

RESEARCH ARTICLE

Anti-Proliferative Effects of *Dendrophthoe 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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Introduction

Dendrophthoe pentandra (L.) Miq. is one of Malaysian mistletoes species; a semi-parasitic plant that commonly found grew on many different species of host plant. In Indonesia, this plant is used as a traditional treatment for cough, diabetes, hypertension, cancer, diuretic, smallpox, ulcer, skin infection and after childbirth (Artanti et al., 2012). In Sulawesi Island, it is used as a medicinal plant to cure cancer (Ishizu et al., 2002). *D. pentandra* leaves extract showed a significant antioxidant and antidiabetic activity (Artanti et al., 2012). The phytochemical screening revealed the presence of flavonoids, saponins, tannins and terpenoids (Zainuddin and Sul'ain, 2015). Quercetin from the flavonoid's group is one of the compounds found in *D. pentandra* that has high antioxidant activity (Widowati et al., 2011).

The reciprocal translocation t(9:22) in chronic myeloid leukemia (CML) generates a BCR/ABL fusion gene that results in the expression of a leukemia-specific oncoprotein, BCR/ABL. BCR/ABL is constitutively active tyrosine kinase that presents in more than 95% patients and results in the underlying process of the

pathogenesis of CML (Sawyers, 1999; Shah et al., 2002). Imatinib mesylate, a tyrosine kinase inhibitor is used as the treatment of CML that specifically targets BCR/ABL protein and induces hematological remission in these patients (Melo and Barnes, 2007; Payandeh et al., 2015). However, the majority of CML patients treated with imatinib would develop resistance under prolonged therapy (Druker et al., 2006).

We previously developed a BCR/ABL-positive imatinib-resistant K562R cell line by an overexposure of K562 cell lines to imatinib to mimics the resistance developed under prolonged therapy (Al-Jamal et al., 2014). The current study highlights the potential of crude *D. pentandra* leaves extract as apoptosis inducer or cell cycle arrester in therapeutic strategy in BCR/ABL-positive and imatinib-resistant leukemic cells.

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

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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 1×10^5 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 at IC₅₀. A total of 1×10^5 cells were collected by centrifugation at 300 x g at 24°C for 5 min. 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).

Statistical analysis

Mann-Whitney test was used for statistical analyses. Data analysis was performed using the SPSS software package (Version 16, SPSS, Armonk, NY, USA) and a p-value <0.05 was considered as significant.

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 increase in G₂/M phase, indicating no arrest of the cell cycle at S phase. In K562R, the G₀/G₁ phase underwent

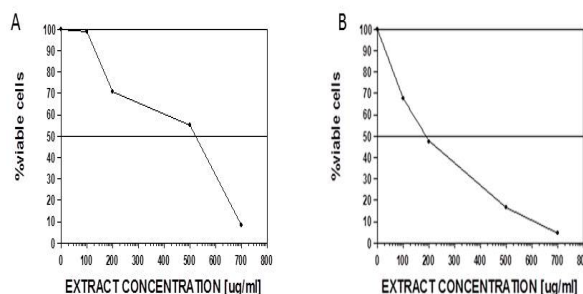


Figure 1. Cell Viability Assay of *D. pentandra* Extract on K562 and K562R Cells.

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).

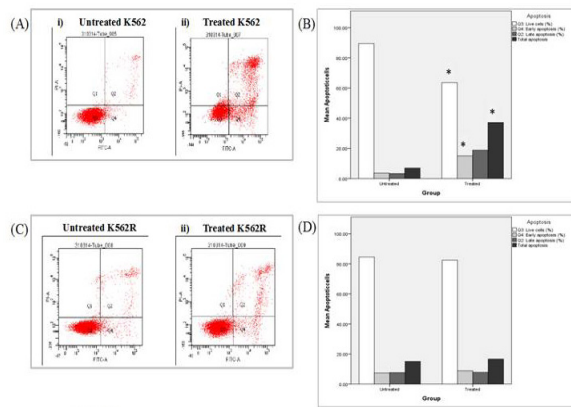


Figure 2. Apoptotic Inductions of *D. Pentandra* Extract on K562 and K562R Cells.

