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Cytotoxic and antiproliferative effects of crude ethanolic extract from *Piper betle* leaves on leukemic cell lines

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ABSTRACT

Background: Leukemia is a group of malignant diseases characterized by the uncontrolled proliferation of abnormal white blood cells in the bone marrow and peripheral blood. These abnormal cells interfere with normal cell growth and development. Nowadays, chemotherapy is the most effective treatment for leukemia but it causes side effects for patients at the same time. To decrease the side effects, medicinal and edible plants are a choice for leukemia treatment. *Piper betle* (betel) leaves are a choice of interest because it is a common material in the recipes of Thai folk medicine.

Objectives: This work aims to investigate cytotoxic and antiproliferative activities of crude ethanolic betel leave extract in leukemic cells.

Materials and methods: The cytotoxic activity and WT1 protein levels were determined using MTT assay and Western blotting, respectively. Hydroxychavecol content in the crude extract was determined using HPLC.

Results: Crude ethanolic betel leave extract had the highest cytotoxicity effects against K562, Molt4, HL60, and U937 cells with IC_{50} values of 36.2±1.1, 19.7±1.6, 28.9±5.6, and 17.3±0.7 µg/mL, respectively. Moreover, it could decrease WT1 protein level and total cell number in a time- and dose-dependent manner compared to vehicle control. Hydroxychavecol was the main compound in crude ethanolic extract following HPLC determination.

Conclusion: Crude ethanolic betel leave extract had high anti-cancer activity *via* WT1 protein suppression in K562 cells.

Introduction

Piper betle, commonly known as betel leaf, is a plant part of the family of Piperaceae which has been used as Thai folk medicine. It is also used in tropical Asia and East Indies. Betel leaves were often chewed together with a little quicklime and areca nut, especially by ancient people. The betel leaves contain carotenes, ascorbic acid, and phenolic compounds. The phenolic compounds of this plant are chavicol,[1] chavibetol, chavibetol acetate,[2] and eugenol.[3] Hydroxychavicol has been reported as the main compound (39.31%), which was determined by GC-MS from crude aqueous extract.[4] In addition, though, in the essential oil extract, the eugenol was reported to be the main compound with a percentage of 36.2 by GC-MS.[5] Betel leaf extract exhibit antioxidative, anti-inflammatory,[6] antimutagenic,[1] antibacterial, antifungal, and antiproliferative activities.[7] Furthermore, the anticancer activity of betel leave extract was reported using aqueous extract (boiling fresh leaves in distilled water) and showed antiproliferative activity in KB cells.[7] However,

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antileukemic cell reports are limited. The Wilms' tumor 1 or WT1, is a protein encoded by the *WT1* gene which has been used as a leukemic cell biological marker to identify prognosis in leukemia patients. Low levels of WT1 protein expression have been found in normal blood cells, and in contrast, WT1 protein have been found overexpressed in leukemic blood cells.[8] Recently, many reports showed that wild-type WT1 is expressed in the majority of Wilms' tumors in addition to a variety of cancers including breast cancer,[9, 10] lung cancer,[11] bone and soft-tissue sarcoma,[12] head and neck squamous cell carcinoma,[13] thyroid,[14] colon,[15] esophageal,[16] pancreatic ductal cancer,[17] and human primary leukemia,[18] and has been implicated as an oncogene in these tumors.[19] *WT1* gene expression has been reported to be suppressed by curcumin), the active compound from turmeric,[20-22] crude kaffir lime leave extract,[23] and crude mangosteen extract.[24] In this study, the anticancer activity of betel leaf extract was examined. The WT1 protein was used as a biological tool to determine the inhibitory mechanism of K562 cells for leukemic cell proliferation.

Materials and methods

Plant material

The betel (*Piper betle* L.) leaf was collected from the Faculty of Pharmacy, Chiang Mai University, Chiang Mai Province, Thailand. A voucher specimen, No. 008612, was deposited at an herbarium, the Northern Research Center for medicinal plants, Faculty of Pharmacy, Chiang Mai University, Thailand. The leave material was washed and chopped into small pieces and then dried by circulating dry air in an oven at 50°C. The materials were then soaked in 95% ethanol and placed in an ultrasonic bath for 15 min, filtered by filter paper No. 1 with 90 mm diameter and concentrated by evaporation under reduced pressure with a rotary evaporator at 50°C.

