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

Summary of the Ph.D. Thesis

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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, phenolics, polysaccharides which are responsible for their therapeutic effects, such as antidiabetic, anti-inflammatory, antioxidant, antibacterial, anticancer, immunomodulatory activities.

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. Macrophages play a key role for regulation of inflammatory responses.

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. Even though the underlying mechanisms of AF is not yet entirely understood, the cardiac potassium ion channels are is considered a novel and attractive target for drug therapy in the treatment of AF.

In the last decades, mushrooms were brought increased attention due to their secondary metabolites with high structural diversity. 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.

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* (Schaeff.) P. Kumm. 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 *H. lateritium*, from aspects of the chemistry and pharmacological properties of the fungi.
- Extraction of *H. lateritium* with various organic solvents (*n*-hexane, chloroform, ethyl acetate 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 vivo* toxicological evaluation of compounds from *H. lateritium* using bdelloid rotifer assays.

- Investigation of the activity of compounds from *H. lateritium* on GIRK and hERG channels.
- Evaluation of anti-inflammatory potential of compounds from *H. lateritium*.

MATERIALS AND METHODS

Fruiting bodies of *H. lateritium* (6.5 kg) were collected in September 2015 from the environs of Bakonybél, Hungary.

In the preparative work, the freeze-dried material was percolated with methanol. After concentration under vacuum, the extract was subjected to liquid-liquid partitioning to yield *n*-hexane, chloroform and ethyl acetate soluble phases.

The investigated extracts were fractionated by flash column chromatography (FCC). Normal- or reversed-phase SiO_2 were applied as stationary phases. The structures of the obtained compounds were characterized by spectroscopic methods (NMR and MS).

The toxicity of compounds were measured by the toxicity and survival lifespan (TSL) assay and mastax contraction frequency (MCF) assay on bdelloid rotifer.

The GIRK and hERG ion channel inhibitor activity of 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 assay was carried out by automated planar patch clamp technology based on the manual patch-clamp method.

The anti-inflammatory properties of extracts and pure compounds were subjected to *in vitro* assays to determine proinflammatory protein levels, such as cyclooxygenase-2 (COX-2), cytosolic prostaglandin E2 synthase (cPGES), and antioxidant nuclear factor (erythroid-derived 2)-like 2 (Nrf2).

RESULTS AND DISCUSSION

Isolation of the compounds of *H. lateritium*

The 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 (**Figure 1**).

The concentrated *n*-hexane phase (25 g) was fractionated by repeated flash column chromatography to obtain five compounds (**3-7**).

The purification of chloroform-soluble phase (18 g) was roughly separated with repeated flash column chromatography followed by reverse phase flash column chromatography resulting in six compounds (**1**, **2**, **8-11**).

The ethyl acetate phase (12 g) was fractionated by repeated flash chromatography to yield compound **12**.

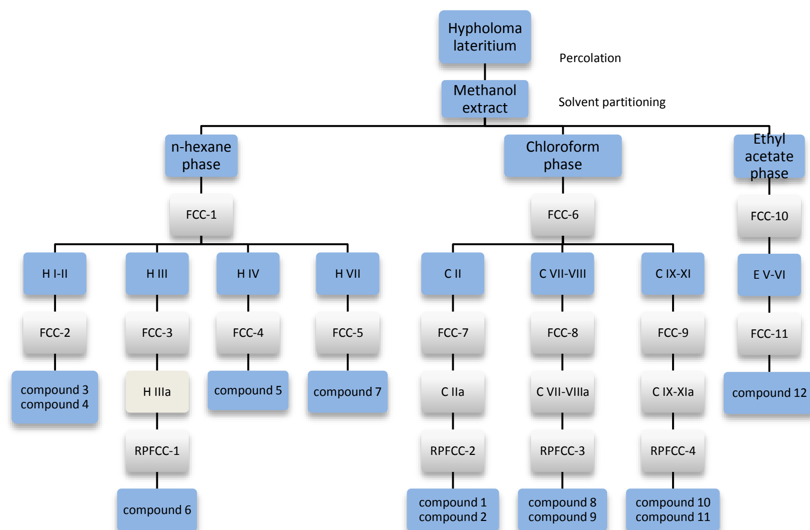

Figure 1. Isolation of compounds from *Hypholoma lateritium*

Compounds from *Hypholoma lateritium*

The structure analysis of compounds isolated from *H. lateritium* led to the isolation of 12 compounds, 8 of them for the first time from this species.

