Short communication: *Canarium odontophyllum* Miq. (dabai) stem bark arrested HCT 116 cell line at G₀/G₁ checkpoint

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Abstract. *Canarium odontophyllum* Miq. is an exotic plant which is native in Borneo and belong to the Burseraceae family. It contains phytochemicals such as saponin, terpenoid, flavonoid and phenolic compound with potential anticancer property. It has been found that the extract of this plant negatively affected colorectal cancer cells by stimulating apoptosis. To elucidate the apoptosis mechanism induced by the plant extract, this study evaluated the effect of *C. odontophyllum* stem bark acetone extract on cell cycle distribution of HCT 116 cell line using propidium iodide assay. For this purpose, IC₅₀ of the acetone extract of *C. odontophyllum* was first determined by treating HCT 116 cells with the extract for 24, 48 and 72 hours. It was found that the acetone extract of *C. odontophyllum* inhibited proliferation of HCT 116 at IC₅₀ value of 55.09 ± 18.29 µg/mL for 24 hours treatment, 37.81 ± 5.09 µg/mL for 48 hours treatment, and 114.9 ± 16.08 µg/mL following 72 hours treatment. Using IC₅₀ value of 48 hours treatment, it was observed that *C. odontophyllum* acetone extract arrested the HCT 116 cells at G₀/G₁ checkpoint. Based on this result, it can be concluded that one of the apoptosis mechanisms induced by *C. odontophyllum* is by arresting cell cycle of HCT 116 cells at G₀/G₁ checkpoint. This finding warrants further investigation on how *C. odontophyllum* causes the cell cycle arrest and its potential to become anticancer agent.

Keywords: apoptosis, *Canarium odontophyllum*, cell cycle, colorectal cancer, HCT 116

INTRODUCTION

Colorectal cancer is among the second common cancer worldwide (World health organization, 2018). It is reported as the second common cancer in males and the third common cancer in females in Malaysia (Veettil *et al.*, 2017). Colorectal cancer is commonly treated with surgery for primary disease and chemotherapy for widespread disease (Haraldsdottir *et al.*, 2014). However,

chemotherapy is often associated with increased toxicities, adverse effects as well as resistance in cancer cells rendering them unsusceptible to chemotherapy. These prompted scientists to conduct studies to find other compounds for alternative source of anticancer agents (Wong *et al.*, 2019).

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Canarium odontophyllum Miq. is an exotic plant of the genus *Canarium*, family Burseraceae, order Sapindales, class Eudicotyledonae. It is also known as “dabai” and can be found in Sarawak and Sabah, Malaysia and Brunei (Ding, 2011). A previous study found that acetone extract of *C. odontophyllum* stem bark is cytotoxic only towards HCT 116 without exerting cytotoxic effects towards human normal colon cells CCD-18CO (Basri *et al.*, 2016). The extract also induced cell death via apoptosis, indicating that *C. odontophyllum* can be further studied as an anticancer agent against colorectal cancer (Basri *et al.*, 2017).

Cell cycle is a process in which an active living cell divides mitotically before producing two identical daughter cells. In the interphase of cell cycle, cells undergo checkpoints at G₀/G₁ or G₂/M to ensure the integrity of DNA and that the nutrients are sufficient to go through the next phase in cell cycle until mitosis occurs. Anticancer agent targets cancer cell proliferation by arresting cancer cells at G₀/G₁ or G₂/M phase, and arrest at either one of these two phases leads to apoptosis in cancer cells. Basri and Subramaniam (2018) observed that the acetone extract of stem bark of *C. odontophyllum* causes DNA damage in HCT 116 cells, which can induce apoptosis. Hence, this study aims to evaluate the effect of the extract on the cell cycle distribution in HCT 116 cells.

