The effect of *Phyllanthus niruri* L extracts on human leukemic cell proliferation and apoptosis induction

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**ABSTRACT**

To investigate the effect of *Phyllanthus niruri* Linn (Euphorbiaceae) in the proliferation of human leukemic cells (MOLT-4 and K562). **Methods:** *Phyllanthus niruri* L (*P.niruri*) was macerated by using various solvents to obtain the crude extracts. Citotoxicity of the extracts against MOLT-4 and K562 cells was tested using MTT assay to find the IC₅₀ value. To analyse cell cycle progression, cellular DNA was measured using propidium iodide (PI) staining. Apoptosis induction was evaluated using Annexin V-FITC and PI stainings and analysed using FACSVerse flow cytometry. Finally, the expression of p53 on MOLT-4 and K562 cell lysate was measured by western blotting, to identify the possible mode of action of the anticancer activity. *P. niruri* crude extracts demonstrated a potential anti-cancer effect towards MOLT-4 cells (IC₅₀ range was 42.21±4.98 to 97.06±18.29 µg/mL). However, against K562 cells, *P.niruri* extracts exhibited a lower inhibitory potency (the IC₅₀ was 120.19±8.48 to 256.55±26.22 µg/mL). The results showed the selectivity of the toxic effect of the extracts against MOLT-4 and K562. To evaluate the possible mechanism of action the anticancer effect, we evaluated *P. niruri* extract action in apoptosis induction and p53 expression. The results showed that methanol and hexane extract inhibited MOLT-4 cell progression from G1 to S-phase, indicating G1 cell arrest. Moreover, apoptotic cell population following treatment of MOLT-4 and K562 cells with methanol extract was markedly increased, showing morphological signs of apoptosis including membrane degradation and chromatin condensation. Furthermore, we found that there was an increase in p53 expression following MOLT-4 treatment with methanol extract, suggesting that p53 induction might be involved in cell apoptosis. The results indicated the involvement of p53 pathway in the mechanism of anti-cancer activity exerted by *P. niruri* extract on MOLT-4 cells. However, for cancer cells lacking P53 expression, such as K562 cells, apoptosis might take place via other pathways.

**Keywords:** *Phyllanthus niruri* L; Leukemia; MOLT-4; K562; Anticancer

**INTRODUCTION**

Since the earliest time, herbal and plant-originated medicines have been exploited for cancer treatment, as recorded in many medicine ancient manuscripts (Nobili, *et al.*, 2009). This practice is still widely used during the modern era, as plant-derived natural compounds have given a new hope in cancer treatment by showing a potency in killing cancerous cells with less side effects (Newman and Cragg, 2007; Nobili, *et al.*, 2009). Plant-derived medicine has also set a fundamental discovery of several important chemotherapy agents such as vincristine, irinothecan, etoposide, and paxitacle (Nobili, *et al.*, 2009).

*Phyllanthus niruri* Linn (*P. niruri*) is one of widely-used medicinal plants in traditional medicine practices, which has demonstrated a variety of pharmacological effects in treating many symptoms (Puspita, 2015; Sabir and Rocha, 2008). From many years of phytochemical studies, researchers have identified more than 50 active
constituents of *P. niruri*, such as flavonoids (Sabir and Rocha, 2008), terpenes (Bagalkotkar, *et al.*, 2006), coumarines (Iizuka, *et al.*, 2007), lignans (Syamasundar *et al.*, 1985), tannins (Than, *et al.*, 2006), saponins, and alkaloids (Sahni *et al.*, 2005). This herb has been extensively studied to evaluate the ethnopharmacological use in treating jaundice, hypertension, urolithiasis, and diabetes. A number of literatures has recorded the therapeutic effects of phytochemicals isolated from *P. niruri*, including hepatoprotective, antioxidant, antihyperuricemic, and antihyperlipidemic activities. For the last few years, researchers have shown growing interest in evaluating the pharmacological activities of this herb as anticancer. *P. niruri* extracts were reported to show a potency in inhibiting cell growth and signalling cascade of various cancer cell lines, including lung cancer (A549), prostate cancer (PC-3), breast cancer (MCF-7), and hepatocellular cancer (HepG2 and Huh-7) (de Araújo Júnior, *et al.*, 2012; Lee, *et al.*, 2006).

