

# Oyster Mushroom (*Pleurotus ostreatus*) Inhibits Migration and Metastasis on 4T1 Breast Cancer Cells

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## Abstract

Metastasis is the main cause of death among breast cancer patient. *Pleurotus ostreatus* is known as anticancer agent that inhibits angiogenesis. Ethanolic extract of *Pleurotus ostreatus* (EEP) which contains lovastatin is predicted to inhibit metastatic cancer through inhibition of MMP-2 and MMP-9. The aim of this study was to determine antiproliferative and anti metastatic activity of EEPw (Ethanolic extract of wet *Pleurotus ostreatus*) and EEPd (Ethanolic extract of dried *Pleurotus ostreatus*) in 4T1 metastatic breast cancer cells line. Qualitative analysis of lovastatin was determined by thin layer chromatography (TLC) using dichloromethane and ethyl acetate as mobile phase and lovastatin standard. Scratch wound healing assay was used to determine migration inhibition ability of EEP while MMP-9 and MMP-2 activity were analysed by gelatin zymography. Molecular docking was performed to know the interaction between lovastatin and MMP-2 & MMP-9. The result showed that EEPw and EEPd contain lovastatin which were proved by spray reaction with anisaldehyde. Each of EEPw and EEPd had cytotoxic activity with  $IC_{50}$  760 and 400  $\mu\text{g/mL}$  respectively. Both of them inhibited closure for about 50 % on 4T1 metastatic breast cancer cells line compared to control. Either EEPw or EEPd decreased MMP-9 expression level compared to control. Lovastatin had higher affinity to bond with either MMP-2 or MMP-9 than native ligand. Overall, EEP could be developed as anticancer agent which was targeted on MMP-2 and MMP-9.

**Keywords :** *Pleurotus ostreatus*, 4T1 metastatic cells, MMP-2, MMP-2, antimetastatic

## INTRODUCTION

In 2014, 21.4% of deaths among women was due to breast cancer (WHO, 2014), especially after the occurrence of metastasis. Metastasis is a complex molecular process which cancer cells leave the first place to grow towards other organs and proliferate to form a new tumor mass (Brooks, *et al.*, 2010). Metastasis activity was due to the activity of metalloproteinases (MMPs), especially MMP-2 and MMP-9 which degraded collagenase VI (gelatin) in cancer cells (Liotta, 1991, Stetler-Stevenson, 1994). Nowadays, treatment of metastatic cancer is targeted to the activity of MMP-2 and MMP-9. Effective and efficient treatment can be done by utilizing potential compounds from natural materials such as oyster mushroom (*Pleurotus ostreatus*).

*P.ostreatus* is an edible mushroom that contains lovastatin (Lakshmanan and Radha, 2013). Lovastatin could decrease angiogenesis by inhibit VEGF activity (Zhao, *et al.*, 2010;

Xiao, *et al.*, 2012). Angiogenesis triggered cancer cell metastasis (Rivolis, *et al.*, 2002). Therefore, lovastatin activity as inhibitors of VEGF could inhibit MMP-2 and MMP-9 activity to prevent metastasis.

This study aims to determine the ability of *P.ostreatus* extract to inhibit cancer cells proliferation and metastasis. The study was conducted by observing cell migration by scratch wound healing assay and protein activity of MMP-2 and MMP-9 by gelatin zymography. At the molecular level, molecular docking was performed between lovastatin and target protein MMP-2 and MMP-9.

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## MATERIALS AND METHODS

### Sample

The *P.ostreatus* were collected from Sleman, Yogyakarta and determined by Biological Pharmacy Laboratory, Faculty of Pharmacy Universitas Gadjah Mada. *P.ostreatus* were extracted in ethanol 96% for 72 h, then the mixture was filtered to remove the marc. The extract was concentrated using rotary evaporator. Sample was dissolved in dimethyl Sulfoxide (DMSO) (Sigma). Both of EEPd and EEPw were diluted in cell culture medium before being applied. DMSO was used as the co-solvent to dissolved samples in culture medium.

### Cell Culture

4T1 cells were obtained from Cancer Cancer Chemoprevention Research Center, Yogyakarta. The cells were maintained in DMEM (Gibco) supplemented with 10 % Fetal Bovine Serum (FBS) (Sigma), 1.5% Penicillin-Streptomycin, and 0.5% fungizone at 37°C in humidified atmosphere of 5 % CO<sub>2</sub>. Trypsin-EDTA 0.25% (Gibco) was used in cell subculture.

