Published online 2022 August 20 **Original Article**

Anti-tumor Effect of the Ethanolic Extract of *Vinca Herbacea* on Human Breast Cancer Cell Line: A Mechanism Study

Somayeh Dehghanipour[1], Sara Saadatmand[1], Nasim Hayati Roodbari[1] and Mehdi Mahdavi[2,3,4,*]

[1]Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran
[2]Department of Advanced Therapy Medicinal Product, Breast Cancer Research Center, Motamed Cancer Institute, Academic Center for Education, Culture, and Research, Tehran, Iran
[3]Recombinant Vaccine Research Center, Tehran University of Medical Sciences, Tehran, Iran
[4]Immunotherapy Group, Institute of Pharmaceutical Science, Tehran University of Medical Science, Tehran, Iran

*** *Corresponding author***: Mehdi Mahdavi, Department of Advanced Therapy Medicinal Product, Breast Cancer Research Center, Motamed Cancer Institute, Academic Center for Education, Culture and Research, Tehran, Iran. Tel: +982188203915; Email: mahdi.research2019@gmail.com

Received 2022 February 07; **Revised** 2022 June 09; **Accepted** 2022 July 18.

Abstract

Background: Breast cancer is extensively studied for its resistance to chemotherapy. Therefore, finding efficient therapeutic agents is vital to increasing the survival rate of patients.
Objectives: Here, we conducted an *in vitro* study on the anti-tumor properties of *Vinca herbacea* extract on SKBR3 cells.
Methods: Apoptotic and anti-invasive activity of *V. herbacea* extract was studied using Annexin FITC-V kit and Matrigel invasive assay, respectively. The expression of angiogenesis genes (*VEGFR-1*, *VEGFR-2*, and *VEGF-A*), apoptotic-related genes (*Bcl-2* and *BAX*), as well as metastatic genes (*MMP2* and *MMP9*) were studied using real-time polymerase chain reaction.
Results: *Vinca herbacea* extract showed significant antiproliferative and cytotoxic effects on human breast cancer cells, compared to human embryonic kidney cells 293. *Vinca herbacea* extract had a great apoptosis-inducing potential in breast cancer cells by activating caspase-3 and increasing the *BAX/Bcl-2* ratio. *Vinca herbacea* extract prevented cancer cell angiogenesis, marked by decreasing the expression level of angiogenesis-related genes, including *VEGF*, *VEGFR-1*, and *VEGFR-2*. In addition, *V. herbacea* extracts reduced cancer cell invasion and noticeably decreased the expression level of metastasis-associated genes, including *MMP2* and *MMP9*.
Conclusion: *Vinca herbacea* extracts exhibited vigorous cytotoxic effects on SKBR3 cells by the alteration of apoptosis, cell adhesion, invasion, and angiogenesis.

Keywords: Angiogenesis, Anti-tumor, Apoptosis, Breast cancer, Metastasis, *V. herbacea* extract

1. Background

Breast cancer has a high frequency in different countries throughout the world (1-3). The GLOBOCAN statistics in 2018 reported 2.3 million new cases from 185 countries with a mortality rate of about 7% (4). In developed countries, the age-standardized incidence rate is more than 80 per 100,000 cases; however, in developing countries, it is estimated to be less than 40 per 100,000 cases (2). Considering Iranian women, breast cancer has a prevalence rate of 24 per 100,000 cases in the last decade (5). According to new statistics in Iran, breast cancer accounts for an estimated 6,160 new cases and 1,063 deaths annually (6, 7). Evidence suggests that the age of the onset of breast cancer is almost 10 years younger in Iranian women than in those in developed countries (5).

Approximately 30% of breast cancers at early stages tend to progress to the advanced metastatic stage, with limited therapeutic options (8). Resistance to chemotherapy drugs is a major complication in this field (9). The response rate to the treatment of metastatic breast cancer with first-line chemotherapies has been calculated at 30-70% (10, 11). Nevertheless, the time for tumor progression following first-line chemotherapies is 6-10 months (10, 11). Furthermore, response to treatment with a single or a combination of therapies decreases to 20-30% in the advanced stages with a survival rate of fewer than 6 months (10).

The dearth of efficient anti-tumor agents, along with high rates of breast cancer incidence and mortality, signifies the critical necessity to treat breast cancer patients (12, 13). Medical plants have been known as the main sources of efficient anti-cancer agents with fewer adverse effects and lower costs for the treatment of breast cancer (14). *Vinca herbacea Waldst*, belonging to the Apocynaceae family, is abundantly grown in the Alborz mountains (15). The alkaloids of *V. herbacea* have been demonstrated to have several biological effects, including longer-term diminution of blood pressure, neuromuscular synapses blocking, bacteriostatic effect, stimulation of the cardiovascular system, and antioxidant and anticancer activity, which are associated with the presence of oxindole alkaloids, such as majdine and isomajdine (16). However, there is limited data on the anti-tumor mechanism of *V. herbacea* extract, principally evidence about its effects on anti-adhesion/invasion and apoptosis pathway.