Flowcytometric scatterplots showing the effect of *D. pentandra* extract and bar graph illustrates the percentage of cell population of each cell cycle phases in (A and B) K562 and (C and D) K562R. Apoptotic induction was upon the treatment of both cell lines with cytotoxic dose (IC₅₀) of *D. pentandra* extract for 48 hours. K562 cells showed a significant increase in the total apoptosis after treatment with the extract but no significant changes were shown in K562R.

a decrease from 29.8% to 27.3%. The S phase of the cells also increased slightly from 62.2% to 64.7% upon the treatment, but G2/M phase of K562R cell lines underwent no changes and maintained at 8.0%.

Discussion

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 μ M (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

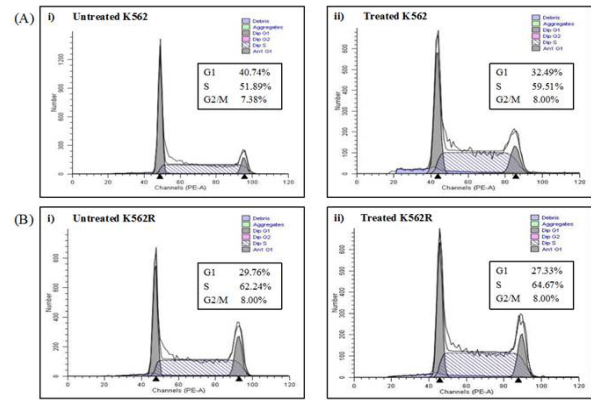


Figure 3. Cell Cycle Progression of K562 and K562R Cells Treated with *D. Pentandra* Extract.

Modfit Software results demonstrate the cell cycle analysis; (A) untreated K562, (B) treated K562, (C) untreated K562R, and (D) treated K562R cells. The DNA content at S phase for both cells were higher with *D. pentandra* extract, but more significant in K562 than K562R. However, cell cycle progressions of K562 and K562R were not arrested due to continuous increased and static reading of cells at G2/M phase respectively.

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 *Pereskia 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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References

- Akan Z, I Garip A (2011). Protective role of quercetin: antioxidants may protect cancer cells from apoptosis and enhance cell durability. *Asian Pac J Cancer Prev*, **14**, 4611-4.
- Al-Jamal HA, Jusoh SA, Yong AC, et al (2014). Silencing of suppressor of cytokine signaling-3 due to methylation results in phosphorylation of STAT3 in imatinib resistant BCR-ABL positive chronic myeloid leukemia cells. *Asian Pac J Cancer Prev*, **15**, 4555-61.
- Artanti N, Firmansyah T, Darmawan A (2012). Bioactivities evaluation of Indonesian mistletoes (*Dendrophthoe pentandra* (L.) Miq.) leaves extracts. *Int J Appl Pharm Sci Res*, **2**, 24.
- Asmaa MJ, Al-Jamal HA, Ang CY, et al (2014). Apoptosis induction in MV4-11 and K562 human leukemic cells by *Pereskia sacharosa* (Cactaceae) leaf crude extract. *Asian Pac J Cancer Prev*, **15**, 475-81.
- Asmaa MJS, Ali AJH, Farid JM, Azman S (2015). Growth inhibitory effects of crude pomegranate peel extract on chronic myeloid leukemia, K562 cells. *Int J Appl Basic Med Res*, **5**, 100-5.
- Dierov J, Dierova R, Carroll M (2004). BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint. *Cancer Cell*, **5**, 275-85.
- DiPaola RS (2002). To arrest or not to G2-M Cell-Cycle arrest. commentary re: A. K. Tyagi et al., silibinin strongly synergizes human prostate carcinoma DU145 Cells to doxorubicin-induced growth inhibition, G2-M Arrest, and apoptosis. *Clin Cancer Res*, **8**, 3311-14.
- Druker BJ, Guilhot F, O'Brien SG, et al (2006). Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*, **355**, 2408-17.
- Endharti AT, Wulandari A, Listyana A, Norahmawati E, Permana S (2016). *Dendrophthoe pentandra* (L.) Miq extract effectively inhibits inflammation, proliferation and induces p53 expression on colitis-associated colon cancer. *BMC Complement Altern Med*, **16**, 374.
- Greene LM, Kelly L, Onnis V, et al (2007). STI-571 (imatinib mesylate) enhances the apoptotic efficacy of pyrrolo-1, 5-benzoxazepine-6, a novel microtubule-targeting agent, in both STI-571-sensitive and-resistant Bcr-Abl-positive human chronic myeloid leukemia cells. *J Pharm Exp Ther*, **321**, 288-97.
- Hoffman R, Graham L, Newlands E (1989). Enhanced anti-proliferative action of busulphan by quercetin on the human leukaemia cell line K562. *Br J Cancer*, **59**, 347.
- Hsiao CJ, Hsiao G, Chen WL, et al (2014). Cephalochromin induces G0/G1 cell cycle arrest and apoptosis in A549 human non-small-cell lung cancer cells by inflicting mitochondrial disruption. *J Nat Prod*, **77**, 758-65.
- Ishizu T, Winarno H, Tsujino E, et al (2002). Indonesian medicinal plants. XXIV. stereochemical structure of perseitol K⁺ complex isolated from the leaves of *scurrula fusca* (Loranthaceae). *Chem Pharm Bull*, **50**, 489-92.
- Mahon FX, Deininger MW, Schultheis B, et al (2000). Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. *Blood*, **96**, 1070-9.
- Melo JV, Barnes DJ (2007). Chronic myeloid leukaemia as a model of disease evolution in human cancer. *Nat Rev Cancer*, **7**, 441-53.
- Nowicki MO, Falinski R, Koptyra M, et al (2004). BCR/ABL oncogenic kinase promotes unfaithful repair of the reactive oxygen species-dependent DNA double-strand breaks. *Blood*, **104**, 3746-53.

