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The Effect Cytotoxic and Apoptotic Propagation Chloroform Fraction of *Ocimum sanctum* L. Towards Hela Cells Line Culture

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ABSTRACT

Materials used in the research are *Ocimum sanctum* L, a herbaceous shrub used in traditional medicine displaying as immunomodulator, anti-stress, hepatoprotective, kemopreventif, and anti-inflammatory. A research to identify its ethanol extract of *Ocimum sanctum* has been conducted, showing that it possesses cytotoxic activities to HeLa cells with IC_{50} value of 209µg/ml. The aim of this research is to find out activities cytotoxic and apoptotic propagation chloroform fraction of *Ocimum sanctum* towards HeLa cells line. The extraction was done using maceration using ethanol 96% solvent and fractionation was done using chloroform. Effects of chloroform fraction on HeLa cells were tested using MTT at various dosages (500, 250, 125, 62.5, and 31.25) µg/ml. Cisplatin (Kalbe) was applied as positive control. The effect apoptotic propagation chloroform fraction of Ocimum sanctum on HeLa cells was observed by DNA painting method using orange ethridium bromide-acridine. The test results showed that the chloroform fraction of *Ocimum sanctum* extract on HeLa cells had an IC_{50} value of 155.67 µg / ml ± 46.99 and Cisplatin had an IC_{50} value of 16.81 µg / ml ± 20.37. Double staining test shows the existence of cells undergoing apoptosis. Based on these results, the chloroform fraction *Ocimum sanctum* has been shown to be cytotoxic and has the possibility of stimulating apoptosis of cervical cancer cells (HeLa). This can be used as a basis for the development of these plants as anticancer agents with specific action targets.

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INTRODUCTION

Cervical cancer is still a health problem for women with high incidence and mortality. Cervical cancer in developed countries ranks second after breast cancer, colorectal, and endometrium (Rasjidi, 2009). Unlike in Indonesia, cervical cancer is the number one cause of death that often occurs in Indonesian women. In Indonesian 270.000 women die each year from cervical cancer or cervical cancer (Rasjidi, 2009). Those treatments cost highly and bring many side effects. Due to those reasons, many researchers hold studies to find new more effective and selective drugs. The ideal development of therapy is needed to reduce mortality, one of which uses natural ingredients. This is meant to minimize the occurrence of resistance and side effects of treatment. One of them is *Ocimum sanctum*.

Ocimum sanctum is a type of medicinal plants that are clinically proven to have an influence on immuno modulator, anti-ulcer, anti-inflammatory as well anti carcinogenic. Anticancer activity in *Ocimum sanctum* caused by the content of apigenin, luteolin, eugenol and ursolic acids (Haryati, 2011). Eugenol compounds can inhibit growth and proliferation in cancer cells and induces apoptosis, eugenol depends on the time and dosage given. Eugenol has a role as a chemopreventive agent (Vidhya, 2011). *Ocimum sanctum* essential oil is cytotoxic with IC_{50} value of 60 µg/mL against MCF-7 cells (Selvi et al., 2015).

Ursolic acid is known to induce amplified apoptosis by reason of its ability to terminate Poly ADP Ribose Polymerase (PARP) as well as reduce Bcl-2 in MCF-7 cells (Bishaye et al., 2011). Research conducted by Haryanti (2011) proves that the chloroform fraction containing ursolic acid had cytotoxic effect on colon cancer cell (WiDr cell line) with IC_{50} value of 25 µg / ml.

The existence of cytotoxic activity in *Ocimum sanctum* L can be caused by one of them apoptotic mechanism. Apoptosis is cell suicide program. Cells are apoptosis will increase shrinkage cell, plasma membrane damage, and occurs it is chromatin condensation. If apoptosis a cell has finished, it will be left behind pieces of dead cells will be recognized with macrophage cells and phagocytosis (Wikanta et al., 2012). Research conducted by Ismiyati (2016) proves that the ethanol extract of *Ocimum sanctum* L. leaves is able to induce apoptosis in HeLa cervical cancer cells. The discovery of new compounds that target apoptosis is wrong one strategy for developing chemopreventive agents from plants as alternative cancer treatments.

