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Neuroprotective effects of melanins and melanin derivatives from *Calvatia Craniiformis* and *Xylaria plebeja* against Parkinsonian toxins

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Fungal melanins have remarkable bioactivities, but their practical applications are limited, partly due to their hydrophobic nature. Melanin modification with amino acids was reported to improve their water solubility. This research aimed to extract, and modify the melanins from Calvatia craniiformis and Xylaria plebeja with arginine, and to evaluate their neuroprotective effects against Parkinsonian toxins. MTT assay showed that all melanins and melanin derivatives could protect SH-SY5Y neuroblastoma cells from 1 mM H2O2. Xylaria plebeja melanin and melanin derivative at 100 µg/mL increased the cell viability about 16.0% compared to the negative control, whereas C. craniiformis melanin and its modified form enhanced the cell viability by 18.4% and 8.3%, respectively and that of the positive control (20 µM resveratrol) was 35.5%. Regarding neuroprotective effects against MPTP, the positive control, C. craniiformis melanin, and modified melanin increased the cell viability with approximately 5.0%, but that of X. plebeja melanin and modified melanin were 13.0%. Both melanins and melanin derivatives from C. craniiformis and X. plebeja showed significant neuroprotective effects against H2O2. However, only X. plebeja melanin and modified melanin displayed significant neuroprotective effects against MPTP and therefore they would be potential materials for follow-up research in Parkinson's disease prevention.

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

Melanins are among the most significant natural pigments that have a wide spectrum of biological activities and are produced by all biological kingdoms[1,2]. Based on the precursors of their synthesis pathways, melanins are devided into three main types including eumelanin (dark/brown/black coloured, commonly found in humans and microorganisms), pheomelanin (yellow/red, found in microorganisms), and allomelanin (normally found in plants). These three types of melanins are found in fungi. However, it should be noted that each fungal group or even each fungal species can produce different types of melanin[3]. Melanin is normally associated with fungal spore cell walls but in some fungi, melanin can be extracellularly produced in the form of granules in the fungal culture broth[3].

Melanins, in general, and fungal melanins, in particular, have powerful antioxidant, thermoregulatory, photoprotective, antimicrobial, antitumor, anti-inflammatory, radioprotective, and immunomodulatory activities[1,4,5]. However, there is little known about the melanin structure, as melanins, and especially fungal melanins are highly heterogeneous and insoluble in most solvents, including water. Nevertheless, initial research suggested that the presence of unpaired electrons in the melanin structure is the key factor that results

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in their wide spectrum of biological properties. The most recognized property is that they are outstanding reactive oxygen species (ROS) scavengers. In fact, their ability to serve as a "sponge" for free radicals is the key factor to enhance fungal survival under harsh conditions[3]. Oxidative stress and apoptosis have been tightly associated with neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (MS)[6]. Hydrogen peroxide (H_2O_2) is a major ROS, produced during oxidative stress, and is often used to create stress-induced damage in PD models. MPTP hydrochloride or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) hydrochloride, a precursor of 1-methyl-4-phenylpyridinium (MPP^+), is known to cause neuropathologic abnormalities as observed in idiopathic PD patients. MPTP hydrochloride is often used as a neurotoxin-based model to induce dopaminergic toxicity for neuroprotective testing and elucidating essential aspects of neuronal diseases[7]. Therefore, neurotoxins, such as H_2O_2 and MPTP, are employed to mimic the symptoms of neurodegenerative diseases since they both trigger oxidative cytotoxicity in dopaminergic neurons[7]. Compounds that enable the protection of neuronal cells from oxidative stress and apoptosis caused by neurotoxins have the potential to prevent or slow down the progression of neurodegenerative diseases[7]. Only a few reports on the cell protection capacity of melanins were found. Hoogduijin et al.[8] reported that melanin could protect human skin cells against H_2O_2-induced DNA strand breaks in both melanocytes and keratinocytes through its ability to bind to Ca^{2+}. In another study, melanins from mycelial culture broth and mycelia of *Aspergillus nidulans* were found to be potential hypochlorous acid (HOCl) scavengers[9]. Berliner et al.[10] reported that the administration of an effective amount of melanin could improve the overall functional ability and secondary motor symptoms in MPTP-induced PD monkeys[10]. The authors also suggested using melanin for the prevention of neurodegenerative diseases, such as PD, because melanin can effectively chelate or scavenge neurotoxins, such as MPTP[10]. More recently, it was found that SH-SY5Y cells, pretreated with a pure soluble melanin pigment (50 µg/mL) from *Streptomyces* sp. and subsequently exposed to 100 µM H_2O_2 had a 38% increase in cell viability compared to those treated with H_2O_2 but without melanin supplementation[5]. Additionally, the positive control (N-acetylcysteine, 20 µg/mL) increased the cell viability only by 12.4%. The researchers also observed that cells subjected to co-treatment with H_2O_2 and *Streptomyces* sp. melanin generated minimal morphological changes, while cells exposed to H_2O_2 alone developed distorted irregular shapes or underwent cell death[5].

Even though fungal melanins have remarkable bioactivities, one of the major obstacles in applying them is that they are insoluble in water and most other common solvents. However, previous studies have shown that modification of melanin with amino acids could increase their water solubility, which subsequently enhances some of their specific bioactivities. For example, arginine-modified intracellular melanin of *Lachnum* (fungus) was found to have better anti-radiation and antioxidant effects, and higher antitumor activity compared to its original form[11,12]. In addition, the glucosamine-modified melanin from this fungus also showed higher hepatoprotective effects on acute alcohol-induced liver injury in mice[13]. The mechanisms of how amino acid modification enhances the activities, however, were not discussed.

Calvatia craniiformis or skulled puffball, is a medium- to large-sized and edible puffball that can be found in different regions of the world[14]. When matured, the fruiting body turns into a yellow-brown color. This color change is anticipated due to the formation of spores with the presence of melanin in their cell wall. Mature specimens have been utilized broadly in traditional medicines of China, Japan and by the Ojibwe people as hemostatic (styptic), wound dressing agents[15], or for the treatment of pityriasis versicolor-associated *Malassezia furfur* infections[16].

Xylaria is one of the ascomycetous genera, commonly discovered on dead wood in different niches of tropical regions. Melanin production has been reported from mycelial cultures of several species of *Xylaria* with amounts of 0.07, 0.32, and 0.38 g/L for *Xylaria hypoxylon*, *X. longipes*, and *X. polymorpha*, respectively[17].

Calvatia craniiformis (collected from Thailand) and *X. plebeja* (collected from Vietnam) are of interest in our research because they form fruiting bodies in quite large sizes (*C. craniiformis*) or numbers (*X. plebeja*) with brown or black colors, which suggest the presence of melanin (Fig. S1). To the best of our knowledge, there are only a couple of studies on neuroprotective effects of bacterial melanin[4,12]. Recently, our group first reported on neuroprotective properties of melanins and arginine modified melanin from fungi (*Daedaleopsis tricolor* and *Fomes fomentarius*)[18]. However, there has not been any study yet on the biological activities, including neuroprotective effects, of melanin from either *C. craniiformis* or *X. plebeja*. In addition, understanding of the neuroprotective effects or biological activities of melanin and amino acid-modified melanin from any organism remains very limited. Therefore, the main purposes of this study were to extract, and modify melanins from *C. craniiformis* and *X. plebeja*, and to evaluate their neuroprotective effects against H_2O_2 and MPTP toxins on neuroblastoma cells (SH-SY5Y cells).