From many years of research, no cytotoxicity effects of this plant to normal cells have been reported until recently. However, many questions remain unanswered regarding the bioactivity of this herb in combating cancer cells, particularly the efficacy against high resistance and recurrence cancer such as leukemia. Leukemia is known as one of common cancer with a high mortality rate owing to high recurrence rate and high resistance rate to chemotherapy (Horner, *et al.*, 2009). As surgery is not favourable, the discovery of more efficient chemotherapeutic agents still holds a major role in the development of leukemia treatment.

In comparison to the other closely related species, crude extracts from other *Phyllanthus* species have demonstrated anti-cancer activities against leukemia cell lines. For example, water extract from *Phyllanthus urinaria* was shown to induce apoptosis in human myeloid leukemia cells (HL-60) through a ceramide related pathway (Huang, *et al.*, 2004). Phyllanthostatin, phytochemical isolated from the roots of *P. acuminatus*, demonstrated a potency to inhibit the proliferation of murine lymphocytic leukemia cell line (P-388) (Pettit, *et al.*, 1990). Extensive evidences have shown that, as one of the aims of cancer therapy, modulation of cell cycle and induction of apoptosis have become one of the crucial keys in terminating leukemia cell proliferation. With regards to *P. niruri* bioactivity, this herb extract has demonstrated an ability to induce cell arrest during cell cycle and subsequent apoptosis in solid tumors such as melanoma and prostate cancer (Tang, *et al.*, 2010). However, despite this initial finding, study in identifying *P. niruri* effect in leukemia cells is very limited as there is no report available to date which elucidate the efficacy of this herb in inducing cell cycle arrest and apoptosis in blood cancer.

To answer these questions, in this study, we investigate the antiproliferative activity of *P. niruri* extracts against leukemia cell lines. Two cell lines from different origin, MOLT-4 and K562, are used in this study. MOLT-4 cell line derived from a patient with acute lymphoblastic leukemia, whilst K562 cell line were obtained from chronic myeloid leukemia. Furthermore, to identify the possible mode of action of anti-cancer effect of *P. niruri* extracts towards leukemia cell lines, we evaluate its action in the cell cycle modulation, and apoptosis induction.

**MATERIAL AND METHODS**

**Material**

Powdered dried *P. niruri* was purchased from PT Haldin Pacific Semesta, Indonesia. Plant material was obtained from selected leaves of *P. niruri* (product specification is attached). The powder was prepared by drying and grinding the plant leaves. All cell lines (MOLT-4, K562, and Swiss 3T3 albino mouse fibroblast) were obtained from ATCC England, UK. Culture medium (RPMI) and all media supplements were obtained from Lonza, Walkersville, MD. Cisplatin, thiazolyl blue tetrazolium bromide (MTT salt), Propidium Iodide (PI), and ribonuclease (RNase)-A were purchased from Sigma-Aldrich, Dorset, UK. FITC Annexin V kit was obtained from BD Bioscience, Oxford, UK.

**Preparation of *P. niruri* extracts**

The extraction of *P. niruri* was performed by solvent maceration (methanol, ethanol, hexane, and chloroform). The extraction was started with 100g of crude material, which was immersed in 200mL of solvent for 24h. Next, the solvent was filtered, and the filtrate was collected. The residue was re-immersed in an equal volume of fresh solvent. The maceration was repeated three times. The dried-crude extract was obtained by drying process using a rotary evaporator. Each extract was prepared for further assays by dissolving in DMSO at a final concentration of less than 0.01M.

**Cell culture**

All cell lines were cultured in RPMI culture medium supplemented with 10% (v/v) Fetal Bovine Serum (FBS), 2mM L-glutamine, 50 IU/mL
penicillin, and 50µg/mL streptomycin. The culture condition was 37°C with 5% CO₂.

