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Mechanism of cytotoxic activity of *Canarium odontophyllum* Miq. (dabai) against human colon cancer cell line HCT 116

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ABSTRACT

Colorectal cancer is among the most common cancers in Malaysia. *Canarium odontophyllum* is a native plant species found in Sarawak. A previous study have shown that *C. odontophyllum* acetone extract caused cell death via primary apoptosis on human colorectal cancer cell line HCT 116. The objective of the present study was to evaluate the mechanism of *C. odontophyllum* stem bark acetone extract against human colorectal cancer cell HCT 116. A MTT assay was used to determine the IC_{50} value of *C. odontophyllum* stem bark acetone extract from concentration 12.5 µg/ml to 200 µg/ml at 48 hrs of treatment against HCT 116. Tetra methyl rhodamine ethyl ester (TMRE) labelling was used to determine the mitochondrial membrane potential (MMP) of HCT 116 cell line after exposure with IC_{50} of the extract at 30 min, 1 hr and 2 hrs. At 48 hrs of treatment, *C. odontophyllum* stem bark acetone extract showed cytotoxic effect towards HCT 116 with IC_{50} of 24 µg/ml ± 3.059. From the MMP result, an increase of population of MMP loss were observed from 1 hr to 2 hrs of treatment with the value of 19 ± 5.3 % and 43 ± 2 % respectively. Furthermore, there was a slight increase level of superoxide anion in extract-treated HCT 116 cell at 30 min, 1 hr and 2 hrs compared to the negative control with values at 3.17 ± 1.4 %, 3.67 ± 2.0 % and 4.5 ± 1.0 % respectively. In conclusion, involvement of superoxide ion and loss of MMP confirmed the function of mitochondria by *C. odontophyllum* stem bark acetone extract in inducing apoptotic mechanism in HCT 116 cell lines. These data provide information on the mechanism of *C. odontophyllum*-induced apoptosis and may have the potential in the development of drug for colon cancer.

KEY WORDS: *Canarium odontophyllum,* dabai, cytotoxic, HCT 116, MMP, Superoxide anion.

1. INTRODUCTION

Colon cancer is defined as an uncontrolled cell growth in the digestive system especially in the large intestine (Underwood & Cross 2009). It is one the most common cancers occurring worldwide especially in Western Countries (Perse, 2013). In Asia, there are 283,596 incidence rate of colorectal cancer with the mortality rate of 144,980 cases (Sankaranarayanan, 2014). The increase in incidence rate of colon cancer in Europe goes along with urbanization and industrialization (Labianca, 2010). Meanwhile in Malaysia, colorectal cancer ranked second common cancer affecting Malaysian (National Cancer Society Malaysia, 2015). Furthermore, colon cancer is common among Chinese rather than Malay and Indian (Lim, 2003).

The treatment for colorectal cancer includes chemotherapy, surgery, radiotherapy, hormone therapy, immune therapy and also sympathetic therapy (Lim, 2002). Plant derived anticancer drugs contribute an important role in cancer chemotherapy (Wang, 2012). There are variety of anticancer agents isolated from plants such as vinblastine, vincristine, and irinotecan (Newman & Cragg, 2005). *Canarium odontophyllum* is an indigenous plant that is also known as "Dabai" or Borneo olive and is available in Sarawak, Malaysia especially in Kanowit, Sarikei, and Kapit (Kueh, 2003). It belongs to the Burseraceae family and genus, *Canarium L*. The fruit of *C. odontophyllum* is oval in shaped, has a thin skin and is blue-black in colour when riped (Ding, 2003). Almost all parts of *C. odontophyllum* were utilised for studies due to its benefits in human health including the pulp, skin of fruit, shell, leaf and stem. The fruit from this plant has antioxidant constituent such as phenolic (Shakirin, 2010). Besides that, the skin, pulp and shell from its fruit contain carotenoid with high antioxidant capacity (Prasad, 2011). The extract from *C. odontophyllum* shell has antimicrobial activity against *Acinetobacter baumannii* although it only inhibits the growth *P. mirabilis* (Basri, 2014). The leaf and bark from *C. odontophyllum* stem have cytotoxic activity against HCT 116 colorectal carcinoma cell line (Basri, 2015).

