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EFFECT OF CANARIUMODONTOPHYLLUM STEM BARK EXTRACT AGAINST HUMAN COLORECTAL CANCER CELL LINE HCT 116

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Keywords: *Canarium odontophyllum, reactive oxygen species (ROS), dihydroethidium test, alkaline comet assay, HCT 116.*

Abstract

*Canarium odontophyllum*stem bark was proven to cause cytotoxicity on colorectal cancer cell line HCT116 by inducing cell death via apoptosis. We aimed to assess cytotoxic mechanism of the acetone extract from stem bark of *C. odontophyllum* against HCT 116. The IC_{50} value of extract against HCT 116 was determined using MTT assay for 48 hours of treatment. ROS assay with dihydroethidium staining was conducted for 30 minutes, 1 hour and 2 hours. The DNA integrity of HCT 116 was evaluated using alkaline comet assay. The extract showed cytotoxic effect on HCT 116 after 48 hours of treatment with IC_{50} value of 82.0 ± 9.3 µg/mL. At 2 hours of post-treatment, the extract induced elevation of ROS level in the cell lines compared to control at 16.0 ± 7.2 % and 10.0 ± 0.9 %, respectively. Elevated tail moment and percentage of DNA in tail revealed DNA damage when the cells were incubated to low doses of extract at IC_{10} and IC_{25} values. Acetone extract from *C.odontophyllum*stem bark has exerted cytotoxic activity against HCT 116 by increasing ROS level and interfering DNA integrity of the cell line therefore, has the potential to be developed as anticancer agent.

Introduction

Colorectal cancer (CRC) has been a leading cause of death globally. There were estimated over 1.2 million new CRC cases and 608,700 deaths in 2008 worldwide [1]. In Malaysia, this second most common cancer mainly affects the Chinese community followed by Malay and Indian community [2]. Colorectal cancer is commonly treated with chemotherapeutic agent consists of fluoropyrimidine in various combination and schedule. However, available chemotherapeutic agent or treatment tend to develop side effects to the patient [3].

Natural products are opted as an alternative treatment to treat cancer and it has been reported that plant extracts have the potential to be developed as anticancer agent besides inhibiting the process of carcinogenesis [4]. Terrestrial plants have been used as medicine in countries like Egypt, China, India and Greece since long time ago and those plants were used as modern medicines as well [5]. *Canarium odontophyllum*, locally known as 'Dabai' is an indigenous plant vastly found in Sarawak and it belongs in the Burseraceae family [6]. A few studies have been conducted on different parts of the plant. Amongst all the studies carried out, *C. odontophyllum* leaves and the stem bark were reported to exhibit cytotoxic activity against colorectal cancer cells. Based on a previous study, the stem bark of *C. odontophyllum* has some phytochemicals which could possibly attribute to the cytotoxic effects. Those phytochemicals include terpenoid, flavonoid, tannin, saponin and phenolic compounds [7].

Previous study reported that acetone extract from the stem bark of *C. odontophyllum* exhibited the highest cytotoxic activity towards HCT 116 cells as compared to methanol and aqueous extracts. Besides that, the study also showed that the primary cell death of HCT 116 was via apoptosis after 48 hours of treatment with acetone extract [7]. Generally, apoptosis involves intrinsic and extrinsic pathways and it requires a specific signal to initiate molecular

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activities which depend on the cascade activity of caspases [8]. Mitochondria play a crucial role in cell death besides generating ATP for cellular metabolisms. Any disruption to the electron transport chain in the mitochondria can result in the production of reactive oxygen species and this gives oxidative stress to the cells. Excessive oxidative stress can induce damage to the macromolecules in the cells such as protein and DNA [9]. Reactive oxygen species is one of the sources which can affect the integrity of DNA and eventually induce damage to the DNA [10]. ROS has direct or indirect mechanisms to initiate cell death either through intrinsic or extrinsic pathways [11]. Therefore, the present study will further investigate the concealing cytotoxic mechanism of acetone extract from the stem bark of *C.odontophyllum* against colorectal cancer cells.

Materials and methods

Plant material

Canarium odontophyllum stem bark was obtained from Sarawak and placed in UKM plant archive with a voucher number of UKMB 40052.

