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**Research Article**

# INHIBITORY ACTIVITY OF ENDOPHYTIC FUNGI ISOLATED FROM SUKABUMI TURMERIC PLANT *(Curcuma longa L.)* TOWARDS MCF-7 CELL LINE

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## ABSTRACT

Kunyit or turmeric plant (*Curcuma longa* L.) is a native southern Asia plant that has been widely used as herbal medicine. In our previous research, we isolated and screened 44 of endophytic fungi of turmeric plant from Sukabumi and Cibinong to determine their antioxidant activity. There were 4 samples isolated from Sukabumi with antioxidant activity for more than 70% at concentration of 100ppm, K.Cl.Sb.R9 (93%), K.Cl.Sb.A11 (81%), K.Cl.Sb.B1 (79%) and K.Cl.Sb.R11 (71%). This research aimed to determine inhibitory activity of the endophytic fungi towards estrogen positive MCF-7 breast cancer cell line. To obtain the filtrate, the broth cultures were filtered and the extracellular fraction was extracted with ethylacetate. The inhibitory activity was determined by using MTT assay. The result showed that the ability of endophytic fungi to inhibit MCF-7 growth was dose-dependent. $IC_{50}$ of endophytic fungi K.Cl.Sb.R9 was 579±38 ppm, K.Cl.Sb.R11 was 542±21ppm, K.Cl.Sb.B1 was 446±15ppm and K.Cl.Sb.A11 was 520±28ppm. Fluorescent double staining based method using calcein AM and ethidium bromide was performed to confirm the inhibitory activity. At 500ppm of filtrate concentration with 24h of cell treatment, treated cell lines showed fewer viable cells compared to untreated cell lines. To conclude, the four isolates of endophytic fungi are able to inhibit proliferation of human breast cancer MCF-7 cell line.

**Key words:** MCF-7, inhibitory activity, endophytic fungi, turmeric plant.

## INTRODUCTION

Turmeric plant is one of the herbal plants which belong to the group of gingers (Zingiberaceae) and originated from the Southeast Asia. Currently, the plant is widely cultivated in the tropical and subtropical area of the world. Turmeric has a rhizome containing curcuminoid, essential oils, carbohydrates and minerals (Krup *et al.,* 2013). There are many studies on turmeric extracts from cell culture to animal trial which reported its potential as therapeutic agents in diseases, such as inflammatory bowel, arthritis, colon cancer, pancreatic cancer and cervical neoplasia. The extract showed various activities as well, including antioxidant, antimicrobial, antiangiogenesis, antitriglyceride, hepatoprotective and cardio-protective (Li *et al.,* 2011; Ikpeama *et al.,* 2014).

*Curcuma longa L.* has associated with endophytic microbes. The endophytic microbe is microorganisms that colonized with plant tissue without causing any adverse effect. It produces biological compounds as well as secondary metabolites in accordance with the host plant. The ability to produce bioactive compounds together with the host is a potential source of medicinal raw materials utilization (Zakiyah *et al., 2015*; Dompeipen, 2015). As the most phytochemically investigated species of Curcuma, *Curcuma longa* resulted in 235 compounds with various activities that have been identified and isolated from the flowers, leaves, roots and rhizomes which primarily are phenolic and terpenoid compounds. Thus, exploring endophytic microbes from *Curcuma longa L* increases the possibility to discover novel compound which is substantial in resistance issues (Alvin *et al*, 2014).

One of the endophytic microorganisms of *Curcuma longa L.* is fungi. *Fusarium nivale*, F.

*Solani*, *Doratomyces stemonitis*, *Penicillium* sp, *Diaphorte* sp were species that found in *Curcuma longa* (Ashraf and Javaid, 2005; Singh *et al,* 2014; Maehara *et al,* 2011). Various studies have reported the potential of endophytic fungi from the plant as antioxidant, antidiabetic anti-microbial and antitumor. Currently, more than 100 compounds have been isolated from fungi with high potential of antitumor activities (Suryanarayanan *et al.,* 2009).

Our previous studies have isolated 44 endophytic fungi isolates of turmeric from Sukabumi and Cibinong. The results of antioxidant activity determination showed that there were 4 isolates from Sukabumi with high antioxidant activity for more than 70%, namely K.Cl.Sb.R9 (93%), K.Cl.Sb.A11 (81%), K.Cl.Sb. B1 (79%) and K.Cl.Sb.R11 (71%)(Bustanussalam *et al.,* 2015; Widowati *et al.,* 2016). This study aims to determine the inhibitory activity of endophytic fungi towards human cancer cell line using human breast cancer cell line MCF-7 as a model.

