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

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LIST OF PUBLICATIONS RELEATED TO THE THESIS

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Bioactivity-guided Isolation of Antibacterial and Antioxidant Metabolites from *Tapinella atrotomentosa*
Molecules **23**, 1082 (2018)

II. **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).
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Chemical Analysis of the Edible Mushroom *Tricholoma populinum*: Steroids and Sulfyniladenosine Compounds
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IV. Ványolós A, Dékány M, **Kovács B**, Krámos B, Bérdi P, Zupkó I, Hohmann J, Béni Z
Gymnopeptides A and B, Cyclic Octadecapeptides from the Mushroom *Gymnopus fusipes*
Organic Letters **18**:(11) pp**.** 2688-2691. (2016)

V. 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
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Antiproliferative Activity of Some Higher Mushrooms from Hungary against Human Cancer Cell Lines
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ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
A2780	ovarian carcinoma cells
A431	human skin epidermoid carcinoma cells
AA	amino acids
COSY	correlated spectroscopy
δ	chemical shift
ESBL	extended-spectrum beta-lactamase
ESI–MS	electrospray ionization mass spectrometry
FCC	flash column chromatography
HeLa	human cervix adenocarcinoma
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HR–MS	high-resolution mass spectrometry
HSQC	heteronuclear single-quantum coherence spectroscopy
JMOD	*J*-modulated spin-echo experiment
MCF-7	human breast adenocarcinoma cells
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
NP	normal-phase
OCC	open-column chromatography
ROESY	rotating-frame nuclear Overhauser effect correlation spectroscopy
RP	reversed-phase
RPC	rotation planar chromatography
TLC	thin-layer chromatography
TOCSY	total correlation spectroscopy
t_R	retention time
UV	ultraviolet
XO	xanthine oxidase

1. 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"[1]. 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 thanks to their unique flavor, high nutritional value and low calories. They are not only rich in minerals, vitamins and fibers, but they also possess a favorable essential amino acid profile[2]. Some mushrooms with psychedelic properties have been applied in religious ceremonies by native peoples of Latin America and Siberia. The so called medicinal mushrooms are popular healing agents which are widely used in traditional medicines of several countries in the Far East (China, Japan and Korea) for thousands of years due to their beneficial pharmacological effects[3].

On the basis of our current scientific knowledge the estimated number of mushroom species is about 140 000, however only 10% of these (approx. 14 000 species) were investigated scientifically[4,5].The higher Basidiomycetes, the most important class of mushrooms, includes about 10 000 species from 550 genera and 80 families. The traditionally used genera are Auricularia, Flammulina, Ganoderma, Grifola, Hericium, Lentinus (Lentinula), Pleurotus, Trametes (Coriolus), Schizophyllum and Tremella[6]. Approximately 700 species of higher Basidiomycetes have been found to possess diverse pharmacological properties, recognized as functional foods and source of medicines and nutraceuticals [7,8].

Mushroom species are a great source of different type of natural products. Among the low-molecular-weight compounds steroids, sesquiterpenes, cerebrosides, quinones, isoflavones, catechols, amines and triacylglycerols were identified so far; while glycans, glycopeptides, proteoglycans, and proteins, were described as commonly occurring mushroom metabolites belonging to high-molecular-weight constituents[7,9].

The spectrum of identified pharmacological activities of Basidiomycetes is very broad. Based on literature data they can possess immunomodulatory, cardiovascular, liver protective, anti-inflammatory, antidiabetic, antiviral, antioxidant, antitumor and antimicrobial properties. Among these bioactivities the antitumor and antimicrobial effects are the most extensively studied, which is not surprising, because nowadays the number of cancer patients and also bacterial resistance has been drastically increased, which urges the need for new sources of antitumor and antimicrobial compounds[10,11].

In the last decades bacterial resistance to first-choice antibiotics have become a problem with global impact on human population. In 2008 infectious diseases were responsible for the death of more than 8.7 million people worldwide according to the report of World Health Organization[12]. The leading problem is certainly the association between multiresistant microorganisms and nosocomial infections, which is further worsened by the fact that in the last decades the antimicrobial research was a fairly neglected area. The abandon of antibacterial research by leading pharmaceutical companies has serious consequences: e.g. in the USA 19 new antibiotics were approved by the F.D.A. from 1980 to 1984, and only 13 were approved from 2000-2014. In this vein, the more intense search for new antimicrobial agents is indispensable. The contribution of mushroom metabolites to antibacterial therapy is low, but they have a large unexplored potential. The appearance of mushroom compounds in antibacterial therapy dates back to 1979 when tiamulin, a semisynthetic derivative of pleuromutilin isolated from *Pleurotus mutilis* in 1951 was approved for veterinary purposes. After a long period of almost thirty years another pleuromutilin derivative has been introduced now to human therapy. In 2007, retapamulin was approved by the US FDA for the topical treatment of impetigo, a skin infection caused by bacteria. Retapamulin is the first molecule originated from a higher mushroom, permitted in human antibacterial therapy. Some other pleuromutilin derivatives designed for systemic antibacterial therapy are waiting for approval in the next future, creating this way a new class of antibiotics[13].

Cancer is a leading cause of death in both more and less economically developed countries. In 2012, 4.1 million new cancer cases occurred and 8.2 million deaths worldwide. Lung, colorectal and prostate cancers are the leading causes of cancer death for males, while among females breast and cervical cancers are the most common cancer diseases[14]. Over 60% of the currently used anticancer agents are derived from natural sources including plants, marine organisms and microorganisms[15]. More recently, some species of higher Basidiomycetes have been found to markedly inhibit the growth of different tumor cell lines. Approximately two hundred species of higher Basidiomycetes were found so far with antitumor activity on different human cancer cell lines[4].

The anticancer and immunomodulatory beta-glucans, lentinan[16] from *Lentinus edodes* and krestin[17] (PSK) from *Trametes versicolor*, are probably the best known medical agents of mushroom origin, which have been integrated into clinical practice in some countries, especially in Japan. 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[18] (sesquiterpenes), ganoderic acids[19] (triterpenes) and gymnopilins[20] (polyisoprenepolyols), among others.

Natural products possess a broad diversity of structures and functions that may be applied for diagnosis, prevention and treatment of human diseases. Among the vast group of natural products, secondary metabolites of mushroom species can be promising starting materials for anticancer and antimicrobial discoveries. Modern isolation and screening technologies can undoubtedly improve the search for new lead molecules, which are essential in our effort to widely expand the number of drugs applied in tumor therapy and infectious diseases.

This thesis summarizes our mycochemical and pharmacological investigations on *Gymnopus fusipes* (Bull.:Fr.) Gray, *Tricholoma populinum* J.E.Lange, *Scleroderma bovista* Fr. and *Tapinella atrotomentosa* Batsch with the goal of finding new natural compounds of promising potential against cancer or infectious diseases.

2. AIMS OF THE STUDY

In 2012 the research group of the Department of Pharmacognosy at the University of Szeged started a screening program in collaboration with the Department of Pharmacodynamics and Biopharmacy and Institute of Clinical Microbiology at the same university. Our goal was 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 of bioactive compounds of the selected four mushroom species belonging to the Basidiomycetes (*Gymnopus fusipes*, *Tricholoma populinum*, *Scleroderma bovista*, *Tapinella atrotomentosa*).

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.
- Extraction of mushroom materials with various organic solvents (*n*-hexane, chloroform and 50% methanol) and with water for screening.
- Investigation of the tumor cell proliferation-inhibitory effect and the susceptibility of Gram-positive and –negative bacteria against the fungal extracts.
- Collect and extract 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 (in collaboration with Gedeon Richter Plc., Hungary), provide characteristic spectral data on the isolated new compounds, and supplement missing NMR data on the already known constituents.
- Evaluate the pharmacological potential of the isolated compounds (in the Department of Pharmacognosy and cooperation with Department of Pharmacodynamics and Biopharmacy, Institute of Clinical Microbiology and Department of Microbiology).

3. LITERATURE OVERVIEW

3.1. MORPHOLOGY AND CHEMISTRY OF INVESTIGATED SPECIES

3.1.1. Morphology and chemistry of *Gymnopus fusipes*

Gymnopus fusipes - also known as spindleshank – is member of the Omphalotaceae family. *G. fusipes* was described by a French mycologist Jean Baptiste François (Pierre) Bulliard in 1791 and gave the species the scientific name *Agaricus fusipes.* Afterwards in 1821 a British mycologist Samuel Frederick Gray transferred this species to the genus Gymnopus, creating the name *Gymnopus fusipes,* which is generally accepted today. However until recently most field guides and other more or less scientific sources referred to this mushroom by the latin name *Collybia fusipes*, a name given to this species by the French mycologist Lucien Quelét in 1872 [21].

G. fusipes is a parasitic mushroom, a common root rot fungus in mature pedunculate oak forests. The species can cause serious damage to the tree root systems of different oak species especially in dry or mildly waterlogged soils[22]. It is native to woodlands of Europe and Asia, but considered an invasive species in North America. It occurs mainly from July to October, being particularly common in European countries with dry and warm climate.

Its cap is convex, brown, often with dark brown spots. Gills are very widely spaced with white or light brown color. The stem is white near the apex and light brown towards the base without any stem ring. Spores are ellipsoidal and smooth. Mycologists in general consider *G. fusipes* an edible species with lower culinary value; however, the old fruiting bodies can produce gastrointestinal symptoms, most frequently diarrhea[23].

Our literature survey on *G. fusipes* revealed that the chemistry and pharmacology of this species are practically unexplored.

3.1.2. Morphology and chemistry of *Tricholoma populinum*

Tricholoma populinum - also known as cottonwood mushroom - is a species of the agaric genus Tricholoma. It was formally described by a Danish mycologist Jakob Emanuel Lange in 1933[24]. The name, "Tricholoma," is derived from two Greek words: „tricho" meaning hairy and „loma" meaning border or fringe, however only a few species (such as *T. vaccinum*) have shaggy caps[25].

Genus Tricholoma contains a large number of white spored, gilled mushrooms, which are located worldwide, existing in a symbiotic relationship with various species of coniferous or broad-leaved trees. Well-known species of the genera are the East Asian *T. matsutake* and

North American species *T. magnivelare.* These are both highly popular among consumers in Asia and America because of their unique spicy odor and taste[25]. Although *T. populinum* has a lower culinary value than the above mentioned species it is consumed in North America by Salish Indian peoples of British Columbia[26] and the Native American tribe of Taos Pueblo in New Mexico[27]. According to Lentini and Venza, fruiting bodies of cottonwood mushroom ("funci di chiuppu") are eaten by locals in Sicily during the fall season[28]. Some other species of Tricholoma are also safe to eat, but there are a couple of poisonous members of the genera, such as *T. pardinum*, *T. tigrinum* and *T. equestre*[25].

T. populinum is widespread in Europe and North America. The species is growing on sandy soil under cottonwood trees near a source of water. It occurs mainly from September to the end of November. Its stipe is whitish or light brownish red, 6-10 cm long. The pileus is light brown; its surface is typically mucous. The gills are first white colored, later brownish[23].

The literature survey on the chemistry and pharmacology of cottonwood mushroom revealed that little is known about the components of the species. Ergosterol peroxide was reported from *T. populinum* by Kreisel et al. in a study from the end of the 80's[29]. This compound with potential immunosuppressive property showed a positive effect in the treatment of urticaria and Buerger's disease in animal models[30]. Based on a study published in 2017, the dichloromethane extract of *T. populinum* decreased the release of the immune cells of the innate and adaptive immune systems, which indicates the immunological properties of the species, though the compounds responsible for these effects could not be identified[31].

3.1.3. Morphology and chemistry of *Scleroderma bovista*

Scleroderma bovista - commonly known as earth ball - is member of Scleroderma genus belonging to the Sclerodermataceae family[32].

S. bovista is widespread all over the world, commonly found also in Hungary. The species occurs in parks, deciduous and pine forests, and in disturbed ground, especially in sandy and clay soils, from June to the end of October. It is fairly small; the fruiting body is 2-5 cm across, round or nearly round being superficially similar to the edible puffballs (Calvatia spp.). Its surface is smooth, developing small pinkish cracks with age. Its peridium is about one mm thick, has gray color, but soon it turns brown, inside the gleba is black. *S. bovista* is a poisonous species, after consumption causes gastrointestinal symptoms and unexplained fainting fit[33]. The species is also known as „false truffle", because previously it has been used to counterfeit truffle species[34].

The ethnomycological profile of Scleroderma species is barely explored; though the available reports indicate that their use in folk medicine is sporadic. According to Guzman et al. in Mexico the dusty gleba of Scleroderma spp. mixed with Lycoperdon spp., Calvatia spp. and Bovista spp. are used to stop the bleed from wounds[34]. In Tanzania Scleroderma spp. are used to avoid bees' sting, when collecting honey from honeycombs[35]. According to literature review, there is no valuable information about the chemical constituents and the pharmacology of the species *S. bovista*.

3.1.4. Morphology and chemistry of *Tapinella atrotomentosa*

Tapinella atrotomentosa was originally described in 1786 by the German naturalist August Johann Georg Karl Batsch, who named it *Agaricus atrotomentosus.* In 1992 *Paxillus atrotomentosus* was transferred from the genus *Paxillus* to the genus *Tapinella* by the Czech mycologist Josef Šutara and named *T. atrotomentosa*[36].

T. atrotomentosa (Tapinellaceae) - velvet rollrim by its vernacular name - has been recorded from Europe, Asia, Central- and North-America. It has brown cap up to 28 cm with depressed center, covered dark brown or black velvety fur. The gills and spores are yellow; the stipe is thick and brown. It is a saprobic fungus, which grows on the roots and stumps of dead pines (*Pinus* sp.) and occasionally other conifers. The species is inedible due to its bitter taste[37].

Previous studies have already revealed the presence of several types of compounds in this species (**Figure 1**). In 1970 Gaylord et al. identified diphenyl-substituted tetronic acid pigments from cultures of *T. atrotomentosa*, namely xerocomic acid and atromentic acid[38]. TLC examination of extracts obtained from fruiting bodies and culture mixtures of *T. atrotomentosa* revealed the presence of thelephoric acid. [39] The species also produces orange-yellow flavomentin and violet spiromentin pigments, which possess terphenylquinone structure[40]. Spiromentins have a unique spiro structure in which benzoquinone is linked to a lactone acetal unit, while flavomentins constitute mono- and diesters of atromentin[41,42]. Atromentin, the 4,4-dihydroxy analogue of polyporic acid, accounts for the reddish-brown color of the external parts of *T. atrotomentosa*, from which it was originally isolated by Thorne[43]. Leucomentins also occur in the species, which are the colorless precursors of atromentin. These compounds mainly constitute esters of leucoatromentin with (2*Z*,4*S*,5*S*)-4,5-epoxy-2-hexenoic acid[44].

From fruiting bodies of *T. atrotomentosa* (+)-osmundalactone was isolated by Buchanan et al. Besides γ-lactone type compounds (e.g. (4*R*,5*S*)-5-hydroxy-2-hexen-4-olide,

(4*R*,5*S*)-5-hydroxyhexan-4-olide) were also identified, among these (3*R*,4*S*, 5*S*)-3-acetoxy-5-hydroxyhexan-4-olide was characterized as a new secondary metabolite[40]. Furthermore a new dimeric lactone, bis-osmundalactone was detected in the species by Hashimoto et al[45].

