

Differentiation and Apoptosis of U937 Leukemia Cells by an Active Compound from *Dendrostellera lessertii*

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ABSTRACT

Background: Regarding the strong differentiation and anti-leukemic activity of *Dendrostellera lessertii* extract, an active agent with similar capabilities was isolated from the crude extract of the plant leaves. The aim of this study was to determine whether the anti-proliferative effect observed for this compound, is differentiation-dependent among the drug-treated cells, using U937 cells. **Methods:** Monocyte differentiation was evaluated by Wright-Giemsa staining, latex particle assay and flow cytometry. Induction of apoptosis was analyzed by Annexin-PI double staining. **Results:** The new compound, at 0.5-2.5 µg/ml inhibited proliferation of U937 cells by more than 70% and their viabilities were decreased by $47 \pm 2.1\%$ after 72 h of treatments. In addition, we found that the effect of the new compound on U937 cells was associated with differentiation toward monocyte/macrophage lineage based on nitroblue tetrazolium reduction assay, morphology change, phagocytic activity and expression of cell surface markers (CD14 and CD11b) as analyzed by flow cytometry. Moreover, our results indicated that the treatment of U937 cells with the new compound for 3 to 4 days induced apoptosis as assayed qualitatively by acridine orange/ethidium bromide and Annexin-V/PI double staining technique using flow cytometry. **Conclusion:** Based on these observations, it is concluded that the anti-proliferative function of the new compound is exerted through differentiation-dependent apoptosis among the treated cells, similar to the function of 3-hydrogenkwadaphnin previously characterized from *D. lessertii* crude extract. *Iran. Biomed. J. 13 (1): 35-42, 2009*

Keywords: Apoptosis, *Dendrostellera lessertii*, Differentiation, U937 cells

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by an increase in the number of myeloid cells in the bone marrow and an arrest in their maturation, frequently resulting in hematopoietic insufficiency [1]. Differentiation induction, as a therapeutic strategy, can have a powerful impact on hematopoietic malignancies, most likely on myeloid leukemia [2]. Several compounds such as DMSO [3] and all-*trans* retinoic acid (ATRA) [4] caused AML cells to differentiate toward granulocytes whereas 12-*o*-tetradecanoylphorbol-13 acetate (TPA) [5] and 1, 25-dihydroxy vitamin D3 [6] differentiated them toward monocyte and macrophage. Besides of differentiation, ATRA and TPA caused apoptosis among the treated leukemia cells [7, 8]. Apoptosis is

a highly-organized physiological process for eliminating the damaged cells without damage to the organisms. It has been originally defined by morphological criteria such as cellular shrinkage, chromatin condensation and nuclear fragmentation associated with endonucleolytic DNA cleavage, cytoplasmic vacuolation, membrane blebbing and finally the formation of apoptotic bodies which are removed by surrounding phagocytic cells [9].

Regarding chemotherapeutic treatments of cancer in general and leukemia in specific, significant attention has been paid to discovery of anti-cancer agents from natural sources in recent years. Indeed, plant-derived compounds have great potential to be developed into cancer drugs due to their well-defined efficient targeting and consequently their low side effects [10]. In that regard, plants from the Thymelaeaceae and Euphorbiaceae families, with

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structurally unique diterpenes belonging to the tigliane, ingenane and daphnane skeletal types, have been found valuable and rich sources for many new anti-cancer agents. The daphnane-type diterpene esters such as genkwadaphnin [11], gnidilatimonoein [12] and 3-hydrogenkwadaphnin (3-HK) [13, 14] from Thymelaeaceae family possess significant anti-leukemic activities with major metabolic effects on DNA and protein syntheses. The inhibition of inosin-5'-monophosphate dehydrogenase (IMPDH) activities, the rate-limiting enzyme of *de novo* guanine nucleotide biosynthetic pathway, by diterpene esters has been proposed to be the main cause of the above mentioned activities [15]. Alterations in the activity of this enzyme have been implicated in the regulation of cellular growth, transformation, differentiation and apoptosis [16, 17]. In fact, several IMPDH inhibitors such as mycophenolic acid and tiazofurin have been used as chemotherapeutic drugs for leukemia cells [16].

