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EXPLORING *Amanita muscaria* (L.) Lam.: CYTOTOXIC, PROAPOPTOTIC AND ANTIFERROPTOTIC EFFECT OF ACETONE EXTRACT ON JAR CHORIOCARCINOMA CELL LINE

Sara D. Milojević[1], Danijela D. Nikodijević[1], Milena G. Milutinović[1], Marijana M. Kosanić[1], Nevena N. Petrović[1], Milica G. Jovanović Krivokuća[2], Mirjana M. Nacka Aleksić[2]*

[1]*University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanović 12, 34000 Kragujevac, Serbia*
[2]*University of Belgrade, Institute for the Application of Nuclear Energy (INEP), Department for Biology of Reproduction, Banatska 31b, 11080 Belgrade, Serbia*
*Corresponding author; E-mail: mnacka@inep.co.rs

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ABSTRACT. The pharmaceutical industry has long focused on nature-derived biologically active compounds to address challenges in cancer treatment, particularly overcoming cancer cell resistance to programmed cell death, including apoptosis and ferroptosis. The cytotoxic effect of *Amanita muscaria* acetone extract on the JAR choriocarcinoma cell line was evaluated using MTT assay and analysis on two selected programmed cell death pathways. The putative ferroptotic effect was evaluated by measuring the main redox parameters and relative expression of key antiferroptotic genes. Our findings indicate that the IC_{50} of the acetone extract of *A. muscaria* was 168.96 (after 24 h) and 43.85 (after 72 h) μg/mL, inducing mainly a proapoptotic effect on choriocarcinoma cells. The extract also demonstrated an antiferroptotic effect. Both the proapoptotic and antiferroptotic effects were achieved regardless of the induction of oxidative stress._ This is the first report on *A. muscaria* acetone extract indicating proapoptotic and antiferroptotic effects on choriocarcinoma cells.

Keywords: *Amanita muscaria*, medicinal mushrooms, cancer treatment, apoptosis, ferroptosis, JAR choriocarcinoma cell line.

INTRODUCTION

Mushrooms are globally recognized not just for their nutritional benefits but also for their significant pharmacological value, serving as rich sources of vital bioactive compounds.

ORCID ID:
S.D. Milojević - 0000-0002-9755-2762; D.D. Nikodijević - 0000-0002-6453-0313;
M.G. Milutinović - 0000-0002-5075-5650; M.M. Kosanić - 0000-0002-5714-7631;
N.N. Petrović - 0000-0002-7983-0597; M.G. Jovanović Krivokuća - 0000-0001-8292-4853;
M.M.N. Aleksić - 0000-0003-0750-9347.

The distinctive appearance of the fungus *Amanita muscaria* (L.) Lam. makes it universally identifiable; however, it is generally acknowledged as poisonous, inedible, and potentially lethal. Further, the mushroom's most well-known application has been associated with the misuse of its psychoactive effects. This is noteworthy, considering the insufficiently explored cytotoxic effects and mechanisms of action associated with various homemade tinctures and extracts derived from this mushroom. Its primary psychoactive components, ibotenic acid and muscimol, are associated with the 'pantherina-muscaria' poisoning syndrome (VOYNOVA *et al.*, 2020). Despite this reputation, *A. muscaria*-containing preparations have been used against various diseases, including cancer, with no apparent adverse effects. So far, various biological effects of mushrooms have been shown, encompassing antitumor and anticancerogenic (FIGUEIREDO and RÉGIS, 2017; PANDA *et al*., 2022), immunosuppressive and antiinflammatory (MIZUNO and MINATO, 2024), hypoglycemic (KAREMPUDI *et al*., 2024), hepatoprotective (AHMAD *et al*., 2023), antimicrobial (TODOROVIĆ *et al*., 2023), antifungal (BERIKASHVILI *et al*., 2023) and other properties. *A. muscaria* is believed to make a substantial contribution to cancer treatment (DUSHKOV *et al*., 2021), despite not displaying strong cytotoxic effects, primarily through its immunomodulatory role. Additionally, the utilization of specific active compounds isolated from *A. muscaria*, such as muscimol, has demonstrated notable effectiveness in easing the symptoms of various neurodegenerative diseases, particularly Parkinson's disease (KONDEVA-BURDINA *et al*., 2019).

Choriocarcinoma, a highly malignant trophoblastic tumor, develops in the uterus following pregnancy, hydatidiform mole, abortion, or ectopic gestation (SILVA *et al*., 2021). The rapid advancement and early metastasis of choriocarcinoma to distant organs, such as the lung, liver, brain, and kidney, can be attributed to the highly proliferative and aggressive nature of trophoblast cells. These cells exhibit a notable permeability to the vascular system, contributing to the development of a highly vascularized and hemorrhagic carcinoma (KAUR, 2021). Despite its high success rate, various antineoplastic drugs used as a treatment for choriocarcinoma often trigger adverse reactions, including liver injury, gastrointestinal issues, mucosal damage, and bone marrow suppression, with an incidence rate exceeding 30% (HUI *et al*., 2023). In order to address and overcome these treatment challenges, it is vital to explore creative solutions, with a preference for those originating from natural sources.

