2 incubator with 5% CO_2 and 95% air for 72 h, at which point they reached optimum confluent. Cells then were harvested for counting of viable cells. The old medium was discarded, the cells were washed once with PBS, and then they were subjected to trypsinisation with 500 µl of 0.25% trypsin/EDTA for approximately 15 min. Next, 1 ml of fresh complete medium was pipetted into each well to neutralize the trypsin. Once the cells were completely detached from the well surface, they were collected into 15 ml centrifuge tubes. The cell suspensions were centrifuged at 200×g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in 1 ml of fresh complete medium (12). The number of viable cells was determined using the trypan blue exclusion assay (TBEA). Graphs of cell viability against FDCW concentrations were plotted for both FDCW varieties. The percentages of cell inhibition were compared with that of the control group.

Measuring cell viability using the TBEA method

The TBEA method to evaluate cell viability was conducted following the manufacturer's protocol with slight modification of volume and dilution factor. In brief, 50 µl of cells from 1 ml of cell suspension were placed in a 1.5 ml eppendorf tube and mixed with 50 µl of 0.4% (w/v) trypan blue dye in a 1:1 ratio. The solution was mixed using a pipette, and 10 µl of the cell suspension were immediately loaded into the heamacytometer chamber and overlaid with a coverslip. By capillary action, the cell suspension was properly loaded into the heamacytometer chamber to avoid air bubbles and overfilling. The number of viable cells was counted under a phase contrast microscope at 100× magnification. The viable cells were distinguished from the dead cells by colour, as viable cells were clear (not stained) and dead cells were stained by the trypan blue dye (13). The number of cells per ml and the total number of cells were calculated based on the following formula:

$$C = \text{Viable cells counted } Q \times Df \times Hf \times V$$

where C is cell concentration; Q is quadrant counted; Df is the dilution factor; Hf is the haemocytometer factor; and V is the sample volume (ml).

Microscopic observation of morphological changes

HeLa cells were seeded at 2.5×10^4 cells in 6-wells plate. Following 24 h of growth, the predetermined concentrations of both FDCW varieties were applied to the cultured cells in each well and incubated at 37 ºC in a 5% CO_2 humidified atmosphere (12). Control cells were exposed to a mixture of fresh complete medium and 10 µl of ultrapure water. Morphological changes of HeLa cells were monitored under a phase contrast microscope at 24, 48, and 72 h after treatment. An Olympus Xcam_Alpha (Tokyo, Japan) camera was attached to the inverted microscope to record morphological changes, and photographs were captured at 40×, 100×, and 200× magnifications. Features related to apoptosis or cell death were identified by comparing treated cells with untreated cells.

Fluorescence microscopy observation using PureBlu™ Hoechst 33342 nuclear staining dye

The nuclear staining procedure using PureBlu™ Hoechst 33342 Nuclear Staining Dye (USA) was conducted according to the manufacturer's protocol. First, 500 µl of deionized water were added to a tube and vortexed briefly to make the 100× stock solution (1.1 µg/ml [2 µM]). Next, the stock solution was diluted to 1:100 with 1× PBS (for fixed cells) to make the 1× staining solution. Hoechst dye is very sensitive to light, thus the procedure was performed in a dark room and the staining solution was wrapped with aluminum foil to avoid exposure to light. Following 72 h of incubation, treated and control cells were rinsed once with 1 ml of PBS. Each well containing cultured cells was fixed with 4% formaldehyde at room temperature for 10 min. The cells then were rinsed with 1× PBS, and 2 ml of staining solution were added to each well for staining. Cells were left to stain for 15 min at room temperature. Each well again was rinsed with 1× PBS thoroughly to remove the dye. Finally, stained HeLa cells were visualized under a fluorescent microscope.

Cell proliferation assay

For the cell proliferation assay, 1.0 x 10^4 cells were seeded in 6-well plates containing 3 ml of fresh complete medium in each well. After 24 h of incubation, HeLa cells were treated with 100 µg/ml of MATAG FDCW or AD FDCW. Untreated HeLa cells exposed to a mixture of fresh complete medium and 10 µl of ultrapure water served as the control. The experiment was carried out in triplicate, and the cells were incubated at 37 ºC with 5% CO_2 and 95% air. The cultured HeLa cells were harvested on days 1, 3, 5, and 8 to evaluate cell proliferation. The medium was replaced on days 3 and 6 with a mixture of fresh complete medium and 100 µg/ml of the respective FDCW varieties (12). On the predetermined days, cells were harvested by removing the old medium, washing once with PBS, and trypsinising with 500 µl of 0.25% trypsin/EDTA in each well. The plates were incubated for about 15 min. To neutralize the trypsin reaction, 1 ml of fresh complete medium was added to each well. All used medium, PBS, and trypsin from each well were collected into centrifuge tubes. The suspended cells from each well were collected into centrifuge tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml of fresh complete medium (12). Subsequently, the TBEA was used to determine the cell number. Graphs of cell viability versus time were plotted. To calculate cell viability, the following formula was applied (14):

$$\text{Cell viability (\%)} = (NV / NT) \times 100\ \%$$

where NV is the number of viable cells and NT is the total number of cells in the population

Statistical analysis

All data were analyzed using the Statistical Package for Social Sciences (SPSS) version 20.0 (SPSS, Inc., Chicago, IL, USA). Values of three measurements (n = 3) were expressed as mean ± standard error of the mean (SEM). Independent Student's t-tests and one-way analysis of variance were used to identify differences between treated cells and untreated control cells. Significance levels of $p < 0.05$ and $p < 0.01$ were considered to be statistically significant. Graphs were plotted using Microsoft Excel 2010.

