Isolation and Structure Elucidation of Diterpenes from Euphorbia pannonica, E. esula and E. falcata

Summary of Ph.D. Thesis

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Introduction

The family Euphorbiaceae is one of the largest families of flowering plants, composed of 5 subfamilies, 49 tribes, over 300 genera and about 8000 species. The genus *Euphorbia* is one of the 6 largest genera of flowering plants, with approximately 1830 species. *Euphorbia* species are widely distributed throughout both hemispheres and range in morphology from large desert succulents to trees, through climbing lianas and even some small herbaceous plant types. 28 Species of this genus have been found in Hungary.

Many Euphorbiaceae species are characterized by the occurrence of highly irritant milky latex. These plants have been used to treat different cancers, tumours and warts from at least the time of HIPPOCRATES.

Diterpenes occurring in plants of the Euphorbiaceae family are of considerable interest from the aspect of natural product drug discovery because of the wide range of their potentially valuable biological activities and their broad structural diversity resulting from the variable acylation of many different skeletons (*e.g.* ingenane, tigliane, daphnane, lathyrane and jatrophane) with numerous aliphatic and aromatic acids. The importance of Euphorbiaceae diterpenes may be demonstrated primarily by the approval granted by the FDA in 2012 for the use of ingenol 3-angelate (ingenol mebutate, PEP005, Picato®, LEO Pharma) in the treatment of actinic keratosis, a precancerous skin condition. Besides ingenol 3-angelate, other promising compounds are currently subjects of drug development projects. Some phorbol and ingenol derivatives, and particularly prostratin, have become of considerable interest in HIV therapy: these compounds reactivate HIV-1 latency by protein kinase C (PKC)-dependent NF-κB (nuclear factor) activation, and avoid the new infection of CD4+ cells. Resiniferatoxin, a compound belonging in the daphnane group, is an ultrapotent capsaicin analogue, which is at present undergoing evaluation in phase II and III clinical trials. Moreover, antileukaemic ingenane diterpenes have been obtained from *Euphorbia esula*, *Croton tiglium* and *Cunuria spruceana*. Further interesting diterpene esters with great structural variety and noteworthy biological activities have been isolated from Euphorbiaceae species.

Aims of the study

The widespread genus *Euphorbia* is the source of a large number of biologically active diterpenes. In 1995, the workgroup of Department of Pharmacognosy, University of Szeged initiated a research programme with the aim of investigating the secondary metabolites of plants of the *Euphorbia* species. The aims of the present work, as part of that programme, were the isolation and structural characterization of new diterpene polyesters, and investigation of their pharmacological effects.

In order to achieve these aims, the main tasks were:

- Screening of *E. pannonica* and *E. falcata* for diterpene content.
- Extraction of the plant materials.
- Isolation and purification of the diterpene esters from *E. pannonica*, *E. esula* and *E. falcata* by a combination of various chromatographic methods (OCC, VLC, RPC, PLC and HPLC).
- Characterization and structure determination of the isolated compounds by different spectroscopic techniques (NMR and HR-MS).
- Evaluation of the pharmacological potential and chemotaxonomical relevance of the isolated diterpenes.

Materials and methods

The compounds were isolated by a multistep separation procedure, including different extraction and chromatographic methods (OCC, VLC, RPC, PLC, and NP- and RP-HPLC). The isolated compounds were characterized and their structures were elucidated by means of different spectroscopic methods (MS and NMR).

Results and discussion

Isolation of diterpenes

The fresh *E. pannonica*, and the frozen *E. esula* and *E. falcata* plant materials were crushed and percolated with MeOH at room temperature. The extracts were concentrated *in vacuo*, and then subjected to liquid-liquid partition with CH_2Cl_2 (*E. pannonica* and *E. esula*) (Figures 1 and 2) or $CHCl_3$ (*E. falcata*) (Figure 3).

The organic phase was first separated by open column chromatography on polyamide with the use of MeOH-H_2O (1:4, 2:3, 3:2, 4:1) solvent systems. The 20–60 % methanol fractions were rich in diterpenes, and subjected to further purifications.

