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Antiproliferative activity of Ethanolic Extract of Kembang Bulan (*Tithonia diversifolia*) Leaf on HeLa Cervical Cancer Cell Line

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Abstract

Tithonia diversifolia has been showed to be cytotoxic and antiproliferative on colon cancer, glioblastoma, hepatoma, kidney cancer, breast cancer, lung cancer, melanoma, leukemia, ovary cancer, prostate cancer, and stomach cancer cell lines, but not on cervical cancer cells yet. Our research aimed to determine the cytotoxicity and antiproliferative activity of *T.diversifolia* leaf ethanolic extract on HeLa cervical cancer cell line. The cytotoxicity and the antiproferative activity assay were done using MTT method for 24 h for cytotoxic assay; and series of 24, 48, and 72 h for antiproliferative assay. The cytotoxic activity was analyzed using IC_{50}, while the antiproliferative assay was analyzed based on the proliferation kinetics. All assays were done in triplicate. *T.diversifolia* leaf ethanolic extract exhibited strong cytotoxic activity on HeLa cervical cancer cell lines with the IC_{50} of 97.839±10.120 µg/mL. The cytotoxic activity was dose dependent. Based on the proliferation assay, the antiproliferative activity was stronger as the incubation time and the dose increases. *T.diversifolia* leaf ethanolic extract showed strong cytotoxic and antiproliferative activity on HeLa cervical cancer cell lines.

Keywords: *T.diversifolia leaf ethanolic extract, cytotoxicity assay, antiproliferative assay, HeLa cervical cancer cells.*

INTRODUCTION

Cancer deaths caused by infection types of cancer (*e.g*. cervical cancer and hepatic cancer) contribute to 25% of death on under developed countries (WHO, 2018). Unsatisfying cancer treatment (CDC, 2021) makes the exploration for new cancer chemoprevention agent, including those from natural origin (Cragg & Pezzuto, 2016).

Tithonia diversifolia is one of those promising cancer chemoprevention agent candidate. Methanolic extract of *T.diversifolia* has been showed to exhibit cytotoxic activity on hepatoma Hep-G2 (Liao, *et al*., 2013), antiproliferative on colon cancer HTC-116 (Goffin, *et al*., 2002), antiproliverative against glioblastoma U373 (Lee, *et al*., 2011), and antiproliferative on kidney cancer cell TK10, breast cancer cell MCF-7, and melanoma UACC62 (Fouche, *et al*., 2008). Aerial ethyl acetate extract of *T.diversifolia* was proven to be cytotoxic on colon cancer cell Col2 (Gu, *et al*., 2002). Ethanolic extract of *T.diversifolia* exhibited chemosuppressive effect on breast cancer

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cell MCF-7, lung cancer cell HT-460, and central nervous system cancer cells SF-268 (Calderón, *et al*., 2006). Water extract of *T.diversifolia* showed to have antiproliferative effect on leukemia P3HR1 (Chiang, *et al*., 2004). Its aerial part 80% ethanolic extract has strong cytotoxic effect on leukemia HL-60 (Kuroda, *et al*., 2007), breast cancer cell BSY-1, central nervous system cancer cells (SF 539, SNB-78), lung cancer cells (DMS273, DMS114), ovary cancer cells (OVCAR-3, OVCAR-5, OVCAR-8, SK-OV-3), stomach cancer cells (MKN1, MKN28, MKN74), and prostate cancer cells (DU-145, PC-3) (Rocha, *et al*., 2012). While *T.diversifolia* essential oils has antiproliferative effect at 72 h treatment on melanoma maligna A375, breast adenocarcinoma MDA-MB231, colon carcinoma HCT116, and multiform glioblastoma T98 G. The effect on A375, MDA-MB231, and HCT116 was equivalent to that of cancer chemotherapeutic agent, cisplatin (Orsomando, *et al*., 2016).

This study was aimed to complete the preliminary data on the potency of *T.diversifolia* as cancer chemoprevention agent, especially on cervical cancer. The desired data obtained were cytotoxic and antiproliferative activity of *T.diversifolia* leaf ethanolic extract on HeLa cervical cancer cells.

METHODS

PEEAfL Preparation

Tithonia diversifolia is a plant that belongs to the Asteraceae family. *T. diversifolia* (*Hemsl.) A. Gray* leaf was collected from Sumbersari, Jember, East Java. We choosed the old and fresh leaves in the end of leaf stalk at completed flowering plant. The leaf was sorted and air dried, then powdered and shived. Approximately, 100 g dried leaf powder was extracted with ethanol (Merck, Darmstadt, Germany) using continuous stirrer for 24 h. The extraction process is carried out with continuous stirring with 3 repetitions. This ethanolic extract was concentrated under reduced pressure using rotary evaporator and dried in oven at 50°C resulting *T.diversifolia* leaf ethanolic extract. The exctract was then suspended with DMSO (Sigma-Aldrich, St. Louis, Missouri, USA) 0.5 % for the *in vitro* assay.