## MATERIALS AND METHODS

### Isolate cultivation, fermentation, and extraction

Each isolate from previous research (Bustanussalam *et al.,* 2015) was cultivated in Potato Dextrose Agar and incubated at room temperature for 48-72h. The isolates were fermented in 2L of Potato Dextrose Broth at 150rpm for 3 days. The broth cultures were vacuum filtered to obtain the filtrates. The filtered filtrates were extracted using ethylacetate and dried by rotavapor. The dried filtrate was redissolved in Dimethyl Sulfoxide (Merck, Germany). Various concentrations of filtrate (100ppm to 800ppm) were used to treat the MCF-7 cell line.

### MCF-7 cell line propagation and treatments

MCF-7 cells (from mammalian cell culture laboratory, Indonesian Institutes of Sciences) were thawed and washed with 9mL of DMEM medium (Gibco, USA) containing 100units/mL of penicillin (Sigma, USA) and 100mg/mL of streptomycin (Sigma, USA). Cells were grown in the same medium containing 20% fetal bovine serum (FBS, Sigma USA) at 37°C and 5% $CO_2$. After 90% of confluency, the cells were washed with phosphate buffer saline (1.15g Na2HPO4; 0.2g KH2PO4; 8g NaCl and 0.2g KCl per liter, pH 7.2) twice and detached with the addition of 500µL of trypsin-EDTA (0.25% trypsin in 0.53mM EDTA) at 37°C for 5min. The cells were transferred into tissue culture flask, propagated in DMEM containing 10% FBS until 70% confluency and applied in 96 well (5000 cell/well) or 6 well plate (15.000 cell/ well) for further study. The cells were grown overnight in the same media, washed with 100µL of PBS and treated with various concentration of extract for 24h. We used anticancer tamoxifen to validate the assay. The treatment conditions were based on a report by Ningrum *et al* (2015) with some modifications.

### MTT assay

Treated cells in 96 well plates were washed twice with 100µL of PBS. 100µL of DMEM with 10% FBS containing MTT (Merck, Germany), with final concentration 0.5mg/mL, was added to each well. Cells were then incubated for 3h and the medium was discarded. Formazan crystals that formed at the bottom of the well were dissolved in 100µL of SDS 10% in 0.01M HCl (Septisetyani *et al.,* 2014). The cells were incubated overnight and measured by spectrophotometery at 570nm. The percentage of the viable cell was compared to untreated control. 30µM of tamoxifen (Sigma, USA) was used as positive control. The experiments were done in triplicates in three dependent times.

$$\text{Growth (\%)} = \frac{\text{OD (sample-blank)}}{\text{OD (control-blank)}} \text{ X } 100\%$$

### Viability staining

Treated cells in 4 well plates were washed twice with 1mL of PBS and stained with 500µL of 2µM calcein AM and 4µM ethidium bromide in PBS. The cells were incubated at room temperature for 45 minutes and observed under fluorescence microscope.

## RESULTS AND DISCUSSIONS

### Isolate Cultivation, Fermentation, and Extraction

It was reported that environmental condition and interaction of endophytic microbe with the pathogens are the most influential factors of morphology variations

(Araujo *et al.,* 2002). In our previous research, we have molecularly identified K.Cl.Sb.B1 as *Colletotrichum sp* (Widowati *et al.,* 2016). Several reports claimed that the species has antioxidant and cytotoxic activities (Tianpanich *et al.,* 2011; Gangadevi and Muthumary, 2008; Chapla, 2014). The isolates were fermented in Potato Dextrose Broth medium. There were two fermentation results of endophytic fungi, biomass, and filtrate. The fungal filtrate was further extracted by ethyl acetate. We used ethyl acetate as a moderately polar solvent due to some advantage properties, such as volatile, relatively non-toxic, and non-hygroscopic (Linn, 1999). The yield quantification of dried fungal filtrate (Table I).

Table I. Yield quantification of the dried filtrate of endophytic fungi isolates from 2 L culture.

| Isolate | Weight (g) |
|---|---|
| K.Cl.Sb.R9 | 0.5877 |
| K.Cl.Sb.R11 | 1.0300 |
| K.Cl.Sb.A12 | 0.2669 |
| K.Cl.Sb.B1 | 0.5805 |

**Inhibitory activity assay**

To validate the assay on MCF-7 cell line, we used commercial anticancer as a positive control. There are some cancer drugs that may be used as assay control, such as leucovorin, vinblastine, 5-fluorouracil, tamoxifen, interleukin-2, retinoic acid or folinic acid (Taylor *et al.,* 1992; Lee *et al.,* 1992; Lindner *et al.,* 1997; Bernhard *et al.,* 1992). Tamoxifen is cell-permeable anticancer and reversible inhibitor of protein kinase C. It also induces cell cancer apoptosis through the induction of p21 protein. Tamoxifen is a potent synthetic anti-estrogenic agent and cytostatic for estrogen-dependent cell line as well (Lippman and Bolan, 1975).

