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Antioxidant, neuroprotective, and neuroblastoma cells (SH-SY5Y) differentiation effects of melanins and arginine-modified melanins from *Daedaleopsis tricolor* and *Fomes fomentarius*

Hoang Anh Thu Nguyen [1], Thien Phu Ho [1], Debby Mangelings[2], Ann Van Eeckhaut[3], Yvan Vander Heyden[2*] and Hanh T.M. Tran[1,2,3*]

Abstract

Background Microbial melanins possess a broad spectrum of biological activities. However, there is little understanding of their neuroprotective and neuronal cell differentiation properties. This study aimed to extract, purify, and modify melanins from two medicinal fungi (*Daedaleopsis tricolor* and *Fomes fomentarius*), and to evaluate their antioxidant activity, as well as their cell protective ability against neurotoxins. In addition, the study also investigated the feasibility of combining melanins or modified melanins with retinoic acid (RA) to induce neuronal differentiation.

Methods Melanin was extracted and purified using alkaline acid-based methods. Antioxidant activities and neuroprotective effects were evaluated using the DPPH (1,1-diphenyl-2-picrylhydrazyl) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assays, respectively. In addition, morphological changes of SH-SY5Y cells were recorded by using a Pannoramic MIDI scanner.

Results All melanins and arginine-modified melanins displayed mild DPPH scavenging activities, which were statistically lower than that of ascorbic acid ($p < 0.05$). In terms of neuroprotection, both melanins and arginine-modified melanins exhibited significant cell protection against H_2O_2 after 24 h exposure ($p < 0.05$). Notably, there is no significant difference between *F. fomentarius* melanin and its modified form as they both increased cell viability by about 20%. Contrarily, while *D. tricolor* melanin enhanced the cell viability with 16%, its modified form increased the cell viability with 21%. These activities, however, are significantly lower than the positive control (*N*-acetylcysteine, $p < 0.05$). Regarding MPTP, only the arginine-modified melanins of the two fungi significantly protected the cells after 24 h

*Correspondence:
Yvan Vander Heyden
Yvan.Vander.Heyden@vub.be
Hanh T.M. Tran
ttmhanh@hcmiu.edu.vn

Full list of author information is available at the end of the article

exposure to the toxin ($p < 0.05$). Specifically, *F. fomentarius* and *D. tricolor* modified melanins enhanced the cell viability with 10.2% and 11.1%, respectively, whereas that of the positive control was 13.2%. Interestingly, combining RA (10 µM) with 20 µg/mL of either *F. fomentarius*, or especially *D. tricolor* arginine-modified melanin, significantly promoted neuroblastoma cell differentiation into mature neuronal cells compared to using RA alone ($p < 0.05$).

Conclusions The arginine-modified melanins of *D. tricolor* and *F. fomentarius* have potential for neuroprotection against Parkinsonian neurotoxins. In addition, the arginine-modified melanin of *D. tricolor* may serve as an excellent material for research in neuroblastoma treatment.

Keywords Neuroprotection, Parkinson's disease prevention, Fungal melanin, Arginine-modified melanin

Introduction

Melanins are dark-brown to black natural macromolecules, built up from a variety of phenolic or indolic monomers. They are found in numerous organisms, including animals, microorganisms, plants, and humans [1]. In humans, melanins occur in the skin, hair, eye, and brain. They protect the skin and hair from reactive damage induced by visible and high-energy ultraviolet photons, shield the retina against excessive light exposure, and protect neurons in the substantia nigra from oxidative stress caused by iron [2, 3]. Although melanin pigments are not required for growth and development, initial studies have revealed that they act as effective antioxidants in living organisms, which may improve the longevity and competitive capacities of species in particular habitats [4]. Melanins have a wide spectrum of bioactivities, such as anti-inflammatory, anticancer, antioxidant, antivenom, antiviral, antimicrobial, and cell protective activities [5]. Studying melanin bioactivities is however challenging, mainly since melanin is neither soluble in water nor in common organic solvents. Previous studies suggested that the solubility and subsequently the bioactivities of melanins (at least their antioxidant activity) were enhanced when they were modified with amino acids [6, 7].

Microbial melanins were found to have powerful 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity. For example, the DPPH scavenging rate of melanin extracted from *Klebsiella* sp. GSK was 74% at 50 µg/mL [8], and 93% at 150 µg/mL for melanin extracted from *Streptomyce* sp [9]. Modification of *Ganoderma lucidum* melanin with arginine slightly increased the DPPH scavenging activity from 69 to 74% at 400 µg/mL [7]. Normally, a dose-dependent pattern in DPPH scavenging activity of melanins is seen [7–9].

Parkinson's disease (PD) is a complicated neurodegenerative disorder that has a large impact on society. Currently, over 8.5 million people are living with PD, and this may exceed 12 million in 2040 [10]. Growing evidence indicates that oxidative stress plays a critical role in the cascade of events that results in the loss of dopaminergic neurons in PD [11]. In particular, H_2O_2 (hydrogen peroxide) is a major contributor to oxidative damage. This toxin is generated by superoxide anion dismutase and peroxisomal oxidases and can be converted by the Fenton reaction to the hydroxyl radical, the most harmful of all reactive oxygen species (ROS) [11]. It is also widely accepted that complex I inhibition stimulates ROS production [12]. The discovery that the complex I inhibitor MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can induce acute and permanent Parkinsonian symptoms provided the first evidence for an association between complex I inhibition with subsequent oxidative stress and the pathogenesis of PD [12]. Compounds that can protect neurons from ROS and other neuron toxins would be potentially interesting for the prevention of neurodegenerative diseases such as PD [13].

SH-SY5Y cells are a human neuroblastoma (NB) cell line derived from an aggressive cancer in children. SH-SY5Y cells are not neuronal, but they have a variety of biochemical and functional characteristics, such as neuronal marker enzymes (dopamine-β-hydroxylase and tyrosine hydroxylase), and neurofilament proteins expression, which are similar to the neuronal cells, and most importantly they are culturable [14]. Therefore, they are widely used as a model for the study of neurodegenerative disorders, typically PD.

There is little understanding of the neuroprotective effects of melanin. According to Li et al. [13], melanin pigment derived from *Streptomyces* sp. ZL-24 possesses the ability to protect SH-SY5Y neuroblastoma cells against H_2O_2-induced oxidative stress. Incubating cells with 100 µM H_2O_2 for 24 h reduced the cell viability to approximately 60%. However, when the cells were pretreated with 1 to 50 µg/mL purified melanin, produced by *Streptomyces* sp. ZL-24, the cell viability increased from 62% up to 98% and the morphological changes were minimal.

Neuroblastomas (NB) or pediatric tumors develop from neural crest-derived cells that cannot differentiate properly because of genomic and epigenetic defects. The main treatments for NB are surgical resection, chemotherapy and/or radiation [15]. However, the latter two are highly toxic [15]. In addition, high risk NBs have developed resistance to chemotherapeutic agents, which leads to repeated treatment(s) and ultimately to death [15]. The

retinoic acid (RA) signaling pathway plays a crucial role in early embryo and neuronal developments, and neuroblastoma (derived from immature nerve cells) is often linked with an interrupted RA pathway. RA administration is used as an effective therapy repairing differentiation in neuroblastoma. In addition, RA is also effectively used as maintenance therapy for high-risk NB (treating the residual cancer cells after the patients have received other treatments such as chemotherapy). RA exists in three isomers: all-trans RA (ATRA), 13-*cis* RA, and 9-*cis* RA of which the first one is the most predominant in the cells and the latter one is the most potent for NB treatment [16].

