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CYTOTOXIC AND APOPTOTIC EFFECTS OF *VINCA* L. EXTRACTS ON MULTIPLE MYELOMA CELL LINES

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Abstract – *Objective:* *Multiple myeloma (MM) is a systemic disease characterized by pathological plasma cells that cause the gathering of carcinogenic cells in the bone marrow. Despite the development of new drugs and the use of herbal preparations as treatment support, MM remains untreatable. In this study, the cytotoxic and apoptotic effects of extracts of species of* Vinca, Vinca herbacea *Waldst. and Kit and Vinca soneri Koyuncu, on MM cell lines were investigated.*

Materials and Methods: *The WST-1 test was used to determine the antiproliferative effects at different doses and time intervals of the applied extracts on MM cells and real-time polymerase chain reaction, caspase enzyme activity, and flow cytometry techniques were used to reveal the apoptotic effects.*

Results: *As a general finding, it can be assumed that* Vinca *extracts may be a potential treatment for MM. The result of the WST-1 test revealed that* Vinca *extracts, especially that of* V. herbacea*, had time- and dose-dependent cytotoxic effects on MM cells. Moreover, an increase in proapoptotic gene expressions, such as BAX, BIK, p53, and casp3, and an increase in caspase 3 enzyme activity indicated that the extracts resulted in apoptosis of MM cells.*

Conclusions: *As a basic contribution, it was determined that extracts from species of* Vinca *have cytotoxic, apoptotic, and invasion inhibitory effects on MM cells.*

KEYWORDS: *Multiple myeloma,* Apocynaceae*, Periwinkle.*

INTRODUCTION

Multiple myeloma (MM) is a malignant neoplasm of plasma cells of B lymphocyte origin that generally results in the over-production of high amounts of monoclonal antibodies[1]. MM is the second most common haematological disorder, characterised by the increment of monoclonal plasma cells in the bone and bone marrow. Despite the emergence of new agents targeting the cell clone and its microenvironment, MM is still tricky cancer to treat due to improved recovery and then relapse phases after treatment, and possible treatment resistance and patient death. Significant advances in the treatment of this disease in the past decade have concluded with higher rates of everlasting complete remission, prolonged event-free survival, and improved general survival rate[1]. Many of the agents used in cancer treatment today are either plant-derived natural products or derivatives of natural products. For more than half a century, phytochemicals have been an important part of antineoplastic drugs and new chemotherapy adjuvants to enhance the effectiveness of chemotherapy in MM. Due to their ability to bind multiple targets, natural products may have an advantage over multidirectional abnormalities with regards to rationally designed monotargeted agents in the treatment of MM [2]. *Vinca* L. (Apocynaceae) is represented by 7 species, spreading across Eu-

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rope, North Africa, and southwest Asia. These species comprise *V. major* L., *V. erecta* Regel and Schmalh, *V. herbacea* Waldst. and Kit. *V. Soneri* Koyuncu, *V. minor* L., and *V. ispartensis* Koyuncu and Ekşi, and the last 5 species grow in Turkey[3-7]. While *Vinca herbacea* has widespread distribution, *Vinca soneri* is an endemic species, and both occur naturally in Turkey. There are at least 150 alkaloids known to have been extracted from species of *Vinca*. The alkaloids of *Vinca* are chemically indole and are known to possess anti-proliferative, apoptotic, antihypertensive and antidiabetic properties[8,9]. In this study, it was aimed to reveal the cytotoxic and apoptosis-inducing effects of the extracts obtained from 2 *Vinca* species, *Vinca herbacea* Waldst. and Kit and *Vinca soneri* Koyuncu, on MM *in vitro.* To achieve this, different approaches were utilized, such as the WST-1 (Water Soluble Tetrazolium-1) viability test, real-time polymerase chain reaction (RT-PCR), caspase-3 enzyme assay, flow cytometry, and invasion assay.

MATERIALS AND METHODS

Plant material

The *Vinca* species were collected from their natural habitats and the voucher specimens were deposited in the Biology Department of Selçuk University, in Konya, Turkey. Their locality information is provided previously[10].

Cell lines

Two MM cell lines, ARH-77 and RPMI-8226, were obtained from the American Type Culture Collection (Manassas, VA, USA) through the Gülhane Military Medical Academy. Cell lines were routinely cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were grown at 37°C and 5% CO_2 and prepared for future experiments.