**MTT Assay**

In 100µL of cell suspensions (containing 10⁵ cells/mL) were plated in 96-wells microplate. *P. niruri* extracts, cisplatin as a positive control, and DMSO as a negative control, was added into each corresponding well. After 5 days incubation, 30µL of MTT solution (containing 5mg/mL of thiazolyl blue tetrazolium bromide) was added into each well. The incubation was continued for a further 4h (37°C, 5% CO₂). Prior to reading, 100µL DMSO was added to dissolve the formazan crystal formed in each well. The optical density was measured using a Multiskan FC® plate reader (absorbance at 590 nm and 690nm).

**Cell cycle analysis**

The cells were seeded at a density of 10⁵ cells/mL in medium containing *P. niruri* extracts. The dose used was the half maximal inhibitory concentration (IC₅₀). As for the control group, the medium was free from *P. niruri* extracts. After incubation for 24, 48, and 72h, the cells were harvested by centrifugation (250 x g for 5min). Subsequently, the cell pellets were washed with phosphate buffer saline (PBS) for three times. The cells were fixed in 70% (v/v) ethanol for 30 minutes at 4°C. After washing with PBS, RNase A (100µg/mL) was added to the cell pellets and incubated at 37°C. Finally, the cell pellets were stained with PI (50µg/mL) and incubated in the dark condition for a minimum of 30min prior to analysis using FACS Verse flow cytometry (BD Bioscience, Oxford, UK) at 488nm excitation and 535nm emission wavelengths. Histogram analysis was performed by using BD FACSuite™ software (BD Bioscience, Oxford, UK).

**Morphological studies**

Following treatment with *P. niruri* extract for 25h, cell slides were prepared from cell suspension (10⁵cells/mL) and stained with hematoxylin-eosin staining. Morphological change was observed to evaluate the signs of apoptosis such as plasma membrane blebbing, nuclear and chromatin condensations, and the appearance of apoptotic bodies (Harikumar, *et al.*, 2009).

**Apoptosis analysis by ANNEXIN V**

Cell staining for apoptosis assay was performed according to the manufacturer’s protocol. Briefly, cell pellet collected after 24h treatment with methanol or cisplatin (IC₅₀ dose) was washed (two times) with cold PBS and re-suspend in 1x Binding Buffer (to obtain cell density of 1 x 10⁶ cells/mL). After washing, the cell pellets were stained with 5µL of Annexin V-FITC and 10µL of propidium iodide (PI) and incubated in the dark condition for 15min. The analysis was performed using a FACS Verse flow cytometry (BD Bioscience, Oxford, UK) 485nm excitation and 535nm emission wavelengths. Histogram analysis was performed by using BD FACSuite™ software (BD Bioscience, Oxford, UK).

**Protein extraction and western blotting**

Cells lysis was performed using high salt lysis buffer (45mM Hepes, 400mM NaCl, 1mM EDTA, 10 % glycerol, 0.5 % ligePal, 2mM sodium orthovanadate, 5mM NaPPi, and 20mM β glycerol phosphate) containing protease inhibitor cocktail. Lysate from cells containing 50µg proteins was fractionated by 10 % SDS-PAGE gel and transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, UK). The transfer process was then blocked in PBS containing 0.1 % Tween 20 and 5% nonfat milk for 1h at room temperature, followed by incubation with primary antibody overnight at 4°C. On the next day, the membrane was washed three times in PBS containing 0.1% Tween 20, and then incubated with horseradish peroxide conjugated secondary antibody (Amersham, GE Healthcare) for 1h. After washing, 1mL of SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoScientific, UK) was added for 30 seconds at room temperature. The bands were detected using gel imaging for fluorescence and chemiluminescence G: BOX (Syngene, UK). The densitometric measurements of protein levels was performed using Image J software (Schneider, *et al.*, 2012). The intensity of the band was analysed and normalised to β-actin intensity.

