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Original Article

The stimulating effects of polyphenol and protein fractions from jelly fig (*Ficus awkeotsang* Makino) achenes against proliferation of leukemia cells

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ABSTRACT

This study aimed to investigate the direct and immune-stimulated antiproliferative activities of jelly fig achenes fractions including pectinesterase inhibitors, crude polyphenols extract, and purified polyphenols extract (PP). Beside the measurement of cell viability of U937, the quantity of cytokines in conditioned medium and morphologic changes in leukemia were observed. After surveying all fractions in jelly fig, the obtained fractions of polyphenol exhibited the highest stimulating effects and directly cytotoxic effects against leukemia with the lowest effect found in protein fractions. The leukemia treated by our PP fraction showed dose-dependent response between the concentration and G2/M cell numbers of the U937 cells. The PP fraction had more pronounced effect on immune-stimulated than direct antiproliferative activities. The finding was also supported by morphological analysis by showing the formation of apoptotic bodies and differentiation from immature U937 cells into mature monocytes/macrophages on cells cultured with PP-conditioned medium. In conclusion, polyphenol fraction of pectinesterase inhibitors from jelly fig showed the immune-stimulated antiproliferative activities against U937 cell.

1. Introduction

Jelly fig (*Ficus awleotsang* Makino) is a native woody vine found in Taiwan. Jelly curd from water extract of jelly fig achenes with added calcium is locally popular as a summer drink. Lin et al [1] found that approximately 98% of jelly fig pectinesterase (PE) exists in the pericarp of achenes, which is involved in the jellification of jelly fig achenes. PE catalyzes the de-esterification of methanol from pectin and converts the

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protopectin into soluble pectin and pectate, which can be turned into gel by addition of calcium. However, the PE activity of jelly fig achenes was eliminated when a homogenized solution of crushed achenes was used with the same PE extraction, which explained the existence of PE inhibitors (PEIs) on the crushed achenes [2]. PEIs prepared from intact jelly fig achenes were previously separated and characterized as polypeptides with molecular weights of 3.5–4.5 kDa, comprising more than 50% histidine [3].

The bioactivities of PEI from the residue of jelly fig used to prepared jelly curd have been reported. Wu et al [4] reported the application of PEI for the reduction of methanol content in carambola wine. The PEI of jelly fig was found to inhibit the growth of *Bacillus cereus* and *Escherichia coli* O157:H7 effectively [5]. Lai et al [5] reported that the superoxide anions radical scavenging activity and the hydrogen peroxide scavenging activity of PEI at 200 μg/mL were equivalent to those of butylated hydroxyanisole (BHA) at 50 μg/mL. In addition, PEI from jelly fig also has been reported to inhibit surface antigen expression by human hepatitis B virus without toxic effects on normal primary hepatocytes [6].

The current cancer treatments include surgery to remove cancerous tissues, and chemotherapy in combination with radiation therapy to kill the remaining cancer cells. However, chemotherapy and radiation therapy can result various side effects on the remaining normal cells rendering lower effectiveness of cancer treatment [7]. Natural compounds derived from foods can act as promising strategies to devitalize malignant cells through apoptosis. Stimulation of immune system by the compounds extracted from food can be an alternative to traditional chemotherapy for treating cancers [7].

Lin et al [8] also reported that PEI was able to reduce the hydroxyl radical-induced DNA damage and inhibited the growth of the three cancer cell lines with CT-26 being the most vulnerable one, HepG2 the second, while MCF-7 the least affected. Moreover, among all the ultrafiltration's filtrate, the 3–10 kDa fraction had the highest inhibition on HepG2 cells [8]. PEI also exerted antiproliferative and apoptotic effects on colorectal cancer COLO205 and HT29 cells by arresting G2/M cell cycle, and caused apoptotic death possibly via mitochondrial pathway involved with exceeding ROS level [9]. PEI has also been reported to potently inhibit proliferation on human leukemic U937 cells via G2/M cell cycle arrest and apoptosis in association with MTP reduction and caspase-3 activation [10].

The immune process, which induces in the lysis of tumor cells, often begins with dendritic cells, which activate lymphocytes and starts the cytotoxic process and tumor cell lysis [11]. This entire cell interaction mechanism is coordinated by different cytokines [12]. The proinflammatory activities of cytokines such as interferon-γ (IFN-γ) are thought to be associated with antitumoral cellular immunity. However, immune response activation involving cytokines such as interleukin (IL)-10 leads to a predominantly humoral immune response [13]. IL-2 is a cytokine that is used as a medicine for leukemia treatment. It can activate killer T-cells and natural killer cell for tumor lysis [14].

