Isolation and structure elucidation of phenanthrenes from *Juncus* species

Summary of PhD Thesis

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INTRODUCTION

Cancer is one of the leading causes of death globally and development of new anticancer agents are in the focus of research worldwide. Although conventional chemotherapy plays an important role in the treatment of cancers, but clinical limitations exist because of dose-limiting side effects and drug resistance. A growing body of combination treatments with natural products has been reported to synergistically prevent tumor growth; therefore, they can be served as a promising therapeutic strategy with a higher clinical efficacy. Besides combination with standard drugs, the efficacy and bioavailability of natural compounds can further increase by applying different formulation techniques. Moreover, natural products are still the best options for finding novel agents/active templates and offer the potential to discover novel structures and new templates that can lead to effective agents in a variety of cancers. Novel biomolecules have an advantage in terms of biosafety, and they can serve as leads for synthetic chemists and pharmacologists. The effective anticancer drugs often work by inhibiting angiogenesis, inducing apoptosis, and blocking cancer cell proliferation. A common feature of phytochemicals is attenuating cancer progression by inhibition of inflammation and induction of apoptosis through caspase-dependent mechanisms or induction of intracellular oxidative stress. Furthermore, natural compounds can target multiple key regulators. Several molecular targets and the mechanisms of action of natural compounds are already explored and great efforts are performed to increase their efficiency by using structure-based drug design strategies. Recent advances in drug delivery systems describe the use of nanoemulsions, nanoparticles, liposomes, and films to carry various phytochemicals such as berberine, curcumin, resveratrol, camptothecins, and celastrol, showing a promising improved anticancer action.

A promising group of natural small molecules are phenanthrenes. Phenanthrenes possess noteworthy pharmacological activities, such as antiproliferative, anti-inflammatory, and antimicrobial properties. Among Juncaceae phenanthrenes, dehydroeffusol, juncusol, and juncuenin B seems to be the most promising ones. All of them showed noteworthy antiproliferative effect against different human cancer cell lines.

In 2014, a research program was initiated in the Department of Pharmacognosy, University of Szeged with the aim of investigating the special metabolites of Juncaceae species. This program involves the phytochemical and pharmacological investigation of rushes, isolation of biologically active phenanthrenes, and semisynthetic derivatization of the most promising ones. To date, more than 20 species were screened in the framework of this project; biologically active phenanthrenes were isolated from *Juncus compressus*, *J. inflexus*, *J. tenuis*, and *Luzula luzuloides* and semisynthetic derivatives were prepared from juncuenin B, juncusol and effusol.

AIMS OF THE STUDY

The family Juncaceae is a plentiful source of phenanthrenes. A few years ago, a research program has been started in the Department of Pharmacognosy, University of Szeged with the aim of investigating the secondary metabolites of plants belonging to the family Juncaceae. The objectives of the present work were the isolation and structural characterization of phenanthrenes, and investigation of their pharmacological effects.

In order to achieve the aims, the main tasks of the presented study were:

- Collection of Juncaceae plant samples (altogether four species).
- Preparation and fractionation of plant extracts.
- Isolation of compounds of *Juncus atratus, J. gerardii, J. maritimus,* and *J. ensifolius* using a combination of different chromatographic methods.
- Structure determination of the isolated compounds by spectroscopic methods (1D and 2D NMR, HR-MS).
- Investigation of the antiproliferative effect of isolated compounds in different test systems.

MATERIALS AND METHODS

Juncus species were collected from different areas of Hungary (*J. atratus*, *J. gerardii*) and Croatia (*J. maritimus*) between 2015 and 2019 or bought from a nursery (*J. ensifolius*) for the preparative phytochemical work. The plants were dried at room temperature, then ground and percolated with methanol. After addition of 50% methanol to the concentrated extracts, solvent–solvent partitions were performed with *n*-hexane, chloroform/dichloromethane, and ethyl acetate. The compounds were isolated by multi-step chromatographic methods, including open-column chromatography (OCC), vacuum-liquid chromatography (VLC), medium pressure liquid chromatography (MPLC), preparative thin-layer chromatography (PLC), gel filtration (GF) and high-performance liquid chromatography (HPLC). Normal (NP) and reversed phase (RP) silica gel, polyamide and Sephadex LH-20 gel were used as stationary phases.

The structure of the isolated compounds was determined using UV spectroscopy, high-resolution mass spectroscopy (HR-MS), and nuclear magnetic resonance spectroscopy (NMR).

The antiproliferative properties of the isolated phenanthrenes were determined on human malignant cell lines using the MTT assay. Cisplatin and doxorubicin were used as positive controls. Reduced MTT was detected at 545 nm using a microplate reader and IC_{50} values were calculated using GraphPad Prism 4.0. All *in vitro* experiments were performed on two microplates with five parallel wells. Stock solutions (10 mM) of the investigated compounds were prepared in DMSO. The highest DMSO content of the culture medium (0.3%) had no significant effect on cell proliferation. In the case of *J. ensifolius*, the active components were tested in combination with doxorubicin on HeLa cell lines.

RESULTS AND DISCUSSION

Extraction and isolation of phenanthrenes

Dried and ground plant materials were extracted with methanol in a percolator at room temperature, and then solvent–solvent partitions were applied with *n*-hexane, chloroform/dichloromethane and ethyl acetate. The organic phases containing the phenanthrenes were subjected to multistep chromatographic procedures in order to isolate the compounds.

Isolation of compounds from *Juncus atratus*

The concentrated CH_2Cl_2-soluble fraction of *J. atratus* was separated on a polyamide column (OCC) with gradient system of MeOH–H_2O [1:1, 2:1, each eluent was collected as a fraction (I. and II.)] (**Figure 1**). Phenanthrenes were enriched in fraction II., therefore, it was subjected to NP-VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH to yield 15 major fractions (II/1–15). As fractions were demonstrated great chemical complexity, more selective methods, including RP-VLC, NP-PLC, GF, and RP-HPLC were applied to extract the components. The purification process led to the isolation of 9 compounds (**1**–**9**).