Results and Discussion

A suppression effect on HeLa cells was seen at lower concentrations of MATAG FDCW, whereas at higher concentrations a growth stimulant effect was noted (Fig.1a). However, the lowest cell growth and cell viability were detected at 100 µg/ml of this FDCWs. At this concentration, cell growth was reduced to 1.88 x10^4 cells/ml and cell viability was decreased to 55%, meaning that 45% of HeLa cells were inhibited. In general, MATAG FDCW seemed to have a suppressive effect because cell growth and viability did not exceed those of the untreated control cells. Significant differences from the control were noted at doses of 60 µg/ml ($p = 0.003$), 100 µg/ml ($p = 0.002$), 140 µg/ml ($p = 0.003$), and 180 µg/ml ($p = 0.013$). HeLa cells exposed to different concentrations of AD FDCW exhibited a pattern similar to that seen for MATAG FDCW (Fig.1b). Cell growth and viability were lowest at 100 µg/ml of AD FDCW. At this dose, cell growth was reduced to 0.68 x10^4 cells/ml and cell viability was decreased to 51%, meaning that 49% of HeLa cells were inhibited. Conversely, at the lowest dose of 20 µg/ml, a stimulatory effect was detected, whereby cell growth and viability exceeded those of the control; however, the change was not statistically significant. Of the concentrations tested, a significant difference from the control was only found at 100 µg/ml of AD FDCW ($p = 0.044$). Nevertheless, the graphs generally show a suppressive effect for both FDCW varieties.

To investigate the proliferative activity of HeLa cells exposed to both FDCW varieties, an 8 d incubation study was conducted. HeLa cells were pre-treated with the IC_{50} of either MATAG FDCW or AD FDCW. Growth inhibition was evaluated at days 1, 3, 5, and 8 (Fig.2a and Fig.2b). Compared to the control, both FDCW varieties demonstrated approximately 50% reduction in cell concentration starting from day 3 and continuing through day 8. HeLa cells exposed to MATAG FDCW demonstrated better time-dependent growth inhibition compared to cells treated with AD FDCW. The graph shows a progressive pattern of cell suppression starting at day 1 and continuing through day 8. However, on days 5 and 8 the suppression remained stable. Conversely, HeLa cells treated with AD FDCW demonstrated an inconsistent temporal suppression pattern in which growth suppression was only observed from days 1 to 3, followed by an increase on days 5 and 8. A significant difference from the control was only noted at day 8 for MATAG FDCW ($p = 0.000$), whereas significant differences were detected at days 3 ($p = 0.000$), 5 ($p = 0.009$), and 8 ($p = 0.003$) for AD FDCW. On day 8 of treatment, MATAG FDCW showed 52% cell inhibition and AD FDCW demonstrated 43% cell inhibition, which suggests that MATAG FDCW had better chemopreventive activity than AD FDCW because it exhibited a better suppression pattern with a higher anti-proliferation effect. Moreover, it was able to suppress HeLa cells continuously even over prolonged exposure.

A vital property of an anticancer drug candidate is its ability to induce apoptosis in cancerous cells (15). Cellular models are reliable tools for toxicity screening and can help identify important aspect of toxicology and in vivo pharmacology (16). In this study, we screened for the IC_{50} dose of both FDCW varieties on HeLa cells and identified it as 100 µg/ml. At this concentration, almost

50% of the HeLa cell population was inhibited. Although both samples had the highest inhibition effect at the same dose, AD FDCW had a greater cytotoxic effect (49% inhibition) compared to MATAG FDCW (45%). Prabhu and co-workers previously reported IC_{50} values for coconut water of 1.25 mM for the 1321N1 cell line and 1.85 mM for the U87MG cell line (17). These different inhibitory concentrations might be due to different cell lines used, different varieties of coconuts used, and variations in

