ORIGINAL ARTICLE

Catharanthus roseus aqueous extract induces apoptosis, cell cycle and alters the associated gene expression in Jurkat cells

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ABSTRACT

Introduction: The alkaloids present in *Catharanthus roseus* (*C. roseus*), vinblastine and vincristine are important anticancer agents that cause cell cycle arrest and apoptosis in various types of cell lines. However, there is no previous reports that emphasized the clear mechanisms of anticancer exerted by a crude aquoeus extract of *C. roseus* although it has been historically used to treat various diseases. **Methods:** The cytotoxicity effects of *C. roseus* aqueous extract on Jurkat cells were evaluated by annexin/PI staining, caspase 3/7 assay, JC-1 assay and cell cycle assay. Gene expression profiling was performed by using SmartChip Real-Time PCR system to evaluate the expression profiles of oncology-related genes of Jurkat cells treated with *C. roseus* aqueous extract. **Results:** Flow cytometry analysis revealed that the extract has caused S-phase arrest and associated with apoptosis through the externalization of phosphatidylserine and depletion of mitochondrial membrane potential in time-dependent manner. The apoptosis mechanism was mediated through the activation of caspase 3/7. From the gene expression analysis, 8 differentially regulated genes were associated with apoptosis which were *CDKN1C, CHI3L2, BIRC8, GFER, ID3-1, BBC3-2, TRAF4* and *VCAN*. Meanwhile, 7 differentially regulated genes were associated with cell cycle progression which were *PIMI-1, CDKN1C, SKP1A, CDC25C, LTBP1, CCNG2* and *RBL1*. **Conclusion:** The recent data may facilitate the identification of specific targeting pathways induced by the extract. The information obtained may be used as diagnostic tools, prognostic markers, and predictors of response to *C. roseus* treatment especially for this particular type of cancer.

Keywords: *Catharanthus roseus*; Jurkat; apoptosis; cell cycle; microarray: RT-PCR

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INTRODUCTION

The application of cytotoxic drugs for cancer chemotherapy that can specifically targeting the apoptosis pathways remains the main therapeutic approach in medical oncology (1, 2). The modulation of apoptosis could be highly useful for cancer treatment since all cancer types are closely related with apoptosis especially in carcinoma and leukemia (3-5). More importantly, the initiation of apoptosis will only restrict to their own destruction with the formation of apoptotic bodies without causing any local damaging inflammatory response (6, 7). However, one downside of conventional cancer therapies particularly involving chemotherapy is the side effects. The non-specificity effects of these chemotherapeutic drugs have caused severe toxicity on non-tumour cells and the intensity can be classified from mild to life-threatening or disabling grade. In pro long chemotherapeutic treatment, chronic toxicity effects include infertility, drug resistance and carcinogenicity (8, 9). These toxicity effects remain to be the major limitation of chemotherapeutic agents and therefore, the search for an alternative cancer treatment is a demand to improve the approach of treatment.

Alternative or complementary drugs that can specifically targeting on the cancer cells but able to preserve or improve immune reponse provides significant approach of cancer treatment (10). Many studies have documented about these differential and synergistic effects of active compounds present in plants that have been widely used as a folkloric medicine. According to the drug discovery report provided by World Health Organisation, majority of the world population relies on plant-based therapies due to its natural properties with significant reaction towards cells (11). Herein, we evaluated the effects of a medicinal plant, *Catharanthus roseus* (L) G. Don on the Jurkat cell line, leukemic T-cells. This plant is formerly known as *Vinca rosea* L. (Apocynaceae) and usually called as Madagascar periwinkle. It is a taxonomical group of plant that constitutes more than 130 different terpenoid indole alkaloids (TIAs), which are pharmacologically active molecules (12, 13).

The anticancer alkaloids, vinblastine and vincristine have been proven can induce the apoptosis through caspase-3 activation. Furthermore, previous studies showed that these compounds can cause cell cycle arrest, particularly metaphase arrest by vinblastine (14) and G2/M phase arrest by vincristine (15). However, the effects of crude aqueous extract of *C. roseus* on Jurkat cells were not intensively studied particularly on the gene expression analysis.

It is generally accepted that Jurkat cell line (leukemic T-cell) is an example of an apoptosis model (16). Furthermore, many studies involving T-cell activation have been performed using either the human leukemic Jurkat cell line (17, 18) or primary T cells. This is due to T-cell activation is usually associated with regulation of multiple intracellular signaling events, mediated by numerous kinases activity (19, 20). Our previous study has shown that the crude aqueous extract of *C. roseus* has significantly inhibited proliferation of Jurkat cells (21). Therefore, understanding the complexity of differential expression of genes in regulating the mechanism of apoptosis and cell cycle in Jurkat cells treated with *C. roseus* extract may rule out an effective treatment strategies.

