

Full Length Research Paper

Cytotoxic effects of crude kaffir lime (*Citrus hystrix*, DC.) leaf fractional extracts on leukemic cell lines

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Crude extracts from the leaves of kaffir lime (*Citrus hystrix*, DC.) were extracted using hexane, ethanol, ethyl acetate, butanol and methanol. The fractions were investigated *in vitro* for their potential cytotoxic activity on HL60, K562, Molt4, U937 cell lines, and normal human peripheral blood mononuclear cells (PBMCs) using the MTT assay. The *in vitro* cytotoxicity bioassays on 4 leukemic cell lines showed that the ethyl acetate fraction exhibited the highest cytotoxicity, with IC₅₀ values of 19.0±0.6, 35.3±1.4, 21.8±0.4, and 19.8±1.0 µg/ml, in response to HL60, K562, Molt4, and U937 cell lines, respectively. These were higher than those of fractions from hexane, ethanol, and butanol. The methanol fraction had no cytotoxic activity (IC₅₀ value > 100 µg/ml). None of the fractions had cytotoxic effects on PBMCs. The active compound in the ethyl acetate fraction should be investigated for possible use in chemotherapy.

Key words: Kaffir lime, *Citrus hystrix*, DC., cytotoxicity, leukemic cell line.

INTRODUCTION

Leukemia has become one of the world's major health problems, due to its high incidence, representing about 53% of all of cancers (Khuhaprema et al., 2007). Chemotherapy is generally effective and is widely used for treating leukemia. However, chemotherapy acts by killing abnormally fast-dividing cells, and thus affects cells that divide rapidly under normal circumstances, causing unwanted side effects. Therefore, naturally occurring plant compounds that have cancer inhibitory effects but cause fewer side effects are a valuable alternative treatment for leukemias. Recently the National Cancer Institute (NCI), USA has launched an extensive program for the development of natural products for treatment of various forms of cancer. Many clinically useful drugs

have been discovered from endogenous plants. These include vinblastine and vincristine from *Catharanthus roseus*, and Taxol (paclitaxel) from the bark of the pacific or American yew tree, *Taxus brevifolia*. Several studies have identified medicinal plant extracts which not only displayed antioxidant activity, but also cytotoxicity to many forms of cancer, including leukemias. Such compounds include curcumin (Sandur et al., 2007), guava extract, and basil leaf extract (Manosroi et al., 2006). In this study, we examined extracts of kaffir lime leaves for cytotoxic effects on leukemic cells. As the leaves of this plant have been used as a spice in many Thai dishes, safety of kaffir lime leaves is highly acceptable. Kaffir lime (*Citrus hystrix*, DC.) is a member of citrus family, and is also known as kieffer lime, Thai bergamot, limau purut or ma-grood. The leaves of kaffir lime trees are a dark green color with a glossy sheen, and are composed of two leaflets. The top leaflet is lightly

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pointed at its tip and is attached to another leaflet beneath that is broader on its upper edge. Kaffir lime leaves are an important ingredient in many Thai dishes, from soups and salads to curries and stir-fries. Kaffir lime originated in Indonesia, Thailand, and Malaysia, and is commonly used in Asian cuisine and folk medicine. Kaffir lime leaves have various medical and culinary uses in South East Asia. Fresh peels and dried fruits are used to relieve nausea, dispel gas, and control normal menstruation.

The fruits are used for cough suppression, and as a shampoo. Kaffir lime leaves and fruit extracts have antioxidant activity (Siripongvutikorna et al., 2005), free radical scavenging ability (Hutadilok-Towatana et al., 2006), antimicrobial activity (Siripongvutikorna et al., 2005), and anti-inflammatory activity (Lertsatitthanakorn et al., 2006). In regard to cancer research, kaffir lime extracts have been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines (Manosroi et al., 2006). Glyceroglycolipids in kaffir lime leaves could inhibit 12-O-tetradecanoylphorbol 13-acetate (TPA) and skin carcinogen activities in mice (Murakami et al., 1995). However, little is known concerning the effect of kaffir lime leaf fractional extracts on human leukemic cells. The objective of this study was to investigate the effects of crude kaffir lime leaf fractional extracts with various solvents (ethanol, hexane, ethyl acetate, butanol, and methanol) on leukemic cell lines using the MTT assay.

MATERIALS AND METHODS

Kaffir lime leaves

Kaffir lime (*C. hystrix* DC.) was purchased from local grocery stores in Chiang Mai, Thailand.

Reagents and chemicals

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide (MTT), and Histopaque®-1077 were purchased from Sigma-Aldrich (St Louis, MO, USA). RPMI 1640 and penicillin-streptomycin were purchased from GIBCO™ Invitrogen (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from Biochrom AG (Berlin, Germany). Ethanol was purchased from Fluka Chemicals (Buchs, Switzerland). All other chemicals were of the highest grade available.

