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RESEARCH ARTICLE

Determination of Amatoxins in *Lepiota brunneoincarnata* and *Lepiota venenata* by High-Performance Liquid Chromatography Coupled with Mass Spectrometry

Pan Long[a]*, Fengxia Fan[a]*, Bin Xu[b], Zhengmi He[a], Yuting Su[a], Ping Zhang[a], Jianwei Xie[b] and Zuohong Chen[a] (ID)

[a]College of Life Science, Hunan Normal University, Changsha, China; [b]State Key Laboratory of Toxicology and Medical Countermeasures, and Laboratory of Toxicant Analysis, Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China

ABSTRACT

Three hepatic failure poisoning incidents caused by *Lepiota brunneoincarnata* and *Lepiota venenata* mushrooms have been occurred in China in 2017, *L. venenata* has been described as a new species. However, the cyclopeptide toxins of these lethal mushrooms remain poorly understood. In this study, the composition and content of amatoxins in *L. brunneoincarnata* and *L. venenata* are analyzed and compared, the analysis of composition and content of amatoxins in *L. venenata* are reported for the first time. The results showed that β-amanitin (β-AMA), α-amanitin (α-AMA), amanin, and amaninamide were identified in *L. brunneoincarnata*, and α-AMA, amanin II (an analog of amanin), and an unknown compound were identified in *L. venenata*. The differences between *L. brunneoincarnata* and *L. venenata* in the identified compounds provide chemical evidence for *L. venenata* as a new species. Quantitative analysis shows that α-AMA concentrations in *L. brunneoincarnata* and *L. venenata* were 0.72–1.97 mg/g dry weight, β-AMA concentrations in *L. brunneoincarnata* were 0.57–0.94 mg/g dry weight, and β-AMA was absent in *L. venenata*.

1. Introduction

The genus *Lepiota* contains approximately 400 species of mushrooms distributed worldwide [1]. It is well-known that several species have caused fatal poisonings after ingestion from Europe, America, and Asia, such as *Lepiota brunneoincarnata* [2–6], *Lepiota helveola* [7,8], and *Lepiota subincarnata* [9]. These poisonous *Lepiota* species contain amatoxins, which are responsible for the hepatotoxicity by irreversibly binding to RNA polymerase II [1,10]. However, in contrast to *Amanita*, there have been relatively few analyses of amatoxins composition and content in *Lepiota* species. In the early years of the last century, the amatoxins detection of *Lepiota* species have been restricted to the Meixner test, thin layer chromatography, and radio-immunoassay; these methods have poor resolution and low sensitivity [11,12]. Until the last few years, the amatoxins analysis in *Lepiota* species has been established using modern high-resolution methods in two related literature. Sgambelluri et al. [12] analyzed the composition of amatoxins present in six Italian species of *Lepiota* using liquid chromatography coupled to UV absorbance and mass spectrometry, and Yilmaz et al. [13] investigated the amatoxins concentration in different parts and in different growth phases of *L. brunneoincarnata* mushroom collected in Turkey using the reversed phase high-performance liquid chromatography (HPLC) method.

In China, mushroom poisoning caused by *Lepiota* species has never been reported; however, three mushroom poisoning incidents caused by *Lepiota* mushrooms occurred in 2017 and we obtained the *Lepiota* species specimens from the poisoning sites. Among these three poisoning cases, two cases were caused by *L. brunneoincarnata*, and one cases was caused by *Lepiota venenata*, which has been described as a new species [14]. The objective of this study was to detect the amatoxins composition and content in *L. brunneoincarnata* and *L. venenata* collected from China using HPLC coupled with high-resolution mass spectrometry (HPLC-HRMS).

2. Materials and methods

2.1. Mushroom collection and sample conditions

Three *Lepiota* samples (*L. brunneoincarnata* MHHNU 31026, MHHNU 31032, and *L. venenata*

CONTACT Jianwei Xie ✉ xiejwbmi@163.com; Zuohong Chen ✉ chenzuohong@263.net
*These authors contributed equally to this work.

