

The cytotoxic test and apoptotic of *Solanum sanitwongsei* Craib against HeLa cells

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Abstract: Cervical cancer is one of many types of cancer that attack women and is a public health problem, especially in developing countries with populations that have a low socioeconomic status. One of the herbs used traditionally for cervical cancer is of the family Solanaceae *Solanum sanitwongsei*. The purpose of this study to determine screening of crude drugs and extracts ethanol, calculate the IC₅₀ values and HeLa cancer cell apoptosis pacing of the most active fraction. The ethanol extract obtained by maceration and fractions obtained by liquid-liquid extraction of ethanol extract of *Solanum sanitwongsei* Craib using the solvent n-hexane and ethylacetate. The extraction fraction obtained from n-hexane, ethyl acetate fraction and water fraction (residual). IC₅₀ value of ethanol extract and fractions of *Solanum sanitwongsei* Craib obtained through testing cytotoxic against HeLa cells using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. The most active fraction continued to test the method flow cytometry apoptotic mechanism.

Results of phytochemical screening of crude drugs and found ethanol extract was found steroids/triterpenoids, alkaloids, flavonoids, tannins, glycosides and saponins. Cytotoxic assay results obtained IC₅₀ value of ethanol extract, fractions n-hexane, ethyl acetate, and water to HeLa cells, respectively for 178 194 µg/ml; 812 954 µg/ml; 85 489 µg/ml and 241.58 µg/ml; and ethyl acetate fraction was the most active fraction. Ethylacetate fraction of *Solanum sanitwongsei* Craib can stimulate apoptosis in HeLa cells.

Keywords : *Solanum sanitwongsei* Craib, HeLa, cytotoxic, MTT, flow cytometry, apoptosis.

Introduction

Cervical cancer is one of many types of cancer that attack women and is a public health problem, especially in developing countries with populations that have a low socioeconomic status. Cervical cancer ranks top of gynecologic cancer include women and 21-30% of all cancers that infect women^{1,2}. In Indonesia, to date cervical cancer is still the issue of women's health with the incidence and mortality rates are high where every day was found 41 new cases and 20 deaths³. One of the medicinal plants that are currently in the research is inggir- inggir plant (*Solanum sanitwongsei*) of the family Solanaceae⁴. *Solanum sanitwongsei* have content glycosides that have activity as anti-HIV and anti-herpes⁵. As for the other ingredients contained in the plant are saponins, tannins, polyphenols, flavonoids and alkaloids⁴.

Plants of the family Solanaceae contains many steroid alkaloids, such as solasodine and solanidine. Solasodine have activity against cervical cancer cells (HeLa) with IC₅₀ of 12.17 ± 3.3 µM whereas 20 µM IC₅₀ cisplatin have used as a comparison. Solasodine also have IC₅₀ values 40 µg/ml against myeloid leukemia U937 cells⁶. Research on anticancer activity through inhibition of cancer cell research has been done by using plants of the family Solanaceae among others the cells P-388, SW-620, MCF-7, NUGC-3. The activity due to the content of chemical compounds solanidine, spironastol, spirosolane glycosides, saponin and many other compounds⁵.

Cell culture HeLa or HeLa cell line is a continuous cell line derived from epithelial cells of cervical cancer (cervical) a women with cervical cancer are named Henrietta Lacks died of cancer in 1951⁷. These cell cultures have inherent spring properties and is used as a model for testing anticancer⁸.

This study is an exploratory study, to determine whether the extracts and fractions of *Solanum sanitwongsei* Craib have anticancer effects against HeLa cells and apoptosis.

Aparatus and Materials

The aparatus were glasses, autoclave (Hirayama), blender (Philips), conical tube, eksikator, Elisa reader (Biorad BenMark), CO2 incubator (Heraceus), an inverted microscope (Olympus), porcelain crucible, laminar air flow (Labconco), micropipette, a rough balance (Home Line), electrical balance (Vibra AJ), oven (Memmert), water bath (Yenaco), rotary evaporator (Haake D1), sentrifugator, a set of water content determination, set of tools distillation, flatbed porcelain cup, porcelain crucible with a lid, desiccator furnace, vortex, 96 - well plate, 6 - well plate and flowcytometry.

