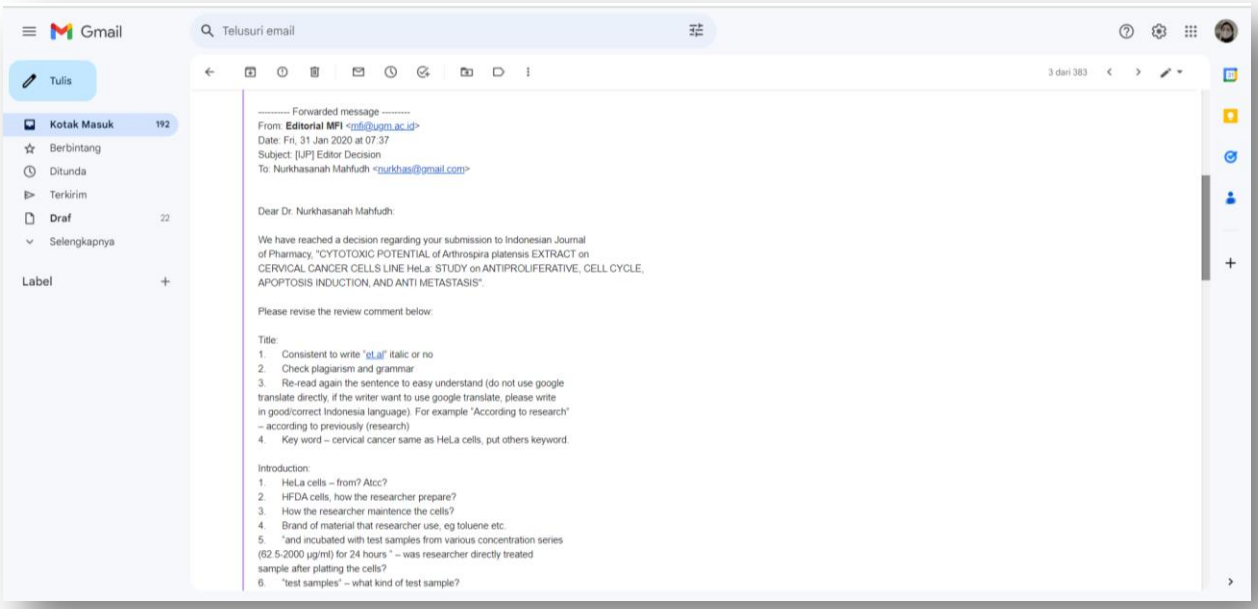
# Daftar Isi

**Bukti Korespondesi Jurnal "Cytotoxic Potential of Arthrospira platensis Extract on Cervical Cancer Cells Line HeLa: Study on Antiproliferative, Cell Cycle, Apoptosis Induction, and Anti Metastasis"**

**Email 1, 31 Januari 2020**



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Tulis

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--------- Forwarded message ---------
From: **Editorial MFI** <mfi@ugm.ac.id>
Date: Fri, 31 Jan 2020 at 07:37
Subject: [UP] Editor Decision
To: Nurkhasanah Mahfudh <nurkhas@gmail.com>

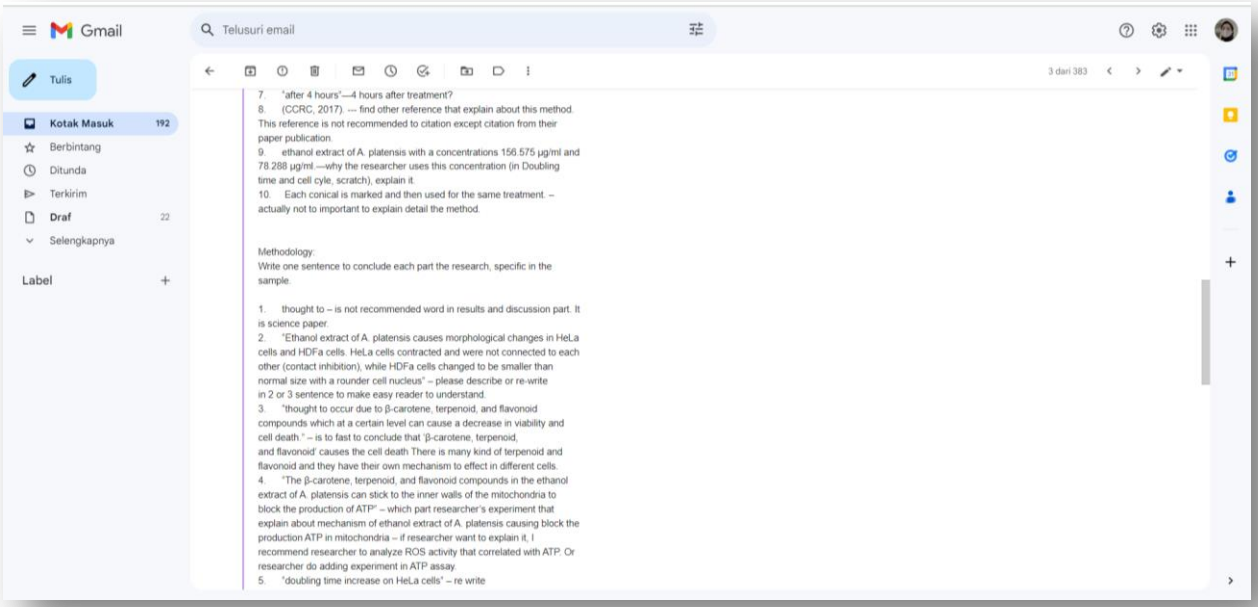
Dear Dr. Nurkhasanah Mahfudh:

We have reached a decision regarding your submission to Indonesian Journal of Pharmacy, "CYTOTOXIC POTENTIAL of Arthrospira platensis EXTRACT on CERVICAL CANCER CELLS LINE HeLa: STUDY on ANTIPROLIFERATIVE, CELL CYCLE, APOPTOSIS INDUCTION, AND ANTI METASTASIS".

Please revise the review comment below:

Title:
1. Consistent to write "et.al" italic or no
2. Check plagiarism and grammar
3. Re-read again the sentence to easy understand (do not use google translate directly, if the writer want to use google translate, please write in good/correct Indonesia language). For example "According to research" – according to previously (research)
4. Key word – cervical cancer same as HeLa cells, put others keyword.

Introduction:
1. HeLa cells – from? Atcc?
2. HFDA cells, how the researcher prepare?
3. How the researcher maintence the cells?
4. Brand of material that researcher use, eg toluene etc.
5. "and incubated with test samples from various concentration series (62.5-2000 µg/ml) for 24 hours " – was researcher directly treated sample after platting the cells?
6. "test samples" – what kind of test sample?



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Tulis

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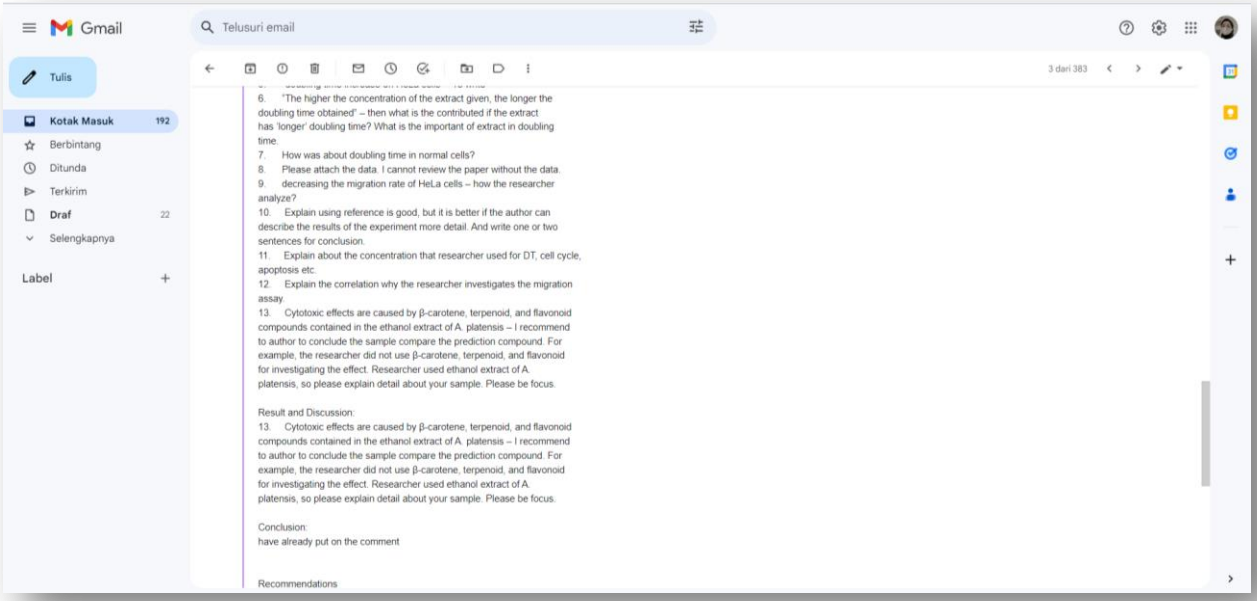
7. "after 4 hours"—4 hours after treatment?
8. (CCRC, 2017). --- find other reference that explain about this method. This reference is not recommended to citation except citation from their paper publication.
9. ethanol extract of A. platensis with a concentrations 156.575 µg/ml and 78.288 µg/ml.—why the researcher uses this concentration (in Doubling time and cell cyle, scratch), explain it.
10. Each conical is marked and then used for the same treatment. – actually not to important to explain detail the method.

Methodology:
Write one sentence to conclude each part the research, specific in the sample.

1. thought to – is not recommended word in results and discussion part. It is science paper.
2. "Ethanol extract of A. platensis causes morphological changes in HeLa cells and HDFa cells. HeLa cells contracted and were not connected to each other (contact inhibition), while HDFa cells changed to be smaller than normal size with a rounder cell nucleus" – please describe or re-write in 2 or 3 sentence to make easy reader to understand.
3. "thought to occur due to β-carotene, terpenoid, and flavonoid compounds which at a certain level can cause a decrease in viability and cell death." – is to fast to conclude that 'β-carotene, terpenoid, and flavonoid' causes the cell death There is many kind of terpenoid and flavonoid and they have their own mechanism to effect in different cells.
4. "The β-carotene, terpenoid, and flavonoid compounds in the ethanol extract of A. platensis can stick to the inner walls of the mitochondria to block the production of ATP" – which part researcher's experiment that explain about mechanism of ethanol extract of A. platensis causing block the production ATP in mitochondria – if researcher want to explain it, I recommend researcher to analyze ROS activity that correlated with ATP. Or researcher do adding experiment in ATP assay.
5. "doubling time increase on HeLa cells" – re write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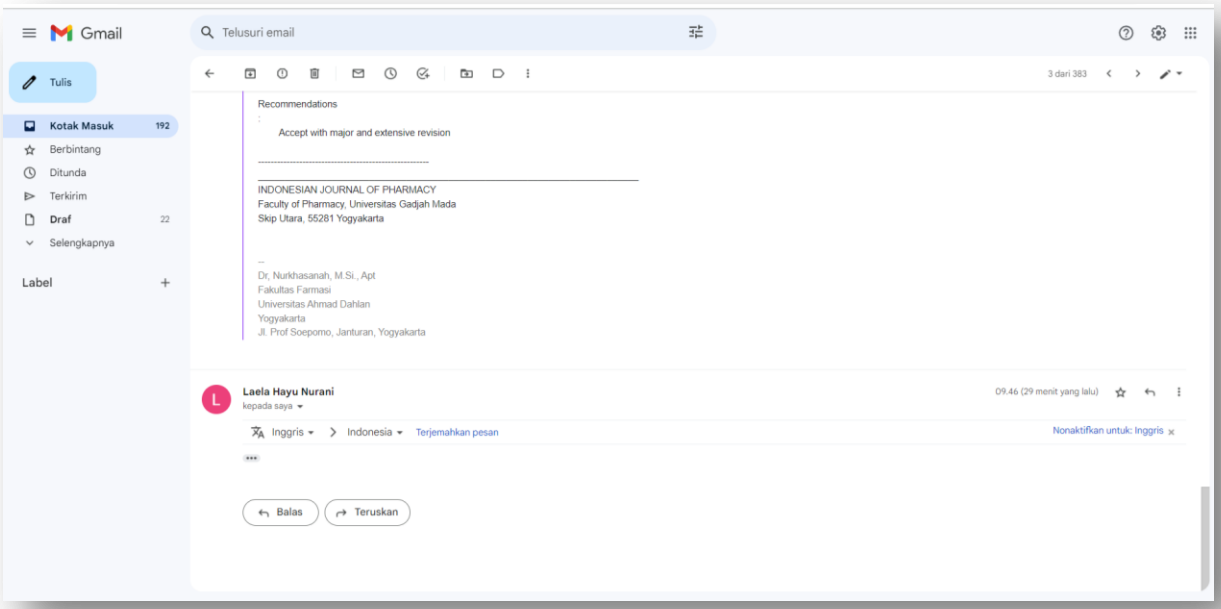
6. "The higher the concentration of the extract given, the longer the doubling time obtained" – then what is the contributed if the extract has 'longer' doubling time? What is the important of extract in doubling time.
7. How was about doubling time in normal cells?
8. Please attach the data. I cannot review the paper without the data.
9. decreasing the migration rate of HeLa cells – how the researcher analyze?
10. Explain using reference is good, but it is better if the author can describe the results of the experiment more detail. And write one or two sentences for conclusion.
11. Explain about the concentration that researcher used for DT, cell cycle, apoptosis etc.
12. Explain the correlation why the researcher investigates the migration assay.
13. Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of A. platensis – I recommend to author to conclude the sample compare the prediction compound. For example, the researcher did not use β-carotene, terpenoid, and flavonoid for investigating the effect. Researcher used ethanol extract of A. platensis, so please explain detail about your sample. Please be focus.
Result and Discussion:
13. Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of A. platensis – I recommend to author to conclude the sample compare the prediction compound. For example, the researcher did not use β-carotene, terpenoid, and flavonoid for investigating the effect. Researcher used ethanol extract of A. platensis, so please explain detail about your sample. Please be focus.
Conclusion:
have already put on the comment
Recommendations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Recommendations
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Accept with major and extensive revision
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INDONESIAN JOURNAL OF PHARMACY
Faculty of Pharmacy, Universitas Gadjah Mada
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Fakultas Farmasi
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Yogyakarta
Jl. Prof Soepomo, Janturan, Yogyakarta
Laela Hayu Nurani
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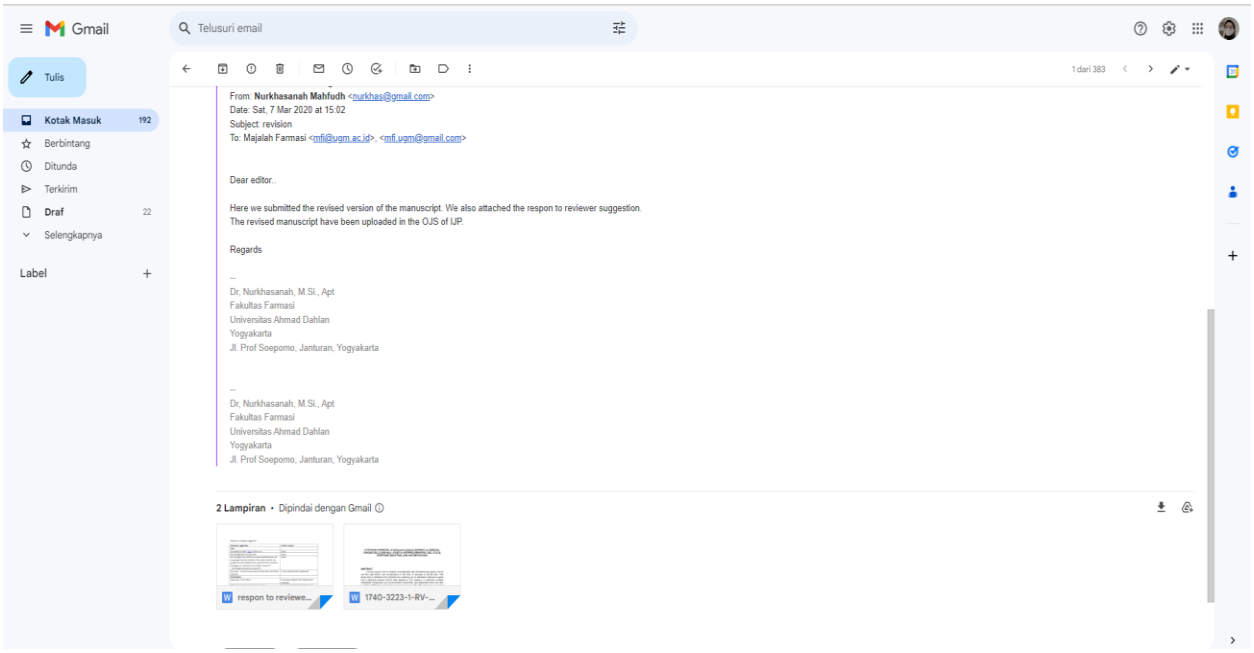
**Email 2, 7 Maret 2020**



