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Full Length Research Paper

Cytotoxicity of extracts from fruit plants against leukemic cell lines

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This study examined the antileukemic activity of non edible parts of 13 common Thai tropical fruits. Their ethanolic extracts were tested for cytotoxic effects on U937, K562, HL60, Molt 4 and normal human peripheral blood mononuclear cells (PBMCs). Three of 20 crude plant extracts (kaffir lime leaves, mangosteen peels, and wampee leaves) had strong cytotoxic effects on K562, U937, and Molt4 cells. The IC_{50} values of kaffir lime leaves on those cells were 26.1, 9.0 and 11.9 µg/ml respectively, whereas those of mangosteen peel were 23.6, 4.5 and 10.1 µg/ml, and those of wampee leaves were 71.9, 13 and 70.4 µg/ml. Furthermore, pomegranate peel extract had a potent cytotoxic effect on HL60 cells (IC_{50} of 8.0 µg/ml), but was non-toxic to normal PBMCs, indicating that as a potential source of antileukemic agents.

Key words: Fruit plants, cytotoxicity, leukemic cell line.

INTRODUCTION

Leukemia was first recognized by the German pathologist Rudolf Virchow and John Bennett in 1845 (Virchow, 1845; Craigie, 1845). Leukemia refers to a group of acute or chronic malignant diseases that involve the blood-forming organs, characterized by an abnormal proliferation, differentiation, and excessive production of white blood cells and their precursors in the bone marrow, with or without a corresponding increase of those in the circulating blood. This results in decreased production and function of normal blood cells. Leukemia can spread to the lymph nodes, spleen, liver, central nervous system, and other organs. To date, the rapid growth of advanced technologies and new discoveries in the diagnosis and treatment of leukemia have made surviving from leukemia more likely than in the past. Chemotherapy is one of the methods used most frequently in the treatment of leukemia. It works by restraining leukemic cells from growing or multiplying and then causing their disruption. Many varieties of fruits and vegetables have been evaluated for their protective activity against cancer. Significant alteration of the incidence of collrectal cancer has been reported as resulting from the comsumption of a diet high in fruits and vegetables. The antioxidative activity of major substances in this diet group has generated interest in fruit and vegetable species as potential sources of chemotherapeutic compounds (McCann et al., 2007). Recently, we investigated antioxidative and cytotoxic activities against Caco-2 and PBMCs cells of several fruit peel extracts. Pomegranate peel had the highest antioxidative activity, followed by the extracts of rambutan, mangosteen, and banana peels. Interestingly, mangosteen peel extract exhibited cytotoxicities against Caco-2 and PBMCs cells. These results were of interest for investigaing the cytotoxicity of these plants against other cancer cells (Okonogi et al., 2007).

Natural antioxidant compounds such as curcumin possess the ability to induce cell death in two leukemic cell lines: K562 and Jurkat cells (Duvoix et al., 2003). Curcumin I and III exhibited *in vitro* cytotoxicity against human chronic myeloid leukemia (Nagabhushan and Bhide, 1992). Kuo et al. (1996) have shown that the dietary component pure curcumin induced apoptosis in human leukemia HL60 cells at a concentration of 3.5 µg/ml. In the course of searching for new chemical entities or remedies from ethnomedicine, the extracts derived from plants, especially fruits or related waste parts

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are of interest as sources of chemotherapeutic agents. Approximately 25% of the drugs prescribed worldwide come from plants, 121 such active compounds being in current use. About 60% of anti-tumor and anti-infectious drugs launched on the market or under clinical trial are of natural origin (Shup, 1998).

In Thailand, tropical fruits are widely grown for domestic consumption and for export. However, their waste products, such as leaves, peels or seeds, are often discarded as garbage. Several types of fruit were selected for this study, based on high levels of consumption in Thailand, ethnomedical use, and their various pharmacological properties. Screening for antileukemic agents from the edible and non edible portions of several fruit plants was conducted on four representative leukemic cell lines (K562, U937, Molt4, and HL60 as well as the normal peripheral blood mononuclear cells (PBMCs)).

