Triterpenes from *Hypholoma lateritium:* Isolation, structure determination and biological activity

Ph.D. Thesis

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TRITERPENES FROM HYPHOLOMA LATERITIUM: ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITY

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Chemistry & Biodiversity **2020** Jun 19.
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LIST OF ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
A2780	ovarian carcinoma cells
Ac	acetyl
AcN	acetonitrile
AF	atrial fibrillation
APD	action potential duration
CC	column chromatography
CH_2Cl_2	dichloromethane
$CHCI_3$	chloroform
CON	control
COSY	correlated spectroscopy
COX	cyclooxygenase
DNA	deoxyribonucleic acid
ESI–MS	electrospray ionization mass spectrometry
EtOAc	ethyl acetate
EtOH	ethanol
FA	fatty acid
FCC	flash column chromatography
GIRK	G protein-coupled inwardly-rectifying potassium channel
HD	high dose
HEK-293	human embryonic kidney cells
HeLa	human cervix adenocarcinoma
hERG	human ether-à-go-go-related gene
HMBC	heteronuclear multiple-bond correlation
HPLC	high-performance liquid chromatography
HR-MS	high-resolution mass spectrometry
HSQC	heteronuclear single-quantum correlation
IKACh	acetylcholine-activated potassium channel
JMOD	*J*-modulated spin-echo experiment
JNK	Jun N-terminal kinase
LD_{50}	median lethal dose
MCF	mastax contraction frequency assay
MCF-7	human breast adenocarcinoma cells
MDA-MB-231	triple-negative breast cancer cell line
MS	mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NMR	nuclear magnetic resonance
NOESY	nuclear *Overhauser* effect spectroscopy
NP	normal-phase
Nrf2	nuclear factor (erythroid-derived 2)-like 2
P38	mitogen-activated protein kinase signaling pathways
RAW 264.7	murine macrophages cells
ROESY	rotating-frame nuclear *Overhauser* effect correlation spectroscopy
RP	reversed-phase
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor alpha
TOCSY	total correlated spectroscopy
TPA	12-*O*-tetradecanoylphorbol 13-acetate
TSL	toxicity and survival lifespan
UV	ultraviolet
XO	xanthine oxidase
δ	chemical shift

1 INTRODUCTION

Fungi are neither plants nor animals, they are considered to constitute the third large kingdom of organisms. Their characteristic features set them uniquely apart from plants and animals[1]. Mushroom is the fruit body of a fungus, the reproductive part which grows either above or below ground and release spores, the seed-like elements from which new fungi are made[2]. Mushrooms have widely been appreciated by humans for their nutritional and health benefits. Fresh and preserved mushrooms are consumed as a delicacy, particularly for their aroma and texture, but also for their low calorie level and high fiber content[3]. A plenty of studies have reported that mushrooms produce a broad range of high- and low-molecular weight primary and secondary bioactive metabolites: terpenoids[4, 5], phenolics[6, 7], polysaccharides[8-10] which are responsible for their therapeutic effects, such as antidiabetic[11, 12], anti-inflammatory[13-15], antioxidant[16, 17], antibacterial[18, 19], anticancer[20, 21], immunomodulatory activities[22, 23].

Inflammation is a biological response to injury, characterized by loss of function and pain, heat, redness and swelling. It is usually linked with the pathogenesis of diseases such as diabetes, arthritis, obesity, metabolic syndrome, cancer and several cardiovascular diseases[24]. Macrophages play a key role for regulation of inflammatory responses. Activated macrophages release a variety of inflammatory mediators, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), reactive oxygen species (ROS), prostaglandin E_2 (PGE_2) and nitric oxide (NO)[25].

Atrial fibrillation (AF) is the most common arrhythmia affecting patients today. Disease prevalence is increasing at an alarming rate worldwide, and is associated with often catastrophic and costly consequences, including heart failure, syncope, dementia, and stroke[26]. In Europe and North America, 2 to 3% of the population had been diagnosed as AF in 2014. In addition, AF occurs more frequently in males and elder people[27]. Even though the underlying mechanisms of AF is not yet entirely understood, the cardiac acetylcholine-activated potassium ion channel (IKACh) is considered a novel and attractive target for drug therapy in the treatment of AF[28]. This ion channel is member of the G-protein coupled inwardly rectifying potassium channel (GIRK) superfamily and is composed of GIRK1/4 (Kir3.1 and Kir3.4) subunits[29]. It has been described that GIRK1/4 is constitutively active in myocytes from patients with chronic AF or from dog model of atrial tachycardia, and, furthermore, these studies have demonstrated spontaneous openings of these channels in myocytes from chronic AF patients[28, 30, 31]. Taking all these findings together, constitutively active GIRK channels in the atrium elevate proarrhythmic risk by causing dispersion of atrial

repolarization and refractoriness, therefore, a selective GIRK blocker without affecting ventricular repolarization is effective and might be useful for the treatment of patients with AF[32, 33].

In the last decades, mushrooms were brought increased attention due to their secondary metabolites with high structural diversity. This can be exemplified by the rotary door-shaped meroterpenoid lingzhiols from *Ganoderma lucidum*[34], highly rearranged triterpenes, spiroinonotsuoxotriols from *Inonotus obliquus*[35], the macrocyclic diterpenoid eryngiolide A with cyclododecane skeleton produced by *Pleurotus eryngii*[36] and cyclic octadecapeptides from *Gymnopus fusipes*[37] among others. However, the medicinal potential of mushrooms has not been fully investigated. Additional biological studies are needed to confirm the pharmacological properties or side effects of mushroom extracts and their metabolites.

The current thesis summarizes the chemical and pharmacological experiments performed on *Hypholoma lateritium* providing a detailed pharmacological and toxicological evaluation of metabolites of *Hypholoma lateritium* in order to identify new natural products of promising potential in the treatment of inflammation and atrial fibrillation.

2 AIMS OF THE STUDY

In the last years the research group of the Department of Pharmacognosy at the University of Szeged started a screening program to investigate the pharmacological activities of Hungarian fungi and to identify the bioactive compounds of the selected mushrooms. The aim of the present work as part of this project was the identification of biologically active compounds from *Hypholoma lateritium* and characterization of their chemical, pharmacological and toxicological profiles.

In order to achieve the aims, the following tasks were carried out:

- A review of the literature of the *Hypholoma lateritium*, from aspects of the chemistry and pharmacological properties of the fungi.
- Extraction of *Hypholoma lateritium* with various organic solvents (*n*-hexane, chloroform and 50% methanol) and with water for screening.
- Collection and extraction the mushroom material of *Hypholoma lateritium* for preparative work.
- Isolation the bioactive compounds responsible for the observed ion channel and anti-inflammatory effects via bioactivity-directed fractionation, using various chromatographic techniques.

- Elucidation the structures of the isolated compounds by NMR and MS methods (in collaboration with Gedeon Richter Plc., Hungary), providing characteristic spectral data on the isolated new metabolites.
- *In vivo* toxicological evaluation of compounds from *Hypholoma lateritium* using bdelloid rotifer assays.
- Investigation of the activity of compounds from *Hypholoma lateritium* on GIRK and hERG channels.
- Evaluation of anti-inflammatory potential of compounds from *Hypholoma lateritium*.

3 LITERATURE REVIEW

3.1 Morphology of *Hypholoma lateritium*

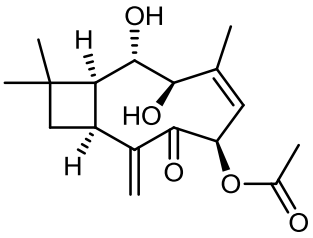
The genus *Hypholoma,* which means "mushrooms with threads", belongs to the family *Strophariaceae*, and includes mushroom species characterized by the presence of well-pigmented pileus and variably developed thread-like veil, which does not form a membranous annulus on the stipe[38]. The genus comprises about 30 species worldwide, distributed from temperate to tropical regions, growing on decomposing wood, living trees, or soil[39]. *Hypholoma* species are recognized as active wood and litter decomposers, and play a significant role in forest ecosystems, being used not only in bioconversion of cellulose, fabric and dye industrial residues[40, 41], but also in biological control of phytopathogenic fungi[42, 43]. *Hypholoma lateritium* ((Huds.:Fr.) P.Kumm. (syn. *Hypholoma sublateritium* (Fr.) Quél. and *Naematoloma sublateritium* (Fr.) P. Karst.)) – commonly known as brick cap or brick top – is member of Hypholoma genus belonging to the Strophariaceae family. *Hypholoma fasciculare* is the most well-known and studied member of the genus, which is identified for its antioxidant and antimicrobial activities[44], producing different types of fungal metabolites, e.g., styrylpyrone-type compounds (hypholomins, fasciculins)[45], steroids (fasciculic acids, fasciculols)[46, 47], and sesquiterpenoids (fasciculatones)[48, 49].

It was described by a German mycologist Jacob Christian Schaeffer in 1762 and he gave the species the scientific name *Agaricus lateritius*. In 1871 *Agaricus lateritius* was transferred from the genus *Agaricus* to the genus *Hypholoma* by German scientist Paul Kummer[50]. This mushroom is distributed throughout most of mainland Europe and also recorded in North America and in some Asian countries. The species is a saprophytic fungus, usually clustered on the dead stumps and logs of broadleaf trees. It occurs mainly from the late of July to the end of November. Its cap is 2-10 cm wide, dark brick red color, lighter at the margin. The gills are first white colored, later purple brown; the stipe is 5-12 cm long, whitish above, reddish brown below[51]. There is some controversy in terms of edibility of this species. The most field guides and other scientific sources regarding to this fungi state that it is edible[52] in Japan, Korea and North America while this species has been recorded as inedible or even poisonous[53] in Europe.

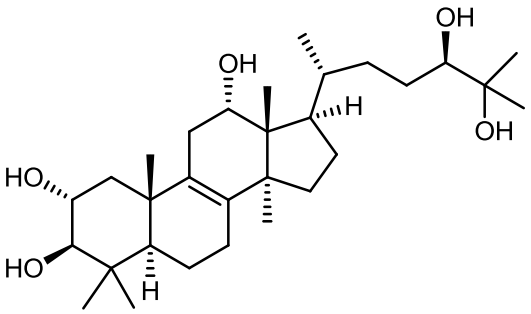
3.2 Chemistry and pharmacology of *Hypholoma lateritium*

In 1984, four fasciculol-type triterpenes such as fasciculol B-D and fasciculol F along with ergosterol, ergosterol peroxide and cerevisterol were reported from *Naematoloma sublateritium* (synonym name of *H. lateritium*) by Bernardi et al.[54]. Based on a study of Backens et al. naematolin, a sesquiterpenoid with caryophyllane skeleton, was isolated from culture of this species[55]. Aqueveque et al. reported that 3,5-dichloro-4-metoxy-benzyl alcohol and marasmal showed antimicrobial activity which have been isolated from mycelial cultures of *Hypholoma sublateritium*[56]. The extensive mycochemical analysis of the fruiting bodies by Yaoita et al. resulted in the identification of three new lanostane type triterpenoids, sublateriols A-C and three known ones, fasciculol A-C. Three of them namely sublateriol C, fasciculols B and C revealed significant cytotoxicity against human cancer cell lines[57] (**Figure 1**). Li et al. reported that *Hypholoma lateritium* mycelia contains phenolic acids namely 4-hydroxybenzoic acid, α-resorcylic acid, 4-coumaric acid, salicylic acid and gentisic acid showing high antioxidant activity[58].

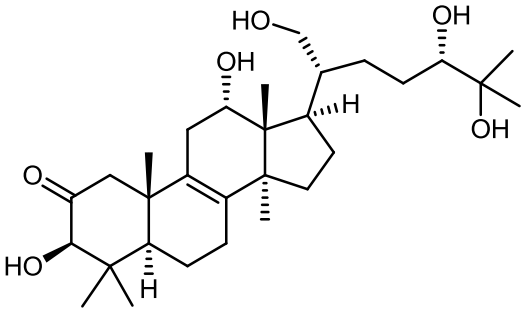
Only a limited number of pharmacological studies have been reported on *Hypholoma lateritium*. Most of them conducted with extracts of different polarities prepared from fungal sporocarps. In 1996, Yasukawa et al. found that the methanol extract of *Hypholoma lateritium* markedly inhibited the inflammatory activity induced by TPA in mice[59]. Choi et al. investigated the ethyl acetate fraction of *H. lateritium*, which showed significant free radical scavenging activity and inhibition of lipid peroxidation while *n*-hexane and dichloromethane fractions of *H. lateritium* inhibited cell proliferation and viability[60]. Lee at al. demonstrated that the extract of this species suppresses TNF-α-induced inflammatory response in human umbilical vein endothelial cells. The *n*-butanol fraction of *H. lateritium* inhibited TNF-α-induced monocyte adhesion to endothelial cells; moreover it dose-dependently decreased the expression of inducible nitrogen oxygen synthase and cyclooxygenase-2[61]. In another paper Lee et al. investigated the inhibitory effect of *H. lateritium* extract on highly invasive and metastatic tumor cells. The *n*-hexane fraction of brick cap significantly inhibited the invasion and migration of MDA-MB-231 breast cancer cells in the Matrigel invasion assay and wound-healing analysis, respectively. The results obtained suggested that *n*-hexane extract of *H. lateritium* inhibits the metastatic potential of MDA-MB-231 cells by inhibiting the phosphorylation of JNK/p38 and reducing AP-1 and NF-κB DNA-binding activities[62]. According to the investigation of Ványolós et al., the aqueous-methanol fraction of *H. lateritium* demonstrated moderate XO inhibitor activity[63].



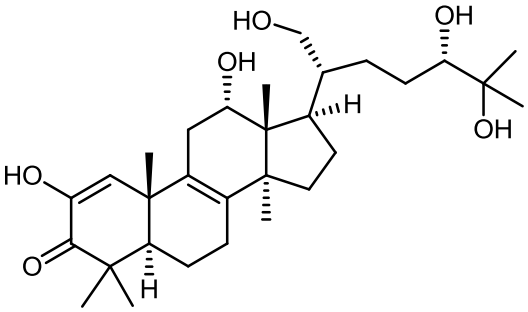
naematolin



fasciculol B



sublateriol C



fasciculol C

Figure 1. Representative of compounds from *H. lateritium*

4 MATERIALS AND METHODS

4.1 Mushroom material

Fruiting bodies of *H. lateritium* were collected in September 2015 from the environs of Bakonybél, Hungary, and identified by Attila Sándor (Hungarian Mycological Society). A voucher specimen (collection number H018) has been deposited in the Department of Pharmacognosy, University of Szeged, Szeged, Hungary. The mushroom material was stored at -20 °C until processing.

4.2 Extraction

4.2.1 Extraction of the mushroom materials for preparative work

The mushroom material (6.5 kg) was freeze dried (Hetosicc, Heto Lab Equipment, Denmark), and then the dry sample (630 g) was crushed in a blender (Retsch-GM200) and percolated with methanol (7 L) for 72 hours at room temperature. The obtained extract was evaporated at 40-45°C under reduced pressure. After concentration, the methanol extract (dry material 92 g) was dissolved in 50% aqueous MeOH and subjected to solvent-solvent partition using *n*-hexane (5×500 mL), chloroform (5×500 mL), and then ethyl acetate (5×500 mL). Each fraction was concentrated by using a Rotavapor R-210 (40 °C, 337 mbar).

4.2.2 Preparation of extracts for pharmacological screening

The fruiting bodies of *H. lateritium* were freeze-dried and ground with a grinder. The lyophilized sample (10 g) was extracted with 3×100 mL methanol by ultrasonication for 3×15 min at room temperature. After filtration, the solutions were combined and evaporated under reduced pressure. The residue was dissolved in 50 mL of 50% aqueous MeOH and was subjected to solvent–solvent partition between *n*-hexane (4×25 mL) (extract A) and $CHCl_3$ (4×25 mL) (extract B) and the residue provided extract C. After extraction with MeOH, the residual mushroom material was dried and extracted with 50 mL of boiling water for 15 min. The filtered extract was lyophilized, giving extract D.

4.3 Purification and isolation of the compounds

4.3.1 Flash column chromatography (FCC)

Flash column chromatography was carried out on a CombiFlash®Rf+Lumen instrument with integrated UV, UV-VIS (detection at 254, 366 nm and all wavelengths) and using 12 g (FCC-4, FCC-5, FCC-7, FCC-8), 24 g (FCC-2, FCC-3, FCC-9, FCC-11) and 80 g (FCC-1, FCC-6, FCC-10) RediSep Rf Gold Normal Phase Silica Flash columns and 4.3 g (RP-FCC-1, RP-FCC-2, RP-FCC-3, RP-FCC-4) Reversed phase C18 columns (Teledyne Isco, Lincoln, USA) at a flow rate 18 mL/min, 30 mL/min, 35 mL and 60 mL/min, respectively. Mobile phases and separation times:

FCC-1: increasing polarity of *n*-hexane-acetone (0% to 100% acetone), *t* = 60 min

FCC-2: increasing polarity of *n*-hexane-acetone (0% to 30% acetone), *t* = 45 min

FCC-3: increasing polarity of *n*-hexane-acetone (0% to 40% acetone), *t* = 50 min

FCC-4: increasing polarity of *n*-hexane-acetone (0% to 40% acetone), *t* = 50 min

FCC-5: increasing polarity of *n*-hexane-acetone (10% to 50% acetone), *t* = 55 min

FCC-6: increasing polarity of *n*-hexane-acetone (0% to 100% acetone), *t* = 60 min

FCC-7: increasing polarity of *n*-hexane-acetone (0% to 40% acetone), *t* = 50 min

FCC-8: increasing polarity of *n*-hexane-acetone (0% to 40% acetone), *t* = 45 min

FCC-9: increasing polarity of *n*-hexane-acetone (0% to 40% acetone), *t* = 50 min

FCC-10: increasing polarity of chloroform-methanol (0% to 100% methanol), *t* = 60 min

FCC-11: increasing polarity of chloroform-methanol (0% to 30% methanol), *t* = 45 min

RPFCC-1: increasing polarity of water-acetonitrile (30% to 60% acetonitrile), *t* = 50 min

RPFCC-2: increasing polarity of water-acetonitrile (30% to 60% acetonitrile), *t* = 50 min

RPFCC-3: increasing polarity of water-acetonitrile (35% to 60% acetonitrile), *t* = 50 min

RPFCC-4: increasing polarity of water-acetonitrile (30% to 70% acetonitrile), *t* = 50 min

4.3.2 Thin layer chromatography

Thin layer chromatography (TLC) was used for monitoring the flash chromatographic separations. TLC examination was carried out on silica gel (20x20 cm Silica gel 60 F_{254}, Merck 105554).

4.4 Structure determination of the isolated compounds

Optical rotation was measured on a Perkin-Elmer 341 polarimeter. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz) and 2D NMR spectra were recorded in all cases, using the pulse sequences available in the VNMRJ 3.2 (Agilent Technologies, Santa Clara, CA, USA) or in TopSpin 3.5 sequence libraries (Bruker). Data analysis and interpretation were performed within

ACD/Labs 2017.1.3 NMR Workbook Suite. NMR data were acquired on a Varian 800, Bruker Avance II HD 500 (Bruker, Billerica, MA, USA) or a Bruker AVANCE II HD 400 MHz spectrometer equipped with a ^{13}C enhanced salt tolerant cryoprobe, a TCI cold probe, or liquid nitrogen cooled Prodigy probe. methanol-d_4 was used as solvent in all cases. Chemical shifts are reported in the δ scale relative to the residual solvent signals (3.31 and 49.15 ppm for ^{1}H and ^{13}C, respectively).

HRMS analyses were performed on a LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) system with ESI ion source using positive or negative of polarity. The samples were dissolved in methanol. HR-MS-MS were acquired using the CID fragmentation method applied to the quasimolecular ion peaks (protonated/deprotonated molecular ion peaks or the sodium adduct ions or the molecular ion peaks with water losses). For data acquisition and analysis Xcalibur software version 2.0 (Thermo Fisher Scientific) was used.

4.5 Pharmacological tests

Pharmacological studies were conducted in collaboration with the Department of Ophthalmology and Department of Pharmacology and Pharmacotherapy (Faculty of Medicine, University of Szeged, Szeged, Hungary), Department of Psychiatry (Faculty of Medicine, University of Szeged, Szeged, Hungary) and Department of Pharmaceutical Botany (Faculty of Pharmacy, Jagiellonian University, Medical College, Kraków, Poland).

4.5.1 Bdelloid rotifer assays

The measurement of test compounds was performed on bdelloid rotifer to gain information about their toxicity and biological activity. Two viability markers of *Philodina acuticornis* have been chosen for the evaluation of investigated compounds. The rotifer culturing, harvesting, and monitoring methods were used based on previous publication by Olah et al[64].

Toxicity and survival lifespan (TSL) assay: The impact of the test compounds on the lifespan of unfed PA rotifers was evaluated. The morphological viability markers are the active motility of the body, normal movement of internal organs, and red eyes.

Mastax contraction frequency (MCF) assay: The mastax (pharynx) is the chewing organ used in feeding which opens and closes periodically. To evaluate and standardize the viability of one-housed rotifers in our experiments, MCF (mastax contraction frequency, contraction/sec) was used as a quantitative viability marker.

4.5.2 GIRK channel inhibitory assay

Investigations of the prepared extracts and natural products were performed on HEK293 (human embryonic kidney) cells stably expressing the GIRK1/4 (Kir3.1/3.4) K^+ channels. The GIRK channel inhibitory assay was carried out by the automated planar patch clamp technology based on the manual patch-clamp method[65]. The cell line originated from UCL Business PLC. Propafenone (Sigma-Aldrich Corporation, St. Louis, USA) was used as a positive control. Cells were maintained in DMEM (Thermo Fisher Scientific Inc., Waltham, USA) medium supplemented with 10% FBS (PAN-Biotech GmbH, Aidenbach, Germany) and 182 μg/mL zeocin (Thermo Fisher Scientific Inc., Waltham, USA).

The following solutions were used during patch-clamp recordings (compositions in mM): external solution: NaCl 140, KCl 4, glucose monohydrate 5, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); high K^+ external solution: NaCl 135, KCl 25, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); K^+-free external solution: NaCl 160, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); internal solution: K-gluconate 40, NaCl 20, KF 60, EGTA 20 and HEPES 10 (pH 7.2, KOH), supplemented with 0.9 mM GTPγS before the experiments to induce channel activation.

The voltage protocol for GIRK ion channel assay started with a depolarizing voltage step to 60 mV for 100 ms before a 500 ms long hyperpolarizing ramp to −140 mV was applied. Then the membrane potential remained at −140 mV for 100 ms before returning to the holding potential of −40 mV. The inward currents were calculated from the −140 mV segment. The pulse frequency was approximately 0.1 Hz. At the beginning of recordings, the normal external solution (4 mM K^+) was replaced to high K^+ (25 mM K^+) external solution in order to increase the current amplitude. After 2–3 min of control period, the test compounds were added to the cells in increasing concentrations, each for approximately 3 min. For further details of GIRK assay please see ref[66].

4.5.3 hERG channel inhibitory assay

hERG measurements were carried out on HEK-293 cells stably transfected with cDNA encoding the hERG (Kv11.1) K^+ channel using planar patch-clamp technology adjusted by Polonchuk[67]. The cell line was purchased from the Cell Culture Service (Hamburg, Germany). 10 μM amitriptyline was used as a reference inhibitor. Cells were maintained in IMDM (PAN-Biotech GmbH, Aidenbach, Germany) medium supplemented with 10% FBS (PAN-Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine (PAN-Biotech GmbH, Aidenbach, Germany), 1 mM Na-pyruvate (PAN-Biotech GmbH, Aidenbach, Germany) and 500 μg/mL G418 (Thermo Fisher Scientific Inc., Waltham, USA).

The following solutions were used during patch-clamp experiments (compositions in mM): external solution: NaCl 140, KCl 4, glucose monohydrate 5, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); internal solution: KCl 50, NaCl 10, KF 60, EGTA 20 and HEPES 10 (pH 7.2, KOH).

