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ORIGINAL ARTICLE

14-Deoxy-11,12-Didehydroandrographolide: A Novel Compound Isolated from *Andrographis paniculata* Nees. Induces Robust Apoptosis in Leukemic Cells

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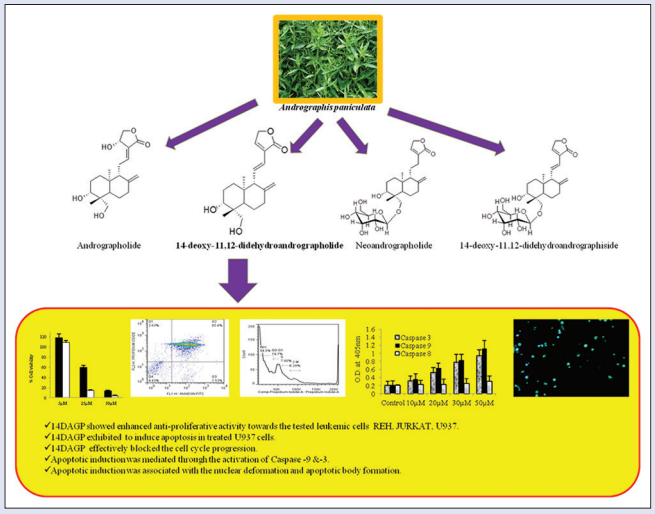
ABSTRACT

Objective: *Andrographis paniculata* is widely cultivated in South and Southeast Asian countries and popularly used in "Ayurveda" medicine. We attempted to investigate antileukemic activity of the biomolecules extracted from this plant and a probable mechanism of action. **Materials and Methods:** Biomolecules from methanolic extract were isolated using silica gel column chromatography and high-performance liquid chromatography. The structures were determined by liquid chromatography-mass spectrometry (LC-MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR. *In vitro* antiproliferative activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The apoptotic efficacy of the most potent molecule was investigated by annexin V and propidium iodide (V/PI) staining and flow cytometry. Caspase activation, cell cycle distribution pattern, and nuclear morphology of the treated leukemic cells were also investigated. **Results:** From the methanolic extract, four biomolecules were isolated, namely 14-deoxy-11,12-didehydroandrographolide (1), andrographolide (2), neoandrographolide (3), and 14-deoxy-11,12-didehydroandrographiside (4). Results of MTT assay exhibited that out of four compounds, compound (1) showed the most potent activity against all the cell lines tested with the lowest IC_{50} values of 13 μM on U937 cells. Annexin V/PI staining revealed that the compound was able to induce apoptosis in concentration-dependent manner with IC_{50} value being 17.66 μM. Apoptotic induction was mediated through elevated activation of caspase-3 and caspase-9. Cell cycle analysis revealed that the compound (1) effectively increased the sub-G0-G1 population in the treated U937 cells (73.25% at 50 μM) in comparison to control set (3.12%). DAPI nuclear staining indicated that compound (1) increased the number of deformed nuclei and an increased level of apoptotic body formation in the treated cells.

Key words: 12-didehydroandrographolide, 14-deoxy-11, *Andrographis paniculata*, antiproliferative, apoptosis, cytotoxicity, leukemia

SUMMARY

- Four biomolecules were isolated from methanolic leaf extract, namely 14-deoxy-11,12-didehydroandrographolide, andrographolide, neoandrographolide, 14-deoxy-11,12-didehydroandrographiside
- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay result showed that 14-deoxy-11,12-didehydroandrographolide was most effective molecule in comparison to the other three molecule
- 14-deoxy-11,12-didehydroandrographolide effectively induced apoptosis in U937 cells which was evidenced by annexin V and propidium iodide assay
- Apoptotic potential of 14-deoxy-11,12-didehydroandrographolide toward U937 cells was also mediated through the activation of caspase-9 and caspase-3
- 14-deoxy-11,12-didehydroandrographolide effectively arrested cell cycle progression at a G0-G1 phase in U937 leukemic cells
- It also induced nuclear fragmentation in U937 cells, which was evidenced by DAPI staining and fluorescence microscopy.



Abbreviations used: LC-MS: Liquid chromatography-mass spectrometry; NMR: Nuclear magnetic resonance; B-ALL: B-cell acute lymphoblastic leukemia; CML: Chronic myeloid leukemia; DAPI: 4′,6-diamidino-2-phenylindole; Bcl_2: B-cell lymphoma 2, Bcl-XL: B-cell lymphoma-extra large; FITC: Fluorescein isothiocyanate; PI: Propidium iodide; RPMI: Roswell Park Memorial Institute, PBS: Phosphate-buffered saline; ROS: Reactive oxygen species; DNA: Deoxyribonucleic acid; XIAP: X-linked inhibitor of apoptosis protein; HPLC: High-performance liquid chromatography.

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INTRODUCTION

Leukemia is the most common childhood cancer associated with an uncontrolled proliferation of immature neoplastic blood cells. As reported by the American Cancer Society (US), 60,300 new cases and 24,370 deaths are expected to occur in 2018 from leukemia.[1] An in-depth study has repeatedly confirmed that the occurrences of a number of mutations in the genome of these neoplastic cells resulting in the upregulation of antiapoptotic proteins such as Bcl-2 and Bcl-XL in concert with the downregulation of different crucial pro-apoptotic proteins such as Bid and Bim that ultimately abrogate apoptotic induction in such cell populations.[2] This basic information on the driving force leading to constant proliferation of these cells brings to conclude that induction of apoptosis is the best strategy to destroy such malignant cells in diseased patients. To combat these neoplastic blood cell proliferation, uses of multitarget drugs are currently being strategized as intervention to cancer therapy. Nowadays, a greater percentage of commercially available drugs contain active principles, e.g., vincristine, podophyllotoxin, etc. particularly from plant sources for treating leukemia because of its multiple targets and least side effects. Therefore, the main focus of our work is to search for a novel antileukemic biomolecule from plant sources, and in this lead, we looked at *Andrographis paniculata* as a potential plant source.

A. paniculata Nees. (*Acanthaceae*), commonly known as "Kalmegh," is a wonder drug and very popular in "Ayurvedic" and "Siddha" systems of medicine in India for its wide spectrum of biological activities.[3] It is an annual herb that grows in the shady places of India, Bangladesh, China, and various other Southeast Asian countries. Different extracts and compounds isolated from this plant have been reported to possess a broad range of therapeutic properties including antihepatic,[4] antitumoral,[5] anti-inflammatory,[6] antiangiogenic[7] effects, etc. Three major labdane-type diterpenoids to which the main biological activities of the plant are attributed are andrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide.[8] Andrographolide, the major diterpenoid of this plant, has reported hepatoprotective, hepatostimulant,[9] anti-inflammatory,[6] antiviral,[10] and anticancer activities.[11] 14-deoxy-11,12-didehydroandrographolide, the second major diterpene of this plant, has reported cardiovascular[12] and anti-inflammatory[13] effects. Recent research reveals the anticancer potentiality of 14-deoxy-11,12-didehydroandrographolide;[14,15] however, the antileukemic activity of this compound has not yet been established. Therefore, through this work, we aimed at unraveling the antileukemic property of this molecule that can be added to the armament of leukemia drug discovery.