The material used were *Solanum sanitwongsei* Craib are yellow colour, is taken from the village of andes derivatives, District Hotanduhan, Siantar City, North Sumatra Province, 96% ethanol, ethyl acetate and n-Hexana distilled, HeLa cancer cells is a collection of the Laboratory of Parasitology Faculty of Medicine Yogyakarta. Media M 199-serum, Fetal Bovine Serum (FBS) 10% (v/v) (Gibco), penicillin- streptomycin 2% (v/v) (Gibco), and Fungizone (amphotericin B) 0.5%. In addition to the above materials are also used 0.25% Trypsin-EDTA (Gibco), Fetal Bovine Serum (FBS), MTT [3-(4,5-dimetiltiazol-2-yl)-2,5 difeniltetrazolium bromide] (Sigma), at a concentration of 5 mg / mL and propidium iodide. Stopper used was sodium dodecyl sulfate in 0.01 N HCl.

Method

Preparation of Extract *Solanum sanitwongsei* Craib.

A total of 10 parts of simplicia was inserted into a vessel, with n-hexane 75 parts, then closed and left for 5 days protected from light, with frequent stirring. After 5 days it was filtered diserkai juice, pulp squeezed and washed with solvents to obtain 100 parts. Maserates moved into a closed vessel, left in a cool area protected from light for 2 days. It was then filtered, evaporated by rotary evaporator and freeze dried. The pulp was dried and macerated again with ethyl acetate and ethanol⁸.

Preparation of Factions *Solanum sanitwongsei* Craib

A total of 5 g of ethanol extract was dissolved in 96 % ethanol until dissolved then add 40 ml of distilled water, put into a separating funnel, then added 100 ml of n - hexane, and then shaken and allowed to stand until there are 2 separate layer. N-hexane layer (upper layer) was taken with a streamed manner, and fractionation performed until a layer of n - hexane gave negative results with LB reagent. Then add 100 ml of ethyl acetate , then shaken, allowed to stand until there are 2 separate layers , ethylacetate layer (upper layer) was taken by way of streamed, and fractionation performed until a layer of ethyl acetate gave negative results with FeCl3 reagent , and water fraction (residual) was taken and all fraction obtained was evaporated with a vacuum rotary evaporator (rotary evaporator) to obtain a viscous fluid

Cytotoxic testing of *Solanum sanitwongsei* Craib

Hela cell viability were assessed using MTT colorimetric assay. The cells were cultured in 96-well plates. Each well contained 5x10 cells. The culture cells were the incubated in a humidified incubator at 37°C in an atmosphere of 5% CO₂ and 95% air for 24 h. after 24 h incubation, the medium was discharge and treated by NEOS, EAEOS and EEOS with concentration of 15.625ug/ml, 31.25ug/ml, 62.5ug/ml, 125ug/ml and 250ug/ml. after incubation for 24h, the cells were incubated with 0,5 mg/mL MTT for 4h in 37°C. Viable cells react with MTT to produce purple formazan crystals. After 4 h, the stopper 10% SDS in 0.01 N HCl was added to dissolve the formazan crystal. The cells were then incubated for 24h in room temperature and protected from light. After incubation, the cell were shaken, and cell absorbance was measured by ELISA reader at λ 595 nm. The experimental data was absorbance of each well and then covered to percentage of viable cell

$$\text{percentage of viable cells} = \frac{B-C}{A-C} \times 100\%.$$

Where A, B and C are absorbances of control group, treatment group and medium respectively.

Flowcytometri assay

The number of cells required for apoptosis is as much a test of 5×10^5 - 1×10^6 cells/wells were then grown in microplate wells 6, and then incubated for 24 hours. The next day the cells of the test sample was added and incubated again for 24 hours. Then the media were taken on each of the wells at each concentration and then put in a 15 ml conical tube and washed with PBS 1x media and accommodated in the same conical. 250 mL of trypsin added to each wells and incubated for 3 minutes at a temperature of 37°C (make sure the cells under a microscope does not gather with each other to get maximum results). Followed by the addition of 1 ml of culture medium and medium accommodated in 15 ml conical tubes. Centrifuged at 6000 rpm for 5 min and the supernatant discarded. Then add as much as 1 ml of PBS and then transferred to media in 1.5 ml conical tube and centrifuged again at 2000 rpm for 3 minutes, after which the supernatant discarded. Then added propidium iodide and measured by means of flowcytometer.

Result and Discussion

Screening Phytochemical Of simplex and ethanol extracts of *Solanum sanitwongsei* Craib.

