ISSN 2395-650X

International Journal of Life Sciences Biotechnology Pharma Sciences

E-mail: editorijlbps@gmail.com editor@ijlbps.org

International Journal of Life Sciences Biotechnology Pharma Sciences

ISSN 2395-650X
Volume 9 Issue3 Aug 2021

Amanita Phalloides Intoxication Gene Expression Patterns In Mice: Apoptotic and Necrotic Pathways

V.Raju, G,Ratnakumari,Vijayalakshmi, Matta Ruthwik

ABSTRACT

The Amanita phalloides mushroom is the most dangerous in the world, and it induces abrupt liver failure, which is the main goal of this study. Hepatotoxicity is caused by the most powerful toxin, α-amanitin (α-AMA), which inhibits RNA polymerase II in hepatocytes and interrupts protein synthesis. Nevertheless, there is a severe lack of sufficient knowledge about the processes that lead to hepatotoxicity produced by α-AMA. This work seeks to uncover the intricate processes of necrosis and apoptosis that take place in mouse hepatocytes as a function of the duration of in vivo exposure to A. phalloides. Methods: The seven-mice BALB-c group was split into five subgroups: control, α-AMA-2, α-AMA-12, α-AMA-72, and α-AMA-96. Mice were given an oral dose of A. phalloides mushroom extract containing 10 mg/kg of α-AMA to simulate poisoning. After 2, 12, 72, and 96 hours, the mice were killed. Afterwards, the RT-qPCR technique was used to measure the amounts of TNF-α, Bax, caspase-3, and Bcl-2 gene expression in liver tissues. Additionally, histological evaluations were conducted to assess damage to liver tissues over time. The findings demonstrated that, when comparing the groups, the levels of the proinflammatory cytokine TNF-α mRNA expression in mouse liver tissues rose at 2 and 12 hours following A. phalloides administration, according to the RT-qPCR data. After ingesting A. phalloides, the levels of Bax mRNA expression spiked at 12 and 72 hours. When comparing the groups at 72 and 96 hours, we found that caspase-3 mRNA expression levels were higher, but Bcl-2 mRNA expression levels were lower. In conclusion, our results demonstrated that apoptotic mechanisms become effective after A. phalloides mushroom poisoning, after which necrotic processes emerge. Finally, novel therapeutic options may be devised by understanding the processes of A. phalloides-induced hepatotoxicity.

Keywords: α-amanitin, TNF-α, Bax, caspase-3, Bcl-2, RT-qPCR

INTRODUCTION

More than 90% of the fatalities caused by mushroom poisoning on a global scale occur as a consequence of Amanita phalloides (Vaill.) Link species, the most lethal poisonous fungus known to man (Vetter, 1998). Liver necrosis and subsequent hepatorenal phase development are clinical features of A. phalloides poisoning. Deterioration of renal and hepatic function, hyperglycemia, delirium, and disorientation may occur in patients over time (Becker et al., 1976). There is no known human mortality threshold for A. phalloides, however 20–79% of poisoned individuals acquire chronic liver disease (Serné et al., 1996; Yilmaz, Er mis, Akata, & Kaya, 2015). The latency phase, which begins after 6–24 hours after consuming

ISSN 2395-650X
Volume 9 Issue3 Aug 2021

the mush meal, is when the gastrointestinal symptoms (GIS) usually begin. Initially, you may have GIS problems, which may last for a few days. In the next twenty-four to thirty hours, clinical and laboratory assessments (the hepato-renal phase) show signs of acute hepatic failure and subsequent damage to many organs (Escudié et al., 2007; Karlson-Stiber & Persson, 2003). It typically takes 5 or 6 days (median duration is 6.1 days) for a deadly poisoning to cause death (Ganzert, Felgenhauer, & Zilker, 2005). According to studies conducted by Jan, Siddiqui, Ahmed, Ul Haq, and Khan (2008) and Enjalbert et al. (2002), the quantity of poison consumed in relation to body weight seems to have an effect on the severity of poisoning. Patients have a better chance of surviving if their liver damage is severe, their liver regeneration rate is high, and their poisoning is well treated (Alldredge B, 2012).