MATERIALS AND METHODS

Reagents

RPMI (Roswell Park Memorial Institute)-1640 was purchased from Sigma-Aldrich (USA). Fetal bovine serum, penicillin–streptomycin and L-glutamine were obtained from Gibco (USA). The kits of Annexin V-FITC/PI apoptosis detection kit II, 5,5', 6,6'- tetrachloro-1, 1', 3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC- 1) mitochondrial membrane potential assay, DNA quality control particles and cycletestTM plus DNA reagent were purchased from BD Biosciences (USA). Caspase 3/7 glo assay kit was purchased from Promega (USA). Vinblastine sulfate was purchased from Tocris (USA) and solubilized in phosphate buffered saline (Sigma-Aldrich, USA) while the camptothecin was purchased from Sigma-Aldrich (USA) and solubilized in dimethyl sulfoxide, DMSO (Sigma-Aldrich, USA) with less than 0.1 % of concentration treated on cells. The High Pure RNA Isolation kit was purchased from Roche, (Germany) and RNA 6000 Nano kit was purchased from Agilent (Germany).

Extraction of plant material

The extraction was performed as according to our previous study (21). Briefly, *C. roseus* plants were collected from Forest Research Institute Malaysia (FRIM), Kuala Lumpur. The identification of the plant with reference number 10933 was verified and deposited at the School of Biological Sciences Herbarium at Universiti Sains Malaysia (USM), Pulau Pinang. The leaves were weighed, washed and dried in a universal hot air oven (Venticell, Germany) at 40 °C. The dried leaves were then grounded, and 50 g of the powdered leaves were mixed with 1 L of distilled water and soaked in a shaker water bath at 40 °C overnight. The mixture was centrifuged at 1560 x g at 25 °C for 15 min. Following the centrifugation, the supernatant was collected and proceed with freeze-drying process using a freeze dryer (Eyela, USA). A concentration of 100 mg/ml working stock was prepared using sterile phosphate buffer saline (PBS) as a solvent.

Cell line

Jurkat cells [American type culture collection (ATCC), USA] were cultured in suspension using RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (v/v), 1% penicillin-streptomycin (v/v) and 1% L-glutamine (v/v). Cells were collected while in the log phase of growth by centrifugation at 400 x g for 10 min, and the pellet was resuspended in RPMI complete growth medium at a concentration of 4 x 10^5 cells/ml. The Jurkat cells were treated with 2.55 µg/ml of *C. roseus* extract (21), 1.5 µg/ml of vinblastine and 4 µM of camptothecin and incubated at various incubation times, as according to the following assays.

Annexin/PI staining

The protocol was performed as described in the manual of Annexin V-FITC/PI apoptosis detection kit II. The Jurkat cells were treated and incubated at 6 and 12 h for early detection of apoptosis. After the incubations, the Jurkat cells were harvested and washed twice with cold PBS. The cells were resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/ml. 100 µl of the solution with a concentration of 1 x 10^5 cells/ml was transferred into a 5 ml culture tube. Then, 5 µl of annexin V-FITC and 5 µl of PI were added and followed by 15 minutes incubation at room temperature (25 °C) in the dark. Afterward, 400 µl of 1X binding buffer was added into each tube and analyzed by for flow cytometer (FACSCalibur, BD Biosciences, USA) with at least 10,000 events per sample. The sample acqusition and analysis were performed by using Cell Quest Pro software.

Cell cycle analysis

The method was performed as described in the manual of cycle test plus DNA reagent kit. The Jurkat cells were treated and incubated at various incubation times which were 6, 12, 24 and 48 h. The untreated cells were used as a control. After each of the incubation time, the cells were harvested and centrifuged at 300 x g for 5 min at room temperature. The supernatant was aspirated and 1 ml of buffer solution containing sodium citrate, sucrose and DMSO was added. The process of centrifugation until the addition of buffer solution was repeated three times. The cell concentration was adjusted to 1.0 x 10^6 cells/ml with buffer solution, followed by centifugation at 400 x g for 5 min at room temperature. The supernatant was discarded and solutions A, B and C were added

accordingly. The optimization of the experimental setting was done by using DNA Quality Control particles prior to sample acquisition. At least 20,000 events were acquired for each sample and analyzed by flow cytometer using CellQuest Pro software. Cell cycle distribution was calculated by using ModFit software (Verity Software House, Topsham, ME).