RA is a natural retinoid in embryos and adult vertebrates, derived from vitamin A [15]. However, because of its hydrophobicity, short half-life, varying plasma concentrations between individuals and cytotoxicity, RA has limited clinical efficiency and is suspected of developing drug resistance [16]. In addition, RA treatment would cause dry skin, cheilitis, lipid abnormalities, headache, muscle and joint pain, and hypercalcemia [17]. Thus, overcoming these limitations of RA or finding a new potential compound with less side effects as a replacement for RA are crucial.

Daedaleopsis tricolor and *Daedaleopsis confragosa* are two morpho-ecological varieties of one species, a wood-rotting fungus of the Polyporaceae family, prevalently found in Europe and Asia [18]. Since Neolithic times, *D. tricolor* fragments, discovered at an archeological site close to Rome that dates back 7,000 years, have been prized for their medicinal and spiritual properties [18]. Although *D. tricolor* has long been known for its therapeutic properties, modern research has not yet fully explored this potential, leading to a paucity of data on its bioactivities. Recently, an untargeted metabolomics study of 40 species of Polyporaceae found that *D. tricolor* would be a promising producer of unique metabolites [19]. Notably, demethylincisterol, a powerful antibiotic against *Helicobacter pylori*, was isolated from *D. confragosa* [19].

Fomes fomentarius, a white-rot fungus of the same family, is widespread across the temperate climate zone of the northern hemisphere. This medicinal fungus has been used for the treatment of various diseases, including gastroenteritic disorders, hepatocirrhosis, oral ulcer, inflammation, and different types of cancers [20]. To the best of our knowledge, there has not been any research on neuroprotective effects and neuronal differentiation of melanins and arginine-modified melanins from fungi in general and from *D. tricolor* and *F. fomentarius*, in particular. In the field, both *D. tricolor* and *F. fomentarius* produce large numbers of (large) fruiting bodies. Especially the fruiting bodies of *F. fomentarius* are remarkably large in size and their mature fruiting bodies are either dark brown or black, indicating the presence of significant amounts of pigments (melanins) (Supplementary Fig. 1). In addition, these two fungi species have been reported to have several medicinal applications in folk medicines. Therefore, the aim of this study was to characterize melanins from *D. tricolor* and *F. fomentarius*, modify them with arginine, and evaluate their antioxidant and cell protective effects against H_2O_2 and MPTP. In addition, the possibility of combining the melanins or arginine-modified melanins with RA to promote neuroblastoma cell differentiation into mature neuronal cells was also investigated.

Materials and methods

Materials

Mature fruiting bodies of *D. tricolor* and *F. fomentarius* were collected in Neder–Over–Heembeek (Brussels, Belgium) and oven-dried (at 50°C) for two days until the samples were completely dry. The samples were previously identified using morphological characteristics and ITS gene sequencing [21].

Fetal bovine serum (FBS), L-glutamine, and penicillin/streptomycin for cell culture were purchased from Invitrogen (Waltham, Massachusetts, USA); DPPH, 13-*cis* RA, hydrogen peroxide, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, and *N*-acetylcysteine were from Sigma-Aldrich (Burlington, Vermont, USA). The other chemicals (sodium hydroxide, potassium hydroxide, ascorbic acid, arginine, hydrochloric acid, dimethyl sulfoxide (DMSO), and potassium bromide) in this study were from Hoa Nam Chemicals - Laboratory Equipment Company (Ho Chi Minh City, Vietnam).

Melanin extraction and purification

The extraction and purification of pigment from dried fungal biomass were done following Rajagopal et al. [22]. The dried fungal sample was ground into powder and suspended in 1 M KOH (1:40, w/v) for 48 h. The mixture was then centrifuged at 3000 g using a HERMLE Z 326 K refrigerated universal centrifuge (Hermle Labortechnik, Wehingen, Germany) for 15 min to collect the supernatant. Subsequently, the supernatant was acidified to pH 2.5 with 2 M HCl and incubated at room temperature for 2 h. The pigment-containing precipitate (crude melanin) was recovered by centrifugation at 3000 g, then rinsed three times with distilled water, and freeze-dried using a Labconco FreeZone 1 L Benchtop Freeze Dry System (Kansas City, Missouri, USA). The crude melanin was purified by acidic hydrolysis with 7 M HCl for 2 h at 100°C. After cooling, the purified melanin was obtained by centrifugation at 3000 g for 15 min, washed three times with 0.01 M HCl and distilled water, and freeze-dried.

Arginine- modification of melanin

Arginine-melanin modification was done following Xu et al. [7] with some minor modifications in temperature and time. Fifty and 75 mg arginine were incubated with 50 mg purified melanin from *D. tricolor* and *F. fomentarius* (Supplementary Fig. 2), respectively, and 5 mL distilled water at 37°C for 45 min, and then centrifuged (10 min, 12000 g). The supernatant was subsequently collected, freeze-dried, and stored at 4°C for further use.

UV-Vis spectrophotometric characterization

The purified melanins and arginine-modified melanins were dissolved in a 0.1 M NaOH solution at a final concentration of 50 µg/mL. The absorbance spectra of the samples were measured between 200 and 800 nm at an interval of 1 nm using a Jasco V-780 Ultraviolet-visible (UV-vis) spectrophotometer (Tokyo, Japan) [23]. As blank solution, 0.1 M NaOH was used.

Fourier transform-infrared (FTIR) spectroscopic characterization

The procedure proposed by Hou et al. [23]. was followed. An amount of 3 mg sample (powder) was mixed with KBr in a ratio of 1:10 and pressed into a tight pellet under vacuum using a Spectra Lab Pelletiser (Spectra Laboratories, Milpitas, California, United States). A blank KBr pellet was also prepared, similarly to the melanin-KBr one. A Tensor 27 FTIR Bruker spectrophotometer (Middlesex, Massachusetts, United States) was used to measure the spectra in the range of 400–4000 cm^{-1}.

Antioxidant activities

The antioxidant activities of the purified melanins and arginine-modified melanins were investigated using the DPPH assay [24]. DPPH radical solution at a concentration of 0.2 mM was prepared in absolute ethanol. Melanin and arginine-modified melanin- samples were dissolved in 1 M KOH to obtain a stock solution of 1000 µg/mL. Diluted working solutions of the test samples were then prepared at different concentrations (20, 40, 80, and 120 µg/mL). Ascorbic acid at different concentrations (2, 4, 8, and 12 µg/mL) in distilled water was used as a positive control and absolute ethanol was used as a negative control. One hundred microliter of ascorbic acid, absolute ethanol or samples at different concentrations were mixed with 100 µL 0.2 mM DPPH free radical solution in 96 well plates. Subsequently, the mixtures were shaken vigorously and left to stand at room temperature for 30 min in the dark. The absorbance of the resulting solutions was measured at 517 nm by a Biotek Synergy HT Microplate Reader (Winooski, Vermont, USA).

The DPPH radical scavenging activity was calculated using formula:

$$\begin{aligned} &DPPH\ radical\ scavenging\ activity\ (\%) \\ &= [(Absorbance\ of\ control \\ &- Absorbance\ of\ test\ sample) \\ &/\ (Absorbance\ of\ control)] \times 100 \end{aligned}$$

Cytotoxicity and neuroprotective effects

The neuroprotective effect was assessed using methods described by Li et al. [13] and Johnson et al. [25]. To evaluate the cytotoxicity (if any) of different concentrations of melanins and modified melanins, and of the neurotoxins H_2O_2 and MPTP, SH-SY5Y cells (Vietnam National University, HCM City, Vietnam) were seeded in 96-well plates at 1×10^5 cells/mL.