According to Garcia et al. (2015), there is currently no effective therapy for poisoning caused by A. phalloides mushrooms. Toxins produced by Acinetobacter phalloides, also known as amatoxins, are the principal cause of the severe liver damage seen in cases of mush room poisoning (Yilmaz et al., 2015). The most poisonous amatoxoid is α-amanitin, also known as α-AMA. It is a bicyclic octapeptide molecule that is very stable under heat and water (Kaya E, 2012; Wieland & Faulstich, 1978). Hepatotoxicity, caused by α-AMA, is the most well-known cause of hepatocellular failure. The primary harmful effect of α-AMA is to hinder protein synthesis in eukaryotic cells by non-competitive nuclear inhibition of RNA polymerase II. Because it inhibits transcription, α-AMA interrupts or impairs several cellular functions. According to research by Lindall, Weinberg, Morris, Roeder, and Rutter (1970) and Wieland (1983), when mRNA levels drop, protein synthesis drops, necrotic processes emerge, and cells die. Various intrinsic regulatory mechanisms govern apoptosis, the regulated programming of cell death that occurs naturally throughout development. In response to certain cellular stressors, mechanisms that hinder DNA transcription, such α-AMA, trigger cell cycle arrest or apoptosis. According to Y. Arima et al. (2005), apoptotic processes are believed to be significant in the development of liver damage in A. phalloides poisoning, and α-AMA is a strong inducer of apoptosis. The in vitro studies conducted by Magdalan et al. (2011) and others on human hepatocyte cultures and nine primary hepatocytes (Magdalan, Ostrowska, Piotrowska, Gomułkiewicz, et al., 2010) suggest that apoptosis could be a key factor in severe α-AMA-induced liver injury.

The underlying mechanisms of the apparent synergistic effect of α-AMA and tumor necrosis factor-α (TNF-α) in inducing apoptosis remain unknown. In addition, α-AMA causes apoptosis by activating the p53 protein (Y. Arima et al., 2005). The development of severe hepatotoxicity by α-AMA may be influenced by the destruction of mitochondrial membrane potential, according to Wang et al. (2018). An rise in hepatic pro-inflammatory TNF-α mRNA levels, death in hepatocytes, and prevention of liver damage in mice treated with anti-TNF-α antibodies were seen when high-dose α-AMA was administered in vivo, according to a study by Leist et al. (1997). Another research that used hepatocyte cultures (Magdalan, Ostrowska, Pi otrowska, Izykowska, et al., 2010) did not establish that α-AMA toxicity is dependent on the presence of TNF-α. In vivo models of inflammatory liver damage, TNF-α has been shown to promote both hepatocellular apoptosis and necrosis (Tiegs & Horst, 2022). Understanding the cellular mechanisms that lead to liver damage is clinically vital, since the intricate process of inducing necrosis and apoptosis by α-AMA is essential. Tragically, A. phalloides poisoning continues to rise in the modern day, and no effective therapy has been

ISSN 2395-650X
Volume 9 Issue3 Aug 2021

discovered (Ertugrul Kaya et al., 2016). Palliative care is the only option for commonly used therapies. To increase the likelihood of survival for patients poisoned with A. phalloides, it is crucial to create novel treatment options that work. Based on the amount of time that mouse hepatocyte cells are exposed to α-AMA cytotoxicity, this study sought to examine the complex apoptotic [Bax, caspase-3, Bcl-2, B-cell lymphoma-2] and necrotic [TNF-α, tumor necrosis factor-α] mechanisms at the gene expression levels. This is how the molecular pathways that cause α-AMA to harm the liver have been tried to be explained. Therefore, our results could help in the search for novel approaches to treating A. phalloides poisoning.

MATERIAL AND METHODS

phalloides mushroom collection

Wild phalloides mushrooms were plucked from the woods near Gümüşova in Düzce, Turkey. The macroscopic characteristics of the gathered mushrooms were used to systematically identify them (Figure 1).

A. phalloides mushroom extraction

The mushrooms were pulverized into a powder after being dried in an airflow of 50-60°C for 24 hours. A mixture of 150 mL of solvent (methanol, water, 0.01 M HCI, in a ratio of 5:4:1, v/v/v) was used to homogenize 10 grams of A. phalloides. The mixture was then incubated for 24 hours. Afterwards, a Soxhlet device was used to centrifuge the solution for 5 minutes at 5000 rpm. After filtering it using syringe filters (0.22 µL), the supernatant was extracted using 150 mL of 50% methanol for 4 hours. Evaporation in a vacuum evaporator was performed at 50°C until the resulting extracts were totally dry (E. Kaya et al., 2015).

