Original Article

Microbial Biosystems 10(1) (2025) 2025.357914
10.21608/mb.2025.352914.1229

Contents lists available at Egyptian Knowledge Bank
Microbial Biosystems
Journal homepage: http://mb.journals.ekb.eg/

Exploring the antioxidant and cytotoxicity perspective of melanin pigment produced by fungi associated with marine macroalgae, Fayed, Suez Canal, Egypt

Reda I. Mansour, Gihan A. El Shoubaky, Eman A. Attia*

Botany and Microbiology Department, Faculty of Science, Suez Canal University, Ismailia 41522, Egypt.

ARTICLE INFO

Article history
Received 14 January 2025
Received revised 15 February 2025
Accepted 16 February 2025
Available online 1 March 2025

Corresponding Editors
Kadhim AL-Ziadi, S. A.
Hashim, H. R.
Hajji-Hedfi, L.

Keywords
Bioactive compounds,
biomedical applications,
free radical scavenging,
marine fungi,
natural pigments,
oxidative stress.

ABSTRACT

This study investigated the physicochemical properties, antioxidant, and antitumor activities of melanin extracted from fungi associated with abundant marine macroalgae collected from Fayed, Suez Canal, Egypt during winter 2023. The epiphytic fungi included *Aspergillus niger*, *Aspergillus flavus*, *Alternaria alternata*, *Cladosporium* sp., and *Curvularia chlamydospora*. that are associated with the red algae *Laurencia obtusa* and *Gracilaria verrucosa*, as well as the green algae *Cladophora crinalis*, *Enteromorpha compressa*, and *Ulva lactuca*. Melanin exhibited a characteristic UV-Vis absorption peak at 235 mm. IR spectra of fungal melanin showed peaks at 3378 cm-1 and 3308 cm-1 (carboxyl, phenolic, hydroxyl, amine), 1720/1705 cm-1 (C=O), 1646/1610 cm-1 (C=C), and 1046/1040 cm-1 (C-O), indicating the presence of various functional groups. All fungal melanin pigments demonstrated significant antioxidant activity in a dose-dependent manner in both DPPH and ABTS assays. *Aspergillus* niger and *Curvularia chlamydospore* showed 50% activity at IC50 values of 12.2 and 13.48 µg/ml in the DPPH assay, and 15.47 and 25.87 µg/ml in the ABTS assay respectively. In vitro, cytotoxicity assays revealed that melanin exhibited differing levels of cytotoxicity against A549 lung cancer cells while showing negligible toxicity to normal cells at varying doses from 31.25 to 1000 µg/ml., significantly reducing tumor cell viability in a dose-dependent mode. *Alternaria alternata* demonstrated the highest cytotoxicity at low concentrations, signifying its potential as a promising anticancer agent. Our findings showed that fungi associated with macroalgae represented a novel source of melanin. This natural pigment exhibits significant potential for various applications, providing a strong theoretical foundation for its utilization.

Published by Arab Society for Fungal Conservation

Introduction

Melanin is a hydrophobic pigment biopolymer produced through the oxidative polymerization of phenolic or indolic chemicals, offering numerous potential applications, particularly in the biomedical field. Although melanin has been extensively studied in humans, animals, and plants, its potential in marine species, especially fungi associated with marine macroalgae, remains relatively unexploited. Microorganisms have evolved sophisticated protective mechanisms to survive like pH fluctuations, temperature variations, high salinity, radiation, and host immune responses. One method is melanization, a biological process involving the synthesis of melanin pigments. Melanin protects against several environmental stresses, such as oxidative stress, ultraviolet radiation, and antimicrobial substances. Microorganisms can enhance

***Corresponding author Email address*: eman_mohamed@science.suez.edu.eg (Eman A. Attia)**

their survival in adverse settings by producing melanin (Belozerskaya et al 2017, Muñoz-Torres et al. 2024).

Fungi demonstrated all the variety of melanin found in nature: eumelanin (black or dark brown), pheomelanin (yellow or red), and the most heterogeneous group of allomelanins, including soluble piomelanins and melanins formed from dihydroxynaphthalene (DHN) compounds (Plonka & Grabacka 2006). Numerous studies have investigated melanin extraction from various fungi genera, such as *Aspergillus, Exophiala, Gliocephalotrichum, Hortea, Inonotus, Penicillium, Phoma, Phyllosticta, Pleurotus, Schizophyllum, Spissiomyces* and *Alternaria* (Elsayis et al.2022, Surendirakumar et al. 2022). This pigment can be localized in different parts of the fungal cell, including the cell wall and spores.

Marine macroalgae are vital in coastal intertidal zones, functioning as critical habitats and resource suppliers for many microbial communities (Manzelat et al. 2018, Korlević et al.. 2021). The macroalgae offer habitats, oxygen, and carbohydrates such as algal polysaccharides to related bacteria (Yang et al. 2019). This spatial closeness promotes intercellular interactions and establishes intricate, diverse microbial communities.

Microorganisms, including bacteria and fungi, establish dynamic associations with macrophytes (Salgado-Castillo et al. 2023). These microbial communities provide a range of beneficial substances, including hormones, vitamins, minerals, carbon dioxide, and novel bioactive metabolites. These resources support the development of macrophytes, growth, and ability to defend against pathogens. Macroalgae and fungi establish symbiotic associations. The macroalgae generate organic matter (food) and oxygen via photosynthesis, which are utilized by the fungi. Furthermore, related microorganisms facilitate the provision of CO2 and minerals. Seaweeds have adapted to flourish in diverse marine ecosystems, ranging from coastal tidal pools to deep-sea conditions. Diverse environments enable seaweeds to obtain adequate sunlight for photosynthesis, even at considerable depths (Fawcett et al. 2017).

Microbial pigments have gained interest due to increasing demand in the market for safer, easily degradable, and eco-friendly goods that do not have adverse consequences (Lopes et al. 2013). Pigment production from microorganisms is considered more advantageous because it is a more efficient and cost-effective process than the chemical synthesis of pigments. Melanin is widely utilized in the derma-cosmetic sector for its UV-protective attributes, which are often employed in the manufacturing of sunscreen and related products (Choi 2021, El-Naggar & Saber, 2022). Moreover, melanin has exhibited remarkable biological functions, such as anti-cancer, antibacterial, anti-inflammatory, and hepatoprotective capabilities (El-Naggar & El-Ewasy, 2017, Ghadge *et al*. 2021, Surendirakumar *et al.* 2022). Melanin, with its inherent antioxidant and radical scavenging properties, has emerged as an optimistic candidate for combating oxidative stress-related diseases. Additionally, its ability to absorb UV radiation makes it a potential sunscreen agent. Moreover, recent studies have highlighted the antitumor potential of melanin, suggesting its role in inhibiting cell proliferation and inducing apoptosis in cancer cells.

Suez Canal, a vital waterway connecting the Mediterranean and Red Seas, offers a unique marine ecosystem with diverse microbial communities. Fungi inhabiting this environment are exposed to many stressors, including fluctuating salinity, temperature, and intense UV radiation (Suthar et al. 2023). To survive and thrive in such harsh conditions, fungi often produce secondary metabolites, such as melanin, which give protective benefits. This study aims to provide a valuable approach to elucidate the potential of melanin produced by marine fungi associated with abundant macroalgae collected from Fayed area, center of Suez Canal, as a natural source of antioxidants and antitumor agents.

Materials and Methods

Area of study

Macroalgal samples were collected from the Fayed area in Ismailia Governorate, Egypt during winter of 2023. This area is approximately situated at the center of Suez Canal. The geographic coordinates of the collection site were Located between two latitude (30.3261° N) and longitude (32.2986°E) lines.

Collection of macroalgae

Five abundant marine macroalgal species were manually collected from the intertidal zone at the low tide during winter 2023. The samples collected included three species of green macroalgae and two species of red macroalgae. The identification of the chosen macroalgae was conducted using the taxonomic keys of Gribb 1983, Aleem 1984 ,Womersley 1984 , 1987.

Isolation and characterization of epiphytic fungus

One centimeter of the selected macroalgae were segmented to isolate the epiphytic fungi, then cultured on potato dextrose agar (PDA) medium supplemented with 0.01% w/v chloramphenicol. The plates were incubated at 28 ± 1°C for 7 days and assessed for fungal growth every 48 hours. Upon observation of fungal development, the cultures were transformed to fresh PDA for continued growing (Karkun Sur & Verma. 2016). Isolated fungi were identified at the genus level according to their macro- and

micro-morphological characteristics. These included colony color, texture, pigment production, as well as microscopic features like hyphae, conidia, stipes, and phialides. Standard mycological and taxonomic keys were used for classification (Ann et al. 2019).

