Hacettepe University, Journal of the Faculty of Pharmacy
Volume 26 / Sayı 2 / July 2006 / ss. 77-85

Cytotoxic Activity on Some *Verbascum* Species Growing in Turkey

Received : 01.06.2006
Revised : 03.11.2006
Accepted : 17.11.2006

I. İrem Tatlı*/ Zeliha S. Akdemir*

Introduction

Plants have been used as folkloric sources of medicinal agents since the beginning of mankind. As the age of modern medicine, single pure drugs emerged, and plant-derived active principles, their semi-synthetic and synthetic analogs have served as a major route to new pharmaceuticals. Since 1961, plant-derived compounds have been approved for use as anticancer drugs: vinblastine (Velban®), vincristine (Oncovin®), etoposide (VP-16®), taxol (Paclitaxel®), etc.[1].

The genus *Verbascum* (Scrophulariaceae) is represented by 228 species, 185 of which are endemic to Turkey[2]. These species are well-known drugs in Turkish folk medicine mainly used due to their expectorant, mucolytic, sudorific, sedative, diuretic and constipate activity[3]. Many studies have so far represented that these species show various kind 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[4].

* H.Ü. Faculty of Pharmacy, Department of Pharmacognosy, 06100 Sıhhiye, Ankara, TURKEY
İletişim için:itatli@hacettepe.edu.tr

Crude extracts from *Verbascum pseudonobile* used in traditional medicine have been screened for potential anticancer bioactive agents, using evaluation of DNA-interaction activity. The extracts proved active in DNA interaction. It was found that there was correlation in DNA-intercalation and the hemolytic effect in plant extracts[5].

Ucar-Turker *et al* reported that the extracts 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[4].

In 1982, it was given a definition for expression of activity, that is, the word cytotoxicity must be used only for *in vitro* activity, the words antineoplastic and antitumor must be used only for *in vivo* test using animal[6].

Development of novel clinically useful anticancer agents would be dependent on the screening system and the sample sources for the bioassay. Improving of simple anticancer pre-screen using convenient and inexpensive cytotoxic assay systems can offer numerous advantages as alternatives to extensive animal testing in the search for new anticancer drugs. The search for potential anticancer agents from natural sources mainly has been carried out with the guidance of bioassay confirmed by Borenfreund *et al* and the screening protocols for each tumor system have been well-established. These screening systems led to fractionate from the plants[7-10].

The objective of this study was to screen for the presence of cytotoxic activity against SK-MEL, KB, BT-549, and SK-OV-3 cell lines in methanol and ethylacetate extracts of five endemic *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 against cancer cell lines. This is the first report on cytotoxic activity of *Verbascum* species using 96-well tissue culture-treated microplate assay protocol.

Material and Methods

2.1. Plant Material

Plant material	**Collection area**	**Date**	**Herbarium no***
Verbascum chionophyllum Hub.-Mor.	C4: Icel: 40 km from Mut to Ermenek, *Pinus brutia* fields, 550-600 m	July 2000	HUEF 00180
Verbascum cilicicum Boiss.	C5: Adana: Between Pozanti and Ulukisla, Alihoca village	July 2000	HUEF 00183
Verbascum pterocalycinum var. *mutense* Hub.-Mor.	C4: İçel: Between Mut and Karaman, *Pinus brutia* and *Pinus nigra* fields, 930-1100 m	July 2000	HUEF 00184
Verbascum pycnostachyum Boiss. & Heldr.	C4: Karaman: From Mut to Karaman	July 2000	HUEF 00182
Verbascum splendidum Boiss.	C5: Konya, Eregli, From Eregli to Karaman, 1150-1200 m	July 2000	HUEF 00181

* The voucher specimens were deposited in the Herbarium of Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

2.2. Extraction and Isolation

2.2.1. Extraction of plant materials

Five samples of air dried aerial parts of the plants (20 g) were extracted with methanol (150 ml) and ethyl acetate (150 ml) using evaporator without vacuum at room temperature. The extracts were evaporated to dryness at 40°C, and stored at 20°C for further analysis. The crude residues were used in cytotoxic assay.

2.2.2. Fractionation of Verbascum pterocalycinum var. mutense Hub.-Mor.

Air-dried and powdered flowers of *Verbascum pterocalycinum* var. *mutense* (485.6 g) were extracted with MeOH (2x2 L) under reflux. The MeOH extract was evaporated to dryness in *vacuo* to yield 43.5 g of crude extract. The methanolic extract was fractionated by open column chromatography on silica gel (500 g) employing hexane, chloroform, ethylacetate, acetone and methanol (each, 4 L), respectively, to yield seven fractions (Frs. A-G).

