

Soursop Effect in Cervical Cancer Apoptosis Mechanism

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Abstract

Cervical cancer is the fifth leading cancer cause of women death in Indonesia. Acetogenin, flavonoids, and tannins in soursop leaves have anti cancer effects through regulated genes which involved in apoptotic process such as in caspase-3. This study aimed to determine the effect of ethanol extract of soursop leaves to apoptosis and caspase-3 gene expression in HeLa cell cultures. This was an *in vitro* study using HeLa cell culture samples divided into 4 groups in laboratory of Biological Science Center Institute Technology Bandung. The first group was HeLa cell cultures without soursop leaves ethanol extract. The 2nd, 3rd, and 4th group were HeLa cells cultures which were given soursop leaves ethanol extract with concentration of 60 µg/mL, 120 mg/mL, and 240 mg/mL, respectively. Apoptosis in each group was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method and the expression of the caspase-3 gene by reverse transcriptase-polymerase chain reaction (RT-PCR). One way analysis of variance (ANOVA) with confidence level of 95% were used as statistical analysis. The result showed the effect of the soursop leaves ethanol extract increased the apoptosis percentage in HeLa cells culture but did not affect the gene expression of caspase-3.

Key words: Apoptosis, caspase-3, soursop leaves

Efek Daun Sirsak dalam Mekanisme Apoptosis Kanker Serviks

Abstrak

Kanker serviks adalah penyebab kematian kelima untuk wanita di Indonesia. Asetogenin, flavonoid, dan tanin dalam daun sirsak terbukti mempunyai pengaruh antikanker dengan regulasi gen melalui proses apoptosis seperti pada *caspase-3*. Penelitian ini bertujuan untuk menentukan efek ekstrak etanol daun sirsak untuk apoptosis dan ekspresi gen *caspase-3* dalam kultur sel HeLa. Penelitian ini adalah penelitian *in vitro* menggunakan sel kultur HeLa yang dibagi menjadi 4 kelompok dilakukan di laboratorium *Biological Science Center* Institut Teknologi Bandung. Grup pertama adalah sel kultur HeLa tanpa ekstrak etanol daun sirsak, sedangkan grup dua, tiga, dan empat adalah sel kultur HeLa yang mendapat ekstrak etanol daun sirsak dengan konsentrasi 60 µg/mL, 120 mg/mL, dan 240 mg/mL secara berturut-turut. Apoptosis dalam setiap kelompok diperiksa menggunakan metode *terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling* (TUNEL) dan ekspresi gen *caspase-3* dengan menggunakan *reverse transcriptase-polymerase chain reaction* (RT-PCR). *One way analysis of variance* (ANOVA) dengan interval kepercayaan 95% digunakan untuk analisis statistik. Hasil penelitian menunjukkan bahwa ekstrak etanol daun sirsak meningkatkan persentase apoptosis dari kultur sel HeLa tetapi tidak berpengaruh pada ekspresi gen *caspase-3*.

Kata kunci: Apoptosis, *caspase-3*, ekstrak daun sirsak

Introduction

Mortality caused by cancer in Indonesia is increasing and now it is the fifth leading cause of death for cancer in Indonesia. Approximately 500,000 women in the whole world are diagnosed with cervical cancer each year and more than 270,000 die.^{1,2} In addition, cervical cancer causes highest fatality with approximately of 75%.³⁻⁷ Two decades of molecular and epidemiological studies shows that human papilloma virus (HPV), especially HPV 16 and HPV 18, causes cancer especially for cervix uteri, because the expression of the HPV oncoprotein (E6 and E7) can deregulate proteins which play a role in the cell cycle.³⁻⁷ The ability of HPV oncoprotein to undermine growth regulatory proteins can affect the CDK inhibitor early in carcinogenesis and tumor invasion. P53 is rarely mutated in human tumors, and its expression level is a significant tumor prognostic factor.⁸

