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Induction of Differentiation and Apoptosis in Three Human Leukemia Cell Lines by a New Compound from *Dendrostellera lessertii*

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Abstract It has previously been shown that *Dendrostellera lessertii* (Thymelaeaceae) has strong anticancer activity. In this study, the antileukemic activity of another new compound from the same plant extract is reported. Promyelocytic (NB4 and HL-60) and erythroleukemia (K562) cells were cultured in the presence of various concentrations of the new compound (0.5–3.0 μg/ml) for 3 d. The cell numbers were then determined by trypan blue exclusion test. The new compound inhibited growth and proliferation of NB4, HL-60 and K562 with IC_{50} values of 1.5, 2.0 and 2.5 μg/ml, respectively. We also found that the new compound inhibited cell proliferation in a dose- and time-dependent manner. At low concentrations and after 48 h of treatment, approximately 50%–70% of NB4 and HL-60 cells were differentiated to monocyte/macrophage lineage and approximately 30%–40% of the treated K562 cells were differentiated in the megakaryocytic lineage, as evidenced by morphological changes and nitro blue tetrazolium reduction assays. Results of Hoechst 33258 staining also indicated that the new compound induced NB4 and HL-60 cell apoptosis at their respective IC_{50} values after 72 h of treatment. Based on the present data, the new compound seems a good candidate for further evaluation as an effective chemotherapeutic agent acting through induction of differentiation and apoptosis.

Key words apoptosis; *Dendrostellera lessertii*; differentiation; macrophage; megakaryocyte

Some leukemia cell lines, such as NB4 (an acute promyelocytic leukemia) and HL-60 (a myeloblastic leukemia), have the potential of bilineage differentiation [1]. Induction of differentiation to granulocytes or monocytes has been achieved after treatment of these two cell lines with dimethylsulfoxide [1], 12-O-tetradecanoylporbol-13-acetate (TPA) [2], all-*trans* retinoic acid (ATRA) [3] or 1α,25-dihydroxyvitamin D3 [4]. Treatment of K562 (erythroleukemia) cells with phobol esters (phorbol dibutyrate and phorbol 12-myristate-13-acetate) can induce differentiation along a megakaryocytic lineage [5,6]. In contrast, treatment of K562 cells with hemin [7], daunomycin [8], or herbimycin A [9] leads them to differentiate along an erythroid lineage. However, it has also been shown that induction of differentiation in some of the leukemia cell lines by ATRA and TPA is associated with apoptosis [10,11]. Apoptosis is a form of self-controlled cell death that is characterized by nucleosomal fragmentation and by several morphological changes, which differ from those of necrotic cells. Induction of differentiation-associated apoptosis in proliferative tumor cells might thus constitute an effective approach for cancer chemotherapies. In that respect, significant emphasis has been placed on identifying new agents, mainly from natural sources, to fight cancer. Herbal therapies have been the center of focus in the past decades.

In the present work, we report on the isolation of a second very active new compound from *Dendrostellera lessertii* crude extract with high differentiation capability among three different cell lines (NB4, HL-60 and K562). Compared with the previously characterized compound from the same plant (3-hydrogenkwadaphnine) [12,13], it appears that the new compound is much more active regarding cell differentiation and apoptosis.

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Materials and Methods

Materials

The RPMI 1640 cell culture medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL Life Technologies (Paisley, UK). The culture plates were obtained from Nunc (Roskilde, Denmark). TPA, propidium iodide, nitro blue tetrazolium (NBT), benzidine and sodium azide were purchased from Sigma (Steinheim, Germany).

