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Cooperative antiproliferative and differentiation-enhancing activity of medicinal plant extracts in acute myeloid leukemia cells

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ABSTRACT

Acute myeloid leukemia (AML) is an aggressive hematopoietic malignancy with poor prognosis and limited treatment options. Sea buckthorn (*Hippophae rhamnoides*) berries, dog rose (*Rosa canina*) rosehips, and garden sage (*Salvia officinalis*) and oregano (*Origanum vulgare*) aerial parts are widely used in traditional medicine and exhibit antitumor effects in preclinical models. However, these plants remain scarcely tested for antileukemic activity. Here, we show that their water-ethanol leaf extracts reduced the growth and viability of AML cells and, at non-cytotoxic doses, potentiated cell differentiation induced by a low concentration of 1α,25-dihydroxyvitamin D_3, the hormonal form of vitamin D, in a cell type-dependent manner. The latter effect was accompanied by upregulation of the vitamin D receptor protein components and its transcriptional activity. Furthermore, at minimally effective doses the extracts cooperated with one another to produce marked cytostatic effects associated with a partial S-phase arrest and a modest induction of apoptosis. In contrast, these combinations only slightly affected the growth and viability of proliferating normal human peripheral blood mononuclear cells. In addition, the extracts strongly inhibited microsomal lipid peroxidation and protected normal erythrocytes against hypoosmotic shock. Our results suggest that further exploration of the enhanced antileukemic effects of the combinations tested here may lead to the development of alternative therapeutic and preventive approaches against AML.

1. Introduction

Acute myeloid leukemia (AML) is the most common acute leukemia in adults and is characterized by the accumulation of highly proliferative immature leukemic cells in the bone marrow which suppresses normal hematopoiesis. Although 50–70% of younger patients with AML achieve complete remission following cytotoxic chemotherapy, most are expected to relapse and die [1]. Furthermore, due to its high general toxicity, standard aggressive treatment is largely unsuitable for elderly individuals representing the majority of patients with AML [2]. Despite a number of new compounds that have been in development for AML treatment, most have failed in clinical trials, and not a single new drug has yet been approved for AML in the last 40 years [3].

Phytochemicals (e.g., vinblastine, paclitaxel, topotecan, etoposide) have been successfully used for the treatment of lymphoid leukemias and solid malignancies [4]. Although the above drugs are not effective in AML therapy, other compounds, e.g., the alkaloid homoharringtonine from the Chinese coniferous tree *Cephalotaxus hainanensis* and the semisynthetic flavonoid flavopiridol from the Indian plant *Dysoxylum binectariferum* are currently evaluated in clinical trials in AML [5,6]. Extracts from various edible and medicinal plants possess therapeutic and preventive potential in hematopoietic cancers and represent rich sources of novel antileukemic drugs [7,8]. Furthermore, we and others have shown that both individual phytochemicals and whole plant extracts can cooperate with one another [9–14] and with

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other natural agents, such as vitamin D derivatives [15–20], in producing enhanced anticancer effects in preclinical models.

Here, we show that leaf extracts from several medicinal plants collected in the Trans-Ili Alatau region of Kazakhstan – sea buckthorn (*Hippophae rhamnoides*), dog rose (*Rosa canina*), garden sage (*Salvia officinalis*) and oregano (*Origanum vulgare*) – exhibited dose-dependent antiproliferative activity in AML cell lines. These extracts were also capable of potentiating the differentiation-inducing effects of 1α,25-dihydroxyvitamin D_3 (1,25D), the active form of vitamin D, which was accompanied by upregulation of the vitamin D receptor (VDR) levels and functional activity. In addition, different combinations of the extracts at minimally effective doses produced marked cooperative cell-type dependent cytostatic effects associated with a partial S-phase arrest and modulation of the levels of cell cycle regulatory proteins.

2. Materials and methods

2.1. Materials

RPMI 1640 medium and fetal calf serum (FCS) were obtained from Gibco-Invitrogen (Carlsbad, CA, USA). Dulbecco's phosphate buffered saline (PBS), HEPES, penicillin and streptomycin were purchased from Biological Industries (Beit Haemek, Israel). Triton X-100, RNAse, Histopaque-1077 and other biochemical reagents were obtained from Sigma-Aldrich (Rehovot, Israel). 1,25D was a gift from Dr. A. Kutner (Pharmaceutical Research Institute, Warsaw, Poland). Antibodies to cyclins A and E, CDK2, $p27^{Kip1}$, VDR, RXRα and β-tubulin were obtained from Santa Cruz Biotechnology (Dallas, TX). Anti-PARP antibody was purchased from BioMol (Plymouth Meeting, PA, USA). Stock solutions of 1,25D (~0.2 mM) were prepared in absolute ethanol and the exact concentration of this agent was then determined spectrophotometrically at 264 nm (ε = 19000).

2.2. Plant materials

Aerial parts of *H. rhamnoides* L. and *R. canina* L. were collected in the foothills of the Trans-Ili Alatau mountains (Almaty region, Kazakhstan) during July 2013. The plants were identified, and voucher specimens No. 5470 (*H. rhamnoides* L.) and No. 3389 (*R. canina* L.) were deposited at the herbarium of the Institute of Botany and Phytointroduction (Almaty, Kazakhstan). Commercial samples of dried leaves of *S. officinalis* and aerial parts of *O. vulgare* (TES, Almaty, Kasakhstan), collected in the Almaty region, were purchased from a local pharmacy.

2.3. Preparation of plant extracts

Crushed dried leaves of *H. rhamnoides*,*R. canina* and *S. officinalis* or aerial parts of *O. vulgare* were extracted with 50% (v/v) aqueous ethanol, at room temperature for 20 h in the dark, as described previously [21]. The mixture was then centrifuged at 20,000*g* for 10 min and the supernatant was dried at 37 °C in a rotary evaporator. Stock solutions of the dried extracts (100 mg/ml) were freshly prepared in 50% ethanol before experiments.

2.4. Determination of total polyphenol and flavonoid content in dried plant extracts

Total phenolic content was determined by the standard Folin-Ciocalteu assay [22]. The absorbance of the samples was measured at a wavelength of 765 nm and the values were expressed as gallic acid equivalents (GAE, μg per mg dried extract). The total flavonoid content was determined using a colorimetric method described by Heimler et al. [23]. The absorption was measured at 510 nm and the amount of total flavonoids was expressed as quercetin equivalents [QE, μg per mg dried extract].