Cells and cell culture conditions

Four types of leukemic cell lines, including HL60 (promyelocytic leukemia), K562 (chronic myelocytic leukemia), Molt4 (lymphoblastic leukemia), and U937 (monocytic leukemia), were cultured in RPMI-1640 medium (GIBCO-BRL) containing 10% fetal calf serum, 1 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. This cell line was maintained in a humidified

incubator with an atmosphere of 95% air and 5% CO₂ at 37°C. When the cells reached confluency, they were harvested and plated for consequent passages or for kaffir lime leaf fractional extract treatments.

Kaffir lime leaf extracts

Two kilograms of fresh kaffir lime leaves were completely dried at 50°C and ground to a powder (~ 980 g). A half portion of dried kaffir lime leaf powder (476 g) was macerated in 95% ethanol for 24 h, and the macerate collected; this was repeated 3 times. The filtrates were combined and evaporated to dryness using a rotary evaporator and subsequently freeze-dried. Another half portion was subjected to sequential maceration with different solvents, starting with hexane for 24 h; the macerate was then collected and this was repeated 3 times. Each day, the filtrate was collected and evaporated to dryness using a rotary evaporator to obtain a crude hexane fraction extract (fraction 1).

The remaining amount of dried kaffir lime leaf powder was placed under the hood to evaporate the hexane. The dried residue was further macerated with ethyl acetate. The filtrate collection and evaporation were performed in the same manner, yielding a crude ethyl acetate fraction extract (fraction 2). The residues from fraction 2 were further extracted with n-butanol and methanol to obtain a crude butanol fraction extract (fraction 3) and crude methanol fraction extract (fraction 4). Crude kaffir lime leaf fractional extracts were kept at -20°C until use. All extracts were dissolved in dimethyl sulfoxide (DMSO) at the concentration of 25 mg/ml to use as stock solutions.

MTT assay

Cell viability was determined by the MTT test method. MTT (5 mg/ml) was dissolved in PBS. Cells were cultured in 96-well plates (1.0 × 10⁴ cells/ well) containing 100 µl medium prior to treatment with crude kaffir lime leaf fractional extracts at 37°C for 24 h. After that, 100 µl fresh medium containing various concentrations (3.1, 6.25, 12.5, 25, 50 and 100 µg/ml) of fractional extracts were added to each well, and incubated for another 48 h. Diluted crude kaffir lime leaf fractional extract solutions were freshly prepared in DMSO prior to each experiment. The final concentration of DMSO in culture medium was 0.4% (v/v). The metabolic activity of each well was determined by the 3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay and compared to those of untreated cells.

After removal of 100 µl medium, MTT dye solution was added (15 µl/ 100 µl medium) and the plates were incubated at 37°C for 4 h in a humidified 5% CO₂ atmosphere. After that, 100 µl of DMSO were added to each well, and mixed thoroughly to dissolve the dye crystals. The absorbance was measured using an ELISA plate reader (Biotek EL 311) at 570 nm with a reference wavelength of 630 nm. High optical density readings corresponded to a high intensity of dye color that is to a high number of viable cells able to metabolize MTT salts. The fractional absorbance was calculated by the following formula:

$$\% \text{ Cell survival} = (\text{Mean absorbance in test wells} / \text{Mean absorbance in control wells}) \times 100$$

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. The 50% inhibition concentration (IC₅₀) of the active substances was

determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture or vehicle control culture (0.4% DMSO in culture medium). The IC_{50} s were compared for their activities. An IC_{50} less than 20 $\mu\text{g/ml}$ of crude extract was considered as an active compound against cancer cells, following the standard National Cancer Institute (NCI) criteria (Chen et al., 1988; Geran et al., 1972)

Preparation of peripheral blood mononuclear cells (PBMCs)

Blood samples from healthy volunteers were collected by venipuncture and transferred into 15 ml heparin coated test tubes. The samples were diluted at 1:1 ratio with PBS, layered onto Histopaque[®]-1077 at a volume ratio of 3:1 and centrifuged at 1,000 x g for 30 min. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. The PBMCs layer was removed and then washed twice with PBS. The supernatant was then removed and the cells were resuspended in RPMI 1640 medium supplemented with 1 mM L-glutamine, 100 units/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin, 10% inactivated FBS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. Cell viability was determined by the trypan-blue dye exclusion method. The PBMC cell density used in the cytotoxicity study was 1×10^5 cells/well of the 96-well tissue culture plate. Dose-response curves between percentage of cell viability and concentrations of the extracts were constructed. The IC_{50} value was determined from the plotted curve.

Statistical analysis

The results were analyzed by using one-way ANOVA, using SPSS version 10.0.