Cells and cell culture conditions

K562 (chronic myelocytic), Molt4 (human lymphoblastic), HL60 (human promyelocytic), and U937 (human monocytic) leukemic cell lines were cultured in RPMI-1640 medium containing 10% fetal calf serum, 1 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen™ Life, Carlsbad, CA, USA). KB-3-1 (human cervical carcinoma) and Caco-2 (human colorectal adenocarcinoma) cells were cultured in Eagle's Minimum Essential Medium (EMEM) and supplemented with 10% fetal bovine serum (FBS) (Invitrogen™ CA, USA), 100 Units/mL penicillin and 100 µg/mL streptomycin. All cell lines will be incubated in humidified 95%, 5% CO_2, and atmosphere at 37°C.

MTT cytotoxicity assay

Cytotoxicity of crude ethanolic betel leave extract was evaluated using MTT assay. Cells (1.0×10^4 cells/well) were cultured in a 96-well plate containing 100 µL medium prior to crude extracts at 37°C for 24 hrs. Afterward, 100 µL of fresh medium containing crude ethanolic betel leaf extract at various concentrations (3-100µg/mL) were added into each well and further incubated for another 48 hrs. After removal of 100 µL medium, 15 µL of MTT dye solution (Sigma-Aldrich, St Louis, MO, USA) at 5 mg/mL was added, and the plate was incubated at 37°C for 4 hrs. Then, aqueous solution was removed, and 200 µL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured using AccuReader™ microplate reader (Metertech-Inc, Taipei, Taiwan) at 545 nm. Metabolic activity of each well was determined and compared to untreated cells (vehicle control). High optical density readings corresponded to a high intensity of dye color, that is, to many viable cells able to metabolize MTT salts. The following formula calculated the fractional absorbance:

$$\% \text{ Cell viability} = \frac{\text{Mean absorbance in test well}}{\text{Mean absorbance in vehicle control well}} \times 100$$

Average cell survival obtained from triplicate determinations at each concentration was plotted as a dose-response curve. The 50% inhibitory concentration (IC_{50}) values of the betel leave and other plant extracts were determined as the lowest concentration, which reduced cell growth by 50% in treated culture, compared to vehicle control. The IC_{50} values were mean±standard error of the mean (SEM) and their activities.

Cell proliferation assay

Total cell numbers were counted using trypan blue exclusion method. Live cells have intact membranes and can exclude trypan blue dye, whereas dead cells with compromised membranes are stained by the trypan blue dye solution. A cell suspension and 0.2% trypan blue were mixed, and viable (unstained) and dead (stained) cells were counted using a hemacytometer. Percentage of viable cells was then calculated.

Protein extraction and Western blotting

Total protein extracts from treated cells were prepared using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5 mM EDTA, 0.1% SDS, and protease inhibitor cocktail). Protein concentration was measured by Folin-Lowry method. Proteins were separated using 12% SDS polyacrylamide gel electrophoresis. Analysis for WT1 protein detection were performed using primary rabbit polyclonal anti-WT1 (Santa Cruz Biotechnology, CA, USA) in the ratio of 1:100, followed by a treatment of HRP-conjugated goat anti-rabbit IgG (Promega, Madison, WI, USA) in a 1:20,000 dilution. The GAPDH protein was probed by primary rabbit polyclonal anti-GAPDH (Santa Cruz Biotechnology, CA, USA) in the ratio of 1:1,000, followed by treatment with HRP-conjugated goat anti-rabbit IgG in a 1:20,000 dilution. The proteins were visualized using the Luminata™ Forte Western HRP Substrate (Merck Millipore Corporation, Billerica, MA, USA) and quantified by the ChemiDoc™ XRS (Bio-Rad, Hercules, CA, USA).

Analysis of crude ethanolic extracts using high performance liquid chromatography (HPLC)

Portions of 0.5-100 mg of samples were dissolved in 1 mL of ethanol for HPLC analysis. The solution was filtered through a 0.22 µm filter (nylon syringe filter, Filtrex, USA). The sample was analyzed by a Dionex ICS-3000 HPLC system with a PDI-100 photodiode array detector. A C18 column (150×4.6 mm, 5 µm particle size, Nucleodur®) with a guard column was used. For elution of the constituents, two solvents denoted as A and B were applied. Solution A was 70%

methanol, whereas B was 30% deionized water with an isocratic program for 20 min. The flow rate was 0.7 mL/min at room temperature, and the injection volume of the extract was 20 µL. The retention times and UV spectra of significant peaks were recorded.

