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RESEARCH ARTICLE

CYTOTOXIC ACTIVITY AND SELECTIVITY INDEX OF BINAHONG (*Anredera cordifolia)* EXTRACTS ON MCF-7 BREAST CANCER CELLS AND VERO NORMAL CELLS LINE

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ABSTRACT

Background: Breast cancers occur because of an impaired balance between proliferation, differentiation, and apoptosis of breast glands. Natural products have potency as cytotoxic agents with less side effects than chemotherapy. One of the potential plants is *Anredera cordifolia* (Ten.) Stennis (binahong), which contains flavonoid 8-glucopyranosyl- 4',5,7-trihydroxy flavone compounds.

Objective: This study aims to determine the potency of binahong leaves extract as an anticancer for breast cancer in vitro and in silico.

Methods: Preliminary tests using molecular docking of 8-glucopyranosyl-4',5,7-trihydroxyflavone compounds on Bcl-2 and HER-2 proteins. The extraction and fractionation were to obtain binahong extract. Thin layer chromatography to identify flavonoid compounds in the extract. DPPH assay was performed to evaluate the antioxidant activity. MTT assay was performed to evaluate cytotoxic activity on MCF-7 breast cancer cells and Vero cells.

Results: In silico test showed a stable bond between 8-glucopyranosyl- 4',5,7-trihydroxylflavone, and Bcl-2 and HER-2 with a docking score of -7.5 kcal/mol and -8.0 kcal/mol, respectively. The binahong extract contain flavonoid compounds that had the retention factor value 0.78; 0.49; 0.35. Antioxidant test resulted IC_{50} value of 4940 µg/mL. Cytotoxic test resulted in IC_{50} value of 1073 µg/mL and 486 µg/mL for Vero cells and MCF-7 breast cancer cells, respectively. The comparison between IC_{50} produced a selectivity index value of 2.149, which shows that binahong extract was selective against MCF-7 breast cancers.

Conclusion: This study concluded that binahong extract has weak potency as anticancer agent on MCF-7 cells.

Keywords : *Anredera cordifolia* (Ten.) Stennis, DPPH assay, Molecular Docking, MTT Assay

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INTRODUCTION

Breast cancer shows the highest number of cases of incidence in Indonesia in 2020. The number of new cases of breast cancer in Indonesia reached 65.858 cases with the second highest number of cases of death after lung cancer.[1] It is a challenge to find safe chemopreventive agents of natural origin.

One of many plants that have a potential to be developed as an chemopreventive agent is Binahong (*Anredera cordifolia* (Ten.) Stennis). Binahong is known as Teng san chi in China and has another name in English, namely heart leaf *Madeira vine*. Previous studies state that there are several anticancer active substances contained in Binahong such as saponins, triterpenoids, alkaloids, polyphenols, flavonoids, and phenolic acids found in Binahong.[2] Previous study showed that 8-glucopyranosyl-4', 5,7-trihydroxyflavone compounds which isolated from methanolic extracts of binahong leaves have a strong antioxidant activity with an IC50 value of 68.07 µg/dl.[3]

MATERIAL AND METHODS

Molecular Docking

The molecular docking process in this study used the Autodock Vina software. Autodock Vina application was supported with other applications such as DS Visualizer, Auto Dock Tools, Python, YASARA, and Open Babel. The HER-2, and Bcl-2 target protein structures are downloaded via the Protein Data Bank (PDB) by PDB ID. Ligand files (without protein) and polar hydrogen-added target protein molecules were prepared too. Then the structure was changed to '.pdbqt' extension. Docking was done by opening a command prompt, and the results of docking were in the form of an affinity value, and RMSD. Then the bond interactions were observed with the DS Visualizer application. Visualization using DS Visualizer was to see the position of ligand and protein as well as a 3-dimensional (3D) interaction picture, and an analysis of data on 8- glucopyranosyl-4',5,7-trihydroxyflavone compounds and doxorubicin (comparison compund) where good stability affinity was expressed by the lowest scoring of a molecule at an RMSD value that was less than 2 Å.[4]

Extraction and Fractionation

The maceration and fractionation method was adapted from Abubakar and Haque.[5] Binahong plants obtained from Wonosobo, Central Java and had determined in Biological laboratory, Faculty of Science and Applied Technology, Universitas Ahmad Dahlan, Yogyakarta. One Kilogram Binahong leaves powder was extracted with ethanol 70% for 5 days. The next 2 days, re-maceration was conducted using ethanol 70%. After the re-maceration, ethanolic extract of binahong leaves was obtained by filtration. Fractionation used a separating funnel with n-hexane solvent to obtain the ethanol fraction. The ethanol fraction was then concentrated with a vacuum rotary evaporator. The heating proceeded by waterbath to optimize the fractionation. At last, the ethanol fraction was weighed using analytical scale, and the was yield calculated. A 76.6 grams of thick extract was obtained with yield value of 7.66%.