Preparation of extract stock solution

One hundred gram of the powdered stem bark *C. odontophyllum* were soaked each in 500 ml acetone in the ratio of 1:5. The mixture was subjected to agitation using electrical shaker for 24 hours at room temperature. The mixture was then filtered using the Whatman filter paper No. 1 to collect the filtrate obtained. The process was repeated using the remaining residue with 500 ml acetone. Both filtrates were then mixed and concentrated under reduced pressure using a rotary evaporator. The extracts obtained were finally pounded to dryness under fume hood in order to produce a crude acetone extract. Stock solution of acetone extract from stem bark of *C. odontophyllum* at 100 mg/mL was prepared by dissolving 100 mg of pounded acetone extract of stem bark in 1 mL of 100% DMSO. The stock solution was mixed using autovortex for five to ten minutes before being centrifuged for five minutes at 20,000g. The stock solution obtained was then filtered using 0.22 µM nitrocellulose membrane filter. The prepared stock solution is then stored at -20˚C until further use.

Preparation of cell culture

HCT 116 human colorectal carcinoma cell line was obtained from American Type Culture Collection Rockville, MD USA. The cell line was maintained in complete growth medium McCoy's 5A media (Sigma Aldrich, USA). The medium was enriched with 10 % of Fetal Bovine Serum (FBS) and 5% of Penicillin-Streptomycin. Cells were cultured at 37 ˚C in 5% CO_2 condition at Biocompatibility and Biotechnology Lab, Faculty of Health Sciences, Universiti Kebangsaan Malaysia.

Cell plating and treatment in vitro

HCT 116 cell line was seeded at an inoculation density of 5 x 10^4 cells/mL. Upon seeding, the cells were allowed for 24 hours of incubation to ensure the cells adherence.

Evaluation of cell viability

MTT [3-(4, 5-dimethyl-2 thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay was performed to determine the IC_{50} value and cytotoxicity effect of acetone extract from stem bark of *C. odontophyllum* against HCT 116. Cells were plated in 96-well plate and the cells were incubated for 24 hours to allow cell attachment. The cells were treated for 48 hours with the extract at concentration ranged between 12.5 µg/mL until 200 µg/mL. Menadione was used as a positive control at concentration ranged from 0.5 µg/mL until 8 µg/mL while untreated cells in the media were used as a negative control. Menadione has been used as positive control in many cancer-based studies as it has anticancer properties [12]. Besides that, the role of menadione as a positive control was basically to check if the model of the experiment is working. Once all the cells have been incubated, 20 µL of 5 mg/mL of MTT was added to each well seeded with the cells and the plate was left for incubation for four hours. Then, about 200 µL of DMSO solution was added to each well to dissolve the formazan crystals and the plate was incubated for 15 minutes. The

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plate was then shaken using an automatic mixer for five minutes prior to measurement at 570 nm with an ELISA microplate reader. Cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \frac{\text{optical density of sample}}{\text{optical density of control negative}} \times 100$$

Assessment of ROS production

Reactive Oxygen Species (ROS) generated in the cells was assessed using Dihydroethidine staining to specifically determine superoxide anion level as employed by previous method [13]. Cells were plated in 6-well plate with each well containing 3 mL of cells and media. The cells were treated with the extract at its IC_{50} value at different time-point intervals of 30 minutes, one hour and two hours. Menadione at concentration 50 µM was used as a positive control to treat the cells for two hours. Untreated cells were used as negative control. Upon treatment, cells were harvested and resuspended in 1 mL of serum-free media and 1µL of 10 mM dihydroethidium stain was mixed into the suspended cells. The cells were then allowed for incubation for 15 minutes at 37˚C in the dark prior to centrifugation at 20,000g for 5 minutes at 4˚C. The stained cells were then washed with 1 mL of cold PBS once before centrifugation. The supernatant was discarded and 500 µL of cold PBS was added to re-suspend the pellet. The samples were then transferred to the falcon tube prior to analysis using FACS Canto II flow cytometer.

