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Amatoxin and phallotoxin concentration in *Amanita phalloides* spores and tissues

Ertugrul Kaya[1], Selim Karahan[1], Recep Bayram[2], Kursat Oguz Yaykasli[3], Serdar Colakoglu[4] and Ayhan Saritas[5]

Abstract

Most of the fatal cases of mushroom poisoning are caused by *Amanita phalloides*. The amount of toxin in mushroom varies according to climate and environmental conditions. The aim of this study is to measure α-, β-, and γ-amanitin with phalloidin and phallacidin toxin concentrations. Six pieces of *A. phalloides* mushrooms were gathered from a wooded area of Düzce, Turkey, on November 23, 2011. The mushrooms were broken into pieces as spores, mycelium, pileus, gills, stipe, and volva. α-, β-, and γ-Amanitin with phalloidin and phallacidin were analyzed using reversed-phase high-performance liquid chromatography. As a mobile phase, 50 mM ammonium acetate + acetonitrile (90 + 10, v/v) was used with a flow rate of 1 mL/min. C18 reverse phase column (150 × 4.6 mm; 5 μm particle) was used. The least amount of γ-amanitin toxins was found at the mycelium. The other toxins found to be in the least amount turned out to be the ones at the spores. The maximum amounts of amatoxins and phallotoxin were found at gills and pileus, respectively. In this study, the amount of toxin in the spores of *A. phalloides* was published for the first time, and this study is pioneering to deal with the amount of toxin in mushrooms grown in Turkey.

Keywords

Amanita phalloides, amanitin, phallotoxin, HPLC

Introduction

The *Amanita phalloides* genus is one of the main causes of fatal mushroom poisoning. Its toxins have been mainly divided into two families: amatoxins and phallotoxins. The toxic effect of phallotoxins is limited, and phalloidin (PHN), phallacidin (PCN), phallolisin, phallin, and phallisasin are some of the members of this group. The amatoxins, on the other hand, are the agents mainly responsible for clinical poisoning (Vetter, 1998). These toxins inhibit RNA polymerase II enzyme by binding to it. α-Amanitin (AA), β-amanitin (BA), γ-amanitin (GA), ε-amanitin, amaninamide, amanin, and amanullin are the members of amatoxins group (Lindell et al., 1970). AA has been the most widely investigated one of all these members. AA is also the first purified toxin, and it can be easily obtained with high purity today (Faulstich et al., 1973; Kaya et al., 2012a). AA rapidly accumulates in the liver after oral intake. Small amounts of toxins in the blood are eliminated through kidneys. For these reasons, clinical toxicity is observed in liver and kidneys. The protein synthesis stops in the cell when RNA polymerase II is blocked. So, liver failure is commonly observed in cases of poisoning (Garrouste et al., 2009;

[1] Department of Pharmacology, School of Medicine, Düzce University, Düzce, Turkey
[2] Department of Pharmacology, Abant İzzet Baysal School of Medicine, Bolu, Turkey
[3] Department of Medical Genetic, School of Medicine, Düzce University, Düzce, Turkey
[4] Department of Anatomy, School of Medicine, Düzce University, Düzce, Turkey
[5] Department of Emergency Medicine, School of Medicine, Düzce University, Düzce, Turkey

Corresponding author:
Ertugrul Kaya, Department of Pharmacology, School of Medicine, Düzce University, Düzce 81620, Turkey.
Email: drekaya@yahoo.com

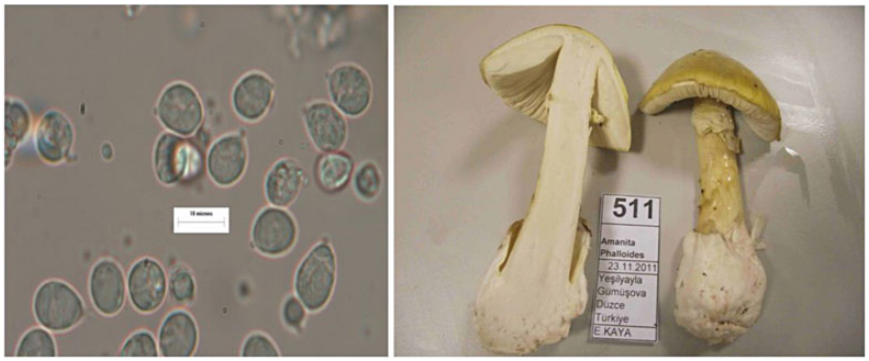

Figure 1. *Amanita phalloides* spores and basidiocarps.

