

# Antiproliferative Effect of Secang Heartwood Ethanolic Extract (*Caesalpinia sappan* L.) on HER2-Positive Breast Cancer Cells

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## Abstract

Human Epidermal Growth Factor Receptor 2 (HER2) plays critical role in the initiation of breast cancer cell proliferation. Inhibition of HER2 overexpression is a promising strategy in the treatment of breast cancer. One of the potential natural substances that possess cytotoxic in cancer cells is Secang (*Caesalpinia sappan* L.). Ethanolic extract of secang heartwood containing brazilein and brazilin shows cytotoxic effect on various cancer cells. Thus this research aims to determine the proliferative inhibition activity of secang heartwood ethanolic extract (SEE) through HER2 expression on MCF-7/HER2 positive. Cytotoxic activity of SEE on MCF-7/HER2 positive cells was observed by MTT assay, meanwhile the inhibition activity of HER2 on MCF-7/HER2 cells was determined by immunofluorescence assay. Furthermore, to confirm the possible mechanism of brazilein and brazilin for its cytotoxic activity was studied through molecular docking using PLANTS software. Based on this study, SEE exhibited cytotoxic effect on MCF-7/HER2 positive cells with IC<sub>50</sub> value of 25 µg/ml, and EES also inhibited HER2 expression, indicated that the antiproliferative effect of SEE was possible through the inhibition of HER2. Docking score of brazilein, brazilin, and native ligand on HER2 were -77; -73; -120 respectively, showed the affinity of brazilein in HER2 was lower than affinity of native ligand, but they had specific amino acid to interaction. Hence, SEE can be developed as one of promising chemopreventive agent from natural products.

**Keywords:** *Caesalpinia sappan* L., cytotoxic, immunofluorescence, molecular docking, MCF7/HER2 cells

## INTRODUCTION

Human Epidermal Growth Factor Receptor 2 (HER2) protein is known to inhibit the anti-apoptotic protein, causing proliferation of breast cancer cells continuously (Siddiqi, *et al.*, 2008). At least, there are 15-20% of breast cancer patients occur show HER2 overexpression (Perou, *et al.*, 2006). HER2 overexpression causes breast cancer cell growth becomes uncontrolled and abnormal (Laskin and Sandler, 2004). Therefore in present research, targeting HER2 protein become one of focuses in anticancer drugs finding. One of the local natural products that have been studied and showed potency as a cancer chemoprevention agent is a Secang (*Caesalpinia sappan* L.). Previous studies

reported that the active compounds in the Secang has possessed cytotoxic effect on MCF-7 breast cancer cells with IC<sub>50</sub> value of 37 µg/mL (Khamsita, 2012). However, possible molecular mechanism which responsible for cytotoxic effect on breast cancer from secang remains unclear. Thus, this study aims to explore secang as antiproliferative agent on breast cancer cells through inhibition of HER2 expression.

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## MATERIALS AND METHODS

### Sample Preparation and Extraction

Secang heartwood were collected from Yogyakarta, Indonesia and determined in Departement of Pharmaceutical Biology, Faculty of Pharmacy UGM.

Dried powder was extracted by maceration for 5 days with 70% ethanol and continued by remaceration for the next 3 days. Collected filtrate were concentrated using rotary evaporator to get viscous extract.

### Cell Culture

MCF-7/HER2 cells were collection of Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, UGM, Indonesia. The cells were kindly given from The Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancrer, Tohoku University and The Japanese Center Research Resources Bank. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Sigma) supplemented with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1.5% Penicillin-Streptomycin and 0.5 % Fungizone (Gibco) and maintained with hygromycin (Gibco). Cells were incubated in 37°C incubator with 5% CO<sub>2</sub>.

### Cytotoxic assay

MTT assay was used to determine the cytotoxic effect of secang heartwood ethanolic extract (SEE) on MCF-7/HER2 cells. Cells were distributed to 96-well plate with the density of  $5 \times 10^3$  cells/well and incubated in 37°C with 5% CO<sub>2</sub> for 24 hours. A serial concentration of SEE was diluted on DMEM with DMSO (Sigma) as cosolvent to determine IC<sub>50</sub> value of EES. After 24 hours of incubation, 3-(4,5-di methyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) was added and followed by 4 hours incubation to form formazan crystal before diluted with 10% v/v SDS in 0.1N HCL (Merck) as stopper reagent. Plate was kept with protection from light for overnight. The intensity of the color

