Original Article

In Vitro Cytotoxic Activity of *Verbascum alceoides* against Cervix Carcinoma Cells

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Abstract

Background: *Verbascum* species showed various pharmacological activities including anti-inflammatory, antitussive, antiulcerogenic, immunomodulatory, antimicrobial, antimalarial, antioxidant, and anticancer activities. **Objectives:** The aim of this work was to evaluate cytotoxicity of different fractions of *Verbascum alceoides*, which belongs to this genus. **Materials and Methods:** Aerial parts of this plant were collected from Doveiseh area in Kordestan province. The plant was extracted using a four-step extraction method with increasing solvent polarity (i.e., hexane, dichloromethane, chloroform-methanol [9:1], and methanol). The methanol extract was finally separated between water and butanol. Hexane, dichloromethane, chloroform-methanol, butanol, and aqueous partitions were then subjected to cytotoxicity evaluation. Showing the most potent cytotoxic effects, the butanolic partition was further fractionated by medium-performance liquid chromatography and similar eluates were pooled to prepare five final butanolic fractions, named A–E. **Results:** *In vitro* cytotoxicity of these fractions against human cervical epithelioid carcinoma (HeLa) and human umbilical vein endothelial cell (HUVEC) was evaluated using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide assay. Fractions D, E, and A showed a significant and dose-dependent inhibition of cell proliferation (half maximal inhibitory concentration [IC_{50}] of 30, 39.8, and 188.6 µg/mL, respectively). According to the preliminary thin-layer chromatography analysis, these cytotoxic effects may be mainly due to presence of saponin and flavonoid compounds. **Conclusion:** Future studies will be aimed to isolate and purify active constituents and investigate the effect of them on more different kinds of cancer cells.

Keywords: *Cytotoxic effect, human cervical epithelioid carcinoma (HeLa), human umbilical vein endothelial cell (HUVEC), Verbascum alceoides*

Introduction

Cancer is the second main cause of death in the world. In 2018, cancer death tolls have been estimated to be 9.6 million. Globally, approximately one sixth of demises are due to cancer, whereas approximately 70% of cancer death tolls happen in countries with low or average incomes.[1] Nowadays, cancer is one of the most lethal diseases and the biggest issue for public health in Iran, and it is the third cause of death after coronary heart diseases and accidents.[2] Although many different types of treatment have been introduced to cope with it, the most common types of cancer are still uncontrollable and costly to the patient and the society.[3]

Medicinal herbs and their derivatives are deemed to be increasingly effective as a supplementary treatment of cancer. Numerous clinical investigations have shown the effectiveness of herbal remedies in the improvement of lifestyle and immune system of the patient when the medicinal herbs are used in conjunction with the conventional treatments.[4] Herbs are one of the most effective medical resources for cancer treatment. Recent studies have shown that use of medicinal herbs has ceased the progress of many different types of cancers and compared to chemical treatment and radiotherapy, it has led to fewer side effects and longer life for patients. Herbal remedies are also generally less expensive and more available.[5] Nowadays, anticancer herbal medicines including *Vinca* alkaloids, etoposide and teniposide (derivatives of podophyllotoxin), taxol, and derivatives of camptothecin are widely used for clinical applications.[6]

Verbascum genus belongs to the family of Scrophulariaceae having 42 species in Iran, among which 14 are endemic.[7] This genus is the biggest genus of Scrophulariaceae family with total 2500 species in the world.[8]

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Members of *Verbascum* genus are specified by yellow flowers and thick fluffy leaves[9] and have been used as herbal medicines for centuries due to their biological active compounds such as flavonoids, saponins, phenylethanoids, neolignans, and iridoid and monoterpene glycosides.[10] Various pharmacological activities have been reported for different species of *Verbascum* including anti-inflammatory, antitussive, antiulcerogenic, immunomodulatory, antimicrobial, antimalarial, antioxidant, and anticancer activities.[11-15] Among the aforementioned *Verbascum* constituents, saponins are the most important natural compounds with anticancer effects[8] as almost all saponins induce apoptosis in tumor cells.[16]

Verbascum alceoides is native to Iran, and in western provinces, aerial parts and seeds of the plant are powdered or made into a decoction to use for numbing and confusing the fish during preying.[17] In spite of wide geographical distribution in western provinces, specially Kermanshah and Kurdistan provinces, there are only a few reports on the biological activities of this plant.[18] However, the aim of this study was bioassay guided fractionation and evaluation of cytotoxic activity of *V. alceoides*, against human cervical epithelioid carcinoma (HeLa) cell line.