Preparation of plant extracts

The leaf samples were dried without sunlight and pulverised under sterile conditions. Subsequently, the prepared 15 g of V. *herbacea* and V. *soneri* samples were extracted with methanol for 6–8 h using a Soxhlet apparatus. The obtained total methanol extracts were evaporated at 40°C in a rotary evaporator. The crude extracts were kept at –20°C until use.

Cytotoxicity assay

The ARH-77 and RPMI-8226 cells were harvested and transferred to the ELISA plate for 24 h, prior to treatment being performed. The *Vinca* extracts were applied at concentrations of 1/1000, 1/2500, 1/5000, and 1/10,000, and the treated MM cells were incubated for 2-time intervals, 24 and 48 h. Cell proliferation experiments were performed via WST-1 (Roche Applied Sciences, Mannheim, Germany) assay. The WST-1 solution was applied to each well. After 2 or 3 h, the plates were measured using an ELISA microplate reader at 420–480 nm. Each experiment was performed in triplicate. Cell proliferation data were analysed using GraphPad Prism 7.0 software (San Diego, CA, USA).

RNA isolation and RT-PCR (Real-Time Polymerase Chain Reaction)

Total RNA isolation was performed via the Axygen RNA isolation kit according to the manufacturer's instructions, and the concentration and quality of the total RNA samples were estimated using Nanodrop 2000 (Wilmington, DE, USA). RNA was aliquoted into small volumes and kept at −86°C for further analysis. Next, 0.5–1 µg total RNA was reverse-transcribed to cDNA via the First-strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The gene expression experiments were performed with RT-PCRs in a final volume of 10 µL mix, which contained 5 µL of SYBR green master mix (Bio-Rad, Hercules, CA, USA), 1 µL of primer, 3 µL of dH_2O, and 1 µL of cDNA. The B-actin gene was used as a housekeeping gene. Vinblastine (VBL) was used as a positive control. The mRNA expression levels of the studied apoptotic gene regions and β-actin were measured via quantitative RT-PCR. The obtained data were analysed using the comparative CT method, and the fold change was calculated using $2^{-\Delta\Delta}$CT.

Caspase 3 enzyme activation assay

The activity of the caspase 3 enzyme was assessed via the Ab caspase 3 assay kit. First, 1–5 × 10^6 cells were prepared for each sample, and the cells were suspended in 50 µL tissue lysis buffer, and then left on ice for 10 min. Next, the homogenate

was centrifuged, after which, the supernatant was transferred into a new tube. Added to each sample were 2X sample buffer and DEVD-PNA. After 1 or 2 h incubation at 37°C, the absorbency of the samples was measured using a BioTech ELISA reader (Winooski, VT, USA) at 400–405 nm. The results were compared to the control group of each cell line and transformed onto a graph.

Apoptosis detection with flow cytometry

Apoptotic cell death in the MM cells was detected using the Annexin V-FITC early apoptosis detection kit (Cell Signalling Technology, Danvers, MA, USA). For each group, cells were dispensed at the amount indicated in the kit's instructions (10^5–10^6 cells/mL), and after 24 h, each extract was added. Following 24 h of incubation with the extracts, the ratio of apoptotic cells was determined by Annexin V-FITC/PI assay (Cell Signalling Technology). At the end of the period, the samples in the wells were pipetted and collected into 2-mL Eppendorf tubes, and then centrifuged at 1000 rpm for 5 min. The supernatants were carefully removed, and then the process was repeated with cold phosphate buffered saline. Subsequently, 1X Annexin-V binding buffer, Annexin V-FITC conjugate, and Propidium iodide (PI) were added, respectively. The samples were left on ice for 10 min, and then the final volume of 250 µL was completed with 1X Annexin-V binding buffer. The prepared samples were analysed by flow cytometry using a BD-Facs-Aria III within 1 h following the staining. The analysis part of the study was performed at the Selçuk University Research and Development Center.

Invasion assay

Cell culture inserts placed in 12-well plates were rehydrated with serum-free cell media. The rehydration solution in the insert was then carefully aspirated. Next, 300 µL of cell suspension (0.5–1.0 × 10^6 cells/mL) and IC_{50} doses of the extracts were added into each insert. After that, the nutrient medium in the insert was carefully aspirated. The ends of 2 to 3 cotton-tipped swabs were wet with water and used to remove non-invasive cells; the interior side of the inserts were gently swabbed. The inserts were transferred into a clean well that contained cell stain solution and left at room temperature for 10 min. The stained inserts were gently washed several times in a beaker of water. Each insert was transferred to an empty well, the extraction solution was added, and the wells were incubated for 10 min on an orbital shaker. Next, 100 µL of each sample was transferred to a 96-well microtiter plate and measured at 560 nm. The invasion capacities of the cells were calculated by comparing them with the control group.