MATERIALS AND METHODS

The stem bark of *C. odontophyllum* was collected from Sarawak and stored in Universiti Kebangsaan Malaysia Herbarium (voucher number: UKMB 40052). Dried acetone extract of stem bark *C. odontophyllum* was prepared according to Basri and Fan (2005). Stock solution is prepared by suspending dried stem bark acetone extract in 100% DMSO and mixed using a vortex. Following this, the solution was centrifuged for 5 mins at 20,000 rpm and filtered through a 0.22 µM nitrocellulose membrane filter. The stock solution was refrigerated at -20°C prior to treatment with colorectal carcinoma cells. HCT 116 human colorectal carcinoma cell (ATCC® CCL-247TM, Rockville, MD USA) was used in this study and

cultured in McCoy's 5A media (Sigma Aldrich, USA) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under 5% CO₂. The cells were cultured to reach 70-80% confluency approximately 2-3 days before subculture.

To determine the cytotoxicity of *C. odontophyllum* extract towards HCT 116 cells, [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MTT) assay (Mosmann, 1983) was carried out were. The cells were seeded in a 96-well plate prior treatment followed by addition with the extract (12.5 - 200 µg/mL) and menadione as positive control (0.781- 12.50 µM) for 24, 48 and 72 hours, separately. Later, 20 µL of MTT (5 mg/mL) solution was added to each well and incubated for 4 hours. Subsequently, 200 µL of DMSO was added to dissolve the formazon crystals formed, incubated for another 15 min and shaken on a mixer for 5 min. The absorbance was read at 570 nm using a microplate reader (Bio-Rad, USA). The viability of cells was calculated using the following formula and half-maximal inhibition concentration (IC₅₀) of the extract was extrapolated from graph, cell viability versus extract concentration:

$$\text{Percentage of viability (\%)} = \frac{\text{Mean Absorbance of Treated Cell}}{\text{Mean Absorbance of Untreated Cell}} \times 100$$

Following this, HCT 116 cells with different densities such as 1.5x10⁵ cells, 1x10⁶ cells, and 5x10⁶ cells per well were cultured in 6-well plates to determine the optimum cell seeding density for cell cycle analysis (Riccardi and Nicoletti, 2006; Jaramillo *et al.*, 2016). Each well was added with complete growth medium to achieve a final volume of 5 mL and incubated overnight to promote cell attachment. The samples were harvested, fixed in 70% cold ethanol overnight, and were thoroughly washed with cold PBS (phosphate-buffered saline) to remove ethanol. Staining of samples was carried out using propidium iodide (PI) method by adding PI staining solution (1 mL) to each sample (Riccardi and Nicoletti, 2006). The samples were transferred to a 5 mL flow cytometer falcon tube and left to stain in the dark for 1 hour before reading using BD FACS CANTO II. The protocol was adapted and modified from Moores Cancer Center, UC San Diego Health Sciences (Moores Cancer Center, 2019).

Based on the optimum experiment above, HCT 116 cells at a density of 5×10^6 cells/well were selected and cultured in a 6-well plate for cell cycle analysis. Each well was added with complete growth medium (McCoy's 5A media with 10% FBS) to obtain a final volume of 5 mL and was incubated 24 hours to allow cell attachment. At 24 hours, the exhausted growth medium was changed with a medium containing acetone extract at IC_{50} value. Positive control of the experiment was carried out by treating the cells with menadione and for negative control, cells were left untreated. Following this, samples were harvested and fixed in 70% cold ethanol overnight. Samples were thoroughly washed with cold PBS to remove ethanol. After that, staining of samples was carried out by adding PI staining solution to every sample. The samples were left in the dark for an hour to stain after transferring them to a 5 mL flow cytometer falcon tube before reading using BD FACS CANTO II.

All data obtained were analyzed using GraphPad Prism 6.0 software. Data were processed through one way-ANOVA and two way-ANOVA to compare for significance. All data were carried out in triplicate and shown as mean $\pm$ SEM at $p < 0.05$.