This study aims to explain the cytotoxic potential of the chloroform fraction *Ocimum bacillicum* L as a chemopreventive agent in HeLa cervical cancer cells. In addition, the effect of the chloroform fraction *Ocimum bacillicum* L was studied on the induction of apoptosis of cancer cells so that later it could be developed as an alternative drug in cancer treatment.

MATERIALS AND METHODS

Materials

Ocimum sanctum, chloroform, ethanol 96%, aquadest, cisplatin (Cisplatin® Kalbe), HeLa cells line, RPMI 1640 (Gibco), 10% fetal bovine serum/FBS (Gibco), 3% penicillin-streptomycin (Gibco), MTT reagent, SDS, 1% fungison, gentamicin (Merck), DMSO (Gibco), DMEM (Gibco), aquabidest, 0.5% trypsin, bufer fosfat salin (PBS) pH (7.4), etidium bromida-akridin oranye (EtBr-AO) (Sigma).

Glassware (Pyrex), rotary evaporator (Heidelph), liquid-nitrogen tank, refrigerator (Samsung), CO_2 incubator (Heraeus), laminar air flow cabinet (Nuaire), microplate 96 wells (Nuclone), micropipette (Socorex), pipette ependorf, inverted mycroscope, filter 0.2 um , hemocytometer (New Bouer), yellow tip, blue tip, white tip, pH meter, glass objects, light microscopes, centrifuges, sonicator (Branson), tissue culture flasks (Olympus), ELISA reader (Bencmark Bio Rad), fluorescence microscope (Zeiss MC 80), cover slip (Nunc).

Preparation of extracts

In the extraction process, *Ocimum sanctum* powder extracted using the method cold is maceration method. In the process this maceration of *Ocimum sanctum* powder is used is 1000 g. This maceration process using ethanol 96% 10.000 ml solvent is done for 5 days, so with contact time longer between solvents and *Ocimum sanctum* powder allows the process of withdrawal of compounds more optimal active. After extracting the results maceration is obtained then carried out concentration extract with rotary tools evaporator until thick extract is obtained with constant weight.

Fractionation using the liquid-liquid extraction method. The ethanol crude extract from *Ocimum sanctum* (10 g) was suspended in aquadest (75 ml) and partitioned with Chloroform 75ml. The fraction obtained was concentrated using rotary vacuum evaporator to obtain fraction of chloroform and water. Chloroform fraction will be used to test cytotoxic to HeLa Cells. Preparation of the test solution with the concentration of the chloroform fraction used was (500, 250, 125, 62.5, and 31.25) μg/mL dissolved using DMSO solvent.

In Vitro Cytotoxicity Studies

Cell culture

HeLa Cell was obtained from the collection of Cancer Chemoprevention Research Center, Pharmacy Faculty, Gajah Mada University is grown in growing media Roswell Park Material Institute (RPMI) supplemented with penisilin–streptomisin 3%, fungison 1% and 10% foetal bovine serum (FBS, Growth medium) at 37°C in 5% CO_2 incubator.

MTT assay

Cytotoxic tests were carried out using MTT Assay. Confluent HeLa cells are harvested and distributed into wells on 96-well plates in the amount of 10 x 10^3 cells / wells. Cells were incubated for 24 hours in a CO_2 incubator for adaptation so that they were ready for treatment. After 24 hours, 100 μL of culture media was added containing samples in various concentration series with 3 replications and re-incubated for 24 hours. As a control used solvent control (DMSO), HeLa cell control, and DMEM culture media control. In solvent control, cells are given DMSO in accordance with the same levels as those contained in the sample. At the end of the incubation, the culture media contained in the plate are removed by turning the plate over, then washed with 100 μL PBS for each well. Then each well was added 100 μL MTT reagent (stock 5 mg / mL). Incubation was continued for 3 hours at 37 ° C until formazan was formed. Living cells will convert MTT becomes formazan which is dark blue / purple. Next, a stopper reagent was added to dissolve formazan crystals and cells were incubated overnight at room temperature and protected from light. At the end of the incubation, the plate is shaken horizontally with a shaker for 10 minutes then read with ELISA reader at a wavelength of 595 nm. The absorbance results that are read are converted as a percentage of cell viability (Ismiyati, 2016).