2.5. Preparation of rat liver microsomes

Healthy Wistar rats (12–14 weeks, 300 ± 50 g) were housed under standard conditions of light and dark cycle with free access to food and water. The experimental protocols were approved by the Institutional Ethical Committee of the Institute of Human and Animal Physiology (Almaty, Kazakhstan). The animals were killed under light ether anesthesia. The liver was isolated, washed, and perfused with chilled saline. The minced tissue was homogenized (1:10 w/v) in 10 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA on ice. The homogenate was centrifuged at 10,000*g* at 4 °C, for 20 min. The supernatant was further centrifuged at 100,000*g*, for 60 min, to obtain the microsomal fraction. Microsomes were suspended in a buffer containing 10 mM histidine (pH 7.2), 25% (v/v) glycerol, 0.1 mM EDTA and 0.2 mM $CaCl_2$, and were kept at −20 °C. The protein content was measured by the Lowry assay using bovine serum albumin as a standard.

2.6. Isolation of rat erythrocytes

Erythrocytes were obtained by centrifuging blood for 10 min at 1000*g*. Plasma and white blood cells were removed, erythrocytes were washed twice with a buffer containing 5 mM Na_2HPO_4 (pH 7.4) and 150 mM NaCl, and used fresh.

2.7. Determination of lipid peroxidation in liver microsomes

Lipid peroxidation (LPO) was assessed by measuring malondialdehyde (MDA) content in the form of thiobarbituric acid-reacting substances (TBARS) by the method of Ohkawa et al. [24]. Briefly, liver microsomes were preincubated with tested agents in a buffer containing 50 mM KH_2PO_4 (pH 7.2) and 145 mM NaCl at 37 °C, for 10 min, under constant stirring. The basal and 0.02 mM Fe^{2+}/0.5 mM ascorbate-induced microsomal LPO was then determined in a reaction mixture containing 0.9 M sodium acetate buffer (pH 3.5), 0.4% SDS, 20 mM thiobarbituric acid and 15 mM phenazine methosulfate following incubation at 95 °C for 60 min. After cooling to room temperature, the mixture was extracted by *n*-butanol:pyridine (15:1, v/v) and centrifuged at 3000*g*, for 5 min. The organic layer was collected and its absorbance was measured at 532 nm. The MDA concentration was expressed as nmol of TBARS per mg protein.

2.8. Determination of osmotic resistance of erythrocytes

Osmotic resistance of erythrocytes (ORE) was measured as described previously [25]. Briefly, isolated erythrocytes were preincubated with test agents at 37 °C, for 10 min, and then subjected to hypotonic solution of NaCl (0.4%), for 20 min at 37 °C and centrifuged. Hemoglobin absorbance was then measured in the supernatant at 540 nm. The level of hemolysis was calculated as the percentage of total hemolysis caused by 0.1% Na_2CO_3.

2.9. Cell culture

HL60 myeloblastic leukemia cells (ATCC-CCL-240) and U937 promonocytic leukemia cells (ATCC-CRL-1593.2) were purchased from American Type Culture Collection (Rockville, MD, USA). Samples of human peripheral blood were collected from healthy donors at Soroka University Medical Center (Beer-Sheva, Israel) after informed consent (Ben-Gurion University Helsinki Committee for Protection of Human Subjects; approval #3587). Peripheral blood

mononuclear cells (PBMC) were isolated on a Histopaque-1077 gradient, as described previously [13]. Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and 10 mM HEPES (pH = 7.4) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

2.10. Cell proliferation and viability assays

AML cells were seeded at 5×10^4 cells/ml onto 24-well plates (Greiner Bio-One GmbH, Solingen, Germany) and incubated with plant extracts or vehicle (≤0.2% ethanol) for 72 h. Cell numbers and viability were determined on the basis of the trypan blue exclusion assay by counting in Vi-Cell XR cell viability analyzer (Beckman Coulter, Fullerton, CA, USA). The number of viable (trypan blue-impermeable) cells was counted directly, and cell viability was calculated as the percentage of viable cells relative to the total (viable + dead) cell count. To determine changes in PBMC proliferation and viability, cells (10^{-4}/well) were treated in 96-well plates with extracts or vehicle (≤0.2% ethanol) in the presence of 35 μg/ml phytohemagglutinin (PHA), for 72 h [13]. Negative control cells were incubated with vehicle in the absence of PHA. Relative amount of viable cells was then determined by both trypan blue exclusion (see above) and the standard XTT assay using Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Average absorbance at 492 nm (650 nm reference wavelength) of PHA only-treated (control) samples was assumed as 100%.

2.11. Cell cycle analysis

Cells (1×10^6) were washed with cold PBS and fixed in 75% ethanol at −20 °C. The cells were then washed twice in PBS and incubated in 1 ml of PBS, containing 0.1% Triton X-100 and 50 μg RNAse at room temperature for 30 min. Cellular DNA content was determined after propidium iodide (10 μg/ml) staining using a Cytomix FC500 flow cytometer equipped with the CXP software (Beckman Coulter). Cell cycle distribution was analyzed using the Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

2.12. Determination of cell surface markers of myeloid differentiation

Cells (5×10^5) were washed with PBS and incubated for 45 min at room temperature with 2 μl CD14-FITC or 2 μl CD11b-PE antibodies (eBioscience, San Diego, CA, USA). The cells were then washed three times with ice-cold PBS, resuspended in 1 ml PBS and analyzed by flow cytometry. IgG1-FITC, and IgG2b-PE isotype controls were used to set threshold parameters.

2.13. Preparation of whole cell lysates and Western blotting

Briefly, cells (≥2×10^6) were lysed in buffer containing 1% (v/v) Triton X-100 at 4 °C, subjected to SDS-PAGE and electroblotted into nitrocellulose membrane. The membranes were exposed to primary antibodies overnight at 4 °C followed by incubation with secondary antibody conjugated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature, as described previously [12]. Protein bands were visualized using the Western Lightning™ Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Inc., Boston, MA). The absorbance of each band was determined using the Image Quant LAS 4000 system (GE Healthcare, Little Chalfont, UK).

