Determination of Cytotoxic and Anticandidal Activities of Three *Verbascum* L. Species from Turkey: *V. cheiranthifolium* Boiss. var. *asperulum* (Boiss.) Murb. Monorg., *V. pycnostachyum* Boiss. & Heldr and *V. orgyale* Boiss. & Heldr

Turkey’s Three *Verbascum* L. Species’ Cytotoxic and Anticandidal Activities Were Determined; *Verbascum cheiranthifolium* Boiss. var. *Asperulum* (Boiss.) Murb. Monorg., *Verbascum pycnostachyum* Boiss. & Heldr and *Verbascum orgyale* Boiss. & Heldr

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**ABSTRACT**

Purpose of this study is to determine cytotoxic and anticandidal activities of *Verbascum cheiranthifolium* Boiss. var. *asperulum* (Boiss.) Murb. Monorg., *Verbascum pycnostachyum* Boiss. & Heldr and *Verbascum orgyale* Boiss. & Heldr belonging to *Verbascum* genus growing in Turkey. The cytotoxic effects of methanolic extract of *Verbascum cheiranthifolium* var. *asperulum*, *V. pycnostachyum* and *V. orgyale* species on the cervical (HeLa) and ovarian cancer (Skov-3) cells were investigated using colorimetric assay. The results indicated that methanolic-extract of *V. pycnostachyum* had a promising toxic effect on both cell lines as compared to the other species. Furthermore, this effect was more significant on Skov-3 cells rather than HeLa cells. Anticandidal effects of the methanolic extracts were evaluated in comparison with standard antifungal agents according to Clinical Laboratory Standards Institute (CLSI) reference methods, for the first time here. *V. pycnostachyum* and *V. orgyale* extracts were demonstrated stronger inhibitory effects than the *V. cheriantifolium* var. *asperulum*. Remarkably, Candida krusei was inhibited by *V. pycnostachyum* extract at the concentration of the 62.5 µg/mL.

**Key words:** Scrophulariaceae, *Verbascum*, Cytotoxicity, Anticandidal activities

**ÖZ**

Bu çalışmada Türkiye’de yetişen *Verbascum* L. cinsine ait üç türün; *Verbascum cheiranthifolium* Boiss. var. *Asperulum* (Boiss.) Murb. Monorg., *Verbascum pycnostachyum* Boiss. & Heldr ve *Verbascum orgyale* Boiss. & Heldr. türlerinin sitotoksik ve antikandidal aktivitelerinin belirlenmesi amaçlanmıştır. *Verbascum cheiranthifolium* var. *asperulum*, *V. pycnostachyum* ve *V. orgyale* türlerinin metanol ekstrilerinin sitotoksik etkileri servikal (HeLa) ve owaryum kanser (Skov-3) hücrelerinde kolorimetrik metod kullanılarak araştırılmıştır. Elde edilen sonuçlar; *V. pycnostachyum* türünün metanol ekstresi diğer türlerle oranla daha yüksek verici toksik etkiye sahip olduğu gösterilmiştir. Buna ek olarak; bu etki Skov-3 hücrelerinde HeLa hücrelerine kıyasla daha anlamlıdır. Üç tür ait metanol ekstresinin antikandidal etkileri “Klinik Laboratuvar Standartları Enstitüsü” (CLSI)’nin mikrodilüsyon standart protokolleri kullanılarak standart antifungal ajanlarla karşılaştırılmış olup, bu çalışma ile ortaya konmuştur. *V. pycnostachyum* ve *V. orgyale* ekstrleri *V. cheriantifolium*’a göre daha kuvvetli inhibitor etkiler göstermiştir. *Verbascum pycnostachyum* ekstresi dikkat çekici olarak *Candida krusei*’yi, 62.5 g/mL konsantrasyonda inhibe etmiştir.

**Anahtar kelimeler:** Scrophulariaceae, *Verbascum*, Sitotoksisite, Antikandidal aktivite

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INTRODUCTION

*Verbascum* L. (1753: 177) (Scrophulariaceae) includes about 360 species throughout the world (1). In Turkey, with the additional 130 hybrids, the genus is represented by 246 species, 6 imperfectly known or doubtful records (2-5). The endemic ratio (80%) of the genus is very high with 196 endemic species (4,5).

In Turkey, the species *V. cheiranthifolium* var. *asperulum*, *V. pycnostachyum* and *V. orgyale* known as “Bozkulak”, “Eğirdir diğer kuyruğu” and “Şöke diğer kuyruğu” respectively (2,4).

Many plant species among the flora of Turkey play an important role in traditional medicine. There are approximately 9000 plant species, some of them are widely used in folkloric medicine due to their antimicrobial and anticarcinogenic properties, in Turkish flora (6,7). One of the well-known *Verbascum* species is *V. thapsus* L., which has been used for the treatment of several diseases including asthma, spasmodic cough, migraine and earache. Moreover, *V. thapsus*, *V. fruticulosum* *V. undulatum* and *V. georgicum* had anti-malarial and antiviral effects that were investigated by both in vitro and in vivo studies (6).

