

Chemopreventive Activity of Roselle's Hexane Fraction against Breast Cancer by In-Vitro and In-Silico Study

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Abstract—Breast cancer is ranked the highest percentage of the new cases and deaths in women around the world. Cancer treatments that currently exist cause side effects that are detrimental to patients triggering the development of cancer treatment that comes from nature or herbal plants. Roselle (*Hibiscus sabdariffa* L.) is a plant that is known to have flavonoids which are potential as anticancer agent. This study was conducted to analyze the roselle's hexane fraction as a chemopreventive agent with an antioxidant test using the DPPH method, in-vitro cytotoxic test of T47D cancer cells line by MTT method, and in-silico test using Vina molecular docking method. The results of the antioxidant test showed the IC₅₀ value of 4259 µg/mL. Cytotoxic activity of the fraction on T47D breast cancer cells showed the IC₅₀ values of 331.86 µg/ml. The molecular docking results obtained a docking score that shows the binding energy of cyanidin 3-O-glucoside to EGFR and HER-2 proteins of -8.2 kcal/mol and 8.1 kcal/mol, respectively. Based on these results, it can be concluded that roselle hexane fraction has a potential as a chemopreventive agent based on its molecular docking and cytotoxic activity against T47D breast cancer cells.

Keywords—*Hibiscus sabdariffa*; cytotoxic MTT, antioxidant DPPH, molecular docking, T47D breast cancer

I. INTRODUCTION

Cancer has become the second largest cause of death in the world, accounting for 8.8 million deaths in 2015^[1]. Breast cancer ranks the highest percentage of new cases and deaths in women worldwide. Every year, there are 43.3% of new cases with 12.9 % percentage of death due to breast cancer^[2]. Therapies that are currently widely used for the treatment of cancer, among others, are surgery, radiation, and chemotherapy. Chemotherapy is an attempt to kill cancer cells by interfering with cellular function and reproduction^[3]. The mechanism of action of chemotherapy agents as anticancer is not selective because it does not only damage the DNA of cancer cells but also the normal cells^[4]. Problems that also arise from cancer treatment with chemotherapy are side effects and also resistance to chemotherapy drugs^[5]. This makes the need for further development and research on cancer drugs, one of which is by using natural ingredients. Roselle (*Hibiscus sabdariffa* L.) from the Malvaceae family is a plant that is widely spread in various countries. This plant is widely used in various countries as traditional medicine. Roselle is a plant that can be developed into an anticancer drug. Roselle petals contain flavonoids that can function as antioxidants i.e. compounds needed by the body to overcome and prevent oxidative stress which play an important role in the pathophysiology of cancer^[6]. Previous studies showed that

roselle had anticancer effects on MCF-7 breast cancer cells^[7] and also showed good antioxidant activity in ovarian cancer cells (Caov-3) and cervical cancer (HeLa). The content of the flowers of roselle includes citric and malic acids; tartaric acids; anthocyanins (delphinidin-entoside-glucoside, delphinidin-3-glucosylglycoside, delphinidin-3-ambubioside, cyanidin-monoglucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside, cyanidin-3-glucosylrutinoside, cyanidin-3-glucoside); flavonol glycoside; gossypitrin; quercetin; myricetin; hibiscetrin; sabbaretin; quercetin; luteolin, a luteolin glycoside and chlorogenic acid; flavonoids (gossypetin, hibiscetin, and their respective glycosides); protocatechuic acid; and sterols (β -sitosterol and ergosterol)^[8].

The purpose of this study was to determine the effect of the roselle's hexane fraction as chemopreventive agent in breast cancer by tracing the antioxidant activity and cytotoxicity of the roselle's hexane fraction in T47D breast cancer cells.