Caspase-3/7 assay

Caspase-3 and -7 activities were measured by using a caspase-glo 3/7® assay kit. Experiments were carried out following the manufacturer's recommended procedures. The Jurkat cells were seeded in white-walled 96-well plates at a concentration of 4 x 10^5 cells/ml. The treated cells were incubated for 6, 12, 24 and 48 h. The untreated cells were used as a control. Following incubation over the indicated time period, caspase-glo 3/7 reagents were added to each well with ratio of sample to reagent 1:1 and the content was gently mixed with a plate shaker at 70 x g for 30 sec. The plate was subsequently incubated at room temperature for 1 h. Luminescence was measured by luminometer with GloMax®- Multi Detection System (Promega, USA). The luminescence of each sample was measured with parameters of 1 min lag time and 0.5 second/well read time. The caspases activity was presented as the means of experiments conducted in triplicate. The unit of the readings is in Relative Light Unit (RLU). The validation of cell death assay was according to Remacle-Bonnet et al. (2005). The formula used is as follows:

Cell death index = Ft/Fc
Ft = Units of RLU in treated cells
Fc= Units of RLU in control/untreated cells

Measurement of mitochondrial membrane potential (ΔΨm)

Changes in the mitochondrial membrane potential were analyzed with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole-carbocyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨm and forms J aggregates which have characteristic absorption and emission spectra. The procedures were as described in the kit of JC-1 mitochondrial membrane potential detection kit. The Jurkat cells were treated and incubated at 6, 12, 24 and 48 h. The untreated cells were used as a control. After the incubation, the cells were harvested and centrifuged at 400 x g for 10 min. The supernatant was discarded and 0.5 ml of freshly prepared JC-1 working solution was added to each pellet. The cells were resuspended gently, followed by 15 minutes of incubation at 37 °C in a CO2 incubator. The cells were washed twice with assay buffer and centrifuged at 400 x g for 5 min. After the centrifugation, the supernatant was discarded and 0.5 ml of assay buffer was added. The cells were resuspended gently and analyzed by flow cytometer (BD Biosciences, USA) using the Cell Quest Pro software. At least 10,000 events were analyzed for each sample.

Gene expression profiling

The Jurkat cells were treated and incubated at 6, 12 and 24 h. The untreated cells were used as a control. Following the incubation time, the cells were harvested and centrifuged at 300 x g for 10 min at room temperature. The supernatant was discarded and the pellet was washed by suspending with sterile PBS, followed by centrifugation. The pellet was resuspended with 200 µl of sterile PBS and proceed with RNA isolation method.

RNA isolation

The total RNA was isolated from cultured Jurkat cells using High Pure RNA Isolation kit and the method was performed as recommended by the kit manual. The extract was then assayed to determine the quality and the concentration of the RNA using RNA 6000 Nano kit and the method was performed as recommended by the manufacturer's protocol.

Reverse transcription

The RT reaction mix was prepared per 20 µl reaction containing 2 µl of 10 x RT buffer, 0.8 µl of 25 dNTP mix (100 mM), 1 µl of 10 RT random primer, 1 µl of multiscribe reverse transcriptase, 1 µl of RNase inhibitor and 4.2 µl of PCR grade water. Then, 10 µl of diluted RNA sample was loaded into the microcentrifuge containing 10 µl of RT reaction mix, indicating the total 20 µl reaction. For negative control tube, 10 µl of PCR grade water was added. The tubes were loaded into the Tpersonal Thermal Cycler (Biometra, USA).

High throughput Real-Time PCR system

Following the reverse transcription, the RNA samples were successfully transcribed into cDNA. The cDNA samples were ready for polymerase chain reaction to amplify the genes. The master mix was prepared (1000 µl per chip) inside a 1.5 ml of microcentrifuge tube, consisting of 500 µl of 2X SYBR green master mix, 10 µl of 10 mg/ml of BSA, 20 µl of samples of cDNA, 2 µl of yeast cocktail and 468 µl of PCR grade water. The chip details and the weight of the empty chip with or without seal were recorded. Each sample was dispensed immediately to the chip by using SmartChip Nanodispenser (WaferGen Biosystems, USA) standard procedure. The weight of the filled chip was recorded to check on the dispensing efficiency. The chips were then centrifuged at 2000 rpm for 15 min. The chips were loaded into the SmartChip cycler (WaferGen Biosystems, USA) and run by using the optimized thermal profile.

Result analysis

Image analysis and data extraction were carried out using the qPCR software. The cycle during which a reaction emits that threshold level of fluorescence is known as the threshold cycle, abbreviated as Ct. Comparison of the expression of each gene between the treated and untreated samples was determined according to the following formulas:

ΔCt = Ct (Gene) – Ct (All Mean)

$\Delta\Delta Ct = \Delta Ct$ (treated) - ΔCt (untreated)
Fold change = $2^{-\Delta\Delta Ct}$

The ΔCt value is calculated for each gene as the difference between the Ct values of the genes against the average of all Ct in the panel (All Mean). The main purpose of calculating All Mean is to normalize the data produced from the same panel. To determine the changes in gene expression level, the differences (ΔΔCt) between the ΔCt values of an experimental sample (treated) and the control sample (untreated) were calculated. The fold-change used in this gene expression study is equal to 2-ΔΔCt by assuming that the PCR replication efficiency for all genes is 100 percent. The genes with at least two fold changed in expression were considered as significantly differentially expressed in this analysis.