In the present paper, we have looked at the antileukemic potential of different biomolecules isolated from *A. paniculata.* Drawing information from the existing literature, we isolated its biomolecules from the methanolic extract by column chromatography and high-performance liquid chromatography (HPLC) by a new protocol and isolated four different compounds (diterpenoids and its glycosides). The structures of these compounds were established by spectral analysis. The isolated compounds were screened for their antiproliferative and apoptotic activities against a panel of human leukemic cell lines. Data gathered through the work proved that 14-deoxy-11,12-didehydroandrographolide possesses a promising antileukemic activity by inhibition of cellular proliferation, externalization of phosphatidylserine, activation of caspases, induction of cell cycle arrest, and deformation of nuclear structure and apoptotic body formation.

MATERIALS AND METHODS

Materials

Roswell Park Memorial Institute (RPMI) 1640 and fetal bovine serum (FBS) were purchased from Gibco/Invitrogen. Penicillin, streptomycin, 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), and thiazolyl blue tetrazolium bromide 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from SIGMA. Silica gel (100–200) and all the solvents used for the preparation of extracts column chromatography and HPLC were purchased from Merck, India. Fluorescein isothiocyanate (FITC) annexin V and PI (V/PI) apoptosis detection kit were purchased from BD Pharmingen, USA.

Collection of plant materials

The leaves of the plants were collected from experimental garden of the Department of Botany, Gurudas College, University of Calcutta during the months of September–October 2014. Plant material was dried, and herbarium specimen was prepared. Voucher specimen was submitted and identified by the Calcutta University Herbarium, Department of Botany, University of Calcutta, India (Voucher specimen no.-20035). All plants were in the same developmental stage and harvested at the same time to avoid variation in the metabolic contents. Plant materials were air dried at 35°C–40°C temperature for several days so that no residual water remains in the sample. Dried samples were ground into fine powder (2 kg) and stored in vacuum desiccators until use.

Preparation of leaf extracts

Two kilogram of ground leaf powder was soaked in 95% methanol at room temperature for 3 days. After that, solvent was evaporated under reduced pressure using a rotary evaporator (BUCHI, Switzerland) at 37°C. Dried extracts were stored in −20°C (Celfrost) until further use.

Isolation of biomolecules from methanolic extract

Dried extract was subjected to silica gel (100–200 mesh) column chromatography and eluted with petroleum ether-chloroform mixtures (1:1 and 1:4) and chloroform-methanol mixture with gradually increasing polarity. Twelve fractions were collected, namely A through L. Fractions C and D were mixed up and subjected to column chromatography over silica gel (100–200 mesh) using chloroform:methanol (99:1, 98:2) solvent system to yield 14-deoxy-11,12-didehydroandrographolide through repeated crystallization. Pure crystals obtained were solid, colorless, needle shaped, yielded approximately 0.5 g in total by weight. Fractions E, F, and G were subjected to column chromatography using chloroform:methanol (98:2, 97:3) solvent system to yield andrographolide through crystallization (approximately 1 g). Pure crystals were colorless, solid, cube shaped. Fractions H and I were subjected to column chromatography over silica gel (100–200 mesh) using chloroform-methanol solvent system (96:4, 95:5, and 94:6) to obtain compound neoandrographolide by crystallization. Pure crystals were solid, needle shaped, colorless, yielded approximately 0.3 g in total by weight [Supplementary Material 1].

High-performance liquid chromatography

Fraction J, which was eluted in chloroform:methanol (93:7, 92:8) mixture from column chromatography, subjected to HPLC (Detector-2489 UV/Vis detector, Waters; wavelength used-300 nm; column-C_{18} semi-preparative column, Waters). Major compound 14-deoxy-11,12-didehydroandrographiside present in this fraction was purified. Solvent system used was water:methanol:acetonitrile:acetic acid in the ratio of 45:45:10:0.5 with 2.5 ml/min flow rate. The compound was yielded about 65 mg.

Cell culture

Four different human leukemic cell lines REH (B-cell acute lymphoblastic leukemia), JURKAT (T-cell acute leukemia), MOLT4 (T-cell acute lymphoblastic leukemia), and U937 (Human leukemic monocyte lymphoma) were maintained in RPMI 1640 media supplemented with 10% heat-inactivated FBS, 100U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of (5%) CO_2 incubator (HF-90).

MTT assay

Cells were seeded at a density of 1×10^5 cells/well in 96-well microtiter plate and treated with each compound at concentrations 10–50 µM for 24 h and 48 h time points. After treatment, 20 µl of 5 mg/ml MTT solution was added to each well and incubated for 3 h at 37°C. After incubation, the plate was centrifuged to pellet down the formazan crystals, and then, the supernatant was discarded. Formazan crystals produced in cells were solubilized in dimethyl sulfoxide. Absorbance was measured at 570 nm using a microplate reader (iMark, Bio-Rad, Japan). Cell apoptosis (100%) was obtained by lysis of cells in 5% sodium dodecyl sulfate lysis buffer. The percentage of cell viability was calculated as mentioned below:

$$\% \text{ cell viability} = 100 \times \left(\frac{(\text{OD sample} - \text{OD 100\%lysis})}{(\text{OD 0\%lysis} - \text{OD 100\%lysis})} \right)$$

Detection of apoptosis

U937 cells were seeded in a 96 well plate. After treating cells with different concentration of compound (1) for 24 h, cells were collected and subjected to annexin V and PI staining using FITC annexin V apoptosis detection kit I (BD Pharmingen), following the protocol provided by the manufacturer. Apoptosis event was then analyzed by flow cytometry (FACSCalibur, BD Biosciences). Postcompensation and analysis of FACS data were done using FlowJo v9 (Ashland, OR, USA).

Caspase-3,-8,-9 activity assay

Involvement of caspase-3,-8,-9 activities was measured spectrophotometrically (UV-Vis spectrophotometer, Shimadzu, Model No.-UV-1800, Wavelength 405 nm). Actively growing cells were seeded in 96 well plate treated with the compound (1) at different concentration. After 24 h of treatment, the cells were lysed in lysis buffer (provided with the caspase assay kit, Sigma). Lysates were centrifuged for 2 min at 10,000 rpm, and the supernatant was collected and used for caspase-3, caspase-9, and caspase-8 according to the manufacturer's protocol.