Statistical analysis

All data were expressed as the mean±standard deviation (SD) or mean±standard error of the mean (SEM) from triplicate samples of three independent experiments. Statistical differences between the means were determined using one-way ANOVA. The differences were considered significant when the probability value obtained was less than 0.05 (p<0.05).

Results

Cytotoxicity of crude ethanolic betel leave extract on leukemic and other cancer cell lines

As shown in Table 1 and Figure 1, crude ethanolic extract of betel leave was treated with various concentrations (3-100 µg/mL) for 48 hrs in K562, Molt4, HL60, U937, KB-3-1, and Caco-2 cell lines. Crude ethanolic of betel leave extract exhibited the strongest cytotoxicity in the U937 cells with an IC_{50} value of 17.3±0.7 µg/mL. When compared to that of its leave extract, the result showed the lower values with IC_{50} values of 36.2±1.1, 19.7±1.6, 28.9±5.6, and 40.2±7.7 µg/mL in K562, Molt4, HL60, and Caco-2 cells, respectively. However, the betel leave extract did not affect KB-3-1 cells (IC_{50} >100 µg/mL).

Table 1 Inhibitory concentration (IC_{50}) of crude ethanolic extracts from Thai traditional plants.

Crude ethanolic extract	Cytotoxicity IC_{50} (µg/mL)					
	K562	Molt4	HL60	U937	KB-3-1	Caco-2
Betel leaves	36.2±1.1	19.7±1.6	28.9±5.6	17.3±0.7	>100	40.2±7.7

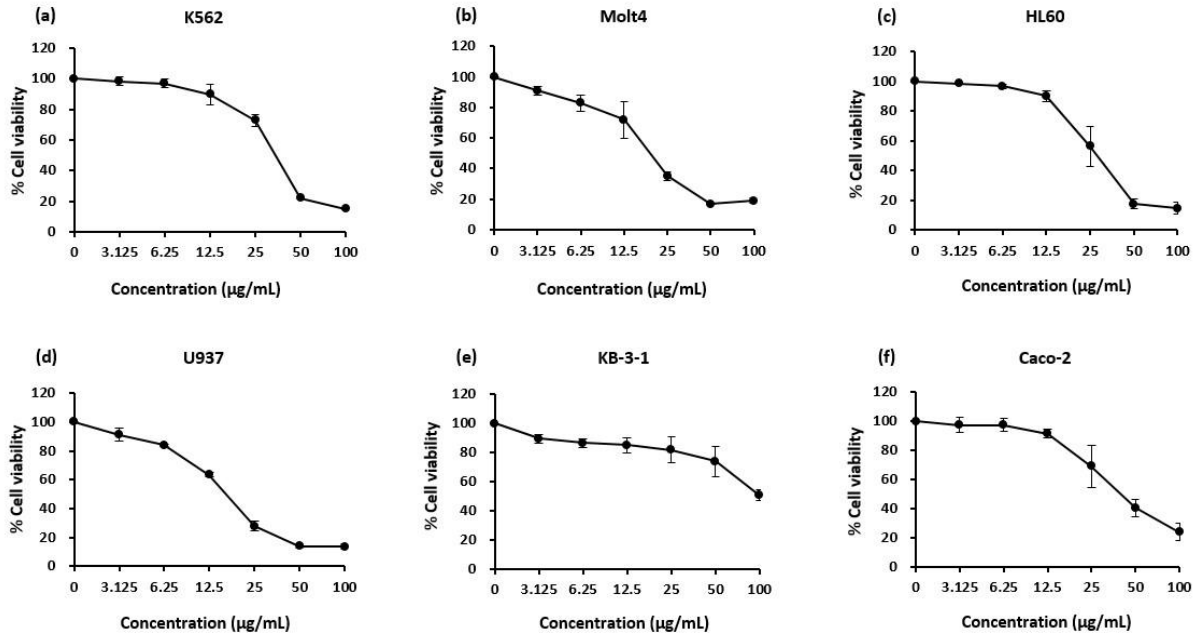

Figure 1. *Inhibitory concentration (IC_{50}) of crude ethanolic betel leaf extract in (a) K562, (b) Molt4, (c) HL60, (d) U937, (e) KB-3-1, and (f) Caco-2 cell lines.*

Inhibitory effects of crude ethanolic betel leaf extract on WT1 protein levels and total cell number in K562 cells

