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Original Article

Apoptosis Induction of Cervical Carcinoma HeLa Cells Line by Dichloromethane Fraction of the Rinds of *Garcinia cowa* Roxb

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ABSTRACT

Objective: This study aims to determine the apoptosis induction of HeLa cervical carcinoma cells death by dichloromethane fraction of the rinds of *Garcinia cowa* Roxb. **Methods:** Apoptosis induction of HeLa cell line was observed using a double staining method. **Results:** The result of double staining observation showed that an apoptosis occurs which marked with yellowish green fluorescence and cell fragmentation. The average percentage of apoptotic cells was higher in the treated variables (70.38%) compared to the control variables (12.26%). Statistical analysis by Independent Sample T-Test showed apoptosis Sig. (2-tailed) = 0.000 (<0.025). **Conclusion:** The dichloromethane fraction of *G. cowa* rind induces apoptosis in HeLa cervical cancer cells.

Key words: *Garcinia cowa* Roxb., Apoptosis induction, HeLa cell line, Double stanining, Carcinoma cells.

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INTRODUCTION

Cancer is one among the leading causes of death in worldwide. Cervical cancer is the second most common cancer in women worldwide, but it is the leading malignancy among women in many developing countries. The death rate of cervical cancer is highest in developing countries, especially Indonesia, which ranks second, while in worldwide it ranks fourth.[1]

Cancer cells have distinctive properties that are able to avoid the mechanism of apoptosis or programmed cell death mechanism. Apoptosis is an active cell suicide controlled by a network of genes and is an important process throughout growth as well as playing a key role in the pathogenesis of diseases including cancer.[2] Apoptosis process has an important role in preventing uncontrolled cell growth. There are at least two communication pathways that are involved in apoptosis, the extrinsic and the intrinsic pathway. The extrinsic route is activated by ligand-bound death receptors of the tumor necrosis factor (TNF) receptor group. The intrinsic route is a signal transduction pathway involving the mitochondria and the Bcl-2 group.[3]

Treatment of cancer generally still relies on chemotherapy, a treatment using cytotoxic which has the effect of inhibiting or kill cancer cells. However, it does not only affect target cells (cancer cells) but also affect normal cells around it.[4] To overcome these problems, a research is needed to find a new anticancer drug that have a selective work against cancer cells without damaging normal cells, especially those derived from natural materials.

Asam kandis (*Garcinia cowa Roxb.*) is known to contain xanthones in almost all parts of it, that show a strong cytotoxic effect that can be used as a potential new cytotoxic agents.[5,6] *G. cowa* is potential as chemopreventive agents have been studied *in vitro* against various types of cancer cells, such as HL-60 (leukemia), MCF-7 (breast), DU-45 (prostate), NCI-H460 (NSCL) and T47D (breast).[7-10]

A preliminary study showed that dichloromethane (DCM) fraction from the rinds of *G. cowa* is known to have a cytotoxic effect on HeLa cervical cancer cells with IC_{50} value of 5.764 µg/mL. Through this study, the apoptosis induction of HeLa cervical cancer cell death by DCM fraction of the rinds of *G. cowa* are studied further to develop cancer therapies, especially herbal medicine. The results of this study are expected to increase the potential and value of *G. cowa.* as a new anticancer with specific action.

EXPERIMENTAL SECTION

Chemicals and Reagents

Chemicals and reagents were all of analytical grade.

Extraction

Fresh rinds of *Garcinia cowa* were collected from from Batu Busuk, Padang, West Sumatra, Indonesia in August, 2015 then chopped into small pieces, air-dried at room temperature for 10 days to a constant weight and subsequently pulverized into fine powder. The powdered sample (500 g) was suspended in 4 liters of 70% ethanol for 24 hrs. The mixture obtained was filtered (with Whatman No. 1 fil-

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ter paper) and the resulting filtrate was concentrated with a rortary evaporator (40°C). Thereafter, the product was lyophilized and extracted with dichloromethane to give 7.0 g of the residue, corresponding to a yield of 1.3%. This was then stored in a dessicator for further use.

