Isolation and structure elucidation of bioactive compounds from *Euphorbia* species

Summary of the Ph.D. Thesis

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Szeged, Hungary
2020

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INTRODUCTION

The genus *Euphorbia* is among the largest genera of flowering plants with approximately 1900 recognized species. Members of this nearly cosmopolitan genus can be characterized by the frequent occurrence of white, milky latex. Previously, several hypotheses have been proposed to explain the function of latex, like storage of plant nutrients, deposit of waste products, or water reserve, but nowadays it is widely considered as a key part of the plant self-defence mechanisms. This hypothesis has been supported by the fact that latices often contain specialized defensive metabolites (e.g. terpenoids, proteins) at much higher concentrations compared to other plant organs. *Euphorbia* species are collectively known as "spurges". Their name has been derived from the Medieval French word "epurger" ("expurgare" in Latin), referring to the purgative properties of the latex and seeds.

Spurges are commonly used in folk medicine for the treatment of microbial (e.g. gonorrhoea, syphilis) and parasitic infections, obstipation, asthma, coughs, rheumatism, snakebites, wounds and haemorrhages, eczema, sores, warts, and other skin disorders. In many cases, traditional applications of the plants were supported by modern pharmacological evidence.

Euphorbia species are prolific producers of macrocyclic diterpenoids. Due to their structural diversity and promising bioactivities, macrocyclic diterpenoids represent an intriguing group of secondary metabolites from the point of view of natural product discovery. In the last few decades macrocyclic diterpenoids have been the subject of extensive phytochemical investigations leading to the isolation of hundreds of new diterpene derivatives with more than 20 different skeletons. Furthermore, cyclic derivatives of the macrocyclic cembrane class are of chemotaxonomic significance because their distribution in the plant kingdom is limited to the Euphorbiaceae and Thymeleaceae families.

AIMS OF THE STUDY

In 1995, Hohmann *et al*. (Department of Pharmacognosy, University of Szeged) initiated a research project with the aim of investigating the secondary metabolites of *Euphorbia* species. As a part of ongoing research, the goals of my work were the isolation and structure determination of new diterpenoids from four selected spurge species (*Euphorbia dulcis* L., *E. taurinensis* All., *E. guyoniana* Boiss. & Reut., *E. davidii* Subils.), followed by the investigation of their bioactivity, executed in collaboration with cooperative partners. In order to meet these objectives, the main tasks were as follows:

- Screening of the plant materials for diterpenoid contents.
- Extraction of the plant materials.
- Chromatographic purification and isolation of diterpenoids.
- Structure elucidation of the isolated compounds.
- Assessment of the chemotaxonomic importance of the isolated compounds.
- Evaluation of the pharmacological properties of diterpenoids.

MATERIALS AND METHODS

The whole plants of *E. dulcis* were gathered in the flowering period at Homoródalmás, Romania. The whole plants of *E. taurinensis* were collected in Budapest, Hungary. The aerial parts of *E. guyoniana* were harvested in the droughty region Grand Erg Oriental of southern Tunisia. The whole plants of *E. davidii* were collected near Igar, Hungary.

The screening of plant materials (excepting *E. guyoniana*) for diterpenoid content started with the extraction of plant samples with MeOH. Diterpenoids have previously been described from *E. guyoniana*, thus screening of this Saharan species was unnecessary. The concentrated extracts were diluted with H_2O, and then partitioned with $CHCl_3$. The $CHCl_3$-soluble phases were separated on polyamide columns by using gradient solvent systems of MeOH–H_2O. The collected fractions

were investigated by thin-layer chromatography (TLC) under UV light (254 nm), then the chromatograms were visualized by *cc*. H_2SO_4 followed by heating the plates (110 ˚C).

The plant materials of *E. dulcis*, *E. taurinensis*, and *E. davidii* were percolated with MeOH, while the air-dried powder of *E. guyoniana* was extracted with $CHCl_3$. The extracts were subjected to liquid-liquid extraction, then the compounds of interest were further purified by multistep chromatographic methods including open-column chromatography (OCC), vacuum-liquid chromatography (VLC), preparative layer chromatography (PLC), and high-performance liquid chromatography (HPLC). Polyamide for OCC, and normal- (NP) and reversed-phase (RP) silica gel for VLC, PLC, and HPLC were used as stationary phase.

