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Activity of Ethanol Fraction Melinjo (*Gnetum Gnemon* L.) Seed on Colonic Cancer (Widr) Cells as Co-Chemotherapy Agent

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Abstract

Cancer deaths increase every year, including in Indonesia. The low selectivity of chemotherapy agents and the resistance of cancer cells against chemotherapy agents is the main cause of chemotherapy treatment failure. It causes serious side effects in sufferers. Beside that, plants produce secondary metabolites which are being investigated for the anticancer activity that is used as new clinical drugs. Therefore we need research that uses plants as co-chemotherapy agents. Melinjo seeds (*Gnetum gnemon* L.) contain gnetin C has the potential to apoptosis WiDr colon cancer cells. The purpose of this study was to determine the potential of Ethanol Fraction Melinjo Seed (EFMS) as a co-chemotherapy agent for colon cancer. The sample was maceration using 70% ethanol and fractionated with ethanol. Phytochemical screening with thin layer chromatography (TLC)-Densitometry, antioxidant test used the DPPH, while the cytotoxic activity of WiDr colon cancer cells and their combination with 5-Fluorouracil chemotherapy agent using the 3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay, and also *in silico* test used molecular docking between gnetin C on EFMS and IKK and COX-2 proteins with the 5-FU. The results showed that EFMS contains gnetin C based on Rf value, has weak antioxidant activity with IC_{50} 1227 µg/mL, weak cytotoxic activity in WiDr colon cancer cells with IC_{50} 681 µg/mL and has combined activity synergistic with 5-Fluorouracil. Molecular docking showed gnetin C strong binding affinity against IKK and COX-2 proteins with scores -12.2 kcal/mol and -9.6 kcal/mol. The result concludes that EFMS has the potential to inhibit the development of cancer cells, especially WiDr colon cancer cells.

Keywords: *Gnetum gnemon L., colon cancer, cytotoxic, molecular docking, WiDr.*

INTRODUCTION

Colon cancer is the second leading cause of death in the United States and the third most common malignancy in the world (World Health Organization, 2021). In Indonesia the incidence of colorectal cancer ranks number 3. The incidence of colorectal cancer in Indonesia is 12.8 per 100,000 adult population with a mortality of 9.5%. (GLOBOCAN, 2012). The low selectivity of anticancer drugs and the resistance of cancer cells

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to chemotherapy agents are often the main causes of treatment failure with chemotherapy, causing serious side effects in colorectal cancer patients (Mutiah, 2015).

Therefore, one of the efforts made is to replace synthetic chemotherapy drugs which have side effects of toxicity on body organs by developing new drugs. So that cancer patients get drugs that are safe and relatively effective and increase endurance through the use of traditional medicines which are considered to have relatively fewer side effects than modern medicines, one of which is through natural exploration (Bredel, 2001). One of the natural ingredients that can be developed as an anticancer agent in colon cancer is melinjo.

Melinjo (*Gnetum gnemon* L.) is one of the plants that are easily found in Indonesia. The fruit, leaves, and flowers are often cultivated and then used as ingredients in many dishes, consumed as food to traditional medicines (Cahyana & Ardiansah, 2016). In addition, this plant has the potential to be developed as an anticancer agent because it contains natural compounds such as stilbenoids that can induce apoptosis of cancer cells so that they have potential as anticancer (Kato, *et al*., 2009).

This study will examine the activity of the ethanol fraction of melinjo seeds (*Gnetum gnemon* L.) against WiDr cancer cells, starting with the identification of the compound content using the thin layer chromatography (TLC)-densitometry method, then *in vitro* research using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and *in silico* methods using molecular docking.