**Statistical analysis**

Data was obtained from three independent experiments and presented as the mean ± Standard Mean Error (SEM). Statistical analysis was performed using Graphpad prism software version 5.0 (GraphPad Software, San Diego California USA). Comparison of means was performed by using one-way ANOVA with Bonferroni's multiple comparison as a post hoc analysis. A p-value smaller than 0.05 was considered statistically significant.
The cytotoxic effect of *P. niruri* extracts on leukaemia cell growth

Cytotoxicity test results showed the potency of the extracts of *P. niruri* in halting the proliferation of MOLT-4 cell line in a dose-dependent manner. The lowest IC\(_{50}\) value was 42.21±4.98 μg/mL (methanol extract), whilst the highest IC\(_{50}\) value 42.89±3.60 μg/mL (ethanol extract) (Table I). The activity shown by each extract against MOLT-4, from the lowest to the highest potency, was ethanol < hexane < chloroform < methanol. Against K562 cell line, the *P. niruri* extracts also demonstrated an inhibitory effect on the cell proliferation. Nevertheless, the cytotoxic effect was lower than that shown against MOLT-4 cell line. The inhibition activity of methanol extract on MOLT-4 cells (IC\(_{50}\) value 42.21±4.98 μg/mL) was higher than the effect on K562 cells (IC\(_{50}\) value 139.28±19.02 μg/mL) (Table I). Moreover, our results demonstrated that the IC\(_{50}\) of cisplatin for MOLT-4 and K562 cells was 0.48±0.03 μg/mL and 1.06±0.01 μg/mL, respectively, showing that K562 is less susceptible towards anti-cancer therapy.

The effect of the extracts on normal cell proliferation was evaluated. Against 3T3 cells, the range of IC\(_{50}\) values was from 0.35±0.08 μg/mL to >500 μg/mL. Treatment with cisplatin demonstrated the lowest IC\(_{50}\) values compared to the herb extracts which means at the low dose cisplatin has given unfavourable effect to normal cells. When the selectivity index (SI) was calculated, cisplatin was shown to have a low selectivity on normal cells (0.73 and 0.33 for MOLT-4 and K562, respectively). With regards to the selectivity of the plant extracts, the range of SI indices was 0.64 to >11.85 for chloroform and methanol extract, respectively. Chloroform extract, as it exerted the low antiproliferative effect on both cell lines tested, also showed the lowest selectivity indices (0.64 and 1.93). The other extracts provided much better selectivity indices (more than 4.16), which suggested that the extracts provided less toxic effect towards normal cells. In overall, methanol extract was shown to be the most active extract, which exerted a significant toxic effect with the lowest IC\(_{50}\) values for MOLT-4. The results have shown the potential anti-proliferation activities of *P. niruri* extracts against MOLT-4.

Table I. IC\(_{50}\) values of *P. niruri* extracts on cancer cell lines (Puspita, 2015)

<table>
<thead>
<tr>
<th>Extract</th>
<th>MOLT-4 (μg/mL)</th>
<th>K562 (μg/mL)</th>
<th>3T3 (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>42.21±4.98</td>
<td>139.28±19.02</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Ethanol</td>
<td>97.06±18.29</td>
<td>148.51±10.81</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Hexane</td>
<td>89.97±3.60</td>
<td>120.19±8.48</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Chloroform</td>
<td>85.08±8.57</td>
<td>256.55±26.22</td>
<td>164.31±8.42</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.48±0.03</td>
<td>1.06±0.01</td>
<td>0.35±0.08</td>
</tr>
</tbody>
</table>

*The IC\(_{50}\) value was calculated using Graphpad Prism from three independent experiments (Puspita, 2015). The negative control used in this study was DMSO, which shown no inhibitory activity on cell proliferation at a concentration less than 0.1M.

