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Original article

Anticancer effects of an extract from a local planarian species on human acute myeloid leukemia HL-60 cells *in vitro*

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ABSTRACT

Current anti-cancer drugs can cause many undesirable side effects to patients. Thus, there is a constant need to develop alternative therapeutic drugs. Bioactive compounds derived from natural products including animals, plants and microorganisms are being actively studied as sources for anticancer treatments. Freshwater planarians are important models for stem cell research and regeneration. However, to date, no studies on the biological activities of planaria extracts on cancer have been published. The aim of this study was to examine the potential antitumoral activity of the extract from planaria species-Malta (PSM) on human acute myeloid leukemia (AML) HL-60 cells. Antiproliferative activity was studied in terms of proliferation, apoptosis and differentiation. The expression of genes involved in the regulation of these important cellular processes was also analyzed using real-time PCR. PSM extract exhibited a selective cytotoxic effect on HL-60 cells when compared to normal lymphocytes. Furthermore, cell cycle analysis and Annexin V/PI assay showed that the extract induced apoptosis in HL-60 cells. The PSM extract induced myeloid differentiation with HL-60 cells showing a decreased nucleo/cytoplasmic ratio, an increase in nitroblue tetrazolium-positive cells, and CD11b- and CD14-positive cells. Finally, we also found that the PSM extract increased the expression of *CEBPA*, *CEBPB*, *CEBPE*, *SPI1*, *BAX*, *CDKN1A* and *CDKN2C*; whereas it reduced the expression of *c-MYC* and *BCL2*.

This is the first study to reveal the antiproliferative, cytotoxic, and differentiation potential of PSM on HL-60 cells and suggests that it may have considerable potential for development as a novel natural product-based anticancer agent against AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of diseases characterised by impaired differentiation and clonal expansion of myeloid progenitors in both the peripheral blood and bone marrow (BM) [1]. In the USA, the age-adjusted incidence of AML is 4.3 per 100,000 with a mean age of 68 years [2]. The overall 5-year survival rate for all AML sub-types with the best available treatment is about 60–75 % for children, 50–60 %, in young patients (15–39 years of age), while in older patients it is approximately 10 % [3]. Even in patients who achieve remission with chemotherapy, relapse occurs in two thirds of them due to chemo-resistance and the persistence of leukemic cells below morphologic detection [4,5]. Moreover, the unsuccessful treatment and persistence of residual cancer stem cells in leukemia may be attributed to the BM microenvironment niches in which these cells are sequestered and protectedfrom the hosts immune response.

Treating acute promyelocytic leukemia (APL) with the differentiating agents, all-trans retinoic acid (ATRA) and arsenic trioxide, has greatly improved prognosis [6]. Therefore, the strategy of inducing malignant cells to overcome their differentiation block and enter the apoptotic pathways has become an attractive alternative to classical cytotoxic chemotherapeutic drugs [7]. In that respect, significant emphasis has been placed on identifying new agents, mainly from natural sources, that are relatively non-toxic to normal cells and effective against cancer cells.

Natural products have been identified as a source of drugs for

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treatment of different diseases including cancer. Natural products derived from plants, marine animals, or microorganisms possess anti-inflammatory, anti-viral and anti-cancer activities. It was reported that 74.8 % of currently used anti-cancer drugs such as taxanes, vinca alkaloids, and the camptothecin class of compounds are derived from natural products [8]. Hence, screening of natural products for novel cancer therapeutic drugs is of utmost importance.

Planarians are important models for stem cell research and regeneration [9]. However, according to our knowledge, there are no studies on the biological activities of these organisms on cancer cells. Regenerative tissues are generally resistant to carcinogenic compounds [10] and the fact that planarians are easily available in nature makes them potentially useful in the development of potential oncology drugs, including agents which induce differentiation and apoptosis. This present work examines the potential antitumoral activity of a Planaria crude extract from a Maltese species on the HL-60 AML human cell line.

2. Materials and methods

2.1. Reagents and antibodies

Dimethyl sulphoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), bisBenzimide H 33342 trihydrochloride (Hoechst 33342), Nitroblue tetrazolium (NBT) and other principal chemicals were purchased from Sigma-Aldrich (Milan, Italy). Roswell Park Memorial Institute (RPMI) 1640 Medium and fetal bovine serum (FBS) were purchased from BIOWEST (Meda, MB, Italy). FITC Annexin V Apoptosis detection kit, antibodies to CD11b, CD14 and corresponding isotype control antibodies were purchased from BD Pharmingen (Buccinasco, Milan, Italy).

2.2. Sample collection and extract preparation

Live planarians, were collected from a woodland area in Malta (Buskett forest). The extract preparation was adapted from McGann et al. [11]. On day 0, starved planarians (n = 40) were placed in a plastic container containing artificial pond water. Planarians were transferred to a sterile 50 mL tube and all manipulations were performed on ice and away from direct sunlight. The planarians were suspended in 10 mL of ice-cold RPMI-1640 medium with inactivated FBS and a mixture of three protease inhibitors (2 μg/mL leupeptin/2 μg/mL Aprotinin/1 mM PMSF). Organisms were homogenized for 1 min and sonicated for 30 s. The homogenate was transferred to sterile 2 mL centrifuge tubes and cell debris was removed by centrifugation and filter-sterilisation. The protein content was assayed using the DC protein assay kit II (Bio-Rad, Laboratories, Milan, Italy) according to manufacturer's protocol. All extracts were stored in 1 mL aliquots in a −80 °C freezer. This extract will be herein referred to as Planaria species – Malta (PSM) extract.

2.3. Cell culture

Human acute myeloid HL-60 cell line (ATCC-CCL-240) was supplied by the America Type Culture Collection (ATCC, Manassas, VA, USA). White cells (buffy coats) of consented healthy donors were collected from the Malta National Blood Transfusion Center. Human peripheral blood lymphocytes were isolated from the buffy coats using density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich) at 500 g and 20 °C for 25 min. Lymphocytes were stimulated to proliferate by addition of 1% phytohaemagglutinin (PHA) (10 mM; Sigma-Aldrich, Milan, Italy).

Both cell types were cultured in RPMI 1640 medium supplemented with 10 % heat-inactivated FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma-Aldrich) at 37 °C in a humidified atmosphere and 5% CO_2.

2.4. Cell proliferation and viability

HL-60 cells (1×10^5/well) were seeded in 48-well plates and treated with different concentrations (1, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) of PSM extract for 24 − 72 h. Total cell number and viability were evaluated by Trypan blue exclusion assay. Additionally, IC_{50} values were calculated for both HL-60 cells and lymphocytes following PSM extract treatment, using the online tool Quest Graph™ IC50 Calculator (AAT Bioquest, Inc., Sunnyvale, CA, USA) [12] to elicit the selectivity index (SI), which describes the selectivity of a compound toward the cancer cells [13]. It was calculated based on the ratio of IC_{50} of proliferating lymphocytes and HL-60 cells.