From *n*-hexane fraction of brick cap mushroom 5 secondary metabolites (**3**-**7**) were detected. One of them is a highly degraded sterol, namely demethylincisterol A_2 (**5**). The other constituents proved to be ergostane triterpenes: ergosterol (**3**), 3β-hydroxyergosta-7,22-diene (**4**), cerevisterol (**6**), and 3β-*O*-glucopyranosyl-5,8-epidioxyergosta-6,22-diene (**7**).

From the chloroform phase of methanolic 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 (**1**) which belongs to lanostane type derivatives, and 8-hydroxy-13-oxo-9*E*,11*E*-octadecadienoic acid (**2**), while **8-11** are fasciculol type triterpenes. Fasciculol E (**9**) was first identified in the sporocarps of *H. fasciculare*, while fasciculol F (**8**), fasciculol C (**10**), and fasciculic acid B (**11**) had already been isolated from this species.

In the ethyl acetate extract of the *H. fasciculare* uridine (**12**) was detected.

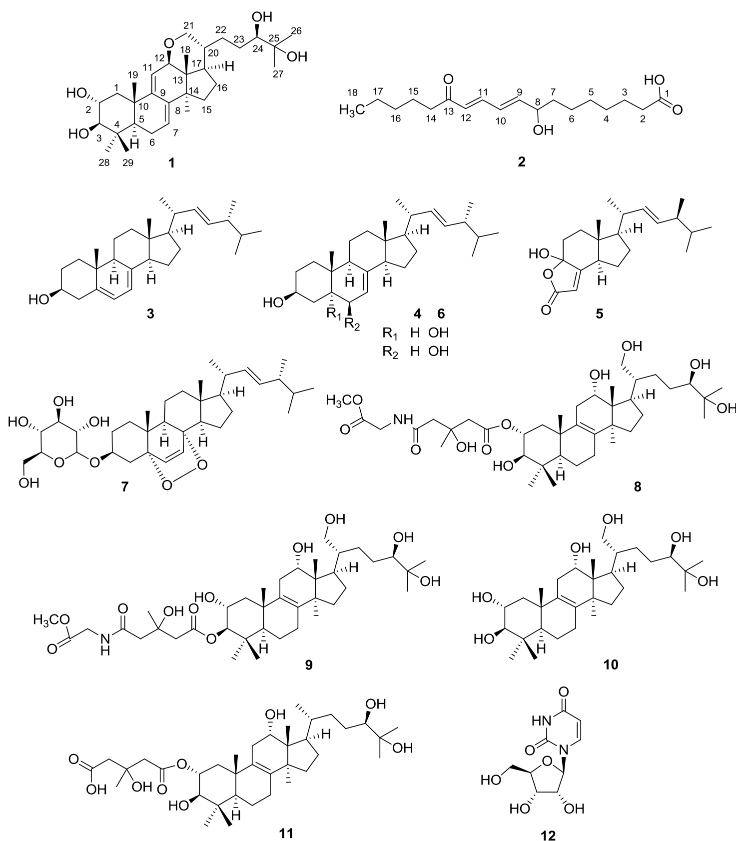

Figure 2. Structures of compounds from *H. lateritium*

Pharmacological investigation of the isolated compounds

Activity of compounds from *H. lateritium* in bdelloid viability assays

The test 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 toxicity and survival lifespan (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. 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 (Fig 3).