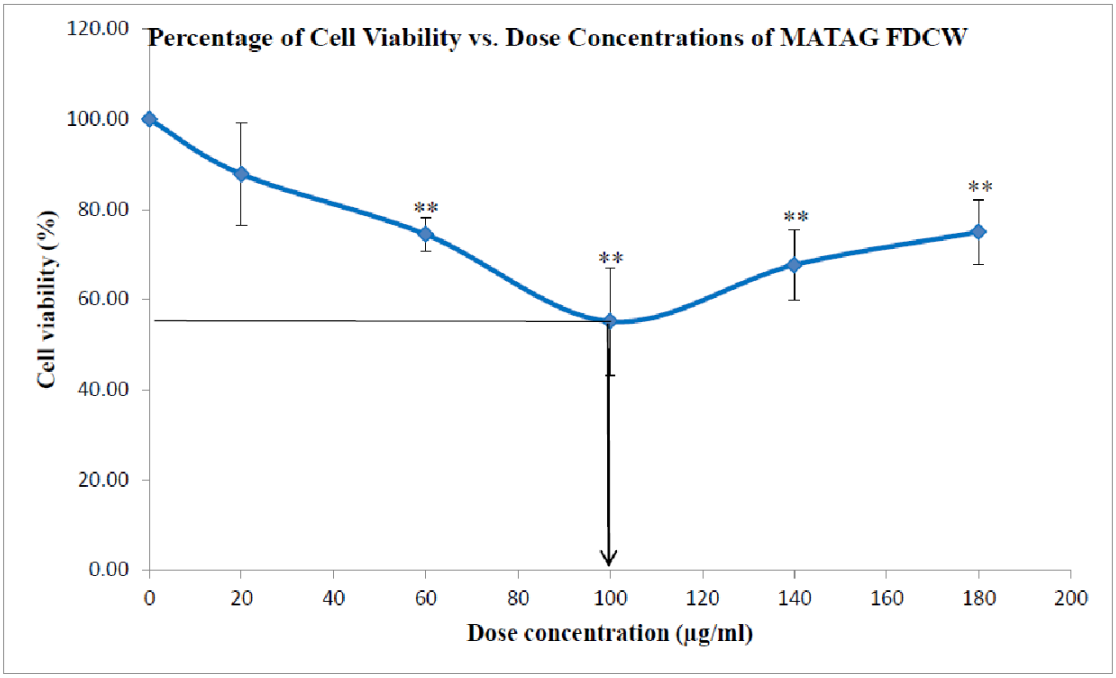


Figure 1a: Percentage of viable HeLa cells in response to different concentrations of MATAG FDCW. The experiment was performed in triplicate, and values are means ± SEM. ** indicates significant difference at $p < 0.05$ with respect to control untreated cells

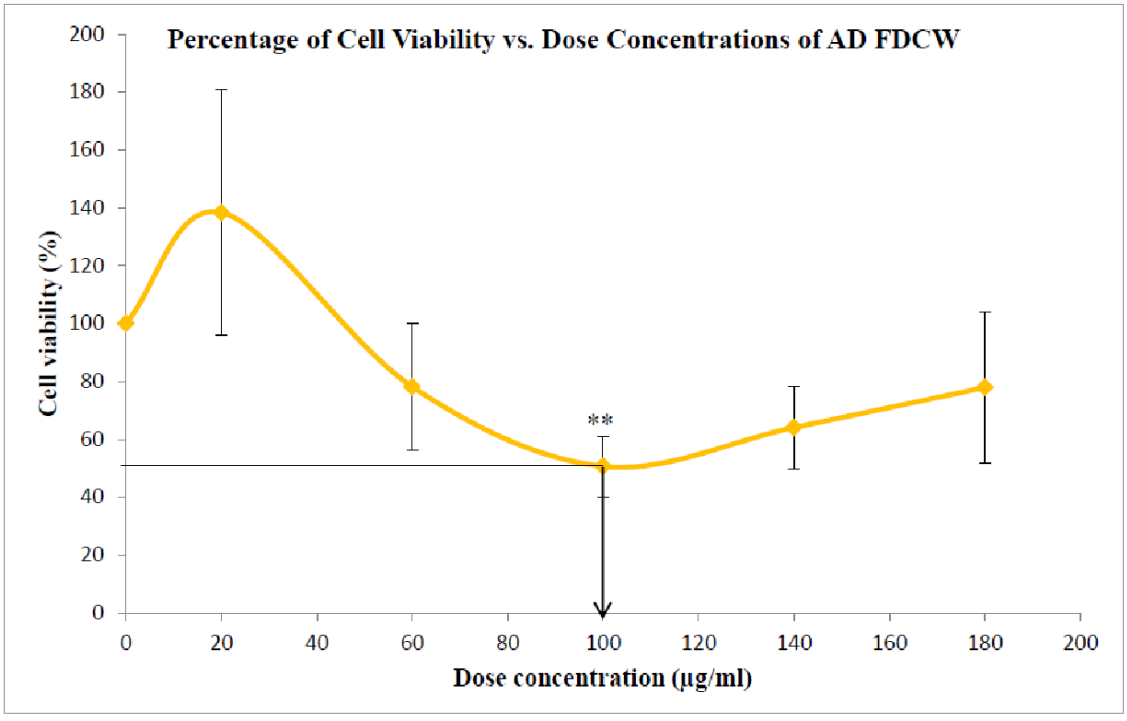


Figure 1b: Percentage of viable HeLa cells in response to different concentrations of AD FDCW. The experiment was performed in triplicate, and data are means ± SEM. ** indicates significant difference at $p < 0.05$ with respect to control untreated cells