The voltage protocol for hERG ion channel started with a short (100 ms) –40 mV step to establish the baseline region. A depolarizing step was applied to the test potential of 20 mV for 3 s, and then the cell was repolarized to −40 mV (1 s) to evoke outward tail current. Holding potential was −80 mV. The pulse frequency was approximately 0.1 Hz. The peak tail current was corrected the leak current defined during the first period to −40 mV.

Recording started in external solution. After this control period, the test compound was applied for approximately 3 min. For further details of hERG assay please see the previous study[66].

4.5.4 Anti-inflammatory assay

4.5.4.1 Cell cultures

Murine macrophages RAW 264.7 (TIB-71, ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% FBS and 1% antibiotic solution (100 IU/mL penicillin, 0.1 µg/mL streptomycin). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in the air and were finally seeded into a 6-well plate (Sarstedt AG&Co., Nümbrecht, Germany) at a density of 5×10^5 cells/well in 2 mL of medium. At every step of the procedure, cell morphology was investigated by an inverted light microscope (Olympus, Tokyo, Japan). Cell viability during culturing was assessed with a Trypan Blue (Thermo Fisher Scientific, Waltham, MA, USA) exclusion test. RAW 264.7 cells were activated with LPS (10 ng/mL; Sigma-Aldrich, Saint Louis, MO, USA) and incubated overnight. After that macrophages were treated with mushroom extracts of *Hypholoma lateritium* at concentrations of 50 and 100 µg/µL for 24 h or with the isolated compounds (1 and 10 µL) for 24 h. After incubation, media and the cells after scrapping were collected.

4.5.4.2 Cell proliferation XTT assay

Cell proliferation was evaluated using a sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2*H*-tetrazolium) inner salt (XTT) with *N*-methyl-dibenzopyrazine methyl sulfate) functioning as the intermediate electron carrier (PMS). RAW 264.7 cells were seeded in 96-well plates (2.5×10^3 cells/well) and incubated for 24 h. The medium was then

removed and 0.5; 1; 2.5; 5; 10; 50 and 100 µg/µL of mushroom extracts as well as isolated compounds were added to FCS-free medium and incubated for the next 24 h. Then XTT solution (50 µL) was added to each well and incubated for 4 h at 37 °C according to the manufacturer instruction (Sigma-Aldrich). The absorbance was measured at 475 nm and 630 nm in Omega plate reader (BMG LABTECH, San Diego, CA, USA). The specific absorbance of the sample was expressed as follows: Specific Absorbance = A_{475nm} (sample)-A_{475nm} (blank)-A_{660nm} (sample). Cell viability was expressed as the percentage of control.

4.5.4.3 Western blot for quantity of COX-2, cPGES and Nrf2 receptor

M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA) with protease inhibitor cocktail set III (Merck, Darmstadt, Germany) was used for cell lysates preparation. Total protein concentrations were determined using the Bradford reaction. 40 µg of proteins per sample were solubilized in a Laemmli buffer with 2% mercaptoethanol (BioRad, Hercules, CA, USA) and subjected to 10% SDS-polyacrylamide gel electrophoresis. Primary antibodies were used: anti-cyclooxygenase-2 (COX-2), anti-β-actin diluted 1:1000 (Thermo Fisher Scientific), anti-prostaglandin E2 synthase (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:1000, anti-Nrf2 receptor (GeneTex, Irvine, CA, USA) diluted 1:200 and secondary antibody anti rabbit IgG (HRP) (Thermo Fisher Scientific, 1:2000). Proteins were detected using the Western blotting detection kit Clarity Western ECL Luminol Substrate (Bio-Rad, USA). The integrated optical densities of the bands were quantified using Chemi Doc Camera with Image Lab software (BioRad). All the results are expressed as means ± standard deviation (SD). The statistical analysis was performed using the one-way ANOVA; $p < 0.05$ was considered significant.

5 RESULTS

5.1 Isolation of the compounds of *Hypholoma lateritium*

The freeze-dried and ground mushroom material (630 g) was extracted with methanol (7 L). After concentration under vacuum, the extract was subjected to liquid-liquid partitioning to yield *n*-hexane, chloroform and ethyl acetate soluble phases (see section **4.2.1.**) (**Figure 2**).

The concentrated *n*-hexane phase (25 g) was fractionated in first step by flash column chromatography (**FCC-1**) on silica gel using an increasing polarity of the mixture *n*-hexane-acetone. The fractions collected were combined based on TLC monitoring to obtain seven major fractions (H I-VII). Four of the united fractions were further fractionated by flash column chromatography (see section **4.3.1.**). The first combined fraction H I+II (2.4 g) was purified using increasing polarity of *n*-hexane-acetone mixtures (**FCC-2**) and led to the isolation of compound **3** (0.92 g) and compound **4** (0.63 g). The combined fraction H III was fractionated in first step by flash column chromatography (**FCC-3**) applying an *n*-hexane-acetone solvent system, then was further purified on reversed phase (**RPFCC-1**) using water-acetonitrile solvent system affording compound **6** (12.4 mg). Investigation of the next fraction H IV (0.49 g) eluted with *n*-hexane-acetone 40:60 (**FCC-4**) yielded compound **5** (3.6 mg). Finally the combined fraction H VII (0.83 g) was purified by flash chromatography (**FCC-5**) with *n*-hexane-acetone solvent system resulting in compound **7** (3.1 mg).

The chloroform-soluble phase was evaporated, and the residue (18 g) was roughly separated by flash column chromatography (**FCC-6**) on a silica gel column using a gradient system of *n*-hexane-acetone. The obtained fractions were combined based on TLC checking, resulting in eleven fractions (C I-XI). The first united fraction C II (0.34 g) was separated by flash chromatography (**FCC-7**) with increasing polarity of *n*-hexane-acetone mixtures. The resulted subfraction C IIa was further purified by reversed phase flash-chromatography (**RPFCC-2**) to obtain compound **1** (3.3 mg) and compound **2** (3.3 mg). The fraction C VII+VIII (1.05 g) was separated by flash chromatography (**FCC-8**) using a mobile phase of *n*-hexane-acetone and further purified by reversed phase flash chromatography (**RPFCC-3**) using a gradient system of water-acetonitrile as mobile phase to result in compound **8** (0.32 g) and compound **9** (0.10 g). The fraction C IX-XI (3.89 g) was purified by flash chromatography (**FCC-9**). The subtraction C IX-XIa obtained here was further subjected to

reversed phase flash chromatography (**RPFCC-4**) and yielded compound **10** (0.42 g) and compound **11** (0.31 g).

Evaporation of the ethyl acetate phase resulted in a dark brown residue (12 g), which was fractionated by flash chromatography (**FCC-10**) on a silica gel column using a gradient system of chloroform-methanol. The fractions with similar compositions were combined into six subfractions (E I-VI) according to TLC monitoring. The combined subfraction E V-VI was subjected to repeated flash column chromatography (**FCC-11**), which resulted in compound **12** (28 mg).

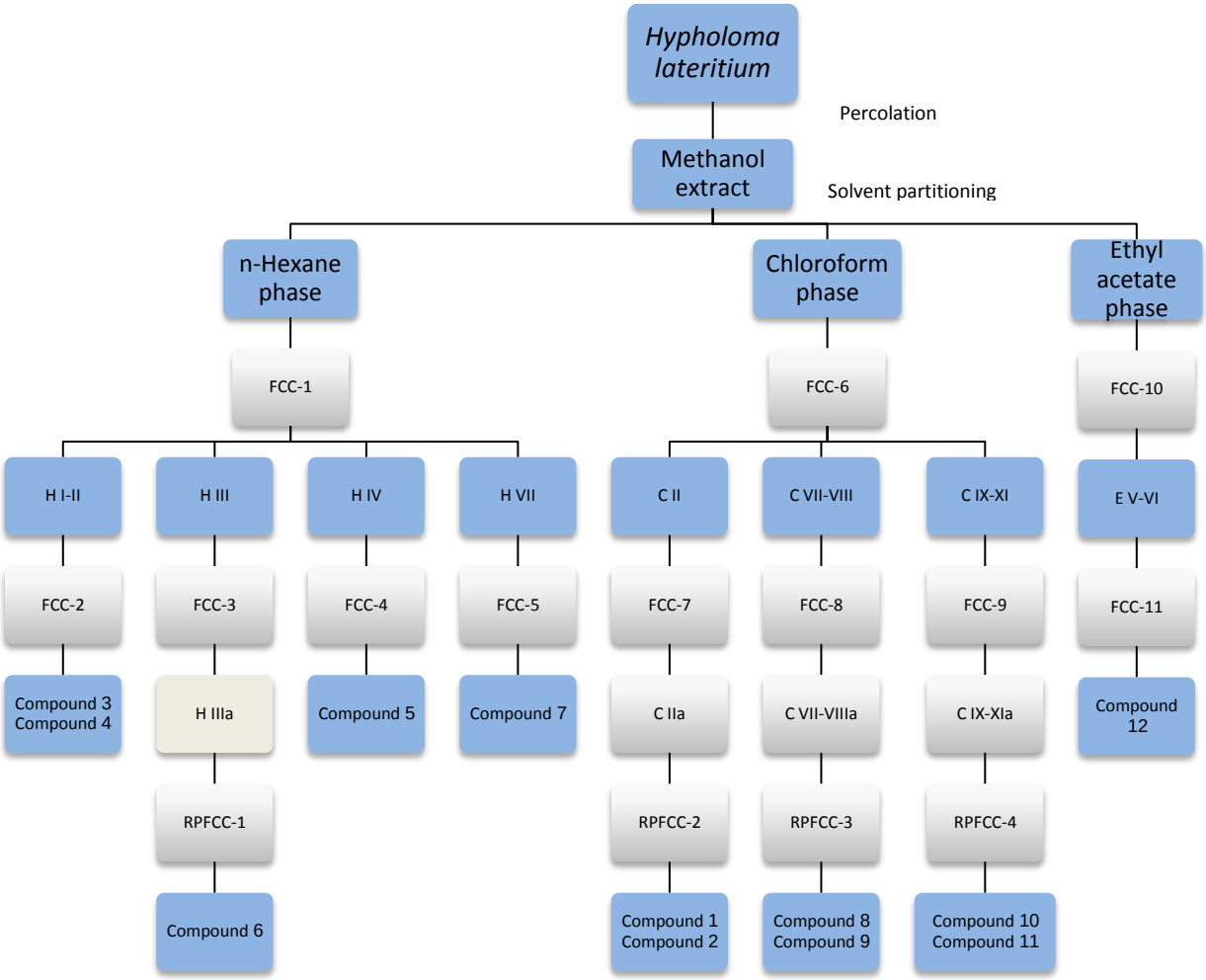


Figure 2. Isolation of compounds from *Hypholoma lateritium*

5.2 Characterization and structure determination of the isolated compounds

The structure elucidation of the isolated compounds was performed by means of NMR and MS measurements. 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.

The investigation of the methanol extract obtained from the freeze-dried fruiting bodies of *Hypholoma lateritium* led to the isolation of 12 compounds, 8 of them for the first time from this species (**Figure 3**).

In the *n*-hexane fraction of wood-decay fungus 5 secondary metabolites (**3**-**7**) were detected. One of them (compound **5)** is a highly degraded sterol, namely demethylincisterol A_2. The other constituents proved to be ergostane triterpenes: ergosterol [compound **3**], 3β-hydroxy-ergosta-7,22-diene [compound **4**], cerevisterol [compound **6**], and 3β-*O*-glucopyranosyl-5,8-epidioxy-ergosta-6,22-diene [compound **7**].

From the chloroform phase of methanol extract of *H. lateritium* 6 compounds were identified. Among the compounds there are two new natural products: lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol [compound **1**] which belongs to lanostane type derivatives, and 8-hydroxy-13-oxo-9*E*,11*E*-octadecadienoic acid [compound **2**], while compounds **8**-**11** are fasciculol type triterpenes. Fasciculol E (**9**) was first identified in the sporocarps of *Hypholoma fasciculare*[68] , while fasciculol F (**8**), fasciculol C (**10**), and fasciculic acid B (**11**) had already been isolated from this species[54].

In the ethyl acetate extract of the species, uridine [compound **12**] was detected. Ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**6**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) were identified by comparing their chromatographic and spectroscopic data with those of authentic samples. Compounds **5** and **8**–**12** were structurally characterized based on NMR and MS spectroscopic data and confirmed by comparing them to those reported in earlier publications[46, 54, 68-73].

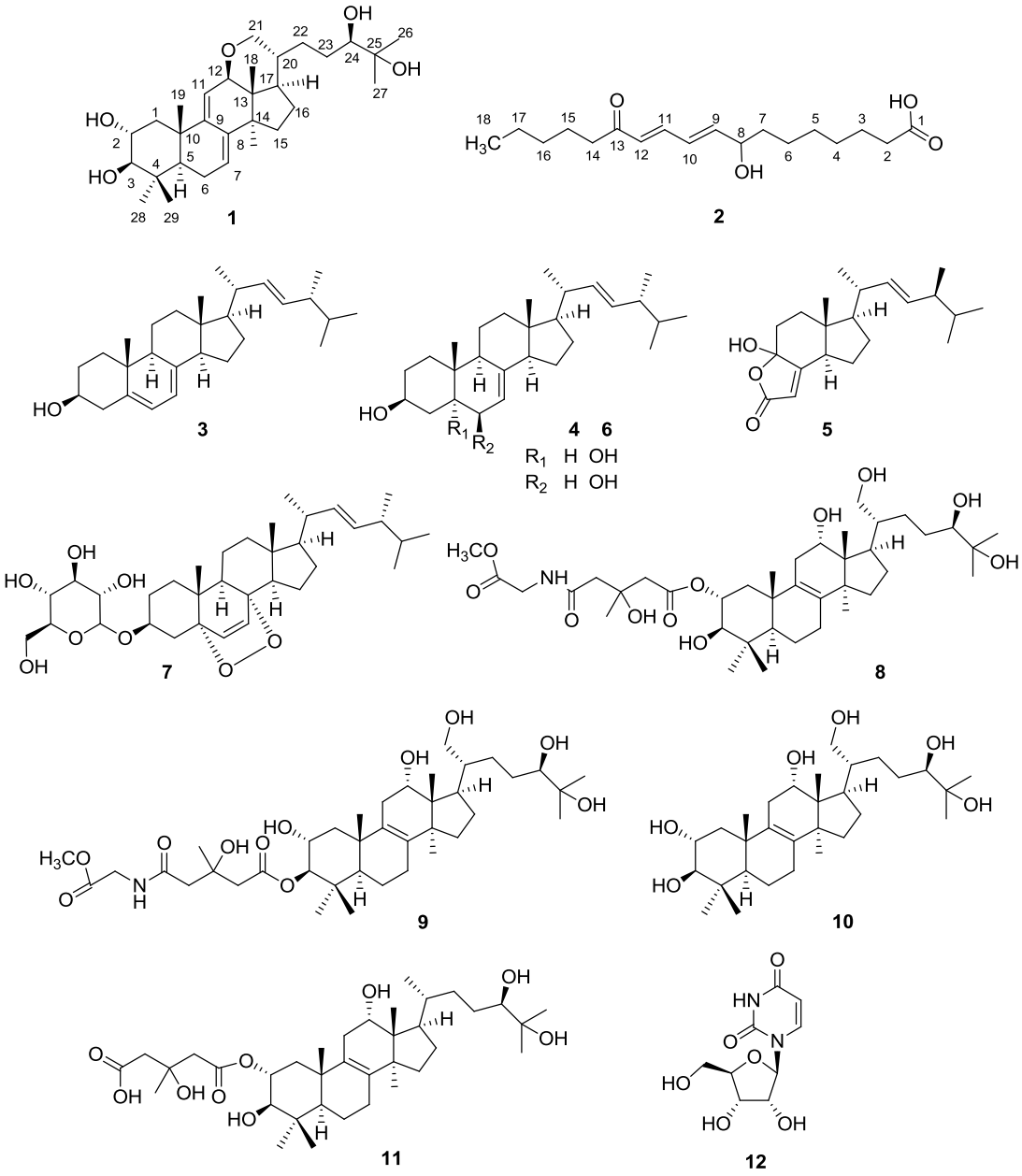


Figure 3. Structures of compounds from *Hypholoma lateritium*

Structure elucidation of the novel natural products from Hypholoma lateritium

Based on the HRESIMS and ^{13}C-NMR data, its molecular formula was determined to be $C_{30}H_{48}O_5$. Consecutive analysis of the ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR spectra suggested the presence of seven tertiary methyl group, two olefinic, four oxygenated, and three aliphatic methines, an oxygenated and six aliphatic methylenes, together with five quaternary carbons. The determined data were quite similar to those reported for sublateriol C[72], except for the ^{1}H and ^{13}C resonances assigned to ring D, and C-12, C-18, C-20, C-21, and C-28 (**Table 1**). The observed HMBC correlations between H-12 and C-21 and between H-21 and C-12 (**Figure 4**) suggested that instead of two hydroxyl groups present in

sublateriol C, an epoxy group between C12 and C21 is present in **1**. This ring closure is in accordance with the H_2O difference obtained between the elementary compositions of sublateriol C and **1**, and explains the chemical shift differences obtained for the protons and carbons close to C-12 and C-20 centers.

Based on the key NOE correlations (**Figure 5**) observed between H-2/H-19, H-3/H-5/H-30, H-12/H-28/H-17, and H-18/H-20, the above presented stereochemistry is suggested. The configuration of C-24 center, however, could not be assigned on this basis, and the OH group is only tentatively given as beta-positioned. This suggestion is firstly based on the assumption that the ring closure does not drastically change the conformation of the side chain. In this case, the observed doublet nature of H-24 with coupling constants of 9.8 and 1.6 Hz, which are closely similar to those reported for sublateriol A[72] or other isolated fasciculic acid and fasciculol derivatives, having the same side chain[46, 68], suggest a similar configuration of the C-24 center. Secondly, making the assumption that similar metabolic pathways lead to sublateriol C and **1** in the same mushroom species, a similar β orientation of 24-OH is suggested. Putting all these together, the structure of compound **1** is suggested as lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol.

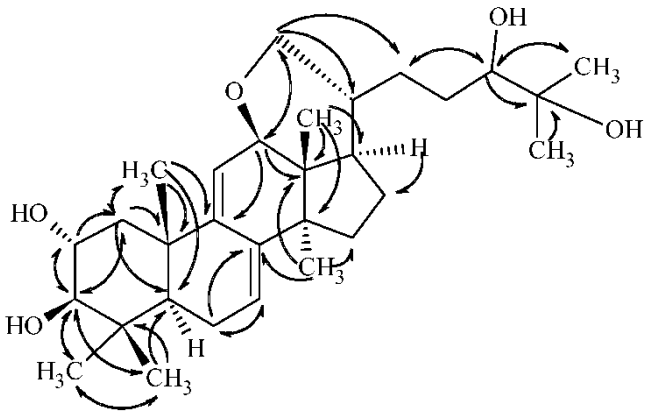


Figure 4. Key HMBC correlations of compound **1**.

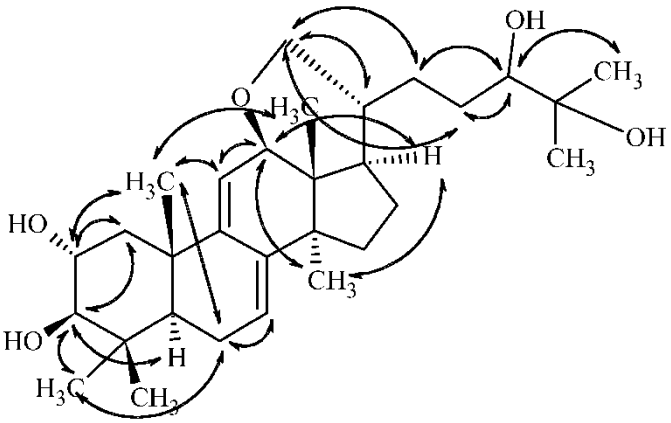


Figure 5. Key NOE correlations determined from the ROESY spectrum of compound **1**.

The molecular formula of compound **2** was established as $C_{18}H_{29}O_4$ from HR-ESIMS measurement giving a pseudomolecular ion peak at *m/z* 309.20673 ($[M-H]^-$) in the negative ion mode. In accordance with this elementary composition, the ^{13}C-NMR spectrum presented eighteen carbon resonances. Based on the ^{1}H and edited HSQC spectra one methyl, ten methylenes, five methines, and two quaternary carbons were present in the isolated compound. The ^{13}C chemical shifts of the quaternary carbons (182.9 and 204.0 ppm) suggested the presence of a carboxylic acid and a ketone functionality in **2**. The multiplicities and coupling constants of the ^{1}H resonances belonging to the methine protons suggested the presence of two conjugated double bonds, both in *E* configuration, connected to a hydroxylated methine and to a keto group. Putting this information together led to the conclusion that the isolated compound is a hydroxyl-oxo-octadecadienoic acid derivative. The positions of the functional groups in the fatty acid chain were unambiguously evidenced on the basis of the ^{1}H-^{1}H COSY and HMBC correlations presented in **Figure 6.** Thus, the HMBC correlations observed between H-14 and C-16, C-12, C-13, and C-15, and between H-18 and C-16 and C-17, suggested that the keto group is at position 13, while the OH is connected to C-8. The HMBC correlation of H-11 and H-12 to C-14, and those of H-9 and H-10 to C-8 confirmed these suggestions. Based on these data, the 8-hydroxy-13-oxo-9*E*,11*E*-octadecadienoic acid structure is suggested for **2**. The absolute stereochemistry of C-8 center was not determined.

Table 1. The ^{1}H and ^{13}C-NMR assignments of compound **1** (δ ppm, J = Hz).

Atom	C Shift	H Shift	H Multiplicity
1	44.6	1.27	m
		2.14	dd (12.7, 4.4)
2	68.0	3.58	m
3	82.4	2.82	d (9.7)
4	38.9		
5	48.8	1.11	m
6	23.0	2.05	m
7	123.8	5.57	d (5.2)
8	142.2		
9	149.2		
10	38.8		
11	117.2	5.30	br s
12	79.7	3.70	br s
13	45.3		
14	47.8		
15	33.0	1.74	m
		1.46	m
16	25.6	1.88	m
		1.49	m
17	46.2	1.44	m
18	13.9	0.50	s
19	22.3	0.97	s
20	38.2	1.75	m
21	73.8	3.91	dd (11.5, 4.3)
		2.95	t (11.1)
22	29.3	1.17	m
		1.30	m
23	28.6	1.54	m
		1.15	m
24	78.4	3.09	dd (9.8, 1.6)
25	72.3		
26	23.4	1.02	s
27	24.4	1.05	s
28	24.1	0.80	s
29	16.1	0.80	s
30	27.8	0.91	s

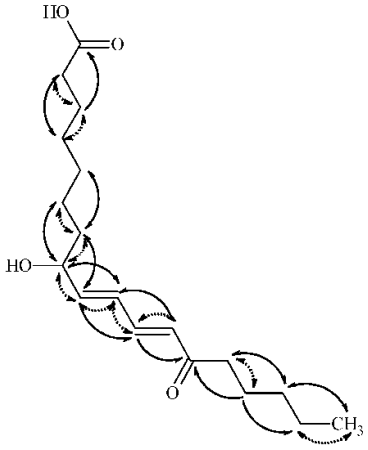


Figure 6. Key HMBC (solid line) and COSY (dotted line) correlations in compound **2**.

Table 2. The ^{1}H and ^{13}C-NMR assignments of compound **2** (δ ppm, J = Hz).