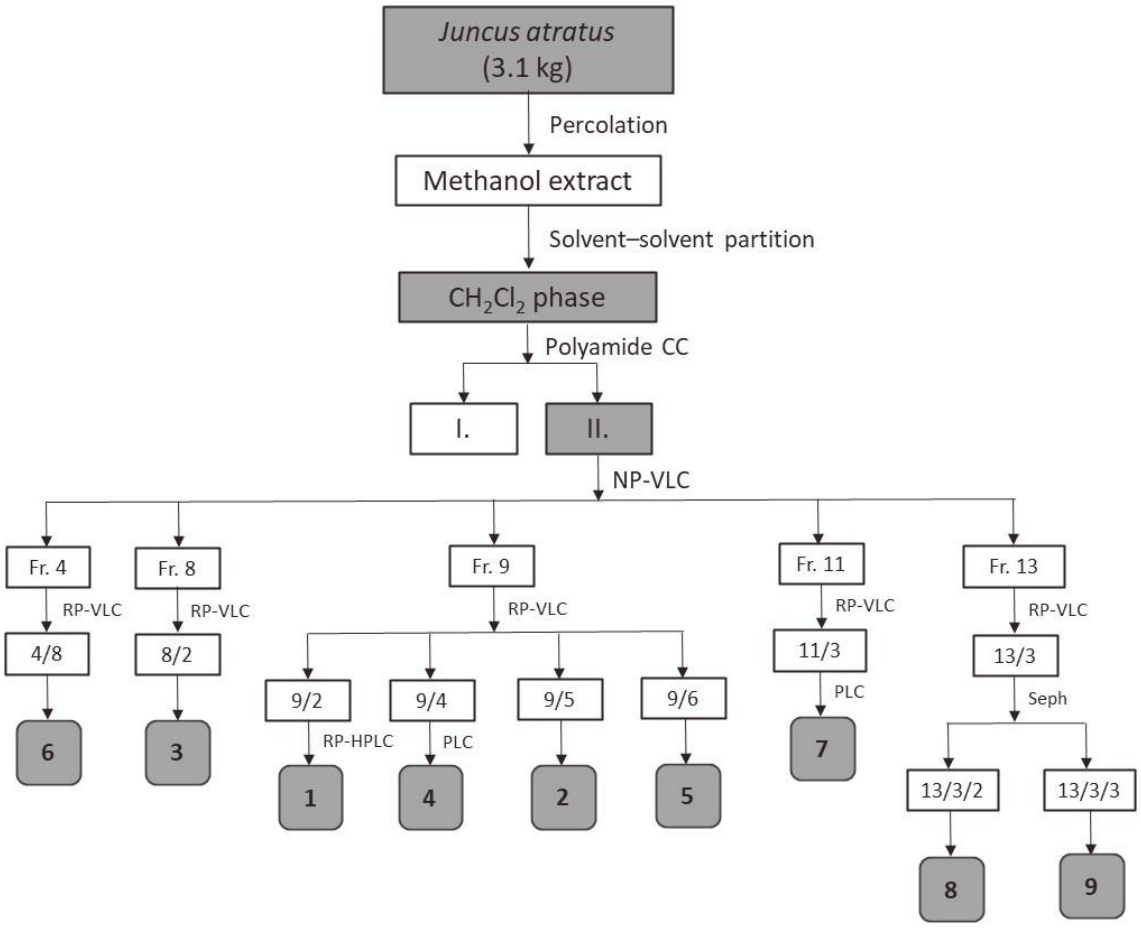


Figure 1. Isolation of the compounds of *J. atratus*

Isolation of compounds from *Juncus gerardii*

The concentrated chloroform-soluble fraction of *J. gerardii* was separated by polyamide OCC with gradient system of MeOH–H_2O [2:3 (A), 3:2 (B), and 2:1 (C, D, E), respectively]. Fractions B and D were subjected to VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH, to yield 16 major fractions (B/1−16) in case of fraction B and 6 major fractions (D/1−6) in case of fraction D. The concentrated EtOAc-soluble fraction (F) was also separated by VLC on silica gel with a gradient system of $CHCl_3$−MeOH, and 15 major fractions (F/1−15) were obtained.

For further separation of the main fractions RP-MPLC, GF, NP-PLC, RP-PLC, NP-HPLC, RP-HPLC methods were used and altogether 23 compounds were isolated (**4**–**7**, **10**–**21**, **22**–**31**) from the plant (**Figure 2**).

Isolation of compounds from *J. maritimus*

After extraction and solvent–solvent partition, the concentrated $CHCl_3$-soluble fraction of *J. maritimus* was separated by VLC on silica gel with a gradient system of cyclohexane–EtOAc–MeOH. This separation yielded 14 main fractions (1–14). All major fractions were purified at first by column chromatography on Sephadex LH-20 gel using CH_2Cl_2–MeOH (1:1) as eluent, and then by RP-MPLC, NP-HPLC, RP-HPLC and NP-PLC to yield 11 compounds (**4**, **24**, **26**, **30**, **32**–**38**) (**Figure 3**).

Isolation of compounds from *J. ensifolius*

After extraction of the plant material with MeOH and solvent–solvent partition with *n*-hexane, $CHCl_3$ and EtOAc, the concentrated $CHCl_3$-soluble fraction was separated by VLC on silica gel using a gradient solvent system of cyclohexane–EtOAc–MeOH to collect 14 major fractions (1–14). In the second step, all major fractions were purified by Sephadex LH-20 gel chromatography using CH_2Cl_2–MeOH (1:1) as eluent. The fractions were then further purified by RP-MPLC and RP-HPLC, and a total of 19 components were yielded (**2**, **3**, **7**, **17**, **39**–**53**) (**Figure 4**).