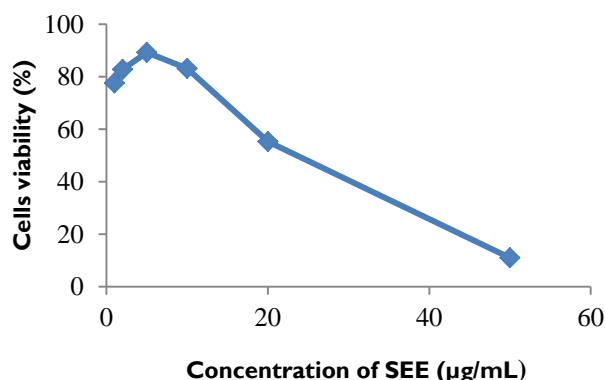
formed was measured by ELISA reader at  $\lambda$  595 nm using ELISA reader (Bio-Rad). IC<sub>50</sub> value, a concentration which inhibit 50% cell population, was calculated from linear regression of log concentration and cells viability.

### Immunofluorescence Assay

HER2 expression was observed by immunofluorescence assay. Confluent MCF-7/HER2 cells were harvested with trypsin-EDTA and distributed with a density of  $5 \times 10^4$  cells/well in 24-wellplate and incubated for 24 h at 37°C, 5% CO<sub>2</sub>. The cells were treated with sample and incubated again. After 15 hours, cell were fixed by 70% ethanol and incubated for 15 min at room temperature. After rinsed with PBS, cells were incubated with blocking serum 1% BSA for 30 min at room temperature. Then, cells were incubated with primary antibody (HER2) for 1 hour at room temperature. After rinsed with PBS, cells were incubated with secondary antibody conjugated by FITC for 1 hour at room temperature in the dark. Then, cell were added by DAPI solution and incubated for 10 min at room temperature in the dark. After rinsed with PBS, cell were added with mounting solution (fluoromount), put on slide glass, and store at 4°C. The protein expressions were observed under fluorescence microscope.

### Molecular Docking

*In silico* study by molecular docking was conducted to examine the affinity of certain ligand to its docking site by evaluating drug-receptor binding energy. Evaluation of the interaction between a molecule and its docking protein involved in particular signal transduction may represent its potential biological activity and allow us to determine the possible mechanism of action. In this study, the docking of brazilin and brazierin as major compound in SEE to HER2 ATP Binding Site was observed, using lapatinib as a comparison. Ligands preparation were done by using Marvin Sketch.



**Figure 1. Cytotoxic effect of SEE on MCF7/HER2 cells.** Briefly  $5 \times 10^3$  cells/well of MCF-7/HER2 cells were seeded in 96 well-plate and incubated for 24h, then treated with SEE. Cells viability was determined by using MTT assay as described in the methods. Cell viability profile after treatment of SEE for 24 h on MCF-7/HER2 cells.

The structure of HER2 protein (3PPO) complex was taken from Protein Data Bank (PDB) ([www.rcsb.org](http://www.rcsb.org)). Protein preparation was done by using YASARA. Molecular docking was conducted by using PLANTS (Protein-Ligand Ant System) Software, giving  $\Delta G$  as docking score result.