II. METHODOLOGY

A. Preparation of Roselle's Hexane Fraction

Roselle simplicia powder was extracted using 70% ethanol by maceration method, with a ratio of 1:10 (1 kg powder: 10 liters 70% ethanol). Simplicia powder was soaked in 70% ethanol for 5 days and stirred every day to make a better extraction process. After 5 days, the obtained maserate was filtered and remacerated for 2 days to optimize the extract. The results of remaseration in the form of extract then fractionated the liquid-liquid partition with hexane solvent in a ratio of 1: 1. Then the hexane fraction was evaporated using rotary evaporation at 60°C to concentrate it. After the hexane fraction was concentrated, it was then thickened with waterbath.

B. Identification of Compounds with Thin Layer Chromatographic

Identification of flavonoid compounds namely anthocyanin using TLC was carried out by staining the test sample namely hexane fraction of roselle flower petals (*Hibiscus sabdariffa* L.) using capillary pipes on GF254 silica gel plate, then eluted with a mobile phase in a tightly closed vessel. The mobile phase used was chloroform. The eluted silica plate was removed from the vessel and dried in an oven at 60°C for 10 minutes, then the plate was evaporated with ammonia and observed under a UV lamp with a wavelength of 254 nm and 366 nm. Furthermore, the spot distance shown was calculated and the R_f value was calculated as well.

C. Antioxidant Activity Test with DPPH Method

Identification of DPPH method is used to test the ability of a component as a free radical catcher in an ingredient or extract. The working principle of the DPPH method is based on DPPH's ability to accept donated hydrogen atoms by antioxidants^[9].

- Making DPPH Raw Solution—A total of 15.8 mg of DPPH powder was weighed and dissolved in methanol to 25 ml. Then a 10 ml DPPH solution was taken and methanol was added to 100 ml, and homogenized with vortex.
- Making Sample Solutions and Standards—Samples of hexane fraction of roselle flower petals were weighed as much as 20 mg and dissolved with methanol to 20 ml, then the mother liquor samples were obtained with a concentration of 1000 µg/ml. A total of 5 mg of quercetin was dissolved in 100 ml of methanol and obtained 50 µg/ml. The mother sample solution was then taken to make a series of levels by dissolving a number of samples with methanol solvent. The series of hexane fraction of roselle flower petals (*Hibiscus sabdariffa* L.) was made in five series levels, namely 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml. The standard parent solution, which is vitamin C, was also made of a series of levels of 1 µg/ml; 2 µg/ml; 3 µg/ml; 4 µg/ml and 5 µg/ml.
- Analysis of Antioxidant Activity—Testing of antioxidant activity was carried out by taking each 5 ml of hexane fraction of roselle and vitamin C solution from each level, then adding 1 ml of DPPH 0.4 mM and homogenizing using vortex, then leaving during operating time in a closed room. Sample absorbance was read with the maximum DPPH wavelength, then the absorbance value was processed into percentage antioxidants to perform IC₅₀ calculations.

D. Cytotoxic Test

Cytotoxic tests are tests using cell tissue culture^[10]. One method of cytotoxicity test is MTT assay. The principle of the MTT assay method is to measure formazan crystals formed from the reaction between MTT salts and the tetrazolium succinic reductase system found in living cell mitochondria^[11]. Cytotoxic tests were carried out using the MTT assay method, and the cells with a density of 5×10^3 cells/100 µL MK were distributed into 96 well plates containing 100 µl with 3 empty wells to be filled with control media, and incubated for 48 hours so the cells could adapt and attach to bottom of the well. The next day the well was taken, and the media was taken and then washed using PBS. Then 50 µl of culture media containing only 0.2% DMSO (control) were added or the test samples were the hexane fraction of roselle flower petals from each series of concentrations and replicated 3 times and added 50 µl of MK. The wells containing control cells were added 100 mL MK after being incubated for 24 hours. Furthermore, making MTT culture media 5 mg/ml was done by dissolving 50 mg of MTT powder in 10 ml PBS, while making MTT reagent was done by diluting the MTT stock of 5 mg/ml in 10 ml MK to obtain a concentration of 0.5 mg/ml. At the end of incubation, the culture media containing the sample was removed and washed with 50 µl PBS. Furthermore, each well was added 100 µl MTT 5 mg/ml, and incubated at 37°C for 4 hours. Cells that live or survive react with MTT to form purple formazan crystals. After 4 hours of incubation, the medium

containing MTT was removed, washed with PBS then added SDS stopper solution in 0.1% HCl 200 µl to dissolve formazan crystals. The plate was shaken over the shaker for 10 minutes then read using an ELISA reader at a wavelength of 595 nm.

