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Phylogeny-Guided Chemical Investigation of the Reclassified Fungus *Commelinaceomyces aneilematis* Reveals Cytotoxic Secondary Metabolites

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Abstract

Commelinaceomyces aneilematis (formerly *Ustilago aneilematis*) is a recently reclassified ascomycete that infects *Murdannia keisak* and is phylogenetically related to the mycotoxin-producing fungus *Villosiclava virens* (= *Ustilaginoidea virens*). Because of this close relationship, *C. aneilematis* was hypothesized to produce cytotoxic secondary metabolites similar to those of *V. virens*. In this study, the cytotoxicity and secondary metabolites of *C. aneilematis* were investigated. The MeOH extract obtained from rice cultures was fractionated by solvent partitioning, and the resulting fractions were evaluated using the WST-1 assay. The CH_3CN fraction exhibited marked cytotoxicity against several human cancer cell lines, particularly HT-29 cells. Chemical investigation of this active fraction led to the isolation of two known cytotoxic metabolites, isochaetochromin B2 (**1**) and ustilaginoidin D (**2**), together with a new isocoumarin glycoside (**3**). Compounds **1** and **2** showed cytotoxicity against PANC-1 and HT-29 cells, whereas **3** was inactive at concentrations up to 100 μM. These results indicate that **1** and **2** are the likely contributors to the cytotoxicity of the CH_3CN extract. The isolation of ustilaginoidin D (**2**), a characteristic metabolite of *V. virens*, provides chemotaxonomic evidence supporting the recent phylogenetic reclassification of *C. aneilematis*. Furthermore, because the host plant of *C. aneilematis*, *Murdannia keisak*, is a weed that can contaminate rice forage, the production of these cytotoxic metabolites suggests a potential toxicological risk to livestock. This study provides the first chemical and

toxicological characterization of *C. aneilematis*, demonstrating that this reclassified fungus is a previously unrecognized producer of biologically active secondary metabolites.

Keywords

Commelinaceomyces aneilematis, Mycotoxins, Cytotoxicity, Isocoumarin Glycoside, Isochaetochromin B2, Ustilaginoidin D, *Villosiclava virens*

1. Introduction

Fungi are prolific producers of structurally diverse secondary metabolites, including mycotoxins, many of which exhibit biological activities such as cytotoxicity, phytotoxicity, and antimicrobial effects [1]. Some fungal secondary metabolites are of particular chemical and toxicological importance because they can enter the food chain through agricultural products or livestock feed, thereby posing potential risks to plants, animals, and humans [2].

Villosiclava virens (= *Ustilaginoidea virens*), the causal agent of rice false smut, is a well-known plant pathogenic fungus that infects rice and causes serious reductions in yield and grain quality [3]. This fungus produces several toxic secondary metabolites, including ustiloxins and ustilaginoidins. Ustilaginoidins are bisnaphtho-*γ*-pyrone derivatives and have been reported to exhibit various biological activities, including cytotoxicity and phytotoxicity [4]-[13]. Therefore, *V. virens* is regarded as a mycotoxin-producing fungus that may pose potential risks to agriculture, the environment, and animal health.

Commelinaceomyces aneilematis is a plant pathogenic fungus infecting *Murdannia keisak*, a weed commonly found in rice paddy fields. This fungus was previously classified as *Ustilago aneilematis* in Basidiomycota. However, recent molecular phylogenetic analysis demonstrated that it belongs to Ascomycota, Hypocreales, Clavicipitaceae, and is closely related to *V. virens*. Based on this finding, the genus *Commelinaceomyces* was established, and the fungus was reclassified as *C. aneilematis* [14].

This taxonomic reclassification is important not only from a phylogenetic viewpoint but also from both chemical and toxicological perspectives. Because *C. aneilematis* is closely related to the mycotoxin-producing fungus *V. virens*, it is reasonable to hypothesize that this fungus also produces cytotoxic secondary metabolites similar to those of *V. virens*. Moreover, the host plant of *C. aneilematis*, *Murdannia keisak*, is a common weed that can contaminate rice forage [15]. Therefore, if *C. aneilematis*-infected plants are incorporated into livestock feed, cytotoxic secondary metabolites produced by this fungus may represent a potential toxicological risk to livestock health. Despite this potential importance, no chemical investigation of *C. aneilematis* has been reported to date. Consequently, neither the secondary metabolite profile nor the toxicological properties of this reclassified fun-

gus have been elucidated.

