Cytotoxic Effect of Ethanolic Extract of Temu Kunci (*Kaempferia pandurata*) and Sirihan (*Piper aduncum* L.) on Breast Cancer Line

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Abstract

Chalcones and their derivates have been shown to have potency as anticancer. Chalcone is a flavonoid compound that is found in temu kunci (*Kaempferia pandurata*) and sirihan (*Piper aduncum* L.). This study aim is to know the cytotoxic effect of the ethanolic extract of *K. pandurata* rhizome and *P. aduncum* L. leaves towards breast cancer cell line T47D. Measurement of ethanolic extract of flavonoid was done by using cellulose for TLC and n-buthanol: acetic acid: water (4:1:5) for the mobile phase and compound cluster detection using citroboric acid reagent and ammonia. Colorimetric test 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay is used to monitor the cytotoxicity effect. The screening for the compound, found in the plants was done using molecular docking. *K. pandurata* rhizome and *P. aduncum* L. leaves ethanolic extract shows a cytotoxic effect toward breast cancer cell with it's IC50 value as much as 66 µM and 68 µM. The cytotoxic effect is then connected to it's result of molecular docking using ArgusLab among it's chalcone compound in plants toward tubulin activity and estrogen receptor α. Furthermore, these docking processes were obtained the lowest scoring value in panduratin A both in tubulin and estrogen receptor α. This results were then compared with other experimental ligands, and showed that the compound interaction was stronger than the colchicine and raloxifen. This shows the probability of chalcone having more of an effect toward tubulin and estrogen receptor α in inducing cytotoxicity.

Key word: breast cancer, chalcone, tubulin, estrogen receptor, docking

Introduction

Chalcones and their derivates are a group of compounds reported to exhibit promising anticancer activity (Achanta, et al, 2006). Chalcone compound which is consisted in temu kunci (*Kaempferia pandurata*) (Sohn, *et al*, 2005) and sirihan (*Piper aduncum)* (Orjala, 1994) was predicted to have potency as an

anticancer agent. The cancer occurred because of the genetic alteration accumulation, especially the growth regulator genes such as oncogene. Besides, there are some important receptors which have a significant role in breast cancer progression such as tubulin protein and estrogen receptors (King 2000).

Based on the characteristics of the chalcone, it is needed to screen the active compound in those plants through molecular docking. The docking software was used to predict the ligand-receptor interaction. The method is designed to know the affinity and interaction between chalcone and its derivates with tubulin and estrogen receptors by ArgusLab. Cytotoxic test was done to the ethanolic extract of *K. pandurata* and *P. aduncum* on T47D cancer cell, to see the correlation between in silico and in vitro analysis method. Further, the result can be used as a basic for the development *K. pandurata* and *P. aduncum* L. as chemoprevention agents.

Materials and Method
Materials
The plants have been determined and obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TO2T) Karanganyar, Central Java. Plant were obtained on March 2007. A collection of breast cancer which was provided from NAIST, Japan. Chemicals are ethanol 70% (General Labora), culture medium DMEM (Gibco), DMSO 100% (Merck), reagent MTT 5 mg/mL, stopper solution Sodium Dodecyl Sulphate (SDS) 10% in HCl 0,01 N (Merck).

Materials for Molecular Docking
1). The chemical structure of chalcone: panduratin A, piperaduncin B, dihydroxi-4'-methoxychalcone (DMC) and 2',6',4–trihydroxi-4' methoxydihydroxychalcone (asebogenin).

2). A set of three dimensional ligand-receptor structure of human tubulin-colchicine and estrogen receptor α-raloxifen were retrieved from the Protein Data Bank (http://www.rcsb.org/pdb); accession codes 1SA0 for tubulin-colchicine, 1GWQ for estrogen receptor α-raloxifen, respectively.

Ethanolic extract
The powdered of the plants were extracted with ethanol 70% through maseration then continued with vacuum evaporation to get the concentrated extract.

Ethanolic Extract of Flavonoid Measurement
Analysis of the ethanolic extract was done by thin layer chromatography using cellulose stationary phase and n-buthanol:

acetic acid: water (4:1:5) as mobile phase. Detection was done by citroboric acid reagent and ammonia. The chromatogram is then visually analyzed under visible ray and UV ray 254 and 366 nm to observe the stain.

Cytotoxic test

Series of concentration of the extract are 500 µg/mL, 400 µg/mL, 300 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, and 10 µg/mL. The concentration of DMSO is 0,125% which is at concentration 500 µg/mL. Breast cancer cell line which was stored in subcultures in nitrogen tank then distributed into the well with density 6.075×10^5 cell/mL, and incubated for 24 hours. At the end incubation, MTT was added to each well and place the well protected from light et room temperature. The live cell will react with MTT creating purple colour. MTT reaction is stopped by adding SDS stopper reagent and then incubated overnight at room temperature. Absorbance is observed by using ELISA reader at 595 nm.

