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Anti-proliferative and pro-apoptotic effect of P.sacharosa, e.elatior and P.granatum aqueous extract on human myeloid leukaemia

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Abstract

Objective: This present study aimed to study the anti proliferative effects of aqueous extract of Pereskia sacharosa, Etlingera elatior and Punica granatum plants and to evaluate proapoptotic ability of the extract conducted on two myeloid leukemia cell lines, MV4-11 (Acute myeloid leukemia) and K562 (Chronic myeloid leukemia).
Methods: MV4-11 and K562 cells were exposed various concentrations of the plants extracts; P.sacharosa (leaves), E.elatior (flowershoots) and P.granatum (peels). Cell proliferation assay and apoptosis assay of MV4-11 and K562 were performed. The cellular morphology of these cells was observed by phase contrast microscopy. Flow-cytometry was performed to analyze cell apoptosis with labeled Annexin V and propidium iodide (PI).
Results: Extracts of P. sacharosa, E.elatior, and P.granatum shown significant inhibition of cell proliferation of MV4-11 and K562 in dose dependent pattern with IC_{50} values of 0.29 (0.1) mg/ml, 0.55 (0.06) mg/ml, 0.36 (0.04) mg/ml, and 0.25 (0.05) mg/ml, 0.35 (0.01) mg/ml and 0.5 (0.04) mg/ml, respectively. The extract also showed no cytotoxic effect on normal cell. In addition, induction of apoptosis was demonstrated in the cell treated with the plant extract by which P.granatum exerted a strongest apoptotic induction in K562 treated cell. However in MV4-11, apoptosis is higher in cell treated with P.sacharosa. E.elatior showed the lowest in apoptosis induction. The extracts also induced apoptotic-like morphological changes such as membrane blebbing, apoptotic bodies, chromatin condensation and cells shrinkage.
Conclusion: This study showed that extract from P. sacharosa, E.elatior, and P.granatum inhibits cell proliferation in MV4-11 and K562 leukemic cells in dose dependent manner mainly via apoptosis mechanism. This preliminary data suggest that, the extracts deserve further investigation to confirm their chemopreventive effects.

Keywords: Leukemia, chemoprevention, apoptosis, medicinal plants

INTRODUCTION

There are new trend in ethnopharmacology that use plants, plant extracts or plant-derived pure chemicals to treat arrays of diseases medical conditions. Medicinal plants or sometime called as traditional or natural medicine has been used for the last two decade especially in East followed by China and India in adherence to herbal medicine . Indeed there is growing interest of public in phytomedicine that staggered over 100 million dollars per year in herbal product sales. Not much investigation has been done on the chemopreventive activities of P.sacharosa, E.elatior and P.granatum thus lead to the hypothesis that our medicinal plants could have greater potential for the chemoprevention activities.

Differentiation-based treatment is closely related with hematological malignancy, particularly leukemia . In this regards, differentiation refers to an ability of leukemia cells to revert to their normal function induces by chemopreventive agents. Leukemia is a genetically heterogenous clonal disorder characterized by the accumulation of acquired somatic genetic alterations in hematopoietic progenitor cells that alter normal mechanisms of self renewal, proliferation and differentiation . The cells fail to differentiate into their normal counterpart, thus cease its function. The heterogenous nature of leukemia has lead to multistep pathogenesis of the disease with different treatment approach. This immature form cannot perform the same function of mature cells mainly for body protection. In this study, we focus on the relevance of the use of Pereskia sacharosa, Etlingera elatior and Punica granatum, as a potential chemotherapeutic agent for hematological malignancies.

P. sacharosa (Cactaceae) has been used as natural remedy in cancer-related diseases for decades. It is a spiny shrub which can reach up 2 to 8 m . Its leaves are either eaten raw or taken as a concoction brewed from fresh plant. It has been found to have cytotoxic activity against various cancer cell lines, such as Human nasopharyngeal epidermoid carcinoma cell line (KB), Human colon carcinoma cell line (HCT 116), Human hormone dependent breast carcinoma (MCF7), Human cervical carcinoma cell line (Hela) and Human lung carcinoma cell line (A549). It is also believed to have anti-tumor, anti-inflammatory, anti-rheumatic and anti-ulcer properties . Previous study shown that methanol extract of P. sacharosa possessed cytotoxic effects against Human Ductal Breast Ephitelial tumor cell line (T- 47D) and the cell death was found to be apoptotic in nature, mainly via the activation of the caspase-3 and c-myc pathways .