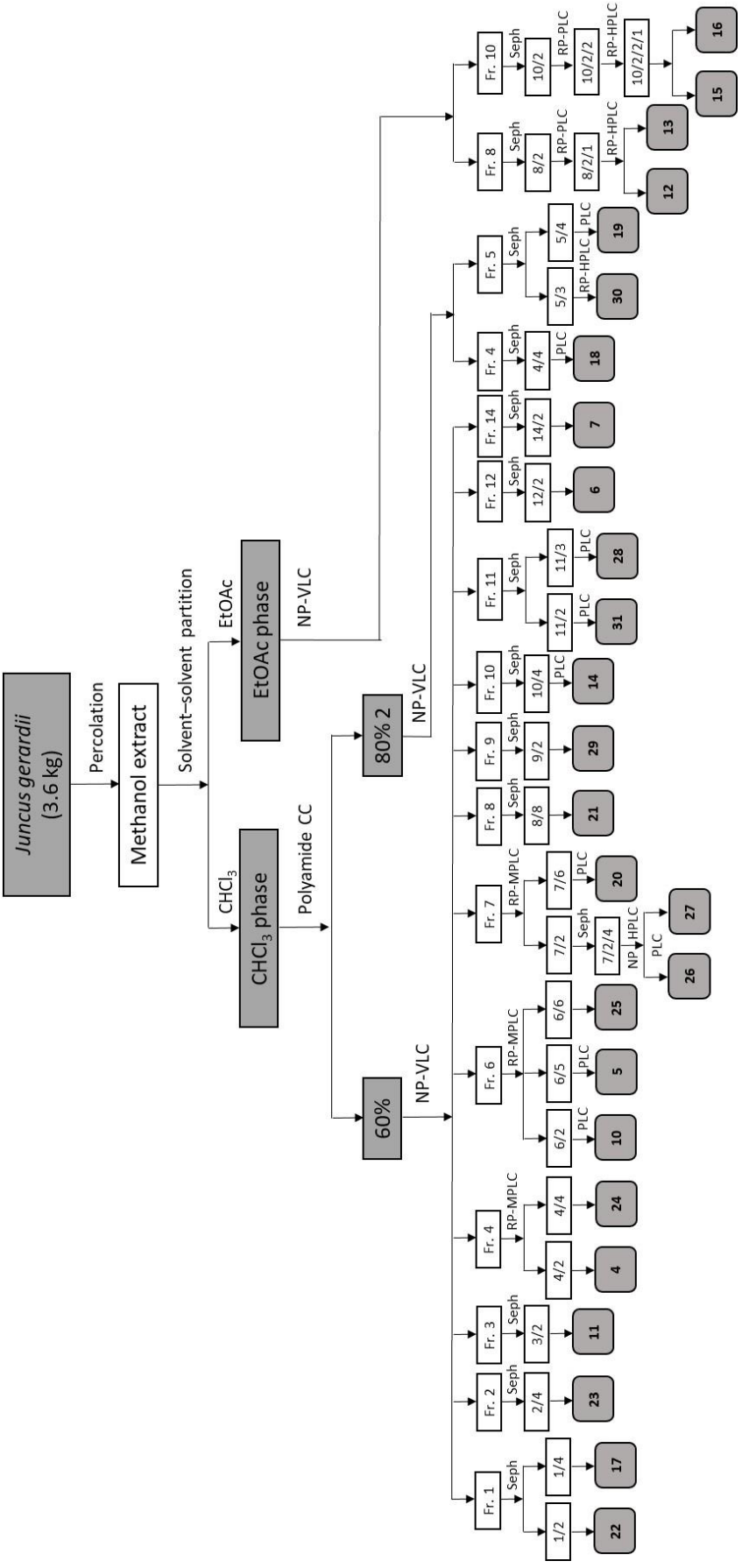


Figure 2. Isolation of compounds from *J. gerardii*

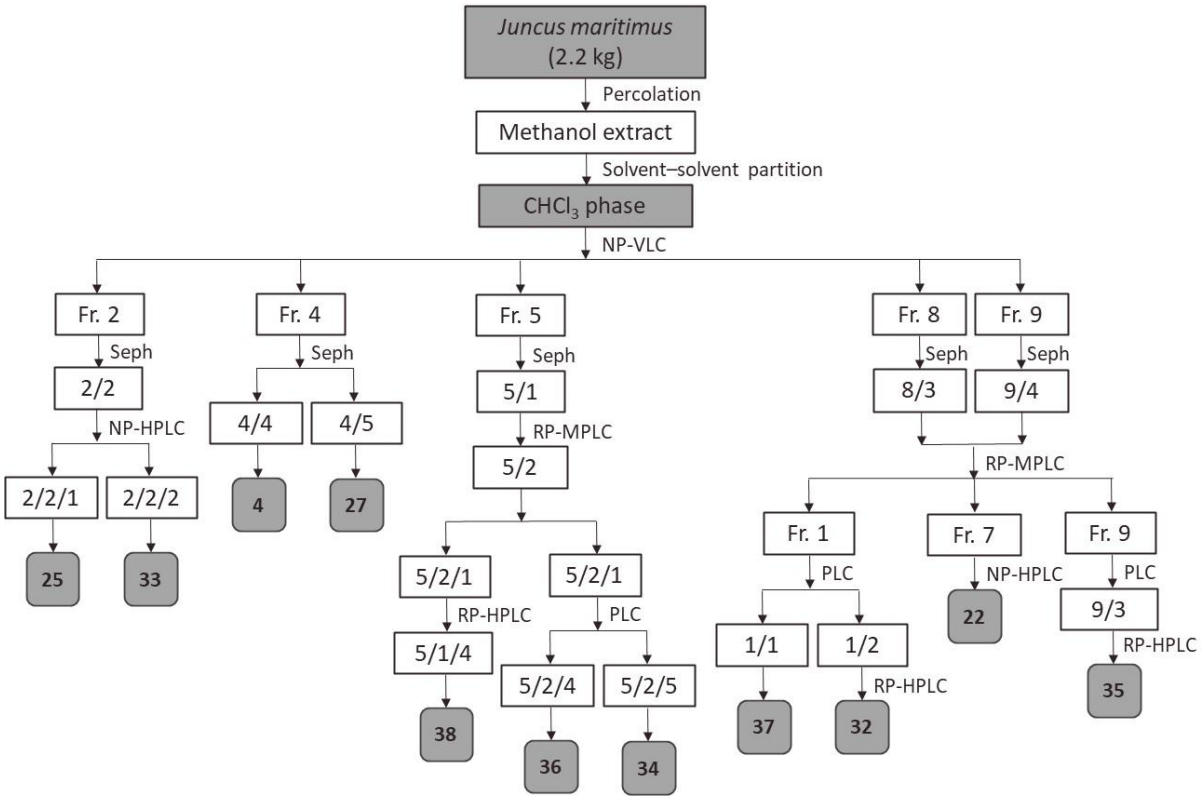


Figure 3. Isolation of compounds from *J. maritimus*

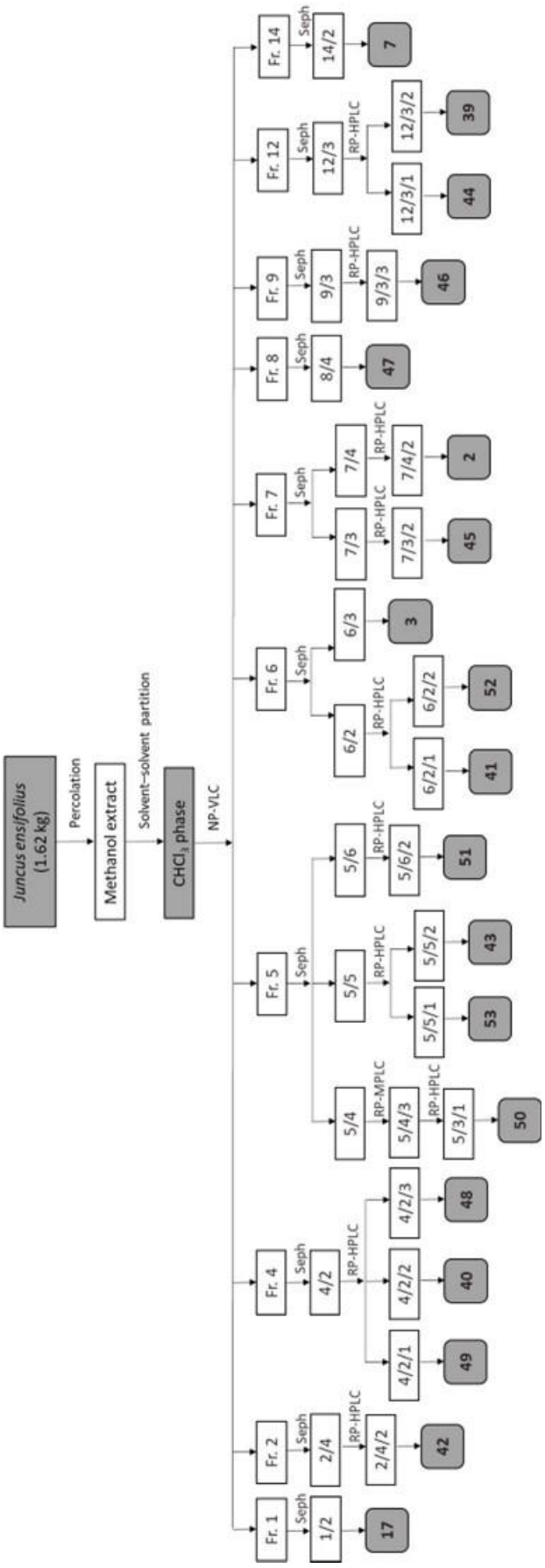


Figure 4. Isolation of compounds from *J. ensifolius*

Characterization and structure elucidation of the isolated compounds

The chemical structures of the isolated compounds were determined by means of spectroscopic methods and by comparison of spectral data with literature values. The molecular masses and compositions were obtained from MS investigations. The results of 1D (^{1}H NMR and JMOD) and 2D (^{1}H–^{1}H COSY, NOESY, HSQC and HMBC) NMR measurements proved to be the most valuable in structure determination. As a result of the NMR studies, complete ^{1}H- and ^{13}C-assignments were made for the new compounds.

Compounds from *J. atratus*, *J. gerardii*, *J. maritimus* and *J. ensifolius*

From the CH_2Cl_2 fraction of *J. atratus* nine compounds (**1**–**9**) were obtained, five of them are phenanthrenes (**Figures 5A**–**C**). Two compounds, juncatrins A (**1**) and B (**2**), are new natural products, substituted with an acetyl and an acetylene group at C-8, respectively, instead of a vinyl group present in juncuenin B (**3**) at the same position. This was the first time that a phenanthrene with an acetylene moiety was isolated from natural source. Regarding the isolation yields of compounds from *J. atratus*, juncuenin B (**3**) is the major phenanthrene of the plant as more than 100 mg was isolated. Most probably, juncatrins A (**1**) and B (**2**) are derived biosynthetically from juncuenin B. Moreover, the phenanthrene dehydroeffusol (**5**) and its 9,10-dihydro analogue effusol (**4**) were also determined from the plant. Also, two flavones [apigenin (**6**) and luteolin (**7**)], the acyclic diterpene phytol (**8**) and 13(*R*)-hydroxyoctadeca-(9*Z*,11*E*,15*Z*)-trienoic acid (**9**) were obtained. All identified compounds (phenanthrenes and other components, **1**–**9**) were isolated for the first time from *J. atratus*.