Alkaline comet assay

Alkaline comet assay was performed to evaluate the extent of DNA damage induced by acetone extract from stem bark of *C. odontophyllum* against HCT 116 at its IC_{10} and IC_{25} values while menadione was used as positive control at IC_{25} value. A total of 3 mL of cell suspension was seeded into each well of the 6-well plate and incubated for 24 hours for cell attachment. The cells were then treated with the extract for 30 minutes before collecting all the media in the well into a centrifuge tube. Cells were washed with PBS and trypsinized with trypsin-EDTA. The solution was then centrifuged at 2000 g for five minutes. The supernatant of each centrifuge tube was discarded and the process was repeated. Comet slides were prepared while preparing the cells. Low melting agar (LMA) and high melting agar (NMA) were preheated until the agar melted. When the NMA reached a temperature of 37˚C, 100 µL of NMA was pipetted onto a frosted slide and a coverslip was placed onto the slide and removed after the agar has hardened. Then, about 80 µL of LMA was added to each sample and then mixed before adding them on top of the hardened NMA slide. A coverslip was then placed and taken out slowly before placing the slides into a Coplin jar filled with lysing solution and cooled at 4˚C for 24 hours. The next day, the slides were placed in the electrophoresis tank filled with chilled electrophoresis buffer. The slides were immersed in the electrophoresis buffer for 20 minutes to allow the DNA strands to unwind. The process of electrophoresis was carried out for 20 minutes at 25 V and 300mA. The slides were then rinsed with neutralising buffer three times for every five minutes. The slides were then stained with 50 µL of ethidium bromide at 20 µg/mL. The slides were then analyzed using a fluorescent microscope.

Statistical analysis

All data were analyzed using SPSS Version 22. All data were done in triplicate and expressed as mean ± S.E.M from three different experiments. One-way ANOVA was used to measure the statistical difference between the mean in all three experiments. The statistical difference was indicated with $p<0.05$.

Results and discussion

Evaluation of cell viability

Figure 1 and 2 showed the result for cytotoxic activity of acetone extract from stem bark of *C. odontophyllum* and menadione against HCT 116. Acetone extract showed an IC_{50} value of 82 ± 9.3 µg/mL. The viability of cells decreased significantly ($p<0.05$) at concentration of 50 µg/mL , 100 µg/mL and 200 µg/mL with percentage viability of 57 ± 8%, 45 ± 9% and 21 ± 7% respectively. As for positive control, menadione showed a prominent

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cytotoxic activity against HCT 116 with an IC_{50} value of 1.7 ± 2.2 µg/mL. Based on MTT assay, acetone extract demonstrated a cytotoxic effect towards HCT 116 after the concentration of 50 µg/mL at 48 hours of post-treatment. The IC_{50} value obtained in the present study was comparable to the IC_{50} value reported by a previous study [7]. The phytochemicals found in plant extract have the potential to act as anticancer agent and inhibit the process of carcinogenesis [14]. Phytochemicals found in acetone extract from stem bark of *C. odontophyllum* are terpenoid, flavonoid, saponin, tannin and phenolic compounds which account for the cytotoxic effect against HCT 116.

