BIOLOGICAL ACTIVITIES OF BACTERIAL MELANIN FROM *STREPTOMYCES SPECTABILIS* AGAINST BACTERIA, FREE RADICALS, ULTRAVIOLET RADIATION, AND HEAVY METAL

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ฤทธิ์ทางชีวภาพของเมลานินที่ได้จากแบคทีเรีย *Streptomyces spectabilis* ต่อการต้านแบคทีเรีย สารอนุมูลอิสระ รังสีอัลตร้าไวโอเลต และโลหะหนัก

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Melanin, a complex biopolymer found in various organisms, has attracted considerable attention due to its diverse biological properties, including antibacterial activity, antioxidant potential, UV protection, and metal ion chelation. In this study, we investigated the melanin produced by *Streptomyces spectabilis* strains AQ2 and CQ2, with a focus on its physicochemical characteristics and biological activities. Both strains were cultured for melanin extraction, and the structure of the extracted melanin was analyzed using Fourier transform infrared spectroscopy (FTIR) and UV-Vis spectroscopy. Melanin yields from both strains were comparable; however, strain AQ2 demonstrated higher melanin production efficiency per unit biomass than strain CQ2, while strain CQ2 produced a greater total amount of melanin when cultivated over a longer period. FTIR analysis revealed that the extracted melanin shared structural characteristics with synthetic melanin, and its UV absorption spectrum was similar to that of standard synthetic melanin. Antioxidant activity was evaluated using the DPPH assay and the DCFH-DA assay to measure intracellular reactive oxygen species (ROS) levels. Melanin from strain CQ2 exhibited stronger free radical scavenging activity than that from AQ2, and melanin from both strains effectively reduced intracellular ROS levels following UV exposure. However, antibacterial activity and ability to enhance cell viability after UV exposure were not particularly promising. Additionally, melanin from both AQ2 and CQ2 showed significant metal ion chelation capability, especially with copper ions, indicating potential for future applications such as environmental detoxification. In conclusion, melanin derived from *S. spectabilis* strains AQ2 and CQ2 demonstrates significant bioactive properties and shows strong potential as a natural agent for UV protection, antioxidant defense, and heavy metal remediation. However, further research is required to optimize its production processes and to enhance the application of its broader biological functions.

Keyword : Melanin, Streptomyces spectabilis, Antioxidant activity, UV protection, Metal ion chelation

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List of Abbreviations

Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma 2
COX	Cyclooxygenase
DCFH-DA	2',7'-Dichlorodihydrofluorescein diacetate
DHN	1,8-dihydroxynaphthalene
DOPA	Dihydroxyphenylalanine
DPPH	2,2-diphenyl-1-picrylhydrazyl
ESR	Electron spin resonance
FTIR	Fourier transform infrared spectroscopy
HGA	Homogentisic acid
L-DOPA	L-3,4-dihydroxyphenylalanine
LOX	Lipoxygenase
McF	McFarland
OD	Optical density
ROS	Reactive oxygen species
THN	1,3,6,8-tetrahydroxynaphthalene
UV	Ultraviolet
UV-Vis Spectroscopy	Ultraviolet-visible spectroscopy

CHAPTER 1

INTRODUCTION

Background

Melanin is a dark-colored biopolymer derived from phenolic and indolic structures, and is broadly present in animals, plants, and various microorganisms. Melanin pigments can be categorized based on their chemical structures, namely, eumelanin, pheomelanin, allomelanin, neuromelanin, pyomelanin and. The precursors of melanin include dihydroxyphenylalanine (DOPA), 1,8-dihydroxynaphthalene (DHN), 5,6-dihydroxyindole, catechol, and homogentisic acid (HGA). Melanin has highly diverse and complex chemical structures.[1, 2]

Melanin comprised several functional groups and a complex structure that contributed to its ability to resist extreme temperatures, ultraviolet (UV), heavy metal ions, and other environmental stresses. It has found widespread applications in fields such as food, cosmetics, biomaterials, optoelectronics, and ecosystem restoration.[3, 4] Numerous studies reported diverse biological activities associated with melanin. For instance, melanin from *Streptomyces* sp. demonstrated antibacterial behavior against *Lactobacillus vulgaris* and *Escherichia coli*.[5] Melanin was highly effective as a scavenger of free radicals and possessed electron transfer properties.[6] Melanin isolated from *Lachnum* YM226 was shown to reduce blood lipid levels and exhibit antitumor activity in a mouse model.[7] Water-insoluble melanin derived from *Inonotus hispidus* had the capability to clear intracellular reactive oxygen species (ROS), enhanced the cell viability of normal embryonic liver cells under hydrogen peroxide treatment, and displayed strong antioxidant activity.[8] Melanin from *Stachybotrys* sp. HSS-1 efficiently absorbed heavy metals, making it suitable for wastewater treatment.[9] Advancements in knowledge and technology have enabled the transformation of melanin pigments into valuable materials.