# Lampiran
# “respon to reviewer”

Respon to reviewer suggestion

| **Reviewer suggestion** | **Author respon** |
|---|---|
| **Title** | |
| Consistent to write "et.al" italic or no | Done |
| Check plagiarism and grammar | Done |
| Re-read again the sentence to easy understand (do not use google translate directly, if the writer want to use google translate, please write in good/correct Indonesia language). For example "According to research" – according to previously (research) | Done |
| Key word – cervical cancer same as HeLa cells, put others keyword. | It was replaced with cytotoxicity |
| **Introduction** | |
| HeLa cells – from? Atcc? | It has been stated in the material and methods |
| HFDA cells, how the researcher prepare? | It has been stated in the material and methods |
| How the researcher maintence the cells? | It has been stated in the material and methods |
| Brand of material that researcher use, eg toluene etc. | It has been stated in the material and methods |
| . "and incubated with test samples from various concentration series (62.5-2000 µg/ml) for 24 hours " – was researcher directly treated sample after platting the cells? | It was stated in the text, there is an incubation before treatment. |
| "test samples" – what kind of test sample? | It has been stated: *A.platensis* extract. |
| "after 4 hours"—4 hours after treatment? | It was explained in the text "…and stand for 4 hours before measuring the intensity" |
| CCRC, 2017). --- find other reference that explain about this method. This reference is not recommended to citation except citation from their paper publication. | It was replaced |
| ethanol extract of A. platensis with a concentrations 156.575 µg/ml and 78.288 µg/ml.—why the researcher uses this concentration (in Doubling time and cell cyle, scratch), explain it. | It was stated in the discussion section |
| Each conical is marked and then used for the same treatment. – actually not to important to explain detail the method. | It was deleted |
| Methodology: | |
| Write one sentence to conclude each part the research, specific in the sample. | done |
| thought to – is not recommended word in results and discussion part. It is science paper. | The sentence has been revised |
| "Ethanol extract of A. platensis causes morphological | It has been revised: |

| | |
|---|---|
| changes in HeLa cells and HDFa cells. HeLa cells contracted and were not connected to each other (contact inhibition), while HDFa cells changed to be smaller than normal size with a rounder cell nucleus" – please describe or re-write in 2 or 3 sentence to make easy reader to understand | The treatment of ethanol extract of *A. platensis* causes morphological changes in HeLa cells and HDFa cells. Some of HeLa cells were shrink and detach to each other (contact inhibition) and decrease in number, as well as HDFa cells (Figure 1) |
| thought to occur due to β-carotene, terpenoid, and flavonoid compounds which at a certain level can cause a decrease in viability and cell death." – is to fast to conclude that 'β-carotene, terpenoid, and flavonoid' causes the cell death There is many kind of terpenoid and flavonoid and they have their own mechanism to effect in different cells. | It has been revised:<br>The cytotoxic activity of ethanol extract of *A. platensis* could be caused by its chemical content including β-carotene, terpenoid, and flavonoid. The previous studies suggested the potency of compounds as cytotoxic agent (Wati *et al.,* 2016). |
| "The β-carotene, terpenoid, and flavonoid compounds in the ethanol<br>extract of A. platensis can stick to the inner walls of the mitochondria to block the production of ATP" – which part researcher's experiment that explain about mechanism of ethanol extract of A. platensis causing block the production ATP in mitochondria – if researcher want to explain it, I recommend researcher to analyze ROS activity that correlated with ATP. Or researcher do adding experiment in ATP assay. | We add some reference in the mechanism of natural product as anticancer through the inhibiting of metabolism and mitochondrial function<br><br>The previous studies showed the potency of flavonoids as anticancer agent (Abotaleb et.al., 2018) through inducing apoptosis. Another mechanism of flavonoid in inhibiting of cancer progression is through its ability to target molecular pathways involved in glucose metabolism and mitochondrial function (Farias & Pozo, 2019). |
| "doubling time increase on HeLa cells" – re write | Done |
| "The higher the concentration of the extract given, the longer the doubling time obtained" – then what is the contributed if the extract has 'longer' doubling time? What is the important of extract in doubling time. | We added in the text:<br><br>This result showed that treatment of *A.platensis* extract could inhibit the cancer cells proliferation. |
| How was about doubling time in normal cells? | We added in the text:<br>In the control group, it was found the doubling time is shorter. It showed that without treatment, Hela cells proliferate faster. |
| Please attach the data. I cannot review the paper without the data. | The data was presented in the supplement |
| decreasing the migration rate of HeLa cells – how the researcher<br>analyze? | Analysis was done from the data presented in Figure 6 |
| Explain using reference is good, but it is better if the author can describe the results of the experiment more | Done<br>In conclusion section |

| | |
|---|---|
| detail. And write one or two sentences for conclusion. | |
| Explain about the concentration that researcher used for DT, cell cycle,<br>apoptosis etc. | We added in the discussion section:<br>"The study of cytotoxicity mechanism of the extract was carried out in concentration under $IC_{50}$ including 156.575 ug/ml and 78,288 ug/ml to allow the cells grow and could be observed." |
| Explain the correlation why the researcher investigates the migration<br>assay. | We added in the discussion: "The scratch wound healing assay was carried out to observed the effect of extract in inhibiting migration of the cancerous cells. The capability of extract to inhibit the cell migration could inhibit the progression of cells, which is the important part of cancerous cells to spread and metastatic." |
| Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of A. platensis – I recommend to author to conclude the sample compare the prediction compound. For example, the researcher did not use β-carotene, terpenoid, and flavonoid for investigating the effect. Researcher used ethanol extract of A. platensis, so please explain detail about your sample. Please be focus. | done |
| Conclusion:<br>have already put on the comment | |

# Lampiran
# “1740-3223-1-RV-R1”

# CYTOTOXIC POTENTIAL of *Arthrospira platensis* EXTRACT on CERVICAL CANCER CELLS LINE HeLa: STUDY on ANTIPROLIFERATIVE, CELL CYCLE, APOPTOSIS INDUCTION, AND ANTI METASTASIS


## ABSTRACT

Cervical cancer can be treated conventionally with chemotherapy agents, but its use has side effects and complications in the form of damage to normal cells. This study aims to determine the potential of A. platensis as an alternative anticancer agent that is selective towards normal cells. Based on TLC analysis, *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, and terpenoids which are able to inhibit proliferation and trigger apoptosis of cancer cells. The study was conducted using cervical cancer cells HeLa and normal cells HDFa. *A. platensis* macerated with 96% ethanol at a ratio of 1:4. Based on probit analysis, it is known that ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with $IC_{50}$ values of 260.444 µg/ml and index selectivity towards HDFa cells of 7.931. The mechanism of cytotoxic activity of ethanol extract of *A. platensis* is related to its ability to extend the doubling time, increase the induction of apoptosis, and reduce the rate of cells migration. Ethanol extract of A. platensis can also increase cells accumulation in the S phase to prevent cells from entering the G2/M phase.


**Key words:** *A. platensis*, cytotoxicity, HeLa cells, HFDa cells


## INTRODUCTION

Cervical cancer can occur due to an infection by the Human Papilloma Virus (HPV) that cause mutations in the p53 gene and increased expression of the protein Bcl-2 which resulted in an imbalance between proliferation and cell (Sari and Syahrul, 2014). One of the methods chosen to treat cervical cancer is by giving chemotherapy agents. However, chemotherapy agents have side effects and complications in the form of normal cell damage (Huang *et al.,* 2018). Therefore, it is necessary to develop alternative anticancer agents which are selective towards cancer cells without affecting normal cells.

Previous research showed that *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, terpenoids, and saponin compounds (Muszynska *et al.,* 2018). Antioxidant compounds are able to regulate antiapoptotic proteins in the mitochondria to increase the induction of apoptosis (Muszynska *et al.,* 2018). In addition, antioxidant compounds also have the ability to repair DNA or RNA damage in normal cells which will trigger the activation of the p53 gene so that there is a cessation of cell cycle and inhibition of cancer cell proliferation (Nugraheni *et al*., 2013). Based on this, it is necessary to investigate *A. platensis* cytotoxicity against HeLa cervical cancer cells. HeLa cells are cervical cell cultures infected with HPV and express 2 oncogens, namely E6 and E7 so that they can multiply in culture media and have cancer properties (Lucey *et al.,* 2009). The selectivity test for *A. platensis* is done using Human Dermal Fibroblast Adult (HDFa) cells, HDFa cells are primary cell cultures that can present responses to stress and other biological approaches that approach in vivo situations in humans (Özen *et al.,* 2005).

The cytotoxic mechanism of an anticancer agent is related to its ability to inhibit proliferation and induce apoptosis (Moningka, 2019). Therefore, an antiproliferation test was performed to determine the doubling time value to show the ability of *A. platensis*

to inhibit HeLa cells proliferation, flow cytometry analysis to observe inhibition patterns in the cell cycle and the magnitude of apoptosis induction in HeLa cells due to *A. platensis* treatment, and cell migration inhibition test with wound healing assay to determine the effect of *A. platensis* on the process of metastatic HeLa cancer cells (Lee *et al.,* 2014).

## MATERIAL AND METHODS

### Plant Material and Cell Culture

*A. platensis* obtained from the Serba Alami Store, Bantul, Yogyakarta in November 2018. The sample has been identified at the Faculty of Biology, Gadjah Mada University. The test subjects used were HeLa cells and HDFa cells which are collections from the in Vitro Cell Culture Laboratory at Yogyakarta Muhammadiyah University. HeLa and HDFa cells were maintained in the RPMI medium with supplemented with FBS and cultured under 5% $CO_2$ and 37$^oC$.

### Extraction and Phytochemical Screening

A total of 100.103 g of *A. platensis* powder was macerated with 400 ml of 96% ethanol. Phytochemical Screening was done by Thin Layer Chromatography (TLC) method, using silica gel GF254 as stationary phase, and various mobile phases such as hexan:acetone (4:1) which are used to analyze β-carotene, toluene:ethyl acetate (93:7) which are used to analyze terpenoids, chloroform:methanol:water (64:50:1) for analyzing saponins, and hexan:ethyl acetate:formic acid (6:4:0.2) for analyzing flavonoids. All of chemicals used were analytical grade from Merck.

### Cytotoxicity Tests on HeLa cells and HFDa cells

Cells were distributed into wells in 96 well plates (each well containing $2x10^4$ cells) and incubated at 37oC, 5% $CO_2$ overnight. Following incubation, the various concentration series (62.5-2000 µg/ml) of *A.platensis* extract were added into each well and incubated for 24 hours. At the end of the incubation, 10µl 0.5% MTT in PBS was added to each well. The reaction was stopped by adding 10% SDS, and stand for 4 hours before measuring the intensity. The intensity of the purple color formed was measured by the ELISA reader at a wavelength of 595 nm (Sulistyani & Nurkhasanah, 2017).

### Doubling Time Test

Cells were starved in culture media containing 0.5% FBS for 24 hours in 96 well plate (each well containing $2x10^4$ cells), then cells were incubated with ethanol extract of *A. platensis* with a concentrations 156.575 µg/ml and 78.288 µg/ml. Cell population in each series of experiments was calculated at 24, 48, and 72 hours' incubation time with ELISA readers at a wavelength of 595 nm, then a curve of cell number vs. incubation time ware made. The doubling time is calculated from the slop after a straight line is drawn on the curve (CCRC, 2017).