The life and death of cells must be balanced in order to maintain homeostasis. One mechanism for homeostatic mammalian's cell death is through apoptosis. Apoptosis is a process of programmed cell death, genetically regulated, active, and characterized by chromatin condensation, cell fragmentation and phagocytosis of these cells by neighboring cells. Disturbances in the control of apoptosis have a clear contribution to the pathogenesis of many human diseases including cancer. Disturbances in the apoptotic pathway may accelerate the expansion of neoplastic cell populations and affect the intrinsic ability to respond to therapy. Apoptosis is a form of cell death that depends on the result of intracellular gene expression. Cell survival is controlled by genes that stimulate and inhibit apoptosis.^{9,10}

The process of apoptosis is divided into two phases namely initiation phase and the execution phase. Initiation of apoptosis occurs because of the signals from different pathways, that are the extrinsic pathway (death receptor pathway initiation) and the intrinsic pathway (mitochondrial pathway). Both pathways equally activate caspase enzymes and related one to the other in some phases.⁹⁻¹²

In recent years, studies on tumor cell biology and molecular biology has been recognized that the incidence and progression of cancer is not only resulting to the proliferation and differentiation disorder, but also closely related

to the abnormal apoptosis. Induction and promotion of apoptosis of cancer cells may also be targeted for cancer therapy.

The development of all chemotherapeutic agents from natural materials which are effective and has low toxicity are rare to find, therefore, scientific research to find other chemotherapeutic cancer agents produced from natural materials in an efficient and affordable way is needed.

Indonesia is known to have abundant of natural ingredients that are assumed can be used to prevent and cure cancer, one of which is *Annona muricata* Linn (Annonaceae) generally known as soursop or graviola. Various chemical compositions have been isolated from its various parts like its roots, barks, leaves, fruits and seeds. Some phytochemicals that are reported to have been isolated and characterized from different parts of this plant are annonaceous acetogenins, lactones, isoquinoline alkaloids, tannins and others.^{13,14}

Annonaceous acetogenins are a large group of phytochemicals that naturally contains a polyketide that have anti-cancer activity.¹³⁻¹⁵ The first generation of annonaceous acetogenins (1, AA005) showed significant activity as a natural product that has a high selectivity between cancer cells to normal cell.¹⁶ Wu et al¹⁷ have isolated from *Annona muricata* leaves two additional types of active substances called monotetrahydrofuran annonaceous acetogenins which are anomuricin (C1) and muricatocin (C2). These substances have cytotoxic effects against human lung cancer A-549 and MCF-7 human breast solid tumor cell lines. Li et al¹⁸ found several types of acetogenins from Annonaceae seed that showed antitumor activity.

Research conducted by Chiu et al¹⁹ reported that bullatacin which is one type of annonaceous acetogenins isolated from *Annona muricata* seeds have the ability to induce apoptosis in human hepatoma cell line. Another study conducted by Liu et al²⁰ concluded that annonaceous acetogenin can inhibit Raji cell proliferation and induce apoptosis in Raji cells by increasing the expression of caspase.

HeLa cell culture or HeLa cell line is a continuous cell line derived from epithelial cells of cervical cancer of a women with HPV-positive cervical cancer, which express the E6 and E7 proteins. These cultured cells have semi-

attached properties used as models for studying cancer cells and cellular signal transduction. The effect of soursop leaves ethanol extract against cell death in cultured HeLa cells as the therapeutic target for uterine cervical cancer can be determined by the TUNEL method of DNA hybridization. The authors hypothesize that the soursop leaves ethanol extracts can enhance the amount of apoptotic cell death and expression of caspase-3 gene.

Methods

Consecutive sampling method was used with object of HeLa cells satisfy the inclusion and exclusion criteria to meet the required sample size which was 12. Using Gomez formula to calculate samples, a minimum of three samples for each experiment groups will be used. HeLa cells samples were divided into 4 treatment groups, with the inclusion criteria of HeLa cell cultures which are uncontaminated, sub confluent and without any morphological changes. The material used in this study are as follows: Soursop leaves, cell medium: RPMI-1640 (Gibco), Fetal bovine serum 10% FBS (Gibco), fungison 0.5% (Gibco), 1% penicillin and streptomycin (Gibco), RNA isolation kit (Tri pure isolation reagent), RT-PCR kit, materials forgel: TAE buffer, agarose, ethidium bromide and RNase-DEPC free content water-treated 0.5% SDS.