The aim of this study was to find whether the anti-proliferative activity of the second isolated compound from *Dendrostellera lessertii* is similar to 3-HK function. In that case, it would be concluded that the stronger anti-proliferative and differentiating activity of the crude extract is the cumulative result of several different agents in the crude extract having similar functions as the crude extract though with lower potencies.

MATERIALS AND METHODS

Materials. Cell culture medium (RPMI-1640), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco BRL (Life Technologies, Paisley, Scotland). Propidium iodide (PI), nitro blue tetrazolium (NBT) and sodium azide were purchased from Sigma Chem. Co. (St. Louis, MO, USA). Ethidium bromide (EtBr) and Acridine orange (AO) were obtained from Pharmacia LKB Biotechnology AB Uppsala (Sweden). The cell line (U937) was obtained from Pasteur Institute of Iran (Tehran).

Plant extraction and purification of the active component. Shoots of *D. lessertii* were collected from the central province of Iran at the end of spring. A voucher specimen was deposited in the Central Herbarium (University College of Science, University of Tehran, Iran). The plant leaves were dried under shade and then powdered. The powder was kept in a closed container in a cold room for

future use. The powdered plant material (250 g) was extracted three times with methanol-water (1:1, V/V). The accumulated alcoholic extract was concentrated under reduced pressure to a final volume of 250 ml. The crude extract was then subjected to CHCl_3 extraction five times. The accumulated chloroform solution was concentrated to 1 ml under reduced pressure. The 1 ml residue was fractionated on a silica gel column (1.4 cm \times 50 cm), using diethyl ether as the eluting solvent, into six fractions. The active compound, with relatively higher differentiation activity relative to other components of the fraction, was purified from the sixth fraction using thin layer chromatography (TLC) technique. The developing chromatography system was a mixture of chloroform and diethyl ether (1:1, V/V). The relative mobility of the compound of interest in the above mentioned system was about 0.3. From 250 g of the plant powder, 1 mg of the active compound was obtained. The structure elucidation of the purified compound is in progress using various spectroscopic techniques.

Cell culture. The U937 cell line was cultured in RPMI-1640 medium supplemented with FBS (10%, v/v), streptomycin (100 $\mu\text{g/ml}$) and penicillin (100 U/ml) and was maintained at 37°C in a humidified atmosphere of 5% CO_2 .

Cell viability assay. The cells (1×10^5 cells/well) were seeded in triplicate into cell culture plates 24 h prior to drug treatment. After treatment with the new compound at different doses for various lengths of times, cell number was established with a hemocytometer and the cell viability was determined by the trypan blue exclusion test. The cells attached to the culture plates were trypsinized with $1 \times$ trypsin-EDTA solution. The numbers of attached and unattached cells were determined using a hemocytometer.

NBT reducing assay. Each cell line was cultured and treated with the drug for various time intervals as explained in the cell viability assay. At the indicated time intervals, the cells were harvested, and NBT reducing activity was determined by the method of Sakashita and colleagues [18] with a slight modification. Briefly, the cells were harvested by centrifugation and suspended in 100 μl of NBT solution (4 mg/ml). After the addition of 100 μl of TPA solution (2 $\mu\text{g/ml}$), the cell suspension was incubated at 37°C for 30 min. The differentiated cells were identified by their intracellular blue

formazan deposits. A minimum of 200 cells of each sample was counted under a light microscope to determine the percent of differentiated cells.

Morphological changes of the treated cells.