The demand for melanin is rapidly increasing due to its broad biological activity. Microbial fermentation offers a quick, cost-effective, and scalable method for extracting

melanin to meet market demand.[10] Streptomycetes exhibit remarkable diversity and bioactive potential, surpassing many other microbial groups. Streptomycetes are extensively distributed in both terrestrial and marine habitats,[11] benefiting from their status as one of the most abundant and widespread bacteria in soil. They are prolific producers of natural antibiotics, contributing significantly to those used in human and veterinary medicine. Actinomycetes, including *Streptomyces*, thrive in highly competitive soil environments, with each isolate having the potential to encode 10–20 secondary metabolites through genes.[12] The genus *Streptomyces* encompasses several unique species that have historically provided more than two-thirds of naturally occurring antibiotics.[13] *Streptomyces* is a vital source of various bioactive substances, including antibiotics, fungicides, enzymes, antioxidants, and compounds with anti-cancer activity.[14]

The isolation of natural microorganisms led to the discovery of two strains of bacteria, *Streptomyces spectabilis* strains AQ2 and CQ2, which can produce a black pigment.[15] Further investigations to ascertain whether the black pigment produced by *S. spectabilis* strains AQ2 and CQ2 is melanin have not yet been conducted. This highlights the need for comprehensive studies aimed at elucidating the pigment's structure and exploring its potential biological activities.

In this study, the cultivation of *S. spectabilis* strains AQ2 and CQ2 will be conducted for the extraction of melanin. The melanin structure will be characterized through Ultraviolet-visible spectroscopy and Fourier transform infrared spectroscopy (FTIR). Additionally, the biological activities of melanin will be investigated, focusing on antibacterial activity, free-radical scavenging ability, UV-protective properties, and the capacity to chelate metal ions. This comprehensive analysis aims to provide insights into the physicochemical properties and biological activities of melanin produced by *S. spectabilis* strains AQ2 and CQ2

Objective

1. To optimize the inoculum preparation for melanin production by *S. spectabilis* strains AQ2 and CQ2

2. To characterize the physicochemical properties of melanin produced by *S. spectabilis* strains AQ2 and CQ2

3. To investigate the biological activities of bacterial melanin from *S. spectabilis* strains AQ2 and CQ2, including antibacterial, free radical scavenging, UV-protective properties, and heavy metal-chelating properties.

Hypothesis

The black pigment produced by *S. spectabilis* strains AQ2 and CQ2 is hypothesized to be true melanin, as confirmed by physicochemical characterization, and it is expected to exhibit significant biological activities such as antibacterial effects, free radical scavenging, UV protection, and metal ion chelation, indicating its potential for biomedical and environmental applications.

Scope of research

This study investigates the production of melanin from *S. spectabilis* strains AQ2 and CQ2 by examining the effects of inoculum preparation and culture conditions on melanin yield. The extracted melanin will be analyzed using UV-Vis spectroscopy and Fourier Transform Infrared Spectroscopy to confirm its structural characteristics and verify its identity as true melanin. Furthermore, the biological activities of the melanin will be evaluated, including antibacterial activity, free radical scavenging potential, UV-protective properties, and metal ion-chelating capacity. Antibacterial activity will be assessed using the disc diffusion method, free radical scavenging ability will be measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, UV-protective properties will be evaluated through resazurin cell viability assay and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) assay to assess intracellular ROS levels, and chelating metal ions will be investigated using an agar plate-based fungal stress response assay.[10] This research aims to provide insights into the production,

properties, and potential applications of melanin derived from the isolated *S. spectabilis* strains AQ2 and CQ2 in biomedical and environmental fields.

Limitations

This study does not investigate the genetic pathways involved in melanin biosynthesis in *S. spectabilis* strains AQ2 and CQ2. Biological activity tests will be conducted in vitro, using a limited selection of bacterial and fungal strains, a few heavy metals, and specific cell lines. All experiments will be performed under controlled laboratory conditions. While this research provides foundational insights, further studies are necessary to fully assess the potential applications of this bacterial melanin.