Apoptosis is indicated by distinct changes in morphology and biochemistry such as cellular shrinkage, membrane blebbing, nuclear and cytoplasmic condensation, fragmentation of DNA, activation of caspase and phosphatidylserine exposure (Nowsheen & Yang, 2012). Generally, the two pathways of apoptosis which are intrinsic and extrinsic represent receptor and mitochondrial mediated apoptosis mechanisms respectively (Wu, 2001; Henry-Mowatt, 2004). Mitochondria also have important role in generating ATP as source of energy (Green & Reed, 1998). The disruption of electron transport chain and loss of mitochondrial membrane potential cause the apoptotic factor to be released from mitochondria (Elmore, 2007).

Furthermore, the incidence and mortality of colon cancer was higher compared to other cancers. Therefore, the significant of the current study is to produce an alternative source of anticancer agent from natural product such

as *Canarium odontophyllum* plant to treat colorectal cancer. This is because synthetic anticancer agents cause side effects to patients when compared with an anticancer agent derived from plants. In addition, this work was undertaken to prevent problems such as resistance to chemotherapeutic agents. Furthermore, no study till date was performed to investigate the effect of *C. odontophyllum* extract on the MMP and superoxide ion in HCT 116 cell lines.

2. MATERIALS AND METHODS

Plant Material: The bark from *Canarium odontophyllum* Miq. stem was flown from Sarawak, Malaysia. All parts of the plants were confirmed its authenticity by Sani Miran and left for deposition in Universiti Kebangsaan Malaysia (UKM) Herbarium, Bangi, Selangor, Malaysia with a voucher specimen number of UKMB 40052. All reagents and chemicals were purchased from Sigma (USA).

Preparation of Acetone Extract: The bark of *Canarium odontophyllum* stem was extracted in acetone solvent. In the preparation of the stock extract solution at 100 mg/ml, 100 mg of acetone extract were dissolved with 1 ml of dimethyl sulfoxide (100 % DMSO). Then the solution was mixed well with an auto vortex until it was completely dissolved. The extract was sterilized by passing through a 0.22 μm membrane filter and was stored in eppendorf tube at -20°C refrigerator until further use.

Preparation of Cell Culture: HCT 116 cell lines were from American Type Culture Collection (ATCC) (Rockville, MD USA with ATCC Number: CCL-247™) and cultured in McCoy 5A media (1x) (Sigma Aldrich, USA). Culturing of HCT 116 cells was done in the sterile laminar flow to avoid any source of contamination. The media was enriched with 10 % fetal bovine serum. Cells were cultured at 37°C in 5% CO_2 humidified condition at Biocompatibility and Toxicology Laboratory, Faculty of Health Sciences, Universiti Kebangsaan Malaysia. Cultured cells were observed daily to see the morphology and cell confluency of 70 % - 80 %. Subculture was done every 2-3 days.

Cell Plating of the Cell and In Vitro Treatment: The cell lines were plated at seeding concentration of 5 x 10^4 cells/mL and were allowed to stand overnight for cell attachment prior to treatment with *C. odontophyllum* acetone extract in a concentration-dependent or time-dependent manner as indicated in each subsection.