Statistical analysis

The representative data is the mean of triplicate and were presented as mean ± S.D. The data analysis was carried out by using SPSS version 20.0. The comparison between control and treated in a particular incubation was tested for significance different using one-way analysis of variance (ANOVA). Differences with probability value less than 0.05 ($p < 0.05$) were considered as significant.

RESULTS

Flow cytometric analysis as shown in Figure 1 exhibits the percentages of non-apoptotic cells (annexin V-FITC$^-$/PI$^-$), early apoptotic cells (annexin V-FITC$^+$/PI$^-$) and late apoptotic or necrotic cells (annexin V-FITC+/PI+) cells following 6 and 12 h incubations. The *C. roseus* extract at 2.55 µg/ml (IC_{50} value) has significantly ($p < 0.05$) induced apoptosis at 12 h, as compared to control with 9.59% versus 6.26% for early apoptotic cells and 8.04% versus 4.45% for late apoptotic or necrotic cells. The standard apoptotic inducer, camptothecin produced the highest rate late apoptotic cells at 6 h (13.03%) and both early (13.67%) and late apoptotic cells (11.01%) at 12 h. Vinblastine sulfate is a commercial purified compound and vinblastine is one of active anticancer compounds in *C. roseus*. A significant increment of early and late apoptotic cells ($p < 0.05$) were observed at 12 h, and have also shown higher percentages in comparison to C.roseus-treated cells. Overall, only camptothecin has induced apoptosis at 6 and 12 h, while the apoptosis induced by *C. roseus* extract and vinblastine was only observed at 12 h.

The flow cytometric assay showed that *C. roseus* induced cell cycle arrest at the S-phase in a time-dependent manner, as shown in Figure 2. Following 6 h exposure, the cell cycle distribution in each of cell cycle for untreated, cells treated with *C. roseus* and vinblastine showed approximately similar percentage in each phase of cell cycle while the cells treated with camptothecin showed arrest in G_0/G_1 phase. After 12 h, *C. roseus* extract started to cause S-phase arrest while

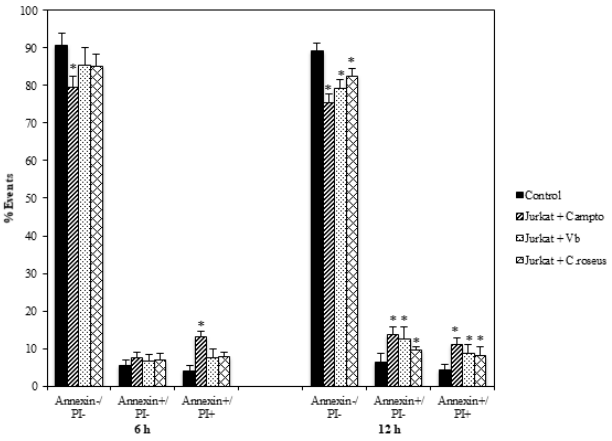

Figure 1: Figure 1: Detection of translocated phosphatidylserine by Annexin V-FITC and PI staining. The Jurkat cells were treated with C. roseus extract, vinblastine, and camptothecin at 6 and 12 h of incubation times and analyzed by flow cytometer. Untreated cells were used as control. The percentages of cell events consist of non-apoptotic cells are (annexin V$^-$/PI$^-$), early apoptotic cells (annexin V$^+$/PI$^-$) and late apoptotic cells or necrotic cells (annexin V$^+$/PI$^+$). Error bars represent mean ± SD from triplicate. Significance value: * $p < 0.05$ compared to untreated cells.

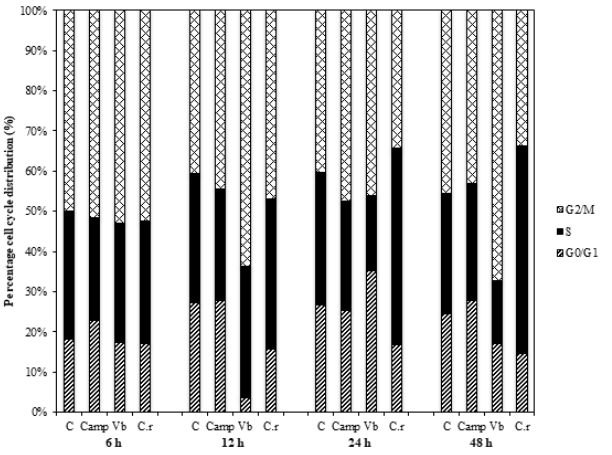

Figure 2: Cell cycle distribution in Jurkat cells. The cells were treated with *C. roseus* extract, vinblastine, and camptothecin for 6, 12, 24 and 48 h. Untreated cells were used as control. Sample acquisition was performed using flow cytometry and further analyzed by ModFit software.

the arrest of G_2/M phase was observed in cells treated with vinblastine with more than 20% difference with control and cells treated with *C. roseus* extract and camptothecin. At 24 h, a higher percentage of S-phase arrest was demonstrated in *C. roseus*-treated cells compared to the untreated while approximately two fold increase as compared to camptothecin-treated cells and vinblastine-treated cells. Vinblastine and camptothecin have caused G_2/M phase arrest. The cells treated with *C. roseus* extract showed the highest percentage of S-phase following 48 h post incubation, which approximately two-fold in comparison with control. Meanwhile the cells treated with vinblastine showed the highest percentage of G_2/M phase. An increased percentage of cells accumulating in the G_0/G_1 phase were observed in camptothecin-treated cells.