2.5. Morphological examination for apoptosis and differentiation

HL-60 cells (2×10^4/well) were seeded in 96-well plates and treated with PSM extract (62.5 μg/mL), DMSO (1.6 and 10 %) or PMA (10 nM) (Sigma-Aldrich) for 48 h.

Cells were stained with Hoechst 33342 (5 μg/mL medium) (Sigma-Aldrich) for 15 min at 37 °C and visualized using an appropriate filter. Cells were evaluated on the basis of their nuclear morphology, noting the presence of homogeneous chromatin, condensed chromatin, and fragmented nuclei. In addition, cytospin preparations stained with Leishman's stain were used to confirm apoptosis and/or differentiation.

2.6. Flow cytometry analysis for cell cycle distribution and apoptosis

HL-60 cells (1×10^5/well) were seeded in 48-well plates and treated with PSM extract (62.5 μg/mL), DMSO (1.6 and 10 %) or PMA (10 nM) for 48 h. For DNA staining, cells were collected, centrifuged, washed with 1 × PBS and fixed with 70 % ethanol overnight at 4 °C. They were then washed twice with 1 × PBS, digested with 100 μL RNAase A (100 μg/mL; for 1 h at 37 °C, and stained with 200 μL PI (100 μg/mL; (Sigma-Aldrich) in the dark for 15 min at 4 °C.

Apoptosis was evaluated using FITC Annexin V Apoptosis Detection Kit (BD Pharmingen, Buccinasco, Milan, Italy) according to manufacturer's instructions. Briefly, cells were collected, centrifuged, washed twice with 1 × PBS and resuspended in 1X Annexin V binding buffer at a concentration of 1×10^6 cells/mL. Then, 100 μL of cell suspension was incubated with 5 μL of Annexin V-FITC and 5 μL of PI for 15 min at a room temperature in the dark. Viable cells were negative for both annexin-V and PI (annexin V^-/PI^-; lower left quadrant), early apoptotic cells were positive for annexin-V staining (annexin V^+/PI^-; lower right quadrant), late apoptotic/necrotic cells were positive for both annexin-V and PI staining (annexin V^+/PI^+; upper right quadrant) and necrotic cells were positive for PI staining (annexin V^-/PI^+; upper left quadrant).

All flow cytometry analyses were performed by FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK), and the data were analyzed utilizing Cell Quest software. At least 1×10^4 events were acquired for each sample.

2.7. Nitroblue tetrazolium (NBT) reduction test

HL-60 cells (2×10^4/well) were seeded in 96-well plates and treated with PSM crude extract (62.5 μg/mL) or DMSO (1.6 %, as a positive control) for 48 h. Next, cells were incubated with 1 mg/mL NBT solution at 37 °C for 30 min. Then, the percentage of cells containing intracellular blue-black formazan deposits were determined. In each count, at least 200 cells were inspected using the EVOS FL Auto 2 Cell Imaging System.

2.8. Flow cytometric analysis of CD14 and CD11b

HL-60 cells (1×10^5 cells/well) were seeded in 48-well plates and treated with PSM crude extract (62.5 μg/mL), DMSO (1.6 %) or PMA

Table 1
Taqman® Gene Expression Assays.

GENE	ASSAY ID	CATALOG NUMBER
BCL2	Hs00608023_m1	4331182
BAX	Hs00180269_m1	4331182
CDKN1A	Hs00355782_m1	4331182
CDKN2C	Hs00176227_m1	4331182
c-MYC	Hs00153408_m1	4331182
SPI1	Hs02786771_m1	4331182
CEBPA	Hs00269972_s1	4331182
CEBPB	Hs00270923_s1	4331182
CEBPE	Hs00357657_m1	4331182
HPRT1	Hs02800695	4331182
YWHAZ	Hs01122445_g1	4331182

(10 nM) for 48 h. Cells were collected, centrifuged, washed twice with pre-cold 1 × PBS and incubated with direct Alexa Fluor 488-labeled anti-CD11b antibody or PE- labelled anti-CD14 antibody (or isotype control antibodies) on ice on a rotating platform in the dark. After 30 min., cells were washed twice and detected by flow cytometry and the data analyzed as above. Expression of cell marker was determined by comparison with isotype control.

2.9. RNA extraction and real-time RT-PCR

Total RNA was isolated using RNeasy Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The concentration of RNA in each sample was determined using the Nanodrop 2000 instrument (Thermo Fisher Scientific, Waltham, USA).

Complementary DNA (cDNA) was produced from 300 ng of RNA from each sample using the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan™Gene Expression Assays (Applied Biosystems.Foster City, California, USA) (Table 1). Two microliters of the diluted c-DNA were amplified in a final volume of 25 µL with 1X TaqMan™ Gene Expression Master Mix. Thermal cycling proceeded for 2 min at 95 °C and 40 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. All real-time PCR reactions were performed in triplicate. To ensure no contamination with genomic DNA, a no reverse transcriptase control (no RT) was included during each run of real-time RT-PCR. A negative control (no cDNA) was also performed. Real-time PCR and data collection were performed on a BioRad CFX-96 thermocycler (Bio-Rad). The relative expressions of mRNAs were calculated using the comparative $2^{-\Delta\Delta Ct}$ method [14] and normalized against the geometric average expression *HPRT1* and *YWHAZ* housekeeping genes [15].

2.10. Statistical analysis

All experiments were performed in triplicate and repeated at least twice. The SPSS statistics software (version 20) was used for statistical analysis. Data are presented as the median and the inter-quartile range (IQR). Normal distribution was assessed by Kolmogorov-Smirnov test. Since data were not normally distribution they were analyzed using the non-parametric Kruskal-Wallis test, followed by Dunn-Bonferroni's *post hoc* analysis. Significance was accepted at a $p < 0.05$.

3. Results

3.1. Planaria extract reduces the growth rate and viability of HL-60 cells

First, we evaluated the potential cytotoxic effects of PSM crude extract on HL-60 cells and lymphocytes by trypan blue assay. HL-60 cells were treated for various times (24 − 72 h) with various doses of PSM extract (1–1000 µg/mL).

As shown in Fig. 1A and B, PSM extract significantly reduced the growth rate of HL-60 cells, even at 24 h, but only at 48 h was a quasi-dose-dependent effect observed with growth inhibition of 18, 51, 64, 71, 73 and 84 % for 31.2, 62.5, 125, 250, 500 and 1000 µg/mL, respectively compared to untreated cells. We also found that growth inhibition caused by PSM extract treatment is, only partly, due to a decreased viability (Fig. 1C). Interestingly, PSM extract exhibited selective cytotoxic effects on the HL-60 cells at 48 h, compared to activated lymphocytes (Fig. 1D). In fact, the IC_{50} value of the extract in HL-60 cells was 61.037 µg/mL, while the IC_{50} value of the extract activated Lymphocytes was 162.53 µg/mL (Table 2).

Based on the obtained data, all subsequent experiments were performed by incubating HL-60 cells with the concentration of 62.5 µg/mL for 48 h.