2. Objectives

This study was conducted to assess various

aspects of *in vitro* anti-cancer effects of *V. herbacea* leaf extract.

3. Methods

3.1. Preparation of extract plant

Vinca herbacea (V. herbacea) leaves were obtained from the Alborz Mountains of Iran and identified in the Pharmacology Department of Tehran University of Medical Sciences, Tehran, Iran [Herbarium #6601 (TEH)]. After cleaning and weighing the leaves, the maceration process was conducted with ethanol 70% at 25°C for 3 days. The ethanolic extract was filtered, concentrated by an evaporator machine, and then aliquoted into 25 ml and stored at -20°C for further steps. Final dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) concentrations (0.5% v/v) were provided from the stock solution and used in the study.

3.2. Cell culture

Human breast cancer cell line, SKBR3, and human embryonic kidney cell line, HEK 293, were purchased from the Pasteur Institute of Iran (Tehran, Iran) and cultivated in 25 cm^2 of Dulbecco's Modified Eagle Medium culture (Gibco, USA) along with 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (FBS; Gibco) and placed at 37°C and 5% CO_2 incubator for 72 h.

3.3. MTT assessment

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method was employed to assess the cytotoxic activity of V. herbacea against SKBR3 and HEK293 cells (17, 18). Concisely, a volume of 1 × 10^5 cells/ml was incubated with 10 to 1,000 µg/ml of V. herbacea extract for 24 and 48 h. The MTT solution (Sigma-Aldrich) was dispensed to V. herbacea-treated cells and incubated for 4 h. Subsequently, after removing the culture medium and replacing it with DMSO solution, the absorbance was measured at 570 nm.

3.4. Apoptosis assessment

Apoptotic cells were identified using Annexin V-FITC/PI staining assay as described by Du et al., (19). Briefly, the cells (2×10^5 cells/well) were treated with an IC_{50} dose of V. herbacea extract for a period of 24 h and subsequently stained with annexin V-FITC solution (BD, USA) and PI (Sigma, USA). The analysis of the cells was performed by a FACScan flow cytometer (BD, USA) and FlowJo software version 9.0 (Tree Star, USA).

3.5. Caspase level

A colorimetric assay kit (Abcam, Cambridge, MA, USA) was applied for caspase-3 and -9 activity assessment. In brief, 2 × 10^5 cells/ml were incubated with an IC_{50} dose of V. herbacea extract for 24 h and then harvested and lysed using lysis buffer. The supernatants were subsequently separated and applied for the assessment of caspase-3 and caspase-9 activity based on the company's instructions.

3.6. Adhesion assay

The assessment of adhesion was performed as explained by Pereira et al., (20). In short, SKBR3 cells (2×10^4 cells/well) in 96-well plates were treated with an IC_{50} dose of V. herbacea extract for 24 h at 37ºC. Un-treated SKBR3 cells were considered negative controls. After the incubation time, the plates were washed twice with phosphate-buffered saline (PBS) to remove unattached cells. Thereafter, the attached cells were fixed with methanol and stained with toluidine blue 1% in sodium tetraborate 1% for 5 min. In the next stage, the wells were washed with PBS and the dye was solubilized in SDS 1% at 37ºC for 20 min. The color density of the plates was read by an enzyme-linked immunosorbent assay plate reader at 540 nm. The un-treated SKBR3 cells were considered 100% adhesion activity, and the adhesion activity of treated SKBR3 cells was calculated as cell adhesion percent in comparison to negative controls.

3.7. Invasion assessment

Invasion evaluation was conducted as explained in a related study (20). In short, the upper and lower transwell chambers (8-mm pore size, Corning Costar Co., MA, USA) were filled with Roswell Park Memorial Institute (RPMI)-1640 diluted Matrigel (Corning) and RPMI-1640 (Gibco) containing 10% FBS, respectively. Cells (2×10^4) were treated with V. herbacea extract, dispensed to the upper chambers, and incubated for 5 h. After the clearance of normal cells, cells underneath the membrane filter were fixed in paraformaldehyde and stained with toluidine blue solution. The suspension was solubilized in 1% SDS, and the absorbance was measured at 600 nm.

3.8. Gene expression assay

The messenger ribonucleic acid (mRNA) expression of angiogenesis (vascular endothelial growth factor A [*VEGF-A*], vascular endothelial growth factor receptor 1 [*VEGFR-1*], and vascular endothelial growth factor receptor 2 [*VEGFR-2*]), metastasis (matrix metalloproteinase-2 [*MMP2*] and matrix metallopeptidase-9 [*MMP9*]), and regulatory genes of apoptosis (B-cell lymphoma 2 [*Bcl-2*] and *Bcl-2* associated X protein [*BAX*]) were measured by a Rotor-Gene 6000 thermocycler (Corbett Research, Australia). Briefly, the number of 5 × 10^5 cells in a 5 ml complete medium were incubated with an IC_{50} dose of V. herbacea extract for 24 h (21). Ribonucleic acids were extracted from the lysed cells, and complementary DNA was synthesized using specific kits (Qiagen, USA) and plied for the real-time polymerase chain reaction (RT-PCR) along with the specific primers (Table 1).