Fungal melanin and modified melanin were dissolved in absolute DMSO, and the final concentrations of the samples were 5, 10, 20, 40, 80, and 100 µg/mL, and DMSO was 0.2%. Upon reaching approximately 80% confluence, cells were pretreated with the prepared samples to determine the cytotoxic effects on neuroblastoma cellular viability. In addition, SH-SY5Y cells were also treated with either H_2O_2 (0.1 and 0.5 mM), MPTP (0.1 and 0.2 mM), *N*-acetylcysteine (NAC) – positive control (1, 5, 10 mM), or 0.2% DMSO – negative control, individually. After 24 h, cell viability was determined using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay [25]. The wells were incubated with MTT for 4 h at 37 °C in the dark. By measuring the absorbance at 570 nm using a Biotek Synergy HT Microplate Reader, the quantity of MTT formazan product was determined.

To evaluate the neuroprotective effect of melanins and arginine-modified melanins against the neurotoxins, SH-SY5Y cells were pretreated with 20 µg/mL melanin and modified melanin samples, or 1 mM NAC for 5 h. Then, cellular oxidative stress was induced in SH-SY5Y by adding H_2O_2 (0.5 mM) or MPTP (0.2 mM) into the cell mixture. Cellular viability of SH-SY5Ycells was determined by the MTT assay at 18 h and 24 h after H_2O_2 treatment, and at 12 h and 24 h after MPTP treatment [26].

Evaluating the effects of melanins and modified melanins on neuroblastoma cell differentiation

SH-SY5Y cells (1×10^5 cells/mL) were cultured in N2B27 medium (Invitrogen), supplemented with 10 ng/mL epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) (N2B27-EGF/bFGF, Invitrogen), for 7 days at 37°C under 5% CO_2 to promote survival and differentiation into neurons. To evaluate cell differentiation effects, 10 µM 13-*cis* RA (Sigma-Aldrich) or 10 µM 13-*cis* RA mixed with 20 µg/mL either melanin or modified melanin was added into the suspension culture in N2B27-bFGF and incubated for 7, 12, and 14 days (d-7, d-12, and d-14) (37°C, 5% CO2). Sham control was treated with 0.2% DMSO.

Morphologies of the cell population were captured at a certain time point using a Pannoramic MIDI scanner (Sysmex, HCM City, Vietnam). Neurite outgrowth was quantified based on the methodologies described by Bui et al. [27] using ImageJ and the NeuronJ plugin, where the longest neurite extending from each neuron was traced and measured. Neurite lengths were categorized into four bins: <50 µm, 50–100 µm, 100–150 µm, and >150 µm. For each treatment group, between 100 and 200 neurons were analyzed to ensure statistical robustness.

Statistical analysis

All experiments assessing antioxidant, neuroprotective, and neuronal cell differentiation effects were conducted in triplication.

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Tukey's test in GraphPad Prism V9.4.1 (GraphPad Software, Boston, MA).

Results are expressed as mean ± SD values.

Results

UV-Vis spectrophotometric characterization

Modified melanin samples were prepared by mixing melanin with arginine at 1.5:1.0 (w/w) ratio for *D. tricolor* melanin and 1.0:1.0 (w/w) for *F. fomentarius* melanin [21].

The absorbances of melanins and arginine-modified melanins in 0.1 M NaOH are shown in Fig. 1.

One of the distinguishing attributes of melanins is that their light absorbance is strongest in the UV region, between 200 and 300 nm, and sharply decreases towards the visible region. UV-absorbance is due to the occurrence of a highly complicated conjugated structure in melanin [28].

Melanins and arginine-modified melanins from *D. tricolor* and *F. fomentarius* presented a broad absorbance peak in the range of 200–300 nm, which was similar to the absorbance of alkaline melanin solutions from other reported sources [29–31]. The maximum absorption wavelength of *F. fomentarius* melanin (238 nm) was similar that of the arginine-modified form (236 nm). *D. tricolor* arginine-modified melanin also possessed a similar absorption maximum (237 nm) as its original form (235 nm) (Fig. 1A). A larger difference in the maximum absorbance wavelengths of melanin (211 nm) and arginine-melanin (202 nm) was previously reported for *Lachnum* sp [6].

Another regularly used parameter for characterizing melanin is that a linear relationship with a negative slope is obtained when plotting the log (absorbance) against

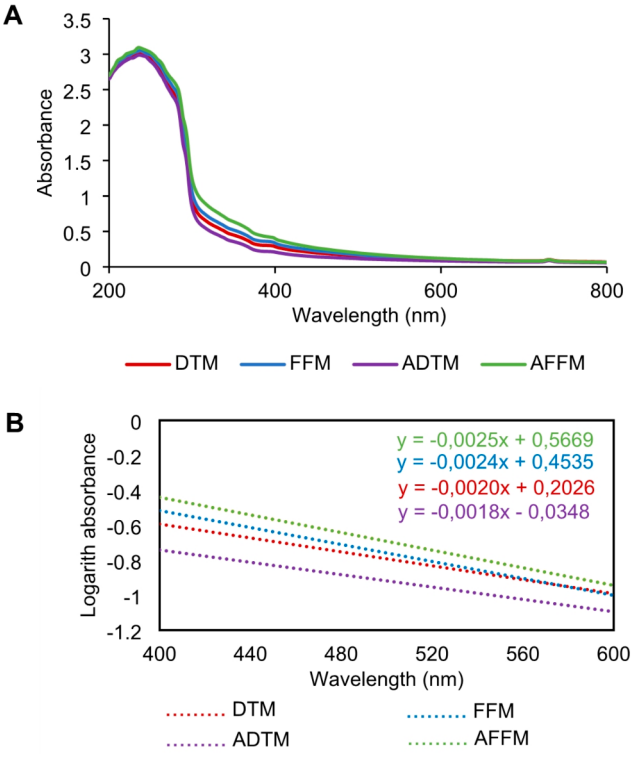

Fig. 1 (**A**) UV–visible spectra in 0.1 M NaOH, (**B**) logarithm of absorbance against the wavelength in a part of the visible light region, for melanins and arginine-modified melanins (50 µg/mL) from *D. tricolor* and *F. fomentarius*. DTM: *D. tricolor* melanin; FFM: *F. fomentarius* melanin; ADTM: arginine-modified *D. tricolor* melanin; AFFM: arginine-modified *F. fomentarius* melanin

the wavelength in the region 400–600 nm [28, 30]. The negative slopes of melanin from *D. tricolor* and *F. fomentarius*, -0.0020 and −0.0024, respectively, were obtained from the straight light equations established through the measured points (Fig. 1B). These values are in accordance with what has been reported for other fungal melanins, of which the slopes ranged from −0.0015 to -0.0030 [7, 30]. In addition, arginine-modified melanins of *D. tricolor* and *F. fomentarius* showed slopes of -0.0018 and −0.0025, respectively (Fig. 1B).

Fourier transform-infrared (FTIR) spectroscopic analysis

Fungal melanins have been reported to have characteristic absorption peaks between 3600 and 3000 cm^{-1}, and between 1650 and 1600 cm^{-1}, due to the presence of C=O and O(N)-H groups in their structure [30]. The melanins of both fungal species in this study exhibited these typical melanin bands (Fig. 2). Specifically, a 3426 cm^{-1} peak was observed with *D. tricolor* melanin, and a 3380 cm^{-1} peak with *D. tricolor* arginine-modified melanin, whereas 3423 cm^{-1} and 3382 cm^{-1} peaks were observed with melanin and arginine-modified melanin from *F. fomentarius*. This demonstrates that all samples have stretching vibrations of amine, amide, aromatic amino, carboxylic acid, and phenolic groups observed in indolic and pyrrolic systems [30, 32]. The peaks at 1545, 1463, 1408, and 1404 cm^{-1} were due to the bending vibration of N-H and the stretching vibration of C-N groups in *D. tricolor* and *F. fomentarius* melanins, and their modified forms, respectively. These indicate that all the samples possess the indole structure [30]. The detected bands at 1643 cm^{-1} and 1660 cm^{-1} from *D. tricolor* melanin and arginine-modified melanin, respectively, and at 1630 cm^{-1} and 1665 cm^{-1} from *F. fomentarius* melanin and arginine-modified melanin, respectively, were attributed to carboxylic functions and due to the vibration of aromatic C=C and C=O groups [30, 33]. *D. tricolor* purified melanin (1034 cm^{-1}), *D. tricolor* arginine-modified melanin (1118 cm^{-1}), *F. fomentarius* purified melanin (1046 cm^{-1}), and *F. fomentarius* arginine-modified melanin (1124 cm^{-1}) also exhibited aromatic ring C-H stretching.