Velvet rollrim belongs to the few mushroom species containing rare ergostane-type ecdysteroids (paxillosterone, its 20,22-*p*-hydroxybenzylidene acetal, atrotosterones A, B and C and 25-hydroxyatrotosterones A and B), besides the most common 20-hydroxyecdysone[46]. In 1997 Haiselova et al. also identified a carbohydrate-binding protein type lectin in the species[47].

Compared to the chemistry of this species, the pharmacology of *T. atrotomentosa* has been less investigated so far. The water extract of mycelia of *T. atrotomentosa* exhibited antitumor activity. However, the extract did not show cytotoxicity, indicating that the antitumor activity is exerted through immunopotentiation[48]. Moreover, in a screening study dichloromethane extract of *T. atrotomentosa* was found to be active against *Bacillus subtilis*, *Escherichia coli* and exhibited molluscicidal property against *Biomphalaria glabrata*[49].

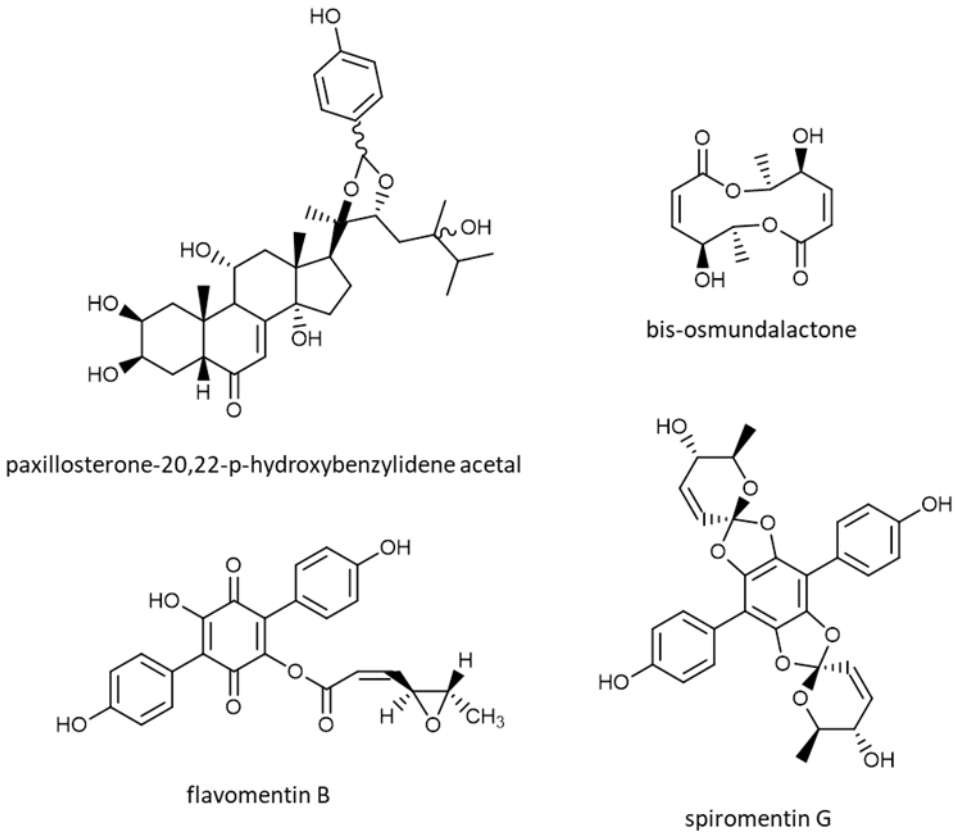


Figure 1. Representative secondary metabolites from *T. atrotomentosa*

4. MATERIALS AND METHODS

4.1. MUSHROOM MATERIALS

Mushrooms for screening for antiproliferative and antimicrobial activities

Mushroom species were collected in north-eastern part of Hungary in 2013–2014 by A. Ványolós and members of Mushroom Society of Miskolc and of Mushroom Society of Zemplén (Hungary). The identification of collected species was performed by Z. Lukács, E. Szilvásy and A. Ványolós. Representative voucher specimens have been deposited in the Department of Pharmacognosy, University of Szeged (Hungary). The identified mushroom samples were stored at –20 °C until processing.

Gymnopus fusipes

Fruiting bodies of *G. fusipes* (2 kg) were collected in northern part of Hungary in July of 2013. Mushroom material was stored at –20 ^{o}C until preparation. A voucher specimen (No.G12) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Tricholoma populinum

Mushroom material (3 kg) for preparative work has been collected in the vicinity of Szeged in 2013. The fruiting bodies of *T. populinum* were stored at –20 °C until processing. A voucher specimen (No.T13) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Scleroderma bovista

Fruiting bodies of *S. bovista* (4 kg) were collected in the vicinity of Szeged in 2014. Mushroom material was stored at –20 ^{o}C until preparation. A voucher specimen (No.S6) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary.

Tapinella atrotomentosa

Mushroom material of *T. atrotomentosa* (2 kg) was collected in the vicinity of Szeged in 2015. Fruiting bodies of *T. atrotomentosa* were stored at –20 ^{o}C until processing. A voucher specimen (No.P7) has been deposited at the Department of Pharmacognosy, University of Szeged, Hungary

4.2. EXTRACTION

4.2.1. Preparation of extracts for pharmacological screening

The fruiting bodies of collected mushrooms were freeze-dried (Hetosicc, Heto Lab Equipment, Denmark) and ground (Braun KSM2 Aromatic Coffee Grinder). Each lyophilized sample (10 g) was extracted with 3 × 100 ml methanol with the use of a VWR ultrasonic bath (type USC500TH) at room temperature. After filtration, the solutions were evaporated to dryness under reduced pressure with a Büchi Rotavapor R-210 (40 °C, 337 mbar). The residues were dissolved in 50 mL of 50% aqueous MeOH and were subjected to solvent-solvent partition between *n*-hexane (4 × 25 mL) (extracts A) and $CHCl_3$ (4 × 25 mL) (extracts B) and the residue gave extracts C. After extraction with MeOH, the residual mushroom materials were dried and extracted with 50 mL of boiling water for 15 min in a multiple water bath (type 1041, GFL). The filtered extracts were evaporated affording extracts D.

4.2.2. Extraction of the mushroom materials for preparative work

Gymnopus fusipes

The fresh mushroom material was freeze-dried, and then the lyophilized sample (210 g) was ground with a Retsch (type GM 2000) grinder and percolated with methanol (2 L) at room temperature. The methanol extract was evaporated by using a Rotavapor R-210 (40 °C, 337 mbar). The dry residue (59.5 g) was dissolved in 50% methanol (400 mL) and subjected to liquid-liquid partition first with *n*-hexane (4 x 200 mL) and then chloroform (4 x 200 mL).

Tricholoma populinum

The freeze-dried mushroom material (310 g) was ground with a Retsch (type GM 2000) grinder and then extracted with methanol (4 L) at room temperature. The methanol extract was concentrated *in vacuo* and the dry residue (92 g) was dissolved in 50% methanol (600 mL). Then solvent-solvent partition was performed first with *n*-hexane (4 x 300 mL) and then with chloroform (4 x 300 mL).

Scleroderma bovista

The lyophilized sample (560 g) was ground with a Retsch (type GM 2000) grinder and percolated with methanol (11.5 L) at room temperature. After evaporation, the methanol extract (78 g) was dissolved in 50% aqueous MeOH (500 mL) and subjected to liquid-liquid partition with *n*-hexane (5 x 500 mL) followed by chloroform (5 x 500 mL) and the residue gave the aqueous MeOH extract.

Tapinella atrotomentosa

The raw mushroom material (2 kg) was blended using Waring Commercial Blender and then was extracted by percolation with methanol (11.5 L) at room temperature. After concentration, the dry methanol extract (90.0 g) was dissolved in 50% aqueous MeOH (600 mL) and solvent-solvent partition was performed with *n*-hexane and chloroform (5 x 500 mL each) yielding *n*-hexane, chloroform and aqueous MeOH-soluble phases.

4.3. PURIFICATION AND ISOLATION OF THE COMPOUNDS

4.3.1. Open-column chromatography (OCC)

Open-column chromatography was performed on silica gel 60 (0.045 – 0.063 mm, Molar Chemicals, Hungary). Amount of sorbent: OCC-1: 220 g; OCC-2: 500 g. Mobile phases (compositions reported as volumetric ratios):

OCC-1: *n*-hexane – acetone [100:0, 98:2, 96:4, 94:6, 92:8, 90:10, 88:12, 86:14, 84:16, 82:18, 80:20, 78:22, 75:25, 70:30, 65:35, 60:40, 50:50, 25:75, 0:100 (2000 mL each)]; 100% methanol (1000 mL); volume of collected fractions 200 mL.

OCC-2: *n*-hexane – acetone [100:0, 98:2, 96:4, 95:5, 94:6, 92:8, 90:10, 88:12, 86:14, 84:16, 82:18, 80:20, 78:22, 75:25, 70:30, 60:40, 50:50, 25:75 (3500 mL each)]; 100% methanol (1000 mL); volume of collected fractions 200 mL.

4.3.2. Rotational planar chromatography (RPC)

RPC was carried out on manually coated SiO_2 (silica gel 60 GF254, Merck 7730, KGaA, Darmstadt, Germany) plates of 1 mm (RPC-3), 4 mm (RPC-1, RPC-2) thickness, at a flow rate of 3 mL/min and 10 mL/min on a Harrison Model 8924 Chromatotron instrument (Harrison Research, Palo Alto, CA, USA). Mobile phases:

RPC-1: dichloromethane – benzene – methanol [28:4:1, 28:8:2, 28:12:3, 28:16:4 (100 mL each)], volume of collected fractions: 10 mL.

RPC-2: dichloromethane – benzene – methanol [25:5:0.1, 25:5:0.25, 25:5:0.5, 25:5:1.0 (100 mL each), 25:5:2.0, 25:5:3.0 (50 mL each)], volume of collected fractions: 10 mL.

RPC-3: dichloromethane – benzene – methanol [25:5:3, 25:5:4, 25:5:5, 25:5:6 (100 mL each)], volume of collected fractions 10 mL.

4.3.3. Flash column chromatography (FCC)

Flash column chromatography was carried out on a CombiFlash®Rf+Lumen instrument with integrated UV, UV-VIS (detection at 254 nm and 366 nm) and ELS detectors using 4 g (FCC-3, FCC-4, FCC-5, FCC-8, FCC-12, FCC-13), 12 g (FCC-1, FCC-2, FCC-7, FCC-10,

FCC-11), 30 g (FCC-16, FCC-17) and 80 g (FCC-6, FCC-9, FCC-14, FCC-15, FCC-18) RediSep Rf Gold Normal Phase Silica Flash columns (Teledyne Isco, Lincoln, USA) at a flow rate 18 mL/min, 35 mL/min, 45 mL and 60 mL/min, respectively. Mobile phases and separation time:

FCC-1: increasing polarity of *n*-hexane – acetone (0% to 40% acetone), *t* = 80 min

FCC-2: increasing polarity of *n*-hexane – acetone (10% to 35% acetone), *t* = 40 min

FCC-3: increasing polarity of *n*-hexane – acetone (10% to 50% acetone), *t* = 60 min

FCC-4: increasing polarity of *n*-hexane – acetone (10% to 20% acetone), *t* = 50 min

FCC-5:increasing polarity of dichloromethane–methanol (0% to 3%MeOH), *t* = 20 min

FCC-6: increasing polarity of *n*-hexane – acetone (0% to 40% acetone), *t* = 80 min

FCC-7: increasing polarity of *n*-hexane – acetone (0% to 50% acetone), *t* = 80 min

FCC-8: increasing polarity of *n*-hexane – acetone (0% to 10% acetone), *t* = 35 min

FCC-9: isocratic eluent system of dichloromethane – methanol (7% MeOH), *t* = 40 min

FCC-10: isocratic eluent system of dichloromethane–methanol (8% MeOH), *t* = 45 min

FCC-11: increasing polarity of *n*-hexane – acetone (0% to 100% acetone), *t* = 60 min

FCC-12: increasing polarity of *n*-hexane – acetone (0% to 10% acetone), *t* = 15 min

FCC-13: increasing polarity of *n*-hexane – acetone (0% to 5% acetone), *t* = 30 min

FCC-14: increasing polarity of *n*-hexane – acetone (0% to 100% acetone), *t* = 80 min

FCC-15: increasing polarity of *n*-hexane – acetone (0% to 20% acetone), *t* =40 min

FCC-16: increasing polarity of *n*-hexane – acetone (0% to 10% acetone), *t* = 40 min

FCC-17: increasing polarity of *n*-hexane – acetone (0% to 10% acetone), *t* = 45 min

FCC-18: increasing polarity of *n*-hexane – acetone (0% to 50% acetone), *t* = 80 min

4.3.4. High-performance liquid chromatography (HPLC)

Reverse-phase-HPLC (RP-HPLC) separations were performed on Jasco LC-2000 Plus system equipped with a Jasco MD-2010 Diode Array Detector (JASCO International Co., Ltd., Tokyo, Japan) (RP-HPLC-1, RP-HPLC-2) and on Agilent 1100 Series pump (Agilent Technolgies, Santa Clara, USA) with Jasco UV-2070 UV/VIS detector (JASCO International Co., Ltd., Tokyo, Japan) (RP-HPLC-3); detection at 254 nm. Applied columns and mobile phases:

RP-HPLC-1: Zorbax Eclipse XDB-C8 column (9.4 x 250 mm, 5 μm; Agilent Technologies, Santa Clara, USA) column. Mobile phase containing 85% acetonitrile in water was applied at a flow rate of 3.0 mL/min.

RP-HPLC-2: Zorbax-ODS column (9.4 mm x 250 mm, 5μm; Agilent Technologies, Santa Clara, USA). Mobile phase was increasing polarity of MeOH – water mixture (45% to 55% MeOH) at flow rate of 2.5 mL/min.

RP-HPLC-3: Zorbax-ODS column (9.4 mm x 250 mm, 5μm; Agilent Technologies, Santa Clara, USA). As mobile phase 70% MeOH in water (isocratic elution) was applied at flow rate of 3.0 mL/min.

Normal-phase HPLC (NP-HPLC) separations were carried out on Wufeng LC-100 Plus HPLC instrument equipped with UV-VIS detector (Shanghai Wufeng Scientific Instruments Co., Ltd., Shanghai, China) at 254 nm. The applied column was a Zorbax-Sil column (250 x 4 mm, 5 μm; Agilent Technologies, Santa Clara, USA), with a flow rate of 3 mL/min. Mobile phases:

NP-HPLC-1: cyclohexane – isopropanol – water 80: 20: 0.2 isocratic eluent system

NP-HPLC-2: cyclohexane – isopropanol – water 80: 20: 0.1 isocratic eluent system

NP-HPLC-3: cyclohexane – isopropanol – water 78:22:0.1 isocratic eluent system

NP-HPLC-4: cyclohexane – isopropanol – water 75:25:0.1 isocratic eluent system

4.4. STRUCTURE DETERMINATION OF ISOLATED COMPOUNDS

Optical rotation was measured on a Perkin-Elmer 341 polarimeter. Standard (^{1}H, ^{13}C, selective 1D-TOCSY, 2D-TOCSY, ROESY, band selective HSQC, band selective HMBC (^{1}H-^{13}C and ^{1}H-^{15}N) and band selective HMBC) NMR spectra were recorded in deuterated chlorofom or methanol (Eurisotop, France) using standard pulse sequences available in the VNMJ 3.2 library. All NMR spectra were recorded at 25 °C on a Varian 800 MHz spectrometer equipped with a ^{13}C sensitivity enhanced salt tolerant $^{1}H/^{13}C/^{15}N$ cryogenically cooled probe head. Chemical shifts are reported in the delta scale using TMS (^{1}H), residual solvent signal (77.0 ppm, ^{13}C) as reference or spectrometer internal reference (using $MeNO_2$ scale for ^{15}N).