MATERIALS AND METHODS

Melinjo ripe seeds (*Gnetum gnemon* L.) obtained in Bantul, Yogyakarta, silica gel GF254, Ethanol 70%, aquadest, ethyl acetate, methanol, chloroform, formic acid, Fungizone 0.5%, penicillin-streptomycin 1% v/v (Gibco, Invitrogen, USA), PBS wash solution, Dulbecco's Modified Eagle Medium (DMEM) containing 10% v/v, Foetal Bovine Serum (FBS) (Gibco), Trypsin-EDTA, WiDr cells from Culture Laborarorium FKIK Universitas Muhammadiyah Yogyakarta, MTT 5 mg/mL in culture media, Dimethyl Sulfoxide (DMSO), 5-Fluorouracil (5-FU), RPMI, SDS stopper reagent in 10% HCL (Merck, Darmstadt, Germany), DPPH, quercetin, laptop set (ACER Aspire V5 171 32364G50ASS LINUX), and IKK and COX-2 structure (PDB file).

Extraction and Fractination

Extraction of simplicia dry powder from melinjo seeds (*Gnetum gnemon* L.) 990 grams using the maceration method with 70% ethanol solvent at a ratio of 1:7 for 5 days and remaceration for 2 days. The fractionation of the extract using a liquid-liquid method with a 1:1 ratio of ethyl acetate. Ethanol fraction as the results of the fractionation is taken and then concentrated with a rotary evaporator at a speed of 100 rpm at a temperature of 60°C, then thickening with a water bath to minimize the residue in the fraction and for the last step the Ethanol Fraction of Melinjo Seeds (EFMS) were measured by calculating the yield.

Thin Layer Chromatography (TLC)-Densitometry

Identification of stilbenoid compounds was carried out in EFMS stock solution with a concentration of 50 mg/mL dissolved in methanol. The stationary phase used was silica GF254 while the mobile phase used was chloroform: ethyl acetate: formic acid (5:4:1). EFMS as the test sample was eluted with the mobile phase in a closed vessel and then observed under visible light, UV light 254 nm, 366 nm and readings of Rf values and chromatographic profiles at wavelengths of 254 nm and 366 nm using densitometry.

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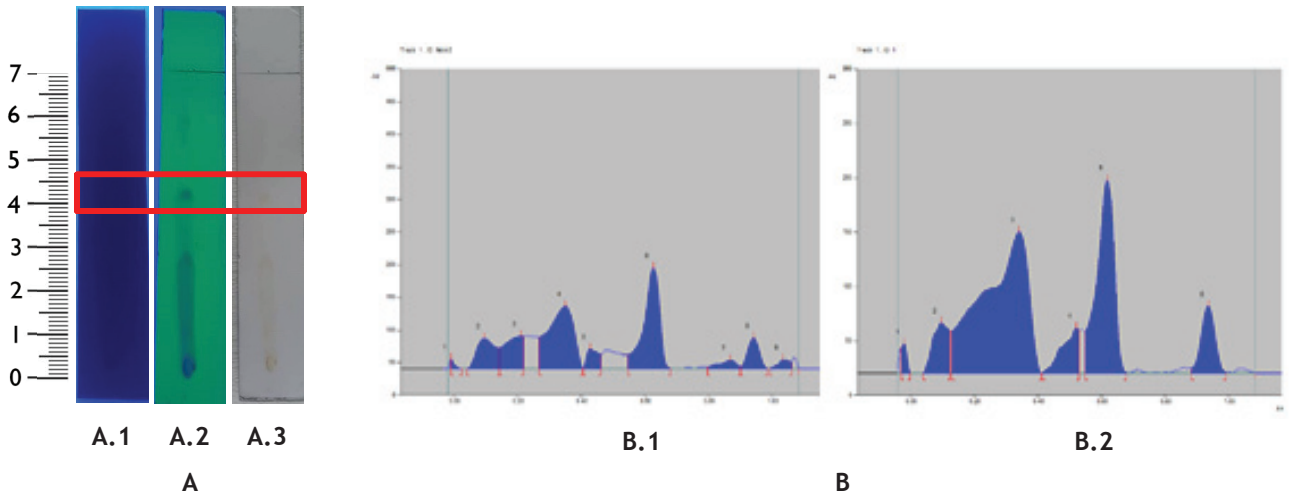

Figure 1. A. Visualization of TLC after Elucidation: A.1. UV 366 nm, A.2. UV 254 nm, A.3. Visible light UV. B. Wavelength Densitometry Profile: B.1. 254 nm; B.2. 366 nm.