Phytochemical screening of simplex and ethanol extract of *Solanum sanitwongsei* Craib. conducted to determine the chemical compounds, can be seen in Table 1.

Table 1 phytochemical screening of crude drug powder and ethanol extracts of *Solanum sanitwongsei* Craib.

No	Parameter	<i>Solanum sanitwongsei</i> Craib.	
		Simplex	ethanol Extracts
1	Alkaloida	+	+
2	Flavonoida	+	+
3	Saponin	+	+
4	Tannin	+	+
5	Glikosida	+	+
6	Steroida/Triterpenoida	+	+
7	Antrakuinon	-	-

Cytotoxic Test of Ethanol Extracts, Fractions n - hexane, Ethyl Acetate and water (residu) of *Solanum sanitwongsei* Craib. against HeLa cells.

Methods MTT [3 - (4,5 - dimethyliazol - 2 -yl) -2,5 - diphenyl tetrazolium bromide] is one quantitative cytotoxicity assay. MTT will be reduced to formazan by succinate tetrazolium reductase system, which is included in the mitochondria of living cells¹⁰. The test results of the test solution cytotoxic HeLa cells IC50 value 178.194 ug / ml for ethanol extract, 812.954 ug / ml for n - hexane fraction, 85.489 ug / ml for ethyl acetate fraction, 241.580 ug / ml for doxorubicin fraction of water and 1,022 mg / m, where the concentration of the extract, fractions and doxorubicin was able to inhibit 50 % growth of HeLa cells. Otherwise potent extract if you have IC 50 values less than 500 ug / ml¹¹, From the test results and calculation of IC50 values extracts and fractions, then that can be categorized into potent extracts and fractions are fractions of ethylacetate as IC50 values below 100 mg / ml. Previously been carried out to determine the chemical class of botanicals and the results are classes of chemical compounds that are suspected anticancer, such as flavonoids, saponins, alkaloids, and triterpenoids/ steroids. In krude ethanol sitotoksiknya activity caused by the presence of this class of compounds triterpenoids/ steroids, alkaloids, flavonoids, glycosides and saponins. Flavonoids are compounds substituted phenyl compounds benzopyran derivatives consisting of the basic framework C15 (C6 - C3 - C6). Some plants that contain flavonoids derivatives, has been used as a preventive and therapeutic agent in

traditional medicine in Asia for thousands of years, such as anticancer. Presence of flavonoids in the ethanol extract and ethyl acetate fraction *Solanum sanitwongsei* Craib. shows the possibility to the extract has anticancer effects.

For n - hexane fraction obtained cytotoxic activity of the smallest among all existing test solution is the IC₅₀ value of 812.954 g/ml, and the results of screening n-hexane fraction class of compounds found triterpenoids / steroids . According to¹², the activity of triterpenoids have to treat inflammation, proliferation, apoptosis, invasion, metastasis and angiogenesis. Since many of these compounds showed good potential in dealing with various mechanisms of cancer , such as the regulation of transcription factors regulate (eg, nuclear factor - kappaB [NF - κB], anti - apoptotic proteins (eg, bcl - 2, bcl - xL) , the originator of cell proliferation metalloproteinases [MMPs], intracellular adhesion molecule- 1 (ICAM - 1) and angiogenic proteins (vascular endothelial growth factor (VEGF). ethyl acetate fraction obtained for IC₅₀ value of 85.489 ug / ml, where the grouping of results fraction of chemical compounds discovered class of compounds alkaloids, flavonoids , saponins and glycosides in the ethyl acetate fraction . alkaloids as anticancer mechanisms include the ability of anti-angiogenic, anti- proliferative, inhibits the activity of topoisomerases , tubulin polymerization and induces apoptosis¹³. flavonoid compounds inhibit cell proliferation in various human cancer cells through the inhibition of oxidative processes that can lead to cancer initiation. This mechanism is mediated decrease xanthin oxidase enzyme, Cyclooxygenase (COX) and Lipooxygenase (LOX) required in the process prooksidasi thereby delaying cell cycle. Flavonoids also inhibit the expression of topoisomerase I and II enzymes that play a role in catalyzing DNA screening. Topoisomerase enzyme inhibitor complex will stabilize DNA topoisomerase and cause cuts and damage¹³. Saponins can recognize cancer cells, because cancer cells have cell membranes and structures are different from normal cells. Cancer cell membranes contain more compounds such as cholesterol .Saponins can bind cholesterol contained in the membrane of cancer cells, thereby disrupting membrane permeability¹⁴. Saponins also reduce the occurrence of reactive oxygen species such as H₂O₂ and inhibit signaling pathways phosphatidyl - inositol-3 kinase which may be the reason for the prevention of chromosomal damage⁵.

