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ORIGINAL ARTICLE

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

Türkiye'den Üç *Verbascum L.* Türünün Sitotoksik ve Antikandidal Aktivitelerinin Belirlenmesi; *Verbascum cheiranthifolium* Boiss. var. *Asperulum* (Boiss.) Murb. Monorg., *Verbascum pynostachyum* Boiss. & Heldr ve *Verbascum orgyale* Boiss. & Heldr

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ABSTRACT

Purpose of this study is to determine of cytotoxic and *anticandidal* activities of *Verbascum cheiranthifolium* Boiss. var. *asperulum* (Boiss.) Murb. Monorg., *Verbascum pynostachyum* 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. pynostactum* and *V. orygale* 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., V. *pycnostachyum* Boiss. & Heldr, ve *V. orgyale* Boiss. & Heldr. türlerinin sitotoksik ve antikandidal aktivitelerinin belirlenmesi amaçlanmıştır. *Verbascum cheiranthifolium* var. *asperulum*, *V. pycnostachyum* and *V. orgyale* türlerinin metanol ekstrelerinin sitotoksik etkileri servikal (HeLa) ve ovaryum kanser (Skov-3) hücrelerinde kolorimetrik metod kullanılarak araştırılmıştır. Elde edilen sonuçlar; *V. pycnostachyum* türünün metanol ekstresinin diğer türlere oranla her iki hücre hattında da umut verici toksik etkiye sahip olduğu gösterilmiştir. Buna ek olarak; bu etki Skov-3 hücrelerinde HeLa hücrelerine kıyasla daha anlamlıdır. Üç türe ait metanol ekstresinin antikandidal etkileri "Klinik Laboratuvar Standartları Enstitüsü" (CLSI)'nün mikrodilüsyon standart protokolleri kullanılarak standart antifungal ajanlarla karlılaştırmalı şekilde ilk kez bu çalışma ile ortaya konmuştur. *V. pycnostachyum* ve *V. orygale ekstreleri V. cheriantifolium*'a göre daha kuvvetli inhibitör 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 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 endemism 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 sığır kuyruğu" and "Söke sığır 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 anti-viral 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 dysmenorrhoea and rheumatalgia. It was also notified the usage for healing wounds in animal care.

Iridoid and neolignan type glycosides, oleanan 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, 37º 02' 53'' N, 30º 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 ddH_2O 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

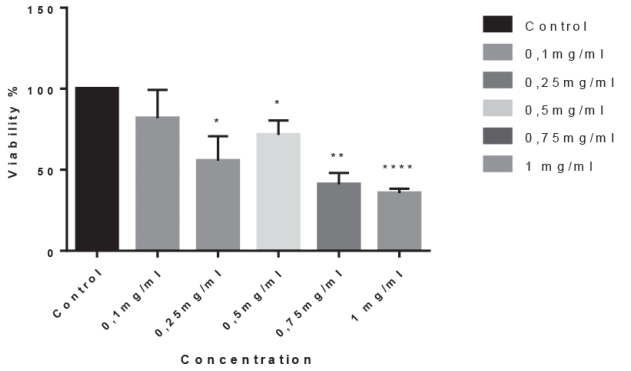
MTT[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] is a non-radioactive assay and measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The reduction of MTT can only occur in metabolically active cells. The assay was performed as mentioned in Mossman. HeLa and Skov-3 cells (2×10^4) were seeded in 96-well plates in the presence and absence of different concentrations of *Verbascum sp*. for 24 hours at at 37°C in a 5% CO_2/95% 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.

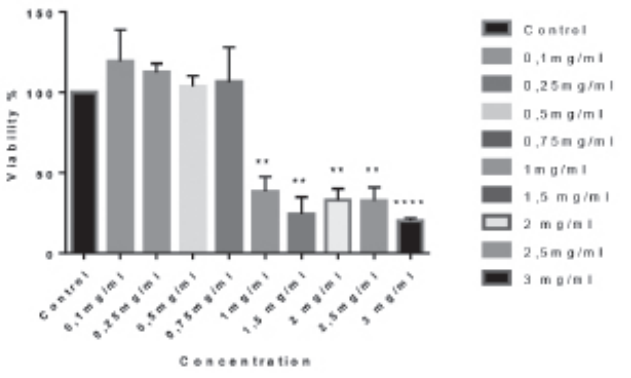
Anticandidal activity

Anticandidal activities of the methanolic extracts were evaluated by partly modified reference method of Clinical and Laboratory Standards Institute (*CLSI*) M27-A2 (21).