Plant extraction and purification of the new active compound

Shoots of *D. lessertii* were collected from a central province in 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×50 cm), using diethyl ether as the eluting solvent, into six fractions. The active compound, with relatively higher differentiation activity in NB4 cells compared with other components of the fraction, was purified from the sixth fraction using the thin layer chromatography (TLC) technique. The developing system of TLC was a mixture of chloroform and diethyl ether (1:1, *V*/*V*). The purity of the isolated compound was further confirmed using the HPLC technique. The relative mobility of the compound of interest in the above mentioned system was approximately 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

Human acute promyelocytic (NB4 and HL-60) and erythroid (K562) leukemia cells were cultured in RPMI 1640 medium supplemented with 10% FBS (*V*/*V*), 100 μg/ml streptomycin and 100 U/ml penicillin [14], respectively. The cells were maintained in an incubator at 37 ºC with 5% CO_2 content.

Viability and differentiation

The cells (1×10^5) were seeded, in triplicate, into culture plates 24 h prior to treatment. After treatment with the new compound at different doses and for various time intervals, the cell numbers were determined using a hemocytometer and the cell viabilities were established by the trypan blue exclusion test. The attached cells were detached by 1×trypsin-EDTA solution [15]. Cell differentiation was studied in terms of morphology using NBT reduction assay for NB4 and HL-60 cells [16,17] and benzidine staining for K562 cells [18].

NBT reduction assay

NBT reduction was assayed as previously described [19]. Briefly, the collected cells (2×10^5 cells per milliliter of medium) were incubated at 37 ºC for 35 min with an equal volume of NBT solution (1 mg/ml) containing 100 μl of TPA solution (2 μg/ml). The percentages of cells containing intracellular blue-black formazan deposits were then determined. In each count, at least 200 cells were inspected and each count was carried out in triplicate.

Morphological evaluation of the differentiated cells

Aliquots of the treated cells were fixed with methanol, stained with Wright-Giemsa and then examined under a light microscope at high magnification (400×). Differentiated cells were identified on the basis of cytoplasmic protrusion and the nuclear pattern.

Morphological study of the apoptotic cells

Cells were seeded in 24-well plates at 1×10^5 cells/well and treated with 1.5 μg/ml (NB4), 2 μg/ml (HL-60) or 2.5 μg/ml (K562) of the new compound for 72 h. Apoptosis was determined morphologically, after staining the cells with Hoechst 3358, using fluorescence microscopy. In brief, cells were washed with cold phosphate-buffered saline (PBS) and adjusted to a density of 1×10^6 cells per milliliter using PBS. Hoechst 3358 solution (1 mg/ml ddH_2O) was added to the cell suspension at a final concentration of 100 μg/ml. The cellular morphology was evaluated by Axoscope 2 plus fluorescence microscopy (Zeiss, Germany).

Latex particle phagocytosis assay

Treated and control cells (NB4 and HL-60) 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 NB4 and HL-60 cells (5×10^4 cells) in 0.1 ml of RPMI 1640 supplemented with 20% FBS. The mixture was incubated for 60 min in the 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.

Results

Cell viability and growth inhibition

As shown in **Fig. 1**, the new compound inhibited the growth of NB4, HL-60 and K562 cells in a dose- and time-dependent manner. The IC_{50} values of the new compound, after 24 h of exposure, were 1.5, 2.0 and 2.5 μg/ml for NB4, HL-60 and K562 cells, respectively. After 24 h of exposure to 1.5 μg/ml of the new compound, growth inhibition was evident in all three cell lines, without substantial loss of viability, especially in K562 cells (**Fig. 1** and **Table 1**). However, at a dose of 2.5–3.0 μg/ml, the new compound showed cytotoxic effects.

Induction of differentiation in NB4 and HL-60 cells

In order to evaluate the possible role of the new drug in differentiation induction in NB4 and HL-60 cells, NBT assay, a reliable marker for differentiation of myeloid leukemia cells, was carried out on the drug-treated cells. As shown in **Table 1**, 72 h after exposure of each cell type to the drug at an indicated concentration (0.5–3.0 μg/ml), approximately 70% of the cells were capable of reducing NBT. This is in sharp contrast to the 2%–3% NBT reducing activities among the untreated respective control cells. Phase contrast microscopy examination of the treated cell morphologies clearly indicated that, 24 h after drug exposure, the cells grew as aggregates (**Fig. 2**) similar to the morphological behaviors of leukemia cells treated with