While the IC₅₀ value for the fraction of water is quite weak latau less can be said to be a potential direction in the search for anticancer compounds.

Apoptosis test

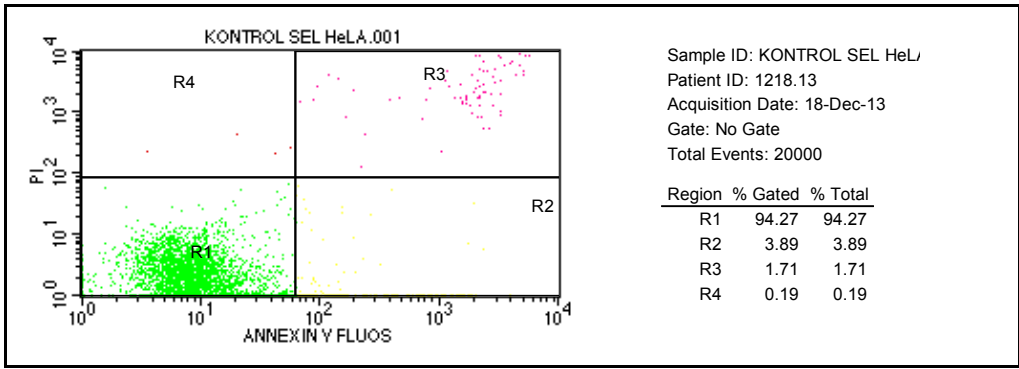
Observations made by the method flowsitometri apoptosis. This method is a method for counting living cells, cell necrosis and apoptosis rapidly. In the current study used an Annexin V protein that can specifically bind to phosphatidylserine found on the plasma membrane of cells during the process of apoptosis. DNA in damaged cells both necrosis and apoptosis will be colored by propidium iodide (PI) which produces orange to red fluorescence. As it passes through the laser beam, the cell will be excited and scatter light to produce light fluorescence^{16,17}.

Testing apoptosis in HeLa done with a variety of treatments. Among them is the control, 1 ½ IC₅₀ and IC₅₀ and doxorubicin are shown in Table 2 and Figure 1 .

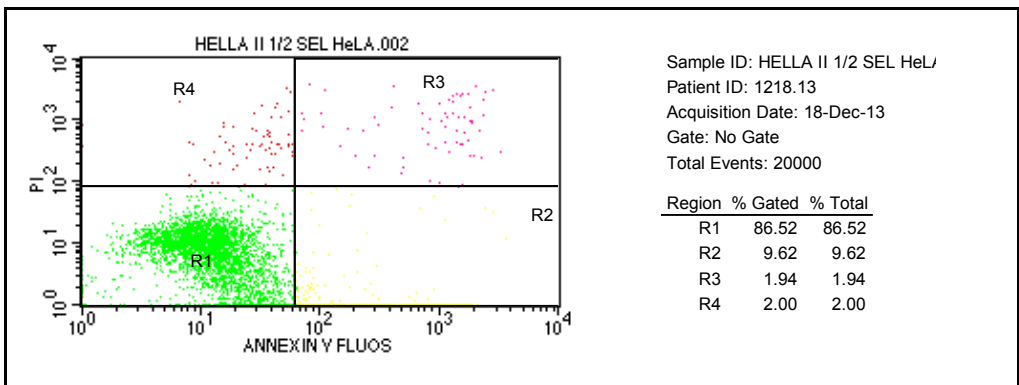
Table 2 apoptosis in HeLa cells

Treatment	Concentration µg/mL	R ₁ (%)	R ₂ (%)	R ₃ (%)	R ₄ (%)
Control	-	94.27	3.89	1.71	0.19
ethylacetate fraction 1X IC ₅₀	86	80,69	11.76	4.27	3.35
ethylacetate fraction 1/2 IC ₅₀	43	86.52	9.62	1.94	2.00
doxorubicin	1	8.24	4.78	10.90	76,14

