Cytotoxic activity of hantap (Sterculia oblongata Mast) leaves extract against breast cancer cells line (MCF7/HER2): the effect on the expression of HER2 mRNA and the apoptosis

Sitti Ayu Suhartina Yahya1*, Mustofa2, Indwiani Astuti2, Woro Rukmi Pratiwi2, Adika Suwarman3
1Master in Biomedical Sciences, Faculty of Medicine, Public Health and Nursing, Universitas Gajah Mada, 2Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gajah Mada, Yogyakarta, Indonesia.
https://doi.org/10.22146/ijpther.3204

ABSTRACT
Hantap leaves (Sterculia oblongata Mast) has been used traditionally to treat breast cancer in Palu, Central Sulawesi. However, its use is just based on empirical evidences rather than scientific evidences. The study aimed to investigate the cytotoxic activity of hantap leaves extracts against breast cancer cells line. The effect of this extract on the HER2 expression and the apoptosis was also evaluated. The hantap (S. oblongata Mast) leaves extracts were prepared by consecutive maceration method using n-hexane, methanol and water, respectively. The cytotoxic activity against MCF7/HER2 breast cancer cells line was evaluated using the MTT assay with doxorubicin as a positive control. The HER2 mRNA expression was examined using RT-PCR and the apoptosis after 24 h incubation was examined using a fluorescence microscope after AO-PI (acridine orange-propidium iodide) staining. Among three extracts tested, the methanolic extract exhibited the most cytotoxic against MCF7/HER2 cells with an IC50 of 91.25 µg/mL. Therefore, the methanolic extract was subjected to further study. The methanolic extract at concentration of 1/2IC50; IC50 and 2IC50 µM induced 6.8; 26.3 and 25.3% apoptosis of the MCF7/HER2 cell lines, respectively. The methanolic extract at concentration of 1/2IC50; IC50 and 2IC50 µM inhibited HER2 mRNA expression to be 0.6; 0.25 and 0.33 compared to control cells. In conclusion, the methanolic extract of hantap leaves (S. oblongata Mast) has cytotoxic activity against MCF7/HER2 breast cancer cell lines by induce cells apoptosis and inhibit HER2 mRNA expression. Further study, will be conducted to isolate active constituents as anticancer.

ABSTRAK
Daun hantap (S. oblongata Mast) telah digunakan secara tradisional untuk mengobati kanker payudara di Palu, Sulawesi Tengah. Namun, penggunaannya masih didasarkan bukti empiris daripada bukti ilmiah. Penelitian ini bertujuan untuk mengetahui aktivitas sitotoksik ekstrak daun hantap (S. oblongata Mast) terhadap sel kanker payudara. Ekstrak daun hantap (S. oblongata Mast) dibuat dengan cara menakar berturut-turut menggunakan pelarut n-heksan, methanol dan air. Aktivitas sitotoksik terhadap sel kanker payudara MCF7/HER2 dikaji menggunakan metode MTT dengan doxorubicin sebagai control positif. Ekstrak HER2 mRNA ditetapkan menggunakan RT-PCR dan apoposis setelah inkubasi 24 jam diamati dengan mikroskop fluoresen setelah pengecatan dengan AO-PI (acridine orange-propidium iodide). Di antara tiga ekstrak yang diuji, ekstrak methanol merupakan ekstrak yang paling toksik terhadap sel MCF7/HER2 dengan nilai IC50 sebesar 91.25 µg/mL. Oleh karena itu, ekstrak methanol digunakan untuk uji selanjutnya. Ekstrak methanol konsentrasi 1/2IC50, IC50 dan 2IC50 menginduksi berturut-turut 6.8; 26.3 dan 25.3% apoptosis sel MCF7/HER2. Ekstrak methanol pada konsentrasi 1/2IC50, IC50 dan 2IC50 menghambat berturut-turut ekspresi HER2 mRNA sebesar 0,6; 0,25; dan 0,33 dibandingkan kontrol. Dapat disimpulkan bahwa ekstrak methanol daun hantap (S. oblongata Mast) mempunyai aktivitas sitotoksik terhadap sel kanker payudara MCF7/HER2 dengan menginduksi apoptosis dan menghambat ekspresi HER2 mRNA. Penelitian lanjutan akan dilakukan untuk mengisolasi kandungan aktifnya sebagai antikanker.