Atom	C Shift	H Shift	H Multiplicity
1	182.9		
2	39.1	2.16	t (7.6)
3	27.7	1.61	m
4	30.9	1.34	m
5	30.6	1.34	m
6	26.6	1.35	m
7	38.2	1.53	m
8	72.7	4.18	q (6.3)
9	148.7	6.24	dd (15.2, 5.8)
10	128.9	6.42	dd (15.2, 10.8)
11	144.6	7.27	dd (15.6, 10.8)
12	130.5	6.18	d (15.6)
13	204.0		
14	41.2	2.61	t (7.4)
15	25.4	1.60	m
16	32.7	1.31	m
17	23.7	1.34	m

5.3 Pharmacological investigation of the isolated compounds

5.3.1 Activity of compounds from *Hypholoma lateritium* in bdelloid viability assays

Compounds **1** and **3**–**11** were examined in *Philodina acuticornis* (PA) viability assays. Administration of cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) resulted in considerable decrease in the number of survivors in the TSL assay. The mastax contraction frequency (MCF) was significantly reduced by 3β-hydroxyergosta-7,22-diene (**4**), demethylincisterol A_2 (**5**), cerevisterol (**6**), fasciculol E (**9**), and fasciculol F (**8**). Cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) were significantly toxic, however the latter in the presence of nutrients proved to be less toxic, and the MCF values

significantly increased in the survivors. The complete opposite was observed with fasciculol C (**10**), which demonstrated toxic effect with feeding, but increased the MCF values without nutrients. Fasciculol E (**9**) produced an overall significant decrease in the viability values, with strong dependence on the presence or lack of nutrients. Among the examined compounds, cerevisterol (**6**) proved to be the most toxic, while lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) had no harmful effect at all. Fasciculic acid B (**11**) exhibited unique effects, since it was used by rotifers simply as a food source (**Figure 7**).

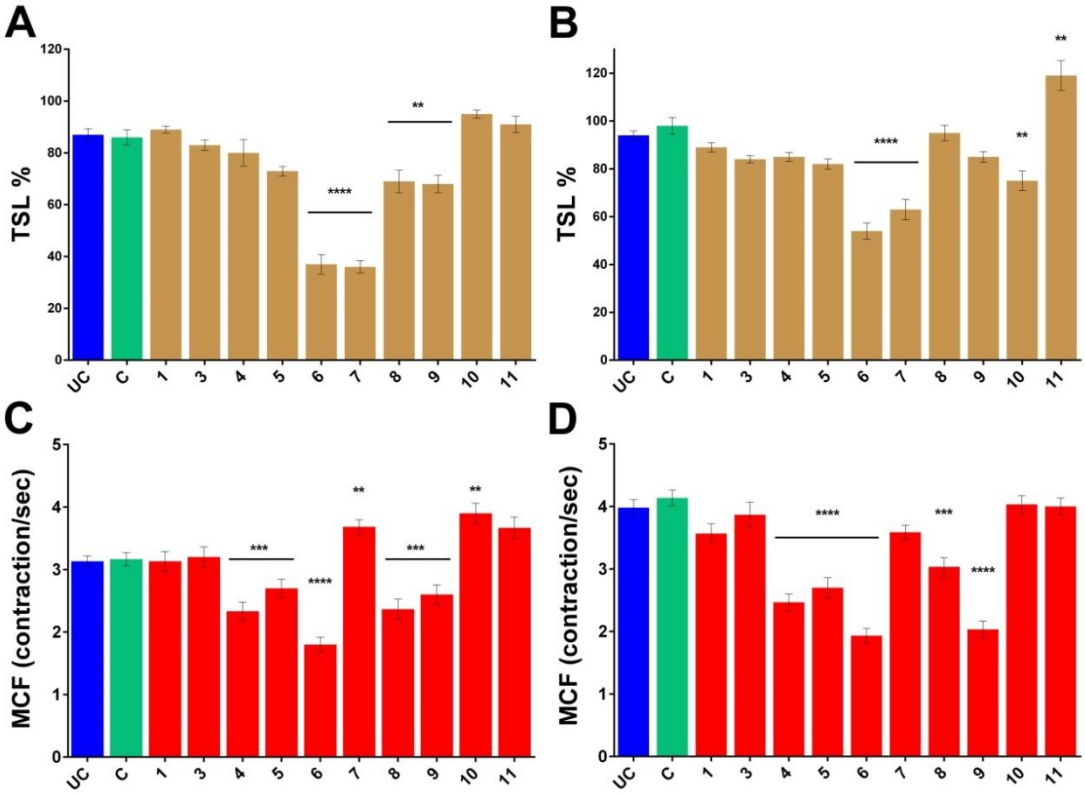


Figure 7. Normalized rotifer characteristics. Changes in the TSL values of the *Philodina acuticornis* after 3-day treatment (**A**) without feeding and (**B**) with feeding, compared to the group C. Changes in the MCF values of the *Philodina acuticornis* after 3-days treatment (**C**) without feeding and (**D**) with feeding, compared to the group C. UC: untreated control, C: control with 0.1% DMSO. **1**, **3-11**: compounds. TSL: toxicity and survival lifespan (n=12, well). MCF: mastax contraction frequency (n=30, individual rotifer). Values are the mean ± SEM; $p^{**} \leq 0.01$, $p^{***} \leq 0.001$, $p^{****} \leq 0.0001$.

5.3.2 GIRK channel inhibitory assay

Investigation of the ion channel activity of mushroom metabolites **1**, **3-11** revealed that lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) exhibits notable blocking activity on GIRK current while it has merely weak inhibitory activity on hERG channel.

Initially, as part of the screening study the GIRK channel inhibitory effects of mushroom extracts (A-D) from *Hypholoma lateritium* have been examined at two concentrations (0.01 mg/mL and 0.1 mg/mL). Among the tested extracts $CHCl_3$ extract (fraction B) revealed to be the most effective (53% decline on GIRK current) at the lower (0.01 mg/mL) concentration (**Figure 8**) (see **Table 3**).

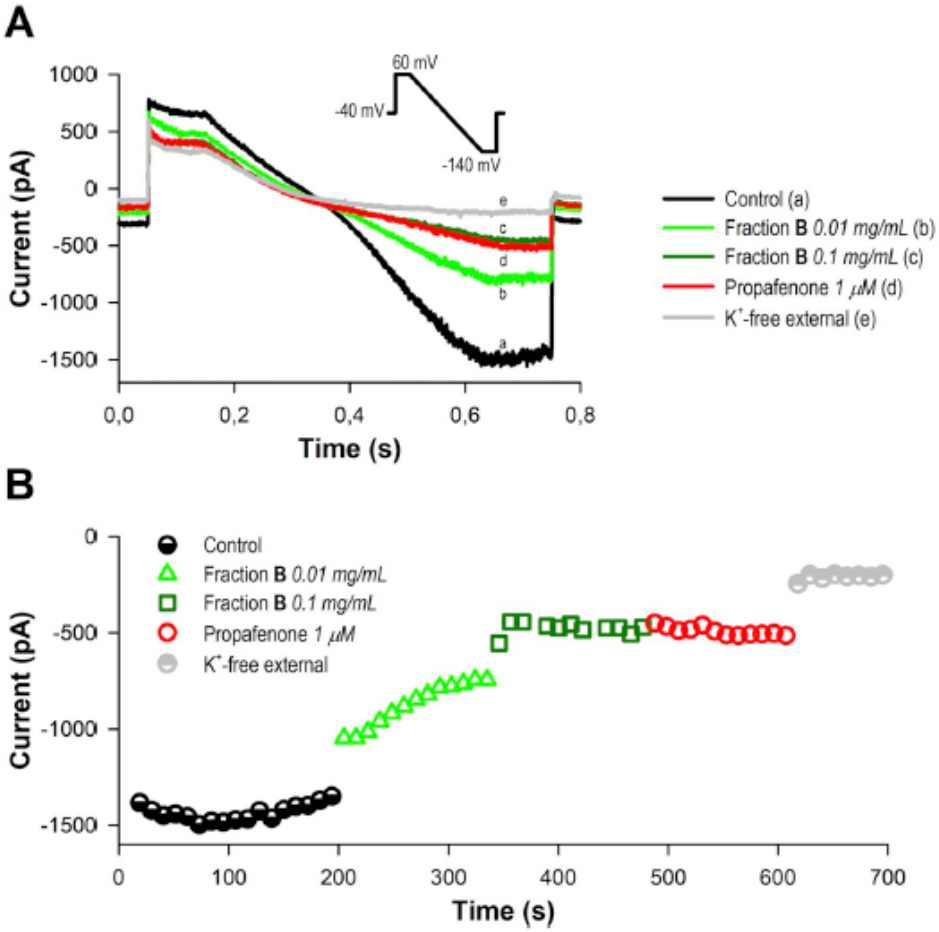


Figure 8. Blocking effects of fraction B of *Hypholoma lateritium* on GIRK current. Panel A shows typical current curves which were recorded during application of fraction B of *Hypholoma lateritium* at 0.01 mg/mL and 0.1 mg/mL concentrations. Inset shows the applied GIRK voltage protocol. The time course of calculated inward currents from the -140 mV segment of the current sweeps is presented on panel B.

Table 3. GIRK channel inhibitory activity of extracts of *Hypholoma lateritium* at 0.01 mg/mL and 0.1 mg/mL concentrations (n=2-3). SEM-Standard error of the mean

Extracts		**Inhibition %**		**SEM**	
		0.01 mg/mL	0.1 mg/mL	0.01 mg/mL	0.1 mg/mL
***n*-Hexane**	(A)	33	69	3	1

Chloroform	(B)	53	79	2	1
Methanol (50%)	(C)	25	46	2	1
Water	(D)	8	14	6	3

On the basis of the results of the preliminary screening *Hypholoma lateritium* was selected for further detailed chemical examination to determine its biologically active metabolites. The GIRK channel modulatory properties of the isolated compounds were measured in two concentrations (1 μM and 10 μM) (see **Table 4**).

Table 4. GIRK channel inhibitory activity of isolated compounds of *Hypholoma lateritium* at 1 μM and 10 μM concentrations (n=2-3). SEM-Standard error of the mean

Compound		**Inhibition %**		**SEM**	
		1μM	10 μM	1 μM	10 μM
Lanosta-7,9(11)-diene- 1 2 ß , 2 1 ì (1) epoxy- 2 Ŭ , 3 ß , tetraol , 2 5		27	60	5	11
Ergosterol	(**3**)	10	19	1	1
3 -hydroxyergosta-7,22-diene	(**4**)	8	16	1	6
Demethylincisterol A_2	(**5**)	11	23	3	1
Cerevisterol	(**6**)	7	13	1	6
3 -O-Glucopyranosyl-5,8-epidioxyergosta- 6,22-diene	(**7**)	14	24	1	5
Fasciculol F	(**8**)	10	27	1	2
Fasciculol E	(**9**)	11	23	2	3
Fasciculol C	(**10**)	10	10	4	7
Fasciculic acid B	(**11**)	13	23	2	8

The most significant inhibitory effect on GIRK channel was possessed by lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) while other compounds proved to exert moderate activity. Thus, further study has been conducted on compound **1** in order to determine its effect on the inward current in four concentrations on five cells.

The GIRK currents were recorded at increasing concentrations (1 μM, 3 μM, 10 μM and 30 μM) of compound **1** after the control period. The test compound exerted $42.0 \pm 6.4\%$ inhibitory activity on GIRK channel at 1 μM. Increase of the concentration of 3 μM to 10 μM led to a further decrease in GIRK current ($60.8 \pm 5.0\%$ and $66.4 \pm 4.5\%$ inhibitions,

respectively) whereas the current did not differ at the highest concentration of 30 µM (66.7±4.6% inhibition) (**Figure 9**).

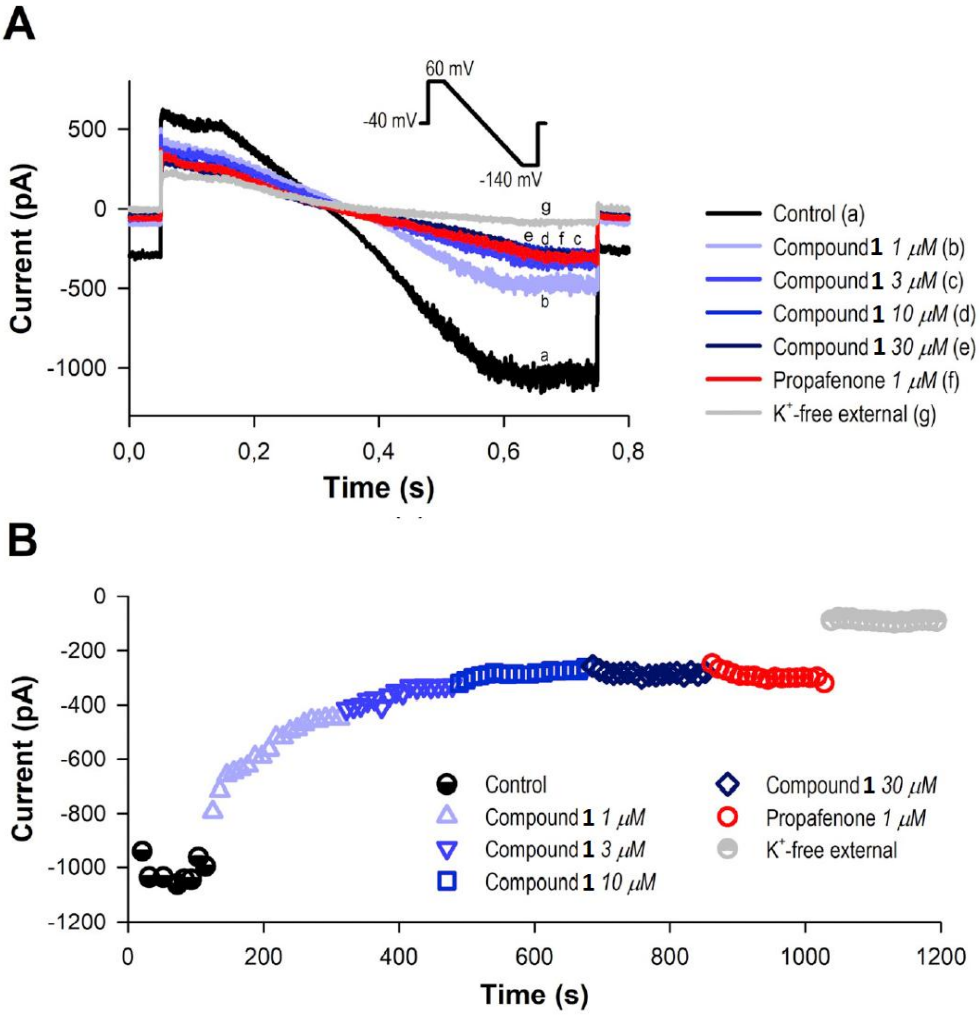


Figure 9. Blocking effects of compound **1** on GIRK current. Panel A depicts original current sweeps which were recorded during application of compound **1** at 1 µM, 3 µM, 10 µM and 30 µM concentrations. Inset shows the applied GIRK voltage protocol. Time course of calculated inward currents from the −140 mV segment of the current curves is presented on panel B.

In the detailed investigations, the dose−response curves of compound **1** were determined where the effect of the compound was tested in four concentrations (0.1 µM, 0.3 µM, 1 µM and 3 µM). The GIRK current was remarkable blocked by compound **1** in a concentration dependent manner. The relative IC_{50} value of compound **1** (the concentration corresponding to a response midway between the estimates of the lower and upper plateaus of the dose-response curve, i.e., 0% and 66.7% inhibition) was calculated to be 395.1±31.8 nM (**Figure 10)**.

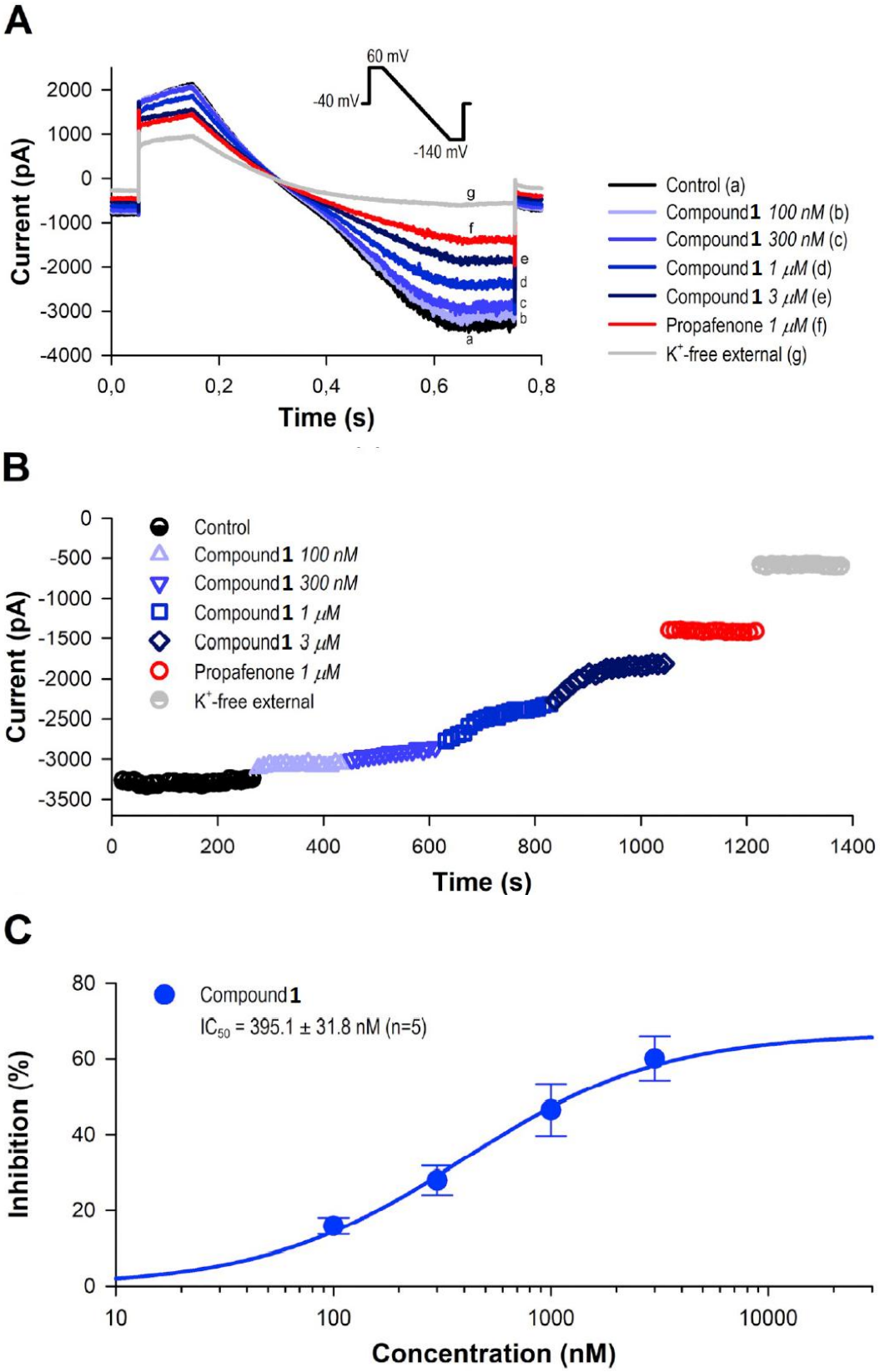


Figure 10. Inhibitory effects of compound **1** on GIRK current. Panel A displays representative current curves which were recorded during application of compound **1** at 0.1 µM, 0.3 µM, 1 µM and 3 µM concentrations. Inset shows the applied GIRK voltage protocol.

Time course of calculated inward currents from the −140 mV segment of the current curves is presented on panel B. Panel C shows the dose-response curves of compound **1**. The GIRK channel inhibitory activity of compound **1** could be characterized by the relative IC_{50} value of 395.1 ± 31.8 nM (*n*=5).

Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) was also tested on HEK-hERG cell line and selectivity of its GIRK blocking effect was evaluated with these experiments. Compound **1** possessed low inhibitory activity (7.9 ± 2.8%) on the hERG channel at 100 μM concentration, while it exhibited a potent GIRK inhibitory, presenting more than three orders of magnitude higher blocking activity on GIRK channel compared to the results obtained on hERG channel. Original hERG current sweeps during the application of compound **1** at 100 μM concentration and the time course of decrease in the peak tail current are shown on **Figure 11**.

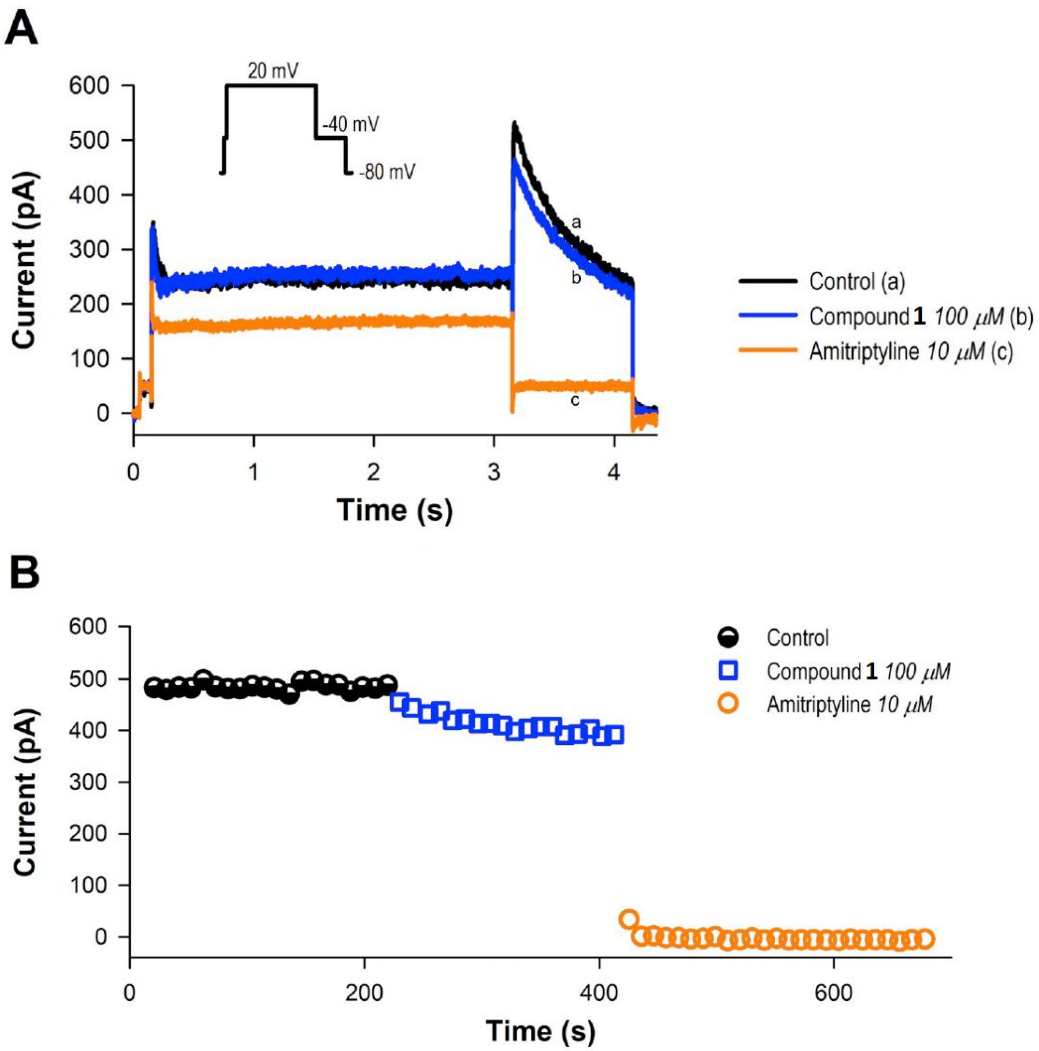
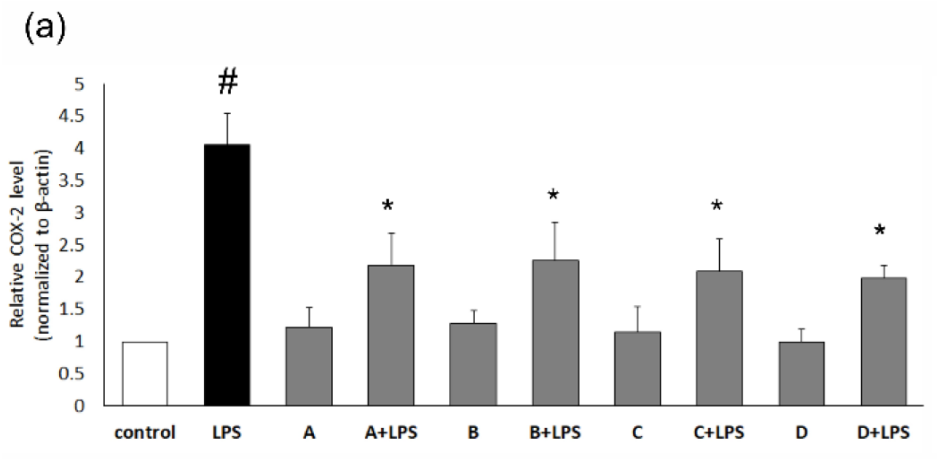


Figure 11. Effects of compound **1** on hERG current. Panel A presents typical hERG current sweeps during the application of 100 μM compound **1**. The inset shows the applied hERG voltage protocol. The original current traces reveal that **1** slightly blocked the hERG channel, while the addition of reference compound amitriptyline (10 μM) fully blocked the current. The time course of decrease in the peak tail current is presented on panel B.