Morphological changes were either microscopically observed in smears stained with Wright-Giemsa stain using high magnification power ($\times 400$) or were stained with AO/EtBr and examined with a fluorescence microscope. For this purpose, the cells were collected and washed with cold PBS and then adjusted to a cell density of 2×10^5 cells/ml using PBS. The AO/EtBr solution (1:1, v/v) was added to the cell suspension in a final concentration of 100 $\mu\text{g/ml}$. The cellular morphology was evaluated by Axoscope 2 plus fluorescence microscope (ZEISS, Yena, Germany).

Latex particle phagocytosis assay. Treated and control cells were assayed for their ability to phagocytize protein-coated latex particles [19]. A protein-coated latex particle suspension, commercially marketed as a pregnancy test (Gravindex-Ortho, Omega House, UK) was used for this assay. The particle suspension was diluted 1:10 with PBS and 0.1 ml of the diluted suspension was mixed with the drug-treated and untreated U937 cells (5×10^4 cells) in 0.1 ml of RPMI-1640 supplemented with 20% FBS. The mixture was incubated for 60 min in a CO_2 incubator. Each cell sample was then washed three times with cold PBS. Each collected cell sample was resuspended in PBS. A minimum of 200 cells was counted in triplicate and those with a minimum of 10 digested particles were considered positive.

Cell surface markers. CD11b and CD14 antigens were detected by two colour immunofluorescence, using commercially available reagents. In brief, the treated and/or untreated cells were incubated simultaneously with fluorescein isothiocyanate (FITC)-conjugated CD11b and phycoerythrin (PE)-conjugated CD14 monoclonal antibodies (IQ product, Netherland) in the phosphate buffered saline (PBS) with 1% FBS and 0.1% sodium azide for 30 min at 4°C . After washing, at least 10^4 cells were analyzed by flow cytometry (Partec PAS, Munich, Germany).

Flow cytometry analysis of apoptosis. Double staining with FITC-Annexin-V and PI for flow cytometry analyses were performed using phosphatidyle serine detection kit including FITC-

Annexin V (IQ product, Netherland). After washing twice with PBS, the treated and/or untreated cells were resuspended in the binding buffer (100 μl of calcium buffer containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl_2). Then, FITC-Annexin-V (10 μl) was added to the cells followed by the addition of 10 μl PI. The samples were incubated for 10 min in the dark at 4°C and then subjected to flow cytometry evaluation.

Statistical analysis. Data are expressed as mean \pm SD of three independent measurements and statistically analyzed using student's *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Cell viability and growth inhibition. The U937 cells were treated with different doses of the drug for various time intervals up to 72 h and their growth patterns were established by trypan blue exclusion test. As shown in Fig. 1A, the new compound from *D. lessertii* inhibited the growth of U937 cells in a dose- and time-dependent manner. The IC_{50} of the new compound after 24 h of exposure was 1.75 $\mu\text{g/ml}$ for U937 cells. Regarding the potency of 3-hydrogenkwadaphnin (3-HK), a previously characterized anti-proliferative compound from *D. lessertii* with IC_{50} of 12.0 nM (7.284 $\mu\text{g/ml}$) and 15.0 nM (9.06 $\mu\text{g/ml}$) in HL60 and K562 cells [20,14], it appears that the new compound has a higher potency compared to 3-HK on the basis of $\mu\text{g/ml}$ of the culture media. Exact potency determination awaits the molecular weight determination of the new compound. Despite the strong growth inhibiting activity of the new compound at 1.5 $\mu\text{g/ml}$, significant changes in the viability of the treated cells was not observed after 24 h of treatment (Fig. 1B), however, after 48 h of incubation viability of the treated cells decreased followed by a massive cell death after 72-96 h.

Induction of differentiation among U937 cells.