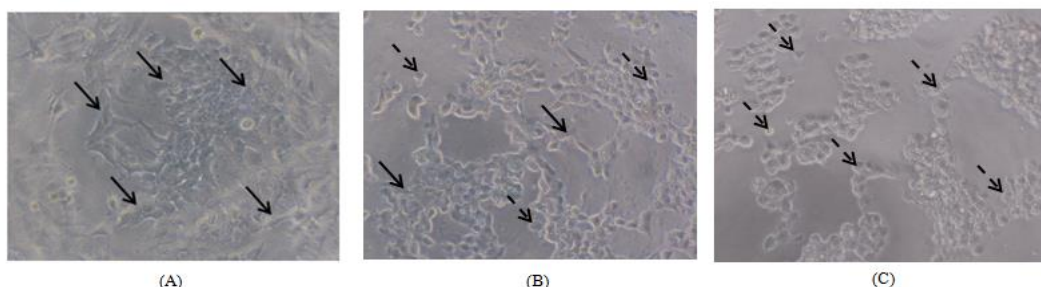
## RESULTS AND DISCUSSION

### Cytotoxic Effect of SEE on MCF-7/HER2 Cells

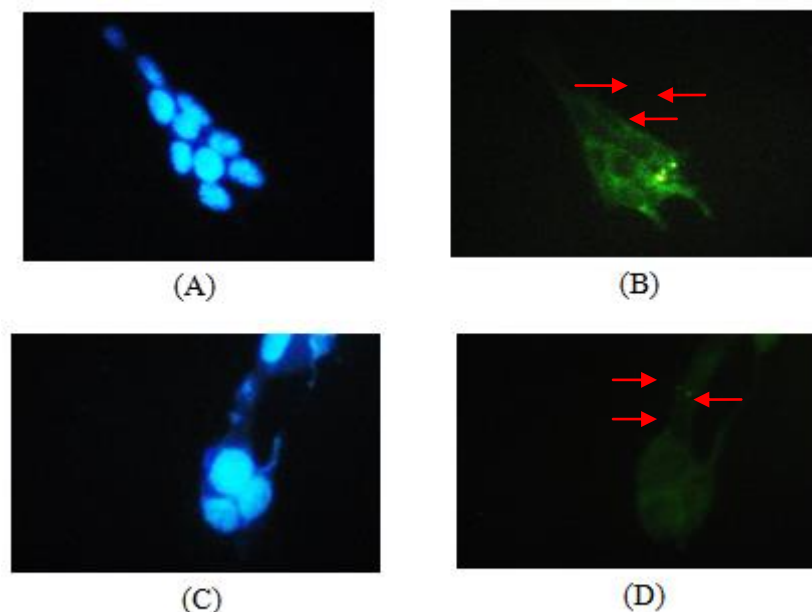
MTT assay was used to evaluate the cytotoxic effect of SEE on breast cancer with HER2 protein overexpression. With  $IC_{50}$  value as parameter. Cytotoxic effect of SEE was observed after 24 h of treatment. Single treatment of K PGV-0 for 24 h induced

morphological changes at dose dependent manner as seen in Fig. 2. Based on MTT assay, SEE was potently inhibited cells with  $IC_{50}$  value of 25 µg/mL on MCF-7/HER2 breast cancer cells.

This result added the potency of SEE as antiproliferative agent towards cancer cells. Previously, single treatment of SEE showed cytotoxic effect on MCF-7 breast cancer cells with  $IC_{50}$  value of 37 µg/mL (Khamsita, *et al.*, 2012). The data is interesting as SEE possessed more cytotoxic effect on breast cancer cells with HER2 overexpression than wild type one. Hence, the possibility of cell proliferation with modulation of HER2 protein was investigated through immunofluorescence assay.



**Figure 2. Cytotoxic activity of SEE on MCF-7/HER2 breast cancer cells.** Cells morphological with or without SEE treatment after 24 h incubation. Cells were observed under inverted microscope and documented with 400x magnification. (A) cell control, (B) SEE 10 µg/mL, and (C) SEE 50 µg/mL. Alive cells were marked by arrow (→) while cells with morphological change were shown with (→).



**Figure 3. The expression of HER2 protein by immunofluorescence after treatment with SEE.** Cells were treated with SEE for 24 h before immunofluorescence as described on method. Cells were observed under fluorescent microscope with 400x magnification. On the picture (A) DAPI control, (B) Alexa 488 anti-mouse control, (C) SEE with concentration of 12,5 µg/mL in DAPI staining, (D) SEE with concentration of 12,5 µg/mL with Alexa 488 anti-mouse staining. The changes in expression was marked with red arrow →

Based on the experiment in MCF-7/HER2 cells, single treatment with SEE with concentration of 12.5 µg/mL reduced the expression of HER2 protein compared to control group (Fig. 3). The result was correlated to the cytotoxic effect of SEE, indicated that there was possibility that SEE played a critical role in HER2 protein expression. The result was supported by previous study done by Laksmiani, *et al* (2014) who did interaction between brazilein as major compound in SEE with HER2 receptor by molecular docking. Brazilein was able to interact with HER2 in ATP-binding site in hydrophobic region with amino acid residue Tyr 91, which had similar interaction with native ligand. Brazilein was also reported to inhibit nfκB, one of transcription factor of HER2 that caused inhibition of several protein that regulate cells proliferation (Konkimalla, *et al.*, 2009; Hsieh, *et al.*, 2013). Hence, SEE possessed cytotoxic effect on breast cancer though inhibition on HER2 protein.

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