Experimental Design

Cell Culture

Cancer cells were removed from the freezer (-80ºC), warmed in a water bath at a temperature of 37ºC for 2-3 minutes. After the that, the cells were transferred into a flask which already contains 8 ml of culture media RPMI-1640, incubated for 3-4 hours at a temperature of 37º C, 5% CO_2, and then observed under a microscope to see if the cell is attached at the bottom of the flask and form a monolayer. Growth medium was replaced once in two days and when the number of cells in the flask reached 70-85%, do sub-culture cells. Medium disposed of in the flask, then add 2 -3 ml trypsin-EDTA and stir gently, incubation for 5-10 minutes at a temperature of 37º C, 5% CO_2, then observe the cells under a microscope. Cells that are ready to be used will be separated from the colony. Then the trypsin-EDTA solution containing the cells were centrifuged at a speed of 1500 rpm for 5 minutes. Discard supernatant and the pellet was suspended in 2 ml of medium. Enter into a new flask, stir gently. Incubation at a temperature of 37ºC, 5% CO_2. At the end of incubation, add 2 ml of trypsin-EDTA into the flask containing the cell culture, incubation again for 5-10 minute. Then the trypsin-EDTA solution containing the cells were centrifuged at a speed of 1500 rpm for 5 minutes. Discard supernatant and the pellet resuspended in 1 ml of RPMI medium. Take 10 µL of cell suspension, put in haemocytometer cell counting. Do it under a microscope and counting the average number of cells is determined to make a cell suspension, ie 50.000 cells in a 24 well plate for observation of apoptosis.

Observation of Apoptosis

Apoptosis was detected by double staining method using ethidium bromide (EtBr) and acridine orange (AO). Coverslips were planted into a 24-well plate and the cells with a density of $5x10^4$ cells/mL are distributed thereon. Then performed incubation at 37ºC for 48 hours in a 5% CO2 incubator. Subsequently added to the test solution with a concentration of 6 µg/mL and incubated again for 24 hours. At the end of incubation, the culture medium was taken. Coverslips containing cells removed, then placed on a glass object. Then, on the coverslip is added EtBr-AO Working Solution. Furthermore, the cover slip readily observed under the microscope fluorescence.

RESULTS

Observation of Apoptosis

A fluorescence color differences were seen between living cells and cells undergoing death by apoptosis and necrosis when observed under a narrow band fluorescein (FITC) filter (520-560 nm). Viable cells (live) have a uniform green fluorescence (Figure 1). Cells undergoing apoptosis death fluoresces yellowish green with rounded cell shape and fragmented, whereas cells undergoing necrosis death fluoresces orange-red with crushed cell morphology (Figure 2).[11] Each of these living cells, apoptosis and necrosis are calculated. The average percentage of apoptotic cells was higher in treatment variables (70.38%) compared to the control variables (12.26%).

The data results tested using the Independent Sample T-Test as follows:

1. The homogeneity of variance by Levene's Test for Equality of Variances obtained the value of viable cells with Sig. = 0.167 (p> 0.05), apoptotic cells with Sig. = 0.582 (p> 0.05), necrosis cells with Sig. = 0.076 (p> 0.05), and Bcl-2 gene expression with Sig. = 0.712 (p> 0.05). This suggests that each of the variances of the percentage from viable, apoptosis, and necrosis cells are similar.
2. The Independent Samples T-Test results showed viable cells with Sig. (2-tailed) = 0.000 (<0.025), apoptotic cells with Sig. (2-tailed) = 0.000 (<0.025), necrosis cells with Sig. (2-tailed) = 0.003 (<0.025). This suggests that there is a significant difference in the percentage of viable, apoptosis, and necrosis cells between the control and treatment variable.

DISCUSSIONS

The results of this study showed that HeLa cervical cancer cells death through apoptosis induction.

The apoptosis observation by double staining on the control variables saw a bright green fluorescence cells which showed the living cells and only a few cells seen orange fluorescence or undergo apoptosis. Apoptosis in control cells is normal because apoptosis is also a part of the cell physiological mechanism. In the treatment variable, almost of all the cells die through apoptosis which characterized with yellowish green fluorescence cells. The yellowish green fluorescence indicated the cells undergo DNA fragmentation and chromatin condensation (Figure 1).

Apoptosis in treatment variable still in the early apoptosis. At the late apoptosis, cells will be observed as apoptotic bodies which it colored as orange yellow. Moreover, it also looked an orange-red fluorescence that is indicated the cells undergo necrosis in treatment variable (Figure 1). Necrosis may occur due to environmental factors. Stress occurred during the preparation of cells with double staining which causes the cells die. This cause was also possible.

From the results of cells counting, the cells reduced in viability and increased in a number of apoptotic cells after the cells were treated and incubated for 24 hours. In contrast to the control, after incubation for 24 hours without being treated, the cell viability was much higher and only a few cells undergoing apoptosis (Figure 2). It indicated that the dichloromethane fraction of *Garcinia cowa* rind has a potential to induce cell death by apoptosis.

The ability of dichloromethane fraction of *Garcinia cowa* rind in triggering apoptosis expected because of the xanthone compounds in *Garcinia cowa*. *Garcinia cowa* contains xanthones in almost of all the parts, such as the fruit, bark, roots, stems, leaves and latex, which showed a strong cytotoxic potential against cancer cells.