HRMS analyses were performed on a LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) system using ESI ionization in positive ion mode. Samples were dissolved in chloroform or MeOH. CID fragmentation experiments were performed using helium as collision gas at normalized collision energy of 35%. For data acquisition and analysis Xcalibur software version 2.0 (Thermo Fisher Scientific) was used.

4.5. MARFEY'S ANALYSIS (Hydrolysis and derivatization)

1 mg of each gymnopeptides was hydrolyzed in 1 ml HCl (6 M) at 110 °C for 24 h. After cooling down to room temperature the resulted solutions were evaporated to dryness and redissolved in 50 μL water. To these solutions (as well as to the reference AA solutions (50 μL; 50 mM)) 100 μL of 1% (w/v) acetone solution of Marfey's reagent (FDAA, Nα-(2,4-dinitro-5-fluorophenyl)-L-alaninamide) and 20 μL of 1 M $NaHCO_3$ were added. The resulted solutions were incubated for 1 h at 40 °C. The reaction was stopped by adding 10 μL 2 M HCl. After evaporating the solvents and redissolving the residue in 1 mL of 50% MeOH, the derivatized gymnopeptide hydrolizates and the derivatized AA standards were subjected to HPLC-HR-ESI-MS analysis[50].

4.6. LC-MS INVESTIGATION OF AMINO ACIDS

The instrument used was a Thermo LTQ FT Ultra coupled to Thermo Accela HPLC. The ionization mode applied was ESI in positive mode, the spray voltage was 4.4 kV, the capillary temperature was 280 °C, and tube lens voltage 250 V. The applied column was a Zorbax Eclipse XDB-C18, 2.1 x 50 mm, 1.8 μm, the eluents used were eluent A (950 mL H_2O + 50 mL MeCN + 1.5 mL formic acid) and B (950 mL MeCN + 50 mL H_2O + 1.5 mL formic acid) with a flow rate of 300 μL/min in gradient elution starting from 90% A and 10% B to 10% A and 90% B in 15 min. The tray oven temperature was set to 10 °C, while the column oven temperature to 30 °C .The detection was performed in UV at 260 nm, 280 nm, and 340 nm.

4.7. MOLECULAR MODELING

The calculations were performed within the Schrödinger software suite, DFT calculations were carried out using Jaguar 9.1.

4.8. PHARMACOLOGICAL ASSAYS

Pharmacological investigations were performed in the Department of Pharmacognosy and in cooperation with the Department of Pharmacodynamics and Biopharmacy, Institute of Clinical Microbiology and Department of Microbiology at University of Szeged (Hungary).

4.8.1. *In vitro* antiproliferative investigations (MTT assay)

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 human breast carcinoma (MCF-7, MDA-MB-231 and T47D) cell lines. All cancer cell lines were purchased from European Collection of Cell Cultures (ECCAC, Salisbury, UK). The cells were cultivated in

minimal essential medium (Lonza Ltd, Basel, Switzerland) supplemented with 10% fetal bovine serum, 1% non-essential amino acids and an antibiotic-antimycotic mixture. Near-confluent cancer cells were seeded onto a 96-well microplate (5000 cells/well) and, after overnight standing, new medium containing the tested extracts and compounds were added. After incubation for 72 h at 37 °C in humidified air containing 5% CO_2, the living cells were assayed by the addition of 5 mg/mL MTT solution (20 μL). MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4 h contact period. The medium was then removed and the precipitated formazan crystals were dissolved in DMSO (100 μL) during a 60 min period of shaking at 25 °C. Finally, the reduced MTT was assayed at 545 nm, using a microplate reader; wells with untreated cells were utilized as controls. Sigmoidal dose–response curves were fitted to the determined data, and the IC_{50} values were calculated by means of GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA). All *in vitro* experiments were carried out on two microplates with at least five parallel wells. Cisplatin (Ebewe Pharma GmbH, Unterach, Austria) was used as reference agent (IC_{50} values 12.4, 0.86, 2.8, 19.1, 5.8, 9.8 μM on HeLa, A2780, A431, MDA-MB-231, MCF-7 and T47D cells, respectively).

4.8.2. *In vitro* antimicrobial investigations of mushroom extracts

The test micro-organisms used were 11 standard and 9 clinical isolates with different antibiotic resistance profile. The standard Gram-positive strains were namely, *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 29213), *Staph. epidermidis* (ATCC 12228), *Streptococcus agalactiae* (ATCC 13813), *Strep. pneumoniae* (ATCC 49619), *Strep. pyogenes* (ATCC 19613) and methicillin-resistant *Staph. aureus* (ATCC 43300). The standard Gram-negative strains were namely, *E. coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 700603), *Moraxella catarrhalis* (ATCC 43617) and *Pseudomonas aeruginosa* (ATCC 27853). Multiresistant *Acinetobacter baumannii* (64060/2 and 61748/2), ESBL-positive *Citrobacter freundii* (63458), ESBL-positive *Enterobacter cloacae* (63033), ESBL-positive *E. coli* (64663), ESBL-positive *Kl. pneumoniae* (63735), multiresistant *Ps. aeruginosa* (61485/1 and 64658) and methicillin-resistant *Staph. aureus* (64326) were applied as clinical strains. The clinical strains were obtained from various departments of Albert Szent-Györgyi Health Center and identified in the Institute of Clinical Microbiology at University of Szeged by MALDI-TOF methods. Microbial cultures were grown on standard Mueller-Hinton agar plates or Columbia agar +5% sheep blood (COS) plates (bioMérieux, Marcy-l'Étoile, France) at 37 °C under aerobic environment. They were maintained at 4 °C throughout the study to use as stock cultures.

Standard disc-diffusion method

Antibacterial activities of extracts against standard bacterial strains and clinical, multiresistant strains were screened for their inhibition zones by standard disc-diffusion method as described previously[51]. The mushroom extracts and the pure compounds were dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA) or water at concentration of 50 mg/mL. The sterile filter paper discs (6 mm diameter) impregnated with the extracts /isolated compounds (10 µL of redissolved extracts) were placed on the plate seeded with the respective bacterial suspension (inoculums 0 5 McFarland, 1-2 x 10^8 CFU ml^{-1}). The solvent (DMSO) was served as negative control, while ampicillin, erythromycin, imipenem, cefuroxime and vancomycin were used as positive control. The plates were incubated at 37 °C for 24 h under aerobic conditions. The diameters of inhibition zones were measured and recorded. All experiments were carried out in triplicate.

Determination of MIC values

The extracts with diameter of inhibition zone ≥10 mm were further subjected to determination of their minimal inhibitory concentration (MICs). The quantification of MICs was performed by microdilution method according to procedures reported earlier[52]. In the 96-well plates the stock solutions of extracts/ pure compounds (50 mg/mL in DMSO) were serially diluted with Bouillon broth medium enriched with glucose (1%) and thiamine (0.005 mg/mL) to obtain a final concentration between 200 and 0.195 µg/mL. About 100 µl of inoculum (0.5 McFarland, 1-2 x 10^8 CFU mL^{-1}) were then added to the wells. A sterility check (medium and DMSO in amount corresponding to the highest concentration), negative control (medium, DMSO and inoculum) and positive control (medium, DMSO, inoculum and standard antibiotics: ampicillin, erythromycin, imipenem, cefuroxime and vancomycin) were included for each experiment. The plates were then incubated at 37 °C for 24 h under aerobic condition. The MICs of fungal extracts were the lowest concentration that completely inhibited the bacterial growth, measuring the optical density at 600 nm using a Fluostar Optima plate reader (BMG Labtech, Ortenberg, Germany). All experiments were performed twice in triplicate.

Double-disc synergy test

Double-disc synergy assay was used for preliminary detection of the positive interaction between extracts and antibiotics, as described earlier[53]. The double-disc test was performed as a standard disc-diffusion assay on Mueller-Hinton agar plates, inoculated as described above. The sterile filter paper discs (diameter 6 mm) containing 10 µL of fungal extracts dissolved in DMSO or water (50 mg/mL), were placed 25 mm apart (centre to centre) around discs

containing standard antibiotics (ampicillin (10 μg), imipenem (10 μg), cefuroxime (30 μg) or vancomycin (5 μg)). After 24 h incubation at 37 °C under aerobic environment, the enhancement of the inhibition zones indicated synergy between test antibiotics and fungal extracts. All double-disc tests were carried out in triplicate. After qualitative evaluation of antimicrobial combination performed with this disc-diffusion method, the determination of fractional inhibitory concentration (FIC) of positive combinations was performed with checkerboard techniques[54]. In brief, all combinations two fold dilutions of the antibiotics and mushroom extracts were dispensed into a 96-well plate in a checkerboard fashion. The inoculum equal to a 0.5 McFarland turbidity standard was prepared from the bacterial isolate. Growth and sterility controls were included in all plates. All checkerboard microdilution synergism tests were carried out in triplicate and evaluated after 24 h of incubation at 37 °C under aerobic conditions. The ∑FIC was then calculated, using fractional inhibitory concentration (FIC) for each drug, as ∑FIC=FICA+FICB, where FICA=MIC of drug A in combination/MIC of drug A when alone, and FICB=MIC of drug B in combination/MIC of drug B when alone. Synergism was interpreted, if ∑FIC was equal or lower than 0.5.

4.8.3. *In vitro* antimicrobial investigations of the isolated compounds

Determination of MIC values

The *in vitro* antibacterial activities were assayed using microdilution method based on the guideline of Clinical and Laboratory Standards Institute[55] against bacterial strains including *Escherichia coli* (SZMC 6271), *Pseudomonas aeruginosa* (SZMC 2329), *Staphylococcus epidermidis* (SZMC 14531) *Staphylococcus aureus* (SZMC 14611), *Bacillus subtilis* (SZMC 0209), *Acinetobacter baumanii* (SZMC 24075), *Escherichia coli* ESBL (SZMC 24090), *Moraxella catarrhalis* (ATCC 25238). The suspensions of each bacteria were prepared from overnight broth cultures cultivated in Luria-Bertani broth (LB, 10 g/L tryptone, 10 g/L sodium chloride, 5 g/L yeast extract) at 37 °C and the concentrations of suspensions were adjusted to 10^5 cells/mL. The DMSO solution of the investigated compounds as well as the reference agents were diluted with the LB media in the final concentration ranging from 1000 μg/mL to 6.25 μg/mL. The 96-well plates were prepared by dispensing into each well 100 μL of LB containing the bacterial cells and 100 μL of dissolved compounds and incubated for 24 h at 37 ˚C. The mixture of 100 μL LB broth and 100 μL sample solvent were used as blank sample for the background correction, while 100 μL of bacterial cultures with 100 μL solvents without the compounds was applied as positive control, while standard antibiotics were used as reference agents. Absorbance was measured at 620 nm after 24 hours with microplate

reader (SPECTROstar Nano, BMG Labtech) and the MIC values were determined as the lowest concentration where the inhibition was higher than 10% of the positive control after the blank correction.

Antibacterial activities in combination with cefuroxime by checkerboard assay

The cefuroxime was used in checkerboard assay in combination with the isolated compounds against MRSA (SZMC 6270). The suspensions of the bacteria were prepared from overnight broth cultures cultivated in LB broth at 37 °C and the concentrations of suspensions were adjusted to 10^5 cells/mL. Into each well of the 96-well plates, 100 µL of LB broth were added containing the bacterial cells, which was completed with 50 µL of each compound and 50 µL of cefuroxime. Double dilutions of both the antibiotic and test compounds were carried out in five levels started from their related MIC values (isolated compounds: 250 µg/mL–15.6 µg/mL; cefuroxime: 300 µg/mL - 18.75 µg/mL). The mixture of 100 µl LB broth and 100 µl sample solvents were used as blank sample for the background correction, while 100 µl of bacterial cultures with 100 µl solvents without the compounds and antibiotic was applied as positive control. The plates were incubated for 24 h at 37 ˚C and the absorbance of the wells were measured at 600 nm with microplate reader (SPECTROstar Nano, BMG Labtech) to observe for growth of the test organism, which was expressed in percentages of the positive control.

The ∑FICIs were calculated as follows: ∑FICI=FIC A + FIC B, where FIC A is the MIC of drug A (cefuroxime) in the combination/MIC of drug A (cefuroxime) alone, and FIC B is the MIC of drug B (isolated compounds) in the combination/MIC of drug B (isolated compounds) alone. The combination is considered synergistic when the ∑FIC is ≤0.5, indifferent when the ∑FIC is >0.5 to <2, and antagonistic when the ∑FIC is ≥2[56].

4.8.4. *In vitro* xanthine oxidase inhibitory investigation

The method is based on a modified protocol of Sigma, a continuous spectrophotometric rate determination: the absorbance of xanthine oxidase (XO)-induced uric acid production from xanthine was measured at 290 nm for 3 min in a 96-well plate, using the plate reader FluoSTAR OPTIMA (BMG LABTECH). The XO-inhibitory effect was determined via the decreased production of uric acid. Reagents: 50 mM potassium phosphate buffer, pH 7.5 with 1 M KOH, 0.15 mM xanthine solution (pH 7.5), and XO solution (0.2 units/mL). XO, isolated from bovine milk (lyophilized powder) and xanthine powder were purchased from Sigma-Aldrich Co. The different isolated compounds (600 µg/mL) were prepared in DMSO. For enzyme-activity control, the final reaction mixture comprised of 100 µL of xanthine, 150 µL

of buffer and 50 µL of XO in a 300 µL well. The reaction mixture for inhibition was made with 100 µL of xanthine, 140 µL of buffer, 10 µL of sample and 50 µL of XO. Allopurinol served as positive control. Fungal samples were added in appropriate volumes so that the final concentration of DMSO in the assay did not exceed 3.3% of the total volume. All the experiments were conducted in triplicate. The reaction was initiated by the automatic addition of 0.050 mL of XO solution to a final concentration of 0.006 units/mL. The IC_{50} values were calculated by analyzing the inhibition (%) of each concentration, by using GraphPad Prism 5.04 software (GraphPad Software Inc.) with non-linear regression.