In this experiment, activity of crude ethanolic betel leaf extract on WT1 protein expression was examined using the IC_{20} value (20 µg/mL) in time and dose responses in K562 cells using Western blotting. In this study, K562 cells was used as a WT1 expressing cell model while HL60 and U937 cells do not express WT1 protein.[22] Molt4 cells express lower level of WT1 than K562 cells.[22] Furthermore, there is no report of WT1 expression in KB-3-1 and Caco-2 cell lines. Different time points (12, 24, and 48 hrs) of crude ethanolic betel leave extract were confirmed their cytotoxicity by MTT assay. The result showed no cytotoxicity at 20 µg/mL at 12, 24, and 48 hrs (Figure 2A). Following crude ethanolic betel leave extract treatment, WT1 protein expression levels were decreased by 42.9, 29.6, and 12.7%, respectively, as compared to vehicle control (Figures 2B, and 2C). The effect of various concentrations of this crude ethanolic extract decreased WT1 protein levels by 14, 23.2, and 37.3% in response to 15, 20, and 25 µg/mL, respectively (Figures 3A and 3B). Moreover, the different time points could significantly decrease the total cell number at 48 hrs by 57.6% in K562 cells (Figure 4). Following various concentrations of crude ethanolic extract treatments, the total cell number was significantly decreased at 12 hrs by 16.7, 23.2, and 34.3%, in response to 15, 20, and 25 µg/mL, respectively, as compared to vehicle control (Figure 5).

Hydroxychavicol content of betel leave extract using HPLC

HPLC chromatogram of crude ethanolic betel leaf extracts showed 5 main peaks at retention times of 1.98, 2.80, 3.36, 4.11, and 5.43 min (Figure 6). At 4.11 and 5.43 min, area under the peak curve was 74.54 and 18.67%, respectively. Retention time of standard hydroxychavicol was 4.10 min, and area percentage under the graph was 87.98%, which was consistent with the time peak of crude ethanolic betel leaf extract curve that was shown. It is possible that retention time at during 4.11 min of ethanolic leaf extract from betel leaves is hydroxychavicol.