### Cell Cycle Analysis by Flow Cytometry

Cells were distributed into 6 well plates (each containing $5x10^5$ cells) and incubated with test samples at a concentrations of 156.575 µg/ml and 78.288µg/ml for 24 hours. At the end of the treatment, the media in each well was put in a different conical. Cells were washed with 500 µL PBS and added with 200 µL trypsin-EDTA 0.25% to the cell, then incubated for 3 minutes to detach the cells and then added with

500 µL of media. Cell suspension was then centrifuged at 600 rpm for 5 minutes. The precipitate was fixed with cold 70% ethanol, and let it for 30 minutes at room temperature and followed by centrifugation at 2000 rpm for 3 minutes and washed with PBS. Following washing, 400 µL PI reagent (propidium iodide) 1 mg/mL (Sigma), 10 mg/mL RNAse (Sigma) and 0.1% (v/v) Triton-X 100 (Sigma) were added. Cells were resuspended and incubated for 5 minutes in a dark room, then read with a flow cytometer with a light beam of 488 nm and a medium speed of 500 cells/second (Mahfudh & Pihie, 2008).

**Annexin V Apoptosis Detection Assay**

For apoptotic observations, Following the treatment, incubation and washing, the cells was then resuspended with 100 µL binding buffer, 2 µL Anexin-V and 2 µL PI, then incubated in a dark room for 10 minutes. Cell suspension is read with a flow cytometer with a light beam of 488 nm and a medium speed of 500 cells/second (Mahfudh & Pihie, 2008).

**Scratch Wound Healing Assay**

Cells were starved in culture media containing 0.5% FBS for 24 hours into 6 well plates (each containing $7.5x10^4$ cells). Then a scratch was made on the surface of the well using sterile yellow tips and given a test sample with a concentration of 156.575 µg/ml and 78.288 µg/ml. Cell condition was observed and documented after incubation of 0, 18, 24 and 48 hours. Then the stroke distance between cell control and treatment was compared using ImageJ Software (Jonkman *et al*, 2014).

**Data Analysis**

Data obtained from ELISA in the form of absorbance is converted as a percentage of cell death. After the percentage (%) of cell death was obtained, it can be calculated that the extract concentration can inhibit the growth of 50% of the cell population ($IC_{50}$ value) by probit analysis according to SPSS version 20. Selectivity is calculated by comparing the $IC_{50}$ value of HDFa cells with $IC_{50}$ cells of HeLa cells. The data of doubling time, % apoptosis, and % cell migration were analyzed by One Way Anova followed by Post Hoc Tukey's Test at $p<0.05$. The cell cycle was analyzed by comparing the largest cell accumulation between treatment and control.

## RESULT AND DISCUSSION

**Phytochemical Screening**

The maceration process produced 2.235 gram of ethanol extract of *A. platensis* with an extraction rate of 2.233%. Based on TLC results, ethanol extract of *A. platensis* contains β-carotene, terpenoids, and flavonoid compounds (Table 1). The previous research showed the potency of these compound to increase apoptosis and inhibit proliferation of cancer cells (Wati *et al.,* 2016).

**Cytotoxicity Tests on HeLa cells and HFDa cells**

The treatment of ethanol extract of *A. platensis* causes morphological changes in HeLa cells and HDFa cells. Some of HeLa cells were shrink and detach to each other (contact inhibition) and decrease in number, as well as HDFa cells (Figure 1). After observing cell morphology, cells are given an MTT solution. The more formazan crystals formed, the absorbance measured in ELISA Reader is higher and more cells are still alive (Suzery and Cahyono, 2014).

The percentage of deaths due to ethanol extract of A. platensis in HeLa cells increases when the concentration increases. The same thing happened with HDFa cells, an increase in the concentration of ethanol extract *A. platensis* also resulted in an increase in the percentage of cell death. However, the dose needed to cause hindrance to HDFa cell viability is higher than the dose needed to cause hindrance to viability in HeLa cells (Figure 2).

The extract has a strong cytotoxic activity if it has $IC_{50}$ <100 µg/ml or moderate if 100 µg/ml<$IC_{50}$<1000 µg/ml against cancer cells, while the extract is toxic to normal cells if it has an $IC_{50}$ value <1000 µg/ml and said not toxic if the $IC_{50}$ value >1000 µg/ml (Benedetti *et al.,* 2010). The present study found that, the $IC_{50}$ values of *A.platensis* extract against Hela cells and HDFa cells were 260.444 µg/ml and 2065.662 ug/ml respectively. Therefore, it can be concluded that the ethanol extract of *A. platensis* has moderate cytotoxic activity against HeLa cells and is not toxic to normal HDFa cells. The cytotoxic activity of ethanol extract of *A. platensis* could be caused by its chemical content including β-carotene, terpenoid, and flavonoid. The previous studies suggested the potency of compounds as cytotoxic agent (Wati *et al.,* 2016). The study of cytotoxicity mechanism of the extract was carried out in concentration under $IC_{50}$ including 156.575 ug/ml and 78,288 ug/ml to allow the cells grow and could be observed.

**The Selectivity of Ethanol Extract of *A. platensis***

Ethanol extract of *A. platensis* has a selectivity index (SI) of 7.9313, so it can be concluded that ethanol extract *A. platensis* is selective against HeLa cancer cells. The mechanism of ethanol extract of *A. platensis* to distinguish cancer cells from normal cells is based on the cells need for Adenosine Triphosphate (ATP). Because cancer cells move, grow and multiply faster and are more active than normal cells, cancer cells need more ATP energy. The previous studies showed the potency of flavonoids as anticancer agent (Abotaleb *et.al*., 2019) through inducing apoptosis. Another mechanism of flavonoid in inhibiting of cancer progression is through its ability to target molecular pathways involved in glucose metabolism and mitochondrial function (Reyes-Carrasco-Pozo, 2019).

**Doubling Time Test**

Based on Post Hoc Tukey's Test stated that ethanol extract of *A. platensis* caused a significant increasing of HeLa cells doubling time compared to the control group (Figure 3). The higher the concentration of the extract given, the longer the doubling time obtained (Table 2). This result showed that treatment of *A.platensis* extract could inhibit the cancer cells proliferation. In the control group, it was found the doubling time is shorter. It showed that without treatment, Hela cells proliferate faster.

Inhibition of proliferation on HeLa cells due to the administration of ethanol extract of *A. platensis* occurs through the mechanism of inhibiting the action of oxidation enzymes that play a role in the production of ATP by flavonoids so that the energy used for cell division is less available (Sahid *et al.,* 2013). After that, β-carotene can increase p53 which gives effect to cell cycle termination (Nugraheni et al., 2013). When the cell cycle is stopped, it is possible for cells to make DNA repairs, which are maximized by terpenoids. Terpenoids have the ability to inhibit cancer cell proliferation by triggering DNA repair mechanisms and inhibiting DNA fragmentation (Thoppil and Bishayee, 2011).

**Cell Cycle Analysis by Flow Cytometry**

Ethanol extract of *A. platensis* can cause accumulation of HeLa cells in the S phase which can cause the inhibition of HeLa cells to enter the G2/M phase (Figure 4). In the G2/M phase the cells are ready to divide, in this phase the process of DNA replication and the biosynthesis of proteins and RNA needed for cell division have been perfected. Barriers to cell cycles in the G2/M phase produce regulatory errors that cause delayed or disrupted cell division (Lacroix and Maddox, 2014).

Inhibition of the cell cycle in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoids compounds contained in the ethanol extract of *A. platensis*. In general, β-carotene compounds have the ability to increase p53 which is able to recognize cells with damaged DNA and stimulate transcription of genes such as p21 or Bax so that the cell cycle stops and DNA has the opportunity to repair itself before entering the next division stage (Nugraheni *et al.,* 2013). While the mechanism of action of flavonoid compounds is to prevent the formation of active CDK-Cyclin complexes thereby suppressing Maturation Promoting Factor (MPF), this results in disruption of checkpoints in the S and G2/M phases so that cells cannot complete their cell cycle (Wati *et al*., 2016). The terpenoid compounds have a mechanism of action in blocking the cell cycle in the G2/M phase by stabilizing the spindle threads in the mitotic phase, thus causing the process of mitosis to be inhibited (Setiawati *et al.,* 2007).

**Annexin V Apoptosis Detection Assay**

Based on flowytometry analysis, ethanol extract of *A. platensis* has the ability to induce cell death through the necrosis pathway, this is reflected in the number of cells undergoing necrosis more than the number of cells undergoing apoptosis in both treatment groups (Figure 5). However, it should be noted that in both treatment groups the range of cells undergoing necrosis and apoptosis is not too far away, so it is necessary to optimize the concentration ethanol extract of *A. platensis* so that the cell population undergoing apoptosis is more optimal. Necrosis can also be caused by an incubation period that is too long (24 hours) so that the apoptotic phase has passed. Tukey's Post Hoc test stated that ethanol extract of *A. platensis* caused a significant increase in apoptosis on HeLa cells compared to the control group.

Apoptosis induction in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoid compounds contained in ethanol extract of A. platensis. Flavonoid compounds have the ability to increase the expression of p53. The p53 gene will stimulate the mitochondria to release cytochrome C to the cytosol and activate the exclusionary caspase so that it can cause apoptotic cell death (Setiawan *et al.,* 2017). The terpenoid compounds will make the bond between the topoisomerase enzyme and the DNA of the cancer cell held longer so that it results in fragmentation or damage to the cancer cell DNA. DNA damage can increase the expression of proapoptotic proteins such as Bax and Bak and decrease the expression of antiapoptotic proteins namely Bcl-2 and Bcl-XL (Susianti, 2016). The mechanism of action is then strengthened by β-carotene compounds which have the ability to reduce the expression of Bcl-2 and induce the release of cytochrome c from mitochondria in HeLa cells which results in increased induction of apoptosis (Palozza, 2005).

**Scratch Wound Healing Assay**

The scratch wound healing assay was carried out to observed the effect of extract in inhibiting migration of the cancerous cells. The capability of extract to inhibit the cell migration could inhibit the progression of cells, which is the important part of

cancerous cells to spread and metastatic.

Based on the Post Hoc Tukey's test stated that ethanol extract of A. platensis caused significant decreasing the migration rate of HeLa cells when compared to the control group (Figure 6). Decreased rate of cell migration in HeLa cells is caused by suppression of MMP-9 expression due to inhibition of NF-κB activity by antioxidant compounds such as β-carotene, flavonoid, and terpenoid compounds, which are contained in the ethanol extract of A. platensis (Sung *et al.,* 2012). Decreased rate of cell migration can illustrate a decrease in the ability of cancer cells to separate from primary tumors into more distant tissues and colonize other organs to form secondary tumors (Zhang *et al.,* 2007). Barriers to cell spreading signify a delay in the metastatic process from cancer (Jiang *et al.,* 2015).

## CONCLUSION

Ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with an IC50 value of 260.444 µg/ml and selective against normal HDFa with SI 7.9313. These cytotoxic properties are related to delays in doubling time, triggers apoptosis, inhibition of the cell cycle in the G2/M phase, and inhibition of cell migration. Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of *A. platensis*.

## ACKNOWLEDGEMENT

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Table 1. The result of phytochemical screening of ethanol extract of *A. platensis* by using TLC method

| Testing | | RF | Detector reagents | Spotting without Detector Reagents | | | Spotting with Detector Reagents | | | Conclusion |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Visible rays | UV rays 254 nm | UV rays 366 nm | Visible rays | UV rays 254 nm | UV rays 366 nm | |
| β-carotene | Ethanol extract of *A. platensis* | 0.16<br>019<br>0.31<br>0.44 | - | Green-yellow | Green | Purple | - | - | - | Positive |
| | β-carotene standard | 0.93 | | Green-yellow | Green | Purple | - | - | - | |
| Terpenoids | Ethanol extract of *A. platensis* | 0.13<br>0.38<br>0.81 | Anisaldehyde-sulfuric acid | - | - | - | Purplish red | Purplish red | Purplish red | Positive |
| | Eugenol standards | 0.56 | | - | - | - | Purplish red | Purplish red | Purplish red | |
| Saponin | Ethanol extract of *A. platensis* | - | Liebermann–Burchard | - | - | - | - | - | - | Negative |
| | Sapogenin Standards | 0.6 | | Brown | Brown | Brown | Brown | Brown | Brown | |
| Flavonoids | Ethanol extract of *A. platensis* | 0.51 | Sitroborat | Green yellow | Green yellow | Green yellow | Green yellow | Green yellow | Yellow fluorescence | Positive |
| | Quercetin Standards | 0.38 | | Green yellow | Green yellow | Green yellow | Green yellow | Green yellow | Yellow fluorescence | |

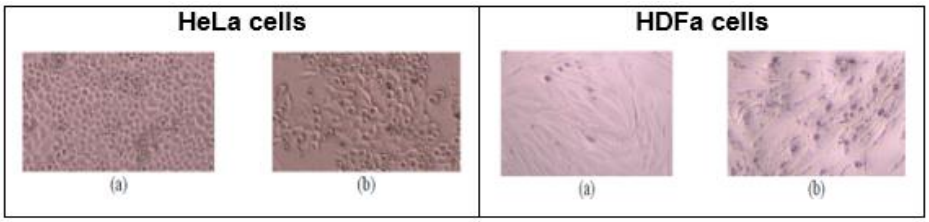



Figure 1. Changes in the morphology of HeLa cells and HDFa cells after 24 hours incubation in the control group (a) as well as those treated with ethanol extract of *A. platensis* (b)

Table 2. Results of doubling time calculations of HeLa cells treated ethanol extract of *A. platensis*

| Treatment | Formula | r | *Doubling time* (hours) |
|---|---|---|---|
| 156.575µg/ml | $Y = 4.1 X 10^{-3} + 4.4540$ | 0.6196 | 36.112 |
| | $Y = 4.1 X 10^{-3} + 4.4534$ | 0.6206 | 36.259 |
| | $Y = 4.1 X 10^{-3} + 4.4534$ | 0.6159 | 36.259 |
| | | | **36.210±0.08*** |
| 78.288 µg/ml | $Y = 4.4 X 10^{-3} + 4.730$ | 0.6000 | 29.332 |
| | $Y = 4.4 X 10^{-3} + 4.731$ | 0.6030 | 29.309 |
| | $Y = 4.4 X 10^{-3} + 4.733$ | 0.6004 | 29.264 |
| | | | **29.302±0.03*** |
| Control | $Y = 4.8 X 10^{-3} + 4.4942$ | 0.5888 | 22.471 |
| | $Y = 4.8 X 10^{-3} + 4.4942$ | 0.5888 | 22.471 |
| | $Y = 4.8 X 10^{-3} + 4.4949$ | 0.5888 | 22.325 |
| | | | **22.422±0.08** |

Note: (a) $p<0.05$ vs concentration of 156.575 µg/ml; (b) $p<0.05$ vs concentration of 78.288 µg/ml; (*) $p<0.05$ vs control.

