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Isolation and characterization of chemical constituents from the mushroom *Clitocybe nebularis*

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ABSTRACT: In the course of our mycochemical studies the extract of *Clitocybe nebularis* was investigated with the aim to identify its bioactive secondary metabolites. Multistep chromatographic purification of the MeOH extract of *C. nebularis* resulted in the isolation of two steroids and an organic acid from the $CHCl_3$ and ethyl acetate soluble fractions. The structures of the compounds were determined by NMR and MS spectroscopy as 5α-ergosta-7,22-diene-3β,5,6β-triol (cerevisterol) (**1**), (22*E*,24*S*)-5α-ergosta-7,22-diene-3β,5,6β,9α-tetraol (**2**), and indole-3-carboxylic acid (**3**). The antimicrobial activity of the compounds was analyzed by agar disc diffusion method against human pathogen strains of *Streptococcus agalactiae, Staphylococcus epidermidis*, *Moraxella catarrhalis*, *Haemophilus influenzae*, and *Proteus mirabilis*. The susceptibility assay revealed that compounds **2** and **3** have weak antimicrobial activity against *M. catarrhalis*. The current study represents the first isolation of compounds **1–3** from *C. nebularis*.

KEYWORDS: *Clitocybe nebularis*; steroids; antimicrobial activity; Tricholomataceae.

1. INTRODUCTION

Edible and even toxic mushrooms are a valuable source of therapeutically important or nutritive compounds. Many mushroom species were reported to produce a large variety of secondary metabolites with unique chemical structures and interesting biological activities. *Clitocybe nebularis* (Batsch) P. Kumm. [syn.: *Lepista nebularis* (Fr.) Harmaja] known as clouded agaric or cloud funnel, member of the family Tricholomataceae is a common species of both conifer and broad-leaved forests in temperate and hemiboreal zones across Europe and North America [1]. *C. nebularis* is generally considered edible, but the strong, aromatic odor dissuades some people and can cause upset after consuming [2,3]. Pharmacological investigations revealed the neuroprotective, antioxidant, antimicrobial and cytotoxic properties of the acetone extract of *C. nebularis* [4] and demonstrated its significant antiproliferative and cytotoxic activities [5,6].

Previously various types of bioactive compounds have been reported from sporocarps of *C. nebularis*. Nebularine a purine riboside with bacteriostatic activity was the first biologically active compound isolated and identified from this mushroom [7]. Besides its antimicrobial activity nebularine displayed a noncompetitive inhibitory effect on xanthine oxidase [8], and possessed plant cytotoxic [9], antifungal and antibacterial properties [10,11]. In addition to nebularine, phenylacetic acid, purine, uridine, adenine, uracil, benzoic acid, and mannitol were also isolated [10]. Clitocypin, the inhibitor of cysteine proteinases has been identified in fruit bodies of *C. nebularis*, and its structure has been determined to have single protein sequence of 150 amino acids [12]. Small and stable proteins named *Clitocybe nebularis* lectin (CNL) were identified in the fruiting bodies along with several similar isolectins. CNL is a β-trefoil type lectin forming homodimers, which displays a remarkable immunostimulation of human dendritic cells, producing a strong T helper cell type 1 response. CNL therefore can be regarded as a promising candidate for different applications in medicine [13].

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The recently identified β-trefoil protein cnispin with protease inhibitory potency proved to be highly specific for trypsin [14]. The examination of free amino acids, fatty acids and sterols of *C. nebularis* revealed that the most abundant free amino acids are alanine, proline, serine and valine, while among fatty acids linoleic and palmitic acids predominated. The presence of ergosterol as the main sterol was also demonstrated [15]. Volatile compounds of *C. nebularis* were analyzed by GC-MS and 1-octen-3-ol and linalool were identified as key components [16].

