

Methanolic Extract of Red Betel Leaves (*Piper crocatum* Ruiz & Pav) Perform Cytotoxic Effect and Antimigration Activity toward Metastatic Breast Cancer

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Abstract

Breast cancer is one type of cancer with a high mortality rate due to its metastatic property. Red betel leaves (*Piper crocatum* Ruiz dan Pav) has been known as herbal medicine containing biophenolic, such as apigenin and luteolin derivatives which has cytotoxic activity toward cancer cells. This study is intended to explore the inhibitory effect of *Piper crocatum* leaves methanolic extract (PCM) on cell proliferation and migration by using 4T1 cells as model of metastatic breast cancer. By using MTT assay, PCM performed cytotoxic activity in a dose dependent manner with IC₅₀ value of 120 µg/mL. Wound healing assay revealed that migration inhibitory activity of PCM on 4T1 cells at the concentration of 30 µg/mL. In conclusion, PCM perform cytotoxic effect and antimigration activity toward metastatic breast cancer cells.

Keywords : breast cancer cells, *Piper crocatum* Ruiz & Pav, cytotoxic, cell mgration

INTRODUCTION

Globally, breast cancer is the most common disease in women with new cases incidence of 1.38 million per year (Eccles, *et al.*, 2013). High mortality rate of breast cancer patients usually due to the late prognosis of the disease, such as recently detected in the metastasis phase. This phase is characterized by high expression of matrix metalloproteinases (MMPs), cell migration, invasion and other phenomena associated with metastatic cascade (Leber, *et al.*, 2009). These conditions cannot be treated only with radiation therapy or by surgery, but rather need to be developed through chemotherapy drugs. However, chemotherapy drugs often also cannot eradicate completely, such as doxorubicin that can trigger the occurrence of metastasis through induction of epithelial-mesenchymal transition (EMT) (Bandyopadhyay, *et al.*, 2010). Therefore to overcome the metastatic cancer it is needed to develop the alternative therapeutic agents that can work more effectively.

Among the medicinal plants in Indonesia, Red Betel or Sirih Merah leaves (*Piper crocatum* Ruiz dan Pav) use to be consumed for healthy purposes, including to cure breast cancer (Manoi, 2007). Sirih

Merah was reported to contain biophenolics such as hydroxychavicol, eugenol, chavibetol, piperol (Gundala and Aneja, 2014). Some of these compound has been shown to exert antimutagenic (Chang, *et al.*, 2002), antiproliferative activity in prostate cancer (Paranjpe, *et al.*, 2013), enhance tumor growth by downregulation of NF-κB pathway (Sarkar, *et al.*, 2008), and also eliminate cancerous cells without harming normal cells (Guha, 2006). Other study revealed that apigenin, luteolin, and flavonoid derivatives present in Sirih Merah leaves (Ferrerres, *et al.*, 2014). Meanwhile, flavonoid compounds are reported to have activity to inhibit cancer cell migration through suppressing the expression of MMP-9 and phosphorylation of FAK (Huang, 2005). Since the expression of MMP-9 and the activation of some kinases involved in cell proliferation and migration closely related to the NF-κB activation, it is suggested that Sirih Merah leaves may potentially to inhibit cell migration and metastasis.

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Previous study showed that Sirih Merah leaves methanolic extract (PCM) inhibited the proliferation of T47D human breast cancer cells (Widowati, *et al.*, 2011) and HeLa cervical cancer cells (Wicaksono, *et al.*, 2009) and exhibited cytotoxic activity in WiDr colon cancer cells (Wulandari, 2015). In this study, we used 4T1 cell culture as the model of metastatic breast cancer cells. This cell line is derived from epithelial tissue of mouse breast gland BALB/cfC3H. The properties of 4T1 cells are aggressive, highly metastatic, low immunogenic and represent characteristics that resemble stage IV breast cancer in humans (Zwolac, 2008) as well as resistant to 6-thioguanin. 4T1 cells can metastasize in several organs affected by breast cancer including the lungs, liver, brain and bone. In addition, 4T1 breast cancer cells are ER, PR and HER-2/neu negatives thus describing the TNBC phenotype (Mehta, *et al.*, 2013). The aim of this study is to observe the activity of PCM in inhibiting the proliferation and migration of 4T1 breast cancer cells.

