Food and Chemical Toxicology 132 (2019) 110687

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Effects of *Amanita muscaria* extract on different *in vitro* neurotoxicity models at sub-cellular and cellular levels

Magdalena Kondeva-Burdina[a,*], Maria Voynova[a], Aleksandar Shkondrov[b], Denitsa Aluani[a], Virginia Tzankova[a], Ilina Krasteva[b]

[a] Laboratory of Drug Metabolism and Drug Toxicity, Department of Pharmacology, Pharmacotherapy and Toxicology, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000, Sofia, Bulgaria
[b] Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000, Sofia, Bulgaria

ARTICLE INFO

Keywords:
Amanita muscaria
Isolated rat brain microsomes
Synaptosomes
Mitochondria
Neuroblastoma cell line SH-SY5Y
Neuroprotection
Human recombinant monoaminoxidase-B enzyme

ABSTRACT

Muscimol is the main compound found in *Amanita muscaria*. Several studies have proven that muscimol has suppressive effects on essential tremor, without impairing speech and coordination. The effects of muscimol in Parkinson-affected patients is also described in a number of studies. These studies describe the free radical scavenging and antioxidant activity of the mushroom extract. We have evaluated the possible neuroprotective effects of a standardized extract from *A. muscaria*, containing high amounts of muscimol, on different models of neurotoxicity in rat brain microsomes, mitochondria, synaptosomes as well as on neuroblastoma cell line SH-SY5Y. The possible inhibitory effect on human recombinant monoaminoxidase-B (hMAOB) enzyme was also studied. The extract revealed statistically significant neuroprotective effects on the *in vitro* neurotoxicity models and no inhibitory activity on hMAOB.

1. Introduction

Mushrooms are widely appreciated all over the world not only for their nutritional properties, but also for their pharmacological value as sources of important bioactive compounds. Various biological effects of mushrooms have been studied, including: antibacterial, antifungal, antiviral, antitumor, cytostatic, immunosuppressive, antiallergic, anti-atherogenic, hypoglycaemic, anti-inflammatory and hepatoprotective effects (Reis et al., 2011). Ferreira et al. (2007) performed studies of radical-scavenging capacity and inhibition of lipid peroxidation from different mushrooms. *Amanita muscaria* (L.) Lam., Basidiomycetes: Amanitaceae (Fly agaric) is considered poisonous with hallucinogenic effect. This mushroom has been known to contain muscimol and ibotenic acid as the main compounds as well as other heterocyclic alkaloids in small amounts. Extracts of *A. muscaria* and *A. pantherina* had the highest antioxidant effects amongst other fungi (Reis et al., 2011). Unilateral infusions of muscimol in the ventral thalamic cores of six patients with tremor led to reversible suppression of the tremor, without influencing the speech and movements of the patients (Ferreira et al., 2007). Another study showed that unilateral infusion of muscimol in the *substantia nigra* (SN) of two patients suffering from Parkinson's disease also significantly decreased tremors (Barros et al., 2007). These studies demonstrated the free radical scavenging and antioxidant effects of *A. muscaria* (Ferreira et al., 2007; Barros et al., 2007).

Parkinson's disease (PD) is the second most common neurodegenerative disorder. It is characterized by a selective and massive neuron loss in *pars compacta* of SN. Dopaminergic neurons control the voluntary movement and therefore, their degeneration causes slowing of movement, muscular rigidity, and resting tremor. Although the etiology and pathogenesis of PD have yet to be established, several predisposing factors have been implicated: oxidative stress (associated with mitochondrial dysfunction), local inflammation, etc. (Stokes et al., 2002). There are still no proven neuroprotective therapies that prevent, slow down or reverse the neurodegeneration or progression of PD. In the near feature, such therapies could possibly include antioxidants, anti-

Abbreviations: AA, ascorbic acid; AME, *Amanita muscaria* extract; GSH, reduced glutathione; LPO, lipid peroxidation; MDA, malonedialdehyde; *t*-BuOOH, *tert*-butyl hydro-peroxide; PD, Parkinson's disease; SN, *substantia nigra*; 6-OHDA, 6-hydroxydopamine; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffer solution; TCA, trichloroacetic acid; TBA, thiobarbituric acid; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ROS, reactive oxygen species; DPPH, 2,2-diphenyl-1-picrylhydrazyl

* Corresponding author. Laboratory "Drug Metabolism and Drug Toxicity", Section "Toxicology", Department "Pharmacology, Pharmacotherapy and Toxicology", Faculty of Pharmacy, Medical University of Sofia, 2 Dunav St., 1000, Sofia, Bulgaria.
E-mail address: magdalenakondeva@gmail.com (M. Kondeva-Burdina).

https://doi.org/10.1016/j.fct.2019.110687
Received 25 May 2019; Received in revised form 15 July 2019; Accepted 16 July 2019
Available online 17 July 2019
0278-6915/

inflammatory agents and other pharmacological interventions (Stokes et al., 2002).

The most common used *in vitro* animal model of inducing and thus examining the pathological and behavioural effects of PD is still the infusion of the neurotoxin 6-hydroxydopamine (6-OHDA). It is relatively fast, cheap and easy to perform. 6-OHDA administration leads to massive destruction of nigrostriatal dopaminergic neurons due to its autoxidation, followed by generation of ROS (especially the superoxide anion). This is another reason why the 6-OHDA model is preferred in our study, which aims to demonstrate the neuroprotective role of antioxidants. In addition, the protocol used in our study was already approved by the Institutional Animal Care Committee.

Muscimol is of great pharmacological interest because of its newly described effects against free radicals. Recent studies describe the reduction of Fe^{3+} to Fe^{2+} potential and great 2,2-diphenyl-1-picrylhydrazyl (DPPH) and NO capturing effects of *A. muscaria* extract (Reis et al., 2011).