Antioxidant activities

The DPPH scavenging activities of melanins and modified melanins (dissolved in 1 M KOH) were tested at different concentrations between 20 and 120 µg/mL; ascorbic acid (2–12 µg/mL) was used as the positive control.

Generally, melanins and modified melanins from *D. tricolor* and *F. fomentarius* had mild antioxidant activities

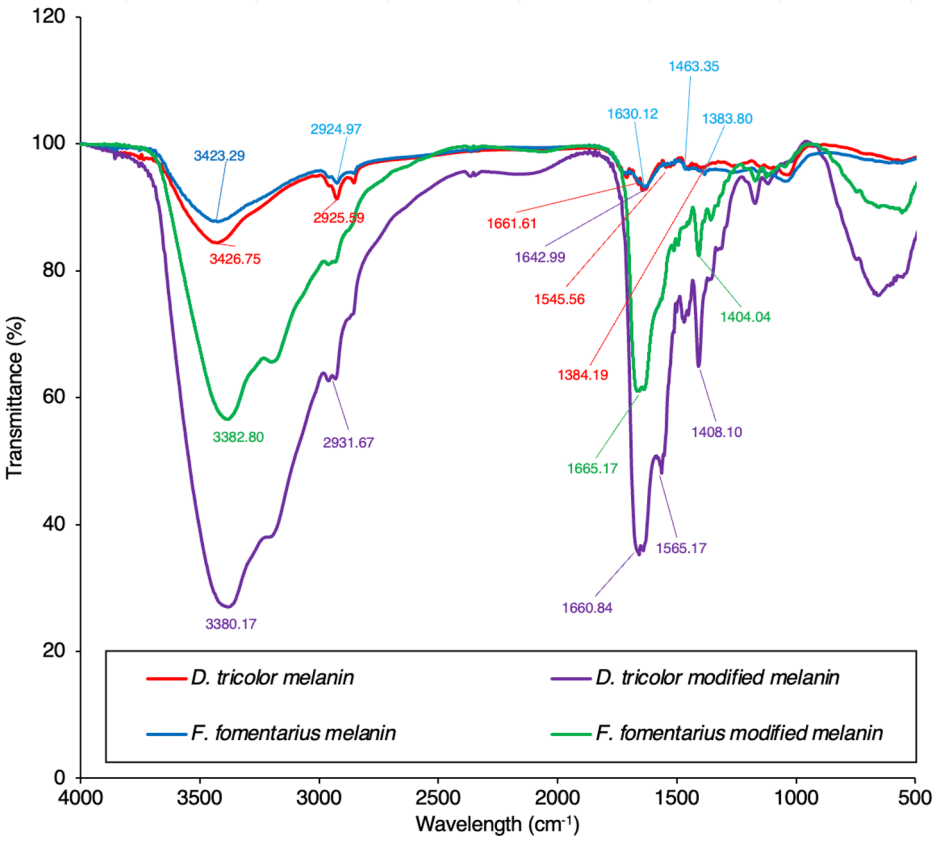

Fig. 2 FTIR spectra of melanins and arginine-modified melanins from *D. tricolor* and *F. fomentarius*

Table 1 DPPH scavenging activities (in %) of melanins and arginine-modified melanins from *D. Tricolor* and *F. fomentarius* at different tested concentrations (20, 120 µg/mL) compared with the positive control (2,12 µg/mL)

Sample	20 µg/mL	120 µg/mL
D. tricolor melanin	15.1 ± 0.04^{a}	33.7 ± 3.3^{b}
F. fomentarius melanin	18.5 ± 2.8^{a}	29.9 ± 1.3^{b}
D. tricolor modified melanin	11.5 ± 1.0^{a}	27.6 ± 1.5^{b}
F. fomentarius modified melanin	15.0 ± 2.5^{a}	27.1 ± 6.0^{b}
Positive control	**2 µg/mL**	**12 µg/mL**
Ascorbic acid	19.8 ± 1.5^{a}	73.6 ± 3.7^{c}

Means sharing a common letter (a, b) are not significantly different ($p > 0.05$, one-way ANOVA followed by Tukey's posthoc test)

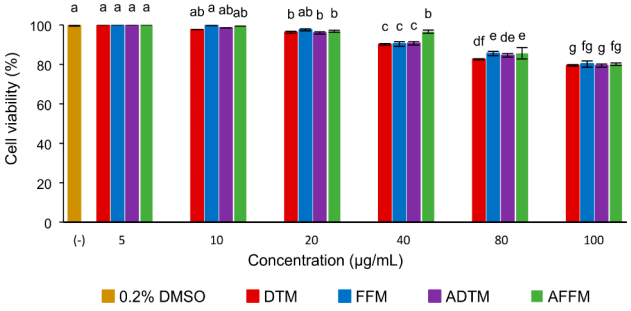

Fig. 3 Effects of different concentrations of melanins and arginine-modified melanins of *D. tricolor* and *F. fomentarius* and 0.2% DMSO solvent (-) on SH-SY5Y neuroblastoma cell survival after 24 h. Means sharing the common letter are not significantly different ($p > 0.05$, one-way ANOVA followed by Tukey's posthoc test). DMSO: Dimethyl sulfoxide; DTM: *D. tricolor* melanin; FFM: *F. fomentarius* melanin; ADTM: arginine-modified *D. tricolor* melanin; AFFM: arginine-modified *F. fomentarius* melanin

and the activities of different concentrations within the range of 20–100 µg/mL were not significantly different from each other (data not shown). However, there was a significant difference in the activities at 20 and 120 µg/mL (Table 1). The scavenging activities of all samples at 20 µg/mL were significantly lower than at 120 µg/mL ($p < 0.05$, Tukey's posthoc test). However, at the same tested concentration (either 20–120 µg/mL), there is no significant difference in the activities between the samples ($p > 0.05$, Tukey's posthoc test). In addition, the activities of all samples were significantly lower than that of 12 µg/mL ascorbic acid ($p < 0.05$, Tukey's posthoc test) (Table 1).

We did not attempt to evaluate the antioxidant activities of melanins and modified melanins above 120 µg/mL because in the subsequent experiment on cytotoxicity of melanin and modified melanin on neuroblastoma cells, the data indicate that the cell viability decreased to 79% when treated with melanins at 100 µg/mL (Fig. 3).

Cytotoxicity on neuroblastoma cells

In this part of the study, the effects of different concentrations of melanins and arginine-modified melanins of *D. tricolor* and *F. fomentarius* as well as of 0.2% DMSO (used as the solvent to dissolve the samples) on SH-SY5Y neuroblastoma cell viability were assessed. The obtained data are shown in Fig. 3.

The toxicity of a tested compound is measured via the cell viability after addition of the compound to the cell medium. The value defining the toxicity varies depending on the context of the study, the type of cells being used, and the specific assay or method employed. However, a reduction in viability generally indicates a toxic effect, while an increase could indicate a protective effect [34].