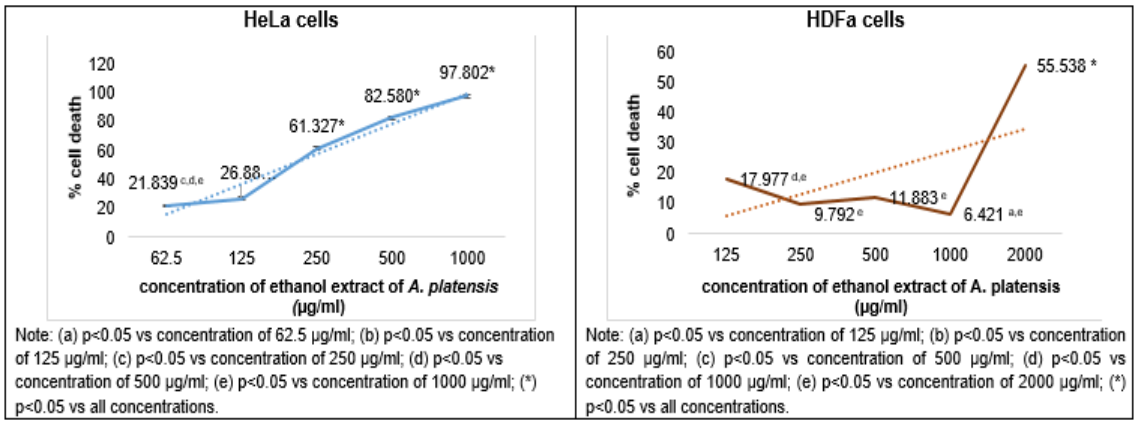



Figure 2. The cytotoxic effect of the ethanol extract of *A. platensis* on HeLa cells and HDFa cells

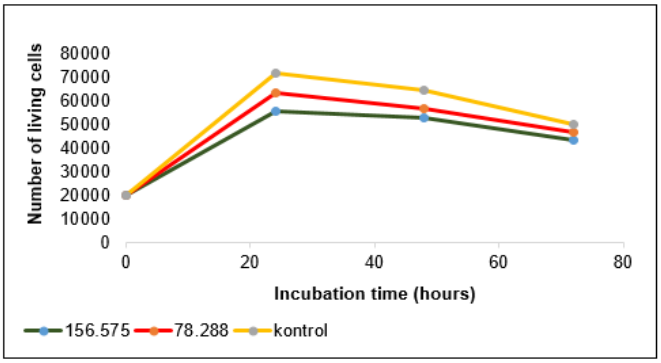



Figure 3. Effect of ethanol extract of *A. platensis* on the number of living cells at various incubation times

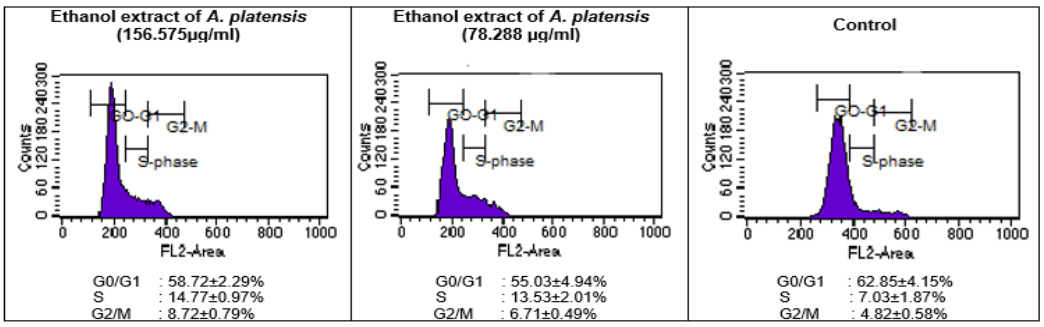
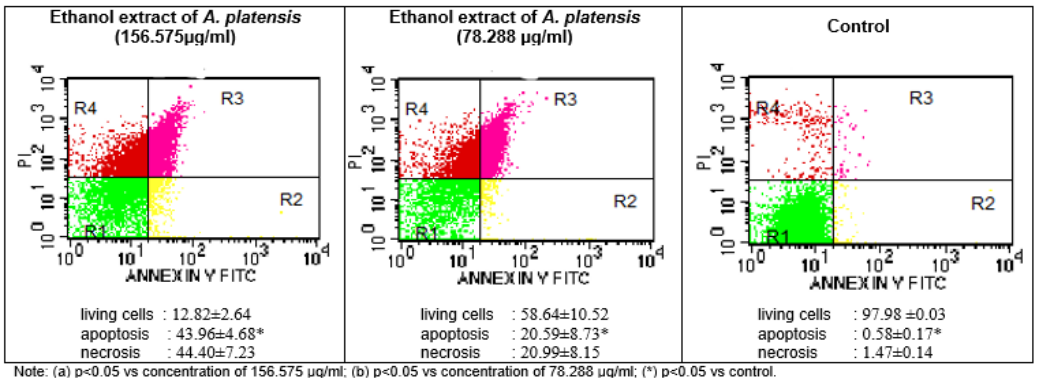



Figure 4. Results of HeLa cell cycle profile analysis treated with ethanol extract of *A. platensis* using the Flow cytometry method




Note: (a) p<0.05 vs concentration 156.575 µg/ml; (b) p<0.05 vs concentration of 78.288 µg/ml; (*) p<0.05 vs control.

Figure 5. Results of HeLa cell population treated with ethanol extract of *A. platensis* using the Flow cytometry method

| Observation | Ethanol extract of *A. platensis* (156.575µg/ml) | Ethanol extract of *A. platensis* (78.288 µg/ml) | Control |
|---|---|---|---|
| 0 hour | | | |
| Migration rate (%) | 0 | 0 | 0 |
| 18 hours | | | |
| Migration rate (%) | 5.510±0.26* | 4.950±0.06* | 4.335±0.11 |
| 24 hours | | | |
| Migration rate (%) | 6.396±0.27* | 5.282±0.06* | 13.464±0.10 |
| 48 hours | | | |
| Migration rate (%) | 7.346±0.13* | 11.362±0.25* | 21.183±0.03 |

Note: (a) p<0.05 vs concentration of 156.575 µg/ml; (b) p<0.05 vs concentration of 78.288 µg/ml; (*) p<0.05 vs control.

Figure 6. The effect of ethanol extract of *A. platensis* on the migration ability of HeLa cells

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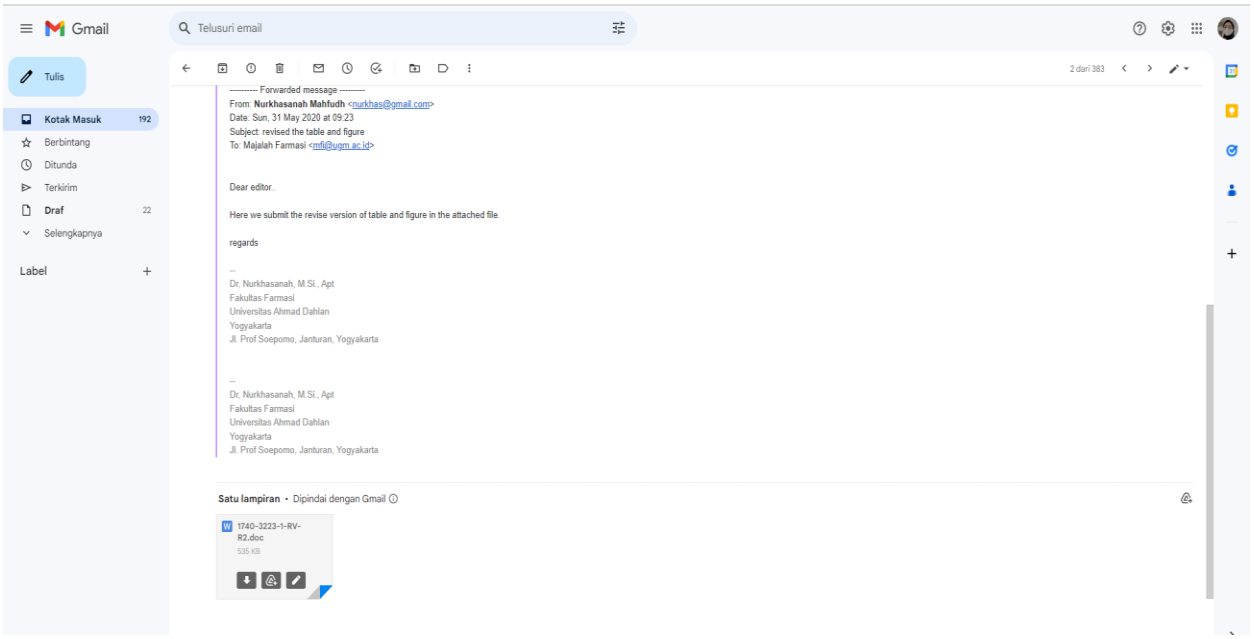

# Lampiran
# “1740-3223-1-RV-R2”

# CYTOTOXIC POTENTIAL of *Arthrospira platensis* EXTRACT on CERVICAL CANCER CELLS LINE HeLa: STUDY on ANTIPROLIFERATIVE, CELL CYCLE, APOPTOSIS INDUCTION, AND ANTI METASTASIS


## ABSTRACT

Cervical cancer can be treated conventionally with chemotherapy agents, but its use has side effects and complications in the form of damage to normal cells. This study aims to determine the potential of A. platensis as an alternative anticancer agent that is selective towards normal cells. Based on TLC analysis, *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, and terpenoids which are able to inhibit proliferation and trigger apoptosis of cancer cells. The study was conducted using cervical cancer cells HeLa and normal cells HDFa. *A. platensis* macerated with 96% ethanol at a ratio of 1:4. Based on probit analysis, it is known that ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with $IC_{50}$ values of 260.444 µg/ml and index selectivity towards HDFa cells of 7.931. The mechanism of cytotoxic activity of ethanol extract of *A. platensis* is related to its ability to extend the doubling time, increase the induction of apoptosis, and reduce the rate of cells migration. Ethanol extract of A. platensis can also increase cells accumulation in the S phase to prevent cells from entering the G2/M phase.


**Key words:** *A. platensis*, cytotoxicity, HeLa cells, HFDa cells


## INTRODUCTION

Cervical cancer can occur due to an infection by the Human Papilloma Virus (HPV) that cause mutations in the p53 gene and increased expression of the protein Bcl-2 which resulted in an imbalance between proliferation and cell (Sari and Syahrul, 2014). One of the methods chosen to treat cervical cancer is by giving chemotherapy agents. However, chemotherapy agents have side effects and complications in the form of normal cell damage (Huang *et al.,* 2018). Therefore, it is necessary to develop alternative anticancer agents which are selective towards cancer cells without affecting normal cells.

Previous research showed that *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, terpenoids, and saponin compounds (Muszynska *et al.,* 2018). Antioxidant compounds are able to regulate antiapoptotic proteins in the mitochondria to increase the induction of apoptosis (Muszynska *et al.,* 2018). In addition, antioxidant compounds also have the ability to repair DNA or RNA damage in normal cells which will trigger the activation of the p53 gene so that there is a cessation of cell cycle and inhibition of cancer cell proliferation (Nugraheni *et al*., 2013). Based on this, it is necessary to investigate *A. platensis* cytotoxicity against HeLa cervical cancer cells. HeLa cells are cervical cell cultures infected with HPV and express 2 oncogens, namely E6 and E7 so that they can multiply in culture media and have cancer properties (Lucey *et al.,* 2009). The selectivity test for *A. platensis* is done using Human Dermal Fibroblast Adult (HDFa) cells, HDFa cells are primary cell cultures that can present responses to stress and other biological approaches that approach in vivo situations in humans (Özen *et al.,* 2005).

The cytotoxic mechanism of an anticancer agent is related to its ability to inhibit proliferation and induce apoptosis (Moningka, 2019). Therefore, an antiproliferation test was performed to determine the doubling time value to show the ability of *A. platensis*

to inhibit HeLa cells proliferation, flow cytometry analysis to observe inhibition patterns in the cell cycle and the magnitude of apoptosis induction in HeLa cells due to *A. platensis* treatment, and cell migration inhibition test with wound healing assay to determine the effect of *A. platensis* on the process of metastatic HeLa cancer cells (Lee *et al.,* 2014).

## MATERIAL AND METHODS

### Plant Material and Cell Culture

*A. platensis* obtained from the Serba Alami Store, Bantul, Yogyakarta in November 2018. The sample has been identified at the Faculty of Biology, Gadjah Mada University. The test subjects used were HeLa cells and HDFa cells which are collections from the in Vitro Cell Culture Laboratory at Yogyakarta Muhammadiyah University. HeLa and HDFa cells were maintained in the RPMI medium with supplemented with FBS and cultured under 5% $CO_2$ and 37$^oC$.

### Extraction and Phytochemical Screening

A total of 100.103 g of *A. platensis* powder was macerated with 400 ml of 96% ethanol. Phytochemical Screening was done by Thin Layer Chromatography (TLC) method, using silica gel GF254 as stationary phase, and various mobile phases such as hexan:acetone (4:1) which are used to analyze β-carotene, toluene:ethyl acetate (93:7) which are used to analyze terpenoids, chloroform:methanol:water (64:50:1) for analyzing saponins, and hexan:ethyl acetate:formic acid (6:4:0.2) for analyzing flavonoids. All of chemicals used were analytical grade from Merck.