Results

Melanin extraction yield

The yields of crude melanin and purified melanin from the dry fruiting bodies of each fungal sample are shown in Table 1.

Table 1 shows that the amount of crude melanin extracted from one gram of *X. plebeja* is about 1.4 times higher than that of *C. craniiformis*, and almost the similar result is observed with their purified forms. The difference in the amounts of melanin can be attributed to the nature of each species. Different fungal species can produce the same or different types of melanin in different amounts[5].

The colors of the crude and purified melanins from *C. craniiformis* and *X. plebeja* are also different (Fig. **S2**): a light-brown color is observed in crude melanin samples from *X. plebeja* while its purified form is black, whereas the crude melanin of *C. craniiformis* and its purified form both have a dark brown color (Fig. **S2**). It should be noted that the colors of melanin can be variable, including dark brown, black, yellow, and red[1] and those of fungal melanin are normally dark brown to black[3]. In this study, the drastic change in color of *X. plebeja*crude

Fungal species	Crude melanin yield (g/g biomass) ($n=3$)	Purified melanin yield (g/g crude melanin) ($n=3$)
C. craniiformis	0.085 ± 0.007	0.185 ± 0.040
X. plebeja	0.120 ± 0.048	0.327 ± 0.053

Table 1. Melanin extraction and purification yield.

melanin from brown to black might result from the removal of contaminants, e.g., carbohydrates, proteins and salts in the crude sample[19]. Mature fruiting bodies or stromata of both *X. plebeja*and *C. craniiformis* were used. However, for the former species, the content of the black spores in the stromata was not high because the spores are stored in asci, which are covered by several layers of walls. On the other hand, in the latter species, the mature fruiting bodies consisted mostly of dark brown spores in spongy dried flesh (containing melanin). Therefore, we assume that there were more impurities in the *X. plebeja* crude melanin, and that their removal made such a large color change.

UV-VIS spectroscopy of the extracted melanin

In this part of the study, UV-VIS spectroscopy was performed to evaluate the absorption characteristics of the fungal melanins. Specifically, melanin samples were dissolved in 0.1 M NaOH, and their absorption spectrum was measured in the wavelength range from 200 to 800 nm. The obtained spectra and the logarithm of the absorbance against the wavelength are presented in Fig. 1.

Further confirmation of melanin identity and purity is described below based on the obtained FTIR spectra.

FTIR analysis of the melanin and melanin derivative samples

FTIR analysis was applied for both purified melanin and melanin derivatives to confirm their identity and the success of the modification with arginine. The FTIR spectra are shown in Fig. 2.

Effects of fungal and modified melanins on SH-SY5Y neuroblastoma cell survival

The SH-SY5Y human neuroblastoma cell line is the most extensively utilized cell line to investigate therapeutic implications on PD in in-vitro models, because it demonstrates many of the molecular and cellular processes of neuronal cells[5].

Different concentrations (10–100 µg/mL) of purified fungal and modified melanins from *C. craniiformis* and *X. plebeja* were tested against SH-SY5Y cells to see whether the substances had any effect on the cells. Results are shown in Fig. 3.

Figure 3 shows that SH-SY5Y cells treated with low concentrations (10–25 µg/mL) of melanins and melanin derivatives resulted in similar cell viability as the untreated cells. On the other hand, when the concentration of melanins and melanin derivatives increased (50–100 µg/mL), a significant decrease in cell viability compared to the untreated cell population *($p < 0.05$)* was seen. However, in those melanins and melanin derivative treatments, the cell viability still remained higher than 95%. This suggested that the fungal melanins and their derivatives at the tested concentrations were not toxic to the neuroblastoma cells.

Effects of neurotoxins on SH-SY5Y neuroblastoma cell survival

SH-SY5Y neuroblastoma cells were exposed to various concentrations of H_2O_2 and MPTP hydrochloride. A one-way analysis of variance (ANOVA) was performed to investigate the difference between the effects of the neurotoxin concentrations on the growth of the cell line. The ANOVA data indicate that there is a significant difference ($p < 0.05$) in the toxicity effects of neurotoxins (H_2O_2 at 0.1, 0.5, and 1mM; MPTP hydrochloride at 0.1, 0.25 and 0.5mM) and the control group (0.2% DMSO) on the SH-SY5Y cells.

The results (Fig. 4) show that incubating the SH-SY5Y cell line with 1 mM H_2O_2 for 24 h was sufficient to induce oxidative stress damage, with a resulting cell viability of around 54%. However, it only took 0.5 mM MPTP hydrochloride to result in approximately 46% SH-SY5Y cell viability.

Neuroprotective effects of melanin and melanin derivatives on SH-SY5Y neuroblastoma cell survival against H2O2 toxicity

SH-SY5Y cells were pretreated with different concentrations of either the fungal melanin or arginine-melanin. Then, the neuroblastoma cells were subjected to 1 mM H_2O_2 and the viability of the melanin and modified melanin-treated cell populations was recorded after 24 h and compared with the untreated population (which was exposed to 0.2% DMSO), the population treated with H_2O_2 alone (negative control) and the 20 µM resveratrol-treated population (positive control) (Fig. 5).

Neuroprotective effects of fungal melanin and modified melanin on SH-SY5Y human neuroblastoma cell survival against MPTP-HCl cytotoxicity

SH-SY5Y cells were pretreated with different concentrations of either the fungal melanin or melanin derivative then subjected to 0.5 mM MPTP-HCl. The cell viability was assessed to determine the neuroprotective effect of the melanins and derivatives against MPTP. The obtained data are displayed in Fig. 6.