Propagation of fungal mycelium

Fungi were cultured in a sterile potato dextrose broth (PDB) medium. Five-millimeter fragments of fungal material were added to Erlenmeyer flasks containing broth and kept at a regulated temperature of 28 ± 1°C for three weeks. Throughout this interval, the broth became darker as a result of pigment synthesis by the fungi. After incubation, the fungi were extricated from the broth using filtration.

Extraction and purification of melanin

An acid-alkali technique was employed to extract melanin pigment from fungal biomass. The desiccated fungal material was subjected to treatment with a potassium hydroxide solution and subsequently autoclaved. The resultant liquid was subjected to filtration, acidification, and centrifugation to precipitate the melanin. The precipitated melanin underwent additional purification via solvent extraction and subsequent drying. The melanin powder was ultimately dissolved in a potassium hydroxide solution for further analysis. This multi-step procedure successfully extracted and purified melanin from the fungal source Rajagopal et al. 2011.

Characterization of melanin

Physiochemical properties

The solubility of the extracted melanin pigment was determined by using several solvents including distilled water (both hot and cold), ethanol, acetone, methanol, chloroform, hexane, ethyl acetate, DMSO (dimethyl sulfoxide), acetic acid, and petroleum ether. The solubility of the substance in 1 M hydrochloric acid and the precipitation behavior of the substance with 1% (w/v) FeCl2 were also evaluated. For comparison, L-DOPA melanin was used as a standard (Zaidi et al. 2014).

UV-Vis spectrophotometric analysis of fungal melanin

Fungal melanin was analyzed using a UV-visible spectrophotometer (T90+UV/VIS Spectrophotometer) to determine its absorption spectrum within the 200-420 nm range. A standard L-DOPA melanin was used as a reference. The method for dissolving fungal melanin (1 mg) in 10% 1M KOH (20 mL) was adapted from (Rajagopal et al.2011). A 10% 1M KOH solution served as the reference blank. The spectrophotometric analysis was carried out at the Central Laboratory and Toxicology Research Unit of Suez Canal University.

Fourier transform infrared (FTIR) spectroscopy

Purified samples were combined with potassium bromide and pressed into discs following the methodology outlined by (Singh et al. 2021) to ascertain the chemical composition of fungi and L-DOPA melanin. Fourier Transform Infrared (FTIR) spectra were obtained for these discs within the 400-4,000 cm^{-1} range by analyzing them with a BRUKER ALPHA 11 spectrometer at a resolution of 4 cm^{-1}. the FTIR analysis conducted at the Central Laboratory and Toxicology Research Unit of Suez Canal University.

Evaluation of antioxidant activity

The antioxidant capacity of melanin was assessed by various *In vitro* assays, including DPPH radical scavenging and ABTS radical cation decolorization. The free radical scavenging activity of various extracts was assessed utilizing the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay. It was utilized for lipophilic antioxidants. A 0.1 mM solution of DPPH in ethanol was prepared. One milliliter of this solution was incorporated into 3 mL of distinct ethanol extracts at varying concentrations (3.9, 7.8, 15.62, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL). Only extracts soluble in ethanol were utilized, and their quantities were modified through dilution. The mixture was agitated vigorously and permitted to rest at ambient temperature for 30 minutes. Absorbance was subsequently quantified at 517 nm utilizing a UV-Vis spectrophotometer (Milton Roy). Ascorbic acid served as the reference standard, and each experiment was conducted in triplicate. The IC_{50} value, indicating the concentration necessary to inhibit 50% of DPPH radicals, was derived from a logarithmic dose-inhibition curve (Fu et al. 2022).. A diminished absorbance of the reaction mixture signified enhanced free radical scavenging activity. The percentage of DPPH scavenging effect was determined using the subsequent formula:

$$\% \text{ inhibition} = (A0 - A1) / A0 \times 100.$$

Where, A0 represented the absorbance of the control reaction, while A1 denoted the absorbance in the presence of a test or standard sample.

ABTS radical scavenging activity

This method can be used for both hydrophilic and lipophilic antioxidants. The ABTS radical scavenging activity was determined following the method of (Re et al.1999) with minor modifications. A 7mM solution of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was reacted with 2.45 mM potassium persulfate to generate the ABTS radical cation (ABTS•+). This solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm. To assess antioxidant activity, 0.07 mL of the extract was mixed with 3 mL of the diluted ABTS solution. After a 6-minute incubation period, the absorbance at 734 nm was

measured. The percentage inhibition of ABTS•+ was calculated using the following formula:

$$\% \text{ inhibition} = (A_{control} - A_{sample}) / A_{control} \times 100$$

Where, $A_{control}$ = Absorbance of negative control at the moment of solution preparation, A_{sample} = Absorbance of sample after 6min

The IC_{50} values were calculated from the graph which represents the concentration of the sample required to scavenge 50% of the ABTS free radicals. The IC_{50} is often used to express the amount or concentration of extracts needed to scavenge 50% of the free radicals. ABTS are expressed as ug GAE/ml (Ma et al. 2023).

Assessment of cytotoxicity

The activity of melanin was evaluated against human lung cancer cells (A549 cells) using the MTT assay to determine cytotoxicity (Cao et al.2020). A 96-well plate was seeded with 1x10^5 cells/ml and incubated for 24 hours to form a monolayer. After removing the growth medium and washing the cells, two-fold dilutions of the melanin sample were prepared in maintenance medium. These dilutions were added to the wells, with control wells receiving only maintenance medium. The plate was incubated at 37°C, and cells were visually inspected for signs of toxicity. Subsequently, MTT solution was added to each well, and the plate was incubated for 4 hours to allow for MTT metabolism. The media was then removed, and the formazan crystals were dissolved in DMSO (Migliorini et al. 2019). The optical density was measured at 560nm, with background subtraction at 620 nm. A higher optical density indicates a greater number of viable cells, thus providing a measure of the antitumor activity of melanin.

Statistical analysis

A bar chart and boxplot for data analysis were generated using PAST: Paleontological Statistics (Version 2.17) with the PAST Model Linear (Hammer & Harper. 2001).

Results and Discussion

Melanin is indispensable for the growth and development of fungi. It is classified as a secondary metabolite that is generated as a survival mechanism in conditions of extreme salinity, temperature, pH, or radiation exposure (Salgado-Castillo et al. 2023). Melanin's potential applications in a variety of disciplines, such as medicine, environmental remediation, and industrial processes, are largely due to its distinctive physicochemical properties and biological functions (ElObeid et al. 2017).

Isolation and identification of epiphytic fungi

In this study, five fungal species were isolated from the selected abundant macroalgae in Fayed, Suez Canal, Egypt (Table, 1) to reveal the relationship between selected macroalgae and fungal species. Some algae, like *Laurencia obtusa*, *Gracilaria verrucosa*, and *Cladophora crinalis* exhibited a higher range of fungal associations, while others, such as *Enteromorpha compressa* and *Ulva Lactuca*, have more specific relationships. Fungi such as *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria alternata* are associated with multiple algae, while *Cladosporium* sp., appeared more selective. These associations could represent symbiotic, parasitic, or commensal relationships, potentially influenced by environmental factors, developmental stages, and the specific ecological roles of the organisms involved.

Figure 1 shows the presence value of fungi that are linked to the chosen species of abundant macroalgae. The error bars on each bar indicated the variability in appearance values. The appearance value reflected the frequency or abundance of specific fungi taxa associated with each macroalga. Some types of green macroalgae, like *Ulva lactuca* and *Enteromorpha compressa*, had more variation in their fungal associations. This was shown by error bars that were bigger. This could be due to factors like environmental conditions, geographic location, or the specific strain of the macroalga. The presence of some fungi can influence various ecological processes, including nutrient cycling, decomposition, and disease resistance. Different macroalgal species appeared to harbor distinct fungal communities.

