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Antioxidant, Cytotoxic Activity and Protein Target Inhibition of Ethyl Acetate Fraction Melinjo Seed (*Gnetum gnemon* L.) by *In Vitro* and *In Silico* Studies on HeLa Cervical Cancer Cells

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ABSTRACT

Cervical cancer is one of the most common cancer suffered in women. Chemotherapy usage often causes physical and psychological side effects in patients. This study aims to determine the antioxidant and cytotoxic effects of the ethyl acetate fraction of melinjo seeds (*Gnetum gnemon* L.) on HeLa cervical cancer cells through *in vitro* and *in silico* assays. Melinjo seed was extracted by maceration using ethanol 70% and fractionated with ethyl acetate to obtain the Ethyl Acetate Fraction of Melinjo Seed (EAFMS). The identification of the active compounds group was done using Thin Layer Chromatography (TLC) method. In vitro studies were conducted on antioxidant tests using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method and cytotoxic activity test using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) Assay. *In silico* test for molecular docking analyzed by Autodock Vina method. The TLC analysis of EAFMS showed the presence of the stilbenoid compounds group. The antioxidant activity of EAFMS is weak, with an IC_{50} value of 175.8 g/ml. Cytotoxic activity of EAFMS is categorized as toxic to HeLa cancer cells with an IC_{50} value of 21.69 g/ml, while EAFMS has a synergistic effect combined with doxorubicin as a standard drug with a combination index (CI) value of 0.24-0.80. A molecular docking test of gnetin C with VHR receptor found a strong and stable bond with a docking score of -8.3 kcal/mol. Thus, EAFMS has the potential to be used as a chemopreventive agent for cervical cancer and can be combined with doxorubicin.

1. Introduction

Cervical cancer is the fourth most common cancer in women. It is estimated that 570,000 women are diagnosed with cervical cancer worldwide, and about 311,000 women die of cervical cancer (WHO 2018). Chemotherapy is the main alternative treatment to adjuvant chemotherapy, which has been shown to reduce side effects and metastasis and reduce genital cell damage (Wu *et al.* 2013). Based on the report, chemotherapy usage often causes physical and psychological side effects in patients, such as nausea and vomiting, pain, weight loss, fatigue, alopecia, decreased appetite, and taste changes (Ambarwati and Wardani 2014). Therefore, this research was conducted to reduce, prevent, or delay the development of cancer cells, especially in cervical cancer, by utilizing plants that are expected to have fewer side effects.

One of the plants that can be used is melinjo fruit (*Gnetum gnemon* L.) which is known to have antioxidant activity and is expected to prevent and reduce the number of cases of cervical cancer. Based on research conducted by Siswoyo (2007), the ethanolic extract of melinjo seeds contains stilbenoid compounds, namely gnetin L, gnetin C, and gnemosides A, C, D. In addition, in the skin of the melinjo fruit there is a resveratrol compound that can inhibit cell migration which is part of cell metastases (Yasmin *et al.* 2018). In 2022, Fatmawati *et al.* observed that the protein extract of melinjo seeds could not inhibit the growth of HeLa cervical cancer cells and has cytotoxic activity against 4T1 breast cancer. Cells with an IC_{50} value of 361.1 µg/ml. However, the protein extract of melinjo seeds could

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not inhibit the proliferation of 4T1 cancer cells. But, in the present study, Sukohar *et al.* (2022) observed that the melinjo seed fractions exhibited antioxidant and cytotoxic activity against HeLa cell lines. That melinjo seeds have the potential as an anticancer based on testing on 3 types of fractions. The ethyl acetate fraction of melinjo seeds had the highest antioxidant and cytotoxic effect compared to the water and n-hexane fractions. Moreover, from the results of the research carried out by Indrayudha *et al.* (2022), it can be found that the protein fraction of melinjo seeds has the potential to be developed as an anticancer compound following antioxidant and cytotoxicity tests. Based on these results, further research is needed to ensure the anticancer activity of the potential active fraction of melinjo seeds and to determine the required mechanism of action by *in silico* and *in vitro* study.

By adhering to previous research, this study will conduct more specific research on cervical cancer with the preliminary test by identification of compounds in the ethyl acetate fraction of melinjo seeds using the TLC method. Hereafter, there was an in vitro study using several methods, such as an antioxidant activity test using the DPPH method and a cytotoxic test on HeLa cancer cells using MTT assay. Subsequently, *in silico* study using molecular docking to determine the binding affinity of the compound in the ethyl acetate fraction of melinjo in inhibiting the VHR protein (Vaccinia H-1 related phosphatase), which plays a role in cervical cancer cell proliferation. Besides that, a combination test with one of the chemotherapy drugs, doxorubicin, will be conducted so that the effectiveness of the combination of the two in inhibiting the growth and development of HeLa cancer cells can be known. So, it is hoped that the results of this study can be used as a reference to develop melinjo as a cancer treatment.

