A Comparison of Antimetastatic Activity between Nerium indicum and Cinnamomum burmannii on 4T1 Cells

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Abstract

Metastatic process becomes a major problem in advanced cancer cases. Natural compounds found in several plants in Indonesia have a potency to be developed as chemotherapeutic agent which are targeted to metastatic process. Jure leaves (Nerium indicum) which contain oleandrin and cinnamaldehyde in cinnamon bark (Cinnamomum burmannii) reported to have cytotoxic activity on several cancer cells, but their activities on metastatic process have never been explored. This research aims to reveal and to compare their anti-metastatic effect toward 4T1 breast cancer cells. The cytotoxicity of jure leaves extract (JLE) and cinnamon essential oil (CEO) was obtained by MTT assay. Metastatic process mainly on cell migration was examined by scratch wound healing assay while MMP-9 expression that described the invasion process was observed by gelatin zymography assay. Molecular interaction between their active compounds and MMP-9 receptor was predicted by molecular docking. The result showed that treatment with JLE and CEO inhibited the growth of 4T1 cells with IC50 value of 125 µg/mL and 2.5 µg/mL, respectively. In addition, JLE performed inhibitory effect of cell migration better than CEO. Meanwhile, both JLE and CEO decreased MMP-9 protein expression. Thus, JLE and CEO have potentials to be developed as an anti-metastatic agent and JLE could be more effective.

Key words: Nerium indicum, Cinnamomum burmannii, anti-metastasis, scratch assay, gelatin zymography

INTRODUCTION

Cancer remains a major burden of disease worldwide. Cancer can be categorized as a "silent killer" disease due to the death of cancer patients that was suffering from cancer at advanced stage (metastasis) and lack of success in the early detection. This data is also supported by the WHO (2009) which states that metastasis is the leading cause of cancer-associated mortality. Therefore, various strategies should be continuously developed to inhibit cancer cells migration, especially from natural compounds.

Natural materials of Indonesia were reported to have anti-cancer activity are jure leaves (Nerium indicum) and cinnamon (Cinnamomum burmannii) which containing oleandrin and cinnamaldehyde as the major compounds (Manna et al., 2000; Siddiqui et al., 1987; Hembing, 1993). Nerium indicum is one such plant which is famed for its therapeutic efficiency for various species. Jure leaves have been reported as anti-cancer agent since 1980 (Hartwel, 1982), cardiac illnesses, asthma, corns, cancer, and epilepsy (Duke and Boca Raton, 1985) hypertension and also diabetes (Tahraoui, 2007). On the other hand, Cinnamon is potential to be developed as chemopreventive agent because it can be inhibitor of angiogenesis protein (Lu et al., 2010). It also inhibits many cancer cells growth and induces G2/M phase arrest (Scoene et al., 2008). Scientific research regarding anti-metastatic of these plants has never been done.

Therefore, this study aims to to reveal and to compare their anti-metastatic effect toward 4T1 breast cancer metastatic cells. This study comprehensively examines the anti-metastatic ability of jure leaves extract (JLE) and cinnamonomum essential oil (CEO).

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Anti-metastatic activity was observed through two parameters: the cell migration by scratch wound healing assay and MMP-9 protein expression by gelatin zymography method. Molecular interactions between the active compound contained in JLE and CEO against MT1-MMP as activator protein MMP which is seen through the molecular docking.

**MATERIALS AND METHODS**

**Cell Culture**

4T1 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) with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1.5% Penicillin-Streptomycin and 0.5% Fungizone (Gibco).

**Sample Preparation**

Jure leaves are obtained from Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. Jure leaves are being dried and extracted by soxhlet with petroleum ether and ethanol 96% respectively. The filtrate is being concentrated by rotary vacuum evaporator. Cinnamons are obtained from Boyolali, Central Java. Cinnamons are being dried and cut off into small pieces. Cinnamon were extracted by distillate.