MATERIALS AND METHODS

Fruit plants

Thirteen species of fruits, grown in Thailand and either are commonly consumed or possess phytopharmaceutical properties were purchased from local grocery stores in Chiang Mai, Thailand. The species examined were: star fruit (*Averrhoa carambola* L.), wampee leaves (*Clausena lansium* (Lour.) Skeels), kaffir lime (*Citrus hystrix* DC.), coconut (*Cocos nucifera* L.), longan (*Euphoria longan* Lam. or *Dimocarpus longan* Lour.), banana (*Musa sapientum* L.), mangosteen (*Garcinia mangostana* L.), dragon fruit (*Hylocereus undatus*), long-gong (*Lansium domesticum* Correa), rambutan (*Nephelium lappaceum* L.), passion fruit (*Passiflora foetida* L.), Guava (*Psidium guajava* L.) and pomegranate (*Punica granatum* L.).

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 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.

Ethanolic extracts

The dried and ground parts of each fruit were extracted with 95% ethanol for three days at room temperature, filtered and concentrated using a rotary evaporator (Eyela N-100, Tokyo, Japan) at 45°C.

The extract was dissolved in dimethyl sulfoxide (DMSO) and diluted with complete RPMI 1640 medium to give final concentrations of total extract ranging from 3.13 - 100 µg/ml.

Cell culture and cytotoxicity

The erythroid leukemic cell line (K562), human promyeloid leukemia (HL60), human monocytic leukemia (U937), and human lymphoblastic cell line (Molt4) were cultured in RPMI 1640 medium supplemented with 1 mM L-glutamine, 100 Units/ml penicillin and 0.1 mg/ml streptomycin, 10% inactivated FCS, and adjusted to pH 7.2 by the addition of 15 mM HEPES. All cell lines were maintained in a humidified incubator with an atmosphere of 95% air and 5% CO_2 at 37°C.

Cell viability was determined by the MTT (3-(4,5 dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) test method. MTT (5 mg/ml) was dissolved in PBS. The solution was filtered through a 0.2 µm filter and stored at 2 - 8°C. Cells were cultured in 96-well plates (3.0 x 10^4 cells/ well) containing 100 µl medium, prior to the treatment with plant extract or vincristine (a chemotherapy drug used for some types of cancer) at 37°C for 24 hrs. After that, 100 µl fresh medium containing various concentrations of plant extracts or vincristine were added to each well, and incubated for another 48 hrs. Diluted plant extract or vincristine solutions were freshly prepared in DMSO prior to each experiment. The metabolic activity of each well was determined by the (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 hrs in a humidified 5% CO_2 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:

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

The average cell survival obtained from triplicate determinations at each concentration was plotted as a dose response curve. The 50% inhibition concentration (IC_{50}) of the active substances was determined as the lowest concentration which reduced cell growth by 50% in treated compared to untreated culture. The IC_{50}s were compared for their activities. An IC_{50} less than 20 µg/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 venepuncture and transferred into 15 ml heparin coated test tubes. It was diluted at 1:1 ratio with PBS, layered onto Histopaque®-1077 at a volume ratio of 3:1 and centrifuged at 1,000 xg 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 0.1 mg/ml streptomycin, 10% inactivated FCS, 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 x 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} was determined from the plotted curve.

Table 1. Percent yield of extracts from different parts of non edible fruit products.