3.2. Planaria extract induces differentiation and apoptotic morphological changes of HL-60 cells

To elucidate the mechanism by which PSM extract reduced cell proliferation and viability in HL-60 cells, morphological analyses were performed. In these experiments, HL-60 cells incubated with DMSO (10 %), DMSO (1.6 %) and PMA (10 nM) were used as positive controls for apoptosis and differentiation (granulocytic and monocytic), respectively. As evidenced by phase-contrast microscopy (Fig. 2A), with respect to untreated cells, incubation with PSM extract (62.5 µg/mL) induced a decrease in the cell number and morphological changes indicative of apoptosis and differentiation. As shown in Fig. 2B, fluorescent microscopy by Hoechst 33342 staining shows that after incubation with the extract, some cells exhibited typical signs of apoptosis such as chromatin condensation and fragmentation. To examine the effect of PSM extract on the differentiation of HL-60 cells, we also analyzed the morphological changes by Leishman staining. As shown in Fig. 2C, untreated cells showed typical myeloid leukemia blast morphology with large nuclei, small cytoplasm, regular cell membrane and large nuclear/cytoplasmic ratio, while PSM extract reduced the nuclear/cytoplasmic ratio in HL-60 cells.

3.3. Planaria extract induces apoptosis and increases the level of sub-G0/G1 of HL-60 cells

To further explore the effect of PSM extract on growth and cell death of HL-60 cells flow cytometry analysis of PI-stained nuclei and Annexin V/PI staining were performed, respectively. In these experiments, HL-60 cells incubated with DMSO (10 %), DMSO (1.6 %) and PMA (10 nM) were used as positive controls for apoptosis and differentiation (granulocytic and monocytic), respectively. As shown in Fig. 3A and B, DNA contents from flow cytometry analysis indicated that among the untreated HL-60 cells, 46.2 % were distributed in G0/G1 phase, 38.1 % were accumulated in S phase, 13.1 % were in G2/M phase and only 2.6 % were sub-G0/G1. When HL-60 cells were incubated with PSM extract (62.5 µg/mL) for 48 h, the sub-G0/G1 peak increased (16.1 %) and the S phase population decreased.

As shown in Fig. 4A and B, flow cytometry analysis showed that PSM extract promoted apoptosis. In fact, after being treated with PSM extract for 48 h, 22.38 % apoptotic cells (early plus late apoptotic cells) were found in HL-60 cells, which were significantly higher than that of untreated cells (6.24 %, $p < 0.05$).

3.4. Induction of differentiation in HL-60 cells after treatment with Planaria extract

In order to support the contention that PSM extract induces myeloid differentiation in HL-60 cells, the NBT assay, a reliable marker for differentiation of myeloid leukemia cells [16], and flow cytometry analysis for myelomonocytic markers CD11b and CD14 [17] were performed. In these experiments, HL-60 cells incubated with DMSO (1.6 %) and PMA (10 nM) were used as positive controls for granulocytic and monocytic differentiation, respectively.

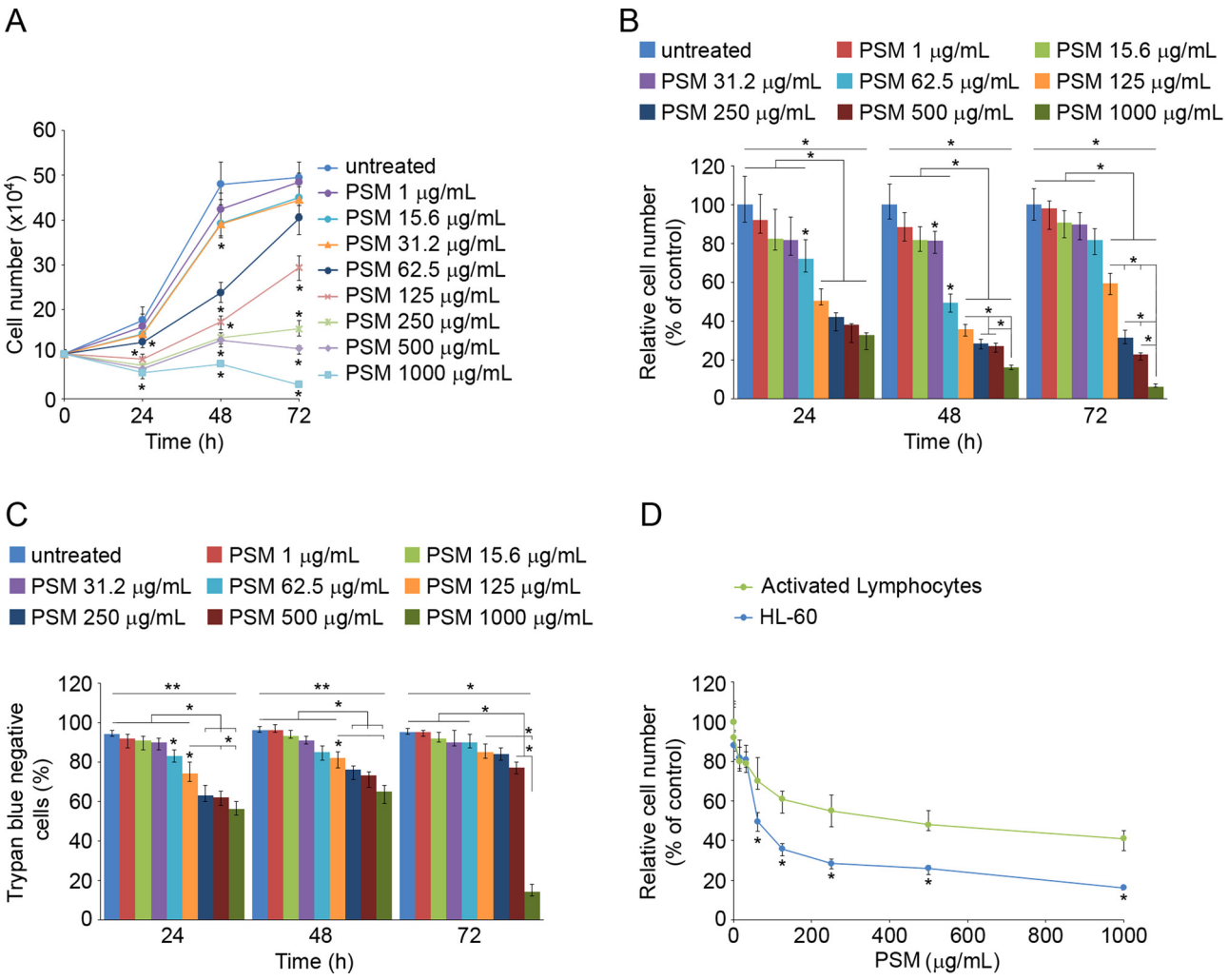

Fig. 1. Effects of PSM extract on cell number (A), relative cell growth (percentage cell number of PSM treated cells relative to untreated cells) (B) and cell viability (C) of HL-60 cells. Cells were treated with PSM extract as indicated for 72 h. (D) Effects of PSM extract on Lymphocytes and HL-60 cells after 48 h treatment. The number of viable cells was determined by trypan blue exclusion. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analyzed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

Table 2
Antiproliferative effects and selectivity of PSM toward HL-60 cells at 48 h.

