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N-hexane Fraction of Bandotan Herb (*Ageratum conyzoides* L.) Increasing Doxorubicin cytotoxic Effect in MCF-7 Breast Cancer Cells Line

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ABSTRACT

Breast cancer is one of the most common cancers that causes the highest morbidity and mortality in humans. Some anticancer medicines are not efficient and also have many side effects. Based on the Previous researches showed That bandotan herb extract contained several compounds which potentially have anti-microbial, anti-inflammatory and anti-cancer effects. This study aimed to analyze the cytotoxic activity and co-chemotherapy effect of n-hexane fraction of Bandotan (NFB) combined with doxorubicin in MCF-7 breast cancer cells line by *in vitro* assay and study the molecular mechanism of the active component NFB by *in silico* assay, with HER-2 as protein target. Antioxidant activity test of NFB measured by DPPH method and cytotoxicity assay of NFB performed with MTT assay method was carried out by molecular docking method between ageratochromene dimer (compound marker of bandotan) on HER-2 protein. Based on the research showed that 1 kg of *Ageratum conyzoides* L. herb powder obtained 1.5g NFB concentrated exctract which would then be utilized in antioxidant and cytotoxic test. The docking molecular method results showed NFB as a potential cytotoxic agent mediated by the ability of ageratochromene dimer inhibited HER-2 protein targets with docking scoreof -6.2 kcal/mol and doxorubicin as comparison with docking score of -6.9 kcal/mol. Antioxidant activity of NFB showed the IC_{50}value was 493 µg/ml. The NFB had low potency as citotoxic activity with IC_{50} value was 306 µg/ml to inhibit the growth of MCF-7 cells line but showed synergistic effect in combination with doxorubicin with Combination Index (CI) value between 0.53-0.90. From the result obtained it could be concluded that the NFB has weak potential as antioxidant and single cytotoxic activity but hassynergistic effect as co-chemotherapy when combined with doxorubicin on MCF-7 breast cancer cells line.

Key words: *Ageratum conyzoides L., Antioxidant, Co-chemotherapy effect, Molecular docking, MCF-7 cells line.*

Introduction

All deaths in the world, 12% of which are caused by breast cancer and become the number 2 as human killer after cardiovascular disease, this is expected to increase every year (NCI, 2012). There are various types of cancers that have been identified, one of them is breast cancer. Breast cancer is very common in women than men with a ratio of 1:1000 (Mulyani, 2013). The level of malignancy of breast cancer is relatively high compared with other cancers that reach 20% of 100,000 in population (Depkes , 2013). The results of preliminary study data conducted at Dharmais Cancer Hospital, Jakarta, obtained data in

2013 showed there are 10 types of cancer that most often occurs, one of which is breast cancer with the incidence rate of 36.9% and is the most common cancer compared with other cancers (Kemenkes, 2014).

One of the most commonly used therapies for cancer treatment is chemotherapy. Chemotherapy agents are anticancer with a narrow security limit. Most of anticancer drug work not selective because it has a mechanism of action to damage DNA both in normal cells and cancer cells (Dai *et al*., 2004). The emergence of a new paradigm in the world of modern medicine "back to nature" making it a challenge to further explore the development of traditional medicine and anticancer medicine derived from plants in particular, anticancer is expected to produce more effective and have harmful side effects (Rahmawati *et al*., 2008). One of the natural ingredients that may be used as a breast cancer co-chemotherapy agent is bandotan (*Ageratum conyzoides* L.). Bandotan had many benefits and easy to find in Indonesia. Bandotan is empirically used as analgesic, antibacterial, anti-inflammation, diuretic and uterine tumors (Wijayakusuma and Dalimarta, 1994).