### Cell Viability Assay

Cell viability was determined using MTT assay. Cells were seeded into a 96-well plate (8000 cells per well) and incubated for 24 h. Cells were treated with various concentration of EEPd and EEPw. Certain concentration of either single or combination of samples were the applied. After 48 h incubation, MTT reagent [3-(4,5-dimethyl-2-thiazoly)-2,5-diphenyltetrazolium bromide in PBS (5mg/mL)] was added to each well. The plate was incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 3 h, formazan crystals were dissolved in 10% SDS and incubated overnight. The absorbance of cells was measured at 595 nm by a microplate reader. The data was presented as percentage of viable cells (%).

### Scratch Wound Healing Assay

Cells (5x10<sup>4</sup> cells/well) were seeded on 24 well plates until 80% confluent. Cells were scratched using microtip. Cells were incubated 24 h hour, then replaced with fresh medium contain EEPw and EEPd. After that incubated

in 37°C for 48 h, cells were observed in 0 h, 18 h, 24 h, and 42 h under the microscope.

### Gelatin-Zymograph Methods

Gel of 20% acrylamid-bis acrylamid containing 1% gelatin was made in the buffer. Cells were washed by PBS and then incubated in 37°C for 24 h. Medium was collected and centrifuged at 400 g for 5 min, 4°C and supernatant was collected. Cells were washed with cold PBS then added with 2 ml cold lysis buffer.

Cells was harvested with a scrapper to collect lysate and incubated on ice for 15 minutes. Lysate then centrifuged for 16000 g for 2 min at 4°C and supernatant was collected. Mix 75 mL of the supernatant with 25 mL of sample buffer (4x). 30 mL of sample was put into sinks. Electrophoresis was performed with the condition of 125 V, 30-40 mA for 90 minutes. The Gel then soaked in coomassie blue dye solution for 1 hour to form a transparent band with a dark blue background.

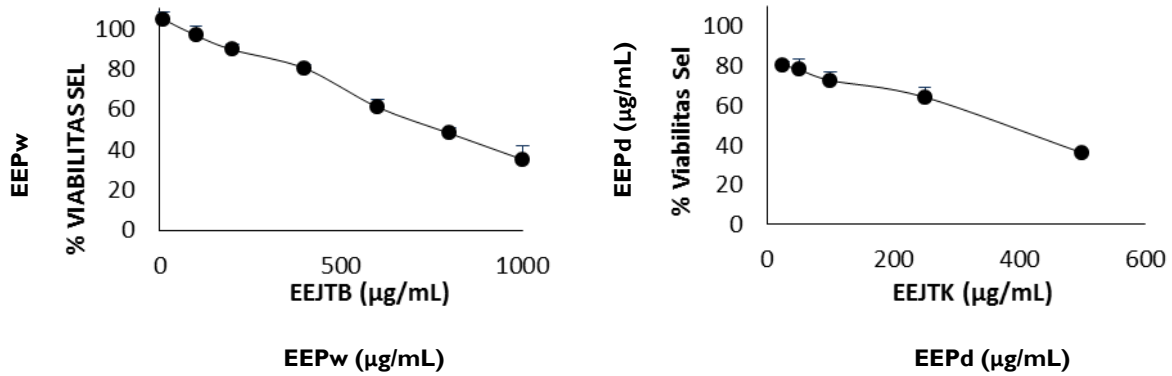
### Molecular Docking

*In silico* study by molecular docking was conducted to examine the affinity of 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 lovastatin in Ligan native. Ligand preparation was done by using Marvin Sketch. 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

### EEPw and EEPd Showed Cytotoxic Activity on 4T1 Cells

Cell viability assay was done to determine the inhibitory concentration (IC<sub>50</sub>) of EEPw and EEPd, on 4T1cells (Fig. 1). All of these compounds shows cytotoxic effect in dose dependent manner. EEPw and EEPd had the IC<sub>50</sub> values of 760  $\mu$ g/mL and 400  $\mu$ g/mL, respectively.



**Figure 1. Cell viability assay of (a) EEPw (b) EEPd in 4T1 cells.** Cells were incubated for 24 h then add with MTT and incubated overnight then read the absorbance using ELISA reader. Result showed that EEPd and EEPw had the IC<sub>50</sub> values 400 µg/mL (a) and 760 µg/mL (b).