Etlingera elatior (Zingiberacea) is generally known as torch ginger. In many parts of Southeast Asia, including Malaysia consume the young influorescence as edible vegetables and eaten raw, or used as flavouring in dishes. Ancient peoples have used the decoction of young flowershoot to reduce body odour after giving birth . Flavonoid content of its young influorescence was estimated to be 286 mg and 21 mg of kaempferol and quercetin

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(per kilogram dry weight) . Previous literature reported that the ethanolic extract of E.elatior possess anti-microbial, anti-tumor and cytotoxicity activity against human cervical cancer, HeLa cells . Preliminary screening showed chloroform ($CHCl_3$) and methanol (MeOH) extracts of this plants possessed high anti-tumor promoting activity with 92.2% and 85.9% respectively. Both hexane and ethyl acetate extracts were cytotoxic to human Burkitt's lymphoma, Raji cells at initial concentration of 200 mg/ml. Ethyl acetate extract showed significant inhibitory activity towards Human T-lymphoblastoid, CEM-SS cells (IC50 4 mg/ml) and Human breast adenocarcinoma, MCF-7 cells (IC50 6.25 mg/ml) .

Pomegranate or P.granatum (Punicaceae) is a fruit tree origin from the Middle East and Asia. The tree is much valued for its red juicy fruit and is ubiquitous. However, various parts of this plants such as peels, seeds, leaves, barks and roots have been reported to have potent medicinal properties . Pomegranate juice and peel contain substantial amounts of polyphenols such as ellagic tannins, ellagic acid and gallic acid . Ellagic acid also found to exhibit anticancer properties towards cervical carcinoma, CaSki cell line by induction of G phase arrest and apoptosis . Whereas, the aqueous extract of peels contain mainly tannins, lesser amount of carbohydrate (glycoside) and a smaller amount of flavonoids . Current research elucidated that the juice and peels have anticancer inhibitory properties that inhibit cell proliferation, cell cycle and angiogenesis . Natural antioxidant compound such as flavonoids in pomegranate fruit extract possess the ability to induce cell death and apoptosis in human prostate carcinoma, PC-3 cell line . Flavonoid-rich fraction from aqueous extract of pomegranate pericarp also promotes differentiation in human HL-60 promyelocytic leukemia cells and has suppressive potential.

In Malaysia, this exotic P. sacharosa E.elatior and P.granatum, are widely grown for domestic consumption, but many are unaware of their medicinal properties. These plants were selected for this study because of their highly conspicuous and have various ethnomedical practices. Therefore the main aims of this study were to screen and to evaluate edible and non-edible portions of P. sacharosa, E.elatior and P.granatum extract for anti-proliferative activity and mode of cell death conducted on two leukemic cell lines, MV4-11 (AML) and K562 (CML).

MATERIALS AND METHODS

Plant materials and preparation of aqueous extracts

Authenticated fresh plants of P.sacharosa, E.elatior and P.granatum were purchased from regular green grocer at Binjal wet market in Kota Bharu, Kelantan, Malaysia. The plant samples were identified at Malaysian Agricultural Research and Development Institute (MARDI), Strategic Resource Research Centre, Serdang, Malaysia. The P.sacharosa's leaves, E.elatior's flowershoot and P.granatum's peels were collected. The samples were washed with distilled water to remove dirt and cut into pieces and freeze dried (at temperature -140°C), to excessively remove their water contents. The dried leaves, flowershoot and peels were grinded for lyophilized powder using grinding machine. 0.05 g of lyophilized powder of P.sacharosa, E.elatior and P.granatum were weighted and were suspended in 50 ml of RPMI media, incubated in 37°C incubator with automatic roller for 24 hours. The insoluble materials were removed by centrifugation at 120 x g for 5 minutes at room temperature. The extracts were filtered using 0.2 ìm Minisart syringe filter (Sartorius Stedim Biotech GmbH, Germany) and were kept under sterile condition and in aliquot. The extracts were stored at 4°C for immediate usage and stored under -20°C for later experimentation.