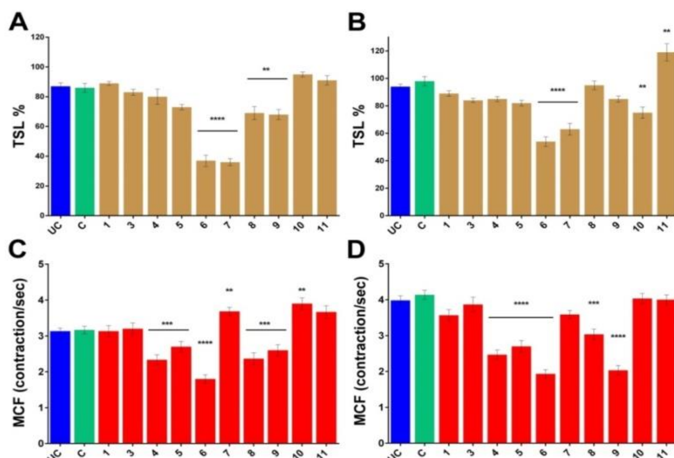

Figure 3. Normalized rotifer characteristics. Changes in the TSL values of the *Philodina acuticornis* after 3-day treatment (**A**) without feeding and (**B**) with feeding, compared to the group C. Changes in the MCF values of the *Philodina acuticornis* after 3-days treatment (**C**) without feeding 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; $p^{**} \leq 0.01$, $p^{***} \leq 0.001$, $p^{****} \leq 0.0001$.

GIRK channel inhibitory assay

Initially, as part of the screening study the GIRK channel inhibitory effects of mushroom extracts (A-D) from *H. 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 (see **Table 3**).

Table 3. GIRK channel inhibitory activity of extracts of *H. 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 *H. 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**).

The most significant inhibitory effect on GIRK channel was possessed by compound **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±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±5.0% and 66.4±4.5% inhibitions, respectively) whereas the current did not differ at the highest concentration of 30 µM (66.7±4.6% inhibition).

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

Compound		Inhibition%		SEM	
		1 µM	10 µM	1 µM	10 µM
Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol	**(1)**	27	60	5	11
Ergosterol	**(3)**	10	19	1	1
3β-hydroxyergosta-7,22-diene	**(4)**	8	16	1	6
Demethylincisterol A_2	**(5)**	11	23	3	1
Cerevisterol	**(6)**	7	13	1	6
3β-*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

In the detailed investigations, the dose-response curves of the test compound were determined where the effect of the compounds 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 (**Fig 4**).

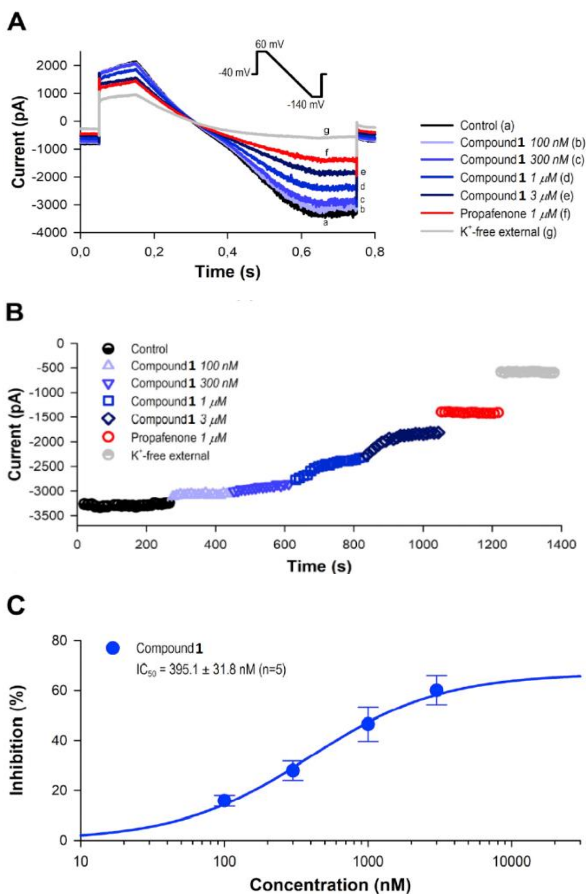

Figure 4. Inhibitory effects of compound **1** on GIRK current. Panel A displays representative current curves which were recorded during application of compound **1** at 0.1 μM, 0.3 μM, 1 μM and 3 μM concentrations. Inset shows the applied GIRK voltage protocol. Time course of calculated inward currents 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).