Statistical analysis

All of the results were presented as mean values. Statistical analysis was performed using GraphPad Prism v.7.0 software (La Jolla, CA, USA). Data were compared using 1-way ANOVA, and $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant.

RESULTS

Cytotoxic effects of the extracts

The cells of 2 human MM cell lines, ARH-77 and RPMI-8226, were used to determine the cytotoxicity of the methanolic *Vinca* extracts against the cancer cells. The WST-1 assay was chosen to compare the antiproliferative effects of V. *herbacea* and V. *soneri* extracts at concentrations of 1/1000, 1/2500, 1/5000 and 1/10,000. The WST-1 assay showed that the ARH-77 and RPMI-8226 cells were reduced significantly with the application of the extracts at different doses and time intervals (24–48 h). The results showed that each extract exhibited a time- and dose-dependent inhibitory effect on the proliferation of the MM cells ($p < 0.05$). The responses of cancer cells against increasing concentrations of the *Vinca* extracts are shown in Figure 1.

When the results of the WST-1 was generally considered for the ARH-77 cell line, it can be said that the V. *herbacea* extract was more effective than the V. *soneri* extract at the applied dosage range. On the other hand, with regards to the effects of the extracts on the RPMI-8226 cell line, less cell death was observed in comparison to the ARH-77 cell line. When the results of the WST-1 for the RPMI-8226 cell line were evaluated in general, it was concluded that the V. *herbacea* extract was more effective than the V. *soneri* extract with regards to the death of the cells in the ARH-77 cell line. The cytotoxic effect of the V. *soneri* extract on the RPMI-8226 cell line occurred with high doses.

Even though the extracts used in this study had a cytotoxic effect on cancer cells, they did not result in any adverse or cytotoxic effects on healthy peripheral blood cells.

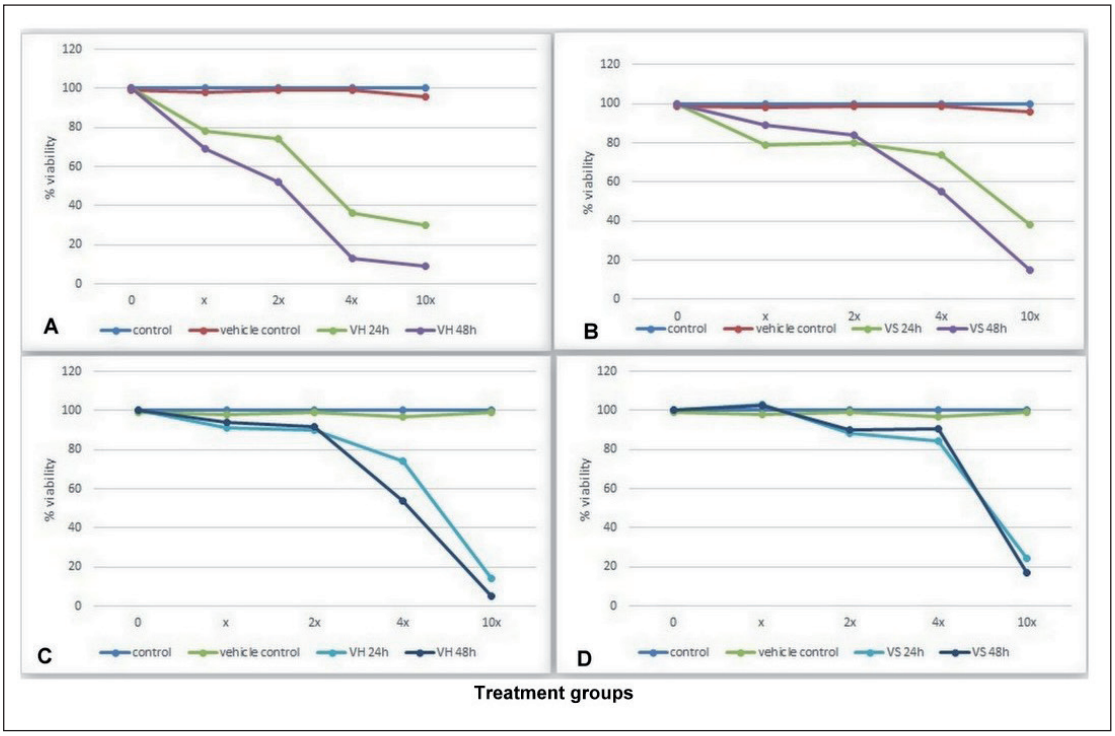

Fig. 1. WST-1 assay results of VH *(V. herbacea)* and VS *(V. soneri)* extract applications (Applied doses x=1/10.000, 2x=1/5000, 4x=1/2500, 10x=1/1000) (Cell lines ***A-B***: ARH-77, ***C-D***: RPMI-8226).