Phase contrast microscopic examination of the drug-treated U937 cells revealed distinct alteration in cell morphology shown in Figure 2. Time-dependent morphological changes in the cell bodies were evident almost 12 h after treatment which was followed for longer exposure time. Twenty four hr after exposure to the drug, a portion of the cells attached to each other forming aggregates within 48-72 h, the majority of the cells developed

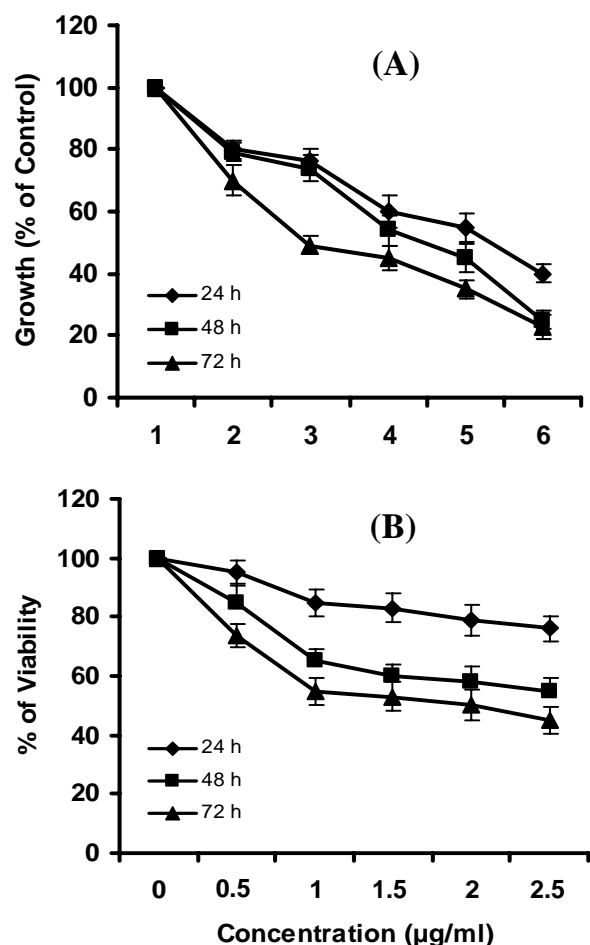


Fig. 1. Effects of the new compound from *Dendrostellera lessertii* on growth and viability of U937 cells. Cells were exposed to the indicated amounts of the new compound ($\mu\text{g/ml}$) for 24, 48 and 72 h. The viable cells were also evaluated by trypan blue exclusion at various time intervals. Cell viability and growth inhibition in each treatment was expressed as a percentage of the control. Each value represents the mean \pm SD of three independent experiments.

pseudopodia extensions and attached to the culture dishes (Fig. 2). These morphological changes seem very similar to the effects of TPA on inducing monocyte/macrophage differentiation of leukemia cell lines [21]. In order to confirm the differentiation ability of the new compound, the production of superoxide anion as a functional evidence of myeloid maturation was measured by the reduction of NBT (Fig. 3). The cells were incubated with various concentrations of the new drug for 24, 48 and 72 h and then were assayed for their NBT reducing activities. As shown in Figure 3A, NBT-positive cells, with blue-black granules, were observed among drug-treated cells after 24 h of treatment; in comparison, very few granule-containing cells were observed among untreated control cells. Figure 3B documents the dose- and time-dependent induction of differentiation among the affected U937 cells.

To further assess and document the differentiation capability of the new compound, the morphologic variations and phagocytic activity were analyzed. Morphological variation of the affected cells following Wright-Giemsa staining is shown in Figure 4A and 4B. Treatment of U937 cells with $1.5 \mu\text{g/ml}$ of new compound resulted in significant changes in cell size and an increase in cytoplasm to nuclear ratio. However, undifferentiated control cells were predominantly promonocytes with round and regular cell margins with large nuclei.

