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Induction of *Helianthus annuus* Leaves Extract to HeLa cell Apoptosis and Cell Cycle Arrest in S, G2-M and M5 Phase

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Abstract

Helianthus annuus L. (*H. annuus*) is a potential medicinal plant for cancer therapy. The aims of this study is to identify profile the anticancer activity of *H. annuus L.* from its leaves, root, stem, and seed as well as to elucidate the apoptosis and cell cycle of the leaves. Ten-gram sample of the powder were extracted by using Ultrasound-Assisted Extraction (UAE) with 200 ml of 96% ethanol by comparison of 1:20 with three times replications. The determination of anticancer activity was used the MTT cell proliferation assay, while apoptosis test and cell cycle were applied with the flow cytometry test. The value of IC_{50} in 96% ethanol extract in the root and stem was >1,000 µg/mL; seed and leaves were 153.76 µg/mL; and 126.6 µg/mL, respectively. The apoptosis induction of *H. Aannuus* leaves extract treatment was 7.17% of apoptosis cells; 90.44% of necrosis, and 2.39% of living cells. The *H. annuus* leaves extract also significantly caused a decrease of cell percentage in G0-G1 phase ($p<0.001$) and an increase in G2-M phase ($p<0.001$). The H. *annuus* leaves extract had greater potential as anticancer instead of other parts. The adding of *H. annuus* leaves extract increased the HeLa cell apoptosis, decreased percentage of HeLa cells in G0-G1 phase, and increased percentage of HeLa cells in G2-M phase. Cell cycle mechanism test showed cell cycle arrest in S, G2-M, and M5 phase in 24 h, hence inhibited the mitosis process.

Keywords: *anticancer, Helianthus annuus L, apoptosis, cell cycle.*

INTRODUCTION

Cervical cancer is the second biggest cancer causing death in the world. about 240000 fatality cases have been confirmed every year (WHO, 2018). Specifically in Indonesia, cancer patients had reached about 347,792 people (1.4% of population) in 2013. The incidence of cervical cancer in Indonesia is 0.8% or around 98,692 people (Health Ministry of the Republic of Indonesia, 2015). Cervical cancer is the fourth most common cancer for women, although they have been significant advances in the screening and treatment (Ferlay, *et al.*, 2015). Cervical cancer occurred in

cervical epithelial cells induced by HPV (Human Papilloma Virus) virus (Nour, 2009). Oncogene E6 binds to E6-AP forming ubiquitin ligase lead to degradation in the p53 gene that averts apoptosis from happening (Muti'ah, 2014). Oncogene in the E7 gene will bind pRb gene, and pRb then next will bind E2F transcription factor to avoid cell cycle regulation (Choudhari, *et al.*, 2013). The complete response rate for primary treatment of early-stage illness ranges from 70% to 90% with overall five-year survival for stage I disease in excess of 90% (Benedet, *et al.*, 2013). An adjuvant cure may improve survival but are associated with various adverse effects and toxicities (Maduro, *et al.*, 2013). Conventional therapies with phytotherapy appear to significantly increase survival rate, tumor remission rate, and decrease common detrimental effects, including vesical complications compared to conventional remedy alone (Xu, *et al.*, 2009).

Based on the World Health Organization (WHO), 70-80% of the world's society today uses herbs as health care because they have a minimum side effect (Al-Jumaily, *et al.*, 2013). During the past decades, a number of clinical trials have been conducted to investigate the value of natural herbs in the treatment of cervical cancer (Xu, *et al.*, 2009). Numerous literatures have reported that about 60% of anticancer agent is obtained from plants derivatives. Accordingly, plants or natural herbs play an essential role on the cancer treatment. One of the plants which can be used is sunflower or *Helianthus annuus* L. (*H. annuus*). Sunflower is one of 60 species in the genus Helianthus and member of the Asteraceae family (The Plantlist, 2010).