Table II. Selectivity of the cytotoxicity of *P. niruri* extracts on MOLT-4 and K562 cells compared with 3T3 cells

<table>
<thead>
<tr>
<th>Extract</th>
<th>Selectivity Index (SI)*</th>
<th>K562</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>&gt;11.85</td>
<td>3.59</td>
</tr>
<tr>
<td>Ethanol</td>
<td>&gt;5.15</td>
<td>&gt;3.37</td>
</tr>
<tr>
<td>Hexane</td>
<td>&gt;5.85</td>
<td>&gt;4.16</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.93</td>
<td>0.64</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.73</td>
<td>0.33</td>
</tr>
</tbody>
</table>

*The selectivity index (SI) is the ratio of the IC\(_{50}\) values of *P. niruri* extracts against 3T3 cells to the IC\(_{50}\) values against leukemia cell lines MOLT-4 and K562.

### RESULT AND DISCUSSIONS

The cytotoxic effect of *P. niruri* extracts on leukaemia cell growth

Cytotoxicity test results showed the potency of the extracts of *P. niruri* in halting the proliferation of MOLT-4 cell line in a dose-dependent manner. The lowest IC\(_{50}\) value was 42.21±4.98 μg/mL (methanol extract), whilst the highest IC\(_{50}\) value 42.89±3.60 μg/mL (ethanol extract) (Table I). The activity shown by each extract against MOLT-4, from the lowest to the highest potency, was ethanol < hexane < chloroform < methanol. Against K562 cell line, the *P. niruri* extracts also demonstrated an inhibitory effect on the cell proliferation. Nevertheless, the cytotoxic effect was lower than that shown against MOLT-4 cell line. The inhibition activity of methanol extract on MOLT-4 cells (IC\(_{50}\) value 42.21±4.98 μg/mL) was higher than the effect on K562 cells (IC\(_{50}\) value 139.28±19.02 μg/mL) (Table I). Moreover, our results demonstrated that the IC\(_{50}\) of cisplatin for MOLT-4 and K562 cells was 0.48±0.03 μg/mL and 1.06±0.01 μg/mL, respectively, showing that K562 is less susceptible towards anti-cancer therapy.
The lower effect towards K562 and high selectivity indices has suggested the selectivity of the anti-cancer activity of this plant.

**Cell cycle analysis in the presence of *P. niruri* extracts**

MOLT-4 cells treated with *P. niruri* extracts and the control groups were analysed after 24, 48, and 72h incubation time. Following 24h incubation, the changes of cell cycle kinetic on all treated groups were observed (Figure 1). The progression of cell cycle from G1 phase to S phase was significantly halted as most of cell population was arrested in G1 phase.

![Cell cycle analysis](image)

Figure 1. MOLT-4 cells were treated with methanol extract (42 µM) for 24, 48 and 72h. During G1-phase (A), the cells are in the preparation stage for entering the S-phase (B), where a single round of DNA synthesis is taking place. After 24h treatment with *P. niruri* extract, the majority of cell population is arrested in G1-phase. After 24 and 72h incubation, cell are accumulated in G2/M phase (C). Data in the graph represent the percentage of the viable cells from three experiment (n=3) as mean ±SEM. Each value was compared to the corresponding control group (*p<0.05, **p<0.001).

After 48h, the control group has completed the first round of cell cycle progression, considering that the doubling time of MOLT-4 cells is 30–32h (Ma, *et al.*, 2014). In this stage, most of the cell population in control group were accumulated in G1 phase for the preparation to S phase. Treatment with *P.niruri* extracts showed a variability of cell arrest phases after 48h incubation. Chloroform, ethanol, and hexane extracts arrested the cell population in G2/M phase, as most of the cell population were accumulated in G2/M phase with a decreased population in S and G1 phase. Different figure was found in the methanol treated group, which showed a lower cell population in G1 phase accompanied by an increased cell population in S phase. The result indicated that MOLT-4 cells which were initially arrested in G-1 after 24h, were able to proceed to the next stage of DNA synthesis in the S-phase after 48h. After 72h, methanol- and hexane-treated groups provided a sound evidence of cell arrest in G2/M phase, accompanied by a decreased G phase population without any rising in G1 cell population. As mentioned earlier, MOLT-4 cells treated with chloroform and ethanol extracts for 24 hours demonstrated and S-phase arrest. However, after 72h treatment, the result is comparable to that shown by untreated cells, which indicated that the cells were able to escape cell cycle arrest and continue the cell proliferation. In overall, our result demonstrated that *P. niruri* extracts caused cell arrest at different phases in MOLT-4 cell cycle.