Compound	HL-60 IC_{50} (µg/mL)	Activated lymphocytes IC_{50} (µg/mL)	SI
Planaria Species-Malta extract	61.037	162.593	2.7[a]

[a] Selectivity Index (SI), calculated based on the ratio of IC_{50} activated lymphocytes and the HL-60 cells.

As shown in Fig. 5A, NBT positive cells, containing blue NBT formazan deposits, were increased after treatment by PSM extract (62.5 µg/mL) for 48 h. Moreover, PSM extract determined an increase in the presence of both CD14- and CD11b-positive cells (Fig. 4B and C).

3.5. Effects of Planaria extract treatment on gene expression in HL-60 cells

We also investigated the molecular mechanisms by which the PSM extract could inhibit proliferation and induce apoptosis and differentiation in the HL-60 cell line. For this purpose, we performed real-time RT-PCR analysis to evaluate the level of expression of genes involved in the regulation of differentiation (*CEBPA*, *CEBPB*, *CEBPE*, *SPI1* and *c-MYC*), apoptosis (*BCL2* and *BAX*) and cell cycle (*CDKN1A* and *CDKN2C*), which are frequently deregulated in leukemia [18–21]. In these experiments, HL-60 cells incubated with DMSO (1.6 %) and PMA (10 nM) were used as positive controls for apoptosis and differentiation, respectively.

As shown in Fig. 6A–C, the treatment with PSM extract (62.5 µg/mL), compared with the untreated counterpart, markedly increased the expression of *CEBPA*, *CEBPB*, *CEBPE*, *SPI1*, *BAX*, *CDKN1A* and *CDKN2C*; whereas strongly reduced the expression of *c-MYC* and *BCL2* after 24 h. The figures also show that, after 48 h, the treatment with PSM extract further increased the expression of *CEBPE* and *BAX*, and decreased *BCL2* and *CDKN1A* expression; whereas the levels of the other genes, except for *CDKN2C*, did not show significant changes compared with untreated counterpart.

4. Discussion

Increasing evidence from cancer studies indicate that bioactive compounds derived from natural sources including animals, plants and microorganisms may act as potentially promising therapeutic agents in the treatment of human cancers [22–25]. In this study, the PSM crude extract exhibited apoptotic and differentiation-inducing activity in human HL-60 cells, a model system for studying human myeloid cell differentiation.

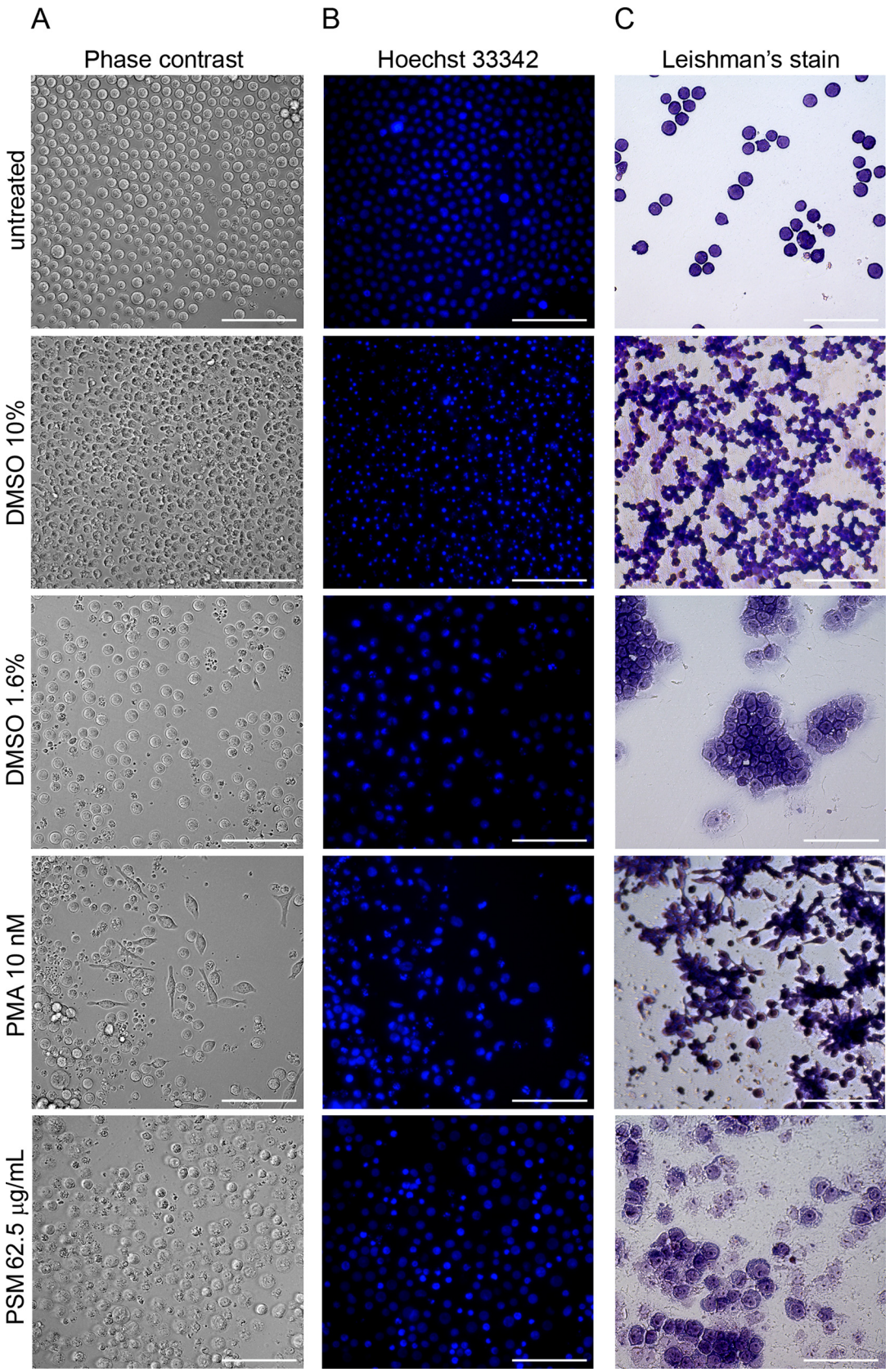

Fig. 2. Morphological changes of HL-60 cells treated with 62.5 µg/mL of PSM extract after 48 h. (A) Phase contrast microscopy images, (B) fluorescence microscopy images after staining with Hoechst 33342 and (C) Leishman staining assay. HL-60 cells incubated with DMSO (10 %), DMSO (1.6 %) and PMA (10 nM) were used as positive controls for apoptosis, granulocytic and monocytic differentiation, respectively. The scale bar is 100 µm. The images were a representative of three independent experiments.