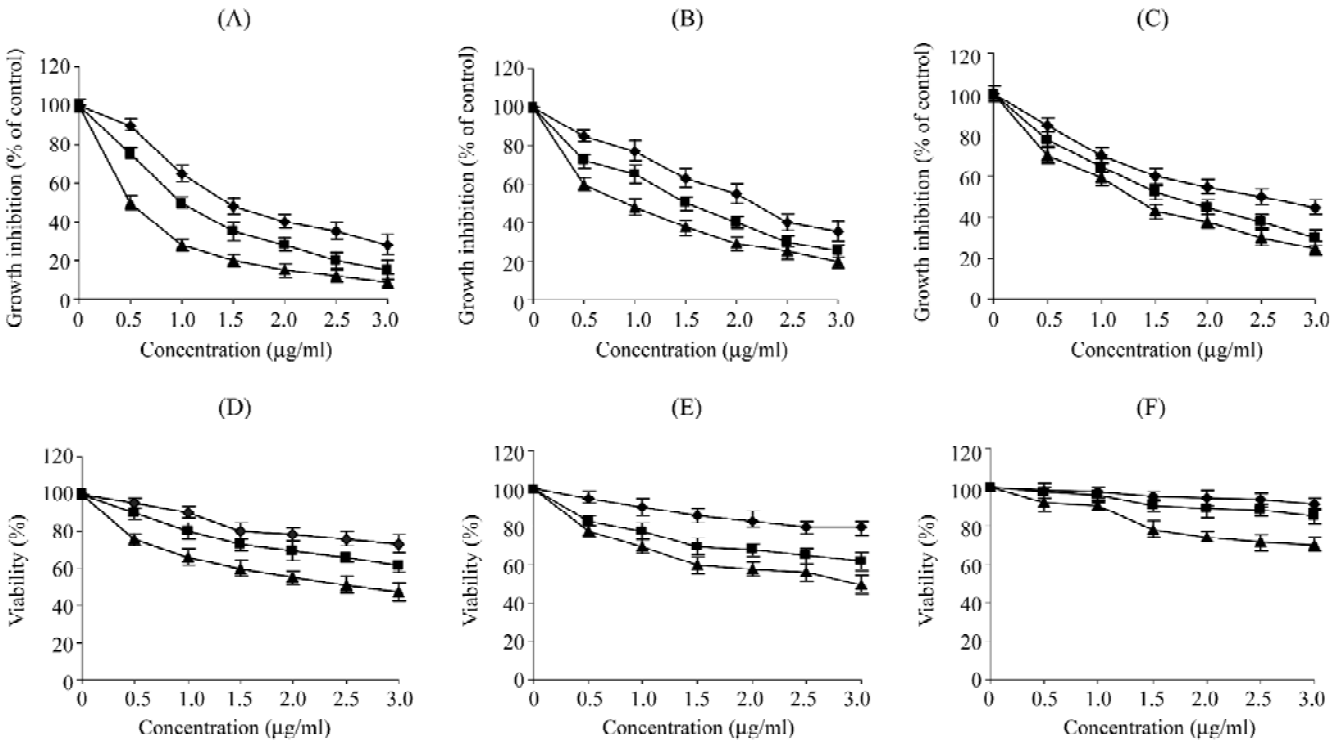

Fig. 1 Effects of the new compound from *Dendrostellera lessertii* on growth inhibition (A–C) and cell viability (D–F) of promyelocytic NB4 (A,D) and HL-60 (B,E) cells and erythroleukemia K562 (C,F) cells

Cells were exposed to the indicated amounts of the new compound (μg/ml) for 24 h (◆), 48 h (■) and 72 h (▲). The number of viable cells was determined by trypan blue exclusion. Cell viability and growth inhibition in each treatment was expressed as a percentage of the control. Each value represents the mean±SD of three independent experiments.