However, the PEI activities reported above were only observed on the crude water extract of jelly curd residue. The compounds involved in this antiproliferative activity have not been studied. Therefore, this study separated the PEI of jelly fig further into crude polyphenol (CP), purified polyphenol (PP), and protein (PR) fractions and observed their direct and immune-stimulated antiproliferative activities.

2. Methods

2.1. Preparation of extracts

Jelly fig achenes were purchased from Tauyuan District office, Kaohsiung City. The PEI solution was prepared from pectin-depleted jelly fig achenes as reported previously [3]. In brief, achenes were rinsed repeatedly in 15 volumes (w/v) of 4% NaCl solution to wash out the major pectin and pectinesterase; subsequently, they were rinsed twice with 20 volumes (w/v) distilled water to wash out residual salt. After drying at 50°C in an oven, achenes were extracted using 15 volumes (w/v) of distilled water for 6 hours and centrifuged (20,000*g*, 30 min) to collect the supernatant. Obtained fraction with PEI activity were lyophilized and stored at 4°C prior to further extraction and analysis.

The lyophilized PEI was extracted with 70% alcohol for 24 hours at 4°C twice. The precipitate was obtained as PR fraction of PEI and the supernatant was collected as CP fraction of PEI. CP was further separated for its polyphenols with Sephadex LH-20 chromatogram (H_2O-MeOH, 0–100%) and 80 tubes (each tube/minute) were collected. Spectrophotometer at 254 nm was used to observe the existence of phenolic compounds and the result is shown in Figure 1. To ensure the content in PP, 1% polyvinylpolypyrrolidone (PVPP) was added into the PP fraction and the phenolic content before and after 1% PVPP treatment were measured. PVPP is a synthetic material that can bind with polyphenolic compounds. After PVPP treatment, total phenolic content reduced to 98.5%. Collected tubes were further purified by HPLC using C-18 column (15 cm × 4 mm) with mobile phase of methanol: acetonitrile: water (55: 30: 15) to collect the PP fraction. All fractions were lyophilized and stored at −20°C until analyses. Extraction rates for PEI, CP, PP, and PR fraction were 2.50 ± 0.20%, 1.20 ± 0.03%, 0.75 ± 0.04%, and 1.30 ± 0.04%, respectively.

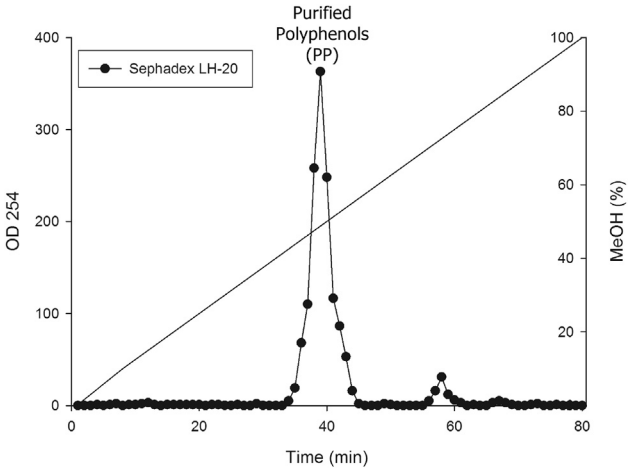

Figure 1 – Elution profile of pectinesterase inhibitor by Sephadex LH-20 chromatogram using different gradients of methanol and water to purify polyphenols.

2.2. Culture of U937 cell

Human monocytoid leukemia cell line U937 (ATCC: CRL-1593.2) purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) was cultured in RPMI-1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA) and 1% penicillin–streptomycin mixture or 1% glutamine. The cultures were maintained in an exponential growth state and subcultured by total medium replacement using centrifugation every 2–3 days, depending on the cell number, and was maintained at a cell concentration between 1×10^5 and 1×10^6 cells/mL by incubation at 37°C in a 5% CO_2 containing humidified incubator.