2.14. Transient transfection and reporter gene assay

The VDRE × 6-Luc reporter plasmid containing a 6-fold direct repeat 3 (DR3) sequence was provided by Dr. L.P. Freedman (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). *Renilla* luciferase expression construct (pRL-null vector) was purchased from Promega (Madison, WI, USA) and served as an internal transfection standard. AML cells were transiently co-transfected with the VDRE × 6-luciferase reporter plasmid (0.8 μg) and *Renilla* luciferase vector (0.2 μg) using JetPEI Reagent (POLYplus-Transfection, Illkirch Cedex, France), as described previously [26]. Transfected cells were exposed to test agents for 24 h followed by measurement of luciferase activity using the Dual Luciferase Reporter Assay system (Promega). The data are presented as the normalized ratios of firefly luciferase to *Renilla* luciferase activity (relative luminescence units, RLU).

2.15. Statistical analysis

Experiments were repeated at least three times. Statistically significant differences between treatments were estimated by unpaired, two-tailed Student's *t*-test. Two compounds (A and B) were considered to show enhancement in the particular experiment if the effect of their combination (AB) was larger than the sum of their individual effects (AB > A + B), the data being compared after subtraction of the respective control values from A, B, and AB [19]. The values are reported as the means ± SD. The significance of the differences between the means of experimental groups was assessed by unpaired two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed with the GraphPad Prism 6.0 Program (Graph-Pad Software, San Diego, CA).

3. Results

3.1. Biochemical properties of H. rhamnoides, R. canina, S. officinalis and O. vulgare leaf extracts

As shown in Table 1, dried 50% ethanol extracts of *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare* leaves contained significant amounts of polyphenolic/flavonoid compounds. All the extracts demonstrated higher potency in inhibiting Fe^{2+}/0.5 mM

Table 1
Properties of tested plant extracts: polyphenol and flavonoid content and the ability to inhibit lipid peroxidation and to increase osmotic resistance of red blood cells.

Scientific name	Family	Plant parts	Total polyphenols (GAE, mg/g dried extract)	Total flavonoids (QE, mg/g dried extract)	a Lipid peroxidation IC50 (μg/mg protein)	b RBC osmotic resistance IC50 (μg/ml RBC)
Hippophae rhamnoides	Hyppericaceae	Leaves	45.9 ± 1.3	23.5 ± 1.5	7.4 ± 3.3* (◆◆)	64.0 ± 8.7*
Rosa canina	Rosaceae	Leaves	45.1 ± 3.0	15.2 ± 1.0	4.8 ± 1.8* (◆◆◆)	67.5 ± 11.0*
Salvia officinalis	Labiateae	Leaves	27.2 ± 1.0	17.7 ± 2.1	2.6 ± 1.5** (◆◆◆)	32.0 ± 5.8**
Origanum vulgare	Labiateae	Aerial parts	36.2 ± 7.6	24.6 ± 1.4	16.6 ± 4.9 (◆)	72.0 ± 9.3
α-Tocopherol	–	–	–	–	14.4 ± 5.8	85.3 ± 7.1
Ascorbic acid	–	–	–	–	43.8 ± 8.2	>100

The data are the means ± SD (n = 3–6). (a) The IC50 values are presented for Fe^{2+}/ascorbate-induced microsomal lipid peroxidation. *, $P < 0.05$ and **, $P < 0.01$ vs. α-tocopherol. ◆, $P < 0.05$; ◆◆, $P < 0.01$ and ◆◆◆, $P < 0.001$ *vs.* ascorbic acid. (b) *, $P < 0.05$ and **, $P < 0.01$ *vs.* α-tocopherol. GAE, gallic acid equivalent; QE, quercetin equivalent; RBC, red blood cells.

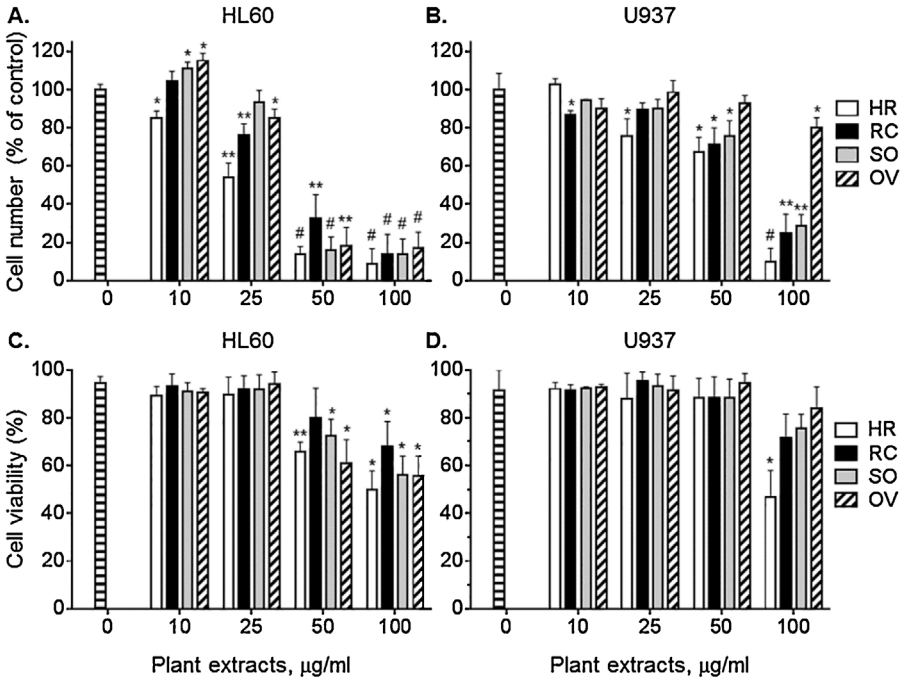

Fig. 1. Concentration-dependent effects of plant extracts on cell growth and viability of AML cells.
Exponentially growing HL-60 and U937 cells were treated for 72 h with increasing concentrations of the indicated plant extracts. The numbers of viable cells (A and B) and cell viability (C and D) were measured by the trypan blue exclusion assay. The data are the means ± SD of 4 experiments performed in duplicate. HR, *H. rhamnoides*; RC, *R. canina*; SO, *S. officinalis*; OV, *O. vulgare*. *, $P < 0.05$; **, $P < 0.01$; and #, $P < 001$ *vs.* untreated control.

ascorbate-induced microsomal lipid peroxidation and protecting erythrocytes from hypotonic hemolysis compared to ascorbic acid and α-tocopherol (except for the *O. vulgare* extract).

