Scholars Academic Journal of Biosciences (SAJB) **ISSN 2321-6883 (Online)**
Sch. Acad. J. Biosci., 2014; 2(5): 336-340 **ISSN 2347-9515 (Print)**

(An International Publisher for Academic and Scientific Resources)
www.saspublishers.com **DOI:** 10.36347/sajb.2014.v02i05.004

Research Article

Cytotoxic and pro-apototic activities of crude alkaloid from root of sengkubak (*Pycnarrhena cauliflora* (Miers) Diels) in human breast cancer T47D cell line

Masriani[1,2*], Mustofa[3] , Jumina[4], Sunarti[5], Eny Enawaty[1]

[1]Faculty of Mathematic and Natural Science Education, Tanjungpura University, Pontianak, West Kalimantan, Indonesia
[2]Doctoral Program, Study Program of Medicine and Health Science, Faculty of Medicine Gadjah Mada University, Yogyakarta 55281, Indonesia
[3]Division of Pharmacology and Therapy, Faculty of Medicine Gadjah Mada University, Yogyakarta 55281, Indonesia
[4]Departement of Chemistry, Faculty of Mathematic and Natural Science, Gadjah Mada University, Yogyakarta 55281, Indonesia
[5]Division of Biochemistry, Faculty of Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia

***Corresponding author**
Masriani
Email: massefku@gmail.com, masrianinugraha@yahoo.com

Abstract: The purpose of this study is to determine the cytotoxicity, cell cycle arrest and apoptotic activity of the crude alkaloid from the root of *P.cauliflora* in human breast cancer cell line T47D. The alkaloid fraction was evaluated cytotoxic activities on T47D and compared with normal African green monkey kidney ephitelial (Vero) cell lines by using MTT assay. The cell cycle arrest and induction of apoptosis were evaluated by flow cytometry. We observed that the crude alkaloid was cytotoxic and selectively the growth of T47D cells compared to untreated control in a dose-dependent manner with an IC_{50} of 1.5±0.2 µg/ml. However, the crude alkaloid was not toxic to normal African green monkey kidney ephitelial (Vero) cells with an selectivity index (SI) of 21.6. Flow cytometry analysis revealed that crude alkaloid caused cell cycle arrest at G2/M phase and induced apoptosis. Hence, *Pycnarrhena cauliflora* displays potential to be further exploited in the discovery and development of new anticancer agents.
Keywords: *Pycnarrhena cauliflora*, crude alkaloid, cytotoxicity, apoptosis, cell cycle

INTRODUCTION

Cancer is known as leading cause of death world wide and has accounted for 7.6 million death (around 13 % of all deaths) in 2008. Cancer related deaths are predicted to increase to over 11 million in 2030 [1]. Breast cancer is one of the most common human malignancies, which accounts for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008 [2]. Although extensive preclinical and clinical studies have in modest success in decreasing the morbidity of breast cancer, to date no successful chemotherapeutic drug is available to control this disease. Every year, several thousands of new chemical entities undergo screening in various cell culture but eventually do not achieve drug status due to severe side effects. Thus, identification of novel agents that are relatively safe but can suppress growth of human breast cancers is highly desirable.

Pycnarrhena cauliflora (Miers) Diels is one of endogenous plant from West Kalimantan that locally well known as Sengkubak [3]. *Pycnarrhena cauliflora* is one of species of Menispermaceae family, plant family that is rich in bisbenzylisoquinoline alkaloids. This alkaloid are known to have various biological activities including cytotoxic [4-6]. Although the leaves of *P. cauliflora* is traditionally used by Dayak and Melayu ethnics as a natural taste substance, its other utilization has not been recognized, yet. A literature review showed that besides a taxonomic description of the species there were no detailed biological investigations describing phytochemical properties of *P. cauliflora* except one report demonstrating the cytotoxicity of *P. cauliflora* extract against HeLa human cervical cancer cell line [7]. As part of our research program consist in evaluation of the biological activities of bisbenzylisoquinoline alkaloids from *P. cauliflora*, we reported here cytotoxic activities of crude alkaloid of *P. cauiflora* roots against human breast cancer cell line T47D. In addition, the ability of crude alkaloid in induction apoptosis and cell cycle arrest we also reported.

