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Antiproliferative Effect of Ethanolic Extract *Eugenia uniflora* Lam. Leaves on T47D Cells

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Abstract

Eugenia uniflora Lam. is one of herbal products developed for anticancer. The aim of the present study was to identify the antiproliferative effect of ethanolic extract of *Eugenia Uniflora* Lam. leaves (EEU) on breast cancer cell line T47D. This Research was initiated by extracting the active contents of *Eugenia uniflora* Lam. leaves by maceration with ethanol 96%. The extract was then analyzed by thin layer chromatography (TLC). Cytotoxic assay of EEU was carried out by using MTT assay. Apoptosis phenomenon was observed with double staining using acridine orange-ethidium bromide. EEU showed cytotoxic effect on T47D cells with IC_{50} value of 65 µg/ml. Moreover, EEU 50µg/ml and 100µg/ml induced apoptosis. TLC examination showed that EEU used in this study contain phenolic, flavonoid, and saponin compounds which were suggested to be responsible for antiproliferative effect. Further molecular mechanism underlying EEU antiproliferative effect needs to be done.

Keywords: *Eugenia uniflora* Lam., T47D cells, antiproliferative, apoptosis

INTRODUCTION

Breast cancer shows the highest cancer case in women worldwide. Breast cancer is the 2nd most frequently cancer in Indonesian woman after cervical cancer (Tjindarbumi and Mangunkusumo, 2002). There are several therapies toward breast cancer, such as biopsy, mastectomy, radiation, hyperthermia, also chemotherapy. However, most of cytostatic agent is not specifically targeted cancer cells. These compounds could be harmful for normal cells which proliferate extensively, such as bone marrow, hair, gastrointestinal, and reproductive cells (Raharja and Tjay, 2002). Thus, research focus to observe drug molecule which is safe, effective, and selective.

National Cancer Institute (NCI) reported that some effective anticancer agents come from nature. One of the potential plant is *Eugenia uniflora* L.. The leaf and branch of *E. uniflora* L. contain flavonoid, saponin, and tannin (Hutapea, 1994). Flavonoid is a natural compound exhibited anticancer activity (Harborne, 1987). Flavonoid contained in leaf extract of *Eugenia uniflora* L. are myricetin, myricitrin, quercetin, gallocatechin, and quercitrin (Scameda-Hirschmann *et al.*, 1987). Whereas, other compounds such as gallocatechin, oenothein B, eugeniflorins D(1) and D(2) could inhibit DNA polymerase of Epstein-Barr Virus (EBV) which play role in replication of EBV (Lee *et al*., 2000). Ethyl acetate fraction and chloroform extract also showed cytotoxic effect on HeLa cells (Handayani, 2006). This study was aimed to observe antiproliferative and induction of apoptosis caused by ethanolic extract of *E. uniflora* L. leaves (EEU) on T47D cells.

MATERIALS AND METHODS

Extraction

Eugenia uniflora L. leaves were collected and determined in B2P2TO2T (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional), Tawangmangu, Central Java. Sample was then dried and powdered. The powder macerated with ethanol 96% for 3x24 hours. Filtrate was concentrated using rotary evaporator at 60^0C.

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Cells Culture

Human breast cancer T47D culture cells culture were a collection of Cancer Chemoprevention Research Center (CCRC), Universitas Gadjah Mada. The cell was a gift from Prof. Kawaichi, Nara Institute of Science and Technology (NAIST), Japan. Cells were cultured in culture medium DMEM (Gibco) supplemented with 10 % Fetal Bovine Serum (Sigma)

Cytotoxic Assay

Cytotoxic assay was done using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Mosmann, 1983). Cells in 80% confluent were harvested and counted, then diluted with complete culture medium. The cells were then transferred into a 96-well plate with a total of 1 x 10^4 cells/well followed by 24 hours incubation. After that, the sample was added to the wells at various concentrations with co-solvent DMSO and incubated at 37^0C in a 5% CO_2 incubator for 24 hours. At the ends of incubation, 100 µL MTT reagent with concentration of 0.5 mg/ml in DMEM was added into each well. The plate was then incubated at 37^0C for 6 hours until formazan was formed. Once formazan was formed, stopper Isopropanol-HCl 4N was added. Plate was then wrapped with paper or alumunium foil and incubated in dark condition overnight. Absorbance was determined with an ELISA reader at λ 550 nm.