This study aimed to determine the potency of n-hexane fraction of bandotan as antioxidant and chemoprevention agent by *in vitro* and *in silico* study. Antioxidant activity of bandotan was tested using DPPH method (1,1-diphenyl-2-picrilhidrazil) was stable to free radical (Molynux, 2004). In the cytotoxic test, the n-hexane fraction of bandotan and combination with doxorubicin were analyzed against MCF-7 cells line by MTT assay. *In silico* study was performed to determine the interaction between Ageratochromene dimer compound on HER-2 protein target.

Materials and Methods

Plant Source and Determination

The Bandotan herb (*Ageratum conyzoides* L.) was obtained in Bantul, Yogyakarta and has been determined at Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta.

Extraction and Fractionation

The extraction was done by maceration method. From 1.2 kg powder of bandotan herb macerated with ethanol 70% (ratio 1:10) during 5 days and remacerated during 2 days. Then Fractionated with n-hexane solvent to separate the active compounds of bandotan herb based on the polarity (ratio 1:1). The extract then evaporated by vacuum rotary evaporator (60^{o} C; 100 rpm) to get n-hexane fraction of bandotan concentrated extract.

Antioxidant Test of NFB by 1,1-Diphenyl-2-Pikrilhidrazil (DPPH) Method

The method started by dissolved 15.8 mg of 1,1-Diphenyl-2-Pikrilhidrazil (DPPH) in 25 ml of methanol (0,4 mM), the control solution made by dissolving of 1 ml of DPPH in 100 ml methanol. The other way weight 20 mg of sample and 5 mg of vitamin C as comparison then dissolve the sample in 20 ml of methanol (1000 µg/ml) and vitamin C in 100 ml of methanol (50 µg/ml). Then made series concentrations from 100; 200; 300; 400 and 500 µg/ml for NFB and from 1;2;3;4 and 5 µg/ml for vitamin C. The 5 mL for each concentration of sample and vitamin C was taken then added by 1 ml of DPPH, then mixed and incubated in dark room during 30 minutes. The absorbance of each solution was read by UV-Vis Spectrophotometer in 517 nmwavelength.

Cytotoxic Activity of NFB by MTT assay

The solution of NFB with concentration 1×10^5 µg/mL and several series concentrations were made. The MCF-7 cells with a density of $1x10^4$ cells/well are distributed into 96 wells plate and incubated for 24 hours to adapt and stick to the bottom of the well. Then, medium was taken, washed with PBS and added 100 µl culture medium containing only 0.2% DMSO (control) or single test sample (NFB and doxorubicin) then incubated for 24 hours. At the end of the incubation, culture medium containing the sample was removed, washed with 100 µl PBS. Then added 100 µl culture medium containing 5 mg/ml of MTT reagen into each well, incubating again for 4 hours at 37 °C. The living cells react with MTT to form a purple formazan crystals. After 4 hours, the medium containing MTT was removed, washed with PBS then added an SDS stopper solution in 200 µl of 0.1% HCl to dissolve the formazan crystals. Rocked over a shaker for 10 minutes then read with an ELISA reader at a 595 nm wavelength. The absorption value is converted into live cell percentage then calculated IC_{50} value with linear regression equation.