The objective of our study was to investigate *in vitro* effects of *A. muscaria* extract (AME) on different models of brain toxicity at subcellular (isolated rat brain synaptosomes, mitochondria and microsomes) and cellular level (neuroblastoma cell line SH-SY5Y), as well as on hMAOB enzyme activity.

2. Materials and methods

2.1. Obtaining and standardisation of AME

A. muscaria caps were collected in May 2018 in Vitosha Mountain and identified by Vladimir Vazharov. A voucher specimen was deposited in his personal collection (Vazharov, 2016). The material (100 g) was dried at room temperature, cut in pieces and then macerated with 70% ethanol (1:1) for 21 days. The extract (AME) was filtered and used for the experiments.

Muscimol chemical reference substance (CRS, Sigma Aldrich, Germany) was dissolved in 50% MeOH (2 mg/ml). Serial dilutions were made as follows: 1 mg/ml; 0.5 mg/ml; 0.1 mg/ml. An aliquot of each standard solution (10 μl) was injected three times in the HPLC. Waters HPLC system (Milford, MA, USA) equipped with binary gradient pump model 1525 EF, manual injector Rheodyne 7725i with 20 μl loop, UV–vis detector model 2489 and Breeze 2 software was used. An optimized HPLC method was developed (Gennaro et al., 1997; Yoshioka et al., 2014). An ODS column (Luna© 250 × 4.6 mm, 5 μm, Phenomenex®, USA) with a column guard and a mobile phase consisted of a 5 mM formate buffer (pH 7.0, A) and acetonitrile (B) with a flow rate of 1 ml/min. The gradient program used was performed as follows: initial 10% B; from 5 to 20 min 10%→100% B, linear; from 20 to 25 min maintained at 100% B; from 26 to 30 min back to 10% B, linear. Separations were monitored at 230 nm. An aliquot of AME (10 μl), filtered through a PVDF filter (0.22 μm) was injected three times for HPLC analysis.

Each experiment was performed in triplicates. MedCalc 12.3 was used for analysis of data. The Krushkal-Wallis one-way analysis of variance was conducted to define the statistical significance of muscimol content. The results were expressed as mean ± SD. Probability value of $p \leq 0.05$ were assumed as statistically significant. The content muscimol was calculated in AME using the equation obtained from the calibration curve $y = 1e^{+07}x + 102446$ ($R^2 = 0.9999$). The accuracy of the assay was established on triplicate injections of standard solutions of muscimol, at the same conditions.

The extract was diluted to achieve different concentration of muscimol as described in the *in vitro* methods.

2.2. Chemicals for pharmacological study

The chemicals used in the experiments were: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Roswell Park Memorial Institute (RPMI) 1640 Medium, Foetal Bovine Serum 10%, L-glutamine, 6-OHDA, Percoll, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sucrose, 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol), Tris hydrochloride, dithiothreitol, phenylmethylsulfonyl fluoride, EDTA, *tert*-butyl hydroperoxide and Tyramine hydrochloride were obtained from Sigma Aldrich, Germany; NaCl, KCl, $CaCl_2x2H_2O$, $MgCl_2x2H_2O$, $NaHPO_4$, D-glucose, trichloroacetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), sulphuric acid and Selegiline were obtained from Merck, Germany; Amplex UltrRed Kit was obtained from Invitrogen, USA.

2.3. Human neuroblastoma cell line SH-SY5Y

2.3.1. Cultivation

Human neuroblastoma cell line SH-SY5Y was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). SH-SY5Y cells were maintained in 75 ml flasks at 37 °C in a humidified atmosphere with 5% CO_2. The cells were cultured in RPMI 1640 medium, supplemented with 10% foetal bovine serum and 2 mM L-glutamine. At 95% confluence they were plated in 96-well plates for the next experiments.

2.3.2. MTT-dye reduction assay

The SH-SY5Y cells were seeded in 96-well microplates at a density 2×10^4 cells/well and allowed to attach to the well surface for 24 h at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, AME diluted with culturing medium (containing 0.15, 1.5, 3, 7.5, 15, 30, 75 μg/ml muscimol) was added to cells and incubated (for 24 h and 48 h). For each concentration a set of at least eight wells were used. The cell viability was estimated by MTT-dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay (Mosmann, 1983).

2.3.3. Lactate dehydrogenase (LDH) leakage

The AME was diluted with growth medium to obtain the tested concentrations (0.15, 1.5, 3, 7.5, 15, 30, 75 μg/ml of muscimol). They were added to SH-SY5Y cells (2 x 10^4 cells/well) and incubated for 24 and 48 h. Eight wells were used for each concentration. LDH leakage from the cells was determined using a commercial LDH cytotoxicity detection kit according to the manufacturer's protocols (Takara Bio Inc). LDH leakage was assessed in the conditioned media and was calculated as a percentage of the solvent-treated control.

2.3.4. Hydrogen peroxide-induced oxidative stress model

The model of oxidative stress damage on neuroblastoma SH-SY5Y cell line was achieved by H_2O_2 treatment of cells. Cells were seeded in 96-well plates at a density of 3 x 10^4/well and allowed to attach overnight. Then they were pre-treated with AME (concentration of muscimol 0.0015, 0.015, 0.15, 1.5, 15 μg/ml) for 1 h. Subsequently, they were subjected to 1 mM H_2O_2 in phosphate buffer solution (PBS) for 15 min. Then the liquids of all wells were changed with culture medium. After 24 h, the amount of attached viable cells was evaluated by MTT assay. Negative controls (cells without H_2O_2 treatment) were considered as 100% protection and hydrogen peroxide-treated cells as 0% protection.