Culture Cell Preparation

HeLa cells used were the collection of Laboratory of Molecular Medicine CDAST University of Jember, Indonesia. Cells were taken from nitrogen container and then thawed. An ampule which had been sprayed with ethanol 70% was used to place the cell into new sterilized conical tube which contain culture media. Cells suspension were centrifuged at 600 rpm during 5 minutes. The supernatant was disposed and the residue was collected and suspended with new culture media slowly until homogen. Cells were grown in tissue culture dish and incubated at 37°C with 5% CO_2. Every 24 h the culture media were replaced and these cells were grown until confluence. After that, the media were disposed and the cells were harvested with trypsin 0.25% and washed with PBS twice. Then these cells were incubated during 3 minutes at 37°C with 5% CO_2. Cells were resuspended with RPMI 1640 culture media supplemented with 10% fetal bovine serum until all cells were released from tissue culture dish. Cells supension were placed into new sterile conical tube. The number cells were counted with haemocytometer and cell counter.

Sample Preparation

Approximately 20 mg sample in microtube were dissolved with 0.1 mL DMSO until homogen to make a stock solution of 200.000 µg/mL. Then the stock solution was diluted with culture media to make a series concentration. The series concentrations used were 20, 40, 80, 160, 320, and 640 µg/mL.

Cytotoxicity Assay

The cytotoxicity assay was conducted using MTT method. Cell which have been counted with haemocytometer were transfered into microplate 96 well. Each well contain 100 µL cell suspension.

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Cell were resuspended again to maintain their homogenity. Then the cells were incubated during 24 h. After that, PBS solution were added into each well and then PBS was disposed. Next, the series concentration of *T.diversifolia* leaf ethanolic extract were added to each well which contain HeLa cells. Then the PBS 100 µL and MTT 100 µL were added to each well. The plate were incubated during 2-4 h. until the formazan was formed. Then SDS 10 % in HCl (Merck) 0.1 N were added as stopper reagent into each well. The plate were wrapped with aluminium foil and incubated in dark room. Calculation of cell viability was conducted using ELISA reader at λ 597 nm as of described at the MTT material protocol. The cell viability was calculated as follows:

Cell Viability=[(absorbance of treated cell- absorbance of medium control)/ (absorbance of cell control-absorbance of medium control)]x100%

(Doyle and Griffiths, 2000).

Antiproliferative Assay

The antiproliferative assay was done using MTT method. The cells proliferation assay was done for 24, 48, and 72 h. resulting the cell viability kinetics.

Data Analysis

The IC_{50} was determined by probit analysis based on the plot of concentration vs cell viability (Doyle and Griffiths, 2000). The IC_{50} were presented as mean + standard deviation (SD) from triplicate.

RESULT

T.diversifolia leaf ethanolic extract yield was 10.12 g from 100.03 g leaf powder, meaning that the rendement was 10.11%. The cytotoxicity assay of *T.diversifolia* leaf ethanolic extract on HeLa cell line resulting IC_{50} value of 97.839±10.120 µg/mL. This result was considered to have strong cytotoxic activity, since the IC_{50} value was less than 100 µg/mL (Prayong, *et al*., 2008). The cytotoxicity profile of *T.diversifolia* leaf ethanolic extract was shown with Figure 1.

The cytotoxic activity of *T.diversifolia* leaf ethanolic extract was dose and time (Figure 2) dependent. We can see that the higher the concentration and the longer the incubation time, the fewer the HeLa cell viability, meaning that this extract showed antiproliferative activity against HeLa cells.

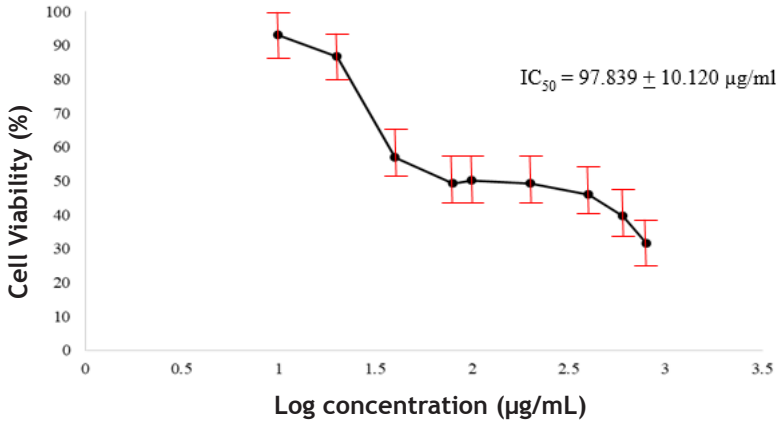

Figure 1. HeLa cells viability upon *T.diversifolia* ethanolic extract treatment.