Compound **1** was also tested on HEK-hERG cell line and selectivity of their 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 inhibition, 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 5**.

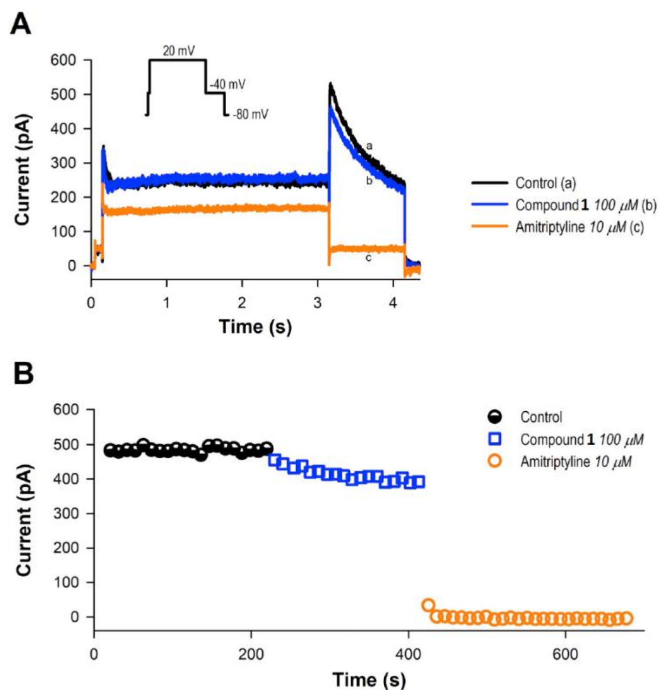

Figure 5. Effects of compound **1** on hERG current. Panel A presents typical hERG current sweeps during the application of 100 µM compound **1**. The inset shows the applied hERG voltage protocol. The original current traces reveal that **1** slightly blocked the hERG channel, while the addition of reference compound amitriptyline (10 µM) fully blocked the current. The time course of decrease in the peak tail current is presented on panel B.

Anti-inflammatory assay

Examination of the anti-inflammatory properties of *H. lateritium* extracts in RAW 264.7 cells activated with LPS and incubated with chloroform, methanol and water extracts of *H. lateritium* revealed an increase of Nrf2. 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 (**Fig 6**).

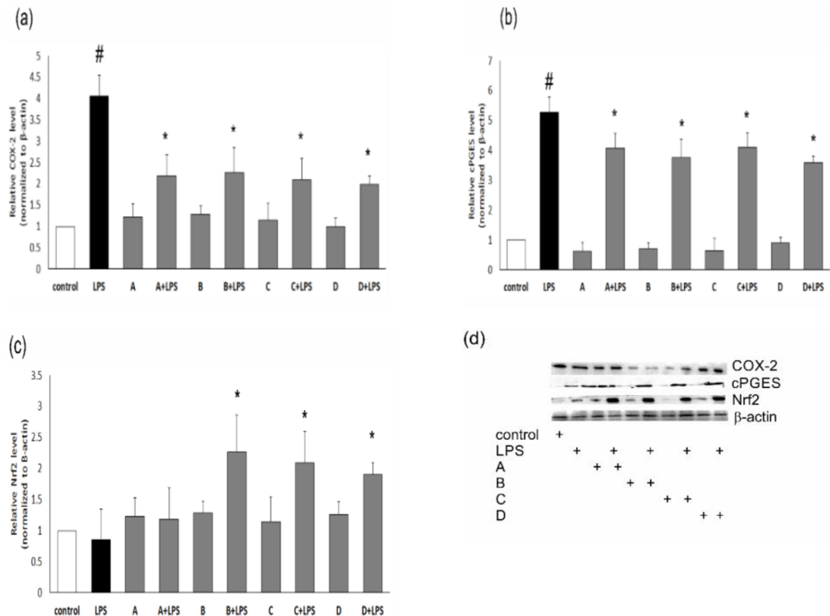

Figure 6. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with extracts of *H. lateritium* and activated with LPS. # vs control, * vs LPS, $p<0.05$. A: *n*-Hexane extract, B: Chloroform extract, C: 50% MeOH extract, D: Water extract

To identify the secondary metabolites of *H. 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 (**Figure 7**). 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.