2.4. Animals

Male Wistar rats with body weight 200–250 g were used. They were housed in plexiglass cages (3 per cage) in a 12/12 light/dark cycle under standard laboratory conditions (ambient temperature 20 °C ± 2 °C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria. Seven days' acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. Vivarium (certificate of registration of

farm № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№A-11-1081/03.11.2011). All performed procedures were approved by the Institutional Animal Care Committee and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) were strictly followed throughout the experiment. Brains were taken from rats immediately after decapitation and stored on ice until needed.

2.5. Subcellular in vitro studies

Two types of buffers were used for the isolation of rat brain synaptosomes and mitochondria. Buffer A was 5 mM HEPES and 0.32 M Sucrose; Buffer B was 290 mM NaCl, 0.95 mM $MgCl_2x2H_2O$, 10 mM KCl, 2.4 mM $CaCl_2x2H_2O$, 2.1 mM NaH_2PO_4, 44 mM HEPES, 13 mM D-Glucose. For the gradient centrifugation, Percoll reagent was used. A stock solution of 90% Percoll was used to prepare 16%, 10% and 7.5% solutions. All solutions were used as described below.

Immediately after harvesting, synaptosomes, microsomes and mitochondria were incubated with different concentrations of *Amanita muscaria* extract (AME) (1.5, 0.15 and 0.015 μg/ml muscimol) for 1 h, based on the results from SH-SY5Y.

2.5.1. Rat brain synaptosomes

2.5.1.1. Isolation and incubation. The synaptosomes were prepared from rat brains by multiple subcellular fractionation using a Percoll gradient (Taupin et al., 1994).

2.5.1.2. Model of 6-OHDA-induced neurotoxicity. This *in vitro* model resembles the neurodegenerative processes occurring in PD. Dopamine metabolism and oxidation lead to the formation of reactive oxygen species (ROS) and reactive quinones. They induce dopamine neurotoxicity and neurodegeneration (Stokes et al., 2002). The synaptosomes were incubated with 150 μM 6-OHDA for 1 h.

2.5.1.3. Synaptosomal viability. After the incubation, the synaptosomes were centrifuged three times at 15 000 × *g* for 1 min. MTT-test was performed to determine synaptosomal vitality by method described by Mungarro-Menchaca et al. (2002).

2.5.1.4. Determination of reduced glutathione (GSH). The level of GSH was determined by measuring the non-protein SH-groups after precipitation of the proteins with trichloroacetic acid (TCA). The presence of thiols in the supernatant was determined using Elmman reagent. The resulting yellow colour was measured spectrophotometrically (λ = 412 nm) (Robyt et al., 1971).

2.5.2. Rat brain microsomes

2.5.2.1. Preparation. The brain was homogenized in 9 vol. of 0.1 M Tris buffer, containing: 0.1 mM Dithiothreitol, 0.1 mM Phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 1.15% KCl and 20% (v/v) glycerol at pH 7.4. The homogenate was centrifuged twice at 17 000 × *g* for 30 min. The supernatants from both centrifugations were combined and centrifuged twice at 100 000 × *g* for 1 h. The resulting pellet was frozen in the Tris until needed (Ravindranath and Anandatheerthavarada, 1990).

2.5.2.2. $FeSO_4$/ascorbic acid-induced lipid peroxidation (LPO). The LPO was started with a solution of 20 μM iron sulphate and 0.5 mM ascorbic acid (Mansuy et al., 1986).

2.5.2.3. Malonedialdehyde (MDA) assay. The quantity of the lipid peroxidation product MDA was assessed, using the method, described by Deby and Goutier (1990).

2.5.3. Rat brain mitochondria

2.5.3.1. Isolation. The mitochondria were prepared by multiple, differential fractionation using a Percoll gradient (Sims and Anderson, 2008).

2.5.3.2. Tert-butyl hydroperoxide (t-BuOOH)-induced oxidative stress. The mitochondria were incubated with 75 μM t-BuOOH (Karlsson et al., 2000).

2.5.3.3. Lipid peroxidation assay. After incubation of the mitochondria, 0.3 ml 0.2% TBA and 0.25 ml sulphuric acid (0.05 M) were added to stop the reaction. The tubes were maintained at 100 °C for 30 min. In the next stage, centrifugation of the tubes was carried out at 3500 × *g* for 10 min.Assessment of the total quantity of MDA formed in each sample was measured in the supernatant at 532 nm (Shirani et al., 2019).

2.5.3.4. Measurement of GSH content. After incubation of mitochondria, 0.04% DTNB was mixed with the mitochondrial suspensions in 0.1M of phosphate buffers (pH 7.4). The absorbance of the yellow product was measured at 412 nm (Shirani et al., 2019).

2.6. Measurement of monoaminoxidase B activity

Monoaminoxidase activity assay of recombinant human MAOB was performed using a fluorometric method by Amplex UltraRed reagent (Bautista-Aguilera et al., 2014) with small modifications (Kasabova-Angelova et al., 2019). Tyramine hydrochloride was used as a substrate. AME diluted to a concentration of muscimol 1 μM. Selegiline (1 μM) was used as a positive control.

2.7. Statistical analysis

Cell viability (SH-SY5Y cell line) tests results were expressed as mean values from at least three independent experiments. The cell survival data and MAOB activity were normalized as percentage of the untreated control set as 100% and the results were expressed as mean values and standard deviation (± SD) (Graph Pad Prizm). Statistical analysis was performed by one-way analysis of variance (ANOVA) with *post hoc* multiple comparisons procedure (Dunnet's test) to assess the statistical differences in case of normal distribution. Values of $p < 0.05$, $p < 0.01$ and $p < 0.001$ were considered statistically significant. Statistical analysis of the results, obtained from brain microsomes, synaptosomes and mitochondria, were performed using statistical programme “MEDCALC”. Results were expressed as mean ± SEM for six experiments. The significance of the data was assessed using the non-parametric Mann-Whitney test. Values of $p < 0.05$; $p < 0.01$ and $p < 0.001$ were considered statistically significant.