Cytotoxic Combination of NFB and Doxorubicin by MTT assay

Cells with a density of $5x10^3$ cells/100 mL in DMEM

medium were distributed into 96 wells plate of 100 ml each and 3 wells for media control then incubated for 2 hours to recover and return to normal after harvesting the cells. After the normal cells returned, a series of NFB and doxorubicin concentration was prepared with ½ IC_{50}, 3/8 IC_{50}, ¼ IC_{50}, and 1/8 IC_{50} concentrations. The well plate containing the cell was taken from the incubator then the medium was discarded and the cell washed with 100 ml PBS, then the PBS was removed and the liquid was drained with a tissue. For the combined treatment group, 50 ml was added per series of NFB concentration into the well and replicated 3 timesthen added of 50 ml of doxorubicin concentration series each well. For a single group, 50 ml was added for each series of concentration of NFB or doxorubicin into the well and replicated 3 times then added 50 ml of DMEM each. For the control of the cell was added 100 ml of DMEM into the wells that already contain cells and replicated 9 times. For media controls added 100 ml of DMEM into an empty well and replicated 3 times. All treatments are incubated in the CO_2 incubator. Prepared 5 mg/mL MTT stock by weight 50 mg of MTT powder and dissolved in 10 ml PBS. The MTT reagent for treatment (0.5 mg/ml) was prepared by taking 1 mL of MTT 5 mg/ml stock and diluted with DMEM up to 10 ml. Cell media was removed and re-washed with PBS and then added 100 mL MTT 0.5 mg/ml to each well including media control. The cells were incubated for 2-4 hours in the incubator until crystals of purple formazan crystals were formed and the crystals were observed under an inverted microscope. When the formazan was clearly formed, a 10% SDS stopper in 0.1 N HCL was added. Plates that have been given SDS are wrapped in paper or aluminum foil and are re-circulated at room temperature overnight. The next day, the absorbance can be read with ELISA reader at l 595 nm then calculated the percentage of the living cell and CI (Combination Index) of the treatment.

Molecular Docking

Molecular docking begins by downloading all required applications and test the active compounds (ageratochromene dimer and doxorubicin are downloaded at Pubchem.com) and target proteins (HER-2 and native ligand are downloaded in Protein Data Bank (PDB) (www.rcsb.org) with PDB ID:3PP0). Further protein target preparation with DS Visualizer application. The HER-2 (PDB ID 3PP0) protein was stored with the file name 3PP0 with the DS visualizer app being converted into .pdb format with the Open Babel app. Each compound to be docked were given 9 conformations with different RMSD values. A valid docking protocol is a docking protocol that generates conformation with a RMSD value less than 2Å then the conformation was docked until an energy affinity or score docking was obtained. Visualization using DS Visualizer for Define Ligand and Ligand Interaction so that it will be seen clearly bonding position of active compound test and protein HER-2 then labeled amino acids that bind to the active compound. Compounds that have good inhibitory potential against the HER-2 protein are compounds with the lowest affinity or the lowest score docking.

Results and Discussion

Plant Determination

The result of the determination showed that the samples tested were bandotan herb (*Ageratum conyzoides* L.) and included in Asteraceae family (Figure 1).

Fig. 1. Bandotan (*Ageratum conyzoides* L.)

Extraction and Fractionation

The bandotan herb (*Ageratum conyzoides l.*) in the wet conditions obtained were cut into smaller sizes to speed up the drying process (Sudewo, 2009). The drying process aimed to stop enzymatic processes that may still occured, so that the degradation of the active substance can be reduced. The maceration of bandotan herb powder with ethanol 70% during 5 days in ratio 1:10, this is the best ratio to get the highest levels of active compounds and antioxidant

activity (Handayani *et al.,* 2016). The ethanol extract of bandotan herb (*Ageratum conyzoides* L.) was obtained of 8.82 L, took 1.1 L of ethanol extract for fractionation. Fractionation was performed to separate polar compounds with non-polar compounds, when the fractionation process, the bandotan ethanol herb fraction (*Ageratum conyzoides* L.) lies at the bottom layer, whereas the n-hexane fraction of bandotan (NFB) is in the upper layer because the ethanol solvent has a bigger density. The n-hexane extract of bandotan showed a result rendement value was 1.103%. These results indicated that the fractionation process of ethanol and n-hexane extract can be separated well because it has different polarity and yields a small rendement value. This result is consistent with the theory, bandotan contains more polar compounds than non-polar polyamide compounds (Okunade, 2002) will be widely dissolved in ethanol extract (Polarity Index: 5.2) than n-hexane solvent with lower polarity index (Polarity index: 0) (Torres *et al*., 2011).