5.3.3 Anti-inflammatory assay

Examination of the anti-inflammatory properties of *Hypholoma lateritium* extracts, in RAW 264.7 cells activated with LPS and incubated with chloroform, methanol and water extracts of *Hypholoma lateritium* revealed an increase of Nrf2 (nuclear factor (erythroid-derived 2)-like 2). In the same way, higher levels of cPGES protein were observed in macrophages co-treated with LPS and in all extracts, but the values obtained were significantly lower compared to those of the LPS-activated cells. The investigations highlighted a decrease in COX-2-levels in RAW 264.7 cells co-treated with mushroom extracts and LPS in comparison with the experiment of LPS-activated macrophages (**Figure 12**).



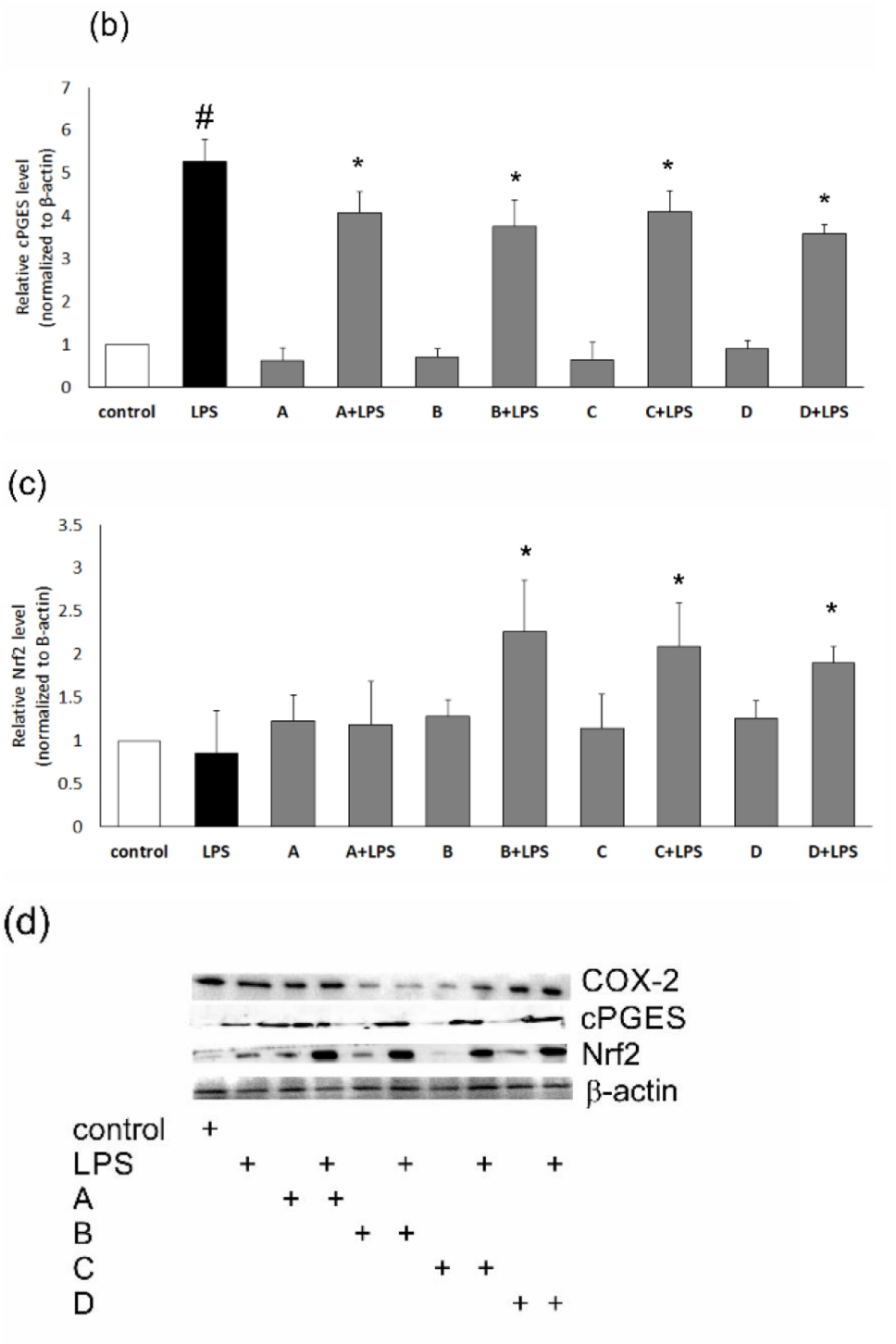
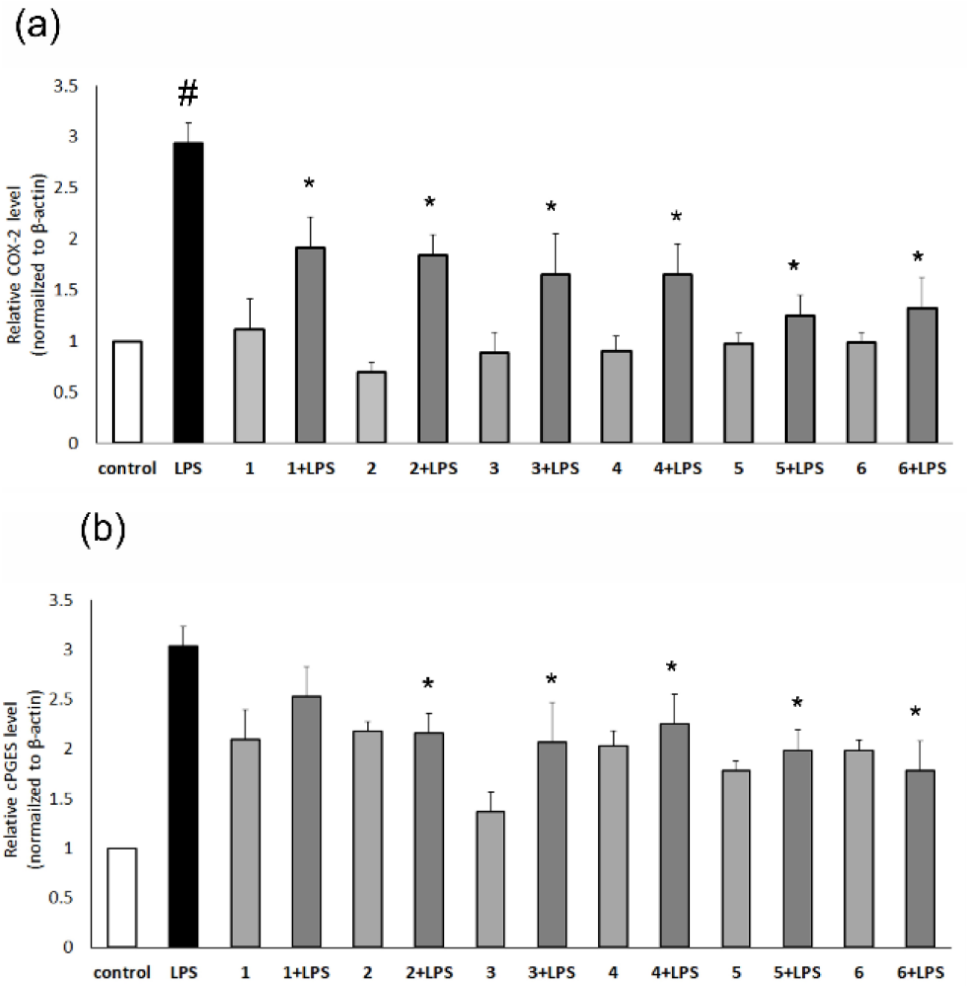


Figure 12. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with extracts of *Hypholoma lateritium* and activated with LPS. # vs control, * vs LPS, $p<0.05$. A: *n*-Hexane extract, B: Chloroform extract, C: 50% MeOH extract, D: Water extract

To identify the secondary metabolites of *Hypholoma lateritium* responsible for the observed anti-inflammatory properties the pharmacological assays for the characteristic compounds of *H. lateritium* have been performed.

The experiments revealed that compounds **1**, **5**, **8-11** activated cPGES, but levels of this protein were lower than those in LPS-activated RAW 264.7 cells. In cells activated with LPS and incubated with compounds **1**, **5**, **8-11** we experienced an increase of Nrf2. Compounds **1**, **5**, **8-11** in general proved to possess similar activities; however fasciculol C (**10**) represents a particular case, because when cells were treated with **10** alone the amount of cPGES was the lowest, while the level of Nrf2 was the highest among the values obtained in all experiments. Macrophages activated with LPS and incubated with fungal metabolites were characterized by decreased COX-2 levels when compared to LPS-activated macrophages (**Figure 13**).



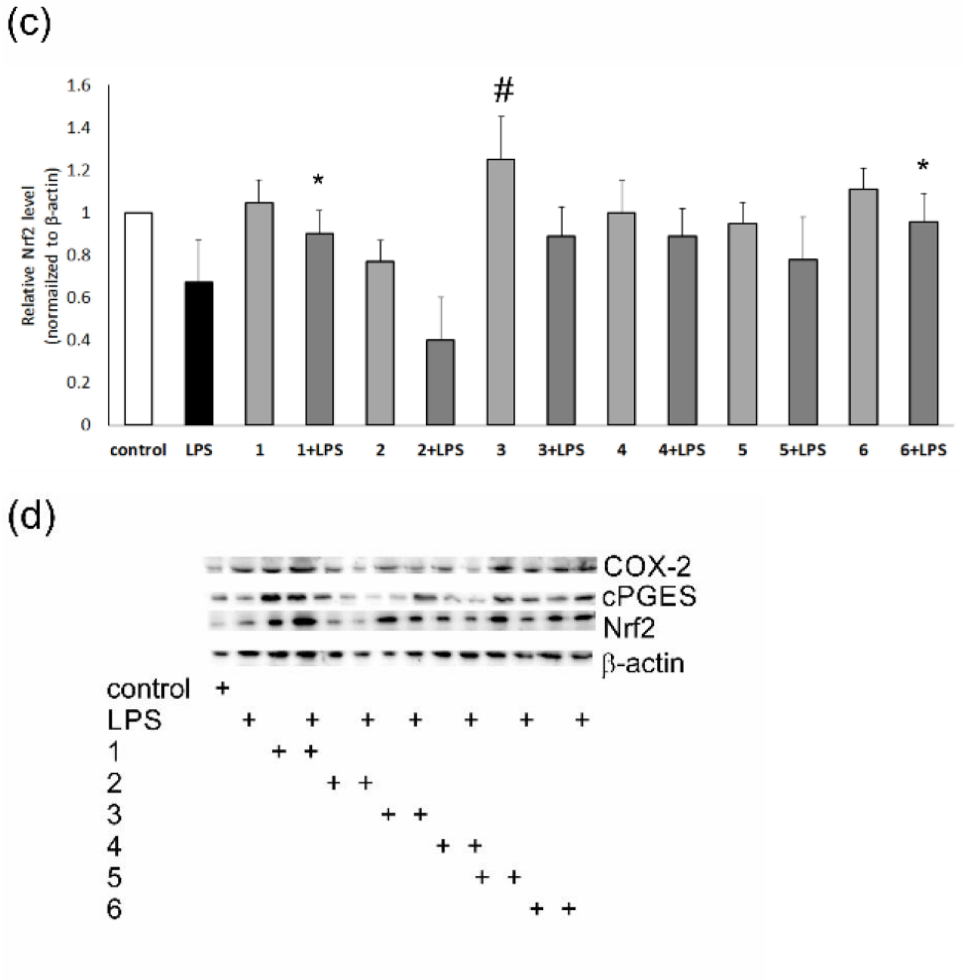


Figure 13. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with compounds isolated from *Hypholoma lateritium* and activated with LPS. # vs control, * vs LPS, $p<0.05$. 1-Fasciculic acid B (**11**), 2-Fasciculol E (**9**), 3-Fasciculol C (**10**), 4-Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**), 5-Fasciculol F (**8**), 6-Demethylincisterol A_2 (**5**)

6 DISCUSSION

6.1 Screening studies

Fungal species were collected from the environs of Bakonybél, 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.

In the study effects of fungal extracts on the G-protein-activated inwardly rectifying potassium channel were screened using the automated patch-clamp method. The organic (*n*-hexane, chloroform, and 50% methanol) and aqueous extracts of *Hypholoma lateritium* were tested in this assay, and among the extracts, $CHCl_3$ extract revealed to be the most effective (53% decline on GIRK current).

Organic (*n*-hexane, $CHCl_3$ and 50% methanol) and water extracts of *H. lateritium* were subjected to *in vitro* assays to determine pro-inflammatory protein levels, such as cyclooxygenase-2 (COX-2), cytosolic prostaglandin E2 synthase (cPGES), and antioxidant nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Fungal extracts demonstrated significant activities on pro-inflammatory protein levels with minor differences among the activities of the fractions of different polarities.

In conclusion, the ion channel activity and anti-inflammatory screening studies demonstrated that the examined mushroom *H. lateritium* and its secondary metabolites could have several beneficial pharmacological properties providing multiple opportunities for the potential therapeutic application of AF and inflammation.

6.2 Investigation of *Hypholoma lateritium*

Mycochemical investigation of fruiting bodies of *H. lateritium* resulted in the isolation of 12 compounds, including 2 new natural products. The structures were elucidated on the basis of spectroscopic methods, including 1D and 2D NMR and MS analyses. Biological investigations revealed that some of the isolated compounds possess noteworthy pharmacological effects.

6.2.1 Isolation of bioactive compounds

Our prior investigations of the extracts with different polarity prepared from the aerial parts of *Hypholoma lateritium* and literature review resulted in the conclusion that the lipophilic, especially chloroform and *n*-hexane extracts, contain the bioactive secondary metabolites.

In the initial step of the mycochemical work, the dried mushroom materials were percolated with an amphipolar solvent (MeOH) at room temperature; then solvent–solvent extraction was used, which led to the *n*-hexane, chloroform and ethyl acetate fractions. The fractions obtained were subjected to normal and reversed phase flash chromatography procedures in order to separate the compounds responsible for the pharmacological activities.

The *n*-hexane phase was fractionated by **FCC** and the combined fractions were further analyzed using repeated **FCC** and **RP-FCC**. Multiple chromatographic separations led to the isolation of five compounds (**3-7**). The purification of the chloroform extract of the species was first carried out by **FCC** followed by **RP-FCC** affording six compounds (**1**, **2**, **8-11**). Two of them (**1**, **2**) were new natural products while four (**8-11**) were previously known fasciculol-type triterpenes. The ethyl acetate fraction of *H. laterirum* was separated by repeated **FCC** steps to give compound **12**.

6.2.2 Structure elucidation

The structures of the isolated compounds (**1-12**) were elucidated by means of spectroscopic methods. The molecular masses and compositions were obtained from MS investigations. The most useful data concerning the structures were furnished by 1D and 2D NMR spectroscopy. From the ^{1}H and ^{13}C NMR, ^{1}H-^{1}H COSY, HSQC and HMBC experiments, the constitutions of the compounds were described and then the relative configurations were characterized with the aid of NOESY and ROESY spectra. As a consequence of the NMR studies, complete ^{1}H- and ^{13}C assignments were made for the characterization of the new compounds and also in the case of some known compounds, where previously published data were incomplete.

Triterpenes from Hypholoma lateritium

The structure determination of compounds isolated from *n*-hexane phases of *H. lateritium* led to the identification of 5 triterpenes.

Ergosterol (**3**) and 3β-hydroxyergosta-7,22-diene (**4**) are common steroids with a widespread distribution in fungal species. Ergosterol is the primary sterol in the cell

membranes of fungi and utilized for fungal growth[73]. It was first isolated from *Claviceps purpurea* over 130 years ago[74]. Cerevisterol isolated from the unsaponifiable fraction of the fat of the common yeast, *Saccharomyces cerevisiae*[75], its chemical structure and some of its properties were described by Honeywell and Bills[76].

According to the spectroscopic measurements compound **7** demonstrated to be 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene possessing a peroxide linkage between C5 and C8. This secondary metabolite was previously detected in the edible mushroom *Hericium erinaceus*[77]. Demethylincisterol A_2 (**5**) identified first from a marine sponge *Homaxinella* sp., is a highly degraded sterol belonging to the class of incisterols[71].

From the chloroform phase of the methanolic extract of *Hypholoma lateritium* 6 compounds were identified. Fasciculic acid B (**11**) was first isolated from the toxic mushroom *Naematoloma fasciculare*, having potent calmodulin antagonist activity[47]. Fasciculol C (**10**) and its depsipeptides fasciculol E (**9**) and F (**8**) were detected from the ethyl acetate extracts of the fruit bodies of *Naematoloma fasciculare* in 1977 by Ikeda et al.[78]. Four years later, compounds **8**-**10** were isolated from *Hypholoma lateritium* [54].

6.2.3 Biological activities of the isolated compounds from *Hypholoma lateritium*

6.2.3.1 Toxicity and viability of compounds on bdelloid rotifers

Based on our screening results, the compounds **1**, **3**-**11** detected in *Hypholoma lateritium,* were evaluated for their toxicity and viability *in vivo* using the bdelloid rotifer assay. Most of the tested compounds demonstrated low toxicity, even though cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) caused significant decrease in the number of animals. According to the mastax contraction frequency values, compound **7** exhibited the highest viability compared to the others. Here we report for the first time preliminary *in vivo* toxicity results for compounds **1**, and **3**-**11**.

Most of the examined compounds have been poorly investigated regarding their toxicity. The median lethal dose (LD_{50}) in mice was available only for fasciculol F (**8**) and E (**9**). Fasciculol F and E gave LD_{50} values of 168 mg/kg and 50 mg/kg, respectively, in intraperitoneal administration in mice[79]. Demethylincisterol A_2 (**5**) isolated from the *Homaxinella* sp. showed a significant cytotoxicity against a panel of five human tumor cell lines. The extensive mycochemical analysis of the aerial parts of *Naematoloma sublateritium* by Yaoitaa et al. resulted in the identification of three new lanostane type triterpenoids, sublateriols A-C and three known ones, fasciculol A-C. Three of them namely sublateriol C,

fasciculol B and C revealed significant cytotoxicity against human cancer cell lines. Ergosterol (**3**) isolated from the fruiting bodies of the fungus *Ganoderma lucidum*, was examined for their inhibitory effects on the induction of Epstein-Barr virus early antigen (EBV-EA) by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in Raji cells, a known primary screening test for anti-tumor promoters. Ergosterol showed low inhibitory effects on EBV-EA induction, with IC_{50} values of 516 mol ratio/32 pmol TPA[80]. 3β-*O*-Glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) identified in the methanol extract of *Scleroderma bovista* Fr. showed significant antiproliferative activity on A2780 human cancer cell line (53.27 ± 6.37%)[81]. Cerevisterol (**6**) identified in the *n*-hexane extract of *T. populinum*, proved to be the most active against T47D human breast cancer cell line (50.2 ± 1.6%)[82].

6.2.3.2 Effects of compounds on GIRK and hERG channels

In the frame of the GIRK channel inhibitory investigation 4 extracts from *Hypholoma lateritium* were tested, among them $CHCl_3$ extract proved to be the most active (53% decline on GIRK current) at 0.01 mg/mL concentration. Ten mushroom metabolites (**1**, **3**-**11**) were investigated in order to determine their GIRK blocking potency. Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) demonstrated remarkable blocking activity on GIRK current (IC_{50} 395.1±31.8 nM). Evaluation of the selectivity of the GIRK inhibitory effect exhibited that lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) has only weak inhibitory activity on hERG channel (7.9±2.8% at 100 μM).

A literature review of the previous investigations suggest that cardiac action of diterpenes[83-85] were mostly studied. However, merely a few of the articles dealing with the cardiac effect of triterpenes are available[86-88]. To the best of our knowledge our research team reported for the first time that a lanostane-type triterpene from *H. lateritium* was able to substantially prevent potassium current through GIRK channels.

6.2.3.3 Anti-inflammatory activity of compounds from Hypholoma lateritium

According to the preliminary studies organic and water extracts of *H. lateritium* exerted significant inhibitory activity on COX-2 and were capable to stimulate the Nrf2 pathway. Our detailed experiments (**Figure 12**) showed that compounds **1**, **5**, **8**-**11** activated cPGES, but levels of this protein were lower than those in LPS-activated RAW 264.7 cells. In cells activated by LPS and incubated with compounds **1**, **5**, **8**-**11** were characterized by increased Nrf2 level. Compounds **1**, **5**, **8**-**11** in general demonstrated similar activities; however fasciculol C (**10**) represents a particular case, because when cells were treated with **10** alone

the amount of cPGES was the lowest, while the level of Nrf2 was the highest among the values obtained in the experiments. Macrophages activated by LPS and incubated with fungal metabolites expressed low level of COX-2 when compared to LPS-activated macrophages. Nrf2 is an essential transcription factor that regulates the expression of antioxidant proteins that protect against oxidative damage caused by injury and inflammation. It is a key participant of cellular defense mechanism; activation of Nrf2 leads to a subsequent production of proteins and antioxidant enzymes, providing the damaged cells and tissues with a complex antioxidant defense. Plenty of studies unequivocally demonstrate that many plant metabolites from fruits and vegetables, e.g. curcumin, resveratrol and sulforaphane are capable of regulating Nrf2[89-91]. Although many plants produce a variety of compounds with Nrf2 activity, the potential of mushroom metabolites in this view is largely unexplored. However, extracts from *Agaricus bisporus* mycelia enriched in α-linolenic acid presented Nrf2 modulating activity[92]. Only a few fungal compounds are known to regulate the Nrf2 pathway, including the benzoid-type antrolone and the ubiquinone derivative antroquinonol identified in *Antrodia* sp., and several steroids from the renowned *Ganoderma lucidum*[93, 94].

Fasciculol C (**10**), E (**9**) and fasciculic acid B (**11**), isolated from the toxic mushroom *H. fasciculare* demonstrated potent calmodulin antagonist activity[47]. A study by Kubo et al. confirmed that fasciculol F (**8**) separated from the same species also had calmodulin-inhibitory activity[95]. Calmodulin is an essential protein regulates a remarkable array of physiological events by binding with calcium in intracellular[96]. Calcium/calmodulin-dependent protein kinase II (CaMKII) is activated in several heart disease with an inflammatory component and regulates pro-inflammatory signaling such as nuclear factor kappa-B (NF-kB)[97].

7 SUMMARY

The aim of our research work was to isolate and characterize the biologically active compounds from *Hypholoma lateritium*, and to evaluate its extracts and compounds for toxicity, GIRK channel inhibitory and anti-inflammatory activities.

The isolation of the compounds was carried out by a multistep separation procedure using FCC and RP-FCC. The structures of the obtained compounds were elucidated by means of spectroscopic methods (MS and NMR). As a consequence of 1D (^{1}H, ^{13}C and JMOD) and 2D NMR studies (COSY, HSQC, HMBC and NOESY), complete ^{1}H and ^{13}C assignments were accomplished for the characterization of the compounds.

Two new natural products lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) and 8-hydroxy-13-oxo-9*E*,11*E*-octa-decadienoic acid (**2**), together with 10 known ones, four ergostane type triterpenes, such as ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**6**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**), a highly degraded sterol demethylinciterol A_2 (**5**), and four fasciculol-type triterpenes, namely fasciculol F (**8**), fasciculol E (**9**), fasciculol C (**10**), and fasciculic acid B (**11**) were isolated from *H. lateritium*; compound **12** was identified as uridine. Six (**4**-**7**, **9**, **12**) of the known compounds were identified in this species for the first time.