Experimental design

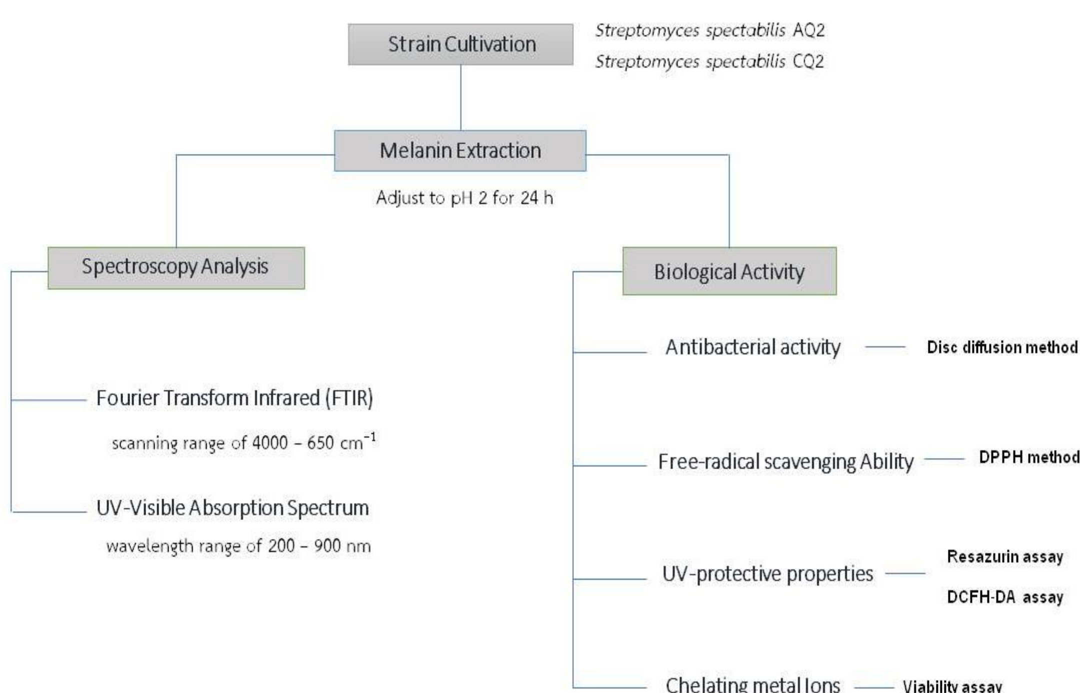


Figure 1 : Experimental design

Benefit of research

This study advances the understanding of *Streptomyces*-derived melanin by presenting a cost-effective and scalable production method. Comprehensive physicochemical characterization confirms its structural integrity, while its demonstrated antibacterial, antioxidant, UV-protective, and metal ion-chelating properties underscore its potential for diverse applications. Moreover, this research paves the way for further exploration of microbial-derived functional materials in pharmaceuticals, cosmetics, and biomaterials. The findings contribute to the development of sustainable and innovative bio-based solutions, promoting the use of natural compounds across various industries.

CHAPTER 2
LITERATURE REVIEW

1. Streptomycetes

Among bacterial lineages, the phylum *Actinobacteria* is notably distinguished as one of the most significant groups, comprising a vast taxonomic diversity with numerous identified species, several of which have become key model organisms in scientific research.[16] The *Actinobacteria* phylum consists of six major classes: *Acidimicrobiia*, *Actinobacteria*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria* and *Thermoleophilia*, covering a broad range of gram-positive bacteria that display diverse characteristics and morphologies.[17] *Actinobacteria* have been recognized as important producers of bioactive natural compounds since the 1950s. Approximately half of all identified secondary metabolites, including enzymes, antibiotics, immunosuppressive agents, and antitumor compounds, are derived from Actinomycetes, members of this phylum. The genus *Streptomyces* is the most notable representative, contributing to over 70 percent of antibiotics used commercially.[18] A significant proportion, approximately 80 percent, of natural products identified from *Actinobacteria* to date have been derived from the genus *Streptomyces*.[19]

Streptomycetes exhibit extensive diversity and possess significant bioactive potential, surpassing many other microbial groups. With over 900 identified species bearing validly published names, Streptomycetes are widely distributed in both terrestrial and marine habitats,[20] and recent studies indicate their presence in extreme environments as well. Characterized by the production of a high number of easily dispersed spores,[21] Streptomycetes are believed to have a cosmopolitan distribution. Moreover, their biosynthetic capacity remains unparalleled, attributed to their large genome size (>7 Mbp),[21] with some strains reported to harbor over 20 gene clusters encoding predicted or known biosynthetic pathways.[22] This unique capability has positioned Streptomycetes as prolific producers of various novel and useful secondary

metabolites, showcasing activities such as antioxidant, anticancer, antimicrobial, and more. Researchers are drawn to explore the bioactive potential of this genus due to its diverse and promising secondary metabolite production.[23]

Although only a small number of compounds isolated from microbes are directly used as effective clinical medicines, Streptomycetes have been identified as producers of several anticancer drugs, including bleomycin (a glycopeptide), dactinomycin (a non-ribosomal peptide), mitomycin C (a quinone), and doxorubicin (an anthracycline).[24]

Furthermore, natural microbial compounds serve as valuable starting points for drug development, offering potential for optimization, modification, and transformation into clinically important drug leads.[25] *Streptomyces*, one of the most abundant and widely distributed soil bacteria, holds a prominent position as a prolific producer of natural antibiotics extensively used in both human and veterinary medicine. These Gram-positive bacteria undergo complex multicellular development, characterized by filamentous growth and sporulation. The genome sequencing of *Streptomyces coelicolor*, a model organism within this genus, has revealed numerous adaptations enabling survival and competition in dynamic soil ecosystems. Notably, each Actinomycete isolate has the genetic potential to encode 10–20 secondary metabolites, highlighting the remarkable biosynthetic capabilities of these organisms.[14]