3. Results and discussion

3.1. Content of muscimol

Muscimol content was 2.94 ± 0.03 mg/ml in the extract. The standard as well as the corresponding peak in the sample retained a t_R = 2.59 ± 0.1 min. There were no peaks with the reported time of ibotenic acid visible in the chromatogram of AME (Gennaro et al., 1997).

3.2. Effects of AME on human neuroblastoma cell line SH-SY5Y

Human neuroblastoma SH-SY5Y cells are characterized by catecholaminergic neuronal properties and human origin, which make them suitable experimental *in vitro* model of different neurodegenerative diseases, e.g. PD, Alzheimer's disease, etc. (Forster et al., 2016; Xicoy et al., 2017). The AME did not present any cytotoxic effect at the

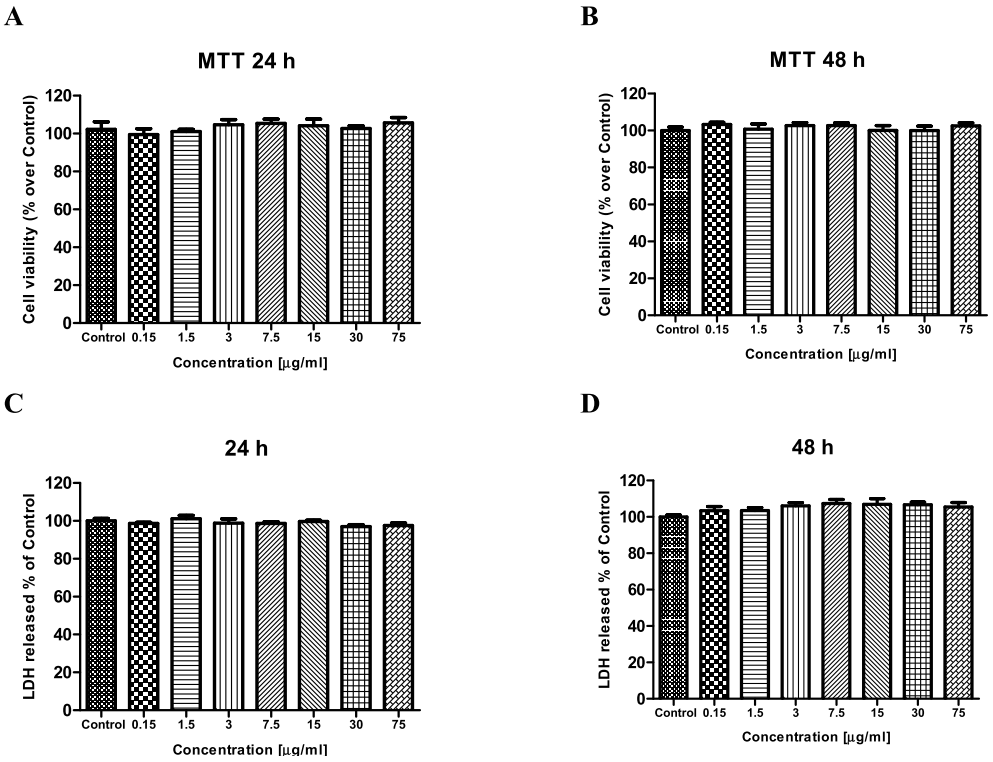

Fig. 1. Effects of AME on SH-SY5Y cell viability (**A, B**) and LDH release (**C, D**).

tested concentrations (Fig. 1). Microscopic observations revealed that the monolayer integrity and cell morphology were preserved (See Supplementary).

The good safety profile of the extract allows us to further evaluate its potential neuroprotective effect in the model of H_2O_2-induced oxidative stress. H_2O_2 treatment caused significant decreased of neuronal cell viability. Considerable neuroprotective effects were observed at muscimol concentrations of 0.015, 0.15, 1.5 µg/ml (Fig. 2). The pre-incubation with AME extract considerably reduced the H_2O_2-induced cell damage, showing statistically significant protection by 68%, 80% and 40%, respectively *vs* H_2O_2-induced cell damage. These results were also confirmed by microscopic observations of the treated cells, as shown in Supplement.

3.3. Effects AME on rat brain synaptosomes

Administered alone, on rat brain synaptosomes, AME did not exert statistically significant neurotoxic effect, compared to the control (non-treated synaptosomes; Fig. 3).

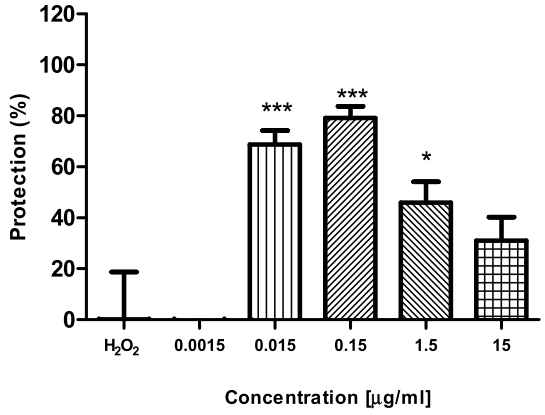

Fig. 2. Protective effects of AME in a model of H_2O_2-induced oxidative damage in SH-SY5Y cells. Groups were compared *vs* H_2O_2 group; * $p < 0.05$; *** $p < 0.001$.

The 6-OHDA *in vitro* model resembles the neurodegenerative processes occurring in Parkinson disease. 6-OHDA metabolism and oxidation lead to the formation of reactive oxygen species (ROS) and reactive quinones. They induce dopamine neurotoxicity and neurodegeneration (Stokes et al., 2002)., The toxic agent (6-OHDA), administered alone on isolated rat brain synaptosomes, showed statistically significant decreases in the synaptosomal viability and GSH level by 45% and 50%, respectively, compared to the control (non-treated synaptosomes; Fig. 4). In combination with 6-OHDA, of the different concentrations of AME (1.5, 0.15 and 0.015 µg/ml muscimol), only the highest concentration revealed statistically significant neuroprotective effects. This concentration preserved synaptosomal viability and GSH level by 29% and by 30%, respectively, compared to 6-OHDA, which was statistically significant. (Fig. 4).