### Cytotoxicity Tests on HeLa cells and HFDa cells

Cells were distributed into wells in 96 well plates (each well containing $2x10^4$ cells) and incubated at 37oC, 5% $CO_2$ overnight. Following incubation, the various concentration series (62.5-2000 µg/ml) of *A.platensis* extract were added into each well and incubated for 24 hours. At the end of the incubation, 10µl 0.5% MTT in PBS was added to each well. The reaction was stopped by adding 10% SDS, and stand for 4 hours before measuring the intensity. The intensity of the purple color formed was measured by the ELISA reader at a wavelength of 595 nm (Sulistyani & Nurkhasanah, 2017).

### Doubling Time Test

Cells were starved in culture media containing 0.5% FBS for 24 hours in 96 well plate (each well containing $2x10^4$ cells), then cells were incubated with ethanol extract of *A. platensis* with a concentrations 156.575 µg/ml and 78.288 µg/ml. Cell population in each series of experiments was calculated at 24, 48, and 72 hours' incubation time with ELISA readers at a wavelength of 595 nm, then a curve of cell number vs. incubation time ware made. The doubling time is calculated from the slop after a straight line is drawn on the curve (CCRC, 2017).

### Cell Cycle Analysis by Flow Cytometry

Cells were distributed into 6 well plates (each containing $5x10^5$ cells) and incubated with test samples at a concentrations of 156.575 µg/ml and 78.288µg/ml for 24 hours. At the end of the treatment, the media in each well was put in a different conical. Cells were washed with 500 µL PBS and added with 200 µL trypsin-EDTA 0.25% to the cell, then incubated for 3 minutes to detach the cells and then added with

500 µL of media. Cell suspension was then centrifuged at 600 rpm for 5 minutes. The precipitate was fixed with cold 70% ethanol, and let it for 30 minutes at room temperature and followed by centrifugation at 2000 rpm for 3 minutes and washed with PBS. Following washing, 400 µL PI reagent (propidium iodide) 1 mg/mL (Sigma), 10 mg/mL RNAse (Sigma) and 0.1% (v/v) Triton-X 100 (Sigma) were added. Cells were resuspended and incubated for 5 minutes in a dark room, then read with a flow cytometer with a light beam of 488 nm and a medium speed of 500 cells/second (Mahfudh & Pihie, 2008).

**Annexin V Apoptosis Detection Assay**

For apoptotic observations, Following the treatment, incubation and washing, the cells was then resuspended with 100 µL binding buffer, 2 µL Anexin-V and 2 µL PI, then incubated in a dark room for 10 minutes. Cell suspension is read with a flow cytometer with a light beam of 488 nm and a medium speed of 500 cells/second (Mahfudh & Pihie, 2008).

**Scratch Wound Healing Assay**

Cells were starved in culture media containing 0.5% FBS for 24 hours into 6 well plates (each containing $7.5x10^4$ cells). Then a scratch was made on the surface of the well using sterile yellow tips and given a test sample with a concentration of 156.575 µg/ml and 78.288 µg/ml. Cell condition was observed and documented after incubation of 0, 18, 24 and 48 hours. Then the stroke distance between cell control and treatment was compared using ImageJ Software (Jonkman *et al*, 2014).

**Data Analysis**

Data obtained from ELISA in the form of absorbance is converted as a percentage of cell death. After the percentage (%) of cell death was obtained, it can be calculated that the extract concentration can inhibit the growth of 50% of the cell population ($IC_{50}$ value) by probit analysis according to SPSS version 20. Selectivity is calculated by comparing the $IC_{50}$ value of HDFa cells with $IC_{50}$ cells of HeLa cells. The data of doubling time, % apoptosis, and % cell migration were analyzed by One Way Anova followed by Post Hoc Tukey's Test at $p<0.05$. The cell cycle was analyzed by comparing the largest cell accumulation between treatment and control.

## RESULT AND DISCUSSION

**Phytochemical Screening**

The maceration process produced 2.235 gram of ethanol extract of *A. platensis* with an extraction rate of 2.233%. Based on TLC results, ethanol extract of *A. platensis* contains β-carotene, terpenoids, and flavonoid compounds (Table 1). The previous research showed the potency of these compound to increase apoptosis and inhibit proliferation of cancer cells (Wati *et al.,* 2016).

**Cytotoxicity Tests on HeLa cells and HFDa cells**

The treatment of ethanol extract of *A. platensis* causes morphological changes in HeLa cells and HDFa cells. Some of HeLa cells were shrink and detach to each other (contact inhibition) and decrease in number, as well as HDFa cells (Figure 1). After observing cell morphology, cells are given an MTT solution. The more formazan crystals formed, the absorbance measured in ELISA Reader is higher and more cells are still alive (Suzery and Cahyono, 2014).

The percentage of deaths due to ethanol extract of A. platensis in HeLa cells increases when the concentration increases. The same thing happened with HDFa cells, an increase in the concentration of ethanol extract *A. platensis* also resulted in an increase in the percentage of cell death. However, the dose needed to cause hindrance to HDFa cell viability is higher than the dose needed to cause hindrance to viability in HeLa cells (Figure 2).

The extract has a strong cytotoxic activity if it has $IC_{50}$ <100 µg/ml or moderate if 100 µg/ml<$IC_{50}$<1000 µg/ml against cancer cells, while the extract is toxic to normal cells if it has an $IC_{50}$ value <1000 µg/ml and said not toxic if the $IC_{50}$ value >1000 µg/ml (Benedetti *et al.,* 2010). The present study found that, the $IC_{50}$ values of *A.platensis* extract against Hela cells and HDFa cells were 260.444 µg/ml and 2065.662 ug/ml respectively. Therefore, it can be concluded that the ethanol extract of *A. platensis* has moderate cytotoxic activity against HeLa cells and is not toxic to normal HDFa cells. The cytotoxic activity of ethanol extract of *A. platensis* could be caused by its chemical content including β-carotene, terpenoid, and flavonoid. The previous studies suggested the potency of compounds as cytotoxic agent (Wati *et al.,* 2016). The study of cytotoxicity mechanism of the extract was carried out in concentration under $IC_{50}$ including 156.575 ug/ml and 78,288 ug/ml to allow the cells grow and could be observed.

**The Selectivity of Ethanol Extract of *A. platensis***

Ethanol extract of *A. platensis* has a selectivity index (SI) of 7.9313, so it can be concluded that ethanol extract *A. platensis* is selective against HeLa cancer cells. The mechanism of ethanol extract of *A. platensis* to distinguish cancer cells from normal cells is based on the cells need for Adenosine Triphosphate (ATP). Because cancer cells move, grow and multiply faster and are more active than normal cells, cancer cells need more ATP energy. The previous studies showed the potency of flavonoids as anticancer agent (Abotaleb *et.al*., 2019) through inducing apoptosis. Another mechanism of flavonoid in inhibiting of cancer progression is through its ability to target molecular pathways involved in glucose metabolism and mitochondrial function (Reyes-Carrasco-Pozo, 2019).

**Doubling Time Test**

Based on Post Hoc Tukey's Test stated that ethanol extract of *A. platensis* caused a significant increasing of HeLa cells doubling time compared to the control group (Figure 3). The higher the concentration of the extract given, the longer the doubling time obtained (Table 2). This result showed that treatment of *A.platensis* extract could inhibit the cancer cells proliferation. In the control group, it was found the doubling time is shorter. It showed that without treatment, Hela cells proliferate faster.

Inhibition of proliferation on HeLa cells due to the administration of ethanol extract of *A. platensis* occurs through the mechanism of inhibiting the action of oxidation enzymes that play a role in the production of ATP by flavonoids so that the energy used for cell division is less available (Sahid *et al.,* 2013). After that, β-carotene can increase p53 which gives effect to cell cycle termination (Nugraheni et al., 2013). When the cell cycle is stopped, it is possible for cells to make DNA repairs, which are maximized by terpenoids. Terpenoids have the ability to inhibit cancer cell proliferation by triggering DNA repair mechanisms and inhibiting DNA fragmentation (Thoppil and Bishayee, 2011).

**Cell Cycle Analysis by Flow Cytometry**

Ethanol extract of *A. platensis* can cause accumulation of HeLa cells in the S phase which can cause the inhibition of HeLa cells to enter the G2/M phase (Figure 4). In the G2/M phase the cells are ready to divide, in this phase the process of DNA replication and the biosynthesis of proteins and RNA needed for cell division have been perfected. Barriers to cell cycles in the G2/M phase produce regulatory errors that cause delayed or disrupted cell division (Lacroix and Maddox, 2014).

Inhibition of the cell cycle in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoids compounds contained in the ethanol extract of *A. platensis*. In general, β-carotene compounds have the ability to increase p53 which is able to recognize cells with damaged DNA and stimulate transcription of genes such as p21 or Bax so that the cell cycle stops and DNA has the opportunity to repair itself before entering the next division stage (Nugraheni *et al.,* 2013). While the mechanism of action of flavonoid compounds is to prevent the formation of active CDK-Cyclin complexes thereby suppressing Maturation Promoting Factor (MPF), this results in disruption of checkpoints in the S and G2/M phases so that cells cannot complete their cell cycle (Wati *et al*., 2016). The terpenoid compounds have a mechanism of action in blocking the cell cycle in the G2/M phase by stabilizing the spindle threads in the mitotic phase, thus causing the process of mitosis to be inhibited (Setiawati *et al.,* 2007).

**Annexin V Apoptosis Detection Assay**

Based on flowytometry analysis, ethanol extract of *A. platensis* has the ability to induce cell death through the necrosis pathway, this is reflected in the number of cells undergoing necrosis more than the number of cells undergoing apoptosis in both treatment groups (Figure 5). However, it should be noted that in both treatment groups the range of cells undergoing necrosis and apoptosis is not too far away, so it is necessary to optimize the concentration ethanol extract of *A. platensis* so that the cell population undergoing apoptosis is more optimal. Necrosis can also be caused by an incubation period that is too long (24 hours) so that the apoptotic phase has passed. Tukey's Post Hoc test stated that ethanol extract of *A. platensis* caused a significant increase in apoptosis on HeLa cells compared to the control group.

Apoptosis induction in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoid compounds contained in ethanol extract of A. platensis. Flavonoid compounds have the ability to increase the expression of p53. The p53 gene will stimulate the mitochondria to release cytochrome C to the cytosol and activate the exclusionary caspase so that it can cause apoptotic cell death (Setiawan *et al.,* 2017). The terpenoid compounds will make the bond between the topoisomerase enzyme and the DNA of the cancer cell held longer so that it results in fragmentation or damage to the cancer cell DNA. DNA damage can increase the expression of proapoptotic proteins such as Bax and Bak and decrease the expression of antiapoptotic proteins namely Bcl-2 and Bcl-XL (Susianti, 2016). The mechanism of action is then strengthened by β-carotene compounds which have the ability to reduce the expression of Bcl-2 and induce the release of cytochrome c from mitochondria in HeLa cells which results in increased induction of apoptosis (Palozza, 2005).

**Scratch Wound Healing Assay**

The scratch wound healing assay was carried out to observed the effect of extract in inhibiting migration of the cancerous cells. The capability of extract to inhibit the cell migration could inhibit the progression of cells, which is the important part of

cancerous cells to spread and metastatic.

Based on the Post Hoc Tukey's test stated that ethanol extract of A. platensis caused significant decreasing the migration rate of HeLa cells when compared to the control group (Figure 6). Decreased rate of cell migration in HeLa cells is caused by suppression of MMP-9 expression due to inhibition of NF-κB activity by antioxidant compounds such as β-carotene, flavonoid, and terpenoid compounds, which are contained in the ethanol extract of A. platensis (Sung *et al.,* 2012). Decreased rate of cell migration can illustrate a decrease in the ability of cancer cells to separate from primary tumors into more distant tissues and colonize other organs to form secondary tumors (Zhang *et al.,* 2007). Barriers to cell spreading signify a delay in the metastatic process from cancer (Jiang *et al.,* 2015).

## CONCLUSION

Ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with an IC50 value of 260.444 µg/ml and selective against normal HDFa with SI 7.9313. These cytotoxic properties are related to delays in doubling time, triggers apoptosis, inhibition of the cell cycle in the G2/M phase, and inhibition of cell migration. Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of *A. platensis*.