Effect of the extracts on apoptotic gene expression

In order to measure the expression level of the proapoptotic and antiapoptotic genes exposed to the extracts and VBL on MM cells, the mRNA levels of these related gene regions (MCL-1, BAX/BCL-2, Apaf, p53, p73, HRK, BAK, DFFA, and caspase-3) were determined via qRT-PCR. After treatment with IC_{50} concentrations of the methanol extracts, the BAX/BCL-2, Apaf, p53, HRK, BAK, DFFA, and caspase-3 expressions were induced in the MM cells. Extract treatment up-regulated the proapoptotic proteins, such as BAX, Apaf, p53 HRK, BAK, DFFA, and caspase-3, and down-regulated the antiapoptotic proteins, such as BCL-2 and MCL-1 (Figure 2) As a whole assessment of RT-PCR data, it was observed that the extracts had different but versatile effects in terms of apoptotic gene expression and regulation. In ARH-77, the V. *herbacea* extract was found to be more effective in terms of inducing apoptosis in molecular pathways, while in the RPMI-8226 cell line, the V. *soneri* extract was found to be more effective than the V. *herbacea* extract.

Caspase 3 activity assay

The caspase 3 enzyme activity is one of the most critical indicators that administered extracts direct MM cells to programmed cell death. The increase in the caspase 3 enzyme activity in the 2 groups was in agreement with the RT-PCR results in this study. The increase in caspase 3 was less at the protein level than at the mRNA level, and this may have been correlated with the presence of posttranslational modifications.

The caspase 3 enzyme activity increased in all of the groups when compared to the control in ARH-77 for 24 and 48 h. Moreover, the highest increase was observed in the ARH-77 cells that had been treated with the V. *herbacea* extract. On the other hand, when the enzyme activity in the RPMI-8226 cell line was observed at 24 h after application, there was only an increase with the V. *soneri* extract with regards to the caspase 3 activity. Moreover, at 48 h after application, the same increase was also observed with the V. *herbacea* extract (Figure 3).

Flow cytometry

After treatment with the IC_{50} concentrations of the *Vinca* extracts for 24 h, the ARH-77 and RPMI-8226 cells were stained with the AnnexinV-FITC and propidium iodide. The flow cytometry analysis of the cells started showing distribution into 4 different groups, as revealed by the analysis: healthy cells (both AnnexinV-FITC- and PI-negative, Q3), early apoptosis (AnnexinV-FITC-positive and PI-negative, Q4), late apoptosis or necro-