Proliferation Assay

Proliferation assay was done using MTT assay. Cells were seeded with a total of 1 x 10^4 cells/well and incubated for 24 hours. After that, the sample was added to the wells in IC_{50} concentration and incubated for various time (0, 24, 48, and 72 hours). MTT assay then was conducted as described in cytotoxic assay methods.

Apoptosis Observation

Apoptosis was observed with double staining method using acrydine orange-ethidium bromide. Cells ($5x10^4$) were seeded at coverslip in 24-wellplate. Cells were then incubated with sample (50 µg/ml and 100 µg/ml) for 24 hours. After incubation, medium was removed and was added with 10 µl acridine orange-ethidium bromide. Cells were observed under fluorescent microscope with 100x magnification.

Data Analysis

Cytotoxic assay. Linear regression between concentration and % cell viability giving the equation y = Bx + A which were used to calculate IC_{50} value, that is the concentration inhibiting 50% of cell growth.

Proliferation assay. Paired sample t-test was used to observe significance difference between control and treated cells' absorbance. Linier regression between incubation time vs cells absorbance was made and the slope was analyzed. Slope in the graph indicating cell cycle arrest phenomenon.

Apoptosis observation. Cells' morphology was observed under fluorescence microscope. Viable cells were in normal shape and green color, while death cells caused by necrosis were orange. Early apoptotic cells were green but brighter in the middle compared to viable cells. Late apoptotic cells were orange until red and formed apoptotic bodies (McGahon *et al.*, 1995).

RESULTS AND DISCUSSION

Cytotoxic Effect of EEU on T47D Cells

Cytotoxic assay was carried out by using MTT assay. Absorbance measured was linier with cells viability. Cytotoxic assay was done using EEU concentration 25, 50, 100, 250, and 500µg/ml. After the treatment, cells were observed under light microscope. EEU altered cells morphology. Increase of EEU concentration caused more cells undergoing morphological alteration, which was indicated by cells becoming rounded form and detached from well (Fig.1(A-D)). Overall, EEU showed cytotoxic activity in dose dependent phenomenon with IC_{50} value of 65 µg/ml (Fig.1(E)).