Therefore, the aim of this study was to investigate the secondary metabolites produced by *C. aneilematis* and evaluate their cytotoxicity. In particular, this study focused on determining whether the reclassified fungus produces cytotoxic metabolites related to those of *V. virens*. We first evaluated the cytotoxicity of the crude extract and solvent fractions of *C. aneilematis*, then isolated metabolites from the active fraction, examined the cytotoxicity of the isolated compounds, and finally elucidated the structure of a newly isolated compound. The findings provide the first chemical and toxicological characterization of *C. aneilematis*, offering new insights into its chemotaxonomic relationship with *V. virens* and its potential implications for livestock health.

2. Materials and Methods

2.1. General Experimental Procedures

For column chromatography, Sephadex LH-20 (GE Healthcare, Chicago, IL, USA) was used. Middle pressure liquid chromatography (MPLC) for isolation of compounds, HPLC pump SSC-3160 (Senshu Science, Tokyo, Japan), RI-UV detector YRU-883 (Yamazen, Osaka, Japan), glass column Ultra pack ODS-S-50B (26 × 300 mm, Yamazen), and chromatographic conditions as follows: flow rate: 8 mL/min, detected by RI-UV detector. HPLC for isolation of compounds, HPLC pump LC-20AT (Shimadzu, Kyoto, Japan), UV detector SPD-20A (Shimadzu), column oven CO-965 (JASCO, Tokyo, Japan) and HPLC column Inertsustain C18 (5 μM, 10 × 250 mm), and chromatographic conditions as follows: flow rate: 2 mL/min, detected wavelength: 210 nm. NMR spectra were measured by ECAII 600 spectrometer (JEOL, Tokyo, Japan). The chemical shifts (δ) are given in ppm, and the coupling constants (J) in Hz. Optical rotation measured by DIP-10000 polarimeter (JASCO). Microplate reader was used as MPR-A100 (AS ONE, Osaka, Japan). ESI-MS were measured at negative mode on T-100LP mass spectrometer (JASCO).

2.2. Fungal Material

The strain *Commelinaceomyces aneilematis* MAFF246963 isolated from *Murdannia keisak* in Japan [14]. This fungus was identified based on morphology and a sequence analysis of the 28S, 18S, TEF, RPB1 and RPB2 regions (GenBank accession No. LC474620, LC474617, LC474623, LC474626 and LC474629). The fungus was maintained on potato dextrose agar medium before use.

2.3. Cell Lines and Cell Culture

Human hepatoma cells (HepG2) were obtained from the National Institutes of Biomedical Innovation, Health, and Nutrition (Osaka, Japan). Human promyelocytic leukemia cells (HL60), human pancreatic carcinoma cells (PANC-1), and human glioblastoma multiforme tumor cells (T98G) were obtained from Riken BioResource Research Center (Ibaraki, Japan). Human colon adenocarcinoma cells (HT29) were

obtained from KAC Co., Ltd. (Kyoto, Japan). Cells were cultured in Dulbecco's modified Eagle medium (DMEM: Nacalai Tesque, Inc., Kyoto, Japan) or RPMI 1640 (Nacalai Tesque, Inc.) supplemented with 10% heat-inactivated fetal bovine serum (Merck, Darmstadt, Germany) and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque, Inc.) at 37˚C in a humid incubator containing ambient air supplemented with 5% CO_2.

2.4. Cultivation and Extraction of *Commelinaceomyces aneilematis*

C. aneilematis was first cultivated on potato dextrose agar (PDA) medium at 25˚C for two weeks to prepare the inoculum. Rice medium (1800 g, with 150 g rice aliquoted into 12 Roux flasks) was pre-sterilized (120˚C, 20 min), and mycelial mats of the fungal inoculum were cut into 1 cm^2 squares with a sterile spatula, with about two pieces placed into each flask, and then statically cultured at 25˚C for three weeks. After cultivation, extraction with methanol was performed, yielding 40.3 g of methanol extract.