Figure 3 reveals that different concentrations of melanin and arginine-modified melanin had profound effects on cell viability. Melanins and arginine-melanins from the two fungal species were shown to be non-toxic to SH-SY5Y cells at concentrations ranging from 5 to 20 µg/mL, as the cell viability remained higher than 95%, which was comparable to the untreated cells. However, when their concentration exceeded 40 µg/mL, the cell viability significantly dropped to 79% ($p < 0.05$, Tukey's posthoc test) (Fig. 3). Thus, in the following experiments, 20 µg/mL was chosen as this concentration is not toxic for the cells and high enough for the neuroprotective and neuronal differentiation activities (if any) to be assessed.

Several studies have demonstrated that high melanin concentrations may result in cellular damage. Eskandari and Etemadifar [35] found that purified melanin from *Dietzia schimae* NM3 at concentrations above 500 µg/mL caused cytotoxic effects in normal human fibroblast cells. Furthermore, melanin from *Amorphotheca* KUC3009 at a concentration above 400 µg/mL significantly decreased the relative cell viability of human keratinocyte cell lines after 96 h of exposure [36].

Cell protective effects

NAC is a strong antioxidant, which has been found to suppress neuronal injury via its ability to alleviate oxidative stress [37]. NAC significantly inhibited H_2O_2-induced ROS production in SH-SY5Y cells [37]. Based on the study of Jurkowska and Wróbel [38], NAC was therefore used as a positive control in this experiment.

The effects of NAC (1–10 mM), H_2O_2 (0.1–0.5 mM), and MPTP (0.1–0.2 mM) on SH-SY5Y cells were evaluated. The obtained data revealed that all components decreased cell survival in a concentration-dependent manner (Fig. 4). At a concentration of 1–5 mM, NAC did not cause toxicity to SH-SY5Y cells as the percentage of cell viability after 24 h was 100%. However, incubating SH-SY5Y cells with 0.5 mM H_2O_2 for 24 h significantly reduced cell viability to around 63% ($p < 0.05$, Tukey's posthoc test), and the viability after treatment with 0.2 mM MPTP was 72% ($p < 0.05$, Tukey's posthoc test). It should be noted that the experiments on toxicity and neuroprotective effects were carried out at different times. Thus, to assure the accuracy, the toxicity of H_2O_2 and MPTP at their chosen concentrations was measured

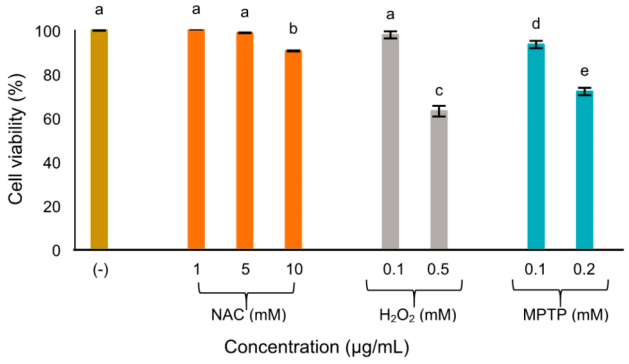

Fig. 4 Effects of DMSO (-), positive control (NAC), and neurotoxins: H_2O_2 and MPTP on SH-SY5Y neuroblastoma cell survival after 24 h. Means sharing the common letter (a) are not significantly different ($p > 0.05$, Tukey's posthoc test)

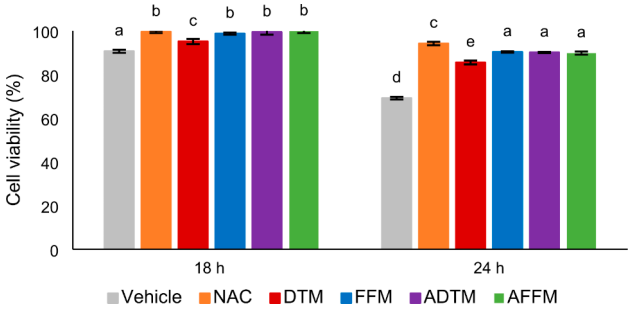

Fig. 5 Cell protective effects of fungal melanin and arginine-modified melanins on SH-SY5Y neuroblastoma cell survival after 18- and 24-hour exposure to 0.5 mM H_2O_2. Means sharing different letters for instance d, e were significantly different at $p < 0.05$ (one-way ANOVA followed by Tukey's posthoc test). Vehicle: 0.5 mM H_2O_2. NAC: *N*-acetylcysteine; DTM: *D. tricolor* melanin; FFM: *F. fomentarius* melanin; ADTM: arginine-modified *D. tricolor* melanin; AFFM: arginine-modified *F. fomentarius* melanin

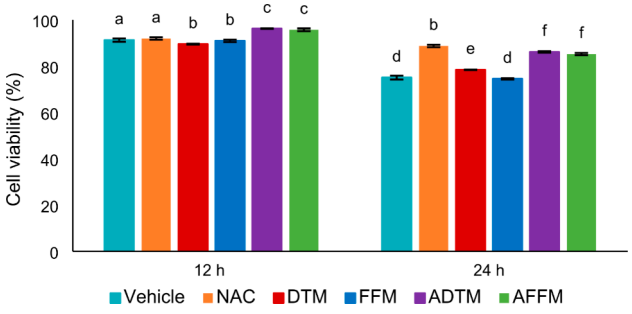

Fig. 6 Protective effects of fungal melanin and modified melanins on SH-SY5Y neuroblastoma cell survival against MPTP (0.2 mM) after 12- and 24-hour exposure to MPTP. Means sharing different letters were significantly different at $p < 0.05$ (one-way ANOVA followed by Tukey's posthoc test). Vehicle: 0.2 mM MPTP. NAC: *N*-acetylcysteine; DTM: *D. tricolor* melanin; FFM: *F. fomentarius* melanin; ADTM: arginine-modified *D. tricolor* melanin; AFFM: arginine-modified *F. fomentarius* melanin

each time when evaluating the neuroprotective effects of the samples (Figs. 4, 5 and 6).

To investigate the effects of the melanins and arginine-melanins against neurotoxins on the cell viability of SH-SY5Y cells, the cells were pretreated with 20 μg/mL of melanin/modified melanin for 5 h and co-treated with 0.5 mM H_2O_2 or 0.2 mM MPTP; 1 mM NAC was used as the positive control. The obtained data on the cell protection ability of melanins and arginine-modified melanins against H_2O_2 and MPTP are displayed in Figs. 5 and 6, respectively.

Effects on neuroblastoma cell differentiation

As mentioned earlier, despite being an effective drug for the treatment of NB, to avoid side effects, 13-*cis* RA is normally used at a low concentration [39], which results in a limited efficiency in inducing NB cell differentiation into mature cells. Therefore, it is crucial to enhance the cell differentiation capacity of this drug without causing side effects.

Previous experiments in this study showed that at low concentrations (around 20 μg/mL) melanin and modified melanin from *D. tricolor* and *F. fomentarius* are safe for cells. In addition, melanin is also present in our brain, and melanin may cross the blood-brain barrier, making it a valuable carrier for therapeutic drugs that need to reach brain tissue to generate and enhance therapeutic responses [40]. To evaluate the effects of melanin and melanin derivatives of *D. tricolor* and *F. fomentarius* in combination with 13-*cis* RA in inducing neuroblastoma cell differentiation, SH-SY5Y cells were exposed to either 10 μM 13-*cis* RA alone, or to a mixture of 20 μg/mL of melanin or modified melanin and 10 μM 13-*cis* RA. Cell growth and morphology of the neuroblastoma cells were assessed at days 7, 12, and 14. The changes in neurite outgrowth length in each group were observed and scored under a Pannoramic MIDI scanner. The percentage of cell differentiation was calculated, and the results are presented in Fig. 7.

