Apoptosis of HeLa Cells via Caspase-3 Expression Induced by Chitosan-Based Nanoparticles of Annona squamosa Leaf Extract: *In vitro* Study

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ABSTRACT

**Background:** Annona squamosa is reported to have a significant cytotoxic activity in some cancer cells. **Objectives:** Thus, this study aim to investigate Annona squamosa leaf extract induced by chitosan nanoparticles (nano-ASLE) to enhance their biological activity as an anticancer agent on HeLa cells. **Methods:** Nano-ASLE (50, 100, 200, 400 µg/mL in DMSO) given on HeLa cells to determined IC₅₀ value by MTT assay. Then, it was divided into three groups as follow IC₅₀, 2IC₅₀, 4IC₅₀ continued with analysis of caspase-3 expression. **Results:** The present study demonstrated that nano-ASLE can suppress HeLa cells proliferation with the IC₅₀ value of 344.48 µg/mL and rapid enhancement of caspase-3 activity has the mean score of 65.3 cell expression and the lowest score shows 45.3 cell expression. **Conclusion:** Nano-ASLE lead to HeLa cell death via the mitochondrial pathway on caspase-3 expression. In addition, the further studies are needed to obtain the loading efficiency, release of drug concentration and *in vivo* study of nano-ASLE to suppress HeLa cells.

**Key words:** Annona squamosa, Cytotoxicity, Apoptosis, Caspase-3, Nanoparticles.

**Key Messages:** Apoptosis effect of *Annona squamosa* nanoparticles.

INTRODUCTION

Cancer has emerged as one of the strongest diseases which causes deaths since many years ago.¹ The second most common gynecological tumors that caused mortality in women worldwide is cervical cancer.² Many studies assess some botanical products as the chemopreventive agent and more than 5000 bioactive compounds have been identified and still continuously increasing for the new one. These novel phytomedicines have shown running as anticancer drug candidate *in vitro* models.³⁻⁵ *Annona squamosa* is called as Srikaya which belongs to Annonaceae family and considered as the medicinal plant for cardiac disease, diabetes and cancer. Bioactive compounds of the leaf extract presence flavonoids, alkaloids, saponins, glycosides, phenolic, tannins, terpenoids, steroids.⁶ *Annona squamosa* has been reported for the discovery of new compounds. Acetogenins have been isolated from the many plants of the Annonaceae.⁷ Acetogenins isolated from *Annona squamosa* is reported to have a significant cytotoxic activity against 9KB, A549, HT-29, 9ASK cancer cells and the caspase-3 activation on HL-60 cells.⁸⁻⁹ Additionally, some studies showed that silver based *Annona squamosa* extract nanoparticle are reported to have the cytotoxic effect and apoptosis induction on MCF-7 cells.¹⁰ Nanotechnology is a promising approach to enhance the bioavailability of herbal medicine. Non-toxic biopolymer material
is one of the nanoparticles colloidal which is expected to protect bioactive compound. A carrier material like the sodium tripolyphosphate (Na-TPP) and chitosan combination are used to preserve releasing of the core. Chitosan is a non-toxic material widely used as nanoparticle-forming polymer. Therefore, this study designed to prepare nano chitosan-Annona squamosa leaf extract to enhance their bioactivities as the anticancer agent on HeLa cells to evaluate the caspase expression as one of apoptosis marker of cells.

MATERIALS AND METHODS

Ethical Approval
All treatment procedures have been tested through Ethics Committee of Gadjah Mada University (KE/FK/0106/EC/2018).

Preparation of Annona squamosa Leaf Extract
The leaves were collected from Lumajang Regency, East Java, Indonesia. 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 hr at 37°C. The extract was evaporated in the waterbath 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 Induced Chitosan
Annona squamosa leaf extract induced with chitosan (nano-ASLE) were prepared in the method of ionic gelation with slight modification. Chitosan (0.1% w/v) was dissolved in acetic acid (0.25% v/v) and sodium tripolyphosphate (0.84% w/v). Annona squamosa leaf extract (1 g) was dissolved in 50 mL distilled water and added with 100 mL chitosan in acetic acid, followed by 350 mL sodium tripolyphosphate dripping in the solution stirring condition at the room temperature. It was stirred for 2 hr and followed by centrifuge 30 min in 6000 rpm, then supernatant was collected then dried for cells treatment.