RESULTS AND DISCUSSION

Yield of crude kaffir lime leaf fractional extracts by organic solvents

Dried kaffir lime leaves (980 g) were obtained from two kilograms of the fresh leaves. One half portion was macerated with 95% ethanol, resulting in a brown sticky extract with 10.36 % yield. Fractionation of another half dried portion was performed with hexane, ethyl acetate, butanol, and methanol, with relative polarities of 0.009, 0.228, 0.602 and 0.762, respectively, compared to water (1.000). The percent yields of crude fractions with increasing polarity were 3.51, 1.12, 2.78 and 10.31%, respectively. In comparison, the percentage yield of extractable components using ethanol from kaffir lime leaves was 2.56% in one study (Hiran et al., 2009). Variation in the percent yield of extracts might be due to different plant materials having different chemical composition, or the nature of the soil and agro-climatic conditions. Other factors could include the effectiveness of the extracting solvent to dissolve endogenous compounds.

Cytotoxicity of crude kaffir lime leaf fractional extracts on leukemic cell lines

Four human-origin cancerous cell lines (K562, U937, Molt4, and HL60) and a normal human cell type (PBMC) were used for the cytotoxicity test. The cancerous cell lines possess differences in their origin, morphology, and genomes, resulting in susceptibility differences to the chemotherapeutic agents. In 1971, the hematopoietic cell line Molt4 was established from the peripheral blood of a patient in relapse from ALL by Minowada et al. (1972). These cells lacked surface and cytoplasmic immunoglobulins. This cell line contains several copies of the Epstein Bar virus (EBV) genome. The most distinctive characteristic of the Molt4 cells was their rosette-forming ability with sheep, goat, horse, and pig erythrocytes. Furthermore, K562, the first myeloid-erythroid cell line, was established from a Ph' chromosome positive CML blast crisis patient (Lozzio and Lozzio, 1975, 1979; Andersson et al., 1979). More than fifty leukemic cell lines have been generated in order to make cells available for leukemia studies, including U937 and HL60 cells. U937, a monocytic cell line, was established from histiocytic lymphoma. HL60, a promyelocytic cell line, was established from AML-M3, M2 (Epstein et al., 1976; Schneider et al., 1977; Gillis and Watson, 1980; Collins et al., 1977). These selected cell lines are representative of four human leukemias, M3 (promyelocytic leukemia; HL60), M4 (monocytic leukemia; U937), erythrocytic leukemic cells derived from chronic myelogenous leukemia (CML; K562), and acute lymphoblastic leukemia (ALL; Molt4).

After cells were treated with crude kaffir lime leaf fractional extracts at various concentrations for 48 h, the cytotoxic effects were investigated using the MTT assay. Cytotoxicity of each crude fractional extracts was determined by an inhibitory concentration at 50% growth (IC_{50}). The IC_{50} values of ethanol, hexane, ethyl acetate, butanol, and methanol fractions on HL60 cell lines were 55.6 ± 3.6 , 21.6 ± 2.3 , 19.0 ± 0.6 , 83.4 ± 10.5 , and > 100 $\mu\text{g/ml}$, respectively (Figure 1). The IC_{50} values of fractions on K562 cells were 91.3 ± 8.4 , 40.1 ± 2.8 , 35.3 ± 1.4 , 97.0 ± 5.3 , and > 100 $\mu\text{g/ml}$, respectively (Figure 2). The IC_{50} values of fractions on Molt4 cells were 91.7 ± 7.6 , 34.9 ± 5.1 , 21.8 ± 0.4 , 94.9 ± 4.5 , and > 100 $\mu\text{g/ml}$, respectively (Figure 3). The IC_{50} values of fractions on U937 cells were 85.8 ± 6.3 , 53.0 ± 9.4 , 19.8 ± 1.0 , 95.8 ± 7.2 , and >100 $\mu\text{g/ml}$, respectively (Figure 4). The IC_{50} values of five crude kaffir lime leaf fractional extracts on four leukemic cell lines are shown in Table 1. The crude ethanol extract contained multiple compounds with a wide range polarity from the kaffir lime leaves. Its cytotoxic activity was lower than those of ethyl acetate and hexane fractions. Thus, the fractional extraction potentiated the efficiency of the kaffir lime leaf extract