3.4. Effects of AME on rat brain mitochondria

Administered alone, on brain mitochondria, AME did not exert statistically significant neurotoxic effect at concentrations: 1.5, 0.15 and 0.015 µg/ml muscimol, compared to the control (non-treated mitochondria; Fig. 5).

Another model, used for oxidative stress, is *t*-BuOOH. Two mechanisms for *t*-BuOOH toxicity were proposed: depletion of cellular stores of GSH and oxidation of functionally important SH groups on mitochondrial enzymes, and/or changes of mitochondrial membrane integrity induced by peroxidation of membrane lipids from ROS (Drahota et al., 2005; O'Donnell and Burkitt, 1994; Öllinger and Brunk, 1995).

On isolated rat brain mitochondria, administered alone, *t*-BuOOH decreased statistically significant the GSH level and increased the MDA production by 50%, compared to the control (non-treated mitochondria; Fig. 6).

In combination with *t*-BuOOH, AME (1.5, 0.15 and 0.015 µg/ml muscimol), again only the highest concentration revealed statistically

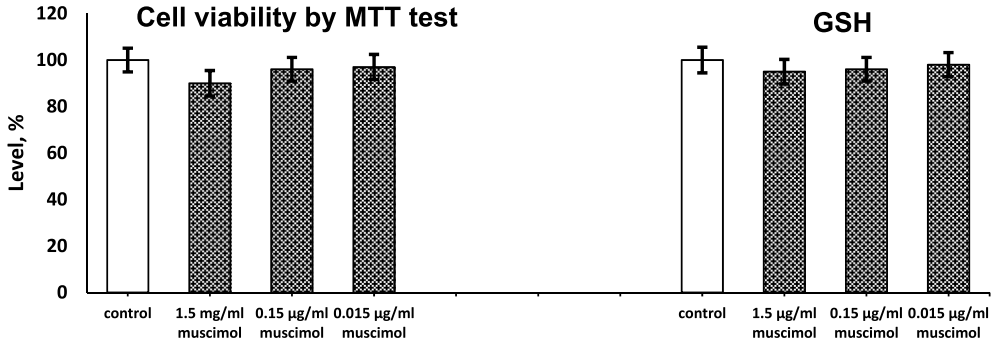

Fig. 3. Effects of AME, administered alone, on isolated rat brain synaptosomes.

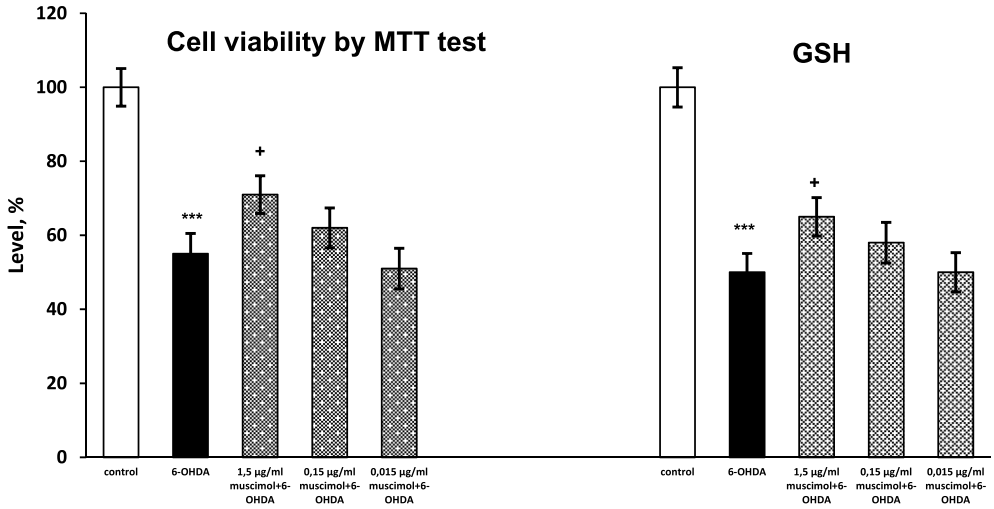

Fig. 4. Effects of AME, in combination with 6-OHDA, on isolated rat brain synaptosomes. *** $p < 0.001$ *vs* control (non-treated synaptosomes); + $p < 0.05$ *vs* 6-OHDA.

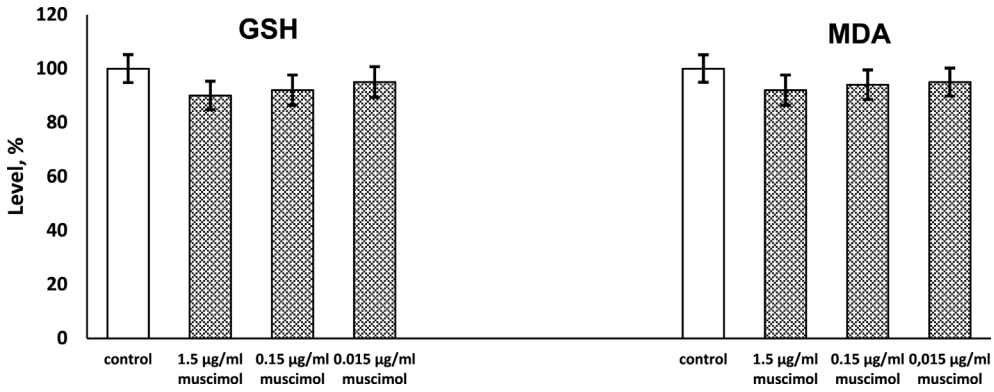

Fig. 5. Effects of AME, administered alone, on isolated rat brain mitochondria.

significant neuroprotective effects. This concentration preserved GSH level by 38% and decreased the MDA production by 23%, compared to the toxic agent (Fig. 6). The protective effects of the extract might be due to the preservation of GSH level or possible ROS scavenger activity.