Table 1. List of samples analyzed in this study.

Scientific name	Species no.	Geographic origin	Poisoning description	Collection time	ITS GenBank accession no.
Lepiota brunneoincarnata	MHHNU 31026	Guangrao County, Shandong Province N37°01'11.73'', E118°26'44.75'', alt. 592 m	A man poisoned, developed acute hepatic failure	2017.8.16	MK095187
L. brunneoincarnata	MHHNU 31030	Dalian City, Liaoning Province N38°54'38.75'', E121°37'47.70'', alt. 60 m		2017.9.6	MK095188
L. brunneoincarnata	MHHNU 31032	Chendou City, Sichuan Province N30°34'22.68'', E104°03'9.10'', alt. 500 m	A woman poisoned, developed acute hepatic failure	2017.9.6	MK095190
L. venenata	MHHNU 31031	Jingzhou City, Hubei Province N30°20'14.40'', E112°14'5.86'', alt.300 m	Two man poisoned, developed acute hepatic failure	2017.9.10	MK095189

MHHNU31031) were collected from the place where the patients had collected the mushrooms under the leadership of the patient's relatives and the mushroom was confirmed by the patients that it was the mushroom they ate. *L. brunneoincarnata* MHHNU 31030 sample was collected from the Labor Park in Daliang, Liaoning. All fresh mushroom carpophores were dried at 50 °C with airflow food dryer (Jintao Electrical Co., LTD, Ningbo, China) for 24 h until completely dry. All samples were deposited in desiccator in the Mycological Herbarium of Hunan Normal University (MHHNU). Information on the collections and their ITS accession numbers in GenBank are given in Table 1 and Figure 1.

2.2. Mushroom identification and phylogenetic analysis based on ITS sequences

All *Lepiota* species were identified by both morphological and molecular phylogenetic analyses. The morphological identification was performed according to the macroscopic and microscopic characteristics using our previous methods [14]. DNA extraction, polymerase chain reaction amplification, sequencing, and alignment of the ITS sequences were performed as described by Zhang et al. [15] and Cai et al. [16]. The phylogenetic tree was generated with both maximum likelihood analyses and Bayesian inference was performed using RAxML 7.2.6 [17] and MrBayes 3.2.4 [18], respectively, with the parameters employed by Cai et al. [16], except that the Bayesian Inference runs were performed for 10 million generations.

2.3. Extraction of the amatoxins

The extraction of amatoxins was performed by our previous described method [19,20]. Pieces of the pileus from the dried carpophores of the samples were cut and dried at 45 °C with airflow food dryer to a constant weight, then ground using a KC-04 pulverizer. For each sample, 0.05 g ground material was weighed and dispensed accurately into individual test tubes, and the peptide toxins were extracted with 2 mL formic acid/methanol/water (0.5:50:49.5, *v/v/v*). The test tubes were then shaken at 200 rpm for 12 h. Following centrifugation at 14,000 rpm for 10 min, the supernatant was decanted and retained while the residue was extracted again as described above. The two supernatants were combined and evaporated, and the residue was dissolved in 1 mL of water. This solution was degreased in petroleum ether, evaporated again, and the residue was redissolved in 100 μL of 10% methanol in water for HPLC analysis. The extraction was performed in triplicate for each sample, and the mean value was determined.

2.4. HPLC-HRMS analysis

HPLC conditions: Waters Acquity UPLC (Waters, Milford, MA, USA) coupled with a variable wavelength detector, CAPCELL PAK C18 (2.1 mm × 100 mm, 2.7 μm), F3643 EXP® GUARD CARTRIDGE CAPCELL CORE C18 S-2.7 (2.1 × 5 mm, 2.7 μm); mobile phase: (A) 0.01 M aqueous ammonium acetate; (B) methanol. Gradient elution: 0–15 min, 0–5% B; 15–35 min, 5–80% B; 35–40 min, 80% B; 40–45 min, 80%–100% B; 45–50 min, 100% B; 50–55 min, 100%–0 B; 55–60 min, 100% A; flow rate: 0.25 mL/min; injection volume: 10 μL; column temperature: 40 °C. The absorbance of the eluates was monitored at 295 nm.