We also evaluated phagocytic activity of the affected cells. It is well established that mature myeloid cells are generally more active in phagocytosis than their corresponding progenitor cells [19]. Exposure of the drug-treated cells to protein-coated latex particles followed by enumeration using a light microscope revealed lower

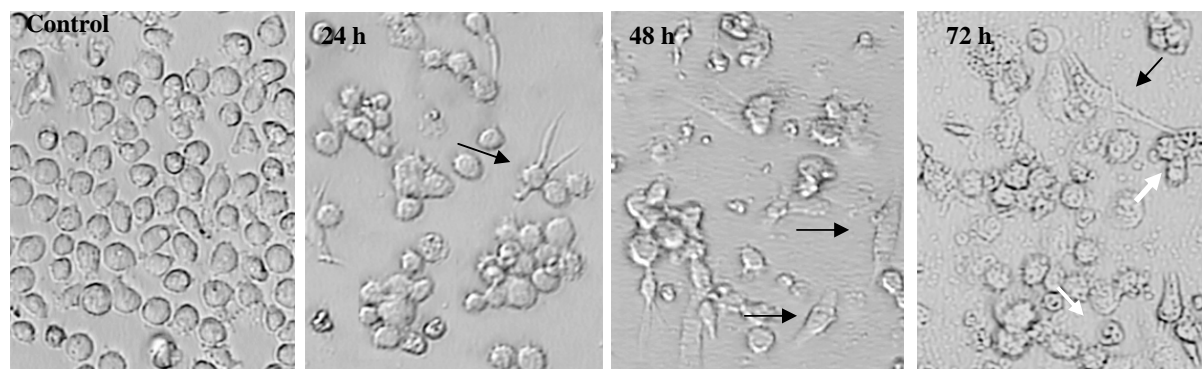


Fig. 2. Morphological changes of U937 cells treated with a single dose of the new compound ($1.5 \mu\text{g/ml}$). Photomicrographs of drug-treated cells were taken by an inverted microscope at $\times 400$ magnifications, after 24, 48 and 72 h of exposures to the drug. Formation of pseudopodia among the differentiated cells and the occurrence of cell death are shown with black and white arrows, respectively.

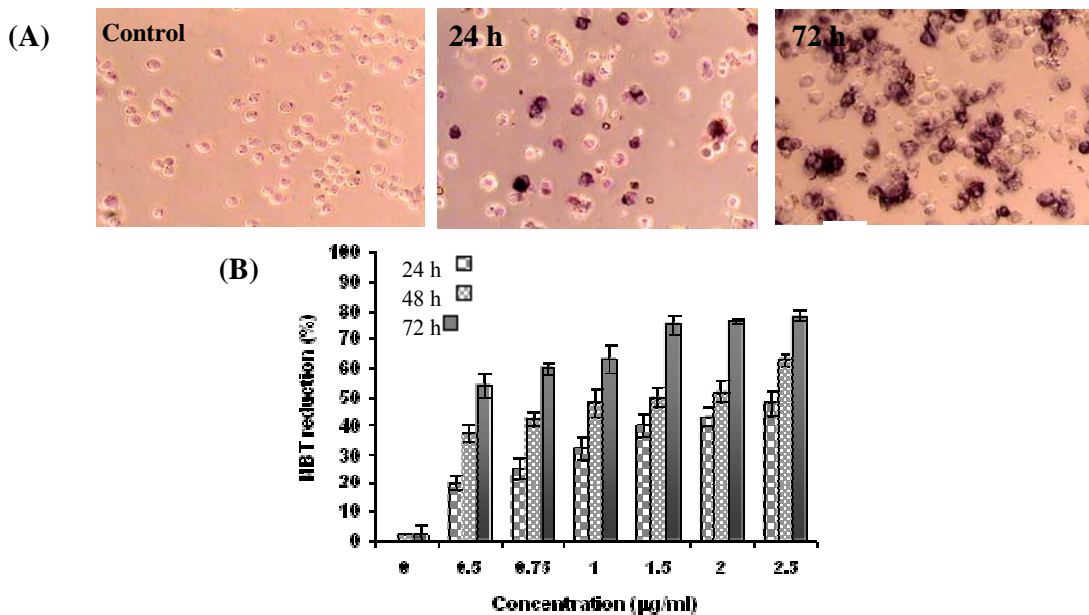


Fig. 3. Effects of the new compound on differentiation of U937 cells. Cells were treated with a single dose of the new compound (1.5 µg/ml). The NBT-positive cells with blue-black granules were observed with an inverted microscope at $\times 200$ magnifications after 24 and 72 h (A). The percentage of cells which could reduce NBT activity upon treatment with different concentration of the drug was indicated at various time intervals (B). The results are the means of three independent experiments \pm SD ($P < 0.05$).

engulfment of the latex particles by the untreated control cells (Fig. 4C) compared to drug-treated cells (Fig. 4D).

