

Anti-metastatic Profiles of *Boesenbergia pandurata* towards MCF-7/HER2 Cells

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Abstract

The development of breast cancer at an advanced stage is signed with metastatic phenomenon, triggering the high mortality, mainly for Human Epidermal Growth Factor Receptor (HER)2 positive cancers. *Boesenbergia pandurata* is well known as medicinal plant possessing anticancer potential due to the cytotoxic and antimetastatic characteristic of its active compound. The aim of this study is to observe the inhibitory effect of *Boesenbergia pandurata* ethanolic extract (BPEE) in combination with doxorubicin on migration of MCF-7/HER2 cells. The BPEE was prepared by 96% ethanol maceration. Under MTT assay, BPEE decreased the cells viability with IC₅₀ value of 23±3.9 µg/mL. Lamellipodia and wound healing assay analysis showed that 5 µg/mL BPEE and its combination with 10 nM doxorubicin inhibited cells migration after 48 hours observation, while gelatin zymography analysis showed that this combination did not affect the expression of Matrix Metalloproteinase (MMP)2 and MMP9, but single treatment of 5 µg/mL BPEE caused lower expression of both MMPs. The combination of 5 µg/mL BPEE and 10 nM doxorubicin inhibited the cells migration but not affect to the cells viability. Thus, BPEE is potential to be developed as an antimetastatic agent. The mechanism underlying the migratory inhibition effect needs to be explored further.

Keywords : *Boesenbergia pandurata*, doxorubicin, MCF-7/HER2, migration

INTRODUCTION

As many as 90% of breast cancer mortality is caused by metastatic phenomena (Lu, et al., 2013a). Approximately 20-30% metastatic breast cancer cases are caused by over-expression of Human Epidermal Growth Factor Receptor (HER)2 (Engel & Kaklamani, 2007) acting through motility induction and cell invasion along with transforming growth factor (TGF)-β1 and TGF-β3 (Seton-Rogers, 2004).

Unfortunately, in some countries, such as Indonesia, detection of breast cancer is just revealed after being at metastasis stage (Indonesian Ministry of Health, 2015). In fact, breast cancer can metastasize

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to the other tissues/organs such as bone, brain and liver (Chiang & Massagué, 2008). The complexity of the disease due to metastasis is still a challenge for the development of cancer drugs focusing on antimetastatic activity.

Doxorubicin is known to be the first-line therapy for treatment of metastatic breast cancer (Ansari, *et al.*, 2007). However, recent studies suggest that the use of doxorubicin precisely triggers cancerous cell metastasis through epithelial-mesenchymal transition (EMT) induction (Bandyopadhyay, *et al.*, 2010), and lamellipodia formation as the initial process of metastatic cells (Putri, *et al.*, 2011). Alternatively, the combination therapy has been developed, such as doxorubicin with cyclophosphamide (Swenson, *et al.*, 2003; Batist, *et al.*, 2006); and trastuzumab with HER2 activated heregulin (Menendez, *et al.*, 2006).

Treatment of metastatic cancer requiring combination therapy indicates that the cancer at this stage is harmful. However, the use of a combination of more than one type of chemotherapeutic agent rises harmful side effects, including blood disorders, nervous system, reproductive system, memory system, until damage to the heart, lungs, liver, kidneys and other vital organs permanently (American Society of Clinical Oncology (ASCO), 2014). Therefore, the exploration of antimetastasis activity from natural plants is still important as an effort to look for the combination therapy in order to reduce the side effects of chemotherapeutic agents.

Boesenbergia pandurata is one of the natural plants that has been widely studied in terms of the anticancer activity. This plant has been reported to have phytochemical properties of more than 51 flavonoid compounds (Chahyadi, *et al.*, 2014) that potentially inhibit the growth of various cancer cells, including MCF-7 breast cancer cells (Kirana, *et al.*, 2007). The potency of *Boesenbergia pandurata* as an antimetastatic agent is also found in some cell models, one of which is human umbilical vein endothelial cells (HUVEC) (Lai, *et al.*, 2015).