The obtained methanol extract was suspended in 300 mL of water and extracted three times with an equal volume of ethyl acetate. The resulting ethyl acetate extract was obtained by evaporation under vacuum, suspended in 300 mL of n-hexane, and extracted three times with an equal volume of acetonitrile (CH_3CN), yielding 2.96 g of hexane extract and 2.33 g of CH_3CN extract. Additionally, the H_2O fraction was partitioned with 1-BuOH and H_2O, yielding 12.50 g of 1-BuOH fraction and 28.56 g of H_2O fraction. All cultivation and extraction were performed at once.

The obtained MeOH extract and solvent fractions were used for cytotoxicity screening.

2.5. Cytotoxicity Screening of Crude Extract and Fractions

The cytotoxicity of the crude MeOH extract and solvent fractions was initially evaluated against human pancreatic carcinoma PANC-1 cells using five independent biological experiments by the WST-1 assay. Data are presented as mean ± SD (n = 5). Each sample was tested at 50 and 100 μg/mL. For crude fraction cytotoxicity tests, cells (T98G 0.2×10^4 cell/well, PANC-1 2.0×10^4 cell/well, HT-29 2.0×10^4 cell/well, HL60 1.0×10^4 cell/well, HepG2 1.0×10^4 cell/well) were seeded at 100 μL per well into 96-well plates and incubated for 48 hours in a CO_2 incubator. For cells other than HL60, various test samples prepared at 50.0 μg/mL and 100 μg/mL were added by replacing the medium, and cells were cultured for 24 hours. For HL60, 5 μL of medium containing various test samples prepared at 1000 μg/mL and 2000 μg/mL were added, and cells were cultured for 24 hours. After 24 hours, 10 μL of Cell Proliferation Reagent WST-1 was added, and absorbance was measured after 4 hours (main wavelength: 450 nm, sub-wavelength: 660 nm) using a microplate reader. The number of samples was 5, and *Dunnett*'s multiple comparisons test was performed to determine statistical significance. 5-Fluorouracil (5-FU) was used as a positive control.

The CH_3CN extract, which showed the strongest cytotoxicity against PANC-1 cells, was further evaluated against additional human cancer cell lines, including T98G, HT-29, HL-60, and HepG2 cells. This screening was performed to identify the active fraction containing cytotoxic metabolites.

2.6. Isolation of Secondary Metabolites from the Active Fraction

Because the CH_3CN extract exhibited strong cytotoxicity, this extract was subjected to further chemical investigation. The CH_3CN extract was separated by repeated chromatographic procedures, including Sephadex LH-20 column chromatography, ODS-MPLC, and preparative HPLC.

Component isolation from the cytotoxic CH_3CN extract was performed using multi-stage chromatography. First, the CH_3CN extract (2.33 g) was chromatographed on LH-20 column chromatography with the mobile phase using 200 mL of *n*-hexane-chloroform (1:4), 200 mL of chloroform-acetone (3:2), 200 mL of chloroform-acetone (1:4), 200 mL of acetone and 500 mL of methanol to obtain 13 fractions.

The ninth fraction (168 mg) obtained from LH20 column chromatography was purified by ODS-MPLC (mobile phase: 60% acetonitrile) and silicagel-HPLC (mobile phase: chloroform-methanol 20:1) to get **3** (12.0 mg).

For the isolation of isochaetochromin B_2 (**1**), Fr. 7, obtained from LH20 column chromatography, was separated by ODS-MPLC using 80% acetonitrile with 0.1% HCOOH as the mobile phase. Fr. 6 from this ODS-MPLC was then separated and purified by ODS-HPLC using 70% acetonitrile with 0.1% HCOOH as the mobile phase, yielding 9.7 mg of isochaetochromin B_2 (**1**) [16] [17].

For the isolation of ustilaginoidin D (**2**), Fr. 6 obtained from LH20 column chromatography, was separated by ODS-MPLC using 60% acetonitrile as the mobile phase, followed by ODS-HPLC using 80% acetonitrile with 0.02% trifluoroacetic acid (TFA) as the mobile phase, yielding 4.2 mg of ustilaginoidin D (**2**) [16] [18].

2.7. Physicochemical Properties of 3

White powder: $[\alpha]^D$ + 12.9˚ (c 0.485, MeOH). HR-ESI-MS (positive mode) m/z: 381.1212, calcd. 381.1186 for $C_{18}H_{21}O_9$, $[M+H]^+$. The ^1H- and ^{13}C-NMR data are shown in Section 3.4.