No.	Common name	Scientific name	Family	Parts	% yield
1	Raw banana	*Musa sapientum* L.	Musaceae	Peels	7.66
2	Ripe banana	*Musa sapientum* L.	Musaceae	Peels	14.44
3	Coconut	*Cocos nucifera* L.	Palmae	Peels (inside)	5.80
4	Dragon fruit	*Hylocereus undatus* (Haw) Britt. & Rose	Cactaceae	Peels	1.40
5	Guava	*Psidium guajava* L.	Myrtaceae	Leaves	8.70
6	Guava	*Psidium guajava* L.	Myrtaceae	Stems	1.12
7	Raw guava	*Psidium guajava* L.	Myrtaceae	Raw Fruit	4.65
8	Kaffir lime	*Citrus hystrix* DC.	Rutaceae	Peels	17.52
9	Kaffir lime	*Citrus hystrix* DC.	Rutaceae	Leaves	7.42
10	Kaffir lime	*Citrus hystrix* DC.	Rutaceae	Stems	1.97
11	Longan	*Euphoria longan* Lam. *Dimocarpus longan* Lour.	Sapindaceae	Seeds	9.59
12	Long-gong	*Lansium domesticum* Correa	Meliaceae	Peels	5.71
13	Mangosteen	*Garcinia mangostana* L.	Guttiferae	Peels	7.21
14	Passion fruit	*Passiflora foetida* L.	Plasifloraceae	Peels	2.60
15	Raw Pomegranate	*Punica granatum* L.	Punicaceae	Peels	6.21
16	Pomegranate	*Punica granatum* L.	Punicaceae	Leaves	11.27
17	Pomegranate	*Punica granatum* L.	Punicaceae	Seeds	4.46
18	Rambutan	*Nephelium lappaceum* L.	Sapindaceae	Peels	10.68
19	Raw star fruit	*Averrhoa carambola* L.	Oxalidaceae	Raw Fruit	11.66
20	Wampee	*Clausena lansium* (Lour.) Skeels	Rutaceae	Leaves	8.66

Microscopic Analysis

Cell morphology of HL60 was examined after being treated with 8 and 50 µg/ml pomegranate extract at 37℃ for 48 hrs under the inverted microscope (Olympus, IX71, Japan).

RESULTS AND DISCUSSION

Yield of extracts

Table 1 shows the percent yield of extracts obtained from different parts of fruit waste products. The yields expressed are based on the dried weight of sample raw materials. Among these extracts, the highest (17.52%) and the lowest (1.12%) yields of extraction were observed from the kaffir lime peels and guava stems, respectively. Sultana et al. (2008) reported that the percent yield of dry matter banana and pomegranate peels after extracting with 80% methanol was 16.4 and 29.9%, respectively. Our result showed that the percent yield of banana peel extract is similar to the ripe banana peel extract (14.4%), whereas the pomegranate peel extract was different in percent yield (6.21%). In contrast, Akamine et al., 2009 found that the percent yield of fresh banana peel extract was 2.3%. In addition, the percent yield of extractable components using ethanol from Kaffir lime leaves as 2.56% (Hiran et al., 2009) and mangosteen peel 29.46% (Bullangpoti et al., 2004). Variation in the percent yield of extracts might be because of different plant materials themselves for example the chemical composition of plant, nature of the soil and agro-climatic condition. The other factors could be the effectiveness of the extracting solvent to dissolve endogenous compounds.

Cytotoxicity test

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; Lozzio and Lozzio, 1979; Andersson et al., 1979). More than fifty leukemic cell lines have been generated in order to make cell available for leukemia studies, including U937 and HL60 cells. U937, a monocytic cell line, was established

Table 2. IC_{50} values (µg/ml) of fruit extracts on four leukemic cell lines and PBMCs.