As mentioned above, HER2 expression contribute to the high incidence of metastatic breast

cancer. However, there have no specific study related to antimetastasis activity of *Boesenbergia pandurata* in breast cancer cells over HER2 expression (MCF-7/HER2). Thus, this study aims to explore the potential of *Boesenbergia pandurata* ethanolic extract (BPEE) and its combination with doxorubicin as an antimetastasis agent in MCF-7/HER2 breast cancer cells.

MATERIALS AND METHODS

Extract Preparation and Thin Layer Chromatography

Rhizome simplicia of *Boesenbergia pandurata* collected from Bina Agro Mandiri Inc. were determined at Biological Pharmacy Laboratory, Universitas Gadjah Mada. Then it was macerated and remacerated with 96% ethanol (1:10) for 5 days. The filtrate was dried using rotary evaporator at 40°C resulting viscous extract.

The viscous extract obtained was then identified using a thin layer chromatography (TLC) method. A total of approximately 10 mg of viscous extract dissolved in 0.5 mL of 96% ethanol. The mobile phase used is n-hexane : ethyl acetate (4:1 v/v).

The chromatogram profile will be observed in the form of color spots appearing under 254 nm UV light, then compared with reference of BPEE in Indonesian Herbs Pharmacopoeia (First Edition, 2009) which uses the same mobile phase and detection method.

Cell preparation

MCF-7/HER2 breast cancer cells were obtained from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, NAIST, Japan). The cells were maintained in Dulbecco's Modified Eagles medium (DMEM) high glucose (Sigma, Saint Louis, USA) with 10% Fetal Bovine Serum (FBS) (Sigma), HEPES, sodium bicarbonate, 1500 U/mL of Penicilin - 1500 µg/mL of Streptomycin and 0.5 µg/mL Fungizone (Gibco, New York, USA).

Cytotoxic Test

Cytotoxic test was carried out by MTT assay adopted from Mosmann, 1983. Briefly, MCF-7/HER2 cells were seeded in 96-well plate with 2.5×10^3 cells/well and were incubated for 24 hours. After incubation, cells were treated with BPEE in increasing concentration of 5, 10, 20, 40 and 50 $\mu\text{g}/\text{mL}$ for 24 hours. Media were removed and washed with 100 μL phosphate buffered saline/PBS (Sigma). MTT reagent was added with the final concentration of 5 mg/mL to each wells then incubated in 37°C for 4 hours then added with sodium dodesyl sulphate 10% HCl 0.01 N. After that incubate over night. The absorbance was determined with enzyme-linked immunosorbent assay (ELISA) reader at λ 595 nm (Biorad).

Lamellipodia Observation

Cells were grown with a density of 7×10^4 cells / wells on 6 well plates. Cells were incubated for 24 hours at 37°C . Cells were added with BPEE with the exception of untreated cell was added only the culture medium. Observation of lamellipodia's cell formation performed at timepoint 0 and 24 hour using inverted microscope at 100x magnification.

Scratch wound healing assay

MCF-7/HER2 cells with concentration of 8.5×10^4 cells/well were seeded in 24-well plate. Cells were incubated for 24 hours. Media were removed and washed with PBS (Sigma). Media with 0.5% FBS were added and incubated for 24 hours. Each well was scratched vertically by using yellow tip and treated with BPEE. The closures of cell migration were observed at 0, 24 and 48 hours after the treatment under an inverted microscope and captured with a digital camera.

Gelatin Zymography

The previously planted cells (3×10^5 cells/wells) were incubated with BPEE for 24 hours, washed with cold PBS and added with 400 μL / well cold lysis buffer. The lysate was centrifuged

at 14,000 rpm for 10 min at 4°C . The calculation of protein content was conducted using Bradford assay method.