*corresponding author: sittiyayusuhartina.yahya8596@gmail.com
INTRODUCTION

Cancer is a disease associated with abnormality and an uncontrolled growth of cells. One of the most common types of cancer in the world is breast cancer. In 2020, it was reported 2.3 million women diagnosed with breast cancer and 685,000 deaths globally. Breast cancer is the most prevalent cancer in the world in the past 5 years with 7.8 million cases. In Indonesia in 2020, 396,914 new cases with 234,511 were reported in 2020. The prevalent cases in the past 5 years was 946,088.

Based on the expression pattern of certain gene, breast cancers are usually divided into five intrinsic or molecular subtypes. HER2+ breast cancer is one of subtype of breast cancers which characterized by the high expression of the HER2 receptor and make up 10-15% of breast cancer. In contrast to normal breast cancer cells which express approximately 20,000 HER2 receptors on the cell membrane, the HER2+ breast cancer cells, express more than 2 million HER2 receptors. This high expression of HER2 receptor is associated malignancy, poor prognosis and high mortality.

Genetic and lifestyle/environmental factors are associated in the aetiology of breast cancer. Genetic mutations in the cancer cells lead to defects in cell-signaling systems that initiate apoptosis. Apoptosis is the process programmed cell death that occur to eliminate unwanted cells. It is mediated by several intrinsic and extrinsic signalling pathways triggered by multiple factors, including cellular stress, DNA damage and immune surveillance. For last decades, apoptosis has been investigated as novel target for cancer treatment.

Medicinal plants have been used traditionally to treat cancer in some regions in the world. Among the medicinal plants have been evaluated their in vitro and in vivo anticancer activity. Hantap leaves (Sterculia oblonga, Mast) has been used traditionally as an alternative therapy for breast cancer in Palu, Central Sulawesi. However, its used as anticancer just based on empirical evidences rather than scientific evidences. A biological activity study reported, hantap leaves extracts have antioxidant activity. Furthermore, phytochemical analysis reported, hantap leaves contained biological active compounds such as tannins (1.24%), alkaloids (10%), flavonoids (3.4%), saponin (4%), oxalate (1.31%), cyanogenic glycoside (0.6%) and phenol (0.006%).

This study aimed to evaluate the cytotoxic activity of the leaves extract of hantap (S. oblonga Mast) against breast cancer cells line (MCF7/HER2). Furthermore, the effect of this extract on apoptosis and HER2 expression were also evaluated.

MATERIALS AND METHODS

Extracts preparation

The protocol of the study was approved by the Medical and Health Research Ethic Committee, Faculty of Medicine, Public Health and Nursing/Dr. Sardjito General Hospital, Yogyakarta (reff. KE/FK/1213/EC on November 5th, 2020). The plant leaves were collected from their natural habitat in Palu, Central Sulawesi and were identified and authenticated in Universitas Tadulako, Palu, Central Sulawesi. The plant leaves were air-dried at room temperature and grounded into powder. Extracts were prepared by consecutive maceration method using n-hexane, methanol and water, respectively. One hundred fifty g of the leaves powder was macerated in n-hexane, with intermittent stirring, for 24 h at room temperature. The extracts of n-hexane were then filtered and concentrated to dryness using a rotary evaporator. Extracts were prepared by consecutive maceration method using n-hexane, methanol and water, respectively. One hundred fifty g of the leaves powder was macerated in n-hexane, with intermittent stirring, for 24 h at room temperature. The extracts of n-hexane were then filtered and concentrated to dryness using a rotary evaporator. Dry n-hexane extracts were stored at 4 °C until further analysis. The residue obtained after maceration in n-hexane was further macerated consecutively with methanol and water. The methanol and water extracts obtained in each maceration step were
collected, dried and stored at 4 °C until further analysis.

Cytotoxicity assay

The cytotoxic activity was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. Briefly, each extract was pre-solubilized in dimethyl sulfoxide (DMSO) at 37 °C to obtained a stock solution. Serial dilutions of the test extracts were prepared from the stock solution to obtained working concentration of 400 – 6.25 µg/mL with the final concentration of DMSO was not higher than 1%. Confluent monolayers of breast cancer cells line (MCF7/HER2) were grown in 96 well microplates for 24 h. Cells were incubated with various concentrations of the test extracts in triplicate at 37 °C in a CO₂ environment for 24 h. The negative control was growth medium cell culture alone instead of plant extract, whereas doxorubicin was used as the positive control. Total RNA was extracted by using the Favorgen kit according to the manufacture's instruction. The extracted RNA was quantified using UV spectrophotometry at λ of 260 and 280 nm and then stored at -80 °C. To synthesize cDNA, 2 µg of the extracted RNA, 1 µL of oligo dT/random primer, 8 µL of DEPC-treated H₂O were mixed in a microtube. After incubation at 70 °C for 5 min ad 4 °C for 1 min, 4 µL 5X RT buffer (dNTPs), 5 µL DEPC-treated H₂O and 1 µL RNase were added for a final volume of 10 µL. The cDNA synthesis conditions were 25 °C for 10 min followed by 37 °C for 50 min and 80 °C for 5 min. The cDNA samples were then stored at –20 °C until analysis.