No.	Common name	Parts	IC_{50} (µg/ml)$^{\Delta}$ (Mean + SD)				
			PBMC	K562	U937	Molt4	HL60
1	Raw banana	Peels	>100	>100	>100	>100	>100
2	Ripe banana	Peels	>100	>100	>100	>100	>100
3	Coconut	Peels (inside)	>100	>100	>100	>100	77.3 ± 0.2
4	Dragon fruit	Peels	>100	>100	>100	>100	>100
5	Guava	Leaves	>100	>100	>100	>100	>100
6	Guava	Stems	>100	>100	>100	>100	>100
7	Raw guava	Fruit	>100	>100	>100	>100	31.8 ± 2.5
8	Kaffir lime	Peels	47.5 ± 0.9	>100	70	38.6 ± 0.9	>100
9	Kaffir lime	Leaves	12.5 ± 0.6**	26.1 ± 1.2	9 ± 0.5**	11.9 ± 0.6**	17.1 ± 0.4**
10	Kaffir lime	Stems	>50	>100	>100	70.4 ± 2.2	36.4 ± 0.8
11	Longan	Seeds	>100#	84 ± 0.7	>100	>100	44.3 ± 1.1
12	Long-gong	Peels	>100	>100	97 ± 0.2	>100	>100
13	Mangosteen	Peels	4.9 ± 0.2**	23.6 ± 0.7	4.5 ± 1.6**	10.1 ± 1.4**	77.3 ± 0.8
14	Passion fruit	Peels	>100	>100	>100	>100	>100
15	Raw pomegranate	Peels	>100#	>100	>100	>100	7.9 ± 1.9**
16	Pomegranate	Leaves	>100	>100	>100	>100	86.4 ± 1.7
17	Pomegranate	Seeds	>100	>100	>100	>100	43.2 ± 1.6
18	Rambutan	Peels	>100	>100	>100	>100	>100
19	Raw star fruit	Fruit	>100	>100	>100	>100	>100
20	Wampee	Leaves	25 ± 1.8	71.9 ± 1.6	13 ± 2.1**	70.5 ± 1.9	59.1 ± 1.5

$^{\Delta}$ Asterisks denote mean of three independent experiment (n = 3).
** Asterisks denote IC_{50} less than 20 µg/ml which are considered as an active compound against cancer cells.
# Asterisks denote effective dose for cell stimulation (ED_{50}): ED_{50} of longan seed extract = 50 ± 1.2 µg/ml, ED_{50} of raw pomegranate peel extract = 44.4 ± 0.4 µg/ml.

from histiocytic lymphoma. HL60, a promyelocytic cell line, was established from AML-M3, M2 (Epatein 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 lymphoblasttic leukemia (ALL; Molt4).

The cytotoxicity and selectivity of the fruit extracts against the selected cancerous cell lines are summarized in Table 2. According to the standard National Cancer Institute criteria (Chen et al., 1988; Geran et al., 1972), crude extracts possessing an IC_{50} less than 20 µg/ml are considered active against the tested cancer cells. The extracts with the IC_{50} less than 20 µg/ml against the cancer cell lines were kaffir lime leaves (9 µg/ml for U937; 11.9 µg/ml for Molt4; 17.1 µg/ml for HL60), mangosteen peels (4.5 µg/ml for U937; 10.1 µg/ml for Molt4), wampee leaves (13 µg/ml for U937) and raw pomegranate peels (8 µg/ml for HL60) (Table 2 and Figure 1). PBMC cells are frequently used as the model for the cytotoxicity test 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 highly cytotoxic activity of the extracts against PBMCs was a generally observed range (4.9 - 25 µg/ml), except the extract from raw pomegranate peels, which exhibited cell stimulatory activity (Table 2 and Figure 2). In addition, pomegranate peel extract had no inhibitory effect on cell proliferation in K562, U937, and Molt4 cell lines. In this regard, the cytotoxicity of the active extracts was highly selective against the HL60 leukemic cell type (Table 2 and Figure 1). Various parts of *Punica granatum* (pomegranate), especially the fruits, have been reported for their pharmacological activities. There have been a long ethnomedical history for the pomegranate fruits, and thus have been of interest to the pharmacological researchers. Juice and peels of pomegranate fruits possess potent antioxidant, anticancer, and antiinflammatory activities (Okonogi et al., 2007; Lansky and Newman, 2007). The stimulatory activity of pomegranate extract on PBMC proliferation indicates selectivity of the extract against specific leukemic cells (HL60) and nontoxicity to normal cells. In order to improve the immune response, such stimulation of PBMC proliferation might be beneficial for patients with immunodeficiency. Longan seeds and pomegranate peels appear to be the best candidates for stimulating PBMCs (Figure 2). Most of the extracts