Stacking and separating gel were prepared and incorporated into electrophoresis chamber. Approximately lysate of 5-7 μL and prestained markers were inserted into the gel wells. Electrophoresis was carried out with a condition of 120 V, 80 mA for 110 min. The electrophoresis gel was immersed in 0.05% brilliant blue coomassie dye solution for 30 minutes to form a transparent band with a dark blue background.

Data Analysis

Absorbance data was obtained from cytotoxicity assay then converted to a percent of cell viability. IC_{50} value calculation is performed by the linear regression method. Lamellipodia observation analysis was done qualitatively through cell photos by magnifying certain spots to observe the lamellipodia formed. To make the display clearer, photo contrast was made in grayscales mode. Scratch analysis was done by measuring the migration distance using ImageJ software by comparing the distance between the untreated and treated cells. Using the same software, gelatin zymograph method was analyzed by comparing the band intensity due to the treatment and the untreated one.

RESULTS

Characterization of BPEE content using TLC

Characterization of the content was performed using TLC and compared with the relevant official reference according to the Indonesian' Herbs Pharmacopoeia (First Edition, 2009). The BPEE chromatogram profile can be seen in Figure 1.

The observed results showed that the spots arising from the test chromatogram were similar in profile with reference chromatogram mentioned above. The similarity of profiles between test and reference chromatogram was indicated by the

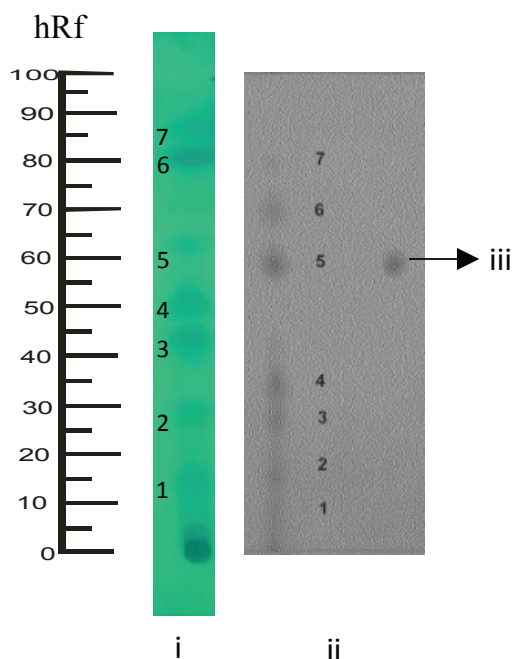


Figure 1. BPEE's chromatogram profile. The chromatogram profile was obtained with TLC method. Silica gel 60 F 254 was used as the stationary phase. Elution was performed using n-hexane : ethyl acetate (4: 1 v / v) as the mobile phase. i: Detection was carried out under 254 nm UV light; ii: chromatogram profile reference of BPEE extract; iii: pinostrobin compound as a comparison.

number of spots. Although not all of the patches on the test chromatogram exhibited a position similar to that of the reference chromatogram, there was a similar position between the chromatogram and the reference chromatogram sequentially in spots 1 to 2 (hRF 19), 2 spots with 3 (hRF 30), 5 spots with 5 (hRF 64), and spots 6 with 7 (hRF 87). With the similarity of the profiles, BPEE used in this study met the official reference standard. Interestingly, in the fifth spots both the test and reference chromatograms have similarities with spot of pinostrobin, a flavonoid, as compounds used by reference. This finding reinforces that the sample used is a *Boesenbergia pandurata* containing flavonoid compounds.

Cytotoxic Activity of BPEE Against MCF-7 / HER2 Cells

In addition to know the cytotoxicity of BPEE against MCF-7 / HER2 breast cancer cells, cytotoxic tests were performed to determine the concentration that can be used for observation of the formation of lamellipodia, scratch wound healing assay and gelatin zymography. The BPEE cytotoxic profile of MCF-7 / HER2 cells is shown in Figure 2.