The composition of the seed is markedly affected by the sunflower variety (Salunkhe, 1992). The seed of *H. annuus* contains flavonoid, carbohydrate, tannin, alkaloid, saponin, phytosterol, terpene, and fixed oils. The seed of *H. annuus* can be used as an anti-inflammatory, anti-pyretic, astringent, diuretic, stimulant, expectorant, and as anticancer in *in vitro* test (Mutiah, 2017). Previous research has reported that the seed of the *H. annuus* shows substantial anticancer activity (83.5

and 166.4 ppm) compared to control ($p<0.05$). The seed of the sunflower could inhibit 27% HeLa cell with 0.001 µM concentration (Zhang, *et al.*, 2006; Al-Jumaily, *et al.*, 2013; Dwivedi, *et al.*, 2015). No systemic toxicity was appeared on sunflower tincture. However, it depended on dose, time, and sex (Boriollo, *et al.*, 2014). The most important part of them are genes that control survival and death of a cancerous cell. Induction of apoptosis is considered as one of the important targets in chemopreventive (Olson, *et al.*, 2001). Tumor suppressor protein p53, a key player in the death cell, has been reported to involve the mitochondrial activating the apoptotic pathway (Schuler and Green, 2001).

MATERIALS AND METHODS

Materials in this study were the roots, stems, seeds, and leaves of *H. annuus*. Solvents used for maceration was 70% ethanol (Merck, Jakarta, Indonesia). Cervix cancer cell line was HeLa obtained from Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada and Prof. Masasi Kawaichi, Laboratory of Gene Function in Animal, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Japan. The reference drug used is cisplatin (PT Dankos Farmas, Jakarta, Indonesia). HeLa cells in a medium of Rosewell Park Memorial Institute (RPMI) supplemented with 10% heatinactivated fetal bovine serum (FCGS) (PAA laboratories, Netherlands), 1% v/v penicillin-Streptomycin (NacalayTesque, San Diego, USA), and 1.0 mM L-glutamine (NacalayTesque). The cells were cultured in the incubator, at 5% CO_2, 95% O_2 at 37°C. Dimethyl sulfoxide (DMSO), were used to dissolve the extract of *H.annuus*. The concentrations used in this study a maximum of 1% in the culture medium. A 0.025% trypsin in the culture medium was used to harvest cells. Phosphate buffered saline (PBS) was used as wash buffer solution. 3- (4,5-dimetiltiazole-2-yl)-2,5-difeniltetrazolium bromide (MTT) was used as a reagent that reacts with the enzyme succinate dehydrogenase in the cell. Material

Testing Regulation to review cell cycle flowcytometry method: A solution propidium iodine (PI) (Sigma) Consisting of 50 µg/mL PI, 20 µg/mL, and 0.1% Triton-X (Pro GC-Merck, Jakarta, Indonesia). Materials for review Testing Methods Induction of apoptosis flowcytometry: Solution PI Consisting of 50 µg/mL PI, 20 µg/mL, and 0.1% Triton-X hearts PBS. Annexin V assay (Annexin V FITC Apoptosis Detection Kit Biovision, USA) and binding buffer (Mutiah, *et al.*, 2017).

Selectivity Index (SI)

The aim of this analysis is to knowing whether the administration of seed, stem, root and leaves of *H. annuus* is selective in killing target cells Hela cell line. A test material said that selectivity is when killing the target cell and the the SI value is >3. SI value obtained from the ratio of IC_{50} concentration of normal cells/vero cells (IC_{50}) compared to HeLa cell IC_{50} (SI= IC_{50} Vero cell : IC_{50} HeLa Cell) (Nurulita, *et al.*, 2014).

Helianthus annuus L. (*H. annuus*) Identification

The roots, stems, seeds, and leaves of *H. annuus* were obtained from Malang area, East Java, Indonesia. The determination of *H. annuus* conducted in Materia Medika, Batu City, Malang, Indonesia. It was proven by the letter, number 074/406/102.7/2017, issued by UPT Materia Medika, Batu City, Malang.

Extraction of *Helianthus annuus* L. (*H. annuus*)

The part of seed, stem, root and leaves of *H. annuus* was cleaned using the flowing water, and dried under the sunlight. The dried organs then grounded into powder (simplisia) and 10 gram powder extracted using Ultrasound-Assisted Extraction (UAE) with 200 mL of 96% ethanol with three times replications The collected filtrates then separated using a rotary evaporator. Next, the extract was dried in oven (40°C).

Thin Layer Chromatography (TLC)

The extract (10 mg) was dissolved in 1 mL of 96% ethanol with UAE. The root, stem, seed, and leaves extract then were spotted on the Thin Layer Chromatography (TLC) silica gel plate F_{254} 10x6 cm. Each spot contained 2 µL of dissolved extract. n-hexane: ethyl acetate (7:3) was used as mobile phase. The stains were observed using Ultraviolet (UV) visualizer at wavelengths of 254 nm and 366 nm. Further, TLC plate was sprayed by H_2SO_4 10% v/v, and it was heated at 105 temperature for five minutes using TLC plate heater.The result of the stain/spot then observed using UV visualizer at wavelength of 366 nm.