This research was an *in vitro* experimental study. The dependent variables are: caspase-3 and the number of apoptosis. The independent variables are concentrations of HeLa cells and the concentration of soursop leaves extract. Variables controlled are HeLa cells, medium and incubation (CO₂) and temperature. MTT toxicity test method is conducted first to determine the IC₅₀ dose.

The experiment cells were divided into 4 groups, each group consisting of 3 samples. Each group had its own treatment. Group I (negative control): 1 mL suspension HeLa cells in RPMI-1640 medium culture. Group II: 1 mL suspension HeLa cells + 60 µg/mL soursop leaves ethanol extract in the RPMI-1640 medium culture. Group III: 1 mL suspension HeLa cells + 120 µg/mL soursop leaves ethanol extract in RPMI-1640 medium culture. Group IV: 1 mL suspension HeLa cells + 240 µg/mL soursop leaves ethanol extract in RPMI-1640 medium culture. IC₅₀ is determined using MTT

method, with the microplate incubated in 5% CO₂ incubator for 24 hours at 37 °C.

Soursop leaves were taken from trees in the area of Bandung. The extraction processes was conducted in the Biological Science Center ITB, Bandung Laboratory, 10 kg wet soursop leaves was cut and washed thoroughly and oven baked dried. After drying, the crude is refined by means of grinding to produce 1 kg of dry powder. The powder was put into the cotton based maserator with added 95% ethanol and it was let to stand for 24 hours. The solution was removed from the maserator. The extract filtered to form a dilute extract. A new ethanol was add into the remains left in the maserator. The addition of ethanol is repeated to the solution until it was colorless (5–6 times). Concentrated aqueous extract obtained from maserator was put to a rotatory evaporator until there are no more solvent dripping from evaporator condenser. The produce obtained was concentrated pasta extract.

HeLa cell growth:

Growth medium used was RPMI-1640 (Roswell Park Memorial instituted), which is a widely used medium for growing mammalian cells, with the addition of 10% FBS (fetal bovine serum), Fungison 0.5% and 1% penicillin-streptomycin. Cell concentrations used were 1x10⁵ cells/mL filled into 15 wells with 1 mL of cell suspension.

Measurement of gene expression:

Once the cells are sub confluent grown, the medium is replaced with the soursop leaves ethanol extract with predetermined concentrations. HeLa cells with soursop leaves ethanol extract is incubated in 5% CO₂ at 37 degrees Celsius within 24 hours. Media cell culture were ready to be harvested by aspiration and isolation of RNA in HeLa cells using Trizol Method.

Reverse transcriptase polymerase chain reaction or RT-PCR into a microcentrifuge tube that contained RNA put 12.5 mL DEPC treated water, Primer {oligo(dT) 18} 1 mL, and 3 mL total RNA template. Add other materials in this order: reverse transcriptase 5xreaction buffer, RNAase inhibitory block TM (0.5 mL: 20 U), dNTPmix, 10 m each 2 mL (1 mM final concentration, M-MuLV reverse transcriptase (2 mL: 40 u). Shuffle and centrifuged. Incubate for 60 minutes at 37 °C.

Primer human caspase-3 (272 bp)²²

Forward

primer:

5'-CAAACITTTTTCAGAGGGGATCG-3'
Reverse primer :

5'-GCATACTGTTTCAGCATGGCAC-3'
Denaturate at 98 °C for 3 min, followed by 60 °C for the addition of 1 U of Taq DNA polymerase, annealing at 72 °C for 30 seconds, 4 cycles of denaturation at 94 °C for 30 seconds, then held at 60 °C for 30 seconds, primer elongation at 72 °C for 30 seconds, 30 cycles.

Primer human beta actin (340 bp)

Forward primer: 5'-GCTGTGCTATCCCTGTA-3'

Reverse primer: 5'-GCCTCAGGGCAGCAGCGG-3'
cycles of denaturation at 98 °C for 3 min, annealing at 72 °C for 30 seconds, 4 cycles of denaturation at 94 °C for 30 seconds, then the extension at 60 °C for 30 seconds, 30 cycles.

