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Cytotoxic Activity of Tegari (*Dianella nemorosa* Lam.) Methanol Extract Against HeLa Cells

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Abstract

Dianella nemorosa Lam. also known as tegari belonging to the Liliaceae family. This plant has been utilized for Papua traditional medicine as well as anticancer agent. This research examined potential cytotoxic activity of tegari (*D. nemorosa*) leaves extract against cervical cancer cell line (HeLa). Methanol extract was obtained by extracting the leaves powder using methanol. Extract was then applied into HeLa cell line to find out the cytotoxic activity. MTT [3-(4,5-dimetilthiazol-2-il)2,5-difeniltetrazolium bromida) assay was used to measure the cytotoxic activity. The result indicated that *D. nemorosa* leaves extract possessed cytotoxic activity in HeLa cell line with IC_{50} values were 685,69 µg/ml, 506,43 µg/ml and 708 µg/ml at the incubation period of 24, 48 and 72 h respectively. The strongest cytotoxic was showed by methanol extract incubated in 48 h.

Keywords : *Dianella nemorosa* Lamk, Cytotoxic, HeLa Cell, MTT Assay

Introduction

Cancer is an abnormal mass of tissue which the cells exhibit an uncontrolled division, leading a progressive increase in the number of dividing cell (Bowman and Rand, 1989; DeVita, *et al.*, 1997; Sudiana, 2008). Cancer is a leading cause of death globally in the world and most cancer are currently increasing in incidence. The World Health Organization estimates that 7.6 million people died of cancer in 2005 and 84 million people will die in the next 10 years if action is not taken.

More than 70% of all cancer deaths occur in low- and middle-income countries, where resources available for prevention, diagnosis and treatment of cancer are limited or nonexistent (Anonim, 2002; Anonim, 2008).

The major treatments currently available are surgery, radiation therapy, chemotherapy and immunotherapy. Chemotherapy often causes severe side effects, particularly reduced resistance to infection, internal bleeding, diarrhea, nausea, hair loss, and insufficient oxygen in the blood, known as anemia. Many people, conversely, attempt to employ traditional therapy, especially from plants (Goldie, 2001; Kintzios and Barberaki, 2004; Aziz *et al.*, 2006; Miller, 2008).

Plants have been an indispensable source of natural products for medicine. An estimation of the World Health Organization states the around 85-90% of the world's population consumes traditional herbal medicines for their primary health care (Tripathi and Tripathi, 2003; Sekar *et al.*, 2007). Many plants used in treatment of

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cancer. The therapeutic effect of natural material is related to the their chemical compounds (Wahyuningsih, 2006).

Dianella nemorosa that has local name Pra Kepey in Papua, is one of the anticancer plants. This is plant commonly used by the local community in Tablasupa village, Sentani, Papua. They generally consumed the leaves of this plant either in the raw form or by drinking the soup made by boiling the leaves in water (Maturbongs *et al.*, 2000; Fitowin, 2006).

Genus *Dianella* commonly known as Tegari in Indonesia consist of 30 known species belonging to the family Liliaceae which are distributed throughout Asian countries such as Indonesia, Taiwan, Bangladesh, Philippina, Japan, and China. It is also found in Polynesia, New Zealand and Australia, (Longid 1992; Xingi *et al.*, 2000; Hegarty *et al.*, 2001; Li, 2006; Uddin and Hassan, 2009).

Some study reported Genus *Dianella* was known to contain naptho- quinone, napthalane, plumbagin, stypandrol, polifenol and saponin (Robinson, 1975; Byrne *et al.*, 1987; Cheeke, 1989; Chung *et al.*, 1998; Jumar and Helda, 2003). Other study show that musizin derivaties of naphthalene, benzoic acid, acetophenone, isougenitol and chromones have been isolated from the roots of *D. ensifolia* (Lojanapiwatna *et al.*, 1982; Chung *et al.*, 1998).

Napthoquinon and plumbagin possess various pharmacological activities such as anticancer (Srinivas *et al.*, 2004; Santos *et al.*, 2003; Wang *et al.*, 2008; Babula *et al.*, 2009) and antitumor (Kavimani *et al.*, 1996). Other study reported that naphthalene, benzoic acid, acetophenone, isougenitol, chromones possess various anticancer activities in a wide variety of cell line (Gul *et al.*, 2002; Tatsuzaki *et al.*, 2006; Gamal *et al*, 2007: Subramaniam *et al.*, 2008; Tumiatti *et al.*, 2009; Nakayama *et al.*, 2009).