An α-AMA standard (1 mg/mL, Sigma Aldrich, St. Louis, MO, USA) was diluted in dH2O at concentrations of 10, 20, 100, 200, 1000, and 2000 ng/mL in the analytical HPLC system. A 6-point calibration curve was then constructed, which was performed three times. Over the target concentration range, the calibration curve exhibited a linear relationship (R2 > 0.99). Following the protocols laid forth by Kaya et al., we ran the chromatographic analysis (E. Kaya et al., 2015; E. Kaya et al., 2013). A Reversed-Phase High-Performance Liquid Chromatography system (RP-HPLC, Shimadzu, Japan) was used to analyze the mushroom extract. The following were the settings used by the RP-HPLC system: Using a C18 column from Agilent Technologies in Palo Alto, CA, with dimensions of 150 x 4.6 mm and 5 µm particles, the DAD detector operates at 302 nm. The mobile phase was a mixture of 0.05 M ammonium acetate (pH 5.5) and 90:10 v/v acetonitrile, and the flow rate was 1 mL/min. The quantity of α-AMA was measured as the mean ± SEM in 1 g of dry mushroom, and the detection limit was set at 2 ng/g orally. To summarize, the semi-preparative RP-HPLC was loaded with 1 mL of the A. phalloides water extract. The percentage was collected by the collector (4.6 x 250 mm C18 ODS column with 5 µm particles) from the start to the finish of the peak at the same retention period as the α-AMA standard. The fraction was subjected to a second round of purification using the preparative HPLC system after being dried in a vacuum evaporator set at 50°C. It was then dissolved in 1 mL of 40% methanol. Next, 20 µL of α-AMA was added to the analytical HPLC equipment in order to determine the concentration and purity of the toxin in that particular fraction. By plugging the analytical peak regions into the calibration curve's equation, we were able to determine the quantity of material. Distilled water was used to dissolve the pure α-AMA that was obtained.

International Journal of Life Sciences Biotechnology Pharma Sciences
ISSN 2395-650X
Volume 9 Issue3 Aug 2021

Figure 1. Amanita phalloides.

conducted a quantitative analysis on the water after the solvent had evaporated. The findings were used to create A. phalloides extracts, which were diluted in distilled water with 20 mg of α-AMA. Worms and medical procedures The male BALB-c mice, which typically weigh between 20 and 30 grams, were procured from DÜSAM, the Dicle University Health Sciences Application and Research Center. All studies with animals were conducted in strict compliance with the regulations set out by the Dicle University Experimental Animals Local Ethics Committee (DUHADEK-2021/45) and with their explicit approval. The animals were kept in an environment with a 12 hour light/dark cycle, consistent humidity (50-55%), and a temperature range of 22±3°C. They also had unrestricted access to food and water. The animals were randomly assigned to one of five groups (n = 7) after a one-week acclimation period. Tong et al. (2007) and Zhao et al. (2006) found that the animal model of intoxication is a valid representation of α-AMA poisoning since it mimics the hepatotoxic symptoms seen in people after amatoxin treatment. Furthermore, the in vivo study's toxin doses and experimental design were crafted using a clinical viewpoint, drawing on data on clinical toxicity procedures and α-AMA pharmacokinetics. Figure 2 depicts the experimental mental model. According to Garcia et al. (2015), Park et al. (2021), and Wieland & Faulstich (1978), a water extract from A. phalloides mushrooms was administered orally by stomach gavage at a concentration of 10 mg/kg α-AMA. Before the studies began, the mice were given a 24-hour fast. Mice in the control group were orally gavaged with 1 mL of 0.9% physiological saline at 0 minutes. The A. phalloides mush room water extract, which contained 10 mg/kg of α-AMA, was used in the α-AMA-2, α-AMA-12, α-AMA-72, and ε-AMA-96 groups.

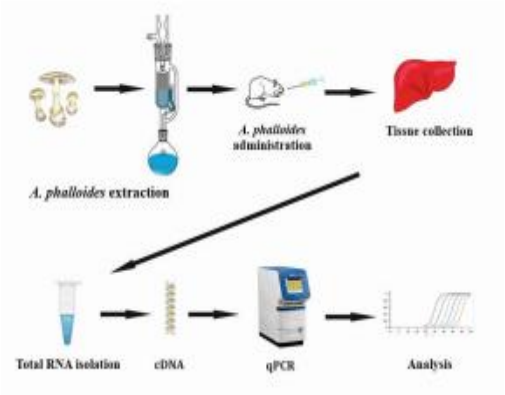


Figure 2. Experimental Design.