MATERIALS AND METHODS

Extract Preparation

Simplicia of *Piper crocatum* Ruiz & Pav leaves were collected from Bina Agro Mandiri Inc., Bantul, Yogyakarta, Indonesia, and determined at Biological Pharmacy Laboratory, UGM. The simplicia were powdered then extracted by soxhlet with 400 mL methanol on temperature 50°C-70°C in 3 cycles of soxhlet. The filtrate was dried using rotary evaporator on 40°C.

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, 1500 U/ml of Penicilin - 1500 µg/mL of Streptomycin and 0.5 µg/mL Fungizone (Gibco).

Cytotoxic Test

Cytotoxic test was carried out by MTT assay (Mosmann, 1983). Briefly, 4T1 cells were seeded in 96-well plate with 4×10^3 cells/well and were

incubated for 24 hours. After incubation, cells were treated with PCM under increasing concentration of 10, 50, 75, 100, 125, 250 µg/mL for 24 hours. Media were removed and washed with 100 µL 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. After incubating, stopper reagent SDS 10% HCl 0,01 N was added and incubating over night. The absorbance was determined with ELISA reader at λ 595 nm (Biorad).

Scratch Wound Healing Assay

4T1 cells planted 7.5×10^4 cells/well in 24-wellplate. Cells were incubated for 24 hours until 80% confluent. Media were removed and washed with 100 µL PBS (Sigma), media was added with 0.5% FBS for starvation and incubated for 24 hours. Each well was scratched vertically by using yellow tip and treated with PCM. Then the closures of cell migration were observed in 0, 18, 24 and 42 hours under inverted microscope and captured with digital camera.

Data Analysis

Absorbance data was obtained from cytotoxicity assay then converted to a percent of cell viability. IC₅₀ value calculation is performed by the linear regression method. Scratch analysis was done by measure the migration distance using ImageJ software by comparing the distance between untreated and treated cells.

RESULTS

Cytotoxic effect of PCM on 4T1 cells

The cytotoxic effects of PCM was conducted by MTT Assay to determine the IC₅₀ value as the parameter, the low IC₅₀ value indicates potential cytotoxicity. IC₅₀ value of PCM is 120 µg/mL on 4T1 cells (Fig. 1). The curved showed that PCM could decrease the cells viability with dose dependent manner.

Based on Prayong, *et al.* (2008), an IC₅₀ value below 100 µg/mL indicates a potent cytotoxic effect whereas over 100 µg/mL indicates moderate cytotoxicity. Accordingly, PCM has potential IC₅₀ cytotoxic value whereas PCM has IC₅₀ value with moderate cytotoxic category. Even though PCM did not possessed potent cytotoxic effect, it is still interesting to observe the cell migration.

(a)

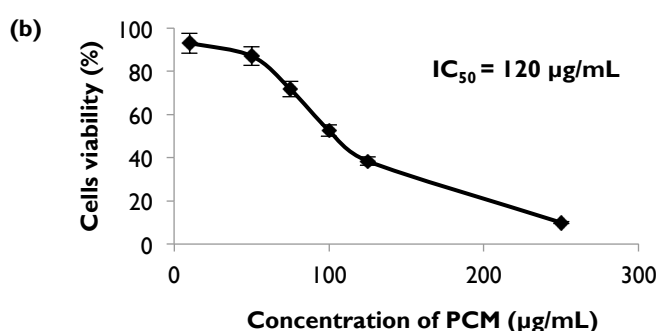
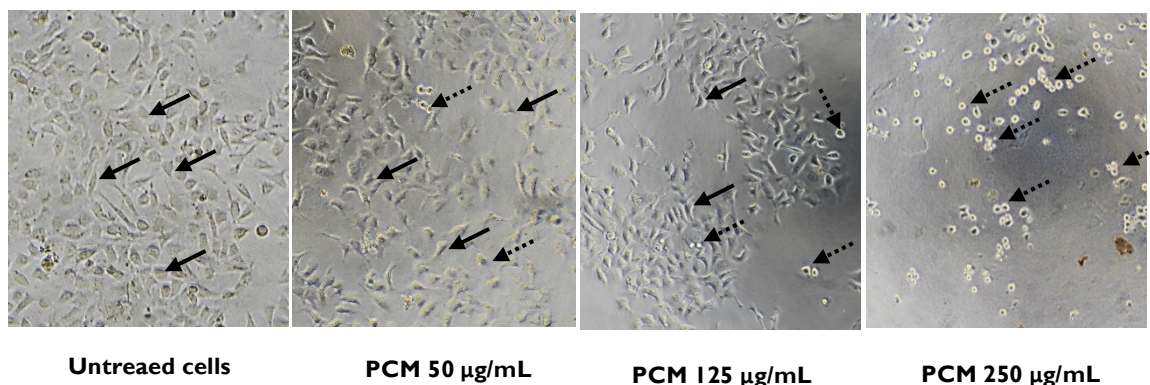


