Research Article:
Assessment of Antitumor Activity of *Vinca herbacea* on Human Ovarian Cancer Cell Line

Somayeh Dehghanipour1, Sara Saadatmand2, Nasim Hayati Roodbari3, Mehdi Mahdavi4*

1. Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.
2. Recombinant Vaccine Research Center, Tehran University of Medical Sciences, Tehran, Iran.
3. Immunotherapy Group, Institute of Pharmaceutical Sciences, Tehran University of Medical Sciences, Tehran, Iran.
4. Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.

* Corresponding Author:
Mehdi Mahdavi, PhD.
Address: Department of Immunology, Pasteur Institute of Iran, Tehran, Iran.
Phone: +98 (21) 44865323
E-mail: mahdavivac@gmail.com

**ABSTRACT**

**Background:** It seems that *Vinca* herbacea has an anti-tumor effect. Here, the immunotherapeutic effect of this compound is assessed against human ovarian cancer (SKOV3) cells because of the high incidence of this tumor in women.

**Materials and Methods:** The cytotoxic activity of *V. herbacea* extract against human ovarian cancer (SKOV3) cells was determined by MTT assay. The apoptosis-inducing potential of *V. herbacea* extract was investigated using the FITC-V Annexin kit. The Matrigel invasion assay was used to investigate the ability of *V. herbacea* extract in reducing ovarian cancer cells invasion. Real-time PCR using specific primers was performed to investigate the expression of angiogenesis (*VEGF*, *VEGFR-1*, and *VEGFR-2*), apoptosis (*Bcl-2* and *Bax*), and metastasis (*MMP2* and *MMP9*) genes.

**Results:** *V. herbacea* caused a significant cytotoxic effect against human ovarian cancer cells in a dose-dependent manner. *V. herbacea* induced apoptosis in SKOV3 cells through caspase-3 activation and an increase in the expression ratio of Bax/Bcl-2. *V. herbacea* inhibited cancer cells’ angiogenesis, which was evident by the significant reduction in the expression of angiogenesis-related genes, including *VEGF*, *VEGFR-1*, and *VEGFR-2*. Besides, *V. herbacea* inhibited cancer cell adhesion and invasion.

**Conclusion:** *V. herbacea* extract elicits a robust cytostatic effect in SKOV3 cells by modulating the activity and or the expression of proteins regulating the process of cellular apoptosis, adhesion invasion, and angiogenesis.

**Keywords:** *Vinca herbacea* extract, Ovarian cancer, Angiogenesis, Apoptosis, Antitumor
Introduction

Ovarian cancer is the third most common and highly lethal gynecological malignant tumor in the female reproductive system. It presents a major challenge to healthcare systems throughout the world, due to its aggressive growth, rapid metastasis, strong invasion, and poor prognosis [1, 2].

The incidence of ovarian cancer is considerable worldwide and mainly diagnosed at an advanced stage. The prognosis of this cancer is poor, which makes this the most lethal gynecological malignancy. In the recent 10 years, an increase in this cancer incidence was mainly observed in Central and South America, Asia, as well as Central and Eastern Europe [3]. However, after 2011 in some countries such as the Netherlands, the incidence declined [4].

At present, chemotherapy is a major therapeutic option for ovarian cancer. Patients are frequently attenuated by developing resistance to a wide spectrum of the classic anticancer agents and considerable adverse side effects such as systemic toxicity. Although primary treatment has enhanced the 5-year survival rate, it has not improved the overall rate of cure, because the percentage of relapsed ovarian cancer patients who developed resistance to taxane- or platinum-based treatment has increased to more than 70% [5-7]. It was reported that 28% of ovarian cancer patients entering their last stage of life, develop malignant ascites resistant to conventional chemotherapy [8]. The high incidence and mortality of ovarian cancer, combined with the paucity of effective anticancer agents [2, 9], highlights designing effective therapeutic approaches, as well as aiding the treatment and control of ovarian cancer patients worldwide. The examination and identification of medicinal plants as major sources of effective anti-cancer agents have been growing to develop new therapeutic agents for metastatic ovarian cancer [10, 11].