RESULTS AND DISCUSSION

The cytotoxicity results showed that *C. odontophyllum* acetone extract inhibited HCT 116 with the lowest viability at 48 hours compared to the 24- and 72-hours treatment with the extract (Figure 1). This indicated that the extract requires 48 hours to express its cytotoxicity. However, higher HCT 116 cell viability is recorded after 72 hours treatment compared to 48 hours. This might be due to the instability of certain phytochemicals in the extract. This finding may be explained by Zheng *et al.* (2016) that reported prolonged exposure of cancer cells at low doses did not inhibit cancer cells' proliferation. In line with this result, plant extract with 48 hours treatment recorded the lowest IC_{50} value ($37.81 \pm 5.09 \mu\text{g/mL}$) while a higher IC_{50} value of $55.09 \pm 18.29 \mu\text{g/mL}$ and $114.9 \pm 16.08 \mu\text{g/mL}$ after 24 and 72 hours treatments, respectively (Table 1). This indicated that the extract exhibited its

maximum cytotoxic effect at such time point (Efferth *et al.*, 2007). However, the IC_{50} value obtained in this study is slightly higher than the value obtained by Basri *et al.* in 2016 ($25 \pm 5.20 \mu\text{g/mL}$) under similar conditions. The difference in IC_{50} value may be due to storage conditions and period of time that could affect the plant extract's phytochemical content as the stem bark has been stored for more than six months. A study conducted by Dinçer *et al.* (2013) found the flavonoid compounds and phenolic acids in *Salvia tomentosa* had decreased after six months of storage. Therefore, a possible decrease in the content of phytochemicals of *C. odontophyllum* stem bark might result in the increase of IC_{50} value in this study as compared to the IC_{50} value obtained in Basri *et al.* (2016). While, IC_{50} of positive control, menadione was $1.6290 \pm 0.99 \mu\text{M}$, $0.3479 \pm 0.18 \mu\text{M}$ and $0.4413 \pm 0.21 \mu\text{M}$ after 24 hours, 48 hours and 72 hours treatments, respectively (Table 2, Figure 2).

Optimization of cell seeding density was performed to obtain the optimum number of seeding cells for cell cycle analysis experiment. Seeding densities were selected based on previous studies, i.e., 1.5×10^5 , 1×10^6 , and 5×10^6 cells/well (Riccardi and Nicoletti, 2006; Basri and Subramaniam, 2018). All plates were set to 24 hours to prevent prolonged treatment, causing apoptosis, resulting in the generation of apoptosis peak or "sub-G1" that will influence the percentage of cell cycle distribution. The software ModFit LT 3.2 was used to observe the narrowing of G_0/G_1 and G_2/M as the cell seeding density increases. The seeding density with the highest CV value is $5.340 \pm 0.03\%$ (1.5×10^5 cells/well), followed by $3.82 \pm 0.47\%$ (1×10^6 cells/well) and $3.147 \pm 0.12\%$ (5×10^6 cells/well) (Table 3). There was a significant difference in CV of 1×10^6 cells/well compared to CV of 1.5×10^5 cells/well (Figure 3). The CV obtained decreased with an increase in cell seeding density, which indicated the analysis's high accuracy. It was also observed that the histogram background at 1×10^6 cells and 5×10^6 cells is less than at 1.5×10^5 cells. This indicated a reduction in debris, proteins, or cell fragments, and DNA fragments decreased as the CV increases. A lower CV of 3% was suggested for cell cycle analysis because a lower CV value yields a narrower G_1 peak, increasing the accuracy of the cell cycle distribution measured

(Darzynkiewicz *et al.*, 2017). In this study, the seeding density that is the nearest to the CV of 3% is 5×10^6 cells/well (CV of $3.147 \pm 0.12\%$). This

suggests a seeding density of 5×10^6 cells/well is the most suitable for cell cycle analysis.

Table 1. IC₅₀ value for extract treatment at three different time points.

