Isolation and structure elucidation of bioactive metabolites from poroid fungi of Hymenochaetaceae and Meripilaceae

Summary of the Ph.D. Thesis

András Sárközy

Department of Pharmacognosy
University of Szeged

Szeged, Hungary
2021

University of Szeged

Graduate School of Pharmaceutical Sciences

Programme of Pharmacognosy

Head: Prof. Judit Hohmann DSc.

Department of Pharmacognosy

Supervisors:

Prof. Judit Hohmann DSc.

Attila Ványolós Ph.D.

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Final Exam Committee:

Head: Prof. Imre Máthé DSc.

Members: Gábor Janicsák Ph.D., Dezső Csupor Ph.D.

Reviewer Committee:

Head: Prof. István Zupkó DSc.

Reviewers: Imre Boldizsár Ph.D., József Deli DSc.

Member: Tamás Papp DSc, Szilvia Berkó Ph.D.

Szeged, Hungary

2021

INTRODUCTION

Although the mushrooms or macroscopic fungi were a significant part of several cultures through the ages, they were always considered mystical, mostly unknown and hard to define. This tradition lives with us even now, as fungi have been chosen not to be a part of the kingdom of Plants or Animals, however, earned its own kingdom with vaguely defined borders. As a part of this group, the mushrooms are widely described as well. According to the definition from Chang and Miles, these organisms are mentioned as "a macrofungi with a distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand".

Despite (or sometimes because of) this mystical reputation, these beings have been used since the early ages of humanity. With regards to their application, probably the most ancient and simplest ones were in terms of nourishment, due to their easy to collect nature, unique taste and high nutritional value. In addition to the benefits detailed above, the mushrooms also tend to contain components that effect the homeostasis in other ways. Correspondingly, the poisonous mushrooms are the most well-known example of that, but some species also contain compounds with psychoactive and beneficial pharmacological effects.

Even the Iceman, a more than 5000 years old mummy found in the Alps collected mushrooms for spiritual-medical purposes, furthermore numerous reports of mushrooms (especially polypores) with beneficial effects are known from this region in the later periods as well. For instance, *Laetiporus sulphureus*, a polypore native to the area was commonly used for the treatment of pyretic diseases, coughs, gastric cancer, rheumatism, insect repellent for mosquitoes and midges, meanwhile the young fruiting body was also considered a delicacy, earning the name chicken polypore. Another great illustration is *Fomes fomentarius* that was used for cauterization since the

age of Hippocrates and applied as a styptic in Austria up to the 19th century. Interestingly, the application of this species was not limited to external use, since this polypore was also believed as a remedy against dysmenorrhoea, haemorrhoids, bladder diseases, pain, and gastric carcinomas. Additionally, a third polypore, *Piptoporus betulinus* also had a role in the history of the European medicinal mushrooms. This edible species was mostly prepared as a tea for enhancing the immune system and reducing fatigue.

Although these results are considerable and the role of polypores in Central European ethnomedicine is notable, further investigations are needed, as this field is far from complete description in terms of both composition and pharmacological effects of the mushrooms. This was the main motivation behind the decision for our workgroup to explore the bioactive metabolites of the native Hungarian mushrooms. Preliminary studies performed by our research group unequivocally demonstrated that polypore species represent an untapped potential in terms of antiproliferative, antioxidant and antimicrobial effects. Based on our preliminary studies and literature search we selected 3 polypores, *Meripilus giganteus*, *Porodaedalea chrysoloma* and *Fuscoporia torulosa* to be the topic of my research and this thesis.