2.8. Cytotoxicity Assay of Isolated Compounds

The cytotoxicity of compounds **1** - **3** was evaluated against PANC-1 and HT-29 cells, because these cell lines were sensitive to the CH_3CN extract. These cytotoxic assays were performed using four independent biological experiments by the WST-1 assay. Data are presented as mean ± SD (n = 4). The compounds were tested at several concentrations, and IC_{50} values were calculated. Doxorubicin was used as a positive control.

For isolated compound cytotoxicity tests, PANC-1 5.0×10^4 cell/well and HT-

29 5.0×10^4 cell/well were seeded at 100 μL per well into 96-well plates and incubated for 48 hours in a CO_2 incubator. After 48 hours, various compounds prepared at 3.125 μM, 12.50 μM, 25.00 μM, 50.00 μM, and 100 μM were added by replacing the medium, and cells were cultured for 24 hours. After 24 hours, the medium was replaced, and 10 μL of WST-1 reagent was added. Subsequently, absorbance was measured after 4 hours. Four independent biological experiments were performed. For each biological replicate, IC_{50} values were independently estimated by linear regression analysis using the linear portion of the dose-response curve. The reported IC_{50} values represent the mean ± standard deviation (SD) of four independent biological experiments.

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.0e (GraphPad Software, San Diego, CA, USA) and multcomp package in R, version 4.1.0 [19]. Comparisons with the respective negative controls were conducted using *Dunnett*'s multiple comparisons test in GraphPad Prism. IC_{50} values were independently estimated for each biological replicate by linear regression analysis using the linear portion of the dose-response curve and are presented as the mean ± SD. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Cytotoxicity of the Extract and Fractions of *Commelinaceomyces aneilematis*

To investigate whether *Commelinaceomyces aneilematis* produces cytotoxic secondary metabolites, the MeOH extract obtained from rice cultures was partitioned into four solvent extractions (MeOH, Hexane, CH_3CN and 1-BuOH extract) (**Figure 1(a)**), and each extract was evaluated for cytotoxicity against PANC-1 cells.

Among the extracts examined, the CH_3CN extract exhibited the strongest cytotoxic activity, significantly reducing cell viability at 50 μg/mL (**Figure 1(b)**). Because this extract showed the highest activity, it was selected for further investigation.

The CH_3CN extract was subsequently evaluated against additional human cancer cell lines (T98G, HT-29, HL-60, and HepG2). Cytotoxicity was observed against all tested cell lines, with particularly strong activity against HT-29 cells (**Figure 1(c)**). These results suggested that the CH_3CN extract contained one or more cytotoxic metabolites responsible for the observed activity.

3.2. Isolation of Secondary Metabolites from the Cytotoxic CH_3CN Fraction

To identify the constituents responsible for the cytotoxicity, the CH_3CN extract was subjected to repeated chromatographic separation using silica gel column chromatography, Sephadex LH-20, MPLC, and preparative HPLC.