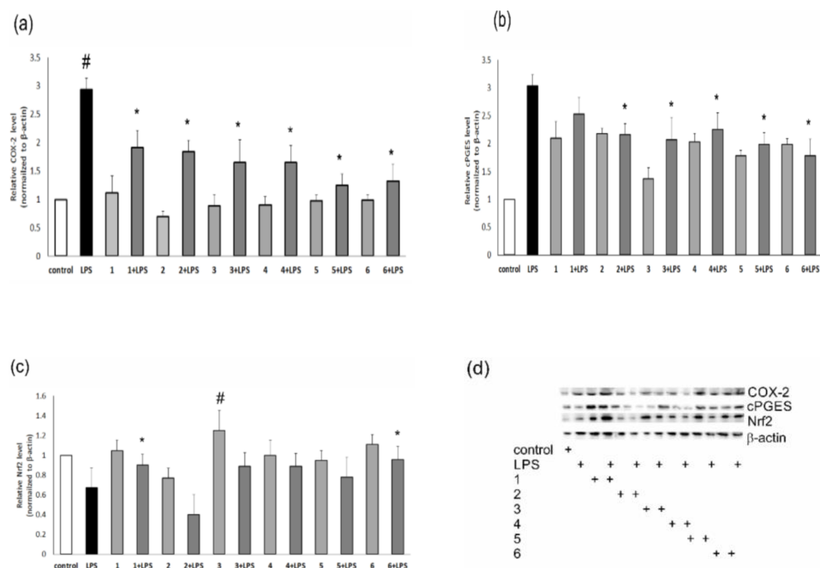

Figure 7. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with compounds isolated from *H. lateritium* and activated with LPS. # vs control, * vs LPS, $p<0.05$. 1-Fasciculic acid B (**11**), 2-Fasciculol E (**9**), 3-Fasciculol C (**10**), 4-Lanosta-7,9(11)-diene-12β,21α-epoxy-2α,3β,24β,25-tetraol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

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

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The thesis is based on the following publications:

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

2. Ványolós, A.; Orvos, P.; **Chuluunbaatar, B**.; Tálosi, L.; Hohmann, J.
   GIRK Channel Activity of Hungarian Mushrooms: From Screening to Biologically Active Metabolites.
   Fitoterapia **2019**, *137*, 104272. If:2.527

3. Ványolós, A.; Muszyńska, B.; **Chuluunbaatar, B**.; G.-Argasińska, J.; Kała, K.; Hohmann, J.
   Extracts and Steroids from the Edible Mushroom *Hypholoma lateritium* Exhibit Anti-inflammatory Properties by Inhibition of COX-2 and Activation of Nrf2.
   Chemistry & Biodiversity **2020** Jun 19. If:2.039*

*The impact factor for the year 2019 is given.

Presentations held in the same theme of the thesis:

1. **Chuluunbaatar, B**.; Béni, Z.; Dékány, M.; Kovács, B.; Sárközy, A.; Datki, Z.; Mácsai, L.; Kálmán, J.; Hohmann, J.; Ványolós, A.
   Steroids from the mushroom *Hypholoma lateritium*: isolation, structure determination and investigation in bdelloid rotifer assays.
   PSE Young Scientists' Meeting on Advances in Phytochemical Analysis (Trends in Natural Products Research)
   Liverpool, 2-5 July 2018.

2. **Chuluunbaatar, B**.; Muszyńska, B.; G.-Argasińska, J.; Hohmann, J.; Ványolós, A.
   Anti-inflammatory activity of steroids from *Hypholoma lateritium*
   PSE Young Scientists' Meeting on Biochemistry, Molecular Aspects and Pharmacology of Bioactive Natural Products
   Budapest, 19-22 June 2019
   .