The *Streptomyces* genus, comprising many well-characterized species, has contributed to the discovery of over two-thirds of naturally occurring antibiotics. In addition to antibiotics, members of this genus are prolific producers of diverse bioactive compounds, including antifungal, enzymes, pigments, agents that promote plant growth, antioxidants, and anticancer molecules. Continued investigation into *Streptomyces* and their secondary metabolites underscores their vital role in medicine, agriculture, and biotechnology.[12]

2. Microbial pigments

Pigments are molecules that absorb specific wavelengths of light while reflecting others within the visible spectrum (380–750 nm), and they represent a notable feature of

many microorganisms.[26] Microbial pigments are not merely coloring agents; they are composed of chemically diverse structures and are associated with a wide range of biological activities. Various classes of microbial pigments include bacteriochlorophylls, carotenoids, flavins, indigoids, melanin, pheomelanin, monascin, phenazostatin D, prodigiosin, quinone precursors, violacein, glaukothalin, pyocyanin, xanthomonadin, phenazines, canthaxanthin, astaxanthin, and beta-carotene, among other.[27-31] These pigments and their derivatives have been reported to exhibit a spectrum of cell-specific biological effects, including inhibitory, lethal, and sublethal activities at various concentrations. The multifunctional nature of microbial pigments underscores their potential contributions beyond coloration, positioning them as valuable compounds for biomedical, industrial, and environmental applications.[32]

Microbial pigments are typically classified into two major types: those secreted into the surrounding medium and those retained intracellularly within microbial cells. For the industrial scale exploitation of these pigments, many of which exhibit promising biological properties, several essential criteria must be fulfilled.

The microbial strain selected should be amenable to consistent cultivation under controlled conditions and exhibit a rapid growth rate to ensure high pigment yields within a short production cycle. Pigment production throughout the year, unaffected by seasonal variability, is crucial for maintaining supply stability. Additionally, the organism must be inherently non-toxic and non-pathogenic to ensure safety during both production and application. An ideal strain should also possess metabolic adaptability, allowing it to grow in diverse and potentially low-cost or unconventional media. Furthermore, robustness under a wide range of physical and chemical stressors, including fluctuations in light intensity and quality, temperature, pH, and osmolarity, is critical for scalable and resilient production. Meeting these criteria is essential to ensure the practical feasibility, economic viability, and overall efficiency of microbial pigment production for industrial applications.[26]

The use of bacteria as biological sources for pigment production offers several notable advantages over fungi. These include shorter life cycles, which enable rapid

biomass and pigment accumulation, and the relative ease of genetic manipulation through advanced molecular techniques. Such characteristics make bacteria highly appealing for large-scale and sustainable pigment production. Consequently, focused research and strategic development in bacterial pigment production are regarded as essential for enabling their successful integration into commercial and industrial applications. Pigment-producing bacteria are ubiquitous, with the ability to thrive in a diverse range of ecosystems, including soil, rhizospheric zones, desert sands, freshwater bodies, and marine environments. Numerous studies have demonstrated that these bacteria exhibit exceptional resilience, tolerating extreme temperatures and salinity, and in some instances, they act as endophytes, colonizing plant tissues without causing pathogenic effects. This physiological and ecological versatility reinforces the potential of pigment producing bacteria as strong candidates for application across various biotechnological and industrial sectors. The bacterial pigments and their application will be presented in Table 1 [33]

Table 1: Bacterial pigments and their application

Bacterial	Pigment	Application	Reference
Micromonospora lupine	Anthraquinone	Antitumor	Igarashi et al., [34]
Streptomyces spp.	Carotenoid and Melanin	Food-grade pigment and Antioxidant	Dharmaraj et al., [35]
Chromobacterium sp.	Violacein	Anti-tumor, anti-microbial and anti-parasite	Duran et al., [36]
Hymenobacter sp. and Chryseobacterium sp.	Caroteniod	Photosensitizers	Ordenes Aenishanslins et al., [37]
Streptomyces glaucescens NEAE-H	Melanin	Anti-cancer and antioxidant	El-Naggar and El-Ewasy[38]

Pseudomonas aeruginosa	Pyocyanin	Anti-microbial	El-Fouly et al. [39]
Hahella chejuensis	Prodiginines	Antibiotic	Kim et al.[40]
Pedobacter	Carotenoid	Antioxidant	Correa Llanten et al.[41]
Vogesella indigofera	Blue pigment	Detect heavy metal	Gu and Cheung,[42]

Over the past decade, growing interest in melanin and the biological process of melanogenesis has been driven by their wide-ranging biomedical and technological applications. A notable reflection of this trend is the rise of polydopamine-based multifunctional coatings in materials science, which exemplify the versatile potential of melanin-like compounds. This is just one of many emerging applications. Others include melanin thin films for organic and bioelectronic devices, advanced drug delivery platforms, functional nanoparticles, engineered biointerfaces, high-performance sunscreens, and environmental remediation technologies. Despite considerable progress in both fundamental understanding and applied development, research on melanin and melanogenesis remains dynamic and evolving. To fully unlock the potential of this field, intersectoral collaboration among academic, clinical, and industrial research entities is essential. Such coordination is crucial to stimulate broader scientific engagement, attract industrial investment, and raise awareness across biomedical, biomaterials, and high-tech sectors about the vast opportunities inherent in pigment cell biology and its associated metabolic pathways.[43]