4.8.5. *In vitro* antioxidant activity investigation

DPPH method

The analysis of free radical scavenging activity was carried out by the modified method of Miser-Salihoglu E. et al[57]. The DPPH method was performed on a 96-well microplate. Microdilution series of extracts and the isolated compounds (concentration of 1 mg/mL dissolved in DMSO), were made starting from 150 µL. To each well 50 µL of DPPH solution (100 µM) was added. The absorbance was measured after 30 minutes at 550 nm with FLUOstar Optima BMG Labtech plate-reader. The less active samples were measured again in 2 mg/mL concentration. Subsequent dilution series were made using the most effective samples, starting from 100 µL, to precisely evaluate half maximal effective concentration (EC_{50} values). As standard, ascorbic acid was used. DPPH (2,2'-diphenyl-1-picrylhydrazyl) and ascorbic acid standard were purchased from Sigma-Aldrich Hungary. The determination of EC_{50} (mg/mL) values were carried out with the help of GraphPad Prism 6.0 software.

ORAC assay

The ORAC assay was carried out on a 96-well microplate according to the method of Mielnik et al[58]. 20 µL of extracts or pure compounds of 0.01 mg/mL concentration were mixed with 60 µL of AAPH (12 mM final concentration) and 120 µL of fluorescein solution (70 nM final concentrations), then the fluorescence was measured through 3 hours with 1.5 minute cycle intervals with FlouStar Optima BMG Labtech plate-reader. All the experiments were carried out in triplicate, as standard Trolox was used. AAPH free radical ((2,2'-azobis(2-methyl-propionamidine)dihydrochloride) and Trolox standard ((±)-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid) were purchased from Sigma-Aldrich Hungary. Fluorescein was purchased from Fluka analytical, Japan. The antioxidant capacity was expressed as mmol Trolox equivalent per g of dry extract (mmolTE g^{-1}), with help of GraphPad Prism 6.0.

5. RESULTS

5.1. SCREENING OF MUSHROOM SPECIES FOR ANTIPROLIFERATIVE ACTIVITY

As part of our screening program for antiproliferative compounds in mushrooms occurring in Hungary, more than 20 macrofungi were screened for their potential antiproliferative activity. The extracts prepared with *n*-hexane (A), $CHCl_3$ (B), aqueous MeOH (C), or H_2O (D) from selected mushroom species, were examined for their activity against HeLa, A431, A2780 and MCF-7 cell lines (see section **4.2.1.** and **4.8.1.**).

Among the samples with different polarities, the fractions A characterized by lipophilic constituents and the fractions B containing compounds with higher polarity proved to be active (see **Appendix I**, in **Table 1**). The fractions C and D did not demonstrate notable antiproliferative effects (>50% growth inhibition) against any cell line. In general, the mushroom extracts exerted the most pronounced activity on the A2780, but exhibited weaker activity against A431 cells. According to our results the most effective species proved to be G. *fusipes*, followed by *S. bovista* (data unpublished), while *T. populinum* exerted moderate inhibition on the investigated cell lines. However, in our pervious study the chloroform extract of *T. populinum* demonstrated remarkable xanthine oxidase inhibitory activity with an IC_{50} value of 85.08±15.02 μg/mL.[59]

Based on our screening studies three mushroom species (*G. fusipes*, *T. populinum* and *S. bovista*) were chosen for further preparative works with the aim of the identification of their biologically active constituents.

5.2. SCREENING OF MUSHROOM SPECIES FOR ANTIMICROBIAL ACTIVITY

In our screening program 160 extracts of 40 mushroom species collected in Hungary, were tested for their antibacterial activities against 11 standard bacterial strains and 9 clinical isolates. The results confirmed that 16 species exhibited moderate to remarkable antibacterial activity against several bacteria (including resistant and multidrug-resistant strains) through use of standard disc-diffusion method at 50 mg/mL concentration (see sections **4.2.1.** and **4.8.2.**). Data obtained in the screening of antimicrobial activity, including the MIC values are shown in **Table 1**.

Table 1. Antibacterial activity of active mushroom extracts: inhibitory zones (mm)/MIC values (μg mL^{-1})

Species	Solvent	Diameter of inhibition zone (mm)/MIC value (μg ml^{-1})						
		B. subtilis ATCC6633	*Mor. catarrhalis* ATCC43617	*Staph. aureus* ATCC29213	MRSA ATCC43300	MRSA 64326	*Staph. epidermidis* ATCC12228	*Strep. pneumoniae* ATCC49619
Amanita rubescens	B	-	9.5	-	-	-	-	9.5
Armillaria mellea	A	-	7.0	-	-	-	-	-
	B	-	11.0/50	-	-	-	-	8.5
Armillaria tabescens	B	-	8.0	-	-	-	-	-
Chalciporus piperatus	D	-	-	-	10.5/25	10.0/25	-	-
Fistulina hepatica	B	12.0/12.5	8.0	10.0/50	10.0/50	10.0/50	8.5	-
Hypholoma fasciculare	B	11.5/12.5	10.5/25	10.0/25	12.5/25	12.0/25	10.5/25	10.0/25
Kuehneromyces mutabilis	B	9.5	8.5	-	-	-	8.5	7.5
Lactarius glaucescens	A	7.5	7.5	8.0	-	-	-	7.5
	B	-	-	8.0	-	-	-	-
Lactarius volemus	B	7.5	10.0/50	-	-	-	-	8.5
Megacollybia platyphylla	B	7.5	12.0/25	-	-	-	-	8.0
Psathyrella candolleana	B	-	9.5	-	-	-	-	8.5
Ptychoverpa bohemica	B	-	9.5	-	-	-	9.5	-
Rhodocybe popinalis	A	-	8.0	-	8.0	8.5	-	-
	B	12.0/12.5	8.0	10.0/12.5	10.7/25	10.0/25	11.0/12.5	-
Russula sororia	A	-	8.0	-	-	-	-	-
Suillus grevillei	B	-	8.5	8.5	-	-	-	-
*Tapinella atrotomentosa**	B	8.0	12.0/25	8.0	8.0	8.0	8.0	-

* *T. atrotomentosa* exhibited inhibitory activity against *E. coli* (8 mm), *Ps. aeruginosa* (8 mm), multiresistant *Ac. baumannii* (10 mm/100 μg ml^{-1}), ESBL-positive *E. coli* (8 mm) and multiresistant *Ps. aeruginosa* (8 mm). Solvent: A: *n*-hexane; B: chloroform; D: water.

It was observed that the studied mushroom extracts demonstrated antimicrobial activity mainly against Gram-positive bacteria, 75% of the investigated strains were susceptible. In general, the chloroform extracts displayed activities in the screening assay. In the cases of three species, namely *Armillaria mellea*, *Lactarius glaucescens* and *Rhodocybe popinalis*, both the *n*-hexane and chloroform extracts demonstrated antibacterial capacity. None of the hot water extracts of mushrooms, except the extract of *Chalciporus piperatus* possessed antimicrobial effects.

In this experiment eight fungal extracts showed diameter of inhibition zone ≥10 mm against one or more bacterial strains. Against *Moraxella catarrhalis* the most potent species were *A. mellea*, *Hypholoma fasciculare*, *Lactarius volemus*, *Megacollybia platyphylla* and *Tapinella atrotomentosa*. The extracts of *Fistulina hepatica*, *H. fasciculare*, *T. atrotomentosa* and *R. popinalis* revealed the highest antibacterial capacity against mainly Gram-positive strains with MICs between 12.5 and 100 μg/mL.

The chloroform extract of *T. atrotomentosa* seems to possess the broadest antibacterial spectrum. It exerted significant activity against Gram-positive bacterial strains, while inhibition zones were also detected against standard and ESBL-positive *Escherichia coli*, standard and multiresistant *Pseudomonas aeruginosa* and multiresistant *Acinetobacter baumannii*. The standard and clinical isolates of methicillin-resistant *Staphylococcus aureus* were susceptible to the extracts of *C. piperatus*, *F. hepatica*, *H. fasciculare* and *R. popinalis* with MICs between 25 and 50 μg/mL.

All active mushroom extracts were selected to evaluate possible synergistic effects with different antibiotics against multiresistant micro-organisms. In the double-disc synergy tests (see section **4.8.2.**), the extracts of *F. hepatica*, *T. atrotomentosa* and *R. popinalis* exhibited synergistic activity with cefuroxime against methicillin-resistant *Staph. aureus*. The combination of *F. hepatica* with cefuroxime revealed the lowest FIC value (0.25, highest synergism), followed by the combination of *T. atrotomentosa* and *R. popinalis* with cefuroxime (FIC = 0.5 and 0.4 respectively).

Based on the results of our preliminary screening programs, the chloroform extract of *T. atrotomentosa* was selected for more detailed mycochemical study, with the aim of the identification of its antibacterial metabolites.

5.3. ISOLATION OF COMPOUNDS FROM THE INVESTIGATED SPECIES

5.3.1. Isolation of the compounds of *G. fusipes*

Lyophilized and ground mushroom material was percolated with methanol. The crude extract was concentrated and subjected to liquid-liquid partitioning with *n*-hexane and then chloroform (see section **4.2.2.**) (**Figure 2**). The concentrated $CHCl_3$ fraction (1.02 g) was separated by rotational planar chromatography (**RPC-1**) on silica gel, using a gradient system of dichloromethane – benzene – methanol. The RPC-1 fractions were ultimately combined into 7 subfractions (B I – B VII) according to the TLC monitoring. The subfraction B III (62.1 mg) obtained with dichloromethane – benzene – methanol 7:2:0.5, was further purified by semipreparative reversed-phase HPLC (**RP-HPLC-1**) using 85% acetonitrile in water as mobile phase to yield compounds **COFUB1** (4.9 mg, t_R 7.6 min) and **COFUB2** (7.8 mg, t_R 10.7 min).

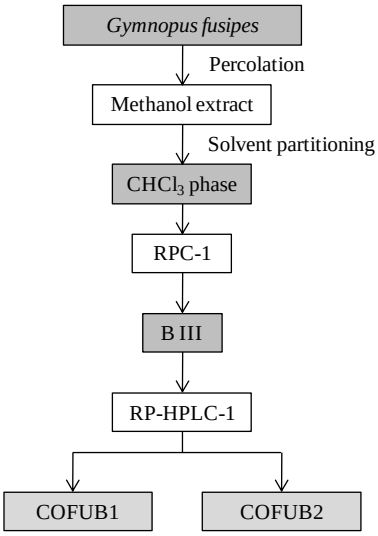


Figure 2. Isolation of compounds from *Gymnopus fusipes*

5.3.2. Isolation of the compounds of *T. populinum*

After percolation of the lyophilized and ground mushroom material, the extract was evaporated and subjected to solvent-solvent partitioning first with *n*-hexane followed by chloroform, and the residue gave the aqueous-methanol extract (see section **4.2.2.**) (**Figure 3**).

Evaporation of the *n*-hexane phase resulted in a dark brown residue (14 g), which was fractionated in first step by open-column chromatography (**OCC-1**) on silica gel using increasing polarity of *n*-hexane – acetone mixtures as eluent. More than 200 fractions (200 mL) were collected, which were combined based on TLC monitoring. Five of the combined fractions (A I - A V) were further analyzed by flash column chromatography (FCC) (see section **4.3.3.**). The combined fraction A I (1.5 g) obtained with *n*-hexane – acetone 96:4,

was purified on flash silica gel column using *n*-hexane – acetone eluent system with increasing polarity (**FCC-1**) and led to the isolation of **TRIPOA4** (0.90 g) and **TRIPOA5** (0.20 g). Fraction A II (140 mg) eluted with *n*-hexane – acetone 8:2, was separated by *n*-hexane – acetone gradient elution (**FCC-2**) to yield **TRIPOA1** (4.2 mg,). The combined fraction A III (60 mg) obtained with *n*-hexane – acetone 75:25, was analyzed in similar chromatographic conditions (**FCC-3**) to give **TRIPOA3** (4.4 mg). The fraction A IV (40 mg) eluted *n*-hexane – acetone 88:12, was purified with *n*-hexane – acetone gradient eluent system (**FCC-4**) resulted in **TRIPOA6** (2.3 mg). Finally the fraction A V (260 mg) obtained with 100% acetone, was further purified by a gradient eluent system of dichloromethane – methanol (**FCC-5**) to yield **TRIPOA2** (3.8 mg).

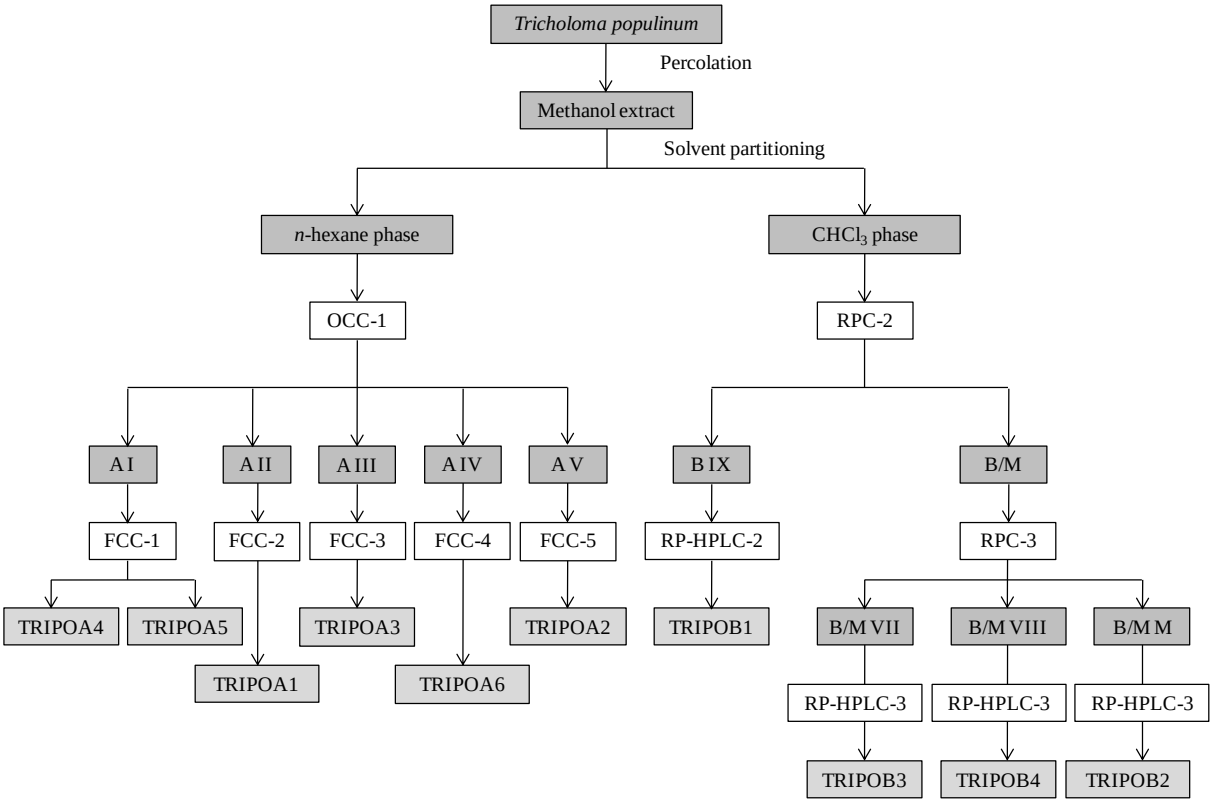


Figure 3. Isolation of compounds from *Tricholoma populinum*

The concentrated chloroform phase (1.0 g) was fractionated with rotational planar chromatography (**RPC-2**) on silica gel using gradient system of dichloromethane – benzene – methanol. RPC-2 fractions were combined into nine major fractions (B I – B IX) according to TLC monitoring. Fraction B IX (13.4 mg) eluted with dichloromethane – benzene – methanol 25:5:2, was further separated by reversed phase HPLC (**RP-HPLC-2**) using MeOH – water gradient elution to obtain **TRIPOB1** (2.0 mg, t_R 5.26 min). The methanol elution of RPC-2 (B/M, 193 mg) was further separated by repeated rotational planar chromatography (**RPC-3**) on silica gel with dichloromethane – benzene – MeOH gradient eluent system and the

fractions obtained were combined into eight subfractions (B/M I - VIII). Finally, subfractions B/M VII, B/M VIII and B/M M (methanol elution) were purified with reversed-phase HPLC (**RP-HPLC-3**) employing 70% MeOH in water to yield **TRIPOB3** (2.4 mg, t_R 7.8 min), and a mixture of epimeric compounds of **TRIPOB2** and **TIPOB4** (8.3 mg, t_R 9.81 min).

