

In vitro anticancer activity *Annona squamosa* extract nanoparticle on WiDr cells

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ABSTRACT

This study aimed to prepare *Annona squamosa* leaf extract-loaded chitosan nanoparticles (nano-ASLE) against human colon cancer (WiDr) cells. Nano-ASLE was made with ionic gelation method. Four concentrations of the nano-ASLE (50, 100, 200, and 400 µg/mL) in dimethyl sulfoxide were prepared on WiDr cells to determine the IC₅₀ value using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Then, it was divided into three groups of concentration of IC₅₀, 2IC₅₀, and 4IC₅₀ and continued with analysis of caspase-3 expression and cell cycle arrest. The results of particles size were obtained 535.1 nm and showed potent cytotoxicity with IC₅₀ 292.39 µg/mL. The expression of caspase-3 increased significantly and caused cell cycle arrest at the G2/M phase and induced apoptosis on WiDr cells. Further studies are needed to obtain the loading efficiency, release of drug concentration, and *in vivo* study of nano-ASLE to suppress WiDr cells.

Key words: *Annona squamosa*, apoptosis, cell cycle, cytotoxicity

INTRODUCTION

Cancer has emerged as one of the strongest diseases of deaths since many years ago. It is caused by many factorials by uncontrolled division cellular and metastasis.^[1] Approximately 7.6 million people die from cancer annually and expected to increase around 13.1 million in 2030.^[2] Colorectal cancer is one of the cancers which have high mortality in both males and females.^[3]

Chemopreventive is a strategy to eliminate cancer selectively to block carcinogenesis process in stages of promotion and progression.^[4] Many studies assessed chemopreventive agent use botanical products because of the capacity as a bioactive compound.^[5] *Annona squamosa*

is considered drug plant for cardiac disease, diabetes, and cancer. *A. squamosa* is called as custard apple in English and belonging to *Annonaceae* family. Phytochemical screening of leaf extract showed the presence of flavonoids, alkaloids, saponins, tannins, glycosides, phenolic, terpenoids, and steroids.^[6] *A. squamosa* has been reported in the discovery of new Annonaceous acetogenins, called as (2,4-cis and trans)-squamolone, (2,4-cis and trans)-9 oxoasimicinone, and bullacin B. Acetogenins have been isolated from the fruit pulp, seeds, twigs, roots, stems, leaves, and bark of many plants in the *Annonaceae* family. Acetogenins isolated from *A. squamosa* seeds reported cytotoxic activity through inhibited proliferation on HL-60 cells and induced apoptosis by the activation of caspase-3 and also against 9KB nasopharynx cells, A549 lung cells, and HT-29 colon cells.^[7] In the past, studies showed that green biosynthesis of silver nanoparticles of *A. squamosa* leaf extract was reported to have cytotoxic effect and induction of apoptosis on MCF-7 cells.^[8]

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Nanotechnology is a promising approach to enhance the bioavailability of herbal medicine. Nontoxic biopolymer material is one of the nanoparticles which are expected to protect bioactive compound. A carrier material like the combination of chitosan and sodium tripolyphosphate (Na-TPP) is used to protect, stabilize, and control the release of the core. Chitosan can easily cross-link with Na-TPP as polyanions to control the release of the drug.^[9] Therefore, this study designed to prepare nano-*A. squamosa* leaf extract (ASLE) to enhance their bioactivities as an anticancer agent on WiDr cells due to induced apoptosis and cell cycle arrest.

MATERIALS AND METHODS

Ethical clearance

All treatment procedures have been tested through The Medical and Health Research Ethics Committee, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia (Approval Reference Number: KE/FK/0106/EC/2018).

Preparation of *Annona squamosa* leaf extract

A. squamosa leaves were collected from Lumajang Regency, East Java, Indonesia. The plant material was identified at Purwodadi Botanical Garden, Indonesian Institute of Sciences, Pasuruan, Indonesia, and approved with the reference number 003/IPH.06.HM/VII/2017. The leaves were cleaned and chopped into small pieces and shade dried. They were powdered using mechanical blender and passed through the coarse sieve (0.2 mm). The powder was macerated with ethanol 96% for 72 h at 37°C. The extract was evaporated in the water bath at the temperature of 60°C. The residue was then stored in a refrigerator at the temperature of 0–4°C.