Cell lines and cell culture medium

MV4-11 cell (human acute myeloid leukemia cell line), AML and K562 (human chronic myeloid leukemia cell line),CML were donated by Department of Hematology, Universiti Sains Malaysia, Malaysia originally purchased from American Type Cell Culture (ATCC) (Rockville, MD, USA). The MV4-11 and K562 cells were culture in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, USA), supplemented with 10% (v/v) of fetal bovine serum (FBS) (Sigma,USA) and 1% (v/v) of penicillin-streptomycin (Invitrogen,USA) in humidified temperature containing 5% carbon dioxide (CO_2) at 37°C in incubator.

In vitro cell proliferation assay

Briefly, the MV4-11 and K562 cells were plated at density of $1x10^5$cells per well in 6 well plates (Becton Dickinson, USA). After 24 hours of culture, the cells were treated with different concentration of complete media (RPMI 1640 media, 10% inactivated FBS, with 100 uL penicillin-streptomycin) dissolved extracts (0, 0.2, 0.4, 0.6, and 0.8 mg/ml) in triplicate. After treatment, the plates were incubated for 72 hours. After incubation, cell proliferation assay was determined by conventional Trypan Blue Exclusion Assay (TBEA). After 72 hours, the cells were collected and resuspended in 1 ml complete medium. One part of 0.4% Trypan blue (Gibco, USA) and one part of cell suspension were mixed (1:1). The cell number in suspension was counted using hemocytometer. Graph was plotted to determine percentage of live cell and dead cell following treatment. Inhibitory concentration to be reduced by half (IC_{50}) was calculated from the graph. To study the potential cytotoxic effect of the extracts on normal cell proliferation, the extracts were further tested on non-cancer cell determined by 3-(4, 5-Dimethylthiazol-2-yl)-5(3-carboxymethonyphenol)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Blood samples were taken from researcher's blood. Mononuclear cells were isolated by Ficoll technique. The cell suspension at $5x10^4$ cells was placed in the 96 six wells plate. Treatment with the plant extract at IC_{50} concentration was added and incubated for 24 hours. The data was acquired based on absorbance reading (OD value) of Sunrise Elisa plate reader (instrument serial number: 03930005221). Graph OD value versus extract concentration were plotted (Data not shown)

Assessment of apoptosis

Apoptosis analysis of MV4-11 and K-562 cells that exposed to the plant extract was carried out using Annexin V labeling BD Annexin-V-FITC assay kit. Flow BDFac Canto II flow cytometer was used for acquiring and analyzing test samples**.** Briefly, the treated cells were washed twice with phosphate buffer saline (PBS). A total of 5×10^5 cells were collected by centrifugation at 1200 x g, 24°C for 5 min. Cells were stained with 5 ìl annexin conjugated with fluorescein isothiocyanate (FITC) and propidium iodide (PI) at room temperature in the dark for 15 min. The cells were analyzed by flow cytometer to measure the fluorescence intensity using the FL-1H channel to detect FITC. The result showed relative granularity at side scattered and relative size of cell at forward scattered with fluorescence stain. Untreated cells served as the negative control. Conventional drug in clinical use, Lestaurtinib or CEP-701 for treatment of AML and Imatinib Mesylate (Gleevec^{TM}) for treatment of CML were served as positive control at IC_{50} concentration of 0.12 ìM and 0.15 ìM,

respectively. A minimum of 10,000 events were collected per sample and analyzed using BD FacDiva software.

Statistical analysis

All the results are expressed as mean ± standard error of mean (S.E.M) of three independent experiments. Statistical analysis was made using one-way ANOVA, followed by post hoc Tukey t-test for cell proliferation assay analysis and Man-Whitney t-test for apoptosis induction analysis with significant level of $p<0.05$.

