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RESEARCH ARTICLE

Brine Shrimp Lethality Bioassay of Some *Verbascum* Species Growing In Turkey

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Summary

Verbascum L. (Scrophulariaceae) species are medicinal plants that have been used for the treatment of inflammatory diseases, asthma, spasmodic coughs and other pulmonary problems in Anatolia and in several countries. The objective of this study was to assess the in vivo cytotoxic activities of thirteen Verbascum extracts including V. chionophyllum Hub.-Mor., V. cilicicum Boiss., V. dudleyanum (Hub.-Mor.) Hub.-Mor., V. lasianthum Boiss., V. latisepalum Hub.-Mor., V. mucronatum Lam., V. olympicum Boiss., V. pterocalycinum var. mutense Hub.-Mor., V. pycnostachyum Boiss. & Heldr., V. salviifolium Boiss., V. splendidum Boiss., V. stachydifolium Boiss. & Heldr and V. uschackense (Murb.) Hub.-Mor. using brine shrimp (Artemia salina) lethality bioassay. The methanolic extracts of V. chionophyllum flowers and leaves, V. cilicicum flowers, V. lasianthum flowers, V. mucronatum flowers, V. pycnostachyum flowers and V. splendidum flowers showed the highest inhibitory activities against the brine shrimp. On the other hand, the rest of the species did not show any remarkable cytotoxic activity.

Key Words: *Cytotoxic activity, Artemia salina, Verbascum, Scrophulariaceae*

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Türkiye'de yetişen bazı Verbascum türlerinin Tuzlu Su Karidesleri Yöntemiyle Sitotoksik Aktivite Çalışmaları

Özet

Verbascum L. (Scrophulariaceae) türleri, Anadolu'da ve bir çok ülkede enflamatuvar hastalıkların, astım, spazmodik öksürük ve diğer akciğer problemlerinin tedavisinde kullanılan tıbbi bitkilerdir. Bu çalışmanın amacı, V. chionophyllum Hub.-Mor., V. cilicicum Boiss., V. dudleyanum (Hub.-Mor.) Hub.-Mor., V. lasianthum Boiss., V. latisepalum Hub.-Mor., V. mucronatum Lam., V. olympicum Boiss., V. pterocalycinum var. mutense Hub.-Mor., V. pycnostachyum Boiss. & Heldr., V. salviifolium Boiss., V. splendidum Boiss., V. stachydifolium Boiss. & Heldr ve V. uschackense (Murb.) Hub.-Mor.'nin dahil olduğu on üç Verbascum türünün ekstrelerinin tuzlu su karidesleri (Artemia salina) yöntemi ile in vivo, sitotoksik aktivitesini araştırmaktır. V. chionophyllum'un çiçek ve yapraklarının, V. cilicicum, V. lasianthum, V. mucronatum, V. pycnostachyum ve V. splendidum'un çiçeklerinin metanol ekstreleri tuzlu su karideslerine karşı en yüksek inhibitor aktiviteyi göstermiştir. Diğer taraftan, çalışılan diğer türler herhangi bir kayda değer sitotoksik aktivite göstermemiştir.

Anahtar Kelimeler: *Sitotoksik aktivite, Artemia salina, Verbascum, Scrophulariaceae*

INTRODUCTION

The genus *Verbascum* (Scrophulariaceae) is represented by 232 species, 196 of which are endemic to Turkey (1). These species are well-known drugs in Turkish folk medicine mainly used due to their expectorant, mucolytic, sudorific, sedative, diuretic and constipation activities (2). Many studies have

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so far demonstrated that these species show various kinds of biological activities. Among these activities, the treatment of haemorrhoids, rheumatic pain, superficial fungal infections, eczema and other types of inflamed skin conditions and diarrhoea as well as asthma, pulmonary complaints, inhibitory activities against the murine lymphocytic leukaemia and influenza viruses A2 and B are considered to be inhibitory to reactive oxygen species and tumor metastasis (3).

Ucar-Turker and Camper (2002) also reported that the extracts of the flowers and leaves of *Verbascum thapsus* showed antitumor activity against *Agrobacterium tumefaciens*-induced tumors on potato disc method as modified by McLaughlin's group. No tumor formation was observed with camptothecin (tumor suppressant), while the tested saponins had moderate tumor inhibition. Thus, saponins are believed to be responsible for these beneficial effects (3).