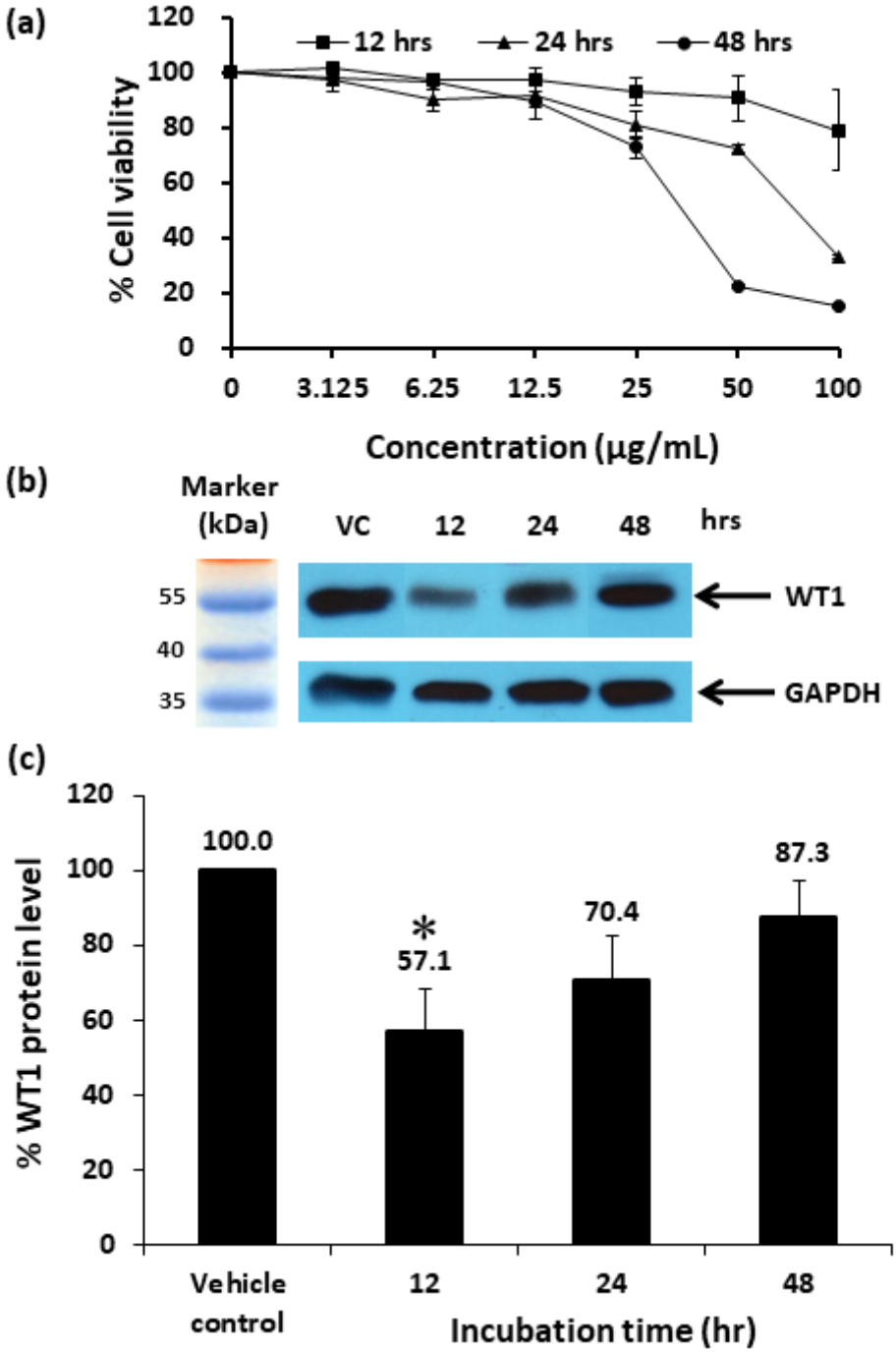

Figure 2. *Times course of measuring crude ethanolic betel leaf extract effect on WT1 protein levels in K562 cells using Western blotting. (a) Effects of cytotoxicity in K562 cells at 12, 24, and 48 hrs. Cells were treated with 20 µg/mL of crude ethanolic betel leave extract for different time points (12, 24, and 48 hrs). (b) Levels of WT1 protein expression were assessed by immunoblotting; GAPDH was used as a loading control. (c) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the crude ethanolic betel leave extracts in the culture medium). Data is reported as the mean value±SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control ($p<0.05$).*

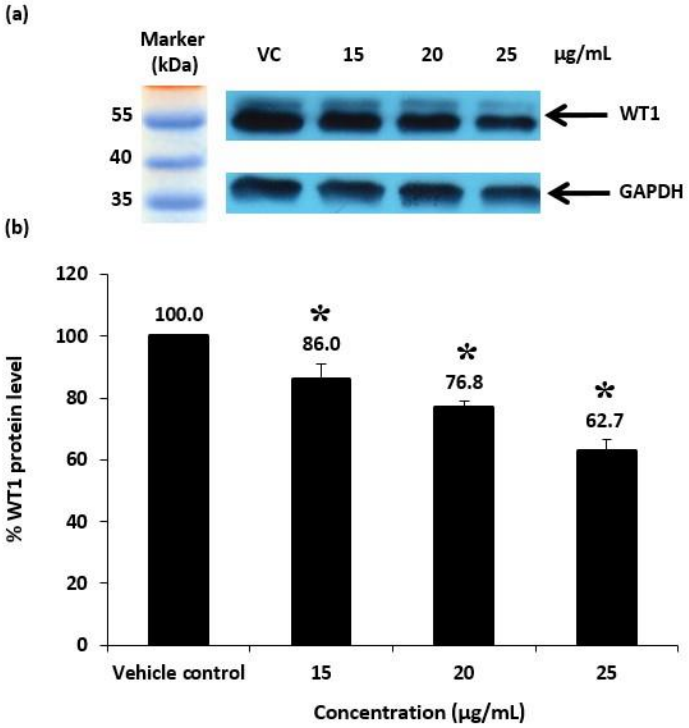

Figure 3. *Effect of various concentrations of crude ethanolic betel leaf extract on WT1 protein levels in K562 cells using Western blotting. Cells were treated with different concentrations of crude ethanolic betel leave extract (15, 20, and 25 µg/mL) for 12 hrs. (a) The levels of WT1 protein expression were assessed by immunoblotting; GAPDH was used as a loading control. (b) Densitometry was used to quantitate the protein levels and graph as the percentage of vehicle control (0.02% DMSO alone without the crude ethanolic betel leave extracts in the culture medium). Data is reported as the mean value±SEM of three independent experiments. Asterisks (*) denote values that were significantly different from the vehicle control (p<0.05).*