Materials and Methods

General

Glass column of LiChroprep RP-18 (25–40μm) and silicon dioxide (SiO_2) plates used for thin-layer chromatography (TLC) were purchased from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), fetal bovine serum (FBS), and penicillin–streptomycin were purchased from Bioidea (Tehran, Iran). All other chemicals and analytical grade solvents were obtained from Merck (Darmstadt, Germany).

Plant material

Aerial parts of *V. alceoides* (locally named "Jaramasi") were collected in April 2018, from "Doveiseh" area, Kordestan province, Iran. The plant was identified by the botanist, Farahnaz Hushidari, and a voucher specimen (No. 2651) was deposited in the herbarium of School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.

Extraction and fractionation

After drying in the shade, aerial parts were grinded using a mill. The powdered plant sample (2000 g) was then extracted at room temperature, using a four-step extraction method with increasing solvent polarity using the following solvents: hexane, dichloromethane, chloroform-methanol (9:1), and methanol. Extraction was carried out using maceration method, performing each step four times with 6 L of solvent under occasional stirring. Methanolic extract was concentrated under vacuum and distributed between water and butanol solvents, using a separator funnel.

Hexane, dichloromethane, chloroform-methanol, butanol, and aqueous partitions were then concentrated completely under vacuum using a rotary evaporator (Heidolph, Schwabach, Germany) and then the remaining H_2O was removed at –40ºC for 24 h using a freeze drier (Snijders, Tilburg, Netherland) to make a fine powder [Table 1]. The final dried samples were subjected to cytotoxicity evaluation against HeLe and human umbilical vein endothelial cell (HUVEC) lines.

Showing the most potent cytotoxic effects, the butanolic partition was further fractionated by medium-performance liquid chromatography (MPLC) (Buchi Gradient System C-605 apparatus), using a glass column of LiChroprep RP-18 (25–40 μm) as the stationary phase and a gradient of water:methanol (100–0) as the mobile phase. Evaluating the elutes by TLC (SiO_2 plates with $BuOH:H_2O:CH_3COOH$ (60:25:15 v/v/v), using cerium sulfate in 2N H_2SO_4 as reagent for visualizing the spots, similar eluates were pooled to prepare five final butanolic fractions, named A–E.

Cell culture

HeLa and HUVEC were purchased from the Pasteur Institute of Iran, Tehran. HUVEC and HeLa cells were cultured in DMEM and RPMI, respectively. Both media were supplemented with 10% (v/v) FBS and antibiotic (100 IU/mL penicillin and 100 μg/mL streptomycin). The cells were kept at 37ºC and in a humidified atmosphere with 5% CO_2.

In vitro cytotoxicity assay

To evaluate cytotoxicity of four different partitions (dichloromethane, chloroform-methanol, butanol, and water) and five butanolic fractions (A–E) on HeLa and HUVEC cells, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was used as described previously.[19] Dimethyl sulfoxide (DMSO) was used to dissolve the dried fractions or partitions and diffident concentrations were prepared from stock solutions through dilution with phosphate buffered saline. To each well of 96-well plates, 180 μL of cell suspension (4×10^4 /mL) was added and incubated overnight. Next day, 20 μL of samples were added to each well (the final concentration of DMSO in the wells was <1%) and the plates were maintained in an incubator at 5% CO_2 and 37ºC for 72 h. Afterward, 20 μL of MTT solution (5 mg/mL) was added to each well and the plates were incubated at 37°C for next 3 h. Finally, the medium was replaced with 150 μL of DMSO to dissolve formazan crystals and the absorbance was measured

Table 1: Mass (g) of partitions recovered and yield (extracted/loaded) of extraction

	Mass recovered (g)	Extraction yield (%)
Hexane	17.3	0.69
Dichloromethane	16.9	1.64
Chloroform-methanol	14.2	0.676
Butanol	193.6	7.74
Water	92.9	0.8

at 570 nm by a microplate reader (PowerWave XS, BioTek Instruments, Winooski, Vermont). All experiments were performed in triplicates and the cell viability was determined using the following equation:

% Cell viability = (absorbance in treated wells – absorbance in blank well)/(absorbance in untreated well – absorbance in blank well).