PCR results are checked using ethidium bromide agarose gel concentration of 0.5%

The results of gene expression electrophoresis images are converted into numeric form using scion image. The software will calculate the points contained in Warba electrophoresis images that had previously been inverted.

Measurement of apoptosis:²³

Cells isolation was done by incubation harvesting. Storing cells on a polylysine slide fixating cells with 1% formaldehyde in PBS (pH 7.4) for 15 min at 40 °C, wash the cells

twice with PBS. Suspended in 70% ice-cold ethanol and stored at -20 °C until used.

Drop 50 mL TUNEL labeling mix (consisting of 5 mL enzyme deoxy nucleo tydil transferase terminal and 45 mL fluorescein-dUTP) and incubate (60 min at 37 °C). Wash with PBS preparations, add anti-fluorescent-POD and incubate (30 min, 37 °C). Wash with PBS preparations, add DAB substrate and incubate (5-20 min, RT). Wash with PBS, add counter stain. Wash with water and dry, wash with absolute alcohol to clear and dry. Close preparations with entelan and silicone cover slip. Observe and count apoptotic cells (brown) using a light microscope. Document each observation
Data analysis: Data will be presented in the form of electrophoresis results of RT-PCR caspase-3 mRNA from the control group and the experiment group and the calculation of the amount of apoptosis in both groups. Effect of soursop leaves extract on the expression of the caspase-3 gene as well as the amount of apoptosis in HeLa cell cultures were tested using one way analysis of variance (ANOVA).

Implications of research ethics:

HeLa cell culture used is a continuous cell line derived from epithelial cells womb (cervix) of a woman with cervical cancer named Henrietta

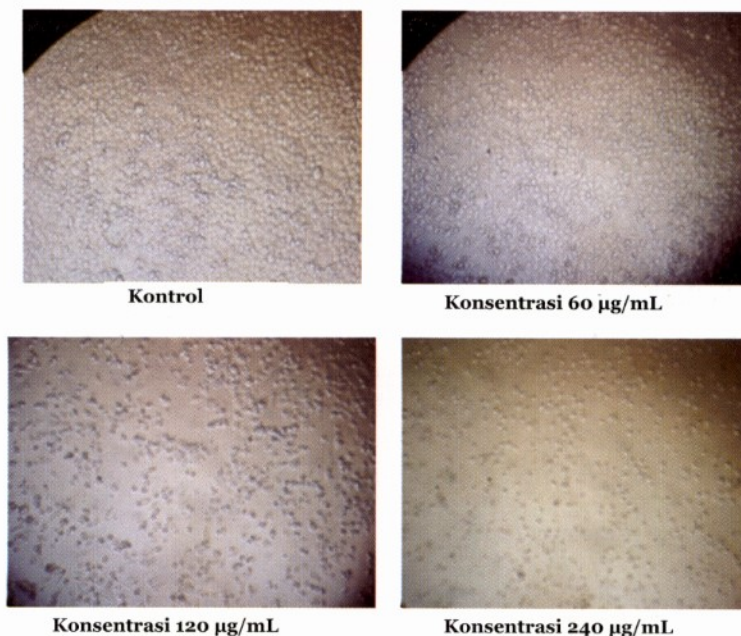


Figure 1 Decreased in Number of Cells on Hela Cell Culture Treated by Soursop Leaves Ethanol Extract