**V**inca **h**erbacea Waldst. et Kit. belongs to the Apocynaceae family, which is an herbaceous perennial plant growing as a trailing vine and distributed in various geographical areas, especially in the Alborz mountains in northern Iran [12-14]. Total alkaloids in various parts of *V. herbacea* are associated with different pharmacological activities, including long-time reduction effects on blood pressure, inhibitory effect on neuromuscular synapses, bacteriostatic effect, and cardio-stimulating activity [15-17]. Oxindole alkaloids establish the main anti-cancer compounds of *V. herbacea*, including vinblastine, vincristine, vindesine, and vinorelbine are widely used antineoplastic drugs. The mechanism of action of these cell cycle-dependent agents is the inhibition of tubulin polymerization into microtubules [18]. Besides, the study of leukopenia-induced mice showed that treatment with *V. herbacea* could restore WBCs count of immunocompetent mice and thereby potentiating the immune response [19].

However, the exact mechanism of antitumor activities of *V. herbacea* leaf extract is unknown, and especially, studies regarding its effects on adhesion, invasion, and apoptosis pathway of ovarian cancer are unavailable. In this study, *in vitro* antitumor activity of *V. herbacea* leaf extract was examined by assessing cell viability, adhesion/invasion, and apoptosis pathway in human ovarian cancer cells.

Materials and Methods

Plant material and extract preparation

The leaves of *V. herbacea* were collected in the flowering phase (May 2017) from Alborz mountain in Alborz Province, Iran. The plant was identified in the Department of Pharmacology at Tehran University of Medical Sciences [Herbarium #6601]. The plant leaves were cleaned, weighed, dried, pulverized, and macerated in ethanol (70%) at room temperature for 3 days with shaking. The extract was passed through a filter paper and liquid sample concentrated in a rotary evaporator (LABORATA 4000 efficient Heidolph, Germany) and finally lyophilized in a freeze dryer (LTE Scientific Ltd, UK). The obtained powder was stored at -20°C until further use. The working solutions were prepared by dissolving the extract in dimethyl sulfoxide 0.5% v/v (DMSO, Sigma-Aldrich, USA) for experiments.

Cell culture

SKOV3 (Human ovarian cancer) and HEK 293 (Human embryonic kidney cells 293) cell lines were purchased from Pasteur Institute (Tehran, Iran) and cultured in tissue culture flask with high glucose Dulbecco’s modified eagle medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin-streptomycin and kept at 37°C in a humidified 5% CO2 incubator for 72 h.

Cytotoxicity assay

Cytotoxicity assay was evaluated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously [20, 21]. Briefly, 1×10⁵ cells/mL was treated with various doses (10 to 1000 µg/mL) of *V. herbacea* extract for 24 and 48 hours.
Subsequently, the cells were treated with MTT solution (Sigma-Aldrich) and then the culture medium was replaced with DMSO and the Absorbance (Ab) was measured at 570 nm using an ELISA microplate reader. The percentage of cytotoxicity activity was calculated as follows: cytotoxicity activity (%)=\[\frac{(\text{Ab of experimental well/Ab of negative control well})\times100}{1}\]. The IC_{50} (concentration that inhibits cell growth by 50%) value of V. herbacea extract was calculated via nonlinear regression of concentration-response curves.

**Cell apoptosis assay**

Annexin V-FITC/PI staining assay was performed to identify apoptotic cells as previously described [22]. Briefly, the cells (2×10^6 cells/well) were treated with an IC_{50} dose of V. herbacea extract. Subsequently, the cells were centrifuged and stained with Annexin V-FITC solution (BD, USA) and PI (Sigma). The cells were analyzed using a FACScan flow cytometer (BD) and FlowJo software version 9.0 (Tree Star, USA).

