

Microscopics diagnostic difficulties and the role of amanitin determination in poisoning caused by *Amanita phalloides*

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ABSTRACT

Introduction: The consumption of mushrooms (Basidiomycota) in Poland is one of the highest in Europe. It is particularly high in the 3rd quarter of each year, which is accompanied by an increase in the number of mushroom poisonings. This study aims to present difficulties with microscopic identification, the most popular method for diagnosing mushroom poisoning in hospital settings, when it comes to detecting *Amanita phalloides* spores in biological material.

Materials and methods: Spore analysis was carried out using aqueous solutions containing reference spores of different mushrooms: death cap (*Amanita phalloides*), parasol mushroom (*Macrolepiota procera*), field mushroom (*Agaricus campestris*), yellow knight mushroom (*Tricholoma equestre*), and green cracking russula (*Russula virescens*). The spore analysis was also carried out for a meal (soup) containing selected spores. Spores were identified using a light microscope and staining with Sudan III

and Meltzer's reagent. A statistical analysis of mushroom poisoning cases was also performed at the Department of Clinical and Forensic Toxicology, Pomeranian Medical University in Szczecin using records for 2015–2019.

Conclusions: Analysis of data from 2015–2019 from the Department of Clinical and Forensic Toxicology at the Pomeranian Medical University in Szczecin showed a marked increase in mushroom poisoning cases in the 3rd quarter of each year. Analysis of materials containing *Amanita phalloides* spores revealed their high similarity to oil drops and other cell structures present in biological material, resulting in the low reliability of microscopic identification. Therefore, as the absence of *Amanita phalloides* spores in the tested biological material does not rule out poisoning with this mushroom, a more advanced instrumental analysis (ELISA, LC/MS) is recommended.

Keywords: toxicology; poisoning; *Amanita phalloides*; amanitin.

INTRODUCTION

Mushrooms are an etiological factor in many cases of poisonings in Poland, a country where mushroom picking and consumption is much more popular than in many other parts of Europe. The 3rd quarter of each year, i.e. the season when the most popular edible species grow, is marked by a significant increase in the number of tests to detect mushroom poisoning.

Mushroom poisoning usually results from the ingestion of wild mushrooms after misidentification of a toxic mushroom as an edible species. The death cap (*Amanita phalloides*) is most often confused with green cracking russula (*Russula virescens*), yellow knight mushroom (*Tricholoma equestre*), parasol mushroom (*Macrolepiota procera*) or field mushroom (*Agaricus campestris*). In some cases, the hospital may admit patients presenting with gastrointestinal upset with a suspicion of mushroom poisoning, but the problems result from the incorrect processing of edible mushrooms.

Depending on the pathomechanism, there are 3 types of mushroom poisoning: cytotoxic, neurotoxic and gastric.

Cytotoxic toxins

The greatest hazard to human life and health is associated with the ingestion of cytotoxic toxins, which cause irreversible damage to the parenchymal organs, mainly the liver, as well as the spleen, kidneys and heart. These toxins have long latency (the time between ingestion and the onset of symptoms), and poisoning is characterised by severe course and high mortality. The most important cytotoxic toxins are amanitins and phalloidins in death cap (*Amanita phalloides*), destroying angel (*Amanita virosa*), spring amanita (*Amanita verna*), deadly galerina (*Galerina autumnalis*) and brown parasol mushroom (*Lepiota helveyana*), but also gyromitrin (in turban fungus – *Gyromitra esculenta*) and orellanine (deadly webcap – *Cortinarius orellanus*) [1, 2, 3, 4, 5].

Neurotoxic poisoning

Neurotoxic poisoning is caused, for example, by the ingestion of fly agaric (*Amanita muscaria*) and panther cap (*Amanita pantherina*), and neurotoxic toxins that affect the nervous system, including muscarine, muscaridine and derivatives of isoxazole and coprine. This type of intoxication is characterized

by a short latency (15 min–2 h) and is manifested by a wide range of symptoms, sometimes including agitation and hallucinations [1, 2, 4, 5].

Gastric poisoning

The yellow staining mushroom (*Agaricus xanthodermus*) and different species of *Russula* contain toxins that cause acute gastroenteritis. These toxins are responsible for gastric poisoning characterised by a relatively short latency and fulminant course (abdominal pain, nausea, vomiting, and diarrhoea). The toxicity of these mushroom species is not very high, but acute intoxication may lead to serious water-electrolyte imbalance, especially in children and the elderly [1, 2, 4, 5].