Santi et al., 2012). Today, many patients can be saved with supportive therapies. However, liver transplantation is a last resort particularly when toxin is taken in high amounts. If the transplantation cannot be done, the patients may die within 5–7 days (Ferreira et al., 2011). BA showed similar characteristics in limited number of studies (Robinson-Fuentes et al., 2008). Relatively little is known about other amatoxins, and there is a strong need for further studies. Although some agents seem to be beneficial in treatment, there is still no specific antidote for amatoxins. Currently, penicillin and silibinin are used for treatment, and the effects of some other drugs like *N*-acetyl cysteine and thioctic acid are also under investigation (Magdalan et al., 2011, Ozbek et al., 2008). It has been observed through some studies on the contents of *A. phalloides* mushroom that toxin levels may alter due to different climatic and environmental conditions. The amount of toxin has also been found to be different in different parts of the mushroom and in different growth phases. The determination of the toxin content in parts of mushroom is important in that it may serve as a guide to the amount of toxin ingested in times of poisoning (Enjalbert et al., 1999; Mcknight et al., 2010). It has been established that no toxin analysis was performed in the spores of *A. phalloides* mushroom in related literature.

Several methods can be used for the analysis of amatoxins and phallotoxins, but reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used method. It is not possible to analyze both types of toxins on the same chromatogram because the ultraviolet absorbance of phallotoxins is different from that of amatoxins. However, it is now possible to analyze several different wavelengths on the same chromatogram using the recently developed diode array detector (DAD) (Brüggemann et al., 1996).

In this study, the quantity of amatoxins and phallotoxins in the spores and other tissues of *A. phalloides* mushrooms, obtained from the province of Düzce, Turkey, were analyzed using RP-HPLC.

Materials and methods

Mushroom collection and preparation

Six pieces of fully developed *A. phalloides* carpophores and surrounding micelles were collected on November 23, 2011 in Yeşilyayla forest (Gümüşova, Düzce, Turkey). Taxonomic classification of mushrooms was made in accordance with microscopic and macroscopic specifications (Figure 1). Spore prints were collected, and carpophores were divided into two pieces sagittally. The weight of fresh pieces was recorded before they were dried at 55°C airflow for 24 h. Then, the dried pieces were weighed, and the lost water ratio was calculated. The toxins were measured at a sagittal part as a whole, and the quantity of toxins in fresh mushroom was calculated through the lost water ratio. The other sagittal part was divided into pileus, gills, stipe, and volva, and then the toxin quantity was measured. All parts of the dried mushroom were ground and homogenized in 1 g/15 mL extraction medium (methanol–water–0.01 M hydrochloric acid (5:4:1, v/v/v) using tissue homogenizer. After incubating for 1 day, all specimens were centrifuged for 5 min at 5000 r/min, and the supernatant was filtered using a 0.22-μL syringe filter.

Standard solutions and chemicals

AA and PHN standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). BA, GA, and PCN standards were obtained from Enzo Life Sciences (Farmingdale, NY, USA). The solvents used in this study were