This procedure afforded a new isocoumarin glycoside (**3**), together with two

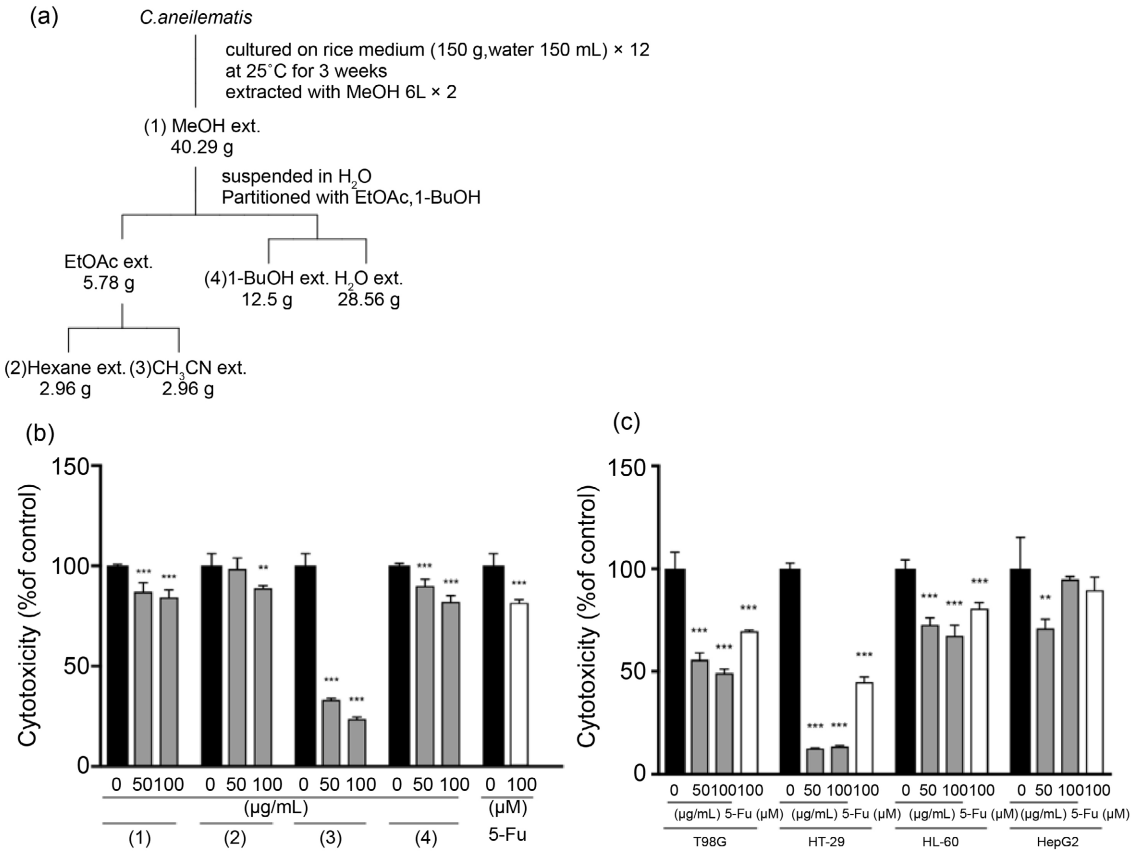

Figure 1. Cytotoxic effect of crude extract of *C. aneilematis* as assessed by WST assay. 5-FU was employed as positive control. Data are shown as means ± SD (n = 5). Comparisons to the negative control (fr.: 0 μg/mL, 5-FU: 0 μM) were performed by *Dunnett*'s multiple comparisons test. Conditions without an asterisk exhibited no significant difference. $^{**}p < 0.01$, $^{***}p < 0.001$. (a) Culture and separation method. (b) Cytotoxicity against PANC-1 cells. (1) MeOH fr. (2) Hexane fr. (3) CH_3CN fr. (4) 1-BuOH fr. (c) Cytotoxicity of CH_3CN fr. against T98G, HT-29, HL-60 and HepG2 cells.

known fungal metabolites, isochaetochromin B_2 (**1**) and ustilaginoidin D (**2**) (**Figure 2**).

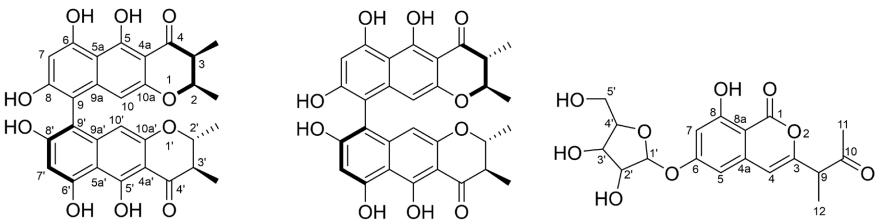

Figure 2. Structure of **1** - **3** isolated from *C. aneilematis*.

3.3. Cytotoxicity of the Isolated Compounds

Compounds **1** - **3** were evaluated for cytotoxicity against PANC-1 and HT-29 cells.

Compound **3** did not exhibit cytotoxicity toward either cell line (IC_{50} > 100 μM). In contrast, isochaetochromin B_2 (**1**) and ustilaginoidin D (**2**) showed significant

cytotoxicity. Compound **1** inhibited the growth of PANC-1 and HT-29 cells with IC_{50} values of 26.0 ± 0.4 and 33.4 ± 2.2 μM, respectively, whereas **2** exhibited IC_{50} values of 12.0 ± 0.7 and 30.4 ± 0.4 μM, respectively. These IC_{50} values are comparable to or even stronger than those of the positive control, doxorubicin (PANC-1: 24.7 ± 1.3 μM, HT-29: 36.0 ± 19.2 μM). The results of the cytotoxicity tests for the isolated compounds are shown in Table 1. These results indicate that the cytotoxicity observed in the CH_3CN fraction is likely attributable to isochaetochromin B_2 and ustilaginoidin D. Furthermore, the isolation of ustilaginoidin D, one of the characteristic metabolites of *Villosiclava virens*, supports the close phylogenetic relationship between *C. aneilematis* and *V. virens* from a chemotaxonomic perspective.