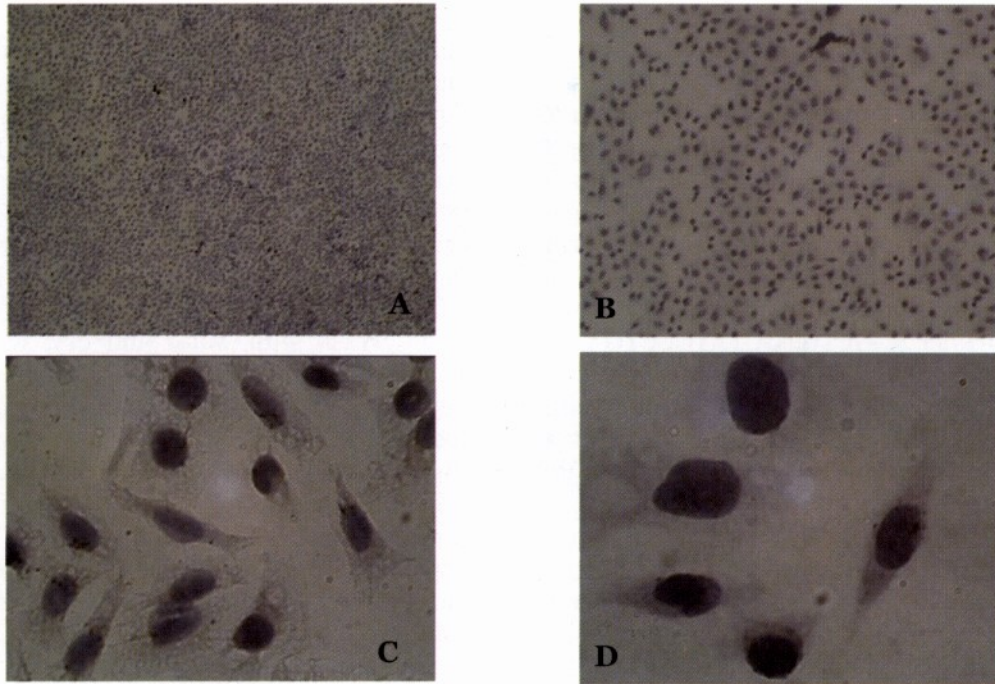


Figure 2 Group I (Control; Untreated HeLa Cell Culture), Magnification by 40x (A), 100x (B) 400x (C), 1,000x (D)

Lacks, who died from cancer in 1951. Cell culture has been widely used as research material. This study used HeLa cells that have long been kept and developed at the Parasitology Laboratory of University of Gadjah Mada. Researchers have obtained permission from the head of Parasitology Laboratorium Gadjah Mada University. Using HeLa cells in this study performed best for the advancement of science, research and the public interest by considering aspects of humanity and respect for Henrietta Lacks.

Results and Discussion

In this study, we administered soursop leaves ethanol extract to see its effect on cell death. The parameter being measured is the percentage of apoptosis and caspase-3 gene expression in HeLa cells culture. Untreated HeLa cells were used as control. To determine the feasibility of the test, HeLa cells β -actin gene functioned as the positive control.

The results of MTT assay showed that IC_{50} of soursop leaves ethanol extract against HeLa cells were much smaller than the water extract. It meant that the soursop leaves ethanol extract is much more potent as anti cancer on HeLa cells.

IC_{50} level of soursop leaves ethanol extract for HeLa cells was 120 microg/mL. The dosages used were 60 μ g/mL ($0,5 \times IC_{50}$), 120 μ g/mL (IC_{50}) and 240 μ g/mL ($2 \times IC_{50}$), respectively.

Observations on HeLa cells culture indicates that there was a decrease in the number of cells. Figure 1 showed HeLa cells culture with 60 μ g/mL ($0,5 \times IC_{50}$), 120 μ g/mL (IC_{50}) and 240 μ g/mL ($2 \times IC_{50}$) of soursop leaves ethanol extract compared with controls.

In control group fibroblast are clearly shown and homogenous and it has clear boundaries, with close distance between cells and cells were intact. In group 2, fibroblast were still found but were not as homogeneous, some had unclear cell borders. The distance between cells were slightly apart, and there were some dead cells, showed by rounded cells. In group 3 (120 μ g/mL) the cells shape were not homogenous, borders were not clear with the distance between cells further apart, and there were many dead cells (rounded cells). In group 4 (240 μ g/mL) round cells were found in all field, with unclear borders and the distance between cells were very far.