Thin Layer Chromatography (TLC)

Analysis of chemical contents of the binahong extract used the TLC method with silica gel GF254 as stationary phase, and the mobile phase using chloroform and methanol with 9:1 ratio was saturated in a

chamber. The TLC plate was put into the chamber, and the next step was to the sample separation. After the separation process, the plate was dried and observed. Physically, spots were detected under visible light on UV 254 and 366 nm.[6] The plate was sprayed with ammonia solvent, so that the resulting spots were seen more clearly.

Antioxidant Test with DPPH Method

The 0.4 mM of DPPH solution with methanol was prepared as a solvent. Test solutions of the binahong extratcs leaves were prepared by making the master solution up to 1000 µg/mL and quercetin standard of 50 µg/mL. The solution obtained was taken using a pipette and dissolved with methanol and was made in a series of 200; 400; 600; 800 and 1000 µg/ml for the ethanol fraction of binahong leaves and a series of 2; 4; 6; 8 and 10 µg/ml for quercetin. A blank solution was made with methanol to be used in determining the maximum wavelength and determining the operating time. The color change that occurs was measured by the absorbance value at a wavelength of 515-520 nm.[7] After DPPH optimization, a wavelength of 516 nm was selected for this study. Antioxidant activity test was conducted by reading the absorbance at the maximum wavelength of DPPH, and the IC_{50} value was calculated by processing the absorbance value into % antioxidants.[8]

Cytotoxic and Selectivity Test with MTT Assay Method

The MTT assay was adapted from van Merloo et al.[9] The sample contained a 5-mg ethanol extract in a 50-ml Dimethyl Sulfoxide (DMSO). The concentration of the test solution was 62,5; 125; 250; 500; 1000 µg/mL. MCF-7 cells were grown in tissue culture flask incubated in CO_2 incubators at 37^0C. Cells were distributed into 96 wells and incubated for 48 hours to adhere to the bottom of the wells. The cells were then washed and incubated using MTT assay reagents. After 4 hours, stopper reagents were added, and shaken for 10 minutes. It was then incubated at room temperature overnight. Each well was read with ELISA reader at a wavelength of 595 nm.