2.3. Direct growth inhibition of U937

Cultures of U937 were incubated in 35-mm petri dishes at an initial concentration of 1×10^5 cells/mL in the presence of different concentration of PEI, CP, PP, and PR at 37°C in a humidified 5% CO_2 incubator for 1–3 days with the untreated cells acting as a control. The numbers of adherent cells after treatment were collected by gently rubbing the dishes with a rubber policeman. Viable cells and cell count were assessed by trypan blue (0.04%) exclusion dye using a hemocytometer to determine the growth inhibition (%). Growth inhibition (%) = (1 − cell number of PEI treatment/cell number of control group) × 100%. A cell viability assay, based on trypan blue dye exclusion by viable cells, was used in order to establish LC_{50} (concentration required to induce 50% of cell viability).

2.4. Cell cycle analysis

After PEI treatment, cells were centrifuged for 5 minutes (300 × *g*) and washed twice with RPMI 1640/10% FBS and PBS. The pellet was suspended, fixed, followed by addition of 1 mL PBS containing 50 mg/mL propidium iodide (PI), 0.1% Triton X-100, and 0.2 mg/mL RNase A, and incubated at room temperature for 30 minutes [10]. Fifteen thousand events were collected with a FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA), and the data were analyzed using the Modfit LT software package (Becton Dickinson). The obtained