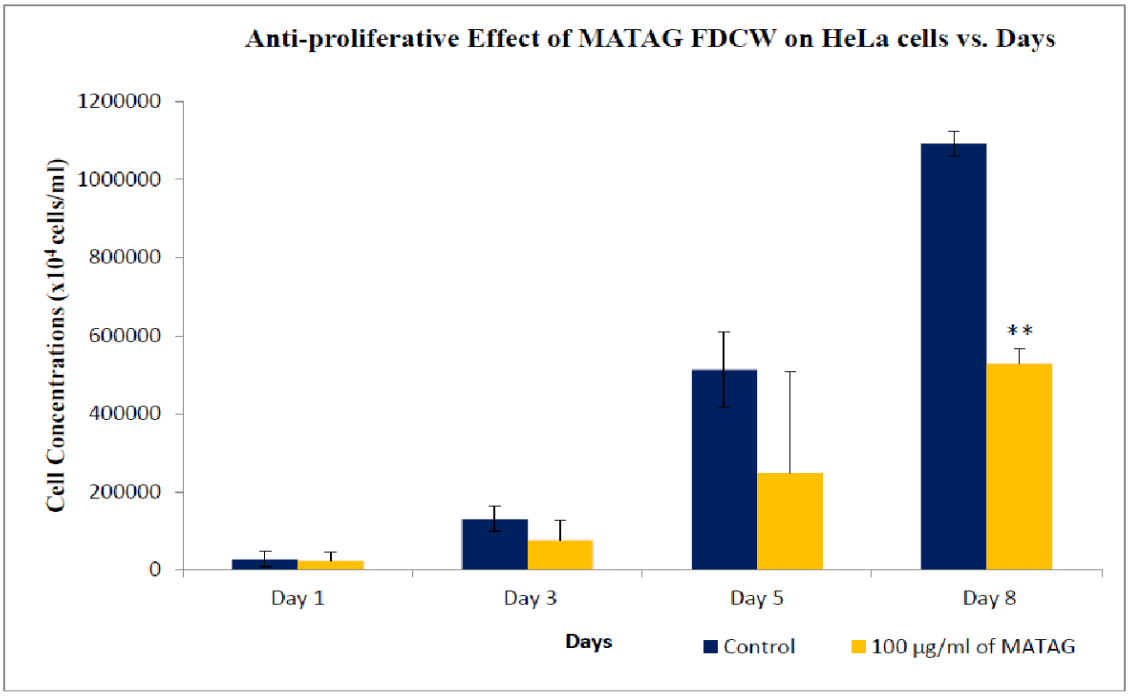


Figure 2a: Anti-proliferative effect of MATAG FDCW over time. The experiment was conducted in triplicate, and data are means ± SEM. ** indicates significant difference at $p < 0.05$ with respect to control untreated cell