E. Molecular Docking

Molecular docking is a method for giving an overview or prediction of the bonding of ligands and complex receptor structures, using computerized methods^[12]. The structure of the protein needed to carry out molecular docking in this study was HER-2 (PDB ID: 3PP0) and EGFR (PDB ID: 1M17) downloaded from the Protein Data Bank) through the website www.rscb.org.

- Molecular Docking with Autodock Vina—Before doing molecular docking, it is necessary to ensure that all files that will be used are in the same folder. First, create a new document and name it conf.txt. Then fill out the form with the information that will be done during the docking process by writing 3pp0.pdbqt and 1m17.pdbqt proteins, the ligands are written with ligand.pdbqt, while center_x, y, z and size_x, y, z are written according to the values listed in grid box, then stored in the same folder. The RMSD value is determined by filling in the Windows Command Prompt with the code and waiting until the process is complete and some conformations will appear. Each conformation shows the affinity of RMSD. The conformation chosen in this study was the one which has an RMSD value of less than 2 Å. Then the output.pdbqt file is split into several separate files according to each conformation, and stored in the same folder to facilitate visualization.
- Visualization of Docking Results—DS Visualizer is used to visualize docking results. Visualization of the results of docking aims to determine the position and description of the bond between proteins and ligands in 3 dimensions .

III. RESULTS AND ANALYSIS

A. Preparation of Roselle's Hexane Fraction

Roselle simplicia dry powder was extracted using maceration method. A total of 1 kg of simplicia powder was immersed in 70% ethanol with a ratio of 1:10 for 5 days, then remacerated at the same ratio for 2 days. Extracted maceration and remaceration results were combined and 7 L liquid extract was obtained, then fractionation was carried out by liquid-liquid partition method using hexane solvent in a ratio of 1: 1. The roselle's hexane fraction obtained as much as 6.3 L was then evaporated with a rotary evaporator. Then the roselle fraction was obtained as much as 3.875 grams.

B. Identification of Compounds with Thin Layer Chromatographic

The identification of compounds was done using TLC with the stationary phase of silica gel GF254, while the mobile phase used was chloroform. Comparative compounds used were flavonoids, quercetin. The observation of the chromatogram carried out on visible light, UV 254 nm and UV 366 nm showed the color spots that can be observed in Figure 1.

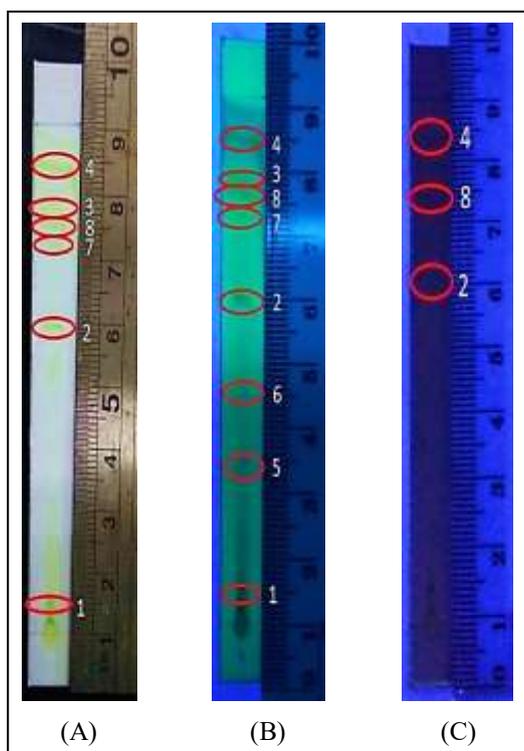


Fig. 1. Identification of Compounds with Thin Layer Chromatographic (A) Visible Light (B) UV 254 nm (C) UV 366 nm.

