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

Ph.D. Thesis

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III. 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

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ABBREVIATIONS AND SYMBOLS

1D	One-dimensional
2D	Two-dimensional
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABCB	ATP binding cassette subfamily B member 1
ABTS	2,2-azino-bis(3-etilbenzotiazolin)-6-sulfonic acid
ACAT	Acyl-CoA:cholesterol acyltransferase
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
CID	Collision-induced dissociation
CLSI	Clinical and Laboratory Standards Institute
COSY	Correlation spectroscopy
CUPRAC	Cupric reducing antioxidant capacity
DM	Diabetes mellitus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Half maximal effective concentration
ELS	Evaporative light scattering
ESI	Electrospray ionization
FAR	Fluorescence activity ratio
FCC	Flash column chromatography
FL-1	Mean fluorescence of the cells
FSC	Forward scatter count
FT	*Fuscoporia torulosa*

GIRK	G protein-coupled inwardly-rectifying potassium channel
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple-bond correlation
HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
HSQC	Heteronuclear single quantum correlation
IC_{50}	Half maximal inhibitory concentration
IL	Interleukin
ISI	Insulin sensitivity index
JMOD	J-modulated spin-echo
LOX	Lipoxygenase
LP	Lipoprotein
MAE	Mean absolute error
MG	*Meripilus giganteus*
MGPS	*Meripilus giganteus* polysaccharide
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
mTOR	Mechanistic target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOE	Nuclear Overhauser effect
NOESY	Nuclear Overhauser effect spectroscopy
NP	Normal phase
OD	Optical density
ORAC	Oxigen radical absorbance capacity
OSI	Oxidative stress index
PBS	Phosphate-buffered saline

PC	*Porodaedalea chrysoloma*
P-gp	P-glycoprotein
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROESY	Rotating-frame nuclear Overhauser effect spectroscopy
ROS	Reactive oxygen species
RP	Reverse phase
RS	RediSep Rf Gold
SARS	Severe acute respiratory syndrome
SDS	Sodium dodecyl sulphate
SSC	Side scatter count
TAS	Total antioxidant status
TBARS	Thiobarbituric acid reactive substance
Te	Telos
TE	Trolox equivalent
TLC	Thin layer chromatography
TMS	Tetramethylsilane
TNF-α	Tumor necrosis factor alpha
TOCSY	Total correlation spectroscopy
TOS	Total oxidant status
UV	Ultraviolet
VIS	Visible

1. 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 creatures are mentioned as "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".[1]

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. Furthermore, they are also rich in vitamins, minerals, fibres and essential amino acids, so the edible mushrooms are considered a delicacy even in our time.[2]

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 pharmacological effects. Although the toxic and hallucination inducing properties of the fungus are generally known, their curative role is also indisputable: medicinal mushrooms are used even nowadays, although their application as a remedy is mostly widespread in the Far East.[3] Despite the popularity in Asia, the tradition of medicinal use of the mushrooms is rare in Europe, however, this knowledge appears being forgotten, rather than being absent.

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. In Central European countries, involving Hungary the carved mushroom was also utilized as caps, charms, and other clothing elements. 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.[4]

Despite the ethnomedical nature of these representative examples, further structural and pharmacological examinations have confirmed the application of these polypores as drugs. Subsequently, steroids, sesquiterpenes, cerebrosides, quinones, catechols, amines, triacylglycerols, glycans, glycopeptides, proteoglycans and proteins are the most common compounds found in the mushrooms.[4–6] Accordingly, several reports examining the effects of the mentioned active ingredients or crude extracts of these mushrooms are available in the literature with typical determination of immunomodulatory, cardiovascular, liver protective, anti-inflammatory, antidiabetic, antioxidant, antitumor and antimicrobial applications.[7–9]

Although these results are impressive and the role of polypores in Central European ethnomedicine is definitely worth, further investigations are needed, as this field is far from complete description in terms of both mushroom composition and pharmacological effects. This was the main motivation behind the decision for our workgroup to explore the physiological effects and the active ingredients 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.[10–13] Based on our preliminary studies and literature search we selected 3 polypores, *Meripilus giganteus*, *Porodaedalea chrysoloma* and *Fuscoporia torulosua* to be the topic of my research and this thesis.

2. 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 Institute of Clinical Microbiology 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.[12] 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 sample 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).

3. LITERATURE OVERVIEW

3.1. Morphology, chemistry and pharmacology of *Meripilus giganteus*

The *Meripilus giganteus* (Pers.: Pers.) Karst. (family Meripilaceae, order Polyporales, phylum Basidiomycota) (known also as *Boletus giganteus* Pers. (Basionym), *Grifola gigantea* (Pers.) Pilát and *Polyporus giganteus* (Pers.) Fr., Riesenporling, Polypore gêant, Giant Polypore, Rooster of the Woods[14] and óriás likacsosgomba) can be found in Europe, Northern Asia and North America in the late summer-early autumn period.[15,16] Due to its parasitic and saprophytic nature this mushroom lives on both living and dead hardwood[15], mainly on Quercus and Fagus species.[16] One of the symptoms of *Meripilus giganteus* contamination of the tree is white-rot, a degradation of lignin caused by oxidative process enhanced by enzymes.[15] Due to this attribution the mushroom is in focus of several forestry and industrial studies.[17–24]

The young tops of *Meripilus giganteus* are soft and considered edible, however the older specimens became harder and tougher and their colour blacken on touch. The name itself originates from the size of the fruiting body which could reach 1 metre in diameter and 10 kg in weight. The fruiting body's upper portion is zoned, furrowed radially and concentrically by streaks of light to dark brown, wrinkled and covered with scales.[25]

Several primary and secondary metabolites were identified in sporocarps of *M. giganteus*. Accordingly, these experiments resulted in the determination of ergosterol,[15] ergosterol peroxide,[15] gallic acid,[25–27] protocatechuic acid,[25–27] the 4 tocopherol isoforms,[16] oxalic acid,[16] malic acid,[16,26] quinic acid,[16] citric acid,[16] fumaric acid,[16,26] *p*-hydroxybenzoic acid,[16,26] p-coumaric acid,[16,26,27] cinnamic acid,[16] palmitic acid, [14,26] oleic acid,[14,26] linoleic acid,[14,26] succinic acid,[26] gentisic acid,[26] vanillic acid,[26,27] caffeic acid,[26,27] ergosterol-5,8-peroxide,[14] a series of odd-numbered *n*-alkanes: docosane ($C_{22}H_{46}$), tricosane ($C_{23}H_{48}$), tetracosane ($C_{24}H_{50}$), pentacosane ($C_{25}H_{52}$), hexacosane ($C_{26}H_{54}$), nonacosane ($C_{29}H_{60}$), triacontane ($C_{30}H_{62}$), hentriacontane ($C_{31}H_{64}$), dotriacontane ($C_{32}H_{66}$);[14] stearic acid, lauric acid, margaric acid, arachnic acid, myristic acid, lignoceric acid, pentadecanoic acid, lauroleic acid,[14] 8-[(1*E*,3*Z*)-undeca-1,3-dien-1-yl]oxocan-2-one,[14] as potentially active components of the mushroom.

A research, utilizing capillary gas-liquid chromatography for finding volatiles produced by flask cultured mycelium, detected 1-butanol, 2-methyl-1-propanol, 2-butoxyethanol, phenylmethanol,

4-hydroxy-4-methyl-2-pentanone, 4-methoxybenzaldehyde, methyl benzoate and 2-methoxy-4-(2-propenyl)phenol in the *M. giganteus* sample.[28]

In further studies mixtures of saturated and unsaturated fatty acids,[14–16] monoglycerols,[15] cerebrosides,[15] carbohydrates,[16] proteins,[16] tannins,[29] saponins,[29] total phenol,[25,26] alkaloid[29] and flavonoid[26,29] content were also examined without exact compound identification.

Moreover, level of inorganic components, such as Mg, K, P, N, Ca, V, Cr, Co, Ni, Cu, Zn, Ga, Rb, Sr, Zr, Nb,[30] Cs, Ba, Ce, Pb,[30,31] Na, Fe,[14,30] Th, U[31,32] and Nd,[32] were determined as well.

Due to its wide-spread nature and easily detectable fruiting body, the *Meripilus giganteus* was the subject of several pharmacological examinations. This research could be divided based on the investigated extracts:

The dichloromethane extract was examined for anti-microbial activity and was found effective against *Bacillus subtilis*, *Escherichia coli* and *Candida albicans*,[15,33] yet the most studies focused rather on the alcohol extracts.