In the previous studies, crude extracts from the aerial parts of *Verbascum pseudonobile* used in traditional medicine have been screened for potential anticancer bioactive agents, using evaluation of DNA-interaction activity. The extracts were proved active in DNA interaction. It was found that there was a correlation in DNA-intercalation and the hemolytic effect in plant extracts (4).

The brine shrimp bioassay was used as an indicator for general toxicity and also as a guide for the detection of antitumor and pesticidal compounds (5).

Phytochemical investigations revealed the presence of iridoid glycosides, oleanane type triterpene saponins and phenylethanoid glycosides from *Verbascum* species in our previous studies (6-8). These compounds are considered responsible for some of the activities. They were shown to have analgesic, antiinflammatory, hepatoprotective, cardiovascular, choleretic, purgative, diuretic, antibacterial, antiviral, immuno-modulator, cytotoxic, cytostatic, anticancer, antitumour activities (9-15).

As related with the above-presented data, this study is undertaken to investigate *in vivo* cytotoxic activity against brine shrimp of the methanol extracts of thirteen *Verbascum* species growing in Turkey. Moreover, the aim of this screening was the selection of the most promising plant species for further bioactivity guided fractionation.

MATERIALS AND METHODS

Plant Materials

Plant materials were collected from different localities between 2000 and 2008 in Turkey. Voucher specimens were authenticated by Prof. Dr. Hayri Duman (Gazi University, Faculty of Science, Etiler, Ankara, Turkey) and were deposited at the Herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University and Herbarium of the Biology Department, Art and Sciences Faculty, Gazi University, Ankara, Turkey. Collection sites, parts used and herbarium numbers of the selected *Verbascum* species as the subject of this study are listed in Table 1.

Preparation of Plant Extracts

Each plant material (the parts used were given in Table 1) was dried under shadow and powdered to a fine grade by using a laboratory scale mill. Dried parts of each plant materials (10 g) were extracted with methanol by maceration at room temperature for two times (2×100 mL). The combined methanolic extracts were evaporated to dryness in *vacuo* to give crude methanolic extracts. The plant parts and the extract yields (w/w) were given in Table 1.

Cytotoxic Studies

Cytotoxicity was evaluated by the brine shrimp lethality bioassay (16, 17). Sea salt (3.8 g) was dissolved in 100 mL water and filtered. Brine shrimp (*Artemia salina* Leach) (San-Francisco Bay Brand Inc., Newark, CA 94560, USA) eggs were placed into the water and left to incubate for 48 h at 28°C in a small tank (Otsuka Pharmaceutical Co. Ltd., Tokyo, Japan). Each extract was tested at 1000, 100 and 10 ppm. Then 20 mg of plant extract was dissolved in 2 mL chloroform (20 mg/2mL). From this solution 500, 50 or 5 µL was transferred to vials corresponding to 1000, 100 or 10 ppm, respectively. Vials including chloroform and extraction solvent (methanol) (500 µL) were prepared as controls. After incubation,

Table 1. The collection of plant parts, collection sites, herbarium numbers and percentage yields of the extracts from *Verbascum* species

Plant name and authors	Collected parts	Collection site	Herbarium number	Yield (w/w, %)
V. chionophyllum Hub.-Mor.	FL, L	Icel, 40 km from Mut to Ermenek, *Pinus brutia* forest, alt.550-600 m	HUEF 00180*	15.0
V. cilicicum Boiss.	FL	Adana, Between Pozanti and Ulukisla, Alihoca Village	HUEF 00183*	17.7
V. dudleyanum (Hub.-Mor.) Hub.-Mor.	AE	Burdur, South shore of Lake Salda, wet places by the lake, alt.1170 m	HUEF 02001*	8.6
V. lasianthum Boiss.	FL, L	Izmir, Urla, Ucahirlar	HUEF 99139*	7.0
V. latisepalum Hub.-Mor.	AE	Burdur, Burdur to Cavdir, *Pinus brutia* forest, alt.800 m	HUEF 02007*	10.7
V.mucronatum Lam.	FL, L	Aksaray, 17 km from Aksaray to Ulukışla, alt. 800-900 m	GAZI 10097*	12.0
V. olympicum Boiss.	AE	Bursa, Uludağ, 1800 m	GAZI 10135*	9.9
V.pterocalycinum var. *mutense* Hub.-Mor.	FL, L	Icel, between Mut and Karaman, 930-1100 m	HUEF 00184*	9.0
V. pycnostachyum Boiss.& Heldr.	FL, L	Karaman, From Mut to Karaman, alt.1300 m	HUEF 00182*	15.2
V. salviifolium Boiss.	AE	Burdur, Yesilova, southwest banks of Burdur Lake, alt.880 m	HUEF 02003*	12.0
V. splendidum Boiss.	FL, L	Konya, Eregli, from Eregli to Karaman, alt.1150-1200 m	HUEF 00181*	16.6
V. stachydifolium Boiss. & Heldr	AE	Konya, Konya to Ankara, 65 km N. of Konya, alt. 900 m	GAZI 10165*	14.9
V.uschackense (Murb.) Hub.-Mor.	AE	Afyon, 5 km from Afyon to Konya, alt. 700-800m	GAZI 10121*	11.4

