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Research Article

Determination of cytotoxic, antioxidant activities and LC/MS-MS profiles of three endemic *Verbascum* L. species

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ABSTRACT

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated.

The cytotoxic effects of methanol extract of *V.detersile*, *V. eskisehirensis,* and *V.gypsicola* species on the cervical (HeLa) and ovarian cancer (SKOV-3) cells were investigated using a colorimetric assay. The results indicated that cytotoxic effect was not observed after treatment of SKOV-3 cells with *Verbascum*. On the other hand, the cytotoxic activity of *V. detersile* was found to be 0.1910 mg/dL and 1.057 mg/dL for HeLa cells after 24 or 48 hours incubation with *V.detersile*, respectively.

The antioxidant activity was determined as 1,1-diphenyl-2-picrylhydrazyl (DPPH•) radical scavenging activity, trolox equivalent antioxidant capacity (TEAC assay), and also the total phenolic content of the samples was found. Total phenols were estimated as Gallic acid equivalents (GAE), expressed as mg Gallic acid/in 1g extract previously described by Singletton. LC/MS profiles separation and detection of phytochemicals of the extract were performed on a Shimadzu UPLC system consisting of a vacuum degasser, an autosampler (SIL20A Shimadzu Autosampler), a binary pump (LC20AD Shimadzu), an oven (CTO20A Shimadzu Column Oven) and DAD dedector (SPD M20A Shimadzu DAD Detector). In terms of antioxidant activity, *V. gypsicola* was found to have the least antioxidant activity among the three extracts, which is also correlated with the total amount of phenolic content in its content. In this way, it differs from other species. *V. detersile* exhibits a different chemical profile from the other two species with the iridoid catalpol derivatives it contains. Apigenin pentoside is the only flavonoid molecule detected in *V. detersille*.

Keywords: Antioxidant, LC/MS-MS, cytotoxicity, Scrophulariaceae, *Verbascum*

1. INTRODUCTION

The *Verbascum* L. (Scrophulariaceae) species are represented in the world with nearly 360 species [1]. In Turkey, the *Verbascum* genus consists of approximately 255 species, 200 of which are endemic (about 80% endemism rate) and it has 130 additional hybrid species [2].

Verbascum species are a popular herb with medicinal uses. In traditional Turkish folk medicine, these medicinal plants are used in the treatment of expectorant, stomachache, stomach ulcer, diabetes, hemorrhoid, rheumatism, and urinary tract infection [3-7]. It has been reported that they are used as respiratory disorders, expectorant, stomach tonic, dyspepsia, diarrhea, diuretic, snake bites, blood clotting of women after childbirth, wound disinfection, and sedative in Iran [8-11]. In addition, the Herbal Medicinal Products Committee (HMPC) reported that Mullein flowers (*Verbascum phlomoides*, *V. thapsus,* and *V. densiflorum*) can be used to soothe the throat in colds and dry cough [12].

In some studies on *Verbascum* has been found to have an antiviral [13], antibacterial [14], enzyme inhibitory activity [15], and wound healing properties [16]. It has also been reported that *V. pycnostachyum* species has a substantial cytotoxic effect against cervical (HeLa) and ovarian cancer (Skov-3) cell lines [17].

It is thought that free radicals may be effective in the formation mechanism of some common diseases and antioxidants prevent cellular damage by preventing these harmful effects [18]. Herbal secondary metabolites such as phenolics have a scavenging effect and so antioxidant activity against these harmful effects of free radicals. [19]. There are studies in the literature proving that some *Verbascum* contains phenolics and has an antioxidant effect [20-22, 29,30].

These and similar studies show that the Verbascum genus has antioxidant properties. However, the results of these species may vary depending on the extra batches, the individual in which they were grown, and the processing methods. Additionally, health effects related to antioxidants require further research.

In Turkey, *Verbascum detersile* Boisse & Heldr., *V. gypsicola* Vural & Aydoğdu and *V. eskisehirensis* Karavel., Ocak & Ekici known as "Zinemit", "Mermer sığırkuyruğu" and "Eski sığırkuyruğu" respectively [23]. In the present study, three endemic *Verbascum* were evaluated for their cytotoxic, antioxidant and LC/MS profiles.

2. MATERIALS AND METHODS

2.1. Plant materials

Information on the localities of three species is given in Table 1.

2.2. Preparation of Extracts

The dried air parts of *V. detersile*, *V. gypsicola,* and *V. eskisehirensis* species were macerated 3 times with 70% ethanol. The dry extracts were kept at +4°C after evaporation and lyophilization.

