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The Ethyl Acetate Extract of Faloak Leaves (*Sterculiaquadrifida* R.Br) inhibits Human Cervical Cancer Cells Proliferation and induce Cell Death

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Abstract

Cervical cancer is the third most common form of cancer in women around the world. Medicinal plants have been used to cure diseases for a long time. Ethyl acetate extract of faloak leaves (EFL) (*Sterculiaquadrifida* R.Br) contains flavonoid, tannins, saponins, and terpenoids that are potentially good as anti-cancer drugs. This study aims to investigate the effect of EFL on HeLa cells of cervical cancer through treatment of a combination of faloak leaves extract and chemotherapy agent paclitaxel (Pac). Cells were treated without Pac of control group, combination treatment group of EFL 25 μg/mL, 50 μg/mL, 100 μg/mL and 5 μg/mL Pac and a single dose of 100 μg/mL EFL. Inhibition of proliferation activity was measured by 3-(4,5 –dimethylthiazol –2–yl)-2,5 diphenyltetrazolium bromide (MTT) assay and HeLa cell death was observed with blue trypan. The anticancer activity compounds fromthis results shown that EFL with or without combination effectively inhibit the proliferation ofHeLa cells. This extract induce HeLa cell death- dependent on the combination of (25, 50, 100 μg/mL) with 5 μg/mL Pac. It can be concluded that ethyl acetate extract of faloak leaves can inhibit proliferation and increase HeLa cell death.

Keywords: faloak leaves, proliferation, ethyl acetate extract, cell dead, phytochemical test.

1. Introduction

Cervical cancer is still the second leading cause of death in women in the world. Chemotherapy is a treatment that uses drugs to kill cancer cells by preventing them from developing and dividing. Cervical cancer is with chemotherapy, one of the drugs that are often prescribed is paclitaxel (Pac). Chemotherapy can cause fatigue, infection risk, nausea and vomiting, hair loss, loss of appetite, and diarrhea, depending on the dosage used. Despite its anti-cancer properties, paclitaxel (Pac) has side effects in clinical use [1].

Various studies have been conducted to improve the efficacy of chemotherapy drugs while reducing their side effects. One of the methods developed is to combine chemotherapy agents either with other substances such as herbs rich in antioxidants[2]. The use of chemotherapy and radiation in the treatment of cancer did not provide significant results and killed cancer cells, instead, it tends to harm the healthy cells and tissues of the patients because of constant exposure to intense radiation over a long period as well as expensive treatment

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[3],reported that the use of herbal therapy is very helpful to improve the general condition of cancer patients. Herbal therapy can be a palliative therapy in pre-cancer patients and advanced stages in improving quality of life[1]. So it is important to develop other treatments that increase the suppression of proliferation activity and cancer cell death by combining chemotherapy agents with therapies based on natural ingredients. One natural ingredient that can be used as an anti-cancer agent such asfaloak (*Sterculiaquadrifida*R.Br). It is a herbal plant that is believed by the Flores, NTT, community as alternative medicine.Rollando and Siswadi (2016) reported the potential of this plant that contains flavonoid, phenolic, tannin, and terpenoid compounds [4]. Each of these compounds has anti-cancer properties for breast cancer, hepatoma, and cell line[5].Research shows evidence that flavonoids act as anti-cancers[6]. Flavonoids can inhibit tyrosine kinase proteins that have potential in the inhibition of cell proliferation and HeLa cell death [7].

Research results to date have not been able to find any scientific evidence that ethyl acetate extract of faloak can decrease proliferation and increase the death of HeLa cells which is a model of cervical cancer line cells. This experimental test aims to explain the molecular mechanism of extract ethyl acetate from faloak leaves through phytochemicals test in the proliferation and death of HeLa cells.

2. Methods andMaterial

2.1. Plant material and extraction

Ethyl acetate extract of faloak leaves (EFL)fresh leaves were collected from plantations in Kupang, East Nusa Tenggara, Indonesia and the identification was authenticated at Laboratory of Ecology and Conservation of Tropical Forest Biodiversity, Faculty of Forestry, UniversitasMulawarman, Indonesia (specimen No. 01/UN17.4.08/LL/2021). The EFL were extracted according to the protocol [2].

2.2. Phytochemical Test Ethyl Acetate Faloak Leaves (EFL)

2.2.1. Flavonoid Test

To prepare flavonoid test,1 g of Ethyl acetate extract of faloak leaves (EFL) was put into the test tube then added 1mL chloroform, and then add 1mL of H_2O, homogenized and leave for a while until a layer of water is formed. Remove the layer of water and added 0.1 g of Mg powder and 5 drops of concentrated HCL. Flavonoid is shown when it forms a red or orange color [8].