Based on these study on chemical compound that might also present in *D. nemorosa*, it was believed that *D. nemorosa* could be developed as a potential anticancer agent for prevention and treatment of different human cancers. Therefore, the objective of this research was to examine the cytotoxic effect of methanol extract of *D. nemorosa* leaves on HeLa cancer cell line.

Materials and Methods

Preparation of extract

D. nemorosa plant was collected from the Tablasupa village-Sentani, Papua. The plant was identified by Laboratory of Plant Taxonomy, Faculty of Biology, Universitas Gadjah Mada and Laboratory of Botany and Herbarium, Indonesian Institute of Science (LIPI). The leaves were washed, dried and chopped finely using a blender. Five hundred grams of dried material were exhaustively extracted with methanol maseration. The methanol extract was filtered and concentrated using a rotary evaporator then the evaporated extract would be dried.

Preparation of cervical cancer cell line (HeLa)

HeLa cell line that was obtained from laboratory stock of LPPT UGM was grown on RPMI (Rosswell Park Memorial Institute) 1640 (Sigma) medium containing 5% v/v Fetal Bovine Serum (FBS) (Sigma) and 1% v/v Fungison (Sigma) in the presence of 1% w/v of Penicillin-streptomycin (Sigma). The cultures were maintained at 37^{O}C in humidified atmosphere of 5% CO_2.

In vitro assay for cytotoxic activity

A hundred microlitre of the cell suspension 2.10^4 cell/ml was plated into 96 well microplate (Nunc, Germany) and treated with different concentration of methanol extract isolated from *D. nemorosa* leaves, in a serial dilution (1000, 500, 250, 125, 62.50, 31.25 dan 15.625µg/ml). Following treatment, plates were incubated in CO_2 incubator at 37^{O}C for 24, 48 and 72 h. After incubation, media was removed and MTT reagent (10µl) in PBS (5mg/ml) was added to each well

and plates were further incubated for 4 h. Reaction was stopped by the addition of 100 µl 10% sodium dodecyl sulfate (SDS) and incubation was continued in CO_2 incubator at 37°C for 15 h. The absorbance was read at wavelength of 540 nm using ELISA reader. The percentage cellular viability was calculated with appropriate control taken into account. The concentration which inhibited 50% of cellular growth (IC_{50}) was determined. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{OD Control-OD treated}}{\text{OD Control}} \times 100$$

Results and Discussion

Cytotoxic test was used to find out a potential anticancer properties of methanol extract isolated from *D. nemorosa* leaves against HeLa cell line. Experiment on cytotoxiciy were designed to determine the maximal concentration of extract compatible with cell survival.

In this study, toxicity data were expressed as IC_{50}, a concentration of extracts that cause 50% inhibition of cell growth and was obtained by plotting the percentage growth inhibition versus concentration of methanol extract sampels. The extract that gave a IC_{50} value of 1000 µg/ml or less was considered as anticancer activities (Doyle and Griffiths, 2000).

The result demonstrated that the extract possessed cytotoxic activity against HeLa cell lines with IC_{50} values of 685.69 µg/ml, 506.43 µg/ml and 708 µg/ml at the incubation of 24, 48 and 72 h respectively. Although the IC_{50} values were higher than the American National Cancer Institute standard that is stated for crude extract of a plant obtained should be less than 20µg/ml (Geran *et al.*, 1972), the methanol extract of *D. nemorosa* still could be able to be developed as a potential chemopreventif agent for prevention and treatment of other cell cancer. As shown in Figure 1 at a concentration of 1000 µg/ml cell, death was almost 100% compared to control and the inhibitory level was decreased when the concentration of the extract was decreased. The result suggest that the methanol extract might be contain bioactive compounds that inhibit the growth of HeLa cell line.

The activities of these extract against HeLa cell line might be due to the presence of highly complex compounds that present in *D. nomorosa*. Several study have reported that chemical constituents or active compounds such as napthoquinone, plumbagin, napthalane,stypandrol, polifenol, saponin, dianellidin, chromones, benzoic acid, aceptophenone have been identified from the roots some of *Dianella* like *D. ensifolia*, *D. revoluta,* and *D. longifolia* (Cooke and Down, 1971; Lojanapiwatna *et al.,* 1982; Colegate *et al.,* 1986; Cheeke, 1989; Chung *et al.,* 1998). Dias *et al.* (2009) reported others Australian medicinal plant *D. callicarpa* contained naphthalene aglycones and glycosides.