Cell cycle analysis

U937 and MOLT4 cells in the exponential phase of growth were treated with compound (1) for 48 h time points and then washed, harvested, fixed with 70% ethanol, and incubated for 30 min in phosphate-buffered saline (PBS) containing 100 µg/ml RNase and 50 µg/ml PI. For each sample, 1×10^4 cells were analyzed on a flow cytometer (FACS Verse, Becton Dickinson, San Jose, CA, USA). Results were expressed as the percentage of cells in each phase of the cell cycle.

Staining of the nuclei of the treated cells with 4′,6-diamidino-2-phenylindole

Cells were seeded at a density 1×10^5 cells/well in 96-well microtiter plate and treated with compound (1) at different concentrations for 24 h and 48 h. After treatment, cells were collected, washed with PBS, and permeabilized with methanol. DAPI (1 µg/mL in PBS) was added to each tube and incubated for 15 min at 37°C in the dark. Stained cells were observed under a fluorescent microscope (Premium FL, Dewinter, Germany).

RESULTS

Characterization of the isolated compounds

Structures of the isolated compounds [Figure 1] were determined by analyzing the spectral data, e.g., liquid chromatography-mass spectrometry (LC-MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR [Supplementary Material 2].

14-deoxy-11,12-didehydroandrographolide

^{1}H NMR ($CDCl_3$, 300MHz): δ 7.18 (1H, s), 6.86 (1H, dd, *J* = 10.2, 15.9 Hz), 6.11 (1H, d, *J* = 15.6 Hz), 4.82–4.78 (3H, m), 4.52 (1H, s), 4.21 (1H, d, *J* = 11.1 Hz), 3.47 (1H, dd, *J* = 4.8, 10.8 Hz), 3.34 (1H, d, *J* = 10.8 Hz), 3.01 (1H, brd), 2.47–2.43 (1H, m), 2.31 (1H, d, *J* = 9.9 Hz), 2.07–2.01 (1 H, m), 1.80–1.73 (4H, m), 1.53–1.49 (1H, m), 1.26 (3H, s), 1.23–1.09 (3H, m), 0.81 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 172.3, 148.1, 143.0, 135.9, 129.2, 121.0, 109.1, 80.7, 69.6, 64.1, 61.6, 54.6, 42.8, 38.5, 38.2, 36.5, 28.0, 22.9, 22.6, 15.8. MS (ESI) 355.27 (M + Na) +.($C_{20}H_{28}O_4$).

Andrographolide

^{1}H NMR (C_5D_5N, 300MHz): δ 7.18 (1H, td, *J* = 1.5, 7.0 Hz), 5.37 (1H, m), 4.88 (1H, d, *J* = 1.0 Hz), 4.85 (1H, d, *J* = 1.0 Hz), 4.60 (1H, dd, *J* = 6.0, 10.0 Hz), 4.50 (1H, dd, *J* = 2.5, 10.5 Hz), 4.43 (1H, d, *J* = 10.5 Hz), 3.64–3.60 (2H, m), 2.73 (t, *J* = 7.0 Hz), 1.90 (1H, m), 1.51 (3H, s), 0.70 (3H, s); ^{13}C NMR (C_5D_5N, 75 MHz) δ 170.7, 147.9, 147.0, 130.2, 108.8, 79.8, 75.4, 66.0, 64.1, 56.3, 55.3, 43.2, 39.1, 38.1, 37.3, 29.0, 25.0, 24.3, 23.7, 15.2. MS (ESI) 373.23 (M + Na) +.($C_{20}H_{30}O_5$).

Neoandrographolide

^{1}H NMR (CD_3OD, 300MHz): δ 7.33 (1H, s), 4.87–4.80 (3H, m), 4.61 (1H, d, *J* = 7.5 Hz), 4.15 (1H, d, *J* = 7.8 Hz), 4.08 (1H, d, *J* = 9.3 Hz), 3.84 (1H, dd, *J* = 1.9, 11.8 Hz), 3.66 (1H, dd, *J* = 5.2, 11.8 Hz), 3.34–3.12 (4H, m), 2.42–2.35 (2H, m), 2.13–2.03 (2H, m), 2.01–1.72 (5H, m), 1.69–1.56 (3H, m), 1.46–1.20 (4H, m), 1.11–1.06 (1H, dd, *J* = 3.6, 12.9), 1.02 (3H, s), 0.98–0.89 (1H, m), 0.7 (3H, s); ^{13}C NMR (C_5D_5N, 75 MHz) δ 174.6, 148.1, 145.3, 134.1, 106.9, 105.5, 78.7, 78.4, 75.3, 72.5, 71.7, 70.6, 62.8, 56.6, 56.2, 39.8, 39.0, 38.7, 38.6, 36.4, 28.1, 24.9, 24.7, 22.0, 19.3, 16.6. MS (ESI) 503.38 [M + Na] +.($C_{26}H_{40}O_8$).

14-deoxy-11,12-didehydroandrographiside

^{1}H NMR (CD_3OD, 300MHz): δ 7.45 (1H, s), 6.89 (1H, dd, *J* = 10, 15.7 Hz), 6.17 (1H, d, *J* = 15.9 Hz), 4.76 (1H, s), 4.60 (1H, s), 4.50 (1H, s), 4.26 (1H, d, *J* = 10.2 Hz), 4.21 (1H, d, *J* = 7.8 Hz), 3.87 (1H, d, *J* = 11.4 Hz), 3.68 (1H, dd, *J* = 4.8, 11.7 Hz), 3.51 (1H, d, *J* = 10.2 Hz), 3.38–3.27 (8H, m), 3.18 (1H, t, *J* = 8.2 Hz), 2.49–2.38 (2H, m), 2.07–1.87 (1H, m), 1.82–1.74 (2H, m), 1.69–1.48 (4H, m), 1.30–1.25 (3H, m), 1.22 (3H, s), 0.91 (3H, s); ^{13}C NMR (CD_3OD, 75 MHz) δ 174.9, 150.3, 146.9, 136.6, 129.6, 122.6, 109.2, 105.0, 80.7, 78.2, 78.0, 75.1, 72.3, 71.7, 71.6, 63.0, 62.8, 56.2, 44.1, 40.0, 39.9, 38.0, 29.0, 24.9, 24.2, 16.2. MS (ESI) 517.39 (M + Na) +.($C_{26}H_{38}O_9$).

