Artikel Penelitian

Potential Eugenol Ethyl Acetate Fractions of Clove Flowers Against Proliferation of Cervical Cancer (HeLa Cell Line)

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Abstrak

Kanker serviks atau disebut juga kanker leher rahim adalah sejenis kanker yang disebabkan terutama oleh Human Papillomavirus (HPV) onkogenik, yang menyerang leher rahim. Salah satu tanaman yang dapat digunakan dalam pengobatan kanker serviks adalah bunga cengkeh. **Tujuan**: Menganalisis pengaruh pemberian fraksi etil asetat eugenol bunga cengkeh untuk proliferasi dan migrasi sel HeLa. **Metode**: Penelitian ini adalah eksperimental *in-vitro*. Fraksi etil asetat dilakukan dengan metode MTT [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Assay. Scratch Assay dilakukan untuk mengetahui daya migrasi dari sel kanker, hingga Trypan Blue untuk melihat (sel mati / sel hidup). Teknik pengumpulan data dilakukan dengan teknik observasi eksperimental. Data yang diperoleh pada uji MTT adalah persentase sel HeLa yang terhambat. Hasil scratch Assay dianalisis dengan software ImageJ dan metode Trypan Blue dengan menggunakan alat TC10. **Hasil**: Pengujian MTT Assay didapatkan nilai IC50 24,51 µg/ml, rerata migrasi sel perlakuan 303 sel dan rerata kelompok kontrol 715 sel. Jumlah perlakuan berproliferasi 6.14 x 105 dan kontrol 12.1 x 105. **Simpulan**: Berdasarkan potensi yang dimiliki, bunga cengkeh dapat menjadi kandidat terapi sel kanker serviks (HeLa Cell Line) dimasa depan.

Kata kunci: anti kanker serviks, kanker serviks, proliferasi sel, MTT Assay, scratch assay

Abstract

Cervix cancer, also known as cervical cancer, is a type of cancer that is caused mainly by oncogenic Human Papillomavirus (HPV), which attacks the cervix. One of the plants that can be used in medicine is clove. ***Objective****: To analyzed the effect of a fraction of ethyl acetate eugenol of clove flower for the proliferation and migration of HeLa cells.* ***Methods****: This was an in vitro experiment. Ethyl acetate fraction was tested in vitro using the MTT [4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) Assay method. A scratch Assay is done to determine the migratory power of cancer cells to Trypan Blue to see (dead cells/living cells). Data collection techniques were carried out using experimental observation techniques. The data obtained in the MTT test is the percentage of inhibited HeLa cells. Scratch Assay results were analyzed with ImageJ software and the Trypan Blue method using the TC10 tool.* ***Results****: The MTT Assay test obtained an IC50 value of 24.51 µg/ml, the average migration of treatment cells 303 and the average control group 715 cells. The number of proliferating treatments is 6.14 x 105 and the control 12.1 x 105.* ***Conclusion:*** *Based on its potential, clove flower can be a candidate for cervical cancer cell therapy (HeLa Cell Line) in the future.*

Keywords*: anti-cervical cancer, cervical cancer, cell proliferation, MTT assay, scratch assay*

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INTRODUCTION

Cervical cancer is cancer that is caused mainly by the oncogenic human papillomavirus (HPV), which occurs at the border between the epithelium that overlooks the ectocervix (porsio) and the endocervix cervical canal called the Squoma-Columnar Junction

(SCJ).[1] Normal cells infected with HPV will transform into cervical cancer cells or HeLa cells, a continuous cell that can be cultivated.[2]

Cervical cancer is the second most chronic disease in the world. In 2018, an estimated 569,000 new cases and 311,000 deaths from cervical cancer were estimated.[3] Population, increasing population age, and increasing risk factors in the population can increase mortality. About 1,00 new cases are diagnosed every year and more than 93 million Indonesian women are at risk of developing the disease.[4] Cervical cancer occurs at the border between the epithelium that covers the ectocervix (porsio) and the endocervical cervical canal called the Squoma-Columnar Junction (SCJ).[1]