In contrast to undifferentiated SH-SY5Y cells, which tend to develop in clusters and may form clumps of rounded cells on top of one another, differentiated SH-SY5Y cells do not cluster and possess a more pyramidal-shaped cell body, and the neurites begin to extend, resembling dendrites and/or axons with lengths exceeding 50 μm [41].

After 7 days, in all treatments, the majority of cells had neurite lengths under 50 μm. Furthermore, none of the treatments had cells with the neurite lengths above 100 μm. Treatment with RA and melanin of *F. fomentarius* had a similar percentage of cells with the neurite lengths in the range of 50 to 100 μm, with that of the treatment with RA alone ($p = 0.1726$, Tukey's posthoc test). However, they were both lower than that of the treatments with RA and arginine-melanin samples (Fig. 7A).

After 12 days, obviously, for all treatments, the percentages of cells with neurite lengths below 50 μm were

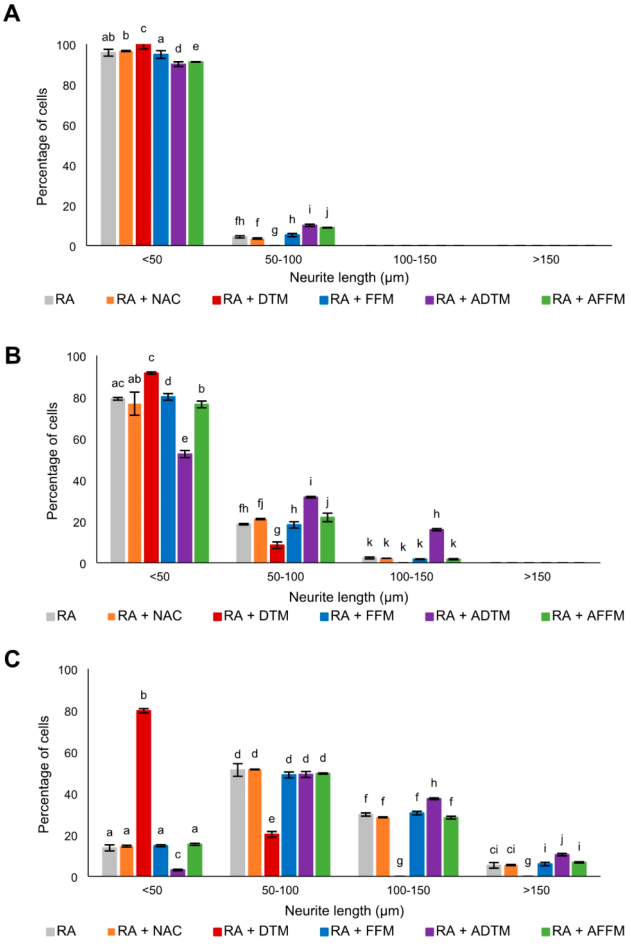

Fig. 7 Quantitation of neurite outgrowth length in cells treated with RA alone, or in combination with 1 mM NAC, melanin or arginine-modified melanin from *D. tricolor* and *F. fomentarius* at day 7 (**A**), day 12 (**B**), and day 14 (**C**). Means sharing different letters (e) were significantly different ($p < 0.05$, one-way ANOVA followed by Tukey's posthoc test). RA: Retinoic acid; NAC: *N*-acetylcysteine; DTM: *D. tricolor* melanin; FFM: *F. fomentarius* melanin; ADTM: arginine-modified *D. tricolor* melanin; AFFM: arginine-modified *F. fomentarius* melanin

significantly lower compared to those at day 7. Remarkedly, the combination of RA with arginine-modified *D. tricolor* melanin resulted in a significantly higher percentage of cells (16%) exhibiting neurite outgrowth lengths between 100 and 150 μm, while the positive control showed only 2.3% in that range ($p < 0.05$, Tukey's posthoc test) (Fig. 7B). None of the treatments showed cells with neurite lengths above 150 μm.

After 14 days, there is no significant difference in neurite outgrowth length over 150 μm between 13-*cis* RA-treatment alone and either the NAC ($p > 0.9999$, Tukey's posthoc test) or *F. fomentarius* melanin combined with RA treatments ($p > 0.9999$, Tukey's posthoc test). However, the combined treatment of RA and *D. tricolor* arginine-modified melanin showed the higher cell differentiation compared to the other treatments ($p < 0.05$, Tukey's posthoc test) (Fig. 7C). Specifically, in this latter treatment, the percentages of cells with neurite lengths below 50 μm, from 50 to 100 μm, from 100 to 150 μm, and over 150 μm were 3.1%, 49.1%, 37.4%, and 10.4%, respectively, whereas those of the treatment with RA alone were 13.7%, 51.2%, 29.8%, and 5.3%, respectively.

Morphologies of cells treated with 13-*cis* RA alone, and in combination with arginine-modified melanins of *D. tricolor*, or *F. fomentarius* are depicted in Fig. 8. After 14 days of differentiation induction, the neurites are visibly elongated (one of the signs that indicates cell differentiation into mature neuronal cells) in 13-*cis* RA-treatment alone and in both treatments with *D. tricolor* or *F. fomentarius* arginine-modified melanins combined with RA. RA-differentiated SH-SY5Y cells had a clear neuronal morphology (Fig. 8A), however, some cells existed individually with shorter processes (limiting them from connecting with surrounding cells) than those treated with RA in combination with *D. tricolor* melanin. The treatment with RA combined with *D. tricolor* arginine-modified melanin resulted in a more evenly distributed cell population on the culture substrate, with numerous long

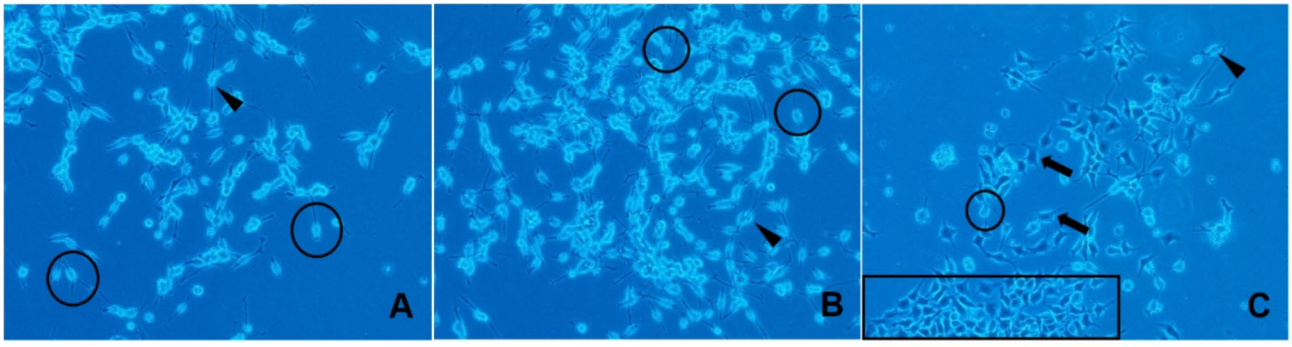

Fig. 8 Cell differentiation after 14 days treatment with 13-*cis* RA alone (**A**), and in combination with arginine-melanin of *D. tricolor* (**B**) and of *F. fomentarius* (**C**), captured by a Pannoramic MIDI scanner at 20x magnification. Circles: neuronal-like cells with a fusiform shape and neuritic processes; Arrows: epithelial-like cells with spread cell margins; Arrowheads: differentiated cells with extensive and elongated neuritic projections; Box: cluster of undifferentiated cells with short projection

neurites projecting to form an interconnected network of neurons (Fig. 8B). On the other hand, in the treatment of RA combined with *F. fomentarius* modified melanin, cells did not differentiate as effectively as when treated with *D. tricolor* modified melanin, even though there were cells processing long neurites, but there still existed a number of cells in clusters, with a few, short processes extending (Fig. 8C). These morphological differences are consistent with the above results (Fig. 7C). The combination of RA with arginine-modified melanin from *D. tricolor* significantly increased the percentage of differentiated cells, with 10.4% exhibiting neurite lengths exceeding 100 µm, compared to 5.3% and 6.7% for RA alone and RA combined with *F. fomentarius* modified melanin, respectively.