## ACKNOWLEDGEMENT

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Table 1. The result of phytochemical screening of ethanol extract of *A. platensis* by using TLC method

| Testing | | Detector reagents | Rf | Spotting without detector reagent | | | Spotting with detector reagents | | | Conclusion |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Visible rays | UV rays 254 nm | UV rays 366 nm | Visible rays | UV rays 254 nm | UV rays 366 nm | |
| B-carotene | Ethanol extract of A platensis | - | 0.16<br>0.19<br>0.31<br>0.44 | Green-yellow | Green | Purple | - | - | - | positive |
| | B-carotene standard | - | 0.93 | Green-yellow | Green | Purple | - | - | - | |
| Terpenoids | Ethanol extract of A platensis | Anisaldehyde-sulfuric acid | 0.13<br>0.38<br>0.81 | - | - | - | Purplish red | Purplish red | Purplish red | positive |
| | Eugenol standard | | 0.56 | - | - | - | Purplish red | Purplish red | Purplish red | |
| Saponin | Ethanol extract of A platensis | Liebermann-Burchard | - | - | - | - | - | - | - | positive |
| | Sapogenin standard | | 0.6 | brown | brown | brown | brown | brown | brown | |
| Falvonoids | Ethanol extract of A platensis | Citric-boric acid | 0.51 | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Yellow fluorescence | positive |
| | Quercetin standard | | 0.38 | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Yellow fluorescence | |

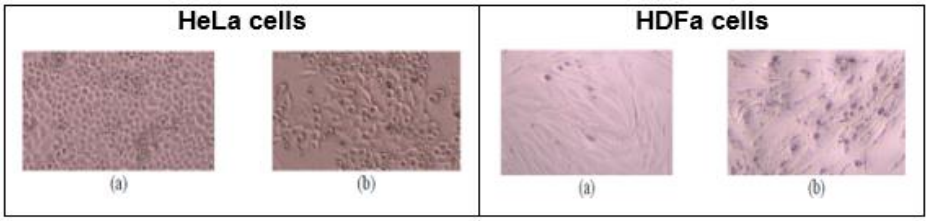



Figure 1. Changes in the morphology of HeLa cells and HDFa cells after 24 hours incubation in the control group (a) as well as those treated with ethanol extract of *A. platensis* (b)

Table 2. Results of doubling time calculations of HeLa cells treated ethanol extract of *A. platensis*

| Treatment | Formula | r | Doubling time (hours) |
|---|---|---|---|
| 156,575µg/ml | $Y = 4,1 X 10^{-3} + 4,4540$ | 0,6196 | 36,112 |
| | $Y = 4,1 X 10^{-3} + 4,4534$ | 0,6206 | 36,259 |
| | $Y = 4,1 X 10^{-3} + 4,4534$ | 0,6159 | 36,259 |
| | | | **36,210±0,08*** |
| 78,288 µg/ml | $Y = 4,4 X 10^{-3} + 4,730$ | 0,6000 | 29,332 |
| | $Y = 4,4 X 10^{-3} + 4,731$ | 0,6030 | 29,309 |
| | $Y = 4,4 X 10^{-3} + 4,733$ | 0,6004 | 29,264 |
| | | | **29,302±0,03*** |
| Control | $Y = 4,8 X 10^{-3} + 4,4942$ | 0,5888 | 22,471 |
| | $Y = 4,8 X 10^{-3} + 4,4942$ | 0,5888 | 22,471 |
| | $Y = 4,8 X 10^{-3} + 4,4949$ | 0,5888 | 22,325 |
| | | | **22,422±0,08*** |

*p<0.05 compare to control

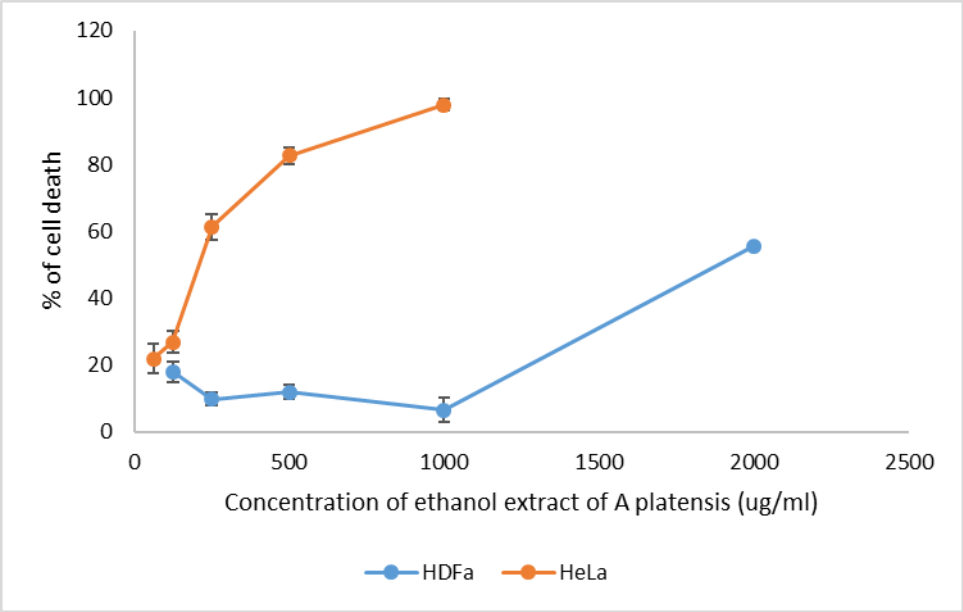



Figure 2. The cytotoxic effect of the ethanol extract of *A. platensis* on HeLa cells and HDFa cells

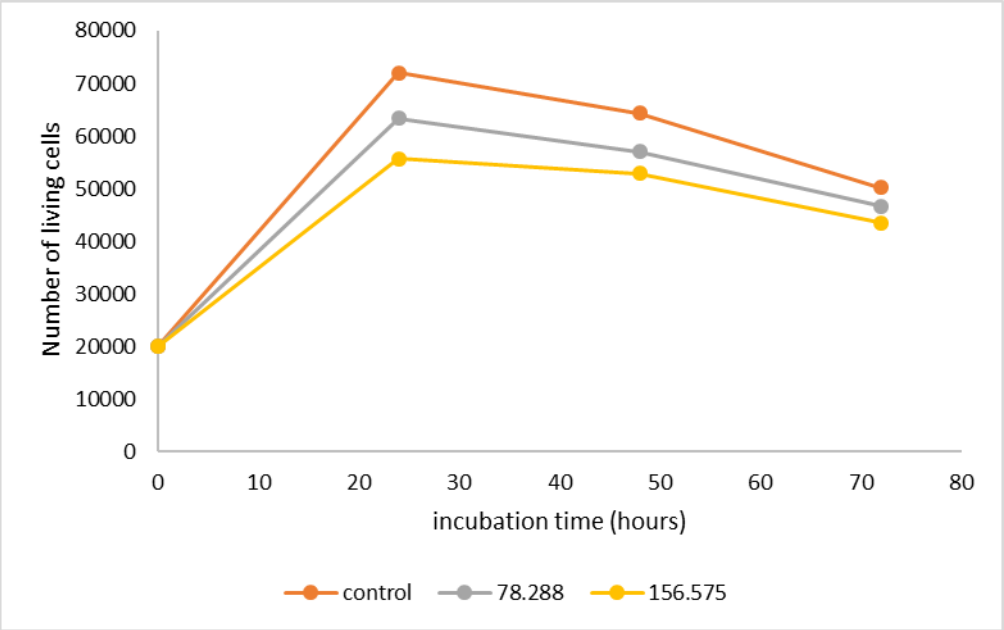



Figure 3. Effect of ethanol extract of *A. platensis* on the number of living cells at various incubation times

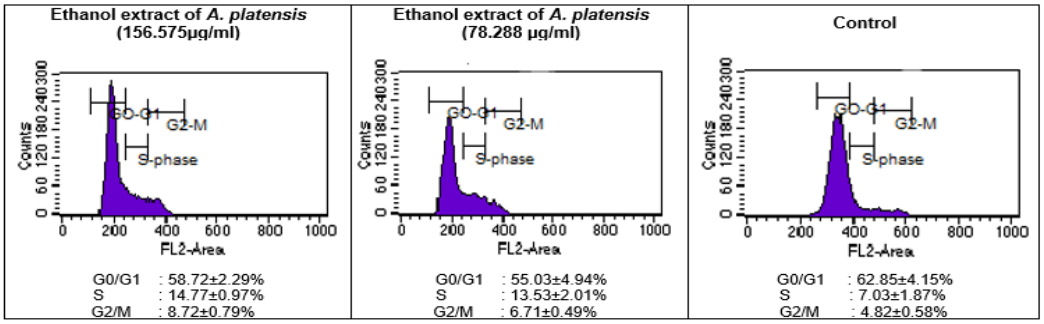
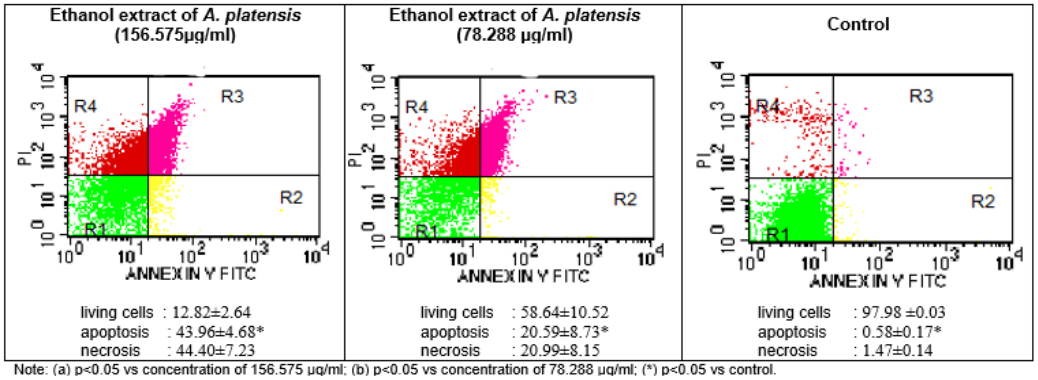
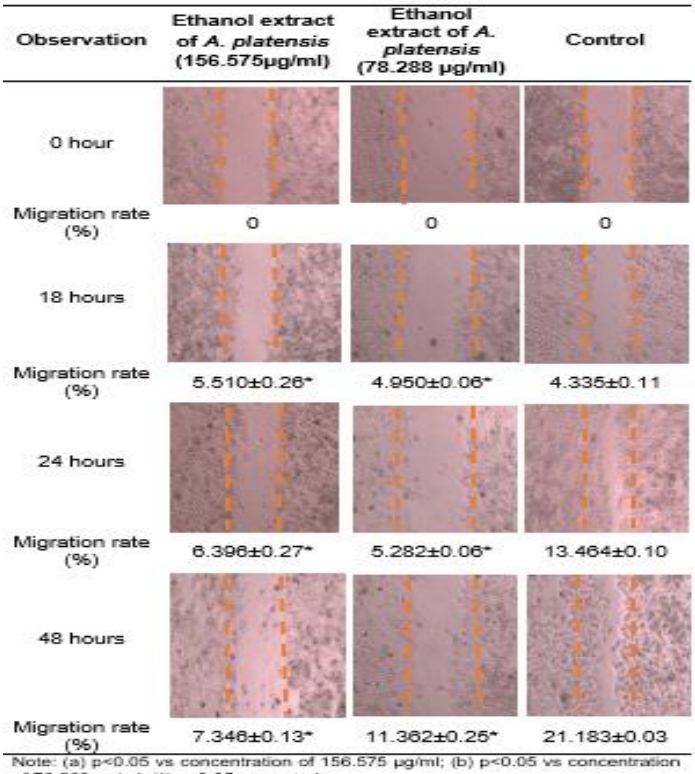



Figure 4. Results of HeLa cell cycle profile analysis treated with ethanol extract of *A. platensis* using the Flow cytometry method




Note: (a) p<0.05 vs concentration 156.575 µg/ml; (b) p<0.05 vs concentration of 78.288 µg/ml; (*) p<0.05 vs control.

Figure 5. Results of HeLa cell population treated with ethanol extract of *A. platensis* using the Flow cytometry method




Note: (a) p<0.05 vs concentration of 156.575 µg/ml; (b) p<0.05 vs concentration of 78.288 µg/ml; (*) p<0.05 vs control.

Figure 6. The effect of ethanol extract of *A. platensis* on the migration ability of HeLa cells

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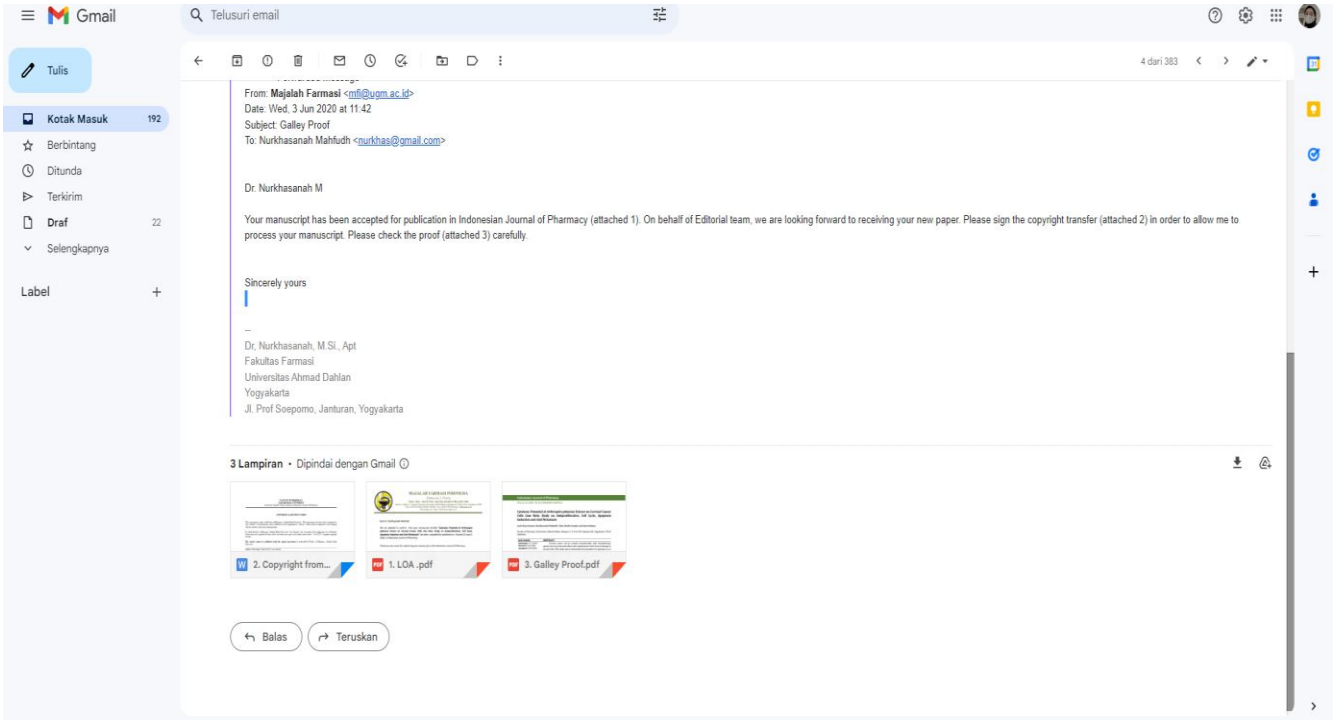

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Dear Dr. Nurkhasanah Mahfudh

We are pleased to confirm that your manuscript entitled "**Cytotoxic Potential of Arthrospira platensis Extract on Cervical Cancer Cells Line Hela: Study on Antiproliferative, Cell Cycle, Apoptosis Induction and Anti Metastasis**" has been accepted for published on Volume 31 Issue 1 2020, in Indonesian Journal of Pharmacy.

Thank you very much for submitting your manuscript to the Indonesian Journal of Pharmacy.