The structures of the isolated compounds were elucidated by means of 1D (^{1}H, JMOD) and 2D (HSQC, HMBC, ^{1}H-^{1}H COSY, NOESY) NMR experiments coupled with HRESIMS measurements. The absolute configuration of one diterpenoid (compound **1**, see later) was determined by single-crystal X-ray diffraction.

Plant extracts from *E. davidii* were prepared for pharmacological screening. The MeOH extract of the plant was partitioned with *n*-hexane, $CHCl_3$, and EtOAc. The dried plant material was subsequently extracted with boiling water.

The isolated diterpenoids were investigated on stable transfected HEK-GIRK1/4 (Kir3.1/3.4) and HEK-hERG (Kv11.1) cell lines. The ion currents were measured by an automated patch-clamp equipment. The MDR-modulating and cytotoxic effects of the diterpenoids were studied on L5178 mouse T-lymphoma cell line using rhodamine 123 accumulation and MTT cell viability assays. The antiproliferative activities of plant extracts of *E. davidii* were evaluated on HeLa (cervix epithelial adenocarcinoma), MCF7 (breast epithelial adenocarcinoma), A2780 (ovarian carcinoma), and A431 (skin epidermoid carcinoma) cell lines by MTT assay.

RESULTS AND DISCUSSION

Screening of the plant materials for diterpenoid content

The MeOH extracts of *E. dulcis* and *E. taurinensis* were separated by OCC. TLC chromatograms of the collected fractions revealed that the plants contain complex mixtures of diterpenoids. The diterpenoids accumulated in the fractions eluted with 60% aqueous MeOH, as suggested by the dense brown, grey, and black spots on the plates with R_f values of 0.15–0.70. TLC chromatogram of the extract of *E. davidii* did not exhibit characteristic spots attributable to diterpenoids.

Isolation of diterpenoids from *E. dulcis*

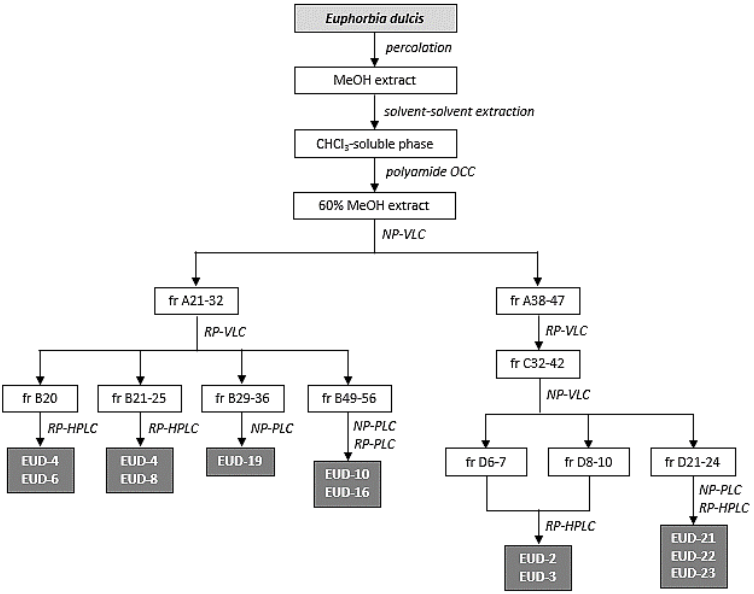


Figure 1. Isolation of diterpenoids from *E. dulcis*

The crude MeOH extract of *E. dulcis* was partitioned with $CHCl_3$ (**Figure 1**). The $CHCl_3$-soluble phase was fractionated by OCC with mixtures of MeOH–H_2O (3:2, 4:1, 1:0). The diterpenoid-rich 60% MeOH fraction was separated by VLC using gradient

cyclohexane–EtOAc–EtOH mobile phases. Further purifications of the combined subfractions A21-32 and A38-47 by various VLC, PLC, and HPLC methods allowed the isolation of 11 diterpenoids (**EUD-2**–**4**, **6**, **8**, **10**, **16**, **19**, **21**–**23**).