Differential antiproliferative potential of the isolated compounds

To fish out the most potent antileukemic biomolecules, we investigated the antiproliferative effects of four different compounds isolated from *A. paniculata* against different leukemic cell lines, e.g., REH, JURKAT, U937, and MOLT4. Each cell line was treated with each of the isolated compounds for 24 h and 48 h time points followed by MTT assay. Results of the MTT assay [Figures 2 and 3] proved that 14-deoxy-11,12-didehydroandrographolidewasmostpotentantiproliferative

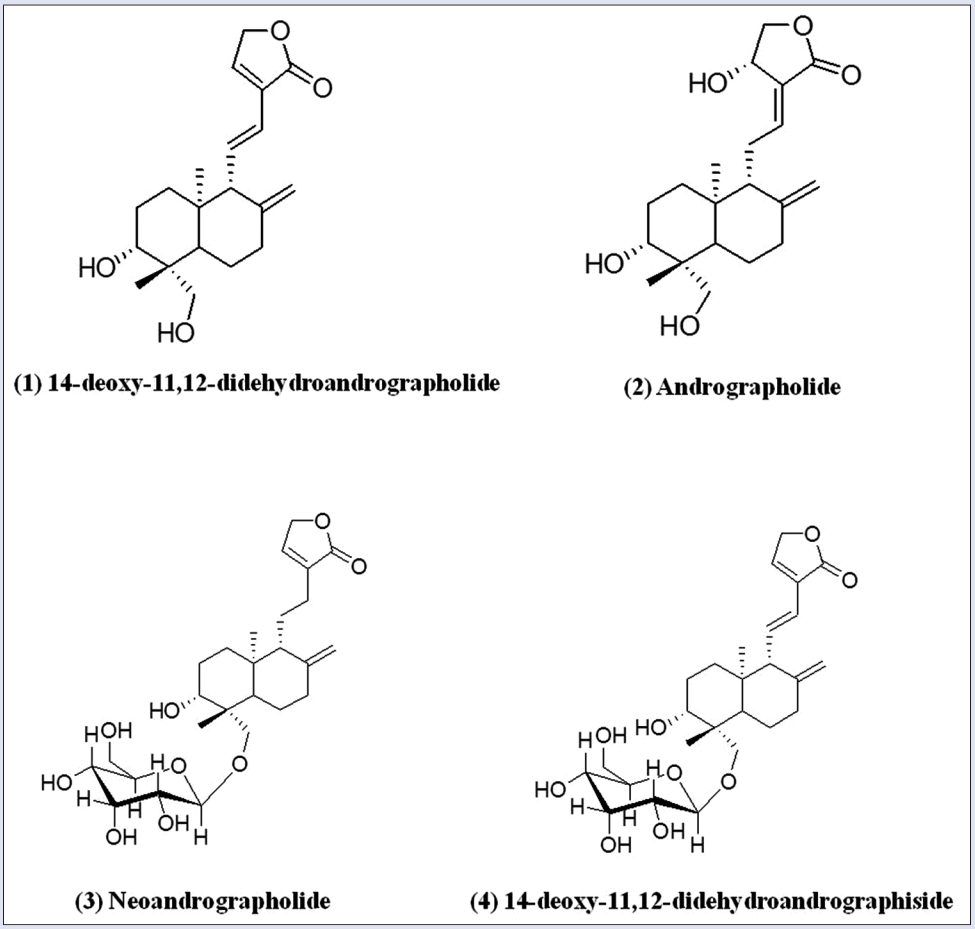

Figure 1: Structures of the isolated diterpenoids from *Andrographis paniculata*. Compounds (1) and (2) are diterpenoids and compounds (3) and (4) are diterpenoid glycosides

molecule on each of the four cell lines tested. Apart from this compound, androdrapholide and 14-deoxy-11,12-didehydroandrodraphiside also exhibited antiproliferative properties against all the cell line tested although the degree of efficacy varies with the type of the cell lines tested. Leukemic cell viability decreased in a concentration and time-dependent manner post 14-deoxy-11,12-didehydroandrographolide, androdrapholide, and 14-deoxy-11, 12-didehydroandrographiside treatment. 14-deoxy-11,12-didehydroandrographolide showed maximum antiproliferative activity with IC_{50} values of 13 μM, 27 μM, and 35 μM on U937, REH, and JURKAT cells, respectively. However, antiproliferative activity of 14-deoxy-11,12-didehydroandrographolide and 14-deoxy-11,12-didehydroandrographiside was found to be moderate in leukemic cells where U937 and REH cells were more sensitive than JURKAT cells [Figures 2a, b and 3a]. The IC_{50} values could not be reached with 14-deoxy-11,12-didehydroandrographolide and 14-deoxy-11,12-didehydroandrographiside in any of the tested leukemic cell lines. MTT data also showed that MOLT4 cells were most resistant [Figure 3b] to antiproliferative effects of all the compounds tested, including 14-deoxy-11,12-didehydroandrographolide. 14-deoxy-11,12-didehydroandrographolide could not reach the IC_{50} value within the concentration range that was tested against MOLT4 cells. Andrographolide showed antiproliferative activity against REH, U937, and MOLT4 cells but not toward JURKAT cells; however, IC_{50} value was not reached in any of the cell lines. Neoandrographolide was found to have the least antiproliferative efficacies against all the leukemic cell lines tested here. In a nutshell from the MTT assay results, we observe that 14-deoxy-11,12-didehydroandrographolide possesses most promising antileukemic potentiality followed by andrographolide and 14-deoxy-11,12-didehydroandrographiside, whereas neoandrographolide does not seem to possess any antileukemic activity [Figures 2 and 3].

14-deoxy-11,12-didehydroandrographolide induces apoptosis in U937 cells

Apoptotic potential of the 14-deoxy-11,12-didehydroandrographolide was analyzed by FITC-annexin V/PI double staining method and flow cytometry and result represented in Figure 4. This compound-induced apoptosis in U937 cells in a dose-dependent manner as shown by an increasing population of cells stained with both annexin V-FITC and PI. The IC_{50} value of 14-deoxy-11,12-didehydroandrographolide was observed to be 17.66 ± 1.15 μM as obtained at 24 h time point in annexin V/PI assay which is very close to MTT assay. A detail data of annexin V/PI assay are given in Table 1. To check if the apoptosis induced by our compound of interest is specific to leukemic cells alone and does not have any cytotoxicity associated toward normal cells, we treated peripheral blood mononuclear cell (PBMC) cells isolated from normal, healthy donors with 14-deoxy-11,12-didehydroandrographolide and performed annexin V/PI assay after 24 h. Interestingly, the compound did not induce apoptosis in normal PBMC, which assures its leukemia-specific activity [Figure 4]. Necrotic event was not observed in U937 cells after treatment.