Statistical analyses

All data are presented as mean ± standard deviation (SD) in triplicate experiments. Significant differences between groups were determined using analysis of variance (ANOVA) followed by a Tukey *post hoc* test (Statistical Package for the Social Sciences [SPSS] software program, version 16.0, Chicago, IL, USA). A value of $P < 0.05$ was regarded as a criterion for significant differences.

Results and Discussion

Verbascum alceoides is one of the Iranian medicinal plants, growing in West regions of the country and has been traditionally used for treatment of mycodermatitis.[10] There are also some reports about antioxidant activity of *V. alceoides*. Souri *et al.*[18] reported that its methanolic extract showed reasonable antioxidant activity comparable to α-tocopherol

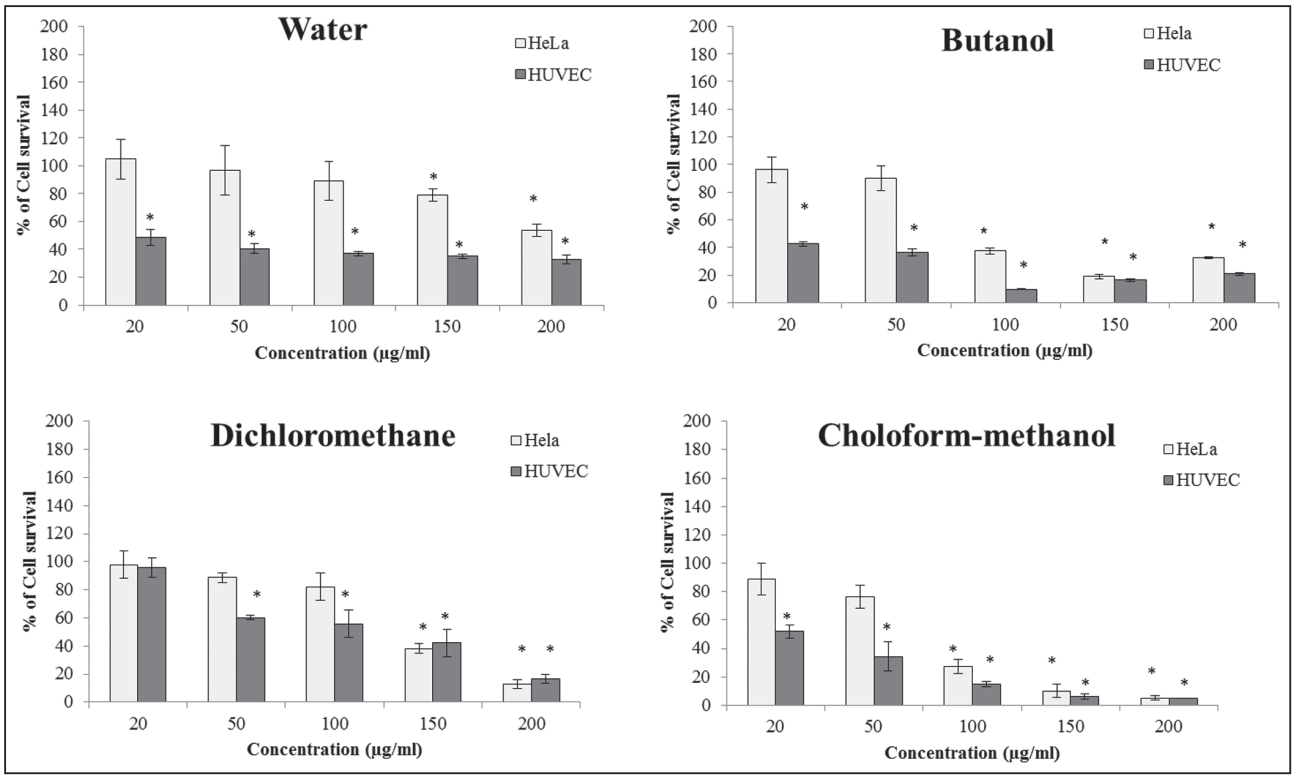

Figure 1: Evaluation of cytotoxicity of different partition of *Verbascum alceoides* against human cervical epithelioid carcinoma (HeLa) and human umbilical vein endothelial cell (HUVEC) using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (mean ± standard deviation, *n* = 4)