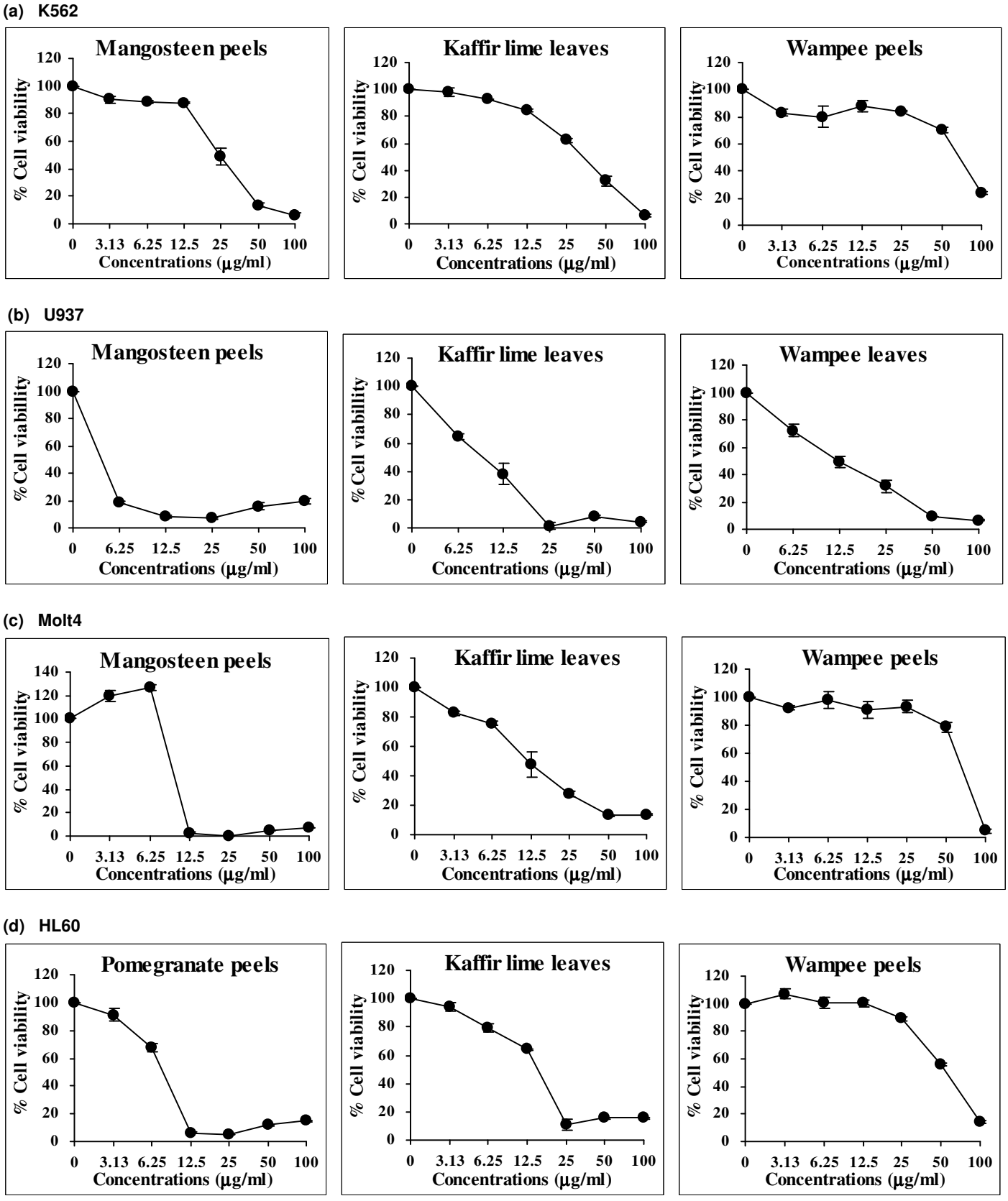

Figure 1. Effect of the active extracts on leukemic cells; (a) K562, (b) U937, (c) Molt4, and (d) HL60.