As illustrated in Figure 3, only camptothecin has significantly ($p < 0.05$) induced the caspase 3/7 activity at 6 h, in comparison with control. An increased caspase 3/7 activity was significantly observed ($p < 0.05$) in all treated cells (camptothecin, vinblastine and *C. roseus*) at subsequent incubation times which were at 12, 24 and 48 h, as compared to untreated.

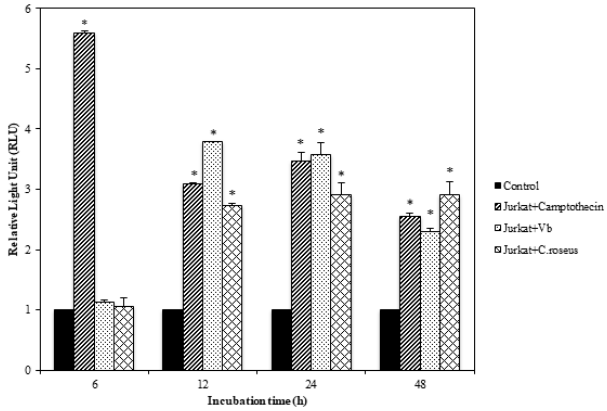

Figure 3: Caspase 3/7 activity in Jurkat cells. The cells were treated with *C. roseus* extract, vinblastine, and camptothecin for 6, 12, 24 and 48 h. Untreated cells were used as control. The caspases activity was determined by glow luminescence and detected by luminometer. Data represents means ± S.D. from triplicate. Significance value: * $p < 0.05$ compared to control.

Figure 4 is the representative histograms for JC-1 stains of Jurkat cells. The data showed that no remarkable changes of the ΔΨm loss in *C. roseus*-treated Jurkat cells at 6 h. The significant changes ($p < 0.05$) were observed at subsequent incubation times, as compared to control. Meanwhile, the camptothecin has remarkably produced the highest ΔΨm loss ($p < 0.05$) in a time-dependent manner. Another positive control, vinblastine sulfate showed no significant changes with control at 6 h. However, the percentages of cells that have lost ΔΨm increased consistently with statistically different in following incubation times.

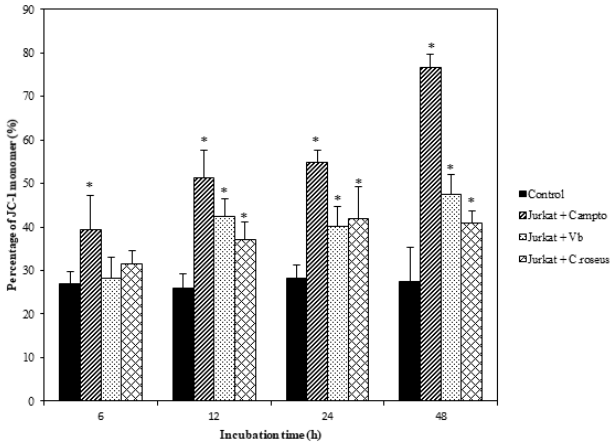

Figure 4: Representative histogram analysis of Jurkat cells by JC-1 staining. The cells were treated with *C. roseus* extract, vinblastine, and camptothecin for 6, 12, 24 and 48 h. Data represents means ± S.D. from triplicate. Significance value: * $p < 0.05$ compared to untreated cells.

Table I has summarized the list of differentially expressed genes, either up or down regulated that associated with apoptosis and cell cycle regulation. For apoptosis, the genes were *CDKN1C, CHI3L2* and *BIRC8* at 6 h; *GFER, ID3-1* and *BBC3-2* at 12 h; *TRAF4* and *VCAN* at 24 h. Meanwhile, for cell cycle regulation which were *PIMI-1, CDKN1C* and *SKP1A* at 6 h; *CDC25C* at 12 h; *LTBP1, CCNG2* and *RBL1* at 24 h.

DISCUSSION

Development of cancer can be due to imbalance between cell cycle arrest and cell death, particularly apoptosis in response to cellular stress in proliferating cells (22). Lack of apoptosis mechanism is believed to play a major role in cancer progression and therapy resistance (23). Therefore, the induction of cell cycle arrest and apoptosis by anticancer agents could have been proposed as efficient mechanisms to prevent the uncontrolled cell proliferation and the survival of the cancer cells (24). Therefore, the potential effects of a crude aqueous extract of *C. roseus* to induce apoptosis and cell cycle arrest on Jurkat cell line were assessed.