Preparation of *Annona squamosa* leaf extract-loaded chitosan nanoparticles

A. squamosa leaf extract-loaded chitosan nanoparticles (nano-ASLE) were prepared by ionic gelation of chitosan (0.1% w/v) and sodium tripolyphosphate (0.84% w/v) anions. One gram of *A. squamosa* was dissolved in 50 mL distilled water and added 100 mL chitosan in glacial acetic (0.25% v/v) and added 350 mL sodium tripolyphosphate in dropwise into the solution stirring condition at room temperature. The pH solution was adjusted by adding 0.1 M NaOH solution to the chitosan-*A. squamosa* complex and stirred for 2 h on magnetic stirrer. Chitosan-*A. squamosa* was centrifuged at 6000 rpm for 30 min and decanted. The supernatant was collected and dried for onward usage as nano-ASLE.^[10]

Particle size analysis

Dynamic light-scattering method was performed to measure the particle size distribution of nano-ASLE using Zetasizer Nano ZS (Malvern Instruments Ltd., UK).

Cell culture of WiDr cells

WiDr cells were collected from the Parasitology Laboratory, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. Cells were cultured in DMEM and supplemented with 10% (v/v) fetal bovine serum, 3% streptomycin-penicillin, and 1% fungizone in 5% CO₂ incubator at 37°C.

Determination of IC₅₀ value

The IC₅₀ value of the nano-ASLE was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. WiDr cells were seeded in 96-well plates at a density of 1×10^4 cells/well with 100 µL of volume and incubated at 37°C and 5% CO₂ for overnight. WiDr cells were added with various concentration of the nano-ASLE for 24 h. Then, the media were removed. Next, it was added with 100 µL of DMEM and 10 µL MTT (5 mg MTT/mL solution) to every single well. The plates were incubated for 4 h. Control cells just received only the media without the nano-ASLE samples. The crystal of formazan which formed in viable cells was solubilized with 100 µL of SDS-stopper HCl 0.1 N. The absorbance 595 nm was measured by ELISA reader by Benchmark Microplate Reader (Bio-Rad, USA) and calculated to determine the IC₅₀ value with linear regression analysis using Microsoft Excel 2016.

Immunocytochemistry staining

WiDr cells were seeded in 24-well plates at a density of 2×10^5 cells/well and incubated for 24 h. WiDr cells were treated with IC₅₀, 2IC₅₀, and 4IC₅₀ (292.39, 584.78, and 1169.56 µg/mL, respectively) of the nano-ASLE for 24 h. WiDr cells were plated in poly-L-lysine slides and fixed with methanol for 3 min, permeabilized for 3 min in PBS, blocked in 2% BSA for an hour and stained with monoclonal antibody caspase-3 for an hour, and then washed with PBS for 3 × 3 min and stained with biotinylated universal secondary antibody for an hour. WiDr cells were incubated with HRP-streptavidin for 10 min, added with DAB for 5 min, washed again with aquadest, then counterstained with hematoxylin for 20 s, and at last, mounted on glass slides. The expression of caspase-3 was evaluated semi-quantitatively based on H-score method.^[11]

Apoptosis observation on WiDr cells

WiDr cells were cultured in 24-well plates at a density of 2×10^5 cells/well and incubated for 24 h. The cells were treated with IC₅₀, 2IC₅₀, and 4IC₅₀ (292.39, 584.78, and 1169.56 µg/mL, respectively) of nano-ASLE for 24 h. The medium was removed and washed using FBS. Coverslips were taken and put on the glass slide before added with 10 µL acridine orange-ethidium bromide and left for 15 min. It was then observed under the fluorescence microscope.

Cell cycle arrest analysis

WiDr cells were cultured in 6-well plates at a density of 1×10^6 cells/well and added with IC₅₀, 2IC₅₀, and 4IC₅₀ (292.39,

584.78, and 1169.56 $\mu\text{g/mL}$, respectively) of nano-ASLE for 24 h. The distribution of cell cycle phase was determined of treated and untreated cells. They were collected and washed twice with PBS (pH 7.4) then centrifuged at 2500 rpm for 10 min. Cells pellet was washed twice with PBS and resuspended with 400 μL of PI/RNase detection kit then incubated at 37°C for 30 min. The distribution of cell cycle phases was determined using BD FACSCalibur Flow Cytometer (BD Biosciences, USA).

Statistical analysis

This project was analyzed using SPSS 21 (SPSS Inc., Chicago, IL, USA) employing one-way analysis of variant ($P < 0.05$), followed by Tukey's honestly significant difference ($P < 0.05$). Tabulated data were presented as the mean \pm standard deviation values.