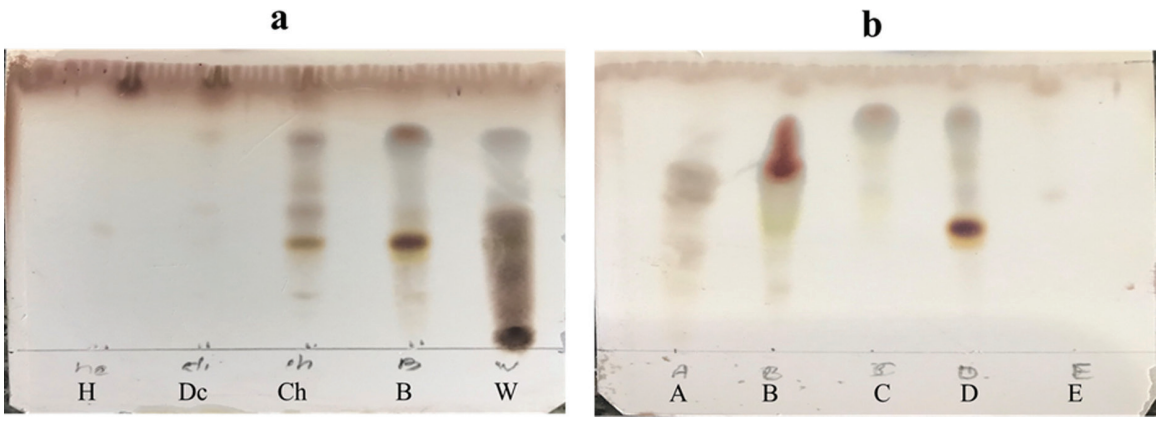

Figure 2: (A) Thin-layer chromatography (TLC) profile of partitions of *Verbascum alceoides*. H, Dc, Ch, B, and W represent hexane, dichloromethane, chloroform-methanol, butanol, and aqueous partitions, respectively. (B) TLC profile of fractions of butanolic partition of *V. alceoides*. Fraction D showed the well-known representative saponins

and concluded that presence of some natural antioxidants such as phenolic and polyphenolic compounds is responsible for this effect. However, to the best of our knowledge, there is no report on evaluation of cytotoxic activity of *V. alceoides*. Here, we first investigated the cytotoxic activity of dichloromethane, chloroform-methanol, butanol, and aqueous extracts of this plant on HeLa and HUVEC cells using MTT assay. As shown in Figure 1, polar and semipolar partitions (i.e., butanol and chloroform-methanol), which mainly consist of saponins and flavonoids, showed more cytotoxic effect. Similar to our findings, other previous studies explored that the polarity of solvent might play at cytotoxic effectiveness of extracts of *Verbascum*. Tatlı and Akdemir[20] compared cytotoxic effect of methanol and ethyl acetate extracts of some *Verbascum* from Turkey and reported the ethyl acetate extract (i.e., less polar) as the more potent cytotoxic against cancerous cell lines.

Fractionation of plant extracts facilities the isolation, purification, and characterization of novel phytochemicals. Through fractionation of the butanolic extract by MPLC, five fractions were obtained which were analyzed by TLC as