Isolation of diterpenoids from *E. taurinensis*

The MeOH extract of *E. taurinensis* was partitioned with $CHCl_3$, then the $CHCl_3$-soluble phase was submitted to polyamide OCC and eluted with mixtures of MeOH and H_2O (3:2, 4:1, 1:0) (**Figure 2**). TLC chromatograms indicated that diterpenoids presented in both 60% and 80% MeOH fractions. The fractions were first separated by VLC using stepwise gradient elution with cyclohexane–EtOAc–EtOH, which yielded 80 (B1-80) and 70 (A1-70) subfractions, respectively. Further purifications of the compounds by means of VLC, PLC, and HPLC resulted in the isolation of 5 diterpenoids (**ETA-1**, **2**, **5**–**7**) from the 80% MeOH fraction, and 2 diterpenoids (**ETA-8**, **9**) from the 60% MeOH portion.

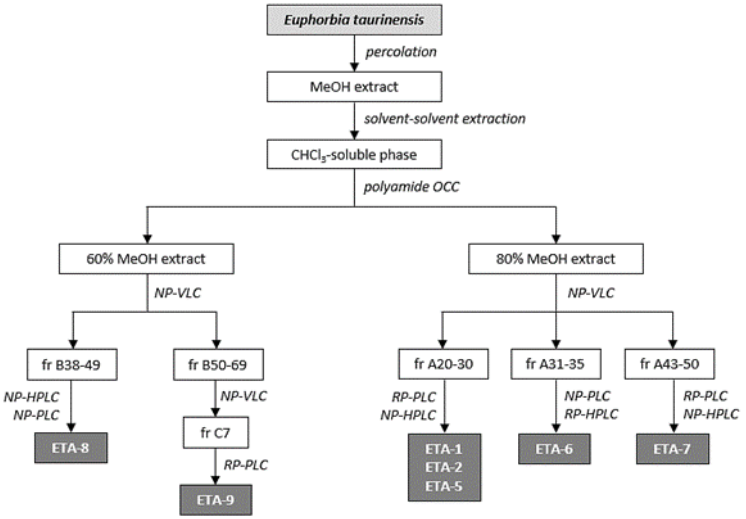


Figure 2. Isolation of diterpenoids from *E. taurinensis*

Isolation of diterpenoids from *E. guyoniana*

The powdered raw material of *E. guyoniana* was extracted with $CHCl_3$ in an ultrasonic bath. Initial fractionation of the lipophilic extract was performed by polyamide OCC using MeOH–H_2O mixtures (3:2, 4:1, 1:0) as mobile phases (**Figure 3**). According to the TLC chromatograms, diterpenoids were selectively enriched in the 60% MeOH fraction. VLC seperation of the 60% MeOH portion was achieved with gradient cyclohexane–EtOAc–EtOH solvent systems. Purifications of the combined subfractions 17-21 and 22-26 were carried out by NP-HPLC and RP-HPLC methods, and yielded 2 diterpenoids (**EGU-3**, **4**).

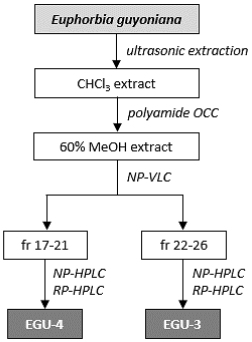


Figure 3. Isolation of diterpenoids from *E. guyoniana*

Isolation of flavonoid glycosides from *E. davidii*

The MeOH extract of *E. davidii* was subjected to repetitive liquid-liquid extraction using $CHCl_3$ and EtOAc (**Figure 4**). As the $CHCl_3$-soluble fraction did not contain any compound worthy of an isolation attempt, the latter and more promising EtOAc phase was chosen for further separation. The isolation procedure started with a VLC step, and mixtures of EtOAc–EtOH–H_2O were applied to elute the compounds. The collected fractions 4, 5, and 6 with high contents of flavonoids were further

separated by different chromatographic techiques (PLC, RP-HPLC). Finally, the plant material afforded 3 flavonoid glycosides (**EDI-17**–**19**).