Ethical Approval

This research was approved by the ethical number 002/EC/KEPK-FKIK/2018 issued by the Research Ethical Commission of Faculty of Medicine and Health Science, Islamic State University of Maulana Malik Ibrahim, Malang.

Cell Culture

The cells that had reached confluent (75%-80% of cell culture flask) were washed twice by PBS before trypsin-EDTA were spread homogeneously and the cell incubated for 3 minutes in the incubator. After incubation, 5 mL of media was added for in-activating trypsin, and the condition of the cells were observed by using the microscope. The cells which had been separated from the cell bind by the matrix then transferred into sterile conical.

HeLa Cell Viability Measurement

HeLa cells counted in a hemocytometer, were diluted in 10,000 cell/well HeLa cell densities. Then, 100 µL/well HeLa cells were moved into 96 well plates. Afterwards, the cells were incubated for 24 h in the temperature of 37°C and 5% CO_2. After 24 h, the media on the plate befell was removed. Then, the 96% ethanol extracts of root, stem, seed, and leaves were dissolved in DMSO 1% v/v. the obtained concentration series were 1000 µg/mL, 500

µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, and 15.625 µg/mL, respectively. The mixtures were placed into well for three times (triple), and eventually incubated for 24 h in 37ºC temperature and 5% CO_2. After incubation, the media on the plate was removed, 100 µL/well of MTT reagent was added, and the extract then was incubated for 3 h. If the formazans was formed, 100 µL/well of 10% SDS in 0.1 N HCl was added. The extracts were denoted for further incubation in a dark room (covered with an aluminum foil) for 24 h. Subsequently, the result was analyzed by using ELISA reader in λ=590 nm. The percentage of the living cell was calculated by Equation 1.:

Living Cell Percentage %=((Intervention Absorbance - Media Control Absorbance))/ ((Cell Control Absorbance - Media Control Absorbance))

HeLa Cell Preparation for Flowcytometry Test

The cell with a density of $5x10^5$cell/well was placed in 6-well plate and then incubated. The *H. annuus* in DMSO solvent (0.25%) is the highest anticancer activity value. The second incubation was carried out for 24 h. The media was taken and transferred into centrifuged and non-centrifuged tube with the velocity of 2,000 rpm for 3

minutes. The supernatant was removed. PBS was attached into the well which was no media on it. Then, transported into the same treated microtube, and the supernatant was removed. This phase was repeated, and the cells were harvested using trypsin. The cells were transferred into the micro tube and centrifuged with 2,000 ppm for three minutes. The remains of the harvested cells on the reservoir were washed using PBS and centrifuged once more, and the PBS was removed. The cells sediment were further tested for cell apoptosis test and cell cycle test by using flow cytometry method.

Measurement of Number of Cells Experiencing Apoptosis

The PI-Annexin Vreagent was added to the sediment of cells. The Microtube containing gel suspense was wrapped with the aluminium foil and incubated in the water bath with 37ºC temperature for five minutes. The gel suspense was re-homogenized and transferred into flowcytometer tube by using nylon filter. Those samples were analyzed by using cell quest program.

The Measurement of the Number of Cells in Each Cell Cycle Phase

Cells were dissolved 500 µL of 60% alcohol. Microtube then was stored in room temperature for an hour. The cells were centrifuged with 2,000
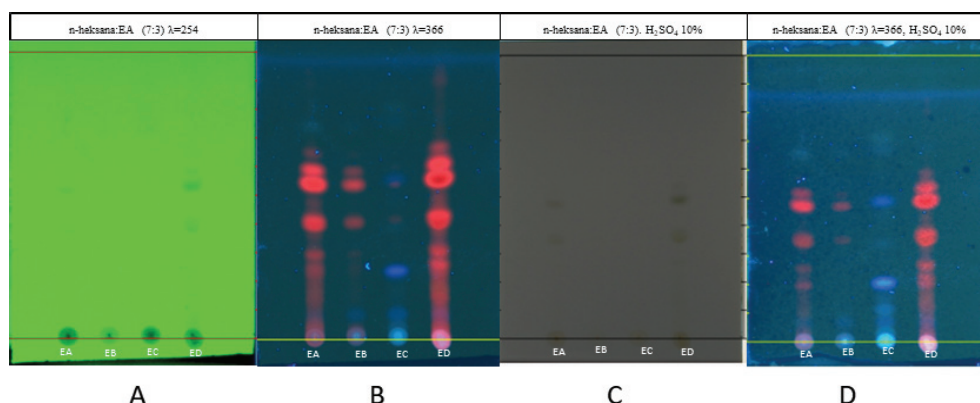