Antioxidant Activity Result of NFB by 1,1-Difenil-2-Pikrilhidrazil (DPPH)

The antioxidant activity of the NFB can be determined by DPPH method, Vitamin C was chosen as a standard comparison in this test. The concentration of Vitamin C and NFB associated with the value of % inhibition will result in a linear regression equation. The linear regression equation of Vitamin C and the n-hexane fraction obtained is y = 16.795x + 0.3778 with R_2 = 0.997 and y = 0.1021x - 0.3132 with R_2 = 0.8011 (Figure 2 and 3). The linear regression equation is used to calculate the value of IC_{50} by entering the value 50 into y line at the obtained linear regression equation. The calculation result showed IC_{50} Vitamin C value of 2.9 µg/ml for IC_{50} value of NFB that is 493 µg/ml (Table 1).

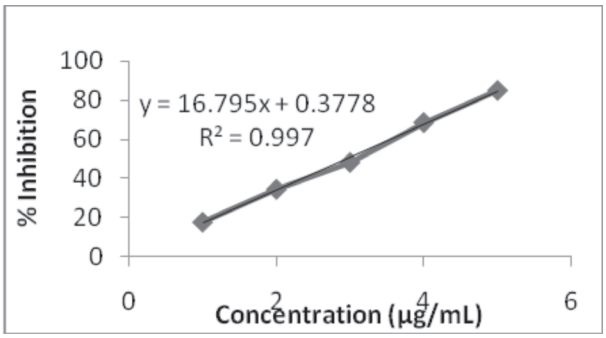


Fig. 2. Antioxidant Activity of Vitamin C

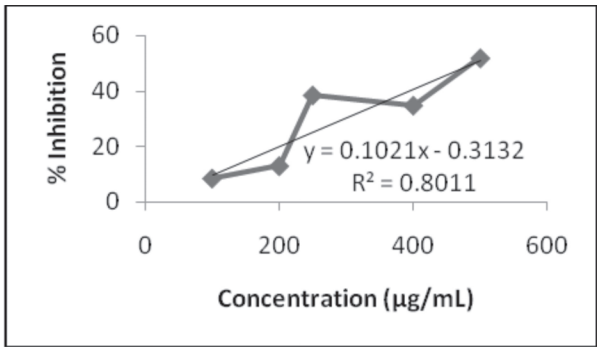


Fig. 3. Antioxidant Activity of NFB

Table 1. Antioxidant Activity of NFB

Sample	Concentration (µg/ml)	% inhibition	IC_{50} Value
Vitamin C	1	17.80744	2.9 µg/ml
	2	34.53074	
	3	48.19579	
	4	68.47087	
	5	84.81392	
NFB	100	8.42674	493 µg/ml
	200	12.94001	
	250	38.48574	
	400	34.77876	
	500	51.8879	

The result showed that IC_{50} of NFB and vitamin C was 493 and 2.9 µg/ml, respectively. The IC_{50} value of NFB was high is thought to be caused by flavonoid compounds binding to side groups that can inhibit antioxidant activity was not contained in the n-hexane fraction, so that flavonoids could not donate hydrogen and electrons to DPPH (Harborne, 1987; Budilaksono *et al*., 2014). In addition, the side groups may also cause the flavonoid to be methylated (group -H to cluster -CH_3), so that the proton source to capture DPPH is reduced (Mikamo *et al*., 2000; Pranata, 2013). The presence of bullies such as proteins and fats in the FNB fraction is also suspected to interfere with DPPH free radical capture (Budilaksono *et al*., 2014). Although IC_{50}values of the fractions fall into the weak category when viewed from the level of antioxidant activity according to Ariyanto (2006) (>150 µg/ml), the IC_{50}value was 200-1000 µg/ml is considered potentially as an antioxidant (Molyneux, 2004; Pranata, 2013).

Cytotoxic Activity of NFB by MTT assay

The cytotoxic test in this study is an advanced test to determine the cytotoxicity activity of NFB by using

MCF-7 cells line. This research use MTT assay method, cytotoxic test result will yield IC_{50} value.