2.3. Cytotoxicity

2.3.1. Cell Culture and reagents

The human cervical adenocarcinoma (HeLa) and human ovarian adenocarcinoma (SKOV-3) cells were obtained from American Type Culture Collection (ATCC). HeLa cells were maintained in Eagle's Minimum Essential Medium (EMEM) (Sigma-Aldrich, UK) supplemented with 20% Fetal Bovine Serum (FBS) (Gibco, UK), 1% penicillin-streptomycin, and 4% sodium bicarbonate as adherent monolayers. SKOV-3 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, UK) supplemented with 10% FBS and 1 % penicillin-streptomycin. The cell lines were routinely subcultured using 0,25 % trypsin-EDTA solution (Sigma-Aldrich, UK). Exponentially

Table 1. Locations of the studied *Verbascum* species

***Verbascum* Species**	**Locations**
V. detersile	B3: Eskişehir 05.07.2019 (ESSE 15614)
V. gypsicola	B4: Ankara 02.07.2019 (ESSE 15615)
V. eskisehirensis	B3: Eskişehir 01.06.2019 (ESSE 15616)

growing cultures were maintained in an incubator with a humidified atmosphere with 5% CO2/95 % air at 37°C.

2.3.2. Cytotoxicity assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is a non-radioactive assay and measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The reduction of MTT can only occur in metabolically active cells. The assay was performed as mentioned in Mosmann [24].

SKOV-3 and HeLa cells, which reached the appropriate density in flasks, were seeded in 96-well plates with $5x10^3$ cells in each well and incubated for 24 or 48 hours. After incubation, various concentrations (0.1; 0.2; 0.6; 1; 1.5; 2; 2.5; 3; 3.5 mg/dL) of *V. detersile*, *V. eskişehirensis* and *V. gypsicola* substances were added to the wells in 4 repetitions. After 24 and 48 hours, 20 µL of MTT dye (stock concentration of 5 mg/mL) was added to each well and then the wells were incubated for 3 hours. After 3 hours, the medium and MTT dye was completely withdrawn from the wells, 100 µL of DMSO was added to each well to dissolve the formazan crystals, and the plates were left in the shaker for 15 minutes. Spectrophotometric measurements of the plates taken from the shaker were performed at a wavelength of 540 nm in a Bio-Tek (ELx800) plate reader. The signal generated is directly proportional to the number of viable (metabolically active) cells in the well. Viability (%) is calculated using the following formula; Viability (%) = (Absorbance of the treated cells) / (Absorbance of the control wells) x 100.

2.3.3. Statistical analysis

The results were obtained from the MTT assay were expressed as mean ± SD. The significant differences were indicated as $p<0.05$ using one-way ANOVA. GraphPad Prism 7 software was used information of graphics.

2.4. Antioxidant activity

2.4.1. 1,1-Diphenyl-2-picrylhydrazyl ($DPPH^{\cdot}$) radical scavenging activity

In this analysis free radical scavenging activity was measured using the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) method. Serial dilutions were prepared with half the concentrations of the previous one, resulting in stock stock solutions (4 mg / ml). DPPH (the same amounts) were added to the diluted solutions and the UV absorbance at 517 nm was measured after 30 minutes. The experiment, extract, and positive standard control were made in triplicate for BHT (Butile Hydroxytoluene). The averages of the absorbances were recorded for each concentration. The percentage of prevention was calculated via Equation 1. The IC_{50} value, which is the concentration of the test material inhibiting 50% of the free radical concentration, was determined as mg / mL using the Sigma Plot statistical program. Microplate dilution method of Kumarasamy was used for the assay [25].

2.4.2. Trolox equivalent antioxidant capacity (TEAC assay)

TEAC assay was performed as in our previous *Verbascum* publication [26]. This assay assesses the capacity of a compound to scavenge the stable ABTS radical in comparison tothe antioxidant activity of Trolox, a water-soluble form of vitamin E that is used as a standard. The blue-greenABTS was produced through the reaction of 7 mM ABTS with 2.5 mM sodium persulfate ($Na_2S_2O_8$) (final concentrations) in the dark at room temperature for 12-16 h before use. The concentrated ABTS solution was diluted with ethanol to a final absorbance of 0.8-0.7 at 734 nm. A 10 µl portion of extract was added to 990 µl of ABTS solution, and the reduction in absorbance was measured 1 min after addition of Trolox (final concentration 1-20 µM) and up to 40 min after addition of the extract. The stock solution of Trolox (2.5 mM) was prepared in ethanol. Absorbance was measured at 734 nm.