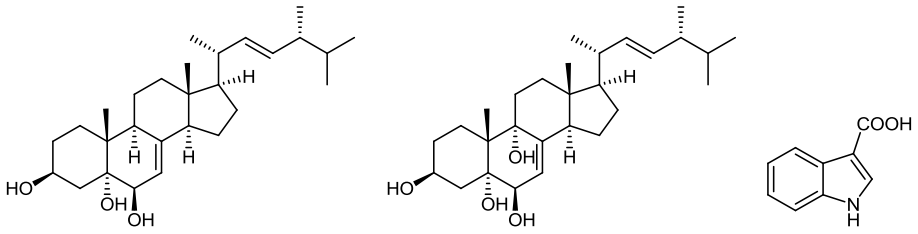
The major objective of the present study was to identify the characteristic lipophilic secondary metabolites of *C. nebularis* and determine their antimicrobial activity.

2. RESULTS AND DISCUSSION

Mycochemical investigation of the chloroform ($CHCl_3$) and ethyl acetate (EtOAc) phases of the methanolic (MeOH) extract of *C. nebularis* resulted in the identification of three compounds (**1–3**) (Figure 1). Identification of the isolated compounds was carried out by 1D and 2D NMR studies. Structure elucidation revealed compounds **1–3** to be 5α-ergosta-7,22-diene-3β,5,6β-triol (=cerevisterol) (**1**), (22*E*,24*S*)-5α-ergosta-7,22-diene-3β,5,6β,9α-tetraol (**2**), and indole-3-carboxylic acid (**3**) [17–19]. Several attempts have been made to identify the major bioactive compounds of different *Clitocybe* species [20, 21], nevertheless, it is important to note that compounds **1**, **2** and **3** were isolated for the first time from *C. nebularis*.

Mushrooms are characterized by accumulation of steroids, and a high number of studies have been reported the isolation of steroids from edible mushrooms, e.g., *Wolfiporia cocos* [22], *Lentinus tigrinus* [23], *Lentinula edodes* and *Tricholoma matsutake* [24]. Steroids of *C. nebularis* have been analyzed by Morelli et al., and cyclolaudenol, 31-norcyclolaudenol, portensterol, ergosterol, and campesterol were found [25]. Interestingly in our experiment these steroids were not identified, but cerevisterol (**1**) and (22*E*,24*S*)-5α-ergosta-7,22-diene-3β,5,6β,9α-tetraol (**2**) could be isolated. Cyclolaudenol, 31-norcyclolaudenol are based on 19-cyclolanostane skeleton, while portensterol, ergosterol, and campesterol have ergostane scaffold, as well as our isolated compounds **1** and **2**. In the study of Senatore steroids of *C. nebularis* were analyzed by GC-MS measurements [15]. It was stated that C_{28} sterols are the principal sterols with lower amounts of C_{27} and C_{29} sterols. Ergosterol was found to be the most abundant sterol comprising 62-68% of the total sterol content. Among the minor compounds ergosterol derivatives ($C_{28}\Delta^{5,7}$, $C_{28}\Delta^{7,22}$ and $C_{28}\Delta^{7}$), $C_{28}\Delta^{5}$, $C_{28}\Delta^{5,22}$, $C_{28}\Delta^{5,24(28)}$ sterols, cholesterol, desmosterol, β-sitosterol, stigmasterol and fucosterol were detected in low or trace amount. Comparing the five studied *Clitocybe* species (*C. aurantiaca*, *C. candida*, *C. cinerascens*, *C. geotropa*, *C. nebularis*), no substantial differences were found by this method among mushrooms belonging to the same genus [15].

This is the first report on the presence of indole 3-carboxylic acid (**3**) in the genus *Clitocybe*, previously only structurally close indole derivatives (indole 3-carbaldehyde) were isolated from a *Clitocybe* species [26]. Indol derivatives were reported as the dominant odorous constituents responsible for the complex odor of the taxonomically related *Tricholom*a species [27].



1 **2** **3**

Figure 1. Compounds isolated from *C. nebularis*.