Evaluation of Cytotoxic Activity: MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Mosmann, 1983) was performed to determine the cytotoxicity of *C. odontophyllum* acetone extract at a concentration range of 12.5 μg/ml to 200 μg/ml at 48 hrs of treatment against HCT 116. Plating was performed in a 96-well plate before incubation of the seeded cells for 24 hrs and treatment with acetone extract. Menadione served as a positive control, while untreated served as negative control. A volume of 20 μL of MTT solution at 5 mg/ml was added to each of the well and incubated for 4 hrs. DMSO solution was pipetted into each well to mix with the formazan crystals at room temperature of 27°C, then further incubated and shaken on an automatic mixer for 15 min and 5 min, respectively. The absorbance was read by using an ELISA plate reader (Bio-Rad, USA) at a wavelength of 570 nm. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean of treated sample}}{\text{Mean of negative control}} \times 100\ \%$$

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

Mitochondrial Membrane Potential Assay: The method was modification of Chan (2010). The cells were plated in 6 well-plates. Each well contained 3 mL of cells and media. As for positive control and purpose of verification of the results, menadione at concentration 50 μM was used and exposed to the cells for 2 hrs while the untreated cells served as negative control. The cells were treated with IC_{50} of *C. odontophyllum* for 2 hrs, 1 hr and 30 min. After treatment, all extract-treated cells were harvested and suspended with one mL serum-free media and 1 μL of 50 μM TMRE (Molecular Probes, Invitrogen) was added to the suspended cells. The cells were incubated in the dark for staining purposes (15 min, 37◦C), followed by centrifugation (2,500 rpm, 5 min, 4◦C). The stained cells were chilled with 1 mL cooled PBS once prior to centrifuge. The supernatant was discarded and 500μL of chilled PBS was added to the pellet. Finally the samples were transferred into a falcon tube and then analyzed with FACS Canto II flow cytometer (BD Bioscience, USA).

Superoxide Anion Assay: The method was employed following Chan (2012) with some modification. The cell was plated in 6 well-plates. Each well contained 3 mL of cells and media. As for positive control, menadione at concentration 50 μM was used and exposed to the cells for 2 hrs while the untreated cell served as negative control. The extract-treated cells at its IC_{50} at different time intervals of 30 min, 1 hr and 2 hrs were harvested and resuspended with 1 mL serum-free media and 1 μL of 10 mM dihydroethidine (HE) (Gibco, Invitrogen) was mixed into the suspended cells. The cells were left for incubation in the dark for staining purposes (15 min, 37°C), followed by centrifugation (2,500 rpm 5 min, 4°C). The stained cells were washed with 1 mL chilled PBS once prior to centrifuge. The supernatant was removed and 500μL of chilled PBS was added to the pellet. Finally the samples were transferred into falcon tube and then analyzed with FACS Canto II flow cytometer (BD Bioscience, USA).

Statistical Analysis: All data were presented as mean ± standard error mean (SEM). Statistical analysis was performed using Statistical Package for Social Science (SPSS) version 22 by employing one-way ANOVA.

3. RESULTS

Assessment of Cytotoxic Activity: The result for cytotoxic effect of *C. odontophyllum* stem bark acetone extract against HCT 116 cell lines was displayed in Figure 1. There was a significant decrease of viable HCT 116 cell lines in comparison to the negative control after 48 hrs of treatment at IC_{50} of 24.00 ± 3.059 µg/ml. At 100 µg/mL concentration of acetone extract, the cell viability of HCT 116 cell lines showed the minimum cell viability with 20 ± 1.00 %. Meanwhile at 200 µg/mL concentration, there is a slight increase of population of HCT 116 cell lines at 5 %. Based on Figure.2, menadione showed a prominent cytotoxic effect towards HCT 116 at IC_{50} of 4.0 ± 1.0 µM.