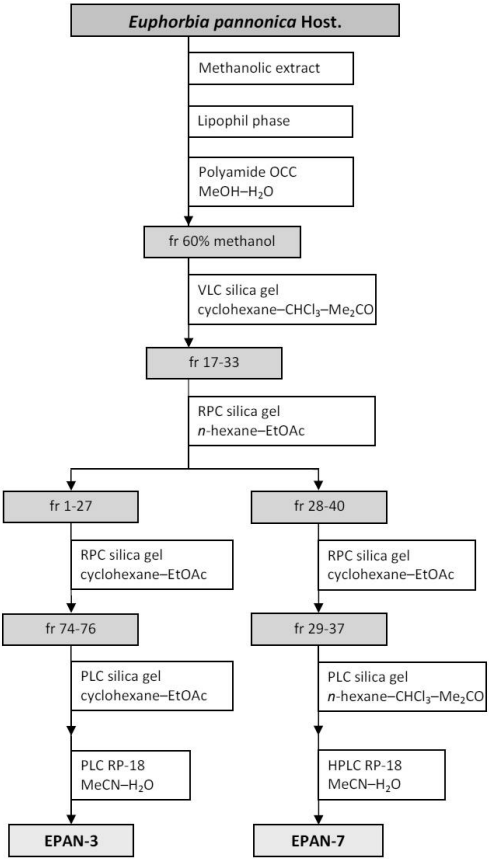


Figure 1. Isolation of diterpenes from *E. pannonica*

In the following steps, even more selective methods (VLC, RPC, PLC and HPLC) were applied. After polyamide OCC, in all experiments the diterpene-containing fractions were separated by adsorption chromatography on silica gel, using a VLC and RPC technique. These chromatographies resulted in a crude fractionation of the main components. For final purification, PLC, NP- and RP-HPLC were applied since these were the most selective and most effective separation methods. Moreover, HPLC provided mild conditions for isolation of light- and heat-sensitive diterpene polyesters.