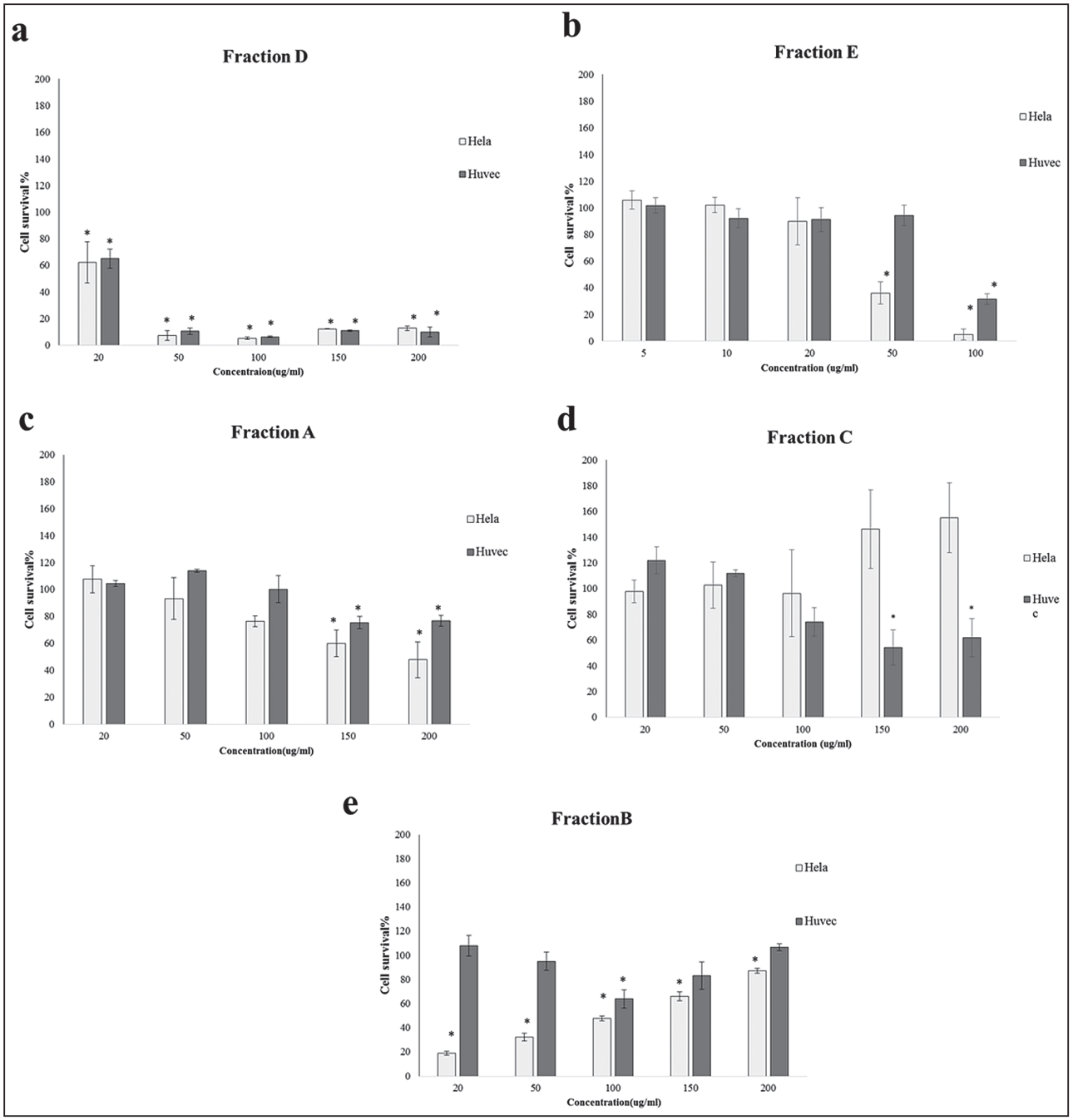

Figure 3: Evaluation of cytotoxicity of different butanolic fractions of *Verbascum alceoides* against human cervical epithelioid carcinoma (HeLa) and human umbilical vein endothelial cell (HUVEC) using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (mean ± standard deviation, *n* = 4)

presented in Figure 2 and their cytotoxic effects against HeLa and HUVEC cells were also evaluated. Fraction D showed the well-known representative saponins on TLC analysis. Cytotoxicity assay revealed that fraction D had the strongest inhibitory effect on viability of cells in a dose-dependent manner [Figure 3A]; however, its cytotoxic effect was not selective for cancer and normal cells [Table 2]. On the basis of our results, fraction E showed the most selective cytotoxic activity (selectivity index of 2.5) particularly at a range of concentration of 50–100 µg/mL [Figure 3B]. As shown in Figure 3C, fraction A also inhibited viability of HeLa cell line more than HUVEC cell line in a dose-dependent manner; however, the cytotoxic activity of fraction A was not strong as fraction E. Interestingly, fraction C showed an opposite effect on HeLa and HUVEC cell lines at high concentrations (≥100 µg/mL); the fraction inhibited the proliferation of HUVEC cell line, whereas it stimulated the growth of HeLa cell line [Figure 3D]. It could be concluded that cytotoxicity of this fraction is dependent to the type of cells and maybe different cellular mechanisms are responsible for this effect. In agreement with our results, another research group also reported cell-dependent effect of methanolic extract of *V. pterocalycinu* as it showed cytotoxic effect against melanoma cell line, whereas the extract was found to be inactive against ovarian cell line.[20] The growth pattern of HUVEC cells treated with fraction B was biphasic [Figure 3E]. A modest cytotoxic effect (approximately 64% cell viability) of the fraction was observed at approximately 100 µg/mL concentration, whereas cell viability increased when its concentration enhanced to 150–200 µg/mL. Similarly, previous studies reported biphasic stimulatory or inhibitory effect for other plant extracts.[11] For example, ethanolic extract of *Foeniculum vulgare* at very low concentrations stimulated cell proliferation of human mesenchymal stem cells, and at higher concentration reduced growth of the cells.[21]