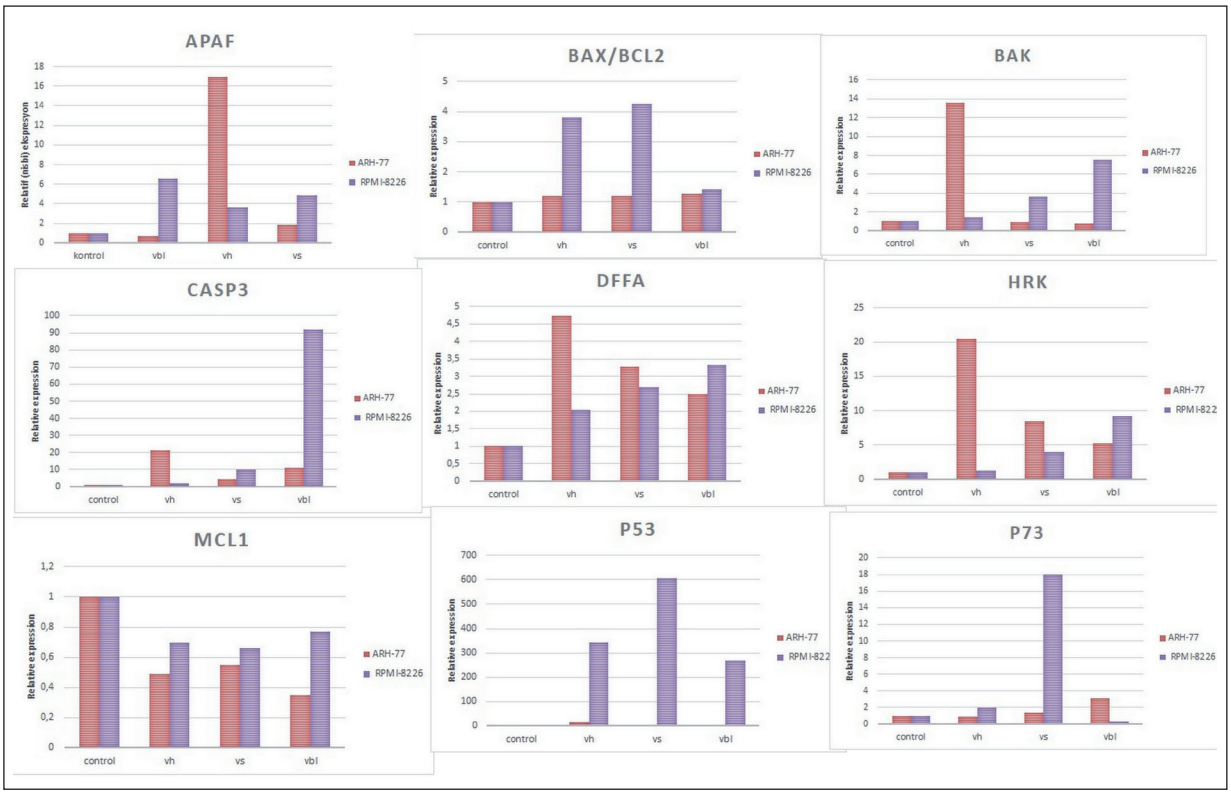

Fig. 2. Relative expression levels of proapoptotic and antiapoptotic genes of extract applied MM cell lines (VH *(V.herbacea)*, VS (V. soneri) VBL (Vinblastine) and control: untreated).

sis (both AnnexinV-FITC- and PI-positive, Q2), and a dead cell population (PI-positive only, Q1). The early apoptotic cell percentage was 1.7 % in the untreated ARH-77 cells. After treatment with the V. *herbacea* and V. *soneri* extracts, the percentages of apoptotic cells were 4.8 % and 5 %, respectively. For the untreated RPMI-8226 cells, the early apoptotic cell percentage was 1.9 %. After treatment with the V. *herbacea* and V. *soneri* extracts, the percentages of apoptotic cells were 3.8 % and 4.9 %, respectively (Figure 4). When the flow cytometry results were evaluated, it was found that the apoptotic cell ratios were increased in the MM cells treated with the extracts when compared to the control. These findings confirmed the results from the gene expression analyses as

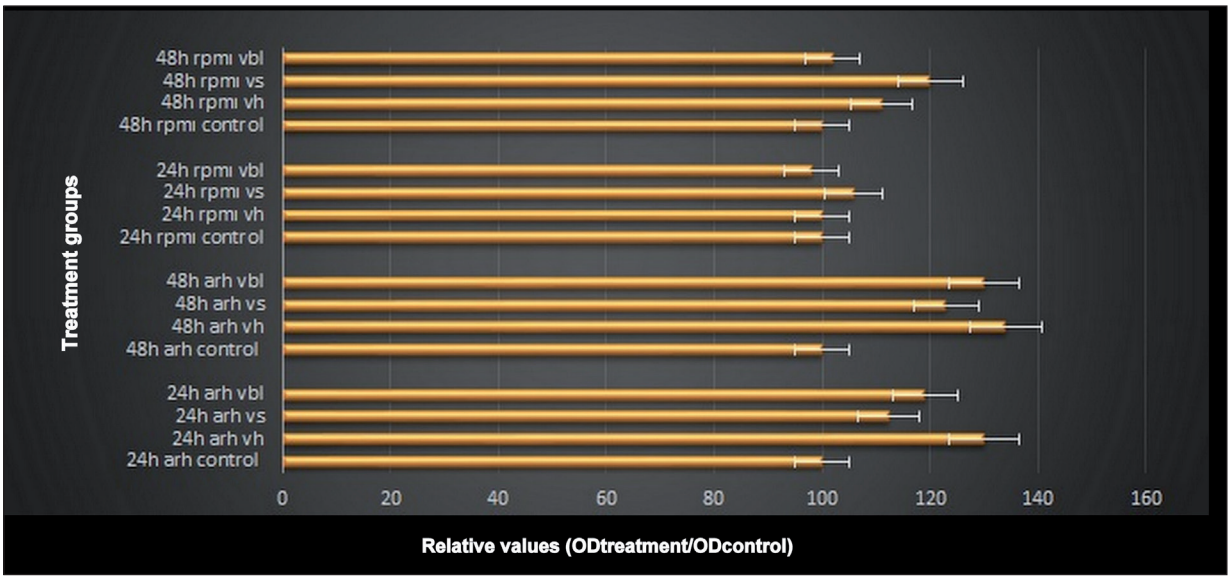

Fig. 3. The caspase-3 enzyme levels of extract applied MM cell lines (VH *(V.herbacea)*, VS *(V. soneri)* VBL (Vinblastine) and control: untreated).