Molecular Docking

The geometric optimization of compound structure test was conducted by using Arguslab program. The in-silico chemical structure validation was conducted by calculating the RMSD value (Root Mean of Standard Deviation) from the docking result of ligand (colchicines and raloxifen) and ligand copy at the receptors. Next, each chemical compound test was docked at a place of cordage experimental ligand-receptor, and then we were *scoring* and identifying its interaction (Anonim, 2004).

Data Analysis

The obtained data (absorbance) are converted into % live cell then statistically analyzed using correlation test method which is continued by signification test to observe the difference of signification among control group and also experimental group. Live cell percentage be counted using formula:

% viable cells = $\dfrac{\text{absorbance cell wih treatment} - \text{absorbance medium}}{\text{Absorbance cell control} - \text{absorbance medium}} \times 100\%$

Then the IC50 concentration were counted using linear regression method. IC50 is the concentration which cause fatal death among it's 50% of all population to show cytotoxicity potential.

The output of the docking process was a form of scoring expressing the strength of ligand-receptors interaction with the parameter RMSD. The software used is good if the RMSD value was smaller than two (≤ 2). The data analysis was conducted by comparing

the scoring from every compound and the experimental ligand and then identified its interaction which was shown with hydrogen bond.

Results

Ligand-Receptor Interaction Study by Molecular Docking

The three dimensional structure of chalcone which is consisted in *K. pandurata* and *P. aduncum* L. were conducted in a geometric optimization using various semi empirical quantum chemical methods. It was conducted to find the stable molecule structure owning the lowest potential energy. The most reliable semi-empirical method which was used for the structure of chalcone and its derivate were the AM1 and PM3 method (Figure 1).
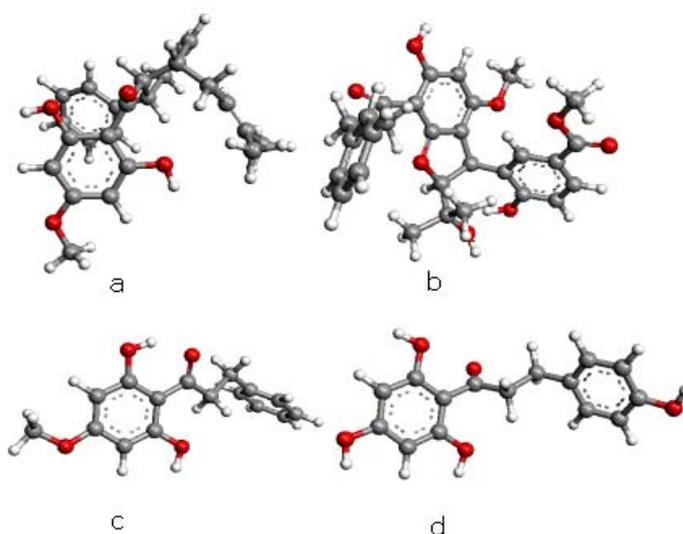

Figure 1. Structure of the chalcone compounds from geometry optimization. (a) panduratin A, (b) piperaduncin B, (c) DMC, (d) asebogenin. Geometry optimization was done using AM1 and PM3 semi-empirical method. The structure that has been stable can be used for docking.

The lowest form of ΔHf value, a compound is asumed stable if it's ΔHf is the lowest. In experiment, optimization is done by using AM1 and PM3 to find its ΔHf value. Then both value is compared with another. Experimental results show that the structure of panduratin A, piperaduncin B and dihydroxi-4'-methoxychalcone (DMC) are most stable with semi-emphirical PM3 method. Meanwhile asebogenin is most stable with semi-emphirical AM1 method (Table 1). The structure with the lowest ΔHf value was then used for docking analysis.

The validation step was conducted to calibrate the docking method in the software we used. The method which was compared to

be Argusdock and GADock (Genetic Algorithma). Both of these

Table 1. Entalphy formation value (ΔHf) compound test

Structure	ΔHf (kcal/mole) based on methode PM 3	ΔHf (kcal/mole) based on methode AM1
panduratin A	-97.7512430	-94.7861763
piperaduncin B	-259.6015037	-254.7305311
DMC	-124.2902851	-123.1297700
asebogenin	-166.9463791	-168.7447479

methods have the difference in the case of the molecular approaching. Argusdock method used the approach of structure and ligand which were docked and only aimed at a certain position. While GADock method, the ligands were docked and aimed at various conductive position, so this method have a non-reproducible character.