Resilience to certain types of programmed cell death, such as apoptosis and ferroptosis, plays significant roles in carcinogenesis and the progression of cancer (WORKENHE *et al*., 2024). As a result, they have attracted increasing interest in the field of oncology. Apoptosis, the most extensively researched form of programmed cell death, is crucial for maintaining tissue homeostasis, and organismal development, and is implicated in various pathologies, including cancer. Targeting apoptosis in cancer treatment is effective across all cancer types because avoidance of apoptosis is a fundamental characteristic of cancer cells, irrespective of its etiology or type (PFEFFER and SINGH, 2018).

Recently, the focal point of interest within cancer research communities is another form of regulated cell death – ferroptosis, which relies on iron and is initiated by the accumulation of toxic lipid peroxides on cellular membranes (JIANG *et al*., 2021). Its uniqueness as a cell death mechanism, distinct from apoptosis and other forms of cell death, makes ferroptosis particularly promising for potential applications in cancer therapy (LEI *et al*., 2022). The glutathione peroxidase 4 – reduced glutathione system (GPX4-GSH system) is a crucial and well-studied cellular defense mechanism that prevents ferroptosis, consequently offering a survival strategy for cancer cells (KUANG *et al*., 2020). Solute carrier family 7 member 11 (SLC7A11), also known as xCT, serves as the transporter subunit in system x_c^- mediated uptake of cystine, an oxidized dimeric form of cysteine – the limiting factor for synthesis of reduced glutathione (GSH) (TANG *et al*., 2021). The SLC7A11–GSH–GPX4 axis is the primary cellular defense against ferroptosis (LI *et al*., 2022). However, even in the absence of GPX4 activity, some cancer cell lines exhibit resistance to ferroptosis, indicating the presence of additional defense mechanisms against this form of cell death, independent of oxidative stress (LEI *et al*.,

2022). Ferroptosis suppressor protein 1 (FSP1), formerly known as apoptosis-inducing factor 2 (AIFM2), protects cells against ferroptosis independently of GSH levels, GPX4 activity, and oxidizable fatty acids, highlighting its role as an additional defense mechanism (DOLL *et al*., 2019).

The rationale behind this research lies in the lack of in-depth studies examining the cytotoxic mechanisms of numerous natural extracts, including the *A. muscaria* mushroom extract. Natural extracts represent a range of substances found in nature that collaborate synergistically, offering a more holistic insight into potential benefits and applications compared to the study of isolated individual compounds and chemically synthesized cytostatics. Our research is focused on the cytotoxic impact of the acetone extract of *A. muscaria*, particularly highlighting the type of cell death triggered by the extract's half-maximal inhibitory concentration (IC_{50}). In addition, considering that choriocarcinoma, characterized by a robust blood supply and abundance of blood elements (BOGANI *et al*., 2023), is susceptible to iron-dependent ferroptotic cell death induction in an iron-rich environment, our study aimed to explore this phenomenon as well.

MATERIALS AND METHODS

Chemicals

All solvents and chemicals in this study were of analytical grade. Chemicals used in this study are: acetone (Beta Chem, Belgrade, Serbia), Dulbecco's Modified Eagle Medium – DMEM (EuroClone, Milano, Italy), Fetal Bovine Serum – FBS (PAN-Biotech, Aidenbach, Germany), Penicillin-Streptomycin – PS (Capricorn Scientific, Ebsdorfergrund, Germany), trypan blue (Sigma-Aldrich, St. Louis, Missouri, USA), 3-[4,5-dimethylthiazol2-yl]-2,5-diphenyltetrazolium bromide – MTT (Sigma-Aldrich, St. Louis, Missouri, USA), DiMethyl SulfOxide – DMSO (Santa Cruz Biotechnology, Dallas, Texas, USA), Acridine Orange – AO (Acros Organics, New Jersey, USA), Ethidium Bromide (Acros Organics, New Jersey, USA), Nitro Blue Tetrazolium – NBT (Sigma-Aldrich, St. Louis, Missouri, USA), SulfoSalicylic Acid – SSA (Loba Chemie Pvt Ltd, Mumbai, India), EthyleneDiamineTetraacetic Acid – EDTA (Sigma-Aldrich, St. Louis, Missouri, USA), trypsin-EDTA (EuroClone, Milano, Italy), 5,5-DiThio-bis-2-NitroBenzoic acid – DTNB (Sigma-Aldrich, St. Louis, Missouri, USA), ThioBarbituric acid – TBA (Sigma-Aldrich, St. Louis, Missouri, US), EURx NG Dart RT Kit (EURx, Gdansk, Poland), EURx SG/ROX qPCR Master Mix (2x) (EURx, Gdansk, Poland), EURx RNA Extracol (EURx, Gdansk, Poland).

A. muscaria collection and extraction method

The samples of fruiting bodies of *A. muscaria* were collected in Southern Kučaj Mountain (Eastern Serbia) in 2021. The specimens were identified by experienced researchers using appropriate literature (UZELAC, 2009). The samples were labeled as AM1 and preserved at the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia.

For the extraction process, the collected samples were first desiccated in the laboratory and then ground into a fine powder. Approximately 100 g of this powder was mixed with 300 mL of acetone, which served as the solvent. The mixture was then extracted in the Soxhlet extractor for 5 h at a temperature of 55 °C. Afterward, it was filtered and concentrated under reduced pressure in the rotary evaporator. The prepared acetone extract was stored in a refrigerator at a temperature of –18°C. For the purpose of the experiment, the extract was dissolved in DMSO and cell culture medium, ensuring that the concentration of DMSO did not exceed 5% in the highest used extract concentration.