Table 1. Primer sequences

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Bax	GGATGCGTCCACCAAGAAG	GGAGGAAGTCCAGTGTCCAGCC
Bcl-2	TGAGTACCTGAACCGGCATCT	GCATCCCAGCCTCCGTTAT
caspase-3	TGCAGAACAAAACCTCAGT	TGTCTCTCTGAGGTTGGCTG
TNF-α	AAATGGGCTCCCTCTCATCAGTTC	TCTGCTTGGTGGTTTGCTACGAC
GAPDH	ACTCCACTCACGGCAAATTC	TCTCCATGGTGGTGAAGACA

given to the animals by orogastric gavage at 0 min. At2,12,72, and 96 hours after sacrifice, the animals' livers were removed. The mice were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) before being slaughtered at the conclusion of the studies. In preparation for RT-qPCR, the liver samples were frozen at -80°C. The liver was also embedded in paraffin for standard histopathologic examination after being fixed in a 10% zinc formalin solution for 24 hours.

The levels of TNF-α, Bax, caspase-3, and Bcl-2 gene expression were assessed using Real Time Polymerase Chain Reaction (RT-qPCR, Applied Bioscience Ste pOnePlus™, Foster City, CA) in order to study the molecular processes of apoptotic and necrotic pathways generated by A. phalloides. The liver tissues were frozen at -80°C and then RNA extraction was carried out. Tissues were treated with RiboZolTM (VWR-Amresco, USA) to isolate total RNA in accordance with the directions provided by the manufacturer. A microvolume spectrophotometer

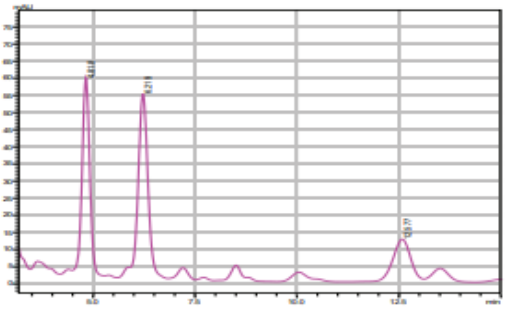
International Journal of Life Sciences Biotechnology Pharma Sciences
ISSN 2395-650X
Volume 9 Issue3 Aug 2021

(Nano-Drop 2000C, Thermo Scientific, USA) was used to measure the amount and quality of the RNA samples. Thermo Fisher, USA's DNAse-I digesting enzyme was used to eliminate genomic DNA contamination. We followed the directions provided by Thermo Fisher, USA, for their cDNA Synthesis Kit to conduct the cDNA synthesis. Using Primer3 software and the sequences found in the NCBI database, we were able to build the primer pairs utilized for qPCR analysis (Table 1). Ten microliters of 2X SYBR Green Master Mix, two microliters of 0.2 µm primer, and one microliter of cDNA were added to a twenty microliter volume of reaction mix for RT-qPCR amplification. Starting with a 10-minute denaturation at 95°C, the real-time PCR thermal cycling conditions consisted of 40 cycles of denaturation (for 30 seconds), reassembly (for 1 minute at 60°C), and extension (for 30 seconds at 72°C). In order to conduct the melting curve study, the temperature was raised from 55°C to 95°C in increments of 1°C. A negative control was established using samples that did not contain cDNA. For normalization, the expression of the housekeeping gene (GAPDH) was used as an internal control. The relative mRNA expressions were assessed using the comparative Ct technique (2−ΔŔCT), as reported by Schmittgen & Livak (2008), for every cDNA sample. PCR amplifications were carried out in triplicate.

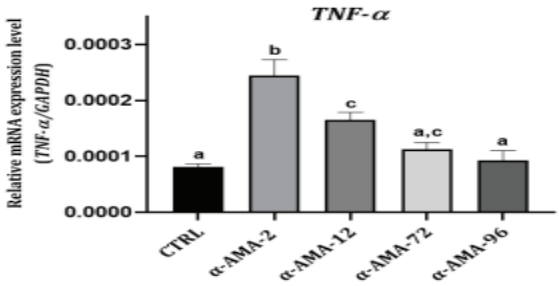
RESULTS

In the RP HPLC chromatogram, the individual retention durations for β-AMA, α-AMA, and γ-AMA were 4.818, 6.219, and 12.577 minutes, respectively. Figure 3 shows the α-AMA analysis RP-HPLC chromatogram.