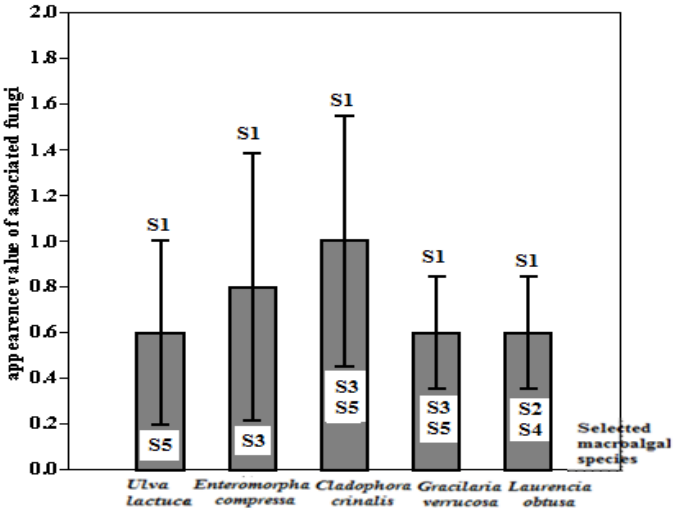

Fig. 1. The presence value of fungi with the selected abundant macroalgal species shows a standard deviation, where S1: *Aspergillus niger*; S2: *Curvularia chlamydospore*; S3: *Aspergillus flavus*; S4: *Cladosporium sp*. and S5: *Alternaria alternata.*

Table 1 Presence of fungi associated with the abundant macroalgal species

	Rhodophyta		Chlorophyta		
Fungal taxa	*Laurencia obtusa*	*Gracilaria verrucosa*	*Cladophora crinalis*	*Enteromorpha compressa*	*Ulva lactuca*
Aspergillus niger	+	+	+	+	+
Aspergillus flavus	+	-	+	+	-
Alternaria alternata	+	-	+	-	+
Cladosporium sp.	-	+	-	-	-
Curvularia chlamydospore	-	+	-	-	-

For instance, the green macroalga *Cladophora crinalis* was seen to have a higher appearance value of associated fungi compared to other species. This displayed that specific macroalgal species may create unique ecological niches that favor certain fungi taxa. In contrast, red algae like *Gracilaria verrucosa* and *Laurencia obtusa* exhibited a lower fungal association. The presence and abundance of specific fungi associated with macroalgae can have significant ecological implications. Fungi can play various roles, such as decomposing organic matter, producing secondary metabolites, or forming symbiotic relationships with the macroalgae. These interactions can influence nutrient cycling, energy flow, and the overall health of marine ecosystems.

Al-Ameen et al. (2024) mentioned that the emergence of free-living fungal species and the presence of fungal colonies in the aquatic environment encompasses a variety of invertebrate organisms, algae, and aquatic plants. This discovery is in accordance with the findings of Bärlocher et al. (2008), who identified a substantial number of fungal species in water samples. These species originated from temporary sources, resulting from air currents bringing fungal spores to the water surface, or from habitats within the water. Furthermore, their spores were dispersed when they matured in water, and they may have been introduced or adhered to plants and animals in the water. This is also in accordance with the results of (Amend et al. 2019).

Fungi are significant biological components of terrestrial ecosystems and are one of the most diverse and expansive kingdoms of eukaryotes (Bridge & Spooner 2012). They reported that there are numerous marine fungi records from Antarctica, but there was limited information available regarding their ecological functions. In the current investigation, certain fungal taxa were identified as species that are mutualists, saprobes, and parasites and were found to be associated with certain macroalgae.

Characterization of melanin

Physiochemical properties

In the present investigation, all fungal melanin pigments precipitate upon the addition of 1% FeCl3, similar to the DOPA melanin standard, so confirming that the extracted pigment is melanin. The solubility results for the extracted melanin indicated that it exhibits relatively low solubility in polar solvents, including water, methanol, acetone, ethanol, ethyl acetate, chloroform, petroleum ether, 1 mol/L HCl, and 1 mol/L NaCl, while demonstrating comparatively high solubility in alkaline conditions such as NaOH and KOH. They have partial solubility in dimethyl sulfoxide (DMSO). These findings were corroborated by (El-Naggar & El-Ewasy 2017, Kamarudheen *et al.* 2019, Elsayis *et al.* 2022).

Spectrophotometric UV analysis of melanin

UV-Vis spectroscopy offers qualitative insights into the presence of chromophores in extracted melanin, yet it cannot deliver detailed structural information. The UV-Vis spectra validated the presence of melanin extract in all five fungal samples (Fig. 2). All five tested fungal extract spectra exhibited broad absorption bands in the UV-Vis region (200-280 nm), analogous to the range of standard melanin pigments (DOPA). The variations in peak positions and intensities among the samples suggest differences in the chemical composition and structure of the melanin, as noted by (Yang et al. 2023).

The absorption spectrum of *Cladosporium sp, Aspergillus niger, Curvularia chlamydospore, Aspergillus flavus,* and *Alternaria alternata* exhibited broad absorption bands, with maximum absorbance values of approximately 229, 235, 231, 232, and 228 nm respectively. The variation in absorbance among the tested fungal extracts may be ascribed to the presence of additional chromophores or distinct structural characteristics in the melanin. Our findings aligned with those reported by Lorquin et al. (2021). Additionally, refer to Fuentes-López et al. (2022) who demonstrated that eumelanin exhibits a maximum absorbance peak at 220 nm. Moreover, Pyo et al. (2016) displayed that pheomelanin shows greater absorption

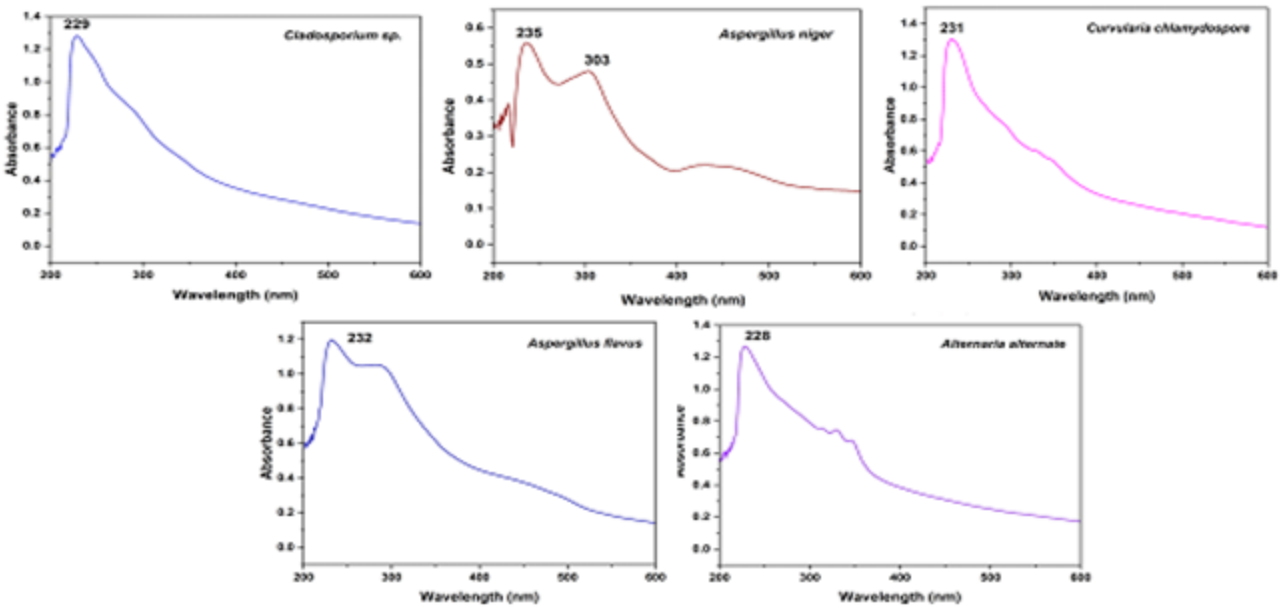

Fig 2. Spectrophotometric UV Analysis of the tested fungal melanin.

across the full spectrum, particularly in the visible region, with the highest absorption wavelength occurring near 233 nm.

The observed bands of the tested fungal melanin were attributable to the π-π* transitions of the aromatic rings within the melanin structure. All five samples exhibited comparable broad absorption bands in the UV-Vis region, indicating the presence of melanin with a similar chromophore system. Nevertheless, there are notable differences in the peak positions and intensities, signifying variations in the degree of conjugation and other structural characteristics of the melanin. Cosmetic companies have used melanin in cream formulations and sunscreens because melanin has high UV light absorption and antioxidant activity as reported by Oh et al. (2021).