Based on the results obtained, BPEE inhibited the growth of MCF-7/HER2 cells in a dose dependent manner. BPEE treatment with higher concentration leads to lower cell viability. The linear relationship between the concentration and cells viability is indicated by the regression value that meets the 95% confidence level requirement for $n=5$. The IC_{50} value of BPEE treatment on MCF-7/HER2 cells was 23 $\mu\text{g/mL}$.

The Effect of BPEE Towards the Lamellipodia Formation Activity of MCF-7 /HER2 Cells

The formation of lamellipodia is an early stage of the metastasis process. Observation of lamellipodia formation was conducted by observing microscopic cell morphology at 24 hours after treatment. The concentration of BPEE used in this observation is 5 $\mu\text{g/mL}$. In addition, a combination treatment of BPEE 5 $\mu\text{g/mL}$ and doxorubicin 10 nM, for further mentioned as combination, was performed. In general, observation at 24 hours (Figure 3) showed the formation of lamellipodia occurring at all treatments. However, single treatment of BPEE and its combination with doxorubicin 10 nM showed less lamellipodia formation than control and single treatment of doxorubicin. In other words, BPEE treatment and its combination with doxorubicin 10 nM have the ability to inhibit the formation of lamellipodia.

The Effect of BPEE Towards Migration Activity of MCF-7/HER2 Cells

Another metastasis marker phenomenon is the ability of cells to migrate. Observation of MCF-7/

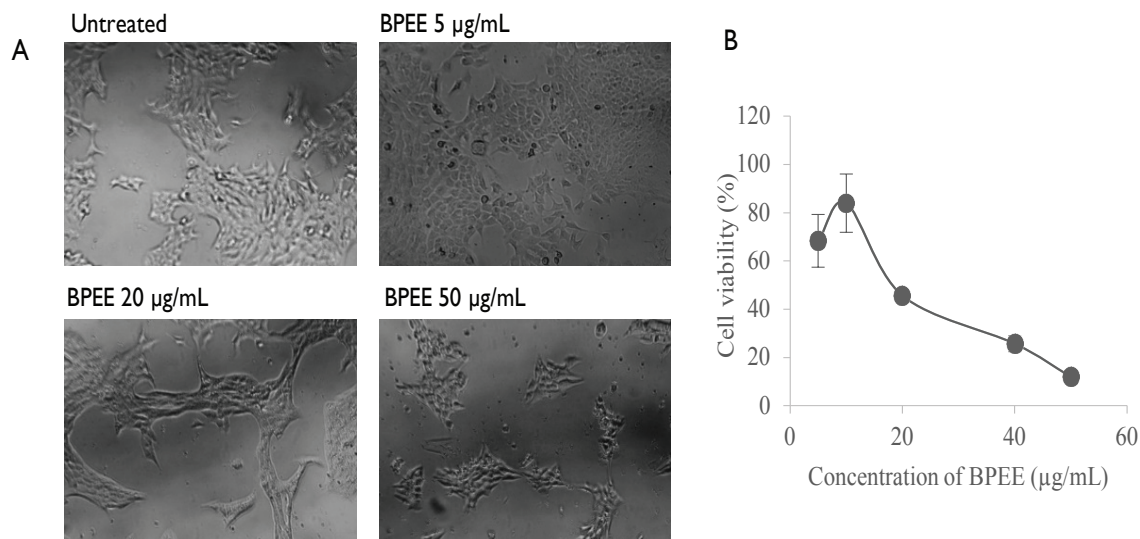


Figure 2. Effects of BPEE treatment on cytotoxic tests. Cells were seeded in 96 well plates with a density of 2.5×10^3 cells/wells and incubated for 24 hours. Cells were starved and treated with BPEE in various concentrations of 5, 10, 20, 40 and 50 µg/mL for 24 hours. A: Cell morphology after treatment with 0, 5, 20, and 50 µg/mL BPEE for 24 hours; B: Quantification of cell viability after treatment with 5, 10, 20, 40 and 50 µg/mL for 24 hours, as measured using MTT method. The graph above is the percentage of cell viability of three independent experiment values with 95% confidence level.