Jurkat cell line which is leukemic T cells was selected since the active compounds in *C. roseus*, vinblastine and vincristine have been clinically used to treat blood cancer particularly leukemia and lymphoma. The limited information on the detailed cytotoxic effects of *C. roseus* crude aqueous extract on T cells has led to this study to fill up the gap. The concentration of the treatment used in this study was based on the IC_{50} value obtained in our previous study (21). IC_{50} or half maximal inhibitory concentration can be defined as the measurement of the potency of substances in inhibiting half of the cell proliferation. Since the potency effects was considered as a midpoint value, thus the IC_{50} value was used in this study.

Flow cytometric analysis demonstrated that the percentages of early apoptotic cells in *C. roseus*-treated cells were increased in time-dependent manner, in comparison with untreated cells. This indicates that apoptosis was the preferential cell death induced by *C. roseus* crude aqueous extract in Jurkat cells. Previous study has shown that any external factors or growth factors that affect the rate of cell proliferation are most likely to act by controlling cell cycle progression (25). Therefore we attempted to study the effects of this crude extract on cell cycle distribution.

Our findings have revealed that vinblastine and *C. roseus* aqueous extract have arrested different phases of cell cycle with G_2/M phase affected by vinblastine while S-phase by *C. roseus* extract, respectively. The G_2/M phase arrest induced by vinblastine has been shown in many previous studies (26, 27). However, this is the first report of the S-phase affected by *C. roseus* crude extract. The cell cycle arrest induced by the extract

Table I: The differential expression of genes in Jurkat cells treated with *C. roseus* extract associated with apoptosis and cell cycle regulation

No	Symbol	Description	Chromosome location	GenBank accession number	UniGene	Significance Log 2 (Fold Change)	Up/down regulated
			Apoptosis				
6 h post-incubation							
1	*CDKN1C*	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	11p15.5	NM_000076	Hs.106070	1.8	Upregulated
2	*CHI3L2*	Chitinase 3-like 2	1p13.3	NM_004000	Hs.514840	1.1	Upregulated
3	*BIRC8*	baculoviral IAP repeat containing 8	19	NM_033341	Hs.348263	-1.7	Downregulated
12 h post-incubation							
4	*GFER*	growth factor, augmenter of liver regeneration	16p13.3-p13.12	NM_005262	Hs.732148	1.2	Upregulated
5	*ID3-1*	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1p36.13-p36.12	NM_002167	Hs.76884	-1.0	Downregulated
6	*BBC3-2*	BCL2 binding component 3	19q13.3-q13.4	NM_014417	Hs.467020	-2.2	Downregulated
24 h post-incubation							
7	*TRAF4*	TNF receptor-associated factor 4	17q11-q12	NM_004295	Hs.8375	1.1	Upregulated
8	*VCAN*	versican	5q14.3	NM_004385	Hs.643801	-2.2	Downregulated
			Cell cycle				
6 h post-incubation							
1	*PIM1-1*	pim-1 oncogene	6p21.2	NM_002648	Hs.81170	2.6	Upregulated
2	*CDKN1C*	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	11p15.5	NM_000076	Hs.106070	1.8	Upregulated
3	*SKP1A*	S-phase kinase-associated protein 1	5q31	NM_006930	Hs.171626	-1.4	Downregulated
12 h post-incubation							
4	*CDC25C*	cell division cycle 25 homolog C	5q31	NM_001790	Hs.656	1.0	Upregulated
24 h post-incubation							
5	*LTBP1*	latent transforming growth factor beta binding protein 1	2p22-p21	NM_206943	Hs.619315	1.3	Upregulated
6	*CCNG2*	cyclin G2	4q21.1	NM_004354	Hs.13291	1.0	Upregulated
7	*RBL1*	retinoblastoma-like 1 (p107)	20q11.2	NM_002895	Hs.207745	-1.0	Downregulated

may rule out the necrosis as a probable cause of cell death. Necrotic cells with degraded DNA have lesser DNA content and distributed across the same region of the flow cytometric histograms. Comparatively, early apoptotic cells have accumulated DNA content and often characterized as a distinct peaks in the histograms of the cell cycle distribution (28). Therefore, our findings clearly demonstrated that apoptosis is most likely to play a part in mediating cell death mechanism induced by the extract. The S-phase arrest induced by *C. roseus* extract suggests that the extract contains compounds that inhibit or disrupt the synthesis of DNA during cell replication that occurs in S-phase. In addition, different phases of cell cycle arrest induced either by vinblastine or *C. roseus* extract shows that there was other compound or more apart from vinblastine that responsible for the S-phase arrest.