**Cytotoxic Test**

Cytotoxic test by MTT is being used to know JLE and CEO cytotoxicity to 4T1 cells. 4T1 cells were seeded in 96-well plate with 5x10^3 cells/well and were incubated in 24 hours. Cells treated with increasing concentrations of JLE (10, 50, 100, 250, 500 µg/mL) and CEO (0.2, 1, 2, 3, 4, 5 µg/mL) for 24 hours. Media were removed and washed with 100 µl PBS (Sigma), add MTT reagen with contration 5 mg/mL to each wells the incubate in 37°C for 4 hours. The living cells can change MTT reagen into purple-colored formazan. After incubating add stopper reagent SDS 10% HCl 0,01 N and incubating over night. Shake the plate in 10 minutes and read the absorbance with ELISA reader at λ 595 nm (Biorad). The single absorbance data converted into viability percent and will be used to measure IC<sub>50</sub>.

**Scratch Wound Healing Assay**

Scratch wound healing assay is method to measures the expansion of a cell population on surfaces. 4T1 cells planted 7.5 x 10^4 cells/well in 24-well plate. Cells incubated for 24h until 80% confluen. Media were removed and washed with 100 µl PBS (Sigma), added media culture with 0.5% FBS for starvation step and incubated for 24 hours. Scratch vertically in each base of the wells using the tip of yellow tip and treated with JLE and CEO. Observed in 0, 18, 24 and 42 hours under inverted microscope and capture it with camera. Scratch analysis done by measure the migration distance using ImageJ software by comparing the distance between untreated and treatment.

**Gelatin Zymography Assay**

This method is for observing enzymatic activity of MMP-9 in 4T1 cells. Medium starvation with concentration of JLE and CEO were collected and become a sample of gelatin zymography. Electrophoresis used 8% SDS-PAGE that contains 0,1% gelatin. After running gel washed with 100 mL aquadest that contains 2% Triton X-10 in 30 minutes so that the SDS can disappear, gel incubated in 100 mL buffer reaction (40 mM Tris-HCl pH 8, 10 mM CaCl₂, 0,02% NaN₃) for 20 hours in 37°C, gel was painted with Coomassie Brilliant blue R-250 and destined with destining solution (20% methanol, 10% acetate acid, and 70% water) (Hsieh et al., 2013). Gelatin degradation by MMP-9 activity observed by the appearance of transparent tape in gel. Next, the intensity of transparent tape analyzed with imageJ software.
Molecular Docking Analysis

The 3D structure of tested compounds, oleandrin and cinnamaldehyde made by MarvinSketch software. The structure of target protein, MMP-2 (PDB ID: 1HOV) and MMP-9 (PDB ID: 2OVX) were downloaded from PDB and the preparation done by YASARA software. Docking validation was performed by Protein-Ligand Ant System (PLANTS) 1.1 manual program. Visualization 2D and 3D performed by MOE 2010 software (Licensed by Faculty of Pharmacy, Universitas Gadjah Mada).

RESULT

Cytotoxic effect of JLE and CEO on 4T1 cells

The cytotoxic effects of JLE and CEO are detected by MTT Assay with IC_{50} value as the parameter, the low IC_{50} value indicates potential cytotoxicity. IC_{50} values on JLE and CEO are 125 µg/mL and 2.5 µg/mL on 4T1 cells (Figure 1).

![Figure 1. Cytotoxic effect of JLE and CEO on 4T1 cells.](image)

Based on Prayong et al. (2008), an IC_{50} value below 100 µg/mL indicates a potent cytotoxic effect whereas over 100 µg/mL indicates moderate cytotoxicity. Accordingly, CEO has potential IC_{50} cytotoxic value whereas JLE has IC_{50} value with moderate cytotoxic category. CEO performed stronger cytotoxic effect than JLE on 4T1 cells.
Antimigration effect of JLE and CEO on 4T1 cells

The inhibitory ability of 4T1 cell migration by JLE and CEO is measured by looking at the closing space inhibition of scratch performed (% closure) in the wound healing assay method. 4T1 cell be used for a model in scratch assay because it is one kind of breast cancer cells that have an ability to do metastasis with parameter % closure. Statistical analysis using SPSS (n:3;p<0.005) showed that treatments of JLE and CEO tended to decrease the width of scratch which was due to cell migration compared to untreated (Figure 2). Accordingly, JLE has potential as a metastatic agent by inhibiting cell migration higher than CEO.