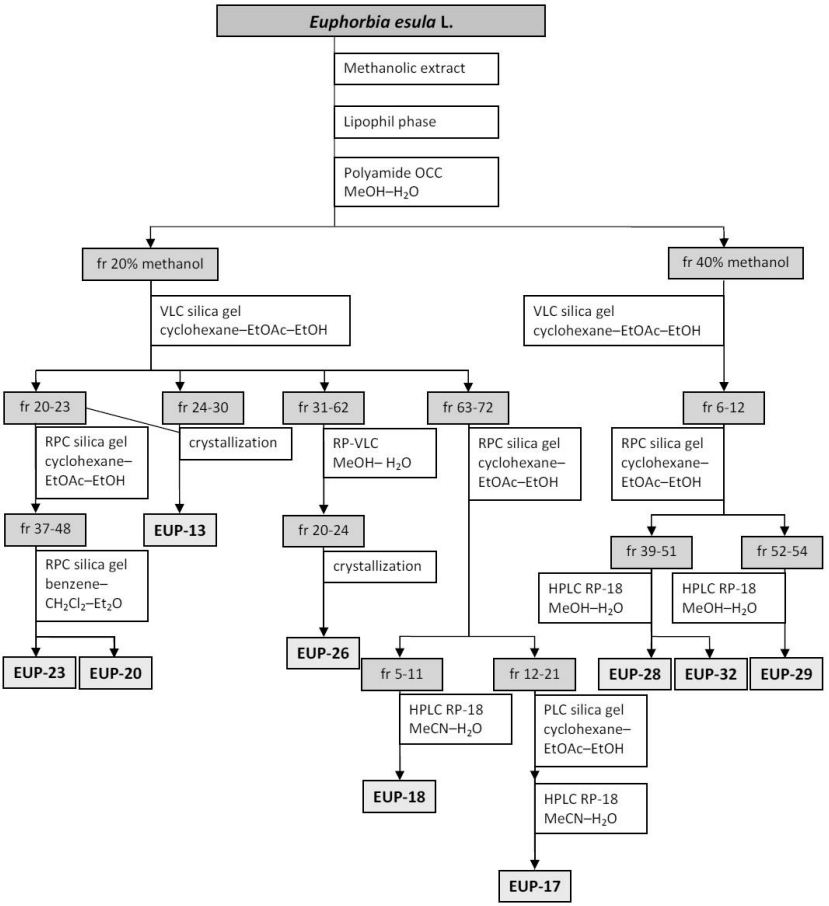


Figure 2. Isolation of diterpenes from *E. esula*

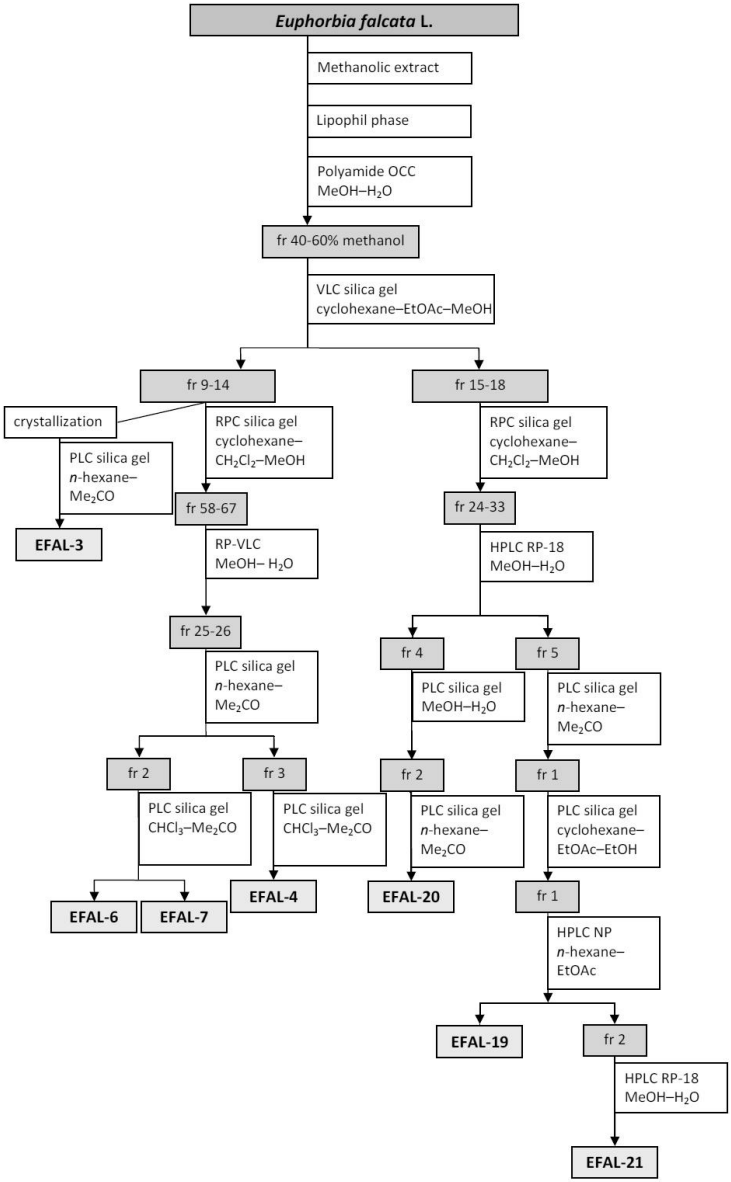


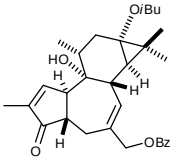
Figure 3. Isolation of diterpenes from *E. falcata*

As a result of the isolation procedure, 18 compounds (occurring in low concentration) were obtained from the multicomponent samples. Usually, compounds with very similar structures were separated per plant, most of the isolated components differing from each other only in the ester groups, and EPAN-3 (**1**) and EPAN-7 (**2**), EUP-23 (**7**) and EUP-26 (**9**), and EFAL–19 (**16**) and EFAL-20 (**17**) differing in only one substituent.

After extensive chromatographic purification, 2 compounds were isolated from *E. pannonica* (EPAN-3 and EPAN-7), 9 from *E. esula* (EUP-13, EUP-17, EUP-18, EUP-20, EUP-23, EUP-26, EUP-28, EUP-29 and EUP-32), and 7 from *E. falcata* (EFAL-3, EFAL-4, EFAL-6, EFAL-7, EFAL-19, EFAL-20 and EFAL-21).