HER2 cell migration activity due to treatments of BPEE, doxorubicin, and its combination was performed with scratch wound healing assay using starvation media.

The result of scratch wound healing assay and quantification of the area of closure due to migration by MCF-7/HER2 is shown in Figure 4.

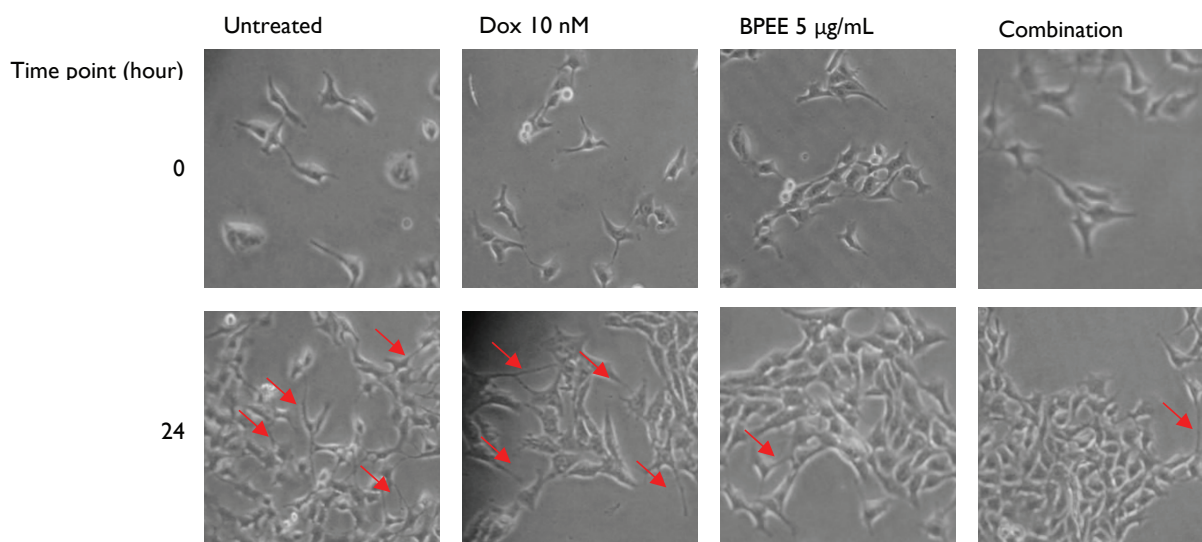


Figure 3. Effect of treatments on lamellipodia formation activity. Cells with a density of 7.5×10^4 cells/well were seeded on 24 well plates. The observations were performed using inverted microscope at 100x magnification after 24 hours treatment incubation. The red arrow shows the formed lamellipodia.