The toxicity of compounds isolated from *H. lateritium* were examined *in vivo* using the bdelloid rotifer assay. On the basis of our screening results, cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) exerted high toxicity, while lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) showed no toxic effect.

In our search for mushroom species for potential GIRK channel inhibitory activity, the chloroform extract of *H. lateritium* proved to be the most active (53% decline on GIRK current) at 0.01 mg/mL concentration. Among fungal metabolites, lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) demonstrated notable blocking activity on GIRK current. Investigation of the ion channel selectivity it was found that compound (**1**) has only weak inhibitory activity on hERG channel.

The anti-inflammatory effects of lipophilic and hydrophilic extracts and compounds **1**, **5**, and **8**-**11** were evaluated *in vitro* using RAW 264.7 cells, which revealed significant activities on pro-inflammatory protein levels, COX-2, cPGES and Nrf2 with minor differences among the activities of the fractions of different polarities. All compounds proved to exert significant inhibitory property on COX-2 and were capable to stimulate the Nrf2 pathway.

In conclusion, in-depth mycochemical and pharmacological investigations of *H. lateritium* afforded promising results, providing multiple opportunities for the potential therapeutic application of the isolated fungal metabolites, particularly regarding their noteworthy GIRK channel inhibitory and anti-inflammatory effects.

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Triterpenes from the Mushroom *Hypholoma lateritium*: Isolation, Structure Determination and Investigation in Bdelloid Rotifer Assays

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Abstract: Twelve compounds (**1**–**12**) were isolated from the methanol extract of brick cap mushroom (*Hypholoma lateritium* (Schaeff.) P. Kumm.). The structures of the compounds were elucidated using extensive spectroscopic analyses, including NMR and MS measurements. Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) and 8-hydroxy-13-oxo-9*E*,11*E*-octa-decadienoic acid (**2**) were identified as new natural products, together with ten known compounds, from which 3β-hydroxyergosta-7,22-diene (**4**), demethylincisterol A2 (**5**), cerevisterol (**6**), 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**), fasciculol E (**9**), and uridine (**12**) were identified in this species for the first time. The isolated triterpenes (**1**, **3**–**11**) were investigated for their toxicity in vivo using bdelloid rotifer assays. Most of the examined steroids in general showed low toxicity, although the effects of the compounds varied in a wider range from the non-toxic lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) to the significantly toxic cerevisterol (**6**), with substantial dependence in some cases on the presence of nutrient in the experimental environment.

Keywords: *Hypholoma lateritium*; mushroom; triterpenes; toxicity; bdelloid rotifer

1. Introduction

Over human history, mushrooms have acquired a good reputation as a popular and valuable foodstuff, being low in calories but high in essential amino acids, vitamins, and fiber. They can be integrated into a versatile and balanced diet, representing not only a tasty and healthy food, but also a rich source of biologically active natural products with therapeutic potential. According to the report of the Food and Agriculture Organization (FAO), there are records of more than 1100 mushrooms with varying degrees of edibility from 85 countries around the world [1]. The question of edibility is fairly subjective; the comestibility of some mushrooms can vary from one region to another, while certain species are eaten only after they are processed in a specific way.

A particular case is represented by *Hypholoma lateritium* ((Schaeff.) P. Kumm. (syn. *Hypholoma sublateritium* (Fr.) Quél. and *Naematoloma sublateritium* (Fr.) P. Karst.)), also known as brick cap, which is a popular edible mushroom in Japan, Korea, and the United States, but in Europe it is

considered inedible or even poisonous [2,3]. *H. lateritium* is a wood-decay fungus growing in clusters on hardwood logs and stumps, widely distributed throughout Europe, North America, and the Far East. Compared to its more common relative, the poisonous sulfur tuft (*Hypholoma fasciculare*), the chemistry and pharmacology of *H. lateritium* are less known. This species produces several triterpenes, e.g., fasciculols B and C, their depsipeptides (fasciculols D and F), and sublateriols A–C [4,5]. Naematolin, a caryophyllane derivative with cytotoxic activity, was isolated from cultures of *H. lateritium* [6]. Attempts have been made to characterize the possible mechanism of action behind the antitumor and anti-inflammatory effects of *H. lateritium* extracts [7,8], as well as to explore the antimicrobial, antioxidant, and xanthine oxidase inhibitory properties of this species [9–11]. The current study was performed to identify the major secondary metabolites of *H. lateritium* and characterize their toxicity in bdelloid rotifer assays. Bdelloid rotifers, as micro-invertebrates, are widely used animal models in toxicity-, aging-, and longevity-related research [12–14]. These organisms are multicellular animals with well-defined anatomical characteristics, possessing a ciliated head structure, bilateral ovaries, mastax, ganglia, muscle, digestive, nervous, and secretory systems, and photosensitive and tactile organs [15,16]. Taking into account the above-mentioned characteristics together with their short lifespan and specific measurable phenotypic features and viability markers [17], bdelloids are useful as in vivo toxicological and lifespan models.

2. Results and Discussion

As part of our ongoing effort to search for biologically-active natural fungal products, our attention has been drawn to the mushroom *H. lateritium*, a species known for its controversial edibility, but investigated to a weaker extent. In this vein, our main goal was to identify the characteristic compounds of *H. lateritium* and to evaluate their toxicity in vivo using bdelloid rotifer assays. The collected fruiting bodies of *H. lateritium* were freeze-dried, and then were extracted with methanol on room temperature. The crude extract was subjected to solvent-solvent partition with *n*-hexane, chloroform, and then ethyl acetate. The obtained *n*-hexane, chloroform, and ethyl acetate phases were applied to an extensive separation process, using a combination of flash chromatography steps on normal and reversed phases to afford 12 compounds (Figure 1).

Compound **1** was isolated as a colorless gum. Based on the HRESIMS and ^{13}C-NMR data, its molecular formula was determined to be $C_{30}H_{48}O_5$. Consecutive analysis of the ^{1}H, ^{13}C, COSY, HSQC, and HMBC NMR spectra (see Supplementary Materials) suggested the presence of seven tertiary methyl group, two olephinic, four oxygenated, and three aliphatic methines, an oxygenated and six aliphatic methylenes, together with five quaternary carbons. The determined data were quite similar to those reported for sublateriol C [5], except for the ^{1}H and ^{13}C resonances assigned to ring D, and C-12, C-18, C-20, C-21, and C-28. The observed HMBC correlations between H-12 and C-21 and between H-21 and C-12 (Figure 2) suggested that instead of two hydroxyl groups present in sublateriol C, an epoxy group between C12 and C21 is present in **1**. This ring closure is in accordance with the H_2O difference obtained between the elementary compositions of sublateriol C and **1**, and explains the chemical shift differences obtained for the protons and carbons close to C-12 and C-20 centers.

Based on the key NOE correlations (Figure 3) observed between H-2/H-19, H-3/H-5/H-30, H-12/H-28/H-17, and H-18/H-20, the above presented stereochemistry is suggested.

The configuration of C-24 center, however, could not be assigned on this basis, and the OH group is only tentatively given as beta-positioned. This suggestion is firstly based on the assumption that the ring closure does not drastically change the conformation of the side chain. In this case, the observed doublet nature of H-24 with coupling constants of 9.8 and 1.6 Hz, which are closely similar to those reported for sublateriol A [5] or other isolated fasciculic acid and fasciculol derivatives, having the same side chain [18,19], suggest a similar configuration of the C-24 center. Secondly, making the assumption that similar metabolic pathways lead to sublateriol C and **1** in the same mushroom species, a similar β orientation of 24-OH is suggested. Putting all these together, the structure of compound **1** is suggested as lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol.

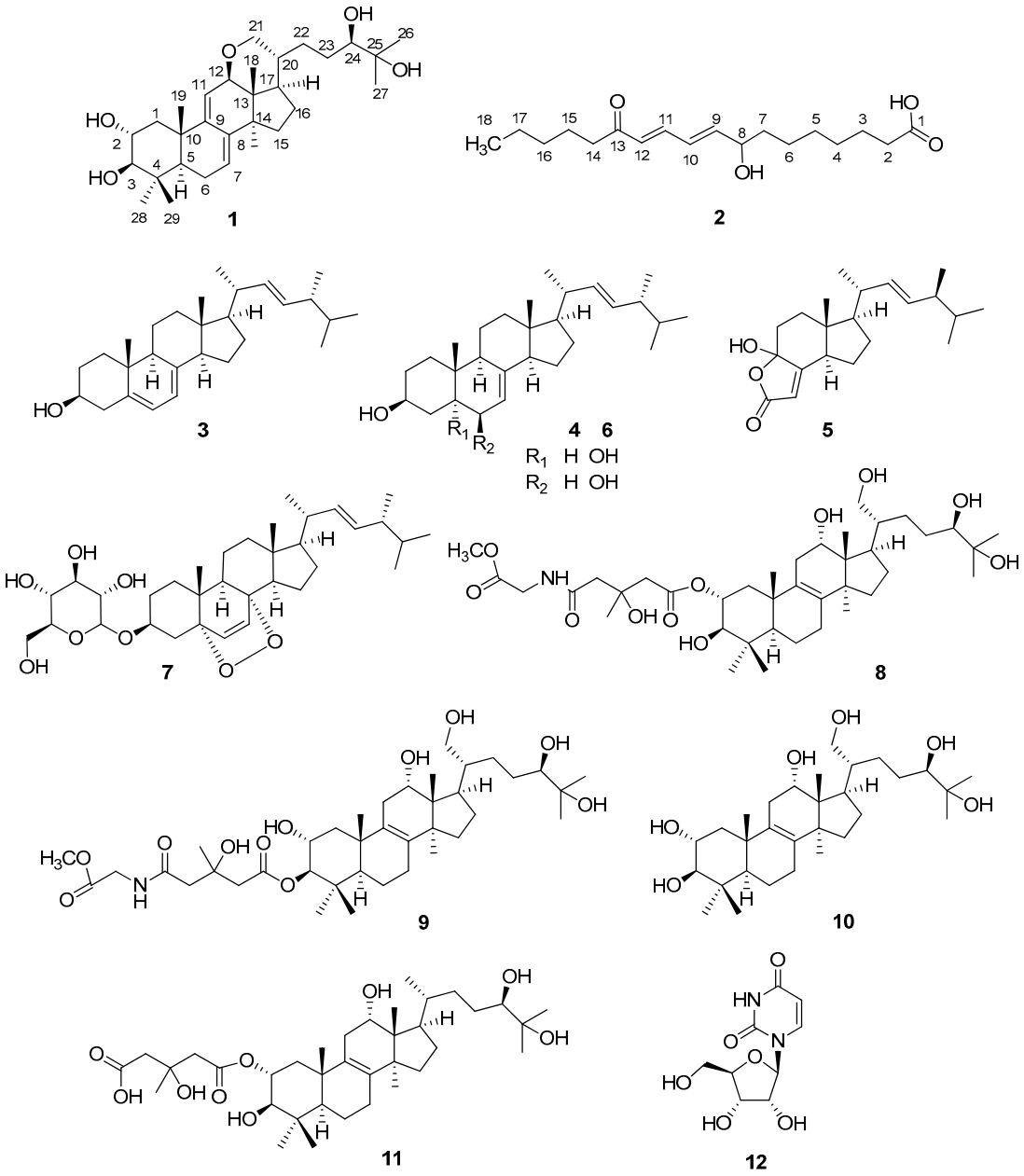

Figure 1. Structures of compounds isolated from *H. lateritium.*

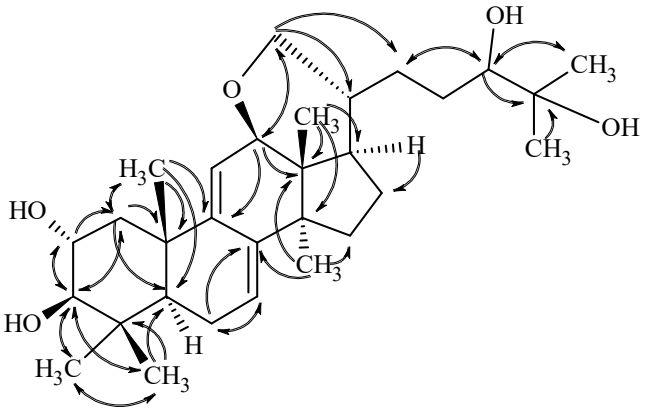

Figure 2. Key HMBC correlations of compound **1.**

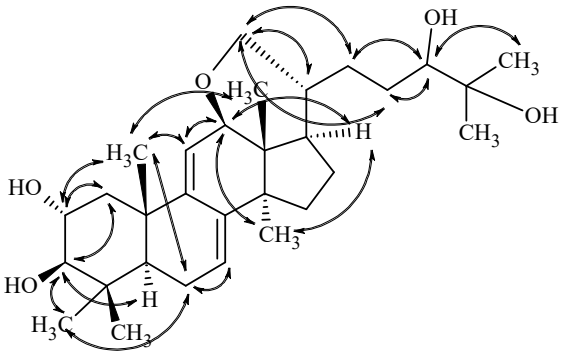

Figure 3. Key NOE correlations determined from the ROESY spectrum of compound **1.**

Compound **2** was obtained as colorless gum. Its molecular formula, $C_{18}H_{29}O_4$, was established from HR-ESI MS measurement giving a pseudomolecular ion peak at *m*/*z* 309.20673 ([M − H]$^-$) in the negative ion mode. In accordance with this elementary composition, the ^{13}C-NMR spectrum presented eighteen carbon resonances. Based on the ^{1}H and edited HSQC spectra one methyl, ten methylenes, five methines, and two quaternary carbons were present in the isolated compound. The ^{13}C chemical shifts of the quaternary carbons (182.9 and 204.0 ppm) suggested the presence of a carboxylic acid and a ketone functionality. The multiplicities and coupling constants of the ^{1}H resonances belonging to the methine protons suggested the presence of two conjugated double bonds, both in *E* configuration, connected to a hydroxylated methine and to a keto group. Putting this information together led to the conclusion that the isolated compound is a hydroxyl-oxo-octadecadienoic acid derivative. The positions of the functional groups in the fatty acid chain were unambiguously evidenced on the basis of the COSY and HMBC correlations presented in Figure 4.

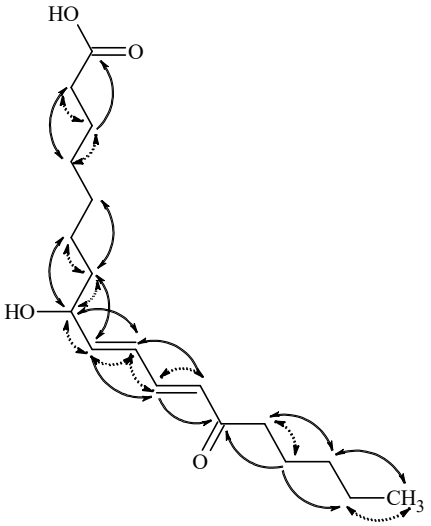

Figure 4. Key HMBC (solid line) and COSY (dotted line) correlations in compound **2.**

Thus, the HMBC correlations observed between H-14 and C-16, C-12, C-13, and C-15, and between H-18 and C-16 and C-17, suggested that the keto group is at position 13, while the OH is connected to C-8. The HMBC correlation of H-11 and H-12 to C-14, and those of H-9 and H-10 to C-8 confirmed these suggestions. Based on these data, the 8-hydroxy-13-oxo-9*E*,11*E*-octadecadienoic acid structure is suggested for **2**. The absolute stereochemistry of C8 center was not determined. Ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**6**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) were identified by comparing their chromatographic and spectroscopic data with those of authentic samples. Compounds **5** and **8**–**12** were structurally characterized on the basis of NMR and MS spectroscopic data (see Supplementary Materials) and confirmed by comparing them to those

reported earlier in the literature [4,5,18–24]. Demethylincisterol A2 (**5**) is a highly degraded sterol isolated first from a marine sponge of *Homaxinella* sp. [24]. Fasciculol E (**9**) was first identified in the sporocarps of *Hypholoma fasciculare* [19], while fasciculol F (**8**), fasciculol C (**10**), and fasciculic acid B (**11**) were previously isolated from *H. lateritium* [4]; compound **12** was determined as uridine.

The isolated compounds were subjected to bdelloid rotifer assay in order to gain information about their toxicity and biological activity. Two viability markers of *Philodina acuticornis* have been used to measure the effect of compounds **1** and **3**–**11** in terms of survival and resilience. Decreases or increases in the toxicity and survival lifespan (TSL) and in the mastax contraction frequency (MCF) are in correlation with the physiological state of individuals. The changes in TSL values, which provides mortality rate, are rather straightforward results. The MCF index assays the chewing organ function, providing information about the effect of the compounds on organs level and gives a more complex image to the results. Compounds which caused significant decrease in the number of survivors were cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) (see Figure 5).

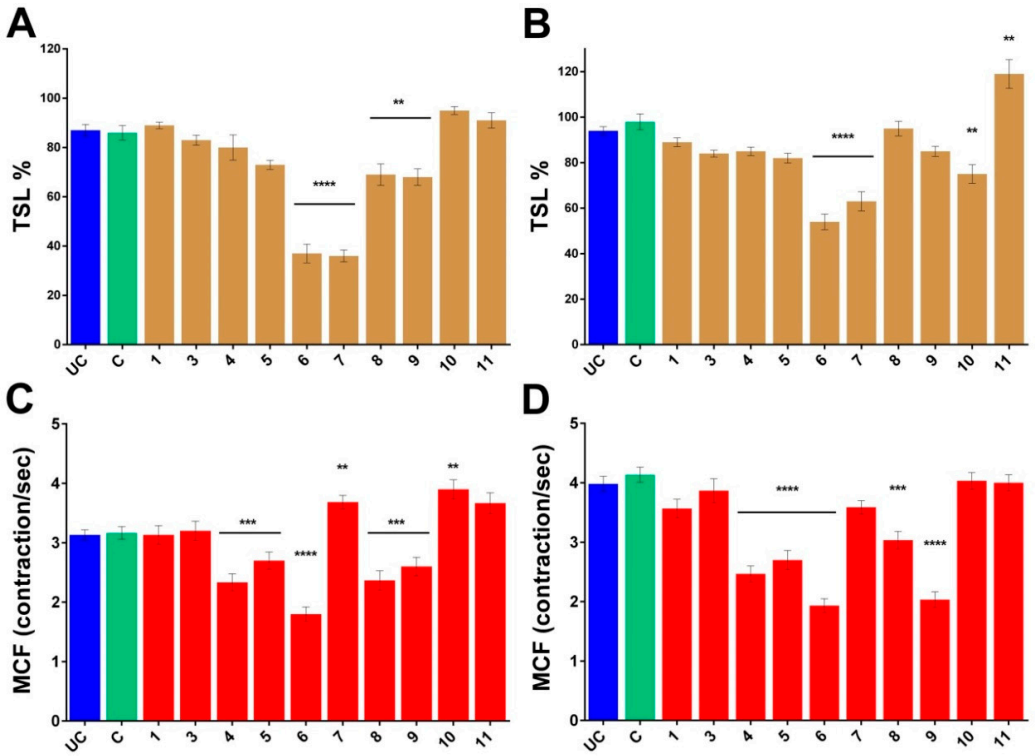

Figure 5. Normalized rotifer characteristics. Changes in the TSL values of the *Philodina acuticornis* after 3-day treatment (**A**) without feeding and (**B**) with feeding, compared to the group C. Changes in the MCF values of the *Philodina acuticornis* after 3-day treatment (**C**) without feeding and (**D**) with feeding, compared to the group C. UC: untreated control, C: control with 0, 1% DMSO. **1**, **3**–**11**: compounds. TSL: toxicity and survival lifespan (n = 12, well). MCF: mastax contraction frequency (n = 30, individual rotifer). Values are the mean ± SEM; p ** ≤ 0.01, p *** ≤ 0.001 and p **** ≤ 0.0001.

Significant decrease was observed in the MCF values of 3β-hydroxyergosta-7,22-diene (**4**), demethylincisterol A2 (**5**), cerevisterol (**6**), fasciculol E (**9**), and fasciculol F (**8**). Cerevisterol (**6**) and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**) were significantly toxic, however the latter in the presence of nutrients proved to be less toxic, and the MCF values significantly increased in the survivors. The complete opposite was observed with fasciculol C (**10**), which demonstrated toxic effect with feeding, but increased the MCF values without nutrients. Fasciculol E (**9**) caused an overall significant decrease in the viability values, with strong dependence on the presence or lack of nutrients. Among the examined compounds, cerevisterol (**6**) proved to be the most toxic, while

lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) had no harmful effect at all. Fasciculic acid B (**11**) exhibited unique effects, since it was used by rotifers simply as a food source. Overall, we can state that most of the investigated steroids in general had low toxicity, although the effect of compounds varied in a wider range from non-toxic lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**) to significantly toxic cerevisterol (**6**), with strong dependence in some cases on the presence of nutrients in the experimental environment.

3. Materials and Methods

Optical rotations were measured with a Perkin-Elmer 341 polarimeter. Flash chromatography was carried out on a CombiFlash®Rf+Lumen Instrument (Teledyne ISCO, Lincoln, NE, USA) with integrated UV, UV-VIS, and ELS detection using RediSep Rf Gold normal and reversed phase flash columns (4, 12, 40 and 80 g) (Teledyne Isco, Lincoln, NE, USA). HRMS analyses were performed on a LTQ FT Ultra (Thermo Fisher Scientific, Bremen, Germany) system. The samples were dissolved in methanol. ESI ionization was used in all cases operating in positive or in case of compound **7** in the negative ion mode. HR-MS-MS were acquired using CID fragmentation method applied on the quasimolecular ion peaks (protonated/deprotonated molecular ion peaks or the sodium adduct ions or the molecular ion peaks with water losses). Data acquisition and analysis were accomplished with Xcalibur software version 2.0 (Thermo Fisher Scientific). NMR data were acquired on a Varian 800, Bruker Avance II HD 500 (Bruker, Billerica, MA, USA) or a Bruker AVANCE II HD 400 MHz spectrometer equipped with a ^{13}C enhanced salt tolerant cryoprobe, a TCI cold probe, or liquid nitrogen cooled Prodigy probe. MeOD-d_4 was used as solvent in all cases. Chemical shifts are reported in the delta scale relative to the residual solvent signals (3.31 and 49.15 ppm for ^{1}H and ^{13}C, respectively). Standard one and two dimensional NMR spectra were recorded in all cases, using the pulse sequences available in the VNMRJ 3.2 (Agilent Technologies, Santa Clara, CA, USA) or in TopSpin 3.5 sequence libraries (Bruker). Data analysis and interpretation were performed within ACD/Labs 2017.1.3 NMR Workbook Suite.

