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CANARIUM ODONTOPHYLLUM STEM BARK EXTRACT EXERT CYTOTOXIC EFFECT IN HCT 116 CELL LINE VIA ATP LOSS AND ROS GENERATION

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ABSTRACT

Background: *Canarium odontophyllum*, had the potential to cause cytotoxicity towards colorectal cancer cell line by inducing cell death via apoptosis. The aim of this study was to further determine the underlying mechanism of acetone extract from the stem bark of *C. odontophyllum* against colorectal cancer cell line HCT 116. **Methods:** The IC_{50} value of acetone extract was determined at concentration range of 12.5 μg/mL to 200 μg/mL, 48 hr upon treatment. The level of Adenosine Triphosphate (ATP) and Reactive Oxygen Species (ROS) was measured using CellTiter-Glo Luminescent Cell Viability assay and dihydroethidium staining assay detected by flow cytometry respectively, upon treatment of the cell line at IC_{50} concentration of the extract at timepoints 30 mins, 60 mins and 120 mins, respectively. **Results:** The acetone extract of *C. odontophyllum* stem bark treatment showed cytotoxic effect against the HCT 116 with IC_{50} value of 84 ± 9μg/mL with significant reduction ($p<0.05$) of ATP level from 139861.67 ± 63650.95 indicated by relative luminescence unit (RLU) at 30 mins of treatment to 29875.5 ± 7284.39 (RLU) after 4 hr of treatment. ROS level showed significant increased in the cell lines at 2 hr of treatment compared to the negative control with the value of 42.75 ± 5.30% and 22.35 ± 5.303301%, respectively. **Conclusion:** In conclusion, acetone extract of *C. odontophyllum* stem bark exerted its cytotoxic effect by a decreased in the intracellular ATP level and increased in the level of ROS.

KEYWORDS: *Canarium odontophyllum*, Colorectal cancer, Cytotoxicity, ROS, ATP.

1. INTRODUCTION

Cancer is uncontrolled growth of abnormal cell that spread to other tissues and lead to death [1] and one example is colorectal cancer associated with malignant neoplasm of the lining gastrointestinal system of large intestine, at the colon and the rectum.[2] According to,[3] the estimated incidence and mortality rates worldwide for colorectal cancer was approximately 746 000, which was ranked as the third most common in men while for women, the estimated number of incidence and mortality was approximately 614,000 and ranked as second most common cancer in women after breast cancer. As for Malaysia, it was ranked as the most common cancer in men and as the second most common cancer in women.[4]

Several treatment modalities for colorectal cancer are available including surgery, chemotherapy, radiotherapy, hormone and immune therapy, targeted therapy and combinatorial therapy.[1] Nevertheless, the treatment comes with adverse effects includes serious bleeding and damage to nearby organ, anemia and fatigue, skin irritation and hair loss. Due to the pitfalls of the treatment modalities available, products from natural sources has extensively been explored for their medicinal potential since they have been used traditionally for the treatment of various diseases.[5]

Canarium odontophyllum, locally known as 'Dabai' or 'Borneo olive' is one of the potential natural source to be developed as an anticancer agent. Taxonomically, it belongs to Burseraceae family and can be found particularly in Sarawak, along river bank of Kanowit, Sarikei and Kapit division.[6] This plant has large pinnate leaves, yellowish brown and cylindrical stem bark, and oval dark purple fruits.

Some studies on different parts of the plant have revealed that the plant has promising medicinal potential. For instance, the leaves of *C. odontophyllum* has antimicrobial and anticancer properties.[7,8] Recently, a study on the stem bark extract of *C. odontophyllum* on human colorectal cancer HCT 116 has shown that it may cause primary cell death via apoptosis with significant DNA damage cytotoxic effect on the colorectal cancer cell line.[9] Subsequently, it was reported that an increase level of superoxide anion and loss of mitochondrial

membrane potential (MMP) confirmed the function of mitochondria by *C. odontophyllum* stem bark acetone extract in inducing apoptotic mechanism in HCT 116 cell lines.[10] The present study was therefore, objectively done to investigate the involvement of adenosine triphosphate (ATP) in exerting the cytotoxic effect of the extract in human colorectal cancer HCT 116 cell line.

The availability and the improvement of the treatment contributes to the increased in the 5-year survival rate from 49% in 1975-1977 to 69% in 2005-2011. Apart from that, further rise in the survival rates and highly effective phytoalternative agent with less side effects have become the main objectives for scientists to develop alternative agent with minimal adverse effect from natural sources. Therefore, this study was performed to characterize further the mechanism of *C. odontophyllum* stem bark extract which have been proven to have cytotoxic action against HCT 116.