Time-dependent expression of TNF-α, Bax, caspase-3, and Bcl-2 genes after A. phalloides administration

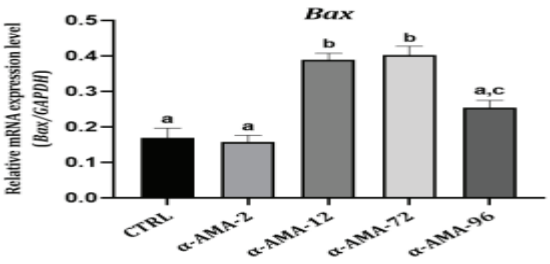


The RT-qPCR findings demonstrated that the α-AMA-2 ($p < 0.01$) and α-AMA-12 ($p < 0.05$) groups had elevated levels of the pro-inflammatory cytokine TNF-α mRNA following A. phalloides administration (Figure 4), indicating that hepatocyte inflammation is induced by exposure to A. phalloides. The levels of TNF-α mRNA in the α-AMA-72 and α-AMA-96 groups steadily declined after the administration of A. phalloides, in contrast to the α-AMA-2 group. In contrast to the control group, the α-AMA-12 and α-AMA-72 groups showed an upregulation in Bax mRNA expression level.



In comparison to the α-AMA-12 and α-AMA-72 groups, the α-AMA-96 group showed a downregulation of the Bax gene expression ($p < 0.01$), as shown in Figure 5. When compared to healthy, untreated mice, our results show that 12 hours after A. phalloides injection, apoptosis is triggered. At 96 hours post-administration of A. phalloides, however, the amount of Bax mRNA expression had dropped. The presence of the pro-apoptotic marker casco

ISSN 2395-650X
Volume 9 Issue3 Aug 2021



The expression of the Bax gene following treatment of A. phalloides varies with time (Figure 5). Animals were given an A. phalloides extract that contained 10 mg/kg of α-AMA. The RT-qPCR was used to examine the Bax gene expression. There are noticeable distinctions between the groupings denoted by different letters. In comparison to the control group, the α-AMA-2 group had a lower level of bppase-3 mRNA expression, however this difference was not statistically significant. Figure 6 shows that the mRNA expression of caspase-3 was considerably increased in the α-AMA-72 ($p < 0.05$) and α-AMA-96 ($p < 0.01$) groups after administration of A. phalloides. Figure 7 shows that when comparing the α-AMA-72 and α-AMA-96 groups, the anti apoptotic marker Bcl-2 showed only a lower expression profile ($p < 0.05$ and $p < 0.01$). Time-dependent histopathology examinations following A. phalloides administration The control group's liver tissue did not show any abnormal findings. Figure 8-a shows that the histological appearance of the hepatic cells and the central vein was normal. Hepatocytes with the pyknotic nucleus and sinusoidal dilatation were seen in the α-AMA-2 group (Figure 8-b). Significant alterations, such as vascular congestion, inflammation, necrosis, sinusoidal dilatation, and hepatocytes with pyknotic nucleus, were seen in the liver tissues of the α-AMA-96 group, as shown in Figure 8-c. The α-AMA-2 group showed less severe histopathological alterations compared to the α-AMA-96 group (Figure 8: a, b, c: H&E staining; x20 magnification).

CONCLUSION

To evaluate time-dependent apoptotic and necrotic processes in mouse liver tissues after A. phalloides injection, the present work looked at the mRNA expression of Bax, caspase 3, Bcl-2, and TNF-α. In the first stage of intoxication, A. phalloides promotes necrotic processes and subsequently causes cell death by apoptotic mechanisms, according to pro-inflammatory, pro-apoptotic, and anti-apoptotic mRNA expression profiles assessed by RT-qPCR. Consequently, our findings demonstrate that apoptotic processes are critical for the development of A. phalloides-induced hepatotoxicity, and that necrosis is also critical for the development of A. phalloides-induced hepatotoxicity in the first stages after ingestion. First of its kind, our findings show that future research on the signaling pathways responsible for A. phalloides hepatotoxicity must focus on studying time-dependent apoptotic and necrotic processes in mice.

REFERENCES

[1]. Alldredge B, C. R., Ernst M, Guglielmo B, Jacobson P, Kradjan, WA, & Williams, BR. (2012). Koda-Kimble and Young's applied therapeutics: The clinical use of drugs (K. W Ed. 10th edition ed.): Lippincott Williams & Wilkins.