Table 1 Cell viability, apoptosis and nitro blue tetrazolium (NBT) reduction activities in human leukemia cells treated with the new compound from *Dendrostellera lessertii* at various doses for 3 d

Dose (μg/ml)	NB4			HL-60			K562	
	Viability (%)	NBT red * (%)	Apoptosis (%)	Viability (%)	NBT red * (%)	Apoptosis (%)	Viability (%)	Apoptosis (%)
0	100.0	2.2±3.1	4.5±3.1	100.0	3.4±1.5	5.5±2.4	100.0	5.2±2.5 [a]
0.5	76.0±2.5	54.0±4.2	44.0±3.6	78.0±4.3	50.0±2.3	35.0±6.3	90.0±3.6	25.0±3.2
1.0	65.0±3.2	63.0±5.6	55.0±5.3	70.0±2.3	56.0±5.4	50.0±4.4	85.0±4.1	38.0±2.6
1.5	58.0±4.1	75.0±2.3	62.0±4.2	61.0±4.1	70.0±4.6	58.0±4.2	75.0±3.5	50.0±3.1
2.0	55.0±3.5	77.0±3.1	68.0±5.2	58.0±5.2	72.0±3.6	60.0±5.1	72.0±2.2	54.0±2.5
2.5	53.0±2.3	78.0±3.4	74.0±2.1	56.0±2.1	75.0±2.3	66.0±3.7	70.0±2.3	58.0±3.6
3.0	50.0±3.2	81.0±3.2	77.0±3.1	54.0±1.5	80.0±1.5	70.0±2.5	68.0±3.6	62.0±2.2

For experiment details see "Material and Methods". * NBT reduction. [a] apoptosis was evaluated after 5 d of drug exposure.

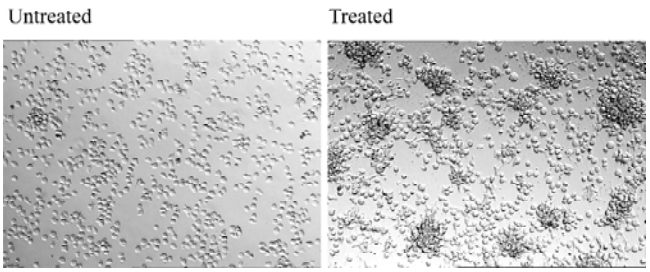

Fig. 2 Morphological changes of promyelocytic NB4 cells treated with 1.5 μg/ml of the new compound from *Dendrostellera lessertii* after 24 h

Aggregates are formed among the treated cells. No aggregation is observed among the untreated cells.

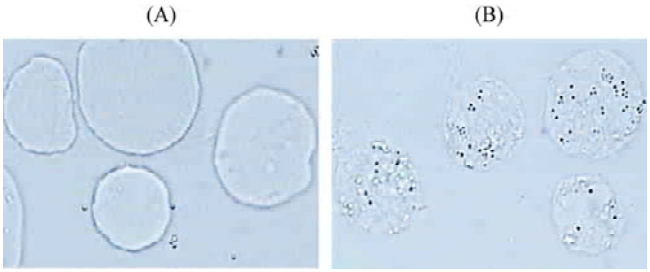

Fig. 3 Phagocytic activity in promyelocytic NB4 cells

The cells were treated at a concentration of 1.5 μg/ml of the new compound from *Dendrostellera lessertii* for 72 h. (A) Control cells treated after 72 h. (B) Treated cells incubated with protein-coated latex particles, as described in "Materials and Methods". Magnification, 400×.