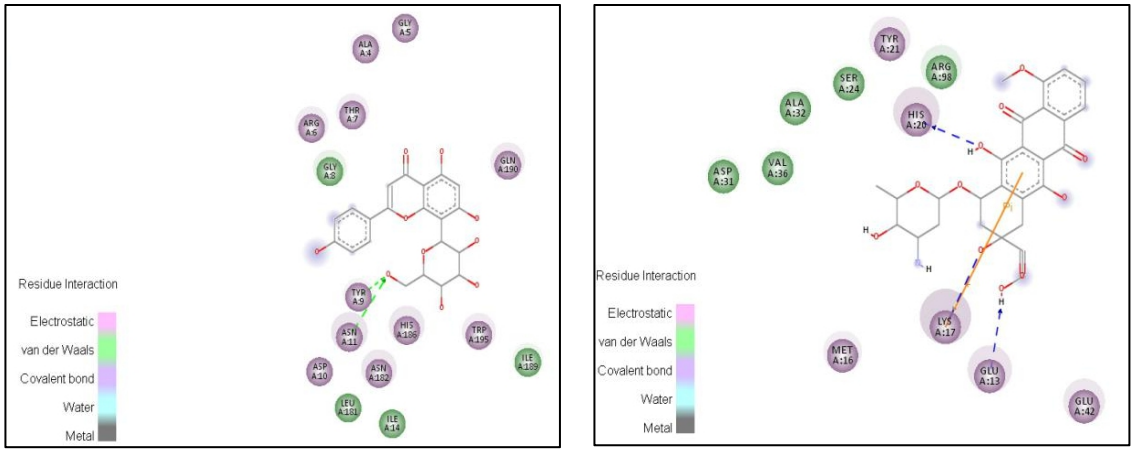
RESULTS

Docking Score Result

Molecular docking test used Autodock Vina application and Open Babel. In this study use Bcl-2 and HER-2 as target proteins and doxorubicin as reference drug. Bcl-2 has a role in apoptosis process as an antiapoptotis, while overexpression of Bcl-2 causes inhibition of the apoptosis process so that cancer cell growth becomes uncontrolled. HER-2 has an important role in regulating cell growth, survival, and differentiation and plays a role in cell proliferation and differentiation. Overexpression of HER-2 was found in breast, ovarian and gastric cancer.[10] Doxorubicin is one of chemotherapy in cancer therapy, it compare to flavonoid because it has a great potential as cytotoxic anticancer agents promoting apoptosis in cancer cells due to the research.[11] The structure was visualized in 2 dimensions with the DS Visualizer application (Figure 1, 2). Docking score of 8-glucopyranosyl-4',5,7-trihydroxyflavone compound against the Bcl-2 protein showed successive values of -7.5 while the HER-2 protein showed a value of -8.0 (Table 1). The affinity value of each bond showed a negative value, which means that the reaction occurred between the ligand with the receptor only required little energy to bind strongly.

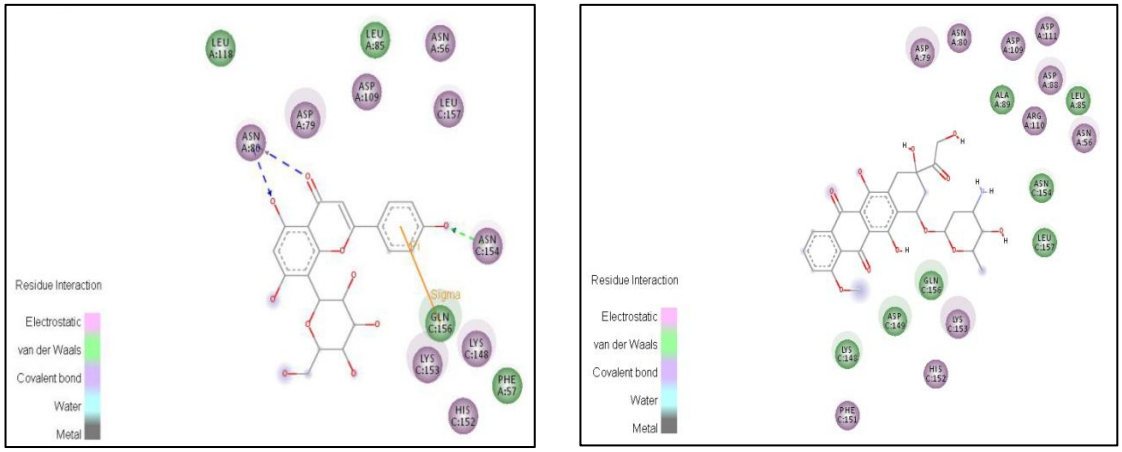
Table 1. The docking results of compounds against Bcl-2 and HER-2

No.	Protein	Name of Compound	Docking Score	RMSD Value	Conformation
1	Bcl-2	8-glucopyranosyl-4',5,7 - trihydroxy flavone	-7.5	1.692	5
2		Doxorubicin	-4.8	1.557	6
3	HER-2	8-glucopyranosyl-4',5,7 - trihydroxy flavone	-8.0	1.313	2
4		Doxorubicin	-6.9	1.258	6



8-glucopyranosyl-4',5,7-trihydroxy flavone Doxorubicin

Figure 1. The 2D visualization of amino acid interactions on Bcl-2 protein. The 8-glucopyranosyl-4',5,7-trihydroxy flavone showed stronger interaction with the amino acid in Bcl-2 protein compared with doxorubicin.



8-glucopyranosyl-4',5,7-trihydroxy flavone Doxorubicin

Figure 2. The 2D visualization of amino acid interactions on HER-2 protein. The 8-glucopyranosyl-4',5,7-trihydroxy flavone showed stronger interaction with the amino acid in HER-2 protein compared with doxorubicin.