RESULTS

Particle size analysis of Nano-*Annona squamosa* leaf extract

The result of particle size analyzer shows that the average of nano-ASLE was obtained at 535.1 nm with a good polydispersity index, 0.365, as shown in Figure 1.

IC₅₀ value of Nano-*Annona squamosa* leaf extract on WiDr cells

The highest reduction of WiDr cells found at the concentration of 400 $\mu\text{g/mL}$ is 58.07 $\mu\text{g/mL}$. Meanwhile, the lowest reduction obtained at the concentration of 50 $\mu\text{g/mL}$ is 29 $\mu\text{g/mL}$. The reductions of cell growth from the two other concentrations, which are 100 $\mu\text{g/mL}$ and 200 $\mu\text{g/mL}$, are 32.18 $\mu\text{g/mL}$ and 45 $\mu\text{g/mL}$, respectively. This study shows that the IC₅₀ value of the nano-ASLE on WiDr cells is 292.39 $\mu\text{g/mL}$. The result of cytotoxic responses of the nano-ASLE on WiDr cells is presented in Figure 2.

Nano-*Annona squamosa* leaf extract-induced caspase-3 expression on WiDr cells

The highest expression of caspase-3 expression is found at the concentration of 1169.56 $\mu\text{g/mL}$ and has the mean score of 59.57 cell expression, and the lowest score shows at the concentration of 292.39 $\mu\text{g/mL}$ and has the mean score of

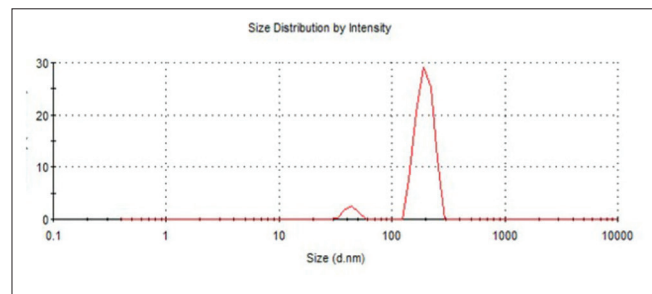


Figure 1: Particle size distribution of nano-*Annona squamosa* leaf extract

43.15 cell expression. While the concentration of 584.78 $\mu\text{g/mL}$ shows the mean of 54.23 cell expression. Furthermore, in control media, there is some little bit expression mean score of 1.2 cell expression. The result of cytotoxic responses of the nano-ASLE on WiDr cells is presented in Figure 3.

Nano-*Annona squamosa* leaf extract-induced apoptosis on WiDr cells

The result due to IC₅₀ value and caspase-3 expression was related to this method. All cells in the untreated group showed green fluorescence meant as viable cells. Orange fluorescence cell meant as apoptotic cells that loss cell membrane permeability and then turn on apoptotic bodies, and several nuclear cells were fragmented and are shown in Figure 4.

Nano-*Annona squamosa* leaf extract-induced G2/M phase on WiDr cells

Cell cycle arrest may mediate induction of apoptosis and shows the inhibition of cell proliferation. As shown in Figure 5 showed that nano-ASLE arrested G2/M phases and increased from 13.72% (control) to 22.89% (1169.56 $\mu\text{g/mL}$). The growth inhibition was associated with a significant cell cycle arrest in G2/M phase. Arrest in G2/M was associated with a concomitant decrease in the cell percentage in G0/G1 phase while in the S phase cells remained at almost at the same levels. When exposed to 584.78 $\mu\text{g/mL}$, the cell population of WiDr cells both in the S and G2/M phases was 9.69% and 22.89%, respectively; they increased compared with the control group.

DISCUSSION

In this study, the cytotoxic effect of the nano-ASLE against WiDr cells is investigated. It reveals that the nano-ASLE has an IC₅₀ value of 292.39 $\mu\text{g/mL}$. It might be caused by the particle size in nanoparticle form for optimizing activity of *in vitro* and increase the bioavailability of uncontrollable molecules. Releasing the bioactive compound with