2. Materials and Methods

2.1. Materials

The samples used in this study were melinjo seeds (*Gnetum gnemon* L.) which, based on previous studies, showed antioxidant and anticancer activity. The seeds from ripe melinjo fruit were obtained from farmers in Bantul, Indonesia, in August 2020.

2.2. Plant Determination

The determination of melinjo seeds (*Gnetum gnemon* L.) was determined at the Biology Laboratory, Faculty of Applied Science and Technology, Ahmad Dahlan University, Yogyakarta.

2.3. Extraction and Fractionation of Melinjo Seed

Melinjo seeds (*Gnetum gnemon* L.) are dried in the sun for 3-5 days and covered with a black cloth to obtain dry Simplicia. After that, they are ground until powder is obtained (Kaulika 2019). Melinjo seed simplicia powder of 990 g was extracted by maceration method using 70% ethanol. Simplicia soaked for 5 days while stirring to maximize the maceration process. After that, maceration for 7 days. The macerate was concentrated with a rotary evaporator to obtain an ethanolic extract of melinjo seeds. The ethanol extract of melinjo seeds was fractionated using a separating funnel with the liquid-liquid method with ethyl acetate and a 1:1 volume of ethanol as solvent. The fraction of ethyl acetate was taken to be concentrated with a rotary evaporator at a temperature of 60°C and in a water bath to obtain a thick Ethyl Acetate Fraction of Melinjo Seeds (EAFMS).

2.4. Analysis of Secondary Metabolites by TLC Assay-Densitometry

EAFMS was eluted on a silica gel plate GF254 with the mobile phase of chloroform: ethyl acetate: formic acid (5:4:1) (Pratiwi 2019). The silica plate is removed from the vessel after reaching the plate boundary and dried. Furthermore, the spots on the dry plate were observed under UV light at 254 nm and 366 nm, and the Rf value was calculated. A test using densitometry was carried out to find out the Rf value in the sample of the ethyl acetate fraction of melinjo seeds (Pratiwi 2019).

2.5. Anticancer Activity using Molecular Docking

The molecular docking applications downloaded include AutoDock Vina (v1.5.7), Biovia DS Visualizer 2021 (v21.1), MGLTools or Autodock Tools (v1.5.7), Python (v3.10.0), Open Babel (v3.1.1.1), and YASARA (v21.6.2). The protein structure selected as the

molecular docking target is the VHR protein structure (PDB ID: 1J4X), which can be obtained from the Protein Data Bank (PDB) by accessing the website www.rcsb.org. Proteins and ligands were prepared using the DS Visualizer application. To be executed, the ligand and protein formats must be converted from pdb to pdbqt. Furthermore, the Grid Box submenu is used to set the protein and ligand docking area. According to Arifin and Febriansah (2022), The RMSD value from the docking process is obtained by filling in the Windows Command Prompt or writing "cmd" in the folder address section, then writing the code vina.exe –config conf.txt –log log.txt then entering. We get a code that will bring up several conformations and docking scores. Each conformation will show the RMSD affinity value as a docking score. Then the output.pdbqt file is separated into several files according to each conformation and visualized using DS Visualizer.

2.6. Antioxidant Activity Analysis Using the DPPH Method

Antioxidant activity was carried out by determining the IC_{50} value of DPPH, according to Kaulika (2019). A standard solution of 0.4 mM DPPH was made as a test reagent. The test sample solutions (EAFMS) were made with five concentration series, namely 100; 200; 300; 400; 500 g/ml; quercetin as standard was made in series with 1; 2; 3; 4; 5 g/ml. The negative control solution was prepared by dissolving 1 ml of 0.4 mM DPPH standard solution with 1 ml of methanol. The Blank solution used was a sufficient methanol solution. The antioxidant activity, the levels of the standard quercetin solution, and the ethyl acetate fraction of melinjo seeds (EAFMS) were taken as much as 2 ml each, then 2 ml of DPPH solution and 6 ml of methanol were added. The solution was homogenized using a vortex and left in a closed room for operating time. Operating time is done with an interval of 20 minutes. The determination of the maximum wavelength of DPPH is 513 nm. Then the results calculated the IC_{50} value.

2.7. Cytotoxic Activity Using MTT Assay

The cytotoxic evaluation was conducted based on the metabolically active cells to convert 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an oxidative form of formazan that can be detected using visible light (Haryanti *et al.* 2022). The cytotoxic test of the Ethyl Acetate Fraction of Melinjo Seeds (EAFMS) on HeLa cancer cells was carried out by inserting cells with a density of 300 cells/6,700 µl MK into 96 well plates of 100 µl each and providing 3 empty wells to be used as controls, then incubated for 48 hours at 37°C. After 48 hours, the culture media was washed using PBS. The washed culture medium was added with 50 µl of culture medium containing only 0.2% DMSO (control) and incubated for 48 hours.