Double evaluation of cell surface expression of CD14 and CD11b by flow cytometry also confirmed the differentiation of affected U937 cells toward monocytes (Fig. 5). The CD14 marker, a glycosyl phosphatidyl inositol-anchored single chain glycoprotein, is unique to monocytes [22, 23] and it is undetectable on the cell surface of monocyte precursors. The cell surface CD11b is another classical marker of myeloid differentiation that is believed to be involved in cell adhesion besides

acting as a complement receptor [24, 25]. Flow cytometric diagrams showed enhanced expression of CD11b and CD14 on the membranes of U937 cells after treatment with the new compound (Fig. 5). By 24 h, the amount of CD11b and CD14 slightly (10-12%) increased (CD11b > CD14) and this process continued to 72 h, so that in comparison to untreated control cells, the amount of CD11b and CD14 increased more than 40% and 20%, respectively among the treated cells. Regarding these data, it is clear that the new compound induces U937 cells differentiation toward monocyte/macrophage destiny.

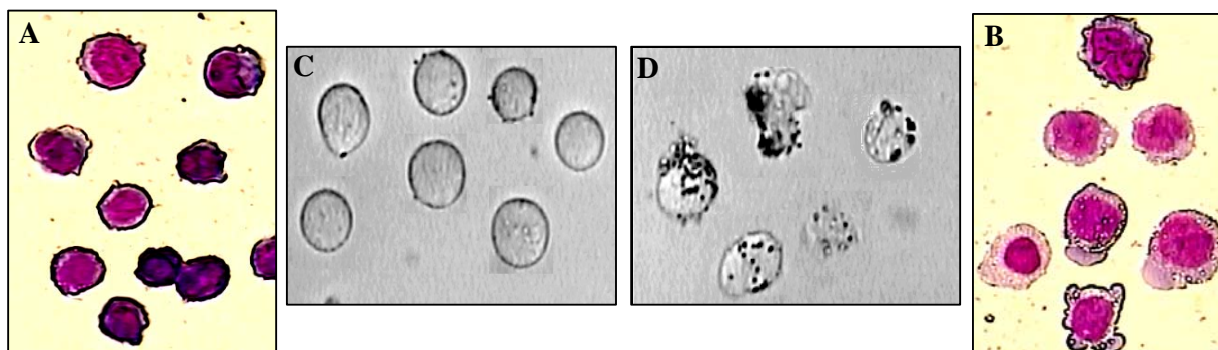


Fig. 4. Wright-Giemsa staining of untreated and treated cells with the new compound (A and B). After 48 h treatment of U937 cells with 1.5 µg/ml of the new compound, the cells were collected, stained by Wright-Giemsa solution and studied using a light microscope. Phagocytic activity of the differentiated cells was also studied after 48 h of treatment (C and D) (Magnification 400 \times).