MATERIAL AND METHODS

Reagents

Rosewell Park Memorial institute 1640 medium (RPMI 1640), M-199 medium, phosphate buffer saline (PBS), penicilin-streptomycin, fungizone/amphotericine B, and Trypsin-EDTA 0.25% were purchased from Gibco

(Invitrogen, USA). Foetal bovine serum (FBS) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Company. PI/Rnase staining buffer was purchased from BD Bioscience (SAN DIEGO, USA). Sodium dodecyl sulphate (SDS) and dimethyl sulphoxide (DMSO) were high purity analytical grade were purchased from Merck (Darmstadt, Germany), while commercial grade n-hexane, dichloromethane, methanol were purchased from CV. General Labora, Yogyakarta, Indonesia.

Extraction

Dried and milled roots of *P. cauliflora* (500 mg) were first defatted with hexane for 3 days at room temperature, then filtered. The plant residues were re-extracted five times. After that they exhaustively extracted with dichlorometane followed with methanol . Finally, the combined extracts were concentrated in vacuum at 40^{o}C using a rotary evaporator. The methanol extract then acidified by the addition of 0.1 N hydrochloric acid solution. The acid solution was then filtered and made alkaline with 0.1 N sodium hydroxide solution to pH 10 and restricted with dichloromethane. The dichloromethane extract was evaporated to dryness to give the crude alkaloid. The crude alkaloid were used for the cytotoxic activity, induction of apoptosis and cell cycle arrest.

Cell Culture

Human breast cancer (T47D) and normal African green monkey kidney epithelial (Vero) cell lines were obtained from Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. T47D and Vero cells were cultured in RPMI 1640 and M199 media, respectively and both media were supplemented with 10% (v/v) foetal bovine serum, 2 % streptomycin–penicillin and 5% amphoterizine. Cells were maintained in 25 cm^2 flasks with 7 ml of media and were incubated in a 5% CO_2 incubator at 37^{o}C. Cells were harvested using 0.25 trypsine-EDTA when they reach 70-80% confluence in culture flasks.

Cell viability assay

The cytotoxicity of T47D and Vero cells was tested by the 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. The T47D and Vero cells were seeded at a density of 1×10^4 cells per well in a volume of 100 µL in 96-well plates and incubated overnight for attachment . Cells were incubated with different concentrations of crude akaloid of *P. cauliflora* for 24 h. After incubation, the media were removed and 100 µL of RPMI and 10 µL MTT (5 mg MTT/mL solution) were added to each well. The plates were incubated for 4 h. Control cells received only the media without the tested samples. The formazan crystals that formed in the viable cells were solubilized with 100 µl of SDS- stopper in HCl 0.1 N. Absorbance at 595 nm was measured by microplate enzyme-linked immunosorbent assay (ELISA) reader. Each measurement was performed in triplicate. The experimental data were absorbance of each well, and then converted to percentage of viable cells. Percentage of viability was calculated with the following formula:

$$\text{Percentage of viability} = \frac{A_{sample}-A_{medium}}{A_{control}-A_{medium}} \times 100\%$$

The cytotoxic dose that kills cells by 50% (IC_{50}) was determined from Probit analysis. The selectivity index (SI) was determined by the ratio between IC_{50} of the crude alkaloid on normal African green monkey kidney epithelial (Vero) cell and IC_{50} of the crude alkaloid on cancerous T47D cell.

Selectivity index (SI) =

$$\frac{\textbf{IC}_{50}\textbf{ of the crude alkaloid on normal African green monkey kidney ephitelial (Vero) cells}}{\textbf{IC}_{50}\textbf{ of the crude alkaloid on cancerous T47D cells}}$$

SI value indicates selectivity of the sample to the cell lines tested. Samples with an SI greater than 3 were considered to have a high selectivity towards cancerous cells [9-10].