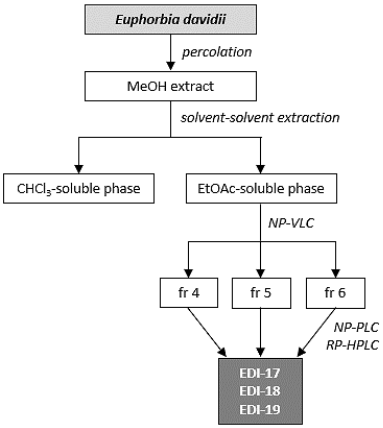


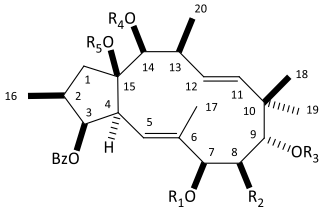
Figure 4. Isolation of flavonoid glycosides from *E. davidii*

Structure determination of the isolated compounds

The flavonoids were identified by comparison of their ^{1}H and ^{13}C NMR data with literature values. The molecular formulas of diterpenoids were determined by means of HRESIMS measurements. The structures of diterpenoids were established by 1D and 2D NMR spectroscopy. The ^{1}H, JMOD, ^{1}H-^{1}H COSY, HSQC, and HMBC spectra revealed the constitutions of the terpenoid scaffolds, then the relative configurations of stereogenic carbons were deduced by relevant NOESY correlations. As a result of the NMR studies, complete ^{1}H and ^{13}C NMR assignments were made for the new natural products. In addition, the absolute configuration of compound **1** was determined by single-crystal X-ray diffraction.

Diterpenoids of *E. dulcis*

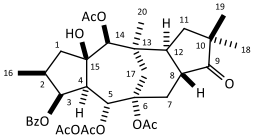
The MeOH extract of *E. dulcis* yielded 9 novel (**1**–**9**) and 2 known (**10**, **11**) jatrophane diterpenoids. Compounds **1**–**11** contain a $\Delta^{(5,6)}$ olefinic bond instead of the regular $\Delta^{(6,17)}$ double bond, and are esterified with acetyl, benzoyl, and tigloyl groups. The diterpenoids of *E. dulcis* differ only in their substitution patterns at C-7, C-8, C-9, C-14, and C-15. The jatrophanes contain a conserved benzoyl moiety at C-3, and interestingly tigloyl groups are found exclusively on C-7 and C-14. Compound **9**, an interesting structural isomer of euphomelliferene B (**10**), have C-14 hydroxy and C-15 acetyl groups. To date just a few jatrophanes have been reported with the same arrangement of substituents.



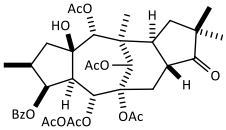
	R_1	R_2	R_3	R_4	R_5
1 (EUD-8)	Tig	H	Ac	Ac	H
2 (EUD-4)	Tig	OAc	Ac	Ac	H
3 (EUD-19)	Tig	OAc	Ac	Tig	H
4 (EUD-16)	H	OAc	Ac	Tig	H
5 (EUD-10)	H	OAc	Ac	Ac	H
6 (EUD-23)	Ac	OH	Ac	Ac	H
7 (EUD-21)	Ac	OH	H	Ac	H
8 (EUD-22)	H	OH	H	Ac	H
9 (EUD-3)	Ac	OAc	Ac	H	Ac
10 (EUD-2)	Ac	OAc	Ac	Ac	H
11 (EUD-6)	Ac	H	Ac	Ac	H

The EUD-series is stereochemically homogeneous, as all jatrophanes are based on a highly oxygenated *trans*-bicyclo[10.3.0]pentadecane core, which comprises 2β and 13β-methyls, and 7β, 8β, 9α, 14β, and 15β acyl or hydroxy functions. Compounds **1** and **11** are not substituted at C-8. Considering the coupling constant pattern and diagnostic NOE correlations, it was deduced that these jatrophanes adopted an *endo*-type conformation, in which the H_3-17 methyl group is perpendicular to the mean plane of macrocycle, and the adjacent protons H-4 and H-5 are antiperiplanar. Furthermore, the absolute configuration of compound **1** was established by single-crystal X-ray diffraction. Compound **1** crystallized in the monoclinic chiral space group $P2_1$ with three crystallographically independent but chemically identical conformers in the asymmetric unit. For all three conformers of **1**, the assignment is most likely (2*S*,3*S*,4*S*,7*R*,9*R*,13*S*,14*S*,15*R*)-9α,14β-diacetoxy-3β-benzoyloxy-15β-hydroxy-7β-tigloyloxyjatropha-5*E*,11*E*-diene.