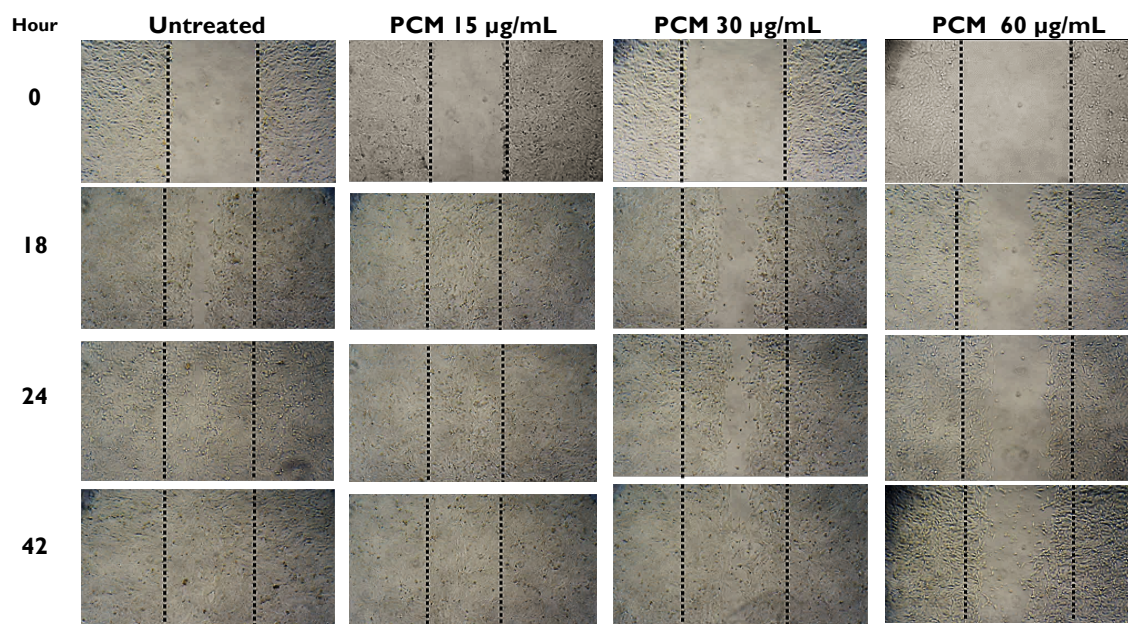
Figure 1. Cytotoxic effect of PCM on 4T1 cells. 4T1 cells (4×10^3 cells/well) were treated with PCM in the concentration as indicated in the figure, then subjected for MTT assay. (a) Morphology of 4T1 cells after treatment of PCM. (b) The cell viability profile showed at various concentrations of PCM. The IC₅₀ value performed triplicate and was calculated by using linear regression in three independent experiments ($p < 0.05$).

Antimigration effect of PCM on 4T1 cells

Cell migration was observed by scratch wound healing assay with various concentration under IC₅₀ value they are $\frac{1}{8}$, $\frac{1}{4}$ and $\frac{1}{2}$ of IC₅₀ (Fig. 2B). These selected concentrations were considered to not affect the growth of 4T1 cells in

order that cell migration is able to be observed collectively. The result showed that PCM performed migration inhibitory activity on 4T1 cells at the concentration of 30 µg/mL after 18th until 42nd hours scratched.