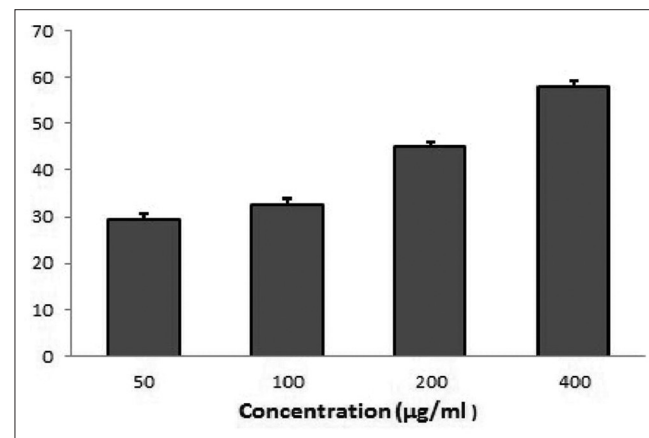


Figure 2: Cytotoxic responses of nano-*Annona squamosa* leaf extract on WiDr cells

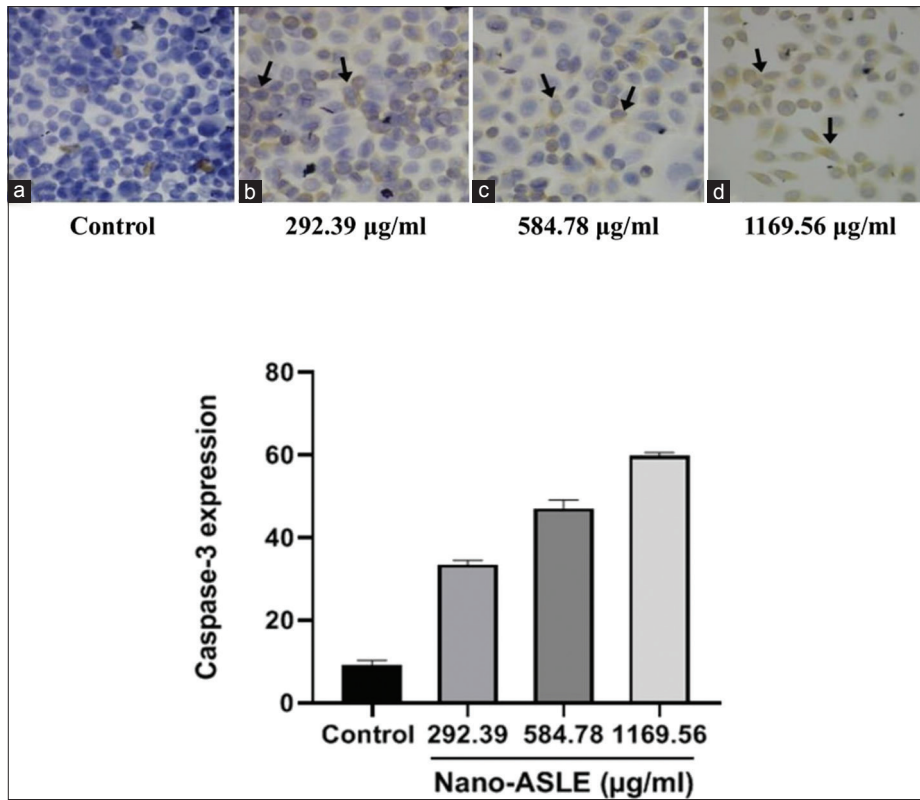


Figure 3: Effect the nano-*Annona squamosa* leaf extract on caspase-3 on WiDr cells ($\times 400$), untreated group (a), caspase-3 expression (black arrow) shown in brown color in cytoplasm of nuclei of the group treated 292.39 µg/ml (b), 584.78 µg/ml (c), 1169.56 µg/ml (d) bar represents mean \pm standard deviation of caspase-3 expression score treated Nano-*Annona squamosa* leaf extract on WiDr cells