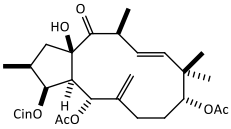
Diterpenoids of *E. taurinensis*



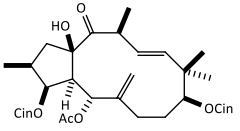
12 (ETA-8)



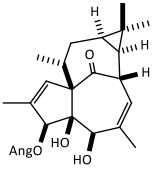
14 (ETA-9)



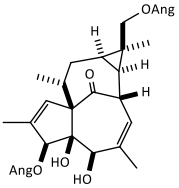
13 (ETA-6)



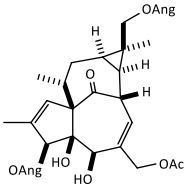
15 (ETA-5)



16 (ETA-1)



17 (ETA-2)



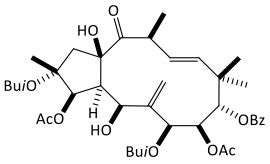
18 (ETA-7)

NMR spectroscopic analysis of the compounds obtained from *E. taurinensis* led to the identification of a novel and a known segetane (**12** and **14**, respectively), a novel

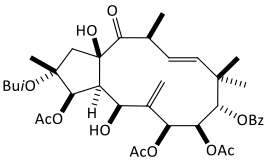
and a known jatrophane (**13** and **15**, respectively), and 3 known ingenane diterpenoids (**16**–**18**). The segetanes represent a unique class of diterpenoids: only 12 compounds had been isolated from *E. segetalis*, *E. paralias*, *E. portlandica*, and *E. peplus* prior to our work. Unlike most of the previously described segetanes, compound **12** contains a β-oriented acetyl group at C-14, and the C-17 bridge is not substituted. In compounds **12** and **14** a rare acetoxyacetyl moiety is attached to C-5. The novel jatrophane **13** bears an α-acetyl group at C-9, while the structurally related diterpenoid (**15**) possesses a C-9 β-cinnamoyl group. Compounds **13** and **15** were determined as *endo*-conformers based the on relevant coupling constant values and NOE interactions.

Diterpenoids of *E. guyoniana*

The $CHCl_3$ extract of *E. guyoniana* yielded 2 new (**19**, **20**) jatrophane diterpenoids. The compounds are highly esterified with acetyl, benzoyl, and isobutyryl groups, and differ from each other in only one substituent: **19** contains a C-7 isobutyryl moiety, while a C-7 acetyl group is found in **20**. Similarly to other jatrophanes previously reported from the plant, **19** and **20** are esterified at C-2, and a keto group is located at C-14. The coupling constant values and important NOE correlations revealed the *exo*-conformation of the diterpenoids, in which the exomethylene points outward, and is parallel with the mean plane of the twelve-membered ring.



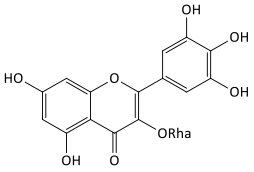
19 (EGU-4)



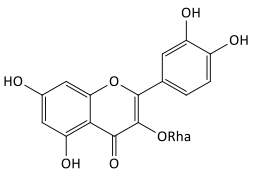
20 (EGU-3)

Flavonoid glycosides of *E. davidii*

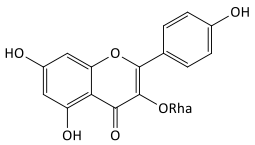
The EtOAc phase of the MeOH extract of *E. davidii* afforded 3 flavonoid glycosides (**21**–**23**). The flavonoids were identified as myricetin-3-*O*-rhamnoside (**21**), quercetin-3-*O*-rhamnoside (**22**) and kaempferol-3-*O*-rhamnoside (**23**).