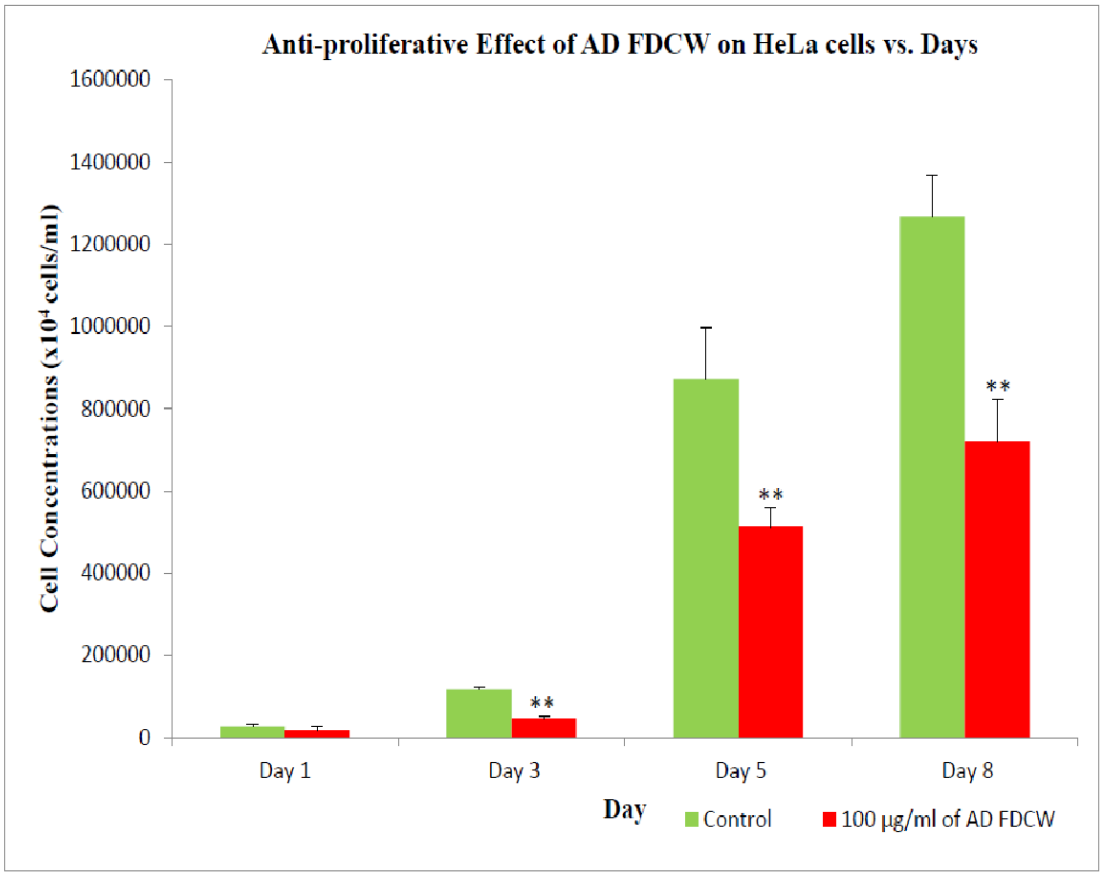


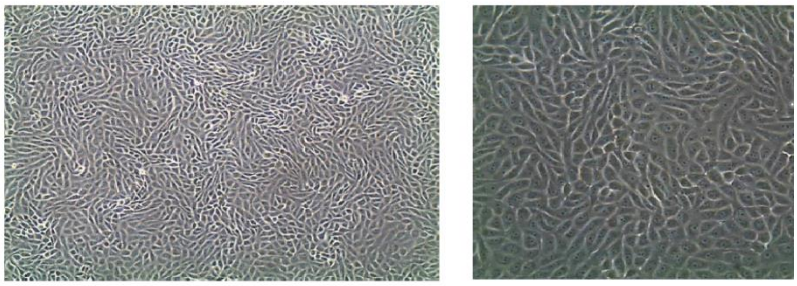
Figure 2b: Anti-proliferative effect of AD FDCW over time. The experiment was conducted in triplicate, and data are means ± SEM. ** indicates significant difference at $p < 0.05$ with respect to control untreated cells

cultivation area and climate factors. According to Doležal and co-workers, coconut water is the richest dietary source of cytokinins (18). They play an essential role in regulating cell growth and averting errors that

can lead to the development of cancer. This might explain the stimulatory effect of the lower concentrations of AD FDCW on HeLa cells. Coconut water is widely used in plant tissue culture as a growth-promoting component in the formulation of media (11). When cytokinins are added to the culture medium, the normal sequence of aging slows down significantly.

The morphological observations in this study were made to identify the mechanism of cell death that resulted from exposure to both FDCW varieties. Exposure to MATAG FDCW resulted in HeLa cell shrinkage, membrane blebbing, narrowing of lamellipodia, and rounding of cells (Fig.3a), and chromatin condensation and nuclear fragmentation also were observed in HeLa cells stained with Hoechst dye and viewed under a fluorescence microscope (Fig.4a). These effects suggest that MATAG FDCW is capable of inducing Type 1 cell death (i.e., apoptosis) in HeLa cells. The condition of the cell culture showed that no more than 50% of cells were rounded (apoptotic bodies), no extensive cell lysis was observed, and 45% of HeLa cell growth was inhibited (Fig.3b). On the other hand, morphological alterations of HeLa cells treated with AD FDCW included rounding of cells (apoptotic bodies), narrowing of lamellipodia, cell shrinkage, membrane blebbing, and vacuolization. Fluorescence microscopy revealed chromatin condensation and nuclear fragmentation (Fig.4b). These features were similar to those seen in cells treated with MATAG FDCW and suggest that cell death was induced via an apoptosis mechanism. However, vacuolization, which is a characteristic of autophagy, was not seen in HeLa cells treated with MATAG FDCW (19). Based on this observation, we suggest that AD FDCW triggered cell death via apoptosis (Type I) and autophagy (Type 2) modes. Based on ISO 10993-5: 2009 - Biological evaluation of medical devices -Part5: Tests for the in vitro cytotoxicity (20), AD FDCW also exhibits mild reactivity, as 49% of HeLa cells were inhibited therefore it falls in Grade 2. Regarding the proposed dual induction of autophagy and apoptosis in AD FDCW treated Hela cells, Gao et al. claimed that autophagy and apoptosis may be triggered by common upstream signals, thus resulting in combined mechanisms (2). Moreover, cells are able to switch between the two responses in a mutually exclusive manner.

(a) Untreated control HeLa cells after 72 hours of incubation.



100x magnification 200x magnification

(b) HeLa cells treated with 100 µg/ml MATAG FDCW after 72 hours of incubation.

