INTRODUCTION

Mushroom-related poisoning is common, but serious toxicities are rarely encountered. Mushrooms that contain cyclopeptide amatoxins are the most toxic species known, and more than 90% of the fatal incidents are due to this type of mushroom poisoning (Karlson-Stiber, Persson 2003; Yilmaz et al., 2015). Amatoxins are present in several Amanita mushroom species, which include several members of the Lepiota and Gallerina mushrooms (Kaya et al., 2013; Kose et al., 2015). Amatoxins can be divided into different subgroups, including alpha-amanitin, beta-amanitin, gamma-amanitin, epsilon-amanitin, amanullinic acid, amanullin, proamanullin, amanin and amaninamide (Vetter, 1998); the toxin constituents vary among and within different species. Furthermore, the quantity of toxins has also been found to vary within different parts of the mushroom and during different growth stages (Yilmaz et al., 2014; Kaya et al., 2013). From the various toxin subgroups, the toxic effect of alpha-amanitin is considered the most potent (Kaya et al., 2014). Here, alpha-amanitin is a strong hepatotoxin made from a cyclic peptide containing eight amino acids. And although alpha-amanitin is primarily held responsible for lethal mushroom poisonings, beta-amanitin and gamma-amanitin have also been shown to have similar cytotoxic actions (Kaya et al., 2014; Bakirci et al., 2015b). These amatoxins cause severe mushroom poisoning by irreversibly binding to RNA polymerase II, which results in the inhibition of DNA transcription and impairment of protein synthesis processes, causing cell death. In this perspective, the mushroom species Amanita phalloides contains these amatoxins, and its ingestion is mainly held responsible for the majority of casualties: caused by delayed-onset hepatocellular necrosis.
and renal cell injury (Kaplan, Larsson, Kornberg, 2008; Yilmaz et al., 2015). Although various hepatoprotective agents are currently available—as monotherapies or in combination with other agents found in experimental studies and clinics—there is still no effective drug for treatment (Kaya et al., 2016; Vetter, 1998).

*Nigella sativa* (also known as black cumin) is a common seasoning that has traditionally been used as a herbal remedy for the treatment of various diseases including fever, cough, bronchitis, asthma, chest congestion, migraine, dizziness, hemiplegia, back pain, dysmenorrhea, diabetes, infection and inflammation, rheumatism, hypertension, and gastrointestinal disorders such as flatulence, dyspepsia, diarrhea. (Gholamnezhad et al., 2016). Thymoquinone (TQ, chemical name: 2-isopropyl-5-methyl-1,4-benzoquinone) is the most bioactive component in the oil and extracts of the herb *Nigella sativa* and has anti-inflammatory, immunomodulatory, anti-histaminic, anti-microbial, analgesic, spasmyelic, bronchodilating, anti-diabetic, anti-hypertensive, anti-tumor, and antioxidant properties (Mabrouk et al., 2016; Darakhshan et al., 2015; Gholamnezhad et al., 2016). More importantly, in various experimental studies, TQ has shown hepatoprotective effects against hypervitaminosis A (Al-Suhaimi, 2012); tert-butyl hydroperoxide toxicity (Daba, Abdel-Rahman, 2004); carbon tetrachloride- (Turkdogan et al., 2003; Kanter, Coskun, Budancamanak, 2005), aflatoxin B(1)- (Nili-Ahmadabadi et al., 2011), and diazinon-induced hepatotoxicity (Nili-Ahmadabadi et al., 2018); surgery-induced hepatic ischemia-reperfusion injury (Tekbas et al., 2018); isoniazid- (Hassan et al., 2012) and paracetamol-induced liver toxicity (Yesmin et al., 2013); high-dose atorvastatin-induced hepatic oxidative injury (Hassan et al., 2018); and cisplatin (Al-Malki, Sayed, 2014), cyclophosphamide (Laskar et al., 2016), and methotrexate-induced hepatotoxicity (Sayeed et al., 2017). However, to the best of our knowledge, there are currently no available studies that investigate the effects of TQ against alpha-amanitin-induced liver damage. As such, the aim of this study was to investigate the possible beneficial effects of TQ regarding its prevention of alpha-amanitin induced hepatotoxicity in human C3A hepatocytes cell line.