3.1. Mushroom Material

Fruiting bodies of *Hypholoma lateritium* were collected in September 2015 from the environs of Bakonybél, Hungary, and identified by Attila Sándor (Hungarian Mycological Society). A voucher specimen (collection number H018) has been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

3.2. Extraction and Isolation

The mushroom material (6.5 kg) was freeze dried and then the dry sample (630 g) was extracted with methanol (7 L) at room temperature. After concentration, the methanol extract (92 g) was dissolved in 50% aqueous MeOH and subjected to solvent-solvent partition using *n*-hexane (5 × 500 mL), chloroform (5 × 500 mL), and then ethyl acetate (5 × 500 mL). The *n*-hexane soluble phase (25 g) was applied to flash chromatography (FC) on silica gel using gradient system of *n*-hexane–acetone (0% to 100%, t = 60 min), affording 7 combined fractions (H 1–7). Fractions H 1–2 (2.4 g) were further separated by FC applying an *n*-hexane–acetone solvent system (0% to 30%, t = 45 min), which resulted in compounds **3** (0.92 g) and **4** (0.63 g). Fraction H 3 was first separated by FC on silica gel using *n*-hexane–acetone eluent with increasing polarity (0% to 40% acetone, t = 50 min), then was further purified on reversed phase using water–acetonitrile solvent system (30% to 60% acetonitrile, t = 50 min), and led to the isolation of **6** (12.4 mg). Fraction H 4 (0.49 g) was subjected to normal phase FC using an *n*-hexane-acetone system (0% to 40% acetone, t = 50 min) to give compound **5** (3.6 mg). The combined fraction H 7 (0.83 g) was analyzed in similar conditions (*n*-hexane–acetone, 10% to 50% acetone, t = 55 min), resulting in compound **7** (3.1 mg). The chloroform soluble phase (18 g) was first separated by normal phase FC, applying a solvent system of *n*-hexane–acetone (0% to 100%, t = 60 min) to obtain 11 major combined fractions (C 1–11), while the ethyl acetate phase (12 g) was subjected to normal phase FC

using a solvent system of chloroform–methanol, which resulted in six major fractions (E 1–6). Fraction C 2 (0.34 g) was separated on normal phase (solvent system *n*-hexane–acetone, 0% to 40% acetone, t = 50 min), followed by reversed phase flash chromatography purification (30% to 60% acetonitrile, t = 50 min) to give compounds **1** (3.3 mg) and **2** (3.3 mg). Fractions C 7–8 (1.05 g) were further purified on normal (*n*-hexane–acetone, 0% to 40% acetone, t = 45 min) and reversed phase (35% to 60% acetonitrile, t = 50 min) flash chromatography in subsequent steps to afford **8** (0.32 g) and **9** (0.10 g). The combined fractions C 9–11 (3.89 g) and E 1 (1.26 g) were separated by subsequent use of normal (*n*-hexane–acetone, 0% to 40% acetone, t = 50 min) and reversed phase (30% to 70% acetonitrile, t = 50 min) flash chromatography to obtain **10** (0.42 g) and **11** (0.31 g). Finally, compound **12** (28 mg) was isolated from fractions E 5–6 using a chloroform–methanol solvent system (0% to 30% methanol, t = 45 min).

Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**1**): colorless gum; $[\alpha]_D^{26}$ +7 (MeOH, *c* 0.2), ^{1}H-NMR and ^{13}C-NMR data, see Table 1. HRESIMS: *m/z* 395.33056 $[M + H\text{-}H_2O\text{-}H_2O]^+$ (delta = −0.3 ppm; $C_{28}H_{43}O$). HR-ESI-MS-MS (CID = 35%; rel. int. %): 377(100); 325(16); 311(51); 307(17); 293(29); 269(12); 251(5).

*8-Hydroxy-13-oxo-9*E*,11*E*-octadecadienoic acid* (**2**): colorless gum; $[\alpha]_D^{26}$ –5 (MeOH, *c* 0.2), ^{1}H-NMR and ^{13}C-NMR data, see Table 2. HRESIMS: *m/z* 309.20673 $[M - H]^-$ (delta = −1.3 ppm; $C_{18}H_{29}O_4$). HR-ESI-MS-MS (CID = 55%; rel. int. %): 291(100); 209(8); 195(21); 171(4).

Table 1. The ^{1}H and ^{13}C-NMR assignments of compound **1.**

Atom	C Shift	H Shift	H Multiplicity
1	44.6	1.27	m
		2.14	dd (12.7, 4.4)
2	68.0	3.58	m
3	82.4	2.82	d (9.7)
4	38.9		
5	48.8	1.11	m
6	23.0	2.05	m
7	123.8	5.57	d (5.2)
8	142.2		
9	149.2		
10	38.8		
11	117.2	5.30	br s
12	79.7	3.70	br s
13	45.3		
14	47.8		
15	33.0	1.74	m
		1.46	m
16	25.6	1.88	m
		1.49	m
17	46.2	1.44	m
18	13.9	0.50	s
19	22.3	0.97	s
20	38.2	1.75	m
21	73.8	3.91	dd (11.5, 4.3)
		2.95	t (11.1)
22	29.3	1.17	m
		1.30	m
23	28.6	1.54	m
		1.15	m
24	78.4	3.09	dd (9.8, 1.6)
25	72.3		
26	23.4	1.02	s
27	24.4	1.05	s
28	24.1	0.80	s
29	16.1	0.80	s
30	27.8	0.91	s

Table 2. The ^{1}H and ^{13}C-NMR assignments of compound **2.**

Atom	C Shift	H Shift	H Multiplicity
1	182.9		
2	39.1	2.16	t (7.6)
3	27.7	1.61	m
4	30.9	1.34	m
5	30.6	1.34	m
6	26.6	1.35	m
7	38.2	1.53	m
8	72.7	4.18	q (6.3)
9	148.7	6.24	dd (15.2, 5.8)
10	128.9	6.42	dd (15.2, 10.8)
11	144.6	7.27	dd (15.6, 10.8)
12	130.5	6.18	d (15.6)
13	204.0		
14	41.2	2.61	t (7.4)
15	25.4	1.60	m
16	32.7	1.31	m
17	23.7	1.34	m

3.3. Bdelloid Rotifer Assays

3.3.1. Model Animal

The culturing, harvesting, and monitoring methods of *Philodina acuticornis* (PA; bdelloid rotifer) have been reported in detail in our prior publication [17]. In brief, the animals were cultured in standard medium (SM), a supervised and semi-sterile environment. Clear cultures of PA were kept in standardized cell culturing flasks (cat. no.: 83.3910.302, Sarstedt AG & Co., Nümbrecht, Germany) at 25 °C and under a light/dark cycle of 12:12 h. Rotifers were chosen approximately 5 days after hatching (determined by body size; length 220 ± 10 µm and width 60 ± 5 µm), 1–2 days before the beginning of the reproductive stage.

3.3.2. Treatment Protocol

As the methodical protocols have been previously reported [17,25], only an overview of the applied techniques is given. After 24 h of the standard isolation process, the rotifers were treated in a 96 well plate (cat. no.: 3695, Costar, Corning Inc., Corning, NY, USA), n = 12/well/compounds. Starting rotifer numbers per well: 25 ± 5. For this in vivo experiment, stock solutions were prepared with 1% aqueous DMSO. The stock solutions were added to SM reaching 100 µM final concentrations for the compounds and 0.1% DMSO content. The untreated control group (UC) was grown in SM, while the control group (C) was kept in SM containing 0.1% DMSO (n = 12, well, respectively). The status of the specimens under treatment was compared to the group C. This period lasted for 72 h without feeding (toxicity interval), or feeding with homogenized yeast solution (50 µg/mL), which is enough for survival but ceases reproduction. The viability of rotifers was assessed with three different assays, utilizing video recordings with a Nikon D5500 DSLR camera (Nikon Corp., Japan).

3.3.3. Viability Assays

The impact of the test compounds on the lifespan of rotifers was assessed. The morphological viability markers chosen for evaluation were defined in our previous work [17].

Toxicity and Survival Lifespan (TSL) Assay. The TSL index is a life-conditional marker of rotifers' existence, provides mortality rate (with or without feeding).

Mastax Contraction Frequency (MCF) Assay. The mastax (pharynx) is part of the digestive system. The function of the mastax is to shred the food by periodic opening and closing. To evaluate the viability of rotifers in our experiments, we used the MCF (contraction/sec) as a quantitative viability marker.

3.3.4. Statistics

Data are presented as means ± SEM. Statistical evaluation was performed with GraphPad Prism 7.0b, using One-way ANOVA with post hoc Bonferroni test. Different levels of significance are indicated as follows: p ** ≤ 0.01, p *** ≤ 0.001 and p **** ≤ 0.0001.

3.3.5. Ethical Approval

Our experiments were performed on micro-invertebrates; therefore, according to the current ethical regulations, no specific ethical permission was needed. The investigations were carried out in accordance with globally accepted norms: Animals (Scientific Procedures) Act, 1986, associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978). Our animal studies comply with the ARRIVE guidelines.

4. Conclusions

Our current study provides the most comprehensive chemical analysis of the mushroom *H. lateritium*, affording not only novel information on the characteristic secondary metabolites of this species, but also valuable results of in vivo toxicity assays of isolated compounds, which can present an essential basis for future pharmacological experiments.

Supplementary Materials: Supplementary materials are available online: Figures S1–S39, Tables S1–S3.

Author Contributions: B.C., B.K., and A.S. performed the extraction and isolation. Z.B. and M.D. performed the spectral analysis and structure determination. Z.D. and J.K. conceived and designed the in vivo bdelloid assays, L.M. performed the bdelloid assays. J.H. and A.V. conceived and designed the experiments. Z.B., L.M., and A.V. wrote the paper. All authors reviewed the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds **1**–**5**, **8**–**12** are available from the authors.

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GIRK channel activity of Hungarian mushrooms: From screening to biologically active metabolites

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ABSTRACT

In the current study effects of fungal extracts on the G-protein-activated inwardly rectifying potassium channel (GIRK1/4) were screened using the automated patch-clamp method. 40 organic (*n*-hexane, chloroform, and 50% methanol) and aqueous extracts were prepared from 10 mushroom species native to Hungary. Among the examined fungal fractions of different polarities some *n*-hexane and chloroform extracts exerted considerable ion channel activity. One of the most active fungal species, *Hypholoma lateritium* was selected for further detailed examination to determine the compounds responsible for the observed pharmacological property. Evaluation of the ion channel activity of mushroom metabolites **1–10** revealed that lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**5**) demonstrates remarkable blocking activity on GIRK current (IC_{50} 395.1 ± 31.8 nM). Investigation of the selectivity of the GIRK inhibitory effect proved that lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**5**) has only weak inhibitory activity on hERG channel (7.9 ± 2.8% at 100 μM), exerting more than three orders of magnitude lower blocking activity on hERG channel than on GIRK channel.

1. Introduction

Atrial fibrillation (AF) is a supraventricular tachyarrhythmia with uncoordinated atrial activation and, accordingly, ineffective atrial contraction. AF is the most common cardiac rhythm disturbance in adults and the prevalence of this arrhythmia increases sharply with advancing age. AF is potentially associated with life-threatening cardiovascular conditions such as thromboembolism, stroke and heart failure, consequently results in significant morbidity and mortality [1].

Even though the underlying mechanisms of AF is not yet entirely understood, the cardiac acetylcholine-activated potassium ion channel (I_{KACh}) is considered a novel and attractive target for drug therapy in the treatment of AF [2]. This ion channel is member of the G-protein-coupled inwardly rectifying potassium channel (GIRK) superfamily and is composed of GIRK1/4 (Kir3.1 and Kir3.4) subunits [3]. It has been described that GIRK1/4 is constitutively active in myocytes from patients with chronic AF or from dog model of atrial tachycardia, and, furthermore, these studies have demonstrated spontaneous openings of these channels in myocytes from chronic AF patients [2,4,5]. Potent selective inhibitors of GIRK, tertiapin and NIP-151 terminated AF, and favorably prolonged the atrial effective refractory period in canine AF model [6,7]. Taking all these findings together, constitutively active GIRK channels in the atrium elevate proarrhythmic risk by causing dispersion of atrial repolarization and refractoriness, therefore, a selective GIRK blocker without affecting ventricular repolarization is effective and might be useful for the treatment of patients with AF.

A selective atrial target is extremely important. GIRK1 and GIRK4 subunits are abundant in atrium, but expressed in very small amounts in ventricle [8,9]. However, many of drugs exert their arrhythmogenic side effects through the ion channel encoded by the human ether-a-go-go-related gene (hERG, Kv11.1), expressed in cardiac ventricular myocytes, which has shown to be extremely promiscuous in its interactions with a wide range of structurally unrelated molecules such as psychiatric, antimicrobial, antihistamine, and even antiarrhythmic drugs [1,10]. hERG represents the α-subunit of the ion channel responsible for rapid delayed rectifier potassium current (I_{Kr}) [11,12]. The I_{Kr} current plays a fundamental role in the phase 3 of repolarization of the action potential; therefore, inhibition of hERG channel delays cardiac action potential repolarization, which lengthens the action potential duration (APD). Prolongation of ventricular action potential

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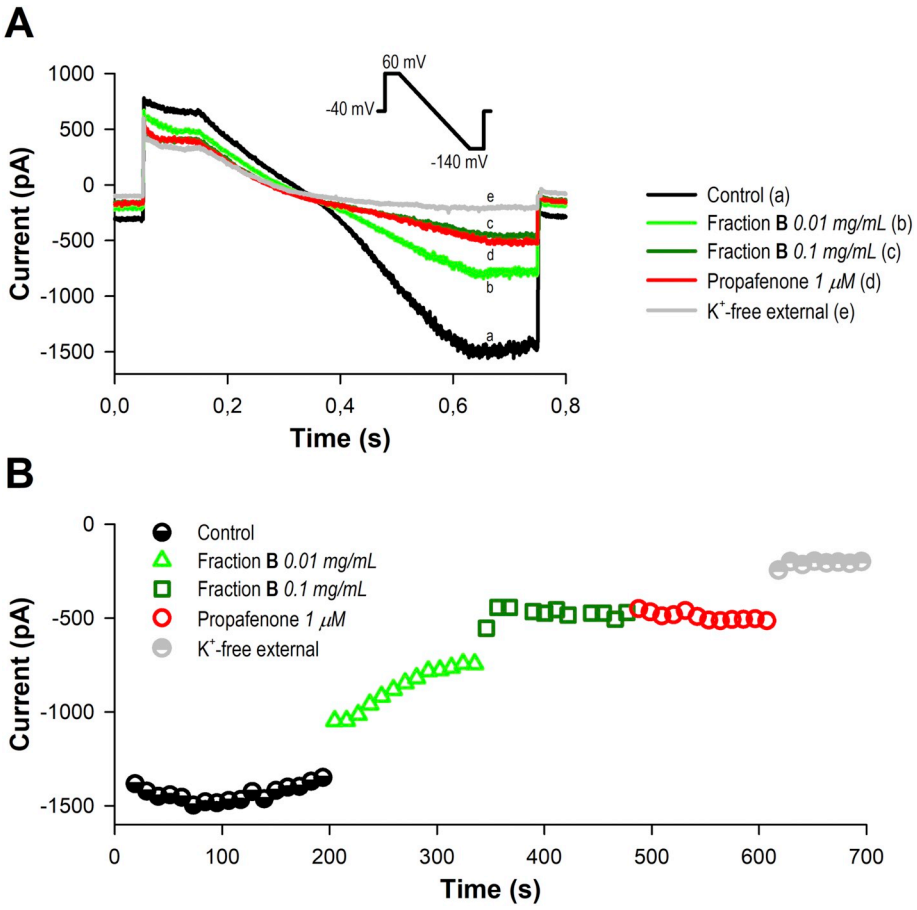

Fig. 1. Blocking effects of fraction B of *Hypholoma lateritium* on GIRK current. Panel A shows typical current curves which were recorded during application of fraction B of *H. lateritium* at 0.01 mg/mL and 0.1 mg/mL concentrations. Inset shows the applied GIRK voltage protocol. Time course of calculated inward currents from the −140 mV segment of the current sweeps is presented on panel B.

Table 1
Information on fungal species collected.

Family	Species	English name	Habitat
Entolomataceae	*Rhodocybe popinalis* (Fr.) Singer	Not known	Mixed woodland
Fomitopsidaceae	*Laetiporus sulphureus* (Bull.) Murrill	Chicken-of-the-woods	Mixed woodland
Marasmiaceae	*Megacollybia platyphylla* (Pers.) Kotl. & Pouzar	Whitelaced shank	Mixed woodland
Omphalotaceae	*Gymnopus dryophilus* (Bull.) Murrill	Russet toughshanks	Mixed woodland
	Gymnopus fusipes (Bull.) Gray	Spindleshank	*Quercus* sp. (parasitic)
Strophariaceae	*Hebeloma sacchariolens* Quél.	Sweet poisonpie	Mixed woodland
	Hypholoma fasciculare (Huds.) P. Kumm.	Sulfur tuft	Mixed woodland
	Hypholoma lateritium (Schaeff.) P. Kumm.	Brick tuft	Mixed woodland
Tricholomataceae	*Tricholoma populinum* J.E. Lange	Cottonwood mushroom	*Populus* sp. (mycorrhizal)
	Tricholomopsis rutilans (Schaeff.) Singer	Plums and custard	*Pinus* sp. (saprobic)

duration might be associated with an increased risk of the polymorphic ventricular tachycardia Torsades de Pointes, which can degenerate into ventricular fibrillation and sudden cardiac death [13–15]. In the past, several drugs have been withdrawn from major markets because of their proarrhythmic effect. At present, to avoid severe cardiotoxicity, every new compound must go through preclinical safety testing determined by the U. S. Food and Drug Administration, the European Medicines Agency and other regulatory entities [16].

The popular term mushroom is applied for macrofungi with distinctive fruiting bodies observable to the naked eye. The number of different mushroom species is estimated to be 140,000, of which only 10% are known to science. They have long been recognized for their therapeutic values, particularly in traditional medicines of Far-Eastern countries. Mushrooms are valuable sources of biologically active compounds possessing antibacterial, anticancer and immunmodulatory properties [17,18]. Extended investigation of active substances originated from mushrooms in different screening systems may result in further discoveries of valuable new compounds.

In the present study, the effects of basidiomycetes mushroom extracts on the G-protein-activated inwardly rectifying potassium channel (GIRK1/4) were investigated using the automated patch-clamp method. For this purpose organic and aqueous extracts were prepared from 10 different mushrooms native to Hungary. The extracts of different polarities were investigated in the cell-based GIRK channel inhibitory assay on HEK-GIRK cell line. One of the most active species was selected for further detailed chemical examination to determine its biologically active metabolites. The GIRK channel modulatory activities of the isolated compounds were also evaluated, with the aim of discovering new promising blockers of the GIRK1/4 channel. In addition, the most potent blocker of GIRK proteins was further tested for its hERG-related cardiotoxicity with automated patch-clamp technology on HEK-hERG cell line.

Table 2
Yields and blocking effects of mushroom extracts at 0.01 mg/mL and 0.1 mg/mL concentrations on GIRK ion channels (n = 2–3).

Species	Solvent	Yield (w/w%)	Inhibition %		SEM	0.1 mg/mL
			0.01 mg/mL	0.1 mg/mL	0.01 mg/mL	
Gymnopus dryophilus	A	1.1	28	72	0	3
	B	0.9	35	80	5	4
	C	7.5	10	15	1	2
	D	5.2	10	20	5	4
Gymnopus fusipes	A	2.8	29	74	4	8
	B	1.3	30	76	6	0
	C	18.3	6	11	1	1
	D	4.1	21	30	1	1
Hebeloma sacchariolens	A	1.7	33	67	4	5
	B	1.6	37	74	2	1
	C	8.2	6	28	3	2
	D	1.6	20	37	6	3
Hypholoma fasciculare	A	1.6	23	53	1	4
	B	3.4	25	60	6	9
	C	7.2	16	35	6	3
	D	4.0	12	20	4	6
Hypholoma lateritium	A	2.2	33	69	3	1
	B	1.0	53	79	2	1
	C	16.4	25	46	0	2
	D	3.9	8	14	6	3
Laetiporus sulphureus	A	1.3	34	74	6	8
	B	0.7	28	68	6	13
	C	5.8	25	43	6	11
	D	3.6	11	24	1	7
Megacollybia platyphylla	A	0.9	39	61	3	15
	B	0.5	29	68	6	3
	C	13.0	11	11	4	6
	D	6.2	8	20	0	6
Rhodocybe popinalis	A	1.4	19	55	3	3
	B	0.8	10	23	3	5
	C	8.8	12	16	1	2
	D	6.2	7	16	1	0
Tricholoma populinum	A	6.0	5	25	0	4
	B	1.7	41	90	2	1
	C	15.0	9	17	1	16
	D	4.6	17	20	3	12
Tricholomopsis rutilans	A	1.3	16	52	4	3
	B	2.7	37	65	4	12
	C	11.5	15	31	8	11
	D	1.3	12	17	0	3

2. Materials and methods

2.1. Samples

Mushroom samples were collected in Hungary in 2012 by A. Ványolós and members of Mushroom Society of Miskolc and of Mushroom Society of Zemplén (Hungary). The collected species were identified by J. Béres (Mushroom Society of Miskolc), J. Kőszeginé Tóth (Mushroom Society of Zemplén) and A. Ványolós. Representative voucher specimens (No. IC 1–10) have been deposited in the Department of Pharmacognosy, University of Szeged. The samples were stored at −20 °C until processing.

2.2. Sample preparation

The sporocarps of collected mushrooms were freeze-dried and ground. Each freeze-dried sample (10 g) was extracted with 3 × 100 mL methanol by ultrasonication for 3 × 15. After filtration, the solutions were evaporated under reduced pressure. The residues were dissolved in 50 mL of 50% aqueous MeOH and were subjected to solvent-solvent partition between *n*-hexane (4 × 25 mL) (extract A) and $CHCl_3$ (4 × 25 mL) (extract B) and the residue gave extract C. After extraction with MeOH, the residual mushroom materials were dried and extracted with 50 mL of boiling water for 15 min. The filtered extracts were freeze-dried, providing extract D.

Isolation, structure determination and characterization of compounds **1–10** are described elsewhere [19].

2.3. Automated planar patch-clamp measurements

GIRK and hERG ion currents were measured using planar patch-clamp technology in the whole-cell configuration with a four channel medium throughput fully automated patch-clamp system (Patchliner, Nanion Technologies GmbH, Munich, Germany; [20]. The pipetting protocols were controlled by PatchControlHT 1.09.30 software (Nanion Technologies GmbH., Munich, Germany). Data acquisition and online analysis were performed with an EPC-10 Quadro patch-clamp amplifier (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany), using PatchMaster 2.65 software (HEKA Elektronik Dr. Schulze GmbH, Lambrecht/Pfalz, Germany). Currents were low-pass filtered at 2.9 kHz using the internal Bessel filter of the amplifier and digitized at 10 kHz.

Automated patch-clamp experiments were carried out at room temperature on suspension of stable transfected cell lines. Suspension of cells for measurements was derived from running cell culture. Cells were maintained in incubator at 37 °C, in 5% CO_2. Before experiments, cells were washed twice with PBS (Thermo Fisher Scientific Inc., Waltham, USA) and then detached with trypsin-EDTA (PAN-Biotech GmbH, Aidenbach, Germany) for 1–3 min. Trypsin was blocked with serum-containing media. The cell suspension was next centrifuged (2 min, 100 *g*), resuspended in serum-free media at a final density of

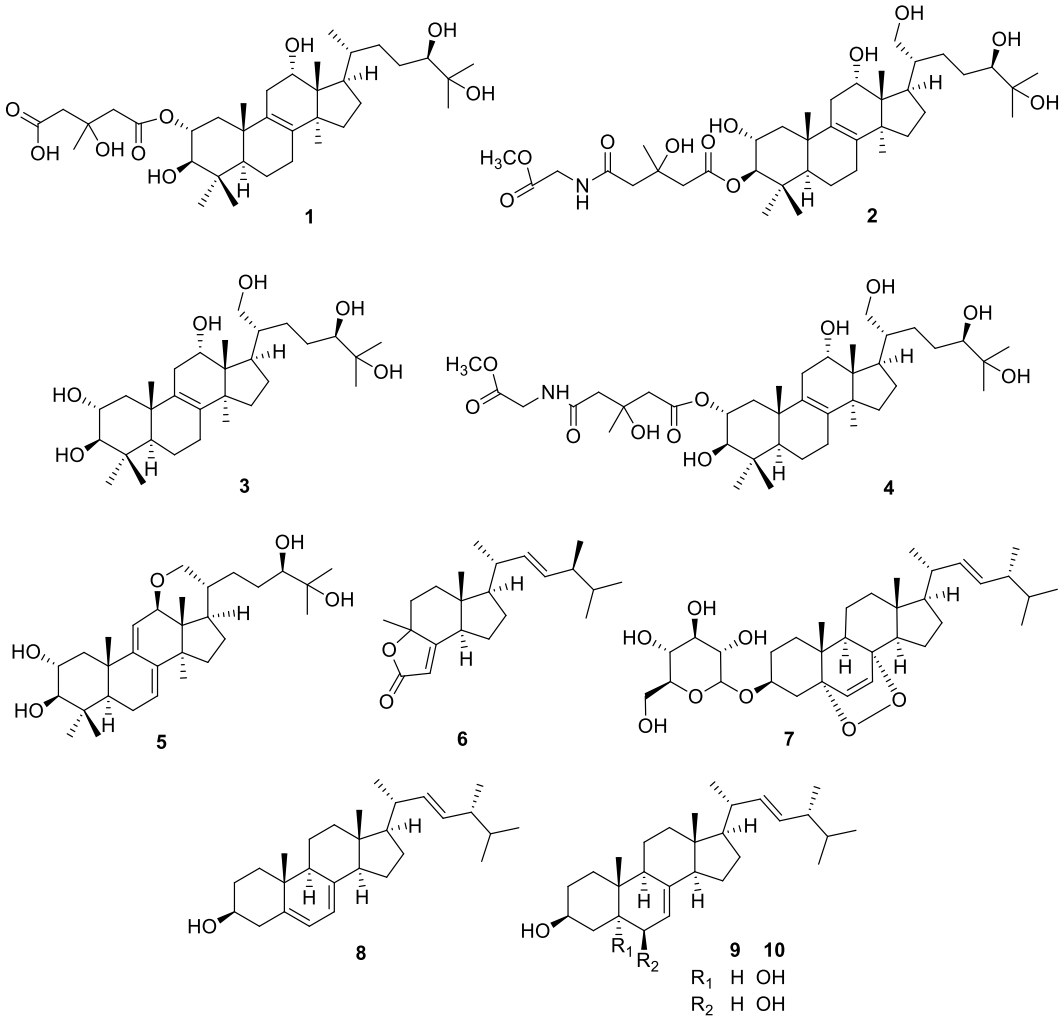

Fig. 2. Triterpenes (**1 − 10**) from *Hypholoma lateritium*.