AIMS OF THE STUDY

In 2012 the research project was started at the Department of Pharmacognosy (University of Szeged) in collaboration with the Department of Pharmacodynamics and Biopharmacy and the Department of Medical Microbiology and Immunology with the aim of investigating the bioactive compounds of Hungarian native mushrooms. The primary goal was to identify the most promising species in terms of pharmacological properties and then isolate the active fungal metabolites responsible for the observed biological activity. A screening study performed by Bernadett Kovács et al.

preceding this present work revealed that some important Central European Phellinus species (including *Porodaedalea chrysoloma* and *Fuscoporia torulosa*) possess significant antioxidant properties, worth for further investigation and compound isolation. These species, complemented with *Meripilus giganteus* formed the basis of my work.

To achieve our goal, the following task were performed:

- Review the available literature data and screening results for the chosen species, with emphasis on the chemical profile and known pharmacological properties.
- Grind and extract the collected mushroom samples with methanol.
- Separate and isolate the pure components utilizing solvent-solvent partition and various chromatographic methods.
- Elucidate the structure of isolated constituents using NMR and MS methods (collaborating with Richter Gedeon Plc., Hungary). Provide characteristic NMR spectroscopic data for the new compounds and supplement the missing NMR data for the known ones.
- Evaluate the pharmacological potential of the isolated compounds (at the Department of Pharmacognosy in collaboration with the Department of Medical Microbiology and Immunobiology).

MATERIALS AND METHODS

For preparative mycochemical work fresh fruiting bodies of *M. giganteus* (12 kg) were collected on Mecsek and Visegrád Mountains between 2014 and 2016. Dry mushroom materials of *P. chrysoloma* (360 g) were collected in Sweden and in the Czech Republic, while samples of *F. torulosa* (1425 g) were found on Gerecse Mountain and in the Botanical Garden of Buda, Hungary.

In the initial step of the preparative work, the dried or raw mushroom materials were percolated with an amphipolar solvent (MeOH). The concentrated extracts were diluted with 50% methanol and liquid–liquid extraction was employed, which resulted in *n*-hexane, chloroform and ethyl-acetate phases.

The investigated extracts were fractionated with different type of multistep chromatographic procedures, like thin-layer chromatography (TLC), flash column chromatography (FCC) and high-performance liquid chromatography (HPLC). Normal- or reversed-phase SiO_2 were applied as stationary phases.

The structures of the obtained compounds were characterized by spectroscopic methods (NMR and MS).

The *in vitro* antioxidant activity was investigated by DPPH and ORAC assays. The studies were carried out on a 96-well microplate using FLUOStar Optima plate reader.

The cytotoxic properties of the isolated compounds were determined by means of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay on U937 human lung (lymphoblast), human colonic adenocarcinoma and embryonic lung fibroblast cells. The interaction of the isolated compounds from *Fuscoporia torulosa* with the chemotherapeutic drug doxorubicin was investigated as well on Colo 320 (colon adenocarcinoma) cell line, while P-gp efflux modulation effect was assessed via Rhodamine 123 accumulation assay on the same type of cells.

Antimicrobial effects were measured against *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus* and the methicillin and ofloxacin resistant *S. aureus*. The minimal inhibitory concentration (MICs) was determined by inspection with naked eye.

RESULTS AND DISCUSSION

Isolation of the compounds of *M. giganteus*

Both *n*-hexane and chloroform fraction of *M. giganteus* yielded pure compounds. Flash chromatography was applied in both cases supported by high performance liquid chromatography (*n*-hexane fraction) and preparative thin layer chromatography (chloroform fraction) (**Figure 1**). This purification process led to the isolation of 9 compounds.