**Caspase-3 and caspase-9 activation assay**

Caspase-3 and caspase-9 activities were measured by using a caspase-3 or caspase-9 colorimetric assay kit (Abcam, Cambridge, MA, USA) according to the manufacturer’s instruction. Briefly, cell suspensions (2×10^6 cells/well) were treated for 24 h with an IC_{50} dose of V. herbacea extract. The cells were harvested and washed three times with PBS and lysed using 50 μL of lysis buffer for 10 min on ice. The lyzed samples were centrifuged at 15000 rpm at 4°C for 1 min and the supernatant was collected and total protein concentration was assessed using Bradford reagent. The samples adjusted to the concentration of 200 μg per 50 μL of lysis buffer and 50 μL of this sample were added to the 96-well plate (Greiner, Germany) as a test and for background well, the lysis buffer was added. Afterward, 50 μL of 2x reaction buffer (10 mM DTT) was added to each well and then 5 μL of the 4 mM DEVD-p-NA substrate (200 μM) was added to the wells and incubated for 2 h at 37°C. The optical density of the reaction was measured at 405 nm on a microplate reader. Fold-increase of caspase-3 and -9 activity was determined by comparing sample (treated) results with untreated wells.

**Adhesion assay**

Adhesion assay was performed as previously described [23]. Briefly, V. herbacea extract-treated and extract-untreated cells were fixed with methanol and subsequently stained with 1% toluidine blue and solubilized in 1% SDS. After color development, absorbance at 540 nm was measured.

**Matrigel invasion assay**

Matrigel invasion assay was performed as previously described [23]. Briefly, the upper and lower transwell chambers (Corning, USA) were filled with RPMI-1640 diluted Matrigel (Corning) and RPMI-1640 containing 10% FBS, respectively, and incubated for 30 min at 37°C. The cells were treated with V. herbacea extract for 24 h, then they were incubated at upper chambers for 5 h at 37°C with 5% CO2. After the removal of non-invading cells with a cotton swab from the top of the membrane, cells underneath the membrane filter were fixed in paraformaldehyde (3.7%) for 15 min, stained with 0.1% toluidine blue solution for 2 min at 37°C. Then the invaded cells underneath the membrane were stained with toluidine blue solution. The suspension was solubilized in 1% SDS and absorbance was measured at 600 nm.

**Reverse transcriptase-polymerase chain reaction**

The mRNA expression of apoptosis regulatory genes (Bax and Bcl-2), angiogenesis genes (VEGF-A, VEGF-R-1, and VEGF-R-2), and metastasis genes (MMP-2 and MMP9) was evaluated by SYBR Green real-time PCR analysis using specific oligonucleotide primers as previously described [24, 25]. GAPDH was used as internal control (Appendix 1).

**Statistical analysis**

GraphPad Prism 6 (GraphPad Prism version 6.01 Software, Inc., USA) was used to perform statistical analyses. The results were analyzed with a One-way analysis of variance and Dunnett’s post hoc test. All values were presented as the Mean±SEM, and a P value of less than 0.05 was considered statistically significant.

**Results**

**V. herbacea extract has high cytotoxic activity against SKOV3**

The cytotoxic activity of V. herbacea extract against SKOV3 cells (Figure 1A) was determined by the MTT assay. Here, HEK 293 cells (Figure 1B) were used as the control. Results show that treatment with V. herbacea extract significantly decreased SKOV3 cell viability. V. herbacea extract at a concentration of 800 μg/mL showed the highest cytotoxicity activity against SKOV3 cells, with cell viability of 19% (P<0.05). The cytotoxicity activity of V. herbacea extract against SKOV3 cells at all concentrations was significantly higher than untreated-cells (P<0.01) and the IC_{50} value of V. herba-
extract was 10.64 µg/mL. As shown in Figure 1B, V. herbacea extract at concentrations of 10 to 150 µg/mL has no significant cytotoxic activity on HEK 293 cells compared to untreated-cells (P>0.05). It is worth mentioning that the IC_{50} value of V. herbacea extract against HEK 293 cells was 251.2 µg/mL, which was significantly higher than the IC_{50} value of V. herbacea extract against SKOV3 cells. Also, there was no significant difference regarding the cytotoxic activity of V. herbacea extract between 24 and 48 h treatments (data not shown).