Calculation of apoptosis cells number:

In group I (Fig. 2), the morphology of cells appeared to be normal such as there was the present of fibroblasts, nucleus located in the

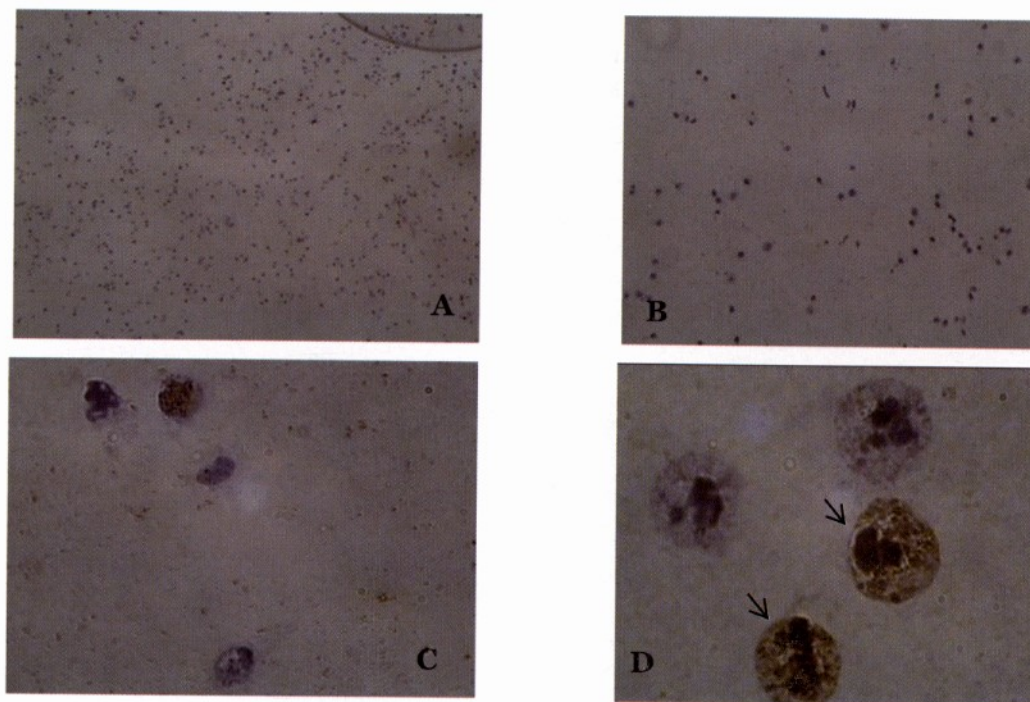


Figure 3 Group II (HeLa Cell Culture Treated with 60 µg/mL Soursop Leaves Ethanol Extract), Magnification by 40x (A), 100x (B), 400x (C), 1,000x (D)