Fourier Transform Infrared (FTIR) Spectroscopy

In the current study, all five analyzed fungal extract spectra exhibited distinct peaks linked to melanin, confirming the existence of this pigment in each sample as shown in figure 3. The peak positions and intensities varied among the examined samples, indicating variations in the chemical composition and structure of the melanin. The peaks in the 3300-3400 cm^{-1} region are characteristic of O-H stretching vibrations, indicating the presence of hydroxyl groups in the melanin structure. All five samples confirmed similar peak patterns, indicating the presence of melanin with a similar chemical structure. However, minor differences in the peak positions and intensities indicated to variances in the extent of oxidation and other structural characteristics of the melanin.

The spectral analysis of various fungal melanin samples revealed that *Cladosporium* sp, *Curvularia chlamydospore*, *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria alternata* exhibited significant peaks at 3378, 3344, 3308, 3322 and 3320cm-1 respectively, corresponding to the stretching vibrations of carboxyl, phenolic, free hydroxyl, and amine groups. Additionally, they displayed wavelengths at 1720 cm-1 (for carboxyl C=O stretching), 1631 cm-1, 1646 cm-1, 1638 cm-1, 1610 cm-1, and 1619 cm-1 (for C=C stretching), as well as 1044 cm-1, 1046 cm-1, 1042 cm-1, 1042 cm-1, and 1040 cm-1 (for C-O stretching), indicating the presence of ether linkages.

Ammanagi et al. (2021), Song et al. (2021) corroborated the previous findings, indicating that Eumelanin exhibited three characteristic absorption peaks: a broad absorption range at 3500–3000 cm-1, associated with the stretching vibrations of carboxyl groups, phenols, free OH, and –NH groups, in addition to intermolecular hydrogen bonding. The C==C stretching of aromatic groups occurs at 1650–1500 cm-1. The C==O stretch in the carboxyl group has a distinct peak at approximately 1710 cm-1. The findings correspond with (Swann et al. 2011). Who established that the peak near 1040 cm-1 is indicative

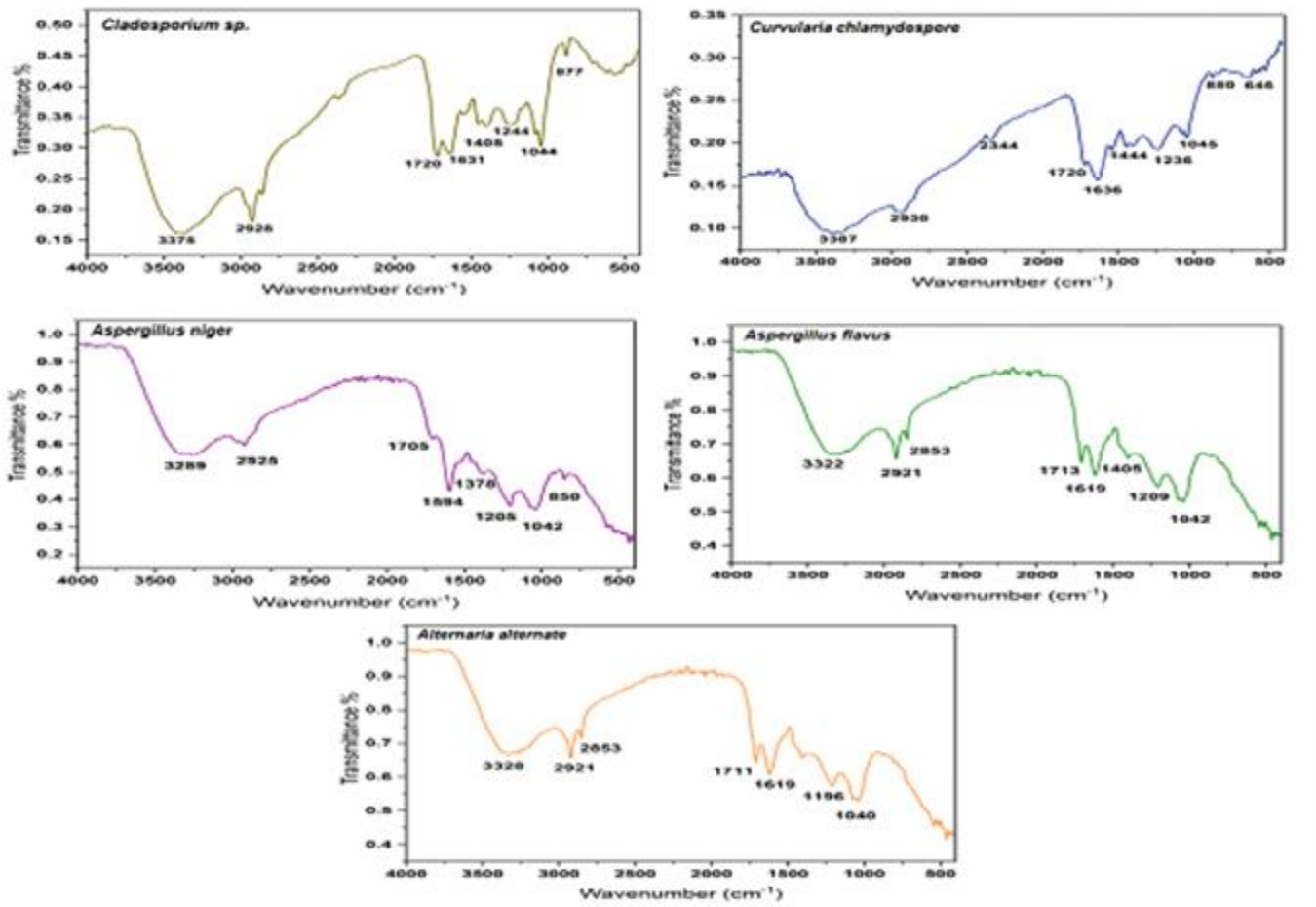

Fig 3. FTIR spectra of the studied fungal melanin samples.

of C-O stretching vibrations, signifying the existence of ether linkages.

Antioxidant activity of fungal melanin

The DPPH radical scavenging assay is a widely utilized method for assessing the *in-vitro* antioxidant properties of various compounds (Roginsky & Lissi. 2005) . This assay demonstrates the capacity of a test compound to neutralize free radicals via electron transfer or hydrogen donation (Wu et al. 2003). This study assessed the DPPH and ABTS radical scavenging abilities of various fungal melanin extracts at concentrations ranging from 3.9 to 1000 µg/ml (Fig. 4 A and B). The scavenging activity percentage was plotted against the concentration of melanin extract in µg/ml, compared to the standards of ascorbic acid and gallic acid. The findings demonstrated that all fungal melanin extracts displayed considerable antioxidant activity in both assays. The antioxidant activity increased with increasing concentration of the melanin extracts, showing a dose-dependent response. *A.niger* and *Curvularia chlamydospore* exhibited moderate DPPH and ABTS scavenging activity, reaching around 80-90% scavenging at higher concentrations. *Cladosporium sp.* and *A. flavus* showed lower DPPH and ABTS scavenging activity in comparison with standard recognizing around 60-70% scavenging at the highest concentration. *Alternaria alternata* recorded the lowest DPPH and ABTS scavenging activity around 40-50% scavenging at the highest concentration.

The present study estimated the IC_{50} concentrations (µg/ml) of ascorbic acid and gallic acid standards and various tested fungal melanin samples (Table 2). IC_{50} values are regarded as the concentration of a substance required to inhibit a biological process by 50% of free radicals. IC_{50} value of Ascorbic acid standard was 2.21µg/ml. The DPPH IC_{50} values for fungal melanin varied significantly. Hence *Aspergillus niger* and *Curvularia chlamydospore* have melanin exhibited IC_{50} values around 12.2 and 13.48µg/ml, demonstrating the highest antioxidant activity. *Cladosporium* sp. and *A. flavus* have low IC_{50} values (48.86 and 33.03µg/ml respectively), indicating moderate antioxidant activity. *Alternaria alternata* has the highest IC_{50} values (62.6µg/ml), signifying the least antioxidant activity among the tested fungi.