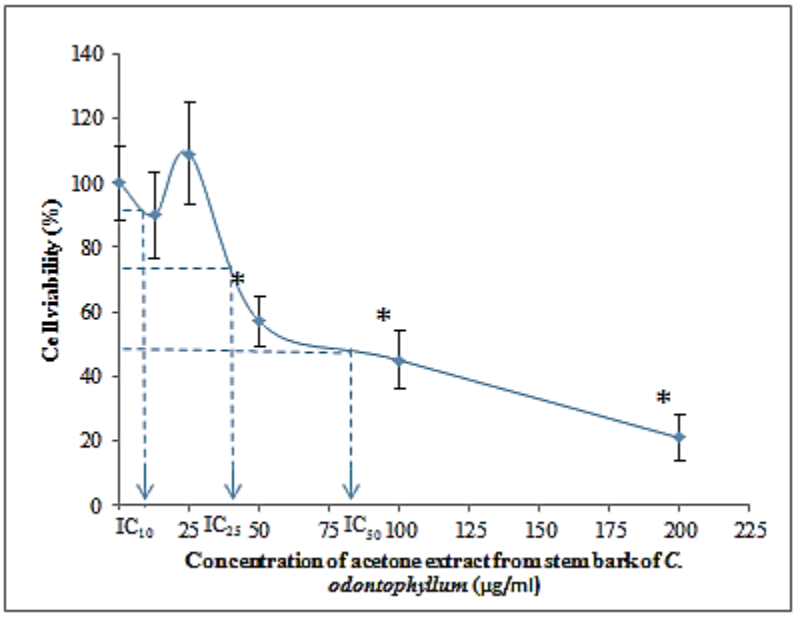

FIG. 1: Cytotoxic activity of acetone extract from stem bark of C. odontophyllum against HCT 116 at concentration ranging from 0 – 200 µg.mL^{-1} after 48 hours of treatment.The data was expressed as mean ± S.E.M. with significant value of p< 0.05. '*' indicates significant difference as compared to the negative control.

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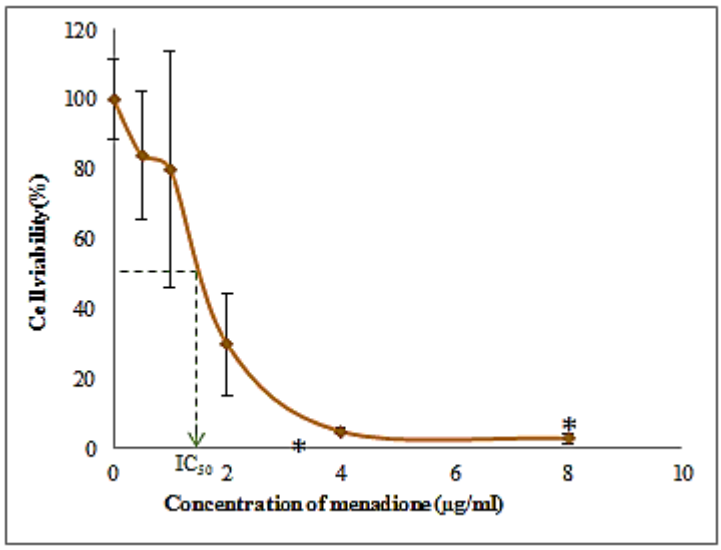

FIG. 2:Cytotoxic activity of menadione concentration ranging from 0 – 8 µg.mL^{-1} after 48 hours of treatment. The data was expressed as mean ± S.E.M. with significant value of p< 0.05. '*' indicates significant difference as compared to the negative control.

Assessment of ROS level

The IC_{50} value obtained from MTT assay was used in this assay to assess the amount of ROS, specifically the superoxide anion level produced by HCT 116 treated with the extract. Figure 3 showed the result of the percentage of cells generated with ROS against time of treatment. Cells treated with the extract showed a slight increase in the level of superoxide anion produced in the cells from the time interval of 30 minutes, one hour and two hours with values 10 ± 2.9 %, 12 ± 0.7 % and 16 ± 7.3 % respectively. Cells treated with menadione again showed a significant increase ($p<0.05$) in the level of superoxide anion produced in the cells. Generally, reactive oxygen species are important for biological systems in human. Cancer cells produce more ROS as compared to normal cells because cancer cells have a higher rate of metabolism than normal cells [15]. However, an abnormal increase of ROS above the threshold level can be harmful towards the cancer cells [16]. Cancer cells are vulnerable towards oxidative stress and easily harmed if any exogenous agents are present to produce ROS and disrupt the antioxidant system in the cancer cells [17]. ROS can cause direct damage to cellular molecules and they seemed to be involved in mediating apoptotic process induced by a variety of stimuli including anticancer agents [18]. Many previous studies reported that plant extracts have a great potential to generate ROS and eventually causing death towards cancer cells. A research reported that ayurvedic *Commiphora mukul* plant had the potential to induce death towards prostate cancer cells through production of ROS [19]. In the present study, level of ROS or specifically the level of superoxide anion was assessed to identify the redox status of HCT 116 after treating with acetone extract from stem bark of *C. odontophyllum*. ROS was chosen as one of the parameters in this study since we aimed to find out the concealing cytotoxic mechanisms of HCT 116 treated with acetone extract from stem bark of *C. odontophyllum*. Moreover, many plant-based extracts are used to investigate the redox status of cancer cell line. In the present study, only a slight increase in the production of superoxide anion level was observed at a time interval from 30 minutes, one hour and two hours. The result obtained in this study contradicts with a previous research [7] where a significant increase in ROS was observed when HCT 116 cells were treated with the sameplant extract. Treatment of traditional Chinese herbs against HCT 116 also showed a significant increase in the production of ROS [20-21]. The difference in the result of the present study and the previous studies may be due to the difference in the phytochemicals present in the plant extract which was used to treat the cells. Terpenoid and flavonoid have the potential to decrease the cell