Real time PCR

To determine the HER2 expression, 1 µg of cDNA were reversed transcribed by using the ExelRT™ Reverse Transcription Kit II according to the manufacture's instruction. PCR reactions used PROMO ExcelTaq™ 2X Fast Q-PCR Master Mix (SYBR). PCR conditions were 2 s at 95°C, 15 s at 95°C, and 60 s at 60°C for 40 cycles. HER2 transcripts were detected using following primers. HER2-F: 5’-CCAGGACCTGCTGAACTGGT-3’; HER2-R: 5’-TGTACGAGCCGCACATCC-3’; and using glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-F: 5’-GAAGATGGTGATGGGATTTC-3’, GAPD-R: 5’GAAGGTGAAGGTCGGAGTC-3’ as endogenous reference. The HER2 expression was quantified following the analysis of a concentration of cDNA. All samples were measured in triplicate. For each experimental sample, the amount of the HER2 and GAPDH were determined from the standard curves.
Apoptosis examination

For the most active extract, its effect on cell apoptotic cell was also examined by using acridine orange/propidium iodide (AO/PI) assay. After seeding in 24 well plate and overnight incubation at 37°C, breast cancer cells line (MCF7/HER2) was treated with 3 different concentration of the test extracts (1/2 x IC<sub>50</sub>, 1 x IC<sub>50</sub> and 2 x IC<sub>50</sub>) and incubated for 24 h. Doxorubicin in concentration of 1 x IC<sub>50</sub> was used as positive control. After being incubation, 20 μL of trypsin was added into each well. When cells had slough off, 25 μL suspensions were transferred to glass slides. Five microliter of dual fluorescent staining solution containing 100 μg/mL AO and 100 μg/mL PI was added to each suspension and then covered with a coverslip. The apoptotic cell in 100 cells was counted within 20 min by using a fluorescent microscope. Viable cells showed green fluorescent, whereas apoptotic cells showed orange fluorescent. The assay was repeated 3 time.

Statistical analysis

Quantitative data were presented as mean ± standard error of the mean (SEM). One way Anova was applied for parametric data and continued by Tamhane Post Hoc test to evaluate significant difference between groups.

RESULTS

Cytotoxicity assay

Curve of relationship between the concentration of hantap leaves (S. oblonga Mast) extracts (A) and doxorubicin (B) and the inhibition of MCF7/HER2 cells line growth after 24 h incubation is presented on FIGURE 1. Based on this curve, the IC<sub>50</sub> value was calculated. Among the three extracts tested, the methanolic extract is the most active with the IC<sub>50</sub> value was 91.25 μg/mL. The IC<sub>50</sub> of hexanic and water extracts were > 400 μg/mL, whereas the IC<sub>50</sub> of doxorubicin was 3.192 μg/mL.
HER2 mRNA expression examination

For the most active extract i.e. methanolic extract, its effect on HER2 mRNA cells expression was evaluated. The HER2 mRNA expression after 24 h incubation with the methanolic extract of hantap leaves (S. oblonga Mast) and doxorubicin is presented in FIGURE 2. In general, the methanolic extract inhibited the HER2 mRNA expression. The methanolic extract at concentration of $1/2IC_{50}$, $1IC_{50}$ and $2IC_{50}$ µM suppressed HER2 mRNA expression to be 0.6; 0.25 and 0.33 compared to control cells. Whereas doxorubicin at concentration of $1IC_{50}$ suppressed HER2 mRNA expression to 0.42.