Table 1. List of primers

Genes	Primers
Bcl-2	For:5'-TGTGTGGAGAGCGTCAACC-3' Rev:5'-TGGATCCAGGTGTGCAGGT-3'
BAX	For:5'-GATGCGTCCACCAAGAAGC-3' Rev:5'-AAGTCCAATGTCCAGCCCAT-3'
VEGFR1	For:5'-AGCCTACCTCACCGTGCAAG-3' Rev:5'-AAAAGAGGGTCGCAGCCAC-3'
VEGFR2	For:5'-GATGCAGGAAACTACACGGTCAT-3' Rev:5'-GGTCCCATACTGGTAGGAATCCA-3'
VEGF-A	For: 5'- TAACGATGAAGCCCTGGAGTG-3' Rev:5'- AGGTTTGATCCGCATGATCTGT-3'
MMP2	For: 5'-CTGACCCCCAGTCCTATCTGCC-3' Rev:5'-TGTTGGGAACGCCTGACTTCAG-3'
MMP9	For: 5'-CTTTGACAGCGACAAGAAGTGG-3' Rev:5'-GGCACTGAGGAATGATCTAAGC-3'
GAPDH	For: 5'-CCCATCACCATCTTCCAGGAGC-3' Rev: 5'-CCAGTGAGCTTCCCGTTCAGC-3'

3.9. Statistical analysis

Variance one-way analysis and Dunnett's post-hoc tests were properly selected for statistical analysis using Graph Pad Prism 6 (version 6.00, Inc., USA). All values were presented as the mean ± standard error of the mean, and the P-values of < 0.05 were considered significant.

4. Results

4.1. Cytotoxic effects of V. herbacea extract

The cytotoxic activity of V. herbacea extract on SKBR3 and HEK 293 cells was determined by the MTT method. As demonstrated in Figure 1, V. herbacea extract at concentrations of 300 µg/ml and above significantly decreased SKBR3 cell viability, compared to the HEK 293 cell line (P<0.05). The IC_{50} value of V. herbacea extract on SKBR3 was 253.9 µg/ml, which was significantly lower than that on HEK 293 cell line (908.3 µg/ml; P<0.01). No significant difference was found between the 24- and 48-hour treated cells.

4.2. Apoptotic properties of V. herbacea *extract*

To assess whether the cytotoxic effect of V. herbacea extract on SKOV3 was due to apoptosis, annexin V/PI staining was carried out (Figure 2A-B).

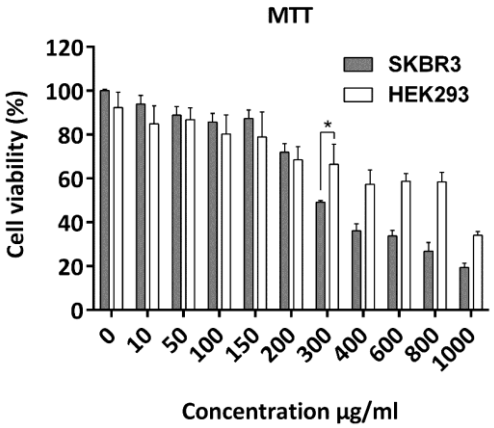

Figure 1. Cytotoxic effects of V. herbacea extract on SKBR3 and HEK 293 cell lines after 24 h of exposure

The number of 1 × 10^5 cells/well were incubated with 10 to 1,000 µg/ml of V. herbacea extract. The MTT solution (Sigma-Aldrich) was used to show cell viability. At the concentrations of 300 µg/ml, a significant correlation was observed regarding the cell viability (*P<0.05).