The study aims to present difficulties concerning the microscopic identification of *Amanita phalloides* spores in biological material, which is the most popular method for diagnosing mushroom poisoning in hospital settings.

MATERIALS AND METHODS

Spore analysis was carried out from previously prepared aqueous of reference mushrooms, i.e. *Amanita phalloides*, *Macrolepiota procera*, *Agaricus campestris*, *Tricholoma equestre* and *Russula virescens*. The spores were also analysed in an edible solution (soup), which served as a sample simulating the milieu of the clinical specimen.

Fungal spores were analysed under a light microscope (Olympus CX21LED) at x40 magnification. There were placed in 10 unstained slides, 10 slides stained with Melzer's reagent (which stains amyloid and dextrinoid structures) and 10 slides stained with Sudan III reagent (which stains droplets of oil). We carried out a statistical analysis of records from toxicology tests performed at the Department of Clinical and Forensic Toxicology, Pomeranian Medical University in Szczecin (DCFT PMU) in the years 2015–2019. The analysis comprised 416 tests for mushroom poisoning.

RESULTS

From 2015 to 2019, 416 tests were carried out at DCFT PMU to detect mushroom poisoning, of which 154 were positive and 262 negative (Fig. 1). A significant increase in the number of positive and negative tests to detect mushroom poisoning was observed from the 2nd half of the 2nd quarter of each year. It should be emphasized that mushroom poisoning is seasonal, and a significant increase in the number of cases was observed between July and September, i.e. in the 3rd quarter of each year (Fig. 2). This is the typical mushroom gathering season in Poland when the most popular edible mushroom species grow.

The identification of the spores from *Amanita phalloides*, *Macrolepiota procera*, *Agaricus campestris*, *Tricholoma equestre* and *Russula virescens* in the prepared aqueous solutions was

unproblematic and it was possible to assign specific spores to the family of mushrooms based on their appearance.

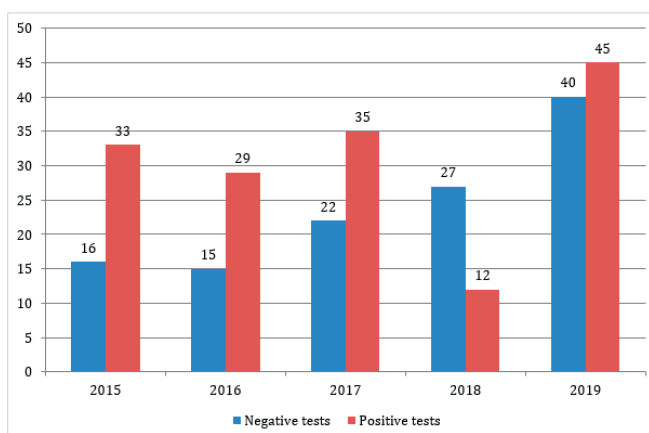


FIGURE 1. Mushroom poisoning tests carried out at the Department of Clinical and Forensic Toxicology, Pomeranian Medical University in Szczecin in 2015–2019

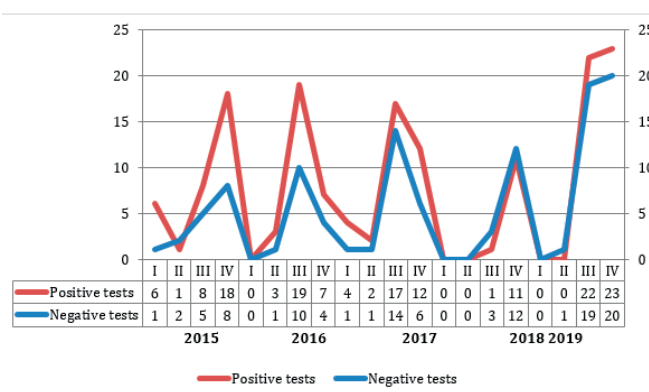


FIGURE 2. Seasonal changes in mushroom poisoning cases diagnosed at the Department of Clinical and Forensic Toxicology, Pomeranian Medical University in Szczecin in 2015–2019; I – 1st quarter of the year (January–March); II – 2nd quarter of the year (April–June); III – 3rd quarter of the year (July–September); IV – 4th quarter of the year (October–December)

Aqueous solutions containing the spores of *Amanita phalloides*, *Macrolepiota procera*, *Agaricus campestris*, *Tricholoma equestre* and *Russula virescens* were prepared, as well as samples of an edible solution (soup), to which spores of reference mushrooms were added. The solution was stained using the 2 most popular reagents, i.e. Melzer's reagent and Sudan III, which allow for the identification of spores.