2.2.2. Saponin Test

To prepare saponin test, 0.5 g of ethyl acetate extract of faloak leaves (EFL)dissolved in 2 mL of H_2O, then heated for 2-3 minutes,and then cooled and following byshaking. A positive test of saponins is indicated when there is stable foam for 30 seconds[9].

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2.2.3. Tannin Test

To prepare tannin test, 1 g of ethyl acetate extract of faloak leaves (EFL) was put in a test tube containing 10 mLH_2O and filtered. Filtrate diluted until it became colorless and then added 3 drops of $FeCl_3$. If a blackish-blue or green-black color is formed, it indicates the presence of tannins [8].

2.2.4. Alkaloid Test

Test of alkaloids was carried out using the Mayer, Wagner, and Dragendrof methods. 0.5 g of ethyl acetate extract of faloak leaves (EFL) added with 1 mL 2M HCl, and 9 mL of aquadest, heated for 2 minutes, cooled and filtered. The filtrate is divided into 3 parts, each added with 2 drop of Mayer, Wagner, and Dragendorff reagents. Mayer's reagent results showed that, when white deposits are formed, it indicates the presence of alkaloid compounds. The results of Wagner's reagent showed when a light brown to yellow sediment is formed indicates the presence of alkaloid compounds. Dragendrof reagents showed that when orange to red-brown formed indicates the presence of alkaloid compounds [8].

2.3. Cell culture

HeLa cells were obtained from the Biomedical Laboratory ofUniversitasBrawijaya, Indonesian. Cells lines was cultured in RPMI-1640 supplemented with 20% FBS v/v, 1% Penicillin-Streptomycin at 37°C in humidified atmosphere containing 5% C02. To ensure proper cell growth and health, a cell line must be sub-cultured when it reaches around 80% confluence.

2.4. Experimental design:

The success rate of sub-cultured was around 80% confluence. After cells sub-cultured, cells were divided into 6 groups. In the control group, cells were treated with or without paclitaxel (Pac) 10 µg/mL, while in the combination treatment group divided separately into four groups with different doses: of extract ethyl acetate faloak leaves (EFL) (25 µg/mL, 50 µg/mL, 100 µg/mL) and paclitaxel (Pac) 5 µg/mL and a single dose of 100 µg/mL extract ethyl acetate leaves (EFL).

2.5. Determination of Total Flavonoid Content (TFC)

Total flavonoid content (TFC)inethyl acetate extract of faloak leaves (EFL) using quercetin standards, was measured according to the previous assay with slightly modified[10]. Briefly, 20 mg of EFL was dissolved in 10 mL ethanol. Then, 300 µl of EFL solution mixed with 60 µl of 5% NaNO2 and 60 µl of 10% AlCl3. After 10 minutes incubation, 400 µl of 1 M sodium hydroxide and 480µl H2O was added. The TFC was evaluated at 510 nm using a spectrophotometer. Quercetin was used as the reference compound to produce the standard curve; results were expressed as mg Q/g.

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2.6. DPPH Assay

Antioxidant potential of ethyl acetate extract of faloak leaves (EFL) was investigated using the 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay, DPPH solution was freshly prepared by dissolving 2 mg DPPH powder in 50 mL ethanol and kept in dark. Dilution series of samples (10 mg) added in the wells of a 96-well plate. Sample tested were prepared in varying concentration using serial dilution method with concentration of 250, 125, 62.5 end 31.25. DPPH solution (0.1 mM) was added to all of wells. The solution was shaken and incubated for 30 minutes at room temperature in the dark. Then the absorbance was read at 517 nm using a spectrophotometer. The scavenging activity of EFL sample solution was calculated by applying the formula:

$$\% \text{ DPPH} = \left[\frac{Abs.Control\ -Abs.Sample}{Abs.Sample}\right] \text{X } 100$$

Absorbancecontrol is the absorbance of DPPH solution without extracts. IC_{50} was calculated from the graph plotted of inhibition percentage against samples concentration and equals to sample concentration providing 50% inhibition.