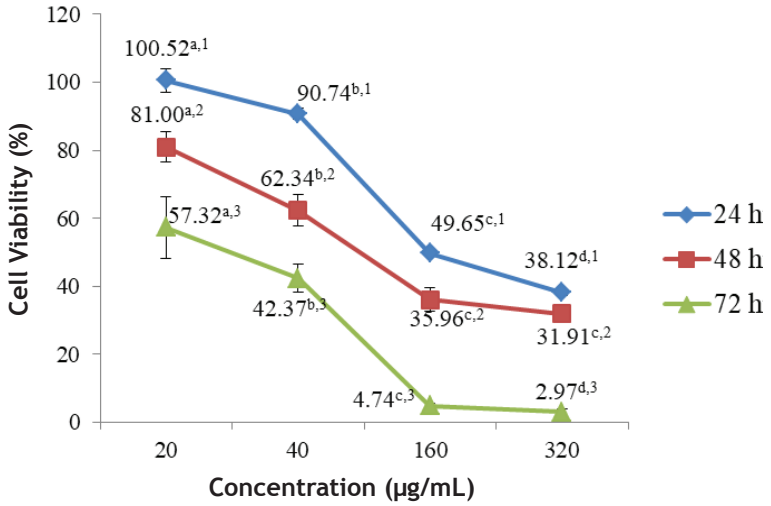

Figure 2. HeLa cell viability kinetics after *T.diversifolia* leaf ethanolic extract treatment for 24, 48, and 72 h. Kinetic Data was showed as mean +SD (n=3). Different alphabet annotation showed sig. difference among concentration, white different number annotation showed sig. difference among incubation time (h).

DISCUSSION

T.diversifolia contains terpenoids, phenols, flavonoids, alkaloids, tannins, and saponins (Olayinka, *et al*., 2015). The flavonoids, sesquiterpenoids, and diterpenoids are weighed as the most outstanding components (Chagas-Paula, *et al*., 2012). The major sesquiterpenoids having isolated are sesquiterpene lactones, *i.e.* germacranolides, eudesmanolides, and guaianolides. The most studied germacranolides were tagitinins that consist of 9 classes (Tagne, *et al*., 2018). On the other hand, flavonoids were found in the leaf trichomes, which include, luteolin, and nepetin (Ambrósio, *et al*., 2008).

T.diversifolia based on several previous studies has been shown to have anti-proliferative activity in some cancer cells. Ethyl acetate extract from *T.diversifolia* especially the content of tagitinin C and 1,2 Repoxytagitinin C has antiproliferative activity against human colon cancer cells (Col2) (Gu, *et al*., 2002). The methanolic extract of *T. diversifolia* had antiproliferative activity against glioblastoma cells U373, with an IC_{50} value of 59.2±3.7 gmL^{-1}, while tagitinin C showed an IC_{50} value of 6.1±0.1 gmL^{-1} (Lee, *et al*., 2011). The ethanolic extract of *T.diversifolia* also showed antifibrotic activity against keloid fibroblast cell proliferation and collagen accumulation (Ranti, *et al*., 2018). In another study, the ethanol extract of *T.diversifolia* at concentrations of 20 gmL^{-1}, 10 gmL^{-1}, and 5 gmL^{-1} for 24 h in keloid fibroblast culture showed slower migration activity compared to untreated keloid fibroblast culture (Wahyuningsih, *et al*., 2019).

The compounds that are proposed to contribute to the cytotoxic and antiproliferative activity of *T.diversifolia* leaf ethanolic extract are tagitinin C and 1β,2α-epoxytagitinin C. These compounds were identified as the compounds having antiproliferative effect on Col2 cells with the IC_{50} value ≤5 µg/mL (Gu, *et al*., 2002). Moreover, survivin, a critical factor of drug resistance in cancer chemotherapy, was dose-dependently downregulated in cells treated with either *T.diversifolia* methanolic leaf extract (dose of 10 µg/mL) or tagitinin C (dose range of 2.5-10 µg/mL). Tagitinin C caused arrest of malignant glioblastoma

cells in G2/M phase (Liao, *et al*., 2011), was found to induce a dose-dependent increase in tumor cell population in sub-G1 phase and their arrest in S phase (Liao, *et al*., 2013).

CONCLUSION

Based on the results, we can conclude that *T.diversifolia* leaf ethanolic extract exhibit strong cytotoxic activity and antiproliferative on HeLa cervical cancer cell lines.

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