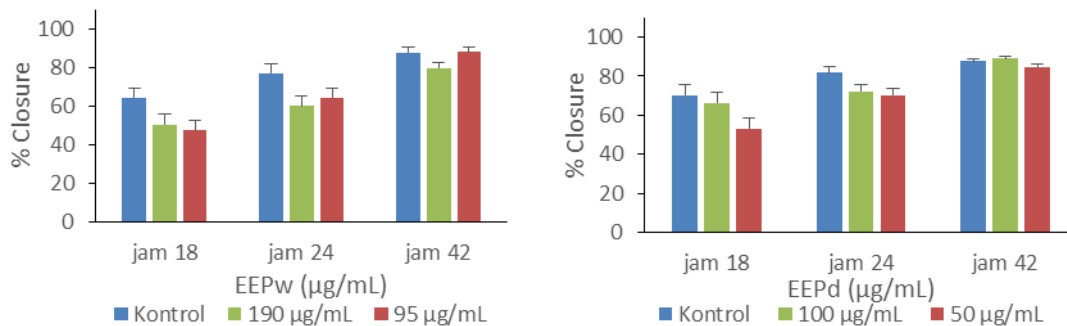
### Scratch Wound Healing Assay

The scratch assay was done to explored EEPw and EEPd effect in migration breast cancer cell 4T1 (Fig.2 and 3). 4T1 cell was used as model in scratch assay due to its ability to performed metastasis.

EEPw and EEPd had the abilit to lowering the closure area on 4T1 breast metastatic cells compared to control cells.

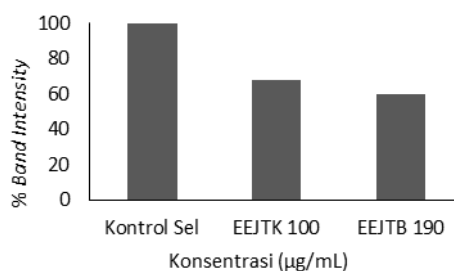
### Molecular docking

*In silico* test by molecular docking was done to predict the ability of oleandrin and synamaldehyd to interacting with MMP-2 and MMP-9 receptor. That interaction is visualized and determinated by amino acid residue which interacting between oleandrin and cynamaldehyd ligand against MMP-2 and MMP-9 (Table 1).



**Figure 2. Effect of EEPd and EEPw against % closure.** Observation area closure was performed using Image-J program to calculate the area of a scratch on 0h , 18h, 24h and 42h with a concentration EEPw or EEPd ¼ and 1/8 then expressed as % closure.

### Gelatin Zymograph assay



**Figure 3. Effect EEPw and EEPd to % the intensity of the band**

**Table 1. Molecular Docking Score**

	MMP-2	MMP-9
RMSD	2,375 °A	1,8338 °A
Lovastatin	-111	-94
Ligan Native	-124	-136

## DISCUSSION

Overexpression of the protein MMP-2 and MMP-9 is one factor leading to breast cancer cell metastasis (Liotta, 1991). The results showed that EEPw and EEPd were cytotoxic against 4T1 breast cancer cells with IC<sub>50</sub> of 760 µg/mL and 400 µg/mL respectively. EEP has intermediate potency as cytotoxic agent.

Inhibition of the closure area show the ability of EEP to reduce breast cancer cell migration. Results of the study showed that EEPw and EEPd with the smallest concentration of IC<sub>50</sub> could of decreased % closures compared to control cells.

MMP-9 is an MMP family members that have been known to have a role in the invasion and metastasis of cancer cells. MMP-9 are classified into type IV collagenase is based on its ability to hydrolyze IV collagen (gelatine) (Wilhelm, *et al.*, 1989). Results of the test using the gelatin zymograph showed a decrease in activity of MMP-9. EEPd and EEPw reduce the expression of MMP-9 protein in compare to control based on the intensity of the band.

Based on molecular docking, lovastatin could to compete with native ligand to interact with MMP-2 and MMP-9 as being shown through the same amino acid residue that interacted with protein MMP-2 and MMP-9. Therefore, bond between lovastatin with MMP-2 and MMP-9 can reduce the chances of continuous proliferation through inhibition of protein expression of MMP-2 and MMP-9. These results indicate that EEP inhibit expression of MMP-2 and MMP-9 protein. Hence, proliferation of 4T1 breast cancer cells could be inhibited.

## CONCLUSION

The results showed that EEP reduce viability of 4T1 breast cancer cells and inhibits cell migration and protein expression of MMP-2 and MMP-9.