RESULTS

Anti proliferative activity of aqueous extract of P. sacharosa, E.elatior and P.granatum on MV4-11 and K-562 cells

As shown in figure 3, there were marked inhibition in cell growth observed in MV4-11 and K562 treated cells. The percent of viable cells was determined by constructing cell viability graph to compare the percent of cell inhibition between treated and untreated cells. Aqueous extracts of P. sacharosa, E.elatior, and P.granatum inhibit cell proliferation in MV4-11 and K562 cell lines with dose dependent manner with IC_{50} values of 0.29 (0.10) mg/ml, 0.55 (0.06) mg/ml, 0.36 (0.04) mg/ml in MV4-11, and 0.25 (0.05) mg/ml, 0.35 (0.01) mg/ml and 0.5 (0.04) mg/ml in K562. Among all extracts, leaves extract of P.sacharosa demonstrated lower IC_{50} value, which showed it has most potent inhibitory properties for each leukemia cells. Lower IC_{50} value demonstrated better cytotoxic activity . Extract from E.elatior showed less potent inhibitory property in MV4-11 cell compared to K562 treated cell. Moreover extract of P.granatum showed less potent in K562 cell but give higher inhibitory effect on MV4-11 cell. There was no inhibitory effect observed in normal cell treated with the extracts (data not shown).

Apoptosis analysis

As shown in figure 2, the apoptosis induction of P. sacharosa, E.elatior, and P.granatum treated cell for 72 hours showed total induction of 25.9%, 18.05% and 21.6%, respectively compare with untreated cell, 6.2% on MV4-11 cell line. While in K562 cells, the percentage of apoptotic cells were increased from 4.8% to 18.7%, 10.2% and 32.8% after treatment with P. sacharosa, E.elatior, and P.granatum, respectively (Figure 4). The response of MV4-11 and K562 cells to the extracts was varied. There were apparent changes of total apoptosis in treated cells compared with untreated cells. However, E.elatior extract showed lesser apoptosis induction among the extracts in both MV4-11 and K562 cells. Cell treated with drug, CEP701 and Imatinib showed 39.0% and 37.8% of apoptosis induction.