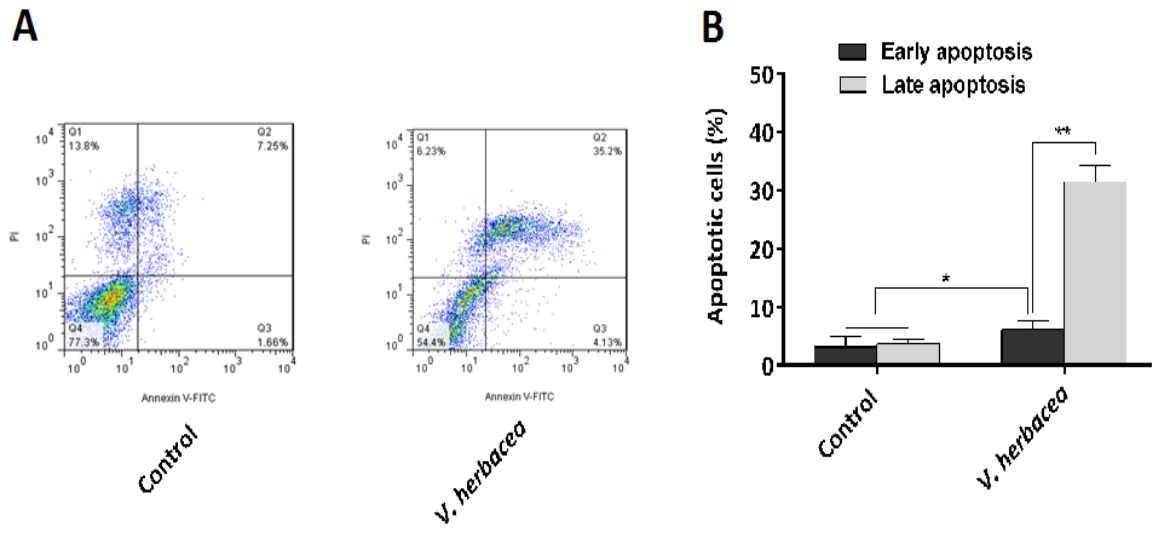

Figure 2. Apoptosis-inducing effects of V. herbacea against SKBR3 cell

(A) The flowcytometry chart shows the cells in the upper right which reveals early apoptosis. (B) Both early and late apoptotic cells are significantly higher in *V. herbacea* than in controls. A student t-test was applied to compare the mean values of relative expression patterns between the two groups. *P < 0.05, ** P < 0.001.

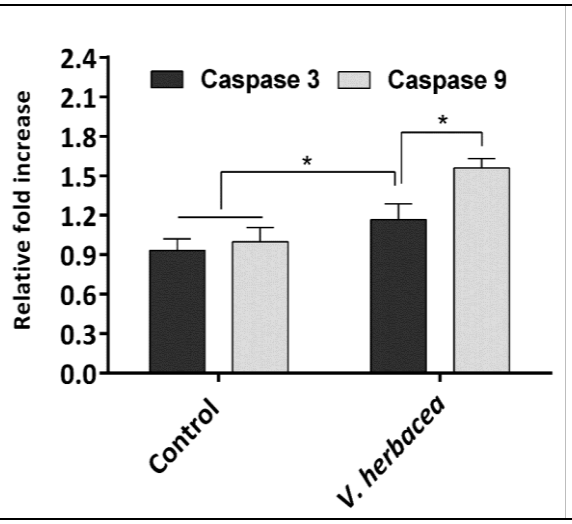

Figure 3. Vinca herbacea extract effects on caspase-3 and-9 activation in SKBR3 cells

Student t-test was employed to compare the mean values of relative fold increase between the two groups. *P < 0.05.

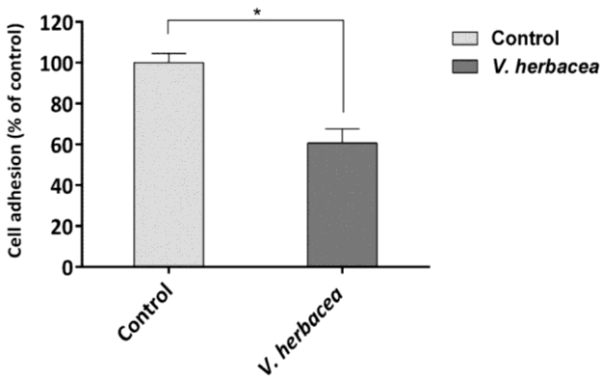

Figure 4. Anti-adhesive effect of V. herbacea extract on SKBR3 cell adhesion

Anti-adhesive effect of V. herbacea extract was higher on SKBR3 cell adhesion than on the control. A student t-test was applied to compare the percentage of cell adhesion between the two groups. *P <0.05.

The percentage of viable cells in V. herbacea extract-treated cells significantly declined in comparison with untreated cells (P<0.05). *Vinca herbacea* extract treatment caused a significant rose in the percentage of early apoptotic cells from 3.1% to 6.1% (P<0.05) (Figure 2A-B). In addition, V. herbacea extracts significantly enhanced the percentage of late apoptotic cells versus early apoptotic ones (P<0.01) (Figure 2A-B). The percentages of early and late apoptotic cells remained unchanged in the control groups (P>0.05).

Moreover, the caspase-3 and -9 activities were measured to assess the cell apoptosis pathway mediated by V. herbacea extract. *Vinca herbacea* extracts significantly increased the caspase-3 and -9 activities 1.3- and 1.6-fold, respectively, compared to that in the untreated cells (P<0.05) (Figure 3). In addition, in V. herbacea -treated cells, the activity of caspase-9 was significantly increased, compared to caspase-3 (P<0.05) (Figure 3).

