

Anticancer Effect and Cell Cycle Analysis of Ethanolic Extract of Gembili (*Dioscorea esculantaa* L. Burkill) on T47D Breast Cancer Cell Line

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Abstract

Gembili (*Dioscorea esculantae*) has been known to contain saponin showing promise as potential candidate for anticancer therapy. Gembili extract has also shown cytotoxic activity against T47D breast cancer cell line. The present study aims to investigate the anti-proliferative effect of ethanolic extract of gembili against T47D breast cancer line. In this study, the T47D breast cancer cell line was divided into 3 groups including the control group and study groups (treated with the ethanolic extract of gembili at the dose of 40 µg/mL and 20 µg/mL). The anticancer activity was determined by cytotoxic test using MTT assay and distribution of each phases in cycle cell was tested against T47D breast cancer cell line by propidium iodine assay. Accumulation of cell in cycle cell were observed using flow cytometry. The study showed ethanolic extract of gembili has moderate cytotoxicity against T47D breast cancer cell line and has a significant difference ($p < 0.05$) in the number of cell accumulation in pre G0 between control and study groups. In conclusion, ethanolic extract of gembili has concentration-dependent anticancer activity with significant alteration on cell cycle pattern. It has anticancer activities and anti-proliferative effect against T47D breast cancer cell line.

Key words: anticancer activity, cell cycle, gembili (*Dioscorea esculantae* L. Burkill), T47D breast cancer cell line.

INTRODUCTION

Cancer is a disease of malignant neoplasms that has a wide broad spectrum and complexity. Cancer progression is characterized by increased proliferative activity and the disruption of apoptosis. Based on data of the world's leading causes of death in 2005, cancer is the second highest cause of death after cardiovascular diseases. Until now almost no cancer can be cured by spontaneous treatment and if the cancer continues to be allowed to grow, it will result in the death of the sufferer (Rasjidi, 2009). Breast cancer is the most frequently diagnosed cancer and causes

most death from cancer among women worldwide yearly. About 1.3 million new cases of invasive breast cancer are expected to occur. Breast cancer incidence has been rising in many developing countries. There have been improved patient survival and quality of life (Rasjidi, 2009).

There are various approaches to breast cancer therapy such as radiotherapy, surgery, and chemotherapy. However, those therapy produces side effect due to its toxicity (Tjindarbumi, 2001). Indonesia has megabiodiversity of plants and some of them are used as traditional medicine. *Gembili* (*D. esculanta*), which is widely

distributed in Indonesia has been shown to contain saponin including diosgenin which is potential as anticancer. In previous study, the ethanolic extract of *gembili* significantly shown antiinflammatory effect through suppression of NFκB (Olayemi and Ajaiyeoba, 2007 and Rizwana *et al.*, 2007). However, the effect of ethanolic extract of *gembili* as anticancer has not been elucidated. In this study we demonstrated that ethanolic extract of *gembili* has anticancer activity against T47D breast cancer cell line by altering cycle cell pattern.

MATERIAL AND METHODS

Plant material

Gembili (*D. esculanta* L. Burkill) were collected from plantation located in Kendal, Central Java, Indonesia during March-May 2011. The *Gembili* (*D. esculanta* L. Burkill) were identified by staff of development structure of plant, Department of biology, Semarang State University, Central Java, Indonesia. The fresh *gembili* (*D. esculanta* L. Burkill) were collected, chopped finely and air-dried at room temperature.

Preparation of Extract

Three hundred grams of dried and chopped materials were extracted with ethanol by percolation method for 3 days, filtered and evaporated using rotary evaporator to produce ethanol extract of *gembili*. The ethanol extracts were dissolved in dimethyl sulfoxide (DMSO-Merck) and subsequently diluted to appropriate working concentrations with RPMI culture medium (RPMI-Sigma) for proliferation inhibitor proliferative.

Cell culture

The human breast cancer T47D cell line was obtained from the Parasitology

Laboratorium of Faculty of Medicine Gajah Mada University, Yogyakarta, Indonesia. The cells were grown and maintained in RPMI supplemented with 10% (v/v) foetal bovine serum (FBS-Sigma Aldrich), 0.5% fungizone (Gibco), 2% penstrep (Gibco), and incubated at 3°C in a humidified atmosphere and 5% CO₂ (Mooney *et al.*, 2002; Tan *et al.*, 2005).