TABLE I. TLC TEST RESULTS AFTER EXPOSURE TO NH₃ VAPOR

Spot	Rf	Color Spots with NH ₃		
		Visible Light	UV 254 nm	UV 366 nm
1	0.06	Green	Purple	-
2	0.62	Green	Green-Brownish	Purple
3	0.85	Yellow	Yellow	-
4	0.92	Green	Green	Purple
5	0.31	-	Purple	-
6	0.43	-	Purple	-
7	0.78	Yellow	Yellow	-
8	0.82	Yellow	Yellow	Purple

The flavonoid compounds in the sample were detected by steaming the silica gel plate with ammonia, then observed in visible light, UV light 244 nm and 366 nm. Observations on spot showed that the intensity of yellow was more concentrated after ammonia evaporation identified the presence of flavonoids. This is because the reaction between flavonoids and ammonia vapor forms the structure of the cinoid in ring B, thus creating a conjugated double bond that is longer and the intensity of the color becomes thicker. Observations on 254 nm UV light showed yellow, greenish yellow, and purple patches. Whereas, observations on 366 nm UV light showed patches that glowed on the silica plate of the sample as well as the silica quercetin plate. Based on this, the hexane roselle fraction is thought to contain flavonoids by showing a deep yellow color after being given ammonia vapor, and spots that glow in the observations on 366 nm rays.

C. Antioxidant Activity Test with DPPH

The method for testing antioxidant activity in this study was 1,1-diphenyl-2-picrylhydrazyl (DPPH) method. The

working principle of the DPPH method is based on DPPH's ability to accept donated hydrogen atoms by antioxidants. After obtaining a hydrogen atom, the ability to absorb DPPH reduces and changes the color of DPPH which was originally purple to yellow. The antioxidant ability of the roselle's hexane fraction can be determined by conducting DPPH free radical capture test. Quercetin is used as a comparison in determining antioxidant activity. The DPPH antioxidant test data can be seen in tables II and III.

TABLE II. PERCENTAGE OF QUERCETIN INHIBITION

Concentration (ug/ml)	Average Absorbance	Absorbance of Blank	% Inhibition
1	0.7998	0.9564	16.37
2	0.7712	0.9564	19.36
3	0.7414	0.9564	22.48
4	0.6776	0.9564	29.15
5	0.6514	0.9564	31.89

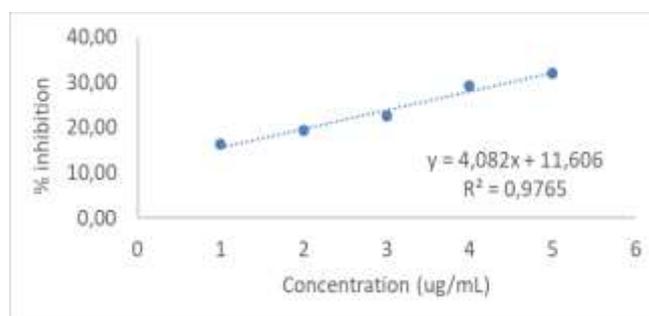


Fig. 2. Quercetin Inhibition Graph

TABLE III. PERCENTAGE OF RHF INHIBITION

Concentration (ug/ml)	Average Absorbance	Absorbance of Blank	% Inhibition
100	0.7398	0.9564	22.64
200	0.7298	0.9564	23.69
300	0.7201	0.9564	24.70
400	0.7163	0.9564	25.10
500	0.7153	0.9564	25.20