Cell cycle and apoptosis analysis

Human breast cancer T47D cells were seeded in a 6-well plate at a density 5 x 10^5 cells/ml and treated with 2 and 4 µg/mL of crude alkaloid of *P. cauliflora* roots after 24 h incubation. The total population of cells, including adhering and floating cells was harvested and washed twice with PBS. After centrifugation at 600 x g for 5 min, the cell pellet was washed twice with PBS and re-suspended with 0.5 ml PI/RNase detection kit. The cells were then incubated at room temperature for 15 min in the dark. Cell cycle phase distribution of nuclear DNA was determined by flow cytometry (Becton Dickinson FACS Calibur) by analyzing at least 20,000 cells per sample. The percentage of cells in the G0/G1, S and G2/M phases were analyzed by ModFit LT 3.0 Cell Cycle analysis software (Becton Dickinson). The percentage of hypodiploid cells (Sub-G1) over total cells was calculated and expressed as percentage of apoptotic cells.

Statistical analysis

Results were expressed as the mean ± SD of data obtained from triplicate experiments.

RESULTS AND DISCUSSION

Cytotoxic effect of the crude alkaloid of *P. cauliflora* roots in T47D cells

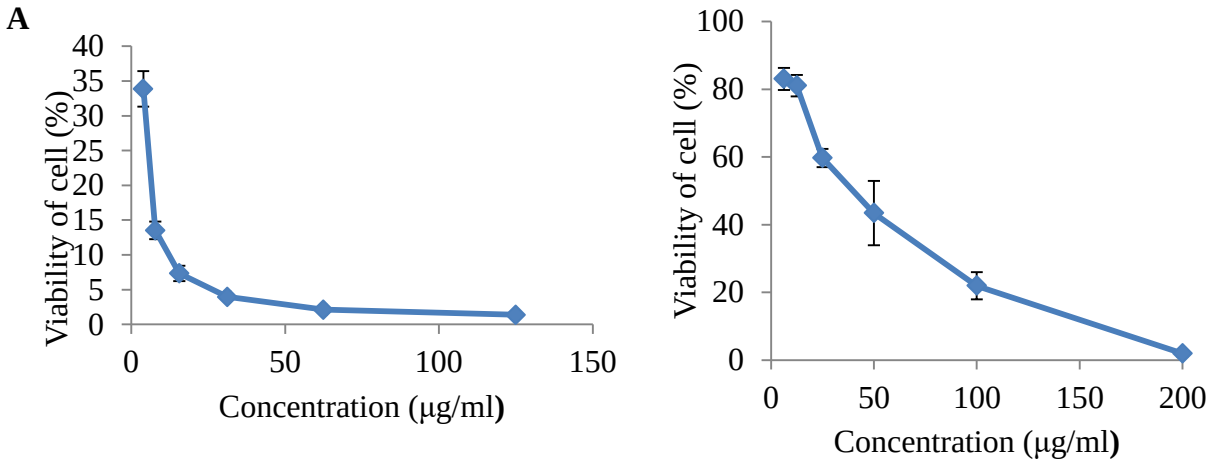

Fig-1: The effect of the crude alkaloid of *P. cauliflora* on viability of T47D (A) and Vero (B) cells. Cells (1 x 10^4 cell/well) were incubated for 24 h with different concentrations of crude alkaloid. Cell viability was evaluated as the ability of cells to reduce MTT to blue formazan crystals.

In this study, we first examined cytotoxicity and selectivity of crude alkaloid of *P. cauliflora* roots on human breast cancer (T47D) and normal African green monkey kidney ephitelial (Vero) cell lines using the 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. As shown in Figure 1 A and 1B, crude alkaloid inhibit the growth of T47D and Vero cells in a dose-dependent manner. The growth of T47D cells decreased with increasing concentrations of crude alkaloid. The half maximal inhibitory concenration (IC_{50}) value was determined from the probit analysis and set as a parameter for cytotoxicity.

