From cyclic peptides to terphenyl quinones: biologically active metabolites from Hungarian mushrooms

Summary of the Ph.D. Thesis

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INTRODUCTION

Mushrooms are macroscopic members of the third largest kingdom on Earth, Fungi. They do not represent a well-defined taxonomic category: according to the definition of Chang and Miles a mushroom is "a macrofungus 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". For a long time, mushrooms have been playing an important role in several aspects of human life. Edible mushrooms are used as part of a regular diet, while some mushrooms with psychedelic properties have been applied in religious ceremonies. The so called medicinal mushrooms are popular healing agents which are widely used in traditional medicines of several countries in the Far East for thousands of years.

On the basis of our current scientific knowledge the estimated number of mushroom species is about 140 000, however only 10% of these are known to science. Approximately 700 species of higher Basidiomycetes have been found to possess diverse pharmacological properties. The spectrum of identified pharmacological activities of Basidiomycetes is very broad. Among these bioactivities the antitumor and antimicrobial effects are the most extensively studied.

In the last decades bacterial resistance to first-choice antibiotics have become a problem with global impact on human population. The appearance of mushroom compounds in antibacterial therapy dates back to 1979, when tiamulin, a semisynthetic derivative of pleuromutilin was approved for veterinary purposes. In 2007, retapamulin, another pleuromutilin derivative was approved by the US FDA for the topical treatment of impetigo.

Cancer is a leading cause of death in both more and less economically developed countries. More recently, some species of higher

Basidiomycetes have been found to markedly inhibit the growth of different tumor cell lines. The anticancer and immunomodulatory beta-glucans, lentinan from *Lentinus edodes* and krestin from *Trametes versicolor*, are probably the best known medical agents of mushroom origin. Besides, low-molecular-weight fungal metabolites with a wide variety of chemical structures have been also identified as antitumor agents in the last decades, such as illudins (sesquiterpenes), ganoderic acids (triterpenes) and terreumols (meroterpenoids), among others.

AIMS OF THE STUDY

In 2012 the research group of the Department of Pharmacognosy at University of Szeged started a screening program to investigate the antiproliferative and antimicrobial activities of the mushroom species native to Hungary with the purpose of obtaining potential antineoplastic and antimicrobial compounds. The aim of the presented study - as a part of this project - the chemical investigation and identification the bioactive compounds of the selected four mushroom species belonging to the Basidiomycetes (*Gymnopus fusipes* (Bull.:Fr.) Gray, *Tricholoma populinum* J.E.Lange, *Scleroderma bovista* Fr., *Tapinella atrotomentosa* (Batsch) Šutara).

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

- Review of the literature on the selected species, from the aspect of the chemistry and pharmacological properties.
- Investigation of the tumor cell proliferation-inhibitory effect and the susceptibility of Gram-positive and –negative bacteria against the fungal extracts.
- Extraction of the mushroom material of the selected species for preparative work.

- Isolate the compounds responsible for the antiproliferative or antimicrobial effects via bioactivity-directed fractionation, using various chromatographic techniques.
- Elucidate the structures of the isolated compounds by NMR and MS methods.
- Evaluate the pharmacological potential of the isolated compounds.

MATERISLS AND METHODS

For antiproliferative and antimicrobial screening, mushroom species were collected in north-eastern part of Hungary in 2013–2014. For preparative mycochemical work fruiting bodies of *G. fusipes* (2 kg) were collected in northern part of Hungary in July of 2013. Mushroom materials of *T. populinum* (3 kg), *S. bovista* (4 kg) and *T. atrotomentosa* (2 kg) were collected in the vicinity of Szeged in successive years of 2013, 2014 and 2015.

In the initial step of the preparative work, the freeze-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 and chloroform phases. For the screening, H_2O-soluble extracts were also prepared.

The investigated extracts were fractionated with different type of multistep chromatographic procedures including open-column chromatography (OCC), rotation planar chromatography (RPC), 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). However, the structure elucidation

of compounds **1-2** required the combination of spectral methods, Marfey's analysis and molecular modeling.

The antiproliferative properties of the extracts and isolated compounds were determined by means of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on a panel of human adherent cancer cell lines including cervix carcinoma (HeLa), ovarian carcinoma (A2780), skin epidermoid carcinoma (A431) and three different human breast carcinoma (MCF-7, MDA-MB-231 and T47D) cell lines.