The isolated compounds were subjected to antimicrobial evaluation with regards to the previous proved activities of *Clitocybe* species against various microorganisms [10,28]. The antibacterial activity of compounds **1–3** was investigated by agar disc diffusion method (Table 1). The antibacterial susceptibility test was measured against *Streptococcus agalactiae* (ATCC 13813), *Staphylococcus epidermidis* (ATCC 12228), *Moraxella catarrhalis* (ATCC 25238), *Haemophilus influenzae* (ATCC 49766), and *Proteus mirabilis* (HNCMB 60076) strains. Compound **2** and **3** inhibited marginally the growth of *M. catarrhalis* strain, whereas this strain shown resistance against compound **1**.

Earlier studies demonstrated that cerevisterol (**1**) inhibited the growth of bacteria other than tested here (MIC value 25 µg/mL against *S. typhi*, *S. aureus* and *A. niger*, and MIC 50 µg/mL against *E. faecalis*) [29], while it was inactive against *Bacillus subtilis*, *B. pumilus*, *S. aureus*, *M. luteus*, *C. albicans* and *A. niger* at 20 µg/mL tested by disk diffusion method [30]. Similarly indol 3-carboxylic acid (**3**) was tested previously against a series of pathogenic fungi (*Fusarium avenaceum, F. graminearum, F. culmorum and Pyricularia oryzae)* and bacteria (*S. aureus*, *E. coli* and *B. subtilis*) but no antifungal or antibacterial activity was observed [31]. For (22*E*,24*S*)-5α-ergosta-7,22-diene-3β,5,6β,9α-tetraol (**2**) no antibacterial data are available in the literature.

Table 1. Antimicrobial activity of compounds **1-3** isolated from *C. nebularis***.** (5 mg/mL concentration).

	Diameter of inhibition zone (mm)				
Compound	*S. agalactiae*	*S. epidermidis*	*M. catarrhalis*	*H. influenzae*	*P. mirabilis*
1	-	-	-	-	-
2	-	-	7	-	-
3[a]	-	-	7	-	-

S. agalactiae (*Streptococcus agalactiae* ATCC 13813), *S. epidermidis* (*Staphylococcus epidermidis* ATCC 12228), *M. catarrhalis* (*Moraxella catarrhalis* ATCC 25238), *H. influenzae* (*Haemophilus influenzae* ATCC 49766), *P. mirabilis* (*Proteus mirabilis* HNCMB 60076); [a] For compound **3** 4 mg/mL concentration was used.

3. CONCLUSION

The present study provides a detailed chemical analysis of the mushroom *C. nebularis* aiming at the isolation of apolar metabolites of the MeOH extract. Further studies are required to determine how the isolated compounds contribute to other bioactivities of this species. Our data together with earlier published ones demonstrate the high diversity of compounds in *C. nebularis* and points to the potential of the mushroom as a natural source of chemical compound for pharmacological applications.

4. MATERIALS AND METHODS

4.1. General

The chemicals used in this research were provided by Molar Chemicals and Sigma-Aldrich Hungary. Flash chromatography was carried out on a CombiFlash® Rf+ Lumen Instrument with integrated UV, UV VIS, and ELS detection using reversed and normal phase flash columns filled with RediSep C18 Bulk 950 (Teledyne Isco, Lincoln, NE, USA USA) and Silica 60 (0.045-0.063 mm) (Molar Chemicals, Halásztelek, Hungary), respectively. Preparative thin layer chromatography (TLC) was performed using silica plates (20x20 cm Silica gel 60 F_{254}, Merck 105554).

HRMS and MS analyses were performed on a Thermo Velos Pro Orbitrap Elite and Thermo LTQ XL (Thermo Fisher Scientific) system. The ionization method was ESI operated in positive (or negative) ion mode. The (de)protonated molecular ion peaks were fragmented by CID at a normalized collision energy of 35%. For the CID experiment helium was used as the collision gas. The samples were dissolved in methanol. Data acquisition and analysis were accomplished with Xcalibur software version 4.0 (Thermo Fisher Scientific). NMR data were collected in methanol-d_4 at 25 °C on a Bruker 500 MHz Avance III HD spectrometer equipped with a liquid helium cooled TCI cryoprobe. Chemical shifts were referenced to residual solvent signals (3.31 (^{1}H) and 49.15 ppm (^{13}C) MeOD-d_4). Standard ^{1}H, ^{13}C, 2D-HSQC, 2D-HMBC, and ROESY data were collected in all cases using the pulse sequences available in the Topspin 3.5 pulse sequence library. The NMR assignments of all isolated compounds were in agreement with those reported earlier in the literature [17–19].