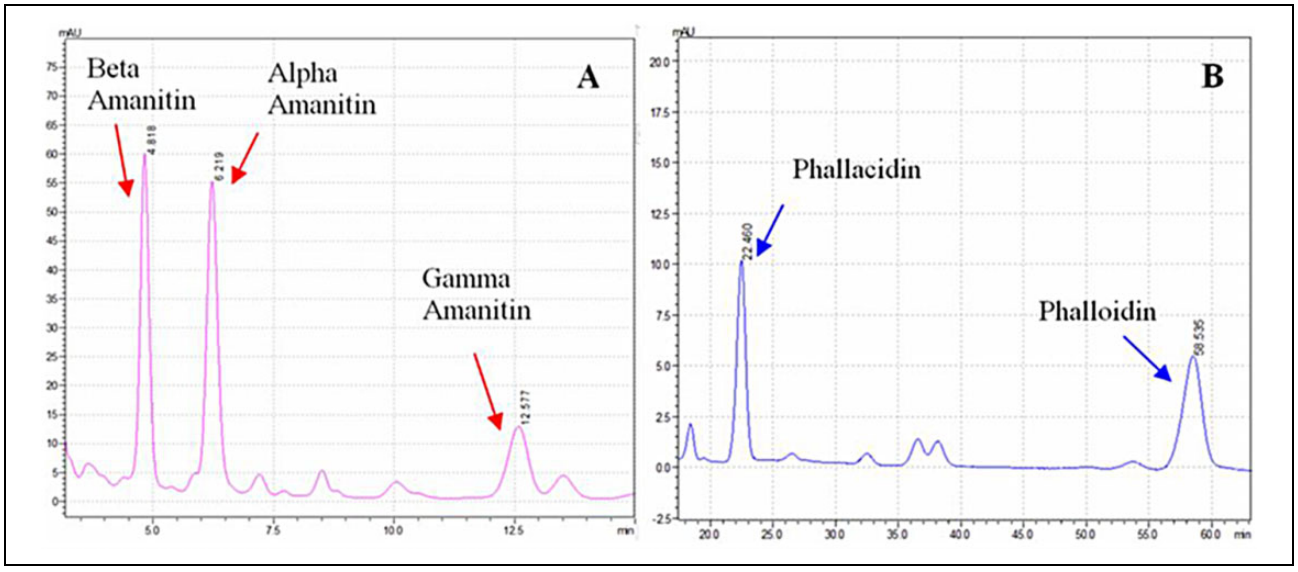

Figure 2. (a) HPLC chromatogram showing amanitin group toxin at 303 nm UV wavelength. (b) HPLC chromatogram showing phallotoxin group toxin at 291 nm UV wavelength. HPLC: high-performance liquid chromatography; UV: ultraviolet.

all HPLC grade. Stock solutions of AA, BA, GA, PHN, and PCN (100 mg/mL) were prepared in deionized water. Calibration standards of all toxins were diluted in the extraction fluid with concentrations of 10, 20, 100, 200, 1000, and 2000 ng/mL. Calibration curves were produced for each toxin and were linear over the range of interest ($R^2 > 0.999$).

RP-HPLC analysis of toxins

Chromatography conditions for the procedure followed in this study have been reported by Mcknight et al. (2010). In short, the authors reported excellent separation of amatoxins and phallotoxins with RP-HPLC and DAD detection. The study was carried out in the laboratory conditions, where RP-HPLC analysis of mushroom extracts was performed on a HPLC system (Shimadzu, Japan). Analytical RP-HPLC analysis of standard solutions of AA, BA, GA, PHN, and PCN and subsequent quantification of mushroom extracts were performed on a 150 × 4.6 mm, 5 µm particle, C18 column (Agilent Technologies, Palo Alto, CA) with 303 nm (for amatoxins) and 291 nm (for phallotoxins) at DAD detector. The mobile phase was used in isocratic profile with a flow rate of 1 mL/min. The content of the mobile phase was 0.05 M ammonium acetate (pH 5.5 with acetic acid)/acetonitrile (90:10 v/v). Detection limits were determined to be 2 ng/g for amatoxins and 2.5 ng/g for phallotoxins. The toxin quantities were given as mean (± SEM) in 1 g dried material for each sample. The fresh mushroom toxin quantity was calculated using the lost water ratio.

Results

The retention times for AA, BA, and GA using RP-HPLC were 6.22, 4.82, and 12.58, respectively, while for PCN and PHN were 22.46 and 58.54, respectively. Sample HPLC chromatograms of toxin analysis are shown in Figure 2.

The results found for each material applying the data obtained from HPLC chromatograms to calibration graph are given in Table 1. The amount of toxin was found to be at very low levels in mycelium (AA: 0.024 mg/g) and spores (AA: 0.087 mg/g). The maximum amount of amatoxins was measured at gills (AA: 3.39 mg/g). A high amount of amatoxins was found at pileus (AA: 2.95 mg/g) and stipe (AA: 2.36 mg/g). A relatively small amount of toxin was found at volva (AA: 1.03 mg/g). The quantities of toxin in unbroken dried mushrooms were also measured (AA: 2.80 mg/g) and toxin quantities of wet mushrooms were calculated proportional to those quantities (AA: 0.33, BA: 0.28, GA: 0.07, PCN: 0.25, and PHN: 0.15 mg/g). All toxins are shown as a graph in Figure 3.

The significant associations for all toxins were observed between different parts of the mushrooms ($p < 0.01$ for AA, $p < 0.01$ for BA, $p < 0.01$ for GA, $p < 0.01$ for PCN, and $p < 0.02$ for PHN).

Discussion

Wide varieties of mushrooms grow in all parts of Turkey, and it is not uncommon to come across poisonous

Table 1. Toxin concentrations obtained using RP-HPLC analysis (in milligram toxin per gram dry mushroom).