Abbreviations: alt.: altitude; AE: Aerial parts; FL: Flower; *Collectors: Akdemir, Duman, Kahraman and Tatli.

10 brine shrimp larvae (nauplii) were introduced into vials containing graded concentrations (ranging from 10 to 1000 ppm) of the extracts. After 24 h, the number of surviving shrimps at each concentration of the extracts was counted and data analyzed with the Finney computer program to determine the LC_{50} at a 95% confidence interval. Sea salt (Sigma 9883) was used in activity tests. The cytotoxic activity of all extracts was compared with umbelliferone and colchicine as the activity cytotoxic substances (18, 19).

RESULTS AND DISCUSSION

For the scientific evaluation of the claimed effect for *Verbascum* species, the methanolic extracts of thirteen *Verbascum* species were investigated *in vivo* against the brine shrimp according to cytotoxic activity procedure. The results are given in Table 2. These extracts showing LC_{50} < 1000 are considered to be toxic in the brine shrimp bioassay. As shown in Table 2, the methanolic extracts of *V. chionophyllum* flowers and leaves, *V. cilicicum* flowers, *V. lasianthum* flowers, *V. mucronatum* flowers, *V. pycnostachyum* flowers and *V. splendidum* flowers showed the highest inhibitory rates against the brine shrimp. On the other hand, the rest of the species did not show any remarkable cytotoxic activity.

Table 2. LC_{50} values of the extracts from *Verbascum* species on brine shrimp lethality bioassay.

Plant materials	Part used and extracts	Concentration (ppm)	LC_{50} (µg/ml)	SD (%) (n=3)
V. chionophyllum	FL, MeOH	1000:100:10	857.48	0.22
V. chionophyllum	L, MeOH	1000:100:10	421.18	0.55
V. cilicicum	FL, MeOH	1000:100:10	119.91	0.89
V. dudleyanum	AE, MeOH	1000:100:10	>1000	0.21
V. lasianthum	FL, MeOH	1000:100:10	3.62	0.56
V.lasianthum	L, MeOH	1000:100:10	>1000	0.11
V. latisepalum	AE, MeOH	1000:100:10	>1000	0.25
V. mucranatum	FL, MeOH	1000:100:10	75.86	0.36
V. mucronatum	L, MeOH	1000:100:10	>1000	0.88
V. olympicum	AE, MeOH	1000:100:10	>1000	0.77
V. pterocalycinum var. *mutense*	FL, MeOH	1000:100:10	>1000	0.98
V. pterocalycinum var. *mutense*	L, MeOH	1000:100:10	>1000	0.45
V. pycnostachyum	FL, MeOH	1000:100:10	749.67	0.89
V. pycnostachyum	L, MeOH	1000:100:10	>1000	0.21
V. salviifolium	AE, MeOH	1000:100:10	>1000	0.17
V. splendidum	FL, MeOH	1000:100:10	6.15	0.58
V. splendidum	L, MeOH	1000:100:10	>1000	0.28
V. stachydifolium	AE, MeOH	1000:100:10	>1000	0.96
V. uschackense	AE, MeOH	1000:100:10	>1000	0.69
Umbelliferon		500:50:5	377.02	
Colchicine		500:50:5	0.0009	

Abbreviations: AE: Aerial parts; FL: Flowers; L: Leaves

In our previous studies, *V. chionophyllum*, *V. cilicicum*, *V. pterocalycinum* var. *mutense*, *V. pycnostachycum* and *V. splendidum* (Scrophulariaceae) were studied for their cytotoxic activities against SK-MEL, KB, BT-549, and SK-OV-3 cell lines. The results were evaluated to compare cytotoxic activity in both their methanol and ethylacetate extracts. The methanol extract of the flowers of *V. pterocalycinum* var. *mutense* showed a weak cytotoxic activity against SK-MEL cell line. Through bioassay-guided fractionation on the methanol extract of this species, seven fractions were obtained; however, none of the fractions had cytotoxic activity against cancer cell lines (20).