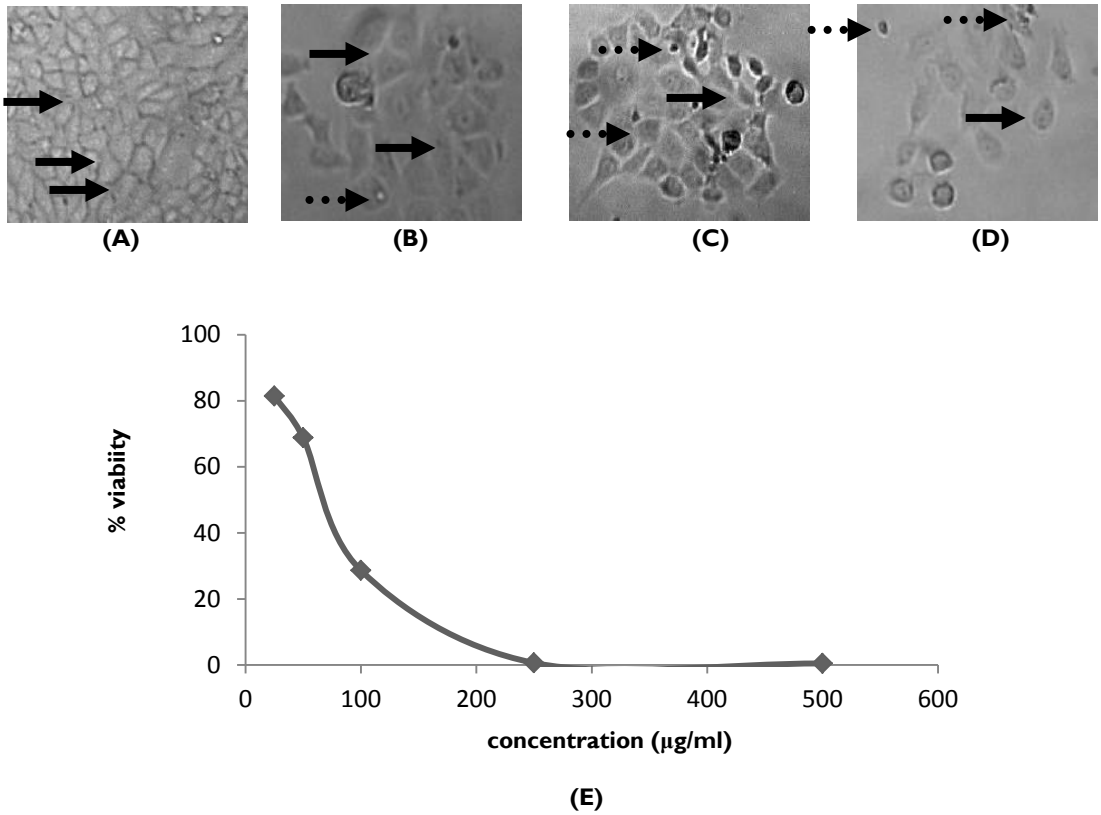


Figure 1. Cytotoxic Effect of EEU on T47D cells. T47D cells (1x10⁴ cells/well) were seeded in 96-well plate and treated with EEU 25, 50, 100, 250, and 500 µg/ml. Cell treated with EEU (A) none (control); (B) 25 µg/ml; (C) 100 µg/ml; and (D) 500 µg/ml, observed under light microscope with 400x magnification. Arrows (➡) indicationg viable cells and dotted arrows (•• ➡) indicating death cells. Cells viability was then measured by using MTT assay as described in the methods and EEU showed cytotoxic effect on T47D cells in dose dependent phenomenon as shown in graph (E).

Effect of EEU on T47D Cells Proliferation

Cell proliferation was also measured by using MTT assay. The absorbance at certain incubation times was measured and correlated with cell viability at these times. Cells were treated with EEU 25, 50, and 75 µg/ml. Proliferation kinetics showed in graph between incubation time versus cell absorbance (Fig.2).

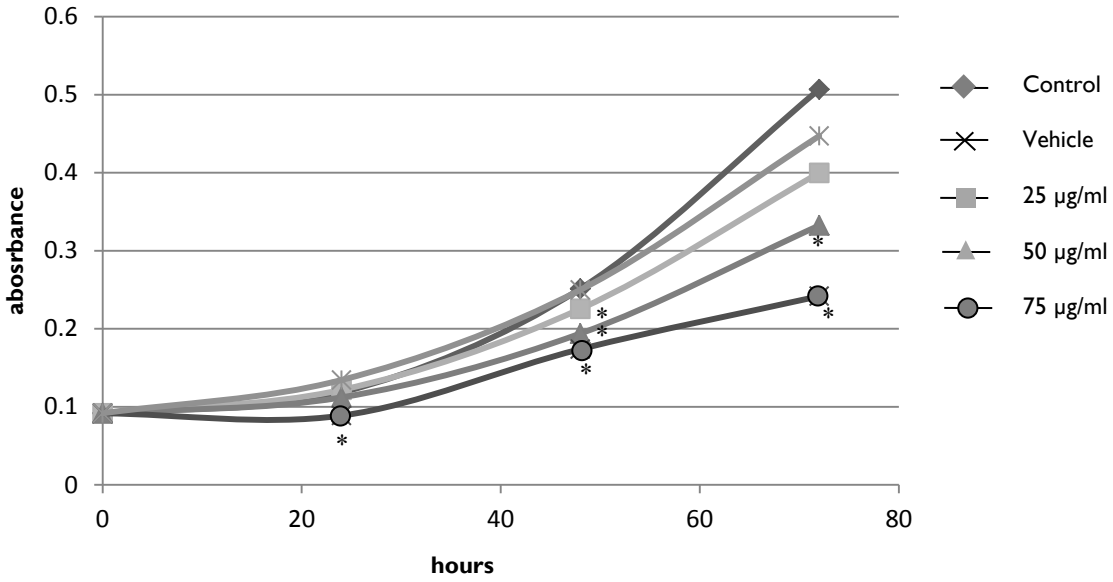


Figure 2. Effect of EEU on T47D Cells Proliferation. T47D cells (1x10^4 cells/well) were seeded in 96-well plate and treated with EEU 25, 50, and 75 µg/ml. Cells viability was determined by using MTT assay and the cells absorbance were measured at 24, 48, and 72 hour of incubation. Absorbance then were plotted in the graph and analyzed statistically by using paired sample t-test ($p<0.05$). (*) indicating significant difference between treated cells and control cells at the same time of incubation.