Flavonoid Identification in the extract

The identification of flavonoid compounds contained in binahong extract was carried out using Thin Layer Chromatography. At each spot formed, the Rf value obtained from the elution distance of the sample was compared with the elution distance of the mobile phase. Spot no. 1 and 2 showed the posibility of flavonoid compounds because the spots showed yellow color under visible light after evaporated the plate using ammonia (Figure 3, Table 2)

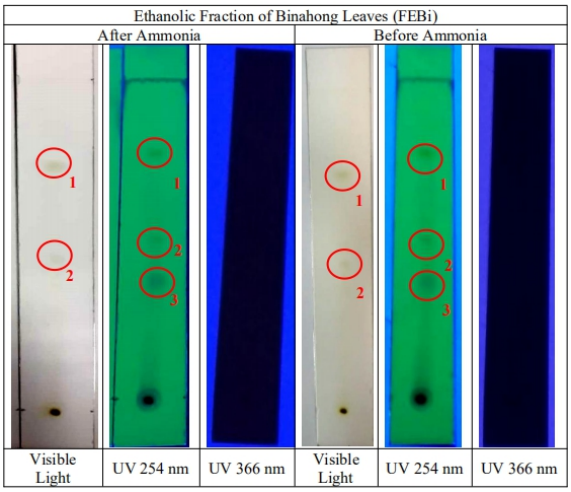


Figure 3. The profile of compound in binahong extract at GF254 silica gel plate by TLC method resulted in three Rf spot values (no. 1, 2 and 3): 0.78; 0.49 and 0.35. Spot no. 1 and 2 showed the posibility of flavonoid compounds because the spots showed yellow color under visible light after evaporated the plate using ammonia.

Antioxidant Test with DPPH Method

The ability of binahong extract as an antioxidant could be seen by stabilizing DPPH free radicals. From the linear regression obtained (Figure 4), the IC_{50} calculation was performed. The IC_{50} value of binahong extract resulted 4940 µg/mL, which means it has weak potency as an antioxidant, and quercetin as comparison compound resulted the IC_{50} value of 6.8 µg/mL showed strong antioxidant activity.

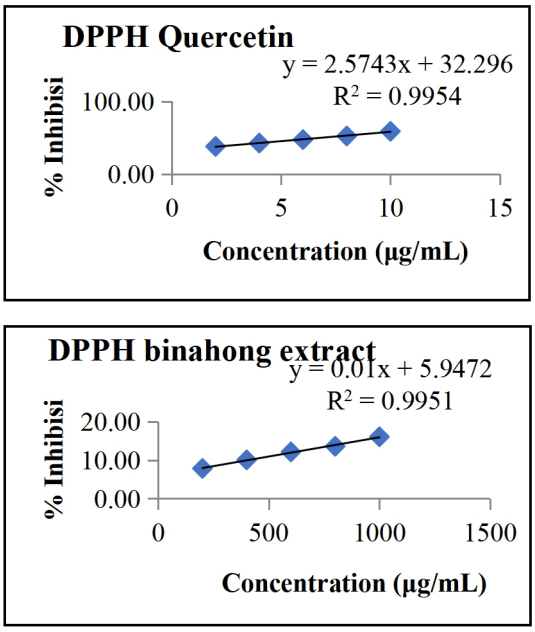


Figure 4. a) Quercetin inhibition **b)** Binahong extract inhibition

Cytotoxic and Selectivity Analysis

The morphology of MCF-7 breast cancer cells was observed using inverted microscope (4x10 magnification). After treating with binahong extract for 24 hours, the cells' morphology was change from perfectly round into irregular spheres and contracted (Figure 5).

The binahong extract could inhibit the growth of MCF-7 cells. In Figure 5c and 5d, Vero cells' morphology did not change significantly after being treated with binahong extract, suggesting that the extract did not kill normal Vero cells. After treatment with binahong extract, a stopper reagent was added to dissolve dark formazan crystals produced by living cells (Figures 5b and 5d), and the absorbance was calculated with ELISA reader.

Based on the Figure 6, the relationship between cell viability and cell concentration obtained a linear regression equation that serves to calculate IC_{50} value. The IC_{50} value of binahing was 486 µg/mL, suggesting a moderate toxic effect on MCF-7 breast cancer cells. In Vero cells

Table 2. The TLC result of binahong extract

Spot Number	Rf Value	Color of the spot					
		Visible light		UV 254		UV 366	
		Before	After	Before	After	Before	After
1	0,78	Green	Yellowish green	Brown	Dark brown	-	-
2	0,49	Faded green	Yellowish green	Faded brown	Brown	-	-
3	0,35	-	-	Brown	Brown	-	-