**MATERIAL AND METHODS**

**Extraction and purification of alpha-amanitin**

The alpha-amanitin was obtained from *A. phalloides* mushroom as purification. Carpophores of *A. phalloides* were gathered in Duzce (Turkey). Taxonomic classification of the mushrooms was made accordingly via microscopic and macroscopic definitions (Kaya et al., 2013). Refinement of alpha-amanitin was performed as described previously (Kaya et al., 2012; 2014). Briefly, the mushrooms were dried and pulverized followed by incubation in 50% methanol at 25°C for 12 h to extract the alpha-amanitin. The dissolved of the obtained extract was then vaporized. Next, the dried extract was dissolved in mobile phase solution [50 mM ammonium acetate + acetonitrile; (90/10; v/v) (both purchased from Sigma-Aldrich, St. Louis, MO)] and the alpha-amanitin purified using the preparative high-performance liquid chromatography (HPLC) system (Shimadzu, Japan) with a C18 column (Advanced Chromatography Technologies Ltd, Aberdeen, Scotland). The obtained extract was purified twice in total with the above HPLC method. The purity and amount of obtained alpha-amanitin toxin were compared with the alpha-amanitin standard (Sigma-Aldrich, St. Louis, MO) using the analytic HPLC system (Shimadzu, Japan).

**Cell culture**

In this study, the cell culture procedures were performed as described previously (Bakirci et al., 2015a,b). Briefly, the C3A human hepatocyte cell line was obtained from the American Type Culture Collection. DMEM/F12 (Dulbecco’s modified Eagle’s medium: Nutrient mix F-12, Invitrogen, Carlsbad, CA) was used as culture medium and fetal bovine serum as serum supplementation (Invitrogen, Carlsbad, CA). The cells were grown in a humidified incubator maintaining 37°C with 5% CO₂. The culture medium was refreshed every three days, and the hepatocytes divided twice a week using trypsin-EDTA (ethylenediaminetetraacetic acid) (Sigma-Aldrich, St. Louis, MO) dissociation.
Experimental procedures

For experimental purposes, the cells were placed in 96-well-plate wells at $3 \times 10^4$ cells/100 µL per well after the dissociation procedure. They were then placed in the incubator and allowed to adhere overnight to the well’s surface. The wells containing cells were divided into multiple groups. The control group was not treated with alpha-amanitin or TQ. The number of living cells of the control group was determined and set as 100% for normalization purposes.

The other groups contained eight TQ subgroups at various concentrations of TQ (obtained from Sigma-Aldrich, St. Louis, MO): 10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 µg/mL. Alpha-amanitin subgroups at different concentrations: 10 and 1 µg/mL. Finally, the main study group was divided into sixteen subgroups. The first half received 10 µg/mL of alpha-amanitin, while the other received 1 µg/mL. After 4 hours, both halves received various concentrations of TQ: 10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 µg/mL.

Cell viability assessment

The overall functional integrity and viability of the groups were assessed with the MTT assay (Sigma-Aldrich, St. Louis, MO) [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] by determining the cell viability 48 hours after the experimental procedure (Bakirci et al., 2015a,b). To this end, enough MTT solution was added to each well to obtain an end concentration of approximately 10% MTT. After 4 hours, formazan (Sigma-Aldrich, St. Louis, MO) was added—dissolved in 100 µL dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO)—to each well. The plates were then incubated overnight in a moisturized drying oven at 37°C. Finally, the absorbance of each well was measured with a microplate ELISA reader using a detection wavelength of 570 nm (after the reference wavelength was adjusted to 690 nm).

Statistical analysis

The average absorbance values of the control group were normalized to 100%, while the other samples were calculated by comparing them to the control groups. Data are given as the mean value ± standard error mean. The statistical analysis for viability values was assessed between the dual groups by using the Mann-Whitney U Test. All values with $P < 0.05$ were considered significant.

RESULTS

According to the MTT assay, for the groups given only TQ at various concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 µg/mL), the cell viability rates at 48 hours post-administration were found at 82.6, 98.3, 102.1, 102.5, 99.4, 99.4, 101.9, and 106.3%, respectively (Figure 1). The cell viability rates at 48 hours post-administration of the groups with only 1 and 10 µg/mL alpha-amanitin were found at 90.5 and 83.1%, respectively (Figure 2).