Antioxidant Activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) Method

DPPH standard solution of 0.4 mM, 1000 g/ml sample stock solution, and 100 g/mL quercetin standard solution were made. Antioxidant tests were carried out with a number of concentration series, 1, 2, 3, 4, and 5 g/mL for quercetin positive control and 100, 200, 300, 400, and 500 g/mL for EFMS. Then, a quercetin took from each concentration and 2 mL of EFMS, then added 2 mL of 0.4 mM DPPH. Then the solution was homogenized using a vortex for 30 seconds and left in a closed room for operating time. To get the absorbance value of the solution, every 5 minutes for 45 minutes, the solution is read at a wavelength of 513 nm. The absorbance value of the sample is read with the maximum wavelength of DPPH, then calculates the IC_{50} value by processing the absorbance value into % antioxidant (Kaulika, 2019).

3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay *In Vitro*

The EFMS was diluted in DMSO by serial concentrations (31.25-1000 µg/mL). The 5-FU (12.5-100 µg/mL) was used as a positive control, untreated cells were used as negative control, and medium control. A number of WiDr colon cancer cells with a density of $5x10^3$ cells/100 µl were inserted and distributed into 96 well plate wells and 3 empty wells were left for media control and incubated for 24 h. Furthermore, the media was removed and washed using PBS, then added 100 µl MTT 0.5 mg/mL. The cells were then incubated at 37°C for 4 h. Live cells will react with MTT to form purple formazan crystals.

Furthermore, the media containing MTT was discarded and washed using PBS after 4 h and added with SDS stopper solution in 0.1% HCl 200 µl

Table 1. IC_{50} and antioxidant activity.

Compound	Linear Regression Equations	IC_{50}	Level
Quercetin	y = 9.8886x + 27.837 R^2 = 0.8411	2.24	Very Strong
EFMS	y = 0.0361x + 5.6998 R^2 = 0.9768	1227	Very Low

Table 2. Live cell percentage data with EFMS treatment.

Concentration (µg/mL)	Average Absorbance	Standar Deviation	% Live Cell
1000	0.135	0.94	19.17
500	0.349	7.91	70.23
250	0.430	2.35	89.37
125	0.472	5.73	99.48
62.5	0.511	5.00	108.70
31.25	0.517	4.04	110.16
Average Absorbance	Cell Control	0.474	
	Media Control	0.055	
Equation		y = -0.0929x + 113.33 R^2 = 0.9967 IC_{50} = 681 µg/mL	

to dissolve the formazan crystals. Above the shaker, the plate was shaken for 10 minutes and read with an ELISA reader at a wavelength of 595 nm. The absorbance value of each well was then converted into percent live cells and data processing was carried out to obtain the IC_{50} value. The IC_{50} value obtained from a single test is used to determine the combined concentration of EFMS and 5-FU with a ratio of ½, ¼, ⅛, and 1⁄16.

Molecular Docking Assay *In Silico*

Molecular docking was carried out using the Autodock Vina application and other supporting software namely, MGLTools or Autodock Tools, Python, Open Babel, and YASARA. Protein IKK (GDP ID: 2GNG) and COX-2 (GDP ID: 6COX) were downloaded from the Protein Data Bank (PDB) via the website www.rcsb.org. Proteins and ligands were prepared and saved in "PDBQT" file. Created a new document and named conf.txt. Then fill in the document by writing the proteins 2GNG. pdbqt and 6COX.pdbqt, the ligands are written as ligands.pdbqt, while center_x,y,z and size_x,y,z are written according to the values listed on the grid box, then saved in the same folder. The Root Mean Square Deviation (RMSD) value from the docking process is obtained by filling in the Windows Command Prompt or writing "cmd" in the folder

Table 3. Live cell percentage data with 5-FU treatment.