3.2. Plant extracts reduce proliferation and viability of AML cells

HL60 and U937 cells were seeded at ~50,000 cells/ml and treated with increasing concentrations (10, 25, 50 and 100 μg/ml) of individual *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare* extracts for 72 h, followed by cell enumeration by the trypan blue exclusion assay. At the end of incubation, the number of untreated control HL-60 and U937 cells increased up to 470,300 ± 52,100 cells/ml and 1,010,000 ± 95,200 cells/ml, respectively, indicating exponential cell growth under these conditions. All the extracts tested reduced viable cell counts (see Section 2) in both cell lines (Fig. 1A and B) in a concentration-dependent manner, HL60 cells being generally more sensitive than U937 cells (see Table 2 for IC_{50} values). *H. rhamnoides* extract was relatively the most effective cell growth inhibitor in both cell lines (Fig. 1 and Table 2). The higher concentrations of all extracts (50 and 100 μg/ml) also significantly decreased the viability of HL60 cells (Fig. 1C) while U937 cells were less susceptible to the cytotoxic effects (Fig. 1D). The latter cell line was also less sensitive to *O. vulgare* extract compared to the other extracts (compare panels A, C and B, D in Fig. 1).

3.3. Plant extracts enhance 1,25D-induced differentiation of AML cells and increase vitamin D receptor levels and functional activity

HL60 and U937 cells were treated with near physiological concentrations of 1,25D (2.5 nM and 1.0, respectively), non-cytotoxic doses of *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare* extract (see Fig. 1C and D) and their combinations, for 96 h. Surface expression of CD11b (a general myeloid marker) and CD14 (a monocytic marker) was then measured by flow cytometry. High dose 1,25D (100 nM) was used as the positive control. The results indicated that in HL60 cells, all the extracts synergistically enhanced 1,25D-induced differentiation while having slight or no effects when added alone (Fig. 2A). However, in U937 cells, only the combinations containing *R. canina* or *O. vulgare* extracts could significantly increase the expression of CD11b and/or CD14 compared to 1,25D alone (Fig. 2B).

Treatment of HL60 cells with *H. rhamnoides* and *R. canina* extracts resulted in a moderate increase in VDR levels and a stronger elevation of RXRα levels, whereas *S. officinalis* and *O. vulgare* extracts were without effect. However, all four extracts were capable of cooperating with a low concentration of 1,25D to further upregulate both components of the receptor for vitamin D (Fig. 2C and Supplemental Fig. 1A). This was also associated with a synergistic transcriptional activation of the vitamin D response element (VDRE), as determined using the luciferase reporter assay in transiently transfected HL60 cells (Fig. 2E). Consistent with their slight differentiation-enhancing effects in U937 cells, the extracts, alone or in combination with 1,25D, did not appreciably affect VDR and RXRα levels in these cells, as compared to control samples (Fig. 2D and Supplemental Fig. 1B). Likewise, a modest increase in 1,25D-induced VDRE transactivation was observed in the presence of *H. rhamnoides*, *R. canina* and *O. vulgare* extracts while *S. officinalis* extract had no effect (Fig. 2F).

Table 2
IC_{50} values for the antiproliferative effects of plant extracts in AML cells (72 h).

Extracts, μg/ml	IC_{50} (μg/ml)	
	HL60	U937
H. rhamnoides	2.5 ± 1.4	65.5 ± 3.3
R. canina	40.0 ± 2.8	77.2 ± 6.9
S. officinalis	39.2 ± 3.1	77.9 ± 7.0
O. vulgare	38.1 ± 1.9	n.d.