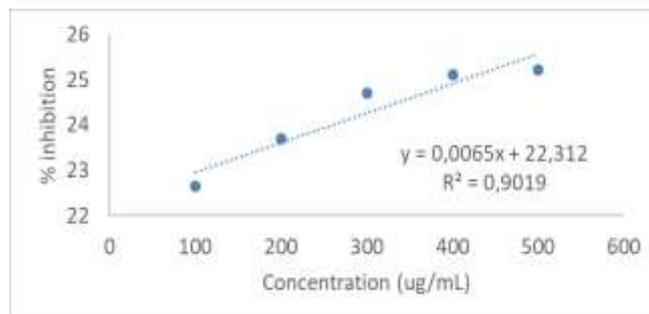


Fig. 3. Hexane Fraction of Roselle Inhibition Graph

TABLE IV. IC₅₀ VALUE

Sample	Linear Regression Equation	IC ₅₀ Value (µg/ml)	Description
Quercetin	$y = 4.082x + 11.606$ $R^2 = 0.9765$	9.4	Very Strong
Hexane Fraction of Roselle	$y = 0,0065x + 22,312$ $R^2 = 0.9019$	4259	Weak

The antioxidant activity of the sample was determined by the magnitude of DPPH radical absorption resistance through calculation of the percentage of resistance to DPPH uptake. The percentage inhibition value of each calculated concentration was then used for the calculation of Inhibitory Concentration 50% (IC₅₀) which shows the concentration value of a material to inhibit 50% of DPPH activity. Based on the antioxidant test in this study, the IC₅₀ value of quercetin was 9,4 µg/ml and was a strong antioxidant. while the IC₅₀ value of the roselle's hexane fraction was 4259 µg/ml and was a weak antioxidant^[13].

D. Cytotoxic Test

Cytotoxic tests were carried out using the MTT assay method to determine the cytotoxic activity of hexane roselle fraction against T47D breast cancer cells. In this test, a comparison of doxorubicin was used as an anticancer agent.

TABLE V. PERCENTAGE CELL LIFE AFTER BEING TREATED BY NFR

Concentration (µg/ml)	Average Absorbance of Sample	Standard Deviation	% of Living Cells
31.25	0.653	2.89	92.63
62.5	0.634	1.78	89.83
125	0.585	0.86	82.34
250	0.292	1.00	37.76
500	0.046	0.16	0.41
Average Absorbance	Cell Control	0.701	
	Media	0.043	
Linear Regression	$y = -0.2082x + 100.94$ $R^2 = 0.9694$		
	IC ₅₀ = 244 µg/ml		

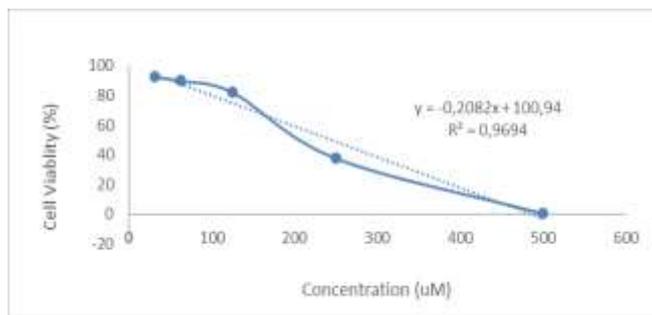


Fig. 4. Percentage of T47D Living Cells After Given Roselle

TABLE VI. PERCENTAGE CELL LIFE AFTER BEING TREATED BY DOXORUBICIN

Concentration (µg/ml)	Average Absorbance of Sample	Standard Deviation	% of Living Cells
2.5	0.099	1.98	22.49
5	0.087	0.61	17.41
10	0.082	1.45	15.54
20	0.078	0.93	13.80
Average Absorbance	Cell Control	0.293	
	Media	0.043	
Linear Regression	$y = -0.4142x + 21.195$ $R^2 = 0.7292$		
	IC ₅₀ = 69 µg/ml		