On the contrary, faction B showed more cytotoxic effect against HeLa cell line at lower concentration [Figure 3E]. It can be suggested that at high concentration of the fraction some components, such as carbohydrates or growth factors, disturbed the cytotoxic activity of compounds such as flavonoids, saponins, and other related compounds and consequently decrease their activity. In agreement with our findings, Garcia-Varela *et al.*[22] evaluated cytotoxic effect of different extracts and solvent fractions of *Rhoeo discolor* on cancer cell lines and reported that extracts and their fractions at lower concentrations showed significant inhibitory effect on proliferation of cancer cells.

There are many studies on antitumor, anticancer, and cytotoxic activity of *Verbascum* species. Marian *et al.*[13] reported significant cytotoxicity of hydroalcoholic extract of *V. phlomoides* L. on melanoma cell line, which might be related to its flavonoid constituents. Küçük *et al.*[23] investigated cytotoxic effect of methanolic extract from three *Verbascum* species on HeLa and Skov-3 cell lines and proposed that saponins are mainly responsible for anticancer activity of *Verbascum* species. Another group evaluated *in vitro* inhibitory effect of aqueous extract of *V. thapsiforme* on protein biosynthesis using the ribosome fraction isolated from rat liver. They suggested that this inhibitory effect is mainly attributed to the saponin fraction.[24]

Klimek and Stepien[25] evaluated the effect of saponins, phenyethanoid, and flavonoid glycosides isolated from four *Verbascum* species growing in Europe on viability of rat spleen lymphocytes and reported that verbascosaponin, verbascoside, luteolin 7-O-glucoside, and forsythoside B showed significant antiproliferative effect. Furthermore, it was found that cytotoxic and cytostatic effects of glycosidic compounds such as verbascoside were dependent on the types of cells.[26]

In conclusion, the cytotoxic effects of different extracts of *V. alceoides* against two cell lines were investigated. The butanolic and chloroform-methanol extracts showed more cytotoxic activity. Five fractions of butanolic extract were also tested against HeLa and HUVEC cell lines and fractions D and E were found to be the most antiproliferative compounds. According to the preliminary TLC analyses, these cytotoxic effects may be mainly due to presence of saponin and flavonoid compounds. Future studies will be aimed to isolate and purify active constituents and investigate the effect of them on more

Table 2: Half maximal inhibitory concentration (IC_{50}) values (µg/mL) for different partitions/fractions of *Verbascum alceoides* against HeLa and HUVEC cells

Partitions/fractions	IC_{50} (µg/mL) HeLa	IC_{50} (µg/mL) HUVEC	Selectivity index
Butanol partition	82.5	11	0.13
Water partition	~200	~20	0.1
Dichloromethane partition	135	111.6	0.82
Chloroform-methanol partition	67.9	17.5	0.25
Fraction A	188.6	~200	–
Fraction B	99	~100	1
Fraction C	–	197.8	–
Fraction D	30	35	1
Fraction E	39.8	~100	2.5

IC_{50} = half maximal inhibitory concentration, HeLa = human cervical epithelioid carcinoma, HUVEC = human umbilical vein endothelial cell
Selectivity indices values were determined as the IC_{50} for HUVEC cell line was divided by the IC_{50} for HeLa cell

different kinds of cancer cells. However, more studies are also required to explore the exact mode of action of the active constituents.

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Conflicts of interest

There are no conflicts of interest.

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