Table 2. Cell viability of MCF-7 with NFB treatment.

Concentration (ìg/ml)	Absorbance Average	Cell Viability (%)	Standard Deviation
1000	0.333	3.26	2.14
500	0.306	1.73	0.69
250	1.54	71.76	4.25
125	1.928	93.78	3.86
62.5	1.992	97.42	6.59
Absorbance Average Media		0.0918	
Cell Control		0.679	
IC_{50}:		306 µg/ml	

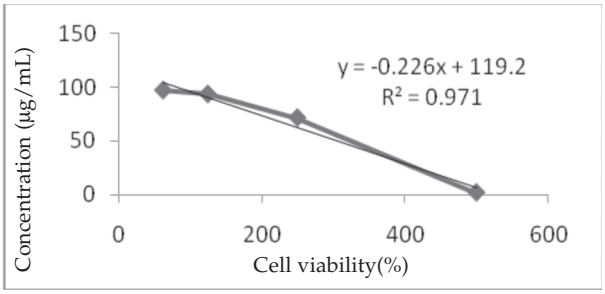


Fig. 4. Chart of Viability of MCF-7 with NFB treatment

Table 3. Cell Viability of MCF-7 with Doxorubicin Treatment

Concentration (µg/ml)	Absorbance Average	Cell Viability (%)	Standard Deviation
200	0.323	4.53	0.21
100	0.495	17.31	1.92
50	0.927	49.41	1.74
25	1.159	66.64	3.13
12.5	1.225	71.55	0.74
6.25	1.276	75.33	0.64
Absorbance Average Media		0.0678	
Cell Control		0.0918	
IC_{50}: 59 µg/ml			

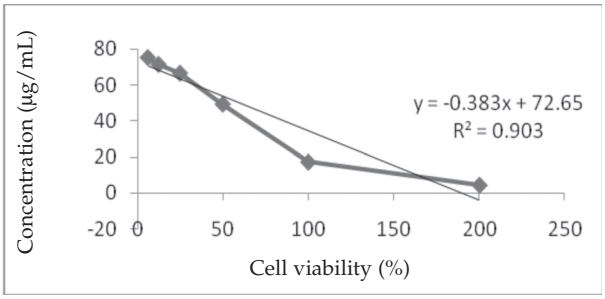


Fig. 5. Chart of cell viability of MCF-7 with doxorubicin treatment

Based on the results of NFB cytotoxic test, the lowest concentration (62.5 µg/ml) was capable to inhibit of 2.59% MCF-7 cells, while at the highest concentration of 500 µg/ml could inhibit 98.27% MCF-7 cells. The IC_{50} value of NFB was 306 µg/ml. High linearity showed the decreasing of cell viability proportional with increasing concentration following equation y= -0.2264x+119.23. Meanwhile, cytotoxic results in doxorubicin showed IC_{50}value of 59 µg/ml. The IC_{50} of doxorubicin value was smaller than the IC_{50} NFB value so the cytotoxic activity of NFB is lower than doxorubicin because it requires a large dose to inhibit 50% viability of MCF-7 breast cancer cells. At the lowest concentration of 6.25 µg/ml viability of MCF-7 breast cancer cells of 75.33%, while at the highest concentration of 200 µg/ml viability of MCF-7 breast cancer cells by 4.53%.

Cytotoxic Combination of NFB and Doxorubicin by MTT assay

From The result of single cytotoxic test, there are 2 results that is single IC_{50} value of each compound and IC_{50} value of combination which was then used to calculate Combination Index (CI) value. From the results of the calculation of a single cytotoxic test, based on the classification according to Weerapreeyakul (2012), it was known that the NFB had a potent enough toxicityto inhibit the growth of MCF-7 breast cancer cells was shown with an IC_{50} value of 306 µg/ml.An extract was said to be active and has great potential for an anticancer agent if its IC_{50} value was less than 100 µg/ml. Doxorubicin which was an antimetabolite cytotoxic medicine has an IC_{50} value of 59 µg/ml.