Apoptotic test

Cover slip is planted into 24 well plates and cells are distributed over it. The cell density used was 5 x 104 cells / well in 1000 μL of culture medium. The incubation is carried out for 24 hours in a CO_2 incubator in order for the cell to be re-adapted. Then the cells were treated with IC_{50} extract and cell control. At the end of the incubation, DMEM culture media was washed with PBS, and the slip cover was removed from the well and placed over a glass object then dyed with 10 μL orange - etidium bromide acridine. Cell morphology observations were performed with a fluorescence microscope using 10x10 magnification (Ismiyati, 2016).

Data analysis

Cytotoxic test

Analysis of test results by collecting data in the form of the absorbance of each wells , then converted to a percent of living cells. Percent of live cells is calculated using the formula :

$$\frac{Absorbance\ samples - Absorbance\ control\ medium}{Absorbance\ control\ cell - Absorbance\ control\ medium}\ x\ 100\%$$

Results of % of cell viability is used to calculate the IC_{50} value by linear regression that can be known potential cytotoxicity. To know the difference between concentration of chloroform fraction do statistically using ANOVA one way, if there is a significant difference followed by Tukey's test. To see the difference between the control IC_{50} (Cisplatin) with IC_{50} chloroform fraction do t-test with a level of confidence (signification level) 95 %.

Apoptotic observation

Observation of apoptosis is done qualitatively by observing HeLa cell morphology under a fluorescence microscope. Live cells will appear normal, intact as the original, and fluorescent green, whereas cells are initial apoptosis will appear condensed, fluorescent green, cells with final apoptosis will appear condensed, fragmented, red fluorescent, and cells that are necrotic will look normal, fluoresce orange to red (Sekti et al., 2010).

RESULT AND DISCUSSION

Results of extraction and fractionation

The making of *Ocimum sanctum* L ethanol extract obtained the results of concentrated extract of 63.5 g with a yield of 6.35%. After the extraction process, then fractionation process from extract is carried out by using extraction methods liquid-liquid (ECC) with chloroform and aquadest solvents aims to separate components compounds based on differences polarity. Based on the results of fractionation obtained the yield of chloroform fraction is 66.74%, and the yield of the water fraction is 13.08%.

Cytotoxic test

Cytotoxic tests provide an overview of the potential of test compounds in inhibiting test cell growth. The parameter used is 50% inhibition concentration (IC_{50}). The concentration of chloroform fraction of basil herbs used in the treatment was 500; 250; 125; 62.5; 31.25 μg/ml. Cisplatin is used as a positive control with concentrations ranging from 100; 50; 25; 12.5; 6.25 μg / ml. Cisplatin as a control positive because it is a platinum complex anti-cancer drug. HeLa cell morphology after incubation 24 hours with the addition of the *Ocimum sanctum* L chloroform fraction and subsequently the administration of MTT reagents was observed using an inverted microscope with a magnification of 100x after 4 hours of incubation can be seen in figure 1.

Based on figure 1, normal HeLa cells appear in the control group in the form of oval with clear nuclei and stick to the bottom tissue culture because cells are able to metabolize using nutrients in the media. The chloroform fraction concentration of 500 μg/mL affected the morphological changes of cells to be more rounded and smaller in size with lower cell density compared to controls. Dead cells are dark in color and do not stick to the bottom of tissue culture because dead cells lose their ability to maintain and provide energy for metabolic function and cell growth. Changes in morphological features in the HeLa cells support the test data cytotoxic that has been done is that the number of living cells is decreasing along with the increasing concentration of *Ocimum sanctum* L chloroform fraction.

Based on the MTT method, living cells will form formazan crystals as seen in figure 1. Reduction of MTT into formazan salt occurs if the enzyme reductase in mitochondria is active. Reduction in cells involves an enzymatic reaction with NADH or NADPH produced by living cells to produce insoluble deposits. MTT breakdown occurs in the cell mitochondria that live by the enzyme succinate dehydrogenase. The absorbance produced is proportional to the concentration of purple formazan dissolved in SDS. Reduction of tetrazolium salts is a reliable way to determine cell proliferation. Yellow MTT tetrazolium salt decreases as a result of cell metabolic activity mainly by the action of the succinate dehydrogenase enzyme (Sliwkal *et.al*, 2016).