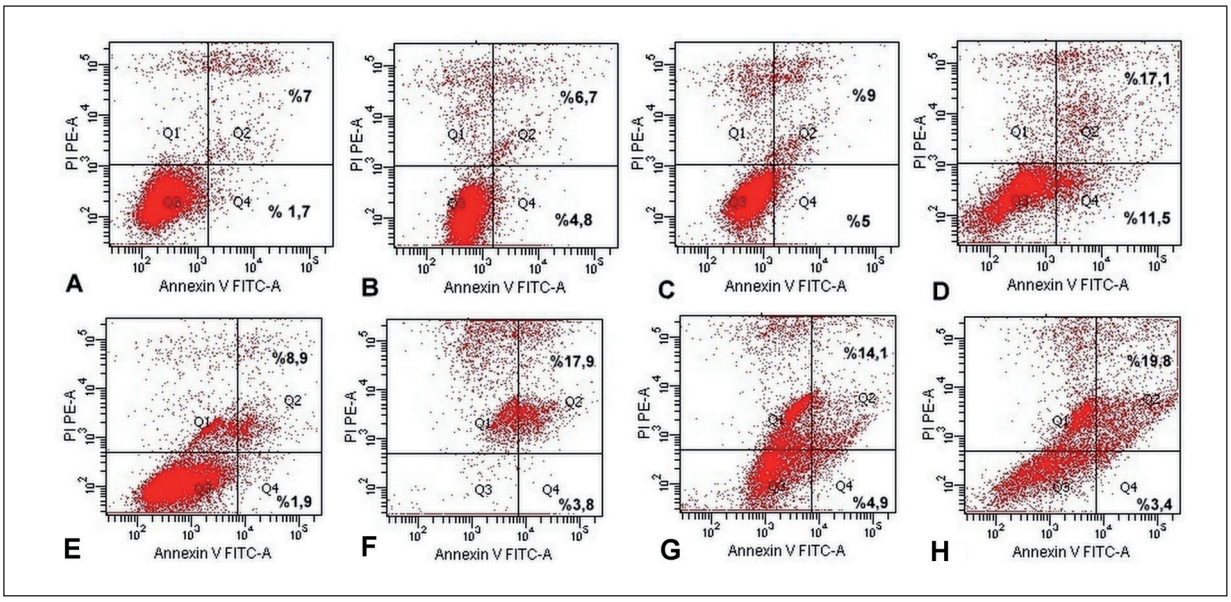

Fig. 4. Flow cytometry graphs of IC_{50} doses of extracts applied MM cell lines (***A***, ARH cell line control; ***B***, VH applied ARH cell line; ***C***, VS applied ARH cell line; ***D***, VBL applied ARH cell line; ***E***, RPMI cell line control; ***F***, VH applied RPMI cell line; ***G***, VS applied RPMI cell line; ***H***, VBL applied RPMI cell line; healthy cells (Q3), early apoptosis (Q4), late apoptosis or necrosis (Q2) and a dead cell population (Q1)).

well as the caspase enzyme activity. In particular, the high early apoptotic cell ratios displayed that the extracts caused apoptosis induction in the MM cells, and this offered substantial potential for the treatment of MM disease in future.

Invasion assay

Invasion refers to the direct extension and penetration by cancer cells into neighbouring tissues. By invasion assay, it was determined that there was a decrease in the invasion capacity of both cell lines. The V. *herbacea* and V. *soneri* extracts significantly reduced the invasion of the ARH-77 and RPMI-8226 cells. The V. *herbacea* extract reduced the invasion capacity of the ARH-77 cells by 20 % and the RPMI-8226 cells by 40 % when compared to control. On the other hand, the V. *soneri* extract reduced the invasion capacity by 10 % and 55 %, respectively (Figure 5). Inhibition of invasion by extract applications may prevent the emergence of secondary cancerous events.