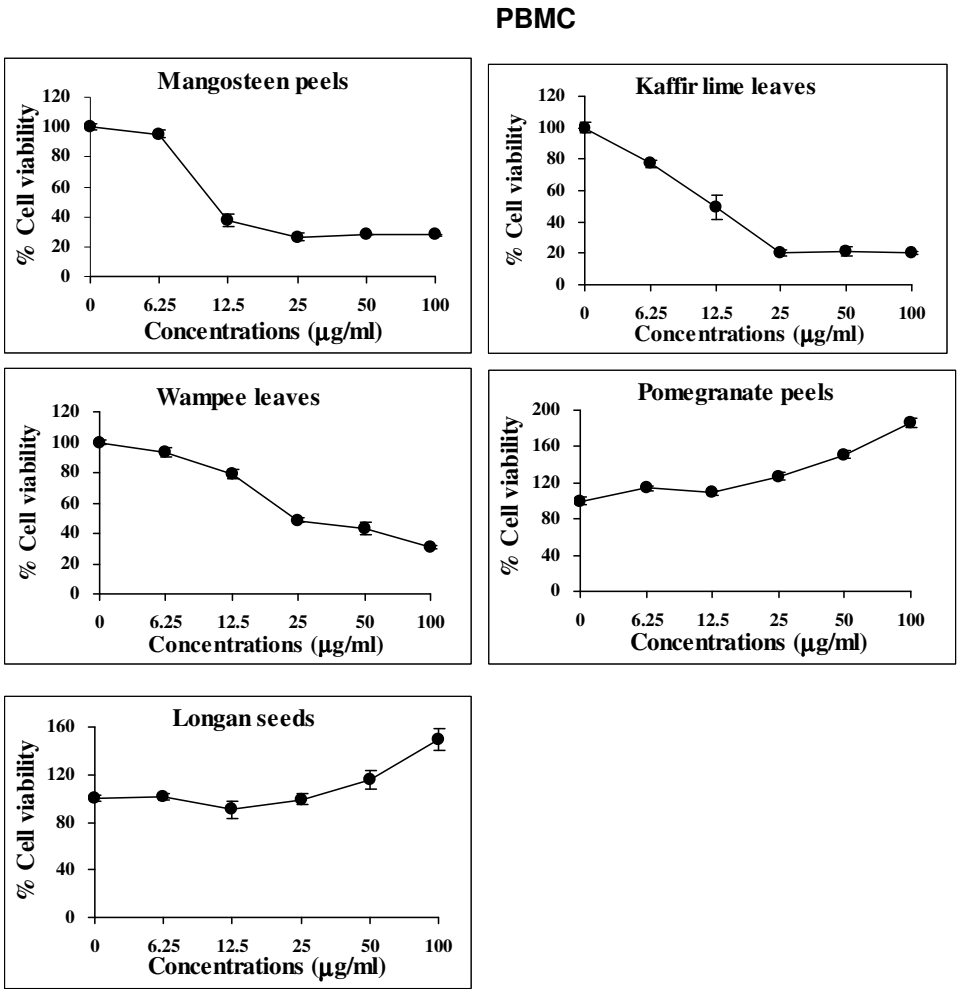

Figure 2. Effect of the active extracts on normal PBMC cells.