The same protocol was used for samples prepared from a meal (soup) containing the above-mentioned spores. There were no difficulties with the identification and classification of spores from edible mushrooms (*Macrolepiota procera*, *Agaricus campestris*, *Tricholoma equestre* and *Russula virescens*) – Figures 3, 4, 5.

However, there were difficulties with the identification of *Amanita phalloides* spores, which were similar to the components of the biological sample, i.e. oil drops. Staining with Sudan III, in some cases, facilitated the discrimination of spores from oil drops (Fig. 6).

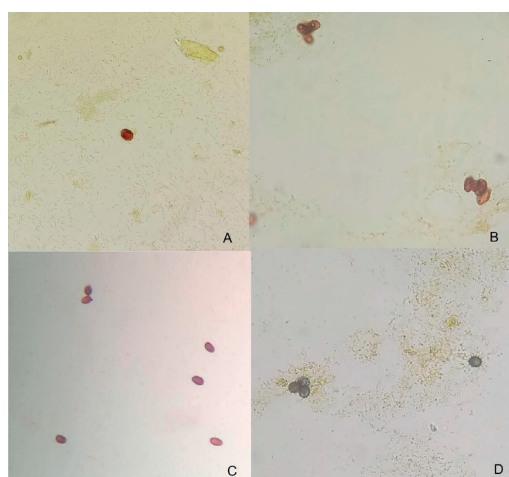


FIGURE 3. Spores of edible mushrooms stained with Melzer's reagent, images acquired from the light microscope (Olympus CX21LED): A – spores of *Tricholoma equestre*; B – spores of *Macrolepiota procera*; C – spores of *Agaricus campestris*; D – spores of *Russula virescens*

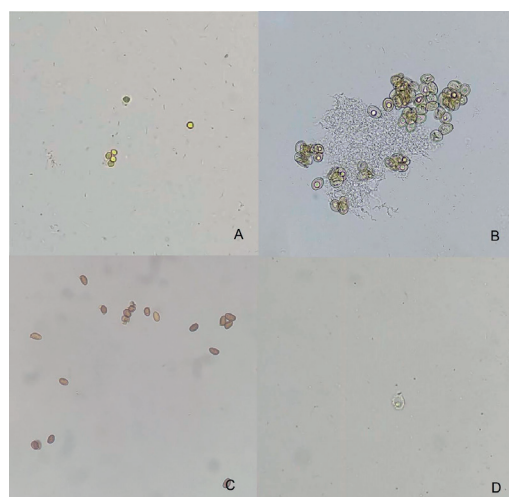


FIGURE 4. Spores of edible mushrooms stained with Sudan III, images acquired from the light microscope (Olympus CX21LED): A – spores of *Tricholoma equestre*; B – spores of *Macrolepiota procera*; C – spores of *Agaricus campestris*; D – spores of *Russula virescens*

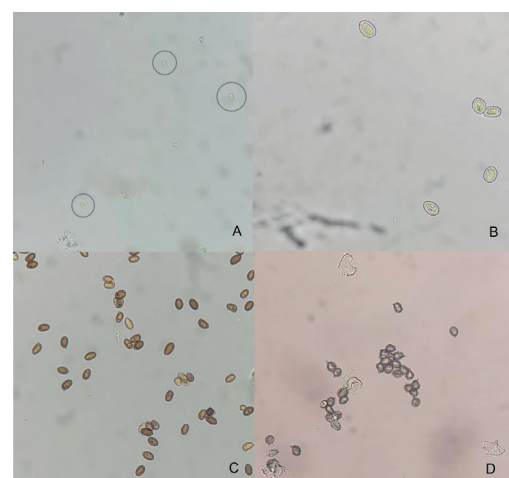


FIGURE 5. Unstained spores of edible mushrooms, images acquired from the light microscope (Olympus CX21LED): A – spores of *Tricholoma equestre* marked in grey; B – spores of *Macrolepiota procera*; C – spores of *Agaricus campestris*; D – spores of *Russula virescens*