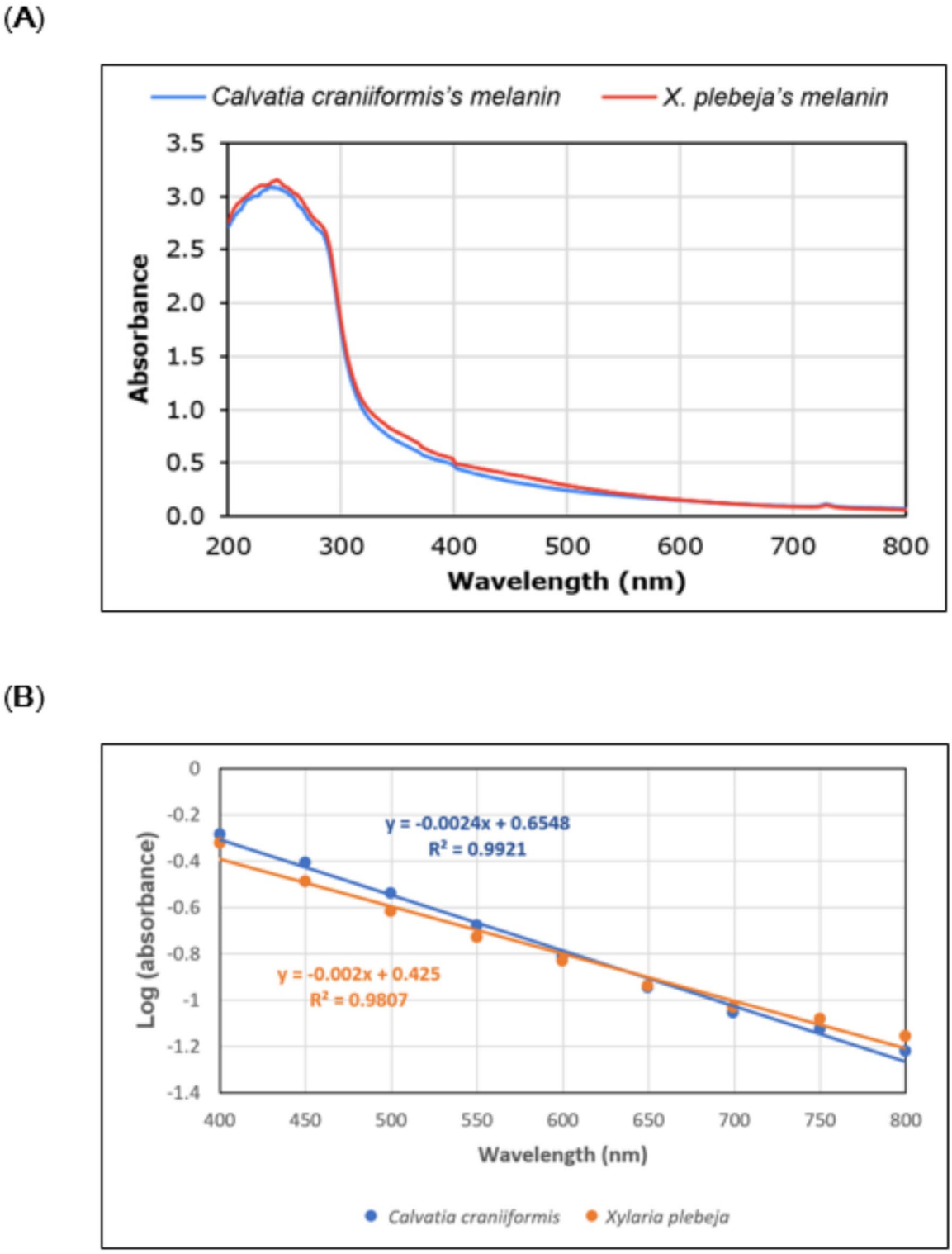

Fig. 1. UV-VIS spectra (**A**) and the logarithm of the absorbance against the wavelength in the visible-light region (**B**) of the melanins (62.5 µg/mL) from *X. plebeja*and *C. craniiformis*.

Discussion

One of the characteristics of melanins is that they have strong absorption in the UV region (200–400 nm), which progressively reduces in the visible-light region[3,13]. As can be seen in Fig. 1A the UV-VIS spectrum of the melanins from *C. craniiformis* and *X. plebeja* are quite similar. Furthermore, both samples exhibit absorption patterns similar to that of melanin from other sources[3,13], with pronounced absorption in the UV region (200–400 nm), followed by a progressive decrease in absorbance as the wavelength increases. This is due to

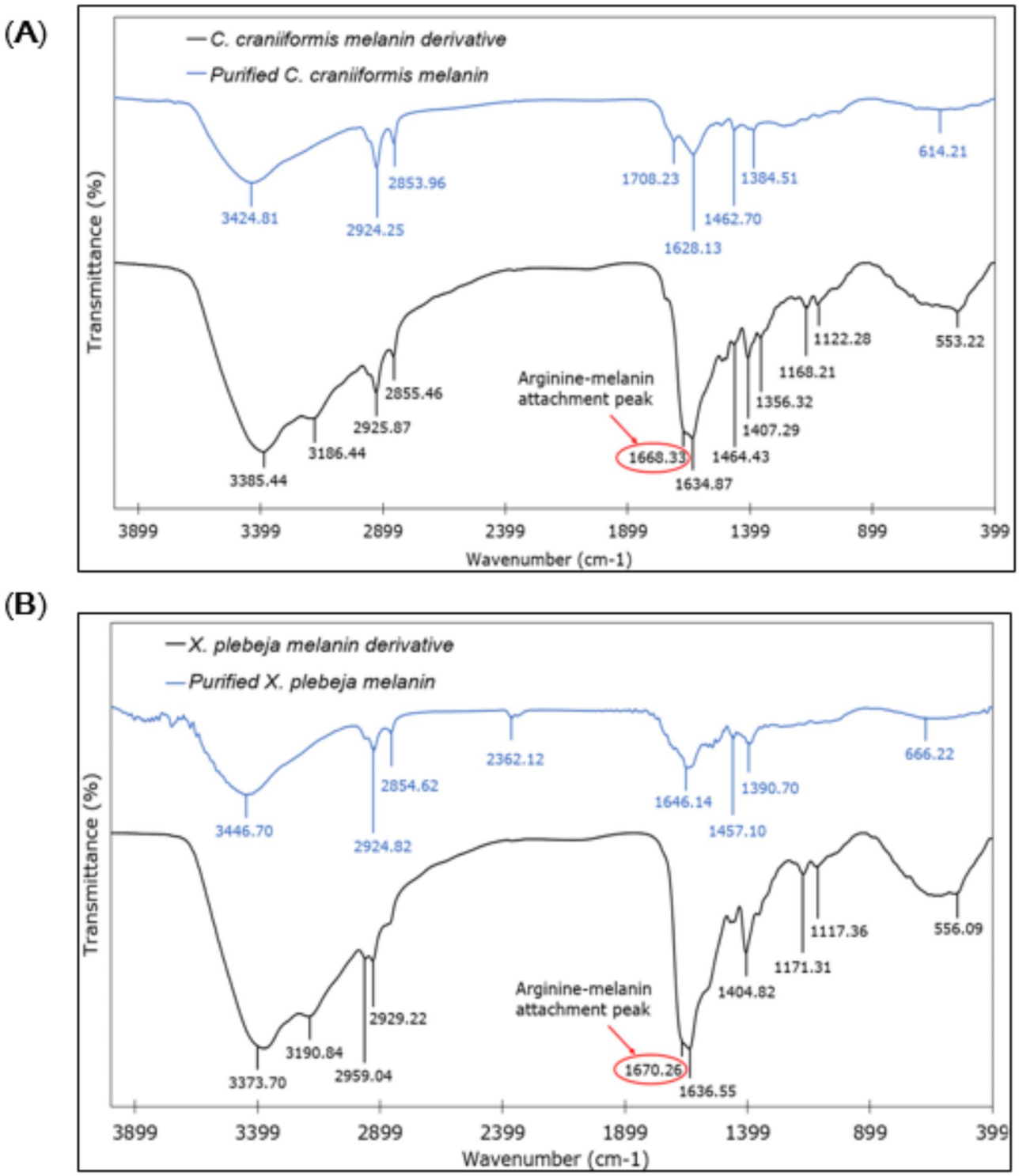

Fig. 2. FTIR spectra of purified melanin and derivative melanin from *C. craniiformis* (**A**), and *X. plebeja* (**B**).

many complex conjugated structures present in melanins[13]. Specifically, *C. craniiformis* melanin and *X. plebeja* melanin both showed strong absorbance values in the UV range with maximum absorption at 239 nm and 243 nm, respectively. These values are similar to those of melanins from other sources[3,20].

Another indicative feature used in the literature for melanin identification is a linear relationship between the log absorbance and the wavelength in the range of 400–800 nm[3]. According to Pralea et al. a straight line with a negative slope is obtained when plotting the absorbance of melanin solution against the wavelength in this visible light zone[3]. For fungal melanin, studies showed that the slopes ranged from − 0.0015 to -0.0030[3,20].