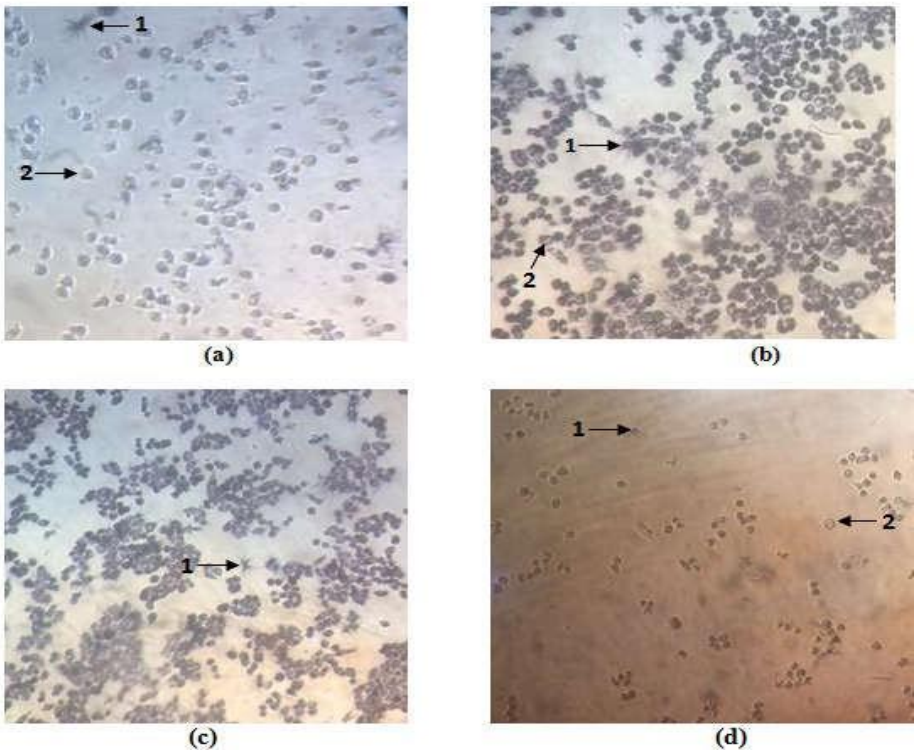

Figure 1. The morphology of HeLa cells is 100x magnification after addition of chloroform fraction *Ocimum sanctum* L and MTT reagent in the treatment of (a) concentration 500µg / ml, (b) concentration of 7.81µg / ml, (c) cell control, (d) Cisplatin concentration 100 µg / ml. Note: (1) living cells, (2) dead cells

Ocimum sanctum L chloroform fraction is able to inhibit the growth of HeLa cells but is not greater than positive control (cisplatin). This can be seen in the amount of formazan crystals produced as well as the percentage of cell death. The greater the content of the test compound, the greater the percentage of cell death. The more formazan crystals produced, the more the number of living cells, so the intensity of the color produced will be more purple.

The results of the cytotoxic test of *Ocimum sanctum* L chloroform fraction showed the viability of HeLa cells decreased % of living cells with increasing levels (figure 2). From this curve, a linear regression equation is made and obtained the equation y = -68.143x + 199.49 with R^2 = 0.9143. IC_{50} values were calculated intrapolated using the equation so that the resulting 155.67 µg / ml.

Several studies have shown that Ocimum sanctum has cytotoxic activity and can inhibit the growth of cancer cells. Ethanol extract and fraction from *Ocimum sanctum* leaves showed activity of cytotoxic effects on breast cancer cells (T47D) through the mechanism of increasing the expression of p53 and able to inhibit the expression of Bcl-2 (Christina, 2019). P53 protein is a transcription factor that activates the transcription of various genes such as p21. P53 protein is responsible for stopping the cell cycle when DNA damage occurs. If DNA damage cannot be repaired, p53 will induce apoptosis by activating pro apoptotic proteins (Syaifudin, 2012).

Ethanol extract of *Ocimum sanctum* leaves can also induce apoptosis of lung cancer cells (A549) through mitochondrial caspase with IC_{50} value of 176 µg/mL (Magesh *et al*., 2009), and effectively induce apoptosis in prostate cancer cells (LNCaP) with IC_{50} values of 116 µg/mL through activation of caspase-9 and caspase-3 which can ultimately cause DNA fragmentation and cell death (Sivanesan *et al*., 2015). Apoptosis is an approach to cell death that contributes to the process of pathogenesis in a disease or cell removal in adult cultures (Wu *et.al*, 2012).