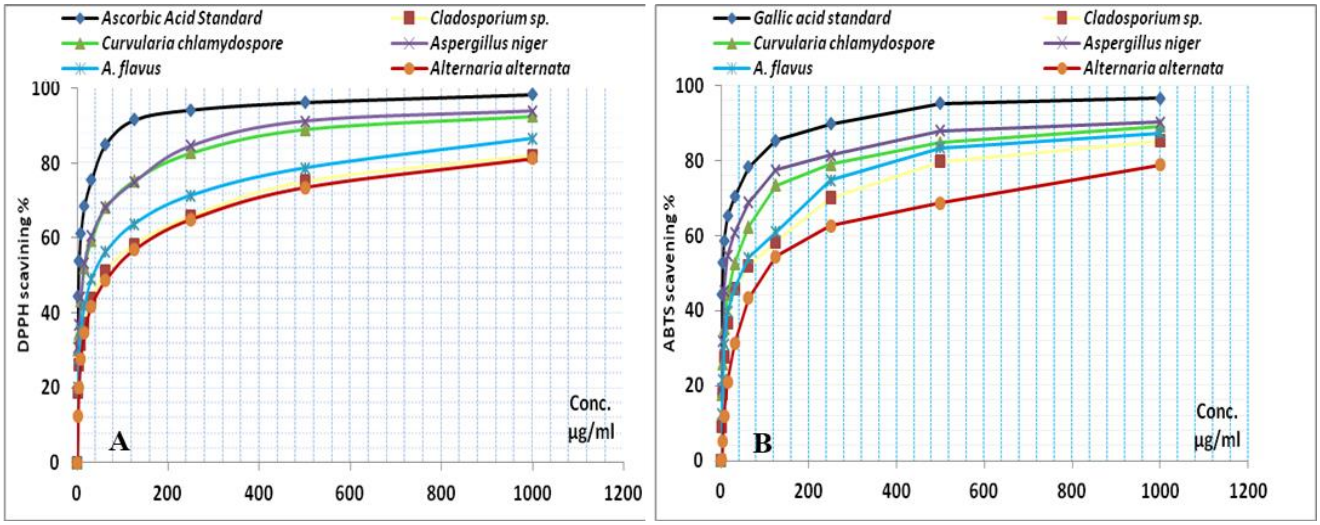

Fig 4. The antioxidant activity of the tested melanin samples using (A) DPPH and (B) ABTS radical scavenging activity assay.

Table 2 The IC50 concentrations (µg/ml) of the tested fungal melanin compared to ascorbic acid and gallic acid standards

IC_{50} ug/ml	Standard	Assay type	*Cladosporium* sp.	*Curvularia chlamydospore*	*Aspergillus niger*	*Aspergillus flavus*	*Alternaria alternata*
Ascorbic acid	2.21±0.05	DPPH	48.86±0.1	13.48±0.1	12.2±0.05	33.03±0.1	62.6±0.1
Gallic acid	2.85±0.1	ABTS	50.8±0.1	25.87±0.1	15.47±0.1	40.12±0.1	111.6±0.1

All melanin samples exhibited concentration-dependent ABTS scavenging activity, similar to DPPH, highlighting their antioxidant potential. The IC_{50} value of gallic acid is 2.85 µg/ml, demonstrating its potency as an inhibitor and its effectiveness in obstructing the target process. The IC_{50} values for the fungal species exhibit considerable variation. *Aspergillus niger* and *Curvularia chlamydospore* exhibited scavenging activities of 50% at IC_{50} values of 15.47 and 25.87 µg/ml, respectively. *Cladosporium* sp. exhibited a concentration of 50.8 µg/ml, while *A. flavus* demonstrated a concentration of 40.12 µg/ml. Finally, *Alternaria alternata* exhibits an IC_{50} value of 111.6 µg/ml, indicating the lowest antioxidant activity among the tested fungal melanin samples.

The DPPH assay typically exhibited greater scavenging activity compared to the ABTS assay at comparable concentrations. This indicates that the DPPH assay may exhibit greater sensitivity in identifying antioxidant activity. In the current study, the FTIR spectra indicated the presence of carboxyl groups, phenols, and free OH groups which accounts for the antioxidant activity of melanin. The findings were corroborated by Shaju et al. (2022), who indicated that the O–H stretching vibrations of carboxylic acids and phenolic compounds in the MFI of cephalopods contribute to their radical scavenging and metal chelating abilities, thereby aiding in the reduction of lipid oxidation processes.

The observed differences in IC_{50} values of the tested melanin may be ascribed to variations in metabolic pathways, the synthesis of specific metabolites with antioxidant properties, or the presence of particular compounds in the composition of the fungal cell wall, as previously reported by Ma *et al*. (2019), Devi et al. (2020). All fungal melanin exhibited substantial antioxidant activity, suggesting their potential utility as natural antioxidants. This area warrants exploration for multiple applications, such as food preservation, cosmetics, and pharmaceuticals. Figure (5) presented box plots that described the distribution of IC_{50} values for various samples in comparison to standard ascorbic acid and gallic acid radicals.

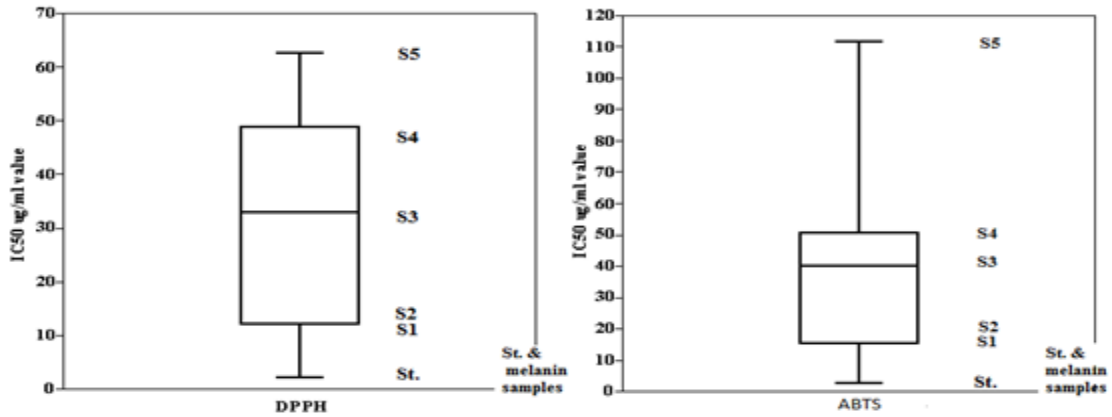

Fig 5. Boxplot of IC50 ug/ml values of DPPH (A) & Gallic acid (B) with melanin samples (±SD) where: standard (St.), S1: *Aspergillus niger*; S2: *Curvularia chlamydospore*; S3: *Aspergillus flavus*; S4: *Cladosporium* sp. and S5: *Alternaria alternata.*

Lower IC_{50} values signified enhanced antioxidant activity. Both box plots indicated that samples *Aspergillus niger* (S1) and *Curvularia chlamydospore* (S2) consistently demonstrated antioxidant activity compared to *Aspergillus flavus* (S3), *Cladosporium* sp. (S4), and *Alternaria alternata* (S5), suggesting a superior antioxidant potential against both radicals. The DPPH data exhibited greater variability among the samples compared to the ABTS data.

In vitro cytotoxicity and anticancer activity of melanin

The evaluation of cytotoxicity is essential for the development of safe and effective pharmaceuticals, as documented by Bin-Jumah et al. (2020), Makvandi et al. (2021). This study was used *in vitro* cytotoxicity assessments of melanin through the MTT assay at varying concentrations ranging from 31.25 to 1000 µg/ml against A549 lung normal and cancer cell line models (Figs. 6- 8). The results demonstrated that pure melanin was non-toxic to the noncancerous A549 cell line, facilitating normal cellular metabolism and proliferation, with an IC_{50} greater than 600 µg/mL. The findings confirmed the superior safety of pure melanin for normal cells as a biocompatible agent, showing negligible effects on the normal cell line. In contrast, all five pure fungal melanin pigments exhibit different levels of cytotoxicity towards A549 lung cancer cells, significantly reducing tumor cell viability in a dose-dependent fashion, as shown in Fig. (8). The photos illustrate a clear concentration-dependent effect of the analyzed fungal melanin samples. As the concentration of the samples increased, the cell density decreased, indicating a cytotoxic effect. Cytotoxicity levels varied among the samples examined.

The IC_{50} values of purified melanin were 95.5 ± 1.87, 112.38 ± 1.16, 110.3 ± 0.83, 75.66 ± 0.75, and 95.85 ± 0.63 µg/ml for *Cladosporium* sp., *Curvularia chlamydospore*, *Aspergillus niger*, *Alternaria alternata*, and *A. flavus*, respectively, as shown in figure 8. The analysis of IC_{50} data indicated that pure melanin demonstrated greater cytotoxicity against cancer cells. The melanin pigment produced by fungi may be a potential natural anticancer agent. previously corroborated by Mohamed *et al.* (2022). Similar results were observed in the HEP2 cancer cell line by Arun et al. (2015), demonstrating that the reduction in cell viability was concentration-dependent, with 60 µg of melanin leading to a 53% decrease in cell viability.