The methanol extract demonstrated cytotoxicity on murine cell line 3LL (IC_{50} 19.8 ± 2.6 μg/mL)[15] and HeLa cells (IC_{50} 0.72 ± 0.27 mg/mL),[16] however, it was non-toxic to zebra fish.[16] It also showed antioxidant activity in several *in vitro* antioxidant tests (DPPH,[16,25] superoxide anion test,[25] Folin- Ciocalteu assay,[16] Ferricyanide/Prussian blue assay,[16] β-Carotene/linoleate assay,[16] TBARS assay[16]). It also had a narrow spectrum of action against Gram-negative and broader against Gram-positive bacteria,[25] however, it was ineffective against *Propionibacterium acnes*,[34] Another experiment, still investigating the antimicrobial effect against 8-8 strains of bacteria and fungi, also highlighted the fact that the Meripilus extract is more effective against Gram-positive bacteria than Gram-negative, moreover, an inhibition on *Aspergillus versicolor* and *Penicillium funiculosum* were demonstrated as well.[16] The same extract was also examined by Maja Karaman et al. for antioxidant and antimicrobial capacity. The former was performed by using 2,2-diphenyl-1-picrylhydrazyl - DPPH - (IC_{50} 175.75 ± 0.43 μg/mL) and OH (IC_{50} 384.83 ± 0.13 μg/mL) assay, the later by applying the extract on 13 different bacterial strain samples and getting 11 positive results.[26] The antioxidant capacity was examined again using the methanolic and chloroform extracts by DPPH assay (EC_{50} 250 ± 1.58 and 62.5 ± 1.79 μg/mL) and OH (pro-oxidative in both cases) assay.[29] The methanolic and ethanol extracts were tested as differentiation inducers on mouse osteoblastic cell line MC3T3-E1 with positive results. The antioxidant activities of the

methanolic and chloroform extracts were measured with DPPH (EC_{50} 155.20 ± 2.36 and 365.3 ± 1.79 μg/mL) and OH (EC_{50} 527.10 ± 1.57 μg/mL– the chloroform showed pro-oxidative effect) and tested on 9 microbial strains.[27] The same extracts showed LP inhibitory effect as well in a different study.[29]

The ethanolic extract was deemed an effective antioxidant as well and was tested as a cytotoxic, pro-apoptotic and anti-proliferative agent on Jurkat and HL-60 cell lines[25]. A later examination found that the extract enhanced apoptosis by reducing the intracellular ROS level and increasing FAS gene expression, moreover, showed selectivity towards leukemic cells. Another study examined the effect of ethanolic and ethyl-acetate extracts as an anti-leishmanial agent resulting moderate activity with IC_{50} values 265.06 ± 0.92 and 306.43 ± 0.28 μg/mL respectively.[35] Both ethanolic and methanolic extracts were considered to be effective against *S. aureus* and *E. coli*.[14] Furthermore, the same experiment included a toxicity screening test demonstrating that neither the methanolic, nor the diethyl ether extract showed cytotoxicity on human bladder cells.[14] The ethanolic extract was tested for its antibacterial and anti-yeast activity on 11 strains (being effective in every cases) and examined as an antioxidant with DPPH assay.[36]

The *Meripilus giganteus* polysaccharide (MGPS; a water soluble glucan with ~1.48×10^5 Da molecular weight) was also an effective antioxidant that showed hydroxide and superoxide radical scavenging, ferrous-ion chelating activity,[37] increased the lymphocyte T-cell subsets, TNF-α and NO production and demonstrated considerable immunomodulatory activity.[38]

3.2. Morphology, chemistry, and pharmacology of *Porodaedalea chrysoloma*

Porodaedalea chrysoloma (Fr.) Fiasson & Niemela (family Hymenochaetaceae, order Hymenochaetales, phylum Basidiomycota) (known also as *Phellinus chrysoloma (Fr.)*, *Polyporus chrysoloma* (Fr.)) distributed on the Northern Hemisphere growing on both living and dead spruce trees,[39] whereas causes white pocket rot leading to a negative reputation on the field of forestry.

Morphologically, it is characterized by the resupinate to effused-reflexed thin basidiocarps, a thin dark layer that separates the upper tomentum from the lower context, and the narrower, shorter setae.

Only a single study focused on the effects of the polysaccharides from the mentioned species and *Phellinus igniarius*. This article, however, found that both polysaccharides has remarkable α-

glucosidase inhibitory activities, capable to increase the insulin sensitivity index (ISI) and demonstrated high α-glucosidase inhibitory activities while decreasing the fasting serum glucose level in type 2 DM mice and improved insulin resistance.[40] The chemistry of *P. chrysoloma* is almost completely unknown so far.

3.3. Morphology, chemistry and pharmacology of *Fuscoporia torulosa*

Fuscoporia torulosa (T. Wagner & M. Fisch.) (family Hymenochaetaceae, order Hymenochaetales, phylum Basidiomycetes) [known also as *Phellinus torulosus* (Bourdot & Galzin) until the split of Phellinus genus; *Polyporus fuscopurpureus* (Boud.), *P. rubriporus* (Quél.), *P. torulosus* (Pers.), *P. marucuccianus* (Lloydand), Tufted Bracket, Rotporiger Feuerschwamm, Wulstiger Feuerschwamm and vörös tapló] is distributed on the Northern hemisphere, living as a parasite or a saprobe of more than 120 different species of wood, causing white pocket rot. Since the mushroom lives in the tree without producing a fruiting body for a relatively long time, it is hard to detect in the early stages of the infection.[41] When the specimen becomes visible, it develops a flat, hemispherical cap with lobed edges. It could grow as much as 30 cm in diameter and coloured a rusty red, but could turn black by age.[42]

It is noteworthy that a new species mainly located in America, *Phellinus coronadensis* was also considered as *Fuscoporia torulosa* previously.[43]

The genus Fuscoporia has a long record in the field of oriental medicine due to its application in treating gastrointestinal problems, inflammations, arthritic pains and even cancer.[44]

Several studies are available describing the chemical constiuents of *F. torulosa*. Deveci et al. identified 11 components (1 of them for the first time), namely 5*α*,8*α*-epidioxyergosta-6,22-dien-3*β*-il-palmitate, ergosta-4,6,8(14),22-tetraen-3-one, ergosterol peroxide, oleanolic acid, 28-norolean-12-en-3*β*-ol, javeroic acid, β-sitosterol, oleanonic acid, 2,3-dihydroxycinnamic acid, 3,4-dihydroxybenzaldehyde, 4-(3,4-dihydroxyphenyl), and but-3-en-2-one.[45] In addition, the following compounds were isolated from the species: pomacerone (furanoid triterpene), ursolic acid, javeroic acid, phellinic acid, senexdiolic acid[46], albertic acid, natalitic acid, torulosic acid (triterpene acids), ergosta-7,22-dien-3*β*-ol, and ergosterol peroxide.[45,47]

Former studies also determined the total carbohydrate and protein levels, the monosaccharide composition in the polysaccharide extract of the mushroom,[48] the phenolic acid composition[44,49]

(gallic acid, catechin, chlorogenic acid, syringic acid and benzoic acid),[49] the average amount of heavy metals (Cd, Cr, Cu, Mn, Zn),[50] boron[51] and trace elements (Na, Mg, Al, P, S, K, Ca, Cl, Fe, V, Cr, Co, Ni, Cu, Zn, Cs, Ba, Ce, Pb, Th, U).[31]

The polysaccharides in the mushroom demonstrated remarkable biological activity in different antioxidant assays, including DPPH, ABTS, CUPRAC and metal chelating assay. In the same study the compounds also showed low anticholinesterase activity in AchE and BChE assays.[48]

Additionally, the antioxidant property of the ethanolic extract was examined by DPPH assay and measuring total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI), meanwhile the fungal sample demonstrated weak cytotoxic effect on A549 cells.[49] Furthermore, the potential cytotoxicity on MCF-7 (breast cancer), PC-3 (prostate cancer), and 3T3 (nontumor) cell lines were also studied. Two (hexane and methanolic) out of the 5 investigated extracts showed cytotoxic activity on all 3 type of cells. Moreover, 11 components were isolated, 10 of which demonstrated toxicity against MCF-7 and 3 of them had a negative impact on PC-3. The identified compounds were scanned for antioxidant activity using ABTS•+, DPPH•, β-carotene–linoleic acid tests, metal chelating activity on Fe^{2+}, and cupric reducing antioxidant capacity (CUPRAC) assays, obtaining promising results.[45] A research group from Thailand examined the antioxidant properties of the mushrooms extracts (water, 50% EtOH, 80% EtOH, EtOH and EtOAc) and crude fractions (EtOAc and CH_2Cl_2) and compared them to other species from the Phellinus genus. In most of the cases *Fuscoporia torulosa* proved to have one of the lowest IC_{50} which could correlate with its relatively high phenolic content.[44]

4. MATERIALS AND METHODS

4.1. MUSHROOM MATERIALS

4.1.1. *Meripilus giganteus*

Samples of *Meripilus giganteus* were collected by Viktor Papp and Kinga Rudolf from the roots of living beech and hornbeam in Mecsek Montains (Southern Transdanubia, Hungary) in September 2014 and 2015, near the villages of Püspökszentlászló (46°10’41” N, 18°21’51” E), Óbánya (46°13’08”N, 18°23’51”E), and Bakonya (46°05’54” N, 18°03’56” E), and in Visegrád Montains (Central Hungary), near the village of Pilismarót (47°44’19” N, 18°50’43” E) in August 2016. The samples were identified based on macro- and microscopic features. The different collections were combined for the preparative mycochemical experiment. The combined amount (12 kg) was processed in fresh condition. Voucher specimens have been deposited in the mycological collection of the Hungarian Natural History Museum (BP 106949-51).

4.1.2. *Porodaedalea chrysoloma*

Although the *Porodaedalea chrysoloma* is native in Hungary, the processed samples were collected from Granbodåsen (Sweden) and Boubínský prales (Czech Republic) from European spruce by Viktor Papp. The two samples were dried, resulting 90 g and 270 g of dry mass, which we combined for extraction. Voucher specimens have been deposited in the mycological collection of the Hungarian Natural History Museum, Budapest.

4.1.3. *Fuscoporia torulosa*

The examined *Fuscoporia torulosa* was collected by Viktor Papp in 2 years time. In March 2017 the mushroom was found on Gerecse Montains, in Central Transdanubia, Hungary living on Austrian oak, black locust and small-leaved linden. The harvested dose was combined with those collected in April 2018 from the Botanical Garden of Buda where it lived on a living black locust. The whole fresh mass weighting 1425 g.

4.2. EXTRACTION

4.2.1. *Meripilus giganteus*

The fresh mushroom material (12 kg) was ground using a Waring industrial blender and percolated with 46 L methanol (Molar Chemicals, Hungary) on room temperature. The combined methanol extract was concentrated *in vacuo* using Büchi Rotavapor R-220 SE equipped with a Julabo FL4003 cooler and a Büchi V-710 Vacuum Pump then a Büchi Rotavapor R-210 combined with a V-100 Vacuum Pump and a B-491 Heathing Bath from the same manufacturer and a Julabo F250 cooler. The residue (145 g) was solved in 50% aqueous methanol and subjected to solvent-solvent partition first with *n*-hexane (5 × 400 mL, A), then chloroform (5 × 400 mL, B). The partitions were dried again using the same method leading to 19.84 g and 7.79 g dry samples, respectively.