Inhibition of MMP-9 protein using gelatin zymography assay

Besides migration cell, metastasis process of cell affected by the MMP-9 and MMP-2 expression and activity. From this result, it can be seen that there is gelatinase activity of MMP-9, but the gelatinase activity of MMP-2 can’t be seen. Its because 4T1 not expression MMP-2 enzyme. Figure 3 showed that JLE and CEO have potentials to be developed as chemoprevention and anti-metastasis agent for advanced stage cancer therapy by decrease expression of MMP-9 protein expression.

Figure 2. Effect of Single Treatment of JLE and CEO on 4T1 Cells Migration. (A) The morphology of the cells after scratch and treated with JLE and CEO. Observations were made after 18, 24 and 42 hour of treatment under an inverted microscope with magnification of 100x. (B) The percentage of 4T1 cells closure after treatment. The area of the scratch were analyzed using ImageJ software then % closure was calculated in accordance with the procedures of the analysis.
**Figure 3. Effect of Single Treatment of JLE and CEO in MMP-9 Expression on 4T1 Cells.** Cells were treated with JLE and CEO. MMP-9 activity assay was conducted using gelatin zymography according to the method. Analysis of the results were done by using ImageJ software to measure the intensity of gelatin degradation by MMP-9 in the gel.

**Molecular docking Result**

Molecular docking is one of in silico test to predict the interaction ability between ligan native (active molecul) with receptor. In this research, molecular docking use to know interaction ability between oleandrin (active molekul in JLE) and cinnamaldehyde (active molekul in CEO) with MMP-2 and MMP-9 receptor. That interaction is visualized and determinated by amino acid residue which interacting between oleandrin and cinnamaldehyde ligand against MMP-2 and MMP-9. Molecular docking analysis based on score docking that represent the needed energy for bounding. The smaller the docking score, the bound between compound and ligand get stronger. The score docking ligand compound in estrogen receptor can be seen in Table 1 and the visualization can be seen in Figure 4.

From the molecular docking result obtained oleandrin and cinnamaldehyde score docking in MMP-2 are -88,2025 and -67,1808. Meanwhile, oleandrin and cinnamaldehyde score docking in MMP-9 are -78,9117 and -68,0184. Therefore, oleandrin and cinnamaldehyde compounds have lower score docking compared to native ligand which signed the compound bound’s strength with ligand is not as strong as native ligand.
DISCUSSION

Metastasis is the major cause of death from cancer. It was found that the patients were aware when the cancer spread into various organs. Thus, it is necessary to develop anti-metastatic agent from natural compounds. The main purpose of this study is to explore and to compare the potential metastatic-inhibitor of JLE and CEO on 4T1 cells. 4T1 cells were used as the model of human metastatic breast cancer cells (Heppner et al. 2000).

Identification of the containing compounds of JLE and CEO under chromatography analysis showed that JLE contains oleandrin and CEO contains cinnamaldehyde as the major compounds. It has been reported that JLE contained many cardenolide compounds such as oleandrin, oleandrigenin, digoxin, digitoin, digitoxigenin, nerizosida, neritalosida, odorosida (Trease and Evans, 2002). On the other hand, under gas chromatography analysis, the result showed that the content of cinnamaldehyde in the CEO was approximately 96.40 %, which is higher than the previous studies by Wang et al. (2009) and Larasati et al. (2014) which reported 60.72 % and 89.40 %, respectively. The presence of oleandrin in JLE and cinnamaldehyde in CEO might be responsible for cytotoxic activity of both compounds.