Melanin exhibits anti-tumor properties, such as inducing apoptosis and inhibiting angiogenesis, while preserving the integrity of healthy cells and tissues (Guo et al. 2023). Morphological assays assess significant changes in cell surface or cytoskeleton, linking these alterations to cell survival. Damage is indicated by significant volume reduction caused by the loss of proteins and intracellular ions due to altered sodium or potassium permeability. Necrotic cells exhibit nuclear swelling, chromatin flocculation, and reduced nuclear basophilia, while apoptotic cells display cell shrinkage, nuclear condensation, and disintegration.

Lung cancer cells subjected to melanin may modify their typical shape. The transition from a rounded morphology to an irregular one due to cytoskeletal disruption, perhaps triggered by apoptotic signaling pathways activated by melanin. Ultimately, melanin pigment was considered a possible anticancer agent. Further research is necessary to validate these findings and explore the potential clinical applications of these natural compounds.

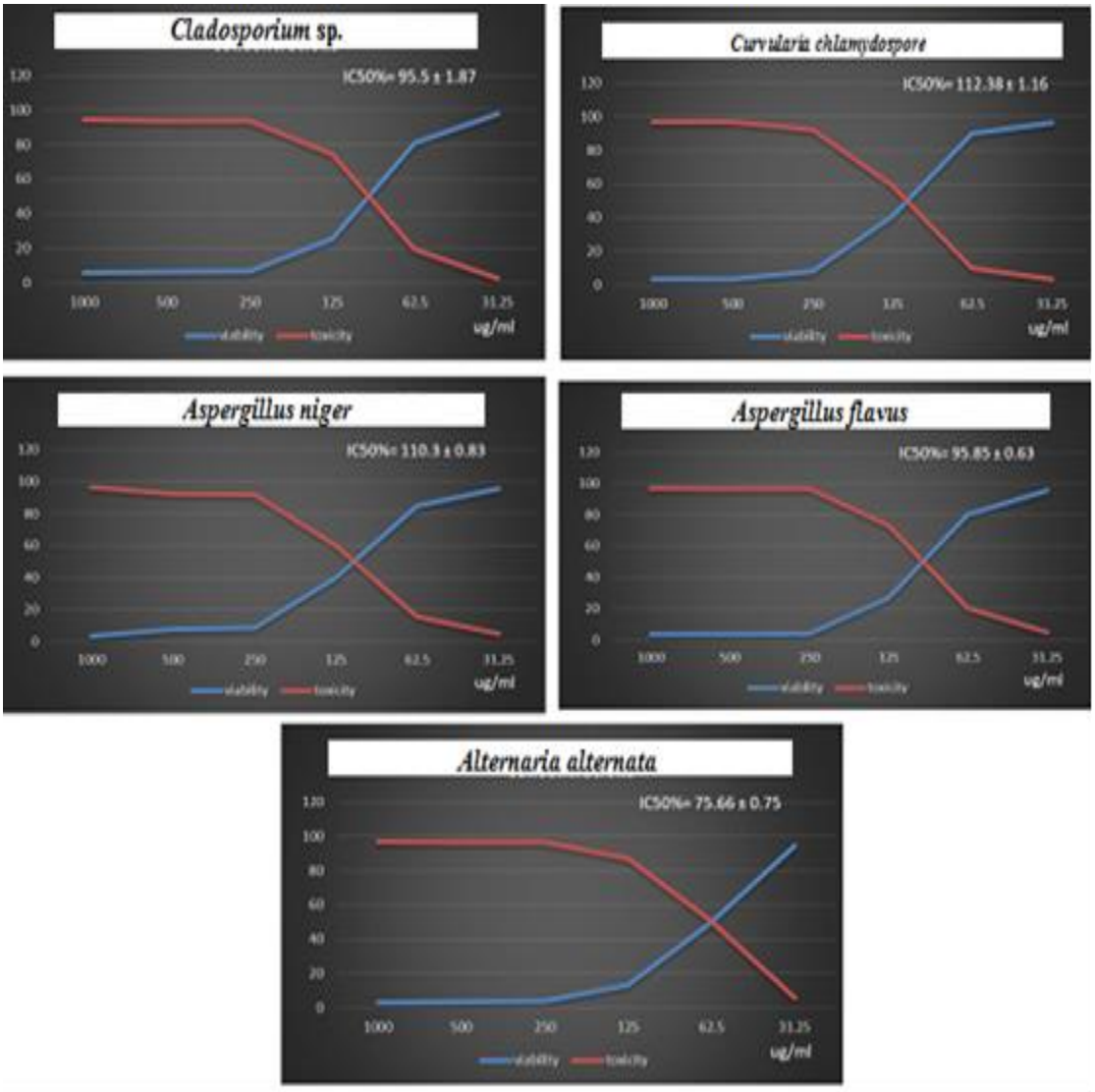

Fig 6. In vitro cytotoxicity activity of melanin against lung A549 cancer cells at different concentrations

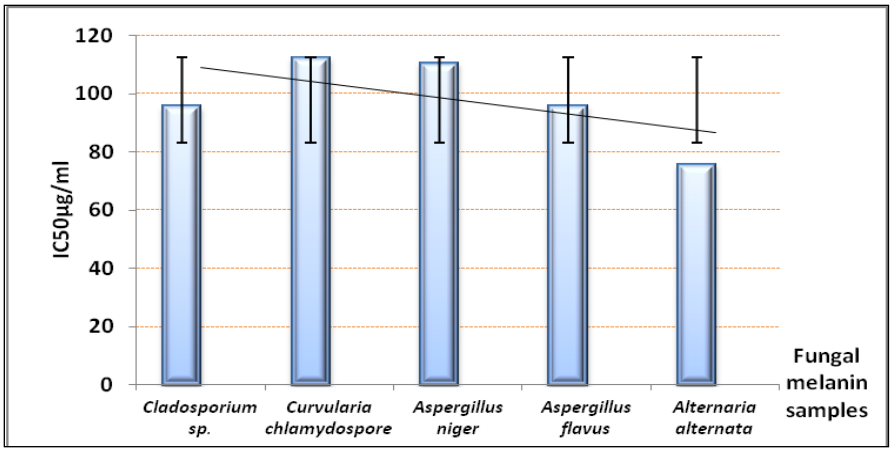

Fig 7. Fungal melanin cytotoxicity (IC50) values against A549 lung cancer cells.