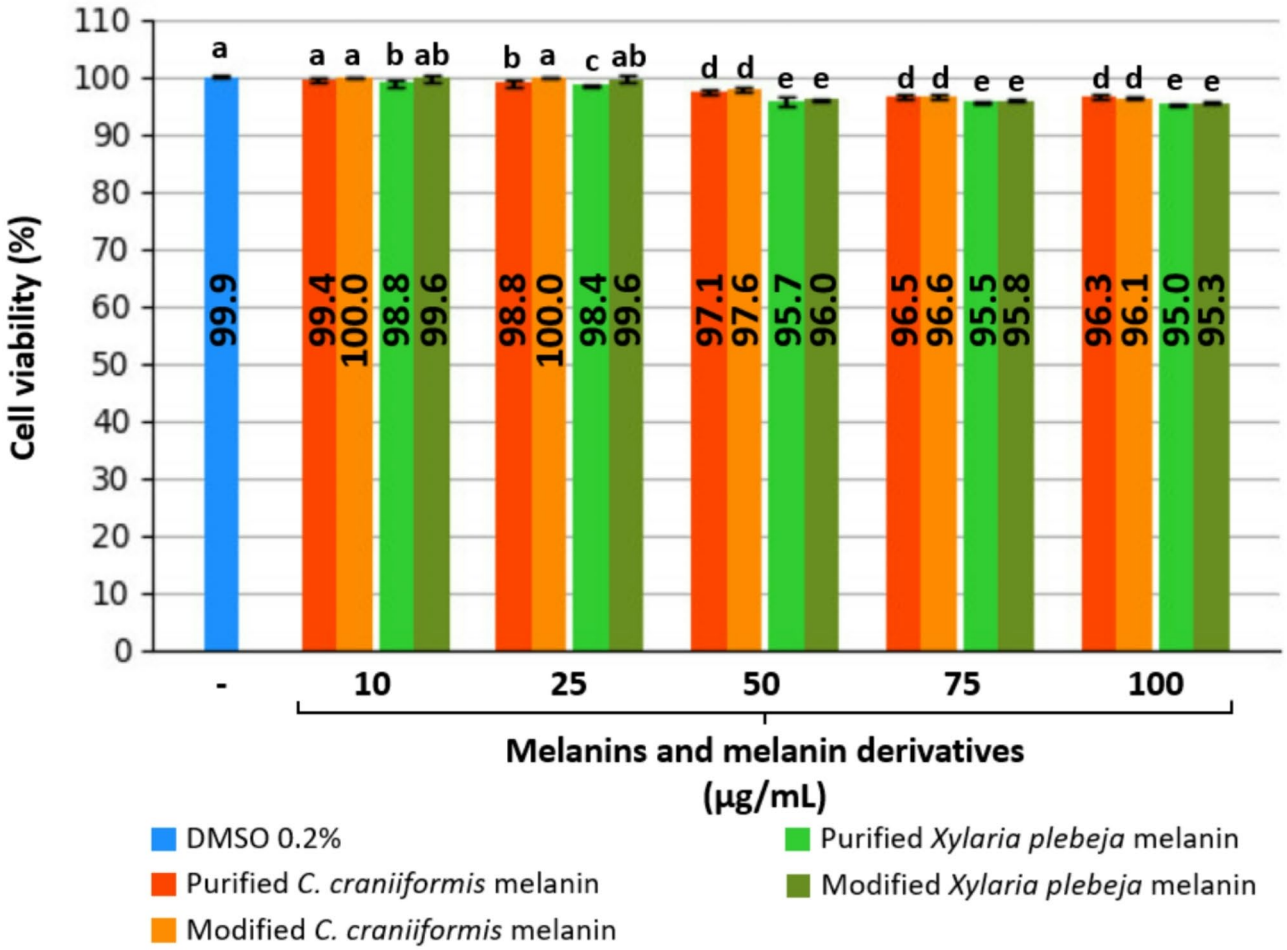

Fig. 3. SH-SY5Y neuroblastoma cell survival at different concentrations (10–100 µg/mL) of fungal melanins and melanin derivatives. Different letters indicate a significant difference *($\rho < 0.05$) (Dunnett's post hoc test).*

The melanin samples in this current study showed the same pattern, with a slope of -0.0024 for *C. craniiformis* melanin and −0.0020 for *X. plebeja*(Fig. 1B).

Apart from the UV-VIS characteristics, initial identification and assessment of melanin purity was also done via FTIR analysis. Generally, FTIR spectra of fungal melanin may exhibit slight variations depending on the type and the extraction method. However, specific characteristic bands can be used to identify some of the functional groups of the melanin macromolecule, such as the peaks observed at 3600–3000 cm^{-1} and 1650–1600 cm^{-1}. More specifically, the absorption band in the range of 3600–3000 cm^{-1} represents stretching vibrations of -OH and -NH groups related to amine, amide, carboxylic acid, phenolic, and aromatic amino functions[11,21]. Several melanin spectra also have absorption bands between 2950 and 2850 cm^{-1} and 1465–1375 cm^{-1}, caused by the stretching vibration of aliphatic C-H groups[11,22]. Moreover, the vibrations of aromatic C = C and C = O stretches of the carboxylic function are often ascribed to the strong, recognizable absorption band between 1650 and 1600 cm^{-1}[13,21,23].

The FTIR spectrum of purified melanin of *C. craniiformis* shows a strong absorbance peak near 3400 cm^{-1} (3424 cm^{-1}) indicating the stretching vibrations of –OH and –NH groups. Another strong absorbance peak, identified as stretching vibrations of C = O or C = C, is observed at 1628 cm^{-1}. The presence of these two characteristic absorbance peaks indicated that the extracted pigment is melanin[11,23]. In addition, the spectra of various melanins in previous studies also showed absorption peaks between 2950 and 2850 cm^{-1} and between 1465 and 1375 cm^{-1}, which were ascribed to the stretching vibration of aliphatic C-H groups[19,24]. The *C. craniiformis* purified melanin spectrum exhibits stretching vibrations of C–H in the saturated hydrocarbons around 2924 cm^{-1}, and a C–S stretching vibration is indicated by the peak at 614 cm^{-1}.

Compared to the purified melanin, the spectrum of the modified melanin of *C. craniiformis* displayed an additional absorbance peak at 1668 cm^{-1}. This is a characteristic absorption peak formed by the binding of the carboxyl group in melanin and the amino group in arginine[13], so this revealed the attachment of arginine to *C. craniiformis* melanin. On the other hand, the spectrum of the melanin derivative still exhibits two characteristic absorbance peaks for fungal melanin at 3385 cm^{-1} and 1634 cm^{-1}.