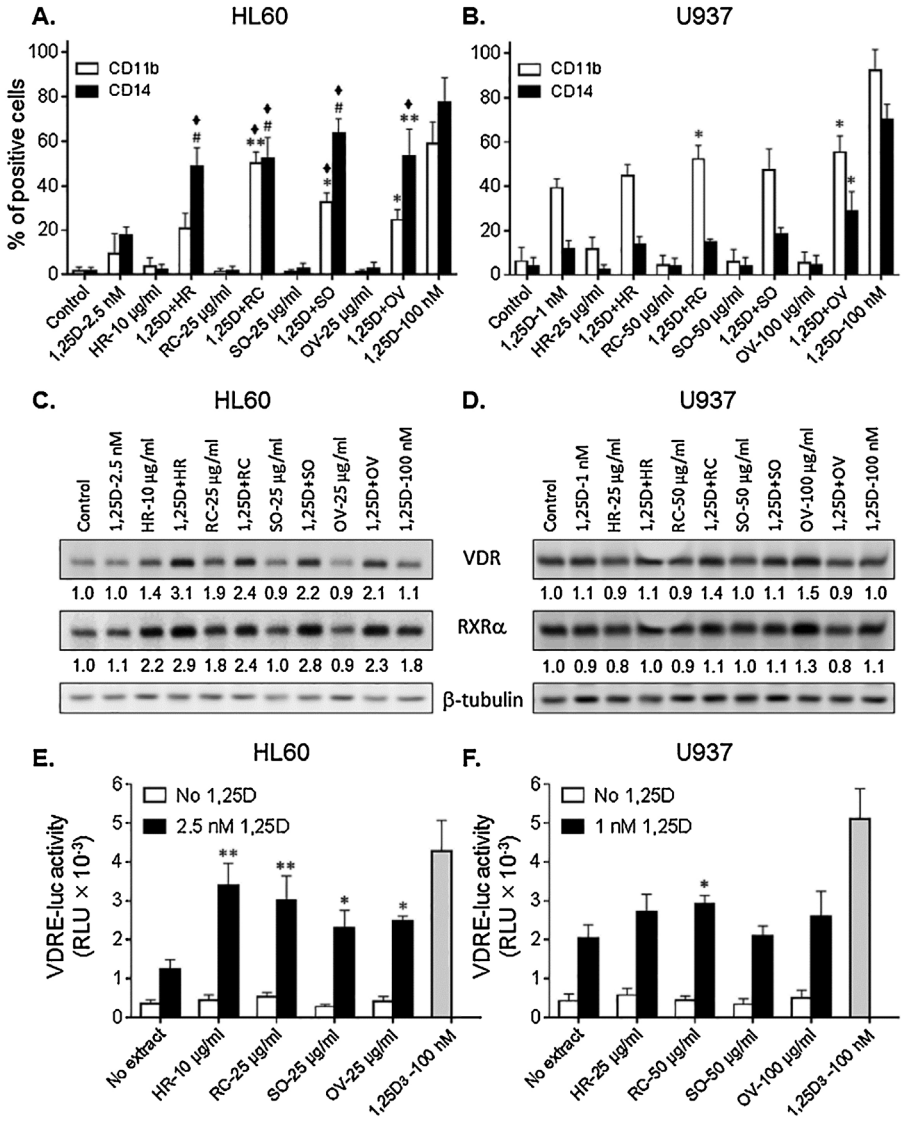

Fig. 2. Effects of plant extracts, 1,25D and their combinations on cell differentiation and the vitamin D receptor levels and transcriptional activity.
HL60 (A and C) and U937 (B and D) cells were incubated with 0.1% ethanol (vehicle control) or the indicated test agents for 96 h. (A and B) Flow cytometric determination of the expression of surface differentiation markers CD11b and CD14. 1,25D (100 nM) was used as a positive control. The data are the means ± SD of 3 experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$; and #, $P < 0.001$ *vs.* 1,25D (1 nM or 2.5 nM) alone; ◆, Synergistic effect of a combination compared with the sum of individual effects. (C and D) Western blot analysis of VDR and RXRα protein levels. Whole cell lysates of HL60 and U937 cells were prepared from the cell samples shown in A and B, respectively. β-Tubulin was used as a protein loading control. Absorbance values for VDR and RXRα normalized to those of β-tubulin are displayed under each protein band. Representative blots of 3 similar experiments are shown. (E and F) Cells were transiently transfected with VDRE × 6-Luc and *Renilla* plasmids, and incubated with the indicated agents for 24 h. The relative VDRE × 6-Luc activity (means ± SD) was calculated from the data of 3 experiments performed in quadruplicate. *, $P < 0.05$ and **, $P < 0.01$ *vs.* $1,25D_3$ alone. Abbreviations are as in the legend to Fig. 1.

3.4. Plant extracts cooperate in inducing cytostatic effects in AML cells but not in normal PBMC

To determine whether different combinations of *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare* extracts can produce enhanced antiproliferative and cytotoxic effects at minimally effective concentrations of each agent (see Fig. 1), AML cells were treated with single and combined extracts for 72 h followed by the trypan blue exclusion assay. As shown in Fig. 3A (*upper panel*), the combinations containing *H. rhamnoides* extract (10 μg/ml) and that from *R. canina*, *S. officinalis* or *O. vulgare* (25 μg/ml) synergistically inhibited proliferation of HL60 cells, whereas different combinations of *R. canina*, *S. officinalis* and *O. vulgare* extracts caused approximately additive inhibitory effects. In U937 cells, all the double combinations tested at 25–100 μg/ml produced marked synergistic or additive antiproliferative effects (Fig. 2B; *upper panel*). Surprisingly, despite their pronounced growth inhibition the combinations exhibited only a slight or no effect on the viability of both cell types (Fig. 2A and B; *lower panels*), suggesting that their antileukemic action is cytostatic rather than cytotoxic.

To examine whether the extracts and/or their combinations affect normal blood cells, freshly isolated PBMC were treated with these agents at relatively high concentrations used in analogous experiments in U937 cells (see Fig. 2C and D) in the presence of a mitogen (phytohemagglutinin; PHA) for 72 h. Relative amounts of viable cells were then measured by the XTT and trypan blue exclusion assays. The data demonstrated that PHA strongly stimulated proliferation of untreated control PBMC (by about 20-fold) (Fig. 3C). In contrast to AML cell lines (Fig. 3A; *upper panel*), only *H. rhamnoides* extract alone or its combination with *O. vulgare* extract moderately (by about 20%; $p < 0.05$) inhibited