2.4.3. Quantitative Determination of the Total Phenolic Contents

Total phenols were estimated as Gallic acid equivalents (GAE), expressed as mg Gallic acid/in 1g extract previously described by Singletton [27]. The stock solutions of the extracts and gallic acid were prepared in methanol. In experiment, 20 µL of the sample (extract/ gallic acid), 1560 µL of ultrapure water and 100 µL FCR were mixed in the 96-deep wells by using 12-channel micropipetor. After 8 min incubation, 300 µL of sodium carbonate solution (20%) was added to the mixture and mixed again. The mixture was incubated during 2 h at 25°C in a dark. Then, 300 mL of the mixture was transferred into 96-well microplate and the absorbance values were measured at 760 nm.

2.5. LC/MS Profiles

LC-MS/MS analyses were carried out using a similar method as the previous study (26), with a slight modification, using Methanol as the organic solvent in gradient elution.

3. RESULTS AND DISCUSSION

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated.

3.1. Cytotoxicity Assay

The cytotoxic effects of all extracts were also examined on HeLa and SKOV-3 cancer cell lines. No effect was observed as a result of the treatment of *V. detersile* (after 24 or 48 hours), *V. eskisehirensis* (after 24 or 48 hours), and *V.gypsicola* (after 48 hours) with SKOV-3 cells. Therefore, statistical analyzes could not be performed. IC_{50} value could not be calculated since cell proliferation occurs in the 24 hour activation of the *V. gypsicola* extract (Figure 1; Table 2). Cytotoxic activity of *V. detersile* was found to be 0.1910 mg/dL and 1.057 mg/dL, respectively, for HeLa cells after 24 and 48 hours of dosing (Fig. 2).

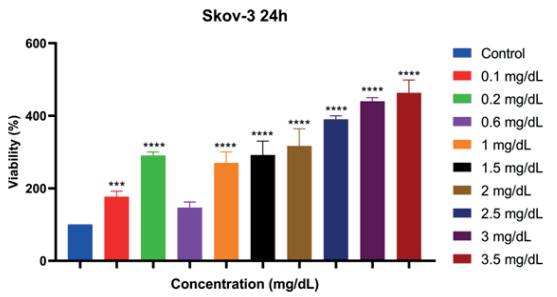

Figure 1. Cytotoxic activity of *V. gypsicola* in SKOV-3 cell lines at 24 h. Each data point is the average of three independent wells. Bars indicate mean ± standard deviation. All comparisons were made relative to untreated control cells (100% cell-viability).

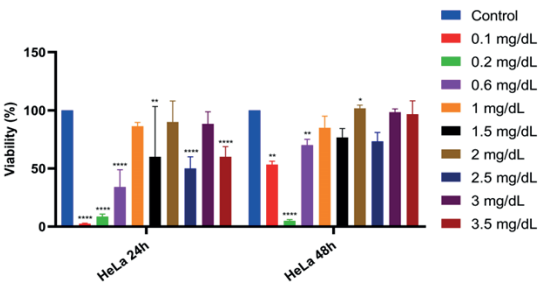

Figure 2. Cytotoxic activity of *V.detersile* in HeLa cell lines after 24 or 48 hours treatment. Each data point is the average of three independent wells. Bars show ± standard deviation. All comparisons were made against untreated control cells (100% cell viability). [based on control, * p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001].

Table 2. Cytotoxic activities (IC_{50}, mg/dL) of *V.detersile, V. eskisehirensis, V.gypsicola*

***Verbascum* Species**	**HeLa**		**SKOV-3**	
	24 h	**48 h**	**24 h**	**48 h**
V.detersile	0.1910	1.057	No effect	No effect
V. eskisehirensis	No effect	No effect	No effect	No effect
V.gypsicola	No effect	No effect	Proliferation	No effect

Table 3. Antioxidant activities of extracts of *Verbascum* species

	DPPH (IC_{50}, Mili molar)	TEAC (mM)	Total Phenolic Contents mg GAE / g extract
V. detersile	0.16	1.96	506.7
V. gypsicola	0.27	0.67	279.1
V. eskisehirensis	0.16	1.17	338.1
Gallic acid	0.002		