Like the purified melanin of *C. craniiformis*, the FTIR spectrum of the purified melanin from *X. plebeja* also shows two strong absorbance peaks that characterize fungal melanin: one peak at 3446 cm^{-1}, indicating

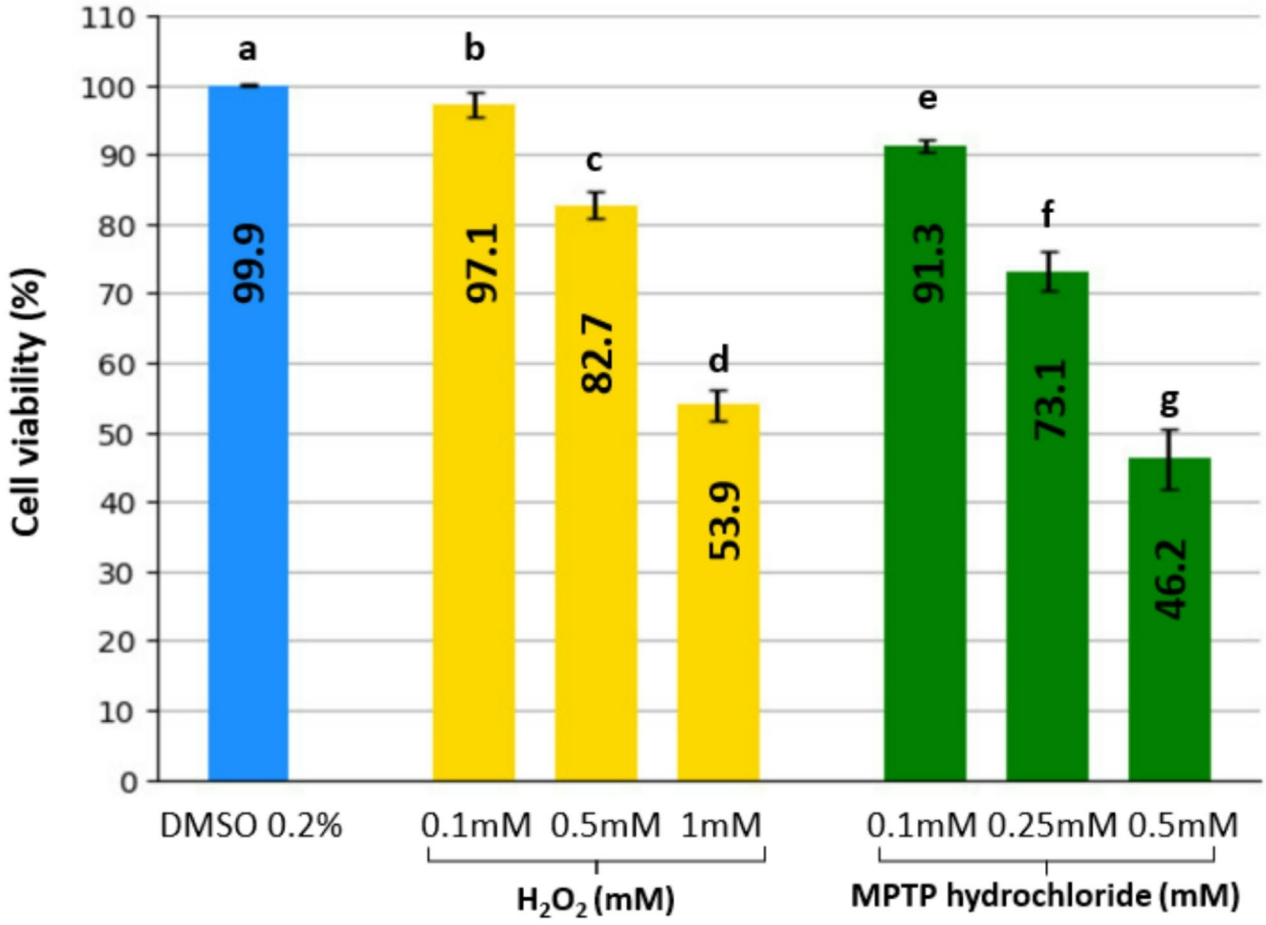

Fig. 4. SH-SY5Y human neuroblastoma cell survival at different concentrations of neurotoxins (H_2O_2 and MPTP). Different letters indicate a significant difference ($p < 0.05$) *(Dunnett's post hoc test)*.

the stretching vibrations of –OH and –NH groups, and another at 1646 cm^{-1} corresponding to the stretching vibrations of C = O or C = C. In addition, the *X. plebeja* purified melanin spectrum exhibits stretching vibrations of C–H in the saturated hydrocarbons around 2924 cm^{-1}, and C–S stretching vibrations are demonstrated by a peak at 666 cm^{-1}. Compared to *the X. plebeja* purified melanin spectrum, the FTIR spectrum of its modified melanin exhibits an additional absorbance peak at 1670 cm^{-1}, which is the characteristic absorption peak formed by binding of the carboxyl group in melanin with the amino group in arginine[13]. Thus, the modification by arginine of *X. plebeja* melanin was also successful. Besides, the modified melanin spectrum also displays two fungal melanin characteristic peaks including 3373 cm^{-1} and 1636 cm^{-1}.

It should be noted that the initial identification of melanin by color, the solubility test, the UV-VIS profile, and the FTIR analysis, confirms that the target pigment, extracted and purified by the methods recommended for melanin extraction and purification, is melanin. These analyses also provide an initial assessment of the sample's purity. However, to further evaluate melanin purity, additional methods such as composition analysis (e.g., HPLC) and structural analysis (e.g., ssNMR) should be employed to have a better assessment. In addition, electron paramagnetic resonance (EPR) spectroscopy would also be a valuable tool for understanding the structure, function, and potential properties of melanin as it provides unique insights into the free radical nature of melanin and its possible interactions with other molecules[1]. Because equipment was unavailable, and the primary aim of this study was to screen for potential sample(s) with neuroprotective activity for follow-up research, a full assessment of melanin purity using these additional methods including EPR was not conducted. This is a limitation of the study.

In our study, the neuroprotective testing was carried out on SH-SY5Y cells using 1 mM H_2O_2 and 0.5 mM MPTP-HCl as a neural oxidative stress inducer, since this concentration was found to result in roughly 50% cell viability (Fig. 4). In Li et al. incubating 100 µM H_2O_2 with SH-SY5Y cells for 24 h resulted in a reduction of cell viability to 60%[5]. However, in our study, a 10-fold higher concentration of H_2O_2 was required to reduce the neuroblastoma cell population to 54%. In Chetsawang et al. SH-SY5Y cells treated with 1 mM H_2O_2 decreased cell viability to 55% as in our study[22]. These differences probably originate from the difference in the quality of the cell lines, and/or the cell density tested, or even the origin of the chemicals utilized in the research.