2.7. Antiproliferation Assay

Cells (6×10^4cells/well) in 96-well plates. The cells were exposed to various concentrations of ethyl acetate extract of faloak leaves (EFL) 25, 50, and 100 µg/mL for 24 hr at 37°C in the 5% CO2 incubator. Subsequently, the anti-proliferation assay was performed according to 3- (4, 5-dimethylthiazol – 2 – yl) - 2, 5 diphenyltetrazolium bromide(MTT)method (TACS MTT Cell Proliferation assay ®4890-25 Cat-K). A 10 µL portion of MTT with 0.5 mg/mL in PBS was added into a well and incubated in a dark for another 4 hr. Then the solution was removed and 100 µl of DMSO was added to each well. Absorbance values were determined at 570 nm using a microplate reader. The effect of the samples on the proliferation of cell lines was expressed as inhibition % cell viability. The inhibition rate was calculated using the following formula:

$$\% \text{ Viability Cell} = \left[\frac{Abs.Sample\ -Abs.Blank}{Abs.Control\ -Abs.Blank}\right] \text{X } 100$$

At 570 nm, determine the absorbance. MTT is metabolized by living cells, resulting in a purple tint. The Absorbancevalue and cell viability have a clear correlation.

2.8. Measuring Cell Death by Trypan Blue

The effect of ethyl acetate extract of faloak leaves (EFL) were investigated on the viability of human HeLa Cells lines by Trypan Blue assay. Briefly, HeLacells were seeded at 6 x 10^4 cells/ mL in 24 well plates. Cells were treated various concentrations of EFL (25, 50, 100 µg/mL) and with or without 5 µg/mL paclitaxel(Pac) The cells were cultured for 24 hours cells were applied of 0.4% trypan blue and put on hemocytometer Cells were counted under light

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microscope. Trypan blue is a dye that is used to determine how many live cells there are by marking only dead cells[11].

2.9. Statistical Analysis

Each experiment was repeated at least 24 times. Data are presented as the mean +s.d (standard deviation). The results were analysed by the one-way Analysis of Variance (ANOVA) test, followed by Post Hoc Tukey test. Data were described as a mean ± standard deviation. Statistical analysis was performed with SPSS software version 20. (SPSS Inc.,Chicago, IL, USA).

3. Results

3.1. Phytochemical Test of EFL

Extract of faloak leaveswere examined by using Mayer, Dragendrof, and Bouchardate reagents. The ethyl acetate extract of faloak leaves (EFL) sample does not form a green colour that turns blue, this indicates the absence of steroid and terpenoid compounds in the ethyl acetate extract.

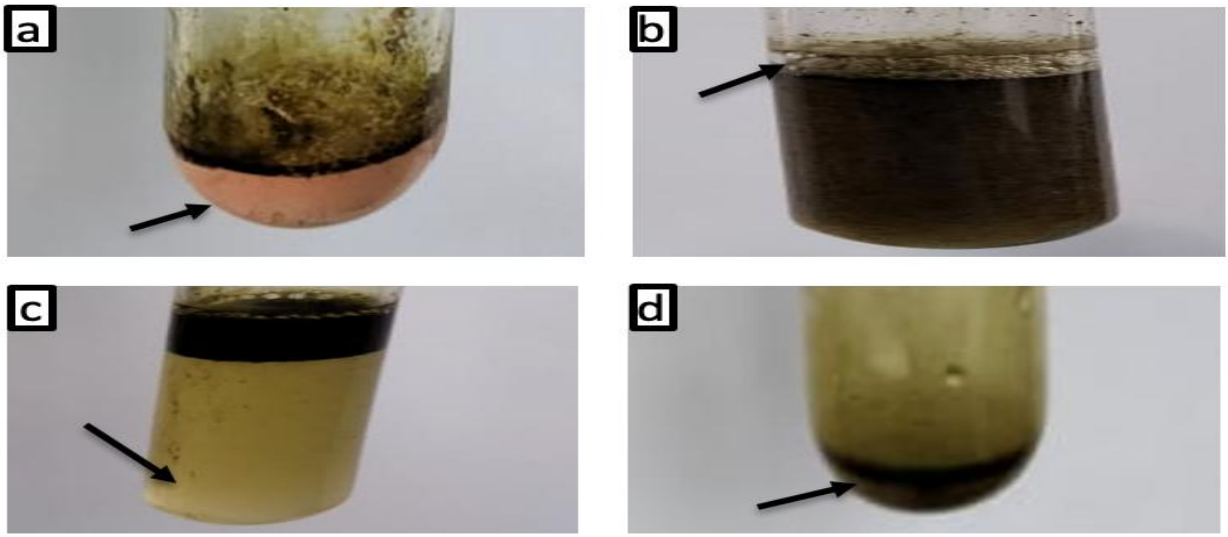

Figure 1.(a) Positive flavonoid compound was indicated by a change in colour from green to orange. (b) Positive saponin bubble forms. (c) Positive tannins solutions are cloudy white. (d) Positive alkaloid from green to orange brown sediment.