According to Haryanti *et al.* (2022), after incubation, discharging the medium, and washing with PBS (Sigma), each well received 0.5 mg/ml MTT (Sigma) in the medium and was incubated for 3–4 h, followed by adding 10% SDS in 0.01 N HCl and incubated overnight without light. Then viewed under an inverted microscope, purple formazan crystals formed, indicating that the surviving cells react with MTT. After 4 hours, the media with MTT was removed, washed, and given a 10% SDS stopper solution in 0.01 N HCl so that the formazan crystals dissolved. The plate was shaken over a shaker for 10 minutes and read on an ELISA reader with a wavelength of 595 nm, and the IC_{50} value was calculated based on the absorbance obtained. Then proceed with a combination test between EAFMS samples and the chemotherapy drug doxorubicin. This test used the concentrations of ½, ¼ , ⅛, and 1⁄16 IC_{50} obtained in the previous cytotoxic test to calculate the percentage of viable cells used to obtain the CI value.

3. Results

3.1. Extraction and Fractionation

The extraction process of 990 g of melinjo simplicia powder by maceration method produced as much as 7,025 ml of liquid extract. Furthermore, the results of the extraction process were concentrated using a rotary evaporator and fractionated using ethyl acetate in a ratio of 1:1. The obtained melinjo seed ethyl acetate fraction (EAFMS) as much as 2.4 ml, then evaporated with a rotary evaporator at 100 rpm and a temperature of 60°C and obtained a thick brown colored fraction of melinjo seed ethyl acetate (EAFMS) as much as 11.46 gr, with an EAFMS yield percentage value of 1.15%.

3.2. Thin Layer Chromatography-Densitometry

The qualitative test used Thin Layer Chromatography-Densitometry using a stationary phase of silica gel plate 254 and a mobile phase of

chloroform: ethyl acetate: formic acid (5:4:1) (Figure 1A). Based on observations under UV light of 254 nm and 366 nm, as shown in Figures 1B and C, five spots on the TLC plate are suspected to be stilbenoid compounds. The spots can be seen in Figure 2, which interprets the plate with the specific Rf Values at Wavelengths of 254 nm in Table 1.

3.3. Molecular Docking Assay

The molecular Docking test was carried out using the Autodocks Vina application. In this test, the interaction between gnetin C, the compound with the most similar results based on the densitometry diagram, from melinjo seeds was obtained to inhibit VHR protein (Table 2). VHR protein levels upregulated in several cervix cancer cell lines compared to normal keratinocytes, including human papillomavirus (HPV) positive cell lines CaSki, HeLa, and SiHa as HPV negative cell lines HT3 and C33. This suggested the research that VHR might be a promising drug target for the treatment of cervical cancer and that small-molecule inhibitors of VHR should be valuable tools to validate this new target.

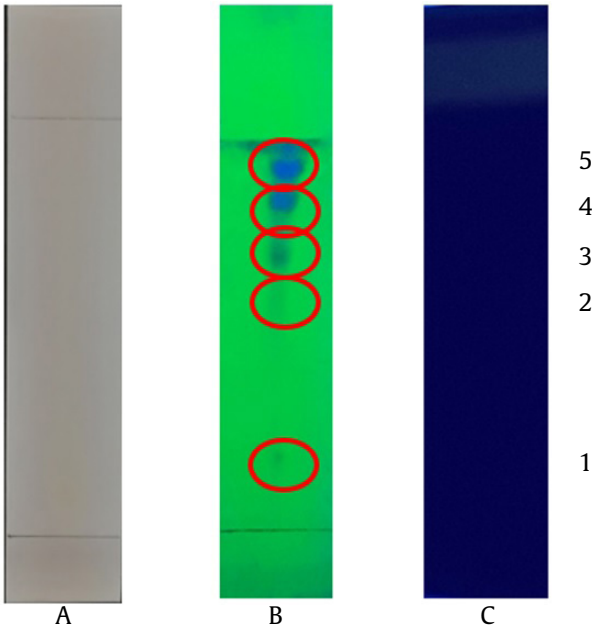


Figure 1. (A) EAFMS thin layer chromatography profile visible light, (B) 254 nm UV light, (C) 366 nm UV light

3.4. Antioxidant Activity by DPPH Method

Based on the antioxidant test using the DPPH method, the EAFMS sample contains antioxidants classified as weak antioxidants with an IC_{50} value of 175.8 g/ml. As for the comparison, quercetin has an antioxidant content classified as a very strong antioxidant with an IC_{50} value of 2.2 g/ml (Table 3).