Cultivation and preparation of cells

JAR cell line, the human choriocarcinoma model, was purchased from ATCC (American Type Culture Collection, USA). Cells were cultured in DMEM cell culture medium supplemented with 10% FBS and 1% antibiotic/antimycotic solution (hereafter complete medium) in a humified atmosphere at 37 ˚C and 5% CO_2.

MTT assay for determining A. muscaria acetone extract cytotoxicity

The MTT assay was employed to assess the cytotoxic effect of varying concentrations of the acetone extract derived from *A. muscaria*. The MTT assay quantifies cell viability through the measurement of cellular metabolic activity, where viable cells reduce the colorless tetrazolium salt to purple formazan crystals, with a decrease in this conversion indicating cytotoxic effects due to compromised cellular viability or proliferation. Prior to treatment, cell suspension was distributed into 96-well plates at a density of 100.000 cells/mL, with each well containing a volume of 100 µL of complete medium. Following the initial 24 h incubation, *A. muscaria* acetone extract was added in 7 different concentrations (0.5–500 µg/mL). Cells treated with medium alone were used as a control. MTT assay was performed after 24 and 72 h of treatment incubation according to MOSMANN (1983). MTT was dissolved in a complete medium at a concentration of 5 mg/mL, and 100 µL of the solution was added to each well following the removal of the previous content. The cells were incubated with the MTT solution for a duration of 3 h. After aspirating the contents of the wells, 150 µL DMSO was introduced, and the absorbances were assessed using an ELISA plate reader set to 550 nm wavelength (ELISA reader RT-2100C, Ivymen). Cell viability percentages were determined by comparing the absorbance of treated cells to that of untreated cells (100%). The CalcuSyn program (BIOSOFT, Cambridge GB – United Kingdom) was used to calculate the IC_{50} from the dose-response curves.

Acridine Orange/Ethidium Bromide staining for determining cell death type

The Acridine Orange/Ethidium Bromide (AO/EB) staining method was employed to identify the type of cell death via fluorescence microscopy (Nikon Ti-Eclipse, Nikon Corporation; Camera Digital Sight DS-Ri1, Nikon Corporation). Cells were seeded into 96-well plates (10.000 cells in 100 µL of complete medium by each well) and after initial 24 h incubation, 24-hour IC_{50} of *A. muscaria* acetone extract was added and incubated for 24 h. Next, cells were stained with both acridine orange and ethidium bromide to determine the percentage distribution of cells with morphological features of viable, apoptotic and necrotic cells, according to BASKIĆ *et al*. (2006). Viable cells displayed uniformly bright green nuclei with organized structures. Necrotic cells were characterized by small volume and uniformly red nuclei. Apoptotic cells exhibited varied characteristics corresponding to different stages of apoptosis, with some appearing uniformly green while others displayed a combination of green and orange-red coloration; nonetheless, their morphological features, such as chromatin condensation, nuclear and cytoplasmic shrinkage, reduced cell volume, and nuclear fragmentation, unequivocally differentiated them from viable and necrotic cells. The percentage of viable, apoptotic and necrotic cells was calculated in relation to the total number of analyzed cells in each treated and control group (a minimum of 300 cells was counted per sample).

Superoxide anion radical (O_2^-), and GSH measurement for determining redox imbalance

To assess the potential redox imbalance, the concentrations of the main free oxygen radical O_2^-, and the crucial component of antioxidant defense, GSH, were measured spectrophotometrically. Following the initial seeding of cells in 96-well plates and a 24 h

incubation with IC_{50} of *A. muscaria* extract, 20 µL NBT (5 mg/mL) was added and allowed to incubate for 45 minutes. In the presence of $O_2^{\cdot -}$, NBT added to samples is reduced to nitroblue-formazan, which was quantified by measuring the absorbance at 550 nm wavelength after adding 20 µL of DMSO (AUCLAIR and VOISIN, 1985). The concentrations of $O_2^{\cdot -}$ were expressed as nmol of $O_2^{\cdot -}$ per milliliter of a sample, concerning the molar extinction coefficient of formazan.

The concentration of GSH was assessed using a method based on the oxidation of GSH with the sulfide reagent DTNB (BEUTLER, 1975). This reagent was dissolved in a solution containing 100 mM phosphate buffer with 1 mM EDTA at pH 7.4. As a result, the samples produced a yellow product 2-nitro-5-thiobenzoic acid (TNB). After 24 h incubation with the IC_{50} of *A. muscaria* acetone extract, cells were treated with 2.5% SSA and then sonicated to release GSH from the cells, improving the detection of TNB color. The absorbances of the samples were measured at 405 nm, using an ELISA plate reader. The concentration of GSH was calculated by dividing the obtained sample absorbance with the factor obtained from the corresponding standard curve.

Malondialdehyde (MDA) concentration measurement for determining lipid peroxidation

MDA is a split product of an endoperoxide of unsaturated fatty acids resulting from oxidation of lipid substrates. Its concentration was measured to determine the presence of lipid peroxidation in cells after the treatment with acetone extract of *A. muscaria*. The assay was conducted *via* the thiobarbituric acid reactive substances (TBARS) protocol described in detail by AQUILAR DIAZ DE LEON and BORGES (2020). The cells were seeded at approximately 5 x 10^5 cells per mL of complete medium before the treatment with 24-hour IC_{50} of *A. muscaria* extract. After 24 h of incubation, the assay was administered. The TBARS, generated in the sample, exhibited a light red-pink color and was quantified using spectrophotometry (Specol 21 M 9521, Iskra) at a wavelength of 532 nm. The TBARS assay was conducted under acidic conditions (pH 4) and at a temperature of 95 °C. The concentration of MDA was calculated using the following formula:

$$C(MDA) = (A / \varepsilon * R) * 1000 / C \text{ (proteins) nmol/mg} \quad (1)$$

C(MDA) – concentration of MDA in each sample, A – absorbance value obtained by subtracting the blank absorbance from the sample absorbance, ε – molar extinction coefficient of MDA (155 $mM^{-1}cm^{-1}$), R – sample dilution, C (proteins) – concentration of proteins in each sample measured by standard curve of BSA (BioPhotometer M 6131, Eppendorf).