It is reported that leaves and flowers of *Verbascum* species have expectorant, mucolytic and demulcent properties, and they are used to treat respiratory disorders such as bronchitis, dry coughs, tuberculosis, asthma in Anatolia (8,9). *Verbascum* species are also used to treat hemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhea. Furthermore these species demonstrate several inhibitory activities against the murine lymphocytic leukemia and influenza viruses A2 and B. Macerated oil prepared from the flowers is used for reducing earache, applied externally for eczema and other types of inflammatory skin disorders (10).

*Verbascum* species have some folkloric usages such as sedative and treatment of dysmenorrhea and rheumatalgia. It was also notified the usage for healing wounds in animal care.

Iridoid and neolignan type glycosides, oleanene type terpenes, flavonoids, polysaccharides, saponins, steroids and alkaloids were major compounds isolated from *Verbascum* species (11). In several bioactivity studies on *Verbascum* sp. reported that crude extracts of roots, leaves, flowers and aerial parts have been shown anti-proliferative (12), anti-inflammatory (13), antioxidant (14,15), anti-histaminic, anti-fungal, anti-bacterial (16), wound healing (17), anti-microbial (18) and anti-cancer effects (19).

In the present study, three species belonging to *Verbascum* genus, were evaluated for their cytotoxic (on cervical and ovarian cancer cell lines) and anticandidal effects for the first time.

EXPERIMENTAL

Plant materials
The plant materials were collected from following localities: *Verbascum cheiranthifolium* var. *asperulum* B3 Eskişehir, Bozdağ region, 18.6.2014, 39° 53' 24'' K- 030° 33' 16'' D, 1267 m, (ESSE:14686); *Verbascum pycnostachyum* C3: Antalya, Korkuteli-Fethiye region, 37° 02' 53'' N, 30° 06' 26'', 1370 m, 20.06.2007, ESSE 14730 (AKDU 6093) and *Verbascum orgyale* C3:Antalya: Antalya-Geyikbayırı region, 36° 52' 41'' N - 30° 26' 37'' E, 1008 m, 15.07.2007, (ESSE 14622, AKDU 6064) in Turkey. Voucher specimens are deposited in the Herbarium of the Faculty of Pharmacy (ESSE), Anadolu University in Eskişehir and Herbarium of the Biology Department, Akdeniz University in Antalya, Turkey (AKDU).

Extraction
Air dried plant materials were macerated with 70% MeOH (MERCK) at 25°C for 24h on orbital shaker. After evaporation and lyophilization steps the dry extract was kept at +4°C until bioactivity studies.

Cell culture
The human cervical adenocarcinoma cells (HeLa) 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. The human ovarian adenocarcinoma cells (Skov-3) 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% tripsin-EDTA solution (Sigma-Aldrich, UK).

Stock solution of extract of *Verbascum* sp. were prepared in sterile ddH2O and that was diluted in culture medium to prepare final concentrations of extracts. The cells were incubated with each *Verbascum* sp. (0,1-3 mg/mL) for 24 hours at 37°C (20).

Cell viability 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 Mossman. HeLa and Skov-3 cells (2 × 10⁴) were seeded in 96-well plates in the presence and absence of different concentrations of *Verbascum* sp. for 24 hours at 37°C in a 5% CO₂/95% air atmosphere. After incubation time, 20 ml of MTT (5 mg/mL) was added to each well and the cells were incubated for a further 2 hours. The reduction of MTT was measured by ELISA (ELX 808 IU) reader at a wavelength of 540 nm.

Viability (%)=(Absorbance of the treated cells) / (Absorbance of the control wells) ×100. Each concentrations was tested in two different experiments run in triplicate.

Sevim KÜÇÜK, Filiz ÖZDEMİR, Gökalp İŞCAN, Zerrin İNCESU 319
Anticandidal activity

Anticandidal activities of the methanolic extracts were evaluated by partly modified reference method of Clinical and Laboratory Standards Institute (CLSI) M27-A2 (21). *Candida albicans* ATCC 90028, *C. utilis* NRRL Y-900, *C. glabrata* ATCC 66032, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as pathogenic test microorganisms. Stock cultures stored in 50% glycerol at -85°C, were inoculated in Mueller Hinton Agar (Acumedia) plates and incubated at 37°C for 24 h for checking purity and viability. After incubation, selected colonies were suspended in 0.85% NaCl solution and adjusted to McFarland No: 0.5. Serial dilutions of the extracts were prepared in range of 4000 to 7 µg/mL. After incubation at 37°C for 24h, MIC values was determined by visual reading of wells without growing. Amphotericin B (Sigma) and Ketoconazole (Sigma) were used as standard antifungal agents.