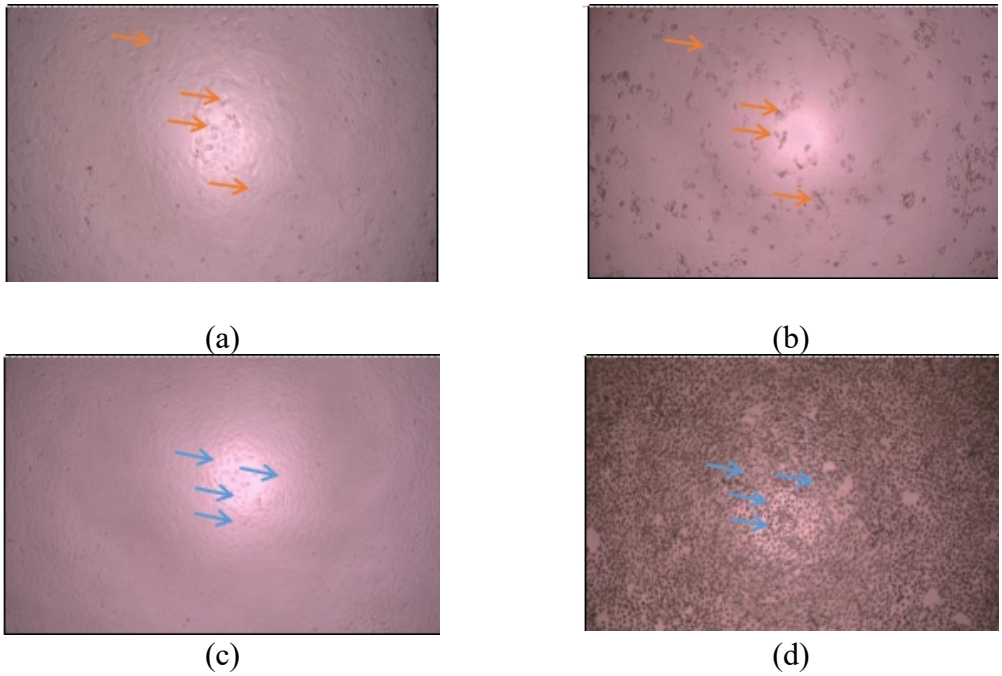


Figure 5. Morphological changes of MCF-7 dan Vero (A) The MCF-7 cell control group (→)(B) After treatment with binahong extract on MCF-7 cell (→) (C) Cell Vero cell control group (→)(D) dan After treatment with binahong extract on Vero cell (→)

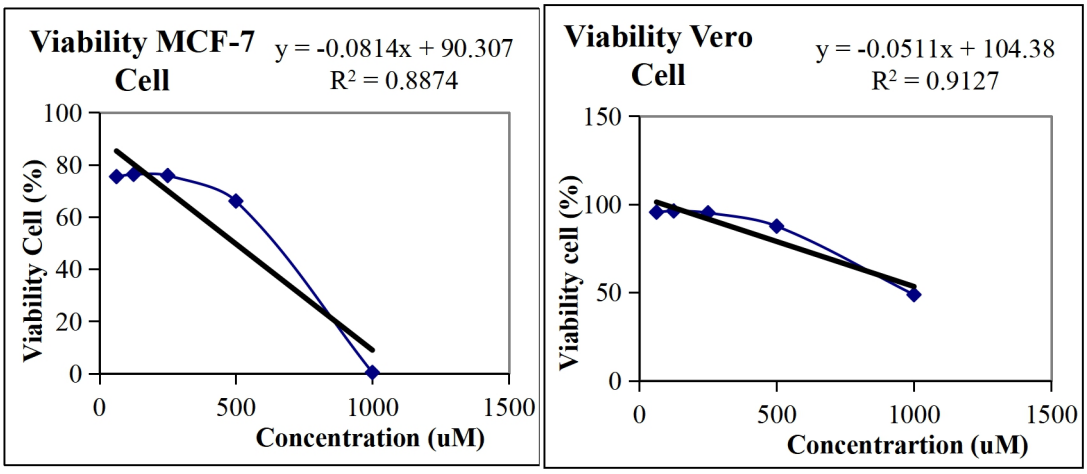


Figure 6. a) Percentage of living cells with binahong extract treatment at MCF-7 cells **b)** Percentage of living cells with binahong treatment at Vero cells

has low cytotoxic effect with IC_{50} value was 1073 µg/mL. Selectivity Index (SI) values were obtained from the comparison of IC_{50} value on normal Vero cells with the IC_{50} value on MCF-7 breast cancer cells. From the data showed that the SI value was 2.15, so the result means that binahong extract has selective activity in inhibiting breast cancer cells.