3.5. Cytotoxic Activity by MTT Assay

The cytotoxic activity of EAFMS against HeLa cancer cells was measured by the MTT Assay method. Based on the graph of the relationship between cell viability and concentration, the linear regression equation EAFMS y = -1.8287x + 89.672 and the linear regression equation doxorubicin y = -3.726x + 69.478 was obtained (Table 4). This equation is then used to calculate the IC_{50} value (Tables 5 and 6). Furthermore, the morphology of HeLa cells was observed before

Table 1. Results of TLC Rf values at wavelengths of 254 nm

Spot number	254 nm	Standard Rf	Predicted compound	Description
1	0.26	0.25	Stilbenoid	+
3	0.64	0.67	Gnetin C	+
4	0.73	0.72	Resveratrol	+

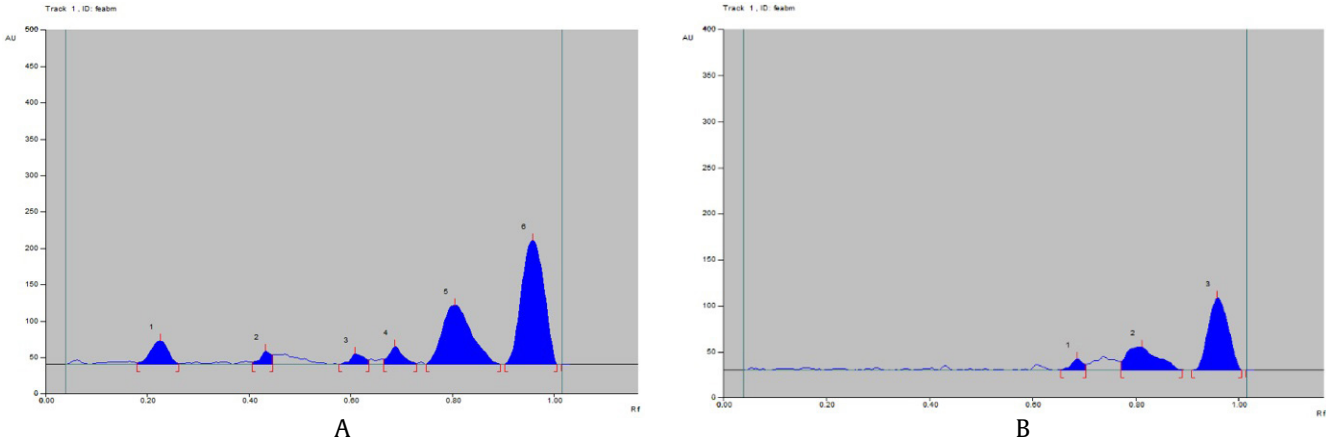


Figure 2. Densitometry profile (A) at wavelength of 254 nm (B) at wavelength of 366 nm

Table 2. Interpretation of Interaction Results of Test Compounds with VHR Protein

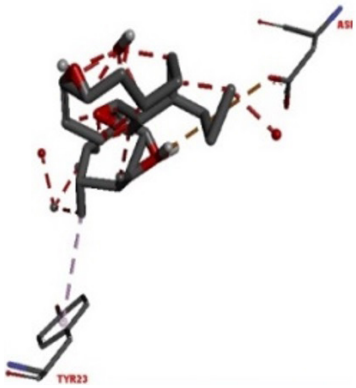
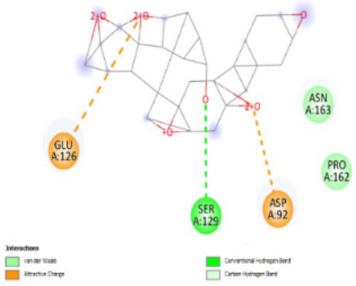
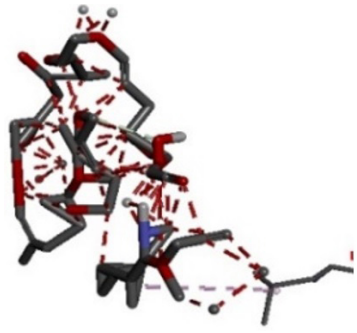
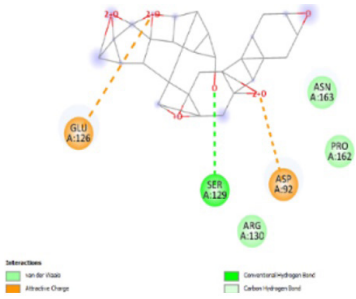
Compound	3D Visualization	2D Visualization	RMSD	Docking score	Conformation
Gnetin C			1.961	- 8.3	2
Paclitaxel			1.872	- 7.8	6