The cytotoxic assay of the combination of NFB and doxorubicin has a synergistic effect because it has a CI value between the range 0.3-0.9 (Table 5). The best value of CI in the combination of NFB with concentration of 150 µg/ml anddoxorubicin concentration of 7.5 µg/ml. These values indicated that the combined concentration gives a synergistic effect as co-chemotherapy in MCF-7 breast cancer cells.

Molecular Docking Result

There are approximately 20,000 HER-2 receptors in normal breast cells. On the surface of breast cancer cells, HER-2 receptors can double to 1.5 million receptors (Nahta *et al.*, 2006). The increased of HER-2 gene expression causes an increase in metastasis, proliferation and breast cells will be induced angiogenesis. HER-2 receptors were capable of forming

Table 4. Viability Cell in Combination Treatment

Sample		Cell viability of doxorubicin (%)				
		0	30	22.5	15	7.5
Cell viability of NFB (%)	0	100	67.98	58.33	71.28	77.52
	150	79.34	22.84	26.13	37.37	33.90
	112.5	81.34	30.72	29.55	47.20	42.61
	75	88.76	36.26	39.14	53.09	54.50
	37.5	91.76	42.44	46.73	59.03	60.92

Table 5. Combination Index (CI) of NFB and Doxorubicin

Combination Index (CI)		Doxorubicin			
		30	22.5	15	7.5
NFB	150	0.60	0.59	0.71	0.53
	112.5	0.68	0.56	0.84	0.55
	75	0.73	0.67	0.86	0.62
	37.5	0.80	0.75	0.90	0.58

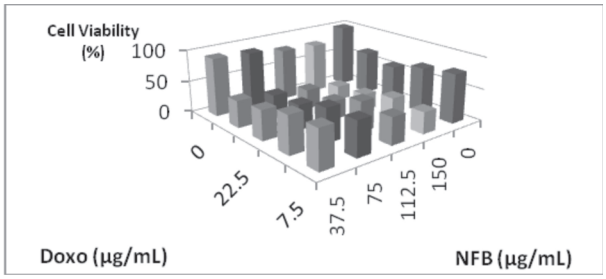


Fig. 6. Chart of Viability in Combination Treatment

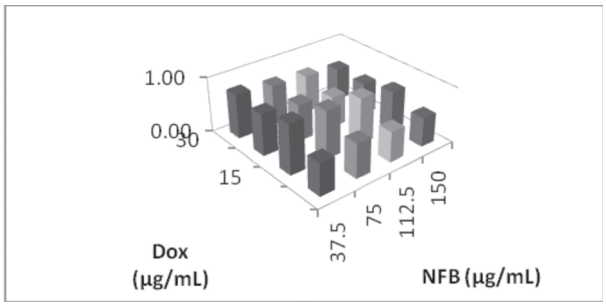


Fig. 7. Chart of Combination Index (CI) of NFB and doxorubicin

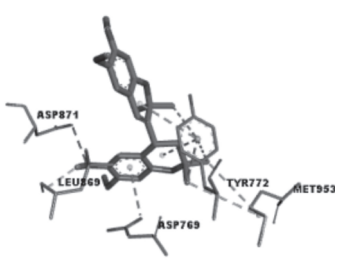
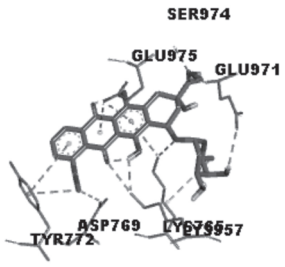
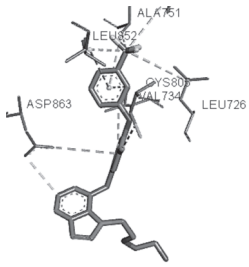
heterodimers which are a combination of HER-2 receptors with various other receptors hence the heterodimer receptor complex and growth factor signals will be sent to the intracellular passage through the cell membrane of the nucleus and the HER-2 gene will be activated (Brennan *et al.*, 2000). HER-2 protein receptor was prepared with ageratochromene. Validation results with Autodock Vina showed that the docking protocol of ageratochromene dimer, doxorubicin and native ligand was acceptable with RMSD <2.00 Å. The RMSD is the value of deviation between a ligand conformation with its comparator, that is, if the deviation was too large then the predictive error of ligand and protein interaction (Korb, *et al.*, 2006).