5.3.3. Isolation of the compounds of *S. bovista*

The freeze-dried and ground mushroom material was extracted with methanol. After concentration under vacuum, the extract was subjected to liquid-liquid partitioning to yield *n*-hexane and chloroform soluble phases (see section **4.2.2.**) (**Figure 4**). The concentrated *n*-hexane phase (19.36 g) was fractionated in first step by open-column chromatography (**OCC-2**) on silica gel using increasing polarity of the mixture *n*-hexane – acetone. The fractions (200 mL) collected were combined based on TLC monitoring. Five of the united fractions were further fractionated by flash column chromatography (see section **4.3.3.**).

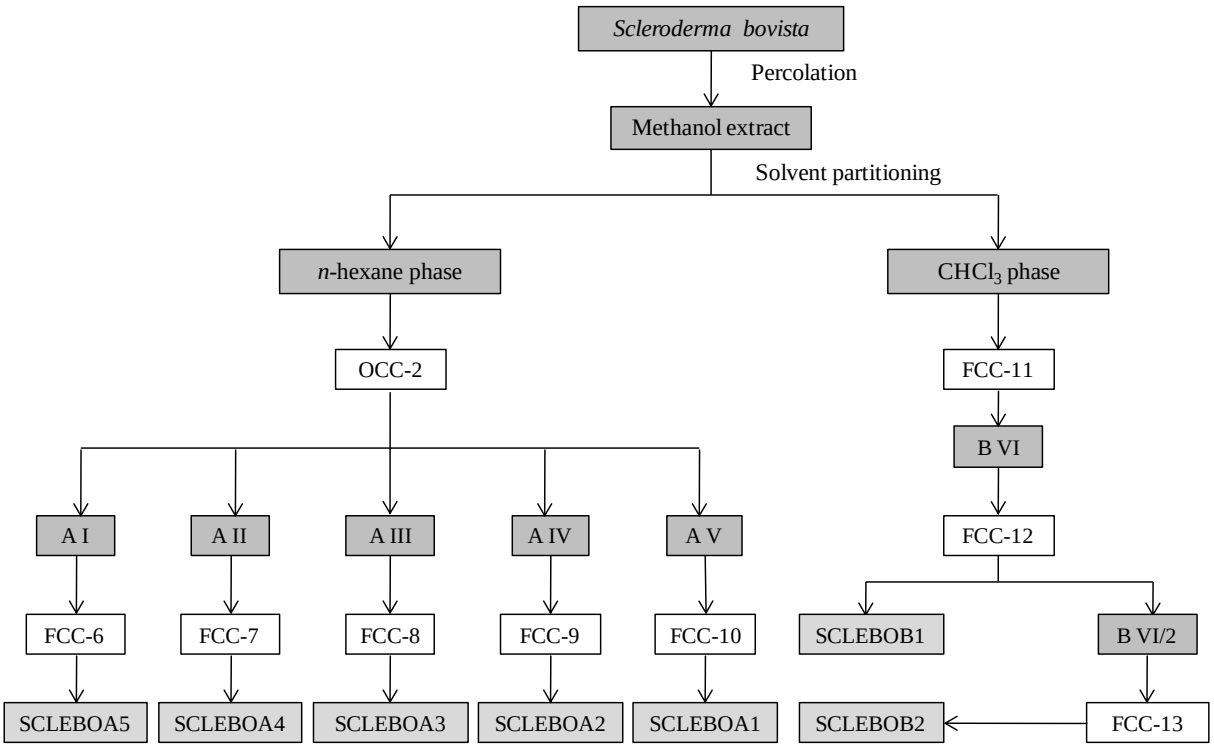


Figure 4. Isolation of compounds from *Scleroderma bovista*

The first combined fraction A I (2.26 g) eluted previously with *n*-hexane – acetone 96:4, was purified using increasing polarity mixtures of *n*-hexane – acetone (**FCC-6**) and led to **SCLEBOA5** (1.8 g). The combined fraction A II (120.4 mg) obtained with *n*-hexane – acetone 92:8, was analyzed by the similar method (**FCC-7**) to isolate **SCLEBOA4** (96.3 mg). Investigation of the next fraction A III (288.9 mg) eluted with *n*-hexane – acetone 90:10 (**FCC-8**) yielded **SCLEBOA3** (11.1 mg). The combined fraction A IV (58.2 mg) obtained

from OCC-2 with *n*-hexane – acetone 25:75, was further purified by flash chromatography (**FCC-9**) with dichloromethane – methanol isocratic solvent system affording compound **SCLEBOA2** (7.1 mg). Finally the combined fraction A-V (159.1 mg) eluted with methanol was analyzed similarly (**FCC-10**), which resulted in **SCLEBOA1** (25.3 mg).

The concentrated $CHCl_3$ phase (1.5 g) was roughly fractionated by flash column chromatography (**FCC-11**) on silica gel column using gradient system of *n*-hexane – acetone. The FCC-11 fractions with similar compositions were combined into seventeen fractions (B I-B XVII) according to TLC monitoring. Fraction B VI (210.0 mg) obtained with *n*-hexane – acetone 7:3, was further separated employing flash column chromatography (**FCC-12**) with *n*-hexane – acetone gradient solvent system. The separation resulted in a pure compound **SCLEBOB1** (99.7 mg) and a subfraction B VI/2. This subfraction (85.7 mg) was subjected to repeated flash chromatography (**FCC-13**) to obtain **SCLEBOB2** (6.1 mg).

5.3.4. Isolation of the compounds of *T. atrotomentosa*

The fresh mushroom material was blended and percolated with methanol. The crude extract was concentrated under vacuum and subjected to solvent-solvent partitioning with *n*-hexane and then chloroform (see section **4.2.2.**) (**Figure 5**). The chloroform-soluble phase was evaporated and the residue (8.56 g) was roughly separated with flash column chromatography (**FCC-14**) on silica gel column using gradient system of *n*-hexane – acetone. The obtained fractions were combined based on TLC checking resulting in seven fractions (B I - B VII). Fraction B II (882.5 mg) eluted wit *n*-hexane – acetone 85:15, was further separated by flash chromatography (**FCC-15**) with increasing polarity of *n*-hexane – acetone. The resulted subfraction B II/7 (332.5 mg), obtained with *n*-hexane – acetone 9:1 and subfraction B II/8 (329.6 mg) eluted with *n*-hexane – acetone 85:15 were further purified by repeated flash-chromatography (**FCC-16** and **FCC-17**). The subfraction B II/7/2 (38.1 mg) eluted with *n*-hexane – acetone 95:5 and subfraction B II/8/2 (194.3 mg) obtained with *n*-hexane – acetone 91:9 were purified with normal-phase HPLC (**NP-HPLC-1** and **NP-HPLC-2**) using cyclohexane – isopropanol – water isocratic eluent system to result in two compounds **PAXATRB1** (21.2 mg, t_R 10.1 min) and **PAXATRB2** (14.0 mg, t_R 5.5 min).

Fraction B IV (1.3 g) obtained previously with *n*-hexane – acetone 75:25, was subjected to flash column chromatography (**FCC-18**) using *n*-hexane – acetone gradient system as mobile phase, resulted in eight subfractions (B IV/1 – B IV/8). The investigation of the subfraction B IV/5 (63.4 mg) eluted with *n*-hexane – acetone 75:25, was performed by HPLC (**NP-HPLC-3**) using mobile phase of cyclohexane – isopropanol – water 78:22:0.1 and

led to the isolation of **PAXATRB5** (15.8 mg, t_R 13.1 min). Finally, the subfraction B IV/6 (38.3 mg), obtained previously with *n*-hexane – acetone 6:4, was analyzed with HPLC (**NP-HPLC-4**) applying an isocratic mobile phase of cyclohexane – isopropanol – water 75:25:0.1 and resulted in **PAXATRB6** (1.3 mg, t_R 16.1 min).

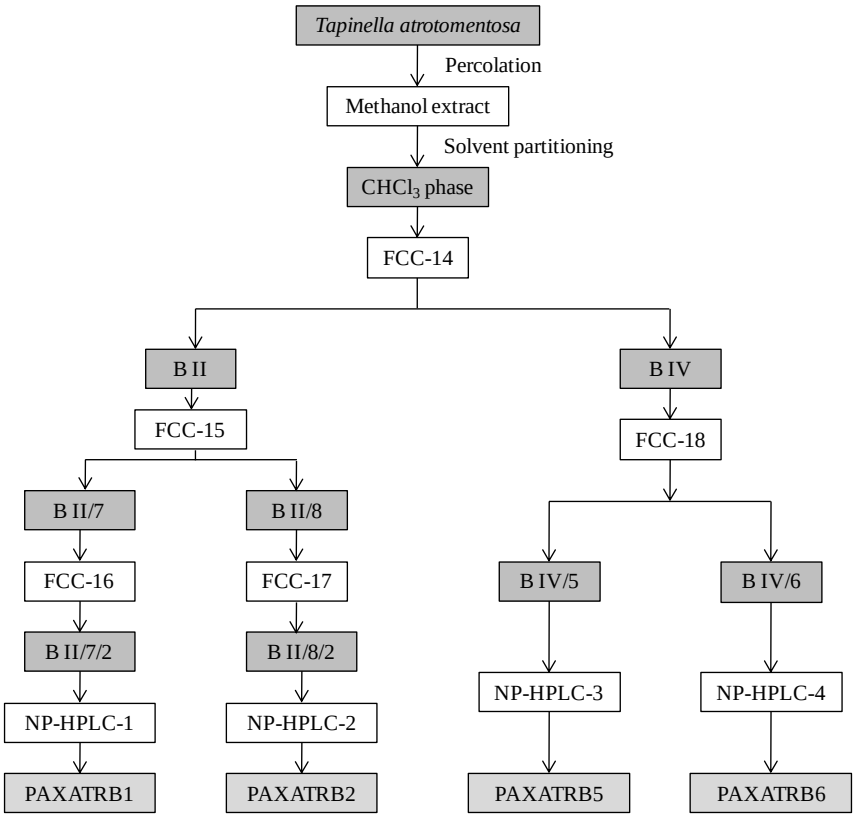


Figure 5. Isolation of compounds from *Tapinella atrotomentosa*

5.4. CHARACTERIZATION AND STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

The structure elucidation of the isolated compounds was performed by means of NMR and MS measurements (see sections **4.4.**, **4.5.** and **4.6.**). Information from 1D (^{1}H-NMR and JMOD) and 2D (^{1}H-^{1}H COSY, TOCSY, NOESY, ROESY, HSQC and HMBC) NMR experiments provided valuable information for the structure determination. HRMS measurements revealed the molecular masses and molecular compositions of the compounds.

5.4.1. Compounds from the chloroform extract of *G. fusipes*

In the chloroform extract of *G. fusipes* two novel natural products were characterized, namely gymnopeptides A [**COFUB1** (**1**)] and B [**COFUB2** (**2**)] (**Figure 6**). Gymnopeptides A and B displayed highly similar ^{1}H NMR spectra in $CDCl_3$. The ^{1}H NMR features confirmed the peptide nature of the two compounds and suggested the presence of a single conformer in

both cases. Consecutive analysis of ^{1}H, ^{13}C, 2D-TOCSY, heteronuclear 2D NMR correlation (HSQC and HMBC), and selective 1D-TOCSY data permitted the identification and complete NMR assignments of the 18 amino acid (AA) residues (see **Annex I**, **II**). This led to the conclusion that the two compounds differed only in the presence of a single AA; a serine (Ser) found in gymnopeptide A was replaced by a threonine (Thr) in gymnopeptide B. The other 17 AA were common in the two compounds: three alanine (Ala), four valine (Val), two *N*-methyl-glycine (sarcosyl, Sar), an *N*-methyl-alanine (NMAla), and seven *N*-methyl-valine (NMVal) (**Figure 6**).

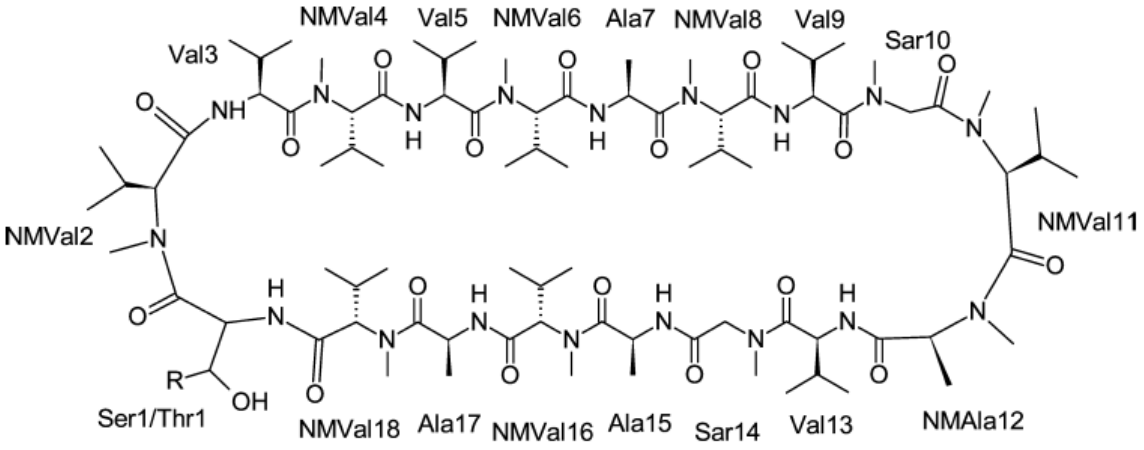


Figure 6. Amino acid sequences of gymnopeptides A (R = H) and B (R = CH_3)

Both are octadecacyclopeptides, where unmodified and *N*-methylated amino acids alternate in the sequences, except in position 11, where instead of an unmodified amino acid an NMVal is present. Our findings suggested that the amide bonds between Ser1 (Thr1 in gymnopeptide B) and NMVal2 and that between NMVal11 and NMAla12 residues are in *cis*, while all other amide bonds are in the *trans* configuration. Taking all these into account, an 18mer cyclic β-hairpin structure (**Figure 6**) is suggested for both peptides.