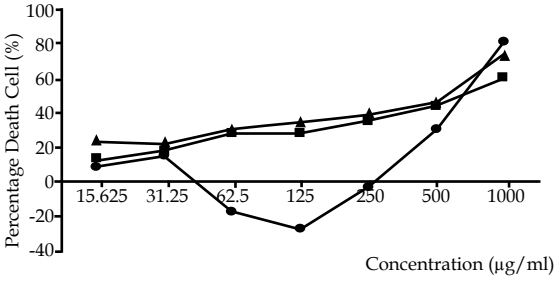

Figure 1. Correlation concentration of methanol extract *D. nemorosa* (µg/ml) with percentage of cell death (%). (■-24 h), (▲-48 h) and (●-72h).

Cell proliferation involves complex combinations of many biochemical processes and different compound might influence different biochemical processes or stages in different manners. Therefore, the presence of the napthoquinone, plumbagin and phenolic group could be assumed to be responsible for the anticancer activities of methanol extract this study. In our preliminary study using paper chromatography also showed that the methanol extract of *D. nemorosa* contained

phenolic, alkaloid, tannin and terpenoid compounds but it doesn't contain saponin.

Several studies had demonstrated the significantly cytotoxic effect of plumbagin against human cervical and breast cancer cell (Srinivas *et al.*, 2004; Ahmad *et al.*, 2008). Chen *et al.*, 2009 reported that the *in vitro* cytotoxicity of plumbagin isolated from ethanol extract of *Plumbago zeylanica L* root have IC_{50} values of 17.9 µmol/L, 12.9 µmol/L, 67.6 µmol/L and 34.7 µmol/L against cancer cell lines 786-O (renal carcinoma), MCF-7 (breast carcinoma), CNE-2 (nasopharyngeal cancer) and HCT116 (colon cancer) respectively. Napthoquinone have an interesting potential as a cytotoxic agent. Its induced cell death on many cancer cell line such as HEPA-3B (hepatic cancer), A-549 (lung cancer), HaCat (keratinocytes) and HeLa (cervical cancer) (Higa *et al.*, 1998; Inbaraj and Chignell, 2004; Srinivas *et al.*, 2004).

Semplea *et al.* (1998; 2001) showed that 250 µg/ml of *D. longifolia* roots extract had been found to inhibit poliovirus types 1 (*Piconarividae*) *in vitro,* and it means that it can be used for cancer treatment that is caused by virus such, as HeLa cell line that was used in this study. Human papilomavirus is considered as virus agent of cervical cancer. The other *D. callicapa* displayed significant antimicrobial and antiviral activities (Dias *et al.*, 2009).

This study showed that the ability of methanol extract to inhibit proliferation of HeLa cell line was estimated by analyzing its effect on the growth of the cells (Figure 2). The growth of the untreated (control) and treated HeLa cell line after incubation for 24, 48 and 72 h were photographed using a phase contrast microscope. Figure 2A shows the untreated cells after 24 h incubation. It can be seen that the cells were growing normally as indicated by the presence of formazan dye formed. The inceasing of the extract concentration caused the decreasing of formazan dye (Figure 2C and 2D). The formation of formazan dye directly was correlated to the number of metabolically active of cell in the culture. It was indicated that methanol extract of *D. nemorosa* leaves proved to possess anticancer properties against HeLa cell lines tested. Therefore, it may has a potential as an anticancer agent since it has IC_{50} values less than 1000 µg/ml. This result supported the MTT experiment as shown in Figure 1.

In our preliminary study showed that chloroform extract from leaves *D. nemorosa* has been done but the chloroform extract did not show any cytotoxic activity on the cell line studies at concentration up to 1000 µg/ml. The result suggested that the methanol extract of *D. nemorosa* may contain bioactive compounds that inhibit HeLa cell line compared to chloroform extract.