(a)



(b)

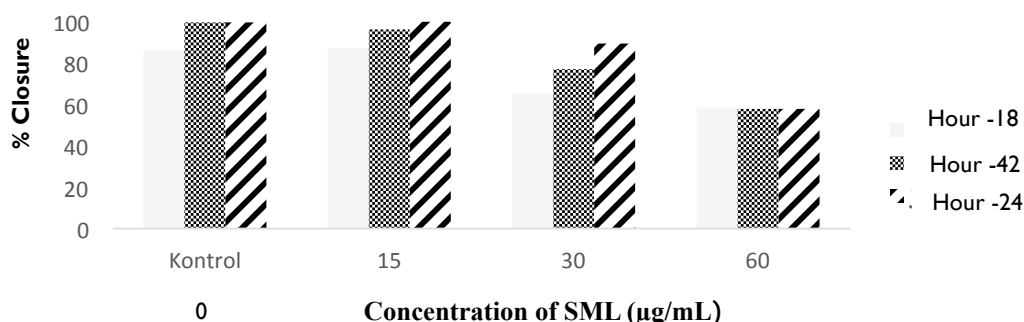


Figure 2. Effect of PCM on 4T1 Cells Migration. 4T1 cells (7.5×10^4 cells/well) were treated with PCM in the concentration as indicated in the figure, then subjected for scratch wound healing assay. (a) The morphology of the cells after scratch and treated with PCM. Observations were made after 18, 24 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.

DISCUSSION

Inhibition of tumor cell migration is crucial in the therapeutic and inhibition of cancer spread, especially in metastasis. Thus, it is necessary to develop anti-metastatic agent. The main purpose of this study is to explore the potential metastasis-inhibitory of *Piper Crocatum* Ruiz & Pav on 4T1 cells. In this study, 4T1 cells were used as the model of human metastatic breast cancer cells (Heppner, *et al.*, 2000).

Previous research has shown that PCM methanolic extract has cytotoxic activity against T47D breast cancer cells through inhibition of p44/p42 phosphorylation associated with cell growth and an important target in cancer therapy (Wicaksono, *et al.*, 2009). Ethanolic extracts of PCM has been also reported to have antiproliferative effects and are able to induce apoptosis in HeLa cervical cancer cells (Wicaksono, *et al.*, 2013).

So far, there have been several studies conducted to determine the inhibitory activity of migration and metastasis of certain compounds in

some cell types. Some of them are compounds of the class of flavonoids and alkaloids. Flavonoids are a class of low molecular weight polyphenol compounds, which are widely distributed in plants and have activity inhibiting cell migration and endothelial activation (Middleton, *et al.*, 2000). Flavonoid compounds are reported to have activity in inhibition of growth and tumor cell metastasis (Piantelli, 2006). Previously, Huang (2005) reported that flavonoid compounds such as luteolin and quercetin are able to suppress the expression and phosphorylation of FAK and MMP-9 expression in vitro that could trigger invasive potential suppression and cell migration. In addition to flavonoid compounds, certain types of alkaloids are also reported to have metastatic inhibitory activity by inhibiting cell migration, such as piperine. It was reported that piperine is able to inhibit the growth and migration of 4T1 breast cancer cells by inhibiting Erk's phosphorylation in vitro and in vivo (Lai, 2012). Flavonoids are known to be also present in PCM (Manoi, 2007; Hartini, *et al.*, 2013). The presence of the compounds in PCM might be responsible for cytotoxic and migration inhibitory activity.

This study described the potency of PCM as an anti-cancer agent which has the cytotoxic potency of 4T1 cell with IC_{50} values of 120 $\mu\text{g/mL}$ (Fig. 1). The decrease in viability of 4T1 breast cancer cells with the phenomenon of dose dependent manner showed that PCM has potential as a chemopreventive agent in breast cancer. However, based on the IC_{50} value obtained in this study, PCM appeared to have a more potent effect when compared to some extracts that had been tested on 4T1 cells in other previous studies. Some extracts of them are methanolic extract of *Prunus africana* with IC_{50} value of 164 $\mu\text{g/mL}$, methanolic extract of *Maytenus senegalensis* leaves with IC_{50} value of 256 $\mu\text{g/mL}$, methanolic extract of *Warbugia stuhlmannii* with IC_{50} value of 123 $\mu\text{g/mL}$ (Nabende, 2015) and water extract of *Potentilla reptans* L. roots with IC_{50} value of 280 $\mu\text{g/mL}$ (Radovanovic, 2013). This showed that PCM also includes a potent extract against 4T1 cells. Potency of methanolic extract of PCM has also been tested in several cancer cells, among others, on T47D breast cancer cells and colon cancer cells WiDr (Wicaksono *et al.*, 2009; Wulandari *et al.*, 2015). In the studies, methanolic extract of PCM was reported to have cytotoxic