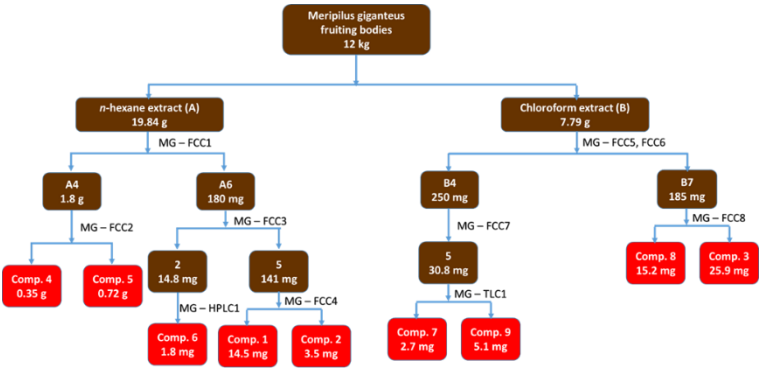


Figure 1. Isolation of compounds from *M. giganteus*

Isolation of the compounds of *P. chrysoloma*

In the case of *P. chrysoloma* the *n*-hexane phase was fractionated, and the combined fractions were further analyzed using repeated FCC (**Figure 2**). Thanks to chromatographic separations five compounds were isolated.

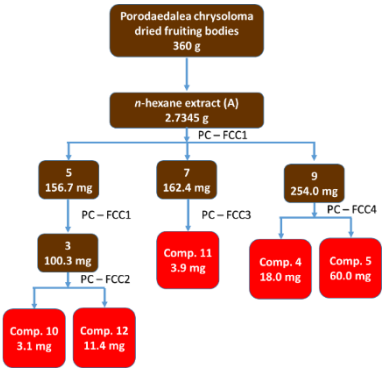


Figure 2. Isolation of compounds from *P. chrysoloma*

Isolation of the compounds of *F. torulosa*

In case of *F. torulosa* the *n*-hexane, chloroform and ethyl-acetate fractions led to the isolation of pure compounds (**Figure 3**). By applying both FCC and HPLC, the *n*-hexane and chloroform fractions yielded 3-3 components, while the ethyl-acetate fraction 2 compounds.

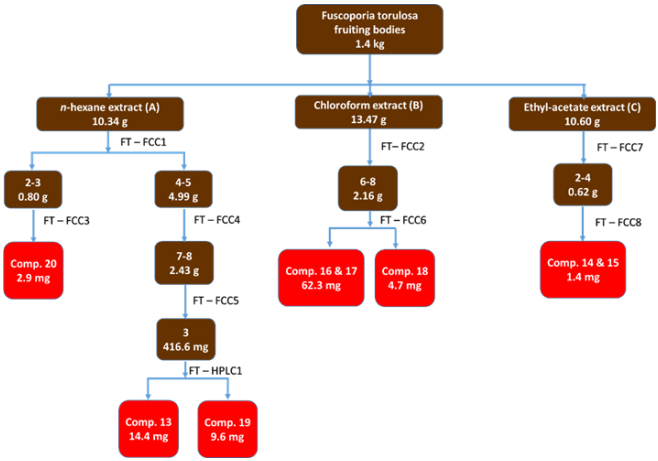


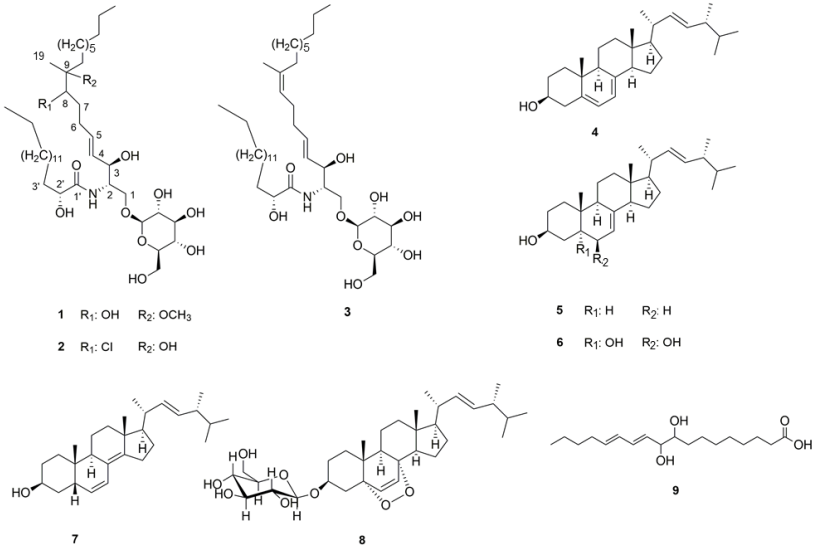
Figure 3. Isolation of compounds from *F. torulosa*