2. MATERIALS AND METHODS

2.1. Plant material

Canarium odontophyllum stem bark was originated from Kuching, Sarawak and being placed in the UKM plant archive with a voucher number of UKMB 40052.

2.2. Chemical and reagent

All chemicals were purchased from Sigma (USA) unless stated otherwise.

2.3. Stock preparation of stem bark acetone extract

A final concentration of 100 mg/mL stock solution of acetone extract from stem bark of *C. odontophyllum* was prepared by dissolving 100 mg of crude stem bark acetone extract in 100% DMSO. The stock solution was mixed using a vortex for 5 to 10 mins before being centrifuged for 5 mins at 20,000g. The stock solution (supernatant) yielded after the centrifuged was then filtered using 0.22 μM nitrocellulose membrane filter. The stock solution was stored at -20℃ until further used.

2.4. Preparation of cell culture

Continuous cell line HCT 116 (ATCC® CCL247™) was obtained from American Type Culture Collection (ATCC), Rockville, MD USA. The cell was cultured in complete growth media McCoy's 5A media (Sigma Aldrich, USA) + 10% (v/v) Fetal Bovine Serum (FBS) at Biocompatibility and Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia, Kuala Lumpur. The cells were cultured until 70-80% confluency in 2 to 3 days and were subjected for subculture.

2.5. Cell plating and treatment *in vitro*

HCT 116 cells were seeded at concentration of 5 x 10^4 cells/mL and were incubated for 24 hr to allow for cell attachment. The cells were treated with acetone extract of *C. odontophyllum* in concentration-dependent and time-dependent manner following the experimental design of each experiment in the study.

2.6. Evaluation of cytotoxic activity

The cytotoxic activity of the extract was determined using [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], MTT assay.[11] The extract with concentration ranging from 12.5 µg/ml - 200 µg/ml was used to treat HCT 116 cells for 48 hr. The assay was performed in a 96-well plate and the seeded cells were incubated for 24 hr before being exposed to acetone extract treatment. Menadione with concentration ranging from 3.125 μM – 50 μM was used as a positive control, while untreated cells were used as negative control. After 48 hr treatment, 20 µL of MTT (5 mg/ml) solution was added to each well and incubated for 4 hr. Following 4 hr incubation, DMSO solution was added to each well to dissolve the formazan crystals and then further incubated for 15 min. The plate was shaken on an automatic mixer for 5 min and the absorbance was read using an ELISA plate reader (Bio-Rad, USA) at 570 nm. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean absorbance reading of treatment group}}{\text{Mean absorbance reading of negative control}} \times 100\ \%$$

The half maximal inhibition concentration (IC_{50}) of acetone extract was selected for further test.

2.7. Assessment on ATP assay

The method was carried out as described by [12] using CellTiter-GLO® Luminescent cell viability assay kit. The cells were seeded in opaque 96-well plates and were incubated for 24 hr to allow for cell attachment. Following 24 hr, the cells were treated with IC_{50} value of the extract for 30 mins, 60 mins, 120 mins and 240 mins. Menadione with concentration of 50 μM was used as positive control while the untreated cells were used as negative control. After the treatment, 100 μL of CellTiter-Glo Assay reagent was added into each well and was shaken using orbital shaker for 2 mins. Incubation was performed for 10 mins prior to determination of ATP level through the luminescent signals produced by using Luminometer.

2.8. Assessment of superoxides anion determination assay

The method was carried out as described by [12] with some modification. Cells suspension (3 ml) was seeded in 6-well plates and was subjected to 24 hr incubation to allow for cell attachment. Menadione at 50 µM at 120 mins incubation was used as positive control as verification of the results, while the untreated cell was used as negative control. The cells were treated with IC_{50} value of the extract for 30 mins, 60 mins and 120 mins. Following the treatment, all treated cells were harvested

and suspended with 1 mL serum-free media and was stained with 1 μL of 10 mM Dihydroethidium (DHE) (Molecular Probes, Invitrogen). The cells were incubated in the dark for 20-30 mins at 37℃, followed by centrifugation at 20,000g for 5 mins at 4℃. The stained cells were washed with 1 mL chilled PBS once prior to centrifuge. The supernatant was removed and 500μL of chilled PBS is added to the pellet. The samples were transferred into a falcon tube and were analyzed using FACSCanto II flow cytometer (BD Bioscience, USA).