HRMS conditions: Waters Xevo G2-quadrupole time-of-flight (QTOF) MS/MS in electrospray positive ionization mode; source temperature: 100 °C; full scan acquisition mode; scan range: *m/z*

Figure 1. Basidiomata of *Lepiota* species. (A) *Lepiota brunneoincarnata* (MHHNU 31026); (B) *L. brunneoincarnata* (MHHNU 31030); (C) *L. brunneoincarnata* (MHHNU 31032); (D) *L. venenata* (MHHNU 31031; photos A, B, and D by Zuohong Chen; photo C by Xiaolan He).

50–1000; capillary voltage: 3 kV; sample cone voltage: 30 V; extraction cone voltage: 4 V; cone flow: 50 L/h; desolvation temperature: 300 °C; desolvation gas: 600 L/h; mass calibration: lockspray with 556.2771 Da of leucine encephalin.

2.5. Standard samples, linear regression equation, and quantitative analysis of α-AMA and β-AMA

Standard samples of α-amanitin (α-AMA, 90% purity, purchased from Merck-Calbiochem, Merck KGaA, Darmstadt, Germany) and β-amanitin (β-AMA, 90% purity, Sigma-Aldrich, St. Louis, MO, USA) were dissolved in doubly distilled water to a concentration of $0.1\,\mathrm{mg\ mL^{-1}}$ stock solution. Each of 50 μL α-AMA and β-AMA was taken and mixed in a tube and a concentration of $0.05\,\mathrm{mg\ mL^{-1}}$ α-AMA and β-AMA solution were obtained. A five-point curve was achieved with injections of 2, 4, 6, 8, and 10 μL (corresponding to 0.09, 0.18, 0.27, 0.36, and 0.45 μg each toxin) of mixed standard samples, and the linear regression equation between the injection amounts of toxins and peak area of each standard toxin and correlation coefficient were calculated using Origin 7.5 software. The regression equations and correlation coefficients were: $Y = 23870X + 1370.5$ and $R = 0.9878$ for α-AMA, $Y = 16272X - 468.3$ and

$R = 0.9938$ for β-AMA. The concentrations of α-AMA and β-AMA in the samples were calculated using peak areas. The concentrations of α-AMA and β-AMA are expressed as the mean ± SD.

3. Results

3.1. Mushroom identification and phylogenetic analysis based on ITS sequences

The macroscopic and microscopic characteristics of *L. brunneoincarnata* are in accordance with the previous literatures [13,21], and the macroscopic and microscopic characteristics of *L. venenata* MHHNU 31031 has been described in our previous paper [14]. The identifications were further confirmed by ITS sequences and the results strongly indicated that the samples of MHHNU 31026, MHHNU 31030, and MHHNU 31032 are *L. brunneoincarnata* with a strong bootstrap support (100%), and *L. venenata* MHHNU 31031 sample represents an independent species (Figure 2).