Concentration (µg/mL)	Average Absorbance	Standar Deviation	% Live Cell
200	0.262	1.27	41.38
100	0.270	0.24	43.28
50	0.346	3.30	59.52
25	0.395	0.63	70.11
12.5	0.412	1.47	73.75
Average Absorbance	Cell Control	0.533	
	Media Control	0.070	
Equation		y = -0.175x + 71.17 R^2 = 0.8006 IC_{50} = 120 µg/mL	

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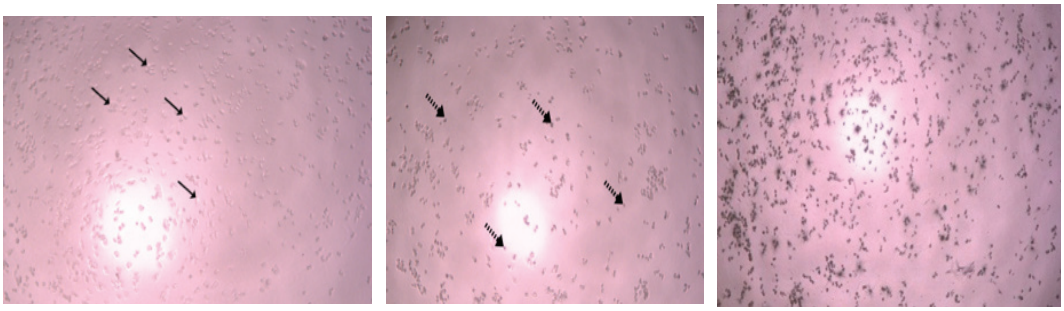


A B C

Figure 2. Changes in cell morphology in treatment with EFMS. (A) Before being treated (B) Immediately after being treated (C) After being treated and incubated and the addition of MTT Reagent. (->1) living cells; (--> 2) dead cells.

address section then writing the code vina.exe –config conf.txt –log log.txt then entering it, to get the code that will bring up several conformations and docking score. Each conformation will show the RMSD affinity value in the form of a docking score. Selected conformation with RMSD value <2 (Arifin & Febriansah, 2019). The best conformation is 2D and 3D visualization using DS Visualizer.

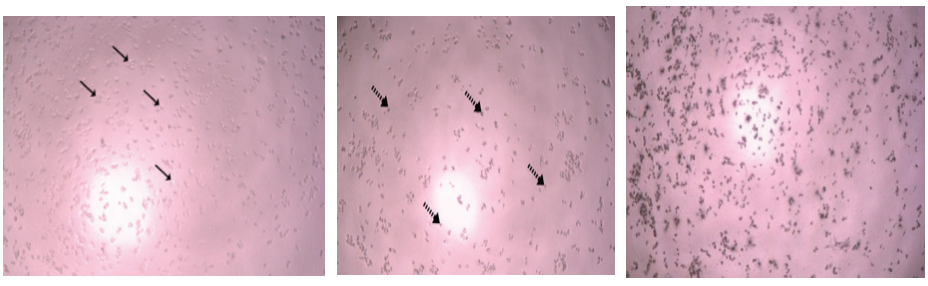
RESULTS

Extraction and Fractination

The extract from the maceration and remaceration was obtained as much as 7.438 mL of liquid extract, then fractionated using the liquid-liquid partition method using ethyl acetate:ethanol (1:1). The EFMS obtained was 1.607 mL then evaporated using a rotary evaporator and thickened with a water bath so that the thick fraction of melinjo seeds was 32.58 grams with a yield of 15%.

Thin Layer Chromatography (TLC)-Densitometry

The EFMS was eluted on a silica plate GF254 with chloroform: ethyl acetate: formic acid (5:4:1) as the mobile phase and the results were observed under visible light, UV 254 nm, and 366 nm (Figure 1A). The test is continued with densitometry to see the Rf value on the plate. Based on the densitometry results, it can be seen that the positive EFMS contains the compound gnetin C with an Rf value of 0.68 at both 254 nm and 366 nm wavelengths which are shown at the highest peaks in Figure 1B. These results are in accordance with previous research conducted by Pratiwi (2019), this study used the Gnetin C standard with an Rf value of 0.67.