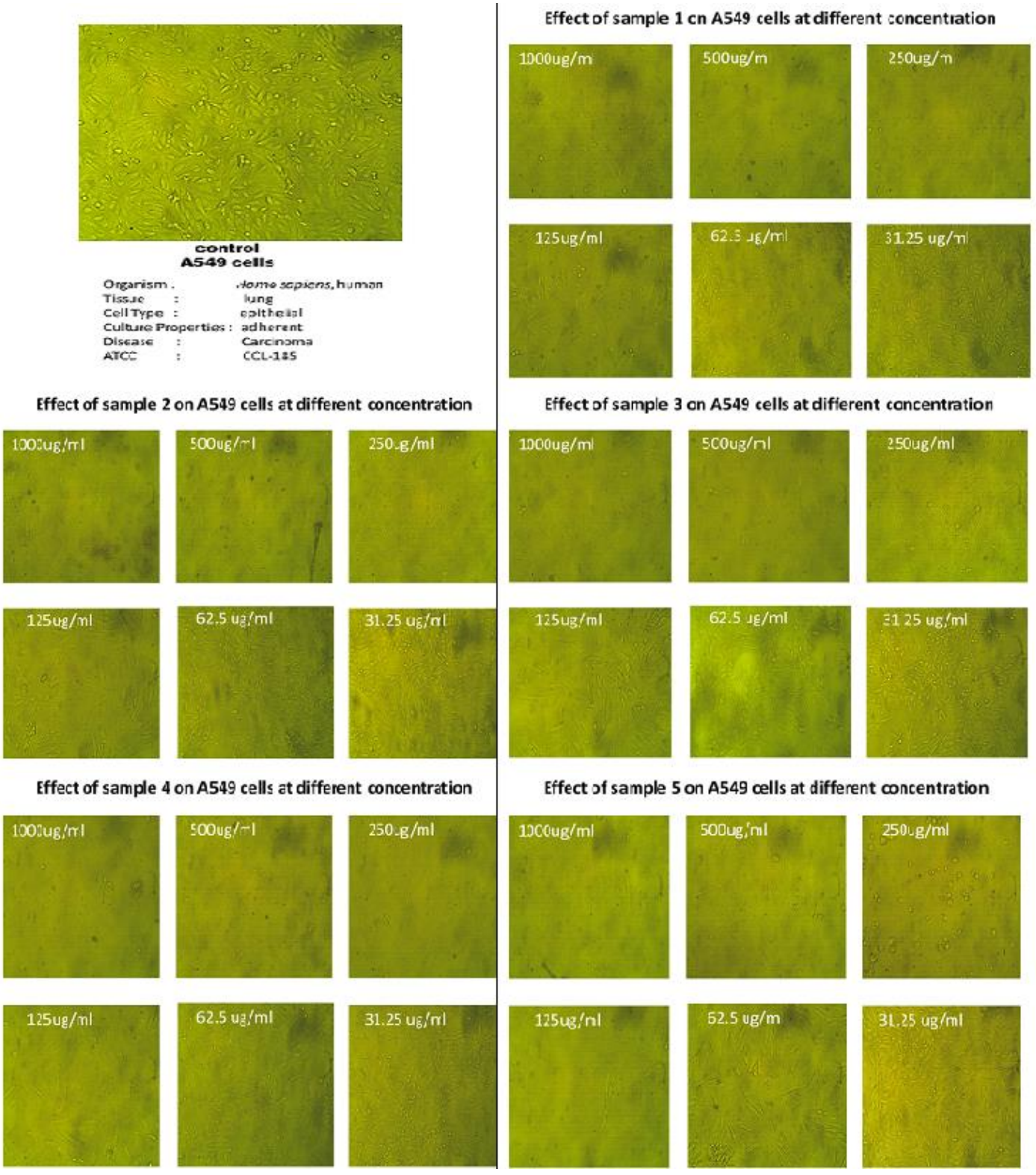

Fig 8. Cytotoxic effects of the tested fungal melanin on A549 lung cancer cell growth at different concentrations (µg/ml) where, S1: *Cladosporium* sp., S2: *Curvularia chlamydospor*e, S3: *Aspergillus niger*, S4: *Aspergillus flavus* and S5: *Alternaria alternata.*

Conclusion

The potential of melanin produced by epiphytic fungi associated with marine macroalgae collected from Fayed area, Suez Canal, Egypt was assessed as a natural source of antioxidants and antitumor agents. Melanin is a complex polymer with a unique structure that allows it to scavenge free radicals effectively. *Aspergillus niger* and *Curvularia chlamydospore* melanin exhibited strong antioxidant activity in both ABTS and DPPH assays. *Cladosporium* sp. and *A. flavus* showed moderate antioxidant activity, particularly in the ABTS assay. *Alternaria alternata* displayed weak antioxidant activity in both assays. The data provided indicates that fungal melanin exhibited varying degrees of cytotoxicity against A549 lung cancer cells. *Alternaria alternata* demonstrated the highest cytotoxicity at low concentration, signifying its potential as a promising anticancer agent. However, further research is necessary to validate these findings and explore the potential clinical applications of these natural compounds.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical Approval

Not Applicable

References

Al-Ameen NI, Mohammad TH, Abd-Alamir SH, Mzahem N A, Khajeek TR. (2024). Isolation and Identification of Fungi from Some Aquatic Invertebrates in Baghdad Al-Jadryia. Egyptian Journal of Aquatic Biology & Fisheries, 28(3).

Aleem A (1984). The Suez Canal as a habitat and pathway for marine algae and seagrasses. Deep Sea Research A, 31(6), 901-918.

Amend A, Burgaud G, Cunliffe M, Edgcomb VP, Ettinger CL, Gutiérrez M, Heitman J, Hom EF, Ianiri G, Jones A C. (2019). Fungi in the marine environment: Open questions and unsolved problems. MBio, 10(2), 10.1128/mbio. 01189-01118.

Ammanagi A, Shivasharana C T, Nagur K, Badiger AS, Ramaraj V. (2021). Functional and structural characterization of melanin from Brevibacillus invocatus strain IBA. Doklady Biological Sciences 500(1),159-169.

Ann V, Freixa A, Butturini A, Romaní A M. (2019). Interplay between sediment properties and stream flow conditions influences surface sediment organic matter and microbial biomass in a Mediterranean river. *Hydrobiologia, 828*, 199-212.

Arun G, Eyini M, Gunasekaran P. (2015). Characterization and biological activities of extracellular melanin produced by *Schizophyllum commune* (Fries). Indian J Exp Biol. Jun;53(6),380-7.

Bärlocher F, Seena S, Wilson KP, Dudley Williams D. (2008). Raised water temperature lowers diversity of hyporheic aquatic hyphomycetes. *Freshwater Biology, 53*(2), 368-379.

Belozerskaya TA, Gessler NN, Aver'yanov AA. (2017). Melanin pigments of fungi. *Fungal metabolites, 8*, 263-291.

Bin-Jumah M, Al-Abdan M, Albasher G, Alarifi S. (2020). Effects of green silver nanoparticles on apoptosis and oxidative stress in normal and cancerous human hepatic cells in vitro. *International journal of nanomedicine*, 1537-1548.

Bridge P, Spooner B. (2012). Non-lichenized Antarctic fungi: transient visitors or members of a cryptic ecosystem? *Fungal Ecology, 5*(4), 381-394.

Barnett HL, Hunter BB. (1986) Illustrated Genera of Imperfect Fungi, 4th Edition. Macmillan Publishing Co., New York.

Cao C, Li Y, Wang C, Zhang N, Zhu X, Wu R, Wu J. (2020). Purification, characterization and antitumor activity of an exopolysaccharide produced by *Bacillus velezensis* SN-1. *International journal of biological macromolecules, 156*, 354-361.

Choi KY. (2021). Bioprocess of microbial melanin production and isolation. *Frontiers in bioengineering and biotechnology, 9*, 765110.

Chou CH, Shrestha S, Yang CD, Chang NW, Lin YL, Liao KW.*et al* (2018). miRTarBase update 2018: a resource for experimentally validated microRNA-target interactions. *Nucleic acids research, 46*(D1), D296-D302.

Crowe LM, Mouradian R, Crowe JH, Jackson SA, Womersley C. (1984). Effects of carbohydrates on membrane stability at low water activities. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 769*(1), 141-150.

Devi R, Kaur T, Guleria G, Rana KL, Kour D, Yadav N, Yadav AN, Saxena AK. (2020). Fungal secondary metabolites and their biotechnological applications for human health. In *New and future developments in microbial biotechnology and bioengineering* (pp. 147-161). Elsevier.

El-Naggar NEA, El-Ewasy SM. (2017). Bioproduction, characterization, anticancer and antioxidant activities of extracellular melanin pigment produced by newly isolated microbial cell factories Streptomyces glaucescens NEAE-H. *Scientific reports, 7*(1), 1-19.

El-Naggar NEA, Saber WI. (2022). Natural melanin: current trends, and future approaches, with especial reference to microbial source. *Polymers, 14*(7), 1339.

ElObeid AS, Kamal-Eldin A, Abdelhalim MA K, Haseeb AM. (2017). Pharmacological properties of melanin and its function in health. *Basic & clinical pharmacology & toxicology, 120*(6), 515-522.

Elsayis A, Hassan SW, Ghanem KM, Khairy H. (2022). Suggested sustainable medical and environmental uses of melanin pigment from halotolerant black yeast Hortaea werneckii AS1. *Frontiers in microbiology, 13*, 871394.

Fawcett D,Verduin J , Shah M, Sharma SB, Poinern GEJ. (2017). A review of current research into the biogenic synthesis of metal and metal oxide nanoparticles via marine algae and seagrasses. *Journal of Nanoscience, 2017*(1), 8013850.

Fu X, Xie M, Lu M, Shi L, Shi T, Yu M. (2022). Characterization of the physicochemical properties, antioxidant activity, and antiproliferative activity of natural melanin from *S. reiliana*. *Scientific reports, 12*(1), 2110.