IC_{50} values (µg/ml) of crude alkaloid of *P. cauliflora* roots on both human breast cancer T47D cells and Vero cells are summarized in Table 1. According to the criteria of the American National Cancer Institute to consider a crude extract promising for further purification based on the IC_{50} values lower than 30 µg/ml in order to discover and develop potential anticancer natural compounds [8]. The crude alkaloid showed strong cytotoxic effect on T47D cells IC_{50} 1.5 ± 0.2 µg/ml. It showed relatively low cytotoxic effect on the non-cancerous cell line Vero with an IC_{50} of 32.4±1.2 µg/ml. Based on this criteria, the crude alkaloid potential to be developed as new anticancer drug.

Idealnya, one of criteria for a drug being good is that it should not exhibt any andesirable side-effects on normal cells. Therefore, selective effect of the anticancer agents according to the tested cell line is very important for the anticancer research. Selectivity of cytotoxic activity of crude alkaloid was determined by comparing the cytotoxic activity (IC_{50}) of crude alkaloid against T47D cancerous cells with that of the African green monkey kidney ephitelial (Vero) cells and results was expressed as a selectivity index (SI). As the SI greater than 3 was concidered as highly selective [9-10]. The crude alkaloid demonstrated highly selective to cancer cells with SI value 21.6.

A relatively low cytotoxic effect on normal Vero cells indicated that the crude alkaloid was not deleterious to non-cancerous cells. It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cell only [11]. Data from this study showed that the crude alkaloid of *P. cauliflora* roots was highly selective towards the T47D cancer cells as compared to normal Vero cells. These data suggest that crude alkaloid specially induces cytotoxicity in T47D cells but not in normal cells.

Table 1. Cytotoxicity and selectivity of crude alkaloid from root of *P. cauliflora* on human breast cancer cell lines T47D using MTT assay

Cell line	IC_{50}[a] (µg/ml)	SI
T47D	1,5±0,2	21,6
Vero	32,4±1,2	

Cells were treated with crude alkaloid at different concentrations for 24 h. IC_{50} values were calculated using Probit analysis. SI >3 indicates high selectivity [9-10]. [a] Data are shown as mean ± SD

Crude alkaloid of *P. cauiflora* roots induce cell cycle arrest followed by apoptosis

To assess the effect of crude alkaloid on cell cycle progression, T47D cells were harvested after 24 h exposure to 0, 2, and 4 µg/ml crude alkaloid and stained with PI for flow cytometri analysis and the results were shown in Fig.2. The sub G1 population indicated apoptotic–associated chromatin degradation. As the treatment concntration increased, the percentage of cells in sub-G1 increase from 4.16% (control) to 5.29% (2 µg/ml) and 11,23% (4 µg/ml). In the non apoptotic popuation, cells in the G2/M phase increased from 20.45% (control) to 20.76% (2 µg/ml) and 24,25% (4 µg/ml), and the G0/G1 phase decreased from 39.81% (control) to 37.40% (2 µg/ml) and 28.51% (4 µg/ml).

The life and death of a cell is controlled by the cell cycle, which is tightly regulated by cyclin, cyclin-dependent kinases (CDK), and CDK inhibitor (CDKI). Thus, deregulation of cell cycle will lead to abnormal proliferation of DNA-damaged cells and evasiveness of apoptosis [12]. Disturbance of the cancer cell cycle is one of therapeutic targets for development of new anticancer drugs[13]. Acumulated evidence has shown that cell cycle arrest might result in apoptosis due to the existence of cell cycle check point and feedback control [14]. Many anti-cancer agents arrest the cell cycle at the G0/G1, S or G2/M phase and then induce apoptotic cell death [15-19]. But several evidence have suggested that some anticancer drug induced apoptosis may occur via signaling path way independent of cell cycle arrest[16]. The result of cell cycle analysis evaluated by flowcytometri showed that crude alkaloid could induce cell cycle arrest in G2/M phase. It suggest that apoptosis induced by crude alkaloid is related to cell cycle arrest.

