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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 (1), fasciculol E (2), fasciculol C (3), lanosta-7,9(11)-diene-12 β ,21 α -epoxy-2 α ,3 β ,24 β ,25-tetraol (4), fasciculol F (5), and demethylcisterol A2 (6). Organic (*n*-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. Compounds (1-6) proved to exert notable inhibitory properties on COX-2 and were capable to stimulate the Nrf2 pathway. Fungal extracts and compounds (1-6) exerted no cytotoxic activities on RAW 264.7 cells.

Keywords: *Hypholoma lateritium* • steroids • anti-inflammatory • 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 (fascicularones).^{[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 sublaterialols^{[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

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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. In their study, Lee et al. demonstrated that the extract of this species decreases TNF- α -induced inflammation 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.^[18] 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 investigations, 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- κ B 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-12 β ,21 α -epoxy-2 α ,3 β ,24 β ,25-tetraol (**4**), fasciculol F (**5**), and demethylcisterol 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 (*n*-hexane, chloroform and 50% methanol) and H₂O extracts of *H. lateritium* were prepared, 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 (**Fig. 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.

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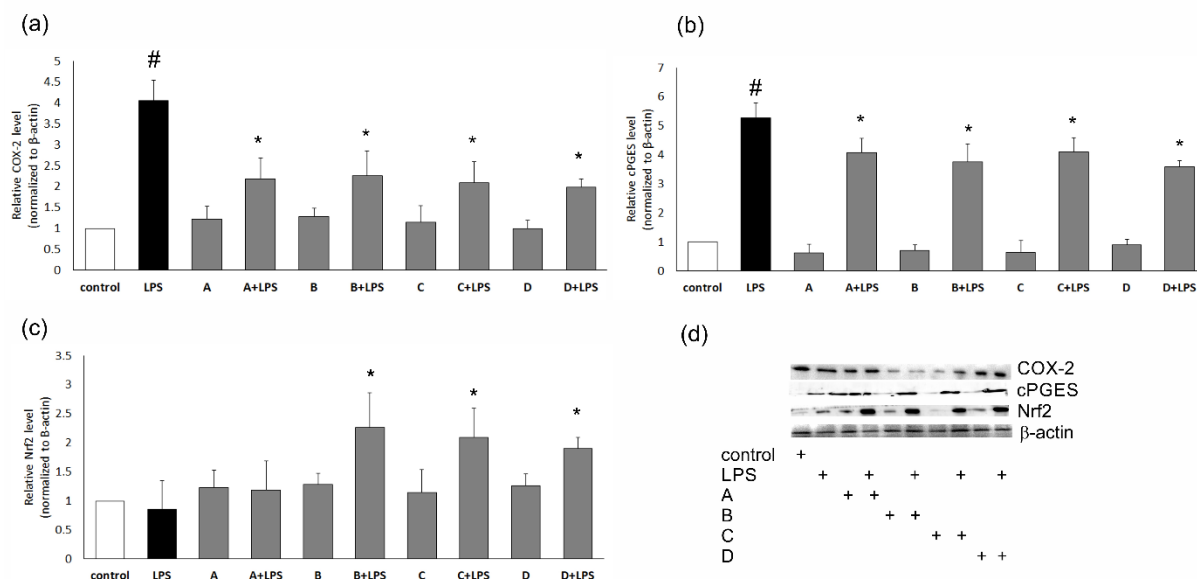


Figure 1. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with extracts of *H. lateritium* (100 µg) and activated with LPS. N=5. # vs control, * vs LPS, $p < 0.05$.

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 (Fig. 2).

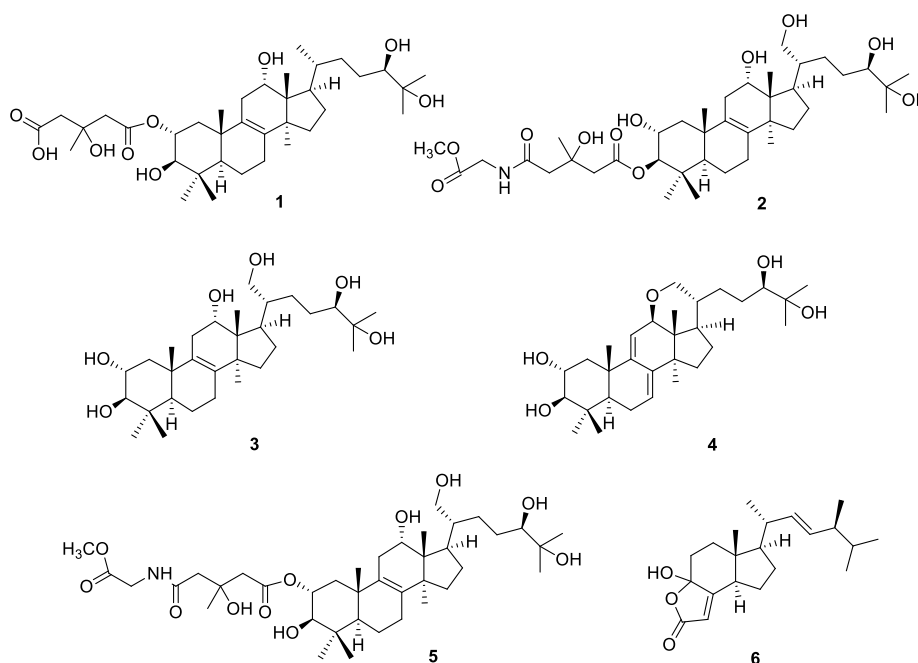


Figure 2. Steroids from *Hypholoma lateritium*

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-12 β ,21 α -epoxy-2 α ,3 β ,24 β ,25-tetraol (4) is a related steroid recently identified in *H. lateritium*, while demethylcisterol A2 (6) is a highly degraded sterol 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 demethylcisterol A2 (6).^{[10],[25]} Our experiments (Fig. 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.