4.3. Effects of V. herbacea extract on tumor cell adhesion, invasion, metastasis, and angiogenesis

As illustrated in Figure 4, the adhesion ability of V. herbacea extract-treated SKBR3 cells was significantly decreased, in comparison to normal cells (P>0.05). To measure the anti-invasion effects of V. herbacea extract on SKOV3 cells, the Matrigel Invasion assessment was conducted (Figure 5). Our results demonstrated that the invasion of V. herbacea extract-treated SKBR3 cells to the Matrigel-coated substrate was significantly reduced by 36%, in comparison to normal cells (P<0.05).

4.4. Expression of the apoptotic-related genes

The mRNA expressions of *Bcl*-2, *BAX*, and apoptosis-associated genes were further assessed using RT-PCR. As demonstrated in Figure 6A, the expression level of anti-apoptotic *Bcl-2* was significantly reduced 1.2-fold in V. herbacea extract-treated cells versus normal cells (P<0.05). SKBR3 cell line treated with V. herbacea extract increased the mRNA expression of the *Bcl-2* gene, in comparison to the untreated cells (P<0.05). Furthermore, as can be seen in Figure 6B, the expressions of *MMP2* and *MMP9* in V. herbacea extract-treated SKBR3 cells were significantly decreased 0.6- and 0.7-fold, respectively, compared to the normal cells (P<0.05). Additionally, RT-PCR analysis revealed that in V. herbacea extract-treated cells, mRNA expression of *VEGF-A*, *VEGFR-1*, and *VEGFR-2* (Figure 6C) was significantly decreased 0.7-, 0.5-, and 0.4-fold, respectively (P<0.05).

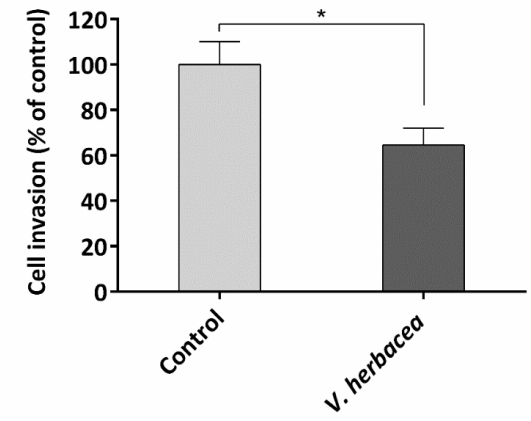

Figure 5. Anti-invasion effect of V. herbacea extract was higher on SKBR3 cell adhesion than on the control. A student t-test was applied to compare the percentage of cell adhesion between the two groups. (P < 0.05).

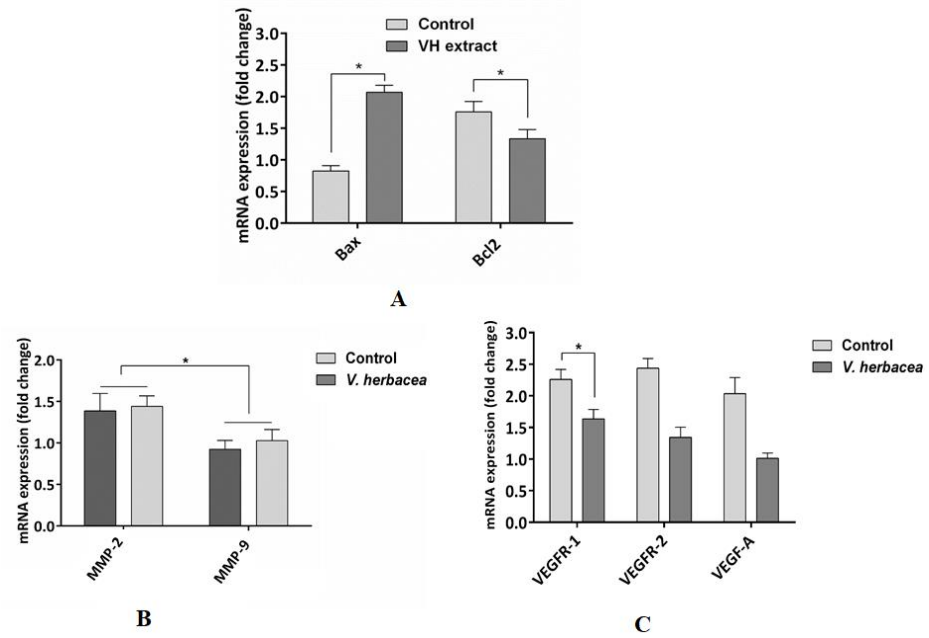

Figure 6. Comparison of mRNA expression as fold change of apoptosis-associated genes in SKBR3 cells
The gene expression was detected by RT-PCR. (A) There was a significant difference in the mRNA levels of BAX and Bcl-2 genes in the treated cells versus controls. (B) Moreover, MMP2 and MMP9 gene expressions and (C) VEGFR-1, VEGFR-2, and VEGFR-A showed a significant difference between the two groups. A student t-test was applied to compare the mean values of relative expression patterns between the two groups. *P < 0.05.