Various therapeutic strategies have been developed in the treatment of cervical cancer, but the results are not so optimal and tend to cause side effects that can harm the patient.[5] Therapies using traditional medicines derived from plants or herbs are also being developed. One of the plants that can be used in medicine is crab.[6] Traditionally, crab flowers are used in the world of medicine because they act as fungicidal, bactericide, analgesic, antioxidant, and anti-inflammatory.[7]

According to a study by Kurnia *et al*. (2019)[8], the eugenol compounds in cranberry oil can enhance the apoptosis of HeLa cells. Most eugenols are found in the flowers of cranberries. The essence oil content in the clenbuterol reaches 21.3% with eugenol levels between 78-95%, of the stalk or flower ginger reaches 6% with eugenol levels between 89-95% and of the leaves of the cranberries reaches 2-3% with a eugenological level between 80-85%.[9]

Cloves can be an alternative to cancer treatment. Clove is one spice plant type with high antioxidant activity due to its fairly high eugenol content.[10] Eugenol compounds in pumpkin oil can enhance the apoptosis process of HeLa cells.[8] Eugenol in pumpkin oil can also act as an antiproliferative in cancer cells.[11] Eugenol's cytotoxic effects on HeLa cells range between 50-200 µM.[12] Some research results show that eugenol has a variety of biological activities, such as antifungal, anticancer, and anti-inflammatory.[13]

Eugenol could be a new alternative to cancer treatment. There is still very little literature today that deals with fractional potential.[14] The objective of this study was to assess how the eugenol fraction influences the flowers on the proliferation of cervical cancer cells.

METHODS

The instruments used were 200µl and 1000µl micropipettes, 25 cm^2 and 75 cm^2 flakes, 0.22 µm filters, 5 ml disposable syringe, 12 well plates, 96 well plate (tissue culture plate), ELISA plate reader, laminar water flow, conical tube, 10 µg/ml streptomycin, incubator, autoclave, centrifuge, object glass, 40 ml tissue culture pumpkin, yellow tip, six-well tissue culture cup, dry block heater, TC10 cell counter, scratch assay, UV-Vis spectroscope, and inverted microscope.

The ingredients used were eugenol extract, MTT powder, phosphate buffer saline (PBS), pH 7.4, FBS, trypsin, EDTA 0.25%, plate and medium culture (DMEM), DMSO, sterile aquades, inverted, HeLa cell line, penicillin-streptomycin 10%, liquid tank, 99% v/v isopropanol, 0.4% b/v blue trypan solution.

The free variable in this research was the level of eugenol extract from cranberry flowers added to the HeLa cell culture at concentrations (0, 15,625, 31,25, 125, 250, and 500 µM). The bound variable was the minimum inhibiting concentration of 50% cells (IC50 value), the percentage of cells that die from the flower's eugenol fraction's cytotoxicity activity and cell migration. The controlled variables are cell culture media, incubation time, temperature, and environmental conditions.

Extraction and fractionation

The Syzygium aromaticum extract was obtained by macerating the flowers using an ethanol solvent of 96%.[15] And stored in a freezer (-10 oC).[16] Then, the liquid-liquid fractioning was continued to obtain an ethyl acetate fraction.[17]

Thin layer chromatography (TLC)

Chemical content identification by KLT was used to determine the chemical content found in the ethyl acetate fraction of cranberry flowers, which is phenolic. Identification was done using silica gel GF254 silica and ethyl acetate: methanol motion phase. (9:1). KLT

plates were detected under UV 254 and 365 nm after delusion. Subsequently, sprayed with FeCl3 spotted impact.

Wake Up HeLa Cell

Cryogenic HeLa cell vials were removed from storage in liquid nitrogen and thawed for a few minutes. Move the fluid into a 15 ml falcon tube containing a complete medium, then centrifuge for 10 minutes at 2000 rpm. The superfood solution was removed, and into the pellets was added medium and FCS 20% to 5 ml, inserted into the culture flask and incubated at a temperature of 37°C, CO2 5% performed observations for 2-4 days under an inverted microscope.