- Payandeh M, Sadeghi M, Sadeghi E (2015). Treatment and survival in patients with chronic myeloid leukemia in a chronic phase in west Iran. *Asian Pac J Cancer Prev*, **16**, 7555-9.
- Potter AJ, Gollahon KA, Palanca BJ, et al (2002). Flow cytometric analysis of the cell cycle phase specificity of DNA damage induced by radiation, hydrogen peroxide and doxorubicin. *Carcinogenesis*, **23**, 389-401.
- Sawyers CL (1999). Chronic myeloid leukemia. *N Engl J Med* **340**, 1330-40.
- Shah NP, Nicoll JM, Nagar B, et al (2002). Multiple BCR-ABL kinase domain mutations confer polyclonal resistance to the tyrosine kinase inhibitor imatinib (STI571) in chronic phase and blast crisis chronic myeloid leukemia. *Cancer Cell*, **2**, 117-25.
- Wang B, Liu J, Gong Z (2015). Resveratrol induces apoptosis in K562 cells via the regulation of mitochondrial signaling pathways. *Int J Clin Exp Med*, **8**, 16926-33.
- Weisberg E, Manley PW, Cowan-Jacob SW, et al (2007). Second generation inhibitors of BCR-ABL for the treatment of imatinib-resistant chronic myeloid leukaemia. *Nat Rev Cancer*, **7**, 345-56.
- Widowati W, Mozef T, Risdian C, et al (2011). The comparison of antioxidative and proliferation inhibitor properties of Piper betle L., Catharanthus roseus [L] G. Don, Dendrophthoe petandra L., Curcuma mangga Val. extracts on T47D cancer cell line. *Int Res J Biochem Bio*, **1**, 22-8.
- Widowati W, Mozef T, Risdian C, et al (2013). Anticancer and free radical scavenging potency of catharanthus roseus, dendrophthoe petandra, piper betle and curcuma mangga extracts in breast cancer cell lines. *Oxid Antioxid Med Sci*, **2**, 137-42.
- William WNJR, Heymach JV, Kim ES, et al (2009). Molecular targets for cancer chemoprevention. *Nat Rev Drug Discov*, **8**, 213-25.
- Zainuddin NASN, Sul'ain MD (2015). Phytochemical analysis, toxicity and cytotoxicity evaluation of dendrophthoe pentandra leaves extracts. *Int J Appl Biol Pharm*, **6**, 109-16.