Toxin name	Spores	Mycelium	Pileus	Gills	Stipe	Volva	Total dry mushroom	Total fresh mushroom
AA	0.087 (±0.001)	0.024 (±0.0001)	2.95 (±0.05)	3.39 (±0.1)	2.36 (±0.03)	1.03 (±0.01)	2.80 (±0.13)	0.33
BA	0.040 (±0.001)	0.01 (±0.001)	2.53 (±0.03)	2.95 (±0.04)	1.75 (±0.01)	0.64 (±0.02)	2.38 (±0.06)	0.28
GA	0.18 (±0.0001)	0.24 (0.0001)	0.62 (0.005)	0.66 (±0.01)	0.5 (±0.019)	0.25 (±0.01)	0.6 (±0.01)	0.07
PCN	0.055 (±0.0001)	0.42 (0.0003)	2.27 (0.021)	2.06 (±0.01)	2.04 (±0.02)	1.88 (±0.01)	2.12 (±0.01)	0.25
PHN	0.018 (±0.0001)	0.01 (±0.0001)	1.4 (±0.03)	1.38 (±0.03)	1.18 (±0.01)	1.25 (±0.004)	1.32 (±0.01)	0.15

RP-HPLC: reversed-phase high-performance liquid chromatography; AA: α-amanitin; BA: β-amanitin; GA:γ-amanitin; PHN: phalloidin; PCN: phallacidin.

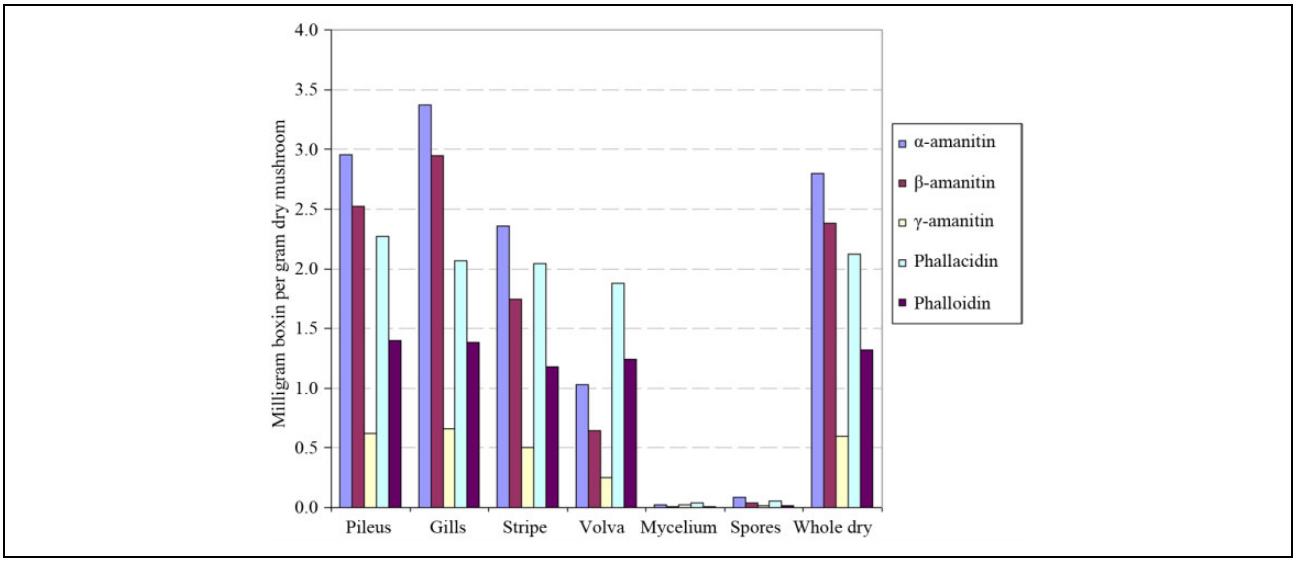

Figure 3. Amatoxin and phallotoxin concentrations in the tissues of *Amanita phalloides*.

mushroom. Therefore, mushroom poisoning is very common, and it may result in death in some cases. In Turkey, the main problem is lack of awareness about toxins in the content of a mushroom. If the content of a mushroom is clarified in detail, deadly mushroom poisoning can be prevented through early diagnosis and effective treatment methods. In this study, the concentrations and distributions of AA, BA, GA, PHN, and PCN in six different mushroom pieces of *A. phalloides* were analyzed using RP-HPLC. According to the experimental data obtained, all toxin concentrations in spores and mycelium were lower than in other parts of mushroom.