Table 1. Cytotoxic effect of **1** - **3** as assessed by WST assay. Doxorubicin was employed as positive control. Data are shown as means ± SD (n = 4).

	IC_{50} (μM)			
Cells	**Isochaeetochromin B2 (1)**	**Ustilaginoidin D (2)**	**3**	**Doxorubicin**
PANC-1	26.0 ± 0.4	12.0 ± 0.7	>100	24.7 ± 1.3
HT-29	33.4 ± 2.2	30.4 ± 0.4	>100	36.0 ± 19.2

3.4. Structure Elucidation of Compound 3

Among the isolated metabolites, compound **3** was identified as a new natural product. Compound **3** was obtained as a white powder. Its molecular formula was determined to be $C_{18}H_{20}O_9$ [m/z: 381.1212, $[M+H]^+$, calcd. 381.1186 for $C_{18}H_{21}O_9$], by HRESIMS, indicating nine degrees of unsaturation. The ^1H and ^{13}C NMR spectra data as shown in Table 2, together with HSQC, COSY, and HMBC analyses, revealed the presence of an isocoumarin glycoside. The HMBC correlation from the anomeric proton (H-1') to C-4 established the attachment of the sugar moiety to C-4 of the isocoumarin nucleus, and the planar structure of **3** was thus established (Figure 3).

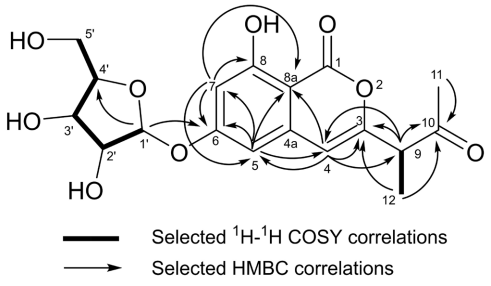

Figure 3. Structure elucidation of **3** by detailed analysis of 2D-NMR.

The sugar moiety was assigned as ribose based on the NMR spectroscopic data and comparison with literature values. Although the absolute configuration of the sugar was not determined experimentally, it was tentatively assigned as D-ribose

because naturally occurring ribosylated secondary metabolites almost exclusively contain D-ribose [20]-[27]. The stereochemistry of the aglycone side chain could not be unambiguously determined because of insufficient spectroscopic evidence. Therefore, only the planar structure of **3** is proposed in the present study, and the absolute configuration of the aglycone remains to be elucidated by further investigation.

Table 2. ^{13}C- and ^{1}H-NMR spectral data of **3**. Measurement solvent: CD_3OD. () indicates coupling constants.

No.	δc	δ_H (*J* in Hz)
1	167.1	
3	156.9	
4	107	6.57 s
4a	140	
5	105.2	6.73 s
6	166.1	
7	104.4	6.70 s
8	164.4	
8a	101.5	
9	52.2	3.77 q (7.3)
10	207.5	
11	28.3	2.23 s
12	14	1.41 d (7.3)
1′	101.7	5.75 d (2.8)
2′	73.5	4.23 t (5.1)
3′	71.1	4.11 m
4′	88.2	4.13 m
5′	63.1	3.65 d (11.9), 3.71 d (11.9)

4. Discussion

The isolation of isochaetochromin B2 (**1**) and ustilaginoidin D (**2**) from *C. aneilematis* is an important finding. Since both compounds have previously been reported from *Villosiclava virens*, these results demonstrate that the close phylogenetic relationship between *C. aneilematis* and *V. virens* is reflected in their secondary metabolite profiles. Thus, the present chemical evidence strongly supports the recent molecular phylogenetic reclassification of *C. aneilematis* from a chemotaxonomic perspective.