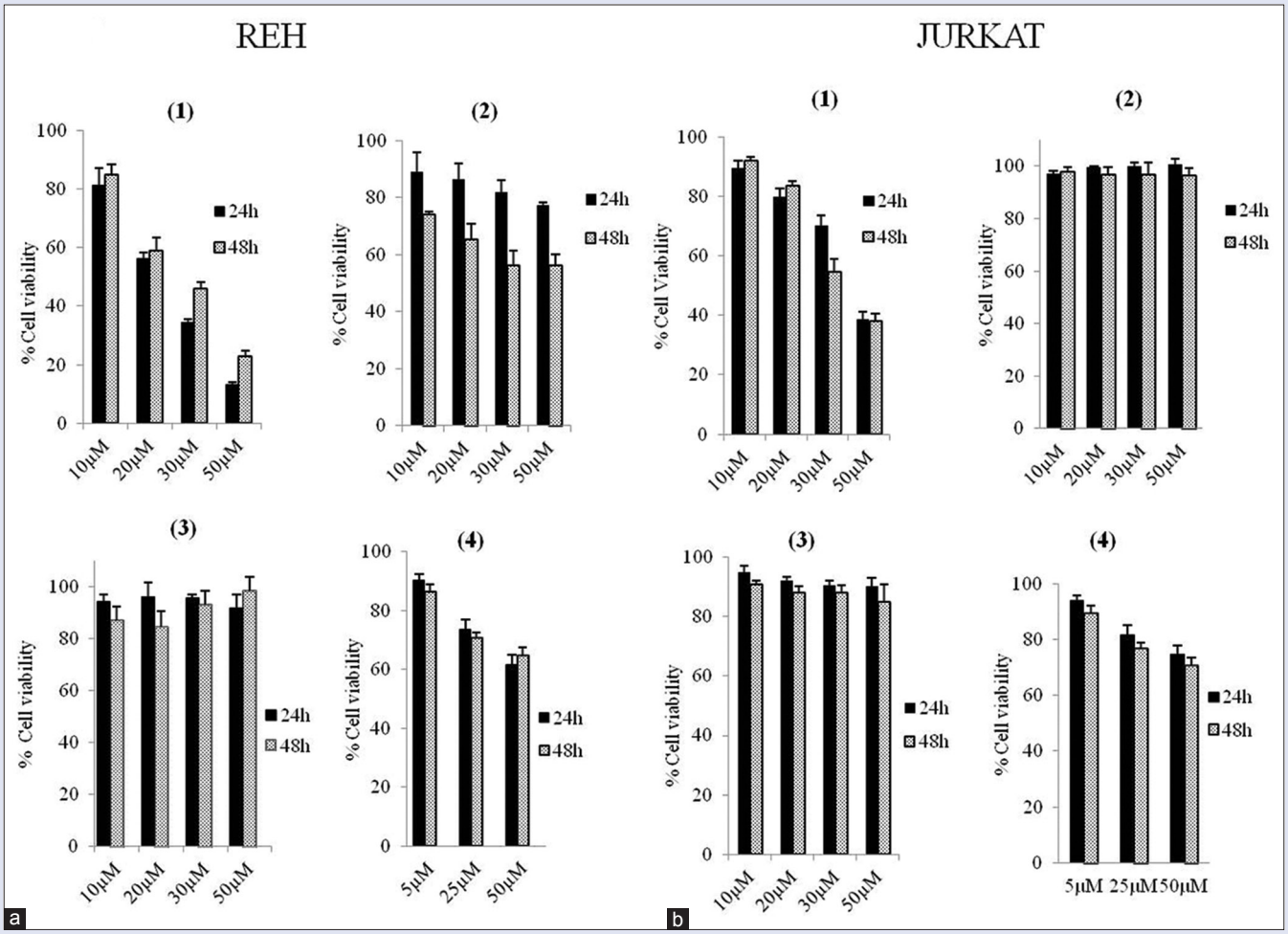

Figure 2: Assessment of antiproliferative activity of the isolated compounds against (a) REH and (b) JURKAT cells. Each cell line was treated with each individual compounds (1, 2, 3, and 4) at different dose for 24 h and 48 h time points. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to evaluate the antiproliferative capacity. Each bar graph represents mean ± standard deviation of three independent experiments

Table 1: Apoptosis analysis by annexin V/propidium iodide assay after compound (1) treatment

Control	10 μM	20 μM	30 μM	50 μM
2.8±1.41	26.41±0.96	57.55±4.78	89±1.46	97.23±1.87

Percentage of annexin V/FITC positive cells after compound (1) treatment at different concentrations. The data represent mean±SD. FITC: Fluorescein isothiocyanate; SD: Standard deviation

Involvement of caspase-3 and caspase-9 in the apoptotic event

Caspase-3, caspase-8, and caspase-9 are the major caspases that indicate the mechanism of apoptosis. The result of the caspase activity study indicated that 14-deoxy-11,12-didehydroandrographolide effectively induced caspase-3 and caspase-9 as evidenced by OD in the activity of the caspases at different concentrations [Figure 5a]. Caspase-8 was not induced during apoptosis after compound (1) treatment. To confirm the involvement of caspases in 14-deoxy-11,12-didehydroandrographolide-induced apoptosis in U937 cells, we pretreated cells with a pan-caspase inhibitor Z-VAD-fmk and finally treated with our compound of interest. We found that the cells were rescued from undergoing apoptosis [Figure 5b]. Altogether the results of this assay confirmed that apoptotic event took place through the activation of caspase-3 and caspase-9 but not the caspase-8.

U937 cells showed cell cycle arrest after compound (1) treatment

Literature study reports the association of cell cycle arrest with the antiproliferative efficacy of biomolecules.[11] We therefore wanted to look at the cell cycle status of U937 cells upon treatment with 14-deoxy-11,12-didehydroandrographolide. To serve the purpose, we performed cell cycle analysis of U937 cells after compound treatment (10 μM, 20 μM, 30 μM, and 50 μM) by PI staining using flow cytometry. Results of flow cytometry showed [Figure 6] that 14-deoxy-11,12-didehydroandrographolide could induce appreciable apoptosis in the U937 cells by mainly targeting the G0/G1 cells and gradually increasing the sub-G0/G_1 cell population with increasing concentration of compound (1) compared to the untreated control cells. Induction of apoptosis was dose dependent in U937 cells, which is consistent with the MTT and annexin V/PI assay results. At 30 μM treatment U937 cells showed 54.75% ±1.11% apoptosis, whereas at 50 μM treatment, U937 cells showed 73.25% ±2.21% apoptosis. Cell cycle analysis after 14-deoxy-11,12-didehydroandrographolide shows that it has considerable potential in arresting cell cycle progression of leukemic cells. A detail data of cell cycle analysis upon the compound (1) treatment has been given in Table 2.