Figure 1. The result of Thin Layer Chromatography (TLC) test on 96% ethanol extract of root, stem, seed, and leaves of *H.annuus*. Profile TLC root extract (EA), Sstem extract (EB), seed extract (EC) and leave extract (ED). GF254 silica gel stationary phase and the mobile phase n-hexane: EA (7:3). (A) after eluasi and viewed under UV 254 nm; (B) observed under UV 366 nm; (C) after being sprayed with 10% H_2SO_4 and heated to 105ºC; (D) after being sprayed, heated and viewed under UV 366 nm.

Table 1. Identification of Thin Layer Chromatography (TLC) profile of *H.annuus* extract.

Sample	Rf Value	UV 366	Compounds	References
EA	0.193	Red	Terpenoid	
	0.348	Red	Terpenoid	
	0.474	Red	Terpenoid	Ismiyah, *et al.*, 2014;
	0.568	Red	Terpenoid	Wagner, *et al.*, 1996
	0.661	Red	Terpenoid	
	0.859	Red	Terpenoid	
EB	0.474	Red	Terpenoid	
	0.856	Red	Terpenoid	Ismiyah, *et al.*, 2014;
	1.046	Blue	Steroid	Wagner, *et al.*, 1996
EC	0.112	Blue	Steroid	
	0.205	Blue	Steroid	
	0.405	Blue	Steroid	Ismiyah, *et al.*, 2014;
	0.49	Blue	Steroid	Wagner, *et al.*, 1996
	0.862	Blue	Steroid	
	1.045	Blue	Steroid	
ED	0.221	Red	Terpenoid	
	0.368	Red	Terpenoid	
	0.452	Red	Terpenoid	
	0.537	Red	Terpenoid	Ismiyah, *et al.*, 2014;
	0.659	Red	Terpenoid	Wagner, *et al.*, 1996
	0.715	Red	Terpenoid	
	0.862	Red	Terpenoid	

rpm for three minutes. The alcohol was removed and 500 µL of PBS was added; the cells were centrifuged with 2,000 rpm for three minutes then washed twice with PBS. The cells were combined with the PI reagent. Microtubes were wrapped with aluminium foil for 30 minutes. The suspense cells were homogenized again then transferred to the flowcytometer tube with nylon filters. Afterwards, cell quest was investigated with the flowcytometer softwarewith wavelengths of 488 nm with rate flow of 500 cell/second.

RESULTS

Thin-Layer Chromatography (TLC) Testing Using Visualizer Method

The result of the chemical compound category showed that the 96% ethanol extracts of *H. annuus* in the root and stem were contained terpenoid and steroid compound as shown in Figure 1. Then, 96% ethanol extracts of seed and leaves were contained terpenoid and steroid compounds, respectively. A previous study reported that *H. annuus* contained terpenoid that is sesquiterpene lactone (Amrehn, *et al.*, 2015; Ismiyah, *et al.*, 2014). Sesquiterpene had a chemical chain derived from three isoprenes. The sesquiterpene compounds had successfully identified in *H. annuus* leaves were artemisinin, heliangolide and eupalinolide C (Mutiah, *et al.*, 2017).

Cytotoxicity and Selectivity Index (SI)

Cytotoxicity and SI were determined in order to determine the extract's ability to inhibit cancer cell growth and the safety of the extract against normal cells. Table 2 shows that the extracts of seed, leaves, and cisplatin had a lower IC_{50} value in HeLa cell than in Vero cell. IC_{50} values in the

Table 2. IC$_{50}$Value of cytotoxicity in 96% ethanol extract of *H. annuus* and anticancer drug employing probit.