Discussion

The UV-Vis data revealed that the melanins and modified melanins from *D. tricolor* and *F. fomentarius* exhibit typical the UV-Vis absorbance characteristics. Arginine modification of melanin results in similar absorbance profiles as of the corresponding melanins. This indicates that the modification retains the essential light absorbance properties of the original melanins.

Based on the FTIR spectra, all functional groups detected were expected in melanin's chemical structures [30]. However, there were some differences in the observed peaks between the arginine-modified and non-modified melanins. Firstly, there was an absorption peak between 1700 and 1600 cm^{-1}, which was 1660 cm^{-1} for *D. tricolor* arginine-melanin, indicating the bond formation of the carboxylic group in melanin and the amino acid group in arginine [6]. Besides, the band at 1665 cm^{-1} which shows the ionized carboxylic group `indicates differences between *F. fomentarius* melanin and *F. fomentarius* arginine-melanin and suggests the binding of arginine to the carboxylic group [7]. Hence, the arginine-based modification of *D. tricolor* and *F. fomentarius* seems to be successful. Remarkably, the arginine-modified melanin spectra exhibit a lower percent transmittance than the non-modified spectra.

Morphology (shape) and size of melanin can be assessed by the scanning electron microscope. However, to get more insights in the melanin structure and composition, several techniques should be combined. For example, to analyze the melanin composition, the sample is cleaved using pyrolysis and the resulting lysates are analyzed using GC/MS [12]. However, to determine the melanin structure, then NMR and other structural analysis techniques should also be used along [5]. Melanin or arginine-modified melanin samples with potential activities obtained from this study will be subjected to further morphological and structural analysis using these techniques in our follow up research.

Generally, the melanins and arginine-modified melanins from *D. tricolor* and *F. fomentarius* had mild antioxidant activities, which were significantly lower than that of ascorbic acid. The free radical scavenging ability of melanins from several fungal species has earlier also been recorded. Available literature suggests that this ability is due to the presence of different functional groups and unpaired electrons in the melanin structure [3]. The melanins and arginine-modified melanins from *F. fomentarius* and *D. tricolor* had higher DPPH scavenging activities than those from other fungal species e.g., *Auricularia auricula* (smaller than 20% at 250 µg/mL) [42] and *Exophiala pisciphila* (13.1% at 100 µg/mL) [43]. There is a close correlation between the structure and chemical composition of melanins and their biological properties, including antioxidant activity [44]. As melanins are highly heterogeneous, different fungal species produce different melanins, thus, their biological activities would also be different [3].

In cellular models, the concentrations of H_2O_2 used to produce oxidative damage are in the millimolar range. Mostly concentrations above 0.1 mM, and often in the 0.1–0.5 mM range, are used [45]. For example, in the study of Li et al. [13]. incubating SH-SY5Y cells with 0.1 mM H_2O_2 for 24 h resulted in around 60% cell viability. In our study, a 5-fold higher concentration of H_2O_2 (0.5 mM) was required to reduce the neuroblastoma cell population to 63%. This is due to occasional differences in the quality of the cell lines, medium compositions, the cell densities, or even in the origin of the chemicals [14].

At 0.5 mM H_2O_2 and 0.2 mM MPTP, the viability of SH-SY5Y cells reduces to 63% and 72%, respectively; this level of stress is sufficient to assess the protective effects of melanin without inducing overwhelming cell death. At higher concentrations of H_2O_2 and MPTP, SH-SY5Y cells may experience severe oxidative stress, with high levels of reactive oxygen species (ROS) causing damage to proteins, nucleic acids, lipids, and membranes, which can lead to the activation of a broad range of cell death processes, such as apoptosis and necrosis [46], complicating the identification of specific protective actions of melanin and its derivatives. In addition, SH-SY5Y cells have endogenous antioxidant defense systems (e.g., superoxide dismutase, glutathione peroxidase, and catalase), which scavenge ROS and free radicals [47]. At increasing H_2O_2 and MPTP concentrations, not only the protective capacity of melanin and its derivatives is exceeded, but the endogenous antioxidant defense mechanisms may also be overwhelmed, leading to cell death. As a result, the level of cell death may be so severe that even if melanin provides some protection, the total viability may remain low, making it difficult to discern the protective effects. Hence, in our study, further neuroprotective testing was

carried out on SH-SY5Y cells using 0.5 mM H_2O_2 and 0.2 mM MPTP as neural oxidative stress inducers.

Based on the cytotoxicity data of different concentrations of melanins and arginine-modified melanins, 20 µg/mL melanins/arginine-modified melanins was used for the evaluation of their cell protective and neuroblastoma cell differentiation effects. In general, all the melanin and arginine-modified melanin samples displayed abilities to protect SH-SY5Y cells from cell damage induced by H_2O_2 after 18 h of incubation, but the cell viabilities in the treatments with melanins and modified melanins compared to vehicle treatment were more profound after 24 h (Fig. 5). There is a statistically significant difference between the samples and the negative control ($p<0.05$, Tukey's posthoc test) both at 18 and 24 h (Fig. 5). After 18 h, except for *D. tricolor* melanin, which modestly increased cell viability by 4.5% in H_2O_2 induced cells ($p<0.05$, Tukey's posthoc test), the remaining samples showed a similar protection as the positive control, of which the cell viability was enhanced by 9%. After 24 h, in the presence of melanin or arginine-modified melanin samples, SH-SY5Y cell survivals increased up to 21%. This is, however, statistically lower than that of the positive control (25%). There is a significant difference in the activities of the melanin from *D. tricolor* and its modified form ($p<0.05$, Tukey's posthoc test), while those from *F. fomentarius* were almost the same and similar to that of *D. tricolor* arginine-modified melanin.

Melanins' ability to occur in two (or potentially more) oxidized or reduced forms explains some of its context-dependent properties [48]. When melanin is reduced, it possesses donatable electrons that may neutralize an oxidative radical [48]. Thus, according to the results above, it is possible that melanin donates electrons to H_2O_2, allowing it to be transformed into water and oxygen. This process reduces the concentration of H_2O_2 and the related oxidative damage within the cell, increasing cell viability.

Regarding the MPTP-treated cells, the cell protection activities of both the positive control and most samples were of low effectiveness. There were considerable differences in the activities of the samples and the positive control at 12 and 24 h.

After 12 h exposure to MPTP, there was essentially no loss of cell viability in SH-SY5Y cells (91.2%) (Fig. 6). No statistically significant differences between the negative control, the positive control and the *F. fomentarius* melanin ($p>0.05$, Tukey's posthoc test) was seen. The cell viability of *D. tricolor* melanin treatment was even significantly lower than the negative control (89.5% versus 91.2%) ($p<0.05$, Tukey's posthoc test). However, both modified melanins displayed significant cell protection with a cell viability of 96.2% for *D. tricolor* arginine-melanin and 95.7% for *F. fomentarius* arginine-melanin treated cells.

After 24 h of MPTP incubation, SH-SY5Y cell viability reduced to 75.1% in the negative control (Fig. 6). It should be noted that both melanin samples showed either low or no cell protection activity, but their modified forms displayed significant activities compared to the negative control ($p<0.05$, Tukey's posthoc test). The positive control (NAC), however, exhibited significantly better neuroprotective activity towards MPTP than all other treatments with a cell viability of 88.7%, whereas those of the treatments with *D. tricolor* modified melanin and *F. fomentarius* modified melanin were 86% and 85%, respectively. Thus, rather similar behavior was observed for both arginine-modified melanins and the positive control.