It was reported that tamoxifen inhibits proliferation of breast cancer cells by apoptosis induction. On MCF-7, Tamoxifen induces rapid death that mediated by extracellularly signal-regulated kinase signaling. The mechanism is ER-dependent and also assisted by ICI182780 insensitive nongenomic mechanisms (Zheng *et al.,* 2007). Therefore, based on its activity, tamoxifen was chosen as a positive control in this research. MCF-7 is ER positive cell line that originated from human breast adenocarcinoma. We used MCF-7 as a model based on several studies which reported that some endophyte fungi had an inhibitory effect on its proliferation (Maemura *et al.,* 1999; Katoch, 2014; Higginbotham, 2013). A study of endophyte fungi fusarium tricintum on MCF-7 cell line reported that the inhibition of cell growth highly associated with low production of inflammatory cytokine TNFα. TNFα has been known as a mediator of the inflammatory response that influence cancer growth (Vasundhara *et al.,* 2016).

Inhibitory activity assay performed by using MTT assay. We used SDS-0.01M HCl as solvent based on our previous optimization of solvent (Septisetyani *et al.,* 2014). The most advantage of using SDS is the elimination of washing step which causes partially dissolved of formazan crystals. We applied a various concentration of samples ranging from 100 to 800 ppm. The 24h treatment was chosen due to several publications of cytotoxic activity of endophytic fungi on MCF-7 cell line (Balakumaran *et al.,* 2015; Vennila *et al.,* 2012; Hulikere *et al.,* 2016; Sadananda *et al.,* 2016).

Table II. $IC_{50}$ of endophytic fungi on MCF-7 cell line growth inhibition

| Isolate | IC50 |
|---|---|
| K.Cl.Sb.R9 | 579±38ppm |
| K.Cl.Sb.R11 | 542±21ppm |
| K.Cl.Sb.B1 | 446±15ppm |
| K.Cl.Sb.A11 | 520±28ppm |

The result showed that the growth inhibition of MCF-7 cell line was dose dependent (Figure 1) with $IC_{50}$ value of endophytic fungi was around 500 ppm (Table II. In our previous research, 100ppm of fungal filtrate results in strong antioxidant activity (Bustanussalam *et al.,* 2015). This denotes that the anti oxidant activity of endophytic fungi possibly more dominant comparing to inhibitory activity. Many studies reported various abilities of antiproliferative and antioxidant activities of endophytic fungi.

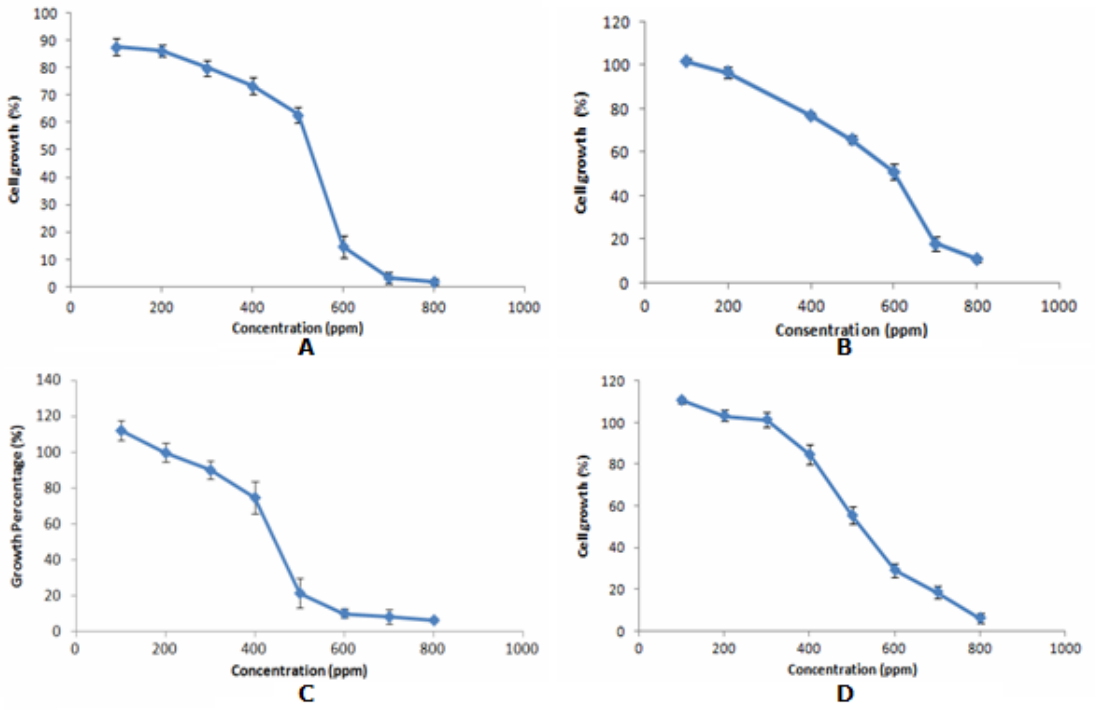


Figure 1. Inhibitory activity of endophytic fungi on MCF-7 growth by MTT assay. The Absorbance was read at 540nm. A=K.Cl.Sb.R9, B=K.Cl.Sb.R11, C=K.Cl.Sb.B1, D=K.Cl.Sb.A11