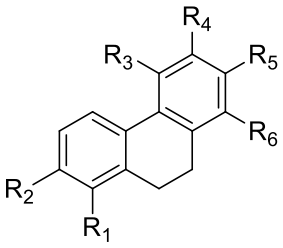
The structure analysis of compounds isolated from *J. gerardii* led to the identification of 23 phenanthrenes, of which 12, members of the gerardiin A-L series (**10**–**21)**, are new natural substances (**Figures 5A**–**C**). Gerardiin A (**10**) and gerardiin B (**11**) are substituted with a methoxymethylene group at C-1 (**10**) or C-7 (**11**). The structure of compound **10** is very similar to that of effusol (**4**), with the only difference being the presence of a methoxy group at C-11. Gerardiins C (**12**) and D (**13**) are glycosides of effusol, substituted with a D-glucose unit at C-2 (**12**) or C-7 (**13**), respectively. Gerardiins F (**15**) and G (**16**) are also substituted with a D-glucose moiety at C-2 (**15**) or C-7 (**16**), but instead of a vinyl group a hydroxyethyl group is joined at C-5 to the skeleton. Similarly, gerardiin E (**14**) contains a hydroxyethyl group at the same position as **15** and **16**. The only difference between gerardiin H (**17**) and juncunol (**36**), isolated from *J. maritimus* and other *Juncus* species (*J. acutus, J. effusus, J. roemerianus, J. subulatus*), is the presence of an unsaturated ring B in the former phenanthrene.

Phenanthrenoid dimers represent a rare class of specialized metabolites; to date, less than 20 have been reported from species in the plant family Juncaceae. In gerardiins I (**18**) and J (**19**), the two effusol (**4**) monomers are connected through their vinyl groups. Gerardiin K (**20**) is composed of two effusol (**4**) monomers that are joined through an ether bond, while in gerardiin L (**21**) an effusol (**4**) and

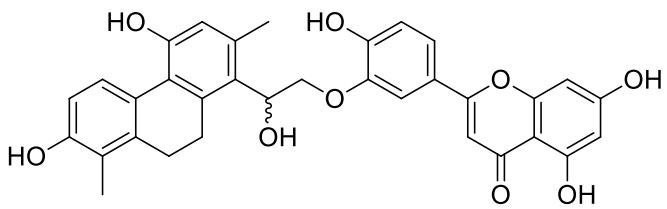
a dehydroeffusol (**5**) unit is attached via a C–C linkage formed between C-8–C-8′ (**Figure 5B**). The individual monomers [effusol (**4**), and dehydroeffusol (**5**)] were also isolated from the plant.