Yogyakarta, 03 June 2209
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Indonesian Journal of Pharmacy

VOL 31 (1) 2020: 19–26 | RESEARCH ARTICLE

# Cytotoxic Potential of *Arthrospira platensis* Extract on Cervical Cancer Cells Line Hela: Study on Antiproliferative, Cell Cycle, Apoptosis Induction and Anti Metastasis

**Laela Hayu Nurani, Nurkhasanah Mahfudh*, Ibnu Gholib Gandjar and Intan Rahayu**

Faculty of Pharmacy, Universitas Ahmad Dahlan, Kampus 3, Jl. Prof. DR. Soepomo Sh, Yogyakarta, 55164 Indonesia

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**ABSTRACT**

Cervical cancer can be treated conventionally with chemotherapy agents, but its use has side effects and complications in the form of damage to normal cells. This study aims to determine the potential of A. platensis as an alternative anticancer agent that is selective towards normal cells. Based on TLC analysis, *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, and terpenoids which are able to inhibit proliferation and trigger apoptosis of cancer cells. The study was conducted using cervical cancer cells HeLa and normal cells HDFa. *A. platensis* macerated with 96% ethanol at a ratio of 1:4. Based on probit analysis, it is known that ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with $IC_{50}$ values of 260.444µg/mL and index selectivity towards HDFa cells of 7.931. The mechanism of cytotoxic activity of ethanol extract of *A. platensis* is related to its ability to extend the doubling time, increase the induction of apoptosis, and reduce the rate of cells migration. Ethanol extract of A. platensis can also increase cells accumulation in the S phase to prevent cells from entering the G2/M phase.

**Keywords:** *A. platensis*, cytotoxicity, HeLa cells, HFDa cells



## INTRODUCTION

Cervical cancer can occur due to an infection by the Human Papilloma Virus (HPV) that cause mutations in the p53 gene and increased expression of the protein Bcl-2 which resulted in an imbalance between proliferation and cell (Sari and Syahrul, 2014). One of the methods chosen to treat cervical cancer is by giving chemotherapy agents. However, chemotherapy agents have side effects and complications in the form of normal cell damage (Huang *et al.,* 2018). Therefore, it is necessary to develop alternative anticancer agents which are selective towards cancer cells without affecting normal cells.

Previous research showed that *A. platensis* contains antioxidant compounds such as β-carotene, flavonoids, terpenoids, and saponin compounds (Muszynska *et al.,* 2018). Antioxidant compounds are able to regulate antiapoptotic proteins in the mitochondria to increase the induction of apoptosis (Muszynska *et al.,* 2018). In addition, antioxidant compounds also have the ability to repair DNA or RNA damage in normal cells which will trigger the activation of the p53 gene so that there is a cessation of cell cycle and inhibition of cancer cell proliferation (Nugraheni *et al.,* 2013). Based on this, it is necessary to investigate *A. platensis* cytotoxicity against HeLa cervical cancer cells. HeLa cells are cervical cell cultures infected with HPV and express 2 oncogens, namely E6 and E7 so that they can multiply in culture media and have cancer properties (Lucey *et al.,* 2009). The selectivity test for *A. platensis* is done using Human Dermal Fibroblast Adult (HDFa) cells, HDFa cells are primary cell cultures that can present responses to stress and other biological approaches that approach in vivo situations in humans (Özen *et al.,* 2005). The cytotoxic mechanism of an anticancer agent is related to its ability to inhibit proliferation and induce apoptosis (Moningka, 2019). Therefore, an antiproliferation test was performed to determine the doubling time value to show the ability of *A. platensis* to inhibit HeLa cells proliferation, flow cytometry analysis to observe inhibition patterns in the cell cycle and the magnitude of apoptosis induction in HeLa cells due to *A. platensis* treatment, and cell migration inhibition test with wound healing assay to determine the effect of *A. platensis* on the process of metastatic HeLa cancer cells (Lee *et al.,* 2014).

## MATERIAL AND METHODS

### Plant material and cell culture

*A. platensis* obtained from the Serba Alami Store, Bantul, Yogyakarta in November 2018. The sample has been identified at the Faculty of Biology, Gadjah Mada University. The test subjects used were HeLa cells and HDFa cells which are collections from the in Vitro Cell Culture Laboratory at Yogyakarta Muhammadiyah University. HeLa and HDFa cells were maintained in the RPMI medium with supplemented with FBS and cultured under 5% $CO_2$ and 37°C.

### Extraction and phytochemical screening

A total of 100.103g of *A. platensis* powder was macerated with 400mL of 96% ethanol. Phytochemical Screening was done by Thin Layer Chromatography (TLC) method, using silica gel GF254 as stationary phase, and various mobile phases such as hexan:acetone (4:1) which are used to analyze β-carotene, toluene:ethyl acetate (93:7) which are used to analyze terpenoids, chloroform:methanol:water (64:50:1) for analyzing saponins, and hexan:ethyl acetate:formic acid (6:4:0.2) for analyzing flavonoids. All of chemicals used were analytical grade from Merck.

### Cytotoxicity tests on HeLa cells and HFDa cells

Cells were distributed into wells in 96 well plates (each well containing $2x10^4$ cells) and incubated at 37°C, 5% $CO_2$ overnight. Following incubation, the various concentration series (62.5-2000µg/mL) of *A.platensis* extract were added into each well and incubated for 24h. At the end of the incubation, 10µL 0.5% MTT in PBS was added to each well. The reaction was stopped by adding 10% SDS, and stand for 4h before measuring the intensity. The intensity of the purple color formed was measured by the ELISA reader at a wavelength of 595nm (Sulistyani and Nurkhasanah, 2017).

### Doubling time test

Cells were starved in culture media containing 0.5% FBS for 24h in 96 well plate (each well containing $2x10^4$ cells), then cells were incubated with ethanol extract of *A. platensis* with a concentrations 156.575µg/mL and 78.288µg/mL. Cell population in each series of experiments was calculated at 24, 48, and 72h incubation time with ELISA readers at a wavelength of 595nm, then a curve of cell number vs. incubation time ware made. The doubling time is calculated from the slop after a straight line is drawn on the curve (CCRC, 2017).

### Cell cycle analysis by flow cytometry

Cells were distributed into 6 well plates (each containing $5x10^5$ cells) and incubated with test samples at a concentrations of 156.575 µg/mL and 78.288µg/mL for 24h. At the end of the treatment, the media in each well was put in a different conical. Cells were washed with 500µL PBS and added with 200µL trypsin-EDTA 0.25% to the cell, then incubated for 3min to detach the cells and then added with 500µL of media. Cell suspension was then centrifuged at 600 rpm for 5 minutes. The precipitate was fixed with cold 70% ethanol, and let it for 30min at room temperature and followed by centrifugation at 2000 rpm for 3 minutes and washed with PBS. Following washing, 400µL PI reagent (propidium iodide) 1mg/mL (Sigma), 10mg/mL RNAse (Sigma) and 0.1% (v/v) Triton-X 100 (Sigma) were added. Cells were resuspended and incubated for 5min in a dark room, then read with a flow cytometer with a light beam of 488nm and a medium speed of 500cells/s (Mahfudh and Pihie, 2008).

### Annexin V apoptosis detection assay

For apoptotic observations, Following the treatment, incubation and washing, the cells was then resuspended with 100µL binding buffer, 2µL Anexin-V and 2µL PI, then incubated in a dark room for 10 minutes. Cell suspension is read with a flow cytometer with a light beam of 488nm and a medium speed of 500cells/s (Mahfudh and Pihie, 2008).

### Scratch wound healing assay

Cells were starved in culture media containing 0.5% FBS for 24h into 6 well plates (each containing $7.5x10^4$ cells). Then a scratch was made on the surface of the well using sterile yellow tips and given a test sample with a concentration of 156.575µg/mL and 78.288µg/mL. Cell condition was observed and documented after incubation of 0, 18, 24 and 48h. Then the stroke distance between cell control and treatment was compared using ImageJ Software (Jonkman *et al*, 2014).

### Data analysis

Data obtained from ELISA in the form of absorbance is converted as a percentage of cell death. After the percentage (%) of cell death was obtained, it can be calculated that the extract concentration can inhibit the growth of 50% of the cell population ($IC_{50}$ value) by probit analysis according to SPSS version 20.

Table 1. The result of phytochemical screening of ethanol extract of *A. platensis* by using TLC method

| Testing | | Detector reagents | Rf | Spotting without detector reagent | | | Spotting with detector reagents | | | Conclusion |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Visible rays | UV rays 254 nm | UV rays 366 nm | Visible rays | UV rays 254 nm | UV rays 366 nm | |
| B-carotene | Ethanol extract of *A platensis* | - | 0.16<br>0.19<br>0.31<br>0.44 | Green-yellow | Green | Purple | - | - | - | positive |
| | B-carotene standard | - | 0.93 | Green-yellow | Green | Purple | - | - | - | |
| Terpenoids | Ethanol extract of *A platensis* | Anisaldehyde-sulfuric acid | 0.13<br>0.38<br>0.81 | - | - | - | Purplish red | Purplish red | Purplish red | positive |
| | Eugenol standard | | 0.56 | - | - | - | Purplish red | Purplish red | Purplish red | |
| Saponin | Ethanol extract of *A platensis* | Liebermann-Burchard | - | - | - | - | - | - | - | positive |
| | Sapogenin standard | | 0.6 | brown | brown | brown | brown | brown | brown | |
| Falvonoids | Ethanol extract of *A platensis* | Citric-boric acid | 0.51 | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Yellow fluorescence | positive |
| | Quercetin standard | | 0.38 | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Green-yellow | Yellow fluorescence | |

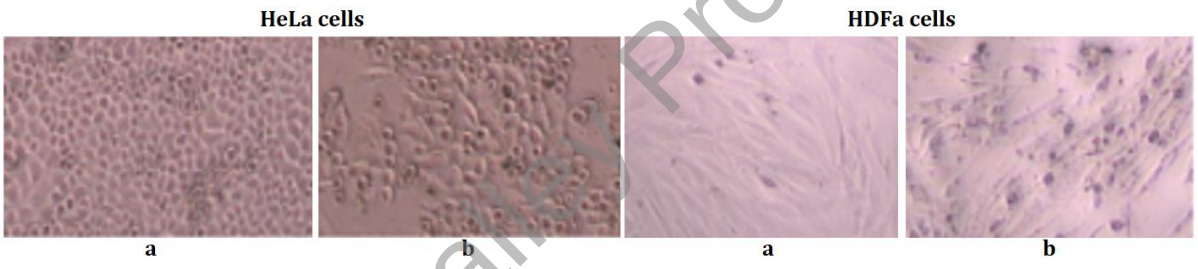




Figure 1. Changes in the morphology of HeLa cells and HDFa cells after 24 hours incubation in the control group (a) as well as those treated with ethanol extract of *A. platensis* (b)

Selectivity is calculated by comparing the $IC_{50}$ value of HDFa cells with $IC_{50}$ cells of HeLa cells. The data of doubling time, % apoptosis, and % cell migration were analyzed by One Way Anova followed by Post Hoc Tukey's Test at $p<0.05$. The cell cycle was analyzed by comparing the largest cell accumulation between treatment and control.

## RESULT AND DISCUSSION

### Phytochemical screening

The maceration process produced 2.235 gram of ethanol extract of *A. platensis* with an extraction rate of 2.233%. Based on TLC results, ethanol extract of *A. platensis* contains β-carotene, terpenoids, and flavonoid compounds (Table I). The previous research showed the potency of these compound to increase apoptosis and inhibit proliferation of cancer cells (Wati *et al.,* 2016).

### Cytotoxicity tests on HeLa cells and HFDa cells

The treatment of ethanol extract of *A. platensis* causes morphological changes in HeLa cells and HDFa cells. Some of HeLa cells were shrink and detach to each other (contact inhibition) and decrease in number, as well as HDFa cells (Figure 1). After observing cell morphology, cells are given an MTT solution. The more formazan crystals formed, the absorbance measured in ELISA Reader is higher and more cells are still alive (Suzery and Cahyono, 2014). The percentage of deaths due to ethanol extract of A. platensis in HeLa cells increases when the concentration increases. The same thing happened with HDFa cells, an increase in the concentration of ethanol extract *A. platensis* also resulted in an increase in the percentage of cell death. However, the dose needed to cause hindrance to HDFa cell viability is higher than the

dose needed to cause hindrance to viability in HeLa cells (Figure 2).

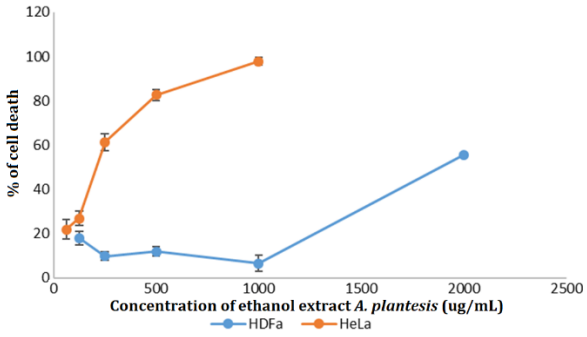


Figure 2. The cytotoxic effect of the ethanol extract of *A. platensis* on HeLa cells and HDFa cells

The extract has a strong cytotoxic activity if it has $IC_{50}$ <100µg/mL or moderate if 100µg/mL <$IC_{50}$<1000µg/mL against cancer cells, while the extract is toxic to normal cells if it has an $IC_{50}$ value <1000µg/mL and said not toxic if the $IC_{50}$ value >1000µg/mL (Benedetti *et al.,* 2010). The present study found that, the $IC_{50}$ values of *A.platensis* extract against Hela cells and HDFa cells were 260.444µg/mL and 2065.662 ug/mL respectively. Therefore, it can be concluded that the ethanol extract of *A. platensis* has moderate cytotoxic activity against HeLa cells and is not toxic to normal HDFa cells. The cytotoxic activity of ethanol extract of *A. platensis* could be caused by its chemical content including β-carotene, terpenoid, and flavonoid. The previous studies suggested the potency of compounds as cytotoxic agent (Wati *et al.,* 2016). The study of cytotoxicity mechanism of the extract was carried out in concentration under $IC_{50}$ including 156.575ug/mL and 78.288ug/mL to allow the cells grow and could be observed.