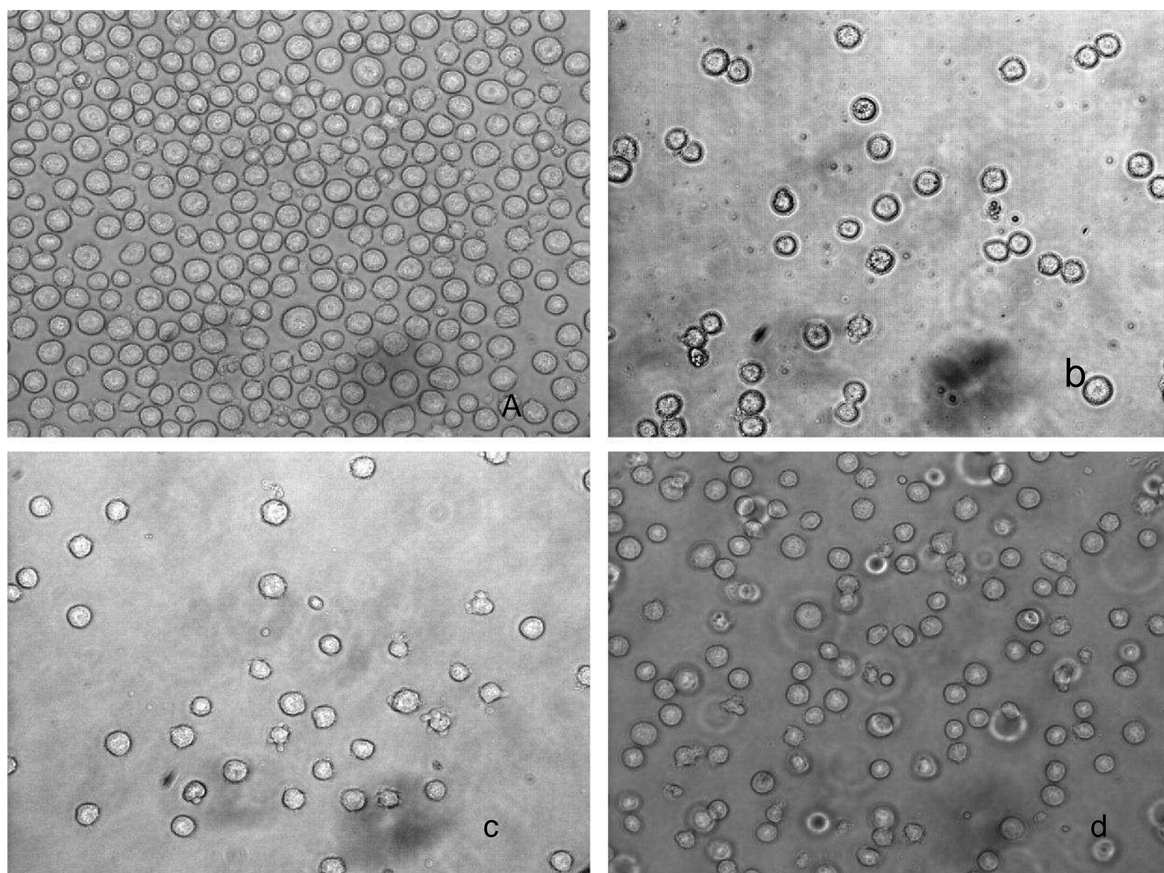

Figure 1 shows photomicrograph of MV4-11 cell treated with P.sacharosa 1(b), E.elatior 1(c) and P.granatum 1(d)

Photomicrograph of MV4-11 cell treated with aqueous extract of P. sacharosa, E.elatior, and P.granatum at IC_{50} concentration for 72 hours. Arrows demonstrated apoptotic bodies observed in P.sacharosa treated MV4-11 cell 1(b), blebbing of the cell membrane in E.elatior 1(c) and chromation condensation observed in P.granatum treated cell 1(d). Untreated MV4-11 cell 1(a) shows minimize number of apoptotic figures. Membrane blebbing, apoptotic bodies and chromatin condensation are the important hallmarks for apoptosis