TPA [20]. In addition, the drug-treated NB4 and HL-60 cells showed the capability to phagocytose latex particles (**Fig. 3**). Phagocytosing of latex particles, which is commonly considered as a criterion of proper function of mature macrophages, indicated that (45.0±3.2)% and (40.0±5.4)% of the treated NB4 and HL-60 cells, respectively, were able to phagocytize latex particles after 72 h treatment with 1.5–2.0 μg/ml of the compound. However, under the same conditions, 3%–5% of the control cells were able to ingest a minimum of 10 particles. Further morphologic assessment obtained after 48 h of treatment clearly showed that both types of cells responded to the new drug by enhancing their attachment to the culture plates and more than 50% of the cells showed monocytic morphologic maturation with cytoplasmic protrusion and increased of cytoplasm/nuclei ratio [**Fig. 4(D,E)**]. As shown in **Fig. 5**, after 24 h of drug treatment, cells started to adhere to the flask surfaces and to form pseudopodia on their surfaces.

Induction of differentiation in K562 cells

The differentiation of human chronic myelogenous leukemia (K562) cells under the influence of the new compound was also evaluated. Treatment of the cells with a single dose of the drug at 2.5 μg/ml induced 15%–20% and 30%–40% differentiation along the megakaryocytic lineage after 48 h and 96 h of exposure, respectively. The conclusion was based on the fact that the control cells consisted, morphologically, of a homogenous population of immature blast-like cells characterized by small size with many cytoplasmic protrusions, a high nuclear-to-cytoplasmic ratio and with round nuclei. However, upon treatment

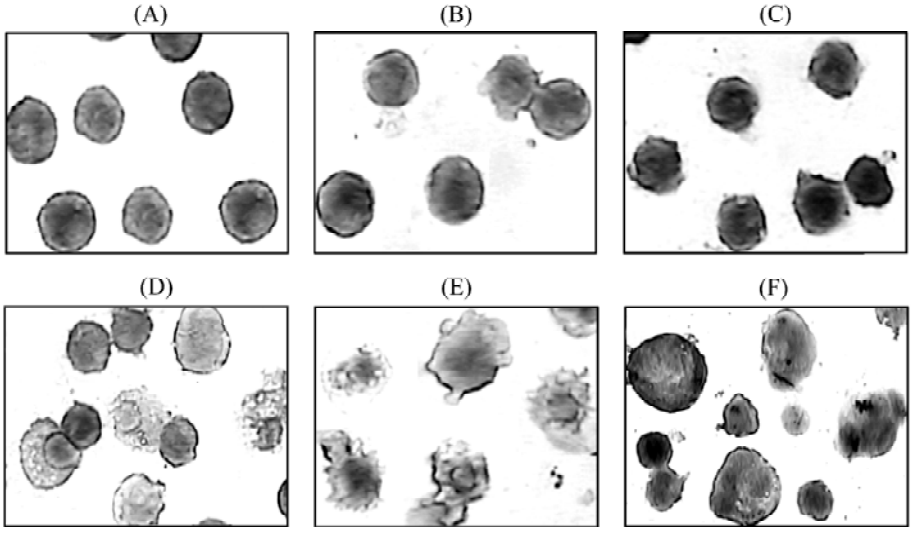

Fig. 4 Morphological changes of promyelocytic NB4 and HL-60 cells and erythroleukemia K562 cells treated with the new compound from *Dendrostellera lessertii*

NB4, HL-60 and K562 cells were treated with the new compound at their corresponding IC_{50} concentrations for 48 h. The cells were fixed and stained with Wright-Giemsa. (A–C) Untreated NB4, HL-60 and K562 cells, respectively. (D–F) Drug-treated NB4, HL-60 and K562 cells, respectively.