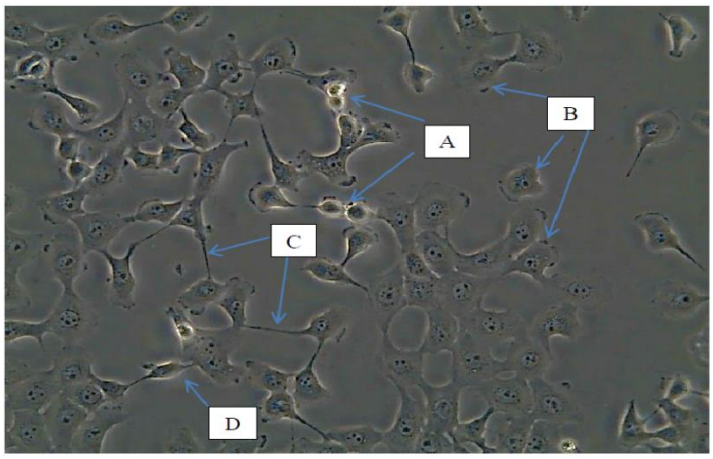
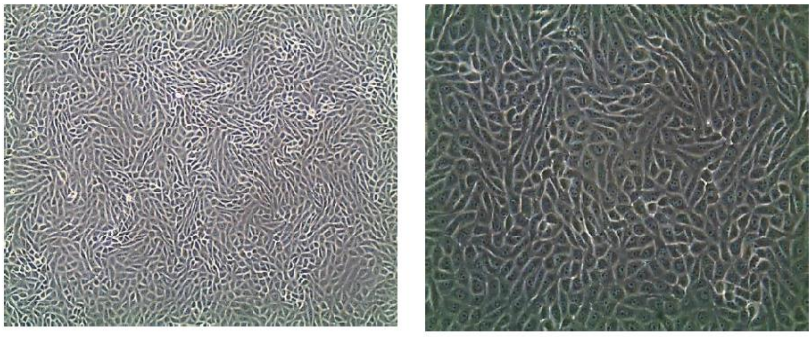


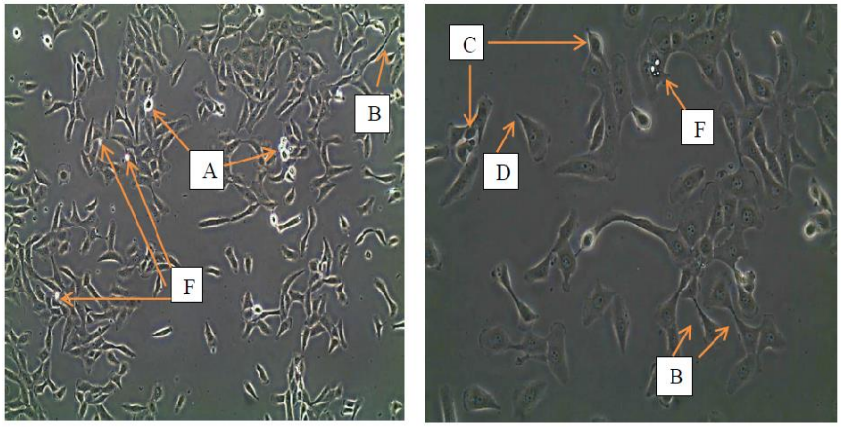
Figure 3a: Photographs taken under an inverted microscope (200x). (a) Control untreated HeLa cells. (b) Morphological changes of treated HeLa cells. A: apoptotic bodies or rounding of cells; B: membrane blebbing; C: narrowing of lamellipodia; D: cell shrinkage

(a) Untreated control HeLa cells after 72 hours of incubation.



100x magnification 200x magnification

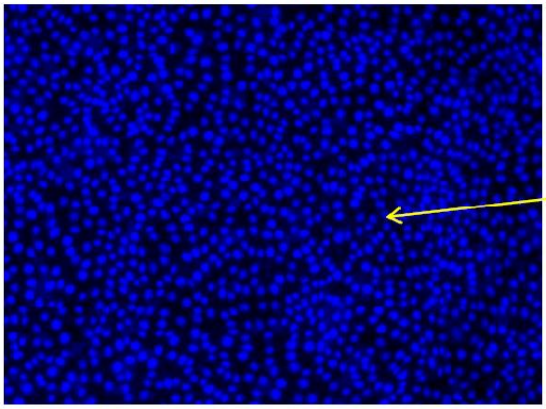
(b) HeLa cells treated with 100 µg/ml AD FDCW after 72 hours of incubation.



100x magnification 200x magnification

Figure 3b: Photographs taken under an inverted microscope (100x and 200x). (a) Control untreated HeLa cells. (b) Morphological changes of treated HeLa cells. A: apoptotic bodies or rounding of cells; B: narrowing of lamellipodia; C: cell shrinkage; D: membrane blebbing; F: vacuolization

(a) Fluorescent stained of untreated HeLa cells after 72 hours of incubation periods.



100x magnification.

(b) Fluorescent stained of HeLa cells treated with 100 µg/ml MATAG FDCW after 72 hours of incubation period.