Sample	HeLa Cell*	Vero Cell*	SI**	SI Category
Root	>1000	>1000	1.163±0.271	Non-selective
Stem	>1000	>1000	0.763±0.552	Non-selective
Seed	153.759±7.738	578.873±6.003	3.770±0.155	Selective
Leaves	126.831±12.612	537.041±4.649	5.647±0.980	Selective
Cisplatin	42.110±13.983	209.449±4.641	5.383±1.882	Selective

*Average Value of IC$_{50}$±SD with three times replication
**SI=Selectivity Index

Vero cell of the extracts of seed, leaves and cisplatin were 578.87 µg/mL; 537.041 µg/mL and 209.449 µg/mL, succesively. The anticancer selection was not only based on the cytotoxicity in HeLa cell but also the selectivity in Vero cell to guarantee its safety. The extract is contemplated if the SI parameter value is more than three (Rollando, *et al.*, 2016). The root and stem extracts of *H. annuus* had low cytotoxicity and toxicity. Cisplatin showed high cytotoxicity in HeLa cell, but cisplatin had a high toxicity in Vero cell. The 96% ethanol of *H. annuus*

leaves extract would have high cytotoxicity compared to other parts of the organ of *H. annuus*. The 96% ethanol *H. annuus* extracts of root and stem were classified as non-active (Table 1). Then, 96% ethanol extracts of the seed and leaves, as well as cisplatin, were classified as selective category.

Root, stem, seed, and leaves extracts were administrated for the treatment of HeLa and Vero cell for 24 h. The figure of HeLa and Vero cell viabilities after the treatment with *H. annuus* extracts is presented in Figure 2.
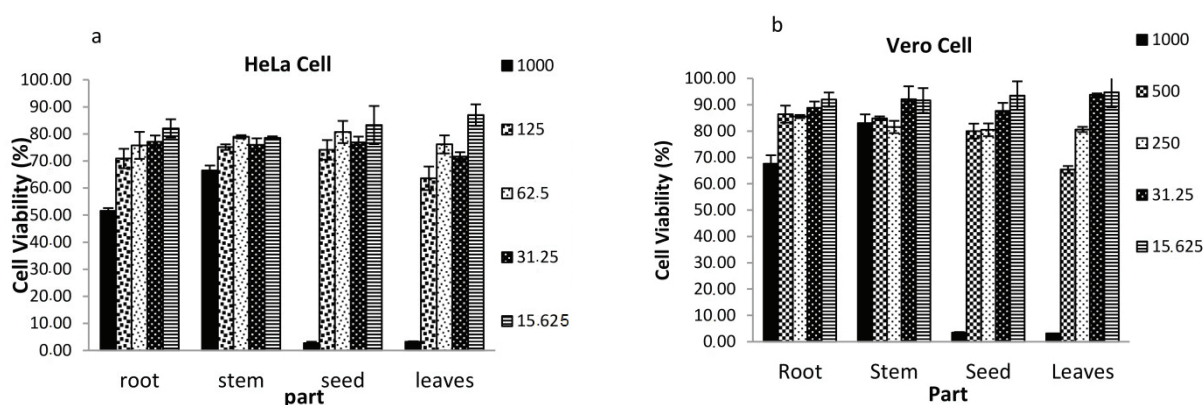

Figure 2. The live cells percentage result graph (a) HeLa cell (b) Vero cell with the administration of 96% ethanol extract of root, stem, seed, and leaves of *H. annuus* for 24 h. The result of the data shows the average percentage of living cells±Standard Deviation with three time replication.
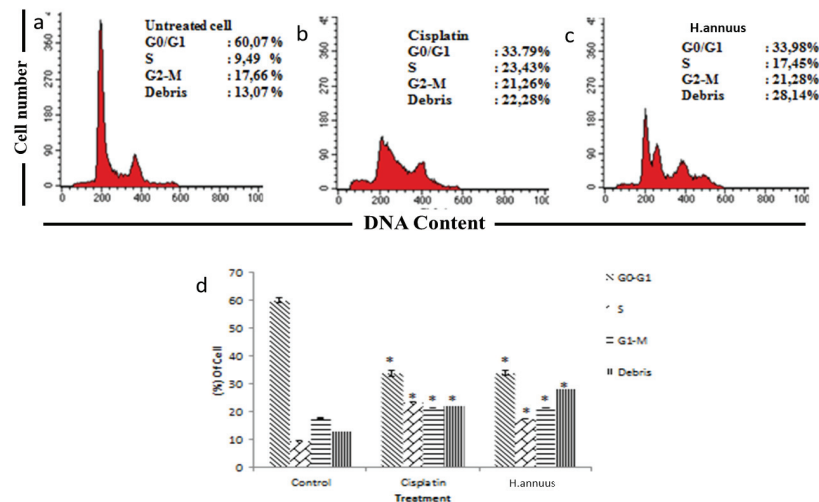
Figure 3. The flowcytometry in cell cycle after 24 h treatment of *H. annuus* leaves extract in HeLa cell. a) Cell cycle histogram HeLa cell control, b) Cell cycle histogram in HeLa cell after *H. annuus* leaves extract treatment, c) Diagram of comparison of cell cycle between the cell control and *H. annuus* leaf extract.