Isolation of RNA, reverse transcription and quantitative polymerase chain reaction (qPCR) for determining gene expression

To assess the expression of the gene of interest, cells were cultured at a density of 500.000 cells/mL in a complete medium. Once reaching the desired confluency, the cells were treated with the 24-hour IC_{50} of *A. muscaria* acetone extract and incubated for 24 h. Next, RNA was isolated from the cells using the instructions provided by the EURx RNA Extracol kit. The isolated RNA (1 µg) was then directly employed in the reverse transcription reaction (Eppendorf Mastercycler EpGradient S PCR) facilitated by instructions of EURx NG Dart RT kit. The obtained cDNA was immediately utilized in a qPCR (7500 Real Time PCR System, Applied Biosystems) to measure the expression levels of the *GPX4*, *SLC7A11*, and *FSP1* genes, with normalization against *GAPDH* as the housekeeping gene. Primer sequences used in qPCR analysis are obtained from KOŠARIĆ (2018) – *GPX4*, ZHANG *et al*. (2020) – *GAPDH* and newly designed – *SLC7A11* and *FSP1* (Table 1). EURx SG/ROX qPCR Master Mix (2x) instructions were followed when preparing the qPCR reaction and 1 µL cDNA was added in a final qPCR reaction volume of 20 µL. Ct values were generated using 7500 Software real-time PCR system

v2.3 (Life Technologies Corp.). Levels of relative gene expression were calculated according the 2^−ΔΔCt method (LIVAK and SCHMITTGEN, 2001).

The specific primers for *SLC7A11* and *FSP1* were designed by identifying optimal candidates with distinct template regions that exhibited the least similarity to other targets, performed by the National Library of Medicine Primer-BLAST online tool (https://www.ncbi.nlm.nih.gov).

Table 1. Primers sequences.

Gene	Forward primer sequence (5`-3`)	Reverse primer sequence (5`-3`)
GAPDH	TGACTTCAACAGCGACACCCA	CACCCTGTTGCTGTAGCCAAA
GPX4	GAGGCAAGACCGAAGTAAACTAC	CCGAACTGGTTACACGGGAA
SLC7A11*	TCCTGCTTTGGCTCCATGAACG	AGAGGAGTGTGCTTGCGGACAT
FSP1*	TGGGTTTGGAAGGTGTGCTT	ACACATGCACATCCCAGAGG

*designed

Statistics

The findings are expressed as mean ± standard error of the mean (SEM) ($p < 0.05$). Biological activity was evaluated through two independent experiments, with each experiment conducted in triplicate for every concentration. Significant differences between experimental groups were determined by unpaired t-test using the SPSS statistical software package (SPSS for Windows, ver. 17).

RESULTS AND DISCUSSION

In this study, our primary aim was to investigate both the cytotoxic effects induced by the acetone extract from *Amanita muscaria* and the underlying mechanisms governing cellular death. We first examined the cytotoxicity of various concentrations of the acetone extract on the JAR choriocarcinoma cell line, using the MTT assay. In brief, JAR cells were incubated with 0.5, 1, 10, 50, 100, 200 and 500 µg/mL of *A. muscaria* acetone extract for 24 and 72 h. Figure 1 displays the results of the MTT assay, illustrating the time and dose dependence of the extract's effect. After 24 h, concentrations greater than 10 µg/mL induced a progressive decline of cell viability, whereas after 72 h, even doses > 1 µg/mL led to pronounced cytotoxic effects (Fig. 1). The 72-hour IC_{50} (43.85 µg/mL) was notably lower than the 24-hour IC_{50} (168.96 µg/mL), indicating that the toxicity of *A. muscaria* acetone extract increases with the duration of exposure.

Previous reports indicate that an alcoholic tincture, derived from dried *A. muscaria* caps, exhibits an anticancer effect in human patients who use it for self-medication (DUSHKOV *et al*., 2023). Similarly, studies evaluating the ethanol extract of *A. muscaria* have demonstrated significant cytotoxic effects on two panels of cancer cell lines *in vitro* (DUSHKOV *et al*., 2021). Among the relatively scarce number of studies on the biological activity of *A. muscaria*, some research has focused on analyzing individual isolated substances from *A. muscaria* like various carbohydrates and acids. In our study, we used acetone extract of *A. muscaria* due to its effectiveness in extracting a wide range of bioactive compounds, both polar and non-polar, essential for comprehensive analysis. For instance, research by ZAVADINACK *et al.* (2021) showed a selective cytotoxic and antiproliferative effect of polysaccharides α-D-galactan and β-D-glucan (isolated from the *A. muscaria*) on B16-F10 melanoma cell line, not affecting non-tumoral BALB/3T3 fibroblast cell line. In their study, even the lowest concentration used (10 µg/ml) exhibited significant cytotoxic activity, with α-D-galactan inhibiting 26.2% of cells and β-D-glucan inhibiting 22.5% of cells at the same concentration.