[2]. Angioi, A., Floris, M., Lepori, N., Bianco, P., Cabiddu, G., & Pani, A. (2021). Extensive proximal tubular necrosis without recovery following the ingestion of Amanita phalloides: a case report. Journal of nephrology, 34(6), 2137-2140. doi:https://10.1007/s40620-021- 01018-w

[3]. Arima, Y., Hirota, T., Bronner, C., Mousli, M., Fujiwara, T., Niwa, S.-i., . . . Saya, H. (2004). Down-regulation of nuclear protein ICBP90 by p53/p21Cip1/WAF1-dependent DNA-damage checkpoint signals contributes to cell cycle arrest at G1/S transition. Genes

to Cells, 9(2), 131-142. doi:https://doi.org/10.1111/j.1356-9597.2004.00710.x

[4]. Arima, Y., Nitta, M., Kuninaka, S., Zhang, D., Fujiwara, T., Taya, Y., . . . Saya, H. (2005). Transcriptional blockade induces p53-dependent apoptosis associated with translocation of p53 to mitochondria. Journal of Biological Chemistry, 280(19), 19166-19176. doi:https://10.1074/jbc.M410691200

[5]. Barbosa, I. A., Machado, N. G., Skildum, A. J., Scott, P. M., & Oliveira, P. J. (2012). Mitochondrial remodeling in cancer metabolism and survival: potential for new therapies. Biochimica et Biophysica Acta, 1826(1), 238-254. doi:https://10.1016/j.bbcan.2012.04.005

[6]. Becker, C. E., Tong, T. G., Boerner, U., Roe, R. L., Sco, T. A., MacQuarrie, M. B., & Bartter, F. (1976). Diagnosis and treatment of Amanita phalloides-type mushroom poisoning: use of thioctic acid. Western Journal of Medicine, 125(2), 100-109.

[7]. Enjalbert, F., Rapior, S., Nouguier-Soulé, J., Guillon, S., Amouroux, N., & Cabot, C. (2002). Treatment of amatoxin poisoning: 20-year retrospective analysis. Journal of Clinical Toxicology, 40(6), 715-757. doi:https://10.1081/clt-120014646

[8]. Escudié, L., Francoz, C., Vinel, J. P., Moucari, R., Cournot, M., Paradis, V., . . . Durand, F. (2007). Amanita phalloides poisoning: reassessment of prognostic factors and indications for emergency liver transplantation. Journal of Hepatology, 46(3), 466-473. doi:https://10.1016/j.jhep.2006.10.013

[9]. Faulstich, H., Talas, A., & Wellhöner, H. H. (1985). Toxicokinetics of labeled amatoxins in the dog. Archives of Toxicology, 56(3), 190- 194. doi:https://10.1007/bf00333425

[10]. Fineschi, V., Di Paolo, M., & Centini, F. (1996). Histological criteria for diagnosis of amanita phalloides poisoning. Journal of Forensic Sciences, 41(3), 429-432.

[11]. Ganzert, M., Felgenhauer, N., & Zilker, T. (2005). Indication of liver transplantation following amatoxin intoxication. Journal of Hepatology, 42(2), 202-209. doi:https://10.1016/j.jhep.2004.10.023

[12]. Garcia, J., Costa, V. M., Carvalho, A. T., Silvestre, R., Duarte, J. A., Dourado, D. F., . . . Carvalho, F. (2015). A breakthrough on Amanita phalloides poisoning: an effective antidotal effect by polymyxin B. Archives of Toxicology, 89(12), 2305-2323. doi:https://10.1007/ s00204-015-1582-x

[13]. Jan, M. A., Siddiqui, T. S., Ahmed, N., Ul Haq, I., & Khan, Z. (2008). Mushroom poisoning in children: clinical presentation and outcome. Journal of Ayub Medical College Abbottabad, 20(2), 99-101.

[14]. Karlson-Stiber, C., & Persson, H. (2003). Cytotoxic fungi--an overview. Toxicon, 42(4), 339-349. doi:https://10.1016/s0041-0101(03)00238-1

[15]. Kaya E, H. M., Karahan S, Bayram S, Yaykaşlı KO, Sürmen MG. (2012). Thermostability of Alpha Amanitin in Water and Methanol. European Journal of Basic Medical Sciences, 2(4), 106-111. doi:https:// doi.org/10.21601/ejbms/9189