21 (EDI-17)



22 (EDI-18)



23 (EDI-19)

Chemotaxonomical aspects

Macrocyclic diterpenoids are considered to be important taxonomic biomarkers because of their structural diversity and limited distribution in the plant kingdom. *E. dulcis* afforded a series of jatrophane diterpenoids bearing a $\Delta^{(5,6)}$ olefinic bond instead of the more frequent 6(17)-exomethylene group. This structural feature has only been found in a minority of jatrophanes and, interestingly, most of those diterpenoids were reported from spurge species of subgenus *Esula*. In light of this, the presence of such compounds might be a useful chemotaxonomic marker for the characterization of members of the subgenus.

Our finding that *E. taurinensis* produces segetanes strongly supports the new taxonomic classification of *E. taurinensis*. The close intra-generic relationships between *E. taurinensis*, *E. segetalis*, and *E. paralias* have also been demonstrated by similarities in their diterpenoid compositions: compounds **14**, **16**, and **17** have earlier been identified in both *E. segetalis* and *E. paralias*, while **15** and **18** were described from *E. segetalis*.

Regarding *E. guyoniana*, in previous studies plant materials of Algerian origin were investigated, while we received the plant sample from Tunisia. The two isolated diterpenoids (**19** and **20**) are not identical with the previously reported jatrophanes.

This finding suggests a great variation in the diterpenoid compositions of populations of *E. guyoniana* grown at different geographical locations.

The latest classification suggests that *E. davidii* belongs to section *Poinsettia* of subgenus *Chamaesyce*. So far diterpenoids have only been detected in species of section *Anisophyllum*, and this fact could provide a reasonable explanation to why we failed to obtain any diterpenoids from *E. davidii* in spite of our best efforts.

The chemical constituents of *E. taurinensis* and *E. davidii* have not been studied previously. Furthermore, all isolated diterpenoids and flavonoid glycosides are described for the first time in the investigated spurges.

Bioactivity of the isolated compounds and plant extracts

Ion channel blocking activity of the diterpenoids

Diterpenoids are characteristic secondary metabolites of the *Euphorbia* species, however, limited data are available on their cardiac effects. Vasas *et al*. reported that myrsinane, premyrsinane, and cyclomyrsinane diterpenoids of *E. falcata* exert a selective GIRK blocking activity, and this finding served as a motivation to explore the electrophysiological effects of the isolated diterpenoids. Compounds **1**–**12** and **15**–**20** were investigated on a HEK-GIRK1/4 (Kir3.1/3.4) cell line. Majority of the tested compounds were found to exert a significant inhibitory effect on the GIRK proteins at 10 μm, and some of them displayed a notable blocking activity even at 1 μm concentrations.

The IC_{50} values of the most potent diterpenoids were determined from the dose-response curves as follows: **1**: 1.3 ± 0.2 μM; **2**:1.6 ± 0.2 μM; **9**: 3.4 ± 0.1 μM; **10**: 1.7 ± 0.2 μM; **11:** 2.6 ± 0.5 μM; **16**: 12.2 ± 0.5 μM; **17**:1.5 ± 0.1 μM. These jatrophane and ingenane diterpenoids were tested for their hERG-related cardiotoxicity on HEK-hERG (Kv11.1) cells. The results of the experiment demonstrated that none of the jatrophanes interfered with the function of the hERG proteins, however, the outward K^+ flow was strongly hampered by compound **17**.

To the best of our knowledge, our group is the first one to evaluate the electrophysiological effects of jatrophanes, segetanes, and ingenanes on GIRK and hERG proteins. Our investigations focusing on the GIRK channels showed no clear correlations between the inhibitory effects and the substitution patterns, so unfortunately, we could not establish any structure-activity relationships for the EUD-series. Nevertheless, jatrophane and ingenane diterpenoids were proven to be potent inhibitors of the atrial GIRK proteins. Considering the selective activities of jatrophanes on GIRK channels, they may represent a group of potential lead compounds for the development of novel therapeutic agents against atrial fibrillation.