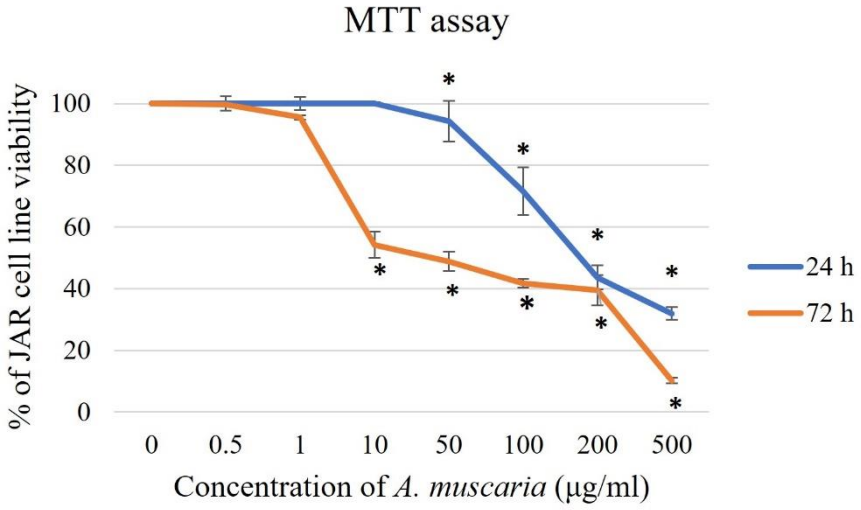

Figure 1. Cytotoxicity of acetone extract of *A. muscaria* on JAR choriocarcinoma cells.

Given the observed cytotoxicity of the acetone *A. muscaria* extract after both, acute and prolonged exposure, we next tried to identify the putative cell death pathways invoked by the extract. For further analysis, only the extract's IC_{50} obtained from results after 24 h incubation, was further used to investigate the mechanisms behind the cytotoxic effects of *A. muscaria* acetone extract.

By staining cells with AO and EB, we were able to determine the percentage of cells with the morphological characteristics of apoptosis and necrosis. The findings revealed a marked increase in the frequency of apoptotic and necrotic cells after adding 24-hour IC_{50} *A. muscaria* acetone extract to the choriocarcinoma culture, in favor of apoptosis (Fig. 2A-C).

Considering the toxicological profile of the *Amanita* species, the proapoptotic activity of the acetone extract of *A. muscaria* may not come as a surprise. Among toxic *Amanita* mushrooms containing mycotoxins – amanitins and others, *A. phalloides* stands out for its notably high concentrations of α-amanitin, and its frequent association with poisoning incidents. Isolated from *A. phalloides*, α-amanitin has been reported as a strong p53- and caspase 3-dependent apoptosis inductor (GARCIA *et al*., 2015). SO *et al*. (2019) reported that compounds derived from another poisonous *Amanita* mushroom – *A. spissacea* exhibited noteworthy cytotoxicity in human lung cancer cells (IC_{50} values ranging from 255.7 to 321.0 µM), primarily through the promotion of proapoptotic effects, highlighting its potential as a valuable natural source for identifying novel anticancer drug candidates. Our results indicated a stronger cytotoxic effect (lower IC_{50}), likely due to the synergistic action of a broader array of compounds present in the acetone extract. Although our findings here reveal that the acetone extract of *A. muscaria* exhibits a significant proapoptotic effect on choriocarcinoma cell cultures *in vitro* (16.33% ± 4.62 apoptotic cells), the active compounds responsible for these effects and the exact apoptotic pathways triggered by the extract remain to be elucidated.

Next, we investigated the oxidizing features of the extract, bearing in mind the fact that higher endogenous ROS levels in cancer cells endow them with increased sensitivity to ROS-inducing therapy and anticancer therapies that manipulate ROS levels show promising results, both *in vitro* and *in vivo* (NAKAMURA and TAKADA, 2021). However, our findings revealed that there was no statistically significant difference in the production of both $O_2^{\cdot-}$ and GSH compared to the control group after 24-hour IC_{50} treatment, although a slight increase in the concentration of both parameters was recorded (Fig. 3). This suggests that the extract does not exert a significant disruptive effect on the redox balance within the cells. Also, these results indicate that the previously detected induction of apoptosis is likely oxidative stress-independent. These findings also highlight the need for a closer study of the oxidative properties

of the *A. muscaria* acetone extract, since the observed antioxidant effect could interfere with radiotherapy and chemotherapy, which are largely dependent on ROS-mediated cytotoxicity, and thus compromise the desired results. This might be particularly relevant for patients prone to self-medication.