Additional studies are required to obtain a more significant reduction in the Bcl-2 gene expression. This can be done with an extended incubation time of cells after treatment with a fraction of *Garcinia cowa* rind.

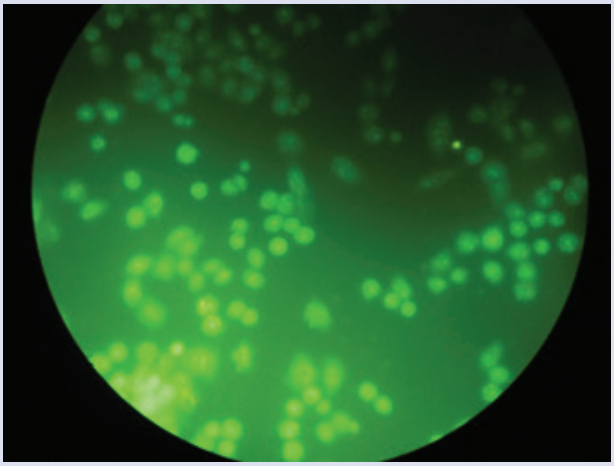

Figure 1: Fluorescence color of cell control after 24 hours incubation. Green fluorescence of viable cells (100x magnification).

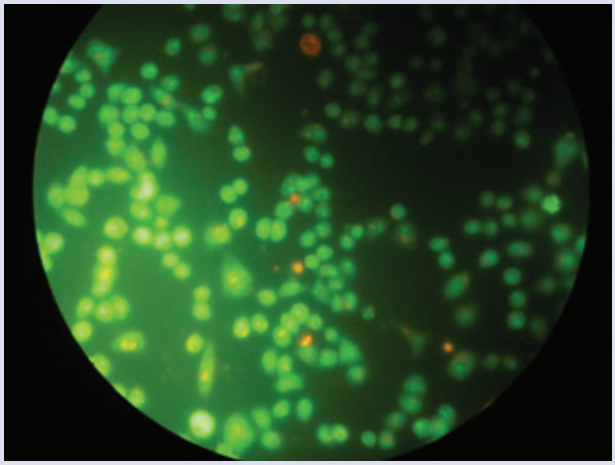

Figure 2: Fluorescence color of the treatment effect fraction dichloromethane rind kandis acid with IC50 6 mg / ml against viable cells, apoptosis and necrosis of HeLa cervical cancer cells at 24 h incubation ( ) viable cells showed green fluorescence, ( ) cells death by apoptosis showed yellowish green fluorescence, ( ) cell death by necrosis showed orange to reddish fluorescence (100x magnification).

Further clarification of molecular action also needs to be done, one of them by observing the proapoptotic gene expression, such as Bax, or their interaction (antiapoptotic and proapoptotic, Bcl-2/Bax) in the process of apoptotic induction. Enhancement of the potential of *G. cowa* as a chemopreventive agent would be very helpful in the development of cancer therapies as well as to minimize the side effects and costs of chemotherapy treatment.

CONCLUSION

The dichloromethane fraction of *G. cowa* rind induces apoptosis in HeLa cervical cancer cells

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CONFLIC OF INTEREST

Authors do not have any conflict of interest.

ABBREVIATION USED

EDTA: Ethylene diamine tetraacetic acid; **EtBr:** Ethidium bromide; **AO:** Acridine orange.

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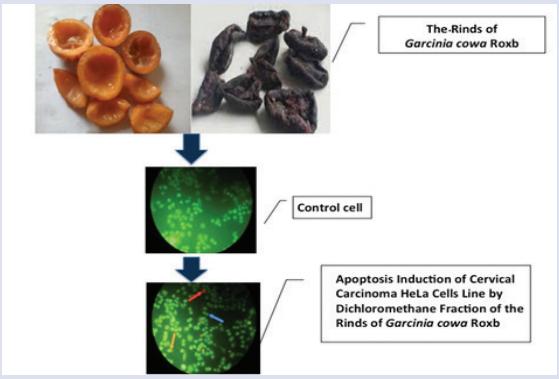
GRAPHICAL ABSTRACT



SUMMARY

The dichloromethane fraction of *G. cowa* rinds provides induces apoptosis in HeLa cervical cancer cells. The average percentage of apoptotic cells was higher in the treated variables (70.38%) compared to the control variables (12.26%). Statistical analysis by Independent Sample T-Test showed apoptosis Sig. (2-tailed) = 0.000 (<0.025).

AUTHOR PROFILE

Fatma Sri Wahyuni: Got her undergraduate degree from Andalas University in 1998 and finished my PhD from University Putra Malaysia in 2010. Her research is in activity studies of Sumatran Plants especially Genus *Garcinia*

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