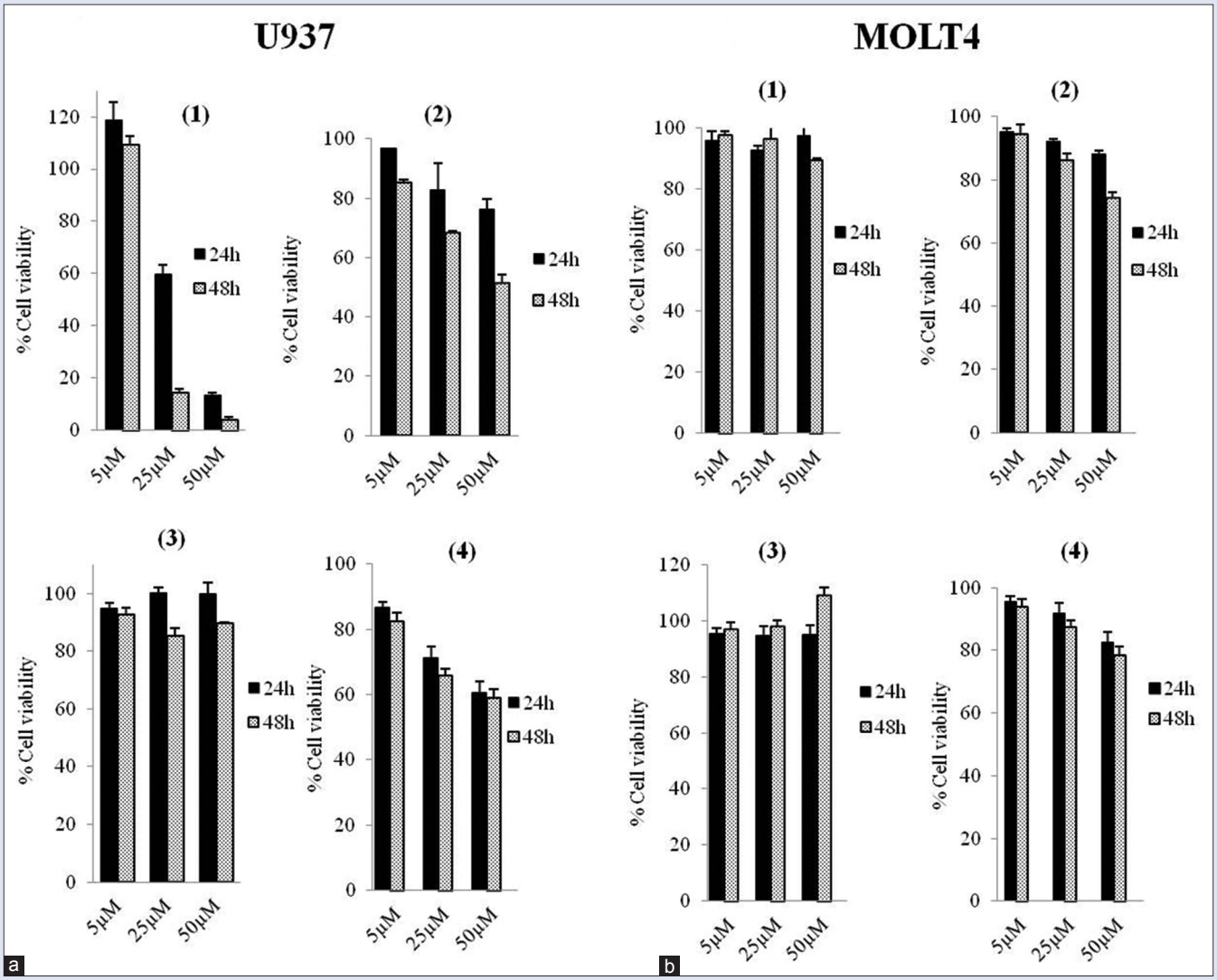

Figure 3: Assessment of antiproliferative activities of the isolated compounds against (a) U937 and (b) MOLT4 cells. Each cell line was treated with each individual compound (1, 2, 3, and 4) at different dose for 24 h and 48 h time points. After incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to evaluate the antiproliferative capacity. Each bar graph represents mean ± standard deviation of three independent experiments

U937 cells exhibit high nuclear deformation and apoptotic body formation upon compound (1) treatment

Nuclear deoxyribonucleic acid (DNA) fragmentation is one of the hallmark events of apoptosis. Although apoptotic efficacy of andrographolide had already been established against a number of cancer type,[16] there was no evidence of apoptotic activity of 14-deoxy-11,12-didehydroandrographolide against leukemic cells, e.g., U937 cells. So far the data that we have obtained advocates apoptotic efficacy of 14-deoxy-11,12-didehydroandrographolide on leukemic cells and hence we further studied if 14-deoxy-11,12-didehydroandrographolide treatment causes nuclear fragmentation in U937 cells. Treated cells were stained with the DNA binding fluorescent dye DAPI (as described in the materials and methods section) and observed under a fluorescence microscope. Results depict that U937 cells showed a higher degree of fragmented and pyknotic nuclei after treatment with 14-deoxy-11,12-didehydroandrographolide (5 μM, 25 μM and 50 μM) in a dose-dependent fashion [Figure 7]. This data further established that 14-deoxy-11,12-didehydroandrographolide could induce nuclear damage in U937 cells and thereby confirmed its apoptotic efficacy.

DISCUSSION

In the present study, we investigated the antileukemic potential of 14-deoxy-11,12-didehydroandrographolide and other biomolecules isolated from Indian medicinal plant *A. paniculata.* Therefore, we attempted to isolate the chemical constituents from the methanolic fractions of the plant. At first, we prepared the methanolic extract of the plant leaves. This methanolic extract was run through the column chromatography to isolate its major biomolecules, namely 14-deoxy-11,12-didehydroandrographolide, andrographolide, and neoandrographolide, and a new HPLC method had been standardized to isolate 14-deoxy-11,12-didehydroandrographiside. Andrographolide and 14-deoxy-11,12-didehydroandrographolide are the major diterpenoids of *A. paniculata*. These diterpenoids alongside neoandrographolide, a diterpenoid glycoside, was obtained through repeated crystallization. 14-deoxy-11,12-didehydroandrographiside,

Table 2: Cell cycle analysis of the cells treated with different concentrations of compound (1)

Phases of cell cycle	Concentration of compound (1)				
	Control	10 µM	20 µM	30 µM	50 µM
Apoptosis	3.12±1.3	9.45±4.47	35.38±5.11	54.75±1.11	73.25±2.21
G0-G1	55.34±3.3	51.67±4.87	27.56±2.89	21.39±4.92	13.12±2.56
S	18.53±4.11	14.45±4.88	11.74±5.11	6.42±4.67	6.75±3.77
G2-M	12.25±2.64	14.33±5.69	10.73±3.52	6.7±3.84	4.69±2.23