4.2.2. *Porodaedalea chrysoloma*

The dry sample (360 g) was ground with a Retsch Grindomix GM 200 and percolated with 15 L methanol (Molar Chemicals, Hungary). This extract was concentrated using a Büchi Rotavapor R-210 combined with a V-100 Vacuum Pump and a B-491 Heathing Bath from the same manufacturer and a Julabo F250 cooler, resulted 19.6 g dry material. This sample was dissolved in 50% aqueous methanol for solvent-solvent partition. For this process *n*-hexane (5 × 250 mL, A), chloroform (6 × 250 mL, B) and ethyl-acetate (9 × 250 mL, C) were applied. After using the same evaporation methods as previously, 2.73 g, 2.58 g and 5.97 g residues were obtained, respectively.

4.2.3. *Fuscoporia torulosa*

The dried sample (1400 g) was stored in room temperature before it was ground with a Retsch Grindomix GM 200. The sample was percolated with 20 L methanol.Concentration of the extract was made by evaporation *in vacuo*, resulting 44.26 g extract. The acquired sample was dissolved in 50% aqueous methanol and subjected to solvent-solvent partition with *n*-hexane (5 × 300 mL, A), chloroform (6 × 300 mL, B) and ethyl acetate (6 × 300 mL, C). Evaporation of the solvents led to 10.34 g, 13.47 g and 10.60 g samples, respectively.

4.3. PURIFICATION AND ISOLATION OF COMPOUNDS

4.3.1. Thin-layer chromatography (TLC)

Thin-layer chromatographic analyses were performed on NP (Merck, TLC Silica gel 60 F_{254}, Darmstadt, Germany) and RP (Merck, TLC Silica Gel 60 RP-18 $F_{254}S$, Darmstadt, Germany) silica gel covered aluminium sheets.

MG-TLC1: Mixture of *n*-hexane:isopropanol (8:2 v/v)

4.3.2. Flash column chromatography (FCC)

The separation with flash chromatography was carried out using a CombiFlash® Rf^+ Lumen instrument equipped with UV, UV-VIS and ELS detectors. We applied RediSep Rf Gold Normal Phase Silica Flash Columns as the stationary phase loaded with 4 (MG, PC, CM), 12 (MG, PC, CM), 60 (MG, CM) and 80 (PC) g of normal-phase silica and 50 g of C18 reversed-phase silica (PC) (Teledyne Isco, Lincoln, NE, USA). For the separations of *F. torulosa* samples we used Telos refillable columns (Kinesis Inc., Vernon Hills, Ill., USA) filled with normal-phase (Merck, Silica gel 60 DF_{254}, 15 μm mean particle size, Darmstadt, Germany) and C18 reversed-phase silica (Teledyne Isco, RediSep C-18 Bulk 950, 40-60 μm mean particle size, Lincoln, NE, USA).

MG-FCC1: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 45%, t: 60 min, RS NP 60 g column)

MG-FCC2: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 25%, t: 60 min, RS NP 12 g column)

MG-FCC3: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 30%, t: 45 min, RS NP 4 g column)

MG-FCC4: Increasing polarity of chloroform:methanol
(methanol 0% to 45%, t: 50 min, RS NP 60 g column)

MG-FCC5: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 100%, t: 60 min, RS NP 12 g column)

MG-FCC6: Increasing polarity of *n*-hexane:acetone
(acetone 5% to 25%, t: 50 min, RS NP 4 g column)

MG-FCC7: Increasing polarity of *n*-hexane:acetone
(acetone 5% to 25%, t: 50 min, RS NP 4 g column)

PC-FCC1: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 50%, t: 60 min, RS NP 80 g column)

PC-FCC2: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 15%, t: 45 min, RS NP 12 g column)

PC-FCC3: Decreasing polarity of water:methanol
(methanol 85% to 100%, t: 50 min, RS RP 50 g column)

PC-FCC4: Decreasing polarity of water:acetonitrile
(acetonitrile 75% to 100%, t: 45 min, RS RP 50 g column)

PC-FCC5: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 35%, t: 60 min, RS NP 4 g column)

FT-FCC1: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 40%, t: 55 min, Te NP 120 g column)

FT-FCC2: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 50%, t: 60 min, Te NP 120 g column)

FT-FCC3: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 25%, t: 50 min, Te NP 80 g column)

FT-FCC4: Increasing polarity of *n*-hexane:acetone
(acetone 0% to 10%, t: 60 min, Te NP 4 g column)

FT-FCC5: Decreasing polarity of water:methanol
(methanol 15% to 50%, t: 60 min, Te RP 4 g column)

FT-FCC6: Increasing polarity of *n*-hexane:acetone
(acetone 5% to 25%, t: 50 min, Te NP 40 g column)

FT-FCC7: Increasing polarity of chloroform:methanol
(methanol 0% to 40%, t: 45 min, Te NP 120 g column)

FT-FCC8: Increasing polarity of chloroform:methanol
(methanol 0% to 40%, t: 50 min, Te NP 12 g column)

4.3.3. High-performance liquid chromatography (HPLC)

For compound purification, we utilized both normal and reverse phase separations on various HPLC devices. The normal phase separation was carried out on a Wufeng LC-100 Plus HPLC instrument equipped with a UV-VIS detector (Shanghai Wufeng Scientific Instruments Co., Ltd., Shanghai, China) set at 254 nm using with a Zorbax SIL column (250 × 4 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA), meanwhile for the reverse phase Zorbex ODS column (250 × 9.4 mm, 5 μm; Agilent Technologies, Santa Clara, CA, USA) was utilized.

MG-HPLC1: Isocratic mixture of *n*-hexane:isopropanol
(isopropanol 35%, SILs NP column)

MG-HPLC2: Isocratic mixture of *n*-hexane:isopropanol
(isopropanol 35%, SILs NP column)

FT-HPLC1: Decreasing polarity of water:methanol
(methanol 91% to 92%, ODS RP column)

4.4. STRUCTURE DETERMINATION

4.4.1. Nuclear magnetic resonance spectroscopy (NMR)

The NMR spectra were recorded on a Bruker Avance NEO 500 MHz (Bruker, Rheinstetten, Germany) - in case of MG, PC and FT -, Bruker 800 MHz Avance III HD (Bruker, Rheinstetten, Germany) - in case of CM and FT - or a Varian 800 MHz (Varian, Inc., Palo Alto, CA, USA) - in case of PC - NMR spectrometer, equipped with a liquid helium cooled 5 mm Prodigy BBO CryoProbe or with a 5 mm HCN ^{13}C-enhanced salt tolerant cold probe. The temperature was set at 298 K and the applied solvents were CD_3OD (MG, FT), $CDCl_3$ (PC, FT), 4:1 and 1:4 mixture of them (CM), or pyridine (CM). Chemical shifts were referenced to residual solvent signals (3.31 ppm for ^{1}H and 49.15 for ^{13}C in case of methanol-d_4). Both one- (^{1}H and ^{13}C) and two-dimensional (COSY, HSQC, HMBC and ROESY) data were recorded with the use of the pulse sequences available in the Bruker Topspin 3.5 or in the VNMRJ 3.2 sequence libraries. For data analysis ACD/Spectrus Processor 2017.1.3 software (ACDLabs, Toronto, ON, Canada) (MG) or MestReNova v6.0.2-5475 software (Mestrelab Research S.L., Santiago de Compostela, Spain) (PC) was applied. Molecular modelling study was performed within the Jaguar software package (Jaguar, version 10.4, Schrodinger, Inc., New York, NY, 2019.)

4.4.2. Mass spectrometry (MS)

With the aim of structure elucidation HRMS and MS-MS methods were used on a Thermo Velos Pro (MG, CM, FT), Thermo Q Exactive Plus (PC) Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA, USA), or LTQ XL (FT) systems with a coupled Agilent 1100 HPLC system. For ionization ESI-operated (MG) or Flow injection analysis (FIA) method (PC) were used in negative or positive ion mode. The protonated, deprotonated and adduct ion peaks were fragmented at a normalized collision energy of 35%–45% by CID with helium as a collision gas. The scan mass range was *m/z* 150–2000 with the resolution of 140,000. The solvent used for every sample was methanol. The resulting data was acquired and analysed with Xcalibur 4.0 (Thermo Fisher Scientific).

4.5. ANTIOXIDANT ASSAYS

4.5.1. 2,2-Diphenyl-1-picryl-hydrazyl-hydrate assay (DPPH)

For determination of the free radical scavenging potential of the isolated compounds, we used a method based on Miser-Salihoglu et al. [52] The examination was performed on a FLUOstar Optima BMG Labtech plate-reader with 96-well microplates. The samples were measured in a DMSO environment with a volume of 150 µL per sample resulting a 1 mg/mL concentration. Every well contained 50 µL of this base solution (100 µM) for the absorbance measurement (30 min., at 550 nm). In case of samples showing no or minor activity, the concentration was doubled for a follow-up measurement. The most active samples were characterized by half maximal effective concentration (EC_{50}), which was determined using a dilution series beginning with 100 µM solution and halving it in every consecutive step. For data evaluation GraphPad Prism 6.0 software was utilized. The DPPH (2,2'-diphenyl-1-picrylhydrazyl) reagent necessary for the process was supplied by Sigma-Aldrich Hungary.

4.5.2. Oxygen radical absorbance capacity assay (ORAC)

The method used in case of the ORAC assays was based on the work of Mielnik et al.[53] For this method a 96-well black microplate using 20-20 µL of the samples with 0.01 mg/mL concentration was applied. These samples were mixed together with 60 µL of AAPH (12 mM final concentration) and 120 µL of fluorescein solution (70 nM final concentration) in each well. The fluorescent characteristic of each sample was measured in triples for 3 hours with 1.5 minute cycle intervals on a FLUOstar Optima BMG Labtech plate-reader. For standard, Trolox ((±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid, Sigma-Aldrich Hungary) was used together with the AAPH [2,2'-azobis(2-methyl-propionamidine)dihydrochloride] reagent. Fluorescein was obtained from Fluka Analytical, Japan. The results of the assay were expressed as mmol Trolox equivalent per g of dry material (mmolTE/g) calculated with GraphPad Prism 6.0 software.