Cytotoxic assay

Cytotoxicity of extract at various concentrations (1,562-1000µg/mL) was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT-Sigma) assay. The MTT colorimetric assay developed by Mosmann with modification was used to screen for cytotoxic activity of all the etanolic extracts of *gembili*. Briefly, the cells were seeded in 96-well plates at a density of 10⁴ cells/well in 100 µl culture medium. Following 24-h incubation, the cells were treated with different concentrations of etanolic extract of *gembili* and doxorubicin (positive control) for 24 h. The potential candidates which resulted in cell survival of less than 50% were further assessed for their IC₅₀ (concentration that inhibits cell growth by 50%) values at the concentration range of 1000 and 1,562 µg/ml. The concentration range used for doxorubicin was 100 to 1,562 µg/mL. Each experiment was performed in triplicate. Then, washing and incubation was performed with MTT solution at 37°C for 4 h. The yellow MTT dye was reduced by succinic dehydrogenase in the mitochondria of viable cells to purple formazan crystals. The formazan crystals was diluted in 10% SDS HCl (0.1%). The result was measured using ELISA reader (595 nm) using a microplate reader (Varioscan Flash, Thermo, Finland) and presented in optical density (OD).

Cell viability (%) =

$$\frac{(\text{control cells absorbance} - \text{media absorbance}) - (\text{treated cells absorbance} - \text{media absorbance})}{(\text{control cells absorbance} - \text{media absorbance})} \times 100$$

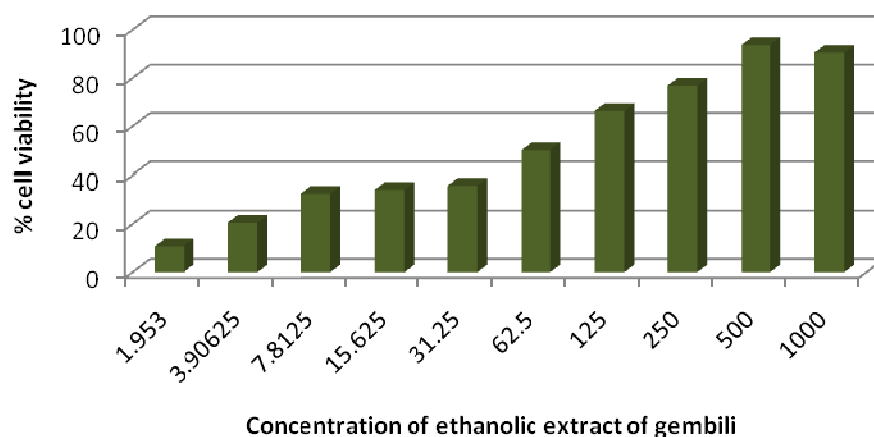


Figure 1. Effect of ethanolic extract of *gambili* on cell growth inhibition of T47D cell line. Cells at density of 1×10^4 cells/well were seeded in 96-well plates. Cell growth inhibition was determined by MTT assay. The data shown are the mean of three independent experiment, each with triplicate well.

The percentage of cytotoxicity compared to the untreated cells was expressed as cell viability (%) (see the equation on the previous page).

Data generated were used to plot a dose-response curve of which concentration of extract required to kill 50% of cell population (IC_{50}) was determined (CCRC, 2009).

Cell cycle distribution of T47F cells treated with ethanolic extract of *gambili* using flowcytometry

The harvested cells (1×10^6) were seeded onto 6 microwell plates (Nunc[®]) and incubated for 24 h at $37^{\circ}C$ in 5% CO_2 . Cells were treated using the sample at final concentration of ethanolic extract of *gambili* 40 $\mu g/mL$, 20 $\mu g/mL$ and incubated for 24 h. Cells were harvested by trypsinization and collected in 15 ml tube, followed by centrifugation at 1200 rpm for 3 minute. The supernatant was discarded and 100 μl of flow reagent was added, followed by incubation for 2 minute at room temperature. Subsequently, RNase solution was added at final concentration 1 mg/ml and Run FACS using Facs Calibur and Cell quest software (Becton and Dickinson).

Statistical analysis

Statistical analysis was performed using SPSS software, version 17. The IC_{50} values were calculated by probit analysis. All data shown as mean \pm standart deviation. For differences between means, one-way analysis of variance (ANOVA) was used with posthoc analysis using either Tukey's HSD test (for equal variances). Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Cytotoxic assay

Cytotoxic assay was observed using MTT assay in T47D cell line exposed to 10 series concentration (1,562 $\mu g/mL$ to 1000 $\mu g/mL$) of ethanolic extract of *gambili* for 24 h and percentage of cell viability was analyzed as shown in figure 1. The cell growth inhibition percentage of 1,953 $\mu g/mL$ was 10.76% and the increase of ethanolic extract of *gambili* concentration.

Figure 1 showed increased concentrations of ethanolic extract of *gambili* causing an increase in the percentage inhibition of cells growth. Cytotoxic assay showed that ethanolic extract of *gambili* had a moderate cytotoxic activity against T47D cell line ($IC_{50}=39.61 \mu g/mL$).