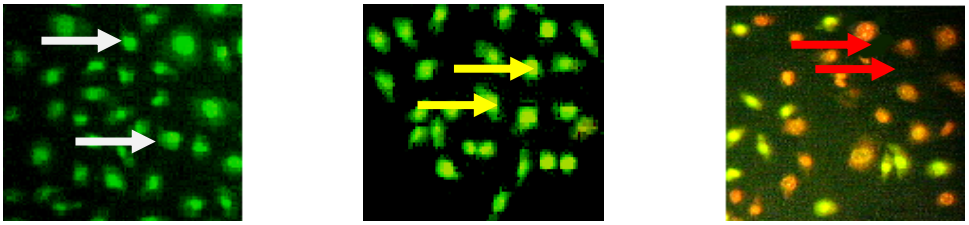
Data obtained were then analyzed by using paired sample t-test with $p<0.05$. EEU 25 µg/ml decreased T47D cells proliferation significantly only at 48 hours of incubation; EEU 50 µg/ml decreased cells proliferation significantly at 48 and 72 hours of incubation; while EEU 75 µg/ml decreased cells proliferation at all incubation time (24, 48, and 72 hours). Inhibition of cells proliferation also could be observed from the slope value of linier regression between times of incubation versus absorbance (Table I). The higher concentration of EEU, the smaller the slope obtained. EEU 75 µg/ml gave the smallest slope, meaning the highest cells proliferation inhibition. Based on the data, EEU inhibited cells proliferation in dose and time dependent manner. The decrease of cells viability could be attributed with cell cycle arrest, apoptosis, necrosis, or combination of them.

Tabel I. Proliferation kinetics of T47D cells at 0-72 hours of incubation.

Groups	Linier Regression	*Slope*	Linearity(R^2)
Control	y = 0,0081x -0,0964	0,0081	0,9678
EEU 25 µg/ml	y = 0,0058x -0,0291	0,0058	0,9791
EEU 50 µg/ml	y = 0,0046x - 0,0076	0,0046	0,9784
EEU 75 µg/ml	y = 0,0032x + 0,0151	0,0032	0,9957
Vehicle	y = 0,0065x - 0,0356	0,0065	0,9778

Effect of EEU on T47D Cells Apoptosis

Double staining using acridine orange-ethidium bromide was done to evaluate the effect of EEU on apoptosis. EEU 50 µg/ml caused cells undergoing green with lighter nuclear (Fig.3). This phenomenon indicated early apoptotic cells (McGahon *et al*., 1995). Moreover, EEU 100 µg/ml caused a lot of cells became orange with fragmented DNA, which indicating the late apoptotic cells.



(A) (B) (C)

Figure 3. Effect of EEU on T47D Cells Apoptosis. T47D cells (5x10⁴) were seeded at coverslip in 24-wellplate, treated with EEU, and stained with acridine orange-ethidium bromide as described in methods. Cells were then observed under fluorescent microscope with 100x magnification. Cells treated with (A) none (control), (B) EEU 50 µg/ml, and (C) EEU 100 µg/ml. White arrows (⇨) showed viable cells, yellow arrows (→) showed early apoptotic cells, and red arrows (→) showed late apoptotic cells.

Induction of apoptosis occurred on T47D cells used in this study may happen through p53-independent pathway, such as via transmembrane death receptor. Gallic acid is a polyphenolic compound which could induce apoptosis through that pathway (Nam *et al.*, 2001). Apoptosis occurred as a result of CD95 or Fas signal transduction (Taraphdar *et al.*, 2001). Fas is a member of TNF receptor which bind to FasL, leading to trimerization of Fas and induce several signal transduction (Kampa *et al.*, 2003). FADD then binds to trimer of Fas and activates caspase-8. Activated caspase-8 would activate caspae-3, an apoptosis effector (Yau, 2004). Flavonoid quercetin and myricetin also induced apoptosis through the release of cytochrome c (Taraphdar *et al.*, 2001), leading to activation of caspase-9 and caspase-3 (King, 2000).

Overall, this study showed that EEU performed cytotoxic effect on T47D breast cancer cells, inhibited T47D cells proliferation, as well as induced apoptosis. Further research need to be conduct is to observe molecular mechanism of EEU and its selectivity on normal cells.

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