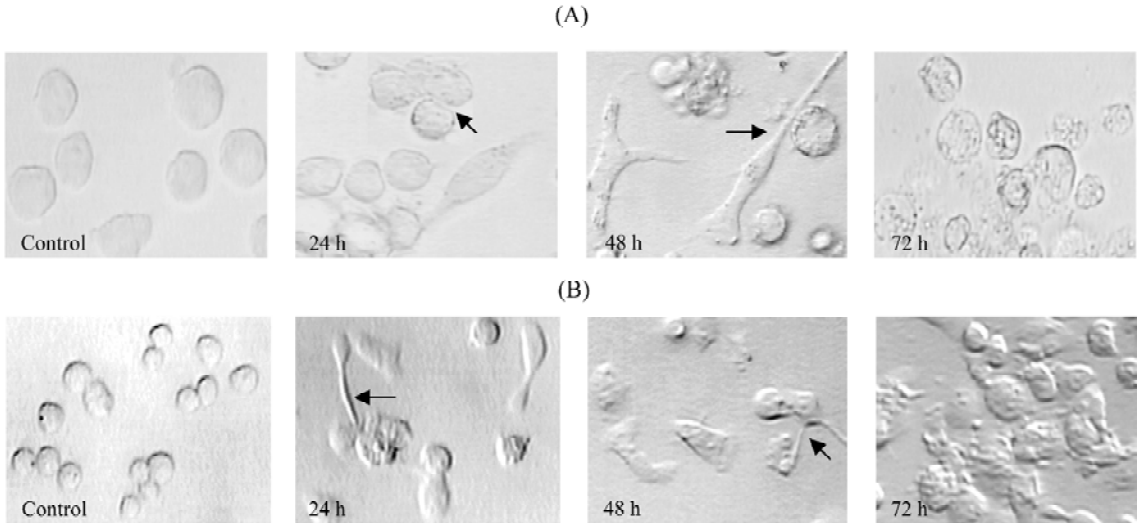

Fig. 5 Morphological changes of promyelocytic NB4 and HL-60 cells treated with the new compound from *Dendrostellera lessertii*, respectively

(A) NB4 cells treated with 1.5 μg/ml of the compound. (B) HL-60 cells treated with 2.0 μg/ml of the compound. Photomicrographs of the cells were taken by an inverted microscope at a magnification of 200×. NB4 and HL-60 cells were treated after 24, 48 and 72 h. At 24–48 h, the cells adhered to the surface of the culture flasks and started to form colonies and pseudopodia (arrows).

with a single dose of the drug, the cells developed megakaryocytic morphology characterized by a lower nuclear-to-cytoplasmic ratio and lobulated nuclei [**Fig. 4(F)**]. Neither NBT reducing activity nor benzidine staining capability was detected in the drug-treated K562 cells, indicating a lack of differentiation toward monocyte, granulocyte or erythroid cell types (data not shown).

Apoptosis and DNA fragmentation

As most leukemia cell differentiators are known to induce apoptosis, we checked whether the induction of differentiation in NB4, HL-60 and K562 cells would lead them to apoptosis. We assessed the nuclear morphological changes by Hoechst 33258 staining. As seen in **Table 1**

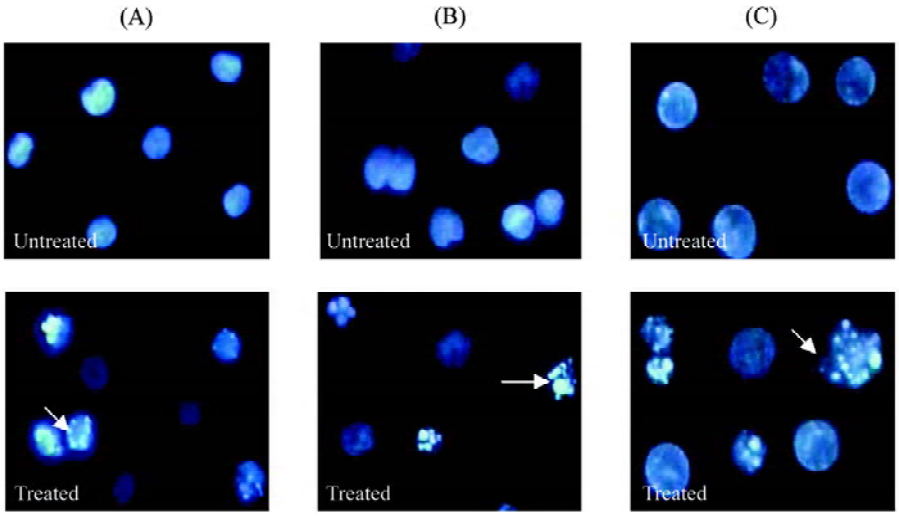

Fig. 6 Effect of the new compound from *Dendrostellera lessertii* on inducing apoptosis in promyelocytic NB4 (A) and HL-60 (B) cells and erythroleukemia K562 (C) cells