V. herbacea extract induces apoptosis of SKOV3

To test whether the cytotoxic effect of V. herbacea extract on SKOV3 cells was due to apoptosis, Annexin V/PI staining was carried out. Treatment of SKOV3 cells with V. herbacea extract at a concentration of 10.64 µg/mL significantly increased the percentage of early apoptotic cells from 3.1% in the control cells to 27.1% in the treated cells (P<0.05). The population of late-stage apoptotic cells after treatment with V. herbacea extract significantly increased to 12.5%, compared to that in the control cells (2%) (P<0.05). In the presence of V. herbacea extract, the number of early cells significantly increased (P<0.05) compared to that in the late apoptotic cells (P<0.01; Figure 2, A and B).

Moreover, to evaluate the cell apoptosis pathway induced by V. herbacea extract, we measured the caspase-3 and caspase-9 activities in the experimental cell lines. Results show that treatment with V. herbacea extract at a concentration of 10.64 µg/mL significantly increased the caspase-3 activity by 1.8 fold, compared to the control cells (P<0.05; Figure. 3A). Also, caspase-9 activity after treatment with 10.64 µg/mL of V. herbacea extract significantly increased by 1.5 folds compared to the control cells (P<0.05; Figure 3B).

Next, we assessed the expression level of apoptosis-related genes, Bax (Figure 3C) and Bcl-2 (Figure 3D) in V. herbacea extract-treated SKOV3 cells by RT-PCR. The relative expression of the genes was determined by dividing its expression amount by that of the GAPDH gene. Results show that incubation of cells with V. herbacea extract resulted in a significant decrease of anti-apoptotic Bcl-2 mRNA expression and also associated with a marked increase in the expression of pro-apoptotic Bax mRNA (P<0.05). The expression level of Bax mRNA was 1.7 folds after the treatment. Moreover, the expression level of Bcl-2 gene was decreased by 0.3 fold when the SKOV3 cells were treated with V. herbacea extract (P<0.05).

V. herbacea extract decreases tumor cell adhesion, invasion, and metastasis

To assess the anti-adhesive effects of V. herbacea extract, SKOV3 cells were incubated with IC_{50} concentration of V. herbacea extract, and attached cells were quantitated colorimetrically using an ELISA plate reader. As shown in Figure 4, the treatment of tumor cells with
10.64 µg/mL of *V. herbacea* extract significantly reduced cell adhesion compared to the control cells (P<0.05).

To determine the inhibitory effects of *V. herbacea* extract on SKOV3 cells invasion, we performed a Matrigel invasion assay. As shown in Figure 5A. *V. herbacea* extract decreased the SKOV3 cell invasion to the Matrigel-coated substrate. Treatment of SKOV3 cells with 10.64 µg/mL of *V. herbacea* extract significantly decreased tumor cell invasion by 30% compared to that in the untreated-cells (P<0.05). Moreover, we assessed the expression level of metastasis-related genes, MMP2 and MMP9, in *V. herbacea* extract-treated SKOV3 cells by RT-PCR. The expressions of MMP2 and MMP9 were both decreased by *V. herbacea* extract treatment versus the control sample. As shown in Figure 5B, the treatment of SKOV3 cells with 10.64 µg/mL of *V. herbacea* extract significantly reduced transcription of MMP2 by 0.7 fold compared with the control cells (P<0.05). Moreover, the expression level of the MMP9 gene decreased by 0.7 fold when the SKOV3 cells were treated as well. Also, the treatment of SKOV3 cells with 10.64 µg/mL of *V. herbacea* extract decreased the mRNA levels of VEGF-A by 0.7 fold as compared with the control cells (P<0.05; Figure 6C).

### Discussion

Ovarian cancer is currently the most common cause of death among other gynecologic cancers in women worldwide [26, 27].

The survival rate of ovarian cancer patients is not only associated with the disease stages but also the ovarian cell types. High-grade serous adenocarcinoma is the most common histologic subtype with the worst prognosis [19, 28, 29]. In the present study, SKOV3 was used for assessing the anti-proliferative and apoptosis-promoting effects of *V. herbacea* in vitro.