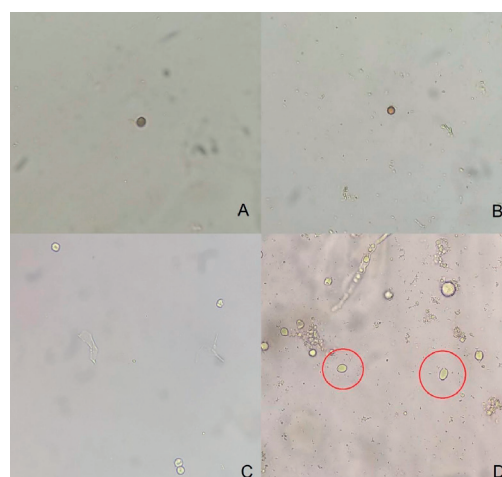


FIGURE 6. Spores of *Amanita phalloides* under the light microscope (Olympus CX21LED): A – spores stained with Sudan III; B – spores stained with Melzer's reagent; C – unstained spores; D – unstained spores marked red in edible solution (soup)

DISCUSSION

The analysis of *Amanita phalloides* spores using a light microscope (Olympus CX21LED) creates many difficulties with the interpretation due to the similarity of the spores to oil drops present in biological material and food. This means that poisoning with *Amanita phalloides* cannot be ruled out even if spores are not detected on the microscopic slides. Intoxication with *Amanita phalloides* can be confirmed or ruled out only by determining amanitin in a toxicological test using instrumental techniques (LC/MS/MS, ELISA) [1, 2, 3].

The observation of characteristic morphological features of the spores is difficult during the microscopic analysis of the specimens prepared from the solutions of reference fungal spores. The staining of specimens visualizes the characteristic features of spores of individual species. However, it is difficult to visualize fungal spores and determine species when testing clinical material. Staining with Melzer's or Sudan III reagents allows for the identification of spore species, but it is much more difficult because of the presence of other cellular and food components that are also stained. Sudan III stains oil drops but also certain plant cells originating from the consumed fruit. When treated with Melzer's reagent, starch grains turn blue and yeast cells turn yellow. The greatest difficulty in the microscopic diagnosis is encountered in the cases of suspected *Amanita phalloides* poisoning. Analysis of *Amanita phalloides* spores in clinical material collected from the patient is extremely difficult because the spores are small and round, resembling oil drops. In addition, neither staining technique used in authors' study allowed for clear discrimination between *Amanita phalloides* spores, artifacts present in biological material, and oil drops. The presence of these features creates difficulties with the identification of spores and the species of mushrooms they originate from. Of note is the fact that in our study analyses were carried out under

conditions created specifically for the purpose of the experiment. One should be aware that in testing material collected from intoxicated patients, identification is even more difficult due to the fact that often material is rich in nutrients, which makes identification more difficult.

Despite the available literature data regarding the appearance of fungal spores [1], they are not sufficient to aid correct identification. *Macrolepiota procera* spores are described as large, oval, hyaline, with a spike on one end, and a characteristic cavity on the opposite end filled with plasma, granular components and vacuoles (Fig. 7). *Tricholoma equestre* spores are small, ellipsoidal, hyaline, with a single thick membrane. The plasma fills the entire spore (Fig. 8). *Agaricus campestris* spores are oval with a dark brown spike on one end (Fig. 9). *Russula virescens* spores are hyaline, ellipsoidal, with a larger spike on one end. The inside of the spore cannot be seen because the whole spore is covered with numerous spikes (Fig. 10). *Amanita phalloides* spores are spherical or slightly ellipsoidal, hyaline, with a spike on one end. The spore plasma is grey and surrounded with a membrane (Figs. 11) [1].

The differences between the actual and reference appearance of the spores (Figs. 7, 8, 9, 10, 11) presented in the photographs and illustrations demonstrate the difficulties with spore analysis under the light microscope encountered during diagnostic procedures. Therefore, this method is biased with a significant error, and cases of *Amanita phalloides* intoxication have to be confirmed using instrumental techniques for toxin detection.