DISCUSSION

Plants have a long history of use in the treatment of cancer as well as many other diseases. The anticancer effects of plants have been recognized for centuries. Several studies have shown that plants and plant-derived natural products have multiple

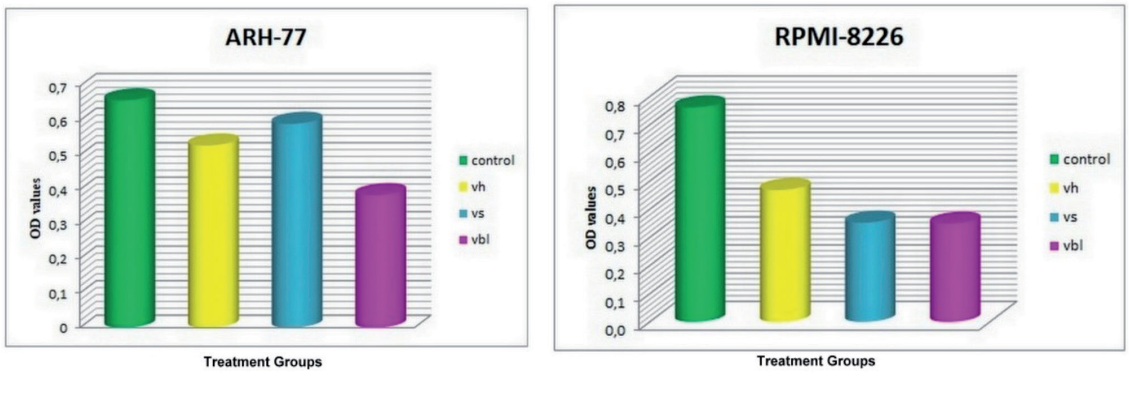

Fig. 5. The invasion capacity graphics of extract applied MM cell lines (VH *(V.herbacea),* VS *(V. soneri)* VBL (Vinblastine) and control: untreated).

TABLE 1. Comparative phenolic content of VH and VS extracts[10].

Number	Compound name	VH (mg/100g plant)	Standard deviation	VS (mg/100g plant)	Standard deviation
1	Gallic acid	3.25	1.13	6.42	0.84
2	Protocathechuic acid	66.30	9.30	64.05	0.92
3	Cathechin	245.31	14.43	246.28	4.10
4	4-Hydroxybenzoic acid	15.33	5.35	29.41	2.30
5	Caffeic acid	107.75	10.66	18.27	1.12
6	Syringic acid	6.42	1.75	128.14	2.16
7	Chlorogenic acid	25.27	8.23	401.23	7.83
8	Routine Trihydrate	1280.25	105.94	277.89	66.82
9	Trans-P-coumaric acid	8.69	9.66	7.31	1.89
10	Trans-Ferulic acid	10.46	6.37	12.77	0.50
11	Myricetin	9.05	7.68	55.01	5.22
12	Trans-Resveratrol	4.66	3.65	8.31	1.30
13	Quercetin	2.85	0.91	18.36	4.87
14	Trans-Cinnamic acid	0.29	0.04	8.35	3.04
15	Naringenin	2.82	0.67	26.48	12.03
16	Kaempferol	-	-	33.35	4.45
17	Isorhamnetin	2.74	0.86	1.54	0.26

antimyeloma activities, including cytotoxicity, *in vivo* and *in vitro* apoptosis induction, inhibition of the cell cycle, inhibition of osteoclastogenesis, and suppression of angiogenesis[11-17]. Compared to the current antimyeloma chemotherapy and agents, plant-derived natural products offer 2 crucial advantages: reducing side effects on healthy cells and reversing drug resistance. Our findings supported the previous reports mentioned above, and the *Vinca* extracts not only had a cytotoxic effect, but they also had *in vitro* apoptosis induction as well as suppression of invasion.