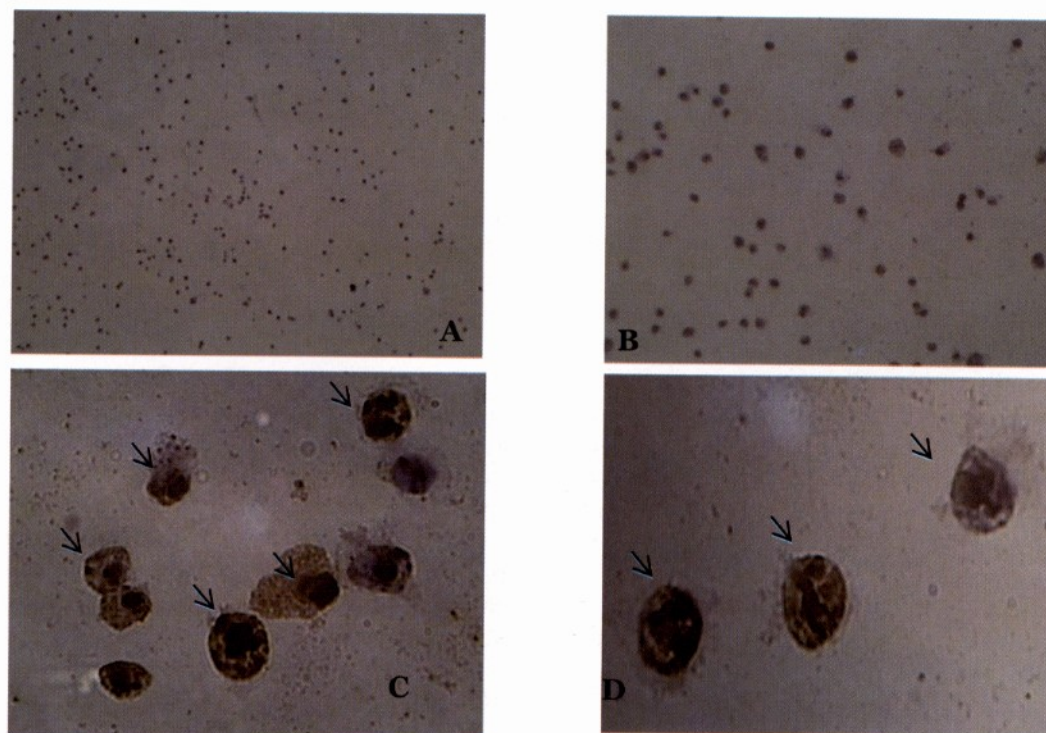


Figure 4 Group III (HeLa Cell Culture Treated with 120 µg/mL Soursop Leaves Ethanol Extract), Magnification 40x (A), 100x (B), 400x (C), 1,000x (D)

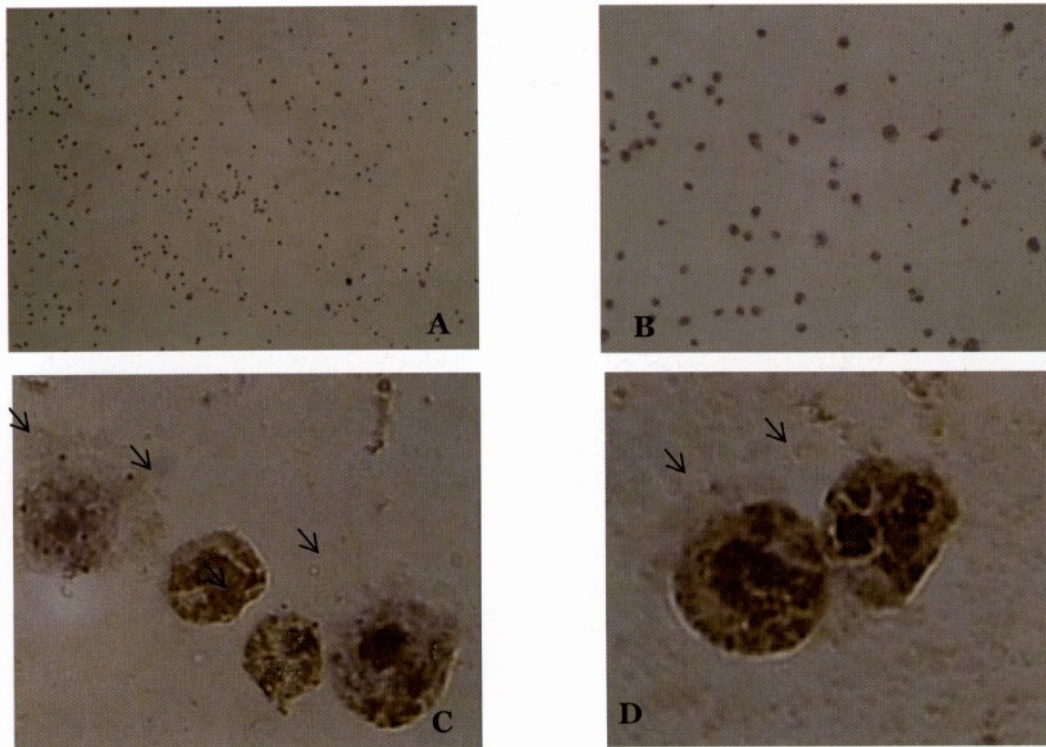


Figure 5 Group IV (HeLa Cell Culture Treated with 240 µg/mL Soursop Leaves Ethanol Extract), Magnification 40x (A), 100x (B), 400x (C), 1,000x (D)

central, cytoplasm looks pale compared to the nucleus and there was no brown cells. These indicate that there were no ongoing apoptosis. In Figure 3, 4 and 5 cells become rounder in shape with some unclear borders, cell spacing was not too tight, there were fragmented nucleus, they were smaller compared to untreated HeLa cell, and many looked brownish. This due to the 3 OH 'results of DNA fragmentation of apoptotic cells which were recognized by the enzyme transferase in TUNEL reagent. These showed the HeLa cells with undergone apoptosis.