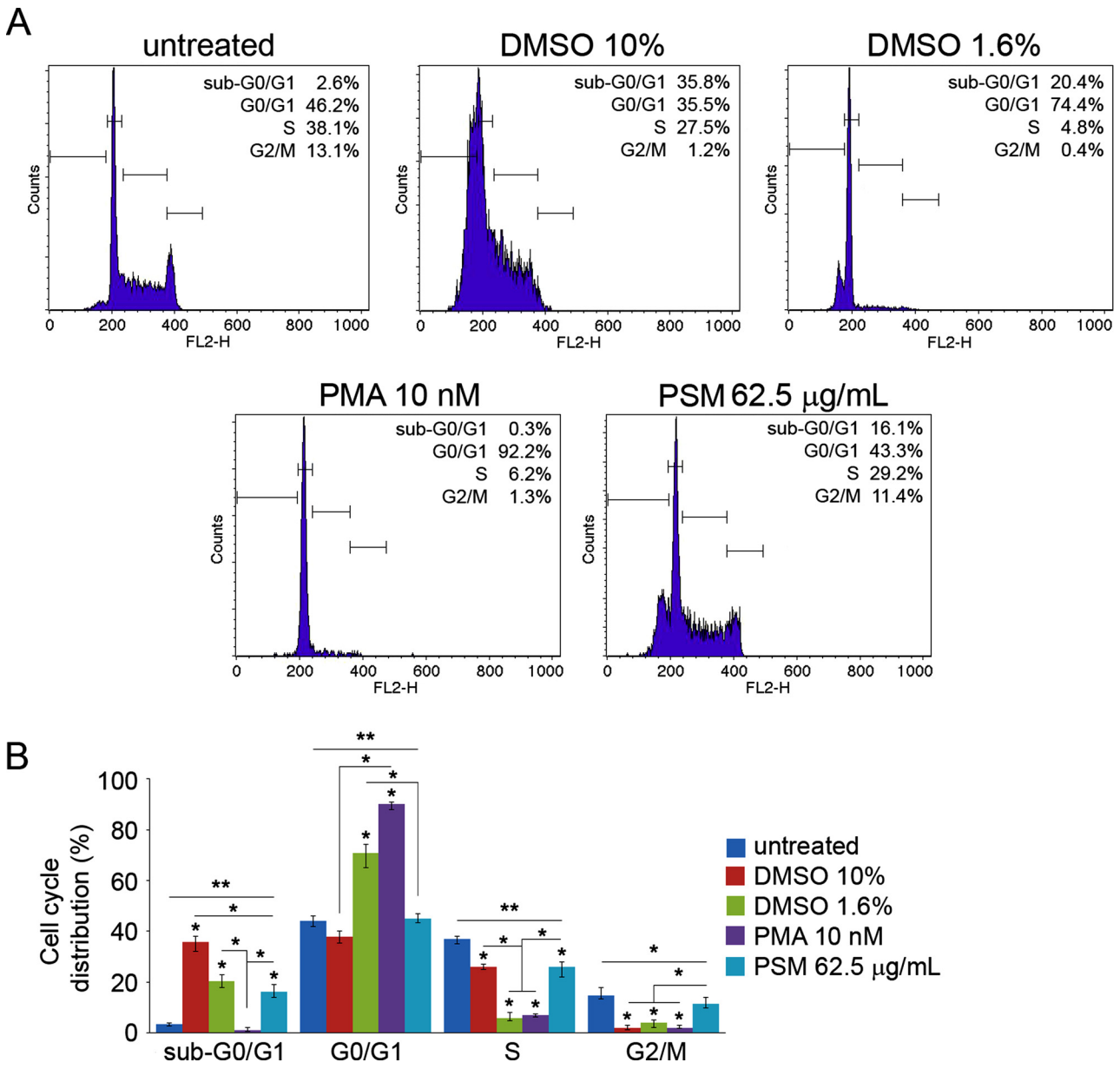

Fig. 3. Effects of PSM extract (62.5 µg/mL) treatment on cell cycle distribution in HL-60 cells after 48 h. (A) Histogram plots of flow cytometry analysis performed in HL-60 cells. (B) Graph summarizing the cell cycle distribution. HL-60 cells incubated with DMSO (10 %), DMSO (1.6 %) and PMA (10 nM) were used as positive controls for apoptosis, granulocytic and monocytic differentiation, respectively. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analyzed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

It is well known that effective and acceptable anticancer agents have to meet various criteria, including its effects on normal cells being relatively harmless [26]. So, the effect of PSM crude extract on HL-60 cells and on human normal proliferating lymphocytes was evaluated. The PSM extract exhibited selective anti-proliferative effect of 2.7-fold against HL-60 cells when compared to activated lymphocytes. This suggests that the PSM extract could have interesting bioactive molecules against cancer cells.

Following PSM treatment, HL-60 cells exhibited morphological changes including decreased N/C ratio with irregular and clear cytoplasm as opposed to the high N/C ratio and regular basophilic cytoplasmic rim in untreated cells. The cells lacked nuclear indentations typical of differentiated cells and resembled 'partially differentiated' HL-60 cells as described following treatment with arsenic trioxide [27]. Moreover, PSM treated cells resembled "paramyeloid' cells described by Zinzar et al. [28] with a granulocyte-like nucleus, but monocyte-like cytoplasm. Paramyeloid cells were also described in patients with chronic myelomoncytic leukemia [29]. These cells had cytoplasmic and nuclear features intermediate between a monocyte and a granulocyte. Even though PSM treated cells exhibited a monocyte-like cytoplasm and increased expression of CD11b and CD14, typical of monocytic differentiation, however, the cells did not show increased adherence to plastic typical of monocytic differentiation as seen with PMA treatment in this study. Interestingly, at the molecular level, studies implicating PU.1 and C/EBPα in myeloid differentiation report that in the first 24 h, PU.1 activates a mixed macrophage/neutrophil gene expression in PUER cells, with cells co-expressing at least one macrophage- and neutrophil- specific gene [30].