Isochaetochromin B_2 (**1**) exhibited significant cytotoxicity against PANC-1 and HT-29 cells, and, to the best of our knowledge, this is the first report describing its cytotoxic activity. Ustilaginoidin D (**2**) has previously been reported to exhibit cytotoxicity against KB cells [4]; however, its activity against PANC-1 and HT-29

cells is demonstrated here for the first time. Although the molecular targets of **1** and **2** remain unknown, structurally related bis-naphtho-γ-pyrones, including ustilaginoidin A and chaetochromin A, inhibit ATP synthase and mitochondrial respiration [5]. These observations suggest that isochaetochromin B_2 and ustilaginoidin D may share a similar mechanism of cytotoxic action.

Compound **3** was characterized as a new isocoumarin glycoside. Ribosylated fungal isocoumarins are relatively rare natural products, and the isolation of **3** expands the structural diversity of this compound class. Structurally related compounds, including daldinisides and orthosporin, have previously been reported [28] [29]. Interestingly, glycosylated analogues generally exhibit weaker biological activities than their corresponding aglycones, suggesting that glycosylation reduces biological activity. This observation is consistent with the present results, in which **3** showed no cytotoxicity, whereas structurally related aglycones have been reported to possess various biological activities.

The recent elucidation of the ustilaginoidin biosynthetic pathway in *V. virens* raises the possibility that *C. aneilematis* possesses homologous biosynthetic genes [30]. Comparative genomic and biochemical studies will be valuable for clarifying whether these closely related fungi share conserved biosynthetic mechanisms for ustilaginoidin production. Ustilaginoidin D has been reported to exhibit hepatotoxicity and teratogenicity in zebrafish [7]. Because the host plant of *C. aneilematis*, *Murdannia keisak*, is a common weed that can contaminate rice forage, the production of cytotoxic metabolites such as ustilaginoidin D and isochaetochromin B_2 suggests that this fungus may represent a potential toxicological risk to livestock. More broadly, the present study demonstrates that recently reclassified fungal taxa can represent previously unrecognized sources of biologically active secondary metabolites, highlighting the importance of integrating molecular phylogeny with natural product chemistry in the search for novel fungal metabolites.

5. Conclusions

In this study, the secondary metabolites and cytotoxicity of the recently reclassified ascomycete *Commelinaceomyces aneilematis* were investigated. Chemical investigation of the cytotoxic CH_3CN extract led to the isolation of two known cytotoxic metabolites, isochaetochromin B_2 (**1**) and ustilaginoidin D (**2**), together with a new isocoumarin glycoside (**3**).

Isochaetochromin B_2 (**1**) and ustilaginoidin D (**2**) exhibited significant cytotoxicity against PANC-1 and HT-29 cells, whereas **3** showed no detectable cytotoxicity.

The isolation of ustilaginoidin D, a characteristic metabolite of *Villosiclava virens*, together with isochaetochromin B_2, provides chemotaxonomic evidence supporting the recent phylogenetic reclassification of *C. aneilematis*. Furthermore, the cytotoxicity of isochaetochromin B_2 is reported here for the first time, and the activity of ustilaginoidin D against PANC-1 and HT-29 cells is newly demonstrated.

Because the host plant of *C. aneilematis*, *Murdannia keisak*, can contaminate rice forage, the production of cytotoxic metabolites by this fungus suggests a potential toxicological risk to livestock. However, further studies are required to evaluate their occurrence in contaminated forage and their relevance to livestock exposure.

More broadly, the present study demonstrates that recently reclassified fungal taxa represent promising targets for natural product discovery, as molecular phylogenetic reclassification can reveal previously unrecognized producers of biologically active secondary metabolites. These findings provide the first chemical and toxicological characterization of *C. aneilematis* and contribute to a better understanding of the relationship between fungal taxonomy, secondary metabolism, and biological activity.

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Author Contributions

Isolation of metabolites, K. Kondo, D. Wakana and Y. Doi; structure elucidation, K. Kondo, D. Wakana and T. Hosoe; cytotoxic assay, K. Kondo, S. Kitaoka, F. Sato, and N. Ikarashi; fungal identification, E. Tanaka; writing—original draft preparation, D. Wakana and T. Hosoe; writing—review and editing, D. Wakana, H. Takeda, S. Kitaoka, F. Sato and T. Hosoe; visualization, D. Wakana; supervision, T. Hosoe; project administration, T. Hosoe; funding acquisition, T. Hosoe. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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