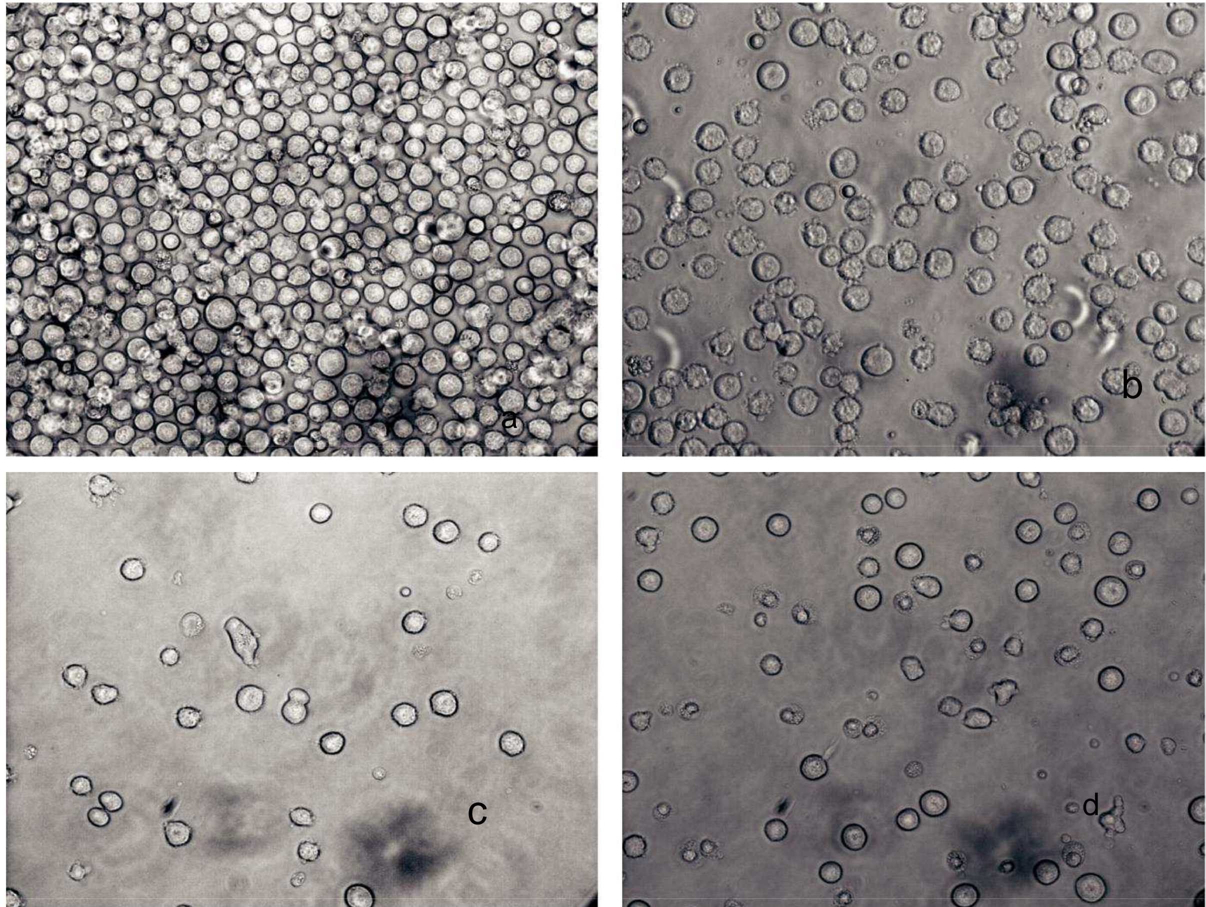

Figure 2 shows photomicrograph of K562 cell treated with P.sacharosa 1(b), E.elatior 1(c) and P.granatum 1(d)

Photomicrograph of K562 cell treated with aqueous extract of P. sacharosa, E.elatior, and P.granatum at IC_{50} concentration for 72 hours. Arrows demonstrated cell shrinkage observed in P.sacharosa treated cell 2(b), apoptotic bodies and chromatin condensation in E.elatior 2(c) and numerous chromation condensation observed in P.granatum treated cell 2(d). Untreated K562 cell 2(a) shows confluent cell population compared with treated

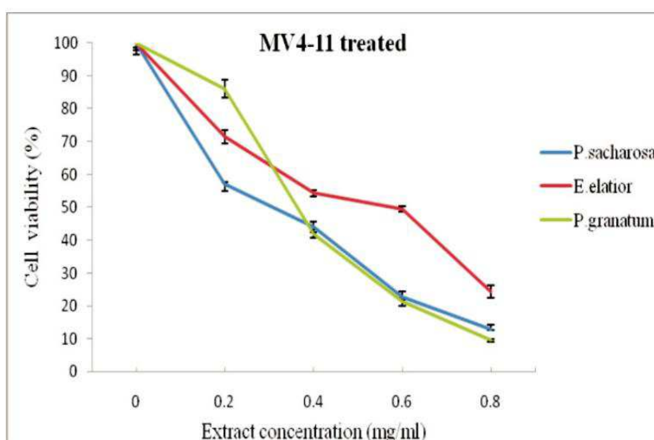

Figure 3A : Antiproliferative activity P. sacharosa, E.elatior, and P.granatum extract on MV4-11 and K562 cells

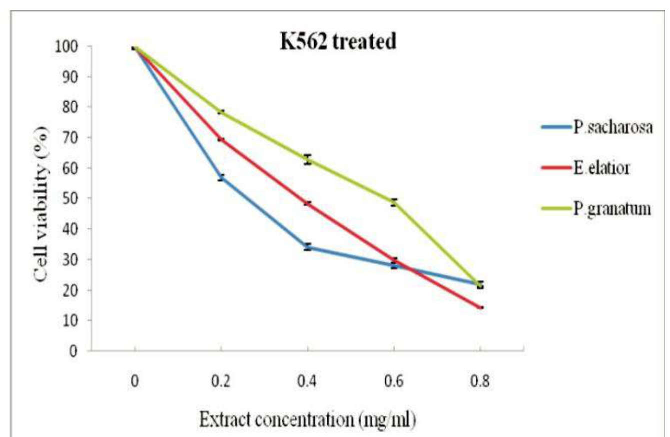

Figure 3B Antiproliferative activities of aqueous extract of P. sacharosa, E.elatior, and P.granatum (peel) treated with various extract concentration (72 hrs) on MV4-11 (A) and K562 cells (B). The cell viability was expressed as a percentage of cell viability (mean ± **SD of triplicate**). Statistical significant different from the negative control are indicated as * $p<0.05$ by one-way ANOVA followed by post hoc Tukey's t-test.