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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.

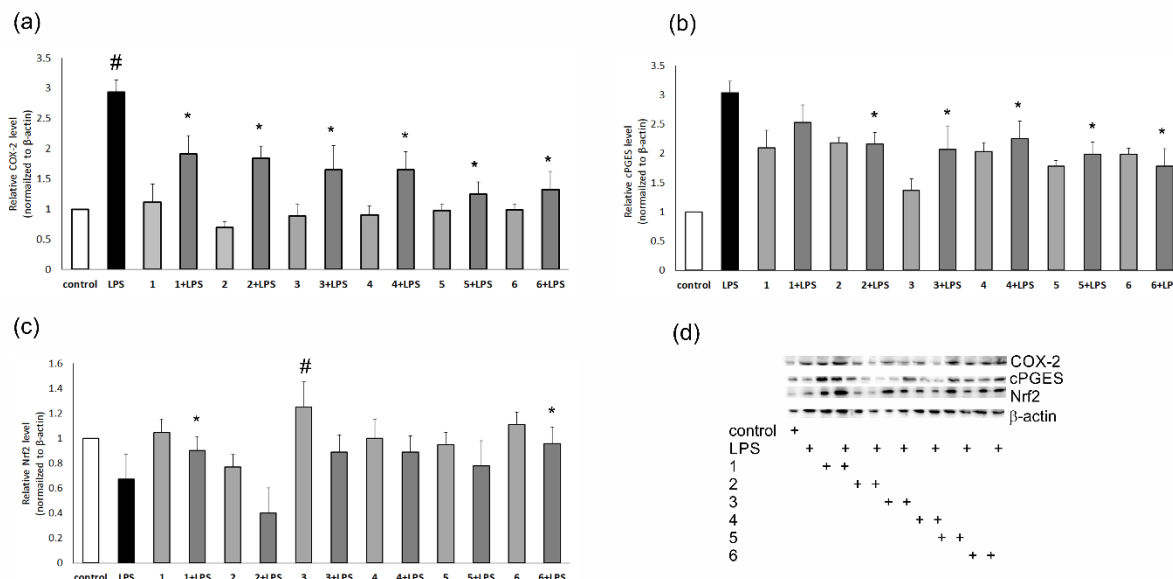


Figure 3. Levels of COX-2 (a), cPGES (b) and Nrf2 (c) and their representative blots (d) in RAW 264.7 cells incubated with compounds **1–6** isolated from *H. lateritium* (10 µg) and activated with LPS. N=5. # vs control, * vs LPS, p<0.05.

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 *n*-hexane (**6**), chloroform (**1–5**) and more polar (**1**, **3**) fractions possess anti-inflammatory activities which warrants to be explored in further pharmacological studies.

Experimental Section

Mushroom material

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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. Ho18) 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 *n*-hexane (4 × 25 mL) (extract A) and CHCl₃ (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 H₂O for 15 min. The filtered extract was lyophilized, giving extract D.

Isolation, identification and characterization of fasciculic acid B (**1**), fasciculol E (**2**), fasciculol C (**3**), lanosta-7,9(11)-diene-12 β ,21 α -epoxy-2 α ,3 β ,24 β ,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 μ g/mL streptomycin). Cells were maintained at 37 °C in humidified atmosphere of 5% CO₂ in air and were finally seeded into a 6-well plate (Sarstedt AG&Co., Nümbrecht, Germany) at a density of 5 × 10⁵ 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 μ g for 24 h or with the isolated compounds **1–6** (1 and 10 μ g) 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-sulphophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium inner salt (XTT) with *N*-methyl-dibenzopyrazine methyl sulfate working as the intermediate electron carrier (PMS). RAW 264.7 cells were seeded in 96-well plates (2.5 × 10³ cells/well) and incubated for 24 h. The medium was then removed and 0.5; 1; 2.5; 5; 10; 50 and 100 μ g 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 μ L) 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 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 μ g 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 \pm standard deviation (SD). The statistical analysis was carried out using the one-way ANOVA; *p* < 0.05 was considered significant.

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Author Contribution Statement

AV designed the study of mushroom extracts and metabolites. JGA, BM, and KK were the authors performing and designing the anti-inflammatory study. BC provided the extracts and isolated compounds for anti-inflammatory studies. BC, JGA, and KK performed the experiments. AV and JH wrote and revised the paper. BM revised the final version of the manuscript.

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Graphical Illustration