A B C

Figure 3. Changes in Cell Morphology in Treatment with 5-FU. (A) Before being treated (B) Immediately after being treated (C) After being treated and incubated and the addition of MTT Reagent. (-> 1) living cells; (--> 2) dead cells

Table 4. Percentage of cell viability in combination treatment.

Compound		5-FU Concentration (µg/mL)			
		60.485	30.243	15.121	7.5606
EFMS (µg/mL)	0	100	58.58	72.10	76.40
	340.85	98.85	18.26	25.47	27.99
	170.43	99.77	21.07	21.22	29.17
	85.213	100.88	20.01	17.87	29.54
	42.606	103.58	21.19	16.97	30.97

Antioxidant Activity by 1,1-diphenyl-2-picrylhydrazyl (DPPH) Method

Antioxidant tests were carried out with a number of concentration series, namely concentrations of 1, 2, 3, 4, and 5 g/mL for quercetin positive control and 100, 200, 300, 400, and 500 g/mL for EFMS and replicated 3 times on each concentration series. The absorbance data of the EFMS antioxidant test results can be seen in the following Table 1. The results show that EFMS has a very weak antioxidant activity with an IC_{50} value of 1227 g/mL while quercetin as a positive control has a very strong antioxidant activity with an IC_{50} value of 2.24 g/mL (Table 1).

3-(4,-5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay *In Vitro*

In terms of the IC_{50} value, the EFMS and 5-FU obtained 681 µg/mL and 120 µg/mL, respectively. The results of the cytotoxic test at the highest concentration of 1000 µg/mL were able to inhibit 80.83% of WiDr cancer cells and and there were 19.17% of live cells (Table 2). Meanwhile the results of the 5-FU cytotoxic chemotherapy agent at the highest concentration of 200 µg/mL were able to inhibit 58.62% of WiDr cancer cells and there were 41.38% of live cells. Based on the results of the treatment of EFMS and 5-FU samples on WiDr

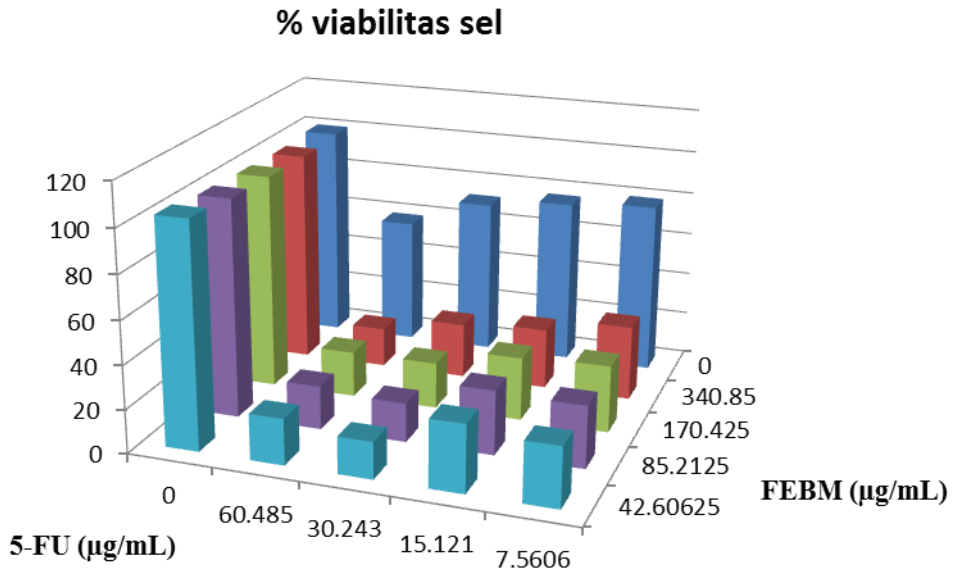


Figure 5. Chart of WiDr cell viability combination treatment of EFMS and 5-FU.