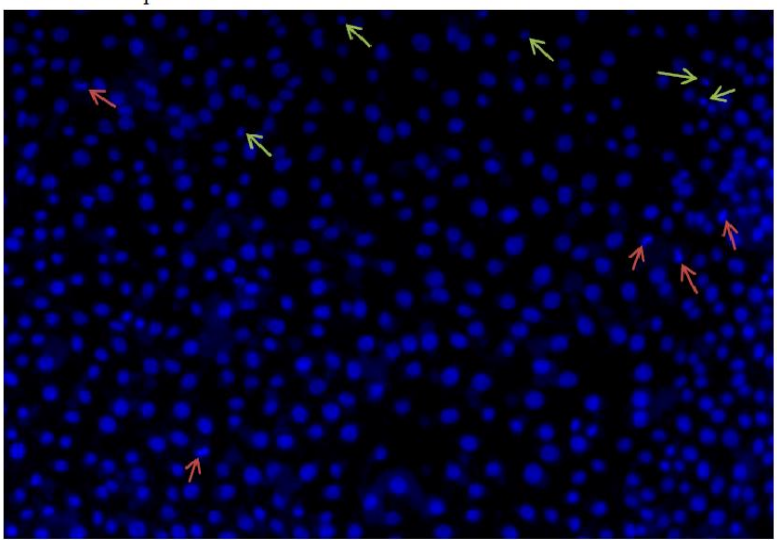
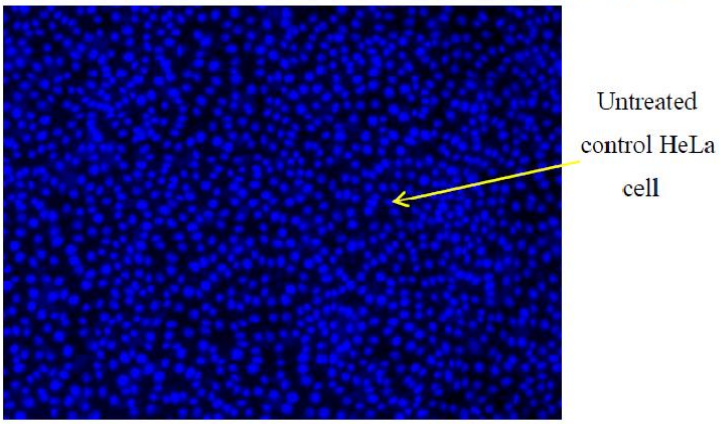


Figure 4a: Chromatin staining using Hoechst 33342 dye as seen under a fluorescent inverted microscope (100x magnification). (a) Control untreated HeLa cells. (b) Stained chromatin of MATAG FDCW treated HeLa cells. Red arrows show nuclear fragmentation, and green arrows show chromatin condensation

(c) Fluorescent stained of untreated HeLa cells after 72 hours of incubation periods.



100x magnification.

(a) Fluorescent stained of HeLa cells treated with 100 µg/ml AD FDCW after 72 hours of incubation period.

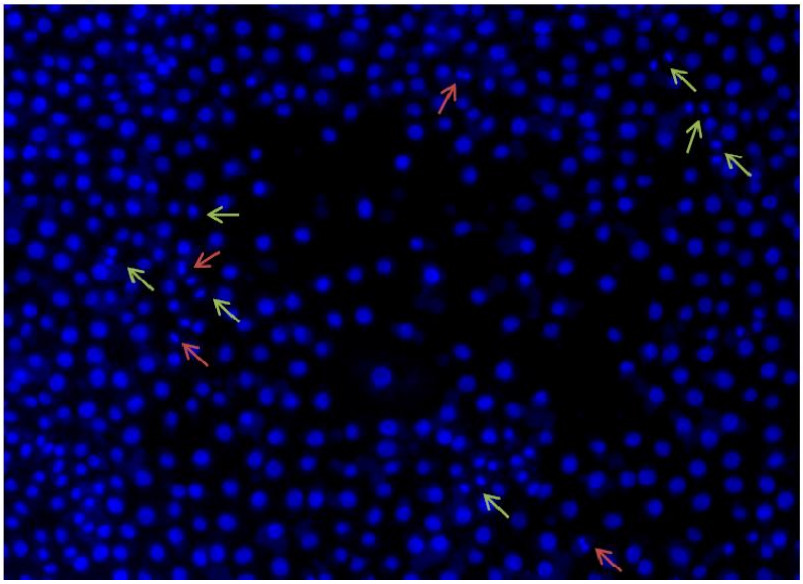


Figure 4b: Chromatin staining using Hoechst 33342 dye as seen under a fluorescent inverted microscope (100x magnification). (a) Control untreated HeLa cells. (b) Stained chromatin of AD FDCW treated HeLa cells. Red arrows show nuclear fragmentation, and green arrows show chromatin condensation

Based on this preliminary study, screening for chemopreventive activity of both FDCW varieties showed positive outcomes. However, MATAG FDCW exhibited better chemopreventive activity compared to AD FDCW. MATAG FDCW showed a better suppression pattern with a higher anti-proliferative effect, and it was able to maintain suppression on HeLa cell proliferation with prolonged time of exposure.

Conclusion

In summary, MATAG FDCW and AD FDCW exhibited chemopreventive activity against cervical cancer cells, as indicated by results of morphological changes after treatment and anti-proliferative effects. Further analysis with more advanced detection techniques, including cell-cycle analysis and apoptotic assays, should be conducted to confirm these results.