3. Melanin pigments

Melanin is a diverse group of naturally occurring pigments found across a broad spectrum of life forms, including animals, plants, fungi, and bacteria. It is synthesized through the oxidative polymerization of tyrosine in higher organisms or phenolic compounds in lower organisms such as fungi and certain bacterial species.[44] In

humans, melanin plays a critical role in determining skin, hair, and eye color. From a chemical standpoint, melanin is composed of high molecular weight, hydrophobic, and negatively charged polymers with an amorphous structure. These features contribute to its notable resilience, as it demonstrates strong resistance to concentrated acids, reducing agents, photodegradation, and thermal decomposition, withstanding temperatures up to 600 °C in certain types. However, despite its stability, melanin is not completely impervious to degradation, as it remains susceptible to oxidation and can be solubilized in alkaline or phenolic environments. Its color spectrum ranges from dark brown to black, although red or yellow variants may occur depending on the biosynthetic pathway. These physical and chemical properties, along with their widespread distribution, have led to increasing interest in melanin as a biological and technologically relevant compound.[38, 45, 46]

The rising demand for melanin pigments has driven interest in alternative sources, especially microorganisms, which are efficient and sustainable producers. Microbial melanin shows promise in various fields such as cosmetics, medicine, and materials science. Recent approaches include AI and statistical optimization, use of low-cost substrates, genetic engineering and mutagenesis, and green synthesis of nano-melanin. These innovations support the development of scalable and eco-friendly melanin production systems.[2, 47-49]

4. Melanin structure

Melanin exhibits a highly diverse and heterogeneous structure due to its origin from various biological sources, resulting in differences in composition, size, color, and function. Its physicochemical properties, such as a strong negative charge, high molecular weight, and hydrophobicity, pose significant challenges for structural analysis. These characteristics complicate conventional methods used for its identification and characterization.[50] Additionally, melanin is insoluble in most solvents and resistant to chemical degradation.[50, 51] Chemical treatments, such as strong bases, can dissolve melanin but often alter its native structure and may break the initial polymer into

fragments. Enzymatic digestion shows limited efficiency in removing proteins and lipids from natural samples. [50] Melanin is commonly described as a heterogeneous polymer formed by the oxidation of phenolic or indolic compounds, followed by the polymerization of intermediate phenols and their corresponding quinones. The chemical classification of melanin pigments includes eumelanin, pheomelanin, allomelanin, neuromelanin, and pyomelanin. The categorization of melanin pigment and a summary of common melanin types, their sources, and corresponding will be presented in figures 2 and 3. Figures 2 and 3 have been adapted from the original published diagrams. [52]

4.1. Eumelanin

Eumelanin is a major subclass of melanin pigments characterized by its black to dark brown coloration. It is predominantly found in human hair and skin, where it contributes to pigmentation and provides protection against ultraviolet radiation. Beyond its presence in humans, eumelanin is also biosynthesized by various microorganisms, including specific bacterial and fungal species. The biosynthetic pathway of eumelanin involves the oxidative conversion of the amino acid tyrosine (or phenylalanine via tyrosine) into L-3,4-dihydroxyphenylalanine (L-DOPA), which is further oxidized to dopachrome. Through a series of enzymatic and spontaneous reactions, dopachrome is ultimately transformed into eumelanin.[53, 54]

4.2. Pheomelanin

Pheomelanin is a major class of melanin pigments characterized by red to yellow coloration and is primarily responsible for the distinctive hue of red human hair. Its biosynthesis shares early steps with eumelanin, beginning with the formation of L-DOPA; however, the pathway diverges through the incorporation of cysteine into the polymer, a process termed cysteinylation. This incorporation introduces sulfur atoms into the pheomelanin structure, differentiating it chemically from eumelanin.[2]

4.3 Allomelanin

Allomelanin refers to a chemically diverse group of nitrogen-free melanin pigments commonly found in fungi and plants. Unlike eumelanin and pheomelanin, which originate from nitrogen-containing precursors, allomelanin is biosynthesized from various

metabolic intermediates such as dihydrofolate, homogentisic acid, and catechols. Notable subclasses include melanin derived from 1,8-dihydroxynaphthalene, formed via spontaneous polymerization of accumulated homogentisic acid, a by-product of tyrosine catabolism.[38, 55]

4.4 Neuromelanin

Neuromelanin is a dark, insoluble pigment located within catecholaminergic neurons of the brain, characterized by a core of pheomelanin surrounded by an outer layer of eumelanin. It is most abundant in humans, present in lower quantities in some non-human primates, and absent in many lower organisms. Neuromelanin has the capacity to bind transition metals such as iron, as well as other toxic substances, implicating it in neuroprotective functions and pathological processes including apoptosis, neurodegeneration, and Parkinson's disease.[56-58]