2.9. Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM Statistical Package for Social Science (SPSS) version 22 by employing one-
way ANOVA. The data were considered statistically significant when $p<0.05$.

3. RESULTS

3.1. Cytotoxic activity assessment

The result of the assessment on the viability of the colorectal cell line was illustrated in Figure 1 and 2. Treatment of the cell with acetone extract of *C. odontophyllum* stem bark at concentration of 12.5 μg/mL and 25 μg/mL had caused a slight and non-significant decreased on cell viability upon 48 hr of treatment. However, the result showed that there was significant decreased ($p < 0.05$) in the percentage of viability of the cell line upon receiving 48 hr treatment at concentration of 50 μg/mL, 100 μg/mL and 200 μg/mL with percentage viability of 80.77%, 40.93% and 26.27% respectively. Based on the result, the acetone extract of *C. odontophyllum* stem bark at the concentration of 84 ± 9 μg/mL has decreased the percentage viability of the colorectal cell line to 50% (IC_{50} = 84 ± 9 μg/mL) as compared to untreated cell (Figure 2). As for menadione, a very prominent cytotoxic activity with IC_{50} value of 7.16 ±1.50 μM (Figure 2) was noted.

3.2. Assessment of intracellular ATP level

The assessment of cytotoxic effect of acetone extract of *C. odontophyllum* stem bark on ATP level of colorectal cancer HCT 116 revealed that the extract at its IC_{50} concentration, 84μg/ mL had cause significant decreased of ATP level in HCT 116 at all the timepoints assessed as compared to the negative control. The ATP level in the cells at 60 mins treatment (132732 ± 8186.36 RLU) showed slight decreased compared to the cells at 30 mins treatment (139861.67 ± 6350.95 RLU). The ATP level has undergone significant decreased at the following treatment time of 120 mins and 240 mins in which the RLU for the ATP level detected was 92708.67 ± 12355.71 RLU and 29875.5 ± 7284.39 RLU, respectively. The ATP level in the cells exposed to 240 mins treatment with the *C. odontophyllum* showed decreased level by 3-fold compared to the cells treated for 120 mins and the value was getting closer to the ATP level in positive control group. The ATP level indicated by Relative Luminescence Unit (RLU) using the CellTiter-Glo Luminescent Cell Viability assay against the treatment time was illustrated in Figure 3. There were significant decreased of ATP level in the colorectal cancer from 30 mins of the treatment to 240 mins of treatment compared to the negative control. Based on the result, the value of ATP level in the cells treated with acetone extract of *C. odontophyllum* stem bark was not as low as the level in the cells treated with menadione at concentration of 50 μM. However, as the time of treatment increased especially in the cells treated for 240 mins, the level of ATP detected in the cells of treatment group was as nearly to the level of ATP in cells of positive control groups.

3.3. Assessment of superoxide anion level

The percentage of peroxide anion level against the treatment of HCT 116 cell line was illustrated in Figure 4. Assessment on the presence of reactive oxygen species in cell line treated with acetone extract of *C. odontophyllum* stem bark at concentration of 84 ± 9 μg/mL showed that there was lower level of superoxide anion presence in the cells treated with the extract at 30 mins and 60 mins. However, there was a significant increase of the superoxide anion detected in the cells exposed to 120 mins treatment which was nearly reaching the value detected in positive control treatment.

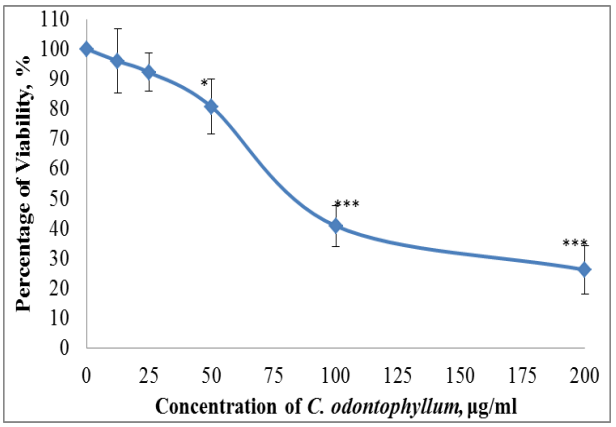

Fig. 1: Cell viability of HCT 116 cells after exposure to acetone extract from stem bark of *C. odontophyllum* at concentration ranging from 0 – 200 µg/ml after 48 hr of treatment.