In other studies, organic extracts of 24 selected plant species, used by Palestinian traditional healers to treat different illnesses and diseases, were tested for their anti-inflammatory and anti-tumoral activities. The plant selection was based on existing ethnobotanic information and interviews with local healers. The extracts of the plants under investigation were tested for their potential anti-tumor (cytotoxic) effect on the murine fibrosarcoma L929sA cells, and on the human breast cancer cells MDA-MB231 and MCF7. Cytotoxicity screening models provide important preliminary data to select plant extracts with potential antineoplastic properties. MTT (Tetrazolium blue) colorimetric assay was used to

evaluate the reduction of viability of cell cultures in the presence or absence of the extracts. The extract from *Verbascum thapsiforme* presented an IC_{50} value at 24 h of 470 μg/mL on L929sA (21).

The influence of the saponins and phenylethanoid glycosides from the flowers of mullein on a spontaneous proliferation of rat spleen lymphocytes have been *in vitro* studied. Verbascosaponin, verbascoside and forsythoside B showed antiproliferative effect and these results have supported their cytotoxic and immunostimulating activities (22).

The effects of some plants on protein biosynthesis *in vivo* have been examined for their antitumor activities. The separation features of the peptide elongation system, isolated from tumoral cells, have been demonstrated. Some elongation factors or ribosomes have been shown to be a target site for the inhibition of protein biosynthesis caused by the substances isolated from various sources. The effect of the fractions isolated from the aqueous extract of the flowers of *V. thapsiforme* on protein biosynthesis was studied. A strong inhibitory effect of the aqueous extract on protein biosynthesis was demonstrated in isolated rat liver ribosomes. The saponin fraction was shown to be responsible for this activity and it was compared to commercial glycyrrhizic acid and its aglycon as the reference drug. It was found that these compounds strongly inhibited the incorporation of [^{14}C]leucine into proteins *in vitro* and that the target site for inhibition was the ribosome fraction from rat liver cells (23, 24).

With the abovementioned studies, our results showed that *Verbascum* species have cytotoxic activities which might explain their beneficial effect on inflammation and cancer diseases. A general correlation was suggested between the anti-inflammatory and antitumor-promoting activities of acylated saponins from Scrophulariaceae plants by Tokuda *et al.* (1991) (25). It was also interesting that while the flowers of *Verbascum* species showed cytotoxic activity, the leaves (except those of *V. chionophyllum*) had no effect against brine shrimp. The reason may arise from the differences and the percentage of secondary metabolites in the flowers and leaves. In our study, the results demonstrated that the saponin glycosides isolated from especially the flowers of *V. lasianthum* and *V. mucronatum* (6-7) were thought to be responsible for the activity. Also, taking the claimed cytotoxic activities of *Verbascum* species into consideration, it is not necessarily only one single compound that is responsible for these effects, which may as well be due to several compounds that act in a synergistic manner or to compounds which regulate one the other.

In a reference survey, no more reports about the cytotoxic activities using brine shrimp lethality bioassay protocol of all mentioned *Verbascum* extracts have been offered so far. It is the first demonstration that the methanolic extracts of *V. chionophyllum, V. cilicicum, V. lasianthum, V. mucronatum, V. pycnostachyum* and *V. splendidum* were shown to possess a significant cytotoxic activity in this study. Further studies on species studied may yield successful results and isolation of active constituents.

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REFERENCES

1. Huber-Morath A. *Verbascum*. In: Davis PH, editor. Flora of Turkey and the East Aegean Islands. Vol. 6. Edinburgh: University Press; 1978, p. 461.
2. Baytop T. Therapy with Medicinal Plants in Turkey (Past and Present). 2nd ed. Istanbul: Nobel Tıp Kitabevleri Ltd.;1999, p. 334.
3. Ucar-Turker A, Camper ND. Biological activity of common mullein, a medicinal plant. *J Ethnopharmacol* 82: 117-125, 2002.
4. Ionkova I, Alferman A. Use of DNA for detection and isolation of potential anticancer agents from plants. *Farmatsiya* 47: 10, 2000.
5. McLaughlin JL, Chang CJ, Smith DL. Bench-top bioassays the discovery of bioactive natural products: an update. *Nat Prod Chem* 9: 383-397, 1991.