Expression studies further confirm that, when compared to untreated HL-60 cells, PSM treated HL-60 cells respond by increased expression of myeloid transcription factors with commitment towards the myelomonocytic route. Identification of molecules leading to activation

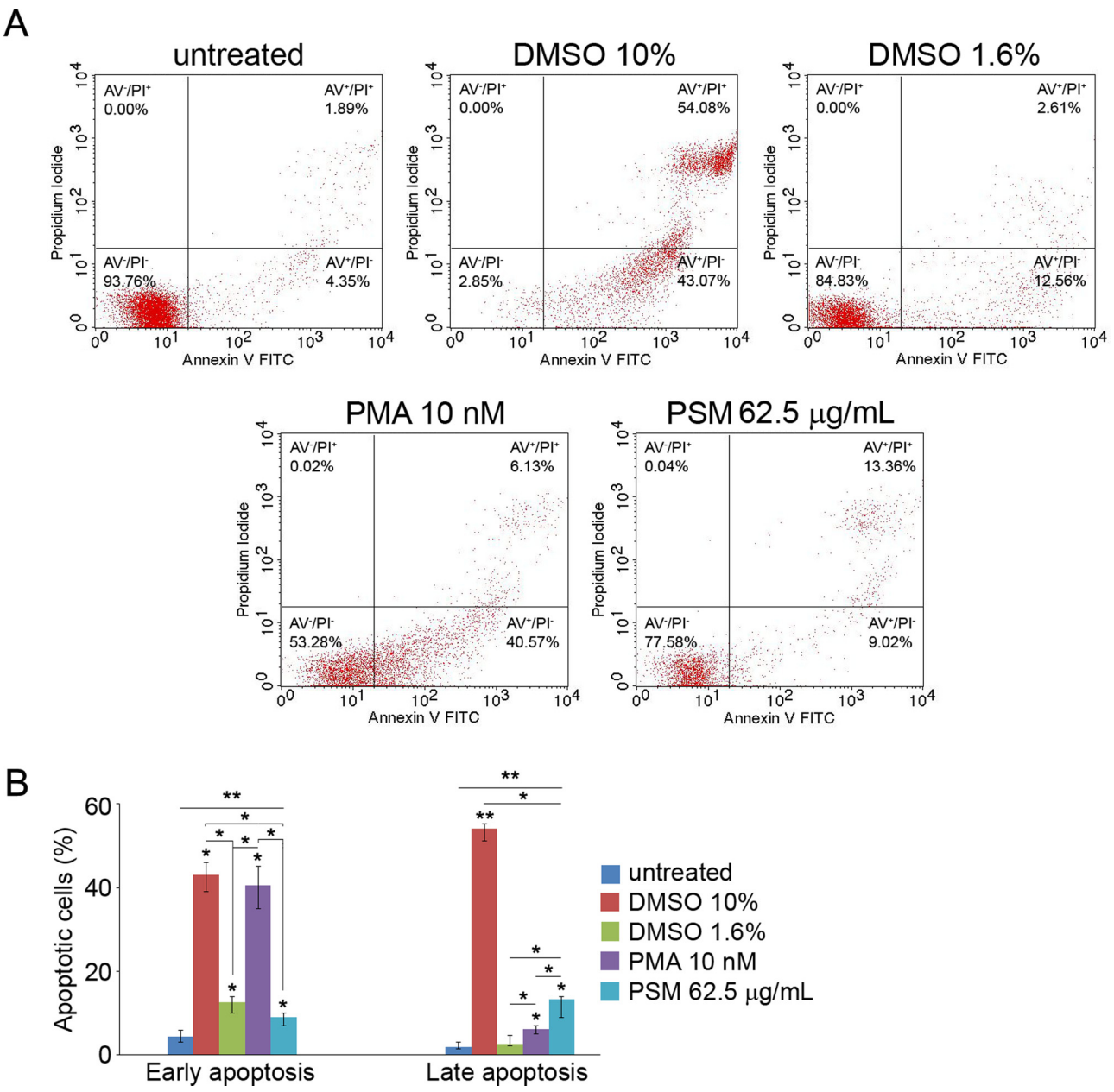

Fig. 4. Apoptotic activity of HL-60 cells in response to treatment with DMSO (10 %), DMSO (1.6 %), PMA (10 nM) and PSM extract (62.5 µg/mL) for 48 h. Apoptotic cells were quantified by flow cytometry after staining with PI and FITC-Annexin V. Four fractions (PI^-/Annexin V^-, PI^-/Annexin V^+, PI^+/Annexin V^+, PI^+/Annexin V^-) were analyzed. (A) Representative flow cytometric dot plots. (B) The summary of populations stained with only Annexin V (early apoptosis) and with both Annexin V and PI (late apoptosis). Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analyzed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

of transcription factors is another strategy that is actively studied for novel therapeutic agents in the fight against cancer.

PSM treatment also caused apoptosis of HL-60 cells with apoptotic cell nuclei evident following Hoechst 33342 staining and the appearance of a cell population in sub-G0/G1 phase of the cell cycle, together with increased and sustained expression of the pro-apoptotic *BAX* gene and significant repression of the anti-apoptotic *BCL2* gene. Even though PSM did not cause cell cycle arrest at the G0/G1 phase typical of differentiation agents such as ATRA and PMA, however, at 48 h treatment, PSM caused partial differentiation of HL-60 cells and induced apoptosis.

Many studies reflect on cell cycle arrest in terms of 'terminal differentiation', when cells no longer divide [31]. However, the exact relationship between cell proliferation and differentiation remains unresolved with actively proliferating cells acquiring characteristics of differentiating cells, including the acquisition of myeloid surface antigens and expression of molecular characteristics [32]. Brown et al. [33] on HL-60 cells, reported that differentiation and cell proliferation are regulated simultaneously but independently, and cells commit to differentiation long before cell cycle arrest, with differentiation not restricted to any particular segment of the cell cycle. In other studies, HL-60 cells traversed two to three cell cycles prior to terminal differentiation and cell cycle arrest following ATRA treatment. [34,35].

The transient expression of *CDKN1A*, *SPI1*, and *c-MYC* could indicate that PSM-induced differentiation of HL-60 cells is unstable, with possible retro differentiation and/or accumulation of PSM resistant HL-60 cells. This might indicate that a constant stimulation by PSM is required to maintain growth arrest and differentiation. Similar results were described following treatment of U937 cells with PMA [36].