Table 3. The IC_{50} value of antioxidant test

Compound	Linier regression equation	IC_{50} value (µg/ml)	Level
EAFMS	y = 0.1083x + 30.978 R^2 = 0.9717	175.8	Low
Quercetin	y = 9.8886x + 27.837 R^2 = 0.8411	22.0	Very strong

Table 4. The IC_{50} value in cytotoxic test

Compound	Linier regression equation	IC_{50} value (µg/ml)	Description
EAFMS	y = -1.8287x + 89.672 R^2 = 0.9157	21.69	Toxic
Doxorubicin	y = -3.726x + 69.478 R^2 = 0.7899	5.20	Very toxic

Table 5. HeLa cell viability data with EAFMS

Concentration (µg/ml)	Average sample absorbance	Cell viability (%)	SD
50	0.065	2.74	0.38
25	0.215	43.66	0.93
12.5	0.242	50.90	4.34
62.5	0.315	71.00	5.20
31.25	0.386	79.40	3.50
1.5625	0.420	99.60	2.96
Average	Cell control	0.421	
Absorbance	Media	0.055	

Table 6. HeLa cell viability data with doxorubicin

Concentration (µg/ml)	Average sample absorbance	Cell viability (%)	SD
10	0.348	35.78	1.38
5	0.442	47.11	1.77
2.5	0.480	51.78	1.66
1.25	0.571	62.73	2.25
6.125	0.696	77.80	2.56
Average	Cell control	0.421	
Absorbance	Media	0.055	

and after being treated with EAFMS (Figure 3) and 5-FU (Figure 4) with an inverted microscope. Based on the results, there was a change in cell morphology in the form of changes in shape which initially tended to be perfectly round to become irregularly rounded and shriveled.

4. Discussion

The seeds of melinjo fruit (*Gnetum gnemon* L.) were extracted by maceration method using 70% ethanol as solvent. The principle of extraction with the maceration method is the diffusion of the filter solution into plant cells containing active compounds, which causes compounds that have the same polarity as the solvent to be pushed out due to differences in osmotic pressure inside the cell and outside the cell (Dean 2010). The extraction result was then fractionated using ethyl acetate solvent so that it is expected to attract stilbenoid group compounds in the melinjo seed sample (*Gnetum gnemon* L.). The extraction and fractionation process results, namely as much as 990 grams of melinjo seed powder, produced the Ethyl Acetate Fraction of Melinjo Seed (EAFMS) of 11.46 grams with a yield percentage of 1.15%.

Preliminary tests using Thin Layer Chromatography (TLC) and Densitometry methods were carried out as a qualitative test to identify the compounds contained in EAFMS. The stationary phase used in this study was silica gel GF_{254}. The mobile phase was chloroform: ethyl acetate: formic acid (5:4:1). Identification is made by comparing the Rf value of the analyte with the standard Rf value (Wulandari 2011). Based on the data obtained in Figure 1, the qualitative test results using the TLC-Densitometry method showed 5 spots on the TLC plate. The compounds suspected to be of the stilbenoid group were spots with Rf values of 0.26, 0.64, and 0.73 cm. These results are in accordance

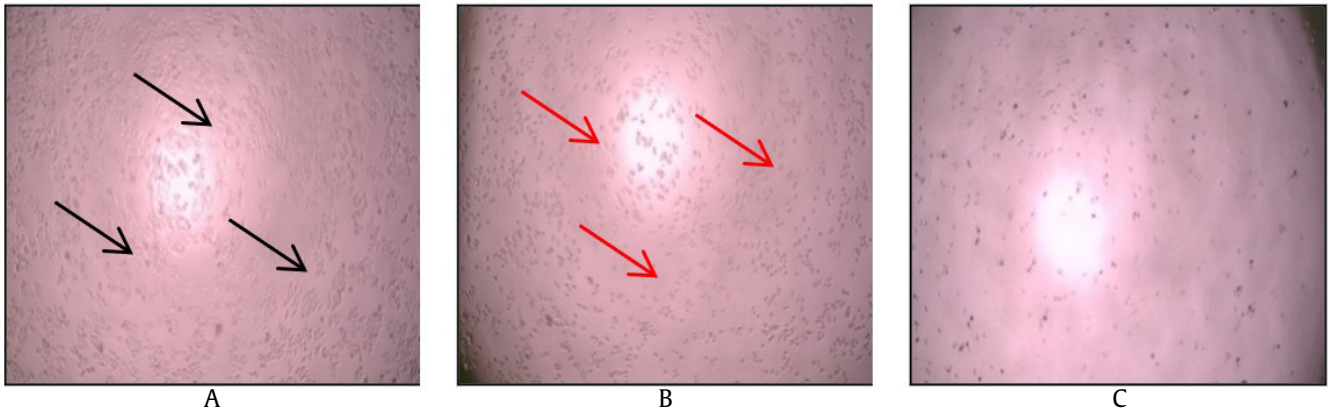


Figure 3. (A) Immediately after being treated with EAFMS (B) after being treated and incubated and adding MTT reagent (C) live cells (→) dead cells (→)