FT-HR-ESI-MS analysis was in accordance with the constitution suggested by NMR spectroscopic analysis (see **Annex III**). Thus, the protonated molecular ions of gymnopeptides A and B were detected at *m/z* 1716.13393 and 1730.15751, respectively. These values corresponded to the molecular formulas of $C_{84}H_{151}N_{18}O_{19}$ and $C_{85}H_{153}N_{18}O_{19}$ (protonated molecular ions), respectively, with high accuracy (Δ = −3.4 and 1.2 ppm, respectively) and were in accordance with the suggested sequences showing a single amino acid difference in the two gymnopeptides. Although MS/MS^{n}-based amino acid sequencing of cyclopeptides is usually complicated by the possibility of multiple and often indiscriminable ring-opening positions[60], in the case of gymnopeptides A and B, the MS/MS data could unambiguously confirm the AA sequences suggested by NMR. In the MS/MS spectra of the

cyclopeptides, the two largest fragment ions corresponded to linear peptides that could be derived from the parent ions by the loss of a Val and a Val plus an NMAla residue. Regarding the suggested sequence, only a single Val-NMAla peptide bond is present. On this basis, a ring opening between Val13 and Sar14 (or between NMAla12 and Val13) could be predicted. Analysis of the fragment ions detected in the MS/MS spectra with regard to the simulated (ProteinProspector 5.12.1) b and y ions derived from these linear peptides supported this ring opening and could already verify most of the suggested sequences in the case of both gymnopeptides. Further analysis of the largest unassigned fragment ions (b15 and y15) suggested another ring opening position between NMVal11 and NMAla12. With the b and y ions derived from these linear peptides, another large set of fragment ions detected in the MS/MS spectra could be explained. Combining the fragmentation information on these two linear peptides, the suggested AA sequence could unequivocally be verified in case of both cyclopeptides.

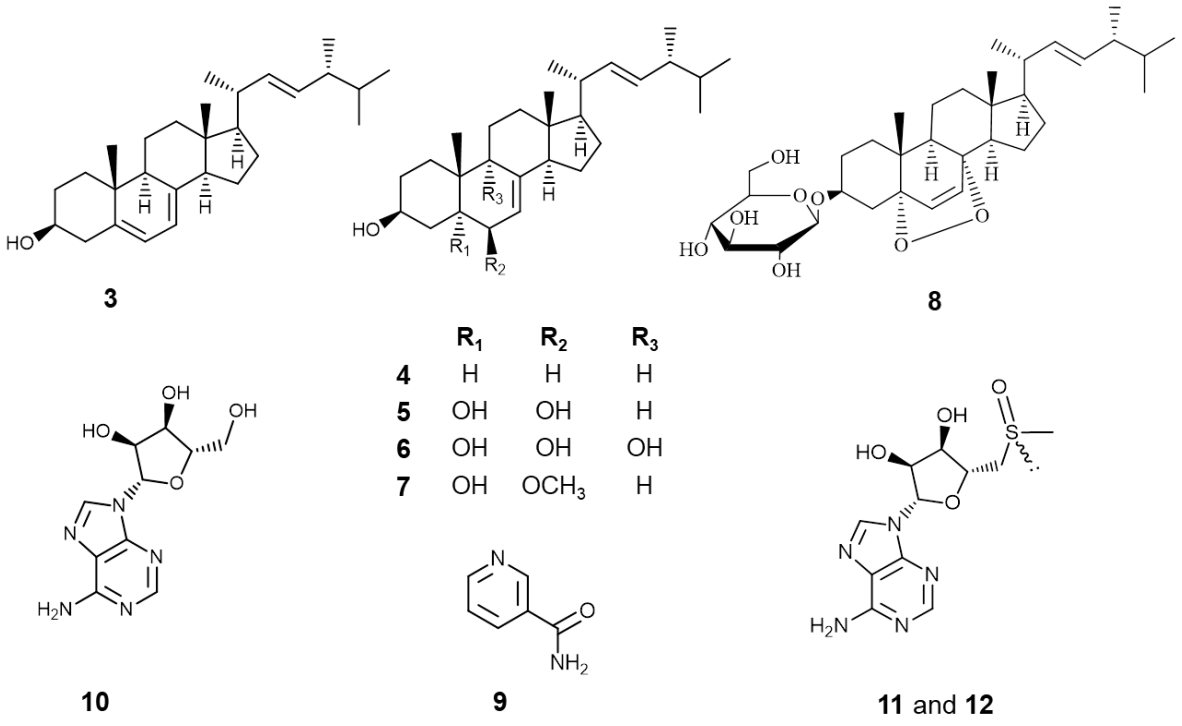
Absolute configurations of the amino acid residues were investigated by HPLC−MS following the methods described in the literature[61,62]. First, acidic hydrolysis of the cyclopeptides was undertaken. This was followed by derivatization of the resulted amino acid mixture with Marfrey's reagent[63]. Finally, HPLC−MS analysis was performed, and the retention times belonging to the accurate mass values of the derivatized amino acids in the extract ion chromatograms were compared to those observed for the derivatized L,D-amino acid pairs prepared and measured using similar experimental conditions (see **Annex IV**). On the basis of the observed data, all Ala, NMAla, Val, and NMVal residues were shown to have the L configuration. Indeed, this finding is in accordance with the conclusion of Gibbs et al.[64] suggesting that a D amino acid residue in the β strand would prevent the formation of β hairpin structures in cyclic peptides. It should be noted that in most cases the epimeric derivatives were detected in low quantities (ca. 1 to 7%) as well. Detection of these derivatives was due to either racemization of the AAs during the long acidic hydrolysis[65-67] or low-level peptide contaminants of the isolated samples. The absolute configurations of serine and threonine could not be determined from the hydrolysates, since neither the serine nor the threonine derivatives could be detected by HPLC−MS. Nevertheless, according to a preliminary molecular modeling study on gymnopeptide A, with L-Ser in position 1 a geometry well agreeing with all NMR data could be obtained by calculations. The calculations were performed within the Schrödinger software suite[68-71]. An initial geometry was calculated for gymnopeptide A at the MM level using the key NOE-derived geometry restraints and conformational search. Finally, a molecular dynamics simulation was

performed to further optimize the geometry and to prove that no significant changes occur during the simulation when no restraints are applied. As mentioned above, the optimized structure (see **Annex V**) is in good agreement with the above-discussed NMR data. Considering all the experimental data, the structures shown in Figure 6 can be proposed for gymnopeptides A and B. These structures were unambiguously confirmed by the study of Pan et al. published in 2017 dealing with the total synthesis of gymnopeptides A and B[72].

5.4.2. Compounds from *T. populinum*

The investigation of the methanol extract obtained from the lyophilized fruiting bodies of *T. populinum* led to the isolation of 10 compounds, 9 of them for the first time from this species. From the *n*-hexane extract of cottonwood mushroom six compounds (**3**-**8**) were isolated, which belong to the group of ergostane triterpene steroids: ergosterol [**TRIPOA5** (**3**)], 3β-hydroxyergosta-7,22-diene [**TRIPOA4** (**4**)], cerevisterol [**TRIPOA1** (**5**)], 3β,5α,6β,9α-tetrahydroxyergosta-7,22-diene [**TRIPOA3** (**6**)], methylated derivative of cerevisterol [**TRIPOA6** (**7**)] and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene [**TRIPOA2** (**8**)].

In the chloroform extract of the species nicotinamide [**TRIPOB1** (**9**)], adenosine [**TRIPOB3** (**10**)] and rare sulfinyladenosine constituents, epimers of 5'-deoxy-5'-methylsulphinyladenosine [**TRIPOB2** (**11**) and **TRIPOB4** (**12**)] were detected.



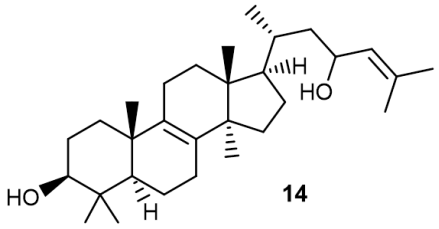
	R_1	R_2	R_3
4	H	H	H
5	OH	OH	H
6	OH	OH	OH
7	OH	OCH_3	H

Compounds **3**–**4** were identified by comparing their chromatographic and spectroscopic data with those of authentic samples. Compounds **5**–**12** were structurally characterized based on NMR and MS spectroscopic data and confirmed by comparing them to those reported earlier for these constituents[73-76].

5.4.3. Compounds from *S. bovista*

As a result of the isolation process 7 compounds were obtained from the fruiting bodies of *S. bovista*. In the *n*-hexane fraction 5 secondary metabolites were identified. One of them [**SCLEBOA1** (**13**)] is ceramide-type compound, namely cerebroside B. The other constituents proved to be ergostane-type steroids: ergosterol [**SCLEBOA5** (**3**)], 3β-hydroxyergosta-7,22-diene [**SCLEBOA4** (**4**)], cerevisterol [**SCLEBOA3** (**5**)] and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene [**SCLEBOA2** (**8**)].

From the chloroform fraction of *S. bovista* 2 compounds with lanostane skeleton were isolated, namely 23-hydroxylanosterol [**SCLEBOB1** (**14**)] and lanosta-8,23-dien-3β,25-diol [**SCLEBOB2** (**15**)]. **8, 13-15** were structurally characterized on the basis of NMR and MS spectroscopic data and were confirmed by comparing them to those reported earlier for these components[77-79]. Compounds **3-5** were identified by comparing their chromatographic and spectroscopic data with those of authentic samples.



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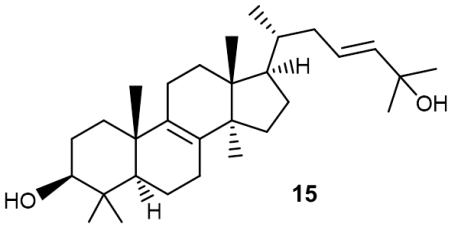

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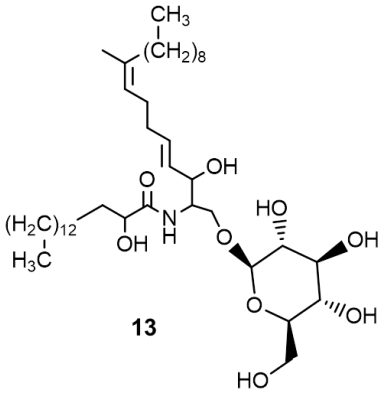

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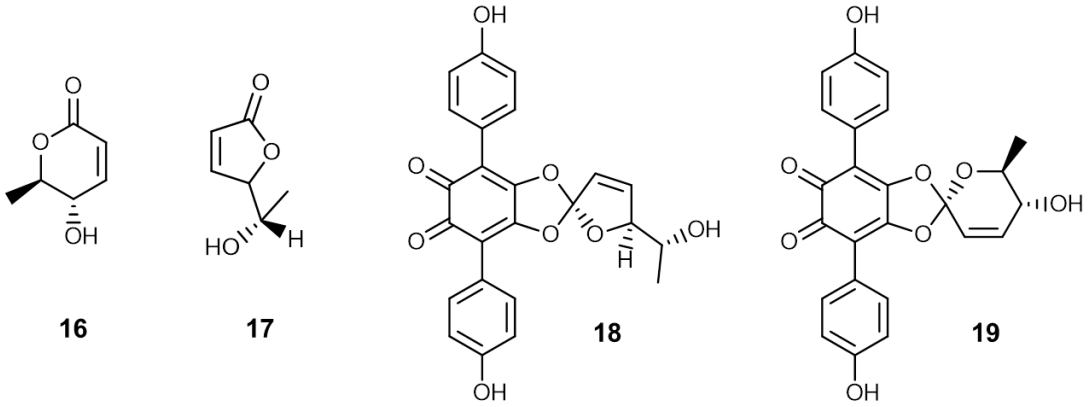
5.4.4. Compounds from the chloroform extract of *T. atrotomentosa*

From the chloroform phase of methanol extract of *T. atrotomentosa* 4 compounds (**16**-**19**) were identified. All of the investigated compounds have already been isolated from natural sources and from the species as well. Among the compounds there are two lactone type components: osmundalactone [**PAXATRB1** (**16**)] and 5-hydroxy-2-hexen-4-olide

[**PAXATRB2** (**17**)], while the other isolated metabolites, namely spiromentin C [**PAXATRB5** (**18**)] and spiromentin B [**PAXATRB6** (**19**)] possess terphenylquinone skeleton. The structure of the components was determined by spectroscopic measurements (NMR and MS); the obtained spectroscopic data were compared to those reported earlier for these compounds.



5.5. PHARMACOLOGICAL ACTIVITIES OF THE ISOLATED COMPOUNDS

5.5.1. Antiproliferative effect of the cyclopeptides of *G. fusipes*

Gymnopeptides A (**1**) and B (**2**) were evaluated for their potential antiproliferative properties against five different human cancer cell lines (HeLa, A431, MCF-7, MDA-MB-231, T47D) by MTT method (see **Appendix III** in **Table 1**). Compounds **1** and **2**, isolated from the chloroform extract of *G. fusipes* demonstrated very strong cell growth inhibitory activity on the studied cancer cells with IC_{50} values in nanomolar range (14-88 nM). Gymnopeptides A (**1**) and B (**2**) proved to be the most active against the breast cancer cells MCF-7 and T47D. In comparison, compound **2** was more potent, than compound **1**, while both of them were at least 2 orders of magnitude more efficient than the reference agent cisplatin.

5.5.2. Biological activity of the compounds of *T. populium*

Compounds **5-8** identified in the *n*-hexane extract of *T. populinum*, were assayed for their potential cytotoxic activity on several human breast cancer cell lines (MCF-7, T47D, MDA-MB-231) by MTT method (see **Appendix IV** in **Table 1**). 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% cell growth inhibition). Among the three cell lines, MCF-7 proved to be the least susceptible against the isolated compounds; however **8** exerted moderate activity on this cell line as well.

3β,5α,6β,9α-tetrahydroxyergosta-7,22-diene (**6**) showed no antiproliferative effect on the studied cancer cells.

Nicotinamide (**9**) and adenosine-type compounds (**10-12**), isolated from the chloroform extract of the species were evaluated for their potential XO inhibitory activity (see section **4.7.3.**), however according to our results that they do not possess inhibitory activity on this enzyme.

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

Compounds **8** and **13-15** were tested for their *in vitro* antiproliferative activity on four different human cancer cell lines (HeLa, A2780, MDA-MB-231, MCF-7) using MTT method (see **Annex VI**). 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**) proved to be the most active against three cancer cells (HeLa, A2780 and MCF-7). Compound **15** exhibited higher antiproliferative property on HeLa (62.21 ± 1.95%) and MCF-7 (73.32 ± 2.76%) cells than **14** (42.88 ± 7.79% and 37.39 ± 5.89%), but the latter was more effective against A2780 cells (68.47 ± 2.94% vs. 54.07 ± 4.22%). 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 on the investigated cancer cells. Among the four cell lines A2780 and MCF-7 proved to be more susceptible against the fungal metabolites tested in this experiment; while MDA-MB-231 demonstrated susceptibility in a weaker extent.