activity against human breast cancer cells T47D with IC_{50} value of 44 $\mu\text{g/mL}$ (Wicaksono, *et al.*, 2009). Meanwhile on WiDr colon cancer cells, it was reported to have cytotoxic activity with IC_{50} value of 100 $\mu\text{g/mL}$ (Wulandari, 2015). The potential of PCM as a chemopreventive agent may continue to be tested further by fractionation in order to obtain an active fraction that has a greater potential as a chemoprevention agent. Moreover, the cytotoxic activity test in this study has not been able to show the mechanism of cytotoxic activity of PCM in 4T1 cells. The mechanism of death or inhibition of cell proliferation by a compound can occur through the process of apoptosis and or cell cycle arrest. So there is a need for further research to find out what mechanism is responsible for PCM cytotoxic effect on 4T1 cells.

In this present study, we also observed the inhibition of cancer cell migration as the one of parts in metastasis process by treatment of PCM through scratch wound healing assay. Based on the percent graph of 4T1 cell closure (Fig. 2B), the treatment of PCM with concentration at 15 $\mu\text{g/mL}$ has not demonstrated any migration inhibitory activity by showing a % closure profile of the cell similar to cell control's (untreated). In the PCM concentration of 30 $\mu\text{g/mL}$, 4T1 cells began to show a decrease in percentage closure which indicated that at this concentration, PCM began to effect inhibition of cell migration time dependent from the 18th hour until the 42nd hour. While at 60 $\mu\text{g/mL}$ concentrations of PCM also showed migration inhibitory activity with a smaller % closure compared to 15 $\mu\text{g/mL}$ and 30 $\mu\text{g/mL}$. Nevertheless, when viewed on the graph of cell closure (Fig. 2B), the % closure profile at 60 $\mu\text{g/mL}$ did not show any increase in time with a % closure being in the 58% range. This revealed that at these concentrations PCM is actually capable of inhibiting 4T1 cell migration.

From the results of this study, PCM is shown to have cytotoxic activity and is also able to inhibit the migration of 4T1 breast cancer cells. In this case, inhibition of 4T1 cell migration by PCM is not affected by its cytotoxic activity. Cytotoxic activity and inhibition of cell migration in this study are two things with different mechanisms. This is demonstrated, although with the treatment of PCM at low concentrations under cytotoxic concentrations, PCM is able to inhibit the migration of 4T1 breast cancer cells starting at a concentration

of 30 µg/mL. That means, through scratch wound healing test on non-cytotoxic dosing or not killing cells PCM is able to inhibit the closure of 4T1 breast cancer-free cell zone starting at a concentration of 30 µg/mL.

The study about methanolic extract of PCM activity in inhibiting cell migration associated with determination of the responsible compound for its activity has never been done before, so there is a need for a continuous series of research in order to infer what mechanisms mediate the inhibition of migrating 4T1 cells by PCM. However, it is possible that the flavonoid compounds contained in PCM are responsible for the inhibitory activity of migrating 4T1 breast cancer cells by inhibiting cell migration in various signaling pathways as described previously. In addition, further studies such as the determination of active compounds on PCM as well as tracing of proteins that play a role in the inhibitory activity of 4T1 cell migration are necessary in order to obtain sufficient and valid interpretation of data to be able to trace the potential of PCM to the metastasis of cancer cells.

CONCLUSION

PCM has cytotoxic activity on 4T1 metastatic breast cancer cells with IC₅₀ value of 120 µg/mL and exhibit the migration inhibitory activity on the cells at 30 µg/mL concentration (1/4 of IC₅₀).

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