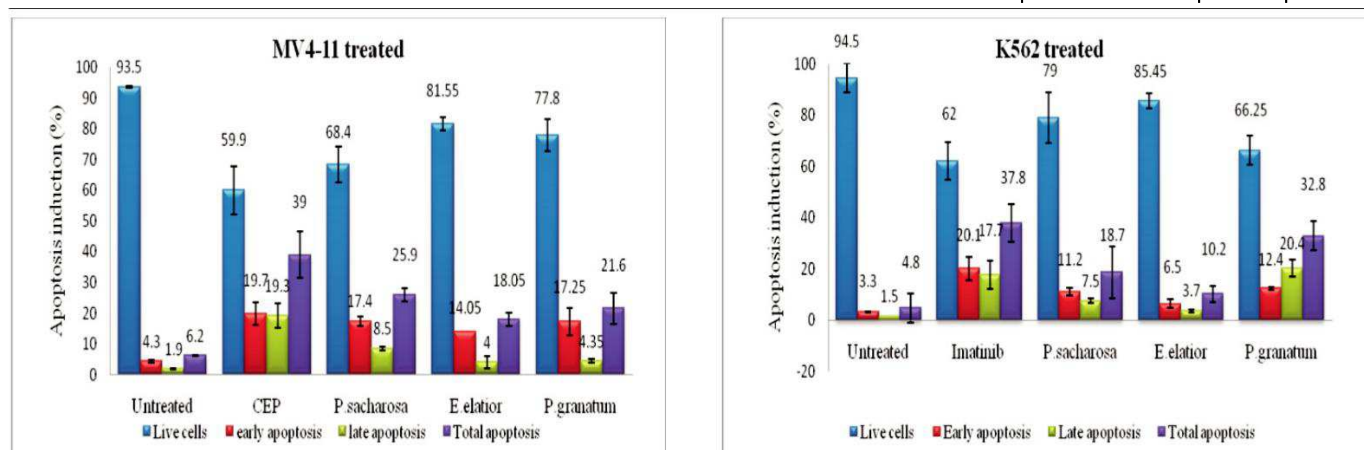

Figure 4 Apoptotic induction of aqueous extract of P. sacharosa, E.elatior, and P.granatum (peel) on MV4-11 (C) and K562 (D) cells. Results are expressed as percentage in apoptosis (mean in triplicate). Untreated cells served as negative control. Statistical significant different from the negative control are indicated as $p<0.05$ non-parametric Man-Whitney t-test

DISCUSSION

The extraction method using cell culture media as applied in this study has not been applied in previous study. It was our trial to replace the conventional aqueous extraction using water by culture medium to look for any growth inhibitory effects, as well as to minimize observational error or perceptual bias while interpreting .This was due to our previous trial demonstrated that presence of at least forty to fifty percent of water content in the extract can inhibit growth of these cells (data not shown), thus lead to false positive result.

Cell death induced by leaves of P.sacharosa, flower shoot of E.elatior and peel of pomegranate were observed for morphological changes. Microscopic evidences of apoptotic features using phase contrast inverted microscopy under high magnification 40 xs. Membrane blebbing, apoptosis bodies and chromatin condensation were the most prominent features (Figure 1, 2). The bleb is a vesicle protruded from plasma membrane that later detached to become apoptotic bodies. Though at increased extract concentration, the nuclei with chromatin condensations were seen in high number.

In our study, it was found that extract of P.granatum and P. sacharosa were the most potent plant for anti proliferative property and apoptosis induction. E.elatior media extract did not show promising result in regards of apoptosis with only little apoptosis induction. The anti proliferative activity of P.granatum peel extract was in good correlation with their good source of polyphenol especially tannin and condensed tannins that have been associated with cancer. Biochemical constituents of P.granatum peel or pericalp are phenolic punicalagin, gallic acid, catechin, quercetin, flavonone and anthocyanidin that have therapeutically beneficial to exhibits powerful anticarcinogenic and antioxidant properties . Ethyl acetate extract of P. sacharosa produced 2,4-Di-tert-butylphenol, ? -tocopherol, ß-sitosterol that reported to possess high antioxidant properties and phenolic content . In addition, phytochemical screening of 95% ethanol (1:10 w/v) soxhlet extraction of E.elatior flowershoot revealed to have significant quantity of phenolic compounds and flavonoids but no tannins was detected . We can pre-assume that P. sacharosa, E.elatior and P.granatum peel contained amounts of phenolic compounds that cause their anti proliferative effect. Phenolic compounds is a potential antioxidant that scavenged free radicals . This natural antioxidant commonly found in most medicinal plants associated with polyphenolic compounds .

