Anti-Proliferative Effects of D. Pentandra Methanol Extract on BCR/ABL-Positive and Imatinib-Resistant Leukemia Cell Lines

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Abstract

Background: Imatinib mesylate, a tyrosine kinase inhibitor specifically targeting the BCR/ABL fusion protein, induces hematological remission in patients with chronic myeloid leukemia (CML). However, the majority of CML patients treated with imatinib develop resistance with prolonged therapy. Dendrophthoe pentandra (L.) Miq. is a Malaysian mistletoe species that has been used as a traditional treatment for several ailments such as smallpox, ulcers, and cancers. Methods: We developed a resistant cell line (designated as K562R) by long-term co-culture of a BCR/ABL positive CML cell line, K562, with imatinib mesylate. We then investigated the anti-proliferative effects of D. pentandra methanol extract on parental K562 and resistant K562R cells. Trypan blue exclusion assays were performed to determine the IC50 concentration; apoptosis and cell cycle analysis were conducted by flow cytometry. Results: D. pentandra extract had greater anti-proliferative effects towards K562R (IC50= 192 μg/mL) compared to K562 (500 μg/mL) cells. Upon treatment with D. pentandra extract at the IC50 concentration: K562 but not K562R demonstrated increase in apoptosis and cell cycle arrest in the G2/M phase. Conclusion: D. pentandra methanol extract exerts potent anti-proliferative effect on BCR/ABL positive K562 cells.

Keywords: D. pentandra- BCR/ABL- K562- imatinib- CML

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Materials and Methods

Plant materials

Fresh leaves of D. pentandra were collected from Pasir Puteh, Kelantan, Malaysia. The samples were authenticated by botanists at the Herbarium Unit, School of Health Sciences, University Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia. *For Correspondence: faridjohan@usm.my
of Biological Science, Universiti Sains Malaysia and the voucher specimen number 11289 were obtained.

**Extraction and Isolation**

The leaves of *D. pentandra* were washed, dried and blended into powdered form. The methanol extract was prepared according to Zainuddin & Sul’ain (2015).

**Cell cultures**

The human chronic myeloid leukemia (CML) cell line (K562) originally obtained from American Type Culture Collection (ATCC) and an imatinib-resistant CML cell line (K562R) previously developed by a long-term co-culture of K562 with imatinib mesylate (Al-Jamal et al., 2014). K562 and K562R cell lines were cultured in Roswell Park Media Institute (RPMI) 1640 (Gibco, CA, USA) with 10% of Fetal Bovine Serum (FBS) (Gibco, CA, USA), 100 IU/ml of penicillin (Invitrogen, CA, USA) and 100 µg/ml of streptomycin (Invitrogen, USA) using 25-cm2 flask, in humidified incubator with 5% CO2 at 37°C.

**Trypan Blue Exclusion Assay**

K562 and K562R cells were seeded in 6-well plates at a density of 1 x 105 cells/well in 100 µl of medium and incubated for 24 h. The cells were treated with different concentration of *D. pentandra* extract dissolved in complete media (0, 100, 200, 500, 700 µg/ml) in triplicate and incubated for 48 h. Viable cells were counted using Neubauer chamber and cells viability was recorded as percentage of surviving cells following the treatment compared to the untreated control. The data represented the mean of the three independent experiments. Inhibitory dose concentration (IC50) values were calculated using GraphPad Prism 3.02 (San Diego, California, USA).

**Assessment of Apoptosis by Flowcytometry**

Apoptosis of K562 and K562R were assessed by Annexin V labeling BD Annexin-V-FITC assay kit (Becton Dickinson, NJ, USA) following treatment with *D. pentandra* extract dissolved in complete media (0, 100, 200, 500, 700 µg/ml) for 24 h. The cells were stained with 5 µl annexin conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) at room temperature in the dark for 15 min and analyzed by Flow BDFac Canto II flow cytometer (Becton Dickinson, NJ, USA) to measure the fluorescence intensity. The result showed relative granularity at side scattered and relative size of the cell at forward scattered with fluorescence stain. Untreated cells served as the negative control. A minimum of 10000 events were collected per sample and analyzed.