The concentrations of phallotoxins were similar in spores and mycelium; however, the AA and BA in spores were approximately four times higher in mycelium. This is the first time that the toxin concentration of *A. phalloides* mushroom's spores and mycelium has been published; therefore, these results cannot be compared to any previous results in literature since there is none. On the other hand, Mcknight et al. (2010) found that the AA in spores of *Amanita bisporigera* was lower in other parts of carpophores. Those results are consistent with the results of this study. In contrast, the ratio of AA concentration between spores and other parts of carpophores are not similar (Mcknight et al., 2010). The phallotoxins concentration in spores of *A. phalloides* and *A bisporigera* are similar; however, the ratio of these toxin concentrations between spores and other parts of carpophores are not similar. According to these data, the quantity and ratio of toxins in spores of *A. phalloides* and *A. bisporigera* are different (Mcknight et al., 2010). It has been found that the toxin concentrations in mycelium are 30–100 times lower than the other carpophore parts. This is the first study to investigate the quantity of toxins in mycelium and spores of *A. phalloides*.

The highest concentrations of amatoxins were measured at gills as in the studies of Enjalbert et al.

(1999). However, these toxin concentrations were five to seven times higher than is the case in this study (Enjalbert et al., 1999, 1993). Now that the extraction and analyzing methods are similar in these studies, the discrepancy between the results of Enjalbert et al. (1999) and those of this study might stem from environmental conditions. The results of this study support the assumption that environmental conditions and climate affect the amount of toxins present in mushrooms (Vetter, 1998).

The mushroom parts containing the maximum quantity of phallotoxin are different. Related literature suggests that phallotoxins concentrations may be different in mushroom parts. In this study, the highest phallotoxins concentrations were measured in the pileus. Phallotoxin quantity and distribution in *A. phalloides* parts vary greatly as a result of environmental and climatic conditions (Brüggemann et al., 1996; Enjalbert et al., 1999, 1993; Mcknight et al., 2010).

There are investigations relating to the toxin contents of the mushroom in literature. In one of them, the total concentration of amatoxins was found to be 2.1–7.3 mg/g comprising 43% AA, 49% BA, and 8% GA. The quantity of AA and BA was found to be similar and more than the quantity of GA. Total amount of amatoxin is similar to our study. They also found that toxin levels differ about four times between studies carried out in consecutive years. (Stijve and Seeger, 1979). The reason for this discrepancy might be due to the different methods used in measuring the toxin or the climatic differences in regions where the mushrooms grow up.

In other study, the quantity of toxin in *A. pallidorosea* mushroom, a member of *Amanita* family were analyzed and found approximately three to six times more higher than our results. In addition, BA was not found in some parts of the mushroom (Wang et al., 2011).

Amanita exitialis, a relatively new species in China belong to *Amanita* family. The total quantity and content of toxins in this species were analyzed by Hu et al (2003, 2012). They found similar total quantity of toxin as our results, and the ratio of toxins ranged according to the developmental stage of the mushroom.

It can be concluded from these results that *Amanita* family mushrooms containing the toxic peptides have enough quantity of toxin to cause fatal poisoning, and the amount of toxins alters according to the type and growing conditions of the mushroom.

The fatal mushroom poisoning is an important, serious health problem. The toxin content of these fatal mushrooms growing in our region should be analyzed in terms of the emergency cases to avoid any devastating effects to health (Fedakar and Turkmen, 2008).

In our previous study, in 2010, we collected and measured the amount of AA in the *A. phalloides* mushroom from the same region and obtained similar results (3.52 mg/g in hat, 5.32 mg/g in lamella, 2.58 mg/g in stem, 0.70 mg/g in volva, and 4.81 mg/g in total) (Kaya et al., 2012b). The AA quantity between current and previous studies is very close (just differ by one to two times), when comparing with other studies from different regions. Therefore, it might be speculated that the toxin content of the mushroom depends on environmental conditions and region more than climate.

The main parameter in deadly mushroom poisoning cases is toxin concentrations taken in by the body by eating fresh mushroom (Vetter, 1998). The toxin content of fresh mushroom has also been calculated and published in this study.

In conclusion, the distributions and concentrations of amatoxin and phallotoxin in tissues of *A. phalloides* are different due to environmental and climatic conditions. This study is the first publication concerning not only the toxin contents in *A. phalloides* spores and mycelium but also the toxin contents in *A. phalloides* that grows in Turkey.

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