had no cytotoxic activity (IC_{50} values higher than 100 µg/ml) against the studied cell types. The extract from kaffir lime leaves, mangosteen peels, and wampee leaves contained potential cytotoxic agent (s), as observed from both cancerous and normal cells. Matsumoto et al. (2004) had reported antiproliferative activity of α-mangostin, an active compound from mangosteen pericarp, on several human leukemic cell lines. The compound demonstrated induction of apoptosis through mitochondria as the target site. However, the cytotoxicity on normal cells was not measured in that study. Essential oil from kaffir lime leaves, an essential spice in Thai food, displayed cytotoxic activity against KB and P388 cell lines (Manosroi et al., 2006). The IC_{50} values for KB and P388 were 99.7 µg/ml and 74.6 µg/ml respectively, whereas lower IC_{50} values were observed for the extracts in our study (9 µg/ml for U937 and 11.93 µg/ml for Molt4). Lime oil possessed components those can induce apoptosis-mediated cells death in human colon adenocarcinoma cells (Patil et al., 2009). Moreover, kaffir lime leave extract was known to exert strong anti-promoting activity in a test of promoter-induced Epstein - Barr virus (EBV) activation (Tiwawech et al., 2000). No antiproliferative activity on cancer cells has been reported for wampee leaves. However, the anticancer activity of wampee peel extract had been reported that 50 µg/ml of ethyl acetate fraction exhibited strong anticancer activities against SGC-7901, HepG-2 and A-549 cell lines with the corresponding inhibitory activities of 78.5, 72.4 and 69.2%, respectively (Prasad et al., 2009).

Vincristine, a chemotherapy drug used as a treatment for some cancer types, had 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. However, PBMCs were not affected by vincristine (IC_{50} value higher than 100 µg/ml) (Table 3 and Figure 3). Most of the fruit extracts which exhibited cell inhibition to leukemic cell lines, also expressed cytotoxicity to the PBMCs. Interestingly, the extracts from pomegranate, raw guava and coconut peels inhibited the proliferation of HL60, but no

Table 3. IC_{50} values (ng/ml) of Vincristine on PBMC, K562, U937, Molt4, and HL60.

Chemotherapeutic agent	IC_{50} (ng/ml)*				
	PBMC	K562	U937	Molt4	HL60
Vincristine	>100	8	0.09	0.63	0.39

* Asterisks denote mean of three independent experiment (n = 3).