3.2. Determinattion of Total Flavonoids Content (TFC)

In this study, Flavonoids Content were expressed as quercetin equivalents (mg Q/g) using the following equation Y= 0.0039X + 0.0044 at R2 = 0.9866, and TFC of ethyl acetate extract of faloak leaves (EFL) is 483.61 mg Q/g. The result of TFC indicates that plant extracts has high flavonoid content.

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3.3. DiphenilpicrylHydrazyl (DPPH) Radical Scavenging Effect

DPPHradical scavenging assay is one of the most frequently used approaches to determine the antioxidant potential of different plant extracts. This study aimed to evaluate the antioxidant and anti- proliferative properties of ethyl acetate extract of faloak leaves (EFL). As demonstrated in Fig. 2.

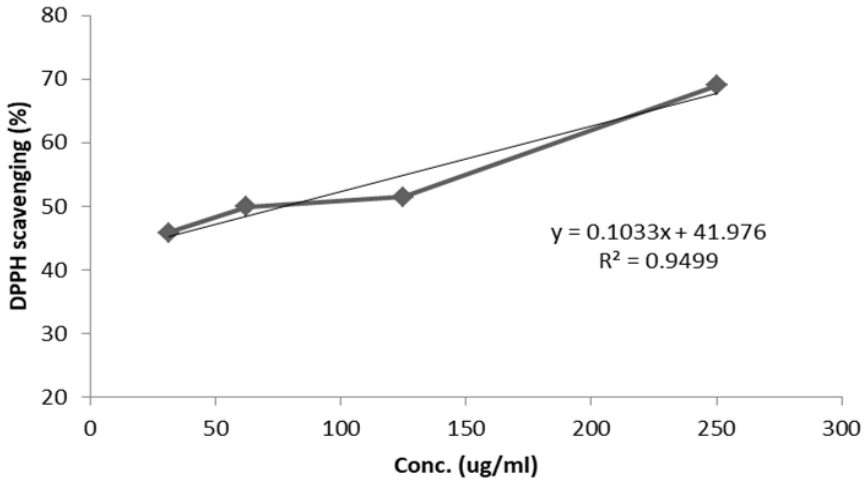

Figure 2. The curve shows that the higher the EFL concentration, the percentage of radical inhibition also increases, with an IC_{50} value 77.67 µg/mL

Results indicate that the IC_{50}value for EFL (IC_{50}: 77.67 µg/mL) demonstrated strong antioxidant activity and the scavenging activity of the different EFL increases in a concentration-dependent manner.

3.4. Antiproliferative activity of the ethyl acetate extract of faloak leaves(EFL)

3- (4, 5– dimethylthiazol–2–yl)- 2, 5 diphenyltetrazolium bromide (MTT) was conducted on HeLa cells to evaluate the antiproliferative effect of ethyl acetate extract of faloak leaves (EFL). Tested cells were treated at various concentrations of EFL (paclitaxel 5 µg/mL and 25, 50, 100 µg/mL) for 24 hours and the results were illustrated in Figure 3. As revealed by the growth curves. After incubation of 24 hours, EFL showed a dose-dependent inhibition from 25 to 100 µg/mL (Fig.3). Cells weretreated with 5 µg/mL paclitaxel and 25 µg/mL EFL for 24 hours reduced cell growth by 50%. The maximum effect was obtained with 5 µg/mL paclitaxel and 100 µg/mL EFL, which indicated EFL anti-proliferation effect to HeLa cells growth decrease with an increasing of concentration.

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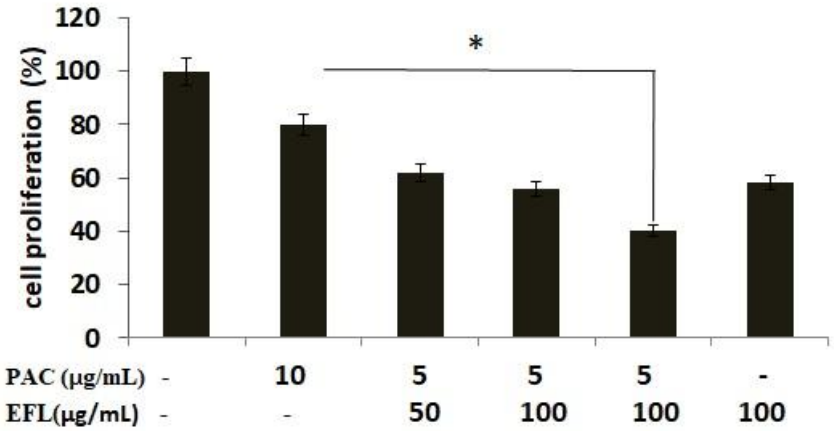