Cell viability assay (MTT *Assay)*

Insert 100 µL of eugenol fractional solution in different concentration series (0-500 ug/mL). As cell controls, 100 µl of culture medium + 100 µ L of cell suspension are added, and as solvent controls, 100µL of DMSO + 100 mL of culture media and 100 µ l of cell suspensions are added with delusions corresponding to the concentration delusion of the test solution. The cells were incubated for 3-4 hours. Add the SDS stopper reagent (100 µL). The microplate was incubated for 48 hours at room temperature and dark room. Living cells react with MTT, forming purple. Test results were read with ELISA reader λ 550 nm.

Cell migration assay *(scratch Assay)*

The basic principle of this method is by (i) growing monolayer cells; (ii) scratching on parts of the cells using a sterile yellow tip; (iii) observing periodically the recolonization of cells in the place where they were originally empty and scratched. (zona yang telah discratch). Determining migration capabilities is done by counting the number of cells that have accumulated in the zone that has been discratched at a specified time interval.

Percentage of dead and alive cells *(Trypan Blue)*

The principle of this method is that normal cells have a cell membrane that is intact and capable of withholding foreign substances that enter the cell, like blue trypan dye, while abnormal cells cannot withstand alien substances prepare ten µL of trypan blue in a sterile microtube, add ten µl of cell suspension into a blue trypan solution and then homogenize. Slowly insert 10µL of blue-trypan solution into one side of the chamber/chamber using a pipette. Count and analyze the number of healthy and dead cells with the TC10 tool.

Data collection techniques were done using experimental observation techniques. The data obtained on the MTT test was the percentage of inhibited HeLa cells. The formula used is as follows:

The relationship between the concentration of the test solution and the cell viability can be displayed in a graph. The price of the IC50 (concentration that can inhibit 50% cell growth) of a test solution can be determined from such a graph. Scratch Assay results were analyzed using ImageJ software and the Trypan Blue method using TC10 tools. Data analysis was performed using variance analysis with a 95% confidence rate.

RESULTS

This research took cloveful flowers from the High Market, Field, West Sumatra. Cloveful flowers were prepared by sorting, planting, drying, and smoothing. Smoothing is done to make it easier for the solvent to penetrate the sample at the time of maceration. Maceration was done by soaking the samples until they were soaked and done twice until a 96% ethanol extract was obtained as follows.

Table 1. Cloveful extract fertilizer

Ekstract		Simplisia (gram)	Yield (%)
Total Extract	Ethanol	900	9,02087

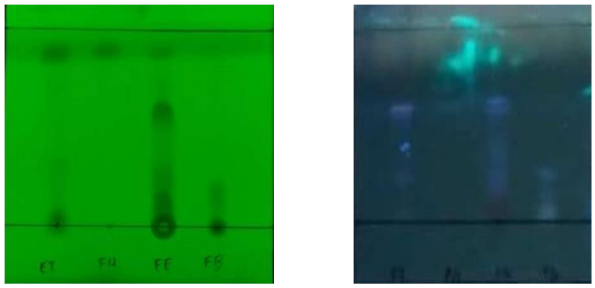
The 96% ethanol extract was continued with fractioning (liquid-aqueous extraction) in stages with n-hexane solvents up to the ethyl acetate solvent. From 30 grams of ethanol extract, 96% obtains the n-hexane fraction, and ethylacetate as follows.

Table 2. Yield of clove flower fraction

No	Fraction	Simplisia(gram)	Yield (%)
1	N-hexane	2,3169	7,7200
2	Ethyl acetate	0,6326	2,1086

The qualitative tests were conducted using Thin-Layer Chromatography (TLC) on 96% total ethanol and fractional extracts to determine the presence of phenolic compounds in cloves. The phenolic compounds are observed under 365 nm UV rays and are marked in black when sprayed with a FeCl3 stain absorber. Separation patterns (chromatograms) can be seen in figure 1.