Electron Microscope Analysis
Morphology of nano-ASLE was evaluated using Scanning Electron Microscope (SEM), 15 kV, model 54160, Hitachi, Japan.

Particle Size Analysis
particle size distribution of nano-ASLE was performed with dynamic light scattering (DLS) method using Zetasizer Nano ZS (Malvern Instrument Ltd., UK).

HeLa Cells Culture
HeLa cells were cultured in DMEM with 10% (v/v) fetal bovine serum, 3% streptomycin-penicillin and 1% fungizone in 5% CO2 incubator at 37°C. Cells were maintained in 25 cm2 flask with 7 mL media then harvested using 0.25 trypsin-EDTA after reaching 80% on confluency.

IC50 Value Determination
The IC50 value of nano-ASLE was determined using MTT assay. HeLa cells were cultivated at a density of 1×104 cells/well in 96-well plates and incubated at 37°C overnight. Cells were added with various concentration of the nano-ASLE for 24 hr. Then, the media were removed. Next, it was added with 100 µL of DMEM and 10 µL of MTT solution to each well and incubated for 4 hr. Control cells received only the media. SDS-stopper HCl 0.1 N were added to evaluated formazan crystal. Moreover, ELISA reader was used to measure the absorbance in 595 nm.

Immunocytochemistry Staining
HeLa cells were cultivated at a density of 2×105 cells/well in 24-well plates and incubated at 37°C overnight. The cells were treated with nano-ASLE 344.48, 688.96, 1377.92 µg/mL for overnight. The cells were layered with poly-L-lysine slides, then fixed using methanol for 3 min, permeabilized for 3 min, blocked in 2% BSA for hour. Monoclonal antibody caspase-3 was added for hour, then washed with PBS and added secondary antibody biotinylated universal for hour. The cells were incubated with HRP-streptavidin for 10 min, then added DAB for 5 min, washed again with aquadest, then counterstained with hematoxylin for 20 sec.

Statistical Analysis
This project was analyzed by using SPSS 21 (SPSS Inc., Chicago, IL), employing one-way analysis of variant (ANOVA) (p < 0.05), followed by Tukey HSD. Tabulated data were presented as the mean ± standard deviation.

RESULTS

Particle Size Analysis of Nano-ASLE
The average particle size of nano-ASLE are obtained at 55.1 nm (Figure 1).

Scanning Electron Microscope (SEM) of Nano-ASLE
The SEM micrograph reveals the rough surface morphology, solid dense cubical and little bit aggregation (Figure 2). The spheres have diameters about 300 nm.
The highest reduction of HeLa cells is 48.25 µg/mL at dose of 400 µg/mL. Meanwhile, the lowest reduction is 8.47 µg/mL at dose of 50 µg/mL. The reductions of cell growth from the two other concentrations, 100 µg/mL and 200 µg/mL are 17.8 µg/mL and 22.96 µg/mL, respectively. This study shows that the IC<sub>50</sub> value of the nano-ASLE on HeLa cells is 344.48 µg/mL. The result of cytotoxic responses of the nano-ASLE on HeLa cells is presented in Figure 3.

**DISCUSSION**

In this study, the cytotoxic effect of the nano-ASLE against HeLa cells is investigated. Moreover, it reveals that the nano-ASLE has IC<sub>50</sub> value of 344.48 µg/mL. Releasing the bioactive compound with nanoparticles actually give alternative therapeutic ways dropping the drug to specific target and decrease the toxicity effects. Chitosan-coating can increase physicochemical stability, controlled release, improvement of cells interaction and bioavailability of drugs and its efficacy. Annona squamosa showed anticancer properties against several cancer cells in evaluation in vitro. In this study we...
suggests that it may be caused by the acetogenin effect that can inhibit the proliferation of cancer cells. Acetogenins selectivity indicated higher NADH oxidase activities and the ATP demand due to their uncontrolled growth.\textsuperscript{14} The prior studies confirmed that the main compounds of annonaceous acetogenins are bullatacin and 12,15-cis-squamostatin-A. Another acetogenin is annonacin. It is a mono-tetrahydrofuran acetogenin isolated from Annonaceae family. The past studies showed that annonacin has cytotoxic effect and induces cell death with apoptosis through caspase-3 related to the pathway to T24 bladder of cancer cells.\textsuperscript{15,16}