Characterization and structure determination of the isolated compounds

The isolated compounds are amorphous solids or crystals. They are optically active. The structures of the isolated compounds were elucidated through spectroscopic methods. From MS measurements, the molecular composition was determined. The most useful data concerning the chemical structures were furnished by 1D and 2D NMR spectroscopy. From ^{1}H NMR, JMOD, ^{1}H-^{1}H COSY, HSQC and HMBC experiments, the constitution of the compounds was determined; then, with the aid of the NOESY spectra, the relative configurations were elucidated. In the isolated compounds, the number of asymmetric carbons were 8–13, and all of them were characterized stereochemically. NMR studies allowed complete ^{1}H and ^{13}C assignments for the characterization of the compounds. Structurally, 9 compounds are polyesters of jatrophane or modified jatrophane, 2 of tigliane, 4 of premyrsinane and 3 of cyclomyrsinane-type. EUP-18 (**6**), EUP-23 (**7**), EUP-29 (**8**), EUP-26 (**9**) and EUP-17 (**11**) isolated from *E. esula* contain a nicotinoyl group, and can therefore be regarded as pseudoalkaloids. The diversity of the ester groups is characteristic for the members of EFAL series, *e.g.* in EFAL-19 (**16**) 4 (acetyl, isobutanoyl, 2-methylbutanoyl and benzoyl), and in EFAL-4 (**13**), EFAL-6 (**14**), EFAL-7 (**15**), EFAL-20 (**17**) and EFAL-21 (**18**) 3 different ester groups were found. EUP-20 (**3**) is the most highly esterified jatrophane diterpenoid, with 8 ester groups. Besides ester groups, hydroxy and keto functions are also present in the molecules. In EUP-20 (**3**), an ether function is to be found between C-11 and C-14, and it therefore possesses an unusual heterocyclic ring system. Such compounds have been isolated earlier only from *E. kansui* and *E. esula*. The EUP series is stereochemically homogeneous, characterized by 2*β*-methyl, 13*α*-methyl and 3*β*, 7*β*, 5*α*, 8*α*, 9*α* and 15*β*-acyl substitution.



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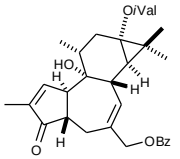

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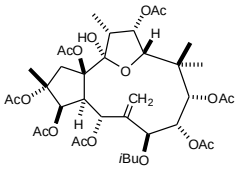

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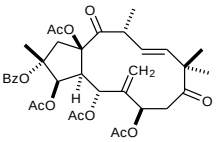

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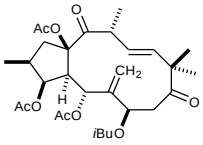

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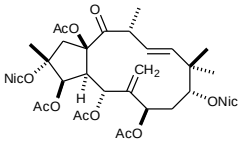

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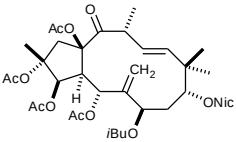

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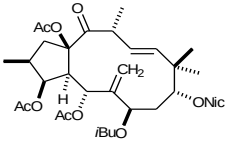

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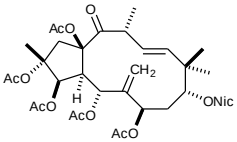

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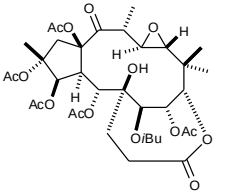

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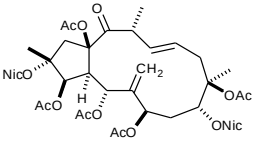

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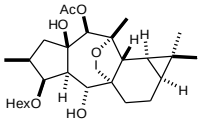

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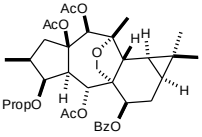

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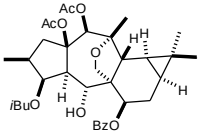

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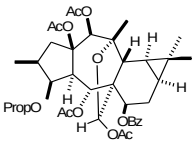

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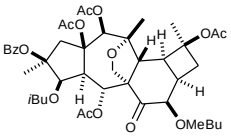

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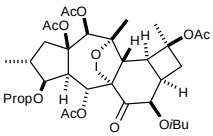

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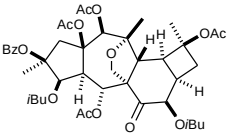