FIGURE 2. HER2 mRNA expression after 24 h incubation with the methanolic extract of hantap leaves and doxorubicin.
**Apoptotic examination**

The effect of the methanolic extract on cells apoptosis was also evaluated. Microscopic examination result after staining by using AO/PI is presented in FIGURE 3. Green-stained MCF7/HER2 cells represent viable cells (VL), whereas red staining represent apoptotic cells (AL). Methanolic extract at concentration of 1/2IC₅₀; 1IC₅₀ and 2IC₅₀ µM induced 6.8; 26.3 and 25.3% apoptosis of the MFC7/HER2 cells, respectively. Whereas doxorubicin at concentration of 1IC₅₀ induced 19.8% apoptosis of the cells (FIGURE 4).

![FIGURE 3. Apoptotic test results on the MCF7/HER2. breast cancer cell line after 24 hours incubation with control cells (a), treatment of S. Oblonga Mast methanol extract with 2IC50 (b), IC50 (c) and 1/2IC50 (d). viable cells (VL/Green), apoptotic cells (AL/Red)](image)

![FIGURE 4. Percentage of MCF7/HER2 cells apoptotic after 24 h incubation with methanolic extract at different concentration.](image)
DISCUSSION

Among three extracts of hantap (S. oblonga Mast) leaves tested, the methanolic extract exhibited the most cytotoxic activity against MCF7/HER2 cells line with an IC_{50} of 91.25 µg/mL. The National Cancer Institute (NCI) of America categorized the cytotoxicity of an extract into high cytotoxic activity if it has an IC_{50} value ≤ 20 µg/mL, moderate cytotoxic activity if it has an IC_{50} value ranged between 21 - 200 µg/mL, weak cytotoxic activity if it has an IC_{50} value ranged between 201-500 µg/mL and no has cytotoxic activity if it has an IC_{50} > 500 µg/mL. Based on this criteria, the methanolic extract of hantap (S. oblonga Mast) leaves could be categorized to have moderate cytotoxic activity.

Several species of the Sterculia genus have been reported to have biological activities such as antioxidant, antiinflammatory, and cytotoxic. Sterculia genus have been also reported to biological active compounds such as tannins, alkaloids, flavonoids, saponin and phenol. Sterculia oblongata Mast rich of phenolic compounds which has strong antioxidant activity. The methanolic extract of S. oblongata Mast was reported to have higher antioxidant activity than that of β-carotene/linoleic acid. Antioxidants are compounds that inhibit oxidation, a chemical reaction that can produce free radicals and chain reaction that may damage the cells or organisms. Antioxidants have been reported to have various biological activities including cytotoxic. The phenolic compounds of the methanolic extract of hantap (S. oblongata Mast) leaves may be responsible for its cytotoxic activity.

The cytotoxic activity of the methanolic extract of hantap (S. oblongata Mast) leaves was supported by its effect on the expression of HER2 mRNA of MCF7/HER2 breast cancer cell lines. In this study, the methanolic extract induced inhibited mRNA expression of the cell lines with the maximal inhibition was observed at the concentration of 91.25 µg/mL (IC_{50}). Human Epidermal Growth Factor Receptor 2 (HER2) is a 185 kDa protein with intracellular tyrosine kinase domain and an extracellular ligand binding domain. It plays important roles in cell growth, survival and differentiation in a complex manner. HER2 is highly expressed in a significant proportion of breast cancer, ovarian cancer, and gastric cancer. HER2 has received great attention as targeted therapy during the past two decades.

The cytotoxic activity of the methanolic extract of hantap (S. oblongata Mast) leaves was also supported by its effect on the apoptosis of MCF7/HER2 breast cancer cell lines. In this study, the methanolic extract induced the cell cancer apoptosis with the maximal induction were observed at the concentration of 91.25 µg/mL (IC_{50}). Apoptosis is a cellular mechanism to eliminate permanent damage of the cells without causing inflammation. It is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate cell number in the body. The discovery of new anticancer targeting induction of cancer cells apoptosis has been focusing in last decade.

CONCLUSION

In conclusion, the methanolic extract of hantap (S. oblongata Mast) leaves has moderate cytotoxic activity against MCF7/HER2 breast cancer cell lines. Furthermore, this methanolic extract may act through induction apoptosis and inhibition HER2 mRNA expression of the cancer cell lines. Further study, will be conducted to isolate active constituents as anticancer.

ACKNOWLEDGMENT

This study is a part of a thesis research in Program of Master Biomedical Sciences, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah...
Mada, Yogyakarta. This study was financial supported by Program of Hibah Dana Masyarakat (DAMAS) 2021, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta.

REFERENCES


16. Murtiningsih T, Pratiwi, Liana, Fathoni A. TLC profiling and