MDR-reversing and cytotoxic activities of diterpenoids of E. taurinensis

Euphorbia diterpenoids are best known for their strong MDR-reversing activity. Therefore, we examined the P-gp modulating and cytotoxic properties of compounds **12** and **15**–**18** on an L5178 mouse lymphoma cell line (**Figure 5**).

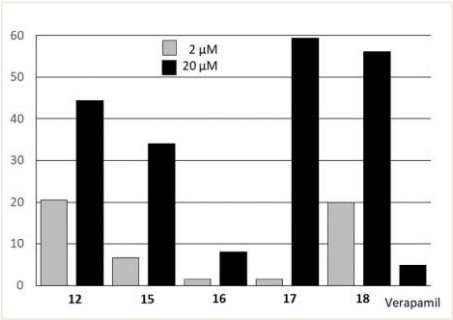


Figure 5. Efflux pump modulating activities of the tested diterpenoids

Compared to the positive control verapamil, all compounds were found to inhibit the P-gp efflux pump on the resistant mouse T-lymphoma cells. Compounds **12**, **17**, and **18** were shown to be the most powerful modulators at a concentration of 20 µM, with an efficacy of 7-9-fold higher compared to verapamil.

Based on our findings, neither the segetane, nor the jatrophane diterpenoids tested exert any cytotoxic activity on the sensitive parent and on the resistant MDR cells in the MTT assay. In contrast, the most active ingenane diterpenoids **17** and **18** displayed a cytotoxic effect on both cell lines. The IC_{50} values of the most active compound **17** on the two cell lines were almost equal, indicating that it has no selectivity towards the resistant cell line, while compound **18** was more potent on the resistant cell line. The presence of a larger ester function at C-17 might enhance the cytotoxicity of the ingenanes, however, further data are needed to confirm this speculation.

Antiproliferative activities of the extracts of E. davidii

The prepared extracts (*n*-hexane, $CHCl_3$, EtOAc, 50% MeOH residue, H_2O) were screened *in vitro* for their antiproliferative activity against HeLa, MCF7, A2780, and A431 cell lines. The *n*-hexane and $CHCl_3$ extracts were found to exhibit a dose-dependent cell growth inhibitory activity on all cell lines. The flavonoid-rich EtOAc extract did not inhibit the proliferation of any cancer cell lines.

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THE THESIS IS BASED ON THE FOLLOWING PUBLICATIONS

1. **Kúsz N**, Orvos P, Bereczki L, Fertey P, Bombicz P, Csorba A, Tálosi L, Jakab G, Hohmann J, Rédei D.
   Diterpenoids from *Euphorbia dulcis* with potassium ion channel inhibitory activity with selective G protein-activated inwardly rectifying ion channel (GIRK) blocking effect
   Journal of Natural Products **2018**, 81: 2483-2492. If: 4.257

2. Rédei D, **Kúsz N**, Sátori G, Kincses A, Spengler G, Burián K, Barina Z, Hohmann J.
   Bioactive segetane, ingenane, and jatrophane diterpenes from *Euphorbia taurinensis*
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4. **Kúsz N**, Szabó M, Pinke Gy, Zupkó I, Hohmann J.
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PRESENTATIONS HELD IN THE SAME THEME OF THE THESIS

1. **Kúsz N**, Rédei D, Dankó B, Hohmann J.
   Euphorbia dulcis, az ígéretes diterpénforrás – Egy szűrővizsgálat eredményei
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2. **Kúsz N**, Dankó B, Pinke Gy, Jakab G, Rédei D, Hohmann J.
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4. **Kúsz N**, Forgó P, Hohmann J, Rédei D.
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   Az MTA Szteroid és Terpenoidkémiai Munkabizottság és az MTA Szegedi Akadémiai Bizottság Szerves és Gyógyszerkémiai Munkabizottság előadóülése
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   Two new jatrophane diterpenes from *Euphorbia guyoniana*
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   Budapest, Hungary, 23-27 August 2015.

6. **Kúsz N**, Rédei D, Orvos P, Tálosi L, Jakab G, Hohmann J.
   Isolation and structure determination of novel jatrophane diterpenes from *Euphorbia dulcis* and their GIRK-channel-inhibitory activity
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