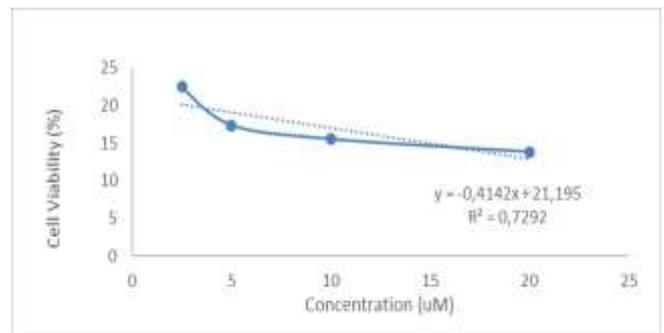


Fig. 5. Graph of Percentage T47D Cells Live after Doxorubicin was given

Based on the results of the FNR cytotoxic test at the lowest concentration of 31.25 µg/ml, it could kill 7.37% T47D cells and the highest concentration of 500 µg/ml could kill 99.59% of T47D cancer cells. The IC₅₀ value obtained from FNR was 224 µg/ml with a linear regression equation $y = -0.2082x + 100.94$ and $R^2 = 0.9694$. Meanwhile the doxorubicin chemotherapy agent at the lowest concentration of 2.5 µg/ml was able to kill 77.51% of T47D cancer cells, and at the highest concentration of 20 µg/ml was able to kill 86.20% of T47D cancer cells. The IC₅₀ value obtained was 69 µg/ml with the equation $y = -0.4142x + 21.195$ and $R^2 = 0.7292$. Cytotoxic tests were also carried out on cell morphology before and after roselle's hexane fraction and doxorubicin treatment. Observations were carried out with an inverted microscope and showed changes in cell morphology after being treated with roselle and doxorubicin. Cells that were originally oval-shaped with pointed ends on both sides changed into irregular after treatment.

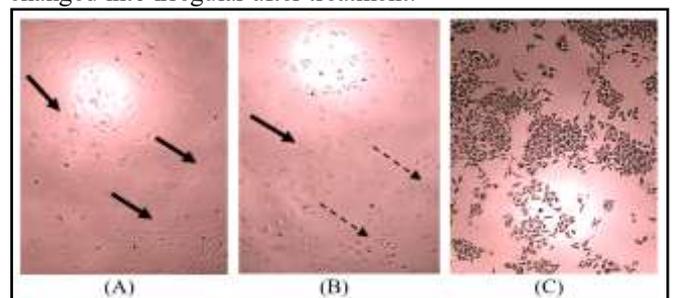


Fig. 6. Changes in Cell Morphology in Treatment with FNR (A) Before being treated (B) Shortly after being treated (C) After being treated and incubated and adding MTT reagent. () Live Cells () Dead Cells

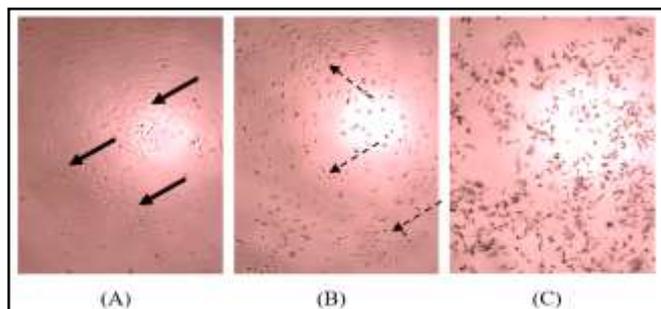


Fig. 7. Changes in Cell Morphology in the Treatment with Doxorubicin (A) Before being given the treatment (B) After a while the treatment is given (C) After being treated and incubated as well as the addition of MTT Reagents. (—) Live Cells (---) Dead Cells

Based on this study, it was shown that the roselle's hexane fraction had a potent anticancer potential for T47D breast cancer cells characterized by inhibition of viability of cancer cells with IC_{50} by 244 $\mu\text{g/ml}$.

E. Molecular Docking

Molecular docking was done by using the Autodock Vina and Open babel applications and then visualizing the 2-dimensional and 3-dimensional structure with the DS Visualizer application. Data from molecular docking results can be seen in the table.