Melanins and arginine-melanins of the two fungal species did not protect SH-SY5Y cells from damage by MPTP as effectively as they did for H_2O_2. Especially, *F. fomentarius* melanin increased cell survival up to 21% in H_2O_2-treated cells, though, the sample had no cell protective effects against MPTP. Regarding MPTP cell protection, the most potential samples are the two arginine-modified melanins.

The difference in the abilities to protect the cells treated with H_2O_2 or MPTP is probably related to the different toxicities caused by H_2O_2 and MPTP. When exposed to MPTP, the neurotoxin is converted to MPP^+ (1-Methyl-4-phenylpyridinium) by the enzyme monoamine oxidase B (MAO-B) in glial cells [49]. MPP^+ is then taken up by dopaminergic neurons via the dopamine transporter and accumulates in mitochondria, where it exerts toxic effects, causing neuronal damage and cell death [50]. The exact mechanism of how melanin could protect cells against MPTP is still unknown. A previous study reported that melanin has a high affinity to MPTP (melanin acts as an effective ionic sponge or chelator due to the presence of amino, carboxyl, and keto functionalities) [40]. Neuromelanin is present in the human brain and its general function is to protect the brain from ROS [2]. There has not been any research in the effect of microbial melanin on human brain. However, administering melanin obtained from *Escherichia coli* recombinant cells helped with the recovery of neuronal cells, which in turn stimulated the synthesis of melanin in the locus coeruleus in Alzheimer's animal models [40]. The authors also suggested the use of melanin for the treatment of neurodegenerative diseases, including Alzheimer and Parkinson disease. However, a contradicting hypothesis implies that neuromelanin with its high affinity toward toxins and its accumulation could be the etiology for neurodegenerative diseases, such as PD [51, 52]. Our opinion is that more evidence is needed in order to conclude whether a too high toxicity level in the brain triggers the synthesis of more melanin molecules for the sequestration of the toxins and at some point, the melanin molecules no

longer reduce the toxicity, which leads to damage of the brain, including neuronal death, or melanins themselves, as effective toxins scavengers, accidentally capture the toxins in the surroundings and over time the accumulation of both melanin and toxins cause the degeneration of the brain. As this study concerns in vitro work, at this stage it is not possible for us to tell how exactly the fungal melanins work to protect the cells. Determining the exact mechanisms of how the melanins and modified melanins in our study protected the cells against H_2O_2 and MPTP requires further study. However, since these substances can protect cells from the two major parkinsonian neurotoxins, this opens possibilities for further research on the use of fungal melanins and modified melanins for the prevention and treatment of neurodegenerative diseases (e.g., PD) in vivo.

It should be noted that the arginine-modified melanins were prepared by simply mixing arginine with melanin (1:1 or 1:1.5, w/w) in distilled water until the melanin was completely dissolved in the solution. Then after centrifugation the supernatant was transferred into another container and freeze-dried. Therefore, some amount of unbound arginine in the modified melanin solution can be present. However, the exact amount was not measured. A possible solution for the removal of arginine is to use dialysis. In a previous study, we found that arginine alone (at the same concentrations as arginine-modified melanin) possessed no antibacterial and biofilm eradication activities [21], whereas those of the arginine-modified melanin were significantly higher. As this is a preliminary study, the occasional neuroprotective effects of different free arginine concentrations were not investigated. However, such experiments are recommended for follow up research.

Previous experiments demonstrated that melanin might neutralize ROS and protect cells from oxidative stress. Excessive intracellular ROS has been found to trigger a switch from the stem cell state to the neuronal differentiation of human embryonic stem cells [53]. However, this would also be one of the reasons for the majority of cell deaths before differentiating into mature neuronal cells [54]. Although RA induces neuronal differentiation, it additionally generates ROS as a byproduct [55], which might have detrimental effects on cell health and differentiation efficiency. The combination of RA and melanin protects cells from oxidative damage, allowing for a more robust and efficient differentiation process. Similarly, arginine-modified melanin can protect neuronal cells from oxidative stress and increase cell survival during differentiation. Arginine-modified melanin may provide additional benefits due to arginine's role in nitric oxide (NO) generation via NO synthase, which can improve neuronal health and function [56, 57]. High NO levels have been shown to accelerate the differentiation of human neuroblastoma cells [58]. This could be one of the reasons why the arginine-modified melanins of the fungi showed better cell differentiation abilities than the original forms. The occasional presence of unbound arginine may potentially also be involved in the observed effects. This is a limitation of the actual study, which requires future research to confirm.

Notably, NAC is a strong antioxidant that can reduce oxidative stress [37], but its effect may be overpowering, disrupting the redox balance required for optimal cell differentiation. Controlled generation of ROS, which can act as signaling molecules, is required for RA-induced differentiation [59]. Excessive ROS scavenging by NAC may disrupt this kind of signal. Melanin and mainly its derivatives, on the other hand, provide a more balanced antioxidant effect, protecting cells while not totally suppressing ROS signaling required for differentiation. However, to confirm this, further experiments to measure the intracellular ROS levels in the presence of melanin should be carried out as in this current paper, only DPPH scavenging activities of the samples were evaluated.

It should be noted that in addition to neurite outgrowth length, and morphological evaluation, metabolic and genetic markers (e.g., β-III Tubulin) assessment should be carried out to confirm the actual conversion of neuroblastoma cells into mature neuronal cells [41].

Conclusions

The dark pigments from fruiting bodies of two medicinal fungi, *D. tricolor* and *F. fomentarius*, were extracted and purified using alkaline and acid-based methods.

The UV-Vis and FTIR analyses indicated that the extracted pigments from *D. tricolor* and *F. fomentarius* were melanin. Melanins and arginine-modified melanins from the two fungal species exhibited mild antioxidant activities. However, all samples showed significant neuroprotective effects against H_2O_2. In addition, the modified melanins also significantly protectd cells from MPTP. Remarkably, combining RA (10 μM) with 20 μg/mL of either *F. fomentarius*, or *D. tricolor* modified melanin, significantly promoted neuroblastoma cell differentiation into mature neuronal cells compared to using RA alone.

The obtained data suggested that the arginine-modified melanins of *F. fomentarius*, and especially that of *D. tricolor* have potential as therapeutic agents and deserve further research for neurodegenerative disease- and brain tumor treatment. The neuroprotective and neuronal differentiation effects of these samples at different concentrations should be investigated in vivo and the mechanisms of their effects should be elucidated. In addition, it

would also be interesting to test the compounds on different types of cell lines.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12896-024-00918-6.

Supplementary Material 1

Supplementary Material 2

Author contributions

Methodology, Investigation, Data analysis, original draft preparation, N.H.A.T. and H.T.P; Editing, M.D; Conceptualization, funding acquisition, editing, V.E.A, V.H.Y, H.M.T.T.

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Data availability

The analyzed data are presented in the manuscript. However, the raw datasets generated from the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]International University – VNU HCM, Applied Microbiology Laboratory, School of Biotechnology, Quarter 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam
[2]Vrije Universiteit Brussel (VUB), Department of Analytical Chemistry, Applied Chemometrics and Molecular Modelling, Laarbeeklaan 103, Brussels 1090, Belgium
[3]Vrije Universiteit Brussel (VUB), Research Group Experimental Pharmacology (EFAR), Department of Pharmaceutical Chemistry, Drug Analysis and Drug Information (FASC), Center for Neurosciences (C4N), Laarbeeklaan 103, Brussels 1090, Belgium

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