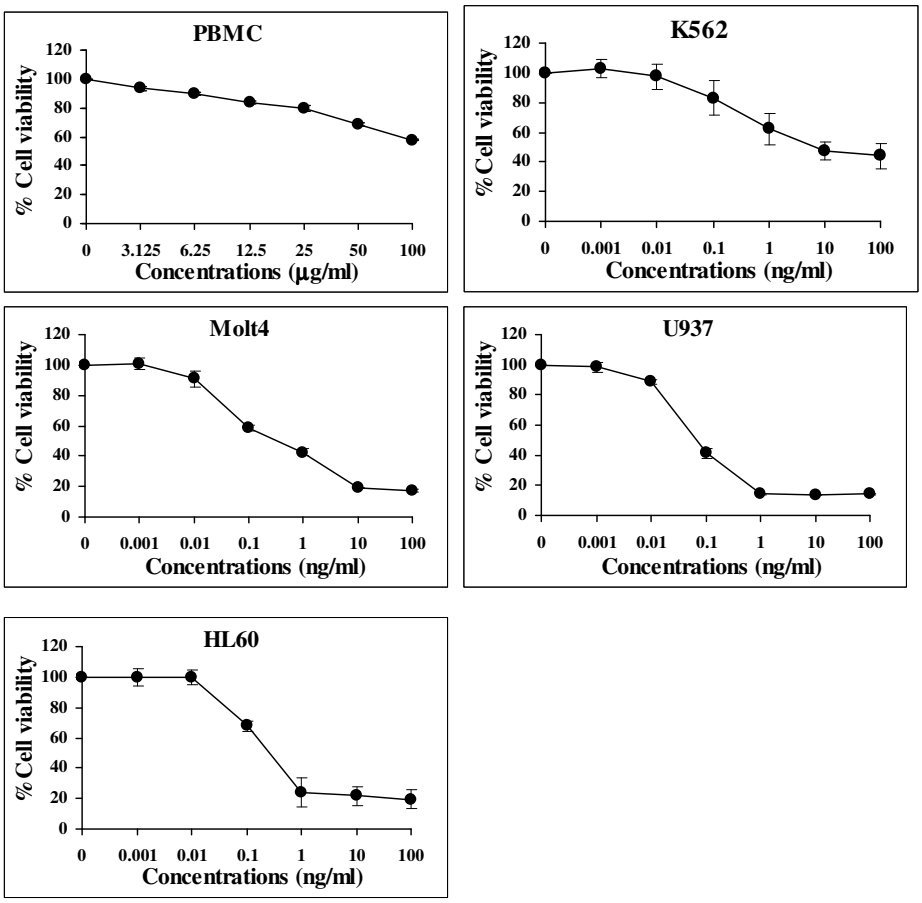

Figure 3. Effect of vincristine on normal PBMC, K562, U937, Molt4, and HL60.

toxicity to the PBMCs.

Regarding the mechanism of cytotoxicity of pomegranate extract in HL60 cells, microscopic analysis showed the blebbing pattern of cell apoptosis at concentrations of 8.0 µg/ml (IC_{50}) and 50 µg/ml as compared to the vehicle control (Figure 4). This suggested that cytotoxicity of pomegranate might involve the apoptosis pathway. Future work will be designed to identify the possible signalling cascade.

The cytotoxicity test is required for future work regarding the target inhibitory protein in order to gain insight into the mechanism of inhibitory effect on the proliferation of the leukemia cells. The noncytotoxic doses in terms of IC_{20} can be used for gene expression determination, especially Wilms' tumor 1 (WT1) mRNA and protein level. 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.

Conclusion

We tested the cytotoxicity to leukemic cells for various fruit extracts. Pomegranate peel extract strongly inhibited HL60 cells, and was non-toxic to normal cells. Interes-

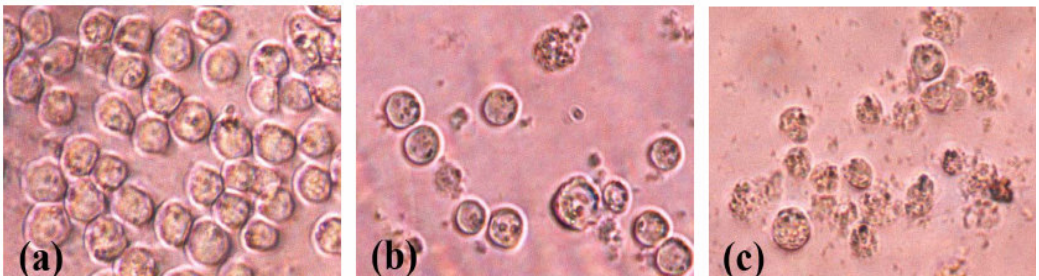

Figure 4. HL60 cell morphology after pomegranate extracts treatment for 2 days. Cell morphology of HL60 was examined after treatment with (a) vehicle control, (b) 8 µg/ml, and (c) 50 µg/ml pomegranate extract.

tingly, we observed a stimulating activity of this extract on PBMC proliferation, indicating the selectivity of the extract against only specific leukemic cells (HL60). The peel of this plant is a promising source of antioxidant or anticancer agents, since the fruits are widely cultivated in different parts of the world. Mangosteen peels, kaffir lime leaves, and wampee leaves also inhibited of K562, U937, and Molt4 cell lines. Even though they were toxic to PBMCs, noncytotoxic doses can be analyzed for their effects on leukemia biological markers, such as the *WT1* gene. However, further study of the activity associated with the different species, cultivation conditions, and investigation the active constituents of these plants may provide useful comparative information in the future.

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