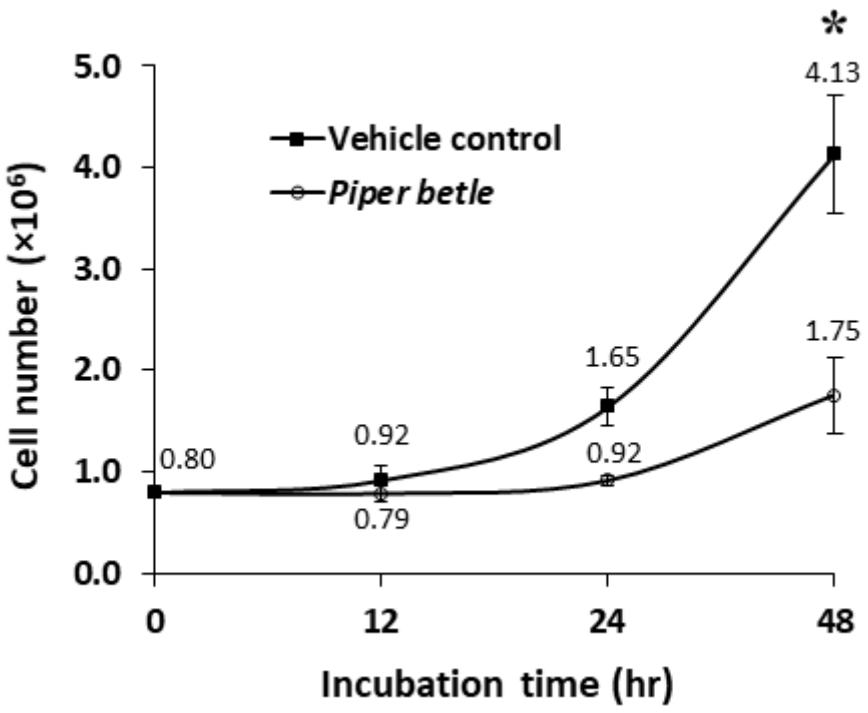

Figure 4. *Total cell number of K562 cells following 20 µg/mL crude ethanolic betel leaf extract treatment during 12-48 hrs. Asterisks (*) denote values that were significantly different from vehicle control (p<0.05) in the same period.*

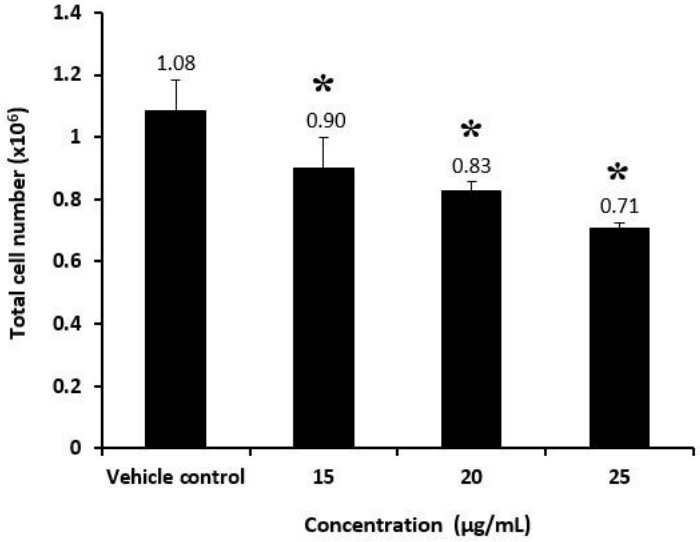

Figure 5. *Total cell number of K562 cells following various concentrations of ethanolic betel leaf extract treatments for 12 hrs. Asterisks (*) denote significant differences from vehicle control (p<0.05).*