4.6. CYTOTOXICITY ASSAY

4.6.1. Cytotoxic effect

The cytotoxic properties of the isolated compounds of *Cordyceps militaris* was determined by utilizing an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on U937 human lung (lymphoblast) cells. The compounds were added in a decreasing concentration to a 96-well cell culture microplate starting with 100 μg/mL. Applied cell density was 6×10^4 cells in a well and the incubation lasted for 24 h on 37 °C. In the next step 20 μL of MTT (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) solution (5 mg/mL) was added to every well. After an additional 4 h on the same temperature 100 μL of 10% sodium dodecyl sulphate (SDS, Sigma-Aldrich Chemie GmbH) was applied. This step was followed by another 12 h incubation period ending in measuring optical density (OD) at 550 nm (ref. 630 nm) with EZ READ 400 ELISA reader (Biochrom, Cambridge, UK) to determine IC_{50} values. The results were calculated by GraphPad Prism software version 5.00 (GraphPad Software, San Diego, CA, USA).

The examination of *Fuscoporia torulosa* compounds was performed similarly as described above with the differences of the starting compound concentration (100 μM) and 10^4 human colonic adenocarcinoma and embryonic lung fibroblast cells were used in 100 μL of RPMI-1640 or EMEM medium in each well. The optical density (OD) was measured with a Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA) at 540 nm (ref. 630 nm) instead of 550 nm.

4.6.2. Checkerboard combination assay

The interaction of the isolated compounds from *Fuscoporia torulosa* with the chemotherapeutic drug doxorubicin was investigated as well. The assay was carried out on Colo 320 (colon adenocarcinoma) cell line with final concentration of the compounds and doxorubicin used in the combination experiment in accordance with their cytotoxicity towards this cell line. The dilutions of doxorubicin were made in a horizontal direction in 100 μL, and the dilutions of the compounds vertically in the microtiter plate in 50 μL volume. Then, 6×10^3 of Colo 320 cells in 50 μL medium were added, except for the medium control wells. The plates were incubated for 72 h at 37 °C in 5% CO_2 atmosphere. The cell growth rate was determined after MTT staining. At the end of the incubation period, 20 μL of MTT solution (from a stock solution of 5 mg/mL) were added to each well. After incubation at 37 °C for 4 h, 100 μL of SDS solution (10% in 0.01 M HCI) were added

to each well and the plates were further incubated at 37 °C overnight. OD was measured at 540 nm (ref. 630 nm) with Multiscan EX ELISA reader (Thermo Labsystems, Cheshire, WA, USA). Combination index (CI) values at 50% of the growth inhibition dose (ED_{50}) were determined using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA) to plot four to five data points at each ratio. CI values were calculated by means of the median-effect equation, according to the Chou–Talalay method, where CI<1, CI=1, and CI>1 represent synergism, additive effect (or no interaction), and antagonism, respectively.

4.6.3. Rhodamine 123 accumulation assay

The evaluation of the Fuscoporia compounds P-gp efflux modulation effect was performed via flow cytometry, thorough measuring the rhodamine-123 accumulation in MDR Colo 320 human colon adenocarcinoma cells. The cell numbers were adjusted to 2×10^6 cells/mL, re-suspended in serum-free RPMI 1640 medium and distributed in 0.5 mL aliquots into Eppendorf centrifuge tubes. The tested compounds were added at 2 or 20 μM concentrations, and the samples were incubated for 10 min at room temperature. Tariquidar was applied as positive control at 0.2 μM. DMSO at 2 v/v % was used as solvent control. Next, 10 μL (5.2 μM final concentration) of the fluorochrome and ABCB1 substrate rhodamine 123 (Sigma) were added to the samples and the cells were incubated for a further 20 min at 37 ºC, washed twice and re-suspended in 1 mL PBS for analysis. The fluorescence of the cell population was measured with a PartecCyFlow® flow cytometer (Partec, Münster, Germany). The fluorescence activity ratio (FAR) was calculated as the quotient between FL-1 of treated/untreated resistant Colo 320 cell line over treated/untreated sensitive Colo 205 cell line according to the following equation:

$$FAR = \frac{Colo320_{treated} / Colo320_{control}}{Colo205_{treated} / Colo205_{control}}$$

4.7. ANTIMICROBIAL ASSAY

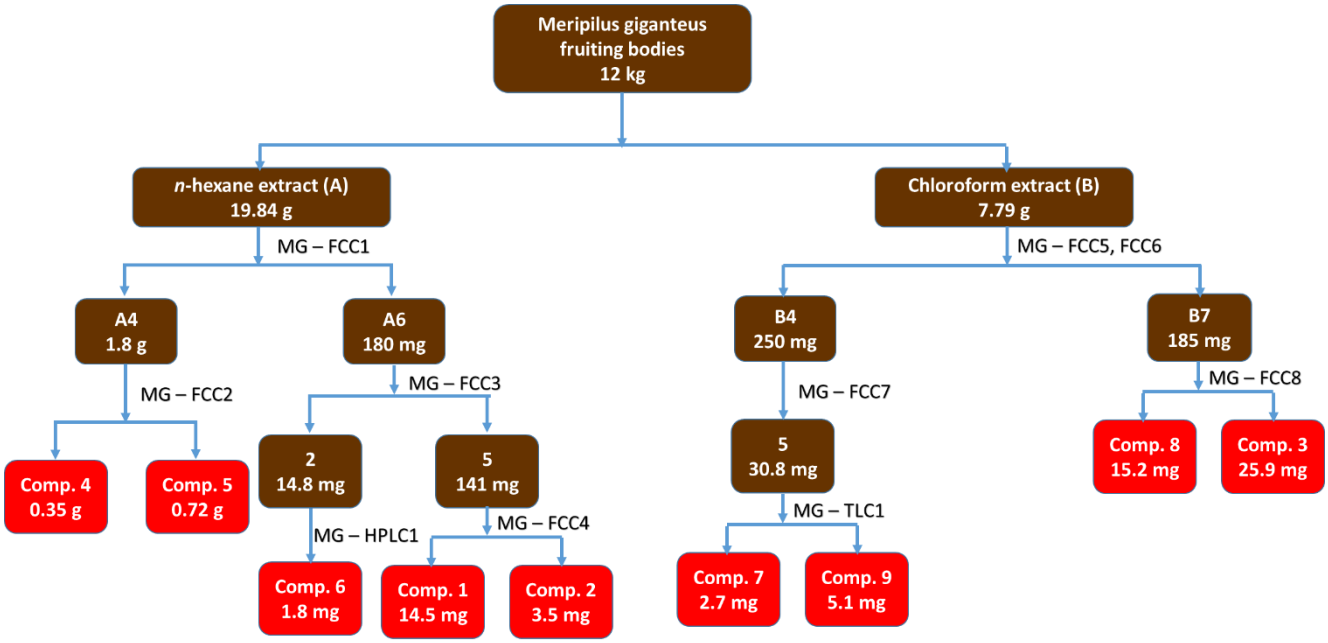
For examining the possible beneficial antimicrobial impact of the tested compounds, *Escherichia coli* ATCC (American Type Culture Collection) 25922, *Salmonella enterica* serovar Typhimurium 14028s, *Staphylococcus aureus* ATCC 25923 and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate were used. The minimal inhibitory concentrations (MICs) were determined in 3 assays according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The measured compounds were diluted in 100 μL of Mueller-Hinton medium using 96-well flat-bottomed microtiter plates. After this step, 10^{-4} dilution of an overnight bacterial culture in 100 μL of medium were then added to each well (except media control wells). These mixtures were incubated for 18 h at 37 ºC before the MIC values were determined by inspection with naked eye.

5. RESULTS

5.1. ISOLATION OF COMPOUNDS FROM THE INVESTIGATED SPECIES

5.1.1. Isolation of compounds of *Meripilus giganteus*

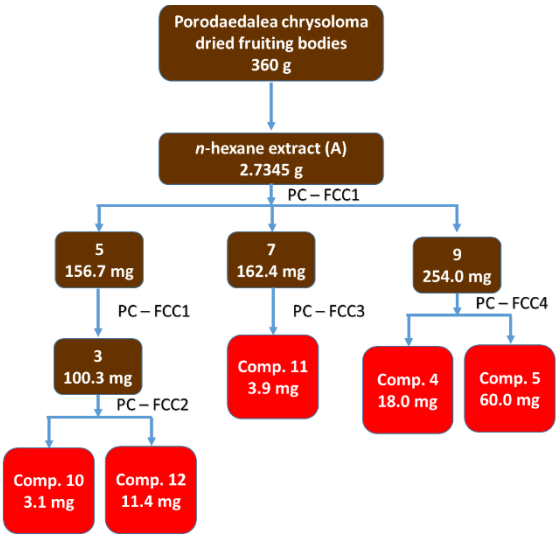
12 kg of fresh fruiting body was grounded and percolated with methanol. The extract was concentrated (145 g), dissolved in 50% aqueous methanol and underwent to a solvent-solvent partition, using *n*-hexane, chloroform and ethyl-acetate. To separate compounds from *n*-hexane fraction, consecutive flash chromatographic steps (MG-FCC 1-4) were used. This combined with a high performance liquid chromatographic separation (MG-HPLC1) yielded the pure compounds **1-2** and **4-6**. The chloroform fraction was processed similarly, utilizing Flash chromatography (MG-FCC5-8) and preparative thin layer chromatography (MG-TLC1) to gain 4 pure compounds, **3** and **7-9**.