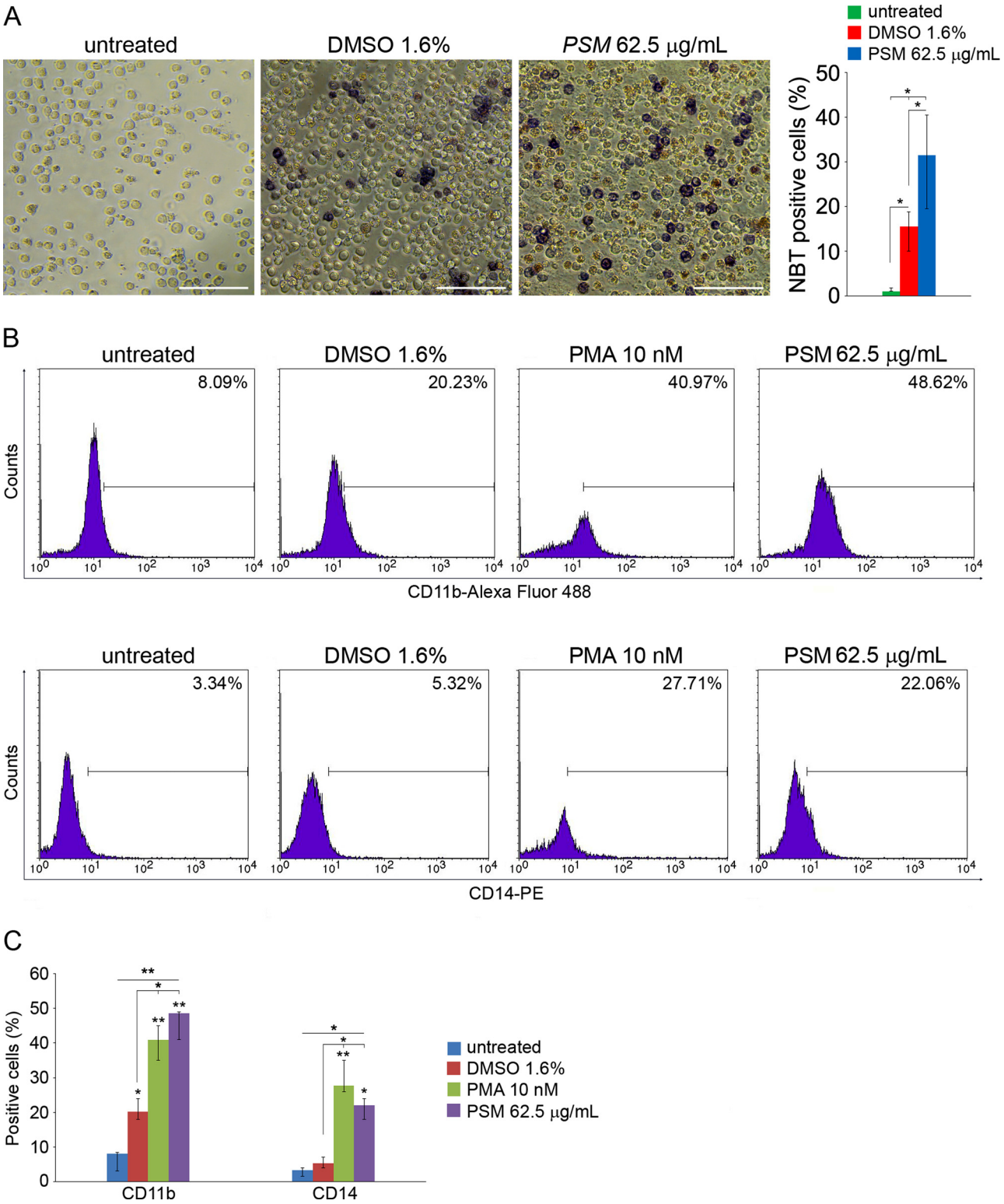

Fig. 5. PSM extract-induced cell differentiation in HL-60 cells. (A) Nitroblue tetrazolium (NBT) reduction assay. Cells were treated with PSM extract (62.5 µg/mL) for 48 h. Microscopy images display cells containing intracellular blue-black formazan deposits (scale bar =100 µm). Graph summarizing the percentages of NBT positive cells. HL-60 cells incubated with DMSO (1.6 %) were used as positive controls for granulocytic differentiation. (B) Cytometric analyses showing cell surface expression of CD11b (top panels) and CD14 (bottom panels) in HL-60 cells treated with DMSO (10 %), DMSO (1.6 %), PMA (10 nM) and PSM extract (62.5 µg/mL) for 48 h. (C) Graph summarizing both CD11b and CD14 reactivity.

Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analyzed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

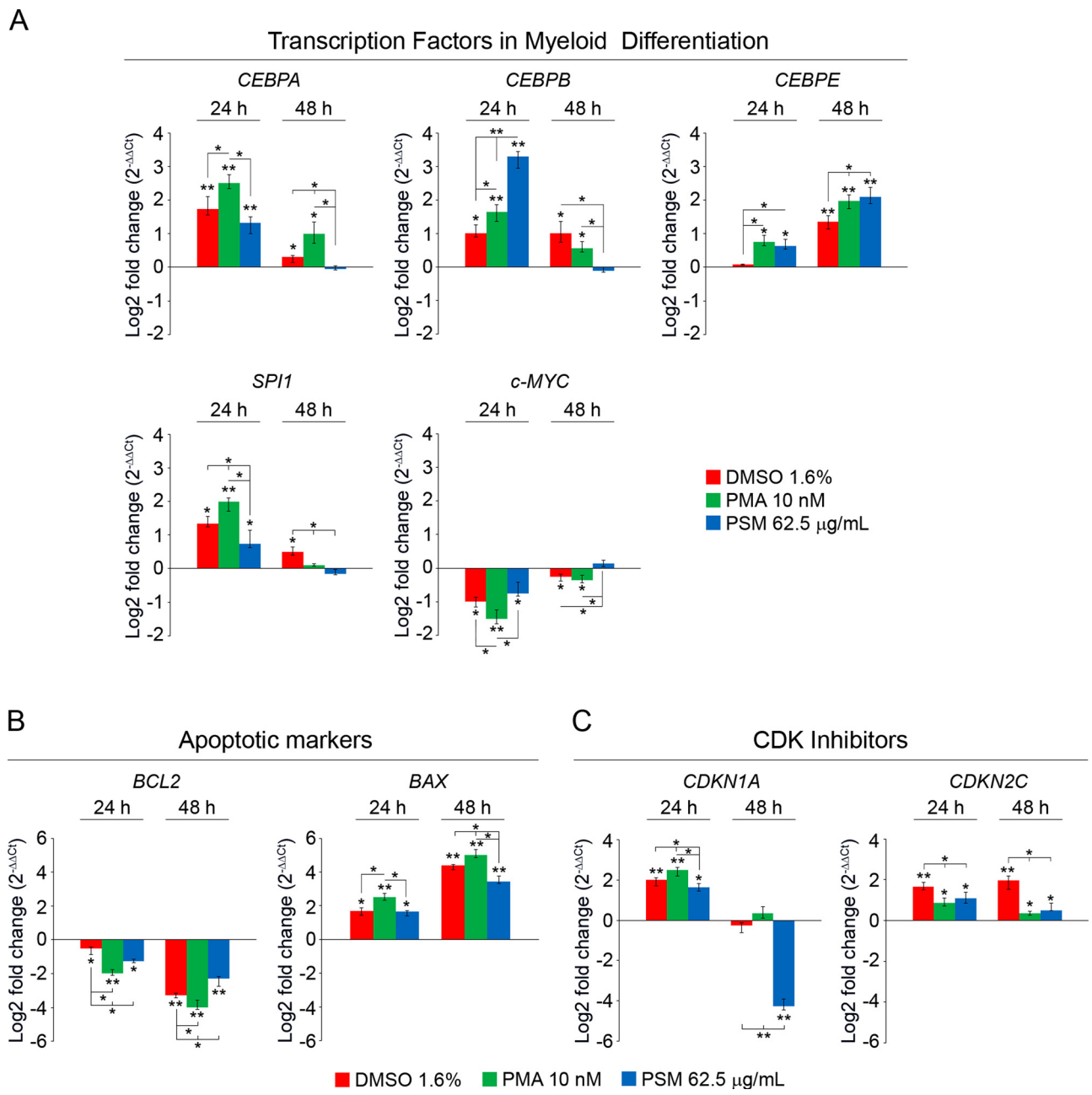

Fig. 6. Differentially expression genes in response to PSM extract treatment in HL-60 cells. Cells were treated with PSM extract (62.5 µg/mL) for indicated times. mRNA expression of genes involved in cell differentiation (A), apoptosis (B) and cell cycle (C) regulation was determined by Real-time RT-PCR. HL-60 cells incubated with DMSO (1.6 %) and PMA (10 nM) were also used as positive controls. Relative transcript levels were determined using the $2^{-\Delta\Delta Ct}$ method and normalized to *HPRT1* and *YWHAZ* reference genes. Expression levels in untreated cells were treated as calibrators. Data are presented as median and inter-quartile range (error bars) (n = 2 independent experiments carried out in triplicate) and were analyzed using nonparametric Kruskal-Wallis, followed by Dunn-Bonferroni's *post hoc* analysis (* $p < 0.05$; ** $p < 0.001$).