Fuentes-López D, Ortega-Zambrano D, Fernández-Herrera M. A, Mercado-Uribe H. (2022). The growth of Escherichia coli cultures under the influence of pheomelanin nanoparticles and a chelant agent in the presence of light. *Plos one, 17*(3), e0265277.

Ghadge A, Er Kara M, Mogale DG, Choudhary S, Dani S. (2021). Sustainability implementation challenges in food supply chains: A case of UK artisan cheese producers. *Production Planning & Control, 32*(14), 1191-1206.

Gribb A. (1983). Marine algae of the southern Great Barrier Reef. *Part I. Rhodophyta*. *Australia Coral Reef Society*.

Guo L, Li W, Gu Z, Wang L, Guo L, Ma S, Li C, Sun J, Han B, Chang J. (2023). Recent advances and progress on melanin: from source to application. *International Journal of Molecular Sciences, 24*(5), 4360.

Hammer Ø, Harper DA. (2001). Past: paleontological statistics software package for educaton and data anlysis. *Palaeontologia electronica, 4*(1), 1.

Kamarudheen, N, Naushad T, Rao KVB. (2019). Biosynthesis, characterization and antagonistic applications of extracellular melanin pigment from marine Nocardiopsis Sps. *Indian J Pharm Educ Res, 53*(2), 112-120.

Karkun Sur A, Verma S. (2016). Study and survey of fungal diversity of borra caves situated in Andhra Pradesh, India. *Int. J. Res. Ayurveda Pharm, 7*, 241-246.

Korlević M, Markovski M,Zhao Z, Herndl GJ, Najdek M. (2021). Seasonal dynamics of epiphytic microbial communities on marine macrophyte surfaces. *Frontiers in microbiology, 12*, 671342.

Lopes FC, Tichota DM, Pereira J Q, Segalin J,de Oliveira Rios A, Brandelli A. (2013). Pigment production by filamentous fungi on agro-industrial byproducts: an eco-friendly alternative. *Applied biochemistry and biotechnology, 171*, 616-625.

Lorquin F, Ziarelli F,Amouric A,Di Giorgio C, Robin M, Piccerelle P , Lorquin J. (2021). Production and properties of non-cytotoxic pyomelanin by laccase and comparison to bacterial and synthetic pigments. *Scientific reports, 11*(1), 8538.

Ma L, Liu Y, Zhang X, Ye Y, Yin G, Johnson B (2019). Deep learning in remote sensing applications: A meta-analysis and review. *ISPRS journal of photogrammetry and remote sensing, 152*, 166-177.

Ma Y, Zhang P, Dai X, Yao X, Zhou S, Ma Q, Liu J, Tian S. Zhu J, Zhang J. (2023). Extraction, physicochemical properties, and antioxidant activity of natural melanin from Auricularia heimuer fermentation. *Frontiers in Nutrition, 10*, 1131542.

Makvandi P, Baghbantaraghdari Z, Zhou W, Zhang Y, Manchanda R, Agarwal T, Wu A, Maiti TK, Varma RS, Smith B R. (2021). Gum polysaccharide/nanometal hybrid biocomposites in cancer diagnosis and therapy. *Biotechnology Advances, 48*, 107711.

Manzelat S F, Mufarrah AM, Hasan BA, Hussain NA. (2018). Macro algae of the Red Sea from Jizan, Saudi Arabia. *Phykos, 48*(1), 88-108.

Migliorini AA, Piroski C S, Daniel TG, Cruz TM. Escher G B. Vieira do Carmo MA. *et al*. (2019). Red chicory (*Cichorium intybus*) extract rich in anthocyanins: chemical stability, antioxidant activity, and antiproliferative activity in vitro. *Journal of food science, 84*(5), 990-1001.

Mohamed AF, Abuamara TM, Amer ME, Ei-Moselhy LE, Gomah TA, Matar ER. etal. (2022). Genetic and histopathological alterations in Caco-2 and huh-7 cells treated with secondary metabolites of marine fungi. *Journal of Gastrointestinal Cancer, 53*(2), 480-495.

Mohamed H, Ebrahim W, El-Neketi M, Awad MF, Zhang H,Zhang Y, Song Y. (2022). In vitro phytobiological investigation of bioactive secondary metabolites from the Malus domestica-derived endophytic fungus Aspergillus tubingensis strain AN103. *Molecules, 27*(12), 3762.

Muñoz-Torres P, Cárdenas-Ninasivincha, S., Aguilar Y. (2024). Exploring the Agricultural Applications of Microbial Melanin. *Microorganisms, 12*(7), 1352.

Oh JJ, Kim J Y, Kim Y J, Kim S, Kim GH. (2021). Utilization of extracellular fungal melanin as an eco-friendly biosorbent for treatment of metal-contaminated effluents. *Chemosphere, 272*, 129884.

Plonka P, Grabacka M. (2006). Melanin synthesis in microorganisms-biotechnological and medical aspects. *Acta biochimica polonica, 53*(3), 429-443.

Pyo J, Ju KY, Lee JK. (2016). Artificial pheomelanin nanoparticles and their photo-sensitization properties. *Journal of Photochemistry and Photobiology B: Biology, 160*, 330-335.

Rajagopal K, Kathiravan G, Karthikeyan S. (2011). Extraction and characterization of melanin from Phomopsis: A phellophytic fungi Isolated from Azadirachta indica A. Juss. *African Journal of Microbiology Research, 5*(7), 762-766.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine, 26*(9-10), 1231-1237. doi: 10.1016/s0891-5849(98)00315-3

Roginsky V, Lissi EA. (2005). Review of methods to determine chain-breaking antioxidant activity in food. *Food Chemistry, 92*(2), 235-254.

Salgado-Castillo SN, López-Peña, H, Díaz R, Peña-Solis K, Ponce-Alquicira E, Soriano-Santos J, Díaz-Godínez, G. (2023). Fungal melanins and their potential applications: A Review. *BioResources, 18*(4), 8688.

Shaju PM Ganesan P, Kingston SD, Murugananthan M. (2022). Fourier transform Infrared characterization of melanin free ink from selected cephalopods for identification of active functional groups responsible for antioxidant activity. *Journal of the Indian Chemical Society, 99*(3), 100375.

Singh S,Malhotra AG,Pandey A, Pandey KM. (2013). Computational model for pathway reconstruction to unravel the evolutionary significance of melanin synthesis. *Bioinformation, 9*(2), 94.

Singh S, Nimse SB, Mathew DE, Dhimmar A Sahastrabudhe H, Gajjar A, Ghadge VA, Kumar P, Shinde PB. (2021). Microbial melanin: Recent advances in biosynthesis, extraction, characterization, and applications. *Biotechnology Advances, 53*, 107773.

Song W, Xing RE, Yang H Liu S, Li P. (2021). Optimization of extractions of eumelanin from cuttlefish ink and the hypoglycemic effects: In vitro enzyme inhibitory activity and glucose consumption in HepG2 cells. *Journal of Food Processing and Preservation, 45*(10), e15868.

Surendirakumar K, Pandey RR, Muthukumar T, Sathiyaseelan A, Loushambam S, Seth A. (2022). Characterization and biological activities of melanin pigment from root endophytic fungus, Phoma sp. RDSE17. *Archives of Microbiology, 204*(3), 171.

Suthar M, Dufossé L, Singh SK. (2023). The enigmatic world of fungal melanin: a comprehensive review. *Journal of Fungi, 9*(9), 891.

Swann N, Poizner H, Houser M, Gould S, Greenhouse I, Cai W, Strunk J, George J, Aron A R. (2011). Deep brain stimulation of the subthalamic nucleus alters the cortical profile of response inhibition in the beta frequency band: a scalp EEG study in Parkinson's disease. *Journal of Neuroscience, 31*(15), 5721-5729.

Wu HC, Chen HM, Shiau CY. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). *Food research international, 36*(9-10), 949-957.

Yang L, Zhang Z, Song Y, Hong S, Xu R, Zhao Y, Zhang W, Cui B, Yang MH. (2023). Diffusion models: A comprehensive survey of methods and applications. *ACM Computing Surveys, 56*(4), 1-39.

Yang X, Liang Q, Chen Y, Wang B. (2019). Alteration of methanogenic archaeon by ethanol contribute to the enhancement of biogenic methane production of lignite. *Frontiers in microbiology, 10*, 2323.

Zaidi KU, Ali AS, Ali SA. (2014). Purification and characterization of melanogenic enzyme tyrosinase from button mushroom. *Enzyme research, 2014*(1), 120739