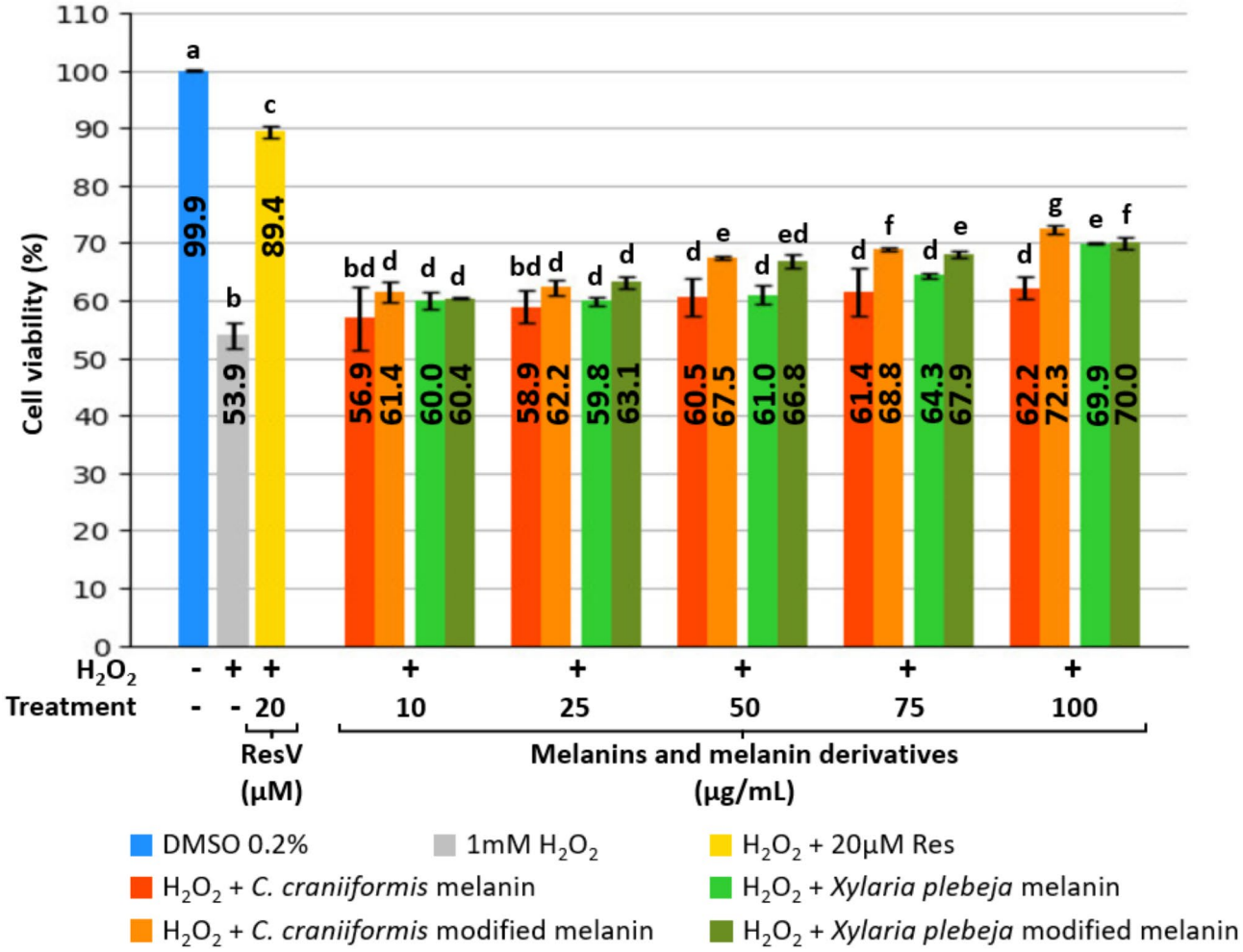

Fig. 5. SH-SY5Y neuroblastoma cell survival against H_2O_2 toxicity after 24 h exposure with and without fungal melanin and modified melanin pretreatment. Different letters indicate a significant difference *(Dunnett's post hoc test)*.

The viability of the SH-SY5Y cells after 1 mM H_2O_2 treatment was 54%, which was significantly lower ($p < 0.001$) (one-way ANOVA and Dunnett's post hoc) than the blank (100%) and the positive control (89%) (Fig. 5).

Both melanins and modified melanins displayed some level of cell protection effect at the tested concentrations (10–100 μg/mL) and the effect was dose-dependent (Fig. 5). Among the melanins of the two species, *X. plebeja* melanin seemed to have slightly higher protection activity than that of *C. craniiformis*, especially at high concentrations (75 and 100 μg/mL). In contrast, the reverse pattern is observed for the modified melanins. Specifically, the modified melanin of *C. craniiformis* displayed slightly better activities.

At the tested concentrations of 10, 25, 50, 75, and 100 μg/mL *C. craniiformis* melanin reduced H_2O_2-induced cell death of SH-SY5Y cells by 3.0%, 5.0%, 6.6%, 7.5% and 8.3%, respectively. ANOVA analysis revealed no significant difference in cell viability between the experimental group of *C. craniiformis* melanin treatment and the control group with melanin concentrations below 50 μg/mL. In the same concentration range, *X. plebeja* melanin increased the cell viability by 6.1%, 6.0%, 7.1%, 10.5%, and 16.0%, respectively. The ANOVA and Dunnett's post hoc test showed that the viability of cells pretreated with *X. plebeja* melanin at all tested concentrations were significantly different from those of the control group exposed to H_2O_2 without any melanin pretreatment ($p < 0.001$). Moreover, the pretreatment of SH-SY5Y cells with modified melanin of both *C. craniiformis* and *X. plebeja* resulted in a significant increase in the percentage of cells protected from H_2O_2-induced damage compared to their purified melanins ($p < 0.001$). As shown in Fig. 5, modified *C. craniiformis* melanin at concentrations of 10, 25, 50, 75, and 100 μg/mL enhanced SH-SY5Y cell viability with 7.5%, 8.6%, 13.6%, 14.9%, and 18.4%, respectively. In a similar manner, modified melanins of *X. plebeja* increased cell viability with 6.5%, 9.2%, 12.9%, 14.0%, and 16.1% at the same concentration range.

A previous study[5] reported that the enhanced cell viability of the H_2O_2-induced SH-SY5Y cell population was 1.6%, 20.3%, 36.2%, 37.3%, and 38.1% when the cells were pretreated with *Streptomyces* sp. melanins at 1, 5, 10, 30, and 50 μg/mL, respectively. It should be noted that in that research the neuroblastoma cells were exposed to 100 μM H_2O_2, which was 10-fold lower than the concentration used in our research (1 mM). As mentioned