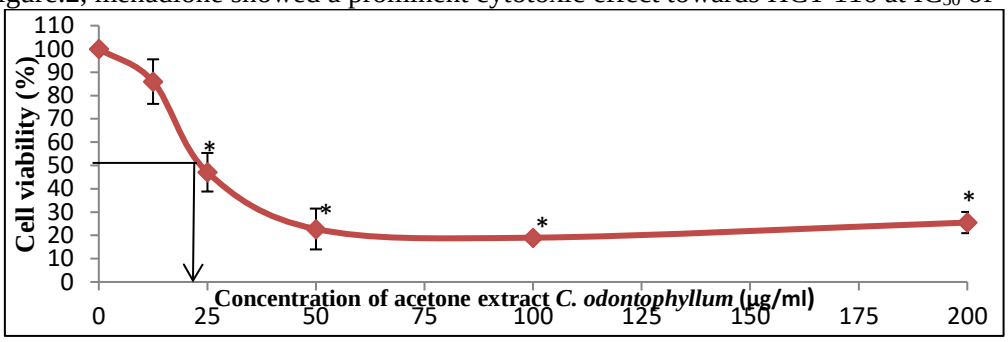

Figure. 1. Cell viability of HCT 116 cells after exposure to acetone extract from stem bark of *C. odontophyllum* at concentration ranging from 0 – 200 µg/ml for 48 hrs of treatment.

The data was expressed in mean ± standard error mean for 3 experiments $p < 0.05$. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA.

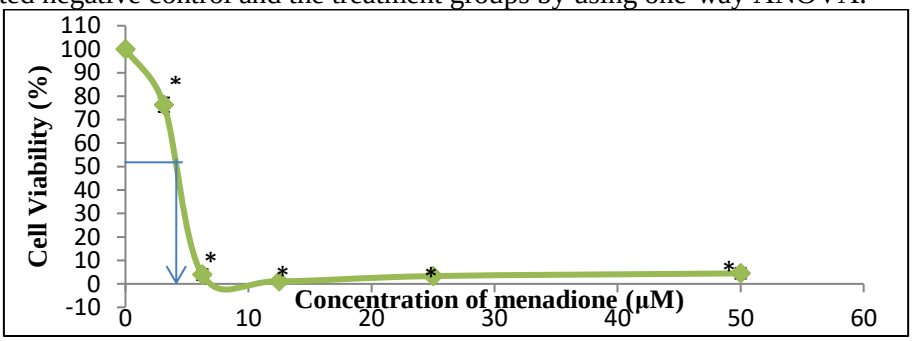

Figure. 2. Cell viability of HCT 116 cells after exposure to menadione for 48 hrs.

The data was expressed in the mean ± standard error mean for 3 experiments, $p < 0.05$. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA.

Assessment of Mitochondria Membrane Potential: Figure.3, showed the result of percentage loss of mitochondrial membrane potential against the duration of treatment. Generally there was an increase of HCT 116 cells population with loss of mitochondrial membrane potential from 1 hr to 2 hrs of treatment time at 19 ± 5.3 % and 43 ± 2.0 % respectively. Based on one-way ANOVA result, there was no significant loss of mitochondrial membrane potential within 30 min and 1 hr of treatment. However, at 2 hrs of treatment, a significant loss of MMP was observed when compared to negative control.

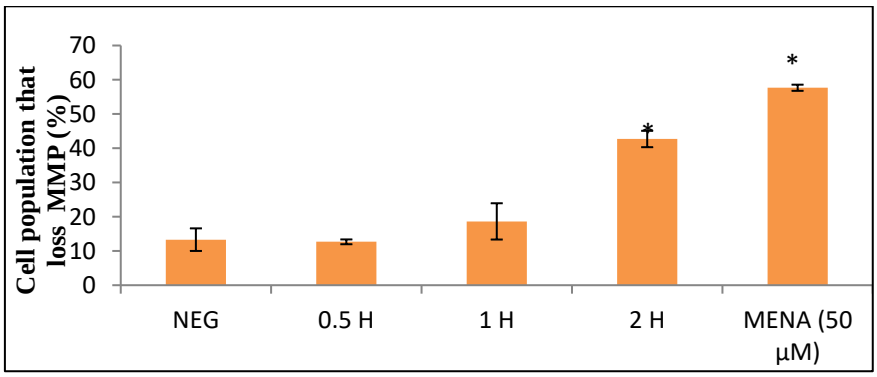

Figure.3. The loss of mitochondria membrane potential in HCT 116 cells after treatment with IC50 for ½ hr, 1 hr and 2 hrs.