The purposes of docking are to study and to compare the potency of chalcone as tubulin blocker. The parameter to configure the interaction strength of ligand-receptor was the scoring. Besides docked to the tubulin, the compounds were also docked to estrogen receptor α. Docking to the experimental ligand was also conducted to compare the interaction strength with the tested compound (Table 2).

Table 2. Docking score of the chalcone compound*

Ligand	Tubulin (1SA0)	ER-Alpha (1GWQ)
Ligand experimental	-6.89	-8.84
Panduratin A	-11.47	-12.65
Piperaduncin B	-9.83	-
DMC	-9.72	-10.12
Asebogenin	-8.94	-9.97

*Docking was run by using ArgusLab with experimental ligand's binding site. The chalcones ligand was docked in ligand's binding site box. Docking was done to receptors: tubulin-colchicine, ERα-raloxifen. Score of the chalcone are compared to experimental ligand's score each receptor.

Based on table 2, the score of panduratin A have the lowest value, indicating that the compound can interacted at tubulin with a larger affinity compared to colchicine. At the estrogen receptor α, score of panduratin A was lower compared to the experimental ligand raloxifen.

The Arguslab program has the ability to show the hydrogen bond between ligand with amino acid residues at receptor, the length of bonding and the ligand structure to the amino residues (Figure 2).

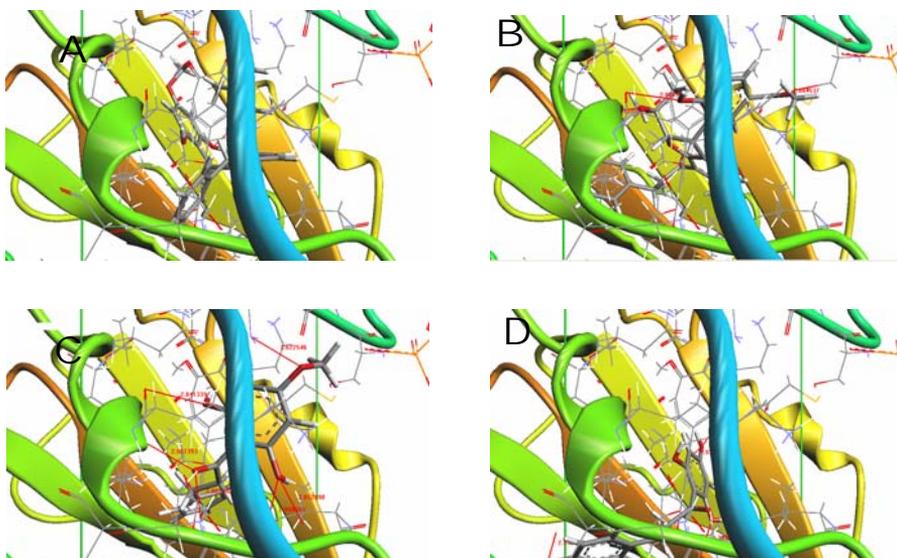The hydrogen bonds represent one of the determinants of the scoring value.

Figure 2. Chalcone compounds interaction on tubulin. Docking was run by using ArgusLab with colchicine's binding site on tubulin. (a) Panduratin A, (b) Piperaduncin B (c) DMC, (d) asebogenin

The Measurement of the Ethanolic Extract of Flavonoid

The measurement is performed to find the active compound having cytotoxic activity. TLC result show that flavonoid compound exists in the ethanolic extract detected by dark red with ammonia and gave positive result with citroboric acid reagent.

Cytotoxic Test using MTT Method

The data obtained was converted to percent of living cell. Cytotoxic parameter used is MTT substrate conversion ability to formazan by sucsinic dehydrogenase. A linear correlation between sample solution concentration and the amount of dead cells is obtained figure 3, shows that there is a dose-dependent phenomenon, which higher sample solution concentration gives lower living cell percentage is also lower.