The physiological form of cell death is induced by a variation of stimuli and several genes have been implicated in the apoptotic cell. Apoptosis is normal physiological process to eliminate the uncontrolled growth of that generate the cell destruction and homeostasis maintenance in healthy tissue. Apoptosis is also characterized by the plasma membrane bleeding, cell shrinkage, increasing cell density, chromatin condensation, karyorrhexis and the formation of apoptotic bodies. One of the apoptosis marker in the early stage is showed by caspase-3 expression.\textsuperscript{17,18} In this study, other compounds of \textit{Annona squamosa} like flavonoid, alkaloid and saponin triggered ROS production that induced apoptosis through actions at different signal transduction pathways such as cyclin-dependent kinases (CDKs), caspases, Bcl-2 family members, epidermal growth factor (EGF)/epidermal growth factor receptor (EGFR), phosphatidylinositol-3-kinase/Akt, MAPK and NF-\textit{k}B, which may affect cellular function by modulating genes or phosphorylating proteins.\textsuperscript{19} The result of caspase-3 expression in immunocytochemistry shows that the increasing doses followed by the increasing amount of caspase-3 expression.

Caspase-3 produces an inactive zymogen, which is a 32 KDa pro-caspase located in the cytoplasm that proteolytically cut at an aspartate residue to generate the cleaved fragments in the terminal and creates 12 KDa and 17 KDa subunit. Two subunits are combined to activate caspase-3 enzyme. This active caspase-3 has a wide gap of activity over the cellular substrates in which it cleaves the cell substrates like DNA repair enzyme and the structural proteins. It also may lead endonucleases and caspase activation which can cause the DNA fragmentation. Furthermore, it cannot be recovered back because of the inactive form of DNA repair enzyme and it can reach apoptosis.\textsuperscript{20}

Mitochondria is another vital organelle in activation of intrinsic pathway of apoptosis. In the present study, we observed damage of mitochondrial membrane potential after the treatment. The pathway is activated by several of factors, including the cytotoxic drugs and oxidative stress. Family of Bcl-2 protein is dominated pathway, which leads to release of cytochrome-\textit{c} from the mitochondria. Next, it binds together with procaspase-9 and apa-1 which contribute to make the formation of apoptosomes and activation of caspase-9, at last procaspase-3 activation to caspase-3 then lead activation of apoptosis formation. 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. Annonacin may deregulate the cell cycle arrest checkpoint to allow a cancer cell to enter mitosis and undergo apoptosis through caspase-mediated DNA fragmentation and also decrease Bcl-2, which eventually reduce cell proliferation of HeLa cells.\textsuperscript{21,22} Future study could be analyzed to find the accurate the value of IC\textsubscript{50} from the nanoparticles form of \textit{Annona squamosa} leaf extract. Thus, it can be concluded that chitosan based-nanoparticles of \textit{Annona squamosa} are found to have anti-cancer activity against the HeLa cells.

**CONCLUSION**

The present study demonstrated that nano-ASLE can inhibit the proliferation of HeLa cells with the IC\textsubscript{50} value of 344.48 µg/mL via the intracellular mitochondrial pathway on caspase-3 expression. In addition, the further studies are needed to obtain the loading efficiency, release of drug concentration and in vivo study of nano-ASLE to suppress HeLa cells.

**ACKNOWLEDGEMENT**

This study was funded by PMDSU grant with the reference number 1341/UN3.14/LT/2018 from KEMENRISTEKDIKTI of Indonesia.

**CONFLICT OF INTEREST**

The authors declare no conflict interests.

**ABBREVIATIONS**

Nano-ASLE: \textit{Annona squamosa} leaf extract induced by chitosan nanoparticles.

**REFERENCES**


**PICTORIAL ABSTRACT**

**SUMMARY**

Annona squamosa is called as Srikaya which belongs to Annonaceae family and considered as the medicinal plant. In this study nanoparticles of Annona squamosa leaf extract was designed to surpass the growth of HeLa cells and evaluate caspase-3 expression as one of apoptosis marker of cells. Chitosan was used as nanoparticle substance to preserve releasing of the bioactive compound. The result of the work are nano-ASLE can surpass the growth of HeLa cell and induce caspase-3 as a apoptosis marker.

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**Indian Journal of Pharmaceutical Education and Research | Vol 54 | Issue 2 | Apr-Jun, 2020**