Our previous study revealed the volatile and phenolic content of *V. herbacea* and *V. soneri* species (Tables 1-3)[10]. With rich phenolic and alkaloid contents, *Vinca* extracts have high potential for the proliferation, apoptosis, and invasion of ARH-77 and RPMI-8226 MM cells. We can unequivocally say that the *Vinca* extracts can inhibit the cell proliferation of MM cells, which is accompanied by the induction of apoptosis. It appears that the applied extracts had different, but mostly positive effects on the 2 different cell lines in terms of cytotoxicity, gene expression, enzyme activity, and invasion. In line with this contribution, it was reported that many plant extracts or plant-derived natural compounds have mainly cytotoxic and antiproliferative effects on myeloma cells[14,18,19]. Aside from the cytotoxic effect, it is essential that the extracts have an apoptosis-inducing capacity. Induction of apoptosis via using plant-derived compounds has been preferred as a target for cancer prevention and treatment[20,21]. In both cell lines, some differences in the level of apoptotic gene expressions were observed after extract application, suggesting that the applied *Vinca* extracts had different but multidirectional effects in terms of gene expression and regulation. The resistance of MM cells to apoptosis involves high expression members of the BCL-2 family. The expression levels of BCL-2, BCL-XL, and MCL-1 are related to the resistance of cells to apoptosis[22,23]. As an important note, a decrease in the expression in a critical survival gene region for MM, MCL-1[24], is crucial for the breakdown of the apoptosis resistance of tumor cells. In addition, previous studies reported the gene expressions of

TABLE 2. GC-MS/MS results of VH extract[10].

Peak	Retention time	Area	Area %	Height	Height %	A/H	Name
1	33.534	1719770	1.36	394279	3.14	4.36	1,4-Metanobenzenecyclodecane
2	48.934	98670595	77.84	4550635	36.22	21.68	Tetrapentacontane
3	53.201	26377727 126768092	20.81 100.0	7618350 12563264	60.64 100.0	3.46	Phenol, 2.2'- metylenebis

TABLE 3. GC-MS/MS results of VH extract[10].

Peak	Retention time	Area	Area %	Height	Height %	A/H	Name
1	39.665	5705684	4.01	1701594	4.38	3.35	2- Pentadecanone
2	41.773	28879622	20.29	9274711	23.88	3.11	Hekzadecanoic acid
3	45.912	8625021	6.06	2607430	6.71	3.31	9,12- Octadecadioenic acid
4	46.064	41069063	28.85	11100585	28.59	3.70	6- Octadecanoic acid
5	46.378	16585337	11.65	3525045	9.08	4.71	Phytol izomer
6	46.681	5624973	3.95	1472724	3.79	3.82	Metyl stearate
7	53.201	27669470	19.44	7442334	19.17	3.72	Phenol, 2.2'- metylenebis
8	54.895	8174202	5.74	1706478	4.39	4.79	Tetrapentacontane
		142333372	100.0	38830901	100.0		

BAX, BCL-2, and p53 can regulate apoptosis of tumor cells[22,25]. The BAX gene is a member of the BCL-2 family that promotes apoptosis, and the ratio of BAX to BCL-2 determines the sensitivity of a cell to apoptosis[26]. In the present study, it was found that the extract applications down-regulated the antiapoptotic gene regions, including BCL-2 and MCL-1, which they up-regulated the BAX/BCL-2 ratio and Apaf, p53, HRK, BAK, and DFFA gene expressions. Caspase-3, the most important caspase-inducing caspase, induces apoptosis through a cleavage of substrates, including proteins and structural proteins, and mediators and regulators of apoptosis, including DNA repair and the cell cycle[27]. The results of the present study have shown that the extracts would also induce the activation of caspase-3 in MM cells. On the level of gene expression, the presence of caspase 3 activity, as well as enzymatic efficiency, may be an indicator that MM cells have gone through apoptosis to cell death. Our results are compatible with several studies about plant extracts or plant-derived compounds that induced apoptosis via an alteration in the apoptotic gene expressions and activated caspase 3 [13, 14, 18, 28-30].

Invasion capacity is the single most important characteristic that distinguishes benign from malignant lesions. It is crucial that plant-derived extracts or phytochemicals prevent tumor cells from migrating to a region different than the region in which they are located. According to the literature, there are limited studies on the blockage of cell invasion via natural plant-derived products. These studies point out that plant extracts may potentially prevent the invasion of myeloma cells; hence, the invasion would be inhibited by applying plant extracts or active phytochemicals on MM cell lines[17,31]. Moreover, the observed reduction in the invasiveness of MM cells, especially in the RPMI-8226 cell line, is promising and essential in preventing the spread of cancer cells to various tissues and organs for the treatment of the disease.

CONCLUSIONS

Despite the development of new drugs and the use of herbal preparations as treatment support, MM remains untreatable. As a general contribution, the regional plant extracts obtained from V. *herbacea*e and V. *soneri* continue to be promising for fighting cancer. More importantly, focus must be aimed at detecting which is the more active agent of all of the contents. Therefore, future studies that are more specific are of great importance for the identification of active or effective substances and the establishment of the pharmaceutical potential of extracts obtained from wild plant sources.

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Conflict of Interests:
The authors declare no conflict of interest

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