This study demonstrated the potency of JLE and CEO as an anti-cancer agent which

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Docking score (kCal/mol)</th>
<th>MMP-2</th>
<th>MMP-9</th>
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<tr>
<td>Native Ligand</td>
<td>-130.558</td>
<td>-110.992</td>
<td></td>
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<tr>
<td>Oleandrin</td>
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<td>-78.9117</td>
<td></td>
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<tr>
<td>Cinnamaldehyde</td>
<td>-67.1808</td>
<td>-68.0184</td>
<td></td>
</tr>
<tr>
<td>RMSD</td>
<td>2.3047 Å</td>
<td>1.8543 Å</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Docking scores of oleandrin and cinnamaldehyde on MMP-2 and MMP-9

Figure 4. Interaction of oleandrin and cinnamaldehyde on MMP-2 and MMP-9. Visualization of 2D ligand interaction on MMP-2 and MMP-9 receptor. These molecular interaction was performed by using MOE software. The sign showed the same amino acid residues at the site of connective.
has the potency of inhibiting 4T1 cell proliferation with IC$_{50}$ values of 125 µg/mL and 2.5 µg/mL, respectively. The presence of cytotoxic activity of JLE and CEO is reinforced with the changes of 4T1 cell morphology along the increasing of the concentrations. Several studies showed that JLE also performed cytotoxic effect on leukemic cells such as HL60 and K562 (Turan et al., 2006), and also lung cancer A549 (Jose et al., 2013). Oleandrin as the major compound of JLE also showed cytotoxic effect on myeloma cancer cells and kidney cancer cells (Wahyuningsih et al., 2000; Wahyuningsih et al., 2006). The previous studies reported that oleandrin in JLE induced apoptosis in prostate cancer cells through inhibition of the Na+, K+-ATPase (McConkey et al., 2000). In other studies proved that aqueous extract of jure leaves were able to block NF-κB and AP-1 induced by TNF, PMA, and LPS (Manna et al., 2001). Blocking NF-κB can cause tumor cells to stop proliferating and induce apoptosis. While cinnamaldehyde in CEO reported by Larasati et al., (2014) on HeLa cells and Koppikar et al., (2010) on SiHa cells, types of cervical cancer, showed that cinnamon performed cytotoxic effect with IC$_{50}$ value of 250 µg/mL and 320 µg/mL, respectively. Cinnamaldehyde has Anti-inflammatory effect by blocking the degradation of IκB leading to suppression of NF-κB activation (Liao et al., 2005). Moreover, cinnamaldehyde is an inhibitor of angiogenesis as a natural VEGF inhibitor (Lu et al., 2010). These results suggested that JLE and CEO showed higher toxicity on 4T1 metastatic breast cancer cells than on cervical cancer cells that could potentially be useful in cancer prevention and/or treatment. Further study focusing on NF-κB activation and expression will elucidate more detail to these phenomena.

In this present study, we also observed and compared the inhibition of cancer cell migration by single treatment of JLE and CEO through scratch wound healing assay. Cell migration is one of parts in metastasis process. Single treatment of JLE tend to inhibit 4T1 cell migration greater than the treatment of CEO solely (Figure 2). We also explored the inhibition of cancer cell invasion by the measurement of matrix metalloproteinase-9 (MMP-9) expression under gelatin zymography assay. The result in inhibitory effect of cell invasion was consistent with cell migration inhibitory which performed that JLE decreased MMP-9 expression higher than CEO.

Molecular docking assay was conducted to compare the interaction between oleandrin in JLE and cinnamaldehyde in CEO with MMP-2 and MMP-9 proteins. Molecular docking result confirmed that the affinity between oleandrin with MMP-2 and MMP-9 was lower than the affinity between cinnamaldehyde with MMP-2 and MMP-9 proteins. Moreover, when compared with cinnamaldehyde, oleandrin bond more amino acid residues in MMP-2 and MMP-9 binding site. Based on the result, we predicted that JLE was more potential as an anti-metastatic agent than CEO.

In general, it can be noted that the treatment of JLE and CEO have a tendency in inhibiting 4T1 cell migration. In this case, the cytotoxic activity of JLE and CEO did not affect the inhibitory effect of 4T1 cell migration because of the different mechanisms and does not influenced each other. It can be shown that cytotoxicity of CEO was very powerful with the lower IC$_{50}$ compared to the JLE, but it did not show inhibitory effect of cell migration. These findings demonstrated that JLE exert stronger anti-metastatic activity than CEO although JLE was less cytotoxic. The mechanism underlying anti-migration effect of JLE and CEO remains to be clarified.
ACKNOWLEDGEMENT

We express our gratitude to Directorate General of Learning and Student Affairs.

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