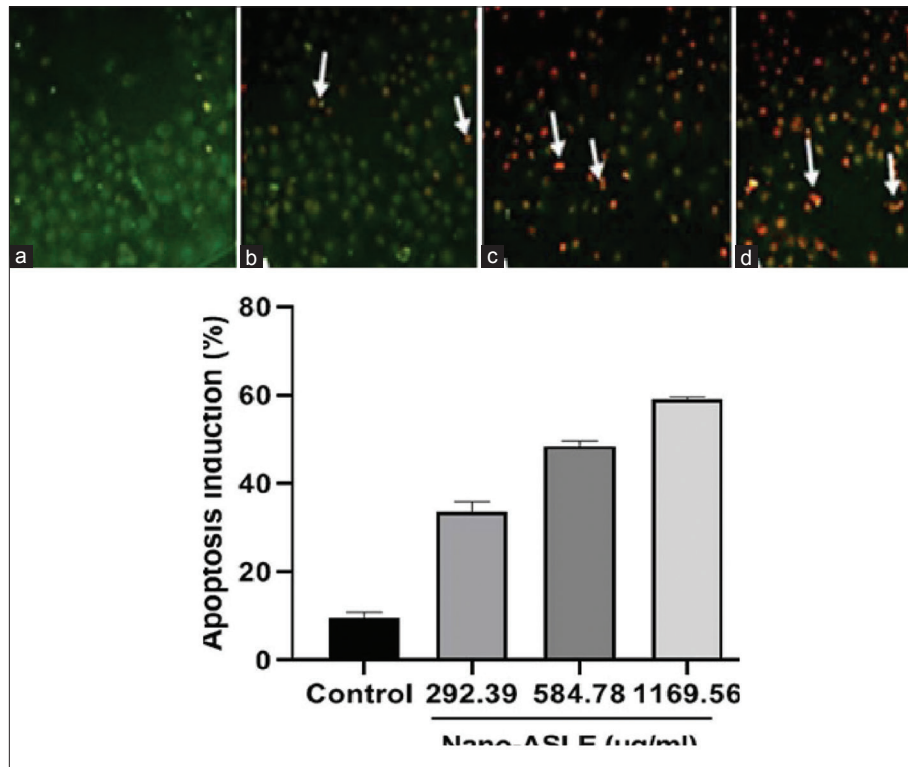


Figure 4: Effect the nano-*Annona squamosa* leaf extract on apoptosis induction on WiDr cells ($\times 400$), untreated group showed green color (a), apoptotic cells showed orange-red color (white arrow) of the group treated 292.39 µg/ml (b), 584.78 µg/ml (c), 1169.56 µg/ml (d), bar represents mean \pm standard deviation of apoptosis induction score treated Nano-*Annona squamosa* leaf extracts on WiDr cells