Antimicrobial effects were measured against 11 standard and 9 clinical isolates by standard disc-diffusion method. In the case of diameter of inhibition zone ≥10 mm the extracts and the compounds were further subjected to determine their minimal inhibitory concentration (MICs) by microdilution method. For the detection of positive interaction between antibiotic drugs and extracts or pure compounds double-disc synergy assay was applied.

The xanthine-oxidase inhibitory activity was evaluated using a spectrophotometric method based on the measurement of uric acid production from xanthine at 290 nm with plate reader.

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.

RESULTS AND DISCUSSION

Screening of Basidiomycetes species for antiproliferative and antimicrobial activities

The aim of our studies was to perform an extensive screening program for the evaluation of mushrooms native to Hungary in terms of their potential antiproliferative and antimicrobial activities.

The organic and water extracts of selected mushroom species were tested *in vitro* for their antiproliferative activity against several human cancer cell lines. The chloroform extract of *G. fusipes* exerted outstanding effect on the investigated human cancer cells. The chloroform extract of *S. bovista* proved to possess notable antiproliferaive activity, while the extracts of *T. populinum* were found to exhibit moderate antiproliferative effects.

In our antimicrobial screening assay 40 species were tested *in vitro* against 11 standard bacterial strains and 9 clinical isolates including resistant and multi-drug resistant bacteria. 16 species exhibited antibacterial effects with moderate to high potential. The chloroform extract of *T. atrotomentosa* demonstrated the broadest antibacterial spectrum, it was found to be effective against Gram-positive bacterial strains, and also against standard and ESBL-positive *E. coli*, standard and multiresistant *P. aeruginosa* and multiresistant *A. baumannii*.

The active mushroom extracts were applied to different multiresistant micro-organisms combined with commercial antibiotics to evaluate their capacity to potentiate the action of standard drugs. The extracts *T. atrotomentosa* exhibited synergistic activity with cefuroxime against methicillin resistant *Staph. aureus*.

Isolation of the compounds of *G. fusipes*

The chloroform fraction of *G. fusipes* was separated first by RPC, then the subfraction B III was further purified with RP-HPLC using acetonitrile – water gradient eluent system (**Figure 1**). This purification process led to the isolation of 2 novel natural compounds COFUB1 (**1**) and COFUB2 (**2**).

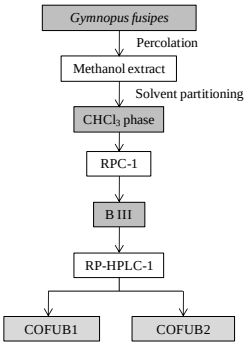


Figure 1. Isolation of compounds from *G. fusipes*

Isolation of the compounds of *T. populinum*

In the case of *T. populinum* the *n*-hexane phase was fractionated by OCC and the combined fractions were further analyzed using repeated FCC (**Figure 2**). Thanks to chromatographic separation six compounds were isolated: TRIPOA5 (**3**), TRIPOA4 (**4**), TRIPOA1 (**5**), TRIPOA3 (**6**), TRIPOA6 (**7**) and TRIPOA2 (**8**). The purification of the chloroform extract of the species was separated with RPC followed by RP-HPLC affording four compounds, two in pure form [TRIPOB1 (**9**), TRIPOB3 (**10**)] and two in a mixture of epimeric forms [TRIPOB2 (**11**), TRIPOB4 (**12**)].

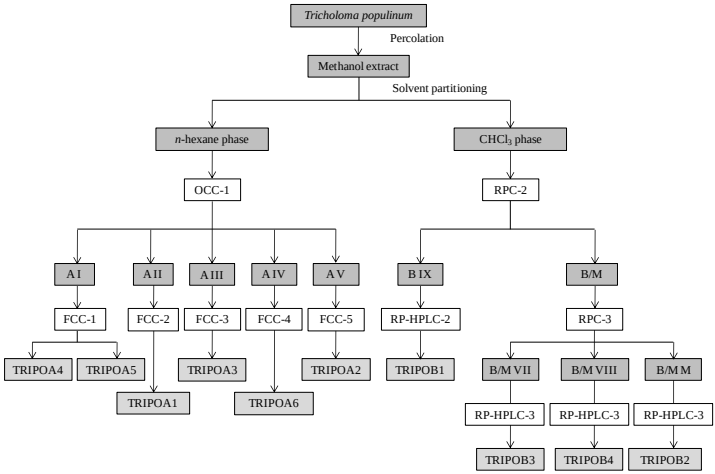


Figure 2. Isolation of compounds from *Tricholoma populinum*

Isolation of the compounds of *S. bovista*

The separation of *n*-hexane extract of *S. bovista* was carried out using open-column chromatography (OCC) (**Figure 3**). The combined fractions were further separated by flash column chromatography (FCC), which resulted in five compounds: SCLEBOA4 (**3**), SCLEBOA5 (**4**), SCLEBOA3 (**5**), SCLEBOA2 (**8**) and SCLEBOA1 (**13**). The analysis of the chloroform