Table 4. Extracts composition of *Verbascum* species

Rt	m/z $[M-H]^-$	Fragments	Identification	Extract	Reference
12.4	401	269, 161	Apigenin pentoside	D	[28]
15.2	495	311, 209, 167	Unknown	G	
16.8	653	491, 377, 309,291, 187, 163, 145	p-Coumaroyl 6-O rhamnosylcatalpol	D	[28]
16.8	507	307, 145	Unknown	G	
17.6	623	461, 161, 135	Verbascoside	D,G	[28]
19.2	579	447, 285,151	Luteolin pentosyl-glucoside	E	[26]
20.6	447	285	Luteolin glucoside	E	[26]
20.9	695	533, 419, 333, 163, 145	p-Coumaroyl acetyl 6-O-rhamnosylcatalpol	D	[28]
21.3	461	369, 327, 285	Luteolin glucuronide	E	[26]
21.6	563	269	Apigenin pentosyl-glucoside	E	[26]
22.8	431	311, 269	Apigenin glucoside	E	[26]
23.4	683	637,445, 361	Unknown	D	
23.4	607	300, 284	Unknown	G	
23.5	461	445, 313, 297, 283,269, 255	Homoplantaginin	E	[29]
27.7	725	679, 487, 403, 311, 215, 163, 151, 147	Unknown	D	
28.7	285	175, 133	Luteolin	G	[26]

D: *V. detersile,* E: *V. eskisehirensis,* G: *V. gypsicola*; Rt: Retention time; m/z: mass-to-charge ratio

3.2. Cytotoxic Effects of Extracts

In cytotoxic studies, it was determined that none of the extracts showed any effect when treated with SKOV-3 cells, whereas only *V. detersile* extract was effective on HeLa cells. No previous cytotoxicity study has been found for this species. However, Küçük et al. conducted a study in 2016 with three different *Verbascum* sp. against HeLa and SKOV-3 cell types. They found that all *Verbascum* sp. extracts dose-dependently reduced cell viability in both HeLa and SKOV-3 cell types [17].

3.3. Antioxidant Activity

In the, the DPPH scavenging effect of the MeOH extract of *V. detersile* collected from Antalya has been reported as IC_{50}: 27 µg/mL [20].

The MeOH extract of *V. eskisehirensis* was reported as DPPH (IC_{50} 176.7 µg/mL) and TEAC assay (0.184 ± 0.08 mM) [26].

Various methods were used to evaluate the antioxidant activity.

According to the total phenolic content results, *V. detersile* was found to have the highest phenolic content among the three species studied. High phenolic content of *V. detersile* provided better results in antioxidant test systems than other extracts. Although the *V. detersile* and *V. eskisehirensis* activity results were the same in the DPPH radical scavenging effect and TEAC experiments, *V. detersile* showed a remarkable effect with antioxidant activity equivalent to approximately 2mM Trolox. In the DPPH radical scavenging effect, no extract was found as effective as the positive control gallic acid. In terms of antioxidant activity, *V. gypsicola* was found to have the least antioxidant activity among the three extracts, which is also correlated with the total amount of phenolic content in its content (Table 3).

In the LC-MS/MS analysis, it was determined that *V. eskisehirensis* was rich in luteolin derivatives and also contained apigenin derivatives. Among the three *Verbascum* species analyzed, *V. eskisehirensis* was the only species that did not contain verbascoside. In this way, it differs from other species. *V. detersile* exhibits a different chemical profile from the other two species with the iridoid catalpol derivatives it contains. Apigenin pentoside is the only flavonoid molecule detected in *V. detersille* (Table 4).

4. CONCLUSION

The *Verbascum* genus includes many species used in folk medicine or the treatment of various diseases. In this study, the cytotoxic, antioxidant activity, and LC/MS-MS profiles of three *Verbascum* species, which are endemic in Eskişehir and its surroundings, were investigated. The findings of this study provide useful information for breeding strategies, and for choosing the best species with high phenolic compound content to produce natural antioxidants for medical and pharmaceutical use. Pronounced antioxidant and rich bioactive compositions determined in this study reveal that *Verbascum* extracts might be a good source for natural health attributing sources.

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Ethical approval

Not applicable, because this article does not contain any studies with human or animal subjects.

Author contribution

Concept: SK, MS; Design: SK, MS; Supervision: SK, MS; Materials: SK, MS; Data Collection and/or Processing: SK, MS, FG, FÖ, ZS; Analysis and/or Interpretation: SK, MS, FG, FÖ, ZS; Literature Search: SK, MS, FG, FÖ, ZS; Writing: SK, MS, FG, FÖ, ZS; Critical Reviews: SK, MS, FG, FÖ, ZS.

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Conflict of interest

The authors declared that there is no conflict of interest.

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