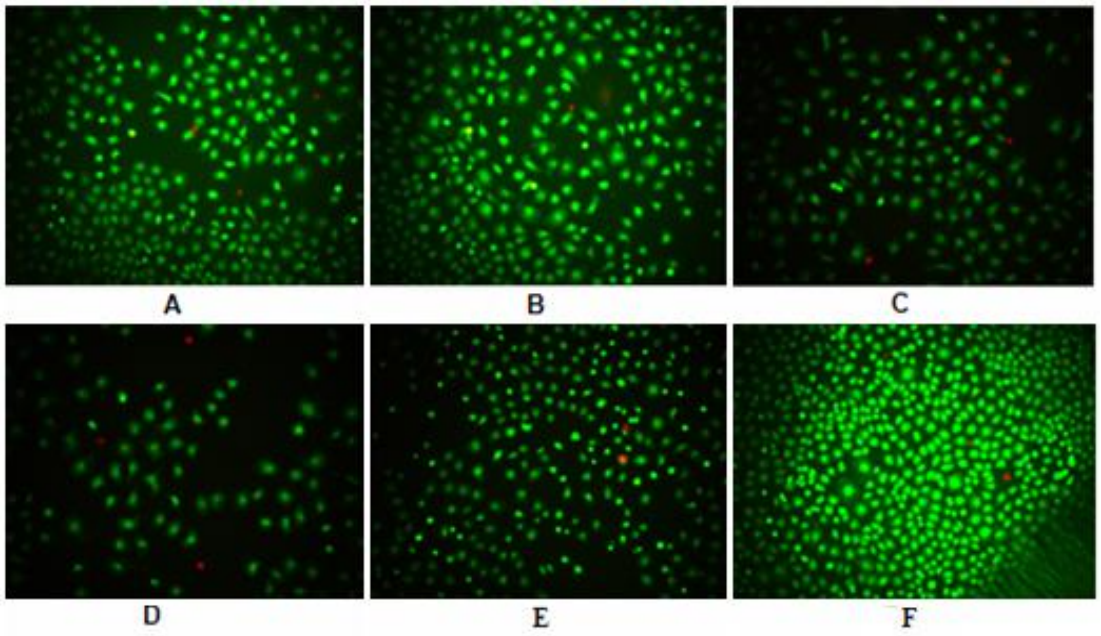


Figure 2.Cell staining based assay of endophytic fungi on MCF-7 cell line. 10X magnification. The cells treated by A=K.Cl.Sb.R9, B=K.Cl.Sb.R11, C=K.Cl.Sb.B1, D=K.Cl.Sb.A11, E=tamoxifen and F= untreated

Paraconiothyrium sp. P83F4 / 1 isolated from Rheedia brasiliensis known to have equal antioxidant and antiproliferation activities (Carvalho *et al.,* 2012). However, endophytic fungi Fusarium tricinctum has more dominant antiproliferative than that of antioxidant (Vasundhara *et al.,* 2016).

Inhibitory activity was furtherly confirmed by staining method using Calcein AM-ethidium bromide III fluorescent agents. The assay principle was based on measurement of two parameters of living cells, intracellular esterase activity as well as plasma membrane integrity. Calcein AM is nonfluorescent cell-permeant that can be enzimatically converted by esterase into intensely fluorescent calcein (Papadopoulos *et al.,* 1994). The polyanionic dye calcein will retain within the living cells producing green fluorescence. Ethidium bromide III is non permeable fluorescent that excluded by intact plasma membrane of live cells. The dead cells will be stained as red colour. The advantage of using ethidium bromide III is 40% brighter than Ethidium bromide I (Papadopoulos *et al.,* 1994).

The results revealed that all cells that treated with 500ppm of endophytic fungi filtrate have less living cell than that of the untreated (Figure 2). It was strongly indicated that the fungi had the ability to inhibit MCF-7 cell proliferation. Further characterization, such as gene expression and flowcytometry based analyses may need to confirm the results.

## CONCLUSIONS

Endophytic fungi K.Cl.Sb.R9, K.Cl.Sb.A11, K.Cl.Sb. B1 and K.Cl.Sb.R11 isolated from *Curcuma longa L* have the ability to inhibit the growth of human breast cancer MCF-7 cell line.

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