fraction of this species was performed by repeated FCC to obtain two compounds SCLEBOB1 (**14**) and SCLEBOB2 (**15**).

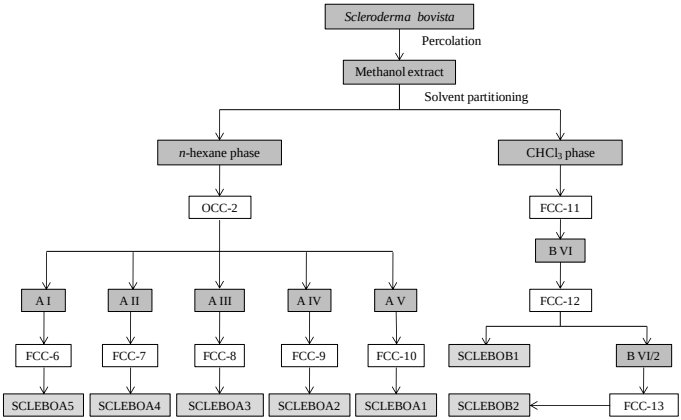


Figure 3. Isolation of compounds from *Scleroderma bovista*

Isolation of the compounds of *T. atrotomentosa*

The chloroform phase of *T. atrotomentosa* was separated by repeated FCC steps (**Figure 4**).

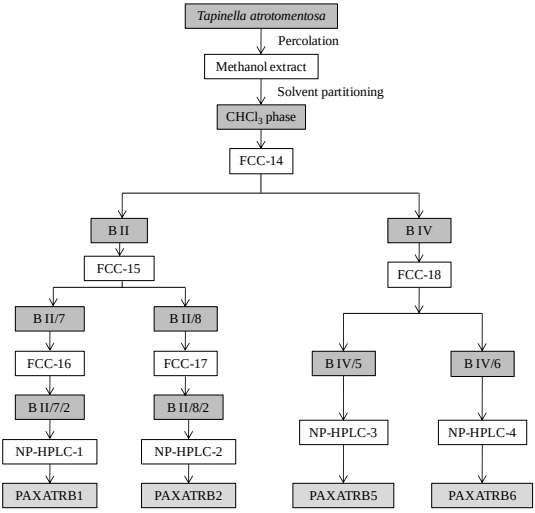


Figure 4. Isolation of compounds from *Tapinella atrotomentosa*

The obtained fractions were purified in each case by NP-HPLC using *n*-hexane – isopropanol – water isocratic eluent system. Utilization of combined chromatographic separation techniques led to the isolation of four compounds: PAXATRB1 (**16**), PAXATRB2 (**17**), PAXATRB5 (**18**) and PAXATRB6 (**19**).

Cyclopeptides from *Gymnopus fusipes*

From the chloroform extract of *G. fusipes* two novel cyclic octadecapeptides were isolated, namely gymnopeptides A (**1**) and B (**2**) (**Figure 5**). To best of our knowledge, they are the largest cyclopeptides among the mushroom metabolites. Gymnopeptides A (**1**) and B (**2**) are constituted of 18 amino acids (three alanine, four valine, two *N*-methyl-glycine (Sar), an *N*-methyl-alanine, seven *N*-methyl-valine and a serine/ threonine residues). The isolated compounds differ only in one amino acid; serine was found in **1** replaced by a threonine in **2**.