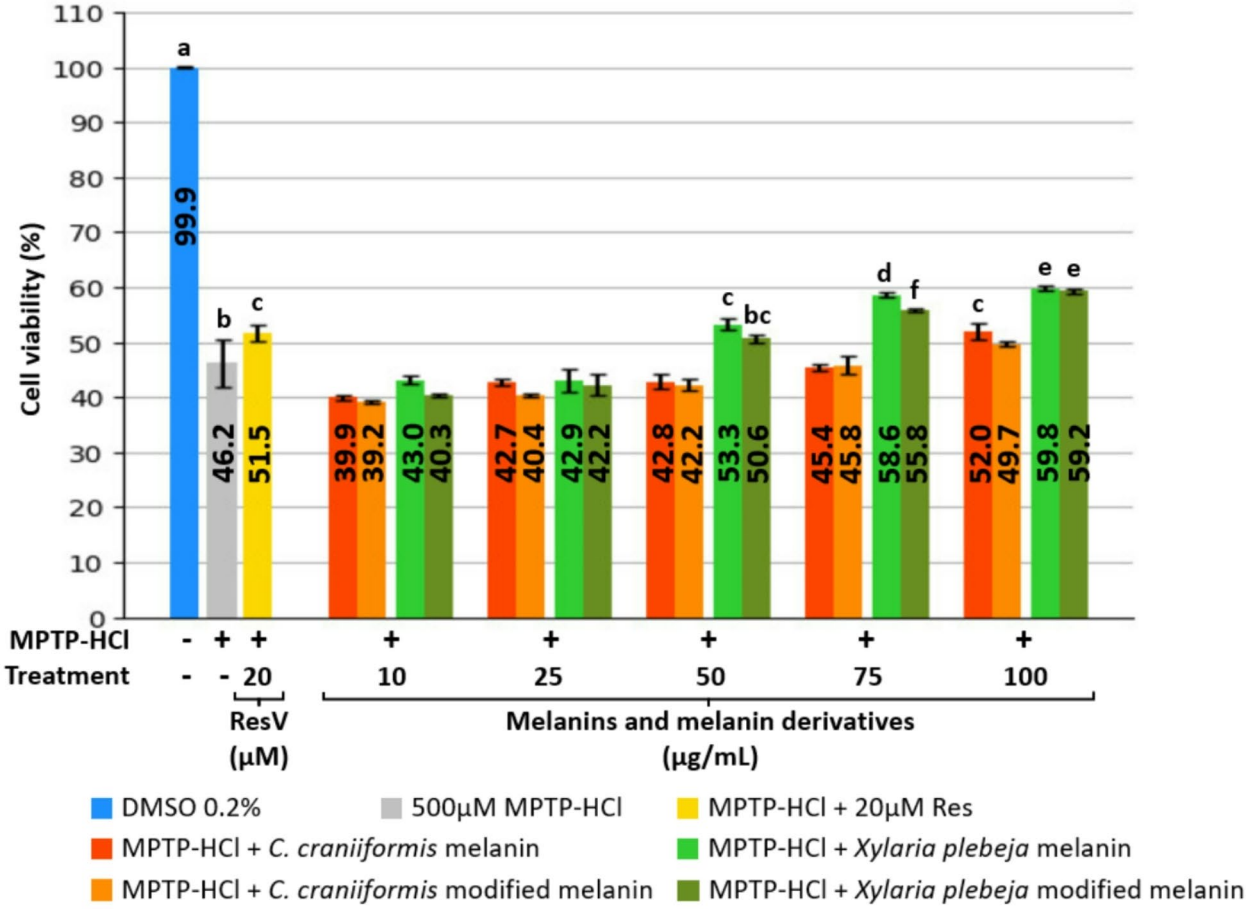

Fig. 6. SH-SY5Y neuroblastoma cell survival against MPTP hydrochloride cytotoxicity after 24 h exposure with and without fungal melanin and modified melanin pretreatment. Different letters indicate a significant difference *(Dunnett's post hoc test)*.

above, differences in the density and/or the quality of the used cell lines also could explain observed differences. In addition, the melanin used by Li et al. was purified by enzymatic treatments (for removal of proteins and nucleic acids) followed by further purification by column chromatography[5]. This may also explain the difference in the neuroprotective activities of the melanin samples. However, this also does not exclude the possibility that the difference in activities is simply because of the nature of the origin of the melanin.

The addition of 0.5 mM MPTP-HCl significantly reduced ($p < 0.001$) (Dunnett's post hoc test) the viability of SH-SY5Y cells to 46.2%. Although known to have a high antioxidant capacity that suppresses cell death, resveratrol at the tested concentration of 20 µM could not protect SH-SY5Y cells from MPTP-HCl as effectively as it did with H_2O_2. Specifically, 20 µM resveratrol protected only an additional 5.3% of cells from cell death caused by the neurotoxic MPTP-HCl (Fig. 6).

MPTP has lipophilic properties, it can cross the blood brain barrier and then be converted into MPP+. This substance then inhibits complex 1 of the mitochondrial respiratory chain in dopaminergic neurons, which leads to mitochondrial dysfunction and increased oxidative stress. This eventually resulted in cell death[7]. ROS (e.g., H_2O_2) on the other hand damages brain tissues via stimulating inflammatory responses and apoptosis and destroying neurovascular units[25]. Therefore, the ability of the melanins and modified melanins as well as the positive control to protect neuronal cells from these two toxins were different due to the difference in the mechanisms of the toxins.

Overall, *C. craniiformis* melanin at concentrations between 10 and 75 µg/mL had essentially no protective impact on the neuroblastoma cells when exposed to MPTP-HCl. Only at the concentration of 100 µg/mL, a significant protective effect was observed ($p < 0.001$) (Dunnett's post hoc test), increasing the cell viability with 5.8%. This is comparable with the effect of the positive control (5.3%) (Fig. 6). Thus, melanin of *C. craniiformis* at the tested concentration range of 10–75 µg/mL had no neuroprotective effect on SH-SY5Y neuroblastoma cells against the damage caused by MPTP hydrochloride. Regarding *X. plebeja* melanin, when applied at concentrations of 10–25 µg/mL, there was no significant difference in cell viability compared with the MPTP-HCl control group ($p = 0.074$ and 0.055, respectively) (Fig. 6). However, the neuroprotective activity of *X. plebeja* melanin against MPTP hydrochloride toxicity was clearly demonstrated at concentrations between 50 and 100 µg/mL ($p < 0.001$).

Xylaria plebeja melanin at 50, 75, and 100 µg/mL increased SH-SY5Y cell viability by 7.04%, 12.4%, and 13.5%, respectively (Fig. 6). These effects were statistically higher than the control group ($p < 0.001$).

The ability to protect cells against MPTP-HCl of *X. plebeja* melanin was comparable to that against H_2O_2, with cell viability improved by 7.1%, 10.5%, and 16.0% at concentrations of 50, 75, and 100 µg/mL (Fig. 6).

The pretreatment of SH-SY5Y neuroblastoma cells with the modified melanins from both *C. craniiformis* and *X. plebeja* did not result in higher protective activities than their purified forms. In contrast, their neuroprotection effects are even slightly reduced. The modified melanin of *C. craniiformis* exhibited no capacity to protect SH-SY5Y cell populations from MPTP-HCl neurotoxin at the tested concentrations till 100 µg/mL. The neuroprotective results of modified *X. plebeja* melanin against MPTP-HCl were somewhat similar. They did not exhibit any significant protective effects on SH-SY5Y cells against MPTP-HCl until they reached a concentration of 50 µg/mL, which slightly increased the cell survival (4.4%) ($p = 0.004$) while at 100 µg/mL, it significantly increased by 13% ($p < 0.001$).

We are aware of only one paper, which dealt with the evaluation of neuroprotective effects of melanin against MPTP in vivo. Berliner et al. reported that melanin (from beef eyes and bacteria) had high affinities toward MPTP. In addition, melanin extracted from *Streptococcus antibioticus* could aid the neuronal recovery in MPTP induced mice after five months of treatment[10]. The exact mechanism of how melanins protect cells from MPP^+ is still unknown, but an initially study revealed that melanins have strong affinity toward MPP^+. Recently, our group found that arginine modified melanin of *D. tricolor* and *F. fomentarius* significantly protected neuroblastoma cells from MPTP in vitro[18].

It is recommended that in addition to measuring the cell viability, and observing the cell morphology, some key biochemical parameters (such as ROS, and glutathione) should be measured, followed by pathways analysis before and after the treatment, in order to fully understand the mechanism(s) of how the melanins/arginine modified melanins protect cells from the neurotoxins (e.g., H_2O_2 and MPTP).

Conclusions

In this study, melanins were extracted from matured fruiting bodies of *C. craniiformis* and *X. plebeja*, as confirmed by UV-Vis spectroscopy and FTIR analysis. Furthermore, both melanins were successfully modified with arginine to increase their water solubility.