Table 3
GIRK channel inhibitory activity of isolated compounds of *Hypholoma lateritium* at 1 μM and 10 μM concentrations (n = 2–3).

Compound	inhibition %		SEM	
	1 μM	10 μM	1 μM	10 μM
1	13	23	2	8
2	11	23	2	3
3	10	10	4	7
4	10	27	1	2
5	27	60	5	11
6	11	23	3	1
7	14	24	1	5
8	10	19	1	1
9	8	16	1	6
10	7	13	1	6

1×10^6–5×10^6 cells/mL, and kept in the cell hotel of the Patchliner. Cells were recovered after 15–30 min and remained suitable for automated patch-clamp recordings for up to 4 h.

Stock of extra- and intracellular solutions were made for automated patch-clamp recordings. Chemicals were purchased from Sigma-Aldrich Corporation (St. Louis, USA). All solutions were sterile filtered. Aliquots were stored at − 20 °C and warmed up to room temperature before use.

Effects of fungal extracts and compounds isolated from *Hypholoma lateritium* were tested. A stock solution of test material was prepared in each case. The concentrations of the examined substances in the stock solutions were 50 mg of dried material/mL for the mushrooms extract and 10 mM for compounds **1–10**. The solubilizing agent was dimethyl sulphoxide (DMSO, Sigma-Aldrich Corporation, St. Louis, USA) in all cases. Aliquots were stored at − 20 °C. Before experiments, stock solutions were further diluted with high K^+ external solution (GIRK assay) or external solution (hERG assay) to give appropriate concentrations for the measurements. The final DMSO concentrations in the tested samples were 1% or less.

2.3.1. GIRK channel inhibitory assay

Experiments were carried out on HEK-293 (human embryonic kidney) cells stably expressing the GIRK1/4 (Kir3.1/3.4) K^+ channels by adapting a method described earlier [21]. Cell line originated from UCL Business Plc. (London, Great Britain). Cells were maintained in DMEM (Thermo Fisher Scientific Inc., Waltham, USA) medium supplemented with 10% FBS (PAN-Biotech GmbH, Aidenbach, Germany) and 182 μg/mL zeocin (Thermo Fisher Scientific Inc., Waltham, USA).

The following solutions were used during patch-clamp recordings (compositions in mM): external solution: NaCl 140, KCl 4, glucose-monohydrate 5, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); high K^+ external solution: NaCl 135, KCl 25, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); K^+-free external solution: NaCl 160, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); internal solution: K-gluconate 40, NaCl 20, KF 60, EGTA 20 and HEPES 10 (pH 7.2, KOH), supplemented with 0.9 mM GTPγS before the experiments to induce channel activation.

The voltage protocol for GIRK ion channel assay (see inset on Panel

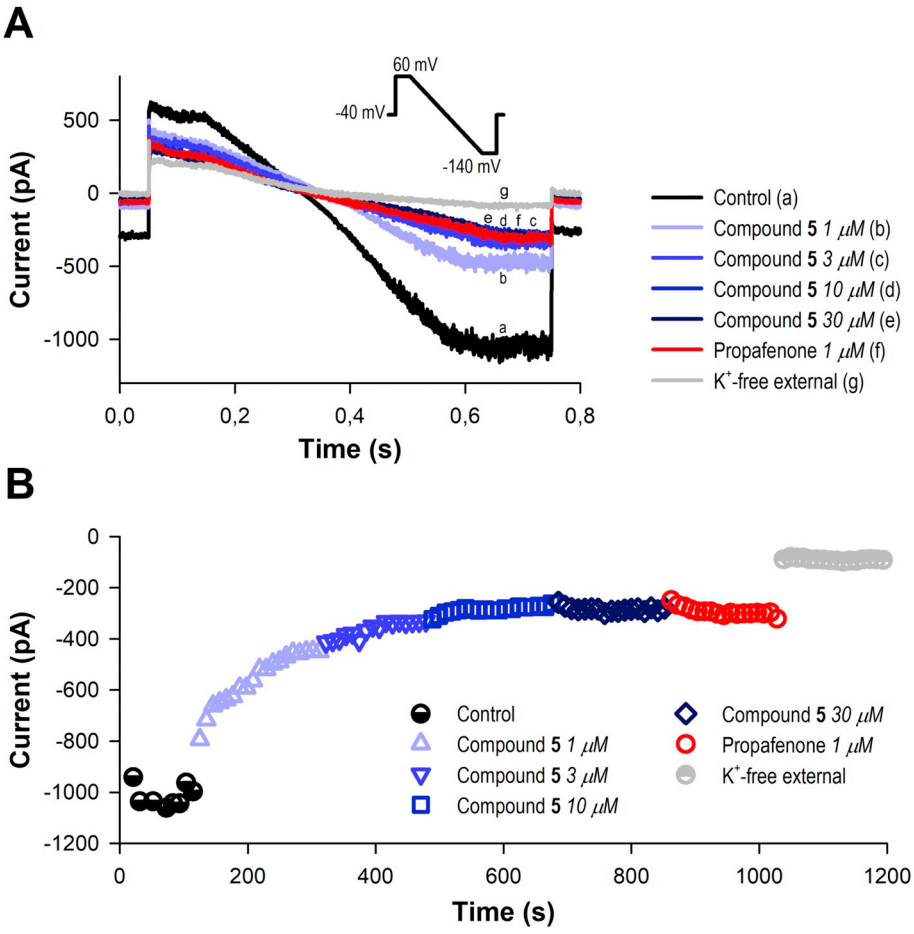

Fig. 3. Blocking effects of compound **5** on GIRK current. Panel A depicts original current sweeps which were recorded during application of compound **5** at 1 µM, 3 µM, 10 µM and 30 µM concentrations. Inset shows the applied GIRK voltage protocol. Time course of calculated inward currents from the −140 mV segment of the current curves is presented on panel B.

A, Figs. 1, 3 and 4) started with a depolarizing voltage step to 60 mV for 100 ms before a 500 ms long hyperpolarizing ramp to −140 mV was applied. Then the membrane potential remained at −140 mV for 100 ms before returning to the holding potential of −40 mV. The inward currents were calculated from the −140 mV segment. The pulse frequency was approximately 0.1 Hz.

At the beginning of recordings, the normal external solution (4 mM K^+) was replaced to high K^+ (25 mM K^+) external solution in order to increase the current amplitude. After 2–3 min of control period, the test compounds were added to the cells in increasing concentrations, each for approximately 3 min. Propafenone (1 µM, Sigma-Aldrich Corporation, St. Louis, USA) was used as a reference compound. In preliminary studies, we measured that the positive control propafenone exerted its effect with 373.0 ± 27.7 nM IC_{50} value and at 1 µM concentration inhibited the GIRK current by 71.4 ± 4.6% (n = 6). Finally, potassium free external solution was applied to completely cease inward potassium currents. The data were corrected with the current values measured in the potassium free external solution, which served as the baseline.

2.3.2. hERG channel inhibitory assay

hERG measurements were performed on HEK-293 cells stably transfected with cDNA encoding the hERG (Kv11.1) K^+ channel as described earlier in detail [21,22]. Cell line was purchased from Cell Culture Service (Hamburg, Germany). Cells were maintained in IMDM (PAN-Biotech GmbH, Aidenbach, Germany) medium supplemented with 10% FBS (PAN-Biotech GmbH, Aidenbach, Germany), 2 mM L-glutamine (PAN-Biotech GmbH, Aidenbach, Germany), 1 mM Na-pyruvate (PAN-Biotech GmbH, Aidenbach, Germany) and 500 µg/mL G418 (Thermo Fisher Scientific Inc., Waltham, USA).

The following solutions were used during patch-clamp experiments (compositions in mM): external solution: NaCl 140, KCl 4, glucose-monohydrate 5, $MgCl_2$ 1, $CaCl_2$ 3 and HEPES 10 (pH 7.4, NaOH); internal solution: KCl 50, NaCl 10, KF 60, EGTA 20 and HEPES 10 (pH 7.2, KOH).

The voltage protocol for hERG ion channel (see inset on Panel A, Fig. 5) started with a short (100 ms) –40 mV step to establish the baseline region. A depolarizing step was applied to the test potential of 20 mV for 3 s, and then the cell was repolarized to −40 mV (1 s) to evoke outward tail current. Holding potential was −80 mV. The pulse frequency was approximately 0.1 Hz. The peak tail current was corrected the leak current defined during the first period to −40 mV.

Recording started in external solution. After this control period, the test compound was applied for approximately 3 min. Following the test compound, 10 µM amitriptyline was used as a reference inhibitor then a wash-out step terminated the pipetting protocol.

2.4. Statistics

All data are expressed as arithmetic means ± standard error (SEM). Statistical analysis was performed with Student's t-test for paired data, and corrected using the Holm-Bonferroni method. Differences were considered statistically significant when P value was < 0.05.

3. Results and discussion

Initially, as part of the screening study the GIRK channel inhibitory effects of some mushroom extracts obtained from species native to Hungary were evaluated. Extracts were examined at two concentrations (0.01 mg/mL and 0.1 mg/mL) on two to three cells. Totally, 40 extracts of 10 higher basidiomycete mushrooms (see Table 1) were assessed: *Gymnopus dryophilus, Laetiporus sulphureus, and Tricholoma populinum*

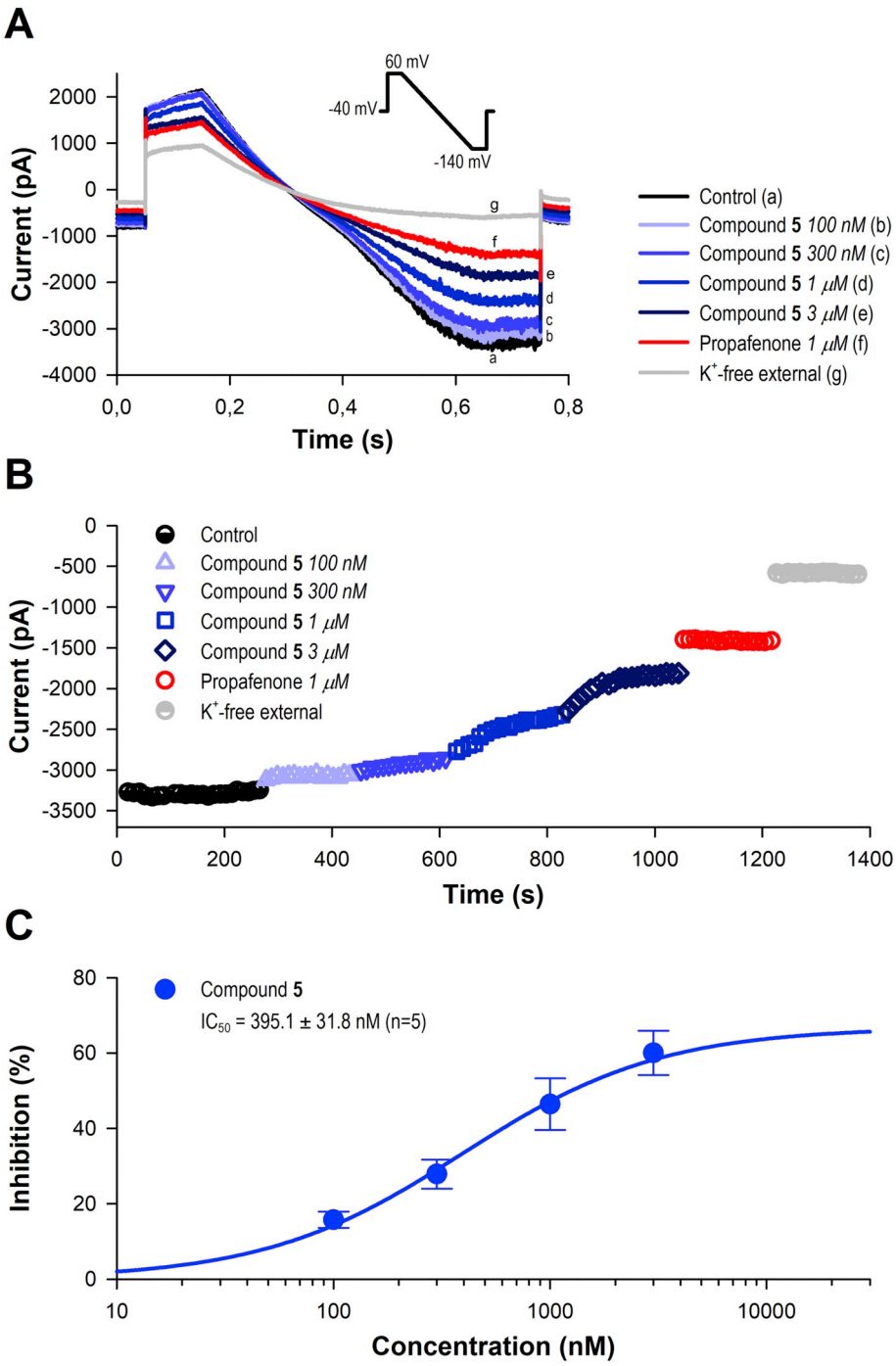

Fig. 4. Inhibitory effects of compound **5** on GIRK current. Panel A displays representative current curves which were recorded during application of compound **5** at 0.1 μM, 0.3 μM, 1 μM and 3 μM concentrations. Inset shows the applied GIRK voltage protocol. Time course of calculated inward currents from the −140 mV segment of the current curves is presented on panel B. Panel C shows the dose-response curves of compound **5**. The GIRK channel inhibitory activity of compound **5** could be characterized by the relative IC_{50} value of 395.1 ± 31.8 nM (n = 5).

are edible; *Gymnopus fusipes*, *Hypholoma lateritium*, *Megacollybia platyphylla, Rhodocybe popinalis* and *Tricholomopsis rutilans* are either not recommended for consumption or there are conflicting reports on their edibility; while *Hebeloma sacchariolens* and *Hypholoma fasciculare* are poisonous species. To the extent of our knowledge no other study has previously investigated the potential GIRK channel activity of mushrooms. According to the results obtained some fungal species display notable inhibitory activity on GIRK channel. *G. dryophilus* and *G. fusipes* are related mushrooms, which have not been extensively studied, the former contains a water-soluble polysaccharide, which has a potential immunomodulatory property based on the inhibition of NO production [23,24]; the latter is a parasitic, root-decay fungus, which has been recently identified as a source of unique cyclic octadecapeptides. [25]. Very little is known about the chemistry of *H. sacchariolens*, a small mushroom with a bitter taste, but a sweet and flowery smell. According to Wood et al., the source of the specific, pleasant odor is 2-aminobenzaldehyde [26]. *H. lateritium*, brick cap by its vernacular name, is a fairly popular edible mushroom in Japan, Korea and the United States, but in Europe it is considered inedible. Compared to its more common relative, the poisonous sulfur tuft (*H. fasciculare*), the chemistry and

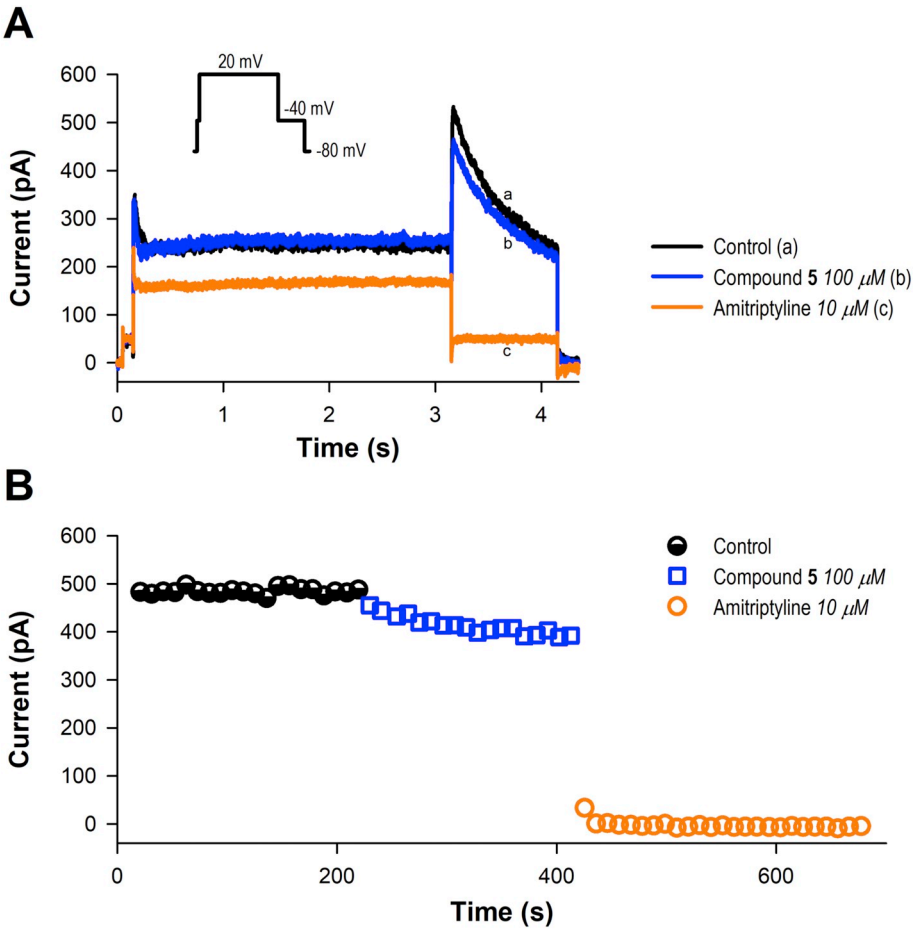

Fig. 5. Effects of compound **5** on hERG current. Panel A presents typical hERG current sweeps during the application of 100 μM compound **5**. The inset shows the applied hERG voltage protocol. The original current traces reveal that **5** slightly blocked the hERG channel, while addition of reference compound amitriptyline (10 μM) fully blocked the current. Time course of decrease in the peak tail current are presented on panel B.

pharmacology of *H. lateritium* are explored in a weaker extent. This species was found to produce several triterpenes e.g. fasciculols, fasciculic acids and sublateriols as well as sesquiterpenes eg. naematolin, a caryophyllane derivative with cytotoxic activity [27–29].

L. sulphureus, commonly known as sulphure polypore, is a delicious bracket fungus, growing on dead or living trees. It has a vast scientific literature, many of its chemical constituents (polysaccharides, triterpenes, polyene pigments) have been described [30–32], possessing a variety of pharmacological properties (anticancer, antimicrobial, antioxidant and hypoglycemic) [31,33,34]. *T. populinum* - also known as cottonwood mushroom - is a mycorrhizal fungus with lower culinary value which produces ergostane type steroids and methylsulfinyladenosine derivatives [35].

Among the fractions with different polarities, fraction A (*n*-hexane fractions with the more lipophilic constituents) and fraction B ($CHCl_3$-soluble compounds) proved to be active (i. e., at least 50% decrease in the current at 0.1 mg/mL concentration). The aqueous (fraction D) and aqueous MeOH (fraction C) extracts did not demonstrate considerable activity on GIRK channel (< 40% decrease in the current at 0.1 mg/mL) (see Table 2.)

However, there are some exceptions: neither fraction B of *R. popinalis* nor fraction A of *T. populinum* exerted remarkable blocking effect on the GIRK channel, whereas fractions C of L. *sulphureus* and *H. lateritium* proved to have notable inhibitory activities.

Fraction B of *H. lateritium* proved to be the most effective (53% decrease on GIRK current) at the lower (0.01 mg/mL) concentration among the tested fungal extracts (Fig. 1).

Therefore, this species was chosen for in-depth chemical analysis in order to identify the secondary metabolites responsible for the observed ion channel activity. Thanks to combined chromatographic techniques we have previously identified a series of triterpenes (see Fig. 2): fasciculic acid B (**1**), fasciculol E (**2**), fasciculol C (**3**), fasciculol F (**4**), lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**5**), demethylincisterol A2 (**6**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**), ergosterol (**8**), 3β-hydroxyergosta-7,22-diene (**9**), and cerevisterol (**10**) [19].

The fungal metabolites were involved in the GIRK channel inhibitory assay at 1 μM and 10 μM concentrations. Compounds were tested on two to three cells (Table 3).

Most of the isolated metabolites possessed moderate activity on GIRK channel except compound **5**, which found to be an active inhibitor of GIRK channel. Therefore, the dose-response curve and IC_{50} value of **5** were characterized in detailed experiments where its effect was tested in 4 concentrations on 5 cells. Initially, investigations were performed at 1 μM, 3 μM, 10 μM and 30 μM concentrations. GIRK current was considerably reduced by these concentrations; the effects on the inward current were statistically significant in all tested concentrations. Application of compound **5** at 1 μM concentration inhibited the GIRK current by 42.0 ± 6.4%. Elevation of the concentration of **5** to 3 μM and to 10 μM resulted in further decrease in GIRK current (60.8 ± 5.0% and 66.4 ± 4.5% inhibitions, respectively). Further increase in test compound level did not alter the current (66.7 ± 4.6% inhibition at 30 μM). Consequently, the maximum effect was determined to this value, and, presumably, the drug may only inhibit 66.7% of the current even at the highest doses (E_{max} = 0.6675). Original GIRK current curves and the time course of decrease in the current are shown on Fig. 3. Compound **5** was also tested in detailed experiments on GIRK cell line at 0.1 μM, 0.3 μM, 1 μM and 3 μM concentrations, in order to acquire the complete dose-response curve. GIRK current was considerably reduced by compound **5** in concentration-dependent manner. The relative IC_{50} value (the concentration corresponding to a response midway between the estimates of the lower and

upper plateaus of the dose-response curve, i.e., 0% and 66.7% inhibition) was determined to be 395.1 ± 31.8 nM (Fig. 4) [36].

Compound **5** was also tested on HEK-hERG cell line and selectivity of their GIRK blocking effect was evaluated with these experiments. Selectivity study was performed at 100 μM concentration on 12 cells. Compound **5** exhibited only slight inhibitory activity (7.9 ± 2.8%) on hERG channel even at this high concentration, showing more than three orders of magnitude higher blocking activity on GIRK channel compared to the results obtained on hERG channel. Original hERG current sweeps during the application of compound **5** at 100 μM concentration and the time course of decrease in the peak tail current are shown on Fig. 5.

Atrial fibrillation (AF) is the most common serious abnormal heart rhythm. In the last two decades, AF has become a very important public health issue, and responsible for huge and rapidly growing healthcare expenditures in Western countries [1,37]. AF is present in 0.12%–0.16% of those younger than 49 years, in 3.7%–4.2% of those aged 60–70 years, and in 10%–17% of those aged 80 years or older [37]. > 750,000 hospitalizations happen because of AF, and it contributes to an estimated 130,000 deaths each year just in the U.S. [38]. Current antiarrhythmic drugs have numerous disadvantages, particularly the lack of atrial selectivity associated with an increased risk of side effects especially proarrhythmia and the poor efficacy rate (e.g., 50–60% for amiodarone, which is generally considered as the most effective drug). With this background, more targeted approaches are required to improve therapy of AF [1,39].