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Table 5. Combination Index (CI) of WiDr cells combination treatment of EFMS and 5-FU.

EFMS Concentration (µg/mL)	5-FU Concentration (µg/mL)			
	60.485	30.243	15.121	7.5606
340.85	24.59	9.34	4.75	2.59
170.43	10.74	2.83	1.51	0.84
85.213	4.95	0.87	0.48	0.27
42.606	2.36	0.29	0.16	0.08

cells, it was shown that as the dose increased, the percentage of survival of WiDr cells decreased (Table 3).

In the cytotoxic test, the morphology of WiDr cells was observed before and after being treated with EFMS (Figure 2) and 5-FU (Figure 4) with an inverted microscope. Based on these observations, 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.

The best combination results based on the level of viability of WiDr cells were the combination of EFMS and 5-FU at levels of 42.606 g/mL and 15.121 g/mL with 16.97% live cells, and also at levels of 85.213 g/mL and 15.121 g /mL with 17.87% live cells (Table 2).

The results of the Combination Index (CI) calculation in the Table 3 show that the combination of EFMS 42.606 g/mL (1⁄16 IC_{50}) and 5-FU 7.5606 g/mL (1⁄16 IC_{50}) is 0.08 which is classified as strong synergistic. So that the best results of the combination of EFMS and 5-FU were obtained at the lowest concentration.

Molecular Docking Assay *In Silico*

The result obtained is the docking score, the value of the energy required to interact with the target protein. The smaller the value of the docking score, the smaller the energy required to bind or

Table 6. Interpretation of interaction results between ligands and IKK proteins.

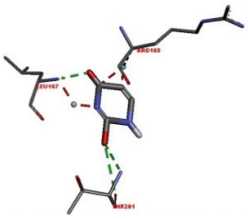
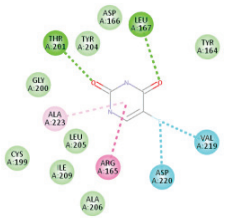
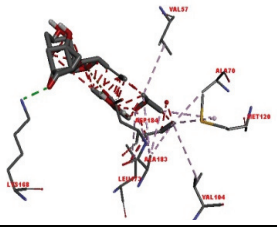
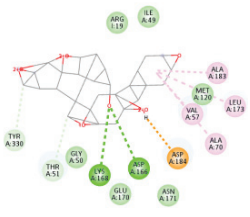
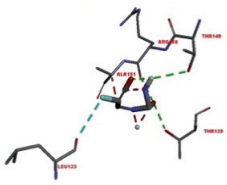
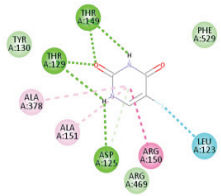
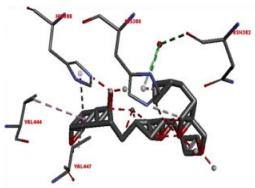
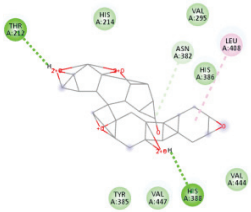
Compound	3D Visualization	2D Visualization	RMSD	*Docking score* (kkal/mol)	Conformation
5-FU			1.717	-5.7	2
Gnetin C			1.412	-12.2	2

Table 7. Interpretation of interaction results between ligands and COX-2 proteins.

Compound	3D Visualization	2D Visualization	RMSD	*Docking score* (kkal/mol)	Conformation
5-FU			1.483	-6.1	2
Gnetin C			1.102	-9.6	2

interact. The best RMSD value and docking score from the interaction of the test compound and the target protein can be seen in Table 6 and Table 7. Molecular docking results between Gnetin C were higher than 5-FU. Gnetin C has a docking score of -12.2 kcal/mol, while the docking score of 5-FU is -5.7 kcal/mol. Based on these results, it means that Gnetin C has the potential as an anticancer through interaction with the IKK protein in WiDr cells. The visualization in the Table 6 is a conformation 2 as the best conformation from the interaction. Furthermore, the Table 7 is visualization of interaction between ligands test and COX-2 protein as receptor. Based on the molecular docking results between Gnetin C were higher than 5-FU. Gnetin C has a docking score of -9.6 kcal/mol, while the docking score of 5-FU is -6.1 kcal/mol. Based on these results, it means that Gnetin C has the potential as an anticancer through interaction with the COX-2 protein in WiDr cells.