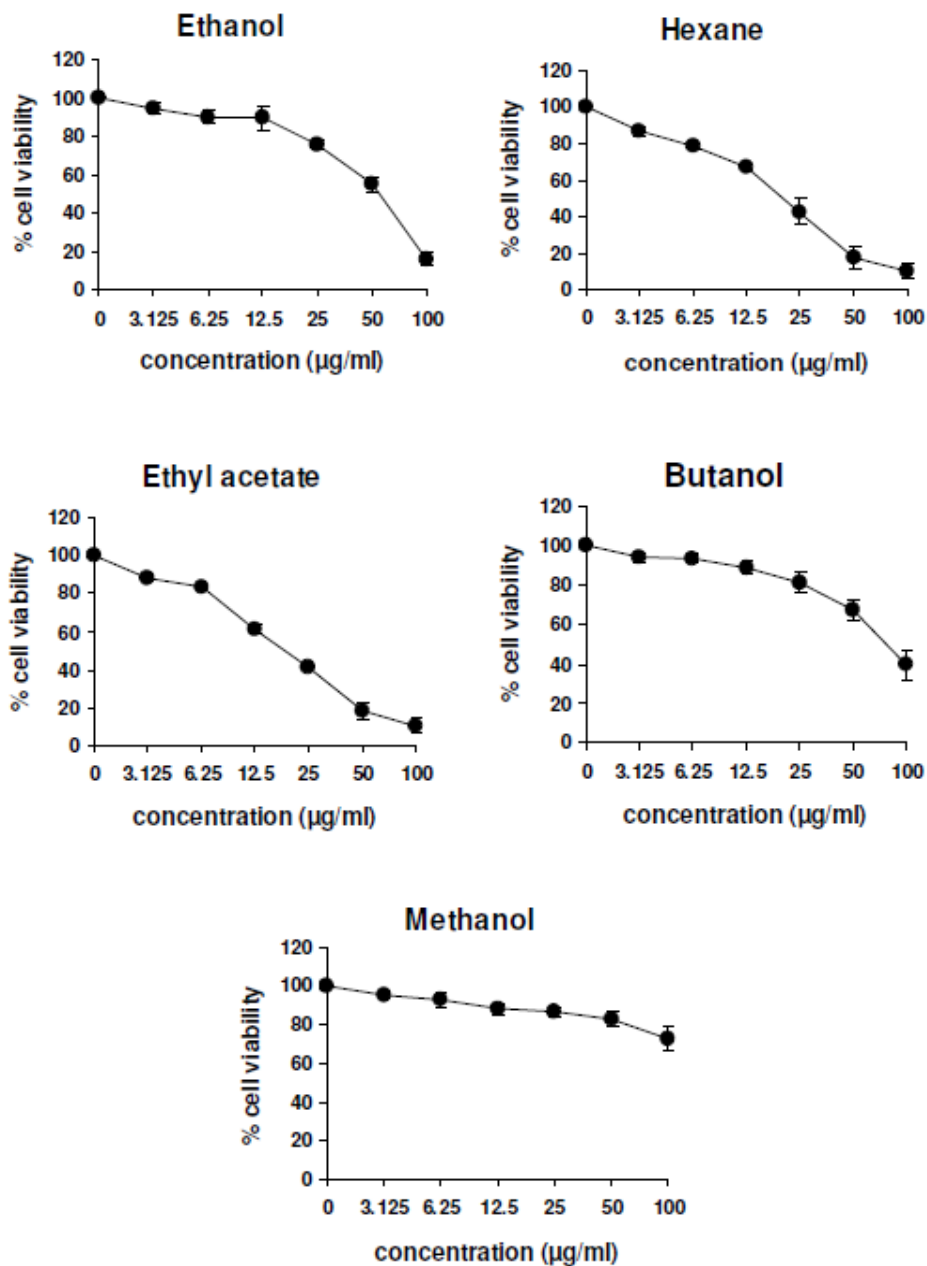


Figure 1. Effect of kaffir lime leaf extracts on HL60 cell line.

compared to a single extraction with 95% ethanol. The statistical analysis supported that those IC_{50} values of both ethyl acetate and hexane fractions were significantly different compared to ethanol extract ($p < 0.05$). The ethyl acetate fraction was considered as the most effective fraction as compared to other fractions while hexane fraction was the minor effective fraction (Table 1). The fractional extracts with the cytotoxicity ($IC_{50} \leq 20$

$\mu\text{g/ml}$) against the leukemic cell lines were fractions of ethyl acetate and hexane in HL60 and U937 cells. Ampasavate et al. (2010) found that ethanolic extract had a cytotoxicity of 9 $\mu\text{g/ml}$ for U937; 11.9 $\mu\text{g/ml}$ for Molt4; 17.1 $\mu\text{g/ml}$ for HL60. The IC_{50} values for the ethanolic fraction in the same cell type were different due to the individual differences of kaffir lime plant, location, and harvesting time. Manosroi et al. (2006) prepared the