(a) (b)



(c)

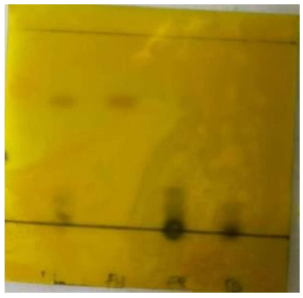


Figure 1. Chromatogram with eluen (Toluene: Ethyl Acetate: Fromatic Acid 7:2,5:0,5) Description: (FH): n-hexagonal fraction, (FE): Ethyl acetate fractures, (FB): Butanol fractions, (FA): Water/Residue fractured, (a): 254 nm UV rays, (b): 365 nm UV radiation, (c): $FeCl_3$ spotted impact. Image (a) shows a rising stain from the ethyl acetate (FE) fraction. In picture (b), there are also rising nodes, and then in photo (c), it is done with a $FeCl_3$ stain impact to prove the phenolic content of the (Fe) ethylacetate fraction that causes a dark stain. This proves that the ethyl acetate fraction of cranberry flowers has eugenol content.

Cell viability assay (MTT *Assay)*

Different concentrations of ethyl acetate fractions were treated against HeLa cells using MTT assay. MTT tests can be helpful for determining cell proliferation, viability, and activation. MTT will measure the ability of living cells to reduce the tetrazolium salts of MTT into formations with NADH reductase and other enzymes that produce a linear relationship between the number of living and dead cells. The number of formations produced would describe the number of cell viability.[18]

Giving different ethyl acetate fraction concentrations will result in different viability. The smallest cell viability is obtained from a concentration of 500 µg/ml, which is 33.8%, while the largest cell viability is at a concentration of 15,625 µg / ml, which is 90,65%. The relationship between the ethyl acetate fraction concentration and the cell viability can be used by using the concentration log chart with the cell viability, resulting in the IC_{50} value of the regression equation.

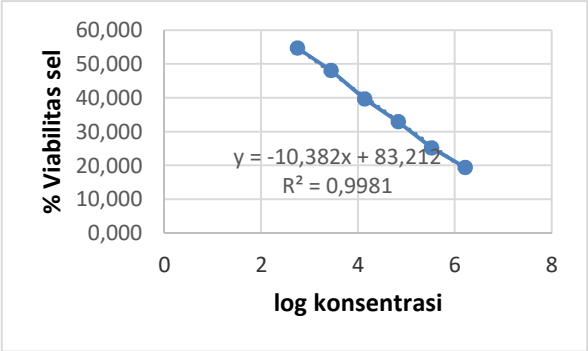


Figure 2. Diagram of log concentration relations with viability

Based on figure 1, the resulting regression equation is y = - 10,382x + 82,212 with a value of 83,212 and b value of -10,382. In this equation, enter the y value of 50% to find the value of IC_{50} and get the x value of 3,199. The IC_{50} value indicates the potential toxicity used as a cytotoxic potential parameter. The IC50 is a value that can inhibit cell proliferation in 50% of the population.[19] According to the NCI (National Cancer Institute) and Geran, IC_{50} values are ≤ 20 µg/mL = highly cytotoxic, 21-200 µg / mL = moderate, 201- 500 µ g / ml = weak and if > 501 µg (mL) = inactive.20 The lower the IC50, the greater the potential to be developed as an anticancer agent.[20] Ethyl acetate fractions are high in antioxidants because they have an IC value of <50 µg/ml.

Observations with a microscope in Figure 4.2.2 show that at a concentration of 500 µg/ml, almost all cancer cells die, whereas at concentrations of 15,625 µg / ml, some of the cancerous cells are still alive. This proves that the ethyl acetate fraction is potentially anticancer. Fractionation has an advantage over other

methods because it can separate bioactive compounds based on the radiation rate because the polar compound is soluble in polar solvents. In contrast, the semi-polar compound is a solute in semi-polar solvents, and non-polar compounds are non-polar solvents.[21]

Cell migration assay *(scratch Assay)*

Observations were performed quantitatively by observing the number of cells accumulating in the previously scratched zone under an inverted microscope. The cells undergoing migration will move toward the scratch zone that was previously empty when it was not incubated. In this study, there was an influence on HeLa cell migration by giving the fraction of the cranberry flowers and finding a decrease in the number of cells accumulating in the scratch zone in the given fractional cell group compared to the control group. After a 48-hour incubation period, an average of 303 cells accumulated into the scratch zone at a fractional administration at a concentration of 24.51 µg/ml (IC_{50}) can be seen in table 3.