In this study doxorubicin was used as standard agent of which the range of cytotoxicity and its possibility of anti-cancer mechanism have been known.

Table 1. Average distribution of T47D cells in each cell cycle after treatment with ethanolic extract of *gembili* for 25 hours.

Treatment	Number of cell (%) ± sd				
	preG0	G0-G1	S	G2-M	M
kontrol	3,81±3.03 *	49,75±2,29*	21,8±5,08	22,4±0,69*	2,53±0,81
EEG 40 µg/ml	23,95±10,83*	34,37±5,57*	19,26±3,22	19,57±1,09*	4,08±0,33
EEG 20 µg/ml	4,17±2,60*	45,1±2,15*	22,19±5,37	26,37±0,89*	3,89±0,36

Note : * indicated p value <0,05 using anova analysis with confidence interval 95%. The data shown are the mean ± deviation standard of triplicate. EEG was ethanolic extract of *gembili*.

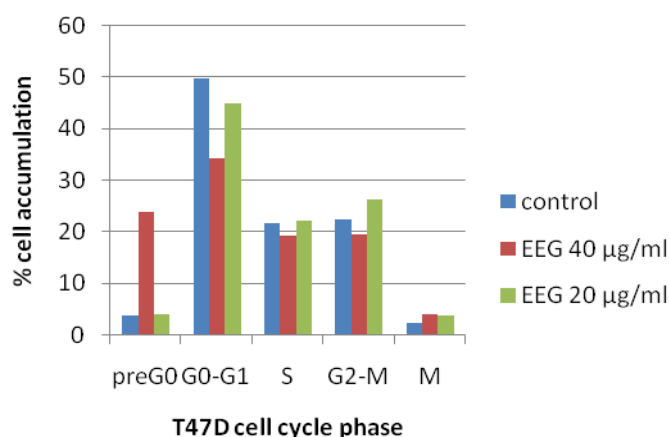


Figure 2. Cell cycle phase distribution of T47D cells. T47D cells (1×10^6 cells/ml) treated with ethanolic extract of *gembili* (EEG) at various dose as well as RPMI were prepared for cell cycle analysis after 24 hours exposure. Cells stained with Propidium Iodide (PI) reagent were analysed with cell quest program using flow cytometer Becton and Dickinson). The data represent the mean of 3 independent experiments.

The IC_{50} value of doxorubicin on T47D cell line was 9.96 µg/mL and showed a strong cytotoxicity ($IC_{50} < 20$ µg/mL). The previous findings showed that diosgenin in *gembili* had anticancer activity on breast cancer cell lines through antiproliferative activity and induction apoptosis (Riswana *et al.*, 2010 and Podolak *et al.*, 2010). In addition, Olayemi and Ajaiyeoba (2007) and Carnago *et al.* (2011) found an antiinflammatory and antioxidant effect of ethanolic extract of *gembili* in mice and in vitro respectively.

Cell cycle distribution

The ethanolic extract of *gembili* inhibited cell proliferation at various concentrations in dose dependent manner. In addition, the mechanism behind the anticancer activity of ethanolic extract of

gembili on cell cycle phases of T47D were investigated. The T47D cell lines were treated with extract at different concentrations of 20 µg/mL, 40 µg/mL for 24 h and percentage of cell distribution in each phase of cell cycle was shown in table 1.

Table 1 showed the number of accumulated T47D cells in each cell cycle after treatment with ethanolic extract of *gembili* for 24 hours. Treatment with 40 µg/ml of ethanolic extract of *gembili* showed most of cell accumulated in pre G0. Our previous study found that doxorubicin at concentration 10 µg/mL on T47D showed most of cell accumulated in pre G0. In contrast, Meiyanto *et al.* (2011) showed that doxorubicin at 7.5 nM induced G2/M arrest on T47D.

Cell cycle modulation by various natural and synthetic agents is gaining widespread attention in recent years. Given that disruption of cell cycle plays a crucial role in cancer progression, its modulation by phytochemicals seems to be a logical approach in controlling carcino-genesis (Singh *et al.*, 2002). T47D cell cycle modulation was indicated by accumulation of cells in pre G0 phase 6.2 fold higher compared to control (3.80% to 23.95%). Pozarowski (2004) mentions that the cells undergoing apoptosis often have fractional DNA due to DNA fragmentation. In the sub-G0 phase is only a fraction of DNA in the cell apoptosis-looking.

These result suggests that ethanolic extract of *gembili* has antiproliferative effect indicated by accumulation of percentage cell distribution in pre G0 (Figure 2). The higher number of cells accumulated in pre G0 phase was probably due to most of cells have undergone apoptosis but the mechanism need to be explored further in more details.

CONCLUSION

Ethanolic extract of *gembili* have concentration dependent anticancer activities with significant alteration on cell cycle pattern in comparison to control.

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