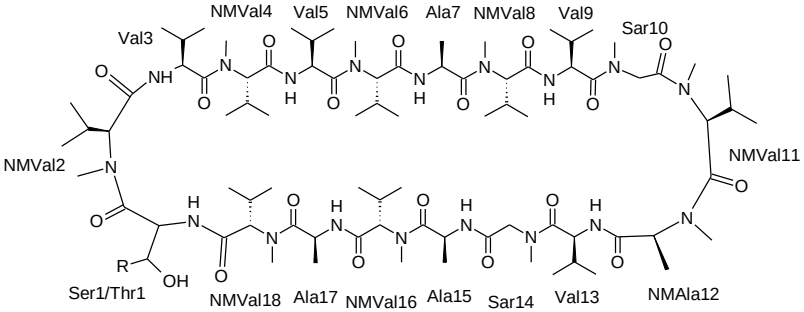


Figure 5. AA sequences of gymnopeptides A (**1**) (R = H) and B (**2**) (R = CH_3)

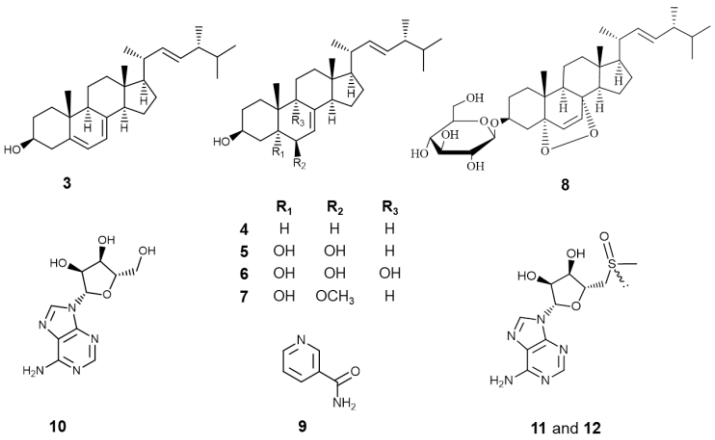
Gymnoppetides A and B are highly methylated, the number of *N*-methylated amino acids is 10 out of 18 in each cyclopeptide. The unmodified and *N*-methylated amino acids alternate in the sequences of the two cyclopeptides, except in position 11. Among the secondary metabolites of higher mushrooms cyclic peptides are fairly rare compounds. Apart from

(−)-ternatin, a highly *N*-methylated cyclic heptapeptide isolated from the medicinal mushroom *Trametes versicolor*, cyclopeptides have been identified in few genera of poisonous species (Amanita, Conocybe, Galerina, Lepiota and Omphalotus).

Steroids and sulfinyladenosine compounds from *Tricholoma populinum*

In the methanol extract of *T. populinum* ten compounds (**3-12**) have been identified, 9 of them for the first time in this species. The structure determination of compounds revealed that **3-8** are triterpene steroids possess ergostane structure: ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**5**), 3β,5α,6β,9α-tetrahydroxyergosta-7,22-diene (**6**), methylated derivative of cerevisterol (**7**) and glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8).**

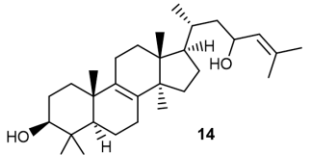
Nicotinamide (**9**) has been previously detected by Turner et al. (1987) in *T. populinum* samples from British Columbia. Besides, compounds **10**-**12** proved to be adenosine type compounds. The methylsulfinyladenosine structures of **11** and **12** (epimers of 5'-deoxy-5'-methylsulphinyladenosine) represent a fairly rare subclass of secondary metabolites.

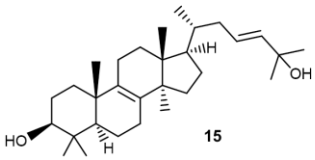


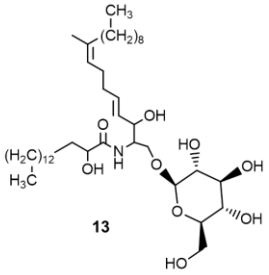
	R_1	R_2	R_3
4	H	H	H
5	OH	OH	H
6	OH	OH	OH
7	OH	OCH_3	H

Ceramide and steroid type compounds from *Scleroderma bovista*

The mycochemical examination of *S. bovista* led to the isolation of seven compounds (**3**-**5**, **8**, **13**-**15**). In the *n*-hexane phase of *S. bovista* a ceramide type compound linked to a glucose molecule, namely cerebroside B (**13**) was detected. The investigation of the species resulted in the identification of 6 triterpene steroids: ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**5**), 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**), 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**).