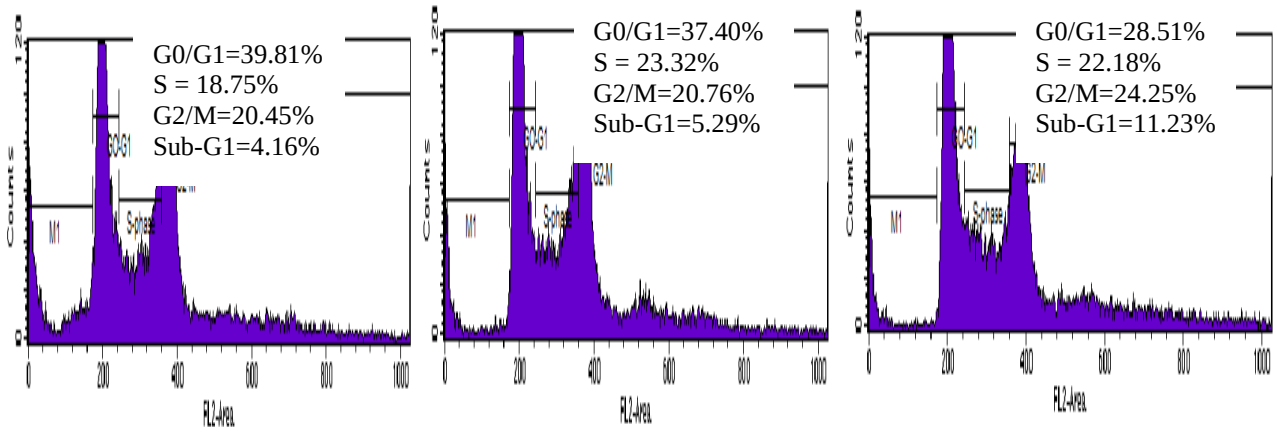

Fig-2: Effect of crude alkaloid on cell cycle progression. T47D cells were untreated or treated with 2 µg/ml and 4 µg/ml crude alkaloid, respectively. Cells washed and stained with PI, and then DNA contents was analysed by flow cytometry.

The G2/M transition is positively regulated by the CDC2 and cyclin B complex [20]. The G2/M arrest in T47D induced by crude alkaloid at concentration 2 and 4 µg/ml may be related to down regulation of Cyclin B and CDC2, whereas apoptosis that occured could be due to the down-regulation of NF-κB. Expression of several NF-κB regulated genes including Bcl-2, Bcl-XL, cIAP, surviving, TRAF1 and TRAF 2 have been reported to function primarily by blocking the apoptosis pathway [21].

CONCLUSION

The crude alkaloid of *P. cauliflora* root exhibited strong cytotoxic and selectivity effect on human breast cancer T47D cell lines. The cytotoxic effects caused by inducing apoptosis and causing cell cycle arrest at G2/M phase.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We gratefully thank to DP2M DIKTI (Directorate of Higher Education) Ministry of Education and culture, Indonesia through “Hibah Bersaing” Research Grant 2013 for financial support in this study.

REFERENCES

1. World Health Organization; WHO Library Cataloguing-in-Publication Data Worls Health Statistic 2010. World Health Organization. [NLM classification:WA 900.1]
2. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D; Global cancer statistics, Cancer J. Clin., 2011;61(2):69–90.