A carefully collected medical history is important for establishing the diagnosis of mushroom poisoning. The type of mushrooms (with gills or pores, or both types) and the amount of these mushrooms ingested by the patient should be determined. The patient should be asked if mushrooms were fresh, dried or previously preserved, and what cooking method was used to prepare a meal that contained mushrooms. Information on the sequence of symptoms should be acquired, including the time of their onset and the time after which the patient arrived at the hospital. It is also important to identify other people who ingested these mushrooms and whether they have experienced symptoms of intoxication [1, 6]. Patients should also be interviewed for their medical history, with a special focus on chronic diseases and currently taken medications.

A detailed medical interview is followed by mycological tests of the mushroom meal leftovers eaten by the patient and the contents of the digestive tract such as vomit, extracted gastric content, lavage fluid, faeces or rectal effluent [1, 4, 6, 7, 8]. The analysis of collected material is focused on fungal spores, because it is possible to determine the type of mushrooms ingested by the patient based on the shape and size of spores. Vomit produced after the ingestion of mushrooms is the optimal material for analysis since it most likely contains identifiable spores. Unfortunately, such material is often unavailable for analysis because the patient does not collect it before coming to the hospital. Therefore, the most popular materials used for toxicology tests are gastric lavage fluid, rectal effluent or faeces [1, 2, 3].

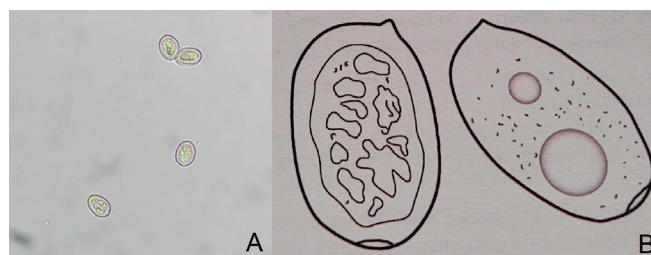


FIGURE 7. *Macrolepiota procera*: A – spores in reference solution, unstained. Image acquired from the light microscope (Olympus CX21LED); B – drawing of a spore presented in reference materials

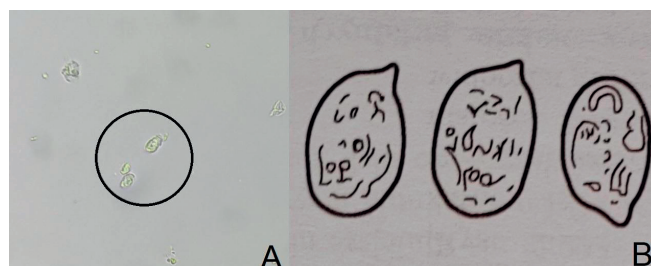


FIGURE 8. *Tricholoma equestre*: A – spores in reference solution, unstained. Image acquired from the light microscope (Olympus CX21LED); B – drawing of a spore presented in reference materials

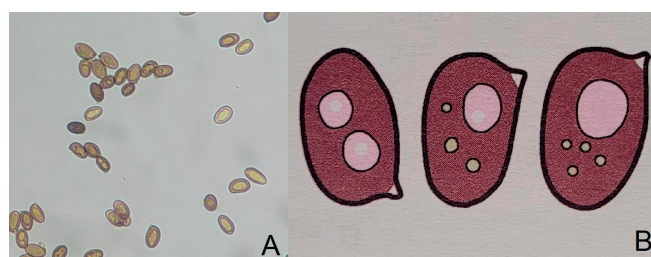


FIGURE 9. *Agaricus campestris*: A – spores in reference solution, unstained. Image acquired from the light microscope (Olympus CX21LED); B – drawing of a spore presented in reference materials

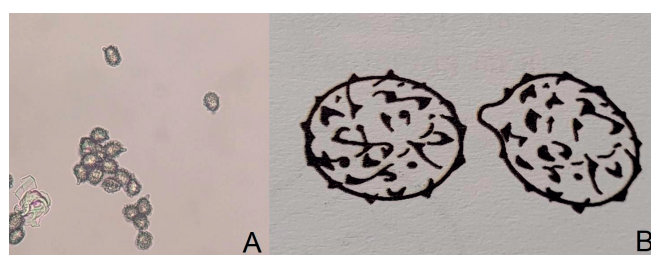


FIGURE 10. *Russula virescens*: A – spores in reference solution, unstained. Image acquired from the light microscope (Olympus CX21LED); B – drawing of a spore presented in reference materials