2.3. Cytotoxicity assay

The *in vitro* cytotoxic activity was determined against four human cancer cell lines, SK-MEL (malignant melanoma), KB (epidermoid carcinoma), BT-549 (ductal carcinoma) and SK-OV-3 (ovary carcinoma) obtained from the American Type Culture Collection (ATCC, Rockville, MD). For initial (primary) evaluation, extracts and fractions were screened at a single concentration (100 and 10 µg/ml, respectively). Follow-up secondary assays were then conducted at three concentrations (10, 3.3 and 1.1 µg/ml). The assay is performed in 96-well tissue culture-treated microplates. Cells (25000 cells/well) were seeded to the wells of the plate and incubated for 24 h. Samples were added and plates were again incubated for 48 h. The assay is based on the accumulation of neutral red dye in the lysosomes of viable cells. A subsequent addition of 2-propanol will lyse the cells releasing the dye into solution and the absorbance is measured at 490 and 630 nm. Corresponding growth inhibition was calculated and graphed. IC_{50}'s are determined from logarithmic graphs of growth inhibition values. The results are compared to results from fibroblast VERO cell line (African green monkey kidney) testing with the same substances in order to determine level of cytotoxicity. Doxorubicin was used as a positive control, while DMSO was used as the negative (vehicle) control[7-10].

Results and Discussion

For the scientific evaluation of the claimed effect for *Verbascum* species, methanol and ethyl acetate extracts prepared from *Verbascum chionophyllum, V. cilicicum, V. pterocalycinum* var. *mutense, V. pycnostachyum* and *V. splendidum* were investigated for cytotoxic activity against SK-MEL, KB, BT-549, and SK-OV-3 cell lines.

The extracts were administered in a standard single dose of 100 µg/ml for cytotoxic activity on a primary assay. The samples showing % cytotoxicity <80 are considered inactive at 100 µg/ml in primary assay. However, the extracts showing % cytotoxicity >80 are confirmed in secondary assay[6-9]. As shown in Table I, the methanol extracts of the flowers of *Verbascum* species more active than those of the leaves of the titled plants in this assay. The methanol extract of the flowers of *V. pterocalycinum* var. *mutense* was weak active against SK-MEL cell line while the other extracts were found to be inactive. Hence, bioassay-guided fractionation procedures were conducted with this extract. The methano-

TABLE I
Cytotoxic activity of the extracts of *Verbascum* species

Species	Used Parts	Solvents	% Cytotoxicity				
			SK-MEL	KB	BT-549	SK-OV-3	VERO
V. chionophyllum	flowers	MeOH	0	8	0	0	3
V. cilicicum	flowers	MeOH	0	6	0	0	0
V. pterocalycinum var. mutense	flowers	MeOH	52	23	7	0	3
V. pycnostachyum	flowers	MeOH	17	20	11	0	1
V. splendidum	flowers	MeOH	0	0	0	0	0
V. chionophyllum	leaves	MeOH	0	8	0	0	0
V. cilicicum	leaves	MeOH	0	2	0	0	0
V. pterocalycinum var. mutense	leaves	MeOH	0	11	0	0	0
V. pycnostachyum	leaves	MeOH	0	12	0	0	2
V. splendidum	leaves	MeOH	0	2	0	0	0
V. chionophyllum	flowers	EtOAc	12	11	0	0	0
V. cilicicum	flowers	EtOAc	8	14	0	0	0
V. pterocalycinum var. mutense	flowers	EtOAc	7	15	0	0	0
V. pycnostachyum	flowers	EtOAc	8	13	2	0	1
V. splendidum	flowers	EtOAc	3	14	0	0	0
Doxorubicin			<1.1	<1.1	<1.1	<1.1	NC

SK-MEL: malignant melanoma; KB: epidermoid carcinoma; BT-549: ductal carcinoma; SK-OV-3: ovary carcinoma; VERO: kidney, African green monkey-normal cell line. "NC"-No Cytotoxicity.

lic extract of *V. pterocalycinum* var. *mutense* flowers was fractionated by open column chromatography on silica gel after removing chlorophyll to afford seven main fractions. Each fraction was then administered to cell line at a single dose of 10 µg/ml. The results were given in Table II. For fractions, IC_{50} values >20 are generally not pursued unless high selectivity is observed, while IC_{50} values ≤ 20 can be pursued for follow up[6-9]. None of the fractions showed cytotoxic activity in primary assay.