Exploiting the potential biological activity of macrofungi, extracts of basidiomycete mushrooms were investigated in our GIRK screen system, with the aim to identify natural sources of promising ion channel blocking compounds. Extracts of different polarity were screened, and one of the most active species, *H. lateritium* was further investigated for active compounds. Among fungal metabolites **1–10**, compound **5** demonstrated notable blocking activity on GIRK current. Considering its intense blocking effect and high selectivity, **5** is a potential promising agent in treatment of AF, despite no complete inhibition was observed. The current study unambiguously confirms that higher macrofungi deserve special attention in terms of secondary metabolites with valuable pharmacological properties.

Declaration of Competing Interest

The authors declare they have no conflict of interest.

Acknowledgements

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FULL PAPER

CHEMISTRY & BIODIVERSITY

Extracts and Steroids from the Edible Mushroom *Hypholoma lateritium* Exhibit Anti-Inflammatory Properties by Inhibition of COX-2 and Activation of Nrf2

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Hypholoma lateritium is an edible macrofungus with a common distribution in Europe, North America, and the Far East. The aim of this study was to investigate the potential anti-inflammatory effects of *H. lateritium* extracts and its isolated steroids: fasciculic acid B, fasciculol E, fasciculol C, lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol, fasciculol F, and demethylincisterol A2. Organic (hexane, chloroform and 50% methanol) and water extracts of *H. lateritium* were subjected to *in vitro* assays to determine pro-inflammatory protein levels, such as cyclooxygenase-2 (COX-2), cytosolic prostaglandin E2 synthase (cPGES), and antioxidant nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Fungal extracts demonstrated significant activities on pro-inflammatory protein levels with minor differences among the activities of the fractions of different polarities. All the compounds proved to exert notable inhibitory properties on COX-2 and were capable to stimulate the Nrf2 pathway. Fungal extracts and the compounds exerted no cytotoxic activities on RAW 264.7 cells.

Keywords: *Hypholoma lateritium*, steroids, anti-inflammatory activity, COX-2, Nrf2.

Introduction

The genus *Hypholoma*, which means 'mushrooms with threads', belongs to the family Strophariaceae and includes mushroom species possessing characteristic well-pigmented pileus and variably developed thread-like veil, which does not form a membranous annulus on the stipe.[1] The genus consists of about 30 species worldwide, occurring in temperate to tropical regions, growing on decomposing wood, living trees, or soil.[2] *Hypholoma* species are recognized as active wood and litter decomposers and play a significant role in forest ecosystems, being used not only in bioconversion of cellulose, fabric and dye industrial residues,[3,4] but also in biological control of phytopathogenic fungi.[5,6] *Hypholoma fasciculare* is the most widespread and investigated member of the genus, which is known for its antioxidant and antimicrobial activities,[7] producing different types of fungal metabolites, e.g., styrylpyrone-type compounds (hypholomins, fasciculins),[8] steroids (fasciculic acids, fasciculols),[9,10] and sesquiterpenoids (fasciculones).[11,12]

Apart from *H. fasciculare*, there is *H. lateritium* (brick cap mushroom), a less known related species, but still with a quite common distribution in Europe, North-America and the Far East. It is a saprobic macrofungus, occurring regularly in small tufts or sometimes singly on hardwood stumps and exposed roots of dead hardwood trees. *H. lateritium* was reported to contain

steroid compounds, e.g., fasciculols, fasciculic acids and sublateriols[13,14] as well as sesquiterpenes, e.g., naematolin, a caryophyllane derivative with antiproliferative property.[15] We have recently explored the chemistry of this species and identified a series of steroids with remarkable structural diversity including ergostane and lanostane derivatives, along with highly degraded sterols with ion channel modulating properties.[16,17]

As regard the pharmacology of *H. lateritium*, previous investigations revealed that this species possesses considerable biological properties; however, these experiments were performed with crude extracts without identifying the major fungal metabolites responsible for the observed biological activity. Lee at al. demonstrated that the extract of this species decreases TNF-α-induced inflammation in human umbilical vein endothelial cells. The butanol fraction of *H. lateritium* inhibited TNF-α-induced monocyte adhesion to endothelial cells; moreover, it dose-dependently decreased the expression of inducible nitrogen oxygen synthase and cyclooxygenase-2.[18] In another article, Lee et al. investigated the inhibitory effect of *H. lateritium* extract on highly invasive and metastatic tumor cells. The hexane fraction of brick cap significantly inhibited the invasion and migration of MDA-MB-231 breast cancer cells in the Matrigel invasion assay and wound-healing investigations, respectively. The results obtained suggested that hexane extract of *H. lateritium* inhibits the metastatic potential of MDA-MB-231 cells by inhibiting the phosphorylation of JNK/p38 and reducing AP-1 and NF-κB DNA-binding activities.[19]

Despite of its wide geographical distribution and richness in various fungal metabolites with pharmacological potential, the ethnomycological profile of *H. lateritium* is rather unexplored. Nonetheless, this mushroom was used in Swedish folk medicine as an anti-inflammatory agent in alleviating symptoms of rheumatic disorder.[20] Therefore, we conducted a research to explore the anti-inflammatory properties of *H. lateritium* extracts and its characteristic constituents, fasciculic acid B (**1**), fasciculol E (**2**), fasciculol C (**3**), lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**4**), fasciculol F (**5**), and demethylincisterol A2 (**6**) for the purpose of confirming the traditional use of this species.

Results and Discussion

Basidiomycota mushrooms are known to possess various beneficial pharmacological properties including anti-inflammatory activity.[21] Previous studies revealed that several extracts prepared from certain edible mushrooms have anti-inflammatory potential: *Cantharellus cibarius*,[22] *Imleria badia*,[23] and *Agaricus bisporus*.[24] In the current study, we examined the pro- or anti-inflammatory properties of *H. lateritium* extracts and identified specific fungal metabolites (**1**–**6**) which may contribute to the favorable biological activities of this fungal species. Accordingly, organic (hexane, chloroform and 50% methanol) and H_2O extracts of *H. lateritium* were prepared, and then, they were subjected to *in vitro* tests in order to determine the pro-inflammatory protein levels, such as COX-2, cPGES as well as Nrf2 using Western blot techniques. Regarding the cytotoxic effect, no such activities were observed in RAW 264.7 cells incubated with mushroom extracts and fungal metabolites **1**–**6**. Cell viabilities were around 100% after treatment. According to results, all fractions demonstrated significant biological activities in the assays performed, however, minor differences were observed among the activities of the fractions with different polarities (*Figure 1*).

In RAW 264.7 cells activated with LPS and incubated with mushroom extracts A–D, an increase of Nrf2 was observed. In the same way, higher levels of cPGES protein were detected in macrophages co-treated with LPS and extracts A–D, but the values obtained were significantly lower compared to those of the LPS-activated cells. The investigations revealed a decrease in COX-2-levels in RAW 264.7 cells co-treated with mushrooms extracts and LPS in comparison with the experiment of LPS-activated macrophages.

To identify the main constituents of *H. lateritium* responsible for the detected anti-inflammatory properties of the crude fungal extracts, we proposed to perform the pharmacological assay of characteristic compounds of *H. lateritium*. The fungal metabolites investigated in the current study belong to the vast class of steroids (*Figure 2*).

Fasciculic acid B (**1**), fasciculol E (**2**), fasciculol C (**3**), and fasciculol F (**5**) represent a special group of compounds known as fasciculols which are specific to mushrooms of the *Hypholoma* genus, especially *H. fasciculare* and *H. lateritium*, lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**4**) is a related steroid recently identified in *H. lateritium*, while demethylincisterol A2 (**6**) is a highly degraded sterol

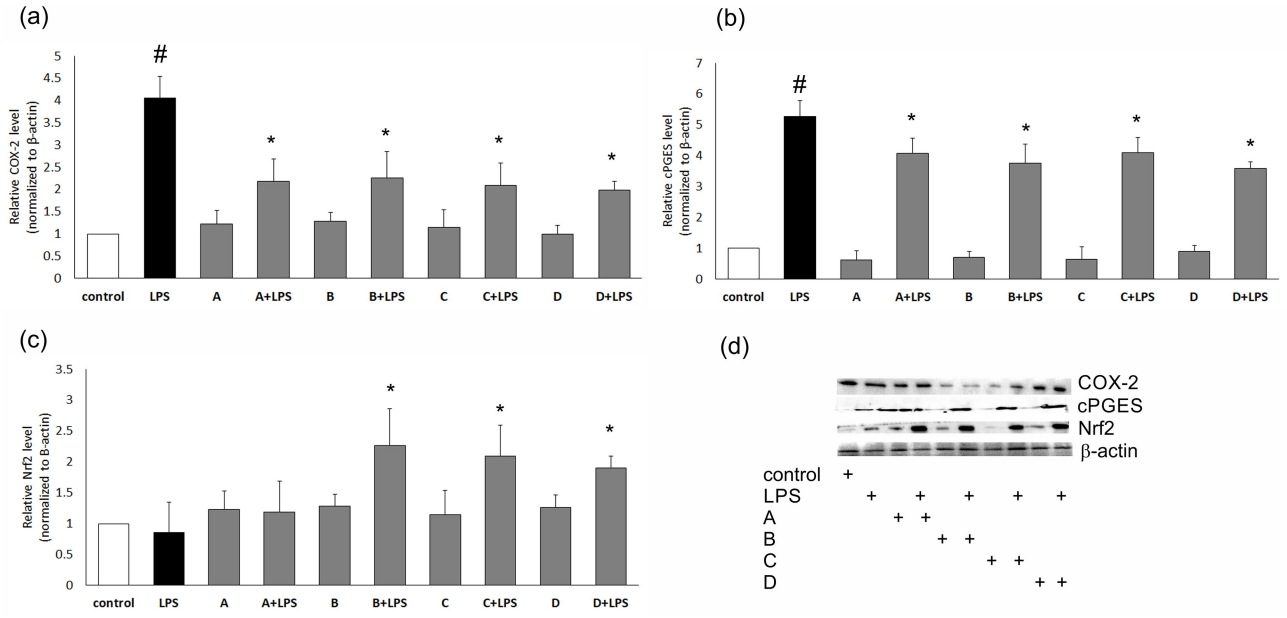

Figure 1. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with extracts of *H. lateritium* (100 μg) and activated with LPS. $N = 5$. # *vs.* control, * *vs.* LPS, $p < 0.05$.

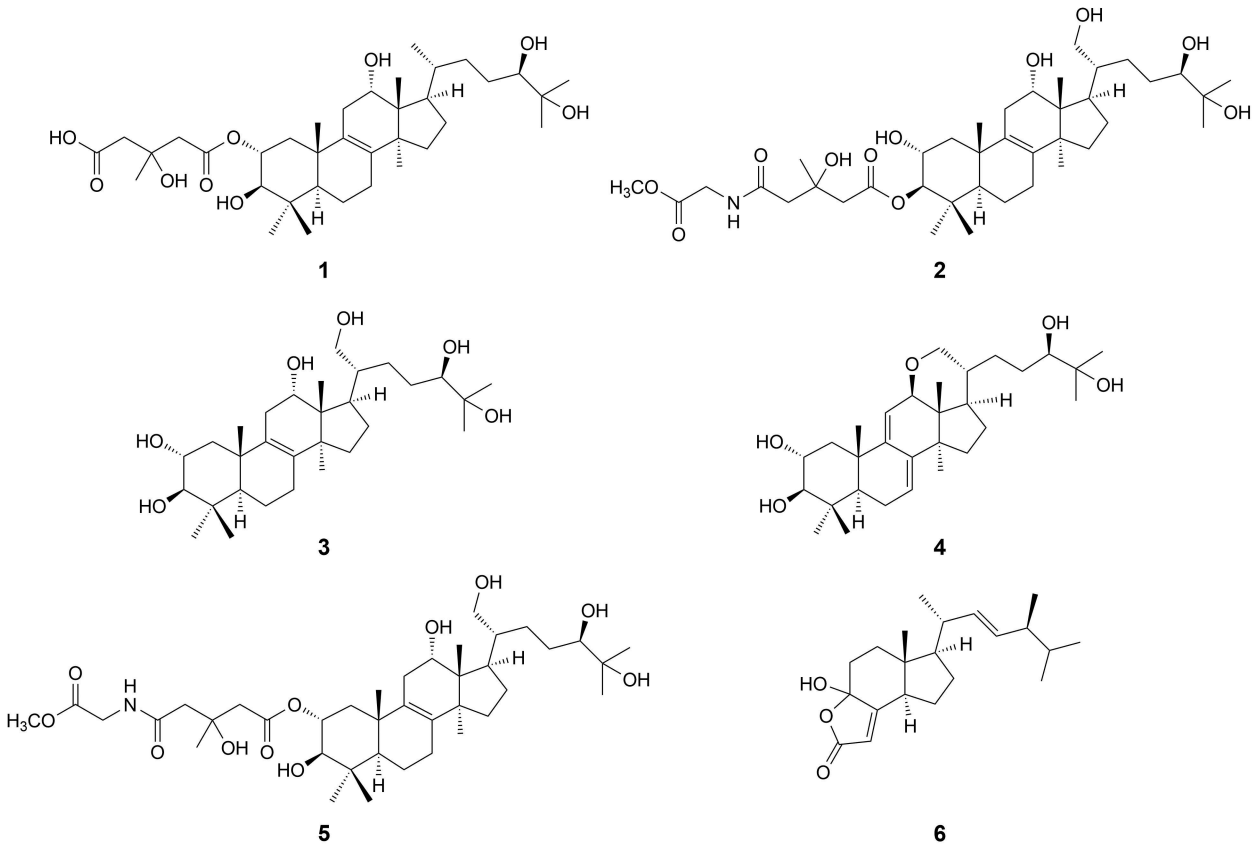

Figure 2. Structures of steroids from *Hypholoma lateritium.*

reported originally from a marine sponge of *Homaxinella* sp.[25] Previous investigations revealed that these compounds could have important pharmacological properties, including the calmodulin antagonistic activity of fasciculic acid B (**1**) and the cytotoxic property of demethylincisterol A2 (**6**).[10,25] Our experiments (*Figure 3*) revealed that **1**–**6** activated cPGES, but levels of this protein were lower than those in LPS-activated RAW 264.7 cells. In cells activated with LPS and incubated with **1**–**6**, we experienced an increase of Nrf2. Compounds **1**–**6** in general proved to possess similar activities, however, fasciculol C (**3**) represents a particular case, because when cells were treated with **3** alone the amount of cPGES was the lowest, while the level of Nrf2 was the highest among the values obtained in all experiments.

Macrophages activated with LPS and incubated with fungal metabolites were characterized by decreased COX-2 levels when compared to LPS-activated macrophages.

Nrf2, or nuclear factor (erythroid-derived 2)-like 2, is an essential transcription factor that controls the expression of antioxidant proteins that protect against oxidative damage produced by injury and inflammation. It is a key participant of cellular defense mechanism; activation of Nrf2 leads to a subsequent production of proteins and antioxidant enzymes, providing the damaged cells and tissues with a complex antioxidant defense. Plenty of studies unequivocally demonstrate that many plant metabolites from fruits and vegetables, e.g., curcumin,[26] resveratrol[27] and sulforaphane[28] are capable of regulating Nrf2. Although many plants produce a variety of compounds with Nrf2 activity, the potential of mushroom metabolites in this view is largely unexplored. However, extracts from *Agaricus bisporus* mycelia enriched in α-linolenic acid presented Nrf2 modulating activity.[24] Only a few fungal compounds are known to regulate the Nrf2 pathway, including the benzoid type antrolone and the ubiquinone derivative antroquinonol identified in *Antrodia* sp., and several steroids from the renowned *Ganoderma lucidum*.[29,30]

The current study demonstrates that the examined fungal steroids could have several beneficial pharmacological properties providing multiple opportunities for the potential therapeutic application of these secondary metabolites.

Conclusions

Organic and water extracts of *H. lateritium* and compounds **1**–**6** proved to demonstrate not only considerable inhibitory properties on COX-2, but they are also capable to stimulate the Nrf2 pathway. Our results provide experimental evidence that extracts of *Hypholoma lateritium* and characteristic compounds of the hexane (**6**), chloroform (**1**–**5**) and more polar (**1**, **3**)

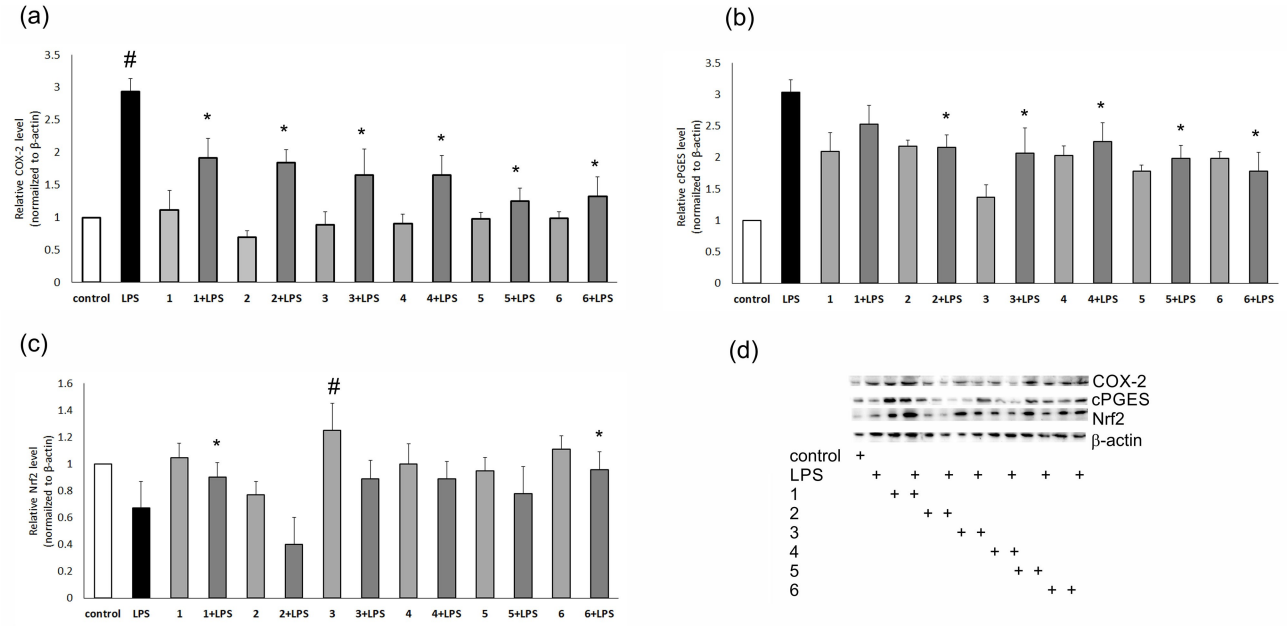

Figure 3. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with compounds **1**–**6** isolated from *H. lateritium* (10 μg) and activated with LPS. $N = 5$. # *vs.* control, * *vs.* LPS, $p < 0.05$.

fractions possess anti-inflammatory activities, which warrants to be explored in further pharmacological studies.

Experimental Section

Mushroom Material

Sporocarps of *Hypholoma lateritium* (Schaeff.) P. Kumm (Strophariaceae family) were gathered in September 2015 in the vicinity of Bakonybél, Hungary. Fungal identification was made by Attila Sándor (Hungarian Mycological Society). A voucher specimen (No. H018) has been deposited at the Department of Pharmacognosy, University of Szeged, Szeged, Hungary.

Sample Preparation

Sporocarps of *H. lateritium* were lyophilized and ground with a grinder, then, a 10 g sample was extracted with 3×100 mL methanol for 3×15 min using ultrasonic bath. Following filtration, the extracts were combined and concentrated in vacuum. The residue was dissolved in 50 mL of 50% aqueous MeOH and was subjected to liquid–liquid partition between hexane (4×25 mL) (extract A) and $CHCl_3$ (4×25 mL) (extract B) and the remaining material provided extract C. After extraction with MeOH, the residual fungal material was dried and extracted with 50 mL of boiling H_2O for 15 min. The filtered extract was lyophilized to give extract D.

Isolation, identification and characterization of fasciculic acid B (**1**), fasciculol E (**2**), fasciculol C (**3**), lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol (**4**), fasciculol F (**5**), and demethylincisterol A2 (**6**) have been previously performed by our research group and are described in a publication by Chuluunbaatar et al.[16]

Cell Cultures

Murine macrophages RAW 264.7 (TIB-71, ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% Fetal Bovine Serum (FBS) and 1% solution of antibiotics (100 IU/mL penicillin, 0.1 μg/mL streptomycin). Cells were maintained at 37 °C in humidified atmosphere of 5% CO_2 in air and were finally seeded into a 6-well plate (Sarstedt AG & Co., Nümbrecht, Germany) at a density of 5×10^5 cells/well in 2 mL of medium. Cell morphology was investigated in every step of the procedure by an inverted light microscope (Olympus, Tokyo, Japan). Cell viability during culturing was assessed with a Trypan Blue (Thermo Fisher Scientific, Waltham, MA, USA) exclusion test. RAW 264.7 cells were activated with LPS (10 ng/mL; Sigma–Aldrich, Saint Louis, MO, USA) and incubated overnight. After that, macrophages were treated with mushroom extracts A–D of *H. lateritium* at concentrations of 50 and 100 μg for 24 h or with the isolated compounds **1**–**6** (1 and 10 μg) for 24 h. Following 24 h of incubation, the cells after scrapping were collected.

Cell Proliferation XTT Assay

Cell proliferation was evaluated using a sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium) inner salt (XTT) with *N*-methyldibenzopyrazine methyl sulfate) working as the intermediate electron carrier (PMS). RAW 264.7 cells were seeded in 96-well plates (2.5×10^3 cells/well) and incubated for 24 h. The medium was then removed and 0.5; 1; 2.5; 5; 10; 50 and 100 μg of mushroom extracts A–D as well as compounds **1**–**6** were added to FCS-free medium and incubated for the next 24 h. Then, XTT solution (50 μL) was added to each well and incubated for 4 h at 37 °C according to the manufacturer instruction (Sigma–Aldrich). The absorbance was measured at 475 nm and 630 nm in a Omega plate reader (BMG LABTECH, San Diego, CA, USA). The specific absorbance of the sample was calculated as follows: Specific Absorbance = A_{475nm} (sample) − A_{475nm} (blank) − A_{660nm} (sample). Cell viability was expressed as the percentage of control.

Western Blot for Quantity of COX-2, cPGES and Nrf2 Receptor

M-PER mammalian protein extraction reagent (Thermo Scientific, Rockford, IL, USA) with protease inhibitor cocktail set III (Merck, Darmstadt, Germany) was used for cell lysates preparation. Total protein concentrations were quantified using the Bradford reaction. Forty μg of proteins per sample were solubilized in a Laemmli buffer with 2% mercaptoethanol (BioRad, Hercules, CA, USA) and subjected to 10% SDS-polyacrylamide gel electrophoresis. Primary antibodies were used: anti-cyclooxygenase-2 (COX-2), anti-β-actin diluted 1:1000 (Thermo Fisher Scientific), anti-prostaglandin E2 synthase (Cayman Chemical, Ann Arbor, MI, USA) diluted 1:1000, anti-Nrf2 receptor (GeneTex, Irvine, CA, USA) diluted 1:200 and secondary antibody anti rabbit IgG (HRP) (Thermo Fisher Scientific,

1:2000). Proteins were determined using the Western blotting detection kit Clarity Western ECL Luminol Substrate (Bio-Rad, USA). The integrated optical densities of the bands were measured using Chemi Doc Camera with Image Lab software (BioRad).

Statistical Analysis

All the results are presented as means ± standard deviation (SD). The statistical analysis was carried out using the one-way ANOVA; $p < 0.05$ was considered to be significant.

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Author Contribution Statement

A.V. designed the study of mushroom extracts and metabolites. J.G.A., B.M., and K.K. were the authors performing and designing the anti-inflammatory study. B.C. provided the extracts and isolated compounds for the anti-inflammatory studies. B.C., J.G.A., and K.K. performed the experiments. A.V. and J.H. wrote and revised the article. B.M. revised the final version of the manuscript.

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