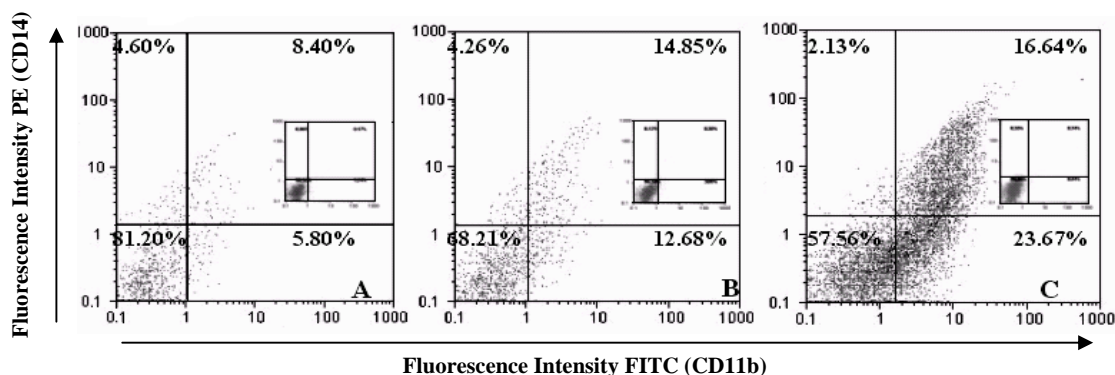


Fig. 5. FACS analysis of CD14 and CD11b antigens expressions among the drug- treated U937 cells. U937 cells (2×10^5 cells/ml) exposed to a single dose of the new compound (1.5 $\mu\text{g/ml}$) for 24 (A), 48 (B) and 72 (C) h. Cells were incubated with CD11b-FITC and CD14-PE antibody and then positive cells were quantified by FACS flow cytometry.

Induction of apoptosis by the new compound. As stated previously, treatment of the cells with the new drug significantly decreased the viability of the cells. To verify whether cell death by the new compound was the result of apoptosis, we evaluated the nuclear morphological changes by fluorescence technique after double staining with acridine orange/ ethidium bromide (AO/EtBr). According to Fig. 6A and 6B, more than 40% of U937 cells underwent apoptosis following 72 h exposure to a single dose (1.5 $\mu\text{g/ml}$) of the drug. Fragmentation of nuclear chromatin was

also evident among 60-70% of the affected cells with 2.5 $\mu\text{g/ml}$ of the new compound. For a further assessment of apoptosis, we examined the exposure of phosphatidylserine on the cell surface using Annexin-V/PI double staining. Flow cytometry analyses revealed that the percentage of Annexin-V/PI positive cells increased gradually to more than 40% after 72 h of treatment (Fig. 6C). These results indicated that the active compound induced apoptosis in U937 cells.