The pathway of apoptosis initiated by the *C. roseus* extract was further examined. Caspases are a family of protease involved as the central component of a proteolytic system in the mechanism of apoptosis. These enzymes play a role in a cascade that results in cell disassembly in response to the pro-apoptotic signals (29). Caspase 3 and 7 are executioner caspases and responsible for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly ADP ribose polymerase (PARP), an important protein involved in DNA repair, maintenance of chromosomal stability, and programmed cell death (30, 31). The result showed that *C. roseus* extract started to significantly induced the activation of caspases 3 and 7 in Jurkat cells cells at 12 h. These findings suggested that the active constituents in the crude *C. roseus* extract have activated the caspase 3/7 activity within 6 to 12 h.

The disruption of mitochondria is presumed to be due to changes in the membrane potential and oxidation-reduction potential of the mitochondria, resulting in the activation of caspase activity (32-35). Our data demonstrated that the apoptosis mechanims was accompanied with the MMP depletion in the *C. roseus*-treated cells. Our studies demonstrated that treatment with the extract increased MMP loss in time-dependent manner, in comparison with untreated cells. Thus, we

can postulate that the apoptosis was mitochondrial pathway with caspase-dependent.

In present study, the gene expression profiles associated with the regulation of apoptosis indicated that 8 genes were differentially expressed in Jurkat cells in response to the *C. roseus* aqueous extract. A predicted diagram of the apoptosis pathway realted with these genes is as shown in Figure 5. The differentially expressed genes that may correlate and support the apoptosis functional analysis were the upregulated *CHI3L2* at 6 h, upregulated *GFER* at 12 h and upregulated *TRAF4* at 24 h.

The roles of *CHI3L2* have been widely discussed in many studies that have shown this gene acts as proliferative, anti-apoptotic, migration and adhesion factor in various types of cells (36-38). This gene might thus, responsible for the inhibition of apoptosis at 6 h. Following 12 h of incubation, the apoptosis was significantly observed through the externalized PS, activation of caspase 3/7 activity and MMP loss. It seemed like these apoptosis events have been mediated by upregulated *GFER*. According to the previous in vivo study, the upregulation

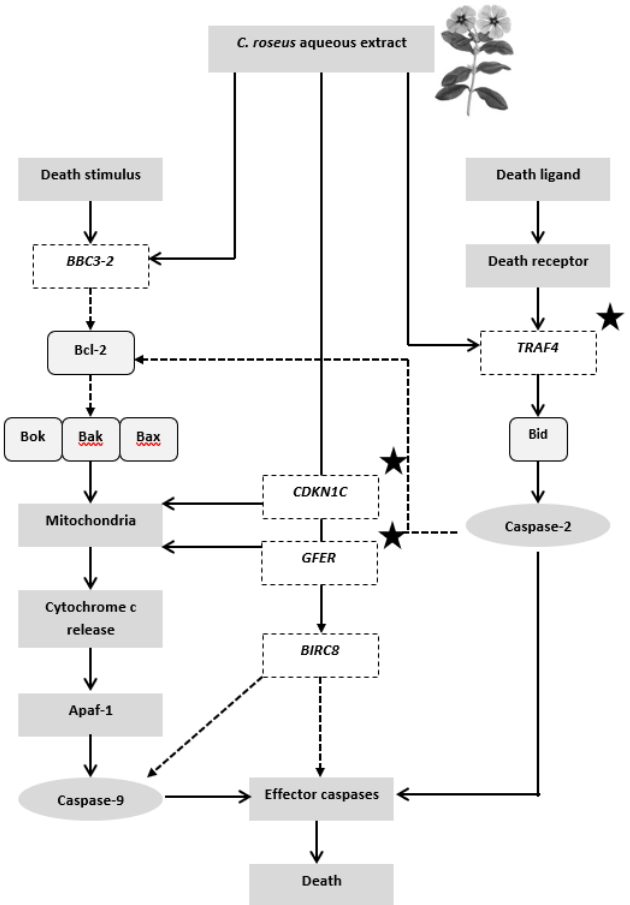

Figure 5: Regulation of apoptosis by differentially expressed genes in C. roseus-treated Jurkat cells. The mitochondrial pathway with caspase dependent was regulated by *BBC3-2, CDKN1C, GFER* and *BIRC8* while the apoptosis associated with death ligand was regulated by *TRAF4*. Bcl-2 is anti-apoptotic protein and the Bok, Bak and Bax are pro-apoptotic proteins. The star symbol labeled next to the gene indicates its involvement in induction of apoptosis. The dashed line indicates inhibition while the straight line indicates activation

of *GFER* gene in mouse ESCs caused the reduction of pluripotency marker gene expression, loss of MMP, excessive fragmentation of mitochondria, elimination of damaged mitochondria through autophagy (mitophagy) and caspase-induced apoptosis (39). Some of these roles are in agreement with the present functional assays that indicated the *C. roseus* has led to MMP loss associated with the activation of caspase 3/7 activity at 12 h. Additionally, the gene expression profile has shown that the upregulation of *GFER* is the only pro-apoptosis gene that has been differentially expressed at 12 h, which further proven its involvement in apoptosis. Further study is required to confirm the central role of *GFER* gene in growth inhibitory effects of *C. roseus*-treated Jurkat cells at 12 h. At 24 h, the gene profile demonstrated that the apoptosis was most likely associated with the upregulation of TRAF4. TRAF4 is one of the protein that involves in the formation of death-inducing signaling complex (DISC) in extrinsic pathway of apoptosis through the initiation of the caspases cleavage and cytochrome c release from the mitochondria (40). The induction of apoptosis through the activation of caspases is in parallel with our findings that suggested a continuous activation of caspase 3/7 activity and MMP loss in Jurkat cells in response to the *C. roseus* treatment at 24 h.