5.5.4. 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 using standard disc-diffusion method and double-disc synergy test (see section **4.8.2.**). The isolated compounds proved to possess antimicrobial properties against Gram-positive and -negative strains as well (**Table 2**). Our investigations revealed that *A. baumanii* and ESBL *E. coli* are the most susceptable against the studied compounds. Among the constituents 5-hydroxy-2-hexen-4-olide (**17**) was the most active, although osmundalactone (**16**) and spiromentin C (**18**) have also shown remarkable effectiveness against *A. baumanii* and ESBL *E. coli*. The investigated compounds possessed moderate activity in the case of *M. catarrhalis*, while the least susceptible strain proved to be MRSA.

Table 2. 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	-	-

Compounds **16-19** were investigated with the aim of evaluating theirs synergistic effect with cefuroxime against MRSA using checkerboard techniques (see section **4.8.2.**), though our results indicate that they do not enhance the activity of the studied antibiotic drug.

Antioxidant activity

The isolated compounds **16-19** from the chloroform extract of the species *T. atrotomentosa*, were evaluated for their antioxidant activity using DPPH and ORAC assays (see section **4.7.4**). In contrast to the results of DPPH test, the metabolites have shown strong antioxidant activity on ORAC assay. Among the isolated compounds **18-19** exhibited the most remarkable antioxidant effect on ORAC assay (**Table 3**), which are higher than that of ascorbic acid used as reference compound. The DPPH assay performed revealed that compounds **16-17** have no antioxidant activity in this assay, while the investigation of compounds **18-19** was not possible due to their purple color which might interfere with the color of the reagent used on 550 nm. The results demonstrate that the isolated compounds possessing marked antioxidant activities have a great contribution to the overall antioxidant property of the original mushroom extract.

Table 3. 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 ± 0.008**

6. DISCUSSION

6.1. SCREENING STUDIES

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. More than 3000 mushrooms are indigenous to Hungary, but to date, however, there have been only few comprehensive screening studies dealing with the anticancer and antimicrobial effects of mushrooms.

Fungal species were collected in north-eastern part of Hungary. The mushroom samples were extracted with an amphipolar solvent (methanol), which permitted the isolation of lipophilic and polar components as well. Solvent–solvent partitioning between the aqueous methanol extract and *n*-hexane, then $CHCl_3$ as well as the extraction with H_2O afforded fractions with different polarities.

The mushroom extracts were tested *in vitro* for their antiproliferative activity against several human cancer cell lines using MTT assay. According to our preliminary studies the chloroform extract of *G. fusipes* exerted outstanding effect on several human cancer cell lines. The chloroform extract of *S. bovista* also proved to possess notable antiproliferaive activity against the investigated cancer cells (>50% growth inhibition). In these experiments the extracts of *T. populinum* were found to exhibit only moderate antiproliferative effect.

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 strains. 16 species exhibited antibacterial effects with moderate to high potential. The studied extracts demonstrated antimicrobial activity mainly against Gram-positive bacteria, being in agreement with the results reported earlier[80,81]. The extracts of *Fistulina hepatica*, *Hypholoma fasciculare*, *Tapinella atrotomentosa* and *Rhodocybe popinalis* revealed the highest antibacterial capacity against mainly Gram-positive strains with MICs between 12.5 and 100 μg/mL. 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 (extended-spectrum beta-lactamase)-positive *Escherichia coli*, standard and multiresistant *Pseudomonas aeruginosa* and multiresistant *Acinetobacter baumannii*.

The active mushroom extracts were applied to different multiresistant micro-organisms combined with commercial antibiotics (ampicillin, imipenem, cefuroxime and

vancomycin) to evaluate their capacity to potentiate the action of standard drugs. The extracts of *F. hepatica*, *T. atrotomentosa* and *R. popinalis* exhibited synergistic activity with cefuroxime against methicillin resistant *Staph. aureus*. So far only few studies have reported positive interaction of fungal extracts and standard antibacterial drugs. The extracts are supposed to increase the efficiency of β-lactam antibiotic through inhibition of β-lactamase production. Nevertheless, further studies should be conducted to investigate the mechanism of action of synergism.

In conclusion, our antiproliferative and antimicrobial screening studies presented a good basis for the selection of promising mushroom species native to Hungary for the discovery of novel compounds with antiproliferative and antimicrobial properties.

6.2. INVESTIGATION OF *G. FUSIPES*, *T. POPULINUM*, *S. BOVISTA*, *T. ATROTOMENTOSA*

The investigated mushroom species were chosen based on our previous screening studies with the aim of the identification of their biologically active secondary metabolites. *G. fusipes* and *S. bovista* were subjected to further mycochemical studies to identify the compounds responsible for the observed antiproliferative property, while *T. atrotomentosa* was included in our preparative work in the perspective of isolation of new antimictobial natural products. Although *T. populinum* exerted moderate antiproliferative activity, our previous studies revealed that the species possesses considerable xanthine oxidase enzyme inhibitory property.[59] Based on these screening results the total mycochemical analysis of the species *G. fusipes*, *T. populinum*, *S.bovista* and *T. atrotomentosa* were carried out.

6.3. ISOLATION OF THE BIOACTIVE COMPOUNDS

Our previous screening programs of the extracts with different polarities prepared from *G. fusipes*, *T. populinum*, *S. bovista*, *T. atrotomentosa* led to the conclusion that *n*-hexane and especially chloroform extracts contain the bioactive fungal metabolites. In the initial step of the preparative work, the mushroom materials were percolated with an amphipolar solvent (methanol) at room temperature; then liquid–liquid extraction was employed, which resulted in *n*-hexane and chloroform phases. All of them were fractionated using different type of chromatographic procedures to isolate the compounds responsible for biological activity.

The chloroform fraction of ***G. fusipes*** was separated first by RPC and subfractions were further purified with RP-HPLC using acetonitrile – water gradient eluent system. This purification process led to the isolation of 2 new natural compounds (**1-2**).

In the case of ***T. populinum*** the *n*-hexane phase was fractionated by OCC and the combined fractions were further analyzed using repeated FCC. During the chromatographic separation six compounds (**3-8**) were isolated. The purification of the chloroform extract of the species was first carried out by RPC followed by RP-HPLC affording four compounds, two in pure form (**9-10**) and two in a mixture of epimeric forms (**11-12**).

The separation of *n*-hexane extract of ***S. bovista*** was started with OCC. The combined fractions were further separated by FCC resulting in five constituents (**3-5**, **8**, **13**). The analysis of the chloroform fraction of this species was performed by repeated FCC to obtain two compounds (**14, 15**).

The chloroform phase of ***T. atrotomentosa*** was separated by repeated FCC steps. The obtained subfractions 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 (**16-19**).

6.4. STRUCTURE ELUCIDATION

The chemical structures of the isolated compounds (**1-19**) were determined by different spectroscopic methods. The molecular masses and compositions were obtained from MS investigations. The most useful data concerning the structures were established by 1D and 2D NMR spectroscopy. The constitutions of the compounds were described via ^{1}H-NMR, JMOD, ^{1}H-^{1}H COSY, TOCSY, HSQC and HMBC experiments, and the relative configurations were then characterized with the aid of NOESY and ROESY spectra. As a result of the NMR studies, complete ^{1}H- and ^{13}C assignments were determined for the new compounds and also in the case of some known compounds, where previously published data were incomplete. In the case of compounds **1** and **2** Marfey's analysis combined with LC-MS investigation proved to be indispensable for determination of absolute configuration of amino acids.

6.4.1. Cyclopeptides from *Gymnopus fusipes*

Two novel cyclic octadecapeptides were isolated from the chloroform extract of *G. fusipes*, namely gymnopeptides A (**1**) and B (**2**). 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, 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**. Gymnopetides A and B are highly methylated, the number of *N*-methylated amino acids is 10 out of 18 in both cyclopeptides, and *N*-methylvaline contributes with 7 monomers to the

overall structure. The unmodified and *N*-methylated amino acids alternate in the sequences of the two cyclopeptides, except in position 11, where instead of an unmodified amino acid an *N*-methylvaline is present.

Among the secondary metabolites of higher mushrooms cyclic peptides are fairly rare compounds; their occurrence seems to indicate a rather specific distribution. Apart from (−)-ternatin, a highly *N*-methylated cyclic heptapeptide[82] isolated from the medicinal mushroom *Trametes versicolor*[83], cyclopeptides have been identified in few genera of poisonous species (Amanita, Conocybe, Galerina, Lepiota and Omphalotus)[84].

The genus Amanita contains about 500 described species, which occur worldwide[85]. Some well-known poisonous species are *Amanita phalloides, A. verna* and *A. virosa* located in Europe and North America, while *A. exitialis, A. fuliginea* and *A. subjunquillea* occur in East Asia[86]. Up to now, ca. twenty-two cyclopeptide toxins from Amanita, Conocybe Galerina and Lepiota species have been identified. They are classified into four major groups: amatoxins (bicyclic octapeptides), phallotoxins (bicyclic heptapeptides), virotoxins (monocyclic heptapeptides)[87] and the non-toxic cycloamanides.

Besides the above mentioned genera, cyclopeptide compounds were detected in the poisonous species of Omphalotus genus. Omphalotins A-D are cyclic dodecapeptides isolated from *Omphalotus olearius*[88,89]. The cyclic dodecapeptides, omphalotins F-I contain unprecedented *N*-hydroxylated tricyclic tryptophan derivative[90].

6.4.2. Steroids and sulfinyladenosine compounds from *Tricholoma populinum*

The structure determination of compounds isolated from *n*-hexane phases of *T. populinum* led to the identification of 6 steroids with ergostane structure. Ergosterol (**3**) and 3β-hydroxyergosta-7,22-diene (**4**) are common steroids with a widespread distribution in fungal species[91]. Ergosterol has been found to be the major sterol of many fungal species since its discovery in the ergot fungus *Claviceps purpurea* by Tanret in 1889[92]. Therefore, like cholesterol in animals, ergosterol has become known as “the fungal sterol”. Based on the analysis carried out by Mitsuhasi and Yokokawa in 1981 ergosterol and 22-dihydroergosterol together comprised over 85% of the total sterols from the studied species[93].

In the *n*-hexane fraction of *T. populinum* cerevisterol (**5**), found first time in the yeast *Saccharomyces cerevisiae*[73], and 3β,5α,6β,9α-tetrahydroxyergosta-7,22-diene (**6**), isolated earlier from *Trametes versicolor*[74] were also detected. They are di- and trihydroxlated derivatives of 3β-hydroxyergosta-7,22-diene (**4**). The methylated derivative of cerevisterol (**7**) has been previously isolated only from *Agaricus blazei* by Kawagishi et al[75]. 3β-*O*-

glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) has a peroxide linkage between C_5 and C_8 and has been identified by Takaishi et al. in 1991 in the edible and medicinal mushroom *Hericium erinaceus*[76]. Our is the second isolation of **8** from natural sources.

From the chloroform phase of methanol extract of cottonwood mushroom 4 compounds were identified. Nicotinamide (**9**) has been previously detected by Turner et al. (1987) in *T. populinum* samples collected in British Columbia (Canada)[94]. Derivatives of **10**, **11** and **12** proved to be adenosine-type compounds. In contrast to the commonly occurring adenosine (**10**), the methylsulfinyladenosine structures of **11** and **12** (epimers of 5'-deoxy-5'-methylsulphinyladenosine) represent a fairly rare subclass of secondary metabolites. To best of our knowledge this type of compounds has been detected only in one fungal (*Ganoderma lucidum*[95]), one plant species (*Sauropus androgynus*[96]), and two marine organisms (*Herdmania momus*[97], *Atriolum robustum*[98]) so far. The marine ascidian *H. momus* synthesizes four complex adenosides with bromines in their structures[97], and the Australian species of *A. robustum* contains a metabolite with methoxyacrylic acid group linked to the sulfinyladenosine moiety[98]. Compounds **11** and **12** were first isolated by Kawagishi et al. in 1993 from *G. lucidum*, however, the fruiting bodies of *T. populinum* contain approx. 80 times higher amount of these sulfinyladenosines than *G. lucidum*.

6.4.3. Ceramide- and steroid-type compounds from *Scleroderma bovista*

In the *n*-hexane extract of earth ball mushroom was detected a ceramide type compound linked to a glucose molecule, namely cerebroside B (**13**). Ceramides are composed of fatty acids and sphingosine, where fatty acids are attached to the amine part of sphingosine. Cerebroside B was isolated first in *Inonotus mikadoi* in 1987 by a Japanese research group[77].

The investigation of *S. bovista* resulted in the isolation of 6 triterpene steroids: ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**5**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) possess ergostane skeletone. The lanostane type metabolites from the chloroform phase of *S. bovista* were identified as 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**). Compound **14** was isolated first from *Scleroderma aurantium* in 1968 by Entwistle et al[78], while **15** was found first in the same species in 1976 by Vrkoc et al[79], however the pharmacological activity of these compounds has not been studied.

In the Basidiomycetes class of fungi the ergosterol is often accompanied by methyl sterols such as lanosterol and 24-methylene-24,25-dihydrolanosterol[93]. These "methyl-sterols" are intermediates in sterol biosynthesis that have methyl groups at C-4 and/or C-14.

6.4.4. Lactone and terphenylquinone derivatives from *Tapinella atrotomentosa*

Based on the spectral data, two compunds were confirmed as lactone type metabolites, isolated from the chloroform phase of *T. atrotomentosa*. Osmundalactone (**16**) was obtained first time from this species in 1995 by a Japanese research group[40]. Previously this compound has been already identified both in free form[99] and as hydrolysis product of osmundalin (a glucoside of osmundalactone) in the fern species of *Osmunda japonica*[100]. Since then the component has also been isolated from the evergreen fern, *Angiopteris caudatiformis* in 2009[101].

According to the spectroscopic measurements compounds **17** proved to be 5-hydroxy-2-hexen-4-olide. This secondary metabolite was first detected in *O. japonica* in 1984 by Numata et al[99].

Thanks to the isolation procedures spiromentin C (**18**) and spiromentin B (**19**) were identified from the chloroform extract of the species. Spiromentin B and C were detected in *T. atrotomentosa* by Besl et al. in 1989, this species is the single source of these pigments described so far [42].

Spiromentines belongs to the group of terphenylquinones, which represent an interesting subclass of secondary metabolites of mushrooms. Natural occurrence of products with a *p*-terphenyl core (also known as diphenylbenzenes or triphenyls) is essentially restricted to fungi and lichens. Based on the literature overview, polyhydroxy-*p*-terphenyls and *p*-terphenylquinones occur in 35 different fungal species (both from fungal cultures and from fruiting bodies of Basidiomycetes) and a total of 115 fungal metabolites were reported, with unusual structures and/or interesting biological properties (e.g. antiproliferative, antibacterial, antioxidant, and anti-inflammatory activity)[102]. *p*-Terphenylquinones are known as fungal pigments for a long time. The prototype of polycyclic-*p*-terphenyl class of fungal metabolites is the bronze-colored polyporic acid, originally isolated from *Polyporus nidulans* by Stahlschmidt[103]. Atromentin, the 4,4-dihydroxy analogue of polyporic acid, accounts for the reddish-brown color of the external part of *T. atrotomentosa*, from which was originally isolated. The colorless precursors of atromentin biosynthesis are leucomentins that are esters of polyporic acid[104]. Flavomentins A-D are orange-yellow terphenylquinones possessing the basic skeleton of atromentin and bearing unsaturated acyl residues[41].