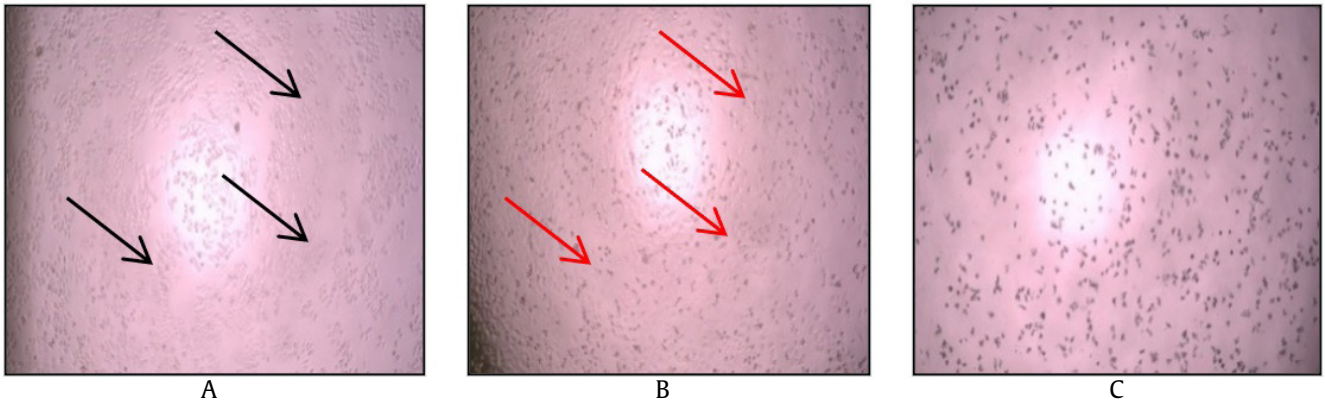


Figure 4. (A) Immediately after being treated with doxorubicin (B) after being treated and incubated and adding MTT reagent (C) live cells (→) dead cells (→)

with the research conducted by Pratiwi *et al.* (2016) using the gnetin C standard with an Rf value of 0.67 cm, the resveratrol standard of 0.72 cm, and the stilbenoid group having an Rf value of 0.26 cm.

The results of the qualitative densitometry test supported the results of the spots on the TLC plate. Figure 2 shows the Rf value, which shows similar results to a previous study conducted by Pratiwi *et al.* (2016) using standard gnetin C so that the spots suspected of being gnetin C on the TLC plate are spot number 3 with an Rf value of 0.64 cm in Table 1 and peak number 3 on the densitometry profile with a wavelength of 254 nm (Figure 2).

The following preliminary test is the *in-silico* test, which uses the molecular docking method. This test aims to determine the interaction in the active compound in the EAFMS, namely gnetin C with the target protein VHR (Vaccinia H-1 related phosphatase) obtained from the Protein Data Bank (PDB) with the protein code IJ4X. VHR is one of the receptors that affect several signaling pathways MAPK, JNK, ERK1, p38, EGFR, and ErbB2 (Pavic *et al.* 2015). The molecular docking results in Table 2 show the RMSD value of gnetin C < 2Å, which is 1.961 with a docking score of -8.3 kcal/mol. Meanwhile, the RMSD and docking scores for paclitaxel were 1.872 and –7.8 kcal/mol, respectively. The docking score describes the energy ligands need to bind with the protein. Therefore, the lower the docking score, the higher the binding affinity of the ligand to the target protein (Ruswanto 2015). The results indicate that gnetin C has a lower binding affinity than paclitaxel, meaning that gnetin C has a stronger binding to the VHR receptor (Table 2). Each receptor is bound by identical amino acid residues, namely SER 129, PRO 162, ASN 163, and GLU 126, so both ligands can inhibit the activity of 1J4X receptors by binding to these amino acids.

A study conducted by Wu *et al.* (2009) reported that VHR is required for the proliferation of HeLa cervical cancer cells. The cell cycle in HeLa cells will stop with the loss of VHR phosphatase, so VHR inhibition can be used to stop cancer cell growth. In this test, the structure of the paclitaxel drug is used to compare one of the first-line drugs in treating cervical cancer (NCCN 2012).