The anti-proliferative effects induced in *C. roseus*-treated Jurkat cells have been shown due to the mechanism of apoptosis and S-phase arrest in the cell cycle distribution. The gene expression profile suggested that the upregulated *CDKN1C* and downregulated *SKP1A* at 6 h, downregulated *RBL1*, upregulated *CCNG2* and *LTBP1* at 24 h might involve in cell cycle arrest at various phases. On the other hand, the upregulated *PIM1* at 6 h and upregulated *CDC25C* at 12 h might accelerate the cell cycle progression. The overall cell cycle mechanism regulated by the related differentially expressed genes in *C. roseus*-treated Jurkat cells is shown in Figure 6.

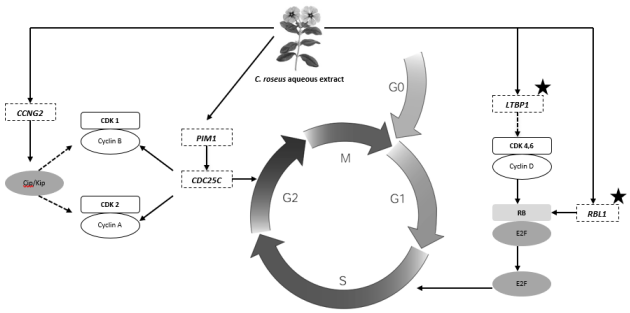

Figure 6: Regulation of cell cycle by differentially expressed genes in C. roseus-treated Jurkat cells. The acceleration of cell cycle was regulated by *PIM1* and *CDC25C*. *PIM1* initiates the activation of CDC25C for the progression G_2 to M phase. The cell cycle arrest was regulated by *LTBP1, RBL1* and *CCNG2*. *LTBP1* inhibits the CDK complex responsible for the S phase gene expression. The downregulated RBL1 may prevent the formation of Rb complex for the S phase progression. *CCNG2* gene inhibits the cell cycle from G_1-S and G_2-M phases of cell cycle. The star symbol labeled next to the gene indicates its involvement in S-phase arrest. The dashed line indicates inhibition while the straight line indicates activation.

Of particular interest amongst the differentially expressed genes causing S-phase arrest is the association between the upregulated *LTBP1* and downregulated RBL1 following 24 h incubation. *LTBP1* gene encodes protein that is closely related with transforming growth factor (TGF) because the TGF requires selective proteins, such as *LTBP1* for its activation, leading to the inhibition of the cyclin-dependent kinase complex responsible for retinoblastoma protein (*RBL1*) phosphorylation that involve in cell cycle progression through the expression of S phase gene (41, 42). The inhibition of the cyclin-dependent kinase and the downregulation of *RBLI* gene may together leading to an inhibition of the S-phase progression. Likewise, the cell cycle study demonstrated that the *C. roseus* extract has remarkably caused S-phase arrest with approximately two-fold increase as compared to control. Therefore, the upregulated of *LTBP1* and downregulated *RBLI* are most likely contributing to a significant S-phase arrest at 24 h.

The contradict mechanism regulated by other apoptosis and cell cycle related genes remain unaswered. However, we speculate that it may be due to various compounds present in the crude *C. roseus* extract. The bioactivity of the extracts may not be solely dependent on a single compound, contributing to a relatively complex mechanism of action. Additionally, it also can be due to the fact that the cascade of apoptosis and cell cycle pathway involves a series of activation of various proteins (43). Hence, the differential expression of a particular gene may not necessarily indicate an induction or inhibition of these mechanisms.

CONCLUSION

Our study has shown that the aqueous extract of *C. roseus* exerted anticancer activity by the induction of apoptosis through caspases activation. Moreover, it affects the cell cycle distribution indicating that the extract has inhibited the growth and proliferation of the Jurkat cells through S-phase arrest. The gene expression profile represents an endless source of data that may be transformed into useful clinical information. Understanding how these pathways can be modulated may thus provide new opportunities to develop effective therapeutics for various diseases, particularly for leukemic patients. The identification of gene signatures with prognostic and predictive value may eventually lead to the discovery of novel cancer target. However, further studies on the normal cells as well as other cancer cell lines are required to rule out the cytotoxicity of *C. roseus* aqueous extract. Additionally, the active compounds responsible for the potency should be identified and must show tolerable level of toxicity in animal models.

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