5.1.2. Isolation of compounds of *Porodaedalea chrysoloma*

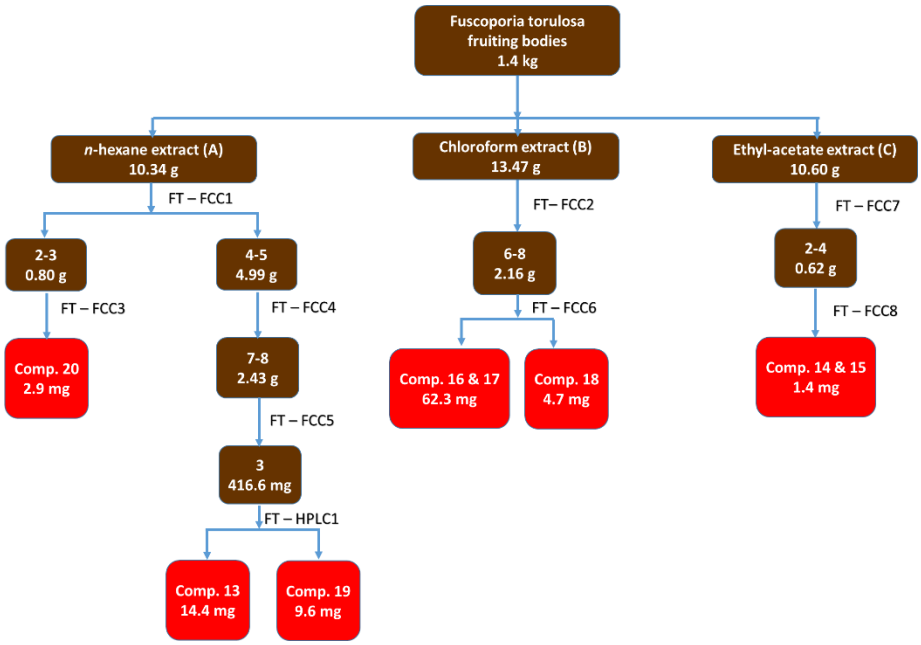
360 g of air-dried sample were grounded and extracted with methanol. After concentration, the extract underwent 3 consecutive solvent-solvent partitions, using *n*-hexane, chloroform and ethyl-acetate, consecutively. Out of these extracts the *n*-hexane became the focus of our examination. After the first step of separation using flash chromatography (PC-FCC1) 3 subfractions were further purified, and led to pure compounds: subfraction 5 yielded compounds **10** and **12** after two

consecutive runs (PC-FCC1 and 2), while subfraction 7 was separated (PC-FCC3) to give compound **11**, subfraction 9 meanwhile led to the isolation of **4** and **5** after one separation step with flash-chromatography (PC-FCC4).



5.1.3. Isolation of compounds of *Fuscoporia torulosa*

1.4 kg of air-dried fruiting bodies were grounded and percolated with methanol. After concentration of the extract, it was resolved in 50% aqueous methanol and solvent-solvent partition were used similarly as mentioned before. After a rough separation with flash chromatography (FT-FCC1), some subfractions of the *n*-hexane fraction resulted in the separation of compound **20** (applying flash chromatography, FT-FCC3), **13** and **19** (applying FCC and HPLC, FT-FCC4-5 and FT-HPLC1). Compounds **16**, **17** and **18** were isolated from a chloroform fraction related subfraction with the application of flash chromatography (FT-FCC2 and 6). The ethyl acetate extract was also processed with flash chromatographic methods (FT-FCC7-8) resulting in the mixture of **16** and **17**.

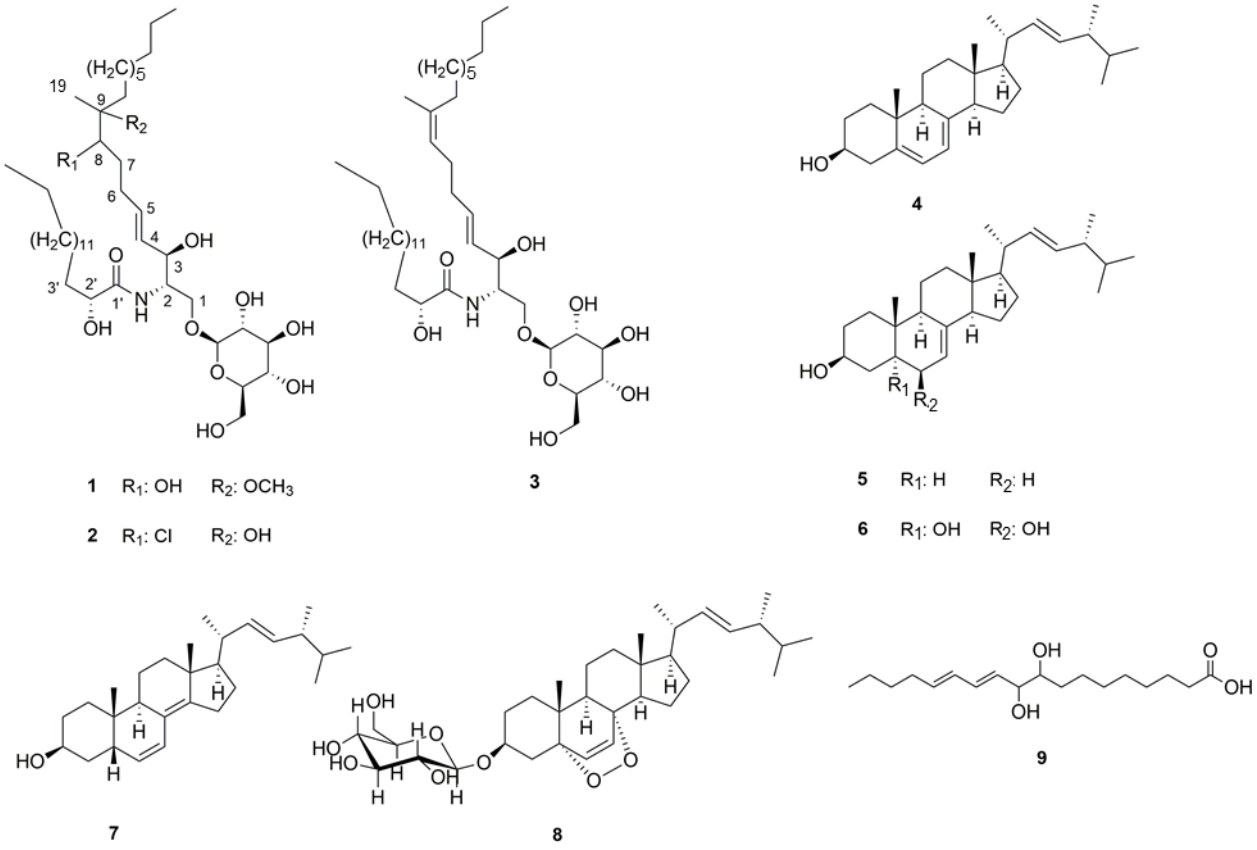


5.2. STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

For the clarification of structures of the isolated compounds, a combination of NMR and MS investigations was used. The information gained from 1D (^{1}H-NMR and JMOD) and 2D (^{1}H-^{1}H COSY, TOCSY, NOESY, ROESY, HSQC, HMBC) NMR led to the structure determinations, whereas HRMS data revealed the molecular masses and compositions of the components.

5.2.1. Compounds from *Meripilus giganteus*

The separation and the structure determination have enabled the identification of 9 compounds. Three of them (compound **1-3**) belong to the group of cerebrosides of which two, mericeramides A (**1**) and B (**2**), are new natural products.



The molecular composition of compounds **1** and **2** found to be $C_{42}H_{81}O_{11}N$ and $C_{41}H_{79}ClO_{10}N$, respectively, highly similar to the composition of cerebroside B (compound **3**). The main different feature of the NMR spectra was the resonance belonging to C7-C9. The readings pointed out that instead of being a double bond in the middle of the sphingadienine chain, a methine group and a quaternary carbon was suggested between C-8 and C-9. The resonances belonging to C-19 also showed dissimilarities compared to cerebroside B. Furthermore, the ^{1}H NMR spectrum indicated the presence of an additional methoxy group in compound **1**. Based on these results and the HMBC correlations 8-hydroxy-9-methoxycerebroside (**1**) and 8-chloro-9-hydroxycerebroside (**2**) structures were identified.

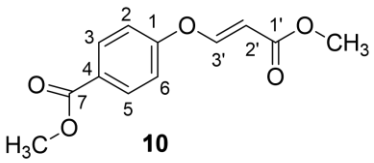
The determination of absolute stereochemistry was impossible based on collected data, however, the high level of similarity between the NMR data of the 2 new compounds and cerebroside B suggests similar stereochemistry in the chiral centres. Compound **1** also showed two peaks in case of ^{13}C resonances assigned for the C4-C8 moiety, suggesting a mixture of C-8 and/or C-9 epimers. 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.[54–56] Unfortunately, based on this method the absolute stereochemistry of (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) could not be determined.

The newly identified components are a part of the group of cerebrosides, neutral glycosphingolipids which are present in animals, plants and also in large numbers of fungi. These compounds consist of a ceramide portion with 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acids, and a carbohydrate moiety, usually glucose or galactose. To the best of our knowledge mericeramids B (**2**) is the first natural halogenated cerebroside identified. The halogenated natural compounds could be considered quite rare, however, there are several examples where the members of the fungi kingdom produced such molecules.[57]

5.2.2. Compounds of *Porodaedalea chrysoloma*

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



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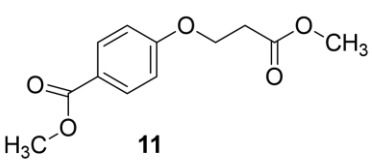

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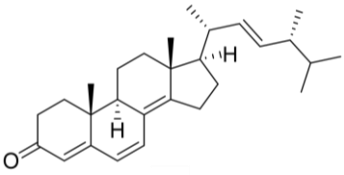


12

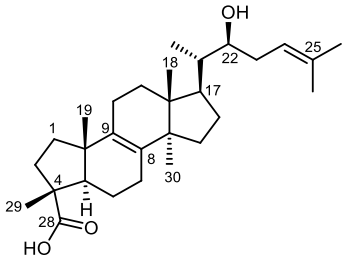
Compound **10** was found to be a white, amorphous solid with the molecular formula of $C_{12}H_{12}O_5$. According to the ^{1}H NMR spectrum signals of mutually coupled protons and the ^{1}H-^{1}H COSY spectrum, the presence of *p*-disubstituted benzene ring and a *trans*-double bond was suspected in 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 ^1H 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.