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viability through the mode of cell death of apoptosis. Previous studies [22-24] reported that plant extracts with terpenoid and flavonoid have the potential to generate ROS and induce ROS-mediated DNA damage leading to cell death against various cancer cells. However, the biological activities of plant extracts cannot be attributed to the presence of individual constituents [25] so the mechanism of cytotoxic activity of acetone extract from stem bark of *C. odontophyllum* could be due to synergistic constituents contained in the extract.

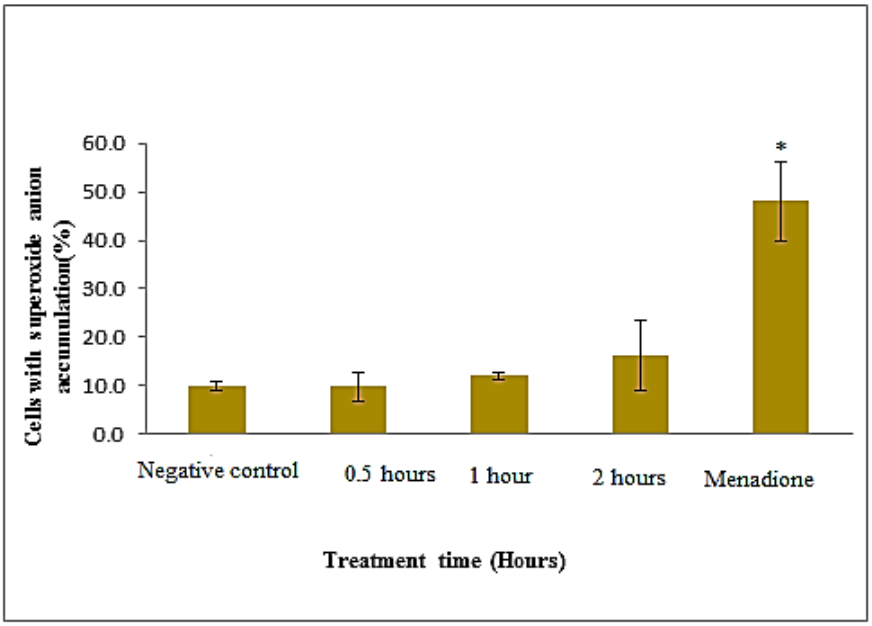

FIG. 3:Percentage of cells with superoxide anion generation in HCT 116 after treatment with IC_{50} for 30 minutes, 1 hour and 2 hours. The data was expressed as mean ± S.E.M. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA. '*' indicates significant difference as compared to the negative control.

Alkaline comet assay

Figure 4 presented the percentage of DNA in the tail while figure 5 presented the tail moment of HCT 116 cells after treatment with acetone extract from stem bark of *C. odontophyllum* at its IC_{10} (22 µg/mL) and IC_{25} (38 µg/mL) values. A concentration-dependent increased manner in the percentage of DNA in tail and tail moment was observed when the cells were incubated with the extract at various concentrations. Cells treated with the IC_{25} value of acetone extract from stem bark of *C .odontophyllum* showed a significant increment in the percentage of DNA in tail and tail moment with the value 15.6 ± 1.1 % and 3.2 ± 0.6 % as compared to cells incubated with IC_{10} value and the negative control. As for menadione, a significant increase in both the percentage of DNA in the tail and the tail moment was observed.