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In the EFAL series, EFAL-7 (**15**) contains a rare hemiacetal moiety, such diterpenes being very rare in the Euphorbiaceae family. EFAL-3 (**12**), EFAL-4 (**13**), EFAL-6 (**14**) and EFAL-7 (**15**) are the first known premyrsinane-type diterpenes containing an acyl moiety instead of a keto group on C-14. Biogenetically, premyrsinanes can be derived from epoxylathyranes by intramolecular cyclization, and they are the precursors of cyclomyrsinanes. Cyclomyrsinane diterpenes are very rare in the plant kingdom; only 7 compounds have been isolated previously from other *Euphorbia* species. Moreover, EFAL-19 (**16**) and EFAL-20 (**17**) are substituted with an ester group at C-2, which is also unprecedented. Similarly to the EUP series, the EFAL series is stereochemically homogeneous, but interestingly the configuration of C-16 is *β* in the premyrsinanes (EFAL-3, EFAL-4, EFAL-6 and EFAL-7) and *α* in the cyclomyrsinanes (EFAL-19 – EFAL-21).

Chemotaxonomical significance

On the basis of the diterpene composition, *E. esula* displays a close relationship with *E. salicifolia*; these species belong in the same section. They contain the same main diterpene components, esulatin A, EUP-13 (salicinolide, **10**) and EUP-17 (euphosalicine, **11**) and other jatrophane diterpenes differing only in the esterification. The diterpenes isolated from *E. esula* in our experiment are not identical with those obtained by other workgroups. It can be observed that samples of different origins (China, North America and Hungary) contain different diterpenes. In the EUP series obtained from the Hungarian collection, the alcohol core of the compounds was different. In this species, the morphological diversity (characteristic of *E. esula*) is manifested in the chemical features (the diterpene profile), too.

The chemical constituents of *E. pannonica* and *E. falcata* have not been investigated previously.

All of the isolated diterpenes were detected for the first time in the given plants.

Biological activity of the isolated compounds

The isolated compounds were tested for their antitumour and MDR-reversing activities.

Antitumour activity: As many experimental data have been published in the past few years on the antitumour activities of Euphorbiaceae diterpenes, the antiproliferative activities of the isolated diterpenes, together with 5 jatrophane diterpenes (esulatins A, B, D–F) identified in our earlier experiments were evaluated against human tumour cell lines (HeLa, Ishikawa, MCF7 and A431) using the MTT test and with cisplatin as positive control. Antiproliferative assays of some

jatrophane (**4**, **5** and esulatins A and E), premyrsinane (**12–15**) and cyclomyrsinane (**17**) esters demonstrated strong activity against human tumour cells (Table 1).

Table 1. Inhibition (%) of tumour cell proliferation by *Euphorbia* diterpenes[a]

Compound	HeLa		Ishikawa		MCF7		A431	
	10 μg/mL	30 μg/mL	10 μg/mL	30 μg/mL	10 μg/mL	30 μg/mL	10 μg/mL	30 μg/mL
EUP-28(**4**)	15.4	23.8	8.0	29.4	12,7	**60.1**	-	-
EUP-32(**5**)	19.1	**64.5**	18.4	**98.4**	46.8	**81.4**	-	-
esulatin A	16.3	**62.6**	20.1	**53.8**	21.4	47.9	-	-
esulatin E	19.5	**58.1**	35.6	**54.1**	30.4	**61.4**	-	-
EFAL-3 (**12**)	12.8	**60.4**	-	-	30.6	**56.0**	23.0	35.6
EFAL-4 (**13**)	22.2	**56.9**	-	-	21.1	49.1	36.2	**81.4**
EFAL-6 (**14**)	25.8	**83.9**	-	-	33.6	**59.2**	38.0	**93.6**
EFAL-7 (**15**)	20.6	47.3	-	-	25.9	38.9	39.2	**69.1**
EFAL-20 (**17**)	16.9	33.7	-	-	17.3	**53.3**	38.9	45.1

[a] Positive control cisplatin: 12.4 μM (HeLa), 3.5 μM (Ishikawa and A431) and 9.6 μM (MCF-7).