3.2. Identification of the amatoxins based on HPLC-HRMS

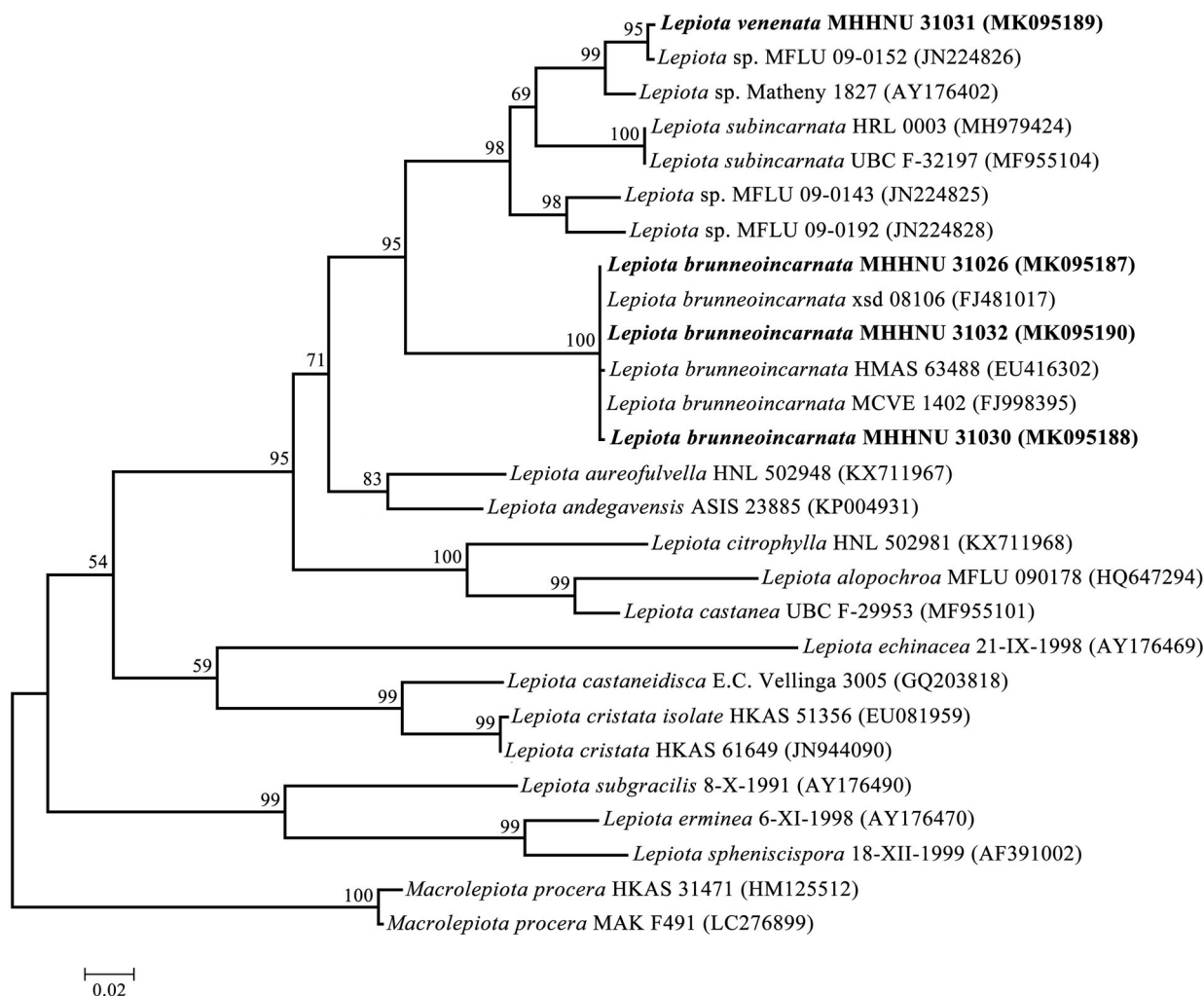The amatoxins extractions of four samples of *Lepoita* species were analyzed using HPLC-QTOF

Figure 2. Phylogenetic tree generated from maximum likelihood analysis based on ITS sequences. Maximum likelihood bootstraps over 50% and Bayesian posterior probabilities over 0.90 are reported on the branches. ITS sequences of four *Lepoita* species in this study are indicated in boldface, and the other ITS sequences of related taxa were retrieved from the GenBank.