TABLE II

Cytotoxic activity of the fractions of the methanolic extract of *V. pterocalycinum* var. *mutense* flowers

Fractions	**IC50 values**				
	SK-MEL	**KB**	**BT-549**	**SK-OV-3**	**VERO**
Fraction A	NA	NA	NA	NA	NC
Fraction B	NA	NA	NA	NA	NC
Fraction C	>83	>100	>100	>77	NC
Fraction D	NA	NA	NA	NA	NC
Fraction E	NA	NA	NA	NA	NC
Fraction F	>60	>100	NA	>100	>100
Fraction G	NA	NA	NA	NA	NC
Doxorubicin	<1.1	<1.1	<1.1	<1.1	NC

SK-MEL: malignant melanoma; KB: epidermoid carcinoma; BT-549: ductal carcinoma; SK-OV-3: ovary carcinoma; VERO: kidney, African green monkey-normal cell line. "NA"-No activity, "NC"- No cytotoxicity.

Conclusion

As results of our screening programme, the methanol extract of the flowers of *V. pterocalycinum* var. *mutense* showed a weak inhibitory effect against SK-MEL cell line, while its fractions and none of the other *Verbascum* extracts are specific inhibitor on cell lines.

Afifi *et al* found that the isolated compounds from *Verbascum sinaiticum* exhibited dose-dependent cytotoxicity when tested against cultured P-388 cells. The cytotoxicity of the ethylacetate extract of *V. sinaiticum* leaves can therefore be related to the presence of the significantly active constituents' chrysoeriol, hydnocarpin, luteolin and sinaiticin. Hydnocarpin also inhibited the growth of Ehrlich ascites carcinomas in CF_1 mice, as well as exhibiting anti-inflammatory and hypolipidemic activities[11].

The influence of the saponins, phenyethanoid and flavonoid glycosides from the flowers of mullein on a spontaneous proliferation of rat spleen lymphocytes have been in vitro studied. Verbascosaponin, luteolin 7-*O*-glucoside, verbascoside and forsythoside B showed antiproliferative effect at the concentration of 100 µg/ml and these results prompt to support on their cytotoxic and immunostimulating properties[12].

The effect of fractions isolated from the extract of *Verbascum* flowers on protein biosynthesis was studied. A strong inhibitory effect of the extract on protein biosynthesis was demonstrated in isolated rat liver ribosome. The saponin fraction was shown to be responsible for this activity and it was compared to commercial drugs. It was found that these compounds strongly inhibited the incorporation of [^{14}C] leucin into proteins *in vitro* and that the target site for inhibition was the ribosome fraction from rat liver cells[13].

Verbascum thapsus L., which has been used as folk medicine in Canada, was evaluated for its anti-hepatoma activity on five human liver-cancer cell lines, i.e. HepG2/C3A, SK-HEP-1, HA22T/VGH, Hep3B and PLC/PRF/5. The hot water extract of crude drug was examined by *in vitro* evaluation for its cytotoxicity. The results showed that the effect of crude drug on hepatitis B virus genome-containing cell lines were different from those against non hepatitis B virus genome-containing cell lines. The inhibitions at 2000 µg/ml dose were 31.0% (HepG2/C3A), 69.9% (HA22T/VGH) and 11.6% (PLC/PRF/5) on HBV (-) and HBV (+) cell lines. *V. thapsus* was observed to be potent effective against the growth of three cell lines[14].

The fractionation of *Verbascum pterocalycinum* var. *mutense* indicated that saponins, phenylethanoids and flavonoids existed in non-polar and polar fractions as compared to spots on TLC plates. Although, these compounds are declared in the literatures[4,11-13] to be responsible for cytotoxic activity, none of the fractions which contain these secondary metabolites has cytotoxic activity against cancer cell lines. However, the results were reproducible and the dose-response behaviour is characteristic of the growth inhibitory effect[15]. Additionally, cytotoxic and cytostatic activity depends on the types of cells. In the case of HeLa cells, cytostatic activity at a low concentration could be interpreted as the cytotoxic activity not being so strong. However, in the cases of dRLh-84 and S-180 cells, the cytostatic activity couldn't be discriminated from cytotoxic activity, because the cytotoxic activity was strong[16].

Furthermore, it wasn't able to be reached correlated results to compare cytotoxic activity in both *V. pterocalycinum* var. *mutense* extract and its fractions. Therefore, it could be said that there was a synergistic effect in total plant extract. In order to correlate the obtained data in the field of cytotoxic activity of *Verbascum* species, further examinations in different assays can be evaluated.

Summary

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.