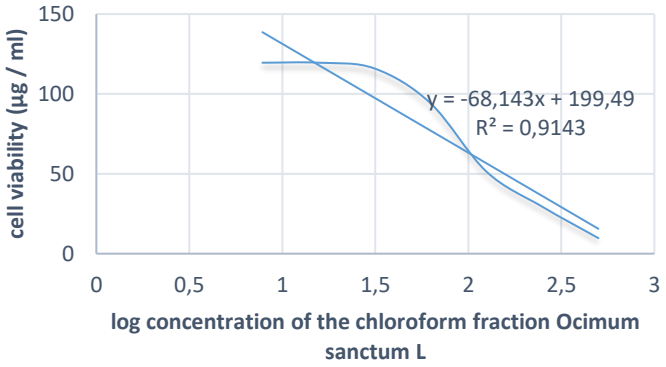

Figure 2. Curve% of HeLa cells living cells vs *Ocimum sanctum* L chloroform fraction level with a concentration of 500; 250; 125; 62.5; 31.25 µg/ml. The count of living cells is based on the MTT method

Based on these results (Table 1), the *Ocimum sanctum* L chloroform fraction has an IC_{50} value greater than the positive control (Cisplatin) which is 16.81 µg/mL so that cisplatin is more toxic than the chloroform fraction. Cisplatin is a high-effectiveness cytotoxic drug that works not selectively because it is toxic to both cancer cells and normal cells, especially normal cells with high proliferation rates such as the spinal cord. The high effectiveness of cisplatin allows it also has a high side effect of nephrotoxicity where the percentage of events is 20-30% (Nissa *et.al.*, 2015). Various side effects that may occur in the use of cisplatin are ototoxicity, gastrotoxicity, bone marrow suppression, allergic reactions and nephrotoxicity (Kurniandari *et.al.*, 2015).

From Table 1, it is known that the *Ocimum sanctum* L chloroform fraction has anticancer activity against HeLa cells with an IC_{50} value of 155.67 µg/mL, this shows that the chloroform fraction classified into anticancer material has moderate cytotoxic activity. Based on the classification of extract cytotoxic activity against cancer cells can be classified as very strong category if the IC_{50} value <10 µg/mL, the strong category if the IC_{50} value is 10-100 µg/mL, and the category is moderate if the IC_{50} value is 100-500 µg / mL (Wzeerapreeyakul *et.al.*, 2012). From the data above means that the chloroform fraction of *Ocimum sanctum* has potential in its activity as a chemoprevention agent. The activity of the chloroform fraction of *Ocimum sanctum* to inhibit HeLa cell growth or its ability to cause cell death, the possibility of pathways and mechanisms can be different. Therefore, the next process is an analysis of apoptotic observations to determine the growth inhibition mechanism.

Table 1. The average absorbance results, percent viability of HeLa cells and IC50 values.

Concentration of test material (µg / mL)		Average absorbance	Average (%) viability of HeLa	IC_{50} (µg / mL)
***Ocimum sanctum* L chloroform fraction**	500	0,157	9,722	155,67
	250	9,223	29,365	
	125	0,297	51,388	
	62,5	0,441	94,246	
	31,25	0,514	115,972	
Cisplatin	100	0,154	8,134	16,819
	50	0,135	9,051	
	25	0,208	24,053	
	12.5	0,356	59,523	
	6.25	0,433	87,797	

Apoptosis test

Observation of apoptosis what happens is done by painting DNA using the double staining method, which is painting using orange acridin and ethidium bromide. Detection of cell death is carried out using a fluorescent microscope.

The observation of morphological images of DNA by fluorescent microscope shows that apoptosis has a profound effect on the development of apoptotic cancer cancer therapy done by painting using Akridin Orange Ethidium Bromide. The cell die will be indicated by fluorescence orange and living cells are shown with green fluorescence (Figure 3B). In the control cell group, membrane the cell is still intact etidum bromide cannot enter inside cells and only orange akridin can enter into flouresence it's green (Figure 3A). Chloroform fraction of *Ocimum sanctum* with concentration 150 µg/mL, visible cells are green and some cells are green with the core or the center of orange (Figure 3). The condition is a sign of early apoptosis. Cells undergoing early apoptosis in double staining painting are less clearly observed.