The result showed that, the average percentage of apoptosis in HeLa cells culture treated with 60 µg/mL, 120 µg/mL, 240 µg/mL soursop leaves ethanol extract was 34.27%, 91.09%, and 95.28% respectively while untreated HeLa cells had 0% apoptosis.

Statistical analysis using ANOVA test at 95% confidence level indicates that there was significant difference of apoptotic cells percentage between group of HeLa cell culture treated by soursop leaves ethanol extract and untreated group, with $p < 0.001$ ($p\text{-value} \leq 0.05$). This results are consistent with previous research conducted by Rachmani and Suhesti

in 2012 in which soursop leaves ethanol extract can induce apoptosis of cancer cells. The results are in accordance with researches by Wang in 2000, Padhasaradhi in 2004, Kojima and Tanaka in 2009, and Hai Bin 2010 which stated that *acetogenin* can induce cell apoptosis by inhibiting ATP cancer.^{16,24,25}

Measurement of caspase-3 gene expression:

The result found out that soursop leaves ethanol extract could not increased caspase-3 gene expression. From the results using Kruskal Wallist test at the 95% confidence level, it was clear that there is no effect of the soursop leaves ethanol extract on the expression of caspase-3 in HeLa cells culture significantly with $p = 1.000$ ($p > 0.05$).

These results are not in accordance with previous studies which showed an increase in caspase-3 in human hepatoma treated with annonaceous acetogenins with lower levels of intracellular cGMP mechanism that would induce apoptosis. Previous study in T24 bladder cancer cells culture showed that annonacin could increased the activity of caspase-3 gene. We used whole soursop leaves which may caused lower levels of acetogenin content than the acetogenin

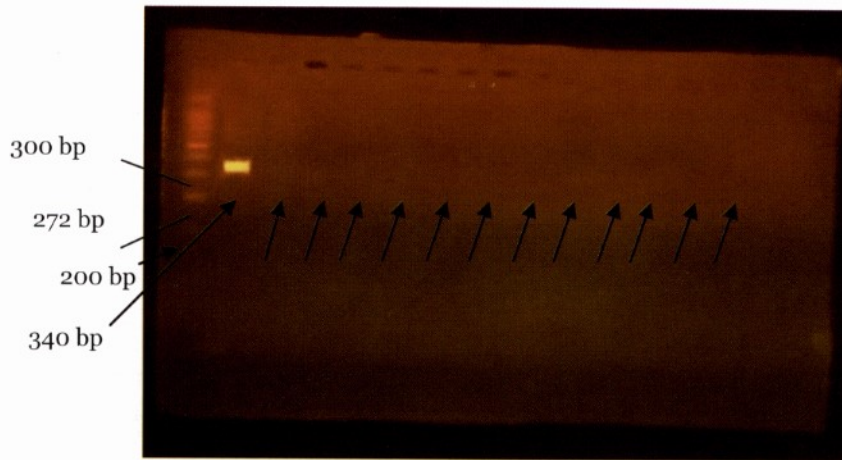


Figure 6 Electrophoresis Results of Caspase-3 Gene Expression

insulation in the previous studies.

Apoptotic cell death that occurs might come via caspase pathway and not through caspase-3 but through caspase-6 or -7, which is also acted as the caspase execution. In the previous study conducted by Yang and colleagues,²⁶ gene expression of caspase-7 in MCF-7 breast cancer cells were increased which lead to induction of apoptosis after administration of flavonoids. That study also found that PARP breakdown were not always came through the activation of caspase-3, but can also come via caspase-7 activation and DNA fragmentation.

Conclusions

There is the effect of soursop leaves ethanol extract in increasing the percentage of the amount of apoptosis in HeLa cells culture. However the soursop leaves ethanol extract do not affect the gene expression of caspase-3 in HeLa cells culture.

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