**Assessment of Cell-Cycle Progression by Flowcytometry**

To study the effect of the extract on cell cycle progression of K562 and K562R cell lines, cell cycle distribution was studied by measuring DNA contents using flow cytometry (Becton Dickinson, NJ, USA). The cells were treated with the extract at IC50 value for 72 h. The treated cells were collected by centrifugation and re-suspended in cold PBS. Cells were stained with PI solution at 37°C in the dark for 30 min. Cell cycle distribution of nuclear DNA was determined by FACS, fluorescence detector using Cell Quest Software (Becton Dickinson, NJ, USA). Analysis of flowcytometric data was performed by Modfit LT 3.2 software (Verity Software House, ME, USA).

**Results**

**Anti-proliferative effect of *D. pentandra***

Cytotoxicity of the methanol extract of *D. pentandra* leaves was tested against a BCR/ABL-positive human CML cell line (K562) and an imatinib-resistant CML cell line (K562R). The IC50 values were used as a parameter for cytotoxicity (growth inhibitory) where it refers to the inhibition of 50% of cells by the extract (Figure 1). The growth inhibitory effects of *D. pentandra* leaves extract on K562 and K562R cell lines were studied by Trypan Blue Exclusion Assay (TBEA). The IC50 values were 500 and 192 µg/mL, respectively.

*D. pentandra* enhanced apoptosis in leukemic cell lines

*D. pentandra* induced total apoptosis of K562 for about 30% after 48 hours treatment at the IC50 concentration of 500 µg/mL, as shown in Figure 2. K562R cells showed no significant increases in early, late and total apoptosis after 48 hours of treatment.

**Cell cycle progression**

The effect of *D. pentandra* methanol extract on cell cycle progression of K562 and K562R upon 48 hours treatment at IC50 concentration were shown in Figure 3. After the treatment period, there was a decrease in the fraction of cells at G0/G1 phase of K562 from 40.7% in untreated to 32.5% in treated cells. The S phase of the cells increased from 51.9% to 59.5%, followed by subsequent increased in G2/M phase, indicating no arrest of the cell cycle at S phase. In K562R, the G0/G1 phase underwent

Figure 1. Cell Viability Assay of *D. pentandra* Extract on K562 and K562R Cells. The effect of various concentrations of *D. pentandra* extract on (A) K562 and (B) K562R cells growth after 48 hours. There was a significant higher in the IC50 of extract concentration on K562 (500 µg/mL) compared to K562R cells (192 µg/mL).
The ability to inhibit apoptosis in the cells, thus resulted in 2000). It leads to instability of the proteins and their in some, but not all resistant cell lines (Mahon et al., 1989). The use of plants in medical research appears to be a great source for the treatment of several cancers. Therapeutic action of phytochemicals could play critical roles in neoplastic transformation, cell proliferation and signaling pathway (William et al., 2009). Leaf crude extract of Pereskia sacharosa plants induces leukemic cell death through induction of apoptosis (Asmaa et al., 2014). The activities of D. pentandra on CML cell lines can be due to the presence of phytochemical compounds such as quercitrine, β-sitosterol, flavonoids, tannins, amino acids, carbohydrates, alkaloids, saponins, and L-asparaginase in the plant that can inhibit cancer cells growth (Widowati et al., 2013)). It was also found that quercetin which is one of the most abundant dietary flavonoids in many fruits and vegetables was able to reduce K562 cell viability and increase the cells apoptosis at doses higher than 200 μM (Akan and i Garip, 2011). Moreover, quercetin acts synergistically with busulphan treatment to enhance the anti-proliferative activities of K562 (Hoffman et al., 1989).