**The selectivity of ethanol extract of *A. platensis***

Ethanol extract of *A. platensis* has a selectivity index (SI) of 7.9313, so it can be concluded that ethanol extract *A. platensis* is selective against HeLa cancer cells. The mechanism of ethanol extract of *A. platensis* to distinguish cancer cells from normal cells is based on the cells need for Adenosine Triphosphate (ATP). Because cancer cells move, grow and multiply faster and are more active than normal cells, cancer cells need more ATP energy. The previous studies showed the potency of flavonoids as anticancer agent (Abotaleb *et al*., 2019) through inducing apoptosis. Another mechanism of flavonoid in inhibiting of cancer progression is through its ability to target molecular pathways involved in glucose metabolism and mitochondrial function (Reyes-Fariaz and Carrasco-Pozo, 2019).

**Doubling time test**

Based on Post Hoc Tukey's Test stated that ethanol extract of *A. platensis* caused a significant increasing of HeLa cells doubling time compared to the control group (Figure 3). The higher the concentration of the extract given, the longer the doubling time obtained (Table II). This result showed that treatment of *A.platensis* extract could inhibit the cancer cells proliferation. In the control group, it was found the doubling time is shorter. It showed that without treatment, Hela cells proliferate faster. Inhibition of proliferation on HeLa cells due to the administration of ethanol extract of *A. platensis* occurs through the mechanism of inhibiting the action of oxidation enzymes that play a role in the production of ATP by flavonoids so that the energy used for cell division is less available (Sahid *et al.,* 2013). After that, β-carotene can increase p53 which gives effect to cell cycle termination (Nugraheni *et al*., 2013). When the cell cycle is stopped, it is possible for cells to make DNA repairs, which are maximized by terpenoids. Terpenoids have the ability to inhibit cancer cell proliferation by triggering DNA repair mechanisms and inhibiting DNA fragmentation (Thoppil and Bishayee, 2011).

**Cell cycle analysis by flow cytometry**

Ethanol extract of *A. platensis* can cause accumulation of HeLa cells in the S phase which can cause the inhibition of HeLa cells to enter the G2/M phase (Figure 4).

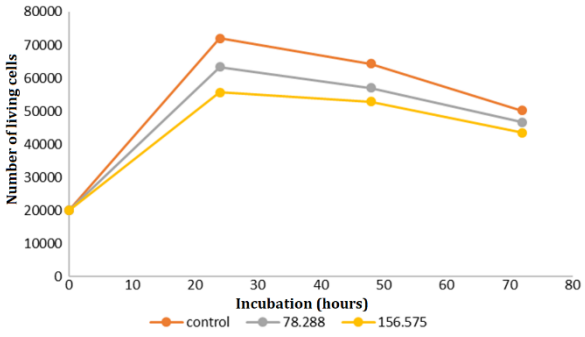


Figure 3. Effect of ethanol extract of *A. platensis* on the number of living cells at various incubation times

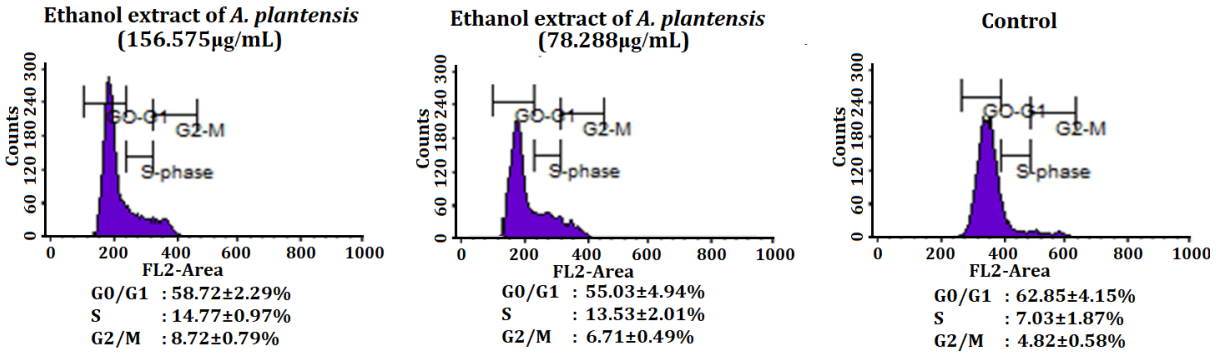


Figure 4. Results of HeLa cell cycle profile analysis treated with ethanol extract of *A. platensis* using the Flow cytometry method

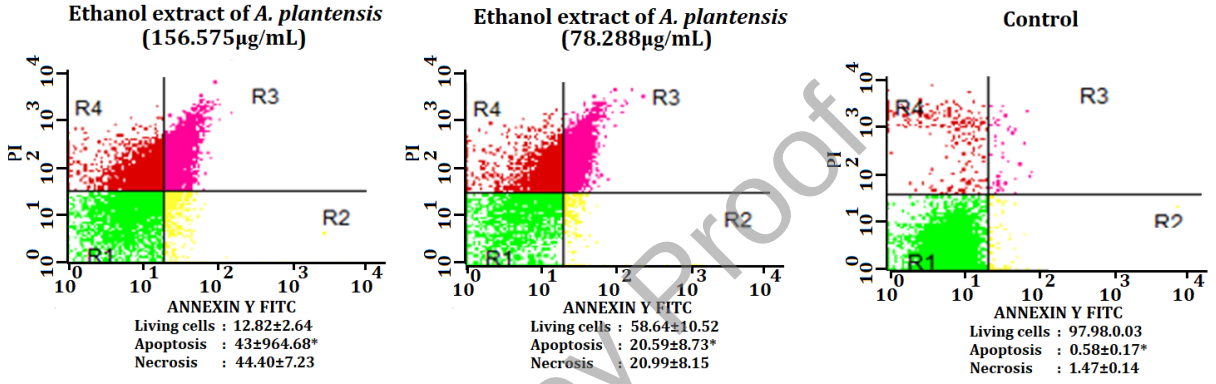


Figure 5. Results of HeLa cell population treated with ethanol extract of *A. platensis* using the Flow cytometry method

Note: (a)p<0.05 vs concentration of 156.575 µg/mL; (b)p<0.05 concentration of 72.288 µg/mL; (*)p<0.05 vs control



In the G2/M phase the cells are ready to divide, in this phase the process of DNA replication and the biosynthesis of proteins and RNA needed for cell division have been perfected. Barriers to cell cycles in the G2/M phase produce regulatory errors that cause delayed or disrupted cell division (Lacroix and Maddox, 2014).

Inhibition of the cell cycle in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoids compounds contained in the ethanol extract of *A. platensis*. In general, β-carotene compounds have the ability to increase p53 which is able to recognize cells with damaged DNA and stimulate transcription of genes such as p21 or Bax so that the cell cycle stops and DNA has the opportunity to repair itself before entering the next division stage (Nugraheni *et al.,* 2013). While the mechanism of action of flavonoid compounds is to prevent the formation of active CDK-Cyclin complexes thereby suppressing Maturation Promoting Factor (MPF), this results in disruption of checkpoints in the S and G2/M phases so that cells cannot complete their cell cycle (Wati *et al.*, 2016). The terpenoid compounds have a mechanism of action in blocking the cell cycle in the G2/M phase by stabilizing the spindle threads in the mitotic phase, thus causing the process of mitosis to be inhibited (Setiawati *et al.,* 2007).

**Annexin V apoptosis detection assay**

Based on flowytometry analysis, ethanol extract of *A. platensis* has the ability to induce cell death through the necrosis pathway, this is reflected in the number of cells undergoing necrosis more than the number of cells undergoing apoptosis in both treatment groups (Figure 5). However, it should be noted that in both treatment groups the range of cells undergoing necrosis and apoptosis is not too far away, so it is necessary to optimize the concentration ethanol extract of *A. platensis* so that the cell population undergoing apoptosis is more optimal. Necrosis can also be caused by an incubation period that is too long (24h) so that the apoptotic phase has passed.

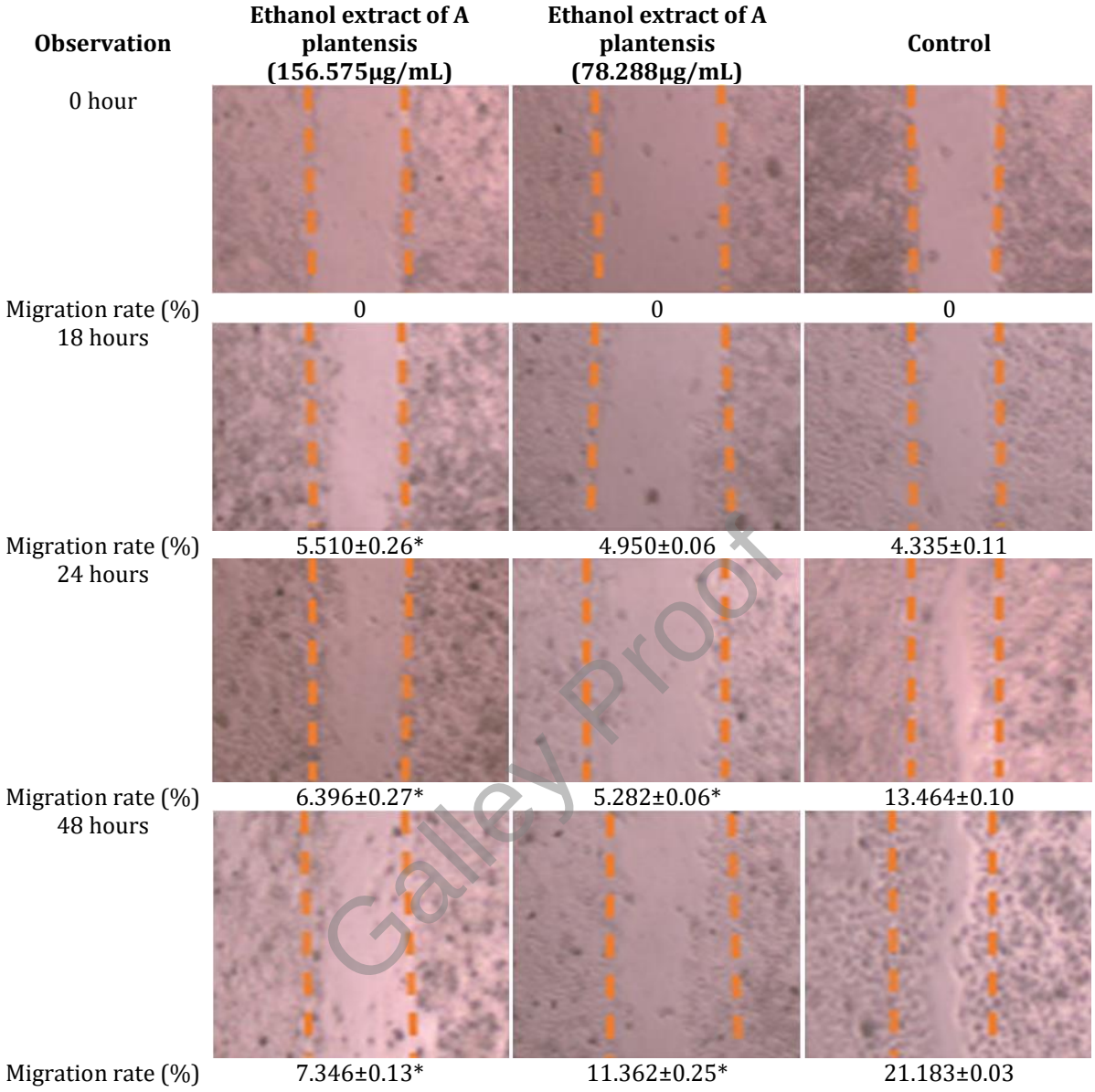




Figure 6. The effect of ethanol extract of *A. platensis* on the migration ability of HeLa cells
Note: (a)p<0.05 vs concentration of 156.575 µg/mL; (b)p<0.05 concentration of 72.288 µg/mL; (*)p<0.05 vs control

Tukey's Post Hoc test stated that ethanol extract of *A. platensis* caused a significant increase in apoptosis on HeLa cells compared to the control group.

Apoptosis induction in HeLa cells is caused by the content of β-carotene, flavonoid, and terpenoid compounds contained in ethanol extract of *A. platensis*. Flavonoid compounds have the ability to increase the expression of p53. The p53 gene will stimulate the mitochondria to release cytochrome C to the cytosol and activate the exclusionary caspase so that it can cause apoptotic cell death (Setiawan *et al.,* 2017). The terpenoid compounds will make the bond between the topoisomerase enzyme and the DNA of the cancer cell held longer so that it results in fragmentation or damage to the cancer cell DNA. DNA damage can increase the expression of proapoptotic proteins such as Bax and Bak and decrease the expression of antiapoptotic proteins namely Bcl-2 and Bcl-XL (Susianti, 2016). The mechanism of action is then strengthened by β-carotene compounds which

have the ability to reduce the expression of Bcl-2 and induce the release of cytochrome c from mitochondria in HeLa cells which results in increased induction of apoptosis (Palozza, 2005).

**Scratch wound healing assay**

The scratch wound healing assay was carried out to observed the effect of extract in inhibiting migration of the cancerous cells. The capability of extract to inhibit the cell migration could inhibit the progression of cells, which is the important part of cancerous cells to spread and metastatic.

Based on the Post Hoc Tukey's test stated that ethanol extract of *A. platensis* caused significant decreasing the migration rate of HeLa cells when compared to the control group (Figure 6). Decreased rate of cell migration in HeLa cells is caused by suppression of MMP-9 expression due to inhibition of NF-κB activity by antioxidant compounds such as β-carotene, flavonoid, and terpenoid compounds, which are contained in the ethanol extract of *A. platensis* (Sung *et al.,* 2012). Decreased rate of cell migration can illustrate a decrease in the ability of cancer cells to separate from primary tumors into more distant tissues and colonize other organs to form secondary tumors (Zhang *et al.,* 2007). Barriers to cell spreading signify a delay in the metastatic process from cancer (Jiang *et al.,* 2015).

## CONCLUSION

Ethanol extract of *A. platensis* has a cytotoxic effect on HeLa cells with an IC50 value of 260.444µg/mL and selective against normal HDFa with SI 7.9313. These cytotoxic properties are related to delays in doubling time, triggers apoptosis, inhibition of the cell cycle in the G2/M phase, and inhibition of cell migration. Cytotoxic effects are caused by β-carotene, terpenoid, and flavonoid compounds contained in the ethanol extract of *A. platensis*.

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