Table 3. Average cell migration

Group	Number of Sample	Number of cells undergoing migration
Control	1	814
	2	589
	3	637
	4	653
	5	798
	6	796
Total		715
Average		
IC_{50}	1	319
	2	578
	3	279
	4	347
	5	122
	6	173
	Total Average	303

An overview of the control group migration and treatment can be seen in figure 3.

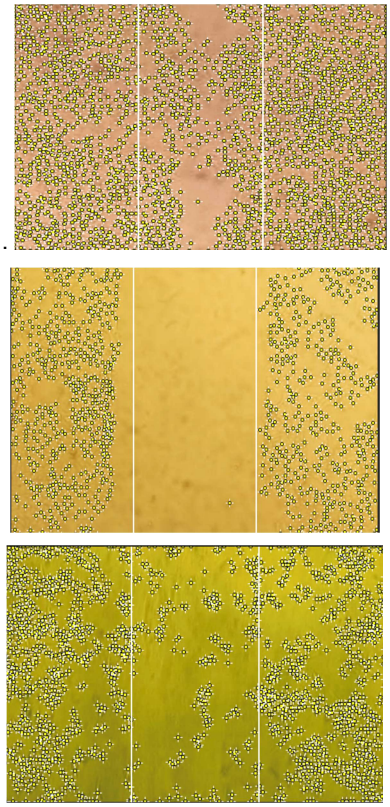


Figure 3. The heLa cell culture migration scratch assay photo shows a) the control group prior to 48 hours of incubation, b) the control groups after 48 hours of incubation, c) treatment group IC50, and d). The cells undergoing migration appear to be gathering into the previously discratched zone.

The preparation was observed using an inverted microscope, which was then read with the help of the ImageJ application. Figure a is a pre-incubation control group, i.e., a group without a clove fraction before incubation. Figure b is a control group that was incubated for 48 hours and was a group to which no cloves fraction was given. In this group, they found cells that migrated to the scratch zone after 48 hours of incubation, but a small number compared with the number of cells migrating in the control group.

A graph of cell migration testing with each treatment can be seen in Figure 4.

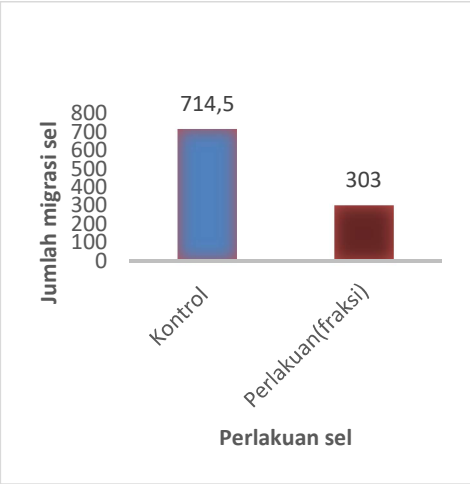


Figure 4. Cell migration testing with each treatment

The normality test used the Shapiro-Wilk test and obtained the results of the control group 0.120 and the treatment group 0.600. The p-value> 0.05, so it is concluded that the data is distributed generally so that the t-dependent test can be performed. The data is then analyzed using a dependent t-test. The test results showed a significant difference in the number of migrated cells after administering eugenol fractions with a $p = 0.009$ ($p < 0.05$).

To determine the influence of cranberry flower fraction giving on HeLa cell migration activity in vitro using scratch assay methods and calculations through observation using an inverted microscope. Observation of cell migration using the scratch assay method is performed using interval imaging or observed periodically according to a specific time interval. The basic principle of this method is to make a scratch on a monolayer cell using a sterile yellow tip to form a gap of a specific size. Determining cell migration capabilities is done by observing cells periodically and counting the number of cells that have accumulated in the discratched zone at a specified time interval.[21] Types of collective cell migration checked with scratch assay tests known as sheet migration.