Furthermore, recovery of *c-MYC* expression has been reported in other studies whereby expression of *c-MYC* was drastically reduced within a few hours during differentiation with nicotinamide and DMSO-treated HL-60 cells with subsequent increase in expression, after which the expression levels remained low [37,38]. It was suggested that two distinct mechanisms of transcriptional control operate on *c-MYC* during differentiation of HL-60 cells with an early reversible downregulation and a late non-reversible downregulation following treatment with differentiating agents [38].

The reduced proportion of PSM treated cells in the S phase and increase in sub-G0/G1 can be indicative of cells that are no longer traversing the S- to G2/M phase transition and hence have undergone cell cycle arrest prior to induction of apoptosis. This could be attributed to a mechanism similar to chemotherapeutic drugs whereby following treatment, DNA breakages accumulate primarily at the S- phase of the cell cycle, unable to proceed to the G2/M phase, with resultant apoptotic cell death [39].

Overall, our findings show that PSM crude extract possesses anti-tumor activity through anti-proliferative, apoptotic and differentiation-inducing actions on HL-60 cells *in vitro* (Fig. 7).

5. Conclusion

In conclusion, we provide evidence showing that a crude extract

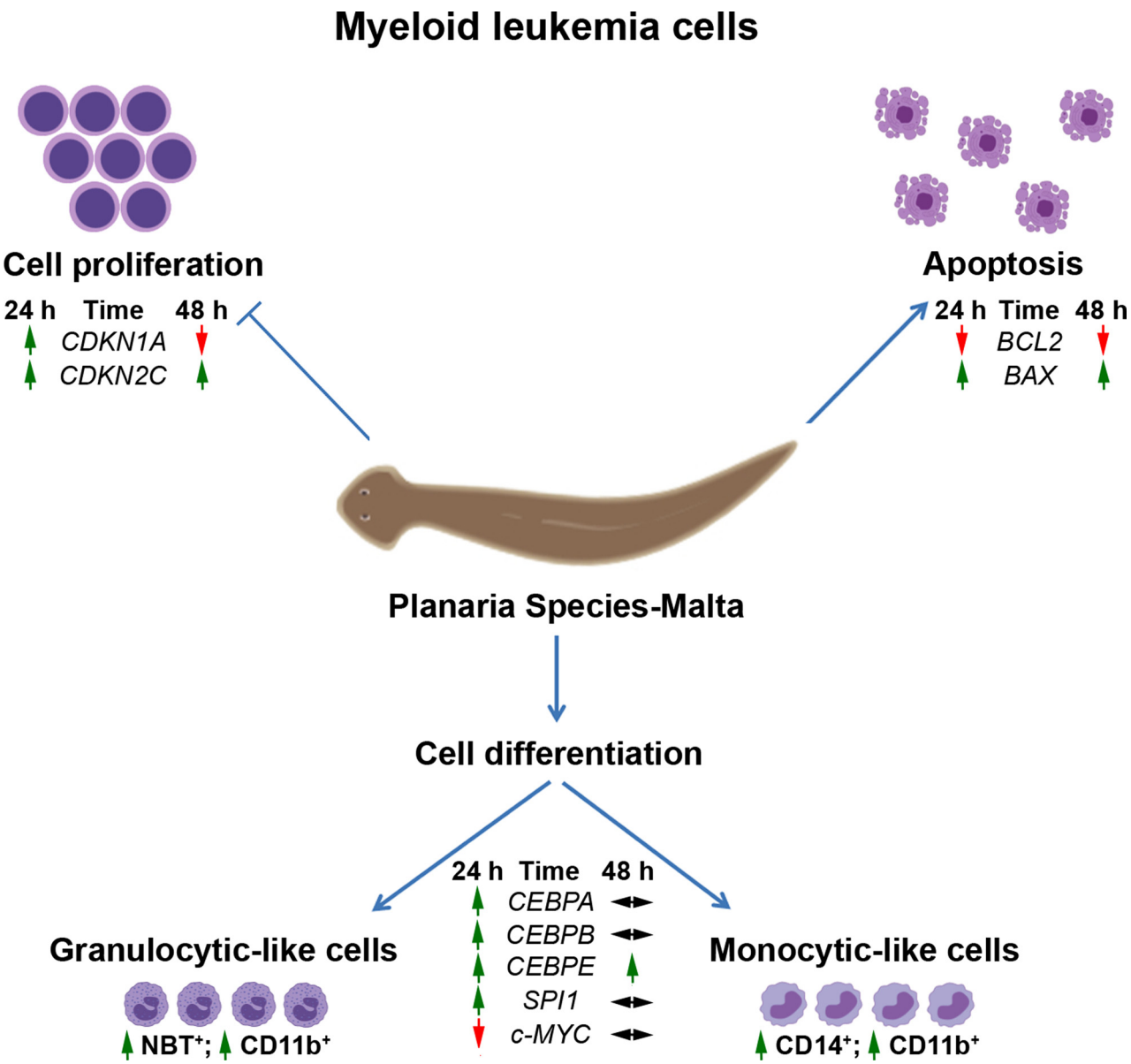

Fig. 7. Schematic representation of the effects of Planaria Species-Malta (PSM) extract in HL-60 cells. Possible mechanisms include inducing apoptosis and cell differentiation, and reducing cell proliferation. Green arrows represent increased expression; red arrows indicate decreased expression; black double-headed arrows indicate unchanged expression.

from a local planarian species could be used to develop new anti-cancer molecules for the treatment of AML. Furthermore, studies have reported on the anti-cancer effects of several regenerative earthworm species, and it is possible that planarians possess similar bioactive molecules including enzymes, proteins, unsaturated fatty acids, peptides, vitamins, among others [40–43]. In the future, the morphological and molecular characterization of the planaria species in Malta will be carried out. This will help not only in species identification but whether different species exhibit different effects on cancer cells. Also, identification of the bioactive molecules will be carried and tested for possible synergistic, antagonistic, and or additive effects on human leukemia cells *in vitro* and *in vivo*.

Ethical approval

All procedures performed in this study involving human participants were covered by ethical approval from the University of Malta research ethics committee, Ref No: FRECMDS_1819_002.

Declaration of Competing Interest

The authors declare no conflict of interest.

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