6. Kahraman, C. Pharmacognostical studies on *Verbascum mucronatum* Lam. Master Thesis, Institute of Health Sciences, Hacettepe University, Ankara, Turkey, 2009.
7. Tatli II, Akdemir ZS. Chemical Constituents of *Verbascum* Species. *Journal of Pharmaceutical Sciences* (FABAD) 29: 93-107, 2004.
8. Tatli II, Schühly W, Akdemir ZS. Secondary Metabolites from Bioactive Methanolic Extract of *Verbascum pycnostachyum* Boiss. & Helder Flowers. *Hacettepe University Journal of the Faculty of Pharmacy* 27 (1): 23-32, 2007.
9. Sticher O. Plant mono-, di- and sesquiterpenoids with pharmacological or therapeutical activity. In: Wagner H, Wolff P, editors. New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity. New York-Berlin: Springer-Verlag; 1977.
10. Jimenez C, Riguera R. Phenylethanoid Glycosides in Plants: Structure and Biological activity. *Natural Product Reports*. 591, 1994.
11. Hostettmann K, Marston A. Saponins. Cambridge, UK: Cambridge University Press; 1995.
12. Vijayavitthal TM, Kanwal R, Amiya PB, Ragini S, Anju P, Lalit MT, Vishwa MLS. Studies on the profile of immunostimulant activities of modified iridoid glycosides. *Bioorganic & Medicinal Chem* 6: 605-611, 1998.
13. Konoshima T, Takasaki M, Tokuda H, Nishimo H. Cancer chemopreventive activity of an iridoid glycoside, 8-acetylharpagide from *Ajuga decumbens. Cancer Lett* 157: 87-92, 2000.
14. Stevenson PC, Simmonds MSJ, Sampson J, Houghton PJ, Grice P. Wound healing activity of acylated iridoid glycosides from *Scrophularia nodosa*. *Phytother Res* 16: 33-35, 2002.
15. Ahmed B, Al-Rehaily AJ, Al-Howiriny TA, El-Sayed KA, Ahmad MS. Scropolioside-D2 and harpagoside-B: two new iridoid glycosides from *Scrophularia deserti* and their antidiabetic and anti-inflammatory activity. *Biol Pharm Bull* 26: 462-467, 2003.
16. McLaughlin JL. Crown gall tumors on potato discs and brine shrimp lethality: two single bioassays for plant screening and fraction. In: Hostetsmann K, editor. Methods in Plant Biochemistry. London: Academic Press; 1991. pp. 1-31.
17. Sarma BK, Pandey VB, Mishra GD, Singh UP. Antifungal activity of berberine iodide, a constituent of *Fumaria indica*. *Folia Microbial* 44 (2): 164-166, 1999.
18. Rao KS, Mishra SH. Antihepatotoxic activity of monomethyl fumarate isolated from *Fumaria indica*. *J Ethnopharmacol* 60 (3): 207-213, 1998.
19. Giliani AH, Bashir S, Janbaz KH, Khan A. Pharmacological basis for the use of *Fumaria indica* in constipation and diarrhea. *J Ethnopharmacol* 96: 585-589, 2005.
20. Tatli II, Akdemir ZS. Cytotoxic Activity of *Verbascum* Species Growing in Turkey. *Hacettepe University Journal of the Faculty of Pharmacy* 26 (2): 77-86, 2006.
21. Kaileh M, Berghe WV, Boone E, Essawi T, Haegeman G. Screening of indigenous Palestinian medicinal plants for potential anti-inflammatory and cytotoxic activity. *J Ethnopharmacol* 113: 510–516, 2007.
22. Klimek B, Stepien H. Effect on some constituents of mullein (*Verbascum* sp.) on proliferation of rat splenocytes *in vitro*. *Eur J Pharm Sci* 2 (1-2): 123, 1994.
23. Paszkiewicz-Gadek A, Grochowska K, Galasinski W. Effect of the aqueous extract and saponin fraction from the flowers of *Verbascum thapsiforme* on protein biosynthesis in a rat liver ribosomal system, *Phytother Res*, 4, 177-181, 1990.
24. Galasinski W, Chlabicz J, Paszkiewicz-Gadek A, Marcinkiewicz C, Gindzienski A. The substances of plant origin that inhibit protein biosynthesis, *Acta Pol Pharm*, 53, 311-318, 1996.
25. Tokuda H, Konoshima T, Kozuka M, Kimura R. Antitumour activities of triterpene saponins from *Verbascum songaricum, Oncology*, 48, 77–80, 1991.