Compounds from *Meripilus giganteus*

The separation and the structure determination have enabled the identification of 9 compounds. Three of them (compounds **1-3**) belong to the group of cerebrosides; two of them, mericeramides A (**1**) and B (**2**), are new natural products, while compound **3** is the known cerebroside B. To the best of our knowledge mericeramide B (**2**) is the first natural halogenated cerebroside identified.

Ergosterol (**4**) and 3β-hydroxyergosta-7,22-diene (**5**) were identified based on the comparison of chromatographic and spectral data with the available standards. Cerevisterol (**6**), 3β-hydroxyergosta-6,8(14),22-triene (**7**), 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) and (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) were

characterized on the basis of HRMS, MS-MS and standard 1D and 2D NMR data compared to those reported in the literature.



Compounds from *Porodaedalea chrysoloma*

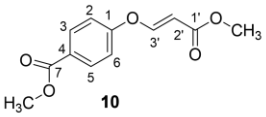
After processing the *Porodaedalea chrysoloma* sample, 5 compounds were isolated, out of which one (**10**) was found for the first time as a natural product.

Compound **10** was isolated as a white, amorphous solid with the molecular formula of $C_{12}H_{12}O_5$. According to the ^{1}H NMR spectrum signals of mutually coupled protons and the ^{1}H-^{1}H COSY spectrum, the presence of *p*-disubstituted benzene ring and a *trans*-double bond was suspected in the molecule. Based on the above mentioned and further results gained from JMOD, HSQC, COSY, HMBC and NOESY spectra, the structure was established as methyl (*E*)-3-(4-methoxycarbonylphenoxy)-acrylate.

Compound **11** was manifested as white amorphous granulates with the composition of $C_{12}H_{14}O_5$. The ^{1}H NMR spectrum was shown high level

of similarity with compound **10**, however, lacking the protons attributed to the *E*-double bond, yet showing the signs of two new methylenes. According to these data an exchange was suggested from the acrylate part to a methyl-propionate, compared to compound **10**. This afforded the structure of methyl 3-(4-methoxycarbonylphenoxy)-propionate, a new compound from natural source.

Ergone (**12**) was characterized by a comparison of the achieved NMR and the available literature data. Ergosterol (**4**) and 3β-hydroxyergosta-7,22-diene (**5**) were identified by matching their chromatographic and spectroscopic data to an authentic sample isolated previously.



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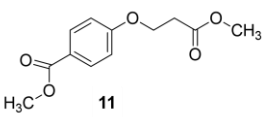

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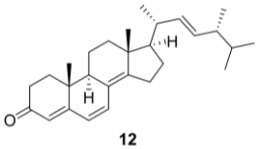

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Compounds from *Fuscoporia torulosa*

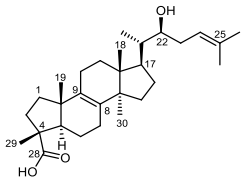
Processing the sample of *Fuscoporia torulosa* allowed the isolation of 8 components. Structure determination identified compound **13** as a novel triterpene, named fuscoporic acid, while **15** proved to be a previously undescribed Z isomer of inoscavin.

Based on ^{1}H and ^{13}C NMR spectra **13** is based on degraded lanosterol skeleton similarly as natalic acid (**19**), which was also isolated from *F. torulosa*.

Compounds **14** and **15** represent a mixture of inoscavin A and its Z isomer in a ca. 5 to 3 molar ratio. To the best of our knowledge the *Z* isomer has not yet been reported in the literature before.