TABLE VII. MOLECULAR DOCKING RESULTS BETWEEN LIGANDS AND HER-2 AND EGFR PROTEINS

Target Protein	Name of Compound	Conformation	RMSD Value	Docking Score (kcal/mol)
HER-2	Cyanidin 3-O-glucoside	2	1.132	-8.1
	Doxorubicin	2	1.619	-7.0
EGFR	Cyanidin 3-O-glucoside	2	1.510	-8.2
	Doxorubicin	2	1.141	-10.0

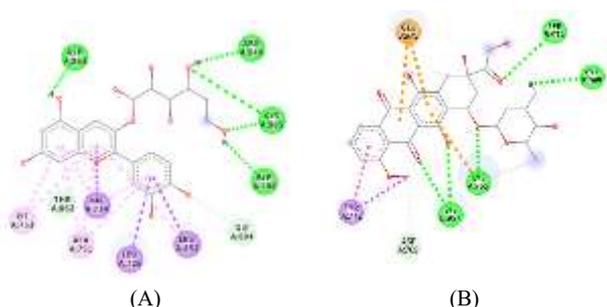


Fig. 8. Interaction of 2 Dimensions of Compounds Test on Protein HER-2 in Pocket Atoms: (A) Cyanidin 3-O-Glucoside (B) Doxorubicin

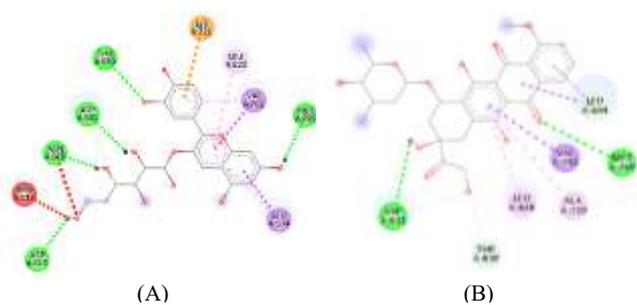


Fig. 9. Interaction of 2 Dimensions of Compounds Test on Protein EGFR in Pocket Atoms: (A) Cyanidin 3-O-Glucoside (B) Doxorubicin

The HER-2 and EGFR receptors found in breast cancer were prepared with cyanidin 3-O-glucoside. Validation results with Autodock Vina showed that the docking protocol of cyanidin 3-O-glucoside, doxorubicin and original ligands could be received with RMSD values $<2 \text{ \AA}$. RMSD is a deviation value between a ligand conformation and its comparison, that is, if the deviation is too large, the greater the prediction of ligand and protein interactions^[14]. Molecular docking results showed that the docking score obtained on HER-2 protein was cyanidin 3-O-glucoside (-8.1 kcal/mol) and the comparison was doxorubicin (-7 kcal/mol) and original HER-2 ligand with -9.4 and -7 kcal/mol proves that the stability of the cyanidin 3-O-glucoside bond with HER-2 is better than the comparison of doxorubicin but not better than the original ligand. Molecular docking results indicate that the docking score obtained on EGFR protein is cyanidin 3-O-glucoside (-8.2 kcal/mol) and its comparison is doxorubicin (-10 kcal/mol) and original EGFR ligand with -7.2 and -7.4 kcal/mol respectively, proves that the stability of the cyanidin 3-O-glucoside bond with EGFR is no better than the comparison of doxorubicin but it is better than the original ligand. It can be said that the energy needed to interact with HER-2 is smaller so that the bonds formed are more stable than doxorubicin. Meanwhile the energy needed to interact with EGFR is greater so that the bonds formed are less stable than doxorubicin.

IV. CONCLUSION

Roselle's hexane fraction has potential as a chemopreventive agent based on cytotoxic and molecular docking tests. Based on cytotoxic tests, the hexane roselle fraction has cytotoxic ability in T47D cancer cells which is quite strong with IC_{50} value 244 $\mu\text{g/ml}$. Molecular docking results show that, cyanidin 3-O-glucoside is better at binding to HER-2 protein than doxorubicin with HER-2 protein.

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