3. Utin Trisna Afrianti. Kajian etnobotani dan aspek konsenrvasi sengkubak [Pycnarrhena cauliflora (Miers)Diels.] di Kabupaten Sintang Kalimantan Barat. Tesis, Sekolah Pascasarjana Institut Pertanian Bogor, Bogor, 2007
4. Wang G, Lemos JR, Ladecola C; Herbal alkaloid tetrandine: from an ion channel blocker to inhibitor of tumor proliferation. Trends Pharmacol. Sci., 2004; 25:120:123
5. Wang JZ, Chen QH, Wang FP;Cytotoxic bisbenzylisoquinoline alkaloids from the roots of cyclea racemosa. J.Nat.Prod., 2010; 73(7):1288-1293.
6. He BC, Gao JL, Zhang BQ, Luo Q, Shi Q, Kim SH, Huang E, Gao Y, Yang K, Wagner ER, Wang L, Tang N, Luo J, Liu X, Li M, Bi Y, Shen J, Luther G, Hu N, Zhou Q, Lu HH, Haydon RC, Zhao Y, He TC;Tetrandine inhibits Wnt/β-signaling and suppresses tumor growth of human colorectal cancer. Mol Pharmacol., 2011; 79(2):211-219
7. Masriani, Mustofa, Jumina, Sunarti;Pycnarrhena cauliflora ethanolic extract induces apoptosis and cell cycle arrest in Hela human cervical cancer cells. Inter. J. Res. Pharm. Biomed. Sci., 2013; 4 (4):1060-1068
8. Itharat A, Houghton PJ, Eno-Amooquaye E, Burke PJ, Sampson JH, Raman A; In vitro cytotoxic activity of Thai medicinal plants used traditionally to treat cancer. J. Ethnopharm, 2004; 90:33-38
9. Prayong P, Barusrux S, Weerapreeyakul N; Cytotoxic activity screening of some indigenous Thai plants. Fitoterapi, 2008; 79:598-601.
10. Mahavorasirikul W, Viyanant V, Chaijaroenkul W, Itharat A, Na-Bangchang K; Cytotoxic activity of Thai medicinal plants against human cholangiocarcinoma, laryngeal and hepatocarcinoma cells in vitro. BMC Compl. Alt. Med., 2010; 10:55
11. Hu X, Zhang X, Qiu S, Yu D, Lin S; Salidroside induce cell induces cell cycle arrest and apoptosis in human breast cancer cells, Biochem. Biophysic. Res. Commun., 2010; 398:62-67
12. Schwartz GK, Shah MA; Targeting the cell cycle: A New approach to cancer therapy, J. Clin. Oncol., 2005; 23:9408-9421
13. Carnero A;Targeting the cell cycle for cancer therapy, Br. J. Cancer, 2002; 87(2):129-133
14. Pietenpol JA, Stewart ZA; Cell cycle checkpoint signaling: cell cycle arrest versus apoptosis. Toxicology, 2002; 181-182:475-481
15. Cheng YL, Chang WL, Lee SC, Liu YG, Chen CJ, Lin SZ; Acetone extract of Angelica sinensis inhibits proliferations of human cancer cells via inducing cell cycle arrest and apoptosis , Life Sci., 2004; 75:1579-1594
16. Hsu YL, Kuo YC, Kuo PL, Ng LT, Kuo YH, Lin CC; Apoptotic effects of extract from Antrodia camphorate fruiting bodies in human hepatocelluler carcinoma cell lines, Cancer Lett., 2005; 221(1):77-78
17. Purohit A, Hejaz HA, Walden L, Morrogh M, Packam G, Potter BV, Reed MJ; The effect of 2-methoxyoestrone-3-O-sulphamate on the growth of breast cancer cells and induced mammary tumors. Int. J. Cancer, 2000; 85:584–589.
18. Fujimoto K, Hosotani R, Doi R, Wada M, Lee JU, Koshiba T, Miyamoto Y, Tsuji S, Nakajima S, Imamura M; Induction of cell cycle arrest and apoptosis by a novel retinobenzoic acid derivative, TAC- 101, in human pancreatic-cancer cells, Int. J. Cancer, 1999; 81:637–644.
19. Sun J, Liu RH;Cranberry phytochemical extracts induce cell cycle arrest and apoptosis in human MCF-7 breast cancer cells. Cancer Lett., 2006; 241:124-134
20. Coqueret O;Linking cyclins to transcriptional control, Gene, 2002; 299:35-35
21. Anggarwal BB; Nuclear factor-kappa B: the enemy within growth factor-induced cell transformation and activator protein, Cancer cell, 2004;16(3):203-208