The analysis of docking results can be seen from the value of RMSD and docking score. The magnitude of the Root Mean Square Deviation (RMSD) value can indicate the accuracy of the calculation if the RMSD value < 2.0 Å. The smaller the RMSD value, it can be said that the better the method used (Ferwadi *et al.* 2017). Docking energy is a parameter used to see the strength of the binding affinity of the ligand to the receptor. The lower the energy produced, the more stable the ligand and the receptor bond. This stability is directly proportional to the ability of the compound to inhibit the proliferation of cancer cells *in silico* (Adelina 2013). A lower binding affinity indicates that a test compound requires less energy to interact with the receptor. In other words, lower binding affinity values have a more significant potential to interact with target proteins (Pangastuti *et al.* 2016).

According to *in vitro* test conducted by Pavic *et al.* (2015), it was proven that with the loss of the Vaccinia-H1-related phosphatase (VHR) receptor, the cell cycle stops at the G1-S and G2-M transitions. VHRs have essential roles in cellular signaling, from cell cycle regulation and DNA damage response to MAPK signaling. Mitogen-activated protein kinase (MAPK) is an enzyme superfamily that has three main families, namely Extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK), and p38 protein kinase (Fakhrudin 2017). VHR receptors are located in the ErbB2, EGFR, ERK 1/2, and P38 pathways in this signal transduction pathway.

The antioxidant test was carried out using the DPPH method (1, 1-diphenyl-2-picrylhydrazi) because it can reduce free radicals. Free radicals are molecules that have one or more unpaired electrons, so they are very reactive and unstable and will continue to react to form chain reactions that can damage cell structures. This method is based on the presence of a hydrogen atom donor (H+) or electrons from the sample tested to the DPPH radical. It becomes a more stable compound characterized by a color change from purple to yellow (Inggrid and Santoso 2014).

The parameter used in this test is the IC_{50} value, a particular concentration that can provide 50% resistance and is compared with the control value used. The IC_{50} value can be calculated based on the graph of the relationship between the percentage of inhibition and concentration. Table 3 shows the calculation results showing that EAFMS has an IC_{50} value of 175.8 µg/ml and quercetin has an IC_{50} value of 22 µg/ml. A lower IC_{50} value indicates a high antioxidant activity, while a high IC_{50} value is related to low scavenging activity (Magena 2021).

This standard quercetin has a smaller IC_{50} value than the EAFMS sample, meaning that the antioxidant activity in the standard quercetin is more potent than in the EAFMS sample. When compared with the previous study conducted by Cahyana and Ardiansyah (2016) in biological test in different compounds that they found on their melinjo, both gnetol and (+)-lirioresinol B have lower radical scavenging activity with IC_{50} was found to be 216.14 µg/ml and 240.13 µg/ml, respectively. Accordingly, gnetin C has higher antioxidant activity than gnetol and (+)-lirioresinol B.

The cytotoxic test was conducted using the MTT Assay method. MTT Assay is one of the methods used to determine the antineoplastic effect of a colorimetric test compound. The measurement principle of this test is based on the mitochondrial activity of viable cells. The MTT salt (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) will be reduced by an enzyme succinate tetrazolium reductase to form formazan crystals. Formazan crystals formed from this reaction will be purple and directly proportional to the number of living cells because the reduction reaction only occurs when respiration in the mitochondria is active (Anggrianti 2008). Dead cells will not be able to metabolize, so they will not be stained by MTT salt and cannot give a purple color to the plate, so the color remains yellow (Freshney 2000).

A cytotoxic test was used to determine the cytotoxic effect of EAFMS samples in inhibiting the growth of HeLa cancer cells. Absorbance measurements using an ELISA reader at a wavelength of 595 nm showed that the EAFMS sample had toxicity that was classified as toxic to HeLa cancer cells, with an IC_{50} value of 21.69 µg/ml (Table 4). The ability of the sample to inhibit or kill cancer cells can be seen from the value of % viability of living cells. If the cell viability is low, the sample can inhibit or kill cancer cells. At a concentration of 50 µg/ml, the value of % viability of living cells was 2.9%, meaning that cells that were able to survive after being given EAFMS samples were 2.91%, or at a concentration of 50 g/ml EAFMS samples were able to kill 97.09% of cells cancer (Table 5). Based on the results obtained, the greater the sample concentration, the smaller the % viability of living cells.

As a comparison in this cytotoxic, doxorubicin is one of the drugs to treat cancer in the community. The data in Tables 4 and 6 showed that doxorubicin was very toxic to HeLa cancer cells with an IC_{50} value of 5.2 g/ml. A compound is declared to have very strong cytotoxic activity if it has an IC_{50} value of <10 g/ml, strong if the IC_{50} value is between 10-100 g/ml, and quite toxic if the IC_{50} value is between 101-500 g/ml (Weerapreeyakul *et al.* 2012).