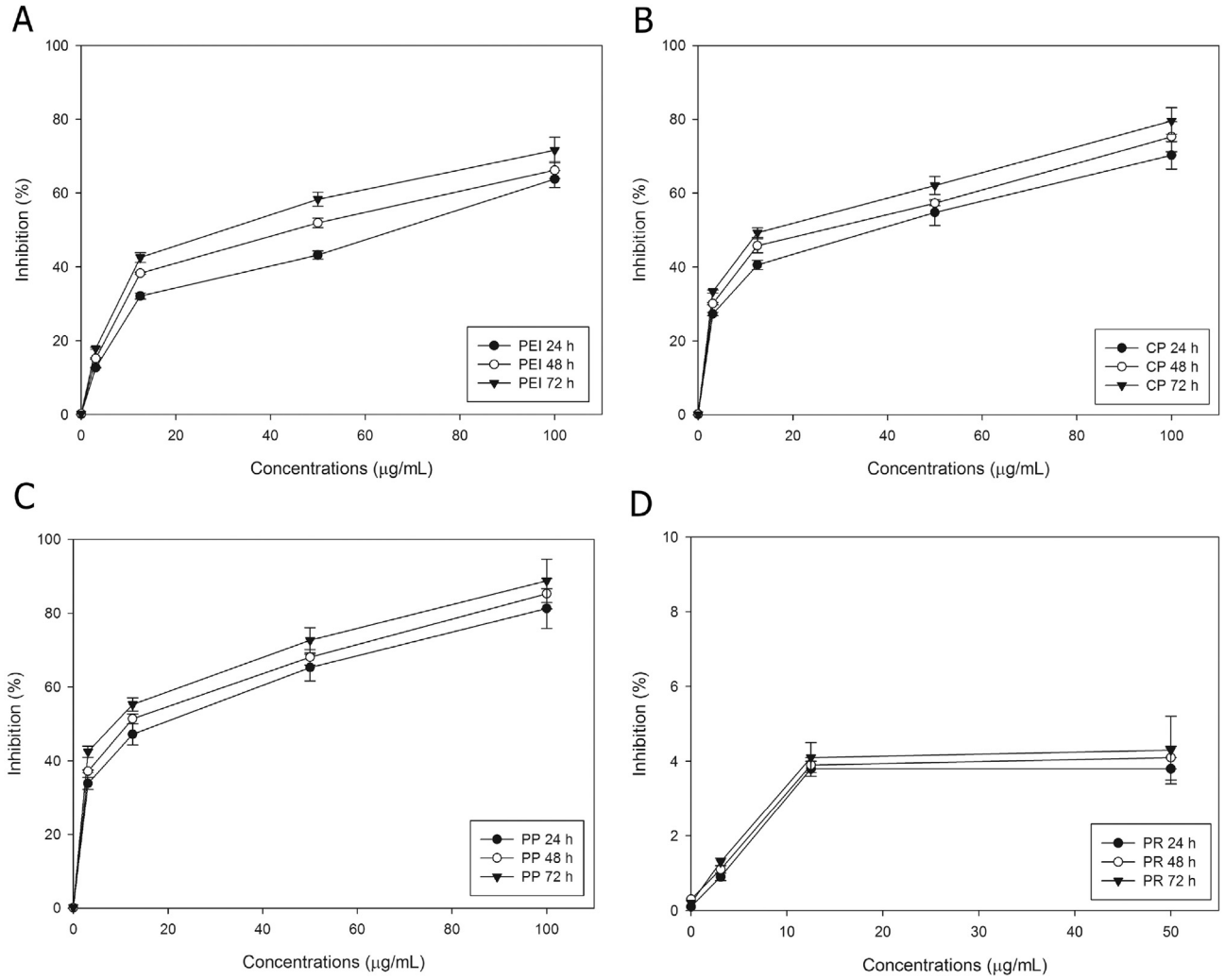

Figure 2 – Growth inhibition of different jelly fig fractions. (A) Pectinesterase inhibitor (PEI), (B) crude polyphenol (CP), (C) purified polyphenol (PP), (D) protein (PR) on U937 monocytoid leukemia cells. Values represents as mean ± standard deviation.

Table 1 – Half maximal inhibitory concentration values (μg/mL) of growth inhibition of pectinesterase inhibitor (PEI), crude polyphenol (CP), purified polyphenol (PP), and protein (PR) fractions from jelly fig on U937 monocytoid leukemia cells.

Incubation time	PEI	CP	PP	PR
24 h	57.99 ± 4.49[a]	29.51 ± 0.61[b]	16.04 ± 3.56[d]	ND
48 h	32.01 ± 2.02[b]	22.29 ± 2.66[c]	11.66 ± 1.03[d,e]	ND
72 h	21.95 ± 2.62[c]	16.10 ± 1.88[d]	7.67 ± 1.19[e]	ND

[a–e] Values (mean ± standard deviation) with different letters were significantly different ($p < 0.05$).
ND = not determined.

data are expressed as the percentage of cells in the G0/G1, S, and G2/M phases [15].

2.5. Immune-stimulated growth inhibition on U937 cells

Immune-stimulated growth inhibition on U937 cells was done following the method Chen et al [7]. Mononuclear cells (MNCs) were isolated from the peripheral blood collected from normal volunteers and a prepared solution of Ficoll–Paque Plus (1.077 g/mL, Pharmacia Fine Chemicals) was gently added to the top layer of the collected blood and then centrifuged at 400 × *g* for 20 minutes at room temperature. The white layer, which contained MNCs, was removed by a dropper and washed twice with buffer.

MNCs were adjusted to 1.5 × 10^6 cells/mL and different fractions of jelly fig (0–100 μg/mL) were added to each culture. Phytohemagglutinin (PHA) at a concentration of 5 μg/mL was used as a positive control. After 24 hours, culture medium of each group was collected by filtering through Millipore of 0.22 μm and designated as conditioned medium (CM). These obtained CM were stored at −80°C for future use as culture media for human leukemic U937 cells treatment.

U937 cell was adjusted to a cell density of 1 × 10^5 cells/mL and cultured in the culture media containing 30% of previously prepared CM. After 3 days of culturing, the viability of U937 cell was estimated using the trypan blue dye exclusion test. The growth inhibition was calculated as by 1-(viable cells of test group/viable cells of control group)] × 100%.

2.6. Assay for cytokines

U937 cell concentration was adjusted to a cell density of 1 × 10^5 cells/mL and cultured in the culture media containing 30% of previously prepared CM. After 3 days of culturing, the cytokine expressions were measured. IL-2, IL-10, and IFN-γ were measured by cytometric bead array assays (human Th1/Th2 cytokine kit; BD Biosciences, San Diego, CA, USA). In short, for this assay, these cytokines were specifically captured by an antibody, bonded to microparticles and detected by the use of a fluorescence-based detection system and flow cytometric analysis. In order to create the standard curves to determine the quantities, a series of 10× dilutions of each cytokine standard was performed together with each sample assay. All data were analyzed in a FACS Caliburflow cytometer (BD Biosciences) using the BD cytometric bead array analysis software [16].