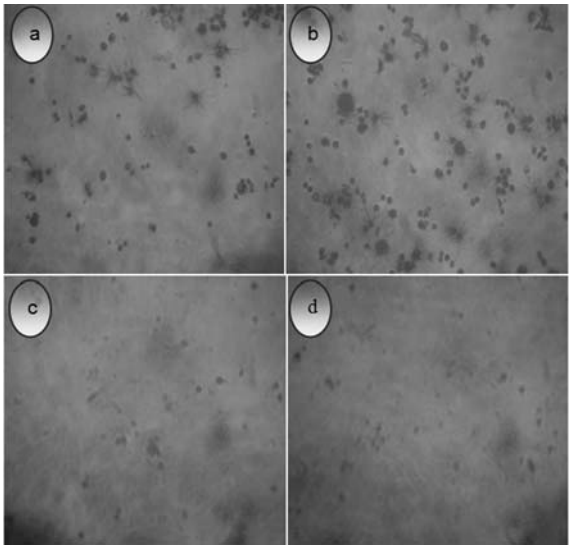

Figure 2. Morphology of Hela cell line after treated with MTT. (A) Untreated cell (B) treated with 15.625 µg/ml extract (C). treated with 125 µg/ml extract and (D) treated with 500 µg/ml extract. Cell treated with methnol extract of *D. nemorosa* after incubation 24 h. Many of formazan dye formed in untreated cell and decreased in 125 µg/ml and 500 µg/ml extract.

The cytotoxicity effect of natural materials is related to the chemical compounds (Cragg and Newman, 2005; James *et al.*, 2007). In *D. nemorosa*, various chemical were present such as phenolic, alkaloid, terpenoid, and tanin. Several studies had shown that these compounds contained in *D. nemorosa* possess various pharmacological properties such as

antioxidant, antiviral and anticancer activity in cultured cell line and animal models (Aslamuzzaman *et al.,* 2003; Ling-Hua *et al.,* 2004; Khalil *et al.*, 2007; Ziying *et al.,* 2009).

Catarina *et al.*,(2003) reported that phenolic compounds inhibit three different human cel line; cervix (HeLa), Mamary gland (MDM-MB-231) adenocarcinomas and lymphoblastic leukemia (MOLT-3). Meiyanto and Endah (2005) showed that screening for phenolic compounds isolated from *Gynura procumbens* found to have chemopreventive potency. Fenolic fraction (IC_{50} 119 µg/ml) of ethanolic extract has antiproliverative effect with the mechanisme of cell cycle delay and induction apoptosis on HeLa cell line. Other study reported some alkaloid can induce apoptosis in HCT-116 (Ling Hua *et al.,* 2009). Identification compounds by TLC and densitometry showed that alkaloid compounds in chloroform fraction from *Carica papaya* have anticancer with the value IC_{50} 104,4 µg/ml on myeloma cell line and induction apoptosis activity (Sukardiman *et al.*, 2006).

Several studies had showed two novel alkaloids, schischkinnin and montamine have been isolated from the seeds of *Centaurea schischkinii* and *Centaurea Montana*. Both of the alkaloids exhibited significant cytotoxicity against human colon cancer cell lines (Shoeb *et al.*, 2005; 2006). Alkaloids like vinblastine and vincristine from the Madagascar periwinkle, *Catharanthus roseus* G. Don. introduced a new era of the use of plant material as anticancer agents. They were the first advanced agents that was used for clinical treatment of cancer. Vinblastine and vincristine are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukemias, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma (Cragg and Newman, 2005).

Phytochemical study of *Irvingia malayana* indicated that methanol extract contained flavonoid, alkaloid, terpenoid, polyphenol, quinone and steroid. This extract was able to inhibit the HeLa cell growth with the IC_{50} of 59 µg/ml after 24 h of incubation (Kusharyanti *et al.,* 2008).

It assumed that methanol extract of *D. nemorosa* contained many compounds like phenolic, alkaloid, terpenoid, and tanin responsible for cytotoxic effect on HeLa cell culture.

In this study, further investigation is needed to determine whether the inhibitory effect of methanol extract on the growth of the cells is due to the inhibition of the proliferation of the cells or the induction of cell death. It has also been suggested that an *in vivo* study should be carried out in conjunction with the *in vitro* study, so that the results can be compared and more information regarding the anticancer properties of methanol extract from *D. nemorosa* leaves will become clear. This is important, because there may be many factors that affect the anticancer activity in the *in vi*vo study. Furthermore, *in vitro* studies may not produce the same results as in vivo experiments.

Studies are necessary to be conducted to investigate the bioactivity compounds, to characterization of the active ingredients (chemical compounds), and to found out the molecular mechanisms involved in anticancer agent of extract methanol *D. nemorosa* leaves is also important for future therapeutic application.

In conlusion, the extract methanol of *Dianella nemorosa* leaves from Papua possess potential cytotixicity activity in cervical cancer cell line (HeLa) with IC_{50} values for 24, 48 and 72 h were 685.69 µg/ml, 506.43 µg/ml and 708 µg/ml respectively. The strongest cytotoxic was showed by methanol extract incubated in 48 h.

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