According to spectral analysis **16** and **17** represent an equimolar mixture of 3,4-dihydroxy-benzaldehyde and osmundacetone. The remaining

constituents are triterpenes, namely senexdiolic acid (**18**), and ergosta-7,22-diene-3-one (**20**).



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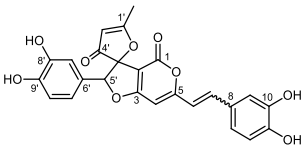
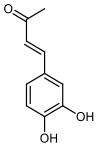


14, 15



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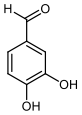

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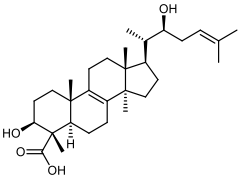

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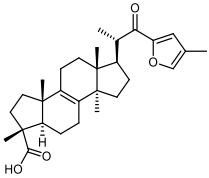

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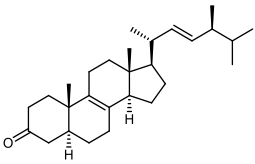

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Pharmacological activities of isolated compounds

Antioxidant activity of the compounds from *Meripilus giganteus*

All 9 of the isolated compounds (**1**-**9**) from *Meripilus giganteus* were examined in terms of antioxidant activity with ORAC assay. With regard to the results, mericeramide B (**2**), 3β-hydroxyergosta-7,22-diene (**5**), and (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) showed considerable antioxidant effect compared to the reference compound ascorbic acid.

Table 1. Antioxidant activity of the compounds from *Meripilus giganteus*

Compounds	ORAC Antioxidant Activity (mmol TE/g)
1	1.81±0.34
2	**2.50**±0.29
3	1.69±0.20
4	1.12±0.06
5	**4.94**±0.37
6	1.94±0.08
7	1.65±0.03
8	1.90±0.05
9	**4.27**±0.05
Ascorbic acid	**6.96**±0.57

Antioxidant activity of the compounds from *Porodaedalea chrysoloma*

The antioxidant activity of the isolated compounds was analysed by ORAC assay. The obtained results showed notable antioxidant properties, furthermore, these values were comparable to that of ascorbic acid used as a reference material. The measurement showed that 2 out of 5 components possess notable antioxidant activity: the methyl (*E*)-3-(4-methoxycarbonylphenoxy)-acrylate (**10**) and 3β-hydroxyergosta-7,22-diene (**5**).

Table 2. Antioxidant activity of the compounds from *Porodaedalea chrysoloma*

Compounds	ORAC Antioxidant Activity (mmol TE/g)
4	1.07±0.04
5	**5.02**±0.47
10	**2.21**±0.34
11	1.58±0.18
12	0.91±0.04
Ascorbic acid	**16.47**±0.01

Antioxidant activity of the compounds from *Fuscoporia torulosa*

Compound **14**-**17** isolated from *F. torulosa* were examined for their potential antioxidant effects using DPPH and ORAC assay. Both measurements concluded similar findings: all the tested compounds showed notable antioxidant effect in the order of compounds **16** + **17** < **14** + **15**, while

compounds **14** + **15** possessed the most potent antioxidant capacity among all the tested compounds discussed in this thesis.

Table 3. Antioxidant activity of the compounds from *Fuscoporia torulosa*

Compounds	DPPH IC_{50} (µg/mL)
14 + 15	**0.72**±0.05
16 + 17	**0.25**±0.01
	ORAC Antioxidant Activity (mmol TE/g)
14 + 15	**2.70**±0.03
16 + 17	**12.20**±0.92

Cytotoxic activity of the compounds from *Fuscoporia torulosa*

Compounds **13** and **18-20** were screened for cytotoxic effects on adenocarcinoma cell lines. Doxorubicin-sensitive Colo 205 and the anticancer agent resistant Colo 320 were applied, together with MRC-5 human embryonic lung fibroblast cell lines. Compounds **13**, **18** and **19** were ineffective, however, the determined IC_{50} for ergosta-7,22-diene-3-one (**20**) was comparable to those determined for doxorubicin used as reference substance. Furthermore ergosta-7,22-diene-3-on (**20**) was more effective as the reference agent on MRC-5 cell lines.