2.7. Morphology imaging

U937 cells (3 × 10^5) were added into 6-mm petri dishes and cultured in the culture media containing 30% of previously prepared CM. After 5 days of treatment, the morphology of U937 cells was observed using an inverted microscope at 400 × magnification. For DAPI (4,6-diamidino-2-phenylindole) staining, harvested cells were washed once with phosphate-buffered saline (PBS), and then resuspended in 4% PBS buffered paraformaldehyde solution for 30 min for cell fixation. After washing with PBS again, 0.6 mL (10 μg/mL) of DAPI (Sigma, St Louis, MO, USA) was added into collected cells for 10 min. Finally, the harvested cells were observed by florescence microscope at 460 nm at 400× magnification [17]. For Liu's staining, 5-day CM treated U937 cells were cytocentrifuged (600*g* for 2 minutes) onto a microscope slide by using a Cytospin^{2R} [18]. Five drops of Liu's A (eosin Y, methanol) reagent (Sigma) was added and let it settle for 15–30 seconds. Afterwards, 10 drops of Liu's B (methylene blue, azure) reagent was added. After mixing with reagent A and B for 2 minutes, the reagent was rinsed with tap water, air-dried, and observed by inverted microscope at ×1000 magnification.

2.8. Statistical analysis

Results are presented as mean ± standard deviation from three replications. Differences between the different treatment groups were assessed by the ANOVA with Duncan's *posthoc* using SPSS program. A confidence level of 5% (p-value < 0.05) was considered to be significant.

3. Results

3.1. Effect of jelly fig achenes fractions on growth inhibition of U937 cells

Figure 2 shows antiproliferative effects (direct growth inhibition) of different jelly fig fractions (PEI, CP, PP, and PR) and

Table 2 – Cell cycle population after 24 hours' treatment of purified polyphenol fractions from jelly fig on U937 monocytoid leukemia cells.

Concentrations	Cell cycle population (%)		
	G0/G1	S	G2/M
Control	37.70 ± 0.80[a]	36.17 ± 1.81[b]	26.13 ± 0.84[d]
10 μg/mL	16.23 ± 1.05[b]	47.60 ± 0.89[a]	36.17 ± 0.74[c]
50 μg/mL	13.67 ± 0.50[c]	34.76 ± 1.77[b]	51.57 ± 1.35[b]
100 μg/mL	10.83 ± 0.60[d]	30.07 ± 1.05[c]	59.10 ± 0.70[a]

[a–d] Values (mean ± standard deviation) with different letters were significantly different among different concentrations ($p < 0.05$).

Table 3 – Immune-stimulated growth inhibition (%) of pectinesterase inhibitor (PEI), crude polyphenol (CP), purified polyphenol (PP), and protein (PR) fractions from jelly fig on U937 monocytoid leukemia cells after 72 hours' treatment.

Concentration (μg/mL)	PEI	CP	PP	PR
Control	0.00 ± 9.21[a]	0.00 ± 9.21[a]	0.00 ± 9.21[a]	0.00 ± 9.21[a]
1	31.33 ± 4.73[b]	46.67 ± 1.53[c]	66.00 ± 6.56[d]	14.33 ± 14.16[a]
10	38.33 ± 1.15[c]	51.00 ± 5.57[c]	69.67 ± 4.52[d]	14.00 ± 12.53[a]
50	45.67 ± 8.06[b]	57.67 ± 7.66[b]	73.67 ± 2.29[c]	11.67 ± 6.81[a]
100	49.67 ± 6.66[b]	61.67 ± 8.74[b]	79.63 ± 2.85[c]	12.33 ± 5.86[a]
PHA (5 μg/mL)	4.67 ± 10.08			

[a–d] Value (mean ± standard deviation) with different letters are significantly different among different fractions ($p < 0.05$).
PHA = phytohemagglutinin.

treatment times (24 hours, 48 hours, and 72 hours) against U937 cell survival. The result revealed growth inhibition with increased concentrations and treatment times. However, differences in growth inhibition as the concentration increased were more obvious than the increase in treatment times. This tendency indicated that the cytotoxicity of fruit extracts was more dosage-dependent than time-dependent.

To compare antiproliferative activity among different jelly fig fraction, half maximal inhibitory concentration (IC_{50}) values were determined. IC_{50} values of growth inhibition of different jelly fig fractions from jelly fig on U937 cells are shown in Table 1. The lower IC_{50} values represented the higher antiproliferative activity. Among different jelly fig fractions, PP showed lowest IC_{50} value, which can be considered as the highest antiproliferative activity. The IC_{50} values of PR were not determined due to low growth inhibition activity. When comparing the antiproliferative activity of jelly fig fractions at the same treatment times, result revealed a consistent order: PP > CP > PEI > PR.