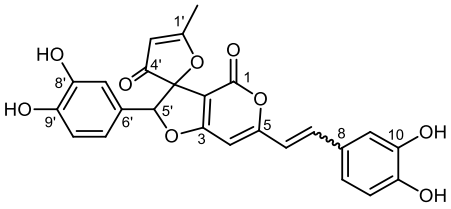
Ergone (**12**) was characterised by a comparison of the achieved NMR and the available literature data. Ergosterol (**4**) and 3β-hydroxyergosta-7,22-diene (**5**) were identified by matching their chromatographic and spectroscopic data to an authentic sample isolated previously.

5.2.3. Compounds of *Fuscoporia torulosa*

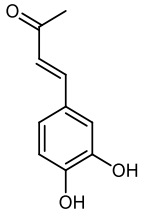
Processing the sample of *Fuscoporia torulosa* allowed us the isolation of 8 components. Structure determination identified compound **13** as a novel triterpene (fuscoporic acid), while **15** proved to be a previously undescribed *Z* isomer of inoscavin A.



13



14, 15



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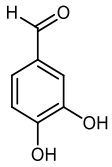

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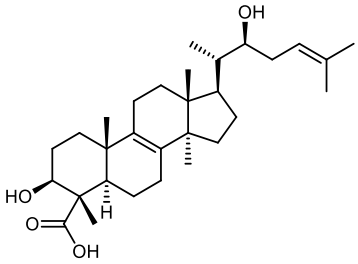

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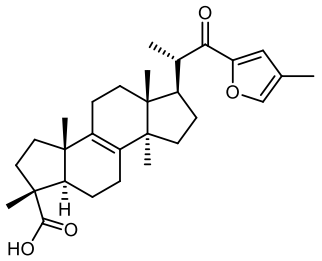

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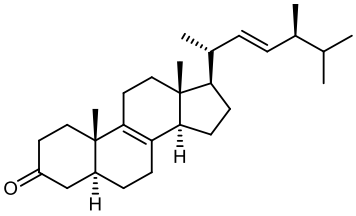

20

The ^{1}H and ^{13}C NMR spectra of **13** presented similar spectral features to those reported for gilvsin D[58] and obtained for natalic acid (**19**).[47] Consecutive analysis of the ^{1}H,^{1}H-COSY, HSQC and HMBC spectra of **13** supported our previous hypothetical suggestion, and enabled the complete ^{1}H and ^{13}C NMR assignments. Due to the spectral data similarities, all three compounds share the same degraded lanosterol skeleton. The structural differences lie in the side chains attached to C-17. HSQC and HMBC data suggested that in case of **13** a 22-hydroxy-24-en side chain was present. 1D and 2D ROESY data were in accordance with the suspected structure and proved that the relative configurations of C-4, C-5, C-10, C-13, C-14, C-17, and C-20 were identical in **13** and gilvsin D as well as in natalic acid (**19**). In addition to this, with regard to the findings of Barrera and coworkers[46] the ca. 3.6 Hz coupling constant observed between H-21 and H-22 suggested an *S* configuration of the C-22 chirality centre. In summary, **13** was characterized as 22*S*-hydroxy-8,24-dien-3-norlanosta-28-oic acid, named fuscoporic acid.

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 *cis* isomer has not yet been reported in the literature before. Considering the agreement of the obtained and published[59] NMR and HRMS data the major component could be assigned as inoscavin A certainly. In accordance with the proposed structure the minor component presented highly similar ^{1}H and ^{13}C NMR features, except those belonging to the hispidine moiety of inoscavin A. Thus, in the ^{1}H NMR spectrum, instead of the two doublet resonances (δ_H 7.47 and 6.75 ppm) with 15.7 Hz coupling constant, two doublets at δ_H 6.87 and 6.11 ppm with a 12.7 Hz coupling constant were obtained for H-7 and H-6, respectively. These differences were in concordance with the proposition that the double bond between C-6 and C-7 was in *cis* configuration in the minor component. ^{13}C NMR, COSY, HSQC and HMBC data

confirmed the proposed structures and enabled the complete ^{1}H and ^{13}C NMR assignments of both components. Homonuclear ROESY data were also in accordance with these structural conclusions.

The relative stereochemistry of the C-4' and C-5' stereogenic centres could not be determined on this basis. Although inoscavin A (**14**) has been known for a long time, no literature data was found that could allow the stereochemical assignment of these centres on a comparative basis either. Unfortunately, the amount of the sample was not enable to collect the specific experimental data (e.g. heteronuclear NOE) that might allow distinguishing between the possible diastereoisomers. In the absence of experimental data, a molecular modelling study was carried out for the determination of the relative stereochemistry. NMR shielding constants and chemical shift values were calculated for the (arbitrary chosen) 4'*R*,5'*S* and 4'*S*,5'*S* epimers by averaging the appropriate values obtained from four representative conformers using the Boltzmann populations derived from the solution phase energies (SI). The resulted unscaled chemical shifts (relative to TMS, using the default Jaguar procedure) are in an exceptional relationship with the experimental data in both cases.

The mean absolute error (MAE) calculated for $^{13}C/^{1}H$ chemical shifts were 1.2/0.17 ppm in the case of the *SS* and 1.3/0.26 ppm for the *RS* epimer, respectively. The scaled shifts[60,61] agreed even better with the experimental values by showing (corrected) MAE values of $^{13}C/^{1}H$ 1.2/0.12 and 1.3/0.21 ppm for the *SS** and *RS** isomers, respectively. Although only small differences were obtained, these were consistently pointing towards the presence of the *SS** isomer. Recently Grimblat et al.[62] showed that the extended and combined application of DP4 probability function introduced by Smith et al.[63] could successfully help in solveing structural questions where other methods failed. Applying their DP4+ method on the calculated isotropic shielding values, in our case the *SS* isomer was predicted as the most probable (with a 100% overall possibility) structural candidate. According to these results the mixture of compound **14** and **15** is described as the mixture of C-4'*S**, C-5'*S** inoscavin A and its *cis* analogue. Considering the obtained optical rotation value of 0, the sample is a racemate.

Compounds **16-20** were structurally characterized by HRMS, and standard one- and two-dimensional NMR data in comparison to those available in the literature. According to spectral analysis **16** represent 3,4-dihydroxy-benzaldehyde and **17** osmundacetone; these compounds were

obtained as an equimolar mixture. The remaining constituents are triterpenes, namely senexdiolic acid (**18**), natalic acid (**19**), and ergosta-7,22-diene-3-one (**20**).

5.3. PHARMACOLOGICAL ACTIVITIES OF THE ISOLATED COMPOUNDS

5.3.1. 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 regards 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

5.3.2. Antioxidant activity of the compounds from *Porodaedalea chrysoloma*

The antioxidant activity of the isolated compounds was analysed by ORAC assay, whereas 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**). It is worth mentioning that the magnitude of antioxidant activity of 3β-hydroxyergosta-7,22-diene was identical to that we measured in the case of *Meripilus giganteus*, however, the ascorbic acid found to be more active in this case.

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

5.3.3. Antioxidant and cytotoxic activity of the compounds from *Fuscoporia torulosa*

Antioxidant activity: Compound **14-17** isolated from *Fuscoporia 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** < compounds **14 + 15**, while compounds **14 + 15** possessed the most potent antioxidant capacity among all the tested compoounds 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 and anti-tumour activity: The components, not examined by DPPH or ORAC assays (**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**) outperformed the reference agent on MRC-5 cell lines.

Table 4. Cytotoxic activity of the compounds from *Fuscoporia torulosa*

Compounds	Colo205 (IC_{50} µM)	Colo320 (IC_{50} µM)	MRC-5 (IC_{50} µM)
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 this rate (compound **20**:doxorubicin 11.2:1), the Combination Index (CI, 0.521±0.15) at the 50% growth inhibition dose (ED_{50}) was indicating a synergism between the examined compounds.

Rhodamine 123 accumulation assay: 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 Colo 320 human colon adenocarcinoma cells. Tariquidar (0.2 μM), a well-known P-gp inhibitor was used as positive control. The compounds were tested at 2 and 20 μM: P-gp modulating effect was obtained at 2 μM concentrations with ergosta-7,22-diene-3-on (**20**), at 20 μM in case of **13**, **18** and **19**. 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. The results presented below show that FAR values were in the range of 0.828-1.139, therefore the tested compounds were ineffective modulators on drug resistant strain Colo 320.

Table 5. Rhodamine 123 accumulation assay results of the compounds from *Fuscoporia torulosa*

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** – calculated by the equation above

Antimicrobial assay: Compounds **13** and **18-20** were investigated for antimicrobial activity on *Escherichia coli* ATCC (American Type Culture Collection) 25922, *Salmonella enterica* serovar Typhimurium 14028s, *Staphylococcus aureus* ATCC 25923 and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate strains, however, none of the compounds were considered efficient. As the mushroom extract of *F. torulosa* showed antimicrobial activity in a screening investigation[12], the active compounds should be others than the isolated compounds.

6. DISCUSSION

6.1. INVESTIGATION OF *MERIPILUS GIGANTEUS, PORODAEDALEA CHRYSOLOMA,* AND *FUSCOPORIA TORULOSA*

The investigated mushrooms were chosen based on a previous screening performed by our research group[10–13], in addition, the availability of biomass and a general literature review was also considered to the decision. The first species, *Meripilus giganteus* was selected by two subsequent factors, whereas *Porodaedalea chrysoloma* and *Fuscoporia torulosa* showed antimicrobial activity and remarkable antioxidant potential both on ORAC and DPPH assays.