As a result of phytochemical investigation of *J. maritimus* 11 phenanthrenes, among them four new ones [maritins A–D (**32**–**35**)] were identified (**Figures 5A**–**C**). The obtained compounds are substituted with hydroxy, methyl, formyl, hydroxymethyl, methoxyethyl, and vinyl groups. From a biosynthetic point of view, maritin C (**34**) was likely formed from a dehydrojuncusol precursor through the modification of its vinylic double bond, followed by a ring closure between C-4 and C-13, forming a rare 4,5-ethanophenanthrene scaffold. The phenanthrene dimer maritin D (**35**) evolves two effusol (**4**) monomers attached through an ether bond between C-2–C-3′, resulted in the formation of a unique diaryl ether skeleton. Seven known phenanthrenes [effusol (**4**), juncusol (**24**), 2,7-dihydroxy-5-formyl-1-methyl-9,10-dihydrophenanthrene (**26**), juncunol (**36**), 2,7-dihydroxy-1,8-dimethyl-5-vinyl-9,10-dihydrophenanthrene (**37**), jinflexin A (**38**) and the dimer effususin A (**30**)] were also isolated from the apolar fraction of the plant. All compounds except for effusol (**4**) were isolated for the first time from the plant.

Finally, 19 compounds, including 17 phenanthrenes, were identified from the methanolic extract of *J. ensifolius* (**Figures 5A**–**C**). 13 Phenanthrenes, namely ensifolins A–M (**39**–**51**), were obtained for the first time from natural source. Ensifolins A (**39**) and B (**40**) are structurally unique phenanthrenes, considering that they are flavonoid- (luteolin, **7**) or benzaldehyde- (**53**) adducts. Compound **41** is a rare 10-hydroxphenanthrene. Similar compound was isolated previously only from *Luzula sylvatica*. Ensifolin D (**42**) is the 11-methoxy derivative of sylvaticin A (**52**) which was also isolated from the plant. Gerardiin H (**17**) can be served as the biogenetic precursor of both **42** and **52**. The dimers isolated from *J. ensifolius* mainly built up from monomers also isolated from the plant; e.g., in ensifolins J (**48**) and L (**50**), two ensifolin E (**43**) units are connected via their C-3 carbons (**48**) or through C-6 and C-3′ carbons (**50**) forming symmetrical or not symmetrical molecules. Similarly, in ensifolin M (**51**) two juncatrin B (**2**) monomers are connected through their C-3 carbons forming a symmetrical dimer, and finally in ensifolin K (**49**) a **43** unit is connected to a dehydrojuncuenin B monomer through a C–C bond formed between C-5–C-3′. Four known phenanthrenes [juncatrin B (**2**), juncuenin B (**3**), gerardiin H (**17**) and sylvaticin A (**52**)], and 4-hydroxybenzaldehyde (**53**) and luteolin (**7**) were also isolated from the plant. All compounds were identified for the first time from *J. ensifolius*.



	R_1	R_2	R_3	R_4	R_5	R_6
1	CH_3	OH	H	OH	CH_3	$COCH_3$
2	CH_3	OH	H	OH	CH_3	CCH
3	CH_3	OH	H	OH	CH_3	$CHCH_2$
4	CH_3	OH	$CHCH_2$	H	OH	H
10	CH_2OCH_3	OH	$CHCH_2$	H	OH	H
11	CH_3	OH	H	H	CH_2OCH_3	$CHCH_2$
12	CH_3	O-glc	$CHCH_2$	H	OH	H
13	CH_3	OH	$CHCH_2$	H	O-glc	H
14	CH_3	OH	$CH(CH_3)OH$	H	OH	H
15	CH_3	O-glc	$CH(CH_3)OH$	H	OH	H
16	CH_3	OH	$CH(CH_3)OH$	H	O-glc	H
22	CH_3	OCH_3	$CHCH_2$	CH_3	OH	H
23	CH_3	OCH_3	$CHCH_2$	H	OH	H
24	CH_3	OH	$CHCH_2$	CH_3	OH	H
25	CH_3	OH	$CHCH_2$	H	CH_2OH	H
26	CH_3	OH	CHO	H	OH	H
27	CH_3	OH	$CH(CH_3)OCH_3$	H	OH	H
28	CH_3	OH	CH_2OH	H	OH	H
32	CH_3	OH	$CHCH_2$	H	OH	CH_2OH
33	CH_3	OH	H	H	CH_3	$CHCH_2$
36	CH_3	OH	H	CH_3	$CHCH_2$	H
37	CH_3	OH	$CHCH_2$	H	OH	CH_3
38	CH_3	OH	$CH(CH_3)OCH_3$	H	OH	H
42	CH_2OCH_3	OH	$CHCH_2$	H	CH_3	H
43	CH_3	OH	OH	H	CH_3	$CHCH_2$
44	CH_3	OH	OH	H	CH_2OH	$CHCH_2$
45	CH_3	OH	OH	H	CH_3	$COCH_3$
46	CH_3	OH	H	OH	CH_2OH	CCH
52	CH_2OH	OH	$CHCH_2$	H	CH_3	H



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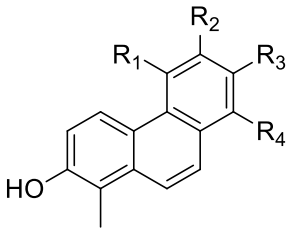
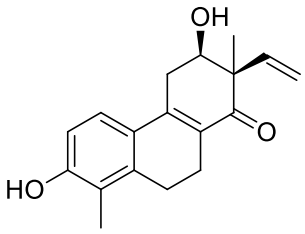


	R_1	R_2	R_3	R_4
5	$CHCH_2$	H	OH	H
17	$CHCH_2$	H	CH_3	H
47	H	OH	CH_3	CCH



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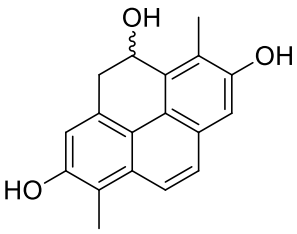

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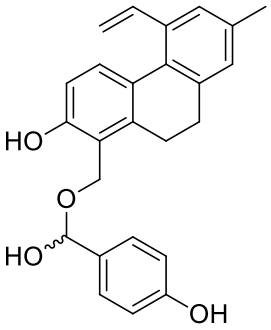

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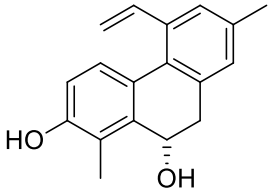

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Figure 5A Structures of the phenanthrene monomers isolated from the four investigated *Juncus* species