**Induction of apoptosis of *P. niruri* in leukemia cell lines**

We further evaluated apoptotic induction caused by *P. niruri*, by observing apoptotic cells after treatment with methanol extract, which demonstrated the most potent anti-proliferative activity. Methanol extract induced apoptosis in MOLT-4 and K562 cells (apoptotic population was 77±6.6% and 36.5±8.5% for MOLT-4 and K562, respectively) (Figure 2). The percentage apoptotic cells significantly increased, characterised by specific morphological changes, including alteration of membrane integrity, cytoplasmic condensation, formation of apoptotic bodies, and DNA fragmentation. Cisplatin, a standard chemotherapy agent used as control in this study, also induced apoptosis in MOLT-4 cells by showing apoptotic cell population of 76±10.4 %. However, K562 cell showed resistance in cisplatin-induced apoptosis (apoptosis population was 6.8±0.3%).
Furthermore, we evaluated the expression of tumour suppressor gene p53 in leukemia cells treated with *P. niruri*. There is an emerging evidence of the importance of p53 in initiating apoptosis, through a complex pathway aiming at eliminating severely damaged cells from continuous replication (Hofseth, *et al.*, 2004).

Our result showed a measurable increase of p53 expression on MOLT-4 after treatment with *P. niruri* extract (Figure 3), indicating the involvement of oncosuppressor p53 in inducing apoptosis. In contrast, K562 has an undetectable level of p53 in untreated and treated groups (Figure 3).

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**Figure 1 - Induction of apoptosis by *P. niruri***
In anti-cancer drugs discovery, more than 60% of chemotherapy agents currently used derived from nature, half of which are listed as plant derived compounds (Cragg and Newman, 2005; David et al., 2012; Shoeb, 2006; Xiao, et al., 2016). In this study, we evaluated the anti-cancer effect of P. niruri crude extracts, which are known as a rich source of phytochemicals (Cragg and Newman, 2005; Sabir and Rocha, 2008; Samali, et al., 2012). Anti-cancer activity possessed by P. niruri has been associated with the presence of polyphenolic compounds in the extracts (Sahni et al., 2005; Samali et al., 2012). Polyphenolic compounds are known to show a potency not only as chemopreventive agents, but also exert their effects in cancer signalling pathways to activate death signals or apoptosis (Cragg and Newman, 2005; Samali, et al., 2012). Previous studies have reported the ability of P. niruri in inhibiting the growth of several types of cancer cells, with no toxic effect towards normal cell lines (Huang, et al., 2010; Lee, et al., 2006; Sahni, et al., 2005; Tang, et al., 2010). The study on the bioactivity of Phyllanthus extracts on leukemia cells is limited, particularly in exploring P.niruri extracts. To our knowledge, our study was the first to report the antiproliferative effect on P.niruri on MOLT-4 and K562. Our results demonstrated that P.niruri crude extracts inhibited the growth of leukaemia cell lines MOLT-4 and K562 in a selective manner, without showing any significant inhibitory effect on the growth of normal cell line.

The results from this study have showed the variation in the efficacy of P.niruri extracts in halting the MOLT4 and K562 cells proliferation. Methanol extract demonstrated the highest inhibitory potential on MOLT4 cells compared to other extracts. Towards K562 cells, hexane extract was found to be more potent compared to methanol extract in inhibiting the cells growth. The variation in the efficacy of P. niruri extracts against MOLT4 and K562 in this study may be attributed to the differences of the bioactive substances present in each extract and/or the characteristic of the cancer cell (Puspita, 2015). It has been argued that the methanolic extracts are more lethal towards cancer cells due to the high solubility of most bioactive compounds in organic solvent such as methanol and ethanol (Tang, et al., 2010; Wu, et al., 2009). Methanol extract is rich of polyphenolic compounds such as gallic acid, rutin, coumaric acid, geraniin, and quercetin (Puspita, 2015; Tang & Sekaran). Martens et all., (2008) reported that polyphenolic compounds such as gallic acid, coumaric acid, flavonoids and resveratrol are responsible for the cytotoxicity of wine extract on MOLT-4 cells in a concentration dependant manner (Mertens-Talcott, et al., 2008). The authors further suggested that antiproliferative effect were more likely to be related to the combination of the polyphenolic compounds rather than the composition of single compound (Mertens-Talcott, et al., 2008). With regards to the anticancer effect of methanolic extracts, a number of studies suggested that anti-proliferative activity of polyphenolic compounds is strongly related to antioxidant activity the compounds, weather in single or combination form (Mertens-Talcott, et al., 2008).