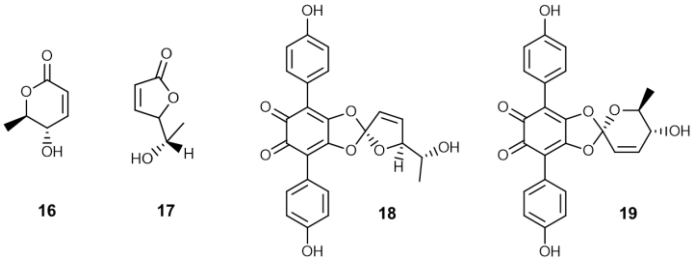






Lactone and terphenyl quinone derivatives from *Tapinella atrotomentosa*

In the chloroform extract of *T. atrotomentosa* four compounds (**16**-**19**) were identified, which had previously been isolated from this species. Two compounds were confirmed as lactone type metabolites, osmundalactone (**16)** and 5-hydroxy-2-hexen-4-olide (**17**), while the other isolated metabolites, namely spiromentin C (**18**) and spiromentin B (**19**) possess terphenyl quinone skeleton. Spiromentins B-C have unique γ- and δ-lactone-acetal spiro structures linked to a 4,5-dihydroxy-1,2-benzoquinone core.



Pharmacological activities of isolated compounds

Antiproliferative effect of the cyclopeptides of *G. fusipes*

Gymnopeptides A (**1**) and B (**2**) were evaluated for their antiproliferative properties against five different human cancer cell lines (HeLa, A431, MCF-7, MDA-MB-231 and T47D). Compounds **1** and **2** demonstrated very strong cell growth inhibitory activity with IC_{50} values in nanomolar range (14-88 nM).

Biological activity of the compounds of *T. populinum*

Compounds **5**-**8** identified in the *n*-hexane extract of *T. populinum*, were assayed for their cytotoxic activity on several human breast cancer cell lines (MCF-7, T47D and MDA-MB-231). Cerevisterol (**5**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) proved to be the most active against T47D cells (50.2 ± 1.6% and 46.0 ± 1.4% cell growth inhibition), while methylated derivative of cerevisterol (**7**) demonstrated significant activity on MDA-MB-231 cells (54.7 ± 1.6%). Compounds **9**-**12** were evaluated for their potential XO inhibitory activity; however they do not possess inhibitory activity on this enzyme.

Antiproliferative activity of the secondary metabolites of *S. bovista*

Compounds **8**, **13-15** were tested for their *in vitro* antiproliferative activity on four different cancer cell lines (HeLa, A2780, MDA-MB-231 and MCF-7). Lanosta-8,23-dien-3β,25-diol (**15**) exhibited higher

antiproliferative property on HeLa (62.21 ± 1.95%) and MCF-7 (73.32 ± 2.76%) cells than 23-hydroxylanosterol (**14**) (42.88 ± 7.79% and 37.39 ± 5.89%), but the latter was more effective against A2780 cells. 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) showed significant activity on A2780 cells (53.27 ± 6.37%), while cerebroside B (**13**) exerted moderate antiproliferative activity.

Bioactivity of the compounds of *T. atrotomentosa*

Antimicrobial activity

Compounds **16-19** obtained from the chlorofom extract of *T. atrotomentosa* were evaluated for their antimicrobial activity against several bacterial strains (**Table 1**). Our investigations revealed that *A. baumanii* and ESBL *E. coli* are the most susceptible against the studied compounds. 5-hydroxy-2-hexen-4-olide (**17**) was the most active against these strains, although osmundalactone (**16**) and spiromentin C (**18**) have also shown significant effectiveness. Compounds **16-19** were investigated their synergistic effect with cefuroxime against MRSA, though our results indicate that they do not enhance the activity of the studied antibiotic drug.

Table 1. Antimicrobial activity of compounds **16-19**

Compounds	**Calculated MIC values (μg mL^{-1})**			
	Acinetobacter baumannii	ESBL *Escherichia. coli*	*Moraxella catarrhalis*	MRSA
16	10	10	-	250
17	6	10	50	250
18	20	10	50	250
19	-	100	-	-

Antioxidant activity

Compounds **16-19** were evaluated for their antioxidant activity using DPPH and ORAC assays. In contrast to the DPPH test, the metabolites have shown strong antioxidant activity on ORAC assay. Spiromentins C (**18**) and B (**19**) demonstrated remarkable antioxidant properties, which were higher than the activity of ascorbic acid used as reference compound (**Table 2**). Although osmundalactone (**16**) and 5-hydroxy-2-hexen-4-olide (**17**) were less active in the ORAC assay, they are still considered compounds with moderate antioxidant property. The DPPH assay performed revealed that compounds **16** and **17** have no antioxidant activity in this assay, while compounds **18** and **19** could not be evaluated in this assay due to their color interference with the applied reagent.