DISCUSSION

Molecular docking is good if the RMSD value <2 Å is obtained. This value interprets the minimum deviation from the interaction and the bonding of tested compounds and proteins. The test results showed that the interaction of the Bcl-2 protein and the 8- glucopyranosyl-4,5,7 trihydroxy flavone was more negative with a docking score of -7.5 kcal/mol compared to doxorubicin of -4.8 kcal/mol. This means that the test compounds require less energy to bind to the Bcl-2 protein than doxorubicin to form a more stable bond. The energy needed to bind 8-glucopyranosyl-4',5,7- trihydroxy flavone with HER-2 protein is also smaller than doxorubicin. It can be seen from the docking score obtained between the HER-2 protein against the compound 8-glucopyranosyl-4',5,7- trihydroxy flavone (-8.0 kcal/mol), which is more negative when compared with doxorubicin (-6.9 kcal/mol). Based on this study, both Bcl-2 and HER-2 proteins have more stable bond to the test active compounds when compared to the doxorubicin drug compounds. Therefore, 8-glucopyranosyl-4',5,7-trihydroxyflavone compound has a good potency to inhibit overexpression of Bcl-2 and HER-2 proteins.

In thin layer chromatography tests, color changes at spots 1 and 2 turning into yellowish-green after being evaporated using ammonia under visible light. To determine the content of flavonoid compounds qualitatively by sprayed or evaporated the plate using ammonia vapor, the positive flavonoid results if the spots are yellow or violet under visible light.[12] At 254 nm UV light, three spots also increasingly experienced a change into dark brown color. More concentrated color occurs because of the reaction between flavonoid compounds and ammonia vapor that forms the structure of the cinoid in ring B, and the conjugated bonds are getting longer.[13] According to Gibbons [14], flavonoids will fluorescence because they have a chromophore group that is bound by an autochrome that reacts with UV. Nevertheless, this study did not reveal the luminescence because it is possible that chromophore groups are undetectable below UV 366 nm. Besides, a quantitative analysis was performed by calculating the value of Rf (Retardation factor). Rf is obtained from the comparison between the distance of the solute and the distance of the mobile phase.[15] TLC of the binahong extract generated three Rf spot values (1, 2 and 3): 0.78; 0.49 and 0.35. Those Rf met the requirements because a good Rf value ranges from 0.2-0.8.[16] Flavonoid glycoside compounds have higher polarity, so that the Rf value will be smaller than that of aglycone compounds (non-glycoside part). In this study, the alleged flavonoid glycoside compounds are spots 2 and 3.

The ability of binahong extract to reduce 50% DPPH is at a concentration of 4940 µg/ml while the comparison of quercetin was at a small concentration of 6,8 µg/ml. If the IC_{50} value obtained is very high, it indicates the ability of the compound as a weak antioxidant. It is thought that the ethanol fraction of binahong leaves was only able to contribute in a small number of hydrogen

and electron atoms to DPPH due to the small content of flavonoid compounds. Besides, the presence of disruptors contained in binahong extract such as protein and fat can reduce its ability in decreasing free radicals.[16] The antioxidant activity of a compound is considered weak if it is in the range of >150 µg/ml.[17] Therefore, the ethanolic fraction of binahong leaves has weak antioxidant activity.

The absorbance is quantitatively read using ELISA reader. The absorbance data obtained are put into a linear regression equation to get the IC_{50} value. IC_{50} values on MCF-7 cells and Vero cells line were 486 µg/ml and 1073 µg/ml, respectively. Based on the classification of toxic levels of IC_{50} values by Weerapreeyakul [18], the compounds are quite toxic in the range of 101-500 µg/mL. It does not have cytotoxic activity if the IC_{50} value is >1000 µg/mL.[19] The binahong extract tested on MCF-7 breast cancer cells in this study has the potential to be non-toxic effect while on normal cells Vero is also non-toxic. The selectivity of an anticancer compound can be measured using selectivity index (SI). SI values were calculated by IC_{50} value on normal cells divided by IC_{50} value on cancer cells.[20] The SI value obtained in this study was 2.15. A compound has selective toxicity to cancer cells if the SI value is high (> 2). It points out that the selective binahong extract test compound has selective effect on MCF-7 breast cancer cells, and it has non-toxic effect on normal Vero cells.

CONCLUSION

The binahong extract has low potency as an anti-breast cancer by in vitro with IC_{50} value of 486 µg/ml but has selective activity on Vero cells line with SI value of 2.15.

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