The data was expressed in mean ± standard deviation with significant value of $p< 0.01$. Data were compared between the untreated negative control (concentration 0 µg/ml) and the treatment groups (concentration 12.5 - 200 µg/ml) by using one-way ANOVA.

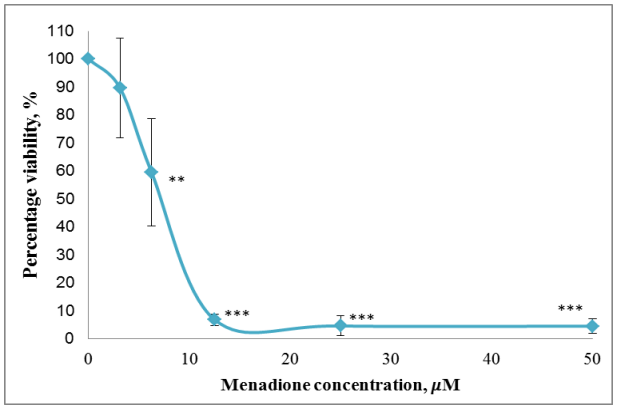

Fig. 2 Cell viability of HCT 116 cells after exposure to menadione at concentration ranging from 0 – 50 µM after 48 hr of treatment.

The data was expressed in mean ± standard deviation with significant value of p< 0.01 and was compared between the untreated negative control and the treatment groups by using one-way ANOVA.

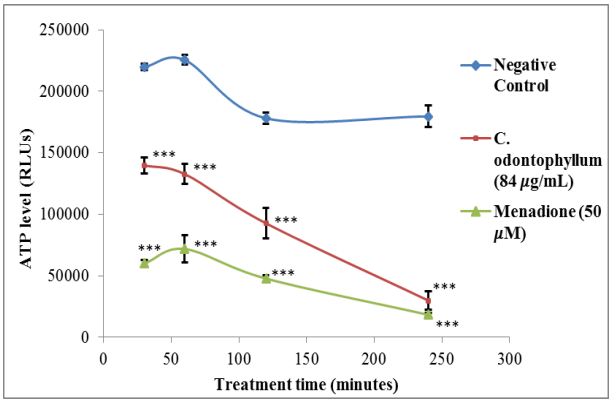

Fig. 3: The ATP level in HCT 116 cells after treatment with IC50 for 30 mins, 60 min , 120 min and and 240 min.

The data was expressed in the mean ± standard deviation with significant value of p< 0.01 and was compared between the untreated negative control and the treatment groups by using one-way ANOVA.

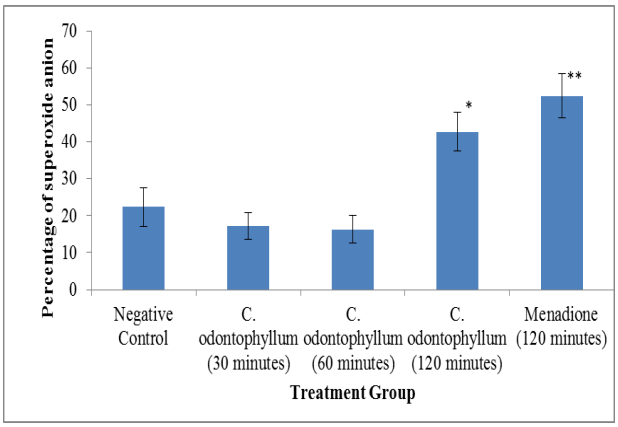

Fig. 4: The level of ROS (superoxide anion) in HCT 116 after treatment with IC50 for 30 mins, 60 mins and 120 mins was detected using DHE staining.

The data was expressed in the mean ± standard deviation with significant value of p<0.05 and was compared between the negative control (untreated cells) and the treatment groups by using one-way ANOVA.

4. DISCUSSION

The active compounds present in the acetone extract of *C. odontophyllum* stem bark might be responsible for the cytotoxicity to the cell line. In this study, terpenoid is believed to exert cytotoxic effect towards the cancer cell as supported by.[13] This is hypothesized by referring to other finding related to the terpenoid for instance, D-limonene (a class of terpenoid derivatives) and artemisinin, which is an active terpenoid isolated from Chinese herb plant, were reported to contribute to anticancer activities.[14] In addition,[14] has also stated that higher amount of terpenoid in a plant contributes to anticancer activity both in vitro and in vivo study.