Both the purified melanins and arginine-melanins obtained from *C. craniiformis* and *X. plebeja* were proven to be harmless to neuroblastoma cells at concentration ranges from 10 to 100 µg/mL. In terms of their ability to protect neuroblastoma cells from H_2O_2, all concentrations displayed protective effects, which were dose dependent. More specifically, melanin from *X. plebeja* displayed significantly higher activities, especially at a concentration of 100 µg/mL, it improved SH-SY5Y cell viability by 16%. The modified melanin of *X. plebeja* displayed a similar activity pattern as its purified form. Interestingly, while *C. craniiformis* melanin showed mild cell protective effects against H_2O_2, the arginine-modified form of this melanin significantly enhanced the activity. Remarkably, at 100 µg/mL, it increased SH-SY5Y cell viability by 18.5%, whereas that of its original form was only 8.3%.

Regarding the ability of cell protection against MPTP-HCl, it is noticed that *C. craniiformis* melanin only displayed a slight effect (5.8%) at 100 µg/mL and the same observation was recorded for its arginine-modified form (3.4%). Melanin from *X. plebeja* showed a better protective activity, but the activity was negligible until its concentration reached 100 µg/mL (13%). Modification of the *X. plebeja* melanin resulted in similar cell viability increase at this concentration.

This study generated the first data set on the neuroprotective effects of melanin and arginine-melanins from *C. craniiformis* and *X. plebeja*. Arginine modification of the melanins significantly enhanced the neuroprotection against H_2O_2. However, when it came to cell protection against MPTP-HCl, the activities seemed to depend more on the nature of the fungal species.

Materials and methods

Fungal materials

Calvatia craniiformis was a generous gift from Dr. Edward Grand (School of Biological and Natural Sciences at Mahidol University, Thailand). The fruiting bodies of this species were collected and identified based on the morphological characteristics and their distribution. The *X. plebeja* fruiting bodies/stromata were collected in Buon Ma Thuot, Vietnam. The stromata were sent to Applied Biotechnology Institute for the molecular identification based on ITS sequencing using ITS1/ITS4 primers and the obtained sequence was compared with the available gene sequences in the GenBank (NCBI) using BLAST search.

Cell lines and cell culture

Human neuroblastoma (SH-SY5Y) cells were kindly provided by Dr. Bui Chi Bao (School of Medicine, Vietnam National University, HCM City, Vietnam). SH-SY5Y cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L- glutamine and 100 U/mL penicillin/streptomycin (Gibco) under a humidified atmosphere of 5% CO_2 at 37 °C.

Melanin extraction and purification

Melanin extraction and purification were performed as described by Selvakumar et al.[19]. Specifically, dried fungal samples were grounded into powder. An amount of 0.5 g fungal sample was submerged in 30–40 mL 1 M KOH and incubated for 48 h, followed by centrifugation (3000 g, 15 min) using a HERMLE Z 326 K refrigerated universal centrifuge (Hermle Labortechnik, Wehingen, Germany). The supernatant was collected and acidified by 2 M HCl to pH 2.5 for melanin precipitation. The mixture was incubated for 2 h, and centrifuged at 3000 g

for 15 min, after which the pellet was collected and washed three times with distilled water. Subsequently, the pellet was freeze-dried using a FreeZone 2.5 L Benchtop Freeze Dry System Labconco (Kansas, Missouri, United States).

The dry crude melanin was purified by an acidic hydrolysis with 7 M HCl at 100ºC. After 2 h, the sample was centrifuged at 12,000 g for 20 min. The pellet was collected and washed with 0.01 M HCl, chloroform, absolute ethanol and distilled water. The purified melanin was then freeze-dried and stored at 4ºC until further use.

UV-VIS spectroscopic characterization

Suspensions of the purified melanins of *X. plebeja* and *C. craniiformis* were prepared in a concentration of 62.5 µg/mL using 0.1 M NaOH as solvent. The solvent was also used as a blank for this test. The absorbance of the prepared samples was scanned in the range of 200–800 nm with intervals of 1 nm using a Biotek Synergy HT Microplate Reader (Winooski, USA)[26].

Melanin modification

The purified melanin of each fungal species was modified by L-arginine (Acros Organics, Geel, Belgium) following the method of Xu et al. (2020)[27]. Melanins of both fungal species were modified by arginine in a mass ratio of 1.0:1.0. An amount of 0.1 g L-arginine was dissolved in 10 mL distilled water, then 0.1 g purified melanin was added. The mixture was incubated at 37ºC for 45 min and centrifuged for 10 min at 7600 g. The supernatant was collected, freeze-dried, and stored at 4ºC until further use.

Fourier-transform infrared spectroscopic (FTIR) analysis

FTIR analysis was applied to confirm the identity of melanins, and the attachment of arginine molecule(s) to the melanin in the modified melanins. An amount of 4 mg of purified melanins or melanin derivatives was ground with KBr in the ratio of 1:10 and pressed into a tablet under vacuum using a Spectra Lab Pelletiser Spectra Laboratories (Milpitas, California, United States). Tablets were then analyzed by a Tensor 27 FTIR spectrometer Bruker (Massachusetts, Middlesex, United States) within the wave number range 4000 –400 cm^{-1}, and KBr without sample was used as background spectrum[13].

Evaluating possible cytotoxicity of melanins and melanin derivatives

SH-SY5Y cells were seeded in white-walled 96-well plates at 1×10^5 cells/mL. The melanin and melanin derivative samples were prepared in 0.2% DMSO at different concentrations of 10, 25, 50, 75, and 100 µg/mL. Upon reaching approximately 80% confluence, cells were pretreated with the prepared melanin and melanin derivative extracts to determine the cytotoxicity effects on neuroblastoma cellular viability. After 24 h, the cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay[28].

Evaluating cytotoxicity of H2O2 and MPTP

SH-SY5Y cells were prepared as described above and then they were treated with H_2O_2 (0.1, 0.5, and 1 mM) or MPTP hydrochloride (0.1, 0.25, and 0.5 mM). After 24 h, the cell viability was also evaluated via the MTT assay[28]. The concentrations of H_2O_2 and MPTP that inhibited about 50% of the cell population were selected for the neuroprotection assay below.

Neuroprotective effect analysis

The neuroprotective effect of fungal melanin extracts and arginine-melanins was assessed using the methods previously described by Johnson et al.[29] and Li et al.[5].

SH-SY5Y cells prepared as above were pretreated for 2 h with either melanin or modified melanin (10, 25, 50, 75, and 100 µg/mL), 20 µM resveratrol (Resv, positive control), or 0.2% DMSO (blank/negative control). Then, cellular oxidative stress was induced in SH-SY5Y cells with 1 mM H_2O_2 or 0.5 mM MPTP for 24 h at 37 °C. The cellular viability of SH-SY5Y cells after treatment was determined after 24 h by the MTT assay.

Statistical analysis

To investigate the differences between different groups, the IBM SPSS Statistics software (IBM, Armonk, New York, United States) was utilized. All analyzed data are presented as mean ± standard deviation of three independent experimental replicates. Statistical significance was determined by using one-way ANOVA and Dunnett's post hoc test.

Data availability

The main data are given in the manuscript and supplementary figures. Additional data can be shared upon request via the corresponding authors.

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Author contributions

D.T.N. (Methodology, Investigation, Data curation, Original draft preparation), L.P.N.V. (Methodology, Investigation, Data curation, Original draft preparation), D.M. (Resources, Editing), A.V.E. (Conceptualization, Funding acquisition, Editing), Y.V.H. (Conceptualization, Funding acquisition, Editing), H.T.M.T (Conceptualization, Funding acquisition, Editing).

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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