4.2. Mushroom material

Samples of *C. nebularis* (Batsch) P. Kumm. were collected in 2017 from the environs of Sándorfalva and Csákányospuszta, Hungary and identified by A. Sándor (Hungarian Mycological Society) and V. Papp. Voucher specimens have been deposited in the mycological collection of the Hungarian Natural History Museum (VPapp-1110171).

4.3. Extraction and isolation

The fresh mushroom material (5.6 kg) was extracted with MeOH (20 L) at room temperature. After concentration, the MeOH extract (54 g) was dissolved in 50% aqueous MeOH and subjected to solvent-solvent partition using *n*-hexane (5 × 500 mL), $CHCl_3$ (5 × 500 mL), and then EtOAc (5 × 500 mL). The $CHCl_3$ soluble phase (2.12 g) was separated by flash chromatography (NP-FC) on silica gel (40 g) using a gradient system of *n*-hexane–acetone (linear from 100:0 to 0:100, t = 60 min). According to TLC monitoring, fractions with similar compositions were combined (C1-C15). Fractions C7 (142 mg), C8 (108 mg) and C9 (69 mg) were further chromatographed by RP_{18}-FC (12 g) using a mixture of H_2O and MeOH (linear gradient from 40 to 90% MeOH, t = 45 min), then further purified by FC on RP_{18} column (4 g) using H_2O–MeOH solvent system (linear gradient from 50% to 95% MeOH, t = 45 min), which resulted in the isolation of compounds **1** (31 mg) and **2** (2.7 mg). The EtOAc phase (4.15 g) was separated by NP-FC (80 g) using *n*-hexane–acetone with increasing polarity (from 100:0 to 65:35, t = 60 min), to obtain 20 major combined fractions (E1–20). Fractions E13 (33 mg), E14 (18 mg) and E15 (20 mg) were further separated by a combination of NP-FC (4 g sorbent, *n*-hexane–acetone (linear gradient from 75:25 to 30:70, t = 50 min) and preparative TLC using a $CHCl_3$–MeOH (97:3) solvent system to isolate compound **3** (2 mg).

4.4. Bacterial strains and culture conditions

The test microorganisms used in this study were 4 standard and 1 clinical isolate with different antibiotic resistance profile. The standard Gram-positive strains were *Streptococcus agalactiae* (ATCC 13813), and *Staphylococcus epidermidis* (ATCC 12228). The standard Gram-negative strains were *Moraxella catarrhalis* (ATCC 25238), and *Haemophilus influenzae* (ATCC 49766). *Proteus mirabilis* (HNCMB 60076) was applied as clinical strain. Bacterial cultures were grown on standard Mueller-Hinton agar (MH) and horse blood (MHF) plates at 37 °C under aerobic environment.

4.5. Determination of antibacterial activity

Screening of antibacterial activity of compounds against standard bacterial strains for their inhibition zones was carried out by standard disc diffusion method [32]. Concisely, the compounds were dissolved in DMSO at concentration 5 (**1** and **2**) and 4 (**3**) mg/mL. The bacterial suspension (inoculums 0.5 McFarland, 1–2×10^8 CFU ml^{-1}) was spread on plate and the sterile filter paper discs (6 mm in diameter) impregnated with 10 µL of the compound solution was placed. DMSO served as negative control, and ampicillin, erythromycin, imipenem, cefuroxime and vancomycin were used as positive control. The plates were incubated (35 +/-2 °C for 24 h) under aerobic conditions. The diameters of inhibition zones created by the compounds (including the disc) were measured and recorded.

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