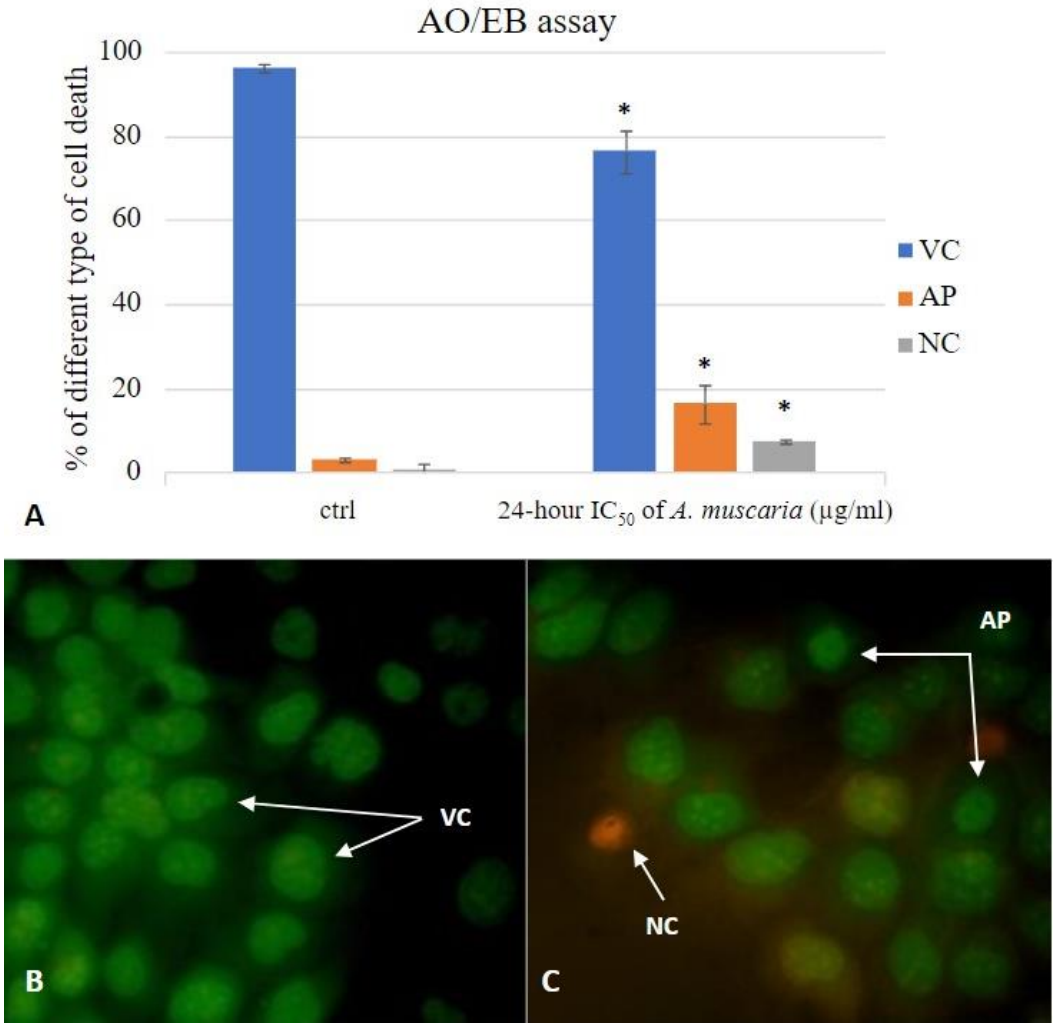

Figure 2. Frequency of apoptotic and necrotic cells in control (ctrl) and after treatment of JAR choriocarcinoma cells with acetone extract of *A. muscaria* (A); representative photomicrographs after AO/EB staining of (B) control (untreated) cells and (C) treated cells – distribution of viable (VC), apoptotic (AP) and necrotic (NC) cells.

Despite the unaltered $O_2^{\cdot -}$ and GSH levels, there was a statistically significant increase in MDA levels after treatment with 24-hour IC_{50} of *A. muscaria* acetone extract. This result indicates increased lipid peroxidation in the cultures treated with 24-hour IC_{50} of *A. muscaria* acetone extract, although low concentrations of MDA were recorded overall (0.26 ± 0.02 nmol/mg) (Fig. 4). The induction of lipid peroxidation could result from a variety of other factors, including direct oxidative stress caused by the extract's components, alterations in ROS generation pathways, and perturbation of GSH-independent antioxidant defense mechanisms. According to AYALA *et al*., (2014), MDA can also be a side product of decomposition of arachidonic acid. In this context, the elevated MDA levels observed in choriocarcinoma cells following treatment with the acetone extract of *A. muscaria* imply a potential influence of the extract on the thromboxane biosynthesis pathway.