The data was expressed in the mean ± standard error mean for 3 experiments $p < 0.05$. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA.

Assessment of superoxide anion level: Figure.4, showed the result of percentage of ethidium positive cells against duration of treatment. The ethidium positive cell indicated the superoxide anion level in HCT 116 cells after treatment with *C. odontophyllum* stem bark acetone extract. In general, there was no significant increase of superoxide anion in HCT 116 in all duration of treatment.

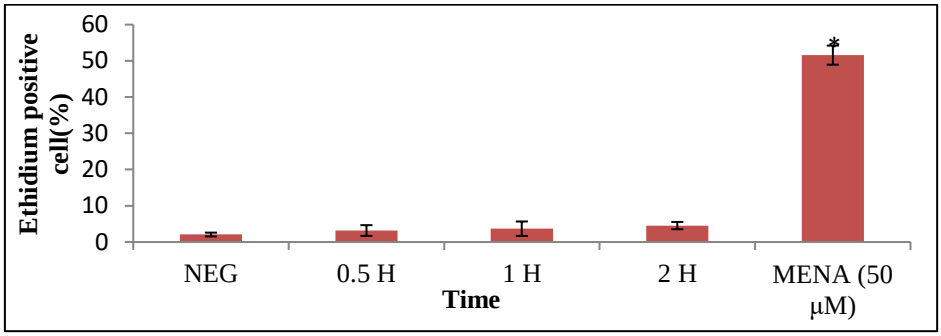

Figure.4. The level of superoxide anion in HCT 116 after treatment with IC50 for ½ hr, 1 hr and 2 hrs.

The data was expressed in the mean ± standard error mean for 3 experiments, $p < 0.05$. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA.

DISCUSSION

Cancer gives a negative impact to the society (Bandopadhyaya, 2015). The search for an alternative treatment for colorectal cancer gives challenges to the scientists with the presence of side effects caused by conventional chemotherapy (Koppikar, 2010). Anticancer agent derived from plant source is more effective for cancer treatment (Ahmad, 2013). In this study, the mechanism of cytotoxic effect of *Canarium odontophyllum* stem bark acetone extract against HCT 116 cell lines was evaluated.

From MTT assay result, *C. odontophyllum* acetone extract demosntrated high cytotoxic effect towards HCT 116 cell lines after 48 hrs treatment. The IC_{50} produced from this study showed a very small difference with the previous study (Basri, 2016) by 1 µg/ml difference in concentration. According to Konarikova (2015), the arrest of cancer cell growth is dependent on the concentration of the treatment used. The higher the concentration used, the lower the cancer cell viability. Furthermore, the lower IC_{50} and high cytotoxic effect also depend on the longer time exposure to the treatment (Ren, 2003). The phytochemical constituents can give cytotoxic effect to cancer cells (Kashani, 2012). *C. odontophyllum* stem bark acetone extract contains flavonoid, terpenoid, tannin, saponin and also phenolic compounds that function as anticancer agents. The potent cytotoxic effect of *C. odontophyllum* acetone extract may be due to flavonoid content in the extract. The flavonoid compound can interfere with function of cytoplasm membrane, form a complex with cell wall and also inhibit the nucleic acid synthesis as the mechanism of action of anti-proliferation agent (Chushnie & Lamb, 2005). For example, the flavonoid compound from citrus inhibits the growth of HL-60 (Manthey, 2001).

Mitochondrion is undoubtedly an important organelle involved in the intrinsic pathway of the apoptotic event (Suen, 2008). Mitochondria have been proposed as a novel molecular target for chemotherapeutic agents to cause cell death by apoptosis (Fulda & Kroemer, 2011). Mitochondria regulate the secretion of apoptotic bodies from intermembrane space to the cytosol by opening the transition pores in the membrane (Gublins, 2003). In the current study, there is a significant loss of mitochondria membrane potential in the HCT 116 cell lines at 2 hrs of treatment suggesting the involvement of mitochondria in *C. odontophyllum* stem bark acetone extract-induced apoptosis. The current result supports the previous study (Basri, 2016) that mitochondria was involved in causing apoptosis HCT 116 treated with *C. odontophyllum* stem bark. The loss of MMP was associated with the change of inner membrane permeability.