Previous studies on cytotoxicity effect of acetone extract from *C. odontophyllum* stem bark against colorectal cancer cell line has reported that the extract would cause the colorectal cancer cell line to undergo cell death primarily via apoptosis.[9] In addition, the extract could damage the DNA of the colorectal cancer cell line as well. This DNA damage might be one of the factors that induce apoptosis cell death to occur in the colorectal cell line as it was considered as common initial event which then will be propagated by the cellular stress response of the cells.[15] The damage of DNA will trigger the apoptotic signals which will be transmitted to mitochondria through the tumor suppressor p53 causing the release of apoptogenic factors which activate the series of events for apoptosis.[16]

The level of ATP detected during the experiment at different timepoints might provide some information and indicator on the mode of cell death. The difference in the ATP level detected in cells undergoing apoptosis and necrosis is related to morphological changes of the dying cells. For instance, cell undergo necrosis will involve rapid swelling, shutting down metabolism and losing membrane integrity which leads to the releasing of cytoplasmic content to the surrounding environment by membrane disruption. These processes do not need ATP to occur and hence, reflected that necrosis is an ATP-independent process as the depletion of ATP itself might induce the necrosis to occur.[17]

In contrast to the apoptosis cell death, it involved series of event such as enzymatic hydrolysis of macromolecules, condensation of chromatin, fragmentation of nucleus which leads to the formation of apoptotic bodies which eventually phagocytosed by other cells.[18] All of these events were referred as energy requiring apoptotic process since energy in the form of ATP was still needed to complete the apoptotic process.[19] This might be explained by the characteristic of apoptosis cell death which is ATP-dependent as supported by.[20,21]

A decreased in cellular ATP level is the characteristic for cell death.[19] However, the severity of ATP level depletion will cause rapid metabolic collapse and necrosis while modest degree of ATP depletion will eventually cause apoptosis.[22] In the present study, gradual depletion of ATP level in the cells following the treatment time from 30 minutes to 240 minutes might indicate that the cells were undergoing apoptosis cell death. Gradual, modest depletion of ATP might indicate that ATP is still needed for apoptosis. Once the ATP depleted to a certain severe level, secondary necrosis might take over.[23] Therefore, since the ATP level in this study was decreasing gradually and modestly upon receiving treatment, it can be concluded that the colorectal cancer cell line in this study was undergoing apoptotic cell death instead of necrosis.

This finding showed higher level of ROS detected in the cells treated with the acetone extract of *C. odontophyllum* at 120 mins treatment in which nearly reaching the level of ROS in cells treated with menadione at the same treatment time. However, the cells treated at lower treatment times did not display any significant increase of ROS when compared to the negative control. The possible reason that might contribute to this observation was due to the presence of endogenous antioxidant such as superoxide dismutase (SOD) which gives protection to the cells at the early time of the treatment.[24] Since the cell was only exposed to the extract for shorter period of time, the effect of the extract in causing the increase of ROS might be halted by the presence of superoxide dismutase which function to stabilize the ROS. However, as the treatment time increases and therefore, contributes the continuous production of ROS, the cells might start to be affected and the endogenous antioxidant might not be sufficient to counteract the ROS produced. This could well be the reason behind the increase of ROS to a very significant level that almost corresponded with the positive control. ROS has been suggested to induce apoptosis through the intrinsic pathway by disrupting the mitochondrial membrane potential through oxidation of the mitochondrial pores which eventually caused the release of apoptogenic protein such as cytochrome c into the cytosol.[25] This in turns promotes the activation of caspases cascade or acting as caspase-independent death effector which leads to the apoptotic cell death event.[26]

5. CONCLUSION

The overall findings of this study postulated that the cytotoxic activity of the acetone extract of *C. odontophyllum* stem bark affects the mitochondria of the cells which cause the gradual depletion of ATP level. In addition, the apoptosis cell death might also be contributed by the higher level of ROS in cells treated with the extract at longer period of time. *Canarium odontophyllum* needs further research to ensure its potential as new anticancer agent. In future, it is highly recommended to identify other parameters which might be involved in the cytotoxic effect against the colorectal cancer cell line for example, identifying the level of GSH, SOD, thiol and hydrogen peroxide associated with colorectal cell. Performing assessment at molecular level of cells such as identifying the involvement of caspase activity in the cytotoxicity of the colorectal cancer cell line might be necessary.

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Disclosure

None.

Competing interests

The authors declare no competing or financial interests.

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