Figure 3. Cells growth inhibition in HeLa cells EFL exposed. Cell proliferation is expressed as a percentage of the maximum value compared with control cells. The control cells weretreated with paclitaxel (Pac)10 µg/mL, combination treatment group of extract ethyl acetate faloak leaves (EFL) 25 µg/mL, 50 µg/mL,100 µg/mL and paclitaxel (Pac) 5 µg/mL and a single dose of 100 µg/mL extract ethyl acetate leaves (EFL).Dose dependent decrease in values was higher in HeLa cells. Data refers to the mean ± SD. n = 3 replicates in each group. *p < .05 and ** p < .01 versus EFL only group.

3.5. Paclitaxel combined with ethyl acetate extract of faloak leaves(EFL) increase cell death with Trypan Blue Assay

The results of cell calculations using the trypan blue assay test showed that the percentage of HeLa cell age increase after being given the extract and the combination of paclitaxel in the concentration of extracts, namely the percentage of HeLa cell death continued to increase in proportion to the increase in the concentration of the extract given. A combined dose of 5µg/mL paclitaxel and 100 µg/mL ethyl acetate extract of faloak leaves (EFL)decreased the number of living cells.

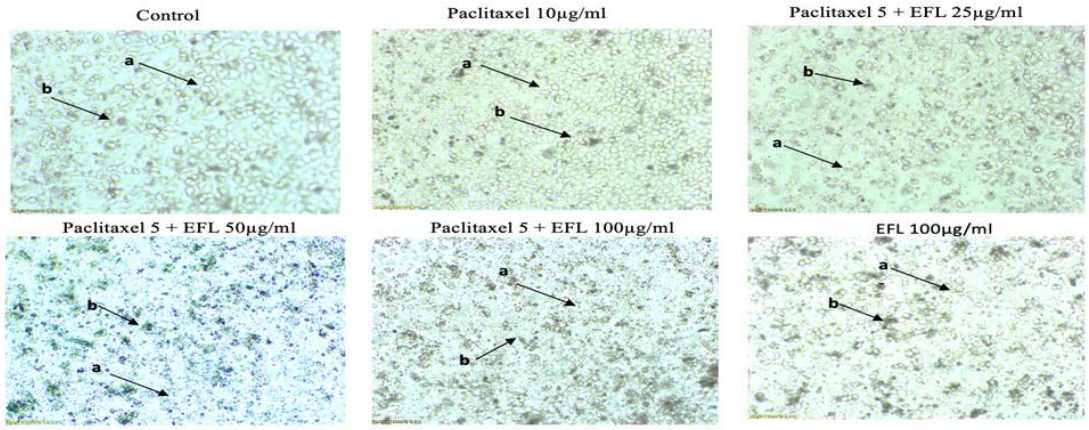

Figure 4. The effect of paclitaxel and EFL combination were culture in HeLa cells. Cells were stained by trypan blue Assay as described in the Materials and Methods. Arrows

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(a) Data show a representative assessment of living cells and (b) cells that appear to dead.