3.2. *Effect of PP fractions of jelly fig achenes on cell cycle arrest of U937 cells*

The effect of PP fraction on U937 cells was further analyzed by flow cytometry on cell cycle population by measuring the DNA contents. An increase in the population of G2/M phase caused by PP treatment was found to be concentration dependent (Table 2). The increase in G2/M phase indicated cell cycle arrest in G2/M phase, implying that there was retardation in passing through G2/M phase to G0/G1 phase in U937 cells.

3.3. *Effect of jelly fig achenes fractions on immune-stimulated growth inhibition of U937 cells*

The immune-stimulated antitumor activity (indirect growth inhibition) of different jelly fig fractions on U937 monocytoid leukemia cells are shown in Table 3. Similar to direct growth inhibition, PP fraction showed the highest growth inhibition activity, whereas PR fraction showed the lowest activity. Moreover, indirect growth inhibition had higher antiproliferative activity compared to direct growth inhibition for 72 hours. PEI, CP, PP, and PR fractions at a concentration of 1 μg/mL had indirect growth inhibitions of 31.33 ± 4.73%, 46.67 ± 1.53%, 66.00 ± 6.56%, and 14.33 ± 14.16%, respectively, while their direct growth inhibitions at higher concentration (3.1 μg/mL) were 17.90 ± 0.29%, 33.43 ± 0.41%, 42.47 ± 0.11%, and 1.30 ± 0.14%, respectively (Figure 2). Compared to PHA (5 μg/mL), PEI, CP, PP, and PR fractions at concentration of 1 μg/mL had higher growth inhibition.

3.4. *Effect of jelly fig achenes fractions on immune-stimulated cytokine expression of U937 cells*

Expression of cytokines (IL-2, IL-10, and IFN-γ) was further observed to study the impact of jelly fig fractions on immunomodulatory function. The result of cytokine expression is shown in Figure 3. The expression of IL-2 after treatment was not significantly different among different jelly fig fractions. There was similar tendency between IL-10 and IFN-γ expression generated by different jelly fig fraction treatments. PP fraction showed the lowest expression and PR fraction showed the highest values in both IL-10 and IFN-γ expression. Although PR fraction (1–100 μg/mL) had higher growth inhibition compared to PHA (5 μg/mL) as shown in Table 3, all cytokine expressions of PP fraction at 100 μg/mL were not significantly different to PHA (5 μg/mL), which indicates that PHA had a more pronounced effect on cytokine expression than growth inhibition.

3.5. *Effect of jelly fig achenes fractions on immune-stimulated cell morphology of U937 cells*

The morphology of U937 cells exposed to jelly fig extracts at their designated concentrations is shown in Figure 4. There was significant cell shrinkage and decrease in density of U937 cells after the PP-CM treatment observed by inverted microscope without staining. Morphological analysis conducted using DAPI staining revealed the presence of nuclei with chromatin condensation and the formation of apoptotic bodies in U937 cells cultured with PP-CM. Beside shrinkage, cells also formed different-sized round clusters or even detached from the media. From Liu's staining results, it showed that PP-CM induced the differentiation from immature U937 cells into mature monocytes/macrophages.

4. Discussion

The PP fraction showed the highest direct antiproliferative activity against U937 cells while the PR fraction showed the