IC ₅₀ (µg/mL)	Treatment time using acetone extract of <i>C. odontophyllum</i> stem bark (hour)		
	24	48	72
	55.09 ± 18.29	37.81 ± 5.09	114.9 ± 16.08

All data shown are in the form of mean $\pm$ SEM. IC₅₀: Half-maximal inhibitory concentration.

Figure 1. Cytotoxic activity of acetone extract of *C. odontophyllum* stem bark towards HCT 116 at concentration ranging from 0 – 200 µg/mL after 24, 48, and 72 hours of treatment. All data shown are in the form of mean $\pm$ SEM at significant level $p < 0.05$. ‘*’ indicates significant difference as compared to untreated cell.

Table 2. IC₅₀ value for menadione treatment at three different time points.

IC ₅₀ (µM)	Treatment time for Menadione (hour)		
	24	48	72
	1.6290 ± 0.99	0.3479 ± 0.18	0.4413 ± 0.10

All data shown are in the form of mean $\pm$ SEM. IC₅₀: Half-maximal inhibitory concentration.

Figure 2. Cytotoxic activity of menadione against HCT 116 at concentration ranging from 0 – 12.5 μM after 24, 48, and 72 hours of treatment. All data shown are in the form of mean $\pm$ SEM at significant level $p < 0.05$. “*” indicates significant difference as compared to untreated cell.

Table 3. Cell seeding density for each well with mean percentage of *Coefficient of Variance*.

Cell Seeding Density (cells/well)	Percentage of Mean of <i>Coefficient of Variance</i> , CV (%)	Standard Error Mean, SEM (%)	Total events achievable (evts)	N
1.5×10^5 cells	5.340 ± 0.03	0.03	10 000	3
1×10^6 cells	3.823 ± 0.47	0.47	20 000	3
5×10^6 cells	3.147 ± 0.12	0.117	20 000	3

All data shown are in the form of mean $\pm$ SEM.

For a more accurate cell cycle analysis, 20,000 events count (evts) was suggested in the cell cycle study by Shen *et al.* (2017). From all the three densities used in this study, only seeding densities of 1×10^6 and 5×10^6 cells/well were able to achieve 20,000 evts, whereas 1.5×10^5 cells/well could collect up to 10,000 evts (Table 3). The different total number of evts observed in this study is because cell seeding density affects cell growth. Ghosh *et al.* (2010) demonstrated a higher cell seeding density increases protein expression and elevates adipose stromal cells' proliferation by the increase in contact point between cells. As HCT 116 cells are adherent cells, high seeding density ensures close contact point with surrounding cells,

enhancing cell proliferation. Less cell seeding density than optimum will reduce close contact that eventually affects cells' growth and influence the number of events achievable for cell cycle analysis. Cell seeding density lower than optimum also influences the kinetics of cancer cells as the efficacy of anticancer agents is controlled by the rate of cell proliferation, enzymatic activity, and the microenvironment (Kelmer and Wilson, 2007; Vadlapatla *et al.*, 2013; Gu and Mooney, 2015). The affected kinetics of cancer cells will influence the cytotoxicity, efficacy, and outcome of cell cycle arrest when treated with the anticancer agent of interest. Based on both the CV value and the evts recorded by each seeding density, 5×10^6

cells/well was used in the cell cycle analysis experiment.