DISCUSSION

Colon cancer is cancer that invades the large intestine. WiDr cells are a type of cell derived from HT-29 cells, isolated from a 78-year-old woman with colon cancer (Chen, *et al*., 1987). Meanwhile, based on research by Kato, *et al.* (2011), melinjo seeds contain stilbenoid derivatives that can induce cancer cell apoptosis, namely transresveratrol and gnetin C which have potential as anticancer. Preliminary test to analyze qualitative compounds in EFMS using TLC and densitometry methods. Based on the tests that have been carried out, EFMS contains a stilbenoid derivative compound, namely gnetin C with an Rf value of 0.68. These results are according to previous research conducted by Pratiwi (2019) using the TLC chromatography system. The mobile phase used was chloroform: ethyl acetate: formic acid (5:4:1), the Gnetin C elution results were seen with an Rf value of 0.67.

In this study, the IC_{50} of DPPH method was 1227 g/mL for EFMS and 2.24 g/mL for quercetin as control. Accordingly, it can be interpreted that with a concentration of 1227 g/mL EFMS and 2.24 g/mL quercetin can stabilize 50% of free radicals. Thus, according to the classification of antioxidants, EFMS is classified as very weak and quercetin as control is classified as very strong. Classification of antioxidants using the IC_{50} value, if the antioxidants in the sample are classified as very strong if the IC_{50} value is <50 ppm, strong if the IC_{50} is in the range

of 50 –100 ppm, while if the IC_{50} is in the range of 100 –150 ppm, weak if IC_{50} is 150 –200 ppm, very weak if the IC_{50} is greater than 200 ppm, and not an antioxidant if the IC_{50} is more than 2000 ppm (Agustini, *et al*., 2015).

Based on the results of the cytotoxicity test EFMS has a cytotoxic effect on WiDr cancer cells with IC_{50} level is 681 g/mL. Cytotoxic activity is divided into three based on the parameter IC_{50} value, namely if IC_{50} <100 g/mL is potential cytotoxic, 100µg/mL <IC_{50}–<1000 g/mL is moderate cytotoxic and if IC_{50}>1000 g/mL it has no cytotoxic activity (Prayong, *et al*., 2008). If the test compound has moderate (moderate) cytotoxic activity, it can be used as a chemoprevention, while the test compound with potential cytotoxic activity can be used as an anticancer agent (Tusanti, *et al*., 2014). Based on this research, EFMS can be used as a chemoprevention and can be combined with a chemotherapeutic agent, namely 5-FU. In addition, based on cell viability, EFMS at a concentration of 1000 µg/mL were able to inhibit 80.83% of WiDr cancer cells and at a 5-FU concentration of 200 µg/mL were able to inhibit 58.62% of WiDr cancer cells. The cytotoxic effect of EFMS depends on the concentration of the dose, the higher the sample concentration, the lower the percentage of cell life. In visual observation through an inverted microscope, it is seen that there is a change in the treatment shown in the image (Figure 3 & Figure 4). Live cells have bright color characteristics, have high density, while dead cells have dark color characteristics, low density, and irregular shapes (Pratama, 2016). Combination therapy may be able to prevent the toxic effects on normal cells while simultaneously producing cytotoxic effects on cancer cells. This may occur if one the drug in the combination regimen is antagonistic, in terms of cytotoxicity, to another drug in normal cells, essentially protecting normal cells from cytotoxic effects (Mokhtari, *et al.*, 2017). Combination chemotherapy agents (co-chemotherapy) is a potential approach method in overcoming the side effects of using chemotherapy agents in cancer patients. Co-chemotherapy allows the control of chemotherapy drugs in low doses so that the toxicity to the surrounding normal cells decreases. The potential that can be utilized in combination is to use natural materials (Tyagi, *et al*., 2004).