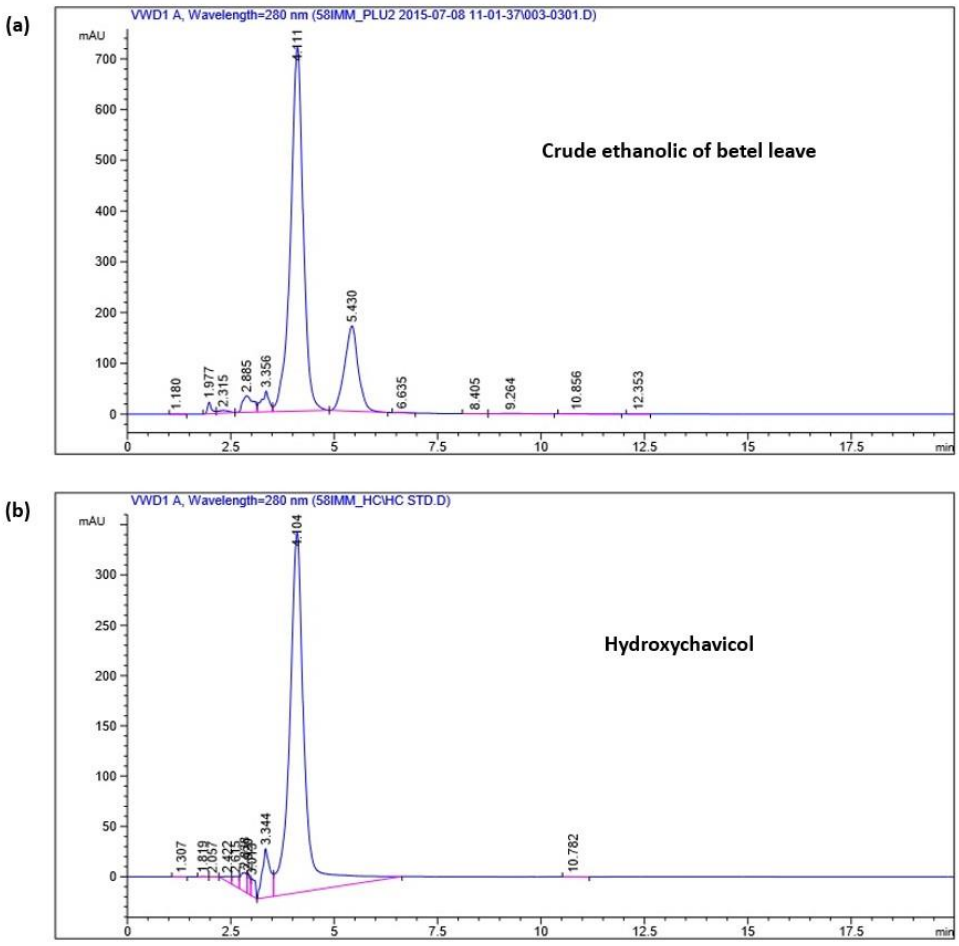

Figure 6. *HPLC chromatogram of the crude ethanolic betel leaf extracts.*

Discussion

Betel (*Piper betle* L.) leaf was selected to study the biological activities because it demonstrated the best activity of anticancer property. This study is the first report of cytotoxic activity of crude ethanolic betel leaf extract in human leukemic cells. It had the strongest activity against human leukemia cell lines. The best activity was shown in U937 cells (IC_{50} value of 17.3±0.7 µg/mL). There was a cytotoxic report of essential oil from betel leaf extract in P388 murine leukemia cell line by MTT assay. The result indicated that essential oil from betel leaf extract exhibited the IC_{50} of over 0.6 mg/mL.[25] Our result was also in line with the effect of kaffir lime leaf extract on U937 cells with IC_{50} of 19.8±1.0 µg/mL. When compared its effect to curcumin in U937 cells, the IC_{50} value after curcumin treatment was 8.7 µg/mL.[26] The cytotoxic effects of crude betel leave extract in both K562 and Molt4 cells were less than that of U937 cells due to the reason for lower levels of WT1 protein in U937 cells.[22] WT1 protein is the transcription factor that promotes cell proliferation in leukemic cells. Thus, high levels of WT1 protein can induce a high rate of cell proliferation in both K562 and Molt4 cells. Furthermore, difference in cell phenotype is one factor that causes differences in cell sensitivities. According to the absence report of the cytotoxic data of crude ethanolic betel leaf extract in human leukemic cells, the cytotoxicity effects of crude ethanolic betel leaf extract in other types of cancer cells have been reported. Crude *Piper betle* leaf extract showed the cytotoxicity with IC_{50} of 0.136 µg/mL at 48 hrs by MTS assay in the HeLa cervical cancer cell line, while *Piper fragile* Benth showed higher cytotoxicity (IC_{50} value of 0.005 µg/mL) than the *Piper betle* leaf extract.[27] However, aqueous *Piper betle* leaf extracts did not show activity in HeLa cells (IC_{50} >100 µg/mL) using neutral red cytotoxicity assay.[7] Cytotoxicities of crude ethanolic betel leaf extracts in U937, K562, Molt4, and HL60 cells were less than vincristine ($9x10^{-5}$, $8x10^{-3}$, $3.9x10^{-4}$, $6.3x10^{-4}$ µg/mL, respectively) in the previous report by $1.92x10^{5}$-, $4.53x10^{3}$-, $7.41x10^{4}$-, and $3.13x10^{4}$-fold, respectively.[28] IC_{50} values of doxorubicin (0.8 ± 0.06 µg/mL) and idarubicin (0.41±0.04 µg/mL) were less than crude ethanolic betel leaf extract in K562 cells by 45.25- and 88.29-fold, respectively.[29]