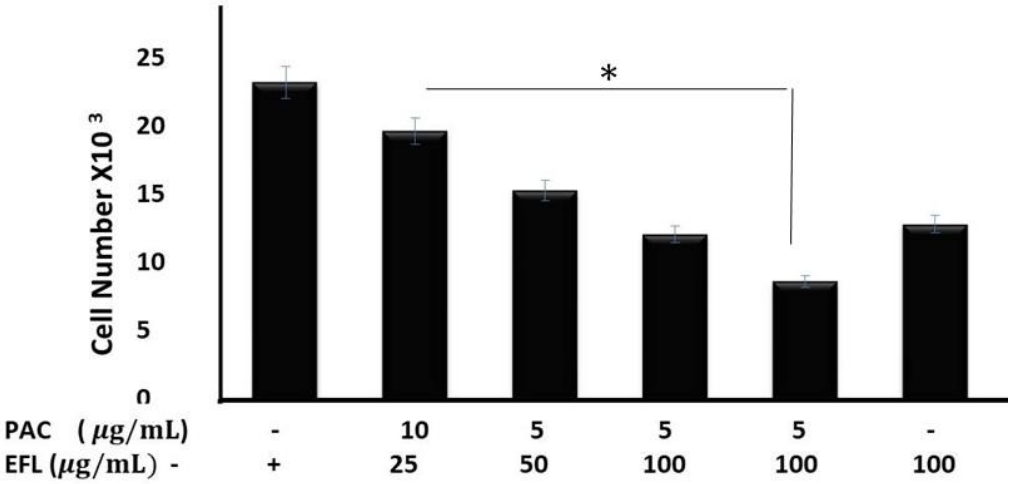

Figure 5.HeLa cell showed a significant rise in the number of HeLa cells post 24 hours. Results shown are mean ± SD, with n = 3 replicates in eachgroup. *P < 0.5, **P < 0.01 versus paclitaxel(Pac) only group.

4. Discussions

The % DPPH scavenging activity of ethyl acetate extract of faloak leaves (EFL) was reported as the percentage of DPPH inhibition, with a higher value is associated to a stronger antioxidant activity. Higher activity of DPPH radical scavenging activity may be attributed to presence of higher levels of total flavonoid (483.61 mg Q/g) as their ability to donate protons and act as inhibitors or scavengers of free radicals is their key role, possibly acting as major antioxidants [12].

The antiproliferative effect of ethyl acetate extract of faloak leaves (EFL) may be due to the flavonoid, saponin, taninand alkaloid, based on the results of our component analysis. Many studies have reported that foods with antioxidant activities have cancer protective effects [13,14]. Zhang et al., (2015) reports the antiproliferative activity of flavonoid in a variety of human cancer cell lines flavonoids can act as anticancer in cell cycle regulation [15].EFL was proven to be able to EFL caused more than 60% inhibition to tested HeLa cell. These results support the previously mentioned observations regarding EFL in arrest of cancer cells. Reports indicate that faloak was rich in various secondary metabolites such as phenols and flavonoids [16.17].

The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situor externally supplied through foods and/or supplements. These antioxidants act as free radical scavengers by preventing and repairing damages caused by ROS and therefore can enhance the immune defence and lower the risk of cancer [15]. Wang et al., (2019) reported that flavonoids and tannins inhibit cell proliferation and cell death of cancer by cell-cycle arrest and can be a good option for cancer treatment (Wang et.al., 2019. Several investigations were conducted indicated that the anticancer effect of alkaloid and terpenoid saponin [19,20,21].

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In this experiment, the number of living cells in HeLa cells was further assessed with the trypan blue. Our results suggest that paclitaxel combination treatments with or without ethyl acetate extract of faloak leaves (EFL)was able to induce HeLa cells. These data prove that the combination of paclitaxel and EFL reduces the survival rate of HeLa cancer cells. Our results support the trypan blue data showed evidence that the combination of paclitaxel and 100 µg/mL EFL is more effective.

According to our results, the combination of ethyl acetate extract of faloak leaves (EFL) and paclitaxel were directly involved in the increasing ofcell death [22]. We suspect that paclitaxel is active when combined with another cytotoxic agent [23,24]whereas EFL reduces living cells due to its antioxidant effect. Our results are in line with previous reports that paclitaxel inhibits cell activity due to antioxidants [25,26]. Cell death and proliferation are one of the mechanisms that occur during the promotion and progression phases of cancer. As a result, it may be used as a source of bioactive compounds with chemotherapy properties. These results indicate that EFLcan successfully inhibit proliferationin HeLa cells. Therefore, it could be used as a novel therapeutic candidate for cervicalcancer treatment.

5. Conclusion

In conclusion our results show that combination ethyl acetate extract of faloak leaves (EFL) and therapy chemotherapy could prevent proliferation and cell death. Therefore, our results suggest that EFL is a promising candidate in cancer therapeutics.

Authors Contributions: ATE performs the concept and design of experiment and performance MTT assay analysis. NC and R have made it easy to measuring cell death use trypan blue and ELISA KIT in real time. LE, EV, S and N do analyse statistical analysis. ATE, T, NR, NC and R revised the manuscript and wrote the script. All authors read and approved the final manuscript.

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Limitations and Future Studies: This research need further analysis to determine how it death mechanism since there is currently very little information. The phytomoleculescouldexpect to transform cancer care in the future.

Ethical Approval: All experiments were conducted according to the principles of Guide for the Care was approved by The Ethical Committee UniversitasBrawijaya, Malang, Indonesia (10/EC/KEPK/01/2021).

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