The drug-treated cells (NB4 and HL-60 after 72 h, and K562 after 120 h) were stained with Hoechst 33258 then observed by fluorescence microscopy (magnification, 200×). The new compound induced chromatin condensation and nuclei fragmentation (arrows).

and **Fig. 6**, more than 50% of NB4 and HL-60 and 30% of K562 cells underwent apoptosis upon a single dose (at IC_{50} value) of the drug after 72 h and in 50%–60% of the cells (NB4 and HL-60) fragmented nuclei were detected by fluorescence. Cells treated with high (2.5–3.0 μg/ml) or low (1.0–1.5 μg/ml) doses of the drug were prone to apoptosis after 24 and 72 h, respectively. In **Fig. 6**, the viable cells are uniformly blue, whereas the apoptotic cells are blue and contain bright blue dots in their nuclei, representing the nuclear fragmentation. These data indicate that the new compound causes apoptosis after induction of differentiation in the three cell lines evaluated.

Discussion

Our previous studies had demonstrated that 3-hydrogenkwadaphnine from *D. lessertii* has anti-leukemic effects and induces monocytic differentiation in HL-60 cells [19]. In the present study, another bioactive compound was isolated from the same plant extract, whose structural elucidation we are currently investigating. The results indicate that the new compound has strong anti-leukemic effects by inducing cell differentiation and apoptosis. NB4, HL-60 and K562 are different types of human leukemia cell lines, whose differentiation to terminal destinies by different specific inducers has been well documented [2–10]. It has been shown that HL-60 [21–23] and NB4 [24, 25] cells have a dual potential because they are competent to differentiate to monocytes in response to TPA and vitamin D3, or to granulocytes in response to ATRA and dimethylsulfoxide. K562 cells can be regarded as pluripotent hematopoietic progenitor cells expressing markers for erythroid, granulocytic, monocytic, or megakaryocytic lineage [26]. The current morphological and biochemical experiments (NBT reduction and benzidine staining) indicated that the new agent at concentrations below its IC_{50} value in each cell type significantly induced NB4 and HL-60 to differentiate along the monocytic/macrophage lineage and K562 along the megakaryocytic lineage. The mechanism by which this compound induces differentiation in these cells remains unknown. Inspection of data presented in **Table 1** clearly indicated that the differentiating potency of the new compound is more evident in NB4 cells compared with HL-60 cells. This is probably due to the fact that HL-60 cells are in a less mature stage than NB4 cells on the myeloid cell differentiation pathway [15]. Indeed, according to a French-American-British classification, HL-60 cells are considered as an AML-M_2 subtype, whereas NB4 cells are a typical cell line belonging to the AML-M_3 subgroup.

Our results have shown that the new compound induces apoptosis at concentrations above that required for differentiation induction. Apoptosis, similar to differentiation, was also dose- and time-dependent. Our observations indicated that, after approximately 12 h, the cells, mainly NB4 and

HL-60, began to differentiate and the peak was reached after 3 d of drug exposure. The treated cells also showed marked enhancement of the ability to phagocytose latex particles. Apoptosis began after 24 h at high drug doses and increased to its highest level (70%) after 96 h. Comparative data clearly indicate that the new compound acts similarly to TPA, ATRA and dimethylsulfoxide in induction of differentiation followed by apoptosis. In conclusion, the results of the present investigation clearly support this view that the biological activity of *D. lessertii* crude extract is due to several bioactive compounds, probably with different mechanisms of action.

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