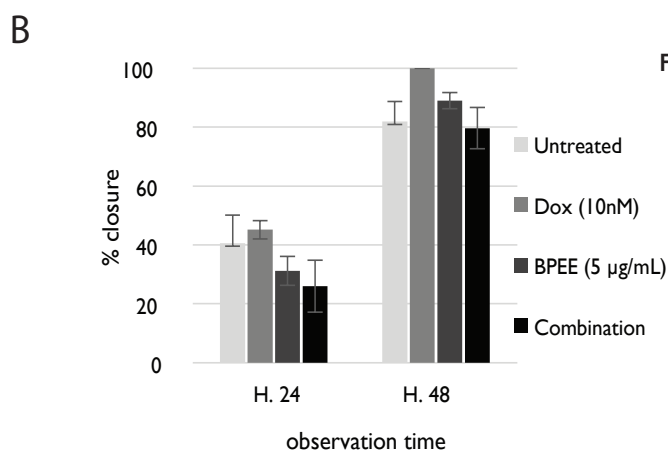
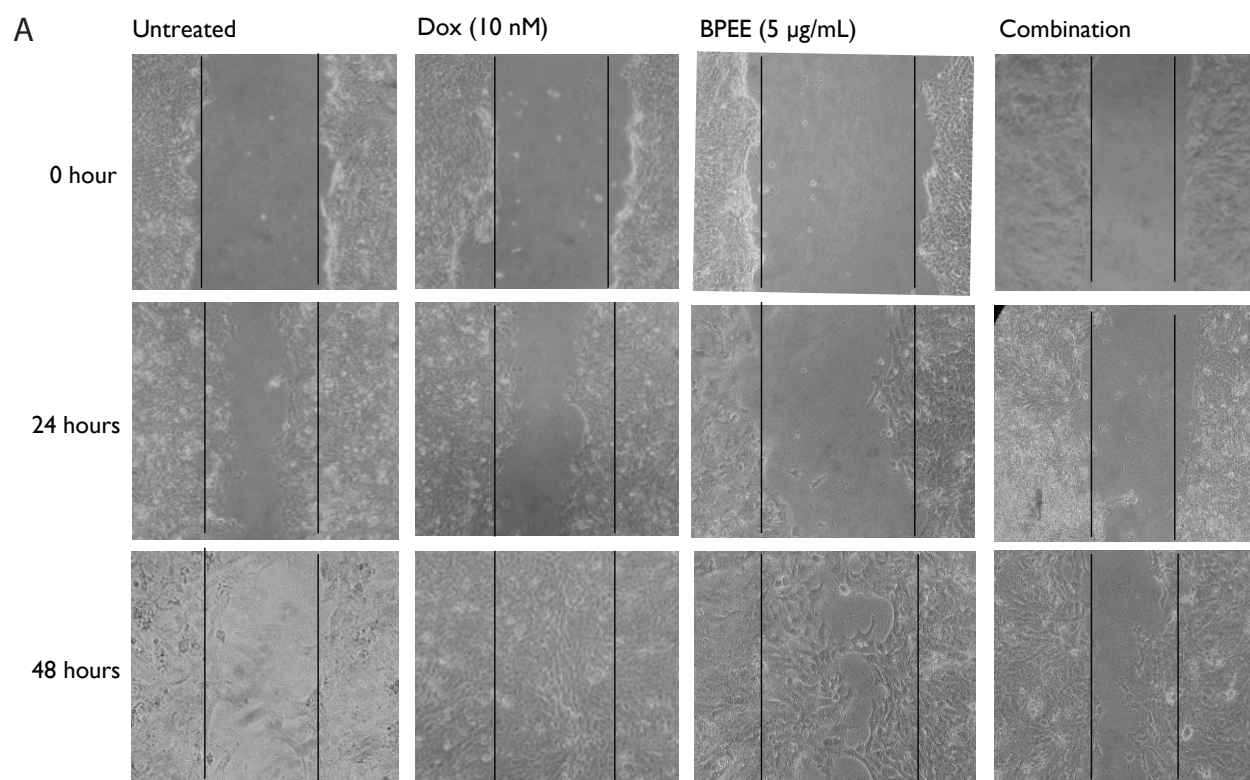


Figure 4. Inhibitory effect of migration. Cells with a density of 7.5×10^4 cells/well were seeded on 24 well plates. A: Cell morphology after scratch wound healing assay and treated with 5 µg/mL BPEE 10nM Doxorubicin and a combination of both. Observations were performed at 0, 24, and 48 hours after treatment under an inverted microscope with 100x magnification. The dashed lines indicate the border of the scratch; B: Percentages of MCF-7/HER2 cell closure at 24 and 48 hours were obtained from average % closure of three observed areas in one well at the same time. The area was quantified using ImageJ® software and calculated. SD values were obtained from three different observations.

The quantification result is done by making a line adjusting the cell boundary using unshaped tools instead of straight lines, so that the area of the cavity also included in the calculation.

At the 24th hour observation, a single treatment of BPEE decreased the % closure when compared with untreated cell and single treatment

of doxorubicin. From the quantification chart it can be seen that the lowest % closure occurred in the treatment of a combination of BPEE with doxorubicin 10 nM, either at the time of 24 or 48 hours of observation. This indicated that the combination treatment of both agents provides better anti-migration effects, yet statistically insignificant.