4.5 Pyomelanin

Pyomelanin is a brown-black phenolic polymer formed through the oxidative polymerization of homogentisic acid (HGA), which is an intermediate in the L-tyrosine degradation pathway. The biosynthesis of pyomelanin involves the accumulation and oxidation of HGA, often facilitated by microbial systems. Its polymerization mechanism is well characterized, involving radical-mediated processes. Although pyomelanin accumulation is associated with tissue damage in alkaptonuria due to the production of ROS, the polymer itself exhibits high chemical stability. Compared to human melanin, pyomelanin is photostable and highly resistant to thermal degradation.[59]

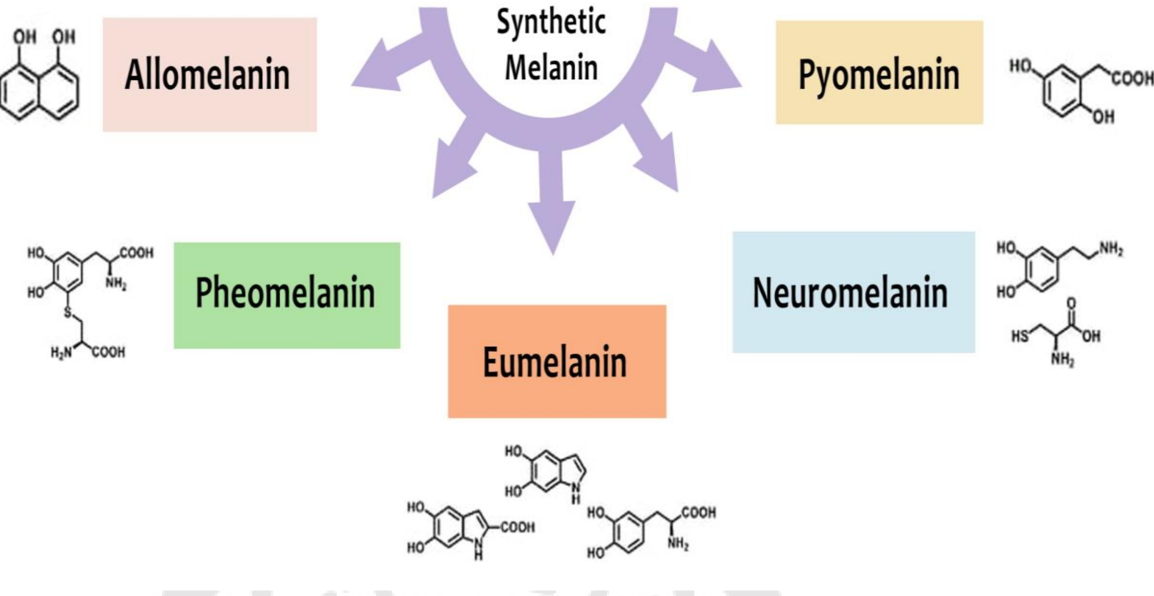


Figure 2: Types of melanin pigment.