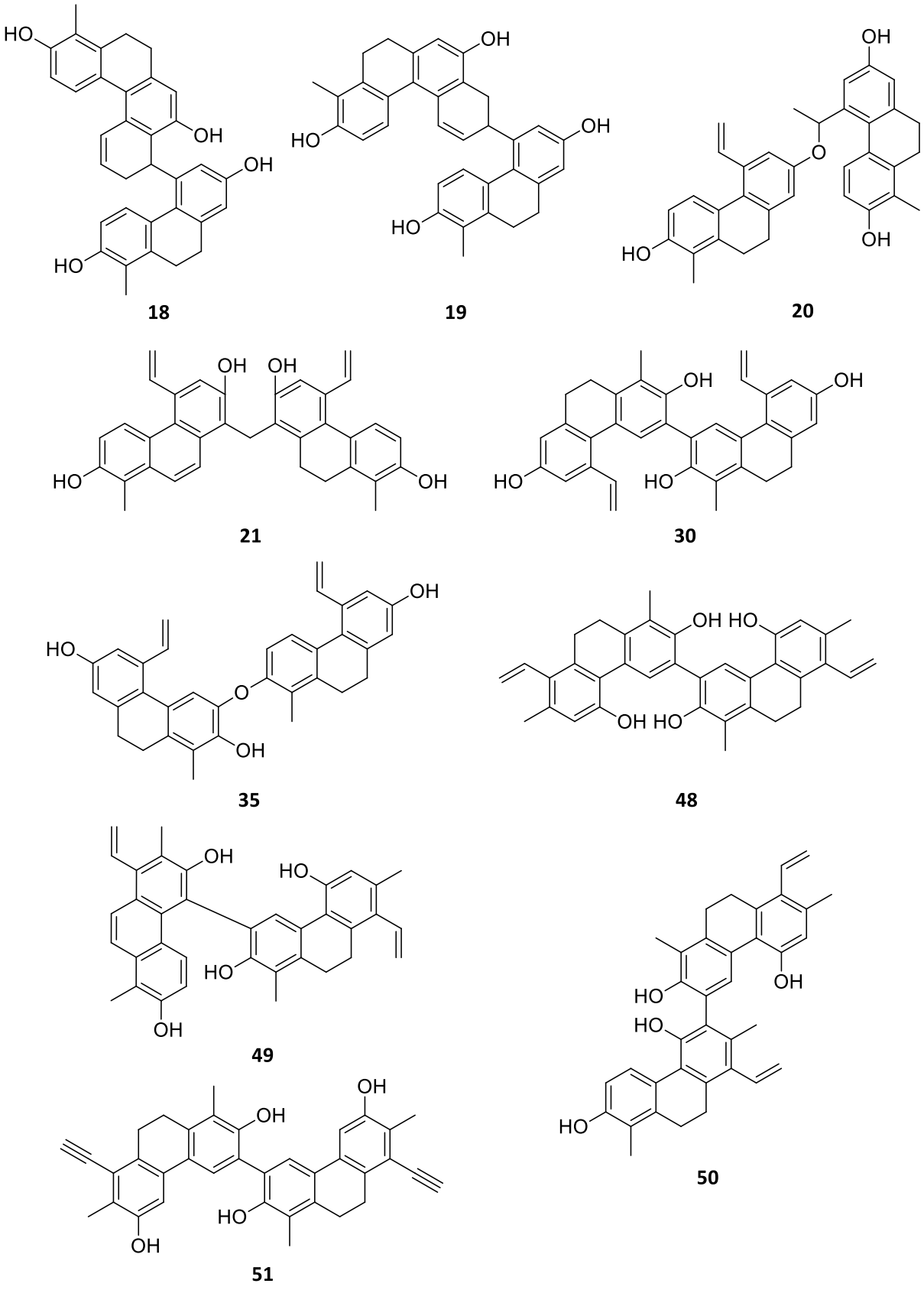

Figure 5B Structures of the phenanthrene dimers isolated from the investigated *Juncus* species

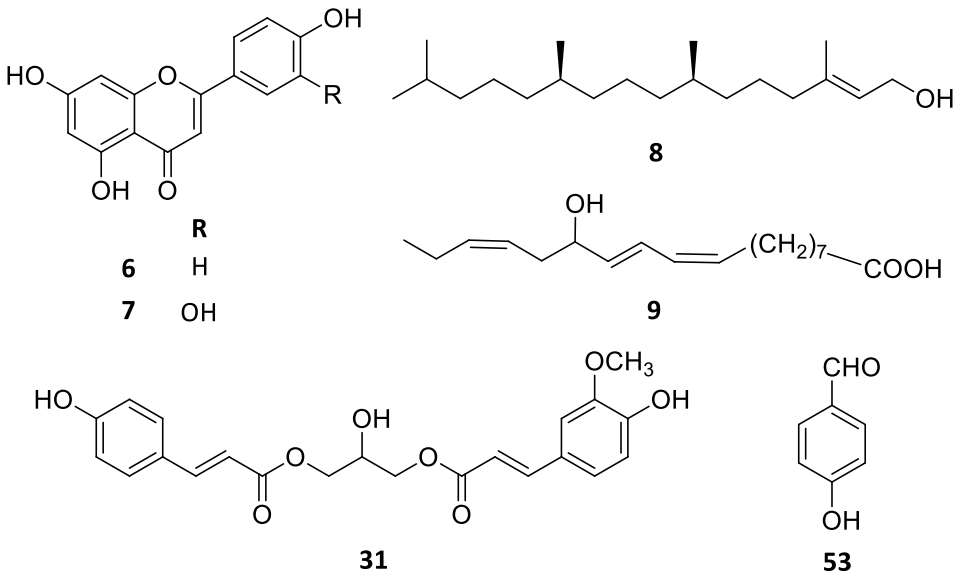

Figure 5C Structures of other compounds isolated from the investigated *Juncus* species

Biological activity of the isolated compounds

Our investigations are focused on the antiproliferative activity of the isolated phenanthrenes to give as many information as possible. Therefore, the isolated phenanthrenes were evaluated for their antiproliferative activity against different human tumor [HeLa, and SiHa (cervix), MDA-MB-231, 4T1, MDA-MB-231, MCF-7, KCR, and HTM-26 (breast), T47D (ductal), A2780, and A2780cis (ovarian), COLO 205, COLO 320 (colon)], and normal [D3 (human cerebral microvascular endothelial), and MRC-5 (human fetal lung fibroblast)] cell lines by MTT assay. DMSO was used as a negative control and cisplatin or doxorubicin as positive controls.