In the study, the greater the concentration of the given cranberry flowers, the smaller the number of cells that can migrate or move to the previously discratched zone. The longer the incubation period also causes the addition of the numbers accumulating to the scratch zone to become smaller. It is also related to Menten's theory of receptor occupation, which states that the effects of a bioactive compound are due to interaction with the receptor in the cell. The intensity of the effect of the bioactive substance is directly compared to the fraction of a bound receptor, and receptor fraction depends on the dose and length of exposure. The longer the exposure and the larger the dose, the intensity will increase.

Based on the results of research conducted by the researchers, it can be stated that the study's hypothesis is accepted because it has been found that the influence of the fraction of clove flowers in inhibiting the migration of HeLa cells in vitro. The research has been in line with existing procedures, but there are still limitations to the research. Research assisted with the ImageJ application in calculating the number of cells undergoing migration.

Proliferation cell (*Trypan Blue*)

The study used a HeLa cell culture of 12 culture discs with details: the first group consisted of 6 discs as a control group, the second group consisted of 6 discs as a group with a fractional concentration of cranberry flowers with IC_{50} 24.51, each group was given a trypan blue dye and a quantitative calculation was performed using the TC10 cell counter.

The results obtained in each study group are presented in table 4.

Table 4. Average cells undergoing proliferation

Group	Number of Sample	Number of Proliferative Cells
Control	1	11.1×10^5
	2	13.1×10^5
	3	14.8×10^5
	4	11.3×10^5
	5	11.5×10^5
	6	10.8×10^5
Total Average		12.1×10^5
IC_{50}	1	4.28×10^5
	2	2.27×10^5
	3	9.57×10^5
	4	6.80×10^5
	5	6.29×10^5
	6	7.60×10^5
Total Average		6.14×10^5

Table 4 describes a known influence of ethyl acetate fraction administration on HeLa cell proliferation by finding a decrease in the number of proliferative cells in the fractioned cell group compared to the control group. After a 48-hour incubation period at a concentration of 24.51 µg/ml (IC_{50}), an average of 6.14 x 10^5 proliferative cells was obtained. The greater the ethyl acetate fraction concentration of the given clove, the smaller the percentage of proliferative cells. Based on Menten's theory of receptor occupation, it is stated that a bioactive compound's effect is due to interaction with the receptor in the cell. The intensity of the effects of the bioactive substance is directly compared to the fraction of the binding receptor, and the receptor fraction depends on the dose and the length of exposure. The longer the exposure and the larger the dose, the intensity will increase.

The normality test used the Shapiro-Wilk test and obtained the results of the control group 0.105 and the treatment group 0.955. The p-value> 0.05 shows that the data is distributed normally so that the t-dependent test can be performed. The data is then analyzed using a dependent t-test. The test results showed a significant difference in the number of migrated cells after administering eugenol fractions with a p = 0.480 (p > 0.05).

Trypan blue is an azo dye derivative of the compound diazine, which is stable and difficult to degrade. Trypan blue coloring tests allow for directly identifying and counting live (uncolored) and dead cells (blue-colored) in a specific population. Trypan blue is impermeable to cell membranes, so it can only enter cells with damaged membranes. Once it enters the cell, trypan blue binds the intracellular protein so that the cell becomes blue.[22]

CONCLUSION

Based on this research, a high cytotoxic activity (IC_{50} = 24.51 µg/ml) against HeLa cells has an influence in inhibiting Hela cell migration with a total of 303 cells undergoing migration and proliferation tests using Trypan Blue obtained 6.14 x 10^5 proliferative cells. The eugenol content in the ethyl acetate fraction was demonstrated by thin-layer chromatography testing using FeCl3 stain impact that gives the dark stain color. The high cytotoxicity of cranberry flowers could be a solution as a future candidate for cervical cancer therapy.

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