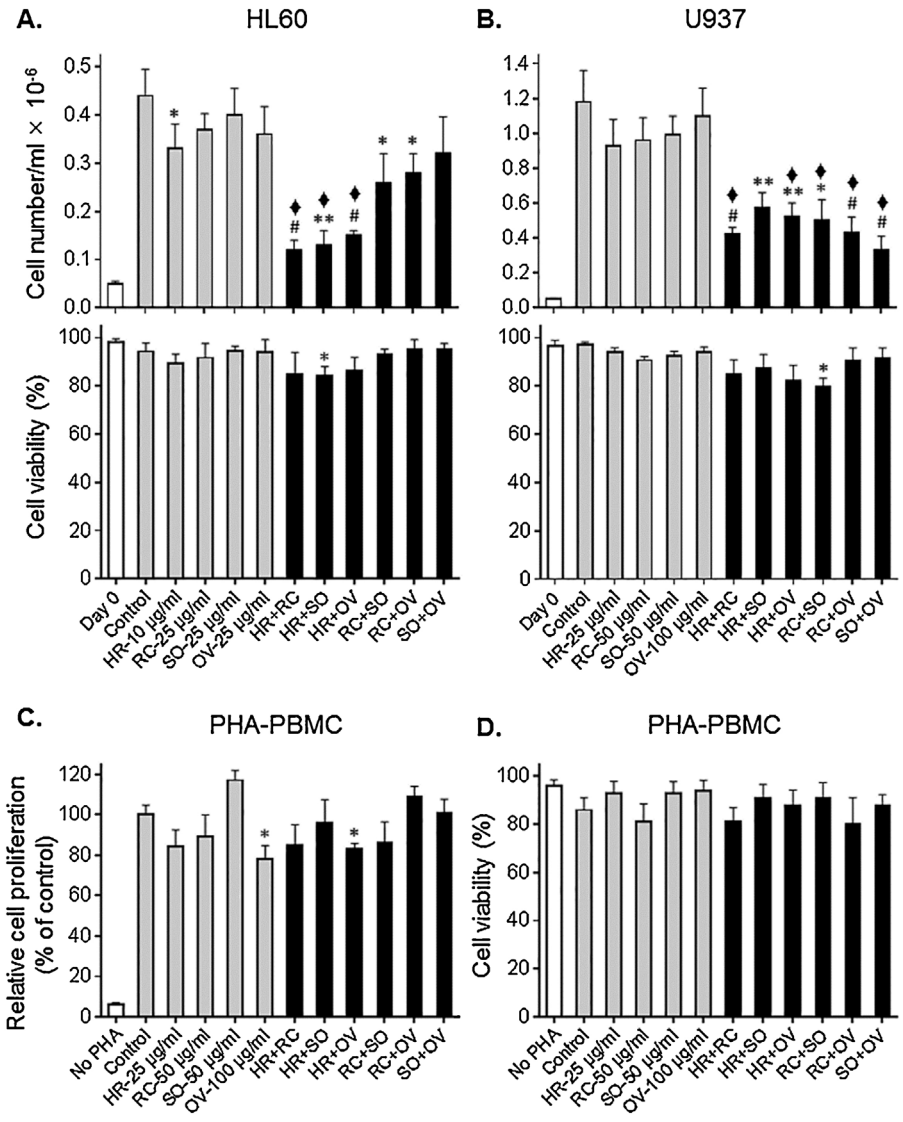

Fig. 3. Cooperative cytostatic effects of plant extracts on AML cells but not on PBMC.
Exponentially growing HL60 (A) and U937 (B) cells were treated for 72 h with the indicated plant extracts, alone and in combination. The numbers of viable cells (*upper panels*) and cell viability (*lower panels*) were determined by the trypan blue exclusion assay. The data are the means ± SD of 3 experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; and #, $P < 0.001$ *vs.* Control; ◆, Synergistic effect of a combination compared with the sum of individual effects. (C and D) PBMC were treated with the indicated extracts or vehicle (Control) in the presence of phytohemagglutinin (PHA), for 72 h. Negative control cells were incubated with vehicle in the absence of PHA. Relative cell proliferation was determined by the XTT assay; (C) PBMC viability was determined by the trypan blue exclusion assay (D). The data are the means ± SD (n = 3). *, $P < 0.05$ *vs.* untreated control cells. Abbreviations as in legend to Fig. 1.

PBMC proliferation (Fig. 3C) while none of the treatments tested significantly affected cell viability (Fig. 3D).

3.5. Effects of plant extracts and their combinations on apoptosis and cell cycle

Consistent with their predominately cytostatic rather than cytotoxic activities in AML cells (see Figs. 1 and 3), the extracts and their combinations caused either slight or no apoptosis-inducing effects. This was evident by only a modest, if any, appearance of the specific apoptotic markers, such as the cleaved 86-kD fragment of the poly(ADP-ribose)polymerase (PARP) protein (Fig. 4A and B) and the sub-G1 (hypodiploid) cell populations (Fig. 4C and D; Tables 3 and 4). On the other hand, the antiproliferative effects of the treatments were associated with a reduction in G0/G1-phase populations and relatively enlarged S-phase populations, as compared to untreated controls. This is exemplified by cell cycle histograms of HL60 and U937 cells treated with vehicle *vs.* the combination of *H. rhamnoides* and *R. canina* extracts (Fig. 4C and D) and summarized in Tables 3 and 4. Compared with U937 cells, HL60 cells were generally more prone to changes in cell cycle distribution, as reflected by marked decreases in the G1/S ratios following most of the combined treatments (Tables 3 and 4). The percentage of cells in the G2/M phase was not significantly affected in HL60 cells (Table 3) or modestly increased in U937 cells after treatment with *H. rhamnoides* and *R. canina* or *S. officinalis* and *O. vulgare* extract combinations (Table 4). Single extracts at the concentrations used in combined treatments only slightly affected distribution of cell cycle phases. Fig. 4E and F and Supplemental Fig. 2A and B, respectively, illustrate changes in cell cycle regulatory proteins induced by the most effective combinations. The data indicate that in both cell lines, the observed partial S-phase arrest was accompanied by a pronounced reduction in the protein levels of cyclins A and E, without altering cyclin-dependent kinase-2 (CDK2)