In this study, cervical carcinoma cell line (KB-3-1) and colon cancer cell line (Caco-2) were analyzed to compare differences amongst the cell types. The crude ethanolic betel leaf extract did not affect the KB-3-1 cells (IC_{50} value >100 µg/mL), while Caco-2 cells had the IC_{50} value of 40.2±7.7 µg/mL. Moreover, aqueous betel leaf extract showed cytotoxicity in human nasopharyngeal epidermoid carcinoma cells (KB) with the IC_{50} value of 29.5±0.3 µg/mL by using neutral red cytotoxicity assay[7] but essential oil extract of *Piper betle* leaf exhibited no antiproliferative activity inhuman mouth epidermal carcinoma cells (KB) and murine leukemia cells (P388) by MTT assay.[25] The aqueous extract of crude betel leaf extract exhibited cytotoxic effect in human epidermoid larynx carcinoma cells (Hep-2).[30] Betel leaf extract inhibited human ductal breast epithelial tumor (T47D) cell proliferation with an IC_{50} value of 55.2 µg/mL using MTS assay.[31] On the opposite, ethyl acetate and hexane extracts showed dose-dependent inhibitory effects on breast cancer (MCF-7) cells with IC_{50} values of 65±0.0 and 163.3±2.89 µg/mL, respectively.[32]

Here WT1 protein was used as a biological marker of leukemic cell proliferation,[8] and as a tool for determining the effect of crude ethanolic betel leaf extract on leukemia cell proliferation. Normally, it has been used to predict leukemia progression. Overexpression of *WT1* gene is important in leukemogenesis because both the RNA and the protein levels of WT1 increase 1,000-10,000 folds in leukemia cells.[8, 33] This suggests it may play an important role in oncogenesis. Pure curcumin extracted from turmeric, a spice grown in Asia's tropical regions, has been shown to decrease WT1 mRNA and WT1 protein levels in human leukemic cell lines.[20-22] *Piper betle* was the herb that presented the cytotoxicity values comparable to other cancer cell lines. This is the first report to show that betel leaves obtain their activity from its bioactive compounds to suppress *WT1* gene expression. Results from Western blotting indicated that the crude betel leaf extract was efficient decreasing levels of WT1 protein in a time- and dose-dependent manner. The maximum value of inhibition (37.3%) was shown at the dose of 25 µg/mL for 12 hrs. The active compound of crude ethanolic betel leaf extract will be further studied. However, previous reports have shown the main compound from betel leaf was hydroxychavicol, which was characterized by GC-MS.[4] Moreover, chavicol was found in crude ethanolic betel leaf extract by HPLC before partitioning into the oil.[34] Their HPLC results showed the same pattern when compared to our results. The biological activity of hydroxychavicol was recently found to induce apoptosis in chronic myelocytic leukemia (CML) cells expressing wild type and mutated bcr/abl with imatinib resistance phenotype.[35] It was also found to induce cell cycle arrest and cell apoptosis of oral KB carcinoma cells.[36] Thus, the active compound from betel leaves will be further studied to elucidate its activity on inhibitory mechanism of the WT1 signaling pathway. However, it has also been reported WT1 signaling involved in protein kinase C alpha (PKCα) and the c-Jun N terminal kinase (JNK) signaling pathway.[37]

Conclusion

Crude ethanolic from *Piper betle* leaf extract had cytotoxic response in leukemic cells. This is the first report to show the inhibitory effect of crude ethanolic from betel leaf via WT1 protein suppression in K562 leukemic cells. Moreover, crude ethanolic extract decreased total cell number in a time- and dose-dependent manner. It is a source of hydroxychavicol, the main active compound product displaying potent inhibitory activity on leukemic cell proliferation, and a promising candidate as a naturally occurring antileukemic drug in the future.

Conflict of interest

There are no conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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