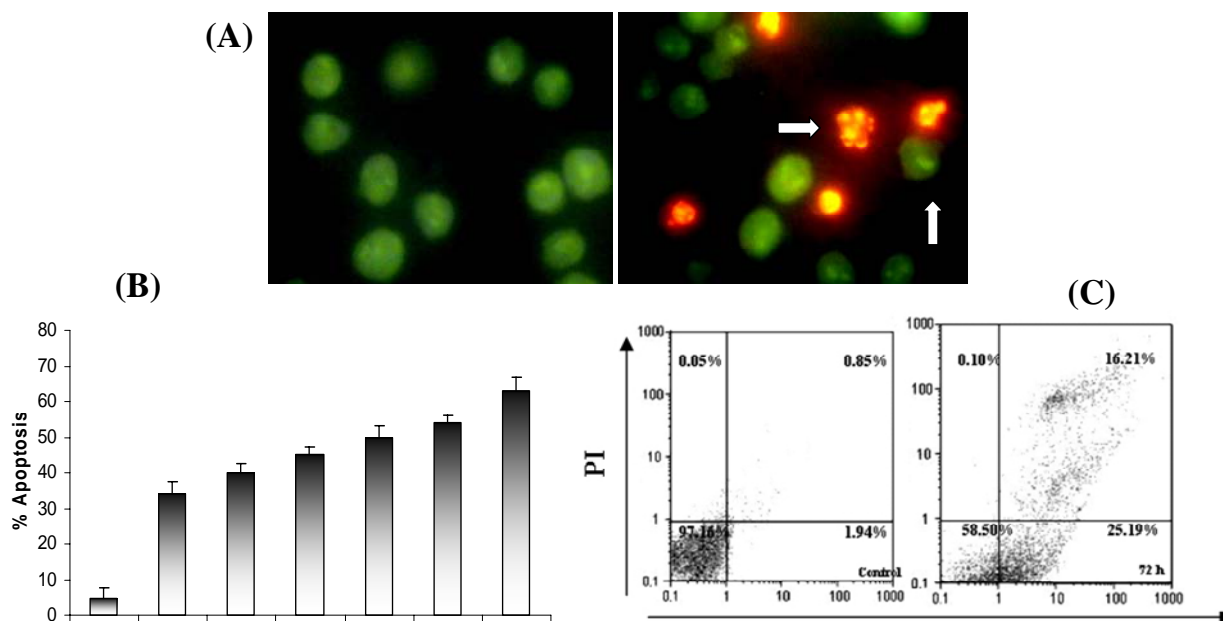


Fig. 6. Induction of apoptosis by the new compound among U937 cells. The drug treated Cells (1.5 $\mu\text{g/ml}$, 72 h) were stained with Acridine orange/ ethidium bromide double staining and then observed by fluorescent microscopy (100 \times). The drug induced condensation and fragmentation of the nuclei (A, arrows) which are typical manifestations of apoptosis and the percentage of apoptotic cells were measured (B). Apoptosis was followed by double Annexin-V/PI staining of the cells treated with a single dose of the drug for different time intervals (C).

DISCUSSION

Leukemia diseases are characterized by a breakdown in the myeloid cell maturation pathway. Hence, a possible therapeutic strategy for treatment of leukemia would be to induce terminal differentiation and eventual senescence. This approach has been very efficient in acute promyelocytic leukemia (APL), where treatment of patients with ATRA improved long-term survival. However, ATRA therapy is usually accompanied by a variety of side effects [26, 27]. Therefore, discovery of new agents with differentiation capability and their use in differentiation therapy has been considered in the clinical fields.

In this investigation, in order to evaluate the differentiation efficiency of the new compound, we selected a typical AML cell line U937. Our results in Figure 1 suggest that the new compound has potent growth inhibitory effects. Attain to this Figure, inhibition of proliferation is the most prominent effect of the drug up to 24 h of treatment, suggesting the drug is cytostatic rather than cytotoxic at early treatment times. However, a massive cell death occurred at longer treatment times (48 and 72 h). On the other hand, the potency of the new compound apparently is not related to p53 status of cells since U937 cells lack functional p53 protein [28].

Further studies revealed that the drug, at low concentrations (below IC_{50}), was capable of inducing differentiation among U937 cells. Occurrence of differentiation in parallel to growth inhibition suggests requirement of cell cycle arrest for differentiation of the cells. Cell to cell adherence and adherence of drug-treated cells to culture plates are criteria of leukemic differentiation toward monocyte/macrophages. In this line, an increase in percent of CD11b-positive cells was detected parallel to an increase in number of adherent cells. In addition, increase in population of CD14-positive cells also confirms that the treated cells have gained the criteria of mature monocyte/macrophages.

In addition, as it is documented by Annexin V/PI staining of the drug-treated cells (Fig. 6), apoptosis is the cause of the cell death. Interestingly, the bulk of the drug-induced apoptosis was occurred by 72 h, the time required to induce differentiation in most cells. These results suggest that apoptosis was probably occurred after differentiation of treated cells. Indeed, it has been reported that apoptosis plays an important role in the elimination of activated granulocytes and monocytes [7,29]. This is

because the mature blood cells have short life span both in vitro and in vivo and, therefore, die as a result of apoptosis [29]. Consistent with these results, most differentiation inducers are known to cause apoptosis following differentiation of the affected leukemia cells.

In conclusion, the results of this investigation clearly indicated that the new and active compound is capable of inducing differentiation and apoptosis in AML cells and therefore could be a valuable candidate for pharmaceutical evaluations.

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