## REFERENCES

- Alarcón,J., Aguila, S., Arancibia-Avila, P., Fuentes, O., Zamorano-Ponce, E. and Hernández M., 2002, Production and Purification of Statins from *Pleurotus ostreatus* (Basidiomycetes) Strains., *Z. Naturforsch C.*, **58**(1-2),62-4.
- Brooks, S.A, Lomax-Browne, H.J., Carter, T.M., Kinch, C.E. and Hall, D.M., 2010, Molecular Interaction in Cancer Cell Metastasis, *Acta Histochem.*, **112**(1), 3-25.
- Green, M. and Riana, V., 2008, Epidemiology Screening and Diagnosis of Breast Cancer in the Asia-Pasific Region: Current Perspectives and Important Consideration, *Asia Pac. J. Clin. Oncol.*, **4**(S3),S5-S13.
- Herndndez, D., Sanchez, J.E. and Yamasaki, K., 2003, A Simple Procedure for Preparing Substrate for *P. Ostreatus* Cultivation, *Bioresour. Technol.*, **90**(2), 145-150.
- Kalmis, E., Azbar, N., Yildiz, H. and Kalyoncu, F., 2008, Feasibility of Using Olive Mill Effluent (OME) as a Wetting Agent During the Cultivation of Oyster Mushroom, *Pleurotus ostreatus* on Wheat Straw, *Bioresour. Technol.*, **99**(1),164-169.
- Lakshmanan, D. and Radha, K.V., 2012, Production of Lovastatin from *Pleurotus ostreatus* and Comparison with Commercial Tablets, *Int. J. Pharm. Pharm. Sci.*, **2**(3), 53-56.
- Liotta, L.A., Steeg, P.S. and Stetler-Stevenson, W.G., 1991, Cancer Metastasis and Angiogenesis: an Imbalance of Possitive and Negative Regulation, *Cell*, **64**(2), 327-336.
- Ramirez, A., Clavijo, C., Palanisamy, M., Soler-Rivas, C., Rodriguez, A., Francisco, M.M., *et al.*, 2011, Edible Mushrooms as Potensial Sources of New Hypocholesterolemic Compound,

- International Conference on Mushroom Biology and Mushroom Products.*
- Ramírez-Anguiano, A.C., Santoyo, S., Reglero, G. and Soler-Rivas, C., 2007, Radical Scavenging Activities, Endogenous Oxidative Enzymes and Total Phenols in Edible Mushrooms Commonly Consumed in Europe, *J. Sci. Food Agric.*, **87**(12), 2272-2278.
- Rivilis, I., Milkiewicz, M., Boyd, P., Goldstein, J., Brown, M.D., Egginton, S., et al., 2002, Differential Involvement of MMP-2 and VEGF During Muscle Stretch - versus Shear Stress-induced Angiogenesis, *Am. J. Physiol. Heart Circ. Physiol.*, **283**(4), H1430-H1438.
- Stelter-Stevenson, W.G., 1994, Progegelatinase A Activation During Tumour Cell Invasion, *Invasion Metastasis*, **14**(1-6), 259-268.
- Toth M, Fridman R. 2011. Assessment of gelatinases (MMP-2 and MMP-9) by Gelatin Zymography, *Methods Mol. Med.*, **57**,163-174.
- WHO, 2014, WHO | Cancer' WHO, <https://www.who.int/mediacentre/factsheets/fs297/en/>, Cited August, 19, 2014.
- Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A. and Goldberg, G.I., 1989, SV40-Transformed Human Lung Fibroblasts Secrete a 92-kDa type IV Collagenase Which is Identical to That Secreted by Normal Human Macrophage, *J. Biol. Chem.*, **264**(29), 17213-17321.
- Wong, W.W., Dimitroulakos, J., Minden, M.D. and Penn, L.Z., 2002, HMG-CoA Reductase Inhibitors and The Malignant Cell: the Statin Family of Drugs as Triggers of Tumor-Specific Apoptosis, *Leukemia*, **16**(4), 508-519.
- Wu, J., Wong, W.W., Khosravi, F., Minden, M.D. and Penn, L.Z., 2004, Blocking the Raf/MEK/ERK Pathway Sensitizes Acute Myelogenous Leukemia Cells to Lovastatin-induced Apoptosis, *Cancer Res.*, **64**(18),6461-6468.
- Xiao, Y., Li, Y., Han, J., Pan, Y., Tie, L. and Li, X., 2012, Transgelin 2 Participates in Lovastatin-Induced- Anti-angiogenic Effectc in Endothelial Through a Phosphorylated Myosin Light Chain-Related Mechanism, *PLoS One*, **7**(10), e46510.
- Zhao, T.T., Trinh, D., Addison, C.L. and Dimitroulako, J., 2010, Lovastatin Inhibits VEGFR and AKT Synergistic Cytotoxicity in Combination with VEGFR Inhibitor, *PLoS One*, **5**(9), e12563.