3.5. Effects of AME on rat brain microsomes

Administered alone, on brain microsomes, AME did not exert statistically significant neurotoxic effect at concentrations: 1.5, 0.15 and 0.015 µg/ml muscimol, compared to the control (non-treated microsomes; Fig. 7). Microsomes incubation with Fe^{2+}/AA resulted in statistically significant increase of the amount MDA by 80% *vs* control (non-treated microsomes; Fig. 7).

In combination with Fe^{2+}/AA, of the different concentrations of AME extract (1.5, 0.15 and 0.015 µg/ml muscimol), only the highest concentration, revealed statistically significant antioxidant effect. This concentration decreased the MDA production statistically significant by 32%, compared to the toxic agent (Fe^{2+}/AA) (Fig. 7). The microsomal fraction, which was prepared by differential centrifugation, contained fragments from endoplasmic reticulum. Microsomes were used as a model of lipid membrane in experiments related to the process of lipid peroxidation (Zaidi et al., 1993). Only the highest concentration of AME showed statistically significant antioxidant effect, which might be due to the possible ROS scavenging activity.

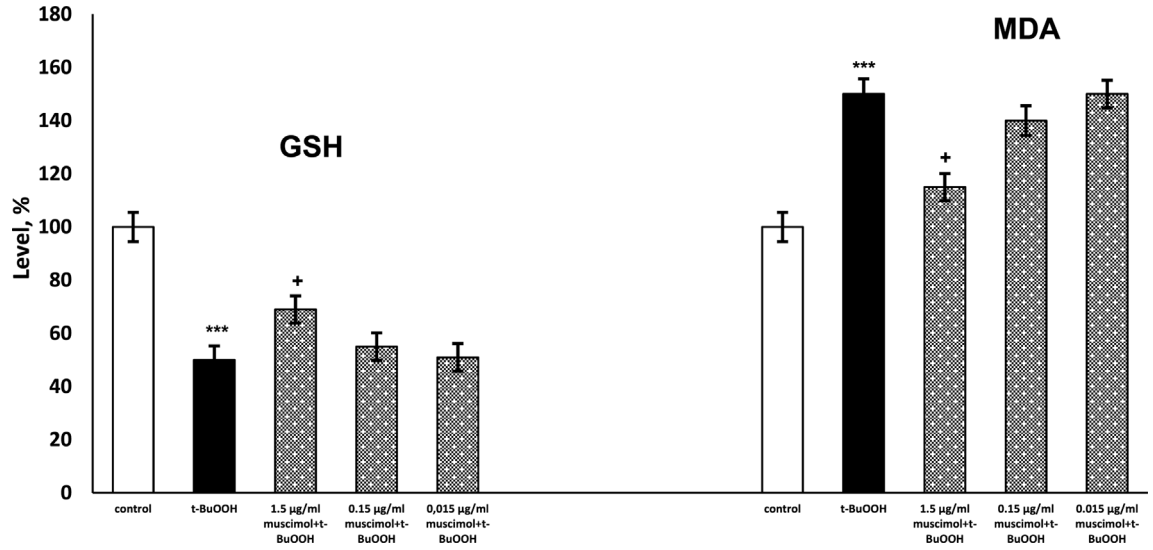

Fig. 6. Effects of AME in combination with *t*-BuOOH, on isolated rat brain mitochondria.
*** $p < 0.001$ *vs* control (non-treated mitochondria); + $p < 0.05$ *vs t*-BuOOH.

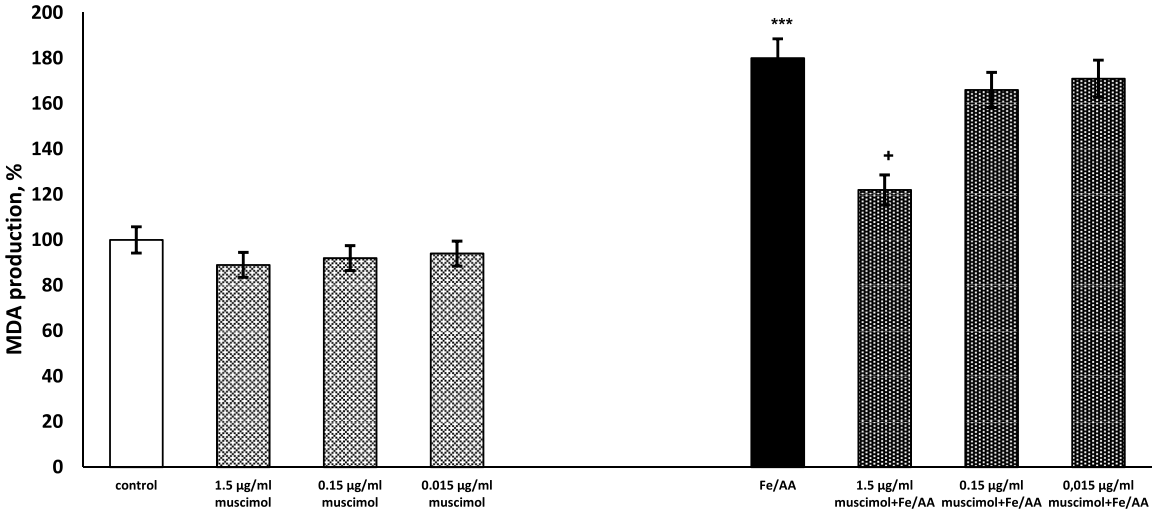

Fig. 7. Effects of AME, administered alone and in conditions of non-enzyme lipid peroxidation (Fe^{2+}/AA), on isolated rat brain microsomes. *** $p < 0.001$ *vs* control (non-treated microsomes); + $p < 0.05$ *vs* Fe^{2+}/AA.