Death of cell through apoptosis can occur due to various factors including DNA damage, chemotherapy agent, ROS and UV radiation (Harris & Thompson, 2000). *Canarium odontophyllum* causes DNA damage to HCT 116 (Basri, 2016). DNA damage causes activation of pro-apoptotic, BH3 that inhibits the anti-apoptotic protein Bcl-2 in mitochondria (Wang & Youle, 2009). The activation of BH3-protein triggers the oligomerization of Bax and Bak, which are speculated to form pores in the outer mitochondrial membrane causing the release of cytochrome c and Smac that induces caspase-dependent apoptosis or releases apoptosis-inducing factor and endonucleases G which eventually triggers caspase-independent apoptosis (Ly, 2003; Wang & Youle, 2009; Wen, 2013). Then, binding of cytochrome c to the adaptor protein Apaf-1 initiates the formation of the apoptosome which activates initiator caspase-9 (Newsheen & Yang, 2012) and triggers the activation of caspase executioner (3, 6, 7) that eventually cleavages variety of substrates and causes morphology and biochemical changes that gave rise to apoptotic cell (Mukhtar, 2012). This event can happen to HCT 116 cell lines treated with *C. odontophyllum* as a result of the phytochemical constituent present in this plant. The polyphenol compound such flavonoid can induce apoptotic intrinsic pathway by modulating Bcl-2 protein and also regulating the opening of mitochondria membrane transition pore (Sandoval-Acuna, 2014). For example, epigallocatechin-3-gallate (EGCG) causes apoptosis on gastric cancer

cell, MKN 45 and also hepatocellular carcinoma cancer cell BEL-7402 and SMMC 7721 by oligomerization of pro-apoptotic Bax protein, depolarization of mitochondrial membrane and down regulation of IAP (Qanuoga, 2005).

Mitochondria also play crucial role in production of intracellular ROS. The production of ROS by mitochondria occurs during the electron transport chain. The reduction of electron transport chain from oxygen molecule causes production of free radicals such as superoxide anion and hydrogen peroxide which eventually change to other ROS molecules (Circu & Aw, 2010). However, the exogenous oxidant such as chemotherapy agent, cigarette, endotoxin, and exposure to environmental also cause ROS production. Generally, ROS causes cell death (Cadenas & Davies, 2000). Natural plants cause generation of ROS on cancer cells for example, *Zingiber officinate Roscoe* causes ROS production by cancerous pancreatic cell (Akimoto, 2015). In this study, *C. odontophyllum* stem bark acetone extract causes low production of superoxide ion on HCT 116 cells as the superoxide anion did not increase in *Canarium odontophyllum*-induced apoptosis. However, it is possible that the superoxide anion was converted to hydrogen peroxide by endogenous superoxide dismutase (SOD) rapidly (Chan, 2010). SOD enzyme is an endogenous antioxidant defense mechanism that acts on ROS (Nijveldt, 2001). The MnSOD enzyme is higher in the malignant cancer such as colorectal cancer, central nervous system lymphoma and mesothelioma (Kuninaka, 2000). However, the mechanism of oxidative stress induce apoptosis by *Canarium odontophyllum* towards HCT 116 cells needs further investigation.

4. CONCLUSION

In conclusion, *C. odontophyllum* stem bark acetone extract showed a potent cytotoxic effect towards HCT 116 cell lines. This plant also causes loss of mitochondrial membrane potential and low production of superoxide ion in human colorectal cancer cell line HCT 116. This plant needs further research to ensure its potential as a new anticancer agent.

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