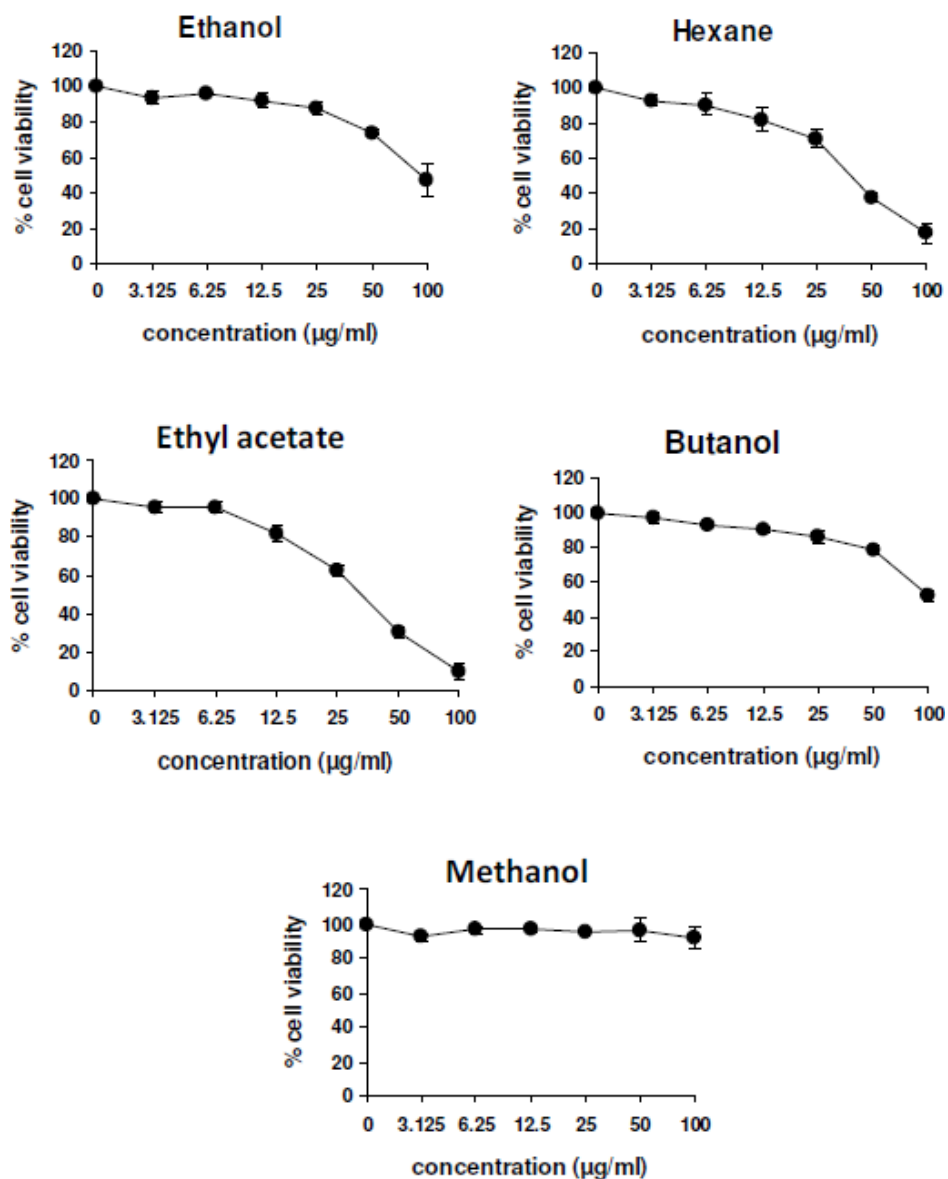


Figure 2. Effect of kaffir lime leaf extracts on K562 cell line.

essential oil from kaffir lime leaves by steam distillation method. The essential oil displayed cytotoxic activity against the human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines. The IC_{50} values for KB and P388 were 1,147.9 and 397.7 µg/ml, respectively, whereas much lower IC_{50} values were observed for the ethyl acetate fractional extracts in our study (19.0 ± 0.6 µg/ml for HL60, 19.8 ± 1.0 µg/ml for U937, 21.8 ± 0.4 µg/ml for Molt4, and 35.3 ± 1.4 µg/ml for K562). The IC_{50} value of the essential oil as compared to our ethyl acetate fraction of kaffir lime leaves was different approximately 10 to 20-fold higher in murine leukemia P388 (397.7

µg/ml) as compared to our human leukemic cell lines (19.0 to 35.3 µg/ml). The reasons of this difference might be from the different compounds and type of cell lines. In K562 cells, ethyl acetate and hexane were the effective fractions. However, their IC_{50} values were higher than those in HL60, Molt4, and U937 cells using the same fractions. This may be due to different in the leukemia phenotype. Lime oil possesses compounds that can induce apoptosis-mediated cells death in human colon adenocarcinoma cells (Patil et al., 2009). Moreover, kaffir lime leaf extract has been shown to exert strong anti-promoting activity in a test of promoter-induced Epstein –