When assessing the in vitro cytotoxic activity of *V. herbacea* extract-induced cell death against SKOV3 cells, we found that *V. herbacea* extract has anti-proliferative activity in a dose-dependent manner, as reflected by the low cytotoxic effects on HEK 293 cells.
In the MTT assay, the number of metabolically active viable cells is associated with the reduction of MTT by mitochondrial dehydrogenase enzyme [30]. The results of the MTT assay indicated that V. herbacea extract has a great potential to inhibit cell proliferation and induce death in human ovarian cancer cells. The study also demonstrates that the V. herbacea extract has poor effects on the growth and metabolism of normal cells, which are the most suitable attributes of a chemotherapeutic drug; therefore, this potential should be considered during further drug development.

We further used Annexin/PI flow cytometric assays to assess the pathways of cell death induced by V. herbacea extract. Analysis of flow cytometric results demonstrated that V. herbacea extract treatment resulted in an increased number of cells undergoing early and late apoptosis. The results suggested that V. herbacea extract induces translocation of phosphatidylserine from inner to the outer surface of the cell membrane, which is generally accepted as one of the biomarkers of apoptosis [31]. Unlike necrosis, apoptosis preserves tissue homeostasis by removing dead cells by immune cells such as macrophages and does not induce inflammatory responses, which destroys normal cells and tissue damage [32]. To maintain their uncontrolled proliferation, cancer cells acquire and show different resistance mechanisms to apoptosis; therefore, because of its apoptosis-inducing potential, V. herbacea extract can be considered a probable chemotherapeutic agent against cancer in future studies [33].

Apoptosis or programmed cell death is a highly complex process and classified into caspase-dependent or caspase-independent mechanisms [34]. The caspase-dependent pathway is further divided into the extrinsic (death receptor) and the intrinsic (mitochondrial) cas-

**Figure 3.** The caspase-3 and caspase-9 activities in the experimental cell lines

A: Caspase-3 and B: Caspase-9 activities in SKOV3 cells after treatment with IC₅₀ concentration of V. herbacea extract

Treatment with V. herbacea extract increased caspase-3 activity by 1.8 fold and caspase-9 activity by 1.5 fold compared to the control cells (P<0.05).

C: Besides, relative mRNA levels of Bax and D: Bcl-2 (in treated SKOV3 cells versus untreated cells. Incubation of the cells with V. herbacea extract at IC₅₀ concentration resulted in a significant decrease in the Bcl-2 mRNA expression while associated with a marked increase in the expression of Bax mRNA (P<0.05). Data are presented as the Mean±SEM of three independent experiments. *P<0.05 indicates the groups, which were significantly different.
cades, as determined by the involvement of caspase-8 or caspase-9, respectively [34]. Both pathways activate caspase-3 that is involved in the final execution of dying cells, while caspase-9 is an initiator caspase that is involved in the intrinsic pathway [34]. A study reported the anticancer effects of *V. herbacea* extract but the mechanisms of cancer cell apoptosis have not been elucidated [35]. In assessing the molecular mechanism underlying the apoptosis process, we found that *V. herbacea* extract induced an increase in caspase-9 and caspase-3 activities, which confirms the contribution of intrinsic caspase pathways in the *V. herbacea* extract-induced cell death. Moreover, the apoptotic effects of *V. herbacea* extract in SKOV3 cells were confirmed by the up-regulation of Bax and down-regulation of Bcl-2, which are the key genes in the intrinsic pathway of apoptosis [36].

The *in vitro* antitumor proliferation and activity of many drug components are accomplished through the Bax/Bcl-2 pathways [37-39]. However, the in vivo association between clinical prognostic factors and Bcl-2 and Bax mRNA expression is variable [40, 41]. These findings support the notion that *V. herbacea* extract reduced the survival and inhibited the growth of ovarian cancer cell via both intrinsic and extrinsic apoptosis pathways, which may have therapeutic potential in the management of ovarian cancer.

It is well known that the development of tumor cell invasion and metastasis is a dynamic multi-step process, including cell adhesion, proteolytic degradation, migration, and angiogenesis [42]. Targeted anti-cancer drugs block cell cycle progression by interfering with specific...
molecules, which have critical roles in tumor cell growth, survival, migration, and invasive spread of cancer [43, 44]. Currently, there is increasing attention toward the combination of multiple anticancer agents, which target and interfere with several pathways [45]. Data from the potency assessment of *V. herbacea* extract showed that the effective inhibition of adhesion and invasion of the treated-SKOV3 cell, which is evident by the significant reduction of the invaded cell to the Matrigel-coated substrate, may be due to the death of the *V. herbacea*-treated cells.