An orange cell consists of cells that have apoptosis and necrosis. An orange-infected apoptotic cell with irregular apoptotic bodies as a distinctive feature of apoptotic (late apoptotic cells) death cells. Whereas in orange cells in all parts of cells and cells appear intact indicates the occurrence of necrosis. Observation of the morphological picture of DNA performed, it can be seen that the fraction of chloroform *Ocimum sanctum* extract can spur the occurrence of death apoptosis on HeLa cells (Ismiyati, 2016).

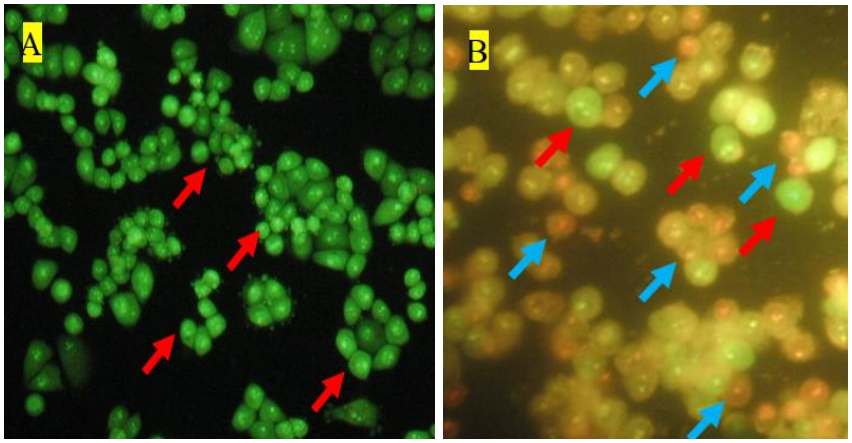

Figure 3. Effect of chloroform fraction treatment on HeLa cells using double staining method. Painting was done using an orange-Etidium bromide Akridin and observed using a fluresense microscope with 10x10 magnification. Cell control (A). Cells that experienced apoptosis after being treated with chloroform fraction of *Ocimum sanctum* with concentration 150 µg / ml (B). The living cell flouresens green (→) while the dead cell is flouresensi orange (→).

The potential of *Ocimum sanctum* chloroform fraction in stimulating apotosis is probably caused by the active compounds contained in fraction. Phytochemical screening of extracts and fractions of *Ocimum sanctum* positive containing steroid, alkaloids and flavonoids compounds (Christina, 2019). Ursolic acid belongs to the triterpenoid class. Ursolic acid is known to have anticancer activity by inducing apoptosis through TNF-releted apoptosis inducing ligand (TRAIL) on cancer cells and JNK-mediated upregulation of death receptors (DR) and decreasing decoy receptor-2 (DcR2) so that it can affect cell survival (Prasad *et al.*, 2011).

Alkaloid compounds are thought to inhibit the activity of the enzyme DNA Topoisomerase II, an enzyme that plays an important role in the process of replication, transcription, DNS

recombination and cancer cell poliferation. With the increase in the number and activity of these enzymes in cancer cells, the process of replication, transcription and poliferation of cancer cells will also increase and with inhibition of the activity of these enzymes there will be a bond between the enzyme and DNA for longer and occur Protein Linked DNA Brake (PLDB) and end with apoptotic death (Sukardiman *et al*., 2006).

Flavonoid compounds can inhibit the expression of P53 protein through inhibition of p53 mRNA translation. Inhibition of p53 protein causes cells to be retained in the G2-M phase of the cell cycle. Check points in this phase will repair DNA and if it is not successful it will spur cells for apoptosis (Ismiyati, 2016).

Possible mechanism of apoptosis still needs to be proven through further studies. Compounds that are definitely responsible for these mechanisms also need further investigation to get a clear scientific basis for the development of active compounds in *Ocimum sanctum* as chemopreventive agents.

CONCLUSION

Based on the results of the research that has been done, it can be concluded that the chloroform fraction of *Ocimum sanctum* has the potential as a chemopreventive agent through cytotoxic activity and induction of apoptosis against cervical cancer cells HeLa.

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