Cell Cycle

H. annuus leaves extract administration had activity in HeLa cervical cancer cell cycle regulation. It caused a decreasing of cell percentage in G0-G1 (33.98%) phase compared to the cell control (60.07%). In the G2-M phase, *H. annuus* leaves extract increased to 21.28% compared to the cell control, 17.66% an increase in M5 phase was 7.31% in the cell control, and 15.27% in

H. annuus leaves extract. The cell distribution result is presented in Figure 3.

Apoptosis

The living cell percentage in a culture without any treatment (cell control) was 95.59%, while 2.9% of the total cell was apoptosis and 1.54% was necrosis cell. Cell culture treated with extract was 7.17% of cell apoptosis, while necrosis was 90.44%
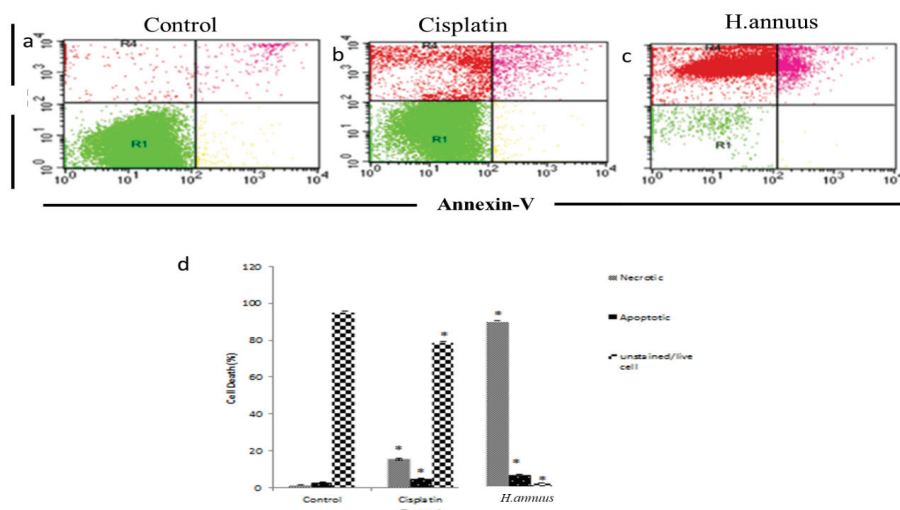

Figure 4. The flowcytometry in apoptosis mechanism after 24 h of *H. annuus* leaves extract treatment in HeLa cell. a) The flowcytometry histogram in HeLa cell control, b) The flowcytometry histogram in HeLa cell after *H. annuus* leaves extract treatment, c) Diagram of apoptosis mechanism comparison of cell control, d) *H. annuus* leaves extract and cisplatin. The data were analyzed employing SPSS. *P<0.01 showed a significant compared to the control.

and 2.39% of the total cells was implied living cells. The picture of the apoptosis cell interpreting result using the flowcytometer method is displayed in Figure 4.

DISCUSSION

The aims of this study is to find out the compound category, an IC_{50} value in HeLa cell, and Vero cell, as well as SI in the extracts of root, stem, seed, and leaves of *H. annuus*. The part of the *Helianthus annuus* plant exhibiting the highest anticancer potential was evaluated a death path, cell cycle.

The HeLa cell viability percentage exhibited a dose-dependent phenomenon that was an existence of the relation between concentrate and cytotoxicity (Figure 2). IC_{50} value is an extracted parameter inhibiting the 50% growth of the cell. The cytotoxicity in 96% ethanol extract in the root and stem of *H. annuus* reached IC_{50}>1000 µg/mL value. It has no anticancer activity since the value of IC_{50} was >500 µg/mL. Meanwhile, the 96% ethanol extracts in the seed, leaves and cisplatin of *H. annuus* revealed IC_{50} values of 153.75±7.738 µg/mL, 125.831±12.612 µg/mL, and 42.11±13.983 µg/mL, respectively. Hence, it has anticancer activity because the value of IC_{50} was <500 µg/mL (Mutiah, *et al*., 2017). The 96% ethanol leaves extract of *H. annuus* had the highest cytotoxicity .