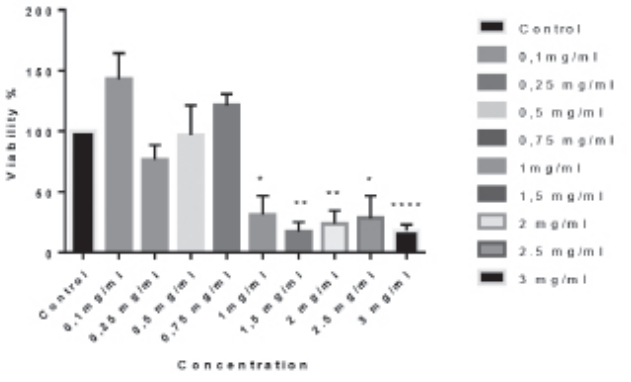
Candida albicans ATCC 90028, *C. utilis* NRRL Y-900, *C. glabrata* ATCC 66032, *C. tropicalis* ATCC 750, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as pathogenic test microorganisms. Stock cultures stored in 50% glycerol at -85˚C, were inoculated in Mueller Hinton Agar (Acumedia) 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.



Verbascum pycnostochyum (A)

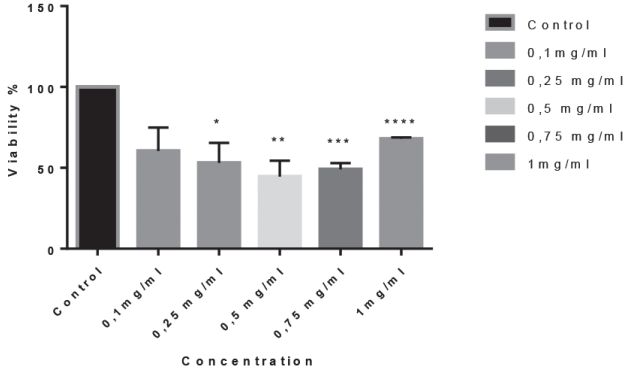


Verbascum cheiranthifolium var. *asperulum* (B)

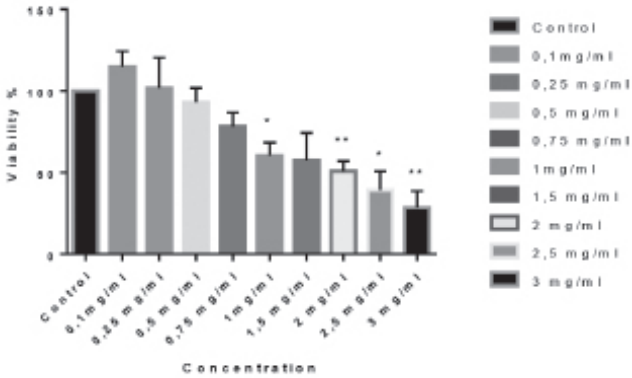


Verbascum orgyale (C)

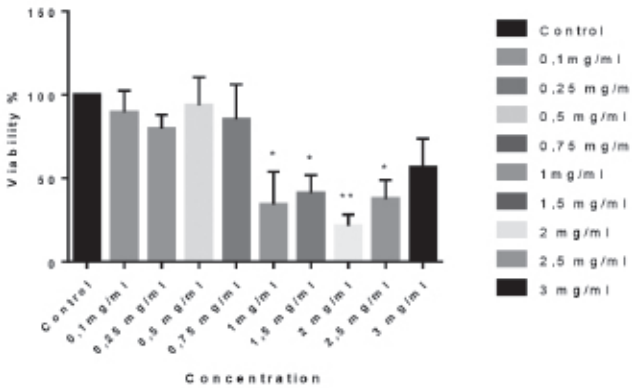
Figure 1. Treatment of either *V. pycnostachyum*, *V. cheiranthifolium* var. *asperulum* or *V. orgyale* extracts with HeLa cells decreased the cell viability in a dose-dependent manner. *Bars* indicate mean ± standard deviation. All comparisons were made relative to untreated control cells. The significant differences were indicated as p< 0.05 using one-way ANOVA. The graphic was created by using GraphPad Prism 6 software. [*p<0.1; **p<0.01; ****p<0.001].



Verbascum pycnostochyum (A)



Verbascum cheiranthifolium var. *asperulum* (B)



Verbascum orgyale (C)

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<0.1; **p<0.01; ****p<0.001].

Table 1. Anticandidal Activity (µg/mL, MIC)

	C. albicans	*C. tropicalis*	*C. parapsilosis*	*C. utilis*	*C. glabrata*	*C. krusei*
V. cherianthifolium var. asperulum	1000	250	>4000	2000	>4000	250
V. orygale	125	250	125	250	>4000	125
V. pycnostachyum	1000	250	125	250	>4000	**62.5**
Amphotericin B	0.031	1.0	0.25	0.5	2.0	1.0
Ketoconazole	0.008	0.031	0.031	0.25	0.25	0.25

RESULTS AND DISCUSSION

Cytotoxicity results

The effects of V. pynostachyum, V. cheiranthifolium var. asperulum and V. orgyale 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. pycnostachyum 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. orgyale methanolic-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. cherianthifolium* 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 cherianthifolium* 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 anticandidal 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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