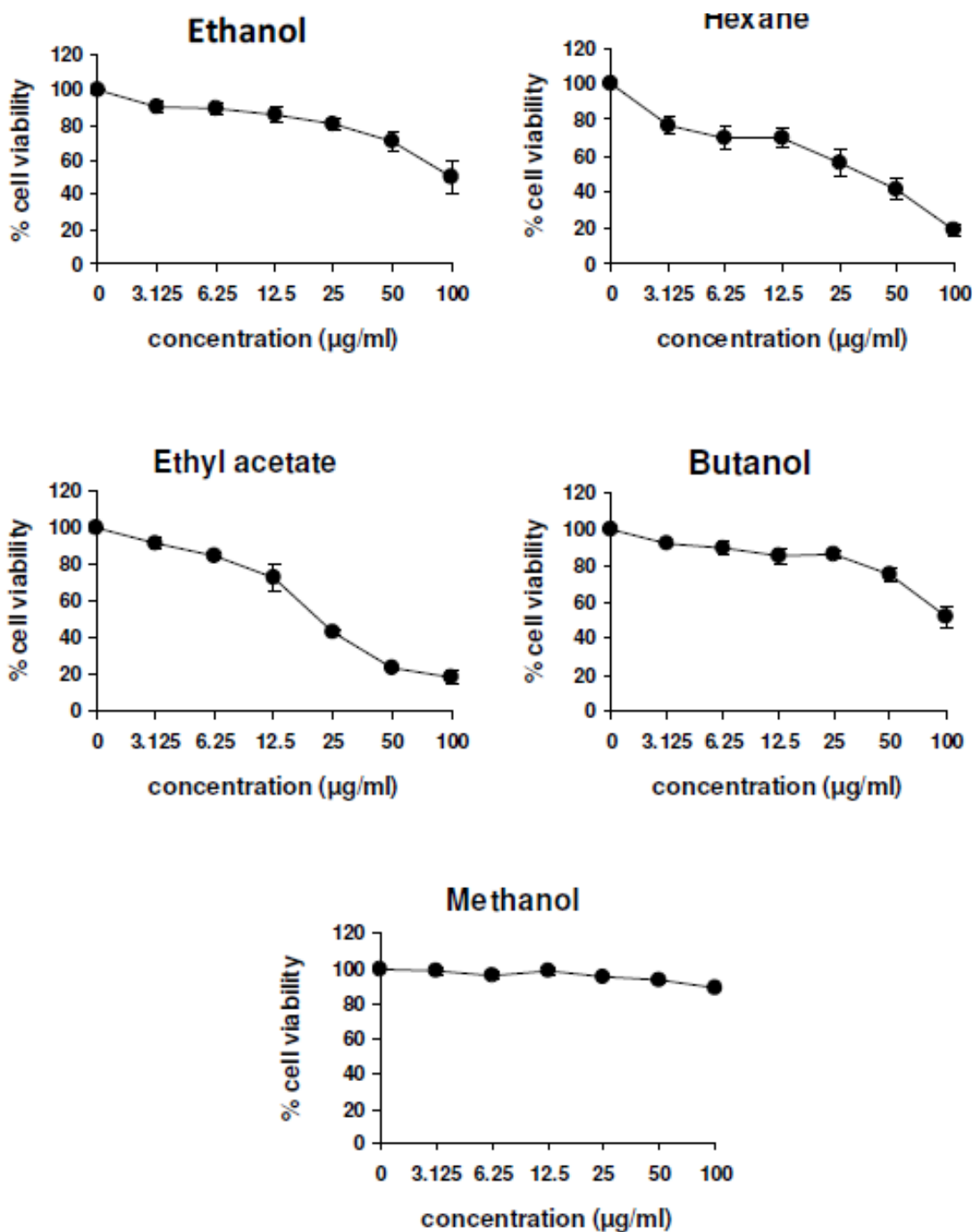


Figure 3. Effect of kaffir lime leaf extracts on Molt4 cell line.

Barr virus (EBV) activation (Tiwawech et al., 2000). PBMCs are frequently used as the model for cytotoxicity testing in normal cells. Many studies have utilized PBMCs to assess the effects of chemicals or extracts on the proliferation of normal cells (Anazetti et al., 2003; Liu

et al., 2004). In this study, the cytotoxic activity of the fractional extracts against PBMCs was observed in more than 80 µg/ml in ethyl acetate and hexane fractions with IC₅₀ values of 86.4 ± 12.8 and 81.6 ± 16.0 µg/ml, respectively but the fractional extracts from butanol,