Among the used human tumor cell lines, HeLa cells were proved to be the most sensitive on phenanthrenes. Phenanthrenes **2**–**5**, isolated from *J. atratus* were more effective [IC_{50}s 3.5 μM (**2**), 2.9 μM (**3**), 3.7 μM (**4**), and 7.8 µM (**5**)] than clinically used reference agent cisplatin (IC_{50} 12.4 µM). They were additionally tested against NIH-3T3 mouse fibroblasts and it was found that all of them exerted only negligible action (less than 35% inhibitory activity) on fibroblasts even at 30 μM, and therefore, the antiproliferative action of the tested molecules can be considered cancer selective. Based on these results, some structure-activity relationships could be obtained. Compounds **1**–**3** differ each other in only the substituent at C-8. Since **1** was markedly less effective than **2** and **3**, presence of an acetyl group instead of a vinyl or an acetylene substituent at the same position seems to be disadvantageous. In case of compounds **4** and **5**, differing only in the saturation of ring B, the dihydrophenanthrene effusol (**4**) proved to be more active. It seems that presence of a methyl group on ring C has no significant effect on the antiproliferative activity of phenanthrenes against HeLa cell line.

Phenanthrene dimers from *J. gerardii* (gerardiins I–L (**18**–**21**), and effususin A (**30**)] reduced significantly the viability of 4T1 cells in a concentration-dependent manner. Since all these compounds

are dimers of effusol (**4**) (compounds **18**–**20** and **30**) or of effusol and dehydroeffusol (**5**) (compound **21**), the cytotoxic effects of the monomers and dimers in both mouse and human tumor cells and in a non-tumor cell line (D3) were compared. The results show unequivocally that the dimeric compounds **18**–**21** and **30** comprising effusol (**4**) and dehydroeffusol (**5**) monomers are cytotoxic to both tumor and non-tumor cell lines, while the monomers (**4** and **5**) alone displayed no or very low cytotoxicity. Among the diphenanthrenes tested, effususin A (**30**) exerted the lowest cytotoxicity, while gerardiins I-L (**18**–**21**) proved to be the most active. Indeed, moderate toxicity of effususin A (**30**) in A2780 human ovarian cancer cells reported by Bús et al. Impedance measurements were in line with the results of the MTT assay, indicating a concentration-dependent toxicity of the dimers.

IC_{50} values of both **18** and **19** were below 10 μM in the two tested tumor cell lines. In case of compound **20**, the concentration that caused 50% inhibition of cell viability was lower than 10 μM only in the mouse (4T1) (IC_{50} 8.1 μM), but not in the human breast cancer (MDA-MB-231) cell line (10.1 μM). On the other hand, compound **21** was more cytotoxic (IC_{50} 7.3 μM) to the human breast cancer cells (IC_{50} was 11.7 μM on 4T1 cells). D3 endothelial cells were the less sensitive to these diphenanthrenes, all of them having IC_{50} values above 10 μM (21.6 μM for **18**, 15.7 μM for **19**, 10.6 μM for **20**, and 13.7 μM for **21**, respectively).

Dimeric phenanthrenes (**30** and **35**) from *J. maritimus*, built up by effusol (**4**) monomers showed substantial antiproliferative activity against all investigated cell lines (HeLa, HTM-26, T-47D, A2780, A2780cis, MCF-7, and KCR). The highest activities were detected on T-47D ductal carcinoma cells (IC_{50} 6.2 μM for **30** and 9.1 μM for **35**, respectively) for both compounds. No significant differences were observed between the effects of dimers on different cell lines. Moreover, other isolated compounds of *J. maritimus* exerted outstanding inhibitory potential against other malignant cell lines, e.g., maritin C (**34**) on MCF-7 cells; maritin B (**33**), juncusol (**24**), and effusol (**4**) on HeLa cells.

Among the phenanthrenes of *J. ensifolius*, the luteolin-substituted phenanthrene ensifolin A (**39**) was found to be the most promising component with substantial antiproliferative effects against all tested cell lines (HeLa, COLO 205 and COLO320, IC_{50} values 3.9–12.7 μM), and showed good selectivity (SI = 4.95) in case of COLO 205 cells. It was more than ten-fold as active as the positive control cisplatin in COLO 205 cells. Interestingly, luteolin (**7**) alone, and compound **45** (ensifolin G), structurally very similar to the phenanthrene unit of ensifolin A (**39**), were inactive for all tested cell lines.

The lowest IC_{50} values against cervical carcinoma (HeLa) cells was found for compounds **3** (IC_{50} = 6.67 μM) and **2** (IC_{50} = 6.65 μM). The only difference between the two compounds is the substituent at C-8, which is a vinyl group in case of **3**, and an acetylene group in **2**. Ensifolin E (**43**) differing from juncuenin B (**3**) only in the position of the hydroxy group (at C-5 in **43**, and at C-6 in **3**), resulted in a significant decrease in the activity against HeLa cells, while changing of methyl group at C-7 in **43** to hydroxymethylene group in ensifolin F (**44**) led to the loss of the activity. Compounds **46** and **52**

possessed moderate antiproliferative activity (IC_{50} values 12.31 μM and 10.56 μM) against HeLa cells. Ensifolin I (**47**) is the dehydroderivative of sylvaticin A (**52**), and this modification resulted in an increased activity in cases of COLO 205 and COLO 320 adenocarcinoma cell lines, while a twofold decrease at HeLa cells. The dimerization of phenanthrene monomers resulted in the decrease of the activity, as it can be seen in case of compounds **47** and **51**, while in case of **48** and **50** which are the dimers of ensifolin E (**43**), neither the monomer nor its dimers showed antiproliferative activity. The best selectivity was obtained for ensifolins D (**42**, SI > 5.15, HeLa), and H (**46**, SI > 8.13, HeLa), and for compounds **2** (SI > 3.91, HeLa), **3** (SI > 5.37, HeLa), and **52** (SI > 9.43, HeLa).

Finally, all phenanthrenes from *J. ensifolius* were tested in a drug combination assay on HeLa cell in order to study the *in vitro* interactions between the compounds and the antineoplastic drug doxorubicin, known to be transported by P-gp. The combination index (CI), based on the Chou and Talalay method, was the main parameter to assess drug-drug interactions as synergistic (CI < 1), additive (CI = 1) or antagonistic (CI > 1). All tested compounds were found to interact synergistically with doxorubicin (CI < 1) on HeLa cell line. Very strong synergisms were observed for ensifolins E (**43**) and H (**46**), with CI values lower than 0.1. Both compounds showed weak or moderated activity (IC_{50}s 25.2–31.2 μM for **43**, and 12.3–63.5 μM for **46**) in case of antiproliferative investigation.