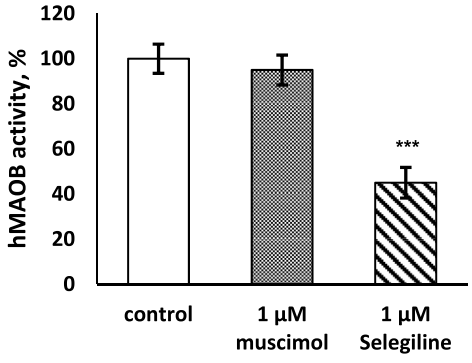

Fig. 8. Effects of AME on hMAOB. *** $p < 0.001$ *vs* control (pure hMAOB).

3.6. Effects AME on the activity of human recombinant monoaminoxidase B enzyme (hMAOB)

Administered alone, the AME (1 μM muscimol) did not exert statistically significant inhibitory effect compared to the control (pure hMAOB; Fig. 8). The classical MAOB inhibitor Selegiline (1 μM) inhibited hMAOB with 55%.

Although a variety of possible pathogenetic mechanisms have been proposed over the years, including excessive release of oxygen free radicals during enzymatic dopamine breakdown, impairment of mitochondrial function and neuroinflammation, the pathogenesis of PD is still largely uncertain. Chronic release of pro-inflammatory cytokines by activated astrocytes and microglia leads to the exacerbation of dopaminergic neuron degeneration. It was found that astrocytes responded to the inflammatory stimulations such as IL-1β and TNF-α, by production of pro-inflammatory cytokines both *in vitro* and *in vivo*. Reactive astrogliosis characterized by the increased expression levels of glial fibrillary acidic protein (GFAP) and hypertrophy of cell body and cell extensions, have been reported in various PD animal models (Wang

et al., 2015).

AME (at different concentrations muscimol), did not exert neurotoxic effects, when administered alone, on sub-cellular (isolated rat brain microsomes, synaptosomes and mitochondria) and cellular (human neuroblastoma cell line SH-SY5Y) levels. It did not inhibit statistically significant hMAOB enzyme. The highest concentration (1.5 μg/ml muscimol), revealed good statistically significant neuroprotective effects on different *in vitro* models of toxicity (H_2O_2-, 6-OHDA- and *t*-BuOOH-induced oxidative stress, as well as model of non-enzyme lipid peroxidation). The neuroprotective effects might be due to different mechanisms: ROS scavenging; influence of the metabolism at mitochondrial level; preservation of GSH level; decreasing the expression of glial fibrillary acidic protein (GFAP), which play role in the PD. Pilipenko et al. (2018) found that muscimol decreased and restored the values of GFAP in an *in vivo* model of Streptozotocin-induced Alzheimer's disease. GFAP play also role in the pathogenesis of PD (Wang et al., 2015).

4. Conclusion

The pharmacological and toxicological effects of the extract from *Amanita muscaria* (different concentrations of muscimol) has been investigated for the first time, using different *in vitro* models of neurotoxicity. Administered alone, AME extract did not revealed neurotoxic effects and did not inhibit the activity of hMAOB enzyme.

Under the conditions of *in vitro* neurotoxicity, AME (only at the highest concentration of muscimol) exerted neuroprotective and antioxidant activity in different models of neurotoxicity on sub-cellular and cellular levels.

The results of the current study will serve as an initial step in revealing the potential use of *Amanita muscaria*, as possible and perspective source of valuable antioxidants, neuroprotectors and as additional therapy of neurodegenerative diseases.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The study was carried out with the financial support of the Council of Medicinal Science at Medical University of Sofia, Contract № D-137/2019, project № 8321/22.11.2018. The authors are grateful to Vladimir Vazharov for collecting and identifying the mushroom.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110687.

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.110687.

References

Barros, L., Ferreira, M.-J., Queirós, B., Ferreira, I.C.F.R., Baptista, P., 2007. Total phenols, ascorbic acid, β-carotene and lycopene in Portuguese wild edible mushrooms and their antioxidant activities. Food Chem. 103, 413–419. https://doi.org/10.1016/j.foodchem.2006.07.038.

Bautista-Aguilera, O.M., Esteban, G., Bolea, I., Nikolic, K., Agbaba, D., Moraleda, I., Iriepa, I., Samadi, A., Soriano, E., Unzeta, M., others, 2014. Design, synthesis, pharmacological evaluation, QSAR analysis, molecular modeling and ADMET of novel donepezil–indolyl hybrids as multipotent cholinesterase/monoamine oxidase inhibitors for the potential treatment of Alzheimer's disease. Eur. J. Med. Chem. 75, 82–95.

Deby, C., Goutier, R., 1990. New perspectives on the biochemistry of superoxide anion and the efficiency of superoxide dismutases. Biochem. Pharmacol. 39, 399–405. https://doi.org/10.1016/0006-2952(90)90043-K.

Drahota, Z., Krivakova, P., Cervinkova, Z., Kmonickova, E., Lotkova, H., Kucera, O., Houstek, J., 2005. Tert-butyl hydroperoxide selectively inhibits mitochondrial respiratory-chain enzymes in isolated rat hepatocytes. Physiol. Res. 54, 67–72.

Ferreira, I.C.F.R., Baptista, P., Vilas-Boas, M., Barros, L., 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: individual cap and stipe activity. Food Chem. 100, 1511–1516.

Forster, J.I., Köglsberger, S., Trefois, C., Boyd, O., Baumuratov, A.S., Buck, L., Balling, R., Antony, P.M.A., 2016. Characterization of differentiated SH-SY5Y as neuronal screening model reveals increased oxidative vulnerability. J. Biomol. Screen 21, 496–509. https://doi.org/10.1177/1087057115625190.