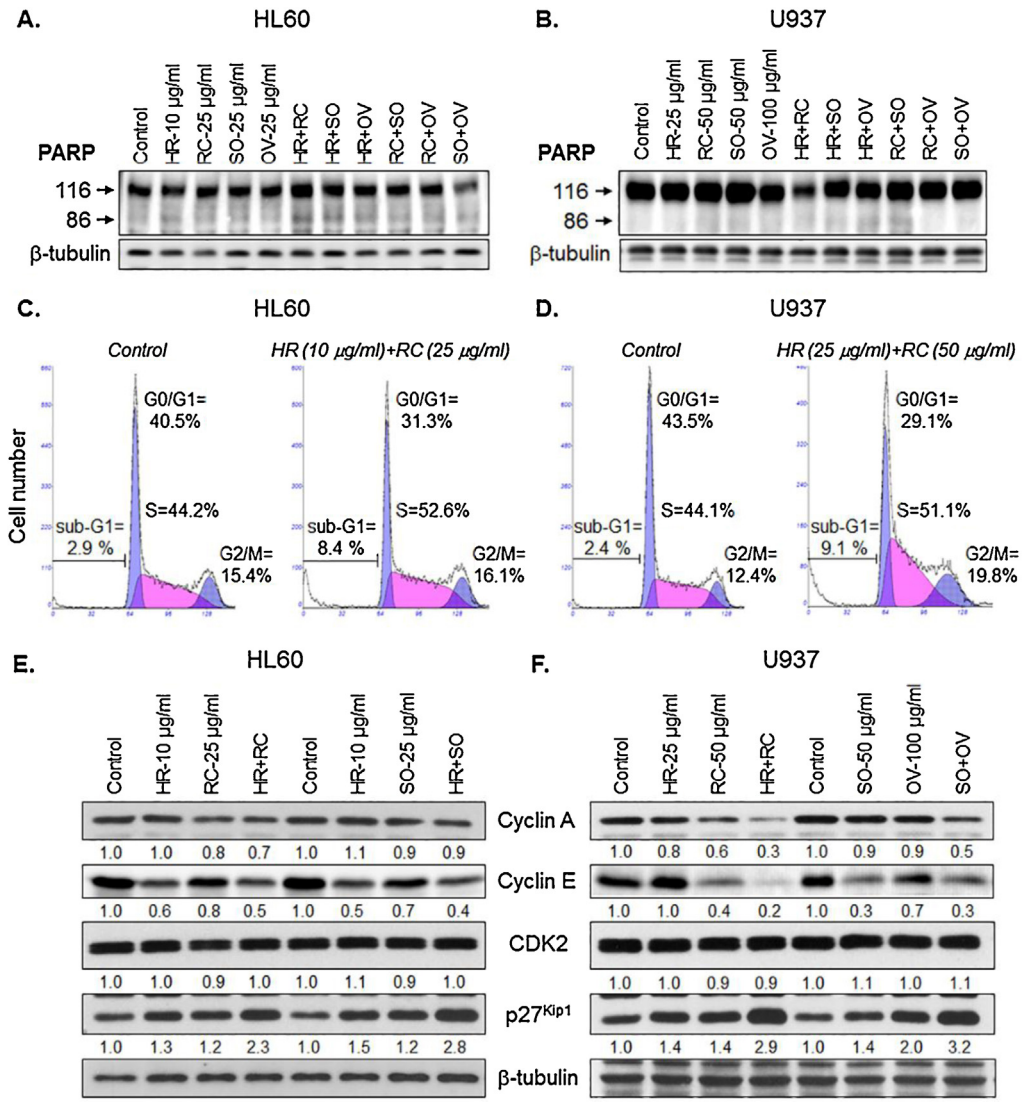

Fig. 4. Effects of plant extracts and their combinations on apoptosis and cell cycle of AML cells.
HL60 (A, C, E) and U937 (B, D, F) cells were treated with the indicated plant extracts, alone and in combination, for 48 h. (A and B) Western blot analysis of PARP cleavage. β-Tubulin was used as a protein loading control. Representative blots of 3 similar experiments are shown. (C and D) Typical examples of cell cycle histograms following treatment with vehicle (Control) and the combination of *H. rhamnoides* and *R.canina* extracts at the indicated concentrations. The percentage of cells in each phase is indicated. (E and F) Western blot analysis of cell cycle regulatory proteins. β-Tubulin was used as a protein loading control. Absorbance values for specific proteins normalized to those of β-tubulin are displayed under each protein band. Representative blots of 3 similar experiments are shown. Abbreviations as in legend to Fig. 1.

levels, and by a marked upregulation of the universal CDK inhibitor $p27^{Kip1}$. Single extracts were generally less efficient than the combinations (Fig. 4E and F and Supplemental Fig. 2).

4. Discussion

In this study we characterized the ability of dried water-ethanol leaf extracts from *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare*, collected in the foothills of Trans-Ili Alatau mountains, to modulate viability, proliferation, cell cycle distribution and differentiation of AML cells *in vitro*. Different extracts obtained from *H. rhamnoides* berries and seeds [27], *R. canina* rosehips [28] as well as *S. officinalis* [29,30] and *O. vulgare* [31,32] herbs have been previously shown to inhibit the growth and induce apoptosis in various solid cancer cell lines. However, to the best of our knowledge, the anticancer activity of *R. canina* leaf extracts has not yet been demonstrated, and our initial publication [21] still remains the only report showing that *H. rhamnoides* leaf extract is effective against cancer (AML) cells in culture. The ability *O. vulgare* extracts to modulate leukemia cell proliferation and differentiation has not yet been shown while only one research group has so far reported the cytotoxic effects of a 70% methanolic extract of *S. officinalis* on KG-1a and U937 AML cells [30].

The four leaf extracts tested here inhibited cell growth in HL60 and U937 AML cell lines, depending on the dose, plant origin and cell type. Furthermore, the extracts were capable of enhancing 1,25D-induced differentiation in a plant- and cell type-dependent manner. The fact that the above effects were generally more pronounced in myeloblastic (HL60) than in more mature, myelomonocytic (U937), cells is intriguing and needs further exploration of an association between the differentiation stage of AML cells and their sensitivity to the extracts and their active components.

1,25D is a powerful differentiation agent which has the potential for AML therapy. However, at concentrations needed to induce terminal differentiation of AML cells it causes life-threatening

Table 3
Cell cycle distribution in HL60 cells treated with plant extracts for 48 h.

Treatment	SubG1	G0/G1	S	G2/M	G1/S ratio
Control	2.9 ± 0.1	40.5 ± 2.0	44.2 ± 2.2	15.4 ± 0.9	0.91 ± 0.05
HR–10 μg/ml	5.1 ± 0.6	39.8 ± 4.4	45.7 ± 5.0	14.5 ± 1.7	0.93 ± 0.11
RC–25 μg/ml	2.7 ± 0.3	40.5 ± 4.5	45.6 ± 5.1	12.8 ± 1.4	0.81 ± 0.10
SO–25 μg/ml	3.3 ± 0.4	39.0 ± 4.3	46.6 ± 3.1	14.8 ± 1.2	0.80 ± 0.09
OO–25 μg/ml	5.0 ± 0.6	41.1 ± 4.9	46.7 ± 5.1	12.2 ± 1.5	0.94 ± 0.12
HR + RC	8.4 ± 0.4*	32.7 ± 1.6*	56.8 ± 4.9*	10.5 ± 0.7	0.60 ± 0.09*
HR + SO	4.4 ± 0.3	31.1 ± 2.3*	58.0 ± 4.9**	10.2 ± 0.5	0.53 ± 0.04**
HR + OV	3.7 ± 0.2	35.5 ± 3.1	52.4 ± 3.7*	12.1 ± 0.8	0.70 ± 0.15
RC + SO	3.2 ± 0.2	32.7 ± 2.0*	51.9 ± 1.6*	15.4 ± 1.1	0.65 ± 0.04*
RC + OV	3.2 ± 0.3	31.4 ± 1.6*	52.6 ± 3.1*	16.1 ± 1.2	0.62 ± 0.08*
SO + OV	3.6 ± 0.2	36.3 ± 2.5	50.2 ± 3.8	13.5 ± 0.7	0.74 ± 0.05*

Data are presented as the means ± SD (n = 3). *, $P < 0.05$ and **, $P < 0.01$ *vs.* Control.