Hexane is a nonpolar solvent; therefore, the bioactive compounds present in hexane extracts is of nonpolar secondary metabolites (Puspita, 2015).
On leukemia cells, hexane extracts from other plant sources have shown anti-proliferative activity on leukemia cells (Harish and Shivanandappa, 2006; Islam, et al., 2008; Parvathaneni, et al., 2014; Sangkaruk, et al., 2017). For example, hexane fraction extracted from M.seamensis flower have shown anti-proliferative activity by inhibiting the growth of K562 via suppression of WT1 and Bcr/Abl proteins (Sangkaruk, et al., 2017). Most of secondary metabolites isolated from hexane extract of P.niruri were of lignans compounds, including hyllanthin, hypophyllanthin, tricontanol and tricontanol (Harish and Shivanandappa, 2006). These lignans are well known for the anticancer properties. Hypophyllanthin and phyllanthin isolated from Phyllanthus amarus showed a dose-dependent inhibitory on breast cancer cell growth (MDA-MB-231 and MCF7) (Parvathaneni, et al., 2014). Lignans from the same herb has exerted a preventive and curative role in Ehrlich Ascites Carcinoma in Swiss albino mice (Islam, et al., 2008). Amongst all extracts tested, our results showed that the hexane extract of P. niruri was most potent in inhibiting the proliferation of K562 cells, which is coherent with that has been reported elsewhere (Huang, et al., 2010; Puspita, 2015). Hexane extract contains triterpenoids, such as glichidione, and lupeol, which has been reported to show cytotoxic effect towards K562 cell lines (Sakkrom, et al., 2010). Moreover, hexane extract of P.amarus, a close species to P.niruri, and its lignans derivate, including phyllanthin, nirtetralin and niranthin, exerted a significant cytotoxic effect on K562 cells (Leite, et al., 2006). The anticancer effect of hexane extracts was suggested due to the ability to inhibit P-glycoprotein (Pgp) activity (Leite, et al., 2006). Pgp is a plasma membrane protein which highly expressed in drug-resistant cells. K562 has an extensive expression of Pgp on the membrane surface (Klein, et al., 1976), therefore might explain the anti-cancer potential of the P.niruri hexane extract K562, as well as the collaborative effect of the containing non-polar compounds in inhibiting the proliferation of K562 cells.

The discovery of effective chemotherapy for cancer is extremely challenging, as cancer cells possess the unique ability to escape apoptosis (Evan and Vousden, 2001; Fulda and Debatin, 2006; Puspita, 2015). Cancer cells have developed resistance towards many anti-cancer drugs because of this unique ability (Brown and Wouters, 1999). Accordingly, one of the goals of chemotherapy is the re-activation of programmed cell death, which cause cell arrest at one phase during the cell cycle (Ma, et al., 2014; Shoeb, 2006). Our result showed that all P. niruri extracts caused the accumulation of MOLT-4 cells in G1-phase and disruption of the cell cycle progress to enter S-phase. Whilst some of cells were able to proceed to the next cell cycle phases, methanol extract of P.niruri demonstrated a potency to halt cell progression to cell division though G2/M phase arrest. Consequently, arrested cells in each phase within cell cycle will undergo a programmed cell death of apoptosis.