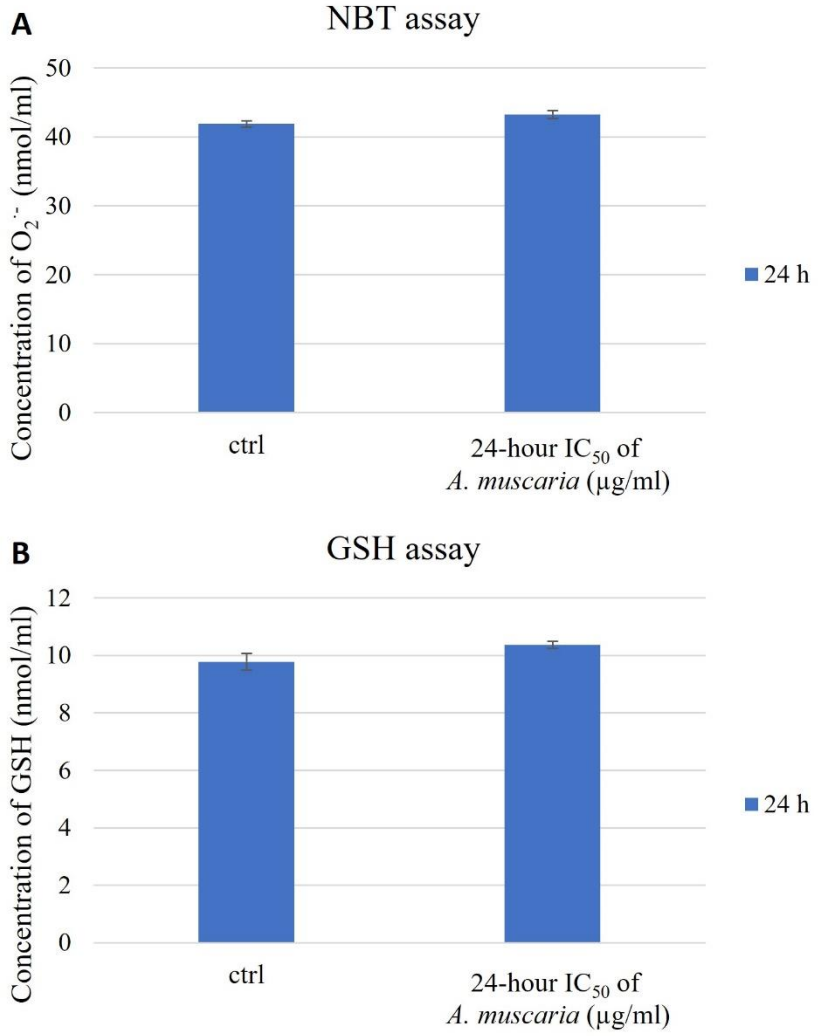

Figure 3. Quantification of redox parameters in JAR choriocarcinoma control cells and cells treated with 24-hour IC_{50} of *A. muscaria* acetone extract. Bar graphs show the concentration of (A) prooxidant superoxid anion ($O_2^{\cdot-}$) and (B) antioxidant GSH.

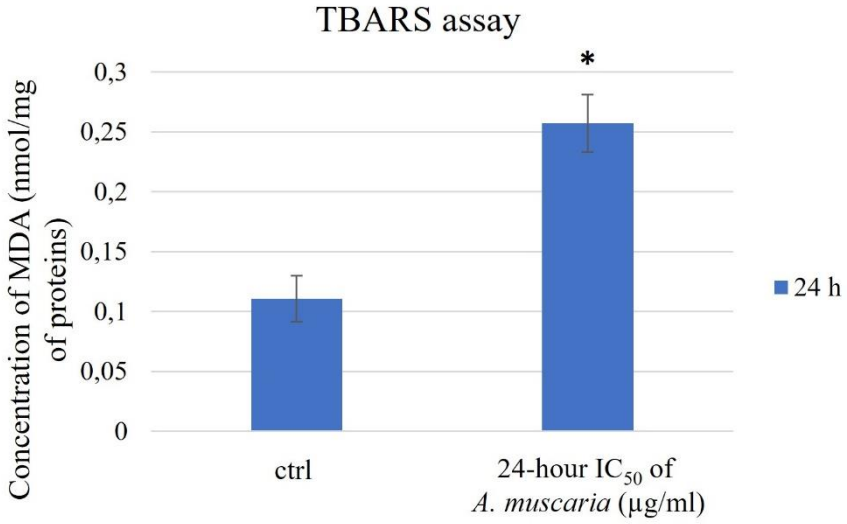

Figure 4. Quantification of lipid peroxidation product (MDA) in JAR choriocarcinoma control cells and cells after treatment with 24-hour IC_{50} of *A. muscaria* acetone extract**.**

In light of lipid peroxidation being a key hallmark of ferroptosis (LEE *et al*., 2021), these findings prompted us to investigate the expression of the crucial genes associated with this process, viz., the SLC7A11-GSH-GPX4 axis. By upregulating GPX4, cancer cells have the ability to prevent the accumulation of lipid peroxides, thus avoiding ferroptotic cell death (WANG *et al.,* 2021). Consequently, inhibiting GPX4 could effectively sensitize cells to ferroptosis (ZHANG *et al.*, 2022). The induction of potent ferroptosis in numerous cancer cell lines can be achieved, among others, by pharmacologically blocking or modifying SLC7A11-mediated cystine transport (LIN *et al.,* 2020). The analysis of relative gene expression in our study revealed a statistically significant upregulation of *GPX4* and *SLC7A11* expression in cells treated with *A. muscaria* acetone extract compared to the control cells (Fig. 5). Considering that i) inactivation of *GPX4* results in uncontrolled lipid peroxidation, initiating robust ferroptosis conditions (LEI *et al*., 2022), and ii) low expression of *SLC7A11* leads to oxidative stress-mediated ferroptosis (LI *et al*., 2022), our findings suggest that the administered treatment most likely does not promote ferroptosis. Instead, we conclude that the extract's predominant effects on cancer cells stem from its antioxidant properties (REIS *et al*., 2011), as evidenced by the heightened expression of the genes encoding for the integral proteins of both, antioxidant and antiferroptotic defense systems, GPX4 and SLC7A11 (LI *et al*., 2022). Additionally, an upregulation of *SLC7A11* has been associated with drug resistance and patient poor prognosis (LIN *et al*., 2020), underscoring the potential adverse effects of the *A. muscaria* acetone extract on cancer chemoresistance development.