SUMMARY

The primary aim of the present work was the phytochemical and pharmacological investigation of Juncaceae species occurring in Hungary, and the isolation and structure determination of biologically active compounds from *Juncus atratus*, *J. gerardii*, *J. maritimus* and *J. ensifolius*.

In the preparative work, the lipophilic extracts (CH_2Cl_2/$CHCl_3$ and EtOAc) were purified by multistep separation procedures, including OCC, VLC, MPLC, GF, PLC and HPLC to yield pure compounds. The structures of the isolated compounds were elucidated by means of spectroscopic methods (HR-MS and NMR). In addition, complete ^{1}H and ^{13}C NMR assignments were made for the characterization of the compounds.

As a result of our work, altogether, 53 compounds were isolated from the four investigated *Juncus* species, 47 of them are phenanthrenes. Five phenanthrenes (**1**–**5**), two flavonoids (**6**, **7**), an acyclic diterpene (**8**) and a fatty acid (**9**) from *J. atratus*, 23 phenanthrenes (**4**, **5**, **10**–**30**) and a glycerol derivative (**31**) from *J. gerardii*, 11 phenanthrenes (**4**, **24**, **26**, **30**, **32**–**38**) from *J. maritimus* and 17 phenanthrenes (**2**, **3**, **17**, **39**–**52**), one flavonoid (**7**) and 4-hydroxybenzaldehyde (**53**) were obtained from *J. ensifolius*. All the isolated compounds except for effusol (**4**) from *J. maritimus* were detected for the first time from the investigated plants. The chemical constituents of *J. atratus*, *J. gerardii* and *J. ensifolius* have not been investigated previously. Among the isolated compounds, phenanthrenes are

the most promising ones from phytochemical and pharmacological points of view. 31 of the identified 47 phenanthrenes are new natural products. 37 Compounds are monomers (33 dihydrophenanthrenes and 4 phenanthrenes) and 10 are dimers. The most interesting monomers are the flavonoid- and 4-hydroxybenzaldehyde adducts **39** and **40**. Compounds **34** and **41** are also unique as in case of **34** a ring closure was occurred between C-4 and C-13 resulted in a tetracyclic ring system while **41** is substituted at C-10. Compounds **12**, **13**, **15** and **16** are glucosides. The methyl group at C-1, a hydroxy group at C-2, and vinyl, methyl and hydroxy substitution on ring C are characteristic features of Juncaceae phenanthrenes. In some cases, the methyl group at C-1 was modified to methoxymethylene (**10**, **42**) or hydroxymethyl moiety (**52**), and the hydroxy group at C-2 was changed to methoxy group in **22** and **23**. Moreover, on ring C, hydroxyethyl (**14**–**16**), methoxyethyl (**27**, **38**), formyl (**26**), hydroxymethyl (**32**), acetyl (**1**, **45**) or acetylene (**2**, **46**) group are presented instead of the vinyl unit. In compound **29**, a carbonyl group can be found in the molecule.

In case of the dimers, mostly two effusol (**4**) monomers are connected in different ways forming C–C bond (**30**) or an ether bond (**20**, **35**). In compounds **18** and **19** the two monomers (**4**) joined through their vinyl groups and a heptacyclic ring system is formed. In **21**, an effusol (**4**) and a dehydroeffusol (**5**) unit are connected. In **48** and **50**, two ensifolin E (**43**) units are joined through their 3–3' or 6–3' carbons, respectively. Compound **43** also formed a dimer with dehydrojuncuenin B in **49**. And finally, in **51** two juncatrin B (**2**) are joined through their C-3–C-3' resulted in a symmetrical dimer.

Based on the phenanthrene content, *J. gerardii* and *J. ensifolius* are considered to be as good sources of phenanthrenes. Juncusol (**24**) and effusol (**4**) are common constituents of *Juncus* species as they were detected in almost all previously investigated species. They can be served as biogenetic precursors of other Juncaceae phenanthrenes.

Vinyl substituted derivatives can be considered as chemotaxonomic markers for plants belonging to family Juncaceae, since these specifically substituted phenanthrenes were reported only from *Juncus* and *Luzula* species. To date, only two *Luzula* species (*L. luzuloides* and *L. sylvatica*) were investigated thoroughly from phytochemical point of view. Previous investigations focused only on the flavonoid content of the plants. As the phenanthrene content of the investigated *Luzula* and *Juncus* species are very similar it can further confirm the close botanical relationship between the two genera.

From pharmacological point of view, the antiproliferative activity and synergistic effect of phenanthrenes with the standard drug doxorubicin can be highlighted. In case of the antiproliferative activity, HeLa cell line proved to be the most sensitive on phenanthrenes. Phenanthrenes **2**–**5**, **24**, **33** and **39** showed remarkable antiproliferative effects [IC_{50}s 2.3 μM (**2**), 2.9 μM (**3**), 3.7 μM (**4**), 7.8 μM (**5**), 0.5 μM (**24**), 11 μM (**33**) and 8.3 μM (**39**)] on HeLa cells. Cytotoxic effect of the dimers of effusol (**18**–**20**), or effusol and dehydroeffusol (**21**) was comparable to that of the positive control doxorubicin to both 4T1 mouse [IC_{50}s 7.8 μM (**18**), 5.6 μM (**19**), and 8.1 μM (**20**)] and MDA-MB-231 human breast

cancer [IC_{50}s 8.0 μM (**18**), 6.6 μM (**19**), and 7.3 μM (**21**)] cells. Interestingly, the monomers **4** and **5** alone displayed no or very low activity. In case of the effusol dimers **30** and **35**, remarkable activities were detected on T-47D (IC_{50} 6.2 μM for **30**, and 9.1 μM for **35**) cells. The luteolin-substituted phenanthrene **39** was found to be promising component especially against COLO 205 cells (IC_{50} 3.9 μM). Finally, in the drug combination assay, the interaction of phenanthrenes isolated from *J. ensifolius* was tested with doxorubicin on HeLa cell line and compounds **43** and **46** possessed very strong synergism with the standard drug with CI values lower than 0.1.

Our findings not only enriched the chemical diversity of phenanthrenes but also provided new natural small molecules with antiproliferative activity for further drug developments.

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THE THESIS BASED ON THE FOLLOWING PUBLICATIONS

I. **Stefkó D**, Kúsz N, Csorba A, Jakab G, Bérdi P, Zupkó I, Hohmann J, Vasas A.
Phenanthrenes from *Juncus atratus* with antiproliferative activity
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