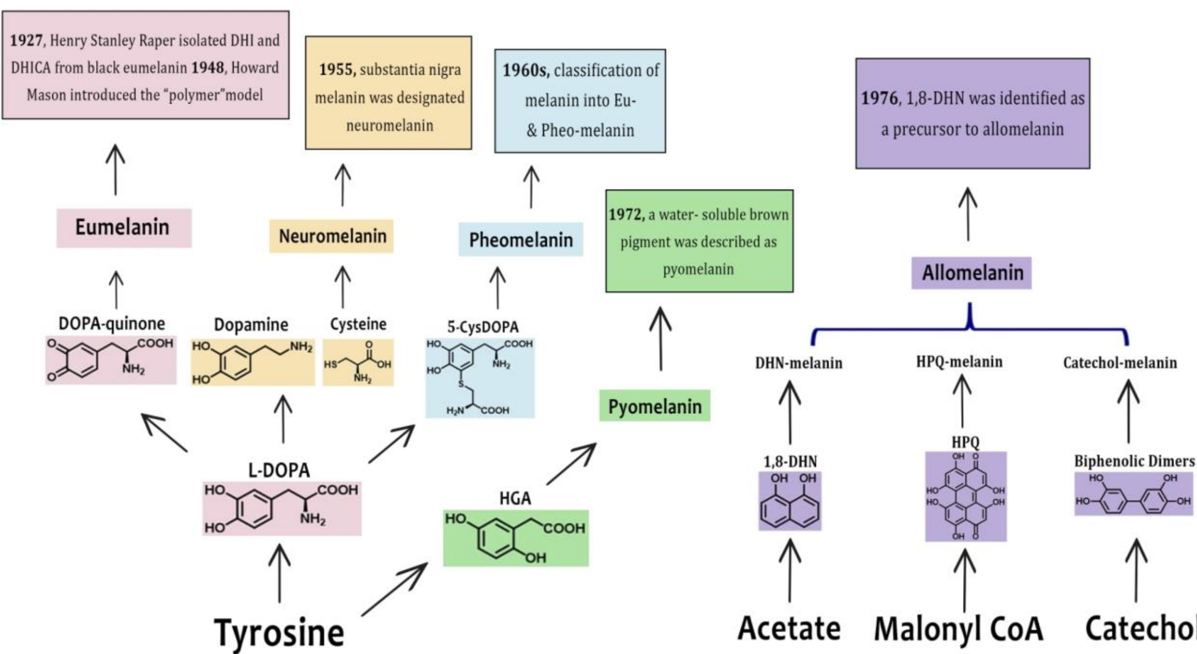


Figure 3: Summary of common melanin, sources, and their corresponding precursors

5. Physicochemical properties of melanin

Melanin is primarily insoluble in water, concentrated acids, and common organic solvents such as methanol, ethanol, benzene, and acetone. However, its solubility can vary depending on several factors, including its biological origin, degree of hydration, purity, and extent of polymerization. These factors collectively influence melanin's dissolution behavior under different environmental conditions. Additionally, melanin's solubility is affected by the ionization state of its functional groups and the presence of hydrophobic interactions between molecules. Lowering the pH of melanin solutions typically induces aggregation and sedimentation, whereas increasing the pH facilitates pigment disaggregation into smaller particles. Melanin is also susceptible to bleaching by oxidizing agents such as sodium hypochlorite and hydrogen peroxide, which degrade the pigment through nucleophilic attack, leading to ring-opening and formation of quinone epoxides. Electron microscopy studies have revealed that natural melanin consists of non-porous, spherical particles approximately 30 nm in diameter that tend to aggregate into larger clusters.[(4)] The UV-Vis absorption properties of melanin vary depending on its source. For example, melanin extracted from *S. glaucescens* exhibits a characteristic absorption spectrum with a peak at 250 nm within the ultraviolet (UV) range (200–700 nm), followed by a gradual decrease in absorption across the visible spectrum. Variations in absorption maxima are observed among different melanin sources: *Chroogomphus rutilus* melanin peaks at 212 nm, *Streptomyces bikiniensis* melanin at 230 nm, and melanin from *Actinoalloteichus sp.* MA-32 shows its highest peak at 300 nm.[(4)] These differences underscore the diversity in the UV-Vis spectral properties of melanin derived from distinct biological origins. FTIR analysis of melanin revealed a broad absorption band around 3273–3422 cm^{-1}, attributed to O-H and N-H stretching vibrations from carboxylic acid and phenolic groups, as well as amino functions in indolic and pyrrolic structures. Additional absorption peaks were observed at 2917, 2839, 1621, 1464, 1374, 1038, and 661 cm^{-1}, corresponding to various functional groups within the melanin structure.[(60, 61)]

6. Source of melanin

Melanin is derived from a wide range of biological and synthetic sources. Naturally, it is found in organisms such as marine animals, plants, and microorganisms. Among marine life, cephalopods, particularly cuttlefish, are considered one of the most prominent sources.[61] These animals expel melanin-rich ink as a defensive mechanism to escape predators. In plants, melanin can be extracted through chemical treatments targeting materials rich in flavonoid monomers or polymers.[62] These natural sources provide melanin with diverse structural and functional characteristics based on their origin.

In addition to natural sources, melanin can be synthesized through chemical methods. One common approach involves the auto-oxidation and polymerization of phenolic or indolic compounds like catechols, producing melanin polymers with UV-Vis absorption that increases from 700 nm to 250 nm.[63] Another synthetic route uses the enzyme tyrosinase to catalyze reactions involving substrates such as tyrosine or L-DOPA, mimicking the natural melanogenesis process.[62]

Microorganisms, especially certain bacteria and fungi, are also significant melanin producers. Their melanin synthesis is often associated with survival under environmental stress. Microbial melanin serves as a protective barrier against ultraviolet radiation, temperature extremes, oxidative damage, lytic enzymes, heavy metals, and antimicrobial compounds.[64, 65] This adaptive advantage underlines the potential of microbial sources for scalable melanin production in biotechnology and industry, due to both their resilience and ability to grow under controlled conditions.

7. Natural melanin and chemical synthetic melanin

Comparing synthetic melanin polymers with natural melanin, especially from an environmental and quality perspective, reveals distinct differences favoring the latter. Natural melanin is considered safer and more cost-effective than its artificial counterpart, particularly when considering proposed scenarios in the current review. However, there has been a growing interest in using microorganisms as an alternative to chemical

melanin production. Various efforts have been made to develop microbial strains capable of producing significant quantities of melanin pigment. Microbially derived melanin offers several advantages over synthetic and those obtained from animals and plants. Unlike other biological-based methods, microorganisms are not influenced by seasonal fluctuations during the production process.[66] Microorganisms possess the ability to modulate their biosynthetic pathways in response to variations in medium composition and growth conditions, rendering melanin pigment production largely independent of external meteorological factors that could otherwise influence yield. Furthermore, these organisms demonstrate rapid and facile growth without adverse effects and can utilize inexpensive substrates for cultivation. Such attributes make microbial synthesis a promising and efficient alternative method for melanin production.[38, 65]