MS/MS. The HPLC profiles are shown in Figure 3. The identification results for the six compounds are shown in Table 2. Peaks 1, 2, 3, and 4 represent masses corresponding exactly to those of β-amanitin, α-amanitin, amanin, and amaninamide, respectively. Among them, Peaks 3 and 6 represent a mass that corresponds to that of amanin (Table 2). We therefore concluded that Peak 6 is likely to be an analog of amanin, which we term "amanin II" hereafter. Peak 4 generated an $[M + H^+]$ ion of m/z 903.3681, which could correspond to either amaninamide or γ-amanitin, both of which have a mass of 902.3592. According to the previous report of Sgambelluri et al. [12], Peak 4 is identified as amaninamide. Peak 5 generated an accurate mass, but there are no corresponding known compounds that have been reported.

3.3. Concentrations of α-AMA and β-AMA in L. brunneoincarnata and L. venenata

The contents of α-AMA and β-AMA of *L. brunneoincarnata* and *L. venenata* are shown in Table 3.

The results show that the *L. brunneoincarnata* samples contain α-AMA and β-AMA and have total toxin contents ranging from 1.29 to 2.67 mg g^{-1} dry weight. By contrast, the *L. venenata* sample contains only α-AMA, β-AMA is absent, the α-AMA content is 1.97 mg g^{-1} dry weight. In addition, the amatoxin content varies significantly among *L. brunneoincarnata* samples collected from different locations, with the amount of amatoxin in tested *L. brunneoincarnata* MHHNU 31032 being twofold more than that of *L. brunneoincarnata* MHHNU 31030.

4. Discussion

Amatoxins, which are responsible for fatal poisonings, are known to be present in some species of *Amanita*, *Galerina*, and *Lepiota* genus [10,22]. The composition and content of amatoxins in lethal *Amanita* species from Europe, North America, and East Asia by means of modern high-resolution methods have been reported in many studies in the past 30 years [19,20,23–25]; however, up to now, only two studies have reported the amatoxins and

phallotoxins in the genus *Lepiota* by liquid chromatography combined with UV absorbance and mass spectrometry [12,13]. Sgambelluri et al. [12] reported that the α-AMA concentrations in *L. brunneoincarnata* and *Lepiota josserandii* were 0.69–0.82 mg/g dry weight and 3.99–4.39 mg/g dry weight, respectively. Yilmaz et al. [13] reported that the α-AMA and β-AMA concentrations in *L. brunneoincarnata* were 2.38 ± 0.70 and 1.97 ± 0.52 mg/g dry weight, respectively. In this study, the α-AMA concentrations in *L. brunneoincarnata* and *L. venenata* were 0.72–1.97 mg/g dry weight, which is in the range of previous reports. The β-AMA concentrations in *L. brunneoincarnata* were 0.57–0.94 mg/g dry weight, and β-AMA was not detected in
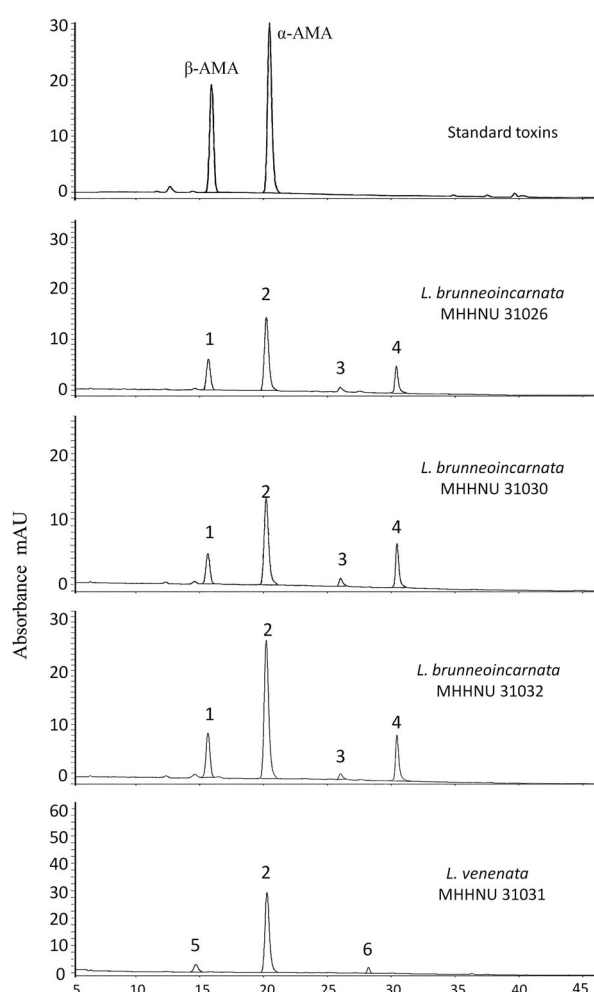
L. venenata. Sgambelluri et al. [12] also reported that *L. josserandii* contained high levels of α-AMA, but β-AMA was absent.