Percentage of cells in different phases of cell cycle, after compound (1) treatment and staining with PI. Data represent mean±SD. SD: Standard deviation; PI: Propidium iodide

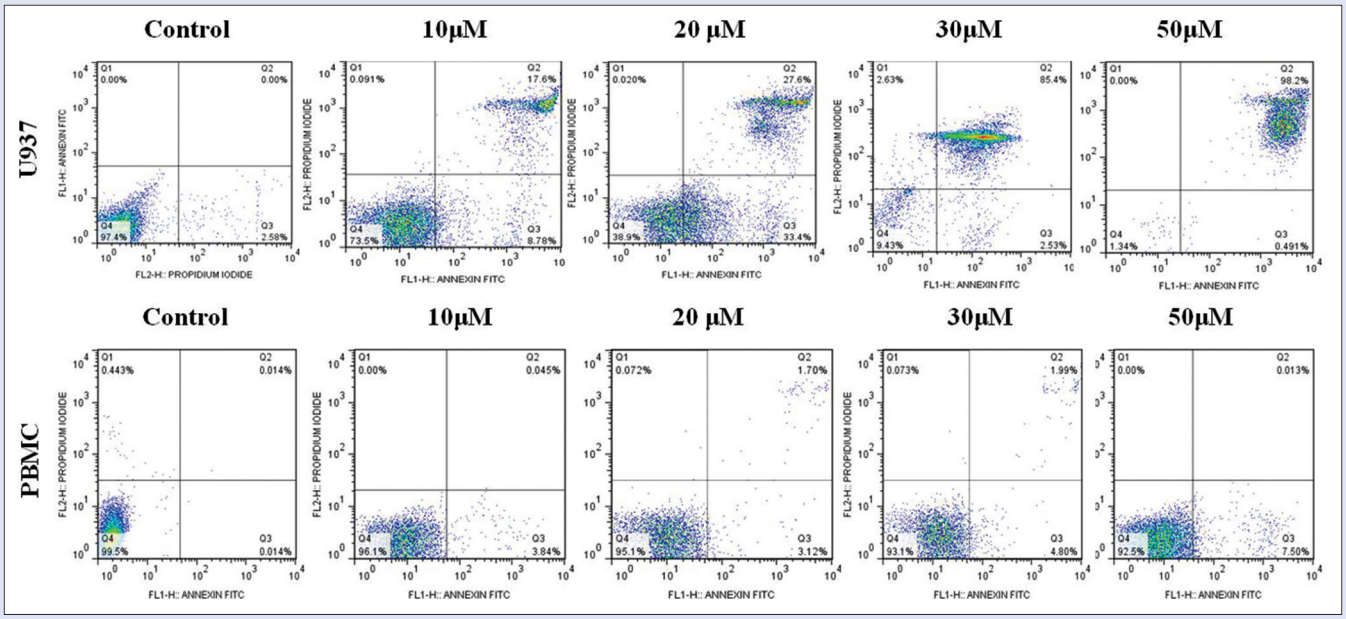

Figure 4: Annexin V and propidium iodide assay of U937 and normal peripheral blood mononuclear cell. U937 and normal peripheral blood mononuclear cell were treated with compound (1) for 24 h. After incubation cells were stained and investigated using flow cytometer

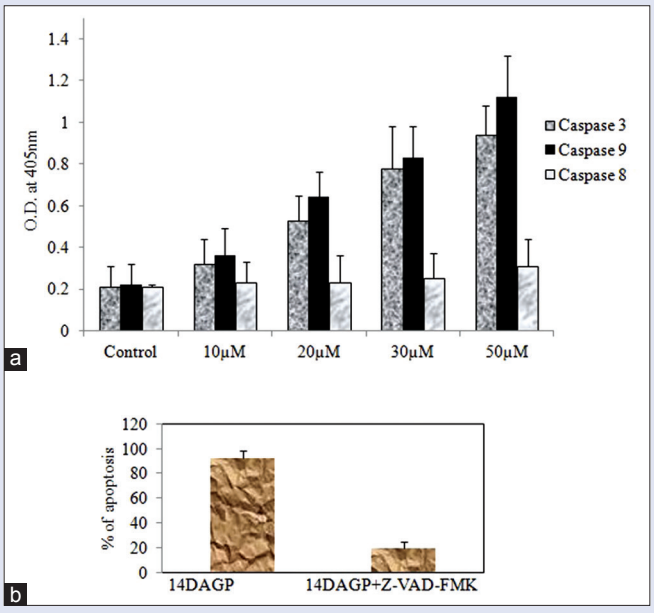

Figure 5: Caspase activity assay after compound (1) treatment. (a) U937 cells were treated with compound (1) for 24 h, and after incubation, activity of different caspase-3, caspase-9, and caspase-8 was investigated spectrophotometrically. (b) U937 cells were treated with or without caspase inhibitor Z-VAD-FMK followed by compound (1) treatment for 24 h and apoptosis level was measured

also a diterpenoid glycoside, however, was isolated by HPLC. Structures of the isolated compounds were determined by spectroscopic methods. The acquired spectral data of these compounds were compared with the published data to confirm the structures and were found to be the same. This indicated that the compounds thus isolated were pure.

Literature study revealed that a number of diterpenoids possess promising antileukemic activities against an array of leukemic cell lines *in vitro* as well as *in vivo,* e.g., oridonin,[17,18] gnidimacrin,[19] melissoidesin G,[20] etc. These diterpenoids induce apoptosis in various cancer cells including leukemia following different mechanisms. Melissoidesin G inhibits HL-60 leukemic cells through loss of the mitochondrial membrane potential ($\Delta\psi_m$), reactive oxygen species generation, caspase activation, and nuclear fragmentation;[20] another diterpenoid, jolkinolide B-induced apoptosis in K562 cells, which was characterized by cell shrinkage, membrane blebbing, loss of microvilli, nuclear condensation, and DNA degradation[21] and it inhibited U937 cells by downregulating the antiapoptotic protein X-linked inhibitor of apoptosis protein, blocking the expression of PI3K/Akt pathway proteins, and activation of effector caspase-3 and caspase-9.[22] This literature study revealed that each of the compounds of a plant may or may not possess antileukemic property and the potential if present can be of various degrees. Therefore, to investigate the antileukemic activities of each of the isolated compounds, we screened their antiproliferative activities against four established human leukemic cell lines REH, JURKAT, U937, and MOLT4 in a concentration and time-dependent manner. Results indicate that 14-deoxy-11,12-didehydroandrographolide showed appreciable antiproliferative activity with an IC_{50} value