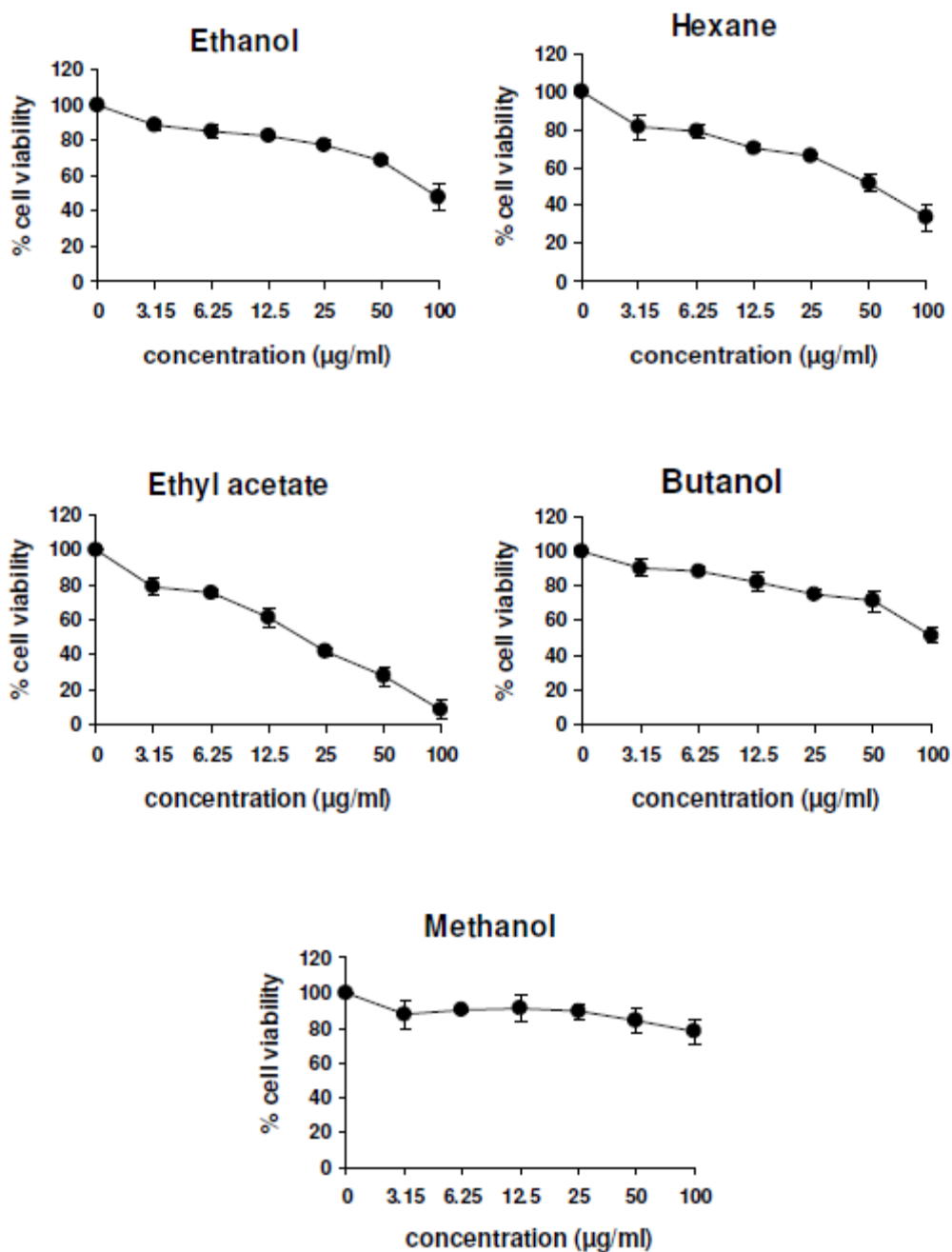


Figure 4. Effect of kaffir lime leaf extracts on U937 cell line.