The proliferation of MV4-11 and K562 were significantly inhibited by the extracts in a dose dependent manner. In separate experiment, time-dependent cell proliferation assay was accomplished to study the effect of treatment to the cells in a long duration. Result demonstrated that, growths of these leukemic cells were not retarded after 5-6 days of culture (data not shown). These finding implied that the extracts anticancer effect have been compromised in long treatment duration. This type of extract preparation would not effective for a long run. A new and freshly prepared extracts were needed in order to get consistency in cell viability assay.

In addition to its effects on cancer cells, the extracts were used against normal peripheral blood mononuclear cells (PBMCs) for cytotoxicity assay (Data not shown). The aqueous extract of P. sacharosa, E.elatior and P.granatum at inhibitory concentration (IC_{50}) of 0.29, 0.55 and 0.36 mg/ml on MV4-11 and 0.25, 0.37 and 0.57 mg/ml on K562, respectively (Table 1) cells had no cytotoxic effects on PBMC cells viability. The results indicate that, unlike cancer cells, the plants extract appeared to be non-toxic towards normal cells.

Several researches have conceived on the base of this plant product, but with considerably distinct inhibitory concentration. Ampasavate et al, 2010 demonstrated the IC_{50} of ethanol extract of P.granatum on HL-60 acute promyeloid leukemia cell was 8.0 ìg/ml (7.9±1.9), compare with this study the IC_{50} was 0.36 and 0.57 mg/ml both cells. In other study made by Habsah et al, 2005 on cytotoxicity activity of E.elatior extract on CEM-SS human T-lymphoblastoid cells, ethanol acetate and methanol extract showed the IC_{50} of (4.0±0.1) and (46.0±0.4) ìg/ml, respectively, with regards in this study, 0.55 and 0.37 mg/ml. From Tan et al, 2004, the EC_{50} of methanol extract of P.sacharosa was 2.0 ìg/ml on T-470 breast cancer carcinoma cells, whereas in this study the IC_{50} of aqueous extract of P.sacharosa on MV4-11 and K562 leukemia cells was 0.29 and 0.25 mg/ml, respectively. These comparative data proved that alcohol based solvent extraction method exerted more therapeutic constituents of the plants rather than aqueous extraction method as the lower the IC_{50}, the better its cytotoxic activity .

In order to examine the mode of cancer cell death, apoptosis assay study was accomplished. From previous research on apoptosis induction in P.granatum (peel), E.elatior and P. sacharosa, acetone extract of E.elatior young inflorescence inhibit cell proliferation by apoptosis induction in HT-29 colorectal cancer cell based on phosphatidylserine (PS)

translocation and caspases-3 activation , P. sacharosa methanol extract induces apoptosis in T-47D human breast carcinoma cell by DNA fragmentation and apoptosis study in P.granatum (peel) was not reported and the study was only confined to antioxidant properties and cytotoxicity assay . In our study, P.granatum (peel), E.elatior and P. sacharosa aqueous extract treatment significantly increased the proportion of annexin V positive cells in MV4-11 and K562 cells compare with untreated that served as negative control. The apoptosis rate determines the amount of cells in early and late apoptosis. The number of apoptotic cell in MV4-11 and K562 treated with P.sacharosa was increased by 4-folds compared with E.elatior and P.granatum extracts. Morover, P.granatum showed the highest fold changes of 7- folds increment in K562 cell outnumbered E.elatior extract effect. The possible reason of being distinct response was MV4-11 and K562 cells carry different molecular and cytogenetics entities that make them respond differently to cellular stress.

CONCLUSION

P. sacharosa (leaves), E.elatior (flowershoot) and P.granatum (peel) aqueous media extract showed anti proliferative effects on MV4-11 and K562 cells. Mode of cell death was proved to be apoptosis in nature. Membrane blebbing, apoptosis bodies and nuclear fragmentation were prominent in all treated cells. To date, P.granatum (peel) was the most potent extract towards leukemia cells with the highest apoptosis induction. However, the aqueous extract of these plants extract was compromised with long treatment duration as it became less efficient. The extracts give non-toxic effects against normal PBMC give signal of potential anti-therapeutic agent. Further study need to accomplish to determine the chemical constituents of the compound responsible for its cytotoxic effects and downstream analysis of proteomic and genomic is greatly needed for further study.

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