D. pentandra extract could induce cell death of K562 only at late apoptosis. Besides, it was seen from the graph that lower apoptotic induction of K562R was conflicting with its higher sensitivity towards D. pentandra, compared to K562. It has been revealed that BCR/ABL tyrosine kinase can be significantly overexpressed in some, but not all resistant cell lines (Mahon et al., 2000). It leads to instability of the proteins and their ability to inhibit apoptosis in the cells, thus resulted in inconsistent apoptotic induction by the treatment. The pattern of apoptotic activity varies between both K562 and K562R because of the difference in their genetic expression and proteins that regulate intracellular signal transduction pathways involved in cell growth, differentiation, migration and survival. LYN is one of Src family tyrosine kinases that is overexpressed and activated in the imatinib-resistant K562R cell line, which help to induce proliferation and survival of K562R (Weisberg et al., 2007) over K562.

The development of drugs for anticancer activity should consider the ability of the drug component to target the regulation cell cycle phases (DiPaola, 2002). The differences in cell cycle pattern of different cells could be due to the ability of DNA to damage and repair differs between cell types, proliferations of the cells or metabolic status of the cells. Moreover, the extent of DNA damage can also differ between treated cells of a apparently homogeneous population, but with the presence of relatively resistant and sensitive subpopulation (Potter et al., 2002).

The significant increase in the S phases for both cells upon treatment might indicate the ability of the cells to repair damages in DNA. It was suggested that BCR/ABL gene can demonstrate two complementary roles in cancer, that are to stimulate signaling pathways that helps in the survival of leukemia cells and to modulate response to DNA damage that induces resistance to drugs or genotoxic damage. It benefits in the cells’ ability to repair any changes occurred in DNA by extending the activation of the G2M cell cycle checkpoint to provide more time for repair and also inhibit pro-apoptotic mechanisms, more than normal cells do (Nowicki et al., 2004; Greene et al., 2007). Moreover, it has been shown that even though a drug can increase DNA damage in cells, the fusion BCR/ABL can completely alter DNA repair reversibly in short amount of time (Dierov et al., 2004). Similar effect was seen when K562 was treated with Pereskeia sacharosa.
extract (Asmaa et al., 2014).

Phase arrest only occurred at G2/M phase in K562, where increased in G2/M phase was followed by decreased in G0/G1 phase. This arrest occurred in cell-cycle checkpoints to allow repair of damaged DNA, thus maintaining the normal content of the cells. Arrest in G2 phase prevents the damaged DNA to enter mitosis until the damage is repaired. Cells that fail to repair after entering G2/M phase will continue to undergo apoptosis (DiPaola, 2002). This is consistent with the high apoptotic induction by the extract on K562 cells. Different effects of the extract on K562R cell cycle progression might indicate the ability of D. pentandra to induce damaged in earlier G0/G1 phase, but unable to reduce the ability to repair DNA damage by the cells, thus allowing DNA to enter S phase. This may be due to the higher levels of the BCR-ABL protein in K562R cells (Greene et al., 2007) and the ability to repair DNA damage quickly, compared to K562 cells.

Many studies have been done on the mechanisms behind cell cycle regulation and apoptosis induction, with the involvement of regulating proteins. Previous studies on K562 have found that the upregulation of pro-apoptotic Bax protein, along with repression of Bcl-2 and inactivation of akt protein as anti-apoptotic proteins, were contributing to the increased of cytochrome-C released from mitochondria and activation of caspase cascade to induce apoptosis after treatment (Asmaa et al., 2014; Asmaa et al., 2015; Wang et al., 2015). The cell cycle arrest in K562 might be related to the expression of p21 and p53 that responsible for checkpoint activation and apoptosis. It has been proven that overexpression of p53 was significant in cells with D. pentandra treatment as a repair mechanism for damaged DNA during cell cycle progression (Endharti et al., 2016), or triggering cell death for not fixable damage.

In conclusion, considering the ability of the methanol extract of D. pentandra to induce apoptosis and cell cycle arrest at G2/M phase in K562, even though at higher IC50 concentration to inhibit 50% of the cell population after 48 hours, D. pentandra leaves extract has a potential anti-proliferative drug. However the major concern was the potential anti-proliferative activity of D. pentandra in imatinib-resistant K562R cell line as it was found to be no significant results. Further study need to be done on K562R cells pertaining the mechanism such as critical signaling pathways involved in the resistance towards imatinib.

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