5. Discussion

The prevention and suppression of tumor metastasis and invasion are hopeful approaches to declining mortality in patients with advanced cancer. Medicinal plant compounds are the main sources of potential chemotherapeutic and antitumor agents. In the current study, SKBR3 was used for assessing the antiproliferative, antiangiogenic, and apoptotic properties of *V. herbacea*.

The MTT results showed that V. herbacea extract prevents cancer cell proliferation, and consequently, stimulates tumor cell death. In addition, V. herbacea extract showed moderate cytotoxic effects on normal cells, which is a proper characteristic for a chemotherapeutic drug. Subsequent experiments were performed at IC_{50} dose. Flow cytometry results revealed that V. herbacea extracts mediated outward translocation of phosphatidyl as a hallmark of apoptosis (22). In contrast to necrosis, immune cells, such as macrophages, remove the dead cells and inhibit adverse inflammatory responses in apoptosis, which causes damage to host cells and tissues (23). Moreover, cancer cells have several resistance mechanisms to apoptosis to maintain uncontrolled proliferation; therefore, the apoptotic effects of V. herbacea extract can be of interest as an anti-cancer agent (24).

Apoptosis occurs via extrinsic and intrinsic pathways, as discovered by the involvement of caspase-9 or caspase-8, respectively (25). Caspase-3 is involved in both pathways to eliminate apoptotic cells, while caspase-9, as the main trigger of the apoptotic cascade contributes to the intrinsic pathway (25). The results of a study by Gulcin et al. demonstrated the anti-cancer effects of V. herbacea extract; however, the apoptosis-inducing potential of V. herbacea extract was not clarified (26). The results of our study showed that V. herbacea extract induced caspase-3 and caspase-9 activities, which indicated the involvement of intrinsic caspase pathways. Moreover, overexpression of *BAX* and down-regulation of *Bcl-2* in V. herbacea extract-treated SKBR3 cells indicate that the intrinsic pathway is involved in apoptosis (27). Our results introduced V. herbacea extract as an effective compound in decreasing the survival of breast cancer cells via activating the intrinsic pathway of apoptosis.

Cell adhesion, proteolytic degradation, migration, and angiogenesis have been widely documented as indications of invasion and metastasis (28). Presently, the use of multiple anticancer agents, which inhibit several pathways of tumor cell invasion and metastasis, has become an interesting area of research (29). The results of our study demonstrated that V. herbacea extract effectively inhibited the adhesion and invasion of the SKBR3 cell, mainly by reducing the invaded cells to the Matrigel-coated substrate. The expression level of angiogenesis-related genes (i.e., *VEGF-A*, *VEGFR-1*, and *VEGFR-2*) was measured in V. herbacea extract-treated SKBR3 cells by RT-PCR. The results showed that the mRNA expressions of *VEGF-A*, *VEGFR-1*, and *VEGFR-2* genes

in V. herbacea extract-treated cells were significantly decreased 0.7-, 0.5-, and 0.4-fold, respectively, compared to the untreated cells. It has been shown that MMP inhibitors have important roles in interfering with tumorigenesis, migration, invasion, metastasis, and inhibition (30). Inhibition of angiogenesis is a critical step for cancer prevention and treatment since it inhibits tumor cell migration (31). RT-PCR results showed that V. herbacea extract reduces the expression of *VEGF-A*, *VEGFR-1*, and *VEGFR-2*, which can prevent tumor angiogenesis. (32). The binding of *VEGF-A* to *VEGFR-1* and *VEGFR-2* induces multiple signaling pathways causing cancer cell proliferation, migration, and survival (33). RT-PCR results showed that V. herbacea extract reduces the expression of *VEGF-A*, *VEGFR-1*, and *VEGFR-2*, which can prevent tumor angiogenesis. The results of the present study supported the idea that V. herbacea extract can inhibit invasion, angiogenesis, and metastasis of SKBR3 cells.

6. Conclusion

The robust cytostatic effect of V. herbacea extract is mediated by inhibiting the process of cellular apoptosis, adhesion/invasion, and angiogenesis. Our results suggested that V. herbacea extract could be used for the eventual development of an effective anti-cancer therapeutic agent aimed at reducing the human morbidity and mortality associated with breast cancer.

Acknowledgments

This paper was extracted from a PhD thesis at Islamic Azad University, Tehran, Iran. The authors would like to thank all the staff and faculty members who kindly supported us through this study.