Cells shape morphology changes are one of the parameters that can be observed when cells undergo toxic conditions, such as the presence of compounds or chemicals (Aisyah *et al.* 2020). Therefore, the cytotoxic activity of EAFMS against HeLa cells can also be observed to spot its morphological change. The cell's morphological differences can be seen in Figures 3 and 4. After being treated and incubated after adding MTT reagent, the cells show morphological changes indicating cell death. According to Tavares-Carreón, *et al.* (2020), the cells morphological changes may also signify the occurrence of apoptosis.

The combination cytotoxicity test of EAFMS samples with the chemotherapy drug doxorubicin to determine the relationship of EAFMS against HeLa cancer cells. This effect can be seen from the combination index (CI) value (Labetubun 2018). The concentration series calculates cell viability and combination index (CI). The smaller the CI value, the stronger the synergistic effect given by the two samples.

The results of the combination of four series of concentrations of EAFMS and doxorubicin obtained varying CI values (Figures 5 and 6), namely strong synergistic, synergistic, and mild synergistic effects. The strong synergistic effect or the smallest CI value resulted from the combination of EAFMS with a concentration of 5.4 µg/ml and doxorubicin 1.3 µg/ml, 0.24 µg/ml. In addition, the EAFMS concentration of 5.4 µg/ml with doxorubicin 2.6 µg/ml and 0.6 µg/mL showed a strong synergistic effect (Table 7). The synergistic effect is one type of interaction that results in more excellent cytotoxic activity from combining two different compounds than only a single compound (Basri and Sandra 2016).

In conclusion, the Ethyl Acetate Fraction of Melinjo Seeds (EAFMS) contains stilbenoid group compounds based on the TLC-Densitometry identification test. One of them is Gnetin C. Gnetin C compound has a strong and stable bond to VHR protein with a docking score of -8.3 kcal/mol based on the molecular docking method. EAFMS has a weak antioxidant activity with an IC_{50} of 175.8 µg/ml based on the DPPH method. EAFMS was toxic to HeLa cancer cells with an IC_{50}

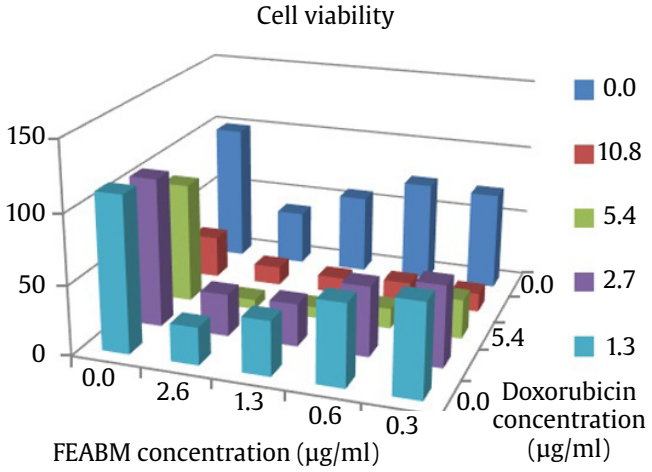


Figure 5. Graph of HeLa cell viability on combination of EAFMS and doxorubicin

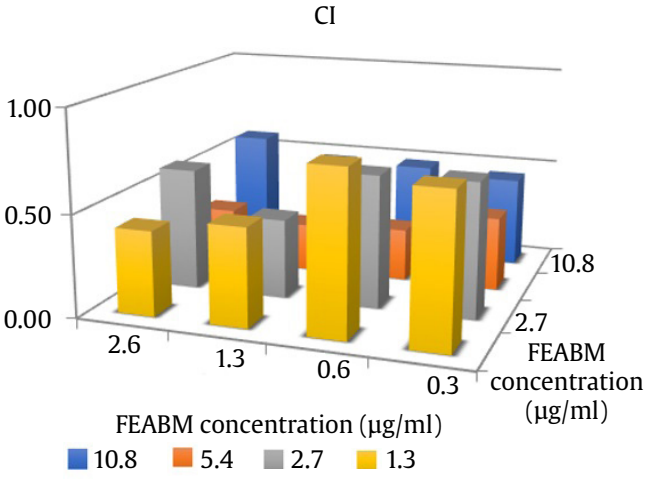


Figure 6. Graph of combination Index (CI) value

Table 7. The CI values of EAFMS with doxorubicin against HeLa cells

EAFMS Concentration (µg/ml)	Doxorubicin concentration (µg/ml)			
	2.6	1.3	0.6	0.3
10.8	0.58	0.47	0.48	0.45
5.4	0.28	0.24	0.26	0.36
2.7	0.60	0.39	0.65	0.66
1.3	0.42	0.48	0.80	0.74

value of 21.69 g/ml. EAFMS and doxorubicin gave a synergistic effect with a CI value range of 0.24-0.80.

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