6.2. ISOLATION OF THE BIOACTIVE COMPOUNDS

Considering the data provided by both the previous screenings, we decided to use *n*-hexane, chloroform and ethyl acetate as solvents for the studied extracts. These fractions were proven to be both effective and easy to handle, yet differed from each other enough in composition and polarity of compounds. As a first step the grinded mushroom samples were percolated with amphipolar solvent (methanol) on room temperature. Secondly, the previously mentioned solvents (*n*-hexane, chloroform, ethyl acetate) were used for a liquid-liquid extraction. The resulting compositions of the samples were examined by TLC and separated by consecutive uses of various chromatographic techniques.

Both *n*-hexane and chloroform fractions of *Meripilus giganteus* were processed using multiple steps of flash-chromatography. This method alone resulted in the separation of compounds **1**-**5** and **8**. In the separation of compound **6** HPLC was involved as well with the use of a *n*-hexane:isopropanol eluent system on normal phase silica column. Finally compounds **7** and **9** were separated by the application of preparative TLC and *n*-hexane:isopropanol solvents.

In case of *Porodaedalea chrysoloma* all separated components were isolated from the *n*-hexane fraction, whereas FCC methods were used both for the rough separation and component purification resulting compounds **4**-**5** and **10**-**12**.

Fuscoporia torulosa was found to be the most complex, yet most rewarding species of the processed samples: every fraction (*n*-hexane, chloroform and ethyl-acetate) yielded fungal metabolites to examine. First the *n*-hexane fraction was separated, resulting in the isolation of **19** with FCC and compounds **13** and **20** with FCC and HPLC. Working with chloroform fraction

allowed us the isolation of the mixture of **16** and **17** and the compound **18** after consecutive steps of FCC. Processing the ethyl acetate fraction also leaned on the FCC technique, resulted a racemic mixture of **14** and **15**.

6.3. STRUCTURE ELUCIDATION

Chemical structures of the isolated compounds were determined by spectroscopic methods. While the MS data provided the molecular masses and compositions, the NMR data furnished essential information in elucidation of structures. The most useful details about the structure were gained from 1D and 2D NMR, however, ^{1}H-NMR, JMOD, ^{1}H-^{1}H COSY, TOCSY, HSQC and HMBC also helped in revealing the exact constitution of the molecules. After gathering enough data for the planar structure, NOESY and ROESY makes us able to find the relative stereochemistry of the compounds. As a result of this process, complete ^{1}H- and ^{13}C-assigments were determined successfully. This proves to be most advantageous in the case of new compounds, however, in some cases the already described molecular data could be supplemented as well.

6.3.1. Cerebrosides, steroids and fatty acids from *Meripilus giganteus*

Processing the extract of *Meripilus giganteus* provided the isolation of 9 compounds, 2 of them were previously undescribed. With regard to the structures, the components can be divided in 3 groups: the novel structures, mericeramides A (**1**) and B (**2**) together with the already known cerebroside B (**3**) belonging to the group of cerebrosides, while ergosterol (**4**), 3-hydroxyergosta-7,22-diene (**5**), cerevisterol (**6**), 3-hydroxyergosta-6,8(14),22-triene (**7**), 3-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) are members of the family of steroids. A fatty acid, (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) was also identified from the mushroom.

Cerebrosides consist of 2 major parts: a ceramide and a linked glucose molecule. The ceramid unit itself could be divided further, since it is a combination of sphingosine and fatty acids binding to the amine part. Mericeramides A (**1**) and B (**2**) are novel natural products, while cerebroside B (**3**) was discovered first in *Inonotus mikadio* by Japanese scientists in 1987.[64] Aside from the novelty, the structure of mericeramides B (**2**) is special for another reason: though there are some halogenated natural products in the fungal kingdom[65], it is the first halogen atom containing cerebroside.

The group of sterols are characteristic metabolites of the fungi. According to a study, the isolated ergosterol and 3-hydroxyergosta-7,22-diene (**5**) are among the most widespread components,[66] and these compounds account for up to 85% of sterols in the studied mushroom species.[67] The discovery of ergosterol dates back to 1889, when Tanret described as part of the characterization of *Claviceps purpurea*.[68] Since then this compound was found in so many fungi that has acquired the name 'the fungal sterol'.

Another common sterol, the hydroxylated derivate of 3-hydroxyergosta-7,22-diene, cerevisterol (**6**) - first isolated from *Saccharomyces cerevisiae*[69] - was also detected in the fungal sample. 3-*O*-Glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) a sterol with a characteristic peroxide linkage between C5 and C8 was identified in this species, its first occurrence was reported in *Hericium erinaceus*, in 1991.[54] The remaining sterol, 3-hydroxyergosta-6,8(14),22-triene (7) was first classified as a product of the oxidation of ergosterol in 1937.[70]

The only fatty acid, (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid was reported previously in a study aimed at investigating a cyanobacterium LOX enzyme.[71]

6.3.2. Phenolic derivatives and steroids from *Porodaeadlea chrysoloma*

The investigation of the chemical composition of *Porodaeadlea chrysoloma* resulted in the isolation of components belonging to two major structural groups: triterpene steroids and phenolic derivatives. Out of the 5 components 3 belong to the former group, namely ergosterol (**4**), 3β-hydroxyergosta-7,22-diene (**5**), and ergone (**12**).

Ergone (**12**) was first isolated by Hiroshi Morimoto et al in 1967 from *Candida utilis*,[72] and meanwhile was reported in several Basidomycetes, including *Scleroderma polyrhizum*,[73] *Astraeus hygrometricus*,[74] *Ganoderma applanatum*,[75] and *G. neo-japonicum*.[76] The component was mostly researched in context of cytotoxic activity and presented beneficial effects in case of renal fibrosis prevention subsequently.[77–82]

The isolated phenolic derivatives proved to be less investigated, since one of the two components was a novel natural product methyl-(*E*)-3-(4-methoxycarbonylphenoxy)-acrylate (**10**), while the other one, methyl 3-(4-methoxycarbonylphenoxy)-propionate (**11**) has not been isolated from natural source until now, just mentioned as product of a synthetic reaction.[83]

6.3.3. Benzaldehyde derivatives and steroids from *Fuscoporia torulosa*

Fuscoporia torulosa contained 8 isolated components, which could be divided into 2 categories: steroids and 3,4-dihydroxybenzaldehyde derivatives. Of the 5 structures based on steroid-scaffold, fuscoporic acid (**13**) as well as the *Z* isomer of inoscavin A (**15**) are considered as novel fungal metabolites. Furthermoer, two other compounds have also relatively limited literature data: natalic acid (**19**) and senexdiolic acid (**18**) were, however, isolated from the currently discussed species by González et al in 1993.[47] While natalic acid was identified from *Fuscoporia torulosa* for the first time , the name of senexdiolic acid comes from *Fomes senex* from which it was first isolated by Batta and Rangaswami in 1970.[84] As a contrast, ergosta-7,22-dien-3-one (**20**) could be considered quite common in fungi. It was isolated from *Polyporus umbellatus*[85], *Coriolus sanguineus*[86], *Ganoderma applanatum*[87], *Pleurotus eous*[88] and *Ganoderma australe*[89] among other species.

The three 3,4-dihydroxybenzaldehyde derivatives showed a similar pattern: both protocatechualdehyde (**17**) and 3,4-dihydroxybenzalacetone (**16**) were known at least since the first half of 1900s[90,91] and were isolated from various organisms, for example insect cuticle[92], *Salvia miltiorrhiza*[93], grapevine leaves[94] and seeds[95] (**17**) and *Inonotus obliquus*[96] (**16**). The *Z* isomer of inoscavin A is a new natural compound, however its *E* isomer was first extracted from *Inonotus xeranticus* by Kim et al. in 1999[59] and was described in several Phellynus species (*P. igniarius*[97], *P. baumii*[98], *P. linteus*[99]), but not in *Fuscoporia torulosa*.

6.4. BIOLOGICAL ACTIVITIES OF THE COMPOUNDS OF INVESTIGATED SPECIES

6.4.1. *Meripilus giganteus*

Nine compounds were isolated out of the extract of *Meripilus giganteus* sample. The antioxidant effect was determined by ORAC assay. 3 of these compounds, mericeramide B (**2**) (2.50±0.29 mmol TE/g), 3β-hydroxyergosta-7,22-diene (**5**) (4.94±0.37 mmol TE/g), and (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) (4.27±0.05 mmol TE/g) showed considerable antioxidant effect compared to the reference compound ascorbic acid (6.96±0.57 mmol TE/g).

Cerevisterol (**4**) was the subject of multiple examinations in previous studies. The GIRK channel inhibitory activity of the compound was determined by using GIRK1/4 (Kir3.1/3.4) K^+ channels

on HEK-293 (human embryonic kidney) cells resulting 7% inhibition at 1 μM and 13% at 10 μM.[100] The compound was also evaluated as an anti-inflammatory agent: although multiple investigation using a nitric oxide production mitigation or DPPH assay found no activity[101–105], the compound has a mild 5-LOX blocking effect (IC_{50} 5.46 μM)[106] and is an effective anti-inflammatory agent.[107] A screening using 12 tumour cell lines indicated cerevisterol to be non-toxic.[108]

Another compound detected in the mushroom, cerebroside B (**7**), inhibited the eukaryotic replicative polymerases without affecting the repair-related polymerase and other DNA metabolic enzymes.[109] A mixture of cerebrosides (containing compound **7**) demonstrated potential anti-inflammatory effect through NO_2^- production inhibition (16,4% at 30 μM, 42,7% at 100 μM)[110] however, another study suggested cerebroside B to be ineffective as an anti-oxidant agent.[111] Renoprotective effect against cisplatin-induced damage on LLC-PK1 cells of cerebroside B (**7**) was also confirmed [112] but its neuritogenic activity was mild.[113] Cerebroside B – along with 3 other analogues – potentiated aculeacin as a cell-wall active anti-fungal agent, without influencing the process in the absence of the drug.[114,115] The compound was considered ineffective as a tyrosinase and hyaluronidase inhibitor or anti-melanoma (B16) substance,[116] however, was moderately effective as antioxidant and had a remarkable activity against *S. aureus* (IC_{50} 323.2 μM) and E. coli (275.1 μM).[117]

6.4.2. *Porodaeadlea chrysoloma*

The detailed chemical investigation of *Porodaeadalea chrysoloma* sample yielded 5 compounds. The antioxidant properties of the isolated compounds were investigated by utilizing ORAC assay. Methyl (*E*)-3-(4-methoxycarbonylphenoxy)-acrylate (**10**) and 3β-hydroxyergosta-7,22-diene (**5**) produced prominent antioxidant results, 2.21±0.34 and 5.02±0.47 mmol TE/g, respectively, which were comparable to 16.47±0.01 mmol TE/g value produced by ascorbic acid.