Multidrug resistance (MDR) reversal activity: The compounds in the EUP and EFAL series were examined for their MDR-reversing activity and it was concluded that they enhance drug retention significantly in tumour cells by inhibiting the efflux pump activity. EUP-32 (**5**), EUP-29 (**8**), EFAL-4 (**13**), EFAL-6 (**14**), EFAL-7 (**15**), EFAL-19 (**16**) and EFAL-20 (**17**) exhibited much stronger effects than that of the positive control verapamil (Table 2). Moreover, members of the EFAL series displayed a synergistic effect with doxorubicin. The ability of premyrsinane and cyclomyrsinane diterpenes to act as potent modulators of MDR has been evaluated here for the first time.

Table 2. Reversal of the MDR of mouse lymphoma cells by diterpenoids isolated from *E. esula* and *E. falcata*, and the results of the combination assay in the case of *E. falcata*

Compound	Conc. μg/mL	FAR[b]	Compound	Conc. μM	FAR[b]	FIX[c]
EUP-32 (**5**)	4 / 40	24.9 / **52.5**	EFAL-4 (**13**)	2 / 20	23.4 / **74.4**	0.23
EUP-29 (**8**)	4 / 40	16.6 / **119.9**	EFAL-6 (**14**)	2 / 20	29.8 / **69.3**	0.40
			EFAL-7 (**15**)	2 / 20	12.6 / **52.9**	0.73
			EFAL-19 (**16**)	2 / 20	46.2 / **52.7**	0.15
			EFAL-20 (**17**)	2 / 20	36.9 / **62.3**	0.32
verapamil	10	23.2	verapamil	22	8.77	

[b] Fluorescence activity ratio (FAR): FAR = (MDR treated/MDR control)/(PAR treated/PAR control); (PAR = parental cell line). [c] Fractional inhibitory index (FIX): FIX = $FIX_{compound}$+ $FIX_{doxorubicin}$; $FIX_{(compound)}$ = $IC_{50(compound + doxorubicin)}/IC_{50(compound)}$ and $FIX_{(doxorubicin)}$ = $IC_{50(compound + doxorubicin)}/IC_{50doxorubicin}$.

Our results open up new opportunities in the design and development of drugs to overcome the MDR of human cancers.

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List of publications

The thesis is based on the following publications:

1. **Sulyok E**; Vasas A; Rédei D; Dombi G; Hohmann J: Isolation and structure determination of new 4,12-dideoxyphorbol esters from *Euphorbia pannonica* Host., *Tetrahedron* 2009; **65**: 4013-4016.
   If: 3.219

2. Vasas A; **Sulyok E**; Rédei D; Forgo P; Szabó P; Zupkó I; Berényi A; Molnár J; Hohmann J: Jatrophane diterpenes from *Euphorbia esula* as antiproliferative agents and potent chemosensitizers to overcome multidrug resistance, *J. Nat. Prod*. 2011; **74**: 1453-1461.
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3. **Sulyok E**; Vasas A; Rédei D; Forgo P; Kele Z; Pinke G; Hohmann J: New premyrsinane-type diterpene polyesters from *Euphorbia falcata*, *Tetrahedron* 2011; **67**: 7289-7293.
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4. Vasas A; **Sulyok E**; Martins A; Rédei D; Forgo P; Kele Z; Zupkó I; Molnár J; Pinke G; Hohmann J: Cyclomyrsinane and premyrsinane diterpenes from *Euphorbia falcata* modulate resistance of cancer cells to doxorubicin, *Tetrahedron* 2012; **68**: 1280-1285.
   If: 3.025*

*The impact factor for the year 2011 is given.

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1. Csupor D; Szendrei K; Tatsimo NJ; **Sulyok E**; Hohmann J:
   A naplemente valódi ereje – Szintetikus potenciafokozó a Pote-Mix Bummban
   Gyógyszerészet 2010; **54**: 526-531.

2. Csupor D; Szekeres A; Tatsimo NJ; Kecskeméti A; Vékes E; Veres K; **Sulyok E**; Szendrei K; Hohmann J:
   Szintetizálnak-e a növények Viagrát? – Szintetikus hatóanyagokkal hamisított „növényi" étrend-kiegészítők
   Magyar Kémikusok Lapja 2011; **66**: 45-49.

Book chapters:

1. **Sulyok E**:
   Csattanó maszlag, Gyömbér, Kasvirág, Maszlagos nadragulya, Máriatövis (monográfiák)
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