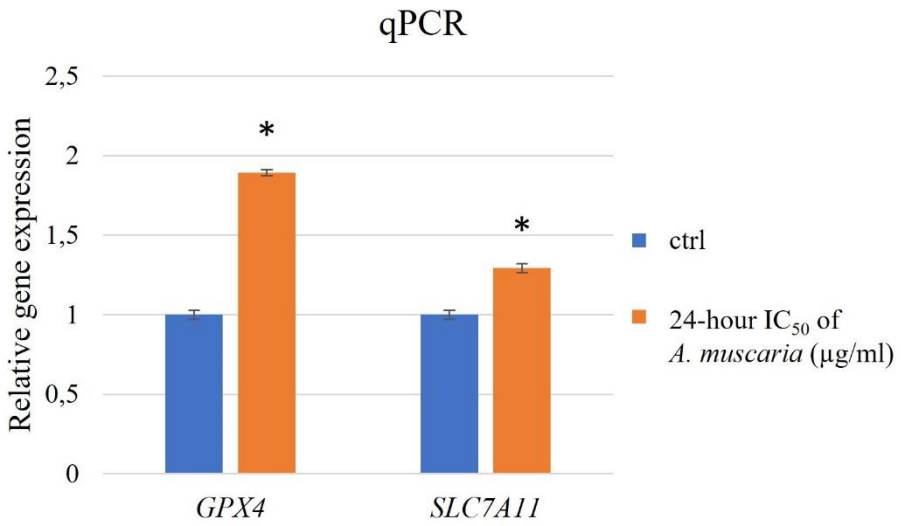

Figure 5. Relative gene expression of *GPX4* and *SLC7A11* in JAR choriocarcinoma control cells and cells after treatment with 24-hour IC_{50} of *A. muscaria* acetone extract.

To investigate the possibility of a proferroptotic effect induced through an oxidative stress-independent pathway, we also evaluated the level of *FSP1* expression. Studies suggest that *FSP1* confers protection against ferroptosis elicited by *GPX4* deletion, and that pharmacological inhibition of *FSP1* strongly synergizes with *GPX4* inhibitors to trigger ferroptosis in several cancer entities (DOLL *et al*., 2019). In the treated choriocarcinoma cells, qPCR analysis revealed that the *A. muscaria* acetone extract significantly enhanced the expression of *FSP1* (Fig. 6). An increase in its expression is associated with decreased sensitization and the general inhibition of ferroptosis, regardless of the GPX4-GSH system (JIN *et al*., 2024). Hence, the observed elevation in *FSP1* gene expression in our study also suggests a possible antiferroptotic effect of the tested extract. Previous studies have demonstrated a correlation between elevated *FSP1* expression and heightened tumorigenicity in thyroid cancer, leading to a poorer clinical prognosis (CHEN *et al*., 2023). Based on this data, it is advisable to

conduct further investigations into the safety of using this mushroom, as there is a potential risk that it could have adverse effects for cancer treatment rather than beneficial ones.

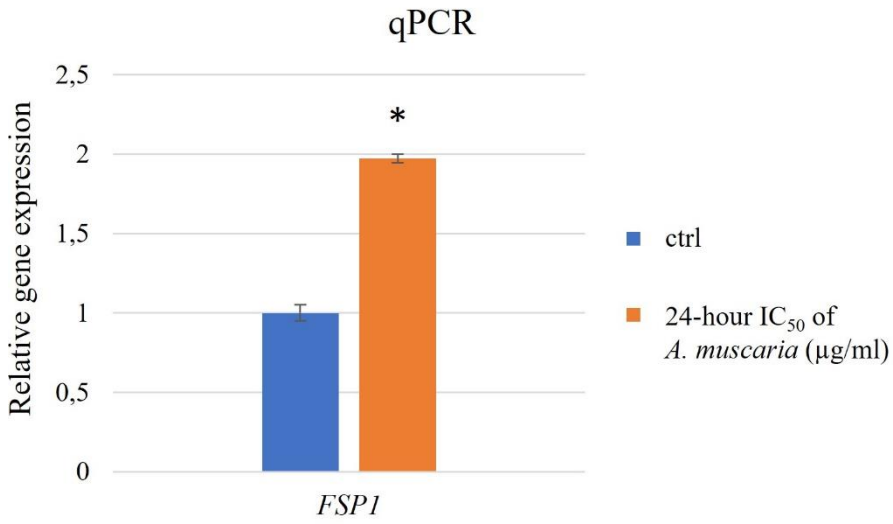

Figure 6. Relative gene expression of *FSP1* in JAR choriocarcinoma control cells and cells after treatment with 24-hour IC_{50} of *A. muscaria* acetone extract.

CONCLUSION

The present study examined the effect of *A. muscaria* acetone extract on the JAR choriocarcinoma cell line. In particular, we examined various types of cell death (induced by the extract's 24-hour IC_{50}) which could serve as potential targets in cancer therapy. Our findings revealed that the acetone extract from *A. muscaria* exhibits moderate cytotoxicity and a proapoptotic effect on JAR choriocarcinoma cells, which is promising. However, our study also demonstrated that this extract exerts an antiferroptotic effect on JAR choriocarcinoma cells by increasing the expression of genes associated with defense mechanisms against ferroptosis, which are not related to oxidative stress.

Based on our findings, we can conclude that the acetone extract from *A. muscaria* is not likely to induce ferroptosis, a novel cancer target, in the JAR choriocarcinoma cell line under the tested conditions. However, it does exhibit some anticancer properties through the induction of apoptosis in choriocarcinoma cells. Therefore, we suggest that further investigation into the anticancer effects of this extract should focus on proapoptotic rather than ferroptotic pathways.

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