This alteration of cell cycle progression is a result form the bioactivity of the active constituents present in P.niruri extracts. As previously mentioned, Polyphenolic compounds exerted a promising potential as chemopreventive and chemotherapeutic agents, mostly due to the ability in interfering with cell cycle kinetics, altering the cell signalling pathways, and activating apoptosis (Cragg and Newman, 2005; Fresco, et al., 2006; Samali, et al., 2012). The definite mode of action of plant extract may not be a result from a single active compound, but rather comprise of multiple phytochemicals action to induce the observe effects. A number of polyphenols, including gallic acid and quercetin, were reported to be abundantly present in P.niruri extracts (Calixto, et al., 1998; Cragg and Newman, 2005). Gallic acid has showed a potency to induce G1-arrest and apoptosis in human prostate carcinoma cell line via Cip1/P21 induction (Agarwal, et al., 2006). Moreover, gallic acid were able to decrease the level of cytokines essential for cell cycle progression, such as CDK4, CDK5, and CDK2 (Agarwal, et al., 2006). It has been previously reported that quercetin showed a potency in inhibiting leukemia cell lines through G2/M phase arrest, resulting in the alteration of cell cycle progression and inducing caspase dependent apoptotic cell death (Lee, et al., 2006). Accordingly, inhibition of MOLT4 and K562 cell cycle may be a result from various active ingredients contains in P.niruri extracts, involving multiple anti-cancer pathways.

In response to anti-cancer therapy, p53 plays an important role during cellular checkpoints within cell cycle to induce apoptosis (Fulda and Debatin, 2006; Lowe, et al., 1994; Ma, et al., 2014). Checkpoint function is mediated by genes it transcriptionally regulated, including P21, and keeps cells from progressing during cell cycle from G1 to S-phase or G2 to M-phase after DNA damage (Fulda and Debatin, 2006; Lowe, et al., 1994). In leukemia pathogenesis, there is a growing evidence of p53 mutation on human lymphoid malignancy,
thus allows leukemic cells to evade apoptosis and progress in uncontrolled manner (Gaidano, et al., 1991). In this study, we showed that apoptotic cell population following treatment of MOLT-4 and K562 cells with methanol extract of P. niruri was markedly increased, showing morphological signs of apoptosis, including membrane degradation and chromatin condensation. It is therefore suggested that methanol extract induced apoptosis in MOLT-4 and K562 as a result from the checkpoints activities and cell arrest during cell cycle. Furthermore, our result demonstrated that p53 expression following MOLT-4 treatment with methanol extract was increased, suggesting that p53 induction might be involved in MOLT-4 apoptosis. However, in K562 the changes in p53 expression was not observed, suggesting that this protein has no significant role in the apoptosis induced by p. niruri extract.

There is still a long debate regarding p53 mechanism in mediating apoptosis (Bunz, et al., 1999), which is associated with several mechanisms, including cross linking with purine bases on the DNA, interrupting DNA repair mechanisms, stimulating DNA damage, and finally causing the apoptosis and cell death (Dasari and Tchounwou, 2014). Nevertheless, our finding has indicated the involvement of p53 pathway in the mechanism of anti-cancer activity exerted by methanol extract of P. niruri on MOLT-4 cells. However, for K562 cells, which are lacking p53 expression, apoptosis might take place through other pathways.

CONCLUSION

The results from this present study have highlighted the potency of P. niruri extracts to alter the proliferation of leukaemia cell lines MOLT-4 and K562 in a selective manner. The selectivity was shown by the evident cytotoxic effects towards cancer cell lines, yet protective towards the normal cell line. Although the actual underlying mode of actions of P. niruri against leukemia cell lines has not been elucidated, the elevated level of p53 has indicated that DNA damage following MOLT-4 treatment with methanol extract has activated the p53 pathway to further initiate apoptosis in leukaemia cells. Our study has provided an initial evidence for the application of P. niruri in the development of plan-derived chemotherapeutic agent for leukemia. Nevertheless, further study on the apoptosis pathway is needed to reveal the mechanism of action of P. niruri against leukemia.

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