Six compounds were identified in this study, of which β-AMA, α-AMA, amanin, and amaninamide were identified by exact mass. An analog of amanin, which we term "amanin II," has the same mass as that of amanin but a different elution time, was identified from *L. venenata*. Additionally, an unknown compound was observed from *L. venenata*. *L. brunneoincarnata* contained β-AMA, α-AMA, amanin, and amaninamide, but *L. venenata* contained only α-AMA, amanin II, and an unknown compound. Lüli et al. [26] recently analyzed the genome of *L. venenata* (the same sample MHHNU 31031) and illustrated that only one MSDIN gene, named LvAMA1 which codes for α-amanitin, exits in the genome, in addition, only α-amanitin was found in the extract by LC-MS. These results showed that the composition of amatoxins of *L. venenata* and *L. brunneoincarnata* is different, which provided a strong chemical evidence for *L. venenata* as a new species, which was described and illustrated based on morphological and molecular phylogenetic data [14].

5. Conclusion

Six compounds were identified from *L. brunneoincarnata* and *L. venenata* by HPLC coupled with mass spectrometry, and the differences in the compounds between *L. brunneoincarnata* and *L. venenata* provide chemical evidence for *L. venenata* as a new species. α-AMA concentrations in *L. brunneoincarnata* and *L. venenata* were 0.72–1.97 mg/g dry weight, β-AMA concentrations in *L. brunneoincarnata* were 0.57–0.94 mg/g dry weight, and β-AMA was absent in *L. venenata*.

Figure 3. HPLC profiles of cyclopeptide toxins extracted from *L. brunneoincarnata* and *L. venenata*.

Table 3. Main amatoxin contents in two *Lepiota* species (mg g^{-1}dry weight).

Scientific name and species no.	α-AMA	β-AMA	Total
L. brunneoincarnata MHHNU 31026	0.85 (±0.05)	0.72 (±0.02)	1.57
L. brunneoincarnata MHHNU 31030	0.72 (±0.05)	0.57 (±0.02)	1.29
L. brunneoincarnata MHHNU 31032	1.73 (±0.01)	0.94 (±0.01)	2.67
L. venenata MHHNU 31031	1.97 (±0.01)	–	1.97

Table 2. Exact masses of compounds in this study.

Peak number	Compounds	True mass (Da)	Observed mass (Da)	Peak identification methods
1	β-Amanitin	919.3382	920.3390 [M + H$^+$], 942.3330 [M + Na$^+$]	Based on standard compounds and references
2	α-Amanitin	918.3542	919.3561 [M + H$^+$], 941.3286 [M + Na$^+$]	Based on standard compounds and references
3	Amanin	903.3432	904.3535 [M + H$^+$], 926.3332 [M + Na$^+$]	Based on references
4	Amaninamide	902.3592	903.3681 [M + H$^+$], 925.3494 [M + Na$^+$]	Based on references
5	Unknown	–	573.9142 [M + H$^+$], 596.4311 [M + Na$^+$]	Based on references
6	Amanin II	903.3432	904.3535 [M + H$^+$], 926.3332 [M + Na$^+$]	Based on references

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Disclosure statement

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ORCID

Zuohong Chen http://orcid.org/0000-0001-7359-0257

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