FIGURE 11. *Amanita phalloides*: A – spores in reference solution, unstained. Image acquired from the light microscope (Olympus CX21LED); B – drawing of a spore presented in reference materials

Mycological analysis under a light microscope is associated with many problems, because the biological material sent for analysis contains morphotic elements that resemble fungal spores, such as elements of human cells, microorganisms, yeast, and food remains. In addition, mycological analysis is biased with a significant error because the absence of fungal spores in the tested material does not rule out the possibility of intoxication. This may happen when a patient has delayed seeing a physician, material for tests was sampled incorrectly, or when the patient suffered severe vomiting and diarrhoea before arriving at the hospital. Therefore, microscopic evaluation of spores in biological material is extremely difficult and requires significant experience and diagnostic skills [1]. Specimens of 3 types of biological material are analysed: gastric lavage fluid, rectal effluent and faeces. Two types of staining techniques are most commonly used, with Melzer's reagent and Sudan III. However, in many cases, staining does not facilitate the identification of spores because of impurities present in the biological material. Importantly, in most hospitals in Poland it is the only method used to diagnose *Amanita phalloides* intoxication.

When intoxication with *Amanita phalloides* is suspected, diagnostic procedures should be initiated as soon as possible, and relevant treatment should be implemented in cases of confirmed intoxication.

One important element of the diagnosis of mushroom poisoning is the toxicological analysis, consisting of the identification of toxins in the clinical material using analytical methods such as high-performance liquid chromatography with electrochemical detection, high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS), capillary electrophoresis-mass spectrometry, capillary electrophoresis with diode array detection, radioimmunoassay, and enzyme-linked immunosorbent assay (ELISA) [1, 2, 3, 8].

The analysis for *Amanita phalloides* intoxication can also be performed using ELISA, which allows for the determination of amanitin in biological material. This method relies on the antigen-antibody relationship and the enzymatic reaction. Enzyme-linked immunosorbent assay is an immunosorbent assay biased with the risk of error due to the different affinity of antibodies to antigens that are not the target toxin in the test. This is caused by the cross-reactivity of the antibodies and antigens in the biological sample, leading to false-positive results and potentially incorrect diagnosis.

Amanitin can also be determined using LC-MS/MS. This technique is used for the separation of high molecular weight substances and selective detection and identification of molecules present in the sample. Liquid chromatography-tandem mass spectrometry allows for the identification and quantification of amanitin in biological material (serum, urine). The LC-MS/MS method is characterized by high sensitivity, resolution and selectivity. It allows the identification and determination of the chemical structure of a chemical compound. It should be emphasized that this is a reference method that allows you to confirm or rule out amanitin poisoning. However, equipment and reagents for LC/MS-MS are expensive, and therefore this technique is not used routinely in analytical laboratories.

Amanitin can be detected in the serum up to 8 h after the ingestion of *Amanita phalloides*, but this test is rarely used in clinical and diagnostic practice. This is caused by the asymptomatic period of the first phase of intoxication, in which patients are very rarely admitted to the hospital. In most cases amanitin is determined in urine samples because in this material it is detected up to 36 h from the moment of *Amanita phalloides* ingestion, i.e. in the 2nd phase of symptomatic poisoning [1, 2, 3, 5].

In addition to a carefully collected interview, mycological and toxicological examination, deviations in basic, publicly available tests, such as: gasometric tests (decrease in pH, decrease in HCO₃, possible decrease in CO₂), determination of prothrombin time (extension to approx. 47 s), international normalized ratio (increase above 4 and even up to 6) of bilirubin level (increase to about 34–513 µmol/L) and transaminases (increase in alanine transferase, aspartate transaminase: 2000–4000 U/L), serum creatinine level (1.9–14.8 mg/dL), ionogram (decrease in electrolytes, i.e. sodium, potassium, chlorine) and hypoglycaemia. These tests, in addition to the diagnostic value, have the greatest importance in the assessment of the effects of poisoning, because they enable early detection of the most common complications of toadstool poisoning, such as acute liver failure, renal failure, and metabolic acidosis. Early detection of these changes enables the introduction of preventive treatment [1, 3, 9, 10, 11].

CONCLUSIONS

1. A negative result obtained with the microscopic method during the diagnostic process in the case of suspicion of *Amanita phalloides* poisoning does not exclude poisoning.
2. Final confirmation of *Amanita phalloides* poisoning should be made using an instrumental method of the LC-MS/MS type.

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