hypercalcemia *in vivo* [33]. In search of approaches to overcome this problem, we and others have found that various phytochemicals, e.g., the carotenoid lycopene [15], the polyphenols carnosic acid [19], curcumin [18,34] and silibinin [17,34] or the sesquiterpene lactones parthenolide [35] and costunolide [36] markedly potentiate the differentiation of AML cells induced by near physiological concentrations of 1,25D. Furthermore, we showed that dietary administration of rosemary (*Rosmarinus officinalis*) leaf extract combined with intraperitoneally injected synthetic analogues of 1,25D resulted in a synergistic suppression of AML development in murine models without hypercalcemic toxicity [20,37]. Thus, the cell line-dependent differentiation-enhancing effects of the four plant extracts tested here strongly encourage the exploration of their components as candidates for combination differentiation therapy of at least some AML subtypes. As an initial step towards elucidating the mechanism of this enhancement, we found that it was associated with upregulation of protein expression of VDR and its heterodimeric partner, the retinoid X receptor (RXRα), concomitant with enhanced transcriptional activity of the receptor complex (Fig. 2). This is consistent with our previous data obtained with combinations of 1,25D and purified polyphenols [19,38].

Another important finding of this study is that in contrast to proliferating normal PBMC, all of the extracts were capable of cooperating with one another at their minimally effective concentrations to produce much greater cytostasis in both AML cell lines than single agents (Fig. 3). This effect was evident irrespective of the potency of each extract. For instance, *O. vulgare* extract that was without effect in U937 cells at any employed dose (Fig. 1) strongly cooperated with the other preparations in this cell line (Fig. 3). These results support the previously reported findings showing that not only individual phytochemicals (e.g., [10,13,14]) but also crude plant extracts and their fractions containing multiple components can cooperate to suppress cancer cell growth (reviewed in Refs. [11,39]).

Notably, despite the enhanced antiproliferative effects of extract combinations, only marginal or no cytotoxicity (Fig. 3) and a modest induction of apoptosis (Fig. 4) were observed in the treated AML cells. This preferential cytostasis correlated in part with a small, but significant, accumulation of cells in S phase on account of a decrease in the percentage of G0/G1 phase. Although this resulted in about 40% reduction in the G1/S ratio in certain samples (Tables 3 and 4), our cell cycle data cannot fully account for the marked antiproliferative effects of most of the combinations tested (Fig. 3A and B). Interestingly, a similar situation was observed by Wang et al. [40] who demonstrated that combinations of 1-desoxy analogues of vitamin D_3 and ketoconazole strongly inhibited proliferation of AML cells without significant changes in cell cycle distribution. Considering the lack of cytotoxicity and the observed changes in cyclins E and A and in $p27^{Kip1}$ (which operate in more than one phase of the cell cycle), we suggest that our combinations may inhibit cell proliferation by generally slowing down the overall cell cycle progression without dramatic redistribution of the G0/G1, S and G2/M phases. The mechanisms of S-phase arrest induced by different agents, including cytotoxic phytochemicals, are complex and is usually associated with the DNA damage response leading to activation of the p53 tumor suppressor (e.g., [41–43]). Since HL60 and U937 cells are p53-deficient, accumulation of cells in the S-phase observed in this study was apparently mediated by an alternative, p53-independent mechanism which involves downregulation of cyclins A and E and upregulation of $p27^{Kip1}$ (e.g., [44,45]).

Leaf extracts from *H. rhamnoides*, *R. canina*, *S. officinalis* and *O. vulgare* contain numerous bioactive phytochemicals, particularly, polyphenolic compounds known for antiproliferative and cytotoxic activities in various cancer cells [29,31,46,47]. Since, as

Table 4
Cell cycle distribution in U937 cells treated with plant extracts for 48 h.

Treatment	SubG1	G0/G1	S	G2/M	G1/S ratio
Control	2.4 ± 0.1	43.5 ± 2.8	44.1 ± 2.6	12.4 ± 2.6	1.06 ± 0.06
HR–25 μg/ml	4.1 ± 0.4	44.8 ± 4.7	43.7 ± 4.2	11.5 ± 1.3	1.05 ± 0.12
RC–50 μg/ml	6.9 ± 1.1*	45.1 ± 5.0	46.5 ± 5.1	8.4 ± 2.4	1.23 ± 0.18
SO–50 μg/ml	4.1 ± 0.5	45.3 ± 5.8	42.6 ± 4.8	12.1 ± 1.3	1.12 ± 0.13
OV–100 μg/ml	6.7 ± 0.7*	47.8 ± 5.5	42.6 ± 5.2	9.6 ± 1.2	1.14 ± 0.11
HR + RC	9.1 ± 0.6*	29.1 ± 1.1**	51.1 ± 2.1*	19.8 ± 2.4*	0.60 ± 0.03*
HR + SO	7.7 ± 0.5*	41.3 ± 2.9	45.9 ± 3.8	12.8 ± 0.9	0.94 ± 0.06
HR + OV	6.1 ± 0.4*	32.2 ± 2.0*	53.5 ± 3.2*	14.4 ± 0.7	0.62 ± 0.04*
RC + SO	6.8 ± 0.4*	39.7 ± 2.1	47.7 ± 3.9	12.6 ± 0.7	0.84 ± 0.11
RC + OV	8.9 ± 0.2*	43.7 ± 2.6	45.2 ± 2.8	11.2 ± 0.7	0.98 ± 0.09
SO + OV	6.7 ± 0.5*	29.2 ± 1.2*	52.8 ± 3.0*	18.1 ± 1.9*	0.61 ± 0.03*

Data are presented as the means ± SD (n = 3). *, $P < 0.05$ and **, $P < 0.01$ *vs.* Control.

mentioned above, at least some of these compounds are capable of cooperating with each other and with different anticancer agents, further research is needed to systematically screen for synergistically acting antileukemic combinations of the extract components. Discovery and exploration of such combination may lead to the development of novel selective therapeutic and/or preventive approaches against AML which is still mostly incurable. Furthermore, the use of polyphenol combinations is likely to mutually improve their bioavailability [48]. This will allow lowering the dosages of active components thereby increasing their specificity towards cancer cells and reducing overall *in vivo* toxicity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biopha.2016.04.062.

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