Conflict of interest statement

Authors declare that there is no conflict of interest.

Acknowledgements

Sincere thanks to Universiti Sains Malaysia in providing fund via the Research University Grant (RUI:USM/812120).

References

1. Rang HP, Dale M, Ritter JM, Flower RJ. Rang and Dale's Pharmacology. 6th Edinburgh: Churchill Livingstone. 2007; 718-735.2.
2. Siegel RL, Miller KD, Fuchs HE and Jemal A. Cancer statistics, 2021. Ca Cancer J Clin. 2021; 71(1), pp.7-33.3.
3. Ferlay J, Colombet M, Soerjomataram I, Parkin DM, Piñeros M, Znaor A, *et al.* Cancer statistics for the year 2020: An overview. Int J Cancer. 2021; 149:778–89.4.
4. Teoh DGK, Marriott AE, Isaksson Vogel R, Marriott RT, Lais CW, Downs Jr LS, *et al.* Adherence to the 2012 national cervical cancer screening guidelines: a pilot study. Am J Obstet Gynecol. 2015;212(1):62.e1- 62.e9.5.
5. El Omari N, Bakha M, Imtara H, Guaouguaoua FE, Balahbib A, Zengin G, *et al*. Anticancer mechanisms of phytochemical compounds: focusing on epigenetic targets. Environmental Science and Pollution Research, 2021; 28(35), pp.47869-47903.6.
6. Shukla S, Meeran SM, Katiyar SK. Epigenetic regulation by selected dietary phytochemicals in cancer chemoprevention. Cancer Lett. 2014;355(1):9-17.7.
7. Fife B. Coconut Water: Dew from the Heavens. Coconut Water for Health and Healing. Colorado, United State: Piccadilly Books. 20088.
8. Prades A, Dornier M, Diop N, Pain JP. Coconut water uses, composition and properties: a review. Fruits. 2012;67(2):87-107.9.
9. Man N and Shah, J. A. (2020). Acceptance of New Coconut Seed Matag Among Coconut Growers in Bagan Datoh, Perak And Bachok, Kelantan. *International Journal of Academic Research in Business and Social Sciences*, *10*(16), 1–26.10.
10. Savalas, L.R.T., Sirodjudin, S. and Gunawan, E.R., 2021. Biochemical Properties of Coconut (Cocos nucifera L.) Lipase. Philippine Journal of Science, 150(5), pp.915-924.11.
11. Rethinam, P. and Krishnakumar, V., 2022. Health Benefits of Coconut Water. In Coconut Water (pp. 385-455). Springer, Cham.12.
12. Priya SR, Ramaswamy L. Tender Coconut Water-Natures Elixir to Mankind. Int J Recent Sci Res. 2014;5(8):1485-1490.13.
13. Wang F. Culture of animal cells: a manual of basic technique. In Vitro Cell Dev Biol. 2006;42(5):169-169. 14.
14. Louis KS, Siegel AC. Cell Viability Analysis Using Trypan Blue: Manual and Automated Methods. In: Stoddart M, ed. Mammalian Cell Viability: Methods in Molecular Biology. New York: Humana Press. 2011:1-6.15.
15. Sini K, Haribabu Y, Sajith M, Surya Sreekumar K. In-vitro cytotoxic activity of Orthosiphon thymiflorus Roth. Sleensen leaf extract against Dalton lymphoma ascites cell line. J Chem Pharm Res. 2012;4(1):917-921.16.
16. Xiao JX, Huang GQ, Zhu CP, Ren DD, Zhang, SH. Morphological study on apoptosis Hela cells induced by soyasaponins. Toxicol In Vitro. 2007;21(5):820-826.17.
17. Astashkina A, Mann B, Grainger DW. A critical evaluation of in vitro cell culture models for high-throughput drug screening and toxicity. Pharmacol Ther. 2012;134(1):82-106.18.
18. Prabhu S, Dennison SR, Mura M, Lea RW, Snape TJ, Harris F. Cn- AMP2 from green coconut water is an anionic anticancer peptide. J Pept Sci. 2014;20(12):909-915. 19.
19. Doležal K, Popa I, Kryštof V, Spíchal L, Fojtíková M, Holub J, *et al.* Preparation and biological activity of 6-benzylaminopurine derivatives in plants and human cancer cells. Bioorg Med Chem. 2006;14(3):875-884.20.
20. Yan, G.E., Elbadawi, M. and Efferth, T., 2020. Multiple cell death modalities and their key features. World Academy of Sciences Journal, 2(2), pp.39-48.21
21. ISO 10993-5:2009 Biological evaluation of medical devices -Part5: Tests for the in vitro cytotoxicity
22. Gao, L., Loveless, J., Shay, C. and Teng, Y., 2020. Targeting ROS-mediated crosstalk between autophagy and apoptosis in cancer. Reviews on New Drug Targets in Age-Related Disorders, pp.1-12.