Alkaline comet assay was used to assess DNA damage by the acetone extract from stem bark of *C. odontophyllum* on HCT 116 cells. This assay is very sensitive to determine DNA strand breaks in individual cells [26]. Most of the drugs which have genotoxic properties able to give anticancer effect by causing damage to DNA of cells either by directly or indirectly [27]. A few previous studies reported that plant extract with phytochemicals like terpenoid and flavonoid have the potential to induce DNA damage towards cancer cells. Ganoderic acid is a form of terpenoid isolated from *Ganoderma lucidum* has been proven to exhibit DNA damage towards breast cancer cells [28]. Besides that, 5-methoxyflavone which is an example of flavonoid compound has been reported to induce DNA damage against HCT 116 [29].

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In this study, IC_{10} and IC_{25} values from acetone extract of *C. odontophyllum* stem bark have been used to avoid false positive results of dying or dead cells [30]. In addition,the percentage of DNA in tail and tail moment has been selected as parameters in this assay as both the parameters give the most precise result for the degree of DNA damage [31]. Interestingly, acetone extract from stem bark of *C. odontophyllum* induced DNA damage on HCT 116 cells treated for 30 minutes at both its IC_{10} and IC_{25} values. Both the concentrations demonstrated an increased in the percentage of DNA in tail and tail moment of HCT 116 as compared to the negative control. Both endogenous sources for example, mitochondrial respiration as well as exogenous sources such as ultraviolet radiation, anticancer agents can directly or indirectly induce DNA damage [32]. Reactive oxygen species are always interlinked when there is DNA damage in cells. ROS can induce oxidative DNA damage and cause breaks in the double-stranded DNA [33]. Damage to DNA can inhibit the process of transcription and replication which eventually lead to cell death [34]. The result of the present study showed that acetone extract from stem bark of *C. odontophyllum* can induce DNA damage towards HCT 116. However, the mechanism and the type of lesions involved in HCT 116 after treatment with acetone extract from stem bark of *C. odontophyllum* need to be further investigated.

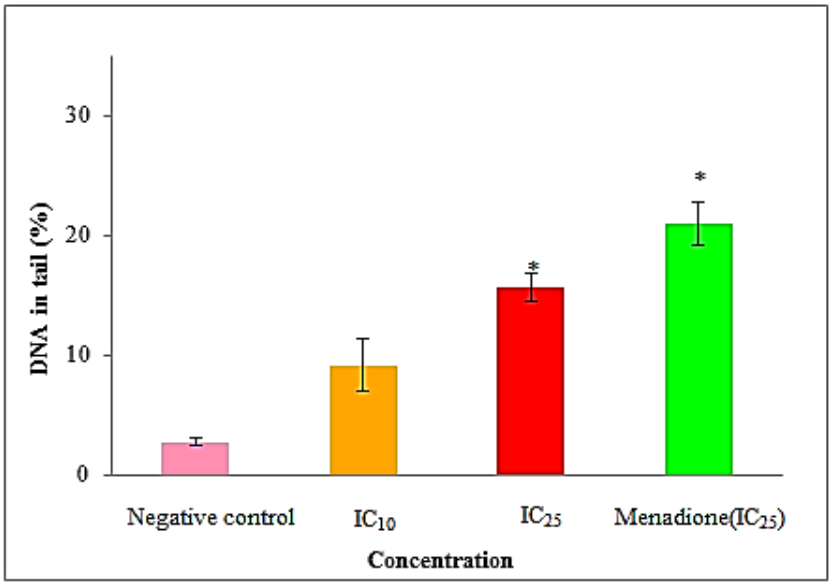

FIG. 4:Percentage of DNA in tail of HCT 116 after treatment with acetone extract from stem abrk of C. odontophyllum and menadione for 30 minutes. The data was expressed as mean ± S.E.M. Data were compared between the untreated negative control and the treatment groups by using one-way ANOVA. '*' indicates significant difference as compared to the negative control.

Conclusion

Acetone extract from stem bark of *C. odontophyllum* exhibited cytotoxic effect against HCT 116 through mechanism involving the production ROS specifically superoxide anion and DNA damage. However, further studies are needed to identify other concealing cytotoxic mechanisms of acetone extract from stem bark of *C. odontophyllum* against HCT 116. This will further aid in identifying the potential of this extract to be developed as anticancer agent.

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