Another isolated component, ergone (**12**) had a vast database of screenings for other effects as well. Although anti-proliferative activity on A375, A2058, HCT116, HBE, THLE and SW620 or 4T-1, A549, HepG-4, and MCF-7 cell lines was not demonstrated, nor inhibited STAT3, neuroprotective and cytotoxic effect on K562 and MDA-MB-231 cell lines were revealed.[118–121]

6.4.3. *Fuscoporia torulosa*

The *Fuscoporia torulosa* sample yielded 2 new compounds - fuscoporic acid (**13**) and *Z* isomer of inoscavin A (**15**) - beside 6, already described molecules.

Fuscoporic acid (**13**), senexdiolic acid (**18**), natalic acid (**19**) and ergosta-7,22-dien-3-one (**20**) were examined for cytotoxicity on Colo 205, Colo 320 (human colon adenocarcinoma) and MRC-5 cell lines, and **20** demonstrated significant results on both Colo 320 and MRC-5 cells, moreover synergistic activity with doxorubicin on Colo 320 cell line.

Unfortunately, an assay on compounds **13** and **18-20** showed negative results in the examinations by Rhodamine 123 accumulation assay and antimicrobial assays on *Escherichia coli* ATCC (American Type Culture Collection) 25922, *Salmonella enterica* serovar Typhimurium 14028s, *Staphylococcus aureus* ATCC 25923 and the methicillin and ofloxacin resistant *S. aureus* 272123 clinical isolate strains.

The antioxidant capacity of **14-17** was determined, where a mixture of **14** and **15** demonstrated considerable activity on DPPH (0.25±0.01 μg/mL) and ORAC (12.20±0.92 mmol TE/g) assays. Compounds **16** and **17** also illustrated signs of antioxidant activity (0.72±0.05 μg/mL and 12.20±0.92 mmol TE/g on the 2 assays, respectively).

According to literature data, ergosta-7,22-dien-3-one (**20**) could not bind to heterokinase 2, nor inhibit SW1990 human pancreatic cancer cell or Vero African green monkey kidney cell growth, however, cytotoxic activity against NCI-H 460 lung carcinoma cells was presented.[76,122] Demonstration of inhibitory effects towards superoxide anion generation and elastase release by human and rat neutrophils in a concentration-dependent manner was reported, despite of nonsignificant results on DPPH and ferrous ion chelating assays.[123]

The antioxidant capacity of protocatechualdehyde (**17**) was measured in a previous research with the result of 59.53±9.70 μmol Trolox/μmol and found effective against A549 and 4T1 cell lines, however, it was non-toxic against human breast carcinoma (MCF-7) and human hepatoblastoma (HepG-2), HT29, HeLa and L1210 cell lines.[124,125] The prolyl endopeptidase inhibitory effect of 3,4-dihydroxybenzalacetone (**16**) and protocatechualdehyde (**17**) was less than 10%, but the antioxidant effect measured with ABTS assay was considerable (EC_{50} 8.23±0.04 μM and 5.88±0.08 μM respectively).[126]

3,4-Dihydroxybenzalacetone (**16**) was reported to activate melanogenesys in B16 melanoma cells even in 10 μmol/L concentration, however, it failed in stimulating tyrosinase activity, making the compound outstanding, but not yet ideal for treatment of vitiligo.[127] The compound was proven to be a less effective herbicide, non-cytotoxic for human breast carcinoma (MCF-7) nor for human hepatoblastoma (HepG-2) cell lines.[124,128] It demonstrated minor antioxidant effect measured by ORAC assay (23.88±0.59 μmol Trolox/μmol), however, was among the most active compounds with similar scaffold in terms of ABTS (EC_{50}: 8.23±0.04 μM), DPPH ($IC_{0.200}$: 7.9 μM) and rat brain homogenate lipid peroxidation inhibition assay (IC_{50}: 12±0.8 μM). [124,126,129] The compound also effective in hydroxysteroid dehydrogenase (3α-HSD), COX-1, COX-2 and XO enzyme assays and showed antioxidant activity on LDL assay (IC_{50}: 0.7 μM).[130,131] 3,4-Dihydroxybenzalacetone (**16**) demonstrated toxic activity against cancer in the case of Bel-7402 (liver), HCT-8 (intestine), MCF-7 (breast), BGC-823 (stomach), Ketr3 (kidney), A549 (lung) and 4TI (mouse breast) tumour cells, but did not affect the HT29 (colon), HeLa (cervix), L1210 (mouse lymphocyte) and HepG2 (liver) cell lines.[125,132]

According to the literature search, inoscavin A (**14**) inhibited supercoiled DNA single strand breakage by both iron chelating and free radical scavenging effect; inhibited rat liver microsomal lipid peroxidation (IC_{50}: 0.3 μg/mL) which was five-fold active as vitamin E (1.5 μg/mL) and also scavenged DPPH radical (IC_{50}: 0.1 μg/mL).[59,133] The compound blocked both H1N1, H5N1 and H3N2 influenza viruses in a noncompetitive way.[98] Inoscavin A (**14**) was found to possess significant RLAR (Rat Lens Aldose Reductase) and HRAR (Human Recombinant Aldose Reductase) inhibitory activity in a study on diabetes mellitus, while also inhibited initial (on haemoglobin A1C formation) and middle phases (on methylglyoxal-medicated protein modification) of protein glycation, for protein tyrosine phosphatase 1β and for propyl endopeptidase. [99,126,134,135] Like the previous compounds, **14** exhibited antioxidant activity on DPPH (IC_{50}: 76.5 μM) ABTS, and superoxide anion radical-scavenging (1.61 nmol Trolox equivalent/mg) assays.[126,136,137] The cytotoxic effect of this compound was evaluated as well, yet only showed activity on Bel7402, L1210, HepG2, SW620, LNCaP, MCF-7 and K562 cell lines.[97,138]

7. SUMMARY

The goal of this study was to highlight the chemical and pharmacological potential hidden within the Central European mushroom species, focusing on previously undescribed compounds with antioxidant, and cytotoxic activity.

For this purpose 3 mushroom species (*Meripilus giganteus, Porodaedalea chrysoloma, Fuscoporia torulosa*) were processed. The samples were grinded, percolated with methanol and then fractions were roughly separated applying liquid-liquid extractions. This led to *n*-hexane, chloroform and ethyl acetate phases. The composition of these extracts was monitored by investigation on TLC.Next, the chosen fractions underwent several steps of separation, using FCC, normal phase and reverse phase HPLC and preparative TLC, affording the isolation of 20 compounds.

In case of *Meripilus giganteus* subsequent preparation of both *n*-hexane and chloroform phases was beneficial: the former led to the isolation of compounds mericeramides A (**1**) and B (**2**), ergosterol (**4**), 3β-hydroxyergosta-7,22-diene (**5**) and cerevisterol (**6**), whereas the latter yielded cerebroside B (**3**), 3β-hydroxyergosta-6,8(14),22-triene (**7**), 3β-O-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) and (11E,13E)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**). Out of these compounds mericeramides A (**1**) and B (**2**), were novel. Their antioxidant activity was measured with the aid of ORAC assay. On this assay mericeramide B (**2**), 3β-hydroxyergosta-7,22-diene (**5**), and (11*E*,13*E*)-9,10-dihydroxy-11,13-octadecadienoic acid (**9**) demonstrated considerable antioxidant activity.

In case of *Porodaedalea chrysoloma*, 5 compounds were isolated from the *n*-hexane fraction. Aside from ergosterol (**4**), 3β-hydroxyergosta-7,22-diene (**5**), the fraction also contained (E)-3-(4-methoxycarbonylphenoxy)-acrylate (**10**), ergone (**12**), furthermore methyl 3-(4-methoxycarbonylphenoxy)-propionate (**11**) was isolated for the first time from natural source. The isolated compounds were investigated with ORAC assay as well. Methyl-(*E*)-3-(4-methoxy-carbonylphenoxy)-acrylate (**10**) was found to be exceptionally active, whereas the results in case of 3β-hydroxyergosta-7,22-diene (**5**) confirmed our previous conclusion about the potent antioxidant effect of the compound.

Fuscoporia torulosa provided the widest range of components in terms of polarity: the *n*-hexane extract (fuscoporic acid (**13**), natalic acid (**19**) and ergosta-7,22-diene-3-one (**20**)), the more polar

chloroform subfractions (3,4-dihydroxy-benzaldehyde (**16**)**,** osmundacetone (**17**) and senexdiolic acid (**18)**) and the ethyl-acetate fraction (E (**14**) and Z (**15**) isomer of inoscavin A) all allow the isolation of pure components. Two of them, fuscoporic acid and Z isomer of inoscavin A were found to be new, undescribed molecules. Although the isolated compounds were ineffective as antimicrobial agents and were inactive on rhodamine 123 accumulation assay, compound ergosta-7,22-diene-3-on (**20**) demonstrated outstanding results on both Colo 320 and MRC-5 cell lines and demonstrated synergistic effect with doxorubicin. The antioxidant capacity of the mixture of **14** and **15** was remarkable in both DPPH and ORAC assays, moreover, **16** and **17** were also proved to be active.

These results demonstrated that the kingdom of fungi is far from fully explored regarding its chemistry or pharmacology. The components described here could provide potent antioxidants to both dietary supplements and part of a healthy diet. Finally, the results also reveales that Hungarian native mushrooms could be as interesting as their oriental relatives in terms of pharmacological effects and composition.

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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.