The Effect of BPEE Towards MMP-2 and MMP-9 Expression of MCF-7/HER2

BPPE was proven to inhibit the expression of matrix metalloproteinase (MMP)-2 and MMP-9 proteins. The method used for MMP-2 and MMP-9 expression test on MCF-7/HER2 cells in this study was gelatin zymography.

Based on this result, it can be seen that there was no huge different of the decrease of MMP-9 expression between BPEE single treatment and combination treatment (Figure 5). While BPEE treatment was more able to decrease MMP-2 expression compared with other treatments. The combination of BPEE-doxorubicin 10 nM was only able to decrease MMP-9 expression when compared with other treatments. This is contrary to the migration observations, single treatment of BPEE has a better migration inhibitory effect in terms of inhibition of MMP-2 expression and MMP-9 than combination treatment.

DISCUSSION

Boesenbegia pandurata in the form of extracts with various solvents have been shown to have anticancer activity in many experimental models of cancer cells, both *in vitro* and *in vivo*. The anticancer potential can be seen through the cytotoxic profile obtained. In this study it was proven that BPEE has anticancer potential in MCF-7/HER2 cells with IC_{50} values lower than 100 $\mu\text{g/mL}$ as described by Prayong, et al. (2008). Cancer mechanism itself can occur differently. Of the ten characteristics of cancer cells (Hanahan & Weinberg, 2011), the phenomenon of metastasis is often targeted as a target for the development of cancer drugs. This is due to many cases of cancer, including breast cancer.

A study related to the effect of *Boesenbergia pandurata* as antimetastasis agent has been done, but no metastatic breast cancer-related studies have been found. This study positioned

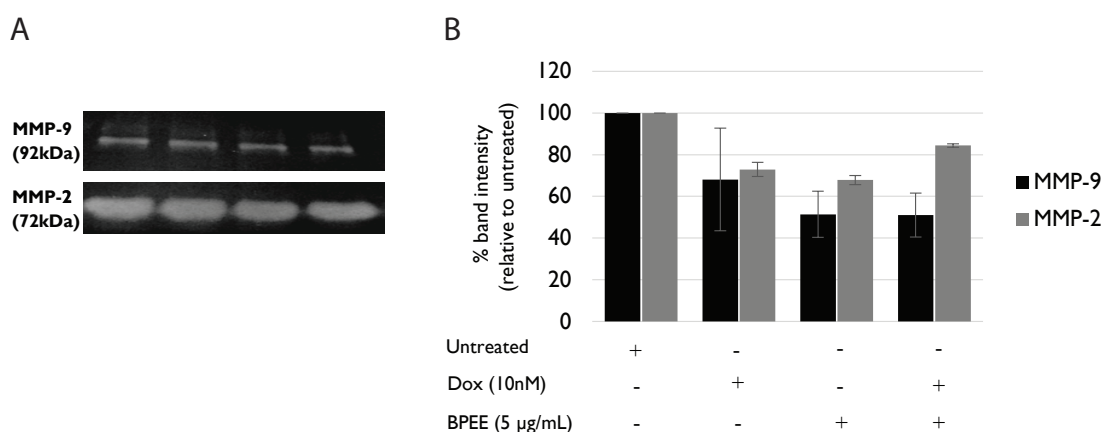


Figure 5. Inhibitory effect of MMP-2 and MMP-9 expression. Cells with a density of 7.5×10^4 cells/well were seeded on 24 well plates. The starvation medium is taken as a lysate after 24 hours of incubation as the sample used. A: Profiles of MMP-2 and MMP-9 expression bands; B: Quantification of transparent band intensity of MMP-2 and MMP-9 by three times of analysis. Observation of MMP-2 and MMP-9 expression was done by gelatin zymography according to the research method. The results analysis was performed using ImageJ® software to measure the intensity of the gelatine degradation bands by MMP-2 and MMP-9 on the gel with three times of calculation.

itself to complement by focusing on the effect of *Boesenbergia pandurata* towards breast cancer cells with overexpression of HER2. The presence of excessive HER2 expression in the cell model used indicates the metastatic characteristics of the cancer cells.