In assessing the mechanism by which *V. herbacea* extract inhibits tumor growth and invasion, we found that *V. herbacea* extract efficiently inhibited ovarian cancer cells’ angiogenesis, which was evident by the significant reduction in the expression of angiogenesis-related genes, including VEGF and its tyrosine kinase receptors, VEGFR-1 and VEGFR-2. Since the exceeding concentrations of angiogenesis inhibitors against those of stimulators could potentially inhibit the tumor growth and dissemination to other organs, inhibition of angiogenesis is a critical step for cancer prevention and treatment [46]. VEGF, VEGFR-1, and VEGFR-2 are identified as common anti-angiogenic target molecules because they are key mediators of angiogenesis [47]. It is worth noting that RT-PCR analysis revealed that *V. herbacea* extract decreases the expression of the VEGF-A, VEGFR-1, and VEGFR-2 genes, which can contribute to its anti-tumor activities through inhibition of tumor angiogenesis. Considering the potential of *V. herbacea* extract to inhibit tumor invasion and metastasis, we found that *V. herbacea* extract markedly reduced the expression level of MMP-2 and MMP-9 in SKOV3 cells. It is well known that the inhibition of MMPs prevents tumor invasion and metastasis [29, 48, 49]. Also, SKOV3 cells, which are identified as high-grade serous adenocarcinoma, have a high metastatic rate [9, 28, 29]. It has been documented that in advanced ovarian serous cancers the elevated MMP level, particularly MMP-2 and MMP-9, associates with tumor progression and metastasis [50-53]. Together, the results of the current study indicate that *V. herbacea* extract can have an inhibitory effect on the invasion and metastasis of SKOV3.

We report that the *V. herbacea* extract may be useful as a therapeutic option against ovarian cancer because it elicits a robust cytostatic effect in SKOV3 cells by affecting the activity and or the expression of genes regulating the process of cellular apoptosis, adhesion, invasion, and angiogenesis. In light of these findings, a thorough assessment of *V. herbacea* extract under controlled clinical settings against different tumor cells seems warranted. Our results reinforce the promise that, perhaps soon, clinical applications of the therapeutic potential of *V. herbacea* extract can provide patients with an extended range of protection, and help reduce the high morbidity and mortality associated with cancer around the world.

**Ethical Considerations**

**Compliance with ethical guidelines**

This is a *in vitro* study. All experiments have performed according to the Helsinki principals.

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**Authors' contributions**
All authors equally contributed in preparing this article.

Conflicts of interest

The authors declare no conflict of interest.

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### Appendix 1. List of primers used in this study

| Gene Symbols | Primer Sequences (5’ -> 3’) |
|--------------|-----------------------------|
| Bcl-2-F      | TGTGTGGAGAGCGTCAACC         |
| Bcl-2-R      | TGGATCCAGGTGTGCAGGT         |
| Bax-F        | GATGCGTCCACCAAGAAGC         |
| Bax-R        | AAGTCCAATGTCCAGCCCAT        |
| VEGFR1-F     | CCAGACCCAGCTCTTACCC         |
| VEGFR1-R     | CGCCAGCACCACCTGATTGT        |
| VEGFR2-F     | GCCTACCTCACGTGTTTCTT        |
| VEGFR2-R     | ACTGTCCTGCTGTGCTCAT         |
| VEGF-A-F     | AGGGCAGAAATCAGACAGAAAGT     |
| VEGF-A-R     | AGGGTCTCGATTTGGATGGCA       |
| MMP2-F       | AGCGGTCACACCTACTCTT         |
| MMP2-R       | GCCAGTCGATTGTGATCTTT        |
| MMP9-F       | CTTTGACTGCGACCAAGAAGTGG     |
| MMP9-R       | GGACTGAGGATTGATCATAAGC      |
| GAPDH-F      | AGGGCTGTCTTTAACTCTGT       |
| GAPDH-R      | CCCACCTGATTTGGAGGGAG       |