Key words: Verbascum species, Scrophulariaceae, Cytotoxic activity

Özet

Türkiye'de Yetişen Bazı Verbascum Türlerinin Sitotoksik Aktivitesi

V. chionophyllum, V. cilicicum, V. pterocalycinum var. *mutense, V. pycnostachycum* ve *V. splendidum* (Scrophulariaceae)'un, SK-MEL, KB, BT-549 ve SK-OV-3 kanser hücrelerine karşı sitotoksik aktiviteleri çalışılmıştır. Sonuçlar, bu türlerin metanol ve etil asetat ekstrelerinin sitotoksik aktiviteleri karşılaştırılarak değerlendirilmiştir. *V. pterocalycinum* var. *mutense* çiçeklerinin metanol ekstresi, SK-MEL kanser hücresine karşı zayıf sitotoksik aktivite göstermiştir. Bu türün metanol ekstresi üzerinde yapılan biyolojik rehberli fraksiyonlama ile yedi fraksiyon elde edilmiş, ancak, fraksiyonların hiçbirisi sitotoksik aktivite göstermemiştir.

Anahtar kelimeler: Verbascum türleri, Scrophulariaceae, Sitotoksik aktivite

Acknowledgements

The authors thank Prof.Dr. Hayri Duman, Gazi University, Faculty of Science, Department of Botany, Etiler, Ankara, Turkey for the authentification of the plant specimen. Cytotoxicity assay was partially supported by NIH grant AI27094.

REFERENCES

1. Lee, K. H.: Novel antitumor agents from higher plants, Medicinal Research Reviews, 19 (6), 569-596 (1999).
2. Huber-Morath, A. Verbascum, in "Flora of Turkey and the East Aegean Islands" (edited by P.H. Davis), Edinburgh, University Press, Vol. 6, 461 (1978).

3. Baytop, T.: Therapy with Medicinal Plants in Turkey (Past and Present), Istanbul, Nobel Tıp Kitabevleri Ltd., 2nd ed., 334 (1999).
4. Ucar-Turker, A., Camper, N.D.: Biological activity of common mullein, a medicinal plant, J. Ethnopharmacol., 82, 117 (2002).
5. Ionkova, I., Alferman, A.: Use of DNA for detection and isolation of potential anticancer agents from plants. Farmatsiya, 47, 10 (2000).
6. Itokawa, H., Takeya, K.: Antitumor sunstances from higher plants. Heterocycles, 35, 1467 (1993).
7. Borenfreund, E., Babich, H., Martin-Alguacil, N.: Rapid chemosensitivity assay with human normal and tumor cells in vitro, In Vitro Cell. Dev. Biol., 26, 1030 (1990).
8. Dou, J., McChesney, J.D., Sindelar, R.D., Goins, D.K., Walker, L.A.: A new quassinoid from Castela texana, J. Nat. Prod., 59, 73 (1996).
9. Muhammad, I., Dunbar, D.C., Khan, R.A., Ganzera, M., Khan, I.A.: Investigation of Una De Gato I. 7-deoxyloganic acid and 15N NMR spectroscopic studies on pentacyclic oxindole alkaloids from Uncaria tomentosa, Phytochemistry, 57, 781 (2001).
10. Muhammad, I., Bedir, E., Khan, S.I., Tekwani, B.L., Khan, I.A., Takamatsu, S., Pelletier, J., Walker, L.A.: A new antimalarial quassinoid from Simaba orinocensis, J. Nat. Prod., 67, 772 (2004).
11. Afifi, M.S.A., Ahmed, M.M., Pezzuto, J.M., Kinghorn, A.D.: Cytotoxic flavonolignans and flavones from Verbascum sinaiticum leaves, Phytochemistry, 34, 839 (1993).
12. 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).
13. 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 (1990).
14. Lin, L.T., Liu, L.T., Chiang, L. C., Lin, Cc.: In vitro Anti-hepatoma Activity of Fifteen Natural Medicines from Canada, Phytother. Res., 16, 440 (2002).
15. Itokawa, H., Takeya, K., Mori, N., Sonobe, T., Mihashi, S., Hamanaka, T.: Studies on antitumor cyclic hexapeptides Ra obtained from Rubia Radix, Rubiaceae. 6. Minor antitumor constituents. Chem. Pharm. Bull., 34, 3762 (1986).
16. Saracoglu, I., Inoue, M., Calis, I., Ogihara, Y.: Studies on constituents with cytotoxic and cytostatic activity of two Turkish medicinal plants, *Phlomis armeniaca* and *Scutellaria salviifolia*. Biol. Pharm. Bull., 18, 1396 (1995).