The molecular docking results showed that the docking score obtained by ageratochromene (-6.2 kcal/mol) was higher than doxorubicin (-6.9 kcal/mol) and HER-2 native ligand (-6.8 kcal/mol). It could be said that the energy required to interact ageratochromenedimer with HER-2 was greater so that the bonds formed are less stable than doxorubicin or the native ligand. The role of HER-2 protein in cell proliferation process using Phospatidyl Inositol 3-Kinase (PI3K) pathway, Kinase/Signal Transduction Activator Transcription (Jak/Stat) and Mitogen Activated Protein Kinase (MAPK), known to ageratochromene can inhibit PI3K. In the process of proliferation of HER-2 protein cells through the Raf proteins are involved and pass through various induced signal pathways. Ageratochromene may damage or degrade HER-2 protein by a mechanism that is still unknown, so cell proliferation can be inhibited. HER-2 protein can activate Ras, Raf, MAPK protein, furthermore increase cell proliferation by activating transcription factor. The proliferation process does not progress to the nucleus due to a decrease in the activity of Ras, Raf and MAPK proteins caused by destruction of HER-2 protein by ageratochromene dimer. Estrogen receptors present in nucleus, cytoplasm and cell membrane account for about 70% of breast cancer cells. Positive estrogen receptors play an important role in cell prolif-

Table 6. Molecular Docking Result

Protein	Test Compound	RMSD (<2.00 Å)	Score*docking*	Conformation
HER-2 (PDB ID: 3PP0)	Ageratochromene Dimer	1.818	-6.2	8
	Doxorubicin	1.572	-6.9	2
	Native Ligand	1.795	-6.8	5

Table 7. Docking Visualization

	Ageratochromene dimer	Doxorubicin	Native Ligand
HER-2			

eration in breast cancer (Chen, 2008).

The types of bonds formed between amino acids and test compounds include covalent bonds and Van Der Waals bonds, in which covalent bonds are stronger than those of other bonds. In addition, covalent bonds are the only irreversible bonds that provide the greatest contribution to the decrease in bonded affinity energy versus hydrogen bonding and Van Der Waals (Patrick, 2001). Covalent bonds tend to be formed from ionic residues (Table 7), polar amino acids form hydrogen bonds, while aromatic and hydrophobic amino acids usually form Van Der Waals bonds, so bonds with ionic residues contribute the most to the decrease in the affinity energy of test compounds because they form bonds covalent (Schneider *et al.*, 2008). Visualization of docking results with DS Visualizer showing that ageratochromene compounds binding to 5 amino acids have the highest number of amino acid ions with 4 amino acids and 1 hydrophobic amino acid, while the original Ligand binds to 8 amino acids with 2 ionic amino acids so that ageratochromene has a less stable bond than doxorubicin or native ligands. Based on affinity energy values and docking visualization, the ageratochromene compound has a stronger and more stable bonding potential of the HER-2 protein.

Conclusion

The n-hexane fraction of bandotan herb has weak potency as antioxidant and single cytotoxic activity but has synergistic effectwith doxorubicin. NFB has potency to develop as co-chemoterapeutic agent with doxorubicin in MCF-7 breast cancer cell line based on *In vitro* assay. The ageratochromene dimer as the active compound has weak potency to develop as anticancer agents based on *in silico* assay.

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