In the cell cycle analysis, the percentage of cell distribution in cell cycle phases depicted a significant increase ($p < 0.05$) of HCT 116 cells at G₀/G₁ phase ($75.99 \pm 0.63\%$) compared to negative control ($65.92 \pm 2.63\%$) (Table 4, Figure 4) after the cells were subjected to the *C. odontophyllum* acetone extract (IC₅₀ at 48 hours, $37.81 \pm 5.09 \mu\text{g/mL}$) indicating there was an arrest at cell cycle checkpoint G₀/G₁. Previously, cell cycle arrest at checkpoint G₀/G₁ has been shown to stimulate apoptosis (Chen *et al.*, 2010; Zorofchian *et al.*, 2014). It is possible that the cell cycle arrest at G₀/G₁ induced by the acetone extract of *C. odontophyllum* also leads to apoptosis as previously demonstrated by Basri *et al.* (2016) in which the extract caused apoptosis in HCT cells. It is noted that there was an increase observed in the G₀/G₁ phase of menadione-treated HCT 116 cells ($73.19 \pm 12.71\%$) in comparison to untreated cells. This showed that cell cycle of menadione-treated HCT 116 cells was arrested at G₀/G₁ checkpoint although the effect of menadione is somewhat weak as the increase was not significant. In spite of this, the ability of menadione to arrest cancer cells is reported by Lee *et al.* (2016) which demonstrated that menadione induced apoptosis in prostate cancer and caused G₀/G₁ checkpoint arrest. Various studies also stated that menadione was cytotoxic and induced G₁ arrest towards cancer cells (Hitomi *et al.*, 2005; Klaus *et al.*, 2010).

The ability of the *C. odontophyllum* stem bark acetone extract to arrest the cell cycle at G₀/G₁ in this study exhibits the potential of this extract to

regulate the modulators in cell cycle, such as the protein cyclin-dependent kinases (CDKs). CDK primarily reduces the level of protein cyclins such as cyclin D-1 that arrest cells at G₀/G₁ phase (Klaus *et al.*, 2010). The importance of acetone extract of *C. odontophyllum* in arresting HCT 116 at G₀/G₁ marked a point of interest for further study of molecular mechanism of cell cycle affected by the extract. Whether *C. odontophyllum* extract affects modulators such as cyclin D and E as well as CDK2, CDK4, and CDK6 that are involved in promoting DNA replication phase and cell division through regulation of G₀/G₁ phase is not known and warrants further investigation (Song *et al.*, 2017).

Figure 3. Graph comparison of Coefficient of Variance between different cell seeding densities. All data shown are in the form of mean $\pm$ SEM at significant level $p < 0.05$. ‘*’ indicates significant difference as compared to control 1.5×10^5 cells ($p < 0.05$).

Table 4. Percentage of cell cycle distribution for three different treatment groups.

Cell cycle phase	Mean of percentage of cell in phases (%)		
	Control (Untreated cell)	IC ₅₀ of acetone extract of <i>C.</i> <i>odontophyllum</i> stem bark treated	IC ₅₀ Menadione
G ₀ /G ₁	65.92 ± 2.63	75.99 ± 0.63	73.19 ± 12.71
S	28.25 ± 0.84	18.51 ± 0.48	20.76 ± 9.75
G ₂ /M	5.84 ± 2.12	5.50 ± 0.28	6.04 ± 2.96

All data shown are in the form of mean $\pm$ SEM. IC₅₀: Half-maximal inhibitory concentration.

Figure 4. Comparison between percentages of cell distribution at different cell seeding densities in negative control (untreated cells) and cells treated with IC₅₀ of extract ($37.81 \pm 5.09 \mu\text{g/mL}$ when treated for 48 hours) and IC₅₀ of menadione ($0.3479 \pm 0.18 \mu\text{M}$ when treated for 48 hours). All data shown are in the form of mean $\pm$ SEM at significant value $p < 0.05$. ‘*’ indicates significant difference as compared to untreated cell.

CONCLUSION

C. odontophyllum Miq. stem bark acetone extract was cytotoxic towards HCT 116 cells at IC₅₀ value of $37.81 \pm 5.09 \mu\text{g/mL}$ when treated for 48 hours. At cell seeding density of 5×10^6 cells/well and treatment with the IC₅₀ of *C. odontophyllum* acetone extract for 24 hours, cell cycle of HCT 116 cells were arrested at G₀/G₁ checkpoint. Hence, *C. odontophyllum* stem bark can be further explored as an anticancer agent against colorectal cancer.

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CONFLICT OF INTERESTS

The authors have declared that no conflict of interest exists.

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