Thanks to the extensive studies of *T. atrotomentosa* and *P. panuoides* carried out in 1989 by Besl et al[42], a new group of *p*-terphenyls were isolated, spiromentins A-D, which possess unique γ-and δ-lactone-acetal spiro structures linked to a 4,5-dihydroxy-1,2-benzoquinone core. These violet pigments can be formed from flavomentins[104]. Later on a

Japanese research group obtained the colorless spiromentins E-J from the fresh fruiting bodies of *T. atrotomentosa*. The same study reports the isolation of (+)-osmundalactone and other three γ-lactones related to the spiromentine structures[40].

Previously some further terphenylquinones were identified in Stibella species, which were determined as diphenyl-benzoquinones and phenyl-dibenzofurandiones. These terphenylquinone derivatives possessed significant activity against the human proto-oncogene src protein tyrosine kinase[105]. Kuhnert et al. isolated novel *para*-terphenyl derivates from the fungus *Hypoxylon rickii,* namely rickenyls A-E with antioxidative and cytotoxic properties[106].

6.5. BIOLOGICAL ACTIVITIES OF THE COMPOUNDS OF THE INVESTIGATED SPECIES

Gymnopus fusipes

The antiproliferative properties of the isolated cyclopeptides, gymnopeptides A (**1**) and B (**2**) were determined by MTT assay on a panel of human adherent cancer cell lines including cervical (HeLa), skin epidermoid (A431), and human breast cancer cells (T47D, MCF-7, and MDA-MB-231). Both cyclopeptides exhibited potent cell growth inhibitory action on all of the studied cell lines at nanomolar concentrations. In comparison, gymnopeptide B (**2**) was more potent than gymnopeptide A (**1**), while both of them were at least 2 orders of magnitude more active than the reference compound cisplatin. Among the cells, MCF-7 and T47D proved to be the most susceptible against the isolated compounds **1-2**.

Cyclopeptides isolated from macrofungi have different biological activities, but their negative effects usually outweigh their positive ones. The cyclopeptides of Amanita genus cause several poisonous symptoms e.g. neurotoxicity, nephrotoxicity and hepatotoxicity[85]. Consumption of the species accounts for over 90% of all fatal mushroom poisonings worldwide[86]. Among the cyclopeptides of Amanita species the amatoxins cause poisoning through inhibiting RNA polymerase II[107], while phallotoxins and virotoxins do not play a role in human poisonings, because they are not absorbed by intestinal cells[87].

Interestingly, a cyclic decapeptide, antamanide,isolated from *A. phalloides* in 1968[108], is acting as a potential anti-toxin against the effects of Amanita's toxins. [109]. Antamanide and a number of its analogues showed immunosuppressive properties as well, which at low doses was comparable with the activity of cyclosporine A, the well-known therapeutic agent from an Ascomycetes fungus, *Tolypocladium inflantum*[110]. Amanexitide, a new cyclic nonapeptides from *A. exitialis,* seems to have the same amino acid residues as in the antamanide, but further

investigation needed to evaluate its potential antidote activity. Amanexitide found to be inactive against cancer cell lines[111].

The investigations of omphalotins possessing cyclic dodecapeptide structure, revealed the nematicidal effect of these compounds. Omphalotin A showed strong and selective activity against the plant pathogenic *Meloidogyne incognita*. Omphalotin A was found to be weakly cytotoxic (at 100 μg/mL), but shows no phytotoxic, antibacterial or antifungal activities[88]. Omphalotins B-I exhibited also strong and selective nematicidal activity against the plant pathogen *M. incognita* with LD_{90} values between 1 and 5 mg/L[90].

(–)-Ternatin, a cyclopeptide identified in *Trametes versicolor* proved to possess antimicrobial properties. Recently its inhibitory effects on fat accumulation in adipocytes and on suppression of the development of hyperglycemia have been discovered[82].

As can be seen from the above, gymnopeptides A and B isolated from *G. fusipes* have a distinct place in group of mushroom cyclopeptides, not only because of their remarkably high antiprolifertive activity, but also because they were detected in a species considered edible and consumed by locals in several European countries. It would be of great interest to conduct further studies to explore other potential biological properties of these peptides and to clarify some aspects of absorption and metabolism of gymnopeptides after consumption of fruiting bodies of *G. fusipes*, as well as to investigate the potential role of these metabolites in the parasitic lifestyle of the mushroom.

Tricholoma populinum

Based on our screening results and the published literature data, the steroids (**5-8**) detected in *T. populinum*, were evaluated for their antiproliferative activity on three different human breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) by MTT method. The compounds proved to be the most active against T47D cells with inhibition values between 23.7 ± 2.4% and 50.2 ± 1.6%. On MDA-MB-231 cells demonstrated significant activity (54.7 ± 1.6% growth inhibition) only the methylated derivative of cerevisterol (**7**), while MCF-7 proved to be the less susceptible cell line against the isolated compounds.

In previous studies, the antiproliferative activity of ergosterol-peroxide isolated from *T. populinum*[29] was evaluated. According to Bok et al., the compound inhibited the growth of erythroleukemia (K562), T-lymphoblastic (Jurkat), promyelocytic leukemia (HL-60), malignant melanoma (WM1341) and multiple myeloma (RPMI 8226) cell lines[112]. In another study ergosterol-peroxide showed mild cytotoxic activity on non-small cell lung adenocarcinoma (A549), ovary malignant ascites (SK-OV-3), skin melanoma (SK-MEL-2)

and colon adenocarcinoma (HCT-15)[113]. The metabolite seems to possess significant neuritogenic activity against PC12 cells suggesting that might be used to prevent neurodegenerative disorders.[114]

Compounds **9-12** obtained from the chloroform extract of the species were evaluated for their potential XO-inhibitory activity, though our results indicate that they do not possess enzyme inhibitory activity.

Scleroderma bovista

According to our screening results and based on the data of the scientific databases, compounds **8**, **13-15** isolated from *S. bovista*, were investigated for their antiproliferative properties on four different human cancer cell lines (HeLa, A2780, MCF-7 and MDA-MB-231) using MTT method. 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**) demonstrated cell growth inhibition against HeLa (42.88 ± 7.79% and 62.21 ± 1.95%), A2780 (68.47 ± 2.94% and 54.07 ± 4.22%) and MCF-7 cells (37.39 ± 5.89% and 73.32 ± 2.76%) at 30 μM concentration, while 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**) exerted significant activity on A2780 cells (53.27 ± 6.37%) at 30 μM concentration.

Based on our investigation, cerebroside B (**13**) seems to possess only weak antiproliferative activity. However, cerebroside-type compounds are frequently described in the scientific literature as compounds with broad anticancer activity thanks to their suspected DNA polymerase enzyme inhibitory properties[7,115]. In a recent study reported by Meng et al., cerebroside B showed significant antimicrobial activity against *Escherichia coli* (IC_{50} 275.1) and *Staphylococcus aureus* (IC_{50} 323.2)[116]. Its effectiveness proved to be approx. five times stronger than that of sorbic acid used as control.

Tapinella atrotomentosa

Compounds **16-19** obtained from the chlorofom extract of *T. atrotomentosa* were evaluated for their antimicrobial activity against several bacterial strains using the microdilution method, and were further studied for their potential synergistic activity combined with cefuroxime. In our antimicrobial assays, the isolated compounds showed antimicrobial activity against Gram-positive and -negative strains, but were no enhanced activities between the fungal samples and the antibacterial drug (see **Table 2**). The results of the antimicrobial experiments revealed that the Gram-negative strains of multiresistant *A. baumanii* and ESBL *E. coli* demonstrated the strongest susceptibility against the studied compounds.

In the recent years both *A. baumanii* and ESBL *E. coli* have become resistant in many cases against the generally used antibacterial drugs causing serious nosocomial infections.

Against these bacterial strains 5-hydroxy-2-hexen-4-olide (**17**) proved to be the most active, although osmundalactone (**16**) and spiromentin C (**18**) also demonstrated remarkable effectiveness. The available data on the activity of mushroom extracts and their metabolites against *A. baumannii* are very scarce. In a study published in 2010 the activity of ethyl acetate extract of *Phellinus merrillii* against *A. baumannii* strains were reported to be fairly low with MIC values in the range of 0.71 and 1.42 mg/mL[117]. Schwan et al. screened more than 300 mushroom species native to North America for their potential activity against *A. baumannii*, but only three fungal species demonstrated more or less activity against this pathogen[118]. Further chemical experiments identified the compound responsible for the observed antimicrobial activity, namely 2-aminoquinoline, isolated from *Leucopaxillus albissimus*. The MIC value of this metabolite determined for *A. baumannii* was 128 μg/mL. In comparison the most effective compound in our experiments, 5-hydroxy-2-hexen-4-olide (**2**) was found to have a MIC value of 6 μg/mL against *A. baumannii.*

Terphenylquinone type compounds possess interesting biological properties (e.g. antiproliferative, antibacterial, antioxidant and anti-inflammatory activity). In a study by Benedict et al. atromentin, a precursor molecule of the biosynthesis of other spiromentins, demonstrated antibacterial activity with MIC values in the range of 25 - 100 μg/mL. [119]. The results of another study by Brewer et al. suggest that the antibiotic activity depends on the *para* substituent of the benzoquinone ring. The activity against *Bacillus subtilis* was found to be higher for 4,4-dimethoxyatromentin (MIC value of 5 μg/mL) with respect to atromentin (MIC value of 500 μg/mL) [120]. In a previous study atromentin demonstrated significant smooth muscle stimulating property and caused contraction of isolated rabbit ileum[121]. According to Zhang et al. 5-hydroxy-2-hexen-4-olide (**17**) proved to be effective as a feeding inhibitor for larvae of the yellow butterfly *Eurema hecabe mandarina*[99].

In the ORAC study spiromentin C (**18**) and spiromentin B (**19**), isolated from the chloroform extract of the species have shown the most remarkable antioxidant effects (16.21 ± 0.38 and 11.23 ± 0.58 mmolTE/g, respectively), which were higher than the activity of ascorbic acid used as reference compound (6.97 ± 0.008 mmolTE/g) (see **Table 3**). Although osmundalactone (**16**) and 5-hydroxy-2-hexen-4-olide (**17**) were less active, they are still considered compounds with notable antioxidant property. Similar antioxidant activity has been observed with the terphenyl type compounds rickenyl A, D and E, isolated by Kuhnert et al. from the macrofungus *Hypoxylon rickii*[106].

7. SUMMARY

The aim of our research work was to evaluate the antiproliferative and antimicrobial activities of mushroom species native to Hungary, and to isolate, characterize and pharmacologically investigate the biologically active compounds of *Gymnopus fusipes*, *Tricholoma populinum*, *Scleroderma bovista* and *Tapinella atrotomentosa*.

In our screening study, lipophilic and hydrophilic extracts of mushroom species were examined *in vitro* against different cancer cell lines (A-2780, A-431, HeLa, MCF-7) using the MTT assay. On the basis of our screening results, the chloroform extract of *G. fusipes* demonstrated the strongest antiproliferative activity, followed by the extracts of *S. bovista*. The extract of *T. populinum* exerted moderate antiproliferative properties, however according to our previous screening assay its chloroform fraction exerted remarkable xanthine oxidase enzyme inhibitory activity. In our search for mushroom species for potential antimicrobial activity, the chloroform extract of *T. atrotomentosa* proved to possess broad antibacterial spectrum. It was found to be effective against Gram-positive bacterial strains, and also against standard and ESBL-positive *Escherichia coli*, standard and multiresistant *Pseudomonas aeruginosa* and multiresistant *Acinetobacter baumannii*. The extracts of *T. atrotomentosa* exhibited synergistic activity with cefuroxime against methicillin-resistant *Staphylococcus aureus*. Our screening results served as a good starting point to carry out bioactivity-guided investigation of selected species in order to identify the compounds responsible for their antiproliferative and antimicrobial activities.

The isolation of the compounds from the species was performed by multistep chromatographic procedures, including OCC, RPC, FCC, NP-HPLC and RP-HPLC. The structures of the obtained compounds were characterized by spectroscopic methods (MS and NMR). The structure elucidation of compounds **1** and **2** required the combination of spectral methods, Marfey analysis and molecular modeling studies.

As a result of the extensive preparative work altogether 19 compounds were isolated from the four mushroom species selected. From the chloroform extract of *G. fusipes* two new cyclopeptides were obtained, namely gymnopeptides A (**1**) and B (**2**). To best of our knowledge, they are the largest cyclopeptides of mushroom origin; both peptides are constituted of 18 amino acids, with a surprisingly high contribution of *N*-methylated amino acids (10 out of 18). Our mychochemical investigation performed on *G. fusipes* gained increased scientific interest, which led to the first total synthesis of gymnopeptides A (**1**) and

B (**2**) in 2017 by Pan et al. The successful synthesis of compounds **1** and **2** paves the way for further pharmacological studies and better characterization of these unique cyclic peptides.

In the *n*-hexane fractions of *T. populinum* and *S. bovista* ergostane type steroids were determined: ergosterol (**3**); 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**5**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**8**). From the *n*-hexane phase of *T. populinum* two further triterpene steroids were isolated, namely 3β,5α,6β,9α-tetrahydroxyergosta-7,22-diene (**6**) and methylated derivative of cerevisterol (**7**). In the chloroform extract of *S. bovista* two steroid compounds with lanostane structure were identified: 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**).

In the *n*-hexane extract of the species *S. bovista* one metabolite proved to be a ceramide-type constituent, cerebroside B (**13**). In the chloroform extract of *T. populinum* one pyridine-type compound, nicotinamide (**9**) and 3 adenosine-type constituents: adenosine (**10**) and epimers of 5'-deoxy-5'-methylsulphinyladenosine (**11** and **12**) were detected. From the chloroform extract of *T. atrotomentosa* two compounds were characterized as lactone-type metabolites: osmundalactone (**16**) and 5-hydroxy-2-hexen-4-olide (**17**), while two compounds were confirmed to have rare terphenylquinone structure: spiromentin C (**18**) and spiromentin B (**19**).

Pharmacological analysis revealed that the isolated compounds possess significant biological activity *in vitro*. Gymnopeptides A (**1**) and B (**2**) exerted remarkable cell growth inhibitory actions on the investigated cancer cells (HeLa, A431, MCF-7, MDA-MB-231, T47D). Among the isolated steroids 23-hydroxylanosterol (**14**) and lanosta-8,23-dien-3β,25-diol (**15**) proved to be most active against HeLa, A2780 and MCF-7 human cancer cells. The antimicrobial investigations showed that osmundalactone (**16**), 5-hydroxy-2-hexen-4-olide (**17**) and spiromentin C (**18**) possess notable antimicrobial activity against *A. baumanii* and ESBL *E. coli* strains and against *M. catarrhalis*. The ORAC assay performed revealed that spiromentin C (**18**) and spiromentin B (**19**) possess stronger antioxidant activity, than osmundalactone (**16**) and 5-hydroxy-2-hexen-4-olide (**17**).

Our results unambiguously demonstrate that mushrooms constitute a rich source of biologically active metabolites with a great structural diversity. The investigated species as well as the isolated fungal metabolites can be regarded as promising starting materials in the search for new pharmaceutical discoveries in the future. In consequence the elucidation of their mechanism of action can be a good basis for developing new effective agents against tumor cells or pathogen microorganisms.

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