Footnotes

Conflicts of Interest: The authors declare that there is no conflict of interest.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

References

1. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer*. 2015;**136**(5):359-86. doi: 10.1002/ijc.29210. [PubMed: 25220842].
2. Power EJ, Chin ML, Haq MM. Breast Cancer Incidence and Risk Reduction in the Hispanic Population. *Cureus*. 2018;**10**(2):1-12. doi: 10.7759/cureus.2235. [PubMed: 29713580].
3. Sadeghipour E, Sahragard N, Sayebani MR, Mahdizadeh. Breast cancer detection based on a hybrid approach of firefly algorithm and intelligent systems. *Indian J Fundam Appl Life Sci.* 2015;**5**(1):468-72.
4. Kashyap D, Pal D, Sharma R, Kumar Garg V, Goel N, Zaguia A, et al., Global Increase in Breast Cancer incidence: risk factors and preventive measures. *Biomed Res Int*. 2022;**2022**:1-16. doi: 10.1155/2022/9605439. [PubMed: 35480139].
5. Rafiemanesh H, Salehiniya H, Lotfi Z. Breast Cancer in Iranian Woman: incidence by age group, morphology and trends. *Asian Pac J Cancer Prev*. 2016;**17**(3):1393-7. doi: 10.7314/apjcp.2016.17.3.1393. [PubMed: 27039778].
6. Ghavami V, Mahmoudi M, Rahimi Foroushani A, Baghishani H, Homaei Shandiz F, Yaseri M. Long-term disease-free survival of non-metastatic Breast Cancer patients in Iran: a survival model with competing risks taking cure fraction and frailty into account. *Asian Pac J Cancer Prev*. 2017;**18**(10):2825-32. doi: 10.22034/APJCP.2017.18.10.2825. [PubMed: 29072428].
7. Zahmatkesh B, Keramat A, Alavi N, Khosravi A, Kousha A, Ghanbari Motlaghet A, et al., Breast cancer trend in Iran from 2000 to 2009 and prediction till 2020 using a trend analysis method. *Asian Pac J Cancer Prev.* 2016;**17**(3):1493-8. doi: 10.7314/apjcp.2016.17.3.1493. [PubMed: 27039796].
8. O'Shaughnessy J. Extending survival with chemotherapy in metastatic breast cancer. *oncologist*. 2005;**10**(3):20-9. doi: 10.1634/theoncologist.10-90003-20. [PubMed: 16368868].
9. Mansoori B, Mohammadi A, Davudian S, Shirjang S, Baradaran B. The different mechanisms of cancer drug resistance: a brief review. *Adv Pharm Bull.* 2017;**7**(3):339-48. doi: 10.15171/apb.2017.041. [PubMed: 29071215].
10. Force J, Henrique Santos Leal J, McArthur HL. Checkpoint blockade strategies in the treatment of breast cancer: where we are and where we are heading. *Curr Treat Options Oncol.* 2019;**20**(4):1-14. doi: 10.1007/s11864-019-0634-5.
11. Ponde N, Aftimos P, Piccart M. Antibody-drug conjugates in breast cancer: a comprehensive review. *Curr Treat Options Oncol.* 2019;**20**(5):1-22. doi: 10.1007/s11864-019-0633-6. [PubMed: 30931493].
12. Coombes C, Page K, Salari R, Hastings PK, Armstrong A, Ahmed S, et al. Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence. *Clin Cancer Res*. doi: 10.1158/1078-0432.CCR-18-3663. [PubMed: 30992300].
13. Ang E, Wewala N, Carroll R, Forgeson G, Anderson M, Fernando J, et al. Neoadjuvant chemotherapy in non-metastatic breast cancer a study on practice trends in a regional cancer treatment service. *Intern Med J.* 2020;**50**(3):315-21. doi: 10.1111/imj.14326. [PubMed: 30989751].
14. Zaid H, Silbermannet M, Amash A, Gincel D, Abdel-Sattar E, Boke Sarikahya N. Medicinal plants and natural active compounds for cancer chemoprevention/chemotherapy. *Evid Based Complement Alternat Med.* 2017:1-2. doi: 10.1155/2017/7952417.
15. Ognyanov I, Pyuskyulev B, Kompis I, Sticzay T, Spiteller G, Shamma M, et al. Alkaloids from Vinca herbacea W.K. X. The structures and stereochemistry of majdine and isomajdine. *Tetrahedron*. 1968;**24**(13):4641-8. doi: 10.1016/s0040-4020(01)98661-2. [PubMed: 5657073].
16. Vachnadze V, Vachnadze N, Gogitidze N, Mushkiashvili N, Mchedlidze K. Biologically active alkaloids from rhizomes with roots of vinca herbacea waldst. Et Kit, growing in Georgia. *Georgian Med News*. 2017;**271**:122-7. [PubMed: 29099714].
17. Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. *Cancer Res.* 1987;**47**(4):936-42. [PubMed: 3802100]
18. Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. *Leuk Lymphoma*. 2003;**44**(11): 1957-62. doi: 10.1080/1042819031000116607. [PubMed: 14738150].
19. Du GJ, Zhang Z, Wen XD, Yu C, Calway T, Yuan CS. Epigallocatechin Gallate (EGCG) is the most effective cancer chemopreventive polyphenol in green tea. *Nutrients*. 2012;**4**(11):1679-91. doi: 10.3390/nu4111679. [PubMed: 23201840].
20. Pereira FV, Ferreira-Guimarães CA, Paschoalin T, Scutti JAB, Melo FM, Silva LS, et al. A natural bacterial-derived product, the metalloprotease arazyme, inhibits metastatic murine melanoma by inducing MMP-8 cross-reactive antibodies. *Plos*