Gennaro, M.C., Giacosa, D., Gioannini, E., Angelino, S., 1997. Hallucinogenic species in Amanita muscaria. Determination of muscimol and ibotenic acid by ion-interaction HPLC. J. Liq. Chromatogr. Relat. Technol. 20, 413–424. https://doi.org/10.1080/10826079708010660.

Karlsson, J., Emgard, M., Brundin, P., Burkitt, M.J., 2000. Trans-resveratrol protects embryonic mesencephalic cells from tert-butyl hydroperoxide. J. Neurochem. 75, 141–150. https://doi.org/10.1046/j.1471-4159.2000.0750141.x.

Kasabova-Angelova, A., Kondeva-Burdina, M., Mitkov, J., Georgieva, M., Tzankova, V., Zlatkov, A., 2019. Neuroprotective and MAOB inhibitory effects of a series of caffeine-8-thioglycolic acid amides. Braz. J. Pharm. Sci. 55 e18255.

Mansuy, D., Sassi, A., Dansette, P.M., Plat, M., 1986. A new potent inhibitor of lipid peroxidation in vitro and in vivo, the hepatoprotective drug anisyldithiolthione. Biochem. Biophys. Res. Commun. 135, 1015–1021. https://doi.org/10.1016/0006-291X(86)91029-6.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63. https://doi.org/10.1016/0022-1759(83)90303-4.

Mungarro-Menchaca, X., Ferrera, P., Morán, J., Arias, C., 2002. β-Amyloid peptide induces ultrastructural changes in synaptosomes and potentiates mitochondrial dysfunction in the presence of ryanodine. J. Neurosci. Res. 68, 89–96.

O'Donnell, V., Burkitt, M.J., 1994. Mitochondrial metabolism of a hydroperoxide to free radicals in human endothelial cells: an electron spin resonance spin-trapping investigation. Biochem. J. 304, 707–713. https://doi.org/10.1042/bj3040707.

Öllinger, K., Brunk, U.T., 1995. Cellular injury induced by oxidative stress is mediated through lysosomal damage. Free Radic. Biol. Med. 19, 565–574.

Pilipenko, V., Narbute, K., Beitnere, U., Rumaks, J., Pupure, J., Jansone, B., Klusa, V., 2018. Very low doses of muscimol and baclofen ameliorate cognitive deficits and regulate protein expression in the brain of a rat model of streptozocin-induced Alzheimer's disease. Eur. J. Pharmacol. 818, 381–399. https://doi.org/10.1016/j.ejphar.2017.11.012.

Ravindranath, V., Anandatheerthavarada, H.K., 1990. Preparation of brain microsomes with cytochrome P450 activity using calcium aggregation method. Anal. Biochem. 187, 310–313. https://doi.org/10.1016/0003-2697(90)90461-H.

Reis, F.S., Heleno, S.A., Barros, L., Sousa, M.J., Martins, A., Santos-Buelga, C., Ferreira, I.C.F.R., 2011. Toward the antioxidant and chemical characterization of mycorrhizal mushrooms from northeast Portugal. J. Food Sci. 76, C824–C830. https://doi.org/10.1111/j.1750-3841.2011.02251.x.

Robyt, J.F., Ackerman, R.J., Chittenden, C.G., 1971. Reaction of protein disulfide groups with Ellman's reagent: a case study of the number of sulfhydryl and disulfide groups in Aspergillus oryzae α-amylase, papain, and lysozyme. Arch. Biochem. Biophys. 147, 262–269.

Shirani, M., Alizadeh, S., Mahdavinia, M., Dehghani, M.A., 2019. The ameliorative effect of quercetin on bisphenol A-induced toxicity in mitochondria isolated from rats. Environ. Sci. Pollut. Res. 26, 7688–7696. https://doi.org/10.1007/s11356-018-04119-5.

Sims, N.R., Anderson, M.F., 2008. Isolation of mitochondria from rat brain using Percoll density gradient centrifugation. Nat. Protoc. 3, 1228–1239.

Stokes, A.H., Freeman, W.M., Mitchell, S.G., Burnette, T.A., Hellmann, G.M., Vrana, K.E., 2002. Induction of GADD45 and GADD153 in neuroblastoma cells by dopamine-induced toxicity. Neurotoxicology (Little Rock) 23, 675–684.

Taupin, P., Zini, S., Cesselin, F., Ben-Ari, Y., Roisin, M.P., 1994. Subcellular fractionation on Percoll gradient of mossy fiber synaptosomes: morphological and biochemical characterization in control and degranulated rat hippocampus. J. Neurochem. 62, 1586–1595.

Vazharov, V., 2016. Medicinal Fungi in Bulgaria, second ed. Biana, Sofia.

Wang, Q., Liu, Y., Zhou, J., 2015. Neuroinflammation in Parkinson's disease and its potential as therapeutic target. Transl. Neurodegener. 4, 1–9. https://doi.org/10.1186/s40035-015-0042-0.

Xicoy, H., Wieringa, B., Martens, G.J.M., 2017. The SH-SY5Y cell line in Parkinson's disease research: a systematic review. Mol. Neurodegener. 12, 10. https://doi.org/10.1186/s13024-017-0149-0.

Yoshioka, N., Akamatsu, S., Mitsuhashi, T., Todo, C., Asano, M., Ueno, Y., 2014. A simple method for the simultaneous determination of mushroom toxins by liquid chromatography–time-of-flight mass spectrometry. Forensic Toxicol. 32, 89–96.

Zaidi, S.I., Agarwal, R., Eichler, G., Rihter, B.D., Kenney, M.E., Mukhtar, H., 1993. Photodynamic effects of new silicon phthalocyanines: in vitro studies utilizing rat hepatic microsomes and human erythrocyte ghosts as model membrane sources. Photochem. Photobiol. 58, 204–210.