The metastasis profiles in this study covered the formation of lamellipodia, % of closure due to scratch wound healing assay, and % band intensity due to gelatin degradation by MMP-2 and MMP-9. In this study, we also enclosed the single treatment of doxorubicin that proven to trigger the formation of lamellipodia (Putri, *et al.*, 2011) with 10 nM concentration without causing death cells (Amalina, *et al.*, 2017). Additionally, the combination of BPEE with doxorubicin was added to their respective concentration in order to know the potential of BPEE as MCF-7/HER2 breast cancer co-chemotherapeutic agent. From the results of the research, it was found that BPEE and its combination with doxorubicin 10 nM could inhibit the formation of lamellipodia. Lamellipodia observation involves a narrower field of view, meaning the observations focused on the cells one by one. Based on the inhibitory effect of lamellipodia formation by BPEE and its combination with chemotherapeutic agents, it shows the potential of BPEE to be developed as an antimetastasis agent.

Beside that, the closure of the scratch wound healing assay represents the movement of the cell with a wider field of view, it can be seen the phenomenon of migration of the observed cells as a whole. This observation also included doxorubicin treatment of 10 nM, whereby it has been known to induce metastasis via induction of epithelial-mesenchymal transition (EMT) (Bandyopadhyay, *et al.*, 2010). Epithelial cells experience a reduction in polarity and adhesion, thus causing epithelial cells to have characteristics such as mesenchymal cells capable of migrating and invading (Kalluri and Weinberg, 2009).

Furthermore, there is also included a combination treatment of both to determine

the potential of BPEE when combined with chemotherapeutic agents in inhibition of MCF-7/HER2 cell migration. BPEE treatment was able to decrease the percentage of closure at 24 hours observation, and the combination of BPEE with doxorubicin 10 nM was known to decrease the percentage of closure compared to untreated cell and single treatment of doxorubicin, either at 24 hours or 48 hours. The decrease effect of % closure becomes important information that can be used as a basis for BPEE development as a combination of cancer chemotherapy or co-chemotherapy agents.

The molecular review of the invasion process was conducted by observing the inhibition of MMP-2 and MMP-9 expression. As far as we explored, there has not been found a study of doxorubicin's effect toward the expression of MMP-2 and MMP-9. So the doxorubicin treatment in this case is an initial investigation to assess the effect of doxorubicin with MMP-2 and MMP-9, since migration and invasion mechanisms are not the same although related. This study showed that all treatments tending to decrease the expression of MMP-2 and MMP-9, except untreated cells. Although one treatment with other treatments showed no significant difference, a single BPEE treatment was more able to decrease the expression of both MMP. Therefore it can be said that BPEE has the potential to be developed as an antimetastasis agent through a decrease in the expression of MMP-2 and MMP-9 in breast cancer cells overexpression of HER2. Nevertheless, the refinement of this study is very necessary to do.

This study shows that BPEE has the ability to inhibit breast cancer cell metastasis over HER2 expression. To accomplish this research, a metastatic study should be conducted through another approach with regard to metastatic cascades, such as intravasation, circulation, extravasation and angiogenesis both *in vitro* and/or *in vivo*. Metastatic studies through migratory inhibition and invasion approaches can also be performed with other methods such as transmembrane assay (Boyden

chamber assay), microfluidic chamber assay, and cell exclusion zone assay (Hulkower, *et al.*, 2011). Molecular studies of MMP-2 expression, MMP-9 and other factors affecting both proteins can be explored more deeply by performing other methods such as Western blot.

CONCLUSION

Overall, the results of this study indicate that BPEE has potential as an antimetastasis agent in MCF-7/HER2 cells, in view of the tendency to inhibit lamellipodia formation, cell migration and MMP-2 and MMP-9 activity.

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