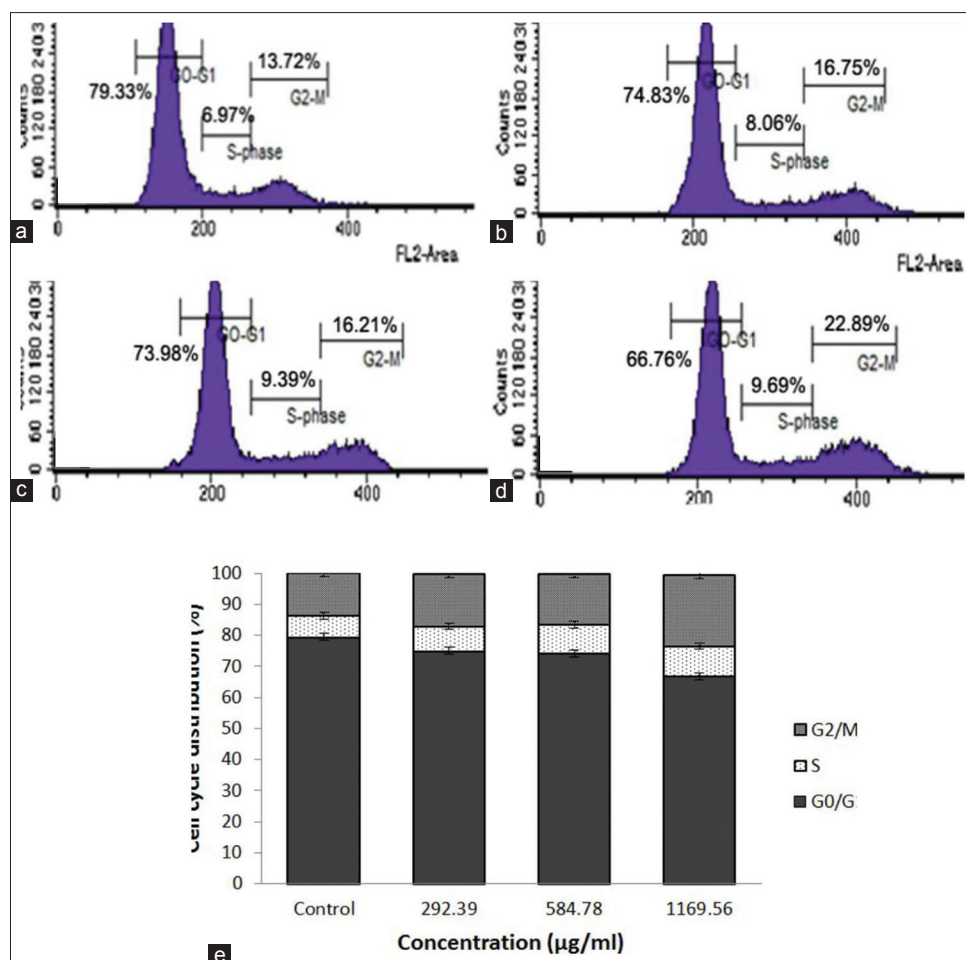


Figure 5: Effect the nano-*Annona squamosa* leaf extract on cell cycle distribution on WiDr cells. Flow cytometry histogram of cell cycle untreated group (a), group treated 292.39 µg/ml (b), 584.78 µg/ml (c), 1169.56 µg/ml (d), graph of analysis of cells number (%) each cell cycle phase relative to total phase (e), for examples, G0/G1 is calculated as percentage of the number cells in the G0/G1 population relative to the number of total cells

nanoparticles actually gives alternative therapeutic ways dropping the drug to specific target and decreases the toxicity effects.^[12]

Acetogenin effect of *A. squamosa* also can inhibit the proliferation of cancer cells. Acetogenin selectivity for tumor cells can be explained both by the higher activities of NADH oxidase and the ATP demand that are peculiar to tumor cells and inherent due to their uncontrolled growth.^[13] The previous studies reported that there are two main compounds of annonaceous acetogenins using HPLC analysis; they are 12, 15-cis-squamostatin-A and bullatacin.^[14] They also showed anticancer properties against various tumor cell lines in evaluation *in vitro*.^[15] Another acetogenin is annonacin. It is a mono-tetrahydrofuran acetogenin isolated from *Annonaceae* family. The past studies showed that annonacin has a cytotoxic effect on T24 cancer cells.^[16]

The result of caspase-3 expression were increased in dose-dependent manner. It is caused by damage of mitochondrial membrane potential after the treatment. The pathway is

activated by a variety of extra- and intracellular stress, including oxidative stress and cytotoxic drugs. The pathway is dominated by a bcl-2 family members that regulate the release of cytochrome-c from the mitochondria then activate caspase protease that dismantle cells to make apoptosis formation and signal efficient phagocytosis of cell corpses.^[17,18] It is consistent with the previous study which showed that annonacin from *Annonaceae* family induces the apoptotic cell death in a caspase-3 related to the pathway on endometrial cancer cells.^[19]

The checkpoint of cell cycle is an important control mechanism to execute of cell cycle activity. Several chemopreventive phytochemicals could restore and alter regulatory checkpoints through inducing cell cycle arrest. Recent investigations suggest that anticancer agents arrest the cell cycle at the G0/G1, S, or G2/M phase.^[20] In this study showed that nano-ASLE arrest G2/M phase on WiDr cells. In the past, studies showed that annonacin isolated from the seed of *Annona reticulata* has a cytotoxic effect to T24 bladder cancer cells by arresting in G0/G1 phase. It induced apoptotic

cell death by upregulating bax and bad expression and increasing caspase-3 activities and procaspase-3 cleavage, but annonacin of *Annona muricata* also induced apoptotic cell death in a caspase-3-related pathway and arrest cell cycle in G2/M phase on endometrial cancer cells.^[16,19] Interestingly, it related to this study that nano-ASLE have a cytotoxic effect, induced apoptosis in caspase-3, and arrest cell cycle in G2/M phase. It could be attention that a key regulator of the G2/M transition of the cell cycle is a complex of CDC1/CDK2 and the cyclin B. If CDC1/CDK-2 was inhibited, it would be expected as an arrest at the G2/M transition.^[21] However, apoptosis induction on WiDr cells may occur via upregulation of pro-apoptotic proteins and a decrease in membrane potential caused by releasing cytochrome-c of mitochondrial and activation of caspase-3.^[22]

CONCLUSION

The research demonstrated that nano-ASLE can inhibit the proliferation of WiDr cells with the IC₅₀ value of 292.39 µg/mL via a mechanism involving the induction of apoptosis and intracellular mitochondrial pathway on caspase-3 expression. It also related to cell cycle arrest at G2/M phase. Further studies are needed to obtain the loading efficiency, release of drug concentration, and *in vivo* study of nano-ASLE to suppress WiDr cells.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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