Finally, for the group with 1 µg/mL alpha-amanitin and various TQ concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 µg/mL), the cell viability rates were found at 74.6, 88.5, 87.4, 88.7, 85.7, 86.8, 88.4, and 92.9%, respectively. For the group with 10 µg/mL alpha-amanitin and various TQ concentrations (10, 5, 1, 0.5, 0.1, 0.05, 0.01, and 0.005 µg/mL), the cell viability rates for each TQ subgroup were found at 65.2, 79.2, 81.4, 81.1, 81.8, 81.8, 82.2, and 91.9%, respectively (Figure 2). Overall, 0.005 µg/mL of TQ showed the best efficacy on cell viability, and when this concentration was compared in proportion to the control alpha-amanitin groups, the differences between them were not statistically significant ($P=0.102, P=0.05$).
DISCUSSION

Over the years, several treatment methods such as cimetidine, penicillin, N-acetyl cysteine, and silibinin have been evaluated for their properties to prevent hepatocyte and renal cell injury after alpha-amanitin poisoning. Despite these efforts, no effective and clear treatment protocol has been defined yet (Tong et al., 2007; Ward et al., 2013; Magdalan et al., 2011).

Oxidative stress is described as the disruption of the equilibration between reactive oxygen species and the antioxidant capacity of living organisms. Here, the increased role of oxidative stress has been verified with various in vitro models using methotrexate, organophosphate, and paracetamol (Ranjbar et al., 2005; Akbulut et al., 2014; Saritas et al., 2012). Recent experimental studies have indicated that alpha-amanitin potentially causes hepatotoxicity by oxidative stress (Magdalan et al., 2011; Nikolova et al., 2010). To further analyze the treatment options, primary human hepatocyte cultures are an appropriate model to investigate various antidotes without the need to consider any of the aforementioned clinical factors.

In regards to hepatotoxicity and oxidative stress, TQ has shown to be a strong antioxidant with protective properties against oxidative organ injury caused by free radical-generating agents (Nagi et al., 2010; Houghton et al., 1995; Bayrak et al., 2008). For example, a rapid assessment for antioxidants, using two thin-layer chromatography screening methods, showed that TQ and the components carvacrol, t-anethole and 4-terpineol showed remarkable radical scavenging effect. In a study by Burits and Bucar (2000), TQ demonstrated antioxidant effects by reducing the oxidative radical
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diphenyl-picrylhydrazyl. In a different study, TQ was also found to have protective effects toward an array of free radical creating compounds caused by doxorubicin treatment (Nagi, Mansour, 2000). In another study, TQ was formulated with a proniosomal formulation and evaluated for its efficacy against hepatotoxicity caused by methotrexate in rats (Sayeed et al., 2017). The results showed that the formulation significantly inhibited the elevated levels of serum marker enzymes such as alanine transaminase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and improved histopathological deformities. Next, in a study by Turkdogan et al. (2003) the hepatoprotective effect of Nigella sativa in rabbits treated with carbon tetrachloride was investigated. The results showed that the hepatocellular degenerative and necrotic changes were mild for the Nigella sativa-treated group and that there were no indications of fibrosis or cirrhotic processes. In a similar study by Kanter, Coskun, Budancamanak, (2005), their results showed that the addition of TQ improved the protecting process against lipid peroxidation caused by carbon tetrachloride-induced hepatotoxicity. Nagi et al. (2010) reported protective effects via antioxidant mechanisms with the prophylactic use of TQ against hepatotoxicity, which was caused by acetaminophen. In another example, Nili-Ahmadabadi et al. (2011) showed that AST, ALT, and ALP levels dropped significantly in mice treated with TQ compared to the ones treated with Aflatoxin B1 (AFB1). Histopathologically, the liver of the AFB1-exposed mice showed inflammation, necrosis, Kupffer cell hyperplasia; and mononuclear cell infiltration, disruption of hepatocytes, and dilation of sinusoids, whereas treatment with TQ helped to normalize the liver structure according to biochemical findings. Here, TQ was found to have an optimal protective effect in mice suffering from AFB1 hepatotoxicity at a dosage of 9 mg/kg. Additionally, Nili-Ahmadabadi et al. (2018) recently showed that TQ attenuated liver toxicity (caused by diazinon) and oxidative damage and improved the antioxidant state of the tissue. They also showed that the therapeutic effects of low TQ dosages (1.25 mg/kg) were relatively better than the ones with higher doses (5 mg/kg), which are results that resemble the ones in our current study.

CONCLUSION

In conclusion, to the best of our knowledge, our study is the first in vitro study that investigates TQ’s effects on alpha-amanitin induced hepatotoxicity. Even though various animal studies have shown the protective antioxidant effects of TQ, our treatment did not significantly increase cell viability in liver damage due to alpha-amanitin toxicity. However, in our study, we showed that the TQ group with the lowest dose (0.005 µg/mL) had the highest viability compared to the control one, indicating that a lower TQ concentration might have a larger effect in vivo as well.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES


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