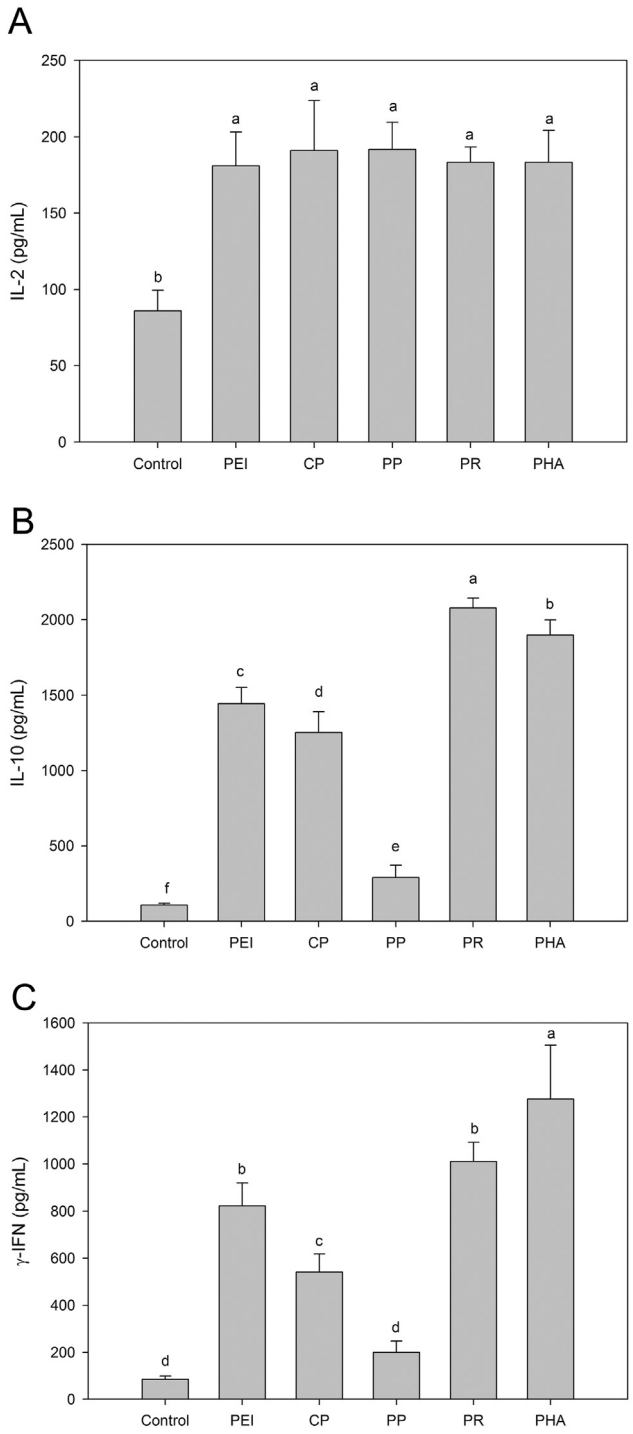

Figure 3 – Cytokine expression. (A) Interleukin-2, (B) interleukin-10, and (C) interferon-γ of U937 monocytoid leukemia cells treated with 100 μg/mL pectinesterase inhibitor (PEI), crude polyphenol (CP), purified polyphenol (PP), and protein (PR) fractions from jelly fig after 72 h treatment. PHA (5 μg/mL) was used as positive control. $^{a-f}$ Value (mean ± standard deviation) with different letters are significantly different ($p < 0.05$).

lowest. The PEI fraction consists of polypeptides with molecular weights of 3.5–4.5 kDa and these positive-charged polypeptide are suspected to be associated with the growth inhibition of U937 tumor cells [10]. However, our study showed that polyphenol contained in the PEI fraction is the major compound involved in the antiproliferative activity against U937 cell. This finding was supported by the result of G2/M cell cycle arrest of the PP fraction on U937 cells, which is the same as the cell cycle arrest found in PEI treatment by Chang et al [10]. Finally, it may be concluded that polyphenols were the compounds involved in the antiproliferative activity against U937 monocytoid leukemia cells.

All fractions had higher immune-stimulated antiproliferative activity than direct antiproliferative activity. Foreign substances could trigger the immunological system in our body [19], thereby exerting their biological actions including antitumor activity [7]. Similar differentiation-inducing effect on U937 cells was observed in the indirect inhibition experiments of polysaccharides from *Monascus purpureus*, *Cordyceps sinensis*, and *Poria cocos* [7,18,20]. Beside polysaccharide, some researches also reported indirect antitumor activity against human myeloid leukemia U937 from nonlectin glycoprotein of *Hypsizygus marmoreus* (Peck) Bigelow mushroom and polyphenol of *Talinum triangulare* leaves and stems [21,22]. This finding showed that polyphenol from the PEI fraction of jelly fig achenes also exerts an immunomodulatory action.