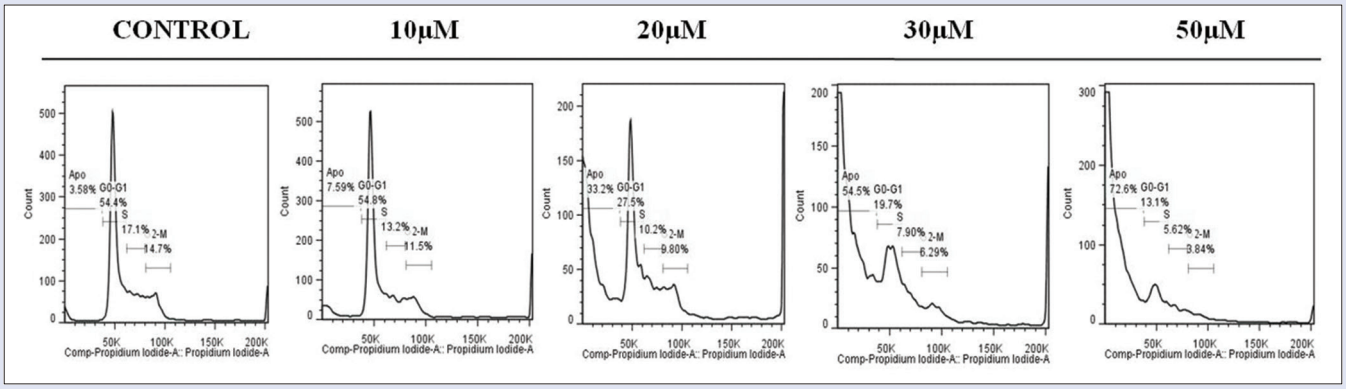

Figure 6: Cell cycle analysis of U937 cell line post compound (1) treatment. U937 cells were treated with different concentrations of compound (1) for 48 h, and DNA was stained with PI and analyzed in flow cytometer

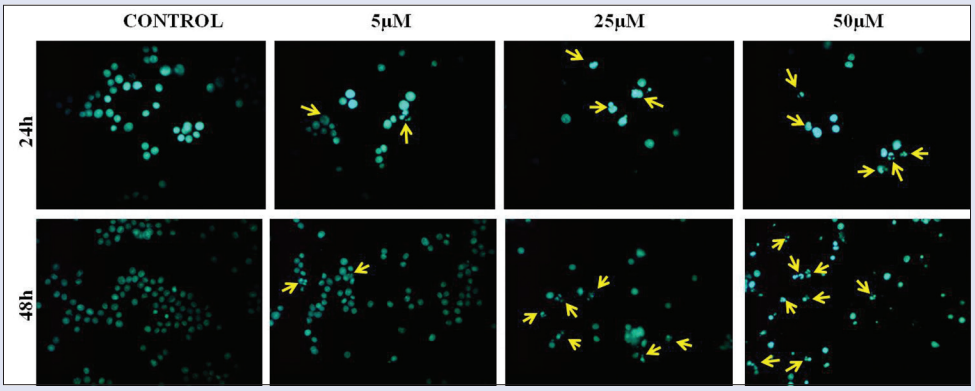

Figure 7: Detection of nuclear deformation an apoptotic body formation by DAPI staining after compound (1) treatment. U937 cells were treated with different concentration of compound (1) for 24 h and 48 h time points. After that, nuclear DNA was stained with DAPI, and cells were observed under fluorescent microscope. U937 cells show high degree of nuclear fragmentation in a concentration and time-dependent manner

of 13 μM at 48 h time point. This experiment also revealed that U937 was the most sensitive, whereas MOLT4 appeared to be most resistent to 14-deoxy-11,12-didehydroandrographolide treatment among all the cell lines tested. Based on this data, we selected 14-deoxy-11,12-didehydroandrographolide to look in-depth at its mechanism of antiproliferative effects shown in U937 cells. At first, we investigated the apoptotic potential of this compound by annexin V/PI assay. The result of this experiment confirmed that our compound of interest induced the programmed cell death or apoptosis in U937, without holding any toxic effects in normal PBMCs as confirmed by our experiments done further. To investigate the apoptotic pathway, we studied the involvement of mitochondrial caspases. Results showed that caspase-9 and caspase-3 were more active than caspase-8 and indicate the involvement of the intrinsic apoptotic pathway. We further studied the effect of 14-deoxy-11,12-didehydroandrographolide on the cell cycle progression of leukemic cells and the PI staining data which we got indicates that the treatment increased significant levels of apoptotic cell population, i.e., sub-G0-G1 population in a dose-dependent manner. Apoptotic activity was further established by studying nuclear fragmentation after 14-deoxy-11,12-didehydroandrographolide treatment. The result again confirmed a greater susceptibility of U937 cells to 14-deoxy-11,12-didehydroandrographolide. Apart from 14-deoxy-11,12-didehydroandrographolide, andrographolide which is a major diterpenoid of *A. paniculata* also has reported anticancer properties against a number of different cancer cells.[11] In our study, this compound also showed differential antiproliferative effects on four different cell lines although the effect was not very significant with respect to 14-deoxy-11,12-didehydroandrographolide. Neoandrographolide is another major diterpenoid glycoside of this plant. The literature review indicates that it can act as a chemosensitizer with suboptimal concentration of etoposide against JURKAT cells although it has negligible antileukemic property when treated alone, which is consistent with our result as well. On the other hand, 14-deoxy-11,12-didehydroandrographiside, another diterpenoid glycoside, we observed that this compound has lower levels of antiproliferative activity against four different leukemic cell lines not reported so far. Altogether the compounds isolated from *A. paniculata* have shown interesting antileukemic effects out of which 14-deoxy-11,12-didehydroandrographolide was found to possess the most significant antileukemic efficacies that have not been reported till date.

CONCLUSION

We found that 14-deoxy-11,12-didehydroandrographolide is the most potent antileukemic compound of all the compounds tested, whereas other compounds although showing much less activity compared to compound (1) also possess antiproliferative effects, which together confer the antileukemic property of the major compounds that were present in the methanolic extract of the plant. We found that 14-deoxy-11,12-didehydroandrographolide being the most potent of all the compounds isolated is equally effective in inducing antiproliferative

effects at concentrations below 40 μM on U937, REH, and JURKAT cells, respectively, whereas in MOLT4 cells, the effects were seen at concentartion 50 μM and above. From the data obtained collectively, it can be said that 14-deoxy-11,12-didehydroandrographolide from *A. paniculata* holds a great promise as a potential antileukemic molecule as the antiproliferative effects seen was only specific for leukemic cells and such effects were not found when tested on normal PBMC cells. Therefore, with further detailed study on the effect of 14-deoxy-11,12-didehydroandrographolide on the molecular pathways activated in leukemia, it could very well become a molecule of therapeutic interest in treating leukemia in the future.

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Conflicts of interest

There are no conflicts of interest.

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