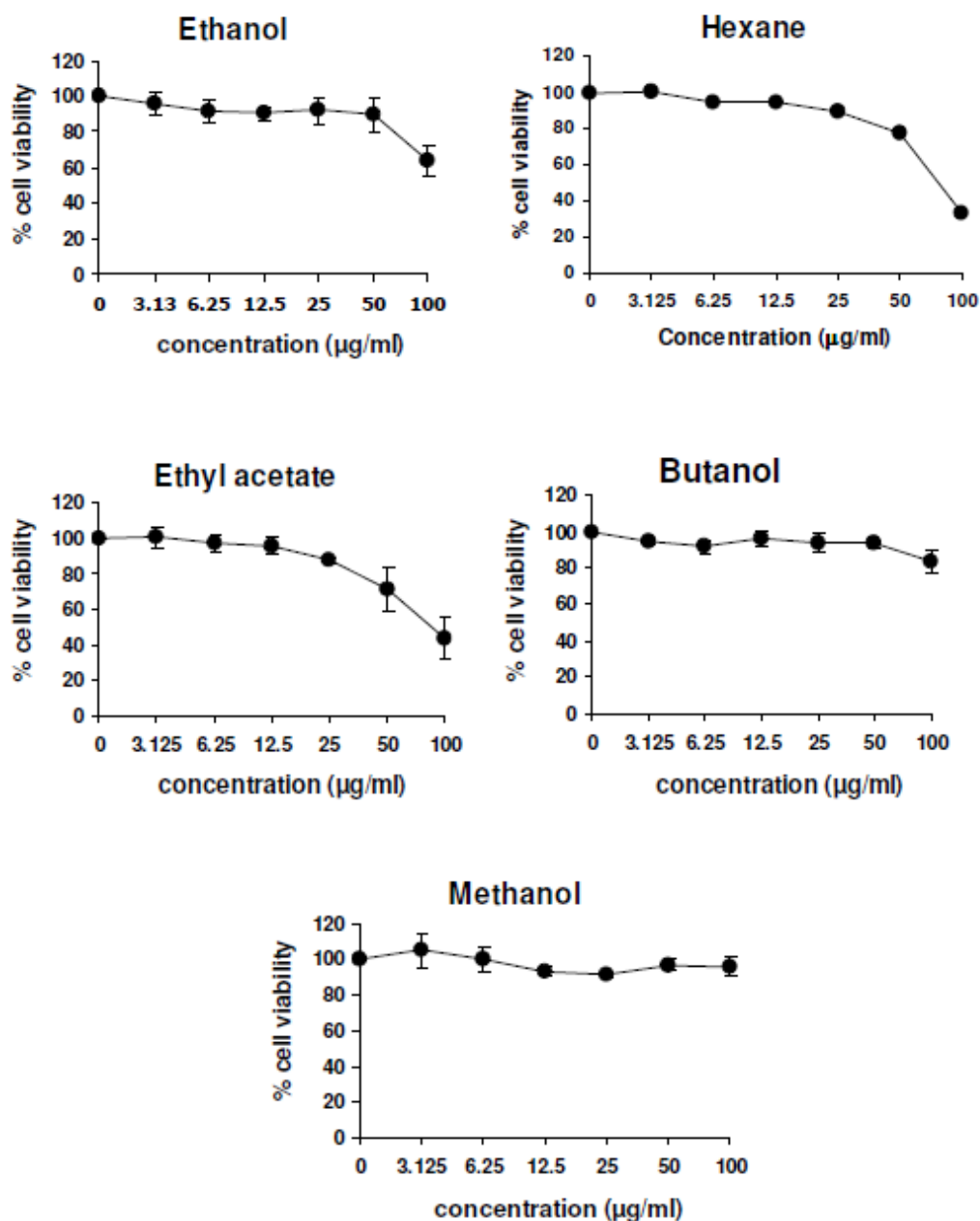
ethanol, and methanol had no cytotoxic activity (IC_{50} value $> 100 \mu\text{g/ml}$) (Figure 5).

Most of the fractional extracts of butanol and methanol had no cytotoxic activity (IC_{50} values $\geq 100 \mu\text{g/ml}$) against the studied cell types. Presumably, the polarities of active compounds are nonpolar to semipolar. As compared to the vincristine, a chemotherapy drug used as a treatment

for some cancer types, there were cytotoxic effects on K562, U937, Molt4, and HL60 cell lines with the IC_{50} values 8, 0.09, 0.63, and 0.39 ng/ml, respectively (Ampasavate et al., 2010). However, the kaffir lime leaf fractional extract used was still a crude fractional extract, and thus should be further identified and examined for its active compounds.

Table 1. IC₅₀ values (µg/ml) of kaffir lime leaf extracts on four leukemic cell lines and PBMCs.

Fraction	IC ₅₀ (µg/ml)				
	HL60	K562	Molt4	U937	PBMCs
Buthanol	83.4 ± 10.5	97.0 ± 5.3	94.9 ± 4.5	95.8 ± 7.2	>100
Ethanol	55.6 ± 3.6	91.3 ± 8.4	91.7 ± 7.6	55.6 ± 3.6	>100
Ethyl acetate	19.0 ± 0.6 [#]	35.3 ± 1.4 [*]	21.8 ± 0.4 [*]	19.8 ± 1.0 [*]	86.4 ± 12.8
Hexane	21.6 ± 2.3 [*]	40.1 ± 2.8 [#]	34.9 ± 5.1 [#]	21.6 ± 2.3 [#]	81.6 ± 16.0
Methanol	> 100	>100	>100	>100	>100

**Figure 5.** Effect of kaffir lime leaf extracts on peripheral blood mononuclear cells.

Future work should focus on the target inhibitory protein in order to gain insight into the mechanism of the inhibitory effect on the proliferation of the leukemia cells.

The noncytotoxic doses in terms of IC₂₀ can be used for gene expression determination, especially Wilms' tumor 1 (WT1) mRNA and protein levels. The *WT1* gene is one promising biological marker for measuring cell proliferation (Anuchapreeda et al., 2006). In addition, further purification to eliminate toxic agent (s) against normal cells is necessary. Testing purified compounds will help to determine the specific cytotoxic chemicals responsible for the observed inhibitory effects on leukemia cell lines. In conclusion, kaffir lime leaf is a promising natural alternative to chemotherapy for leukemia. The active compound in kaffir lime leaves should now be identified.

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