An interesting finding was that the PP fraction showed limited effect on IL-10 and IFN-γ expression. Although the PR fraction had more effect on IL-10 and IFN-γ expression, the anti-proliferation activity determined by MTT assay remained low. Wang et al [23] reported that different extract from the same material can act on different mechanism. The PBS extracts from *Gelidium amansii* gel may initiate innate immunity by increasing macrophage proliferation, NO production, and cytokine secretion, while ethanol extracts have anti-inflammatory activities by suppressing lipopolysaccharide-stimulated tumor necrosis factor-α, IL-1β, and IL-6 secretion [23]. It has been well documented that the IFN-γ acts as a central mediator of the immunoregulatory and antitumor effects [24,25]. However, there are contradicting findings that IFN-γ is both an inducer and an enhancer of induction of human myelogenous leukemia cells [26]. Zaidi and Merlino [27] proposed that IFN-γ is a double-edged sword that can have anti- and protumorigenic activities depending on the cellular microenvironmental or molecular context. It was suspected that the role played by IFN-γ might be protumorigenic activities in this study and PP suppressed this expression of IFN-γ. IL-10 is a potent anti-inflammatory cytokine due to its ability to inhibit production of proinflammatory (IFN-γ and tumor necrosis factor-α) cytokines [28]. However, as humoral immune responses promotor, high IL-10 level in systemic lupus erythematosus patients is considered pathogenic and its blockade ameliorates the disease. Mannino et al [29] also summarized the paradoxical role of IL-10 in immunity and cancer. Thus, the contradicting cytokines expression in this study might be because we observed the combination of immune-modulatory and antitumor activity, not only the single activity. In conclusion, the PP fraction from jelly fig has highest antiproliferative activity against U937 cell and G2/M phase arrest is strongly associated with the U937 cell death and the effect was more pronounced on immune-stimulated than direct antiproliferative activities.

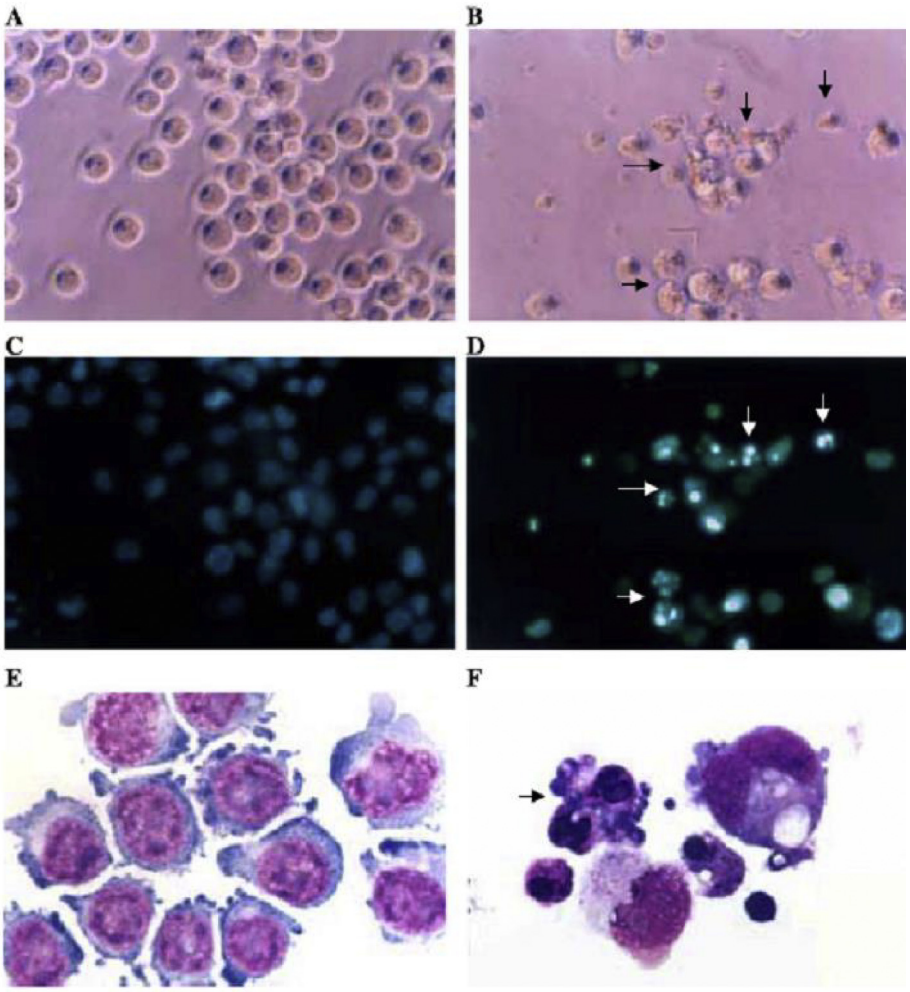

Figure 4 – Morphological of untreated (left row) and 5-day 100 μg/mL purified polyphenol (PP) conditioned medium (CM)-treated U937 cells (right row) observed by inverted microscope at 400× magnification (A, B), confocal microscope using DAPI staining 400× magnification (C, D), and inverted microscope with Liu's staining 1000× magnification (E, F).

Conflicts of interest

All authors have no conflicts of interest to declare.

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