The amount of IC_{50} Vero cell showed lower toxicity in the seed extract compared to leaves extract. Although seed extract had the lower toxicity value than leaves extract, the SI value in the leaves extracts was the higher than the seed extract's. Thus, 96% ethanol of *H. annuus* leaves extract had cytotoxicity and selectivity in Vero cell. Therefore, *H. annuus* leaves extract was chosen for further investigation through apoptosis and cell cycle test. The highest selected value was observed in 96% ethanol extract of *H. annuus* leaves which mean the extract had an anticancer activity without affecting the normal cell. It can further be used as

an anticancer compound. Therefore, this research was administered by investigating the mechanism of apoptosis and cell cycle using the extract of *H. annuus* leaves.

Cell Cycle mechanism testing employs flowcytometry. The method was chosen to determine the cell cycle constraint in HeLa cell. *H. annuus* leaves extract pointed out an increasing result in S, G2-M and M5 phase in 24 h. The G2-M phase delayed in a cell cycle with a long time. It can inhibit the mitosis process. As a result, mitotic catastrophe might happen (Costantino, *et al*., 2013; Kitai, *et al*., 2016). Mitotic catastrophe mechanism is an oncosuppressive mechanism causing damage in mitosis phase. Hence, the cells undergo an anti-proliferation and dead. Mitotic catastrophe mechanism in the sesquiterpene lactone blocks the forming of microtubule from, restraints DNA replication and cross-linking in DNA, particularly in adenine and guanine alkaline (Choudari, 2013; Kitai, *et al*., 2016). The extension in M5 area is a sign of polyploid forming in DNA (Figure 3). The delay was occurred in G2-M phase precipitate damage in DNA, as a sign of cyclin B1 presence. It prompted an abnormality of chromatin number into polyploid (Margarete, 2015; Vakifahmetoglu, *et al*., 2008). So, the inhibition of anaphase process was caused by the occurrence of polyploid by inhibiting the process of the Anaphase Promoting Complex (APC) that failed to degradation cyclin B1.

One of the unique features of mitotic catastrophe in the first 24 h is the size amplification of cell and DNA degradation. The characteristics or features of mitotic catastrophe are similar to necrosis (Kimura, *et al*., 2013). The flowcytometry technique adopted to define the occurrence of apoptosis. The method determines the necrosis and apoptosis process. The results of *H. annuus* leaves extract treatment for 24 h were: 7.17% apoptosis cells, 90.44 % necrosis cells, and 2.39% living cells.

Meanwhile, a cisplatin identified necrosis event with a lower percentage than *H. annuus* leaves extract. Those indicated that the

H. annuus leaves extract death had a similar pathway to cisplatin known as necrosis. However, the number of death percentage between cisplatin and *H. annuus* leaves extract were the highest in *H. annuus* leaves extract. The early signs of death cell in mitotic catastrophe are necrosis by inducing specific biochemistry that has not profoundly studied. Nevertheless, death cell necrosis can be followed by a death process by apoptosis in a long time (Muti'ah, 2014). The pathway of the mitotic catastrophe occurrence can also get into necrosis pathway when there is no help from protein. The ascertainment of the final pathway of mitotic catastrophe depends on the length of treatment given (Kimura, *et al.*, 2013). The majority of chemotherapy works by inducing cell cancer apoptosis. There are two types mechanism of proliferation shrinkage and induced apoptosis particularly the p53-dependent mechanism and p53-independent mechanism (Rachmawati, *et al.*, 2012).

CONCLUSION

In conclusion, from the part seed, stem, root and leaves, *H. annuus* leaves extract had greater potential anticancer than the other parts. The adding of 96% ethanol leaves extract increased the HeLa cell apoptosis. Cell cycle mechanism test showed cell cycle arrest in S, G2-M and M5 phase in 24 h, so that inhibited the mitosis process.

ACKNOWLEDGMENT

CONFLICT OF INTEREST

There is no conflict of interested in this study.

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