From cell living percentage data versus concentration, inhibition concentration 50 can be measured. IC50 value obtained is 66 µg/mL (*K. pandurata*) and 68 µg/mL (*P. aduncum* L.). Showing that *K. pandurata* extract is more potent than *P. aduncum* L extract.
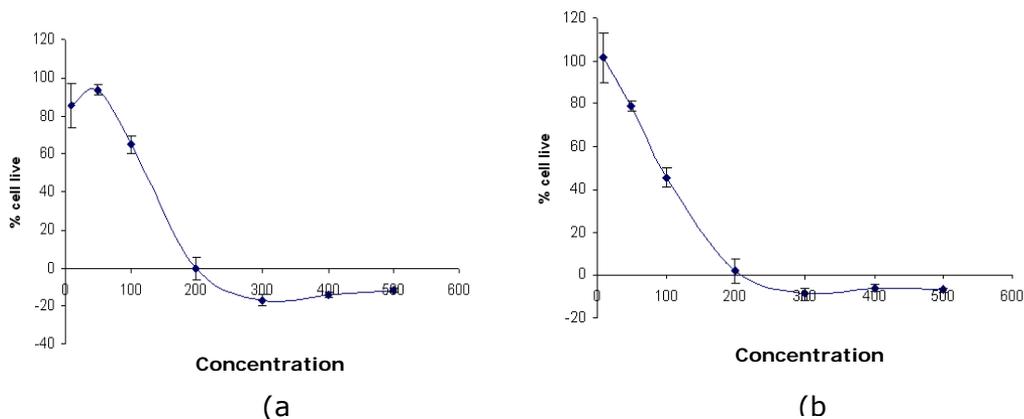
(a

(b

Figure 3. Cytotoxicity effect of tested extract on cell T47D. Cell T47D placed on 96 well-plate then treated with extract on (10; 50; 100; 200; 300; 400; 500) μg/mL preceeded with reading on ELISA reader on λ 595 nm after being incubated for 24 hours. Cytotoxicity of extract stated with % value live shown as mean ± SD from three experiments. The higher concentration of tested solution so the lower live percentage observed. (a) extract of *K. pandurata* rhizome (b) extract of *P. aduncum* L. leaves.

Discussion

This research show that the ethanolic extract of *K. pandurata* rhizome and *P. aduncum* L. leaves, have cytotoxic activity on breast cancer cell T74D. To find the possible mechanism facilitating the effect of the extract to the cell, docking analysis has been performed. The compound with highest interaction on tubulin and estrogen receptor α is panduratin A, which is one of *K. pandurata* constituents. If panduratin A is able to act as an antagonist with colchicine and raloxifen, the signal transduction cascade connected to both receptors can be inhibited. This is based on the antagonist property, and the effect can not be produced if it is binded to the receptor. There are many pathways in breast cancer therapy. However, based on this research, the active compound can be used in the development of anticancer drugs.

K. pandurata extract possesses the least IC50 value among the extracts. This can be explained by the docking result, which shows that chalcone obtained from the extract is highly interact with tubulin

and estrogen receptor α. Panduratin A scoring value is also lower than the experimental ligand on tubulin the probability that chalcone compound is more effective on tubulin and estrogen receptor α in inducing cytotoxicity. The IC50 of *P. aduncum* L. extract possesses is much different from *P. aduncum* L. extract. The docking result shows all of the three chalcone in the extract is lower than the experimental ligand, which is mean that the interaction with receptor is higher. This is indicating that in silico method is consistent with in vitro method.

Docking results on tubulin shows that chemical compounds with chalcone's frame can interact with tubulin on the pocket binding site of colchicine. Beside that, this compound also has high affinity on tubulin. Therefore, it can be predicted that this compound has similar mechanism with colchicine in inhibiting tubulin's action. Competition on colchicine binding site of tubulin by this compound allows chalcone bind on the location that inhibit formation of bending tubulin from straight tubulin so α- and β-tubulin's unity is inhibited (Hadfield, 2003). The result is mitotic spindle during mitotic can not be constructed, while the microtubule has an important role and act as critical point during this process. Therefore chalcone is considered able to stop cell cycle and inhibit proliferation.

T47D cell's proliferation is affected dominantly by estrogen and HER-2, so it's both activities becomes an affective target in producing cytotoxic effect (King, 2000). Estrogen binds with estrogen receptors formed active receptor complex and effect gene transcription which regulate cell proliferation. If reviewed from chalcone and it's derivatives with amino acid on ligand binding site, that compound can binds with estrogen receptors and formed different conformation, compared when estrogen hormone binds with that receptor.

However, those possible mechanism need to be proven through next research. Primarily on testing pure compound obtained from isolation or synthesis on breast cancer's cell. The compound responsible for cytotoxic activity also needed further research.

The future prospect, clinical proves of ethanolic extract of *K. pandurata* and *P. aduncum* L. potency in inducing the cytotoxicity is still need to be followed up. Ethanolic extract of *K. pandurata* and *P. aduncum* L. can acted as a chemoprevention agent and assisting cancer therapy. This research shows that the ethanolic extract of *K. pandurata* and *P. aduncum* L. is a promising chemopreventive agent with potent IC50 value.

Conclussion

Chalcone compound in ethanolic extract of *K. pandurata* and *P. aduncum* L. possess cytotoxic property and potent to fight breast cancer cell. Ethanolic extract of *K. pandurata* and *P. aduncum* L. can act as a chemoprevention agent.

Acknowledgments

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