Table 4. Cytotoxic activity of the compounds from *Fuscoporia torulosa* (IC_{50} µM)

Compounds	Colo205	Colo320	MRC-5
20	11.65±1.67	8.43±1.1	7.92±1.42
Doxo	2.46±0.26	7.44±0.2	>20

After these results, a checkboard combination assay was also performed, looking for potential effect enhancement in case of a combined application of ergosta-7,22-diene-3-on (**20**) and doxorubicin on Colo320 cells. The final concentration of the examined components was determined by the previous results. At compound **20**:doxorubicin 11.2:1 rate, the

combination index (CI, 0.521±0.15) at the 50% growth inhibition dose (ED_{50}) was indicating a synergism between the examined compounds.

The effect of compounds **13** and **18**-**20** on modulation of P-gp efflux was evaluated by flow cytometry, measuring the rhodamine-123 accumulation in MDR Colo320 human colon adenocarcinoma cells. Tariquidar, a well-known P-gp inhibitor was used as positive control. The FAR values were used to assess the P-gp modulating potential. Generally, compounds can be considered to be active when presenting FAR values higher than 2. Therefore the tested compounds were ineffective modulators on drug resistant strain Colo 320.

Table 5. Rhodamine 123 accumulation assay results of compounds **13** and **18**-**20**

Samples	conc. (µM)	FSC	SSC	FL-1	FAR
Tariquidar	0.2	1945	837	64.100	5.533
13	20	2005	851	13.200	1.139
18	20	2074	861	11.900	1.027
19	20	2095	891	12.200	1.053
20	2	2099	857	10.100	0.872
DMSO	2.00%	2073	848	9.590	0.828
Colo 320	-	2052	841	8.870	-

FSC: Forward Scatter Count - provides information about cell size
SSC: Side Scatter Count - proportional to cell granularity or internal complexity
FL-1: Mean fluorescence of the cells
FAR: Fluorescence Activity Ratio

Antimicrobial activity of the compounds from *Fuscoporia torulosa*

Compounds **13** and **18**-**20** were investigated for antimicrobial activity on *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus* and the methicillin and ofloxacin resistant *S. aureus* clinical isolate strains, however, none of the compounds were considered efficient.

ACKNOWLEDGEMENTS

I express my deepest gratitude to my supervisors, Prof. Judit Hohmann (director of Department of Pharmacognosy) and Dr. Attila Ványolós for the management of my work.

I owe special thanks to my co-authors for the pleasant co-operation. My thanks to Dr. Viktor Papp and Dr. Kinga Rudolf for their help in the identification and collection of mushroom samples. I am grateful to Dr. Zoltán Béni, Dr. Kúsz Norbert, Csorba Attila and Dr. Miklós Dékány for the NMR and MS measurements. I am thankful to Dr. Annamária Kincses and Dr. Gabriella Spengler for antimicrobial and cytotoxicity and to Dr. Zoltán Péter Zomborszki for antioxidant activity investigation.

My thanks are likewise due to all my colleagues in the Department of Pharmacognosy for the favorable atmosphere, to my lab partners for the helpful attitude in areas of life and to my fellow graduate, Ph.D. and post-Ph.D. students for every constructive advice and cheerful moment.

I would like to extend my special thanks to my family and my lovely wife for their unending support and understanding attitude during these years and to everyone outside the walls of the University, who supported me in the hard times.