Combination tests between EFMS and 5-FU were carried out at several concentration series using the IC_{50} value of a single cytotoxic test with parameters in the form of cell viability calculations and CI values. Based on the results the combination of EFMS and 5-FU showed good results with cell viability <50%. The best cell viability lies in the combination of levels, namely 42.606 µg/mL EFMS and 15.121 µg/mL 5-FU with 16.97% live cells. Another parameter that shows the effectiveness of the combination is the CI value. The best CI values when combined have results <0.9. The combination that gave a synergistic effect was the combination of 42.606 µg/mL EFMS and 7.5606 µg/mL 5-FU with a CI value of 0.08 and % live cells of 27.58%. The results showed that based on the CI value with a synergistic effect it had % living cells <50%, so based on these results it showed that EFMS had a synergistic co-chemotherapy effect and was more suitable as co-chemotherapy. Combination therapy improves treatment outcomes and results in superior therapeutic effects, especially when a synergistic anticancer activity is achieved. Moreover, the combinational approach overcomes clonal heterogeneity which is further associated with improved response rates. Another advantage of combination therapies is reducing the emergence of drug resistance (Ayoub, 2021)

The *in silico* test used is molecular docking method, it is possible to predict the interaction between the active compound of EFMS, gnetin C, and the chemotherapeutic agent, 5-FU against IKK and COX-2 receptors. The principle of molecular docking is the attachment of the ligand to the target compound. Parameters of molecular docking results in the form of RMSD and docking score. The validation of the molecular docking method based on the best RMSD which produces a value of <2Å used as a parameter for conducting testing of

the active compound (Ruswanto, 2015). The lower the value (<2Å), the better the validity because it approaches the conformation of the original compound (Page, *et al*., 2013).

The best docking score or binding affinity is obtained from the bond between the test compound (active compound from plant) and the target receptor (genom from disease) which has a lower score than other interactions. The lower the docking score, the higher the binding affinity for the test compound with the target receptor (Ruswanto, 2015). The interaction with IKK protein showed that the docking score of gnetin C compound (-12.2 kcal/mol) was lower than that of 5-FU (-5.7 kcal/mol). Furthermore, the interaction with COX-2 protein showed that gnetin C (-9.6 kcal/mol) had a lower docking score than 5-FU (-6.1 kcal/mol).

The relationship between IKK and COX-2 in colon cancer occurs because of increased COX-2 expression associated with NF-κB activation (Brown & Dubois, 2014). COX-2 in the colon is dynamically expressed and is required for the maintenance of colonic homeostasis. However, the increase in COX-2 expression causes the induction of prostaglandin formation and inflammation resulting in increased metastasis, increased cell proliferation, and inhibits the process of apoptosis (Abdullah, *et al*., 2013). Therefore, inhibition of NF-kB activation through suppression of IKK and inhibition of increased COX-2 expression can be one of the specific targets in colon cancer chemoprevention.

CONCLUSION

The EFMS contains stilbenoid derivatives of the gnetin C group. Although, EFMS has weak antioxidant potential with IC_{50} value of 1227 g/mL, but it has moderately toxic activity against WiDr cancer cells with an IC_{50} value of 681 µg/mL. Therefore, EFMS can be combined with the chemotherapy agent 5-FU showing mild to moderate synergistic activity with CI values ranging from 0.08 to 24.59. Besides that, based on the *in silico* molecular docking test, gnetin C compound in melinjo seeds has a stronger binding affinity than 5-FU in inhibiting IKK and COX-2 proteins with docking scores of -12.2 kcal/mol and -9.6 kcal/mol. Therefore, for further research it is necessary to carry out a cell cycle test using the flowcytometry method to determine the inhibition of EFMS on the WiDr cell cycle. Furthermore, it is necessary to do a cytotoxic test of the ethanol fraction of melinjo seeds on normal cells.

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