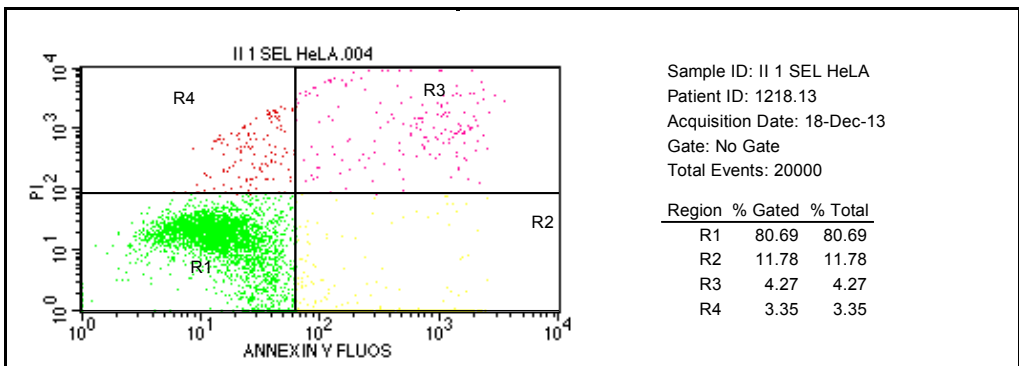
Description: R₁ = live cells, R₂ = cells undergoing early apoptosis, R₃ = cells undergoing apoptosis and necrosis early end, R₄ = cells undergoing late necrosis.



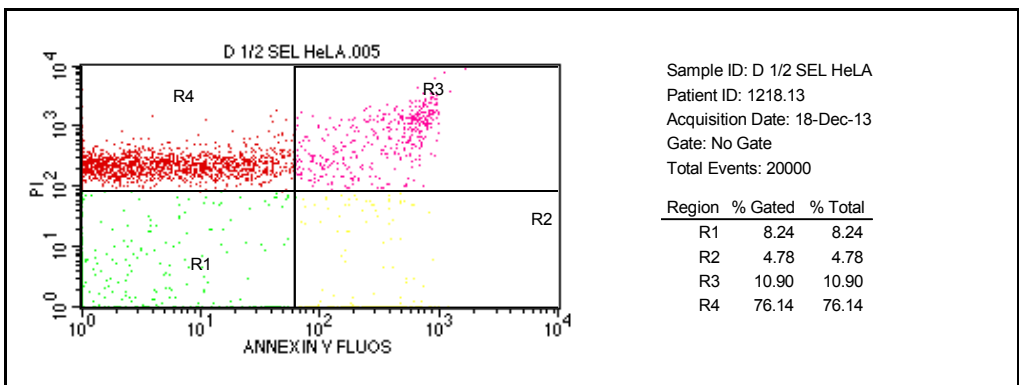
(a)



(b)



(c)



(d)

Figure 1. Percentage Overview hela cell conditions (a) control, (b) 1 IC50 (c) 1/2 IC50 and (d) of doxorubicin

In HeLa cells were given ethylacetate fraction IC50 seen the percentage of cells undergoing early apoptosis (11.76%), ½ IC50 (9.62%) and doxorubicin (4.78) and compared to controls (3.89%). Looks ethyl acetate fraction of *Solanum sanitwongsei* more Craib increase the number of cells undergoing early apoptosis compared with doxorubicin.

The percentage of cells undergoing apoptosis and necrosis beginning at the end of the administration of ethyl acetate fraction of *Solanum sanitwongsei* Craib IC50 , IC50 less than ½ early apoptosis. The number of living cells in the control (94.27), on treatment with ethyl acetate fraction of *Solanum sanitwongsei* Craib IC50 (80.69 %), ½ IC50 (86.52 %) and doxorubicin (8:24 %). Mechanism of action of ethyl acetate fraction of *Solanum sanitwongsei* Craib is likely to be the initial phase of apoptosis.

Conclusion

1. IC50 value of ethanol extract of *Solanum sanitwongsei* Craib, the fraction of n-hexane, ethyl acetate fraction and residual fraction in the treatment of HeLa cells, respectively for 178 194 ug / ml; 812 954 ug / ml; 85 489 ug / ml and 241.58 ug / ml.
2. Fraction of ethyl acetate at a dose of 1 ½ IC50 and IC50 can spur apoptois

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