Table 2. Antioxidant activity of compounds **16-19**

Compounds	**ORAC antioxidant activity (mmolTE/g)**
16	0.74 ± 0.30
17	3.85 ± 0.34
18	**16.21 ± 0.38**
19	**11.23 ± 0.58**
Ascorbic acid	**6.97 ± 0.01**

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The thesis is based on the following publications:

1. Béni Z, Dékány M, **Kovács B**, Csupor-Löffler B, Zomborszki ZP, Kerekes E, Szekeres A, Urbán E, Hohmann J, Ványolós A
   Bioactivity-guided Isolation of Antibacterial and Antioxidant Metabolites from the Mushroom *Tapinella atrotomentosa*
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2. **Kovács B**, Béni Z, Dékány M, Bózsity N, Zupkó I, Hohmann J, Ványolós A
   Isolation and Structure Determination of Antiproliferative Secondary Metabolites from the Potato Earthball Mushroom, *Scleroderma bovista* (Agaricomycetes)
   International Journal of Medicinal Mushrooms **20**:(5) pp. 411-418. (2018) If: 1.211*

3. **Kovács B**, Béni Z, Dékány M, Orbán-Gyapai O, Sinka I, Zupkó I, Hohmann J, Ványolós A
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   Gymnopeptides A and B, Cyclic Octadecapeptides from the Mushroom *Gymnopus fusipes*
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* The impact factor for the year 2017 is given.

5. Liktor-Busa E, **Kovács B**, Urbán E, Hohmann J, Ványolós A Investigation of Hungarian Mushrooms for Antibacterial Activity and Synergistic Effects with Standard Antibiotics against Resistant Bacterial Strains
   Letters in Applied Microbiology **62**:(6) pp. 437-443. (2016) If: 1.575

6. Ványolós A, **Kovács B**, Bózsity N, Zupkó I, Hohmann J Antiproliferative Activity of Some Higher Mushrooms from Hungary against Human Cancer Cell Lines, *International Journal of Medicinal Mushrooms* **17:**(12) pp. 1145-1149. (2015) If: 1.357

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   International Journal of Medicinal Mushrooms **19**:(5) pp. 387-394. (2017) If: 1.211

Presentations held in the same theme of the thesis:

1. Ványolós A, **Kovács B**, Béni Z, Dékány M, Krámos B, Liktor-Busa E, Zomborszki ZP, Zupkó I, Hohmann J
   Hungarian Mushrooms as Untapped Source of Natural Products: from Screening Studies to Biologically Active Metabolites
   65th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research – GA2017; Basel, 03.-07. September 2017.

2. **Kovács B**, Béni Z, Dékány M, Zupkó I, Hohmann J, Ványolós A
   A fakó áltrifla (*Scleroderma bovista* Fr.) tartalomanyagainak vizsgálata
   Fiatal Gyógynövénykutatók Fóruma; Budakalász, 24. June 2016.

3. **Kovács B**, Béni Z, Dékány M, Zupkó I, Hohmann J, Ványolós A
   Isolierung und Strukturaufklärung von Naturstoffen aus *Scleroderma bovista*
   Phytokongress, Bonn, 02.-04. June 2016.

4. Zomborszki ZP, **Kovács B**, Papp V, Hohmann J, Csupor D, Ványolós A
Antioxidative Aktivität von mitteleuropäischen Phellinus-Arten
Phytokongress; Bonn, 02.-04. June 2016.

5. **Kovács B**, Béni Z, Dékány M, Zupkó I, Hohmann J, Ványolós A
A nyárfa-pereszke (*Tricholoma populinum* J. E. Lange) tartalomanyagainak vizsgálata
Fiatal Gyógynövénykutatók Fóruma; Budakalász, 24. June 2015.

6. Ványolós A, **Kovács B**, Béni Z, Dékány M, Hohmann J
Mycochemical Study of the Mushroom *Tricholoma populinum*
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7. Ványolós A, **Kovács B**, Hohmann J
A gombák bemutatkoznak: egy átfogó, farmakológiai szűrővizsgálat eredményei
XIV. Magyar Gyógynövény Konferencia; Pannonhalma, 29.-30. May 2015.

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