![Figure 1](image1.png)

Verbascum pycnostachyum (A)

![Figure 2](image2.png)

Verbascum pycnostachyum (A)

![Figure 3](image3.png)

Verbascum cheiranthifolium var. asperulum (B)

![Figure 4](image4.png)

Verbascum cheiranthifolium var. asperulum (B)

![Figure 5](image5.png)

Verbascum orgyale (C)

![Figure 6](image6.png)

Verbascum orgyale (C)

**Figure 1.** Treatment of either *V. pycnostachyum*, *V. cheiranthifolium* var. *asperulum* or *V. orgyale* extracts with HeLa cells decreased the cell viability in a dose-dependent manner. Bars indicate mean ± standard deviation. All comparisons were made relative to untreated control cells. The significant differences were indicated as p<0.05 using one-way ANOVA. The graphic was created by using GraphPad Prism 6 software. [*p<0.01; **p<0.001; ****p<0.0001*].

**Figure 2.** The percentage of cell viability after treating Skov-3 cells with either *V. pycnostachyum*, *V. cheiranthifolium* var. *asperulum* or *V. orgyale* methanolic-extract. Bars indicate mean ± standard deviation. All comparisons were made relative to untreated control cells. The significant differences were indicated as p<0.05 using one-way ANOVA. The graphic was created by using GraphPad Prism 6 software. [*p<0.01; **p<0.001; ****p<0.0001*].
RESULTS AND DISCUSSION

Cytotoxicity results

The effects of V. pynostachyum, V. cheiranthifolium var. asperulum and V. orygale methanol-extracts were assessed on HeLa (Figure 1) and Skov-3 (Figure 2) cells after 24 hours incubation with each extract using the MTT assay. The results obtained here indicated that all Verbascum sp. reduced the cell viability of both HeLa and Skov-3 cells in a dose-dependent manner. Particularly, the cell viability of both cell lines was significantly declined after treatment of V. pynostachyum extract as compared to other Verbascum sp. that cytotoxic effect was observed at lower concentration (0.5 mg/mL - 44.62% cell viability) on Skov-3 cells rather than HeLa cells (0.5 mg/mL - 71.54% cell viability).

V. orygale methanol-extract was shown a similar effect on both cell lines; HeLa (1 mg/mL - 30.96% cell viability) (Figure 1C) and Skov-3 (1 mg/mL - 34.22% cell viability) (Figure 2C). On the other hand, a dramatic decrease in cell viability for HeLa was observed after incubation of 0.93 mg/mL V. cheiranthifolium var. asperulum methanol-extract (Figure 1B) as compared to the cell viability rate of Skov-3 cells treated with 2.01 mg/mL extract (Figure 2B).

The studies about the isolation of bioactive compounds have been reported that flavonoids, saponins, phenylpropanoid (12) and the phenylethanoid glycosides (22) were isolated although the type of bioactive compounds varies depending on the various Verbascum sp. Specifically, the isolation works on methanolic-extract and structure elucidation studies of V. pynostachyum were shown that it contained iridoids-glycosides, aukubin, ajugol, ajugosid, harpagoside, phenylethanoid glycoside and verbascoside (10). It has been reported that verbascoside has a hydrophilic character (19) and saponins (23) to possess anti-cancer and antimicrobial activity.

In this study, particularly V. pynostachyum species having a significant cytotoxic effect on Skov-3 cells that might be caused by the compounds such as verbascoside. However, in order to explain the relationship between activity-structure, it is necessary to determine the content of bioactive compounds of V. pynostachyum methanolic-extract.

Anticandidal activity results

Anticandidal activities of the methanolic extracts of V. cheiranthifolium var. asperulum, V. orygale and V. pynostachyum were evaluated by using CLSI M27-A2 reference method. Tested Candida species were moderately inhibited by the extracts between the concentrations of the 62.5-4000 µg/mL (minimal inhibitory concentration). Remarkably, V. pynostachyum showed strong effects on Candida krusei having a MIC value of 62.5 µg/mL. V. orygale and V. pynostachyum demonstrated better effects than Verbascum cheiranthifolium var. asperulum against all tested Candida strains. All extracts were assumed to have the MIC values outside of the tested range against Candida glabrata ATCC 66032 (Table 1). In the previous study on Verbascum species, extract of the V. sinuatum L. showed anticanidal effect at the concentration of 32 µg/mL against C. albicans (25). In another study methanolic extract of the V. georgicum which have antimicrobial constituents reported as a novel antimicrobial raw material (6). According to a scientific review on bioactivities of Verbascum species, methanol and ethanol extracts showed strong inhibitory effects on Candida albicans and Gram (+) bacteria strains due to the their saponin content (26).

Today, especially in immunocompromised people, Candida infections causes major health problems. There are few available systemic antifungal drugs, additionally the rate of drug resistance is increasing dramatically to available drugs. The search for new natural antifungal agents against pathogenic Candida species is extremely important (24).

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