One. 2014;**9**(4):1-11. doi: 10.1371/journal.pone.0096141. [PubMed: 24788523].
21. Kumar S, Sharma VK, Yadav S, Dey S. Antiproliferative and apoptotic effects of black turtle bean extracts on human breast cancer cell line through extrinsic and intrinsic pathway. *Chemistry Central journal*. 2017;**11**(1):1-10. doi: 10.1371/journal.pone.0096141. [PubMed: 24788523].
22. Alotaibi MR, Hassan ZK, Al-Rejaie SS, Alshammari MA, Almutairi MM, Alhoshani AR, et al., Characterization of apoptosis in a breast cancer cell line after il-10 silencing. *Asian Pac J Cancer Prev.* 2018;**19**(3):777-83. doi: 10.22034/APJCP.2018.19.3.777. [PubMed: 29582634].
23. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495-516. doi: 10.1080/01926230701320337. [PubMed: 17562483].
24. Mohammad RM, Muqbil I, Lowe L, Yedjou C, Hsu HY, Lin LT, et al., Broad targeting of resistance to apoptosis in cancer. *Semin Cancer Biol*. 2015;**35**:78-103. doi: 10.1016/j.semcancer.2015.03.001. [PubMed: 25936818].
25. Pu X, Storr SJ, Zhang Y, Rakha EA, Green AR, Elliset IO, et al. Caspase-3 and caspase-8 expression in breast cancer: caspase-3 is associated with survival. *Apoptosis*. 2017;**22**(3):357-68. doi: 10.1007/s10495-016-1323-5. [PubMed: 27798717].
26. Gulcin I, Beydemir S, Topal F, Gagua N, Bakuridze A, Bayram R, et al. Apoptotic, antioxidant and antiradical effects of majdine and isomajdine from Vinca herbacea Waldst. and kit. *J Enzyme Inhib Med Chem.* 2012;**27**(4):587-94. doi: 10.3109/14756366.2011.604318. [PubMed: 21883037].
27. Findley HW, Gu L, Yeager AM, Zhou M. Expression and regulation of Bcl-2, Bcl-xl, and Bax correlate with p53 status and sensitivity to apoptosis in childhood acute lymphoblastic leukemia. *Blood*. 1997;**89**(8):2986-93. [PubMed: 9108419].
28. Bashyam MD. Understanding cancer metastasis: an urgent need for using differential gene expression analysis. *Cancer*. 2002;**94**(6):1821-9. doi: 10.1002/cncr.10362. [PubMed: 11920546].
29. D'Arrigo G, Navarro G, Di Meo C, Matricardi P, Torchilin V. Gellan gum nanohydrogel containing anti-inflammatory and anti-cancer drugs: a multi-drug delivery system for a combination therapy in cancer treatment. *Eur J Pharm Biopharm.* 2014;**87**(1):208-16. doi: 10.1016/j.ejpb.2013.11.001. [PubMed: 24215783].
30. Winer A, Adams S, Mignatti P. Matrix metalloproteinase inhibitors in cancer therapy: turning past failures into future successes. *Mol Cancer Ther.* 2018;**17**(6):1147-55. doi: 10.1158/1535-7163.MCT-17-0646. [PubMed: 29735645].
31. Lu J, Zhang K, Nam S, Anderson RA, Jove R, Wen W. Novel angiogenesis inhibitory activity in cinnamon extract blocks VEGFR2 kinase and downstream signaling. *Carcinogenesis*. 2010;**31**(3):481-8. doi: 10.1093/carcin/bgp292. [PubMed: 19969552].
32. Goel HL, Mercurio AM. VEGF targets the tumour cell. *Nat Rev Cancer.* 2013;**13**(12):871-82. doi: 10.1038/nrc3627. [PubMed: 24263190].
33. Abhinand CS, Raju R , Soumya SJ, Arya PS, Sudhakaran PR. VEGF-A/VEGFR2 signaling network in endothelial cells relevant to angiogenesis. *J Cell Commun Signal.* 2016; **10**(4):347-54. doi: 10.1007/s12079-016-0352-8. [PubMed: 27619687].