The thesis is based on the following publications:

1. **Sárközy A**, Béni Z, Dékány M, Zomborszki ZP, Rudolf K, Papp V, Hohmann J and Ványolós A
   Cerebrosides and Steroids from the Edible Mushroom *Meripilus giganteus* with Antioxidant Potential
   Molecules **25**(6):1395 (2020)
   IF: 3.267

2. **Sárközy A**, Kúsz N, Zomborszki ZP, Csorba A, Papp V, Hohmann J and Ványolós A
   Isolation and Characterization of Chemical Constituents from the Poroid Medicinal Mushroom *Porodaedalea chrysoloma* (Agaricomycetes) and their Antioxidant Activity
   International Journal of Medicinal Mushrooms **22**(2):125-131 (2020)
   IF: 1.423

3. Béni Z, Dékány M, **Sárközy A**, Kincses A, Spengler G, Papp V, Hohmann J and Ványolós A
   Triterpenes and Phenolic Compounds from the Fungus *Fuscoporia torulosa*: Isolation, Structure Determination and Biological Activity
   Molecules **26**(6):1657 (2021)
   IF: 3.267

Other publication:

1. Chuluunbaatar B, Béni Z, Dékány M, Kovács B, **Sárközy A**, Datki Zs, Mácsai L, Kálmán J, Hohmann J, Ványolós A
   Triterpenes from the Mushroom *Hypholoma lateritium*: Isolation, Structure Determination and Investigation in Bdelloid Rotifer Assays
   Molecules **24**(2):301 (2019)
   IF: 3.267

Presentations held in the same theme of the thesis:

1. **Sárközy A**, Béni Z, Dékány M, Sipos N, Kúsz N, Wasser S, Hohmann J, Ványolós A
   Isolation of beauveriolides from *Cordyceps militaris* mycelium
   25th International Symposium on Analytical and Environmental Problems; Szeged, 07-08. October 2019.

2. Chuluunbaatar B, Béni Z, Dékány M, Kovács B, **Sárközy A**, Datki Zs, Mácsai L, Kálmán J, Hohmann J, Ványolós A
   Steroids from the mushroom *Hypholoma lateritum*: isolation, structure determination and their investigation in bdelloid rotifer assays
   Young Scientists' Meeting on Advances in Phytochemical Analysis: Trends in Natural Products Research; Liverpool, 02–05. July 2018.

3. **Sárközy A**, Béni Z, Dékány M, Zomborszki ZP, Papp V, Rudolf K, Hohmann J, Ványolós A
   Az óriás likacsosgomba (*Meripilus giganteus* Karst.) tartalomanyagainak vizsgálata
   Fiatal Gyógynövénykutatók Fóruma; Budakalász, 12. May 2017.

Hymenochaetaceae és Meripilaceae családba tartozó taplógombák bioaktív metabolitjainak izolálása és szerkezet meghatározása

Bár a taplógombák a közép-európai népgyógyászat részét képezik, további vizsgálatok szükségesek a megfigyelt hatások igazolására, a hatóanyagok feltérképezésére. Munkacsoportunk fő célkitűzése volt, hogy feltérképezzük a hazai gombafajok pozitív élettani hatásait és a hatásért felelős anyagait. Előzetes vizsgálataink és az irodalom áttekintése után kutatásaim témájául 3 fajt, az óriás bokrosgombát (*Meripilus giganteus*), a *Porodaedalea chrysolomá*t és a vörös taplót (*Fuscoporia torulosa*) választottuk ki. Preparatív munkánknak köszönhetően a *Meripilus giganteus* esetében 7 ismert és 2 új, a *Porodaedalea chrysoloma* feldolgozásakor 4 ismert, illetve 1 új, a *Fuscoporia torulosa* mintából pedig 6 ismert és 2 új vegyületet sikerült izolálni. Ezen vegyületük antioxidáns hatását ORAC, illetve DPPH teszt segítségével eredményesen vizsgáltuk, a *F. torulosá*ból izolált ergoszta-7,22-dién-3-on pedig szinergista hatást mutatott a doxorubicin kemoterápiás hatóanyaggal.