An additional noteworthy advantage of microbial melanin lies in the diversity of microbial sources. Various reports have highlighted the existence of melanin in different bacterial and fungal species, each possessing distinct characteristics and potential applications. In laboratory settings, microbial species with melanogenic capabilities have been studied and optimized for the development of production processes. For instance, *Aeromonas media* and *Pseudomonas maltophilia*[67] have been identified as bacterial species capable of producing authentic 3,4-dihydroxyphenylalanine melanin pigment. Although both bacteria-derived melanin shares many biophysical properties, the yield of *A. media* was found to be significantly higher. Moreover, it demonstrated greater effectiveness in protecting a bioinsecticide against ultraviolet or solar radiation compared to the melanin produced by *P. maltophilia*.[67] In a separate study on Actinomycetes, a newly isolated strain, *S. glaucescens* NEAE-H, demonstrated the capacity to produce significant quantities of black melanin pigment when cultured on an optimized peptone-yeast extract iron agar medium. In Vitro trials revealed melanin has anticancer activity against skin cancer cell lines. These examples underscore the richness of microbial sources for melanin production and the potential for diverse applications stemming from different microbial melanin.[38] The list of bacteria and

Actinomycetes producing microbial melanin pigments will be presented in tables 2 and 3.[64]

Table 2: List of bacteria that produce microbial melanin pigments.

Microorganism	Objective	Main Finding	Reference
Bacillus thuringiensis	High-temperature-induced melanin formation	The bacterium synthesized melanin when cultivated with L-tyrosine at 42 °C.	Ruan et al.[68]
Burkholderia cepacia	Reduction in monocyte oxidative burst response	The antioxidant properties of melanin may offer protective benefits to melanin-producing organisms.	Zughaier et al.[69]
Klebsiella sp. GSK	Isolation and analysis of the physicochemical properties of melanin	High melanin yield (250 mg/L) was achieved in 3 days by a bacterium utilizing L-tyrosine as a precursor.	Sajjan et al.[70]
Pseudomonas stutzeri	Melanin biosynthesis by *P. stutzeri* derived from the red alga *Hypnea musciformis*	Approximately 6.7 g/L of melanin was produced by this strain even in the absence of L-tyrosine.	Ganesh Kumar et al.[71]

Table 3: List of Actinomycetes that produce microbial melanin pigments.

Microorganism	Objective	Main Finding	
Nocardiopsis dassonville	Extraction of functional melanin pigments from actinobacteria inhabiting marine environments	Melanin production from a marine source was first observed in *N. dassonvillei*.	Kamarudheen et al.(72)
Streptomyces cyaneus	Application of response surface methodology to optimize culture conditions for melanin production and gamma radiation-assisted synthesis of copper oxide nanoparticles	A high melanin yield (9.898 mg/mL) was achieved under optimized conditions, further amplified by 2.0% faba bean seed peel and gamma radiation-induced stimulation.	El-Batal et al.(73)
Streptomyces spp.	Melanin extraction and biochemical identification from *Streptomyces* cultures	Melanin biosynthesis using L-tyrosine or L-DOPA as precursors could serve as a useful taxonomic marker for *Streptomyces* species.	Dastager et al.(74)

8. Melanin production by microorganisms

Investigations into the control of melanin synthesis in various microorganisms have recently gained attention. Microorganisms provide several advantages for melanin production, such as being unaffected by seasonal growth limitations, exhibiting cost-efficiency, and presenting an environmentally sustainable option. Natural melanin derived from microorganisms is typically synthesized through the transformation of either tyrosine (via the DOPA pathway) or malonyl-coenzyme A (via the DHN pathway), each facilitated by distinct sets of enzymes. In the first pathway, which closely resembles mammalian melanin synthesis, the melanin precursor, tyrosine, undergoes conversion to L-DOPA and then to dopaquinone through the actions of tyrosinase and laccase. Dopaquinones, highly reactive molecules, undergo spontaneous oxidation and autopolymerization, leading to the formation of melanin.[44, 75, 76] The melanin produced through the DOPA pathway is known as DOPA-melanin or eumelanin. However, during the catabolic process, enzymatic imbalances or interruptions can lead to the accumulation of other hydroxylated aromatic compounds, such as HGA, resulting in the formation of different types of melanin.

In the second pathway, malonyl-coenzyme A is produced endogenously as a precursor. Through a sequential decarboxylative condensation of five malonyl-coenzyme A molecules, 1,3,6,8-tetrahydroxynaphthalene (THN) are formed. THN subsequently undergoes a series of reduction and dehydration steps to yield DHN. The polymerization of DHN then results in the formation of DHN-melanin as the final product. It is important to note that both pathways occur in bacteria and fungi. However, The DOPA pathway is primarily used by most bacteria and Basidiomycetous fungi for melanin synthesis, whereas the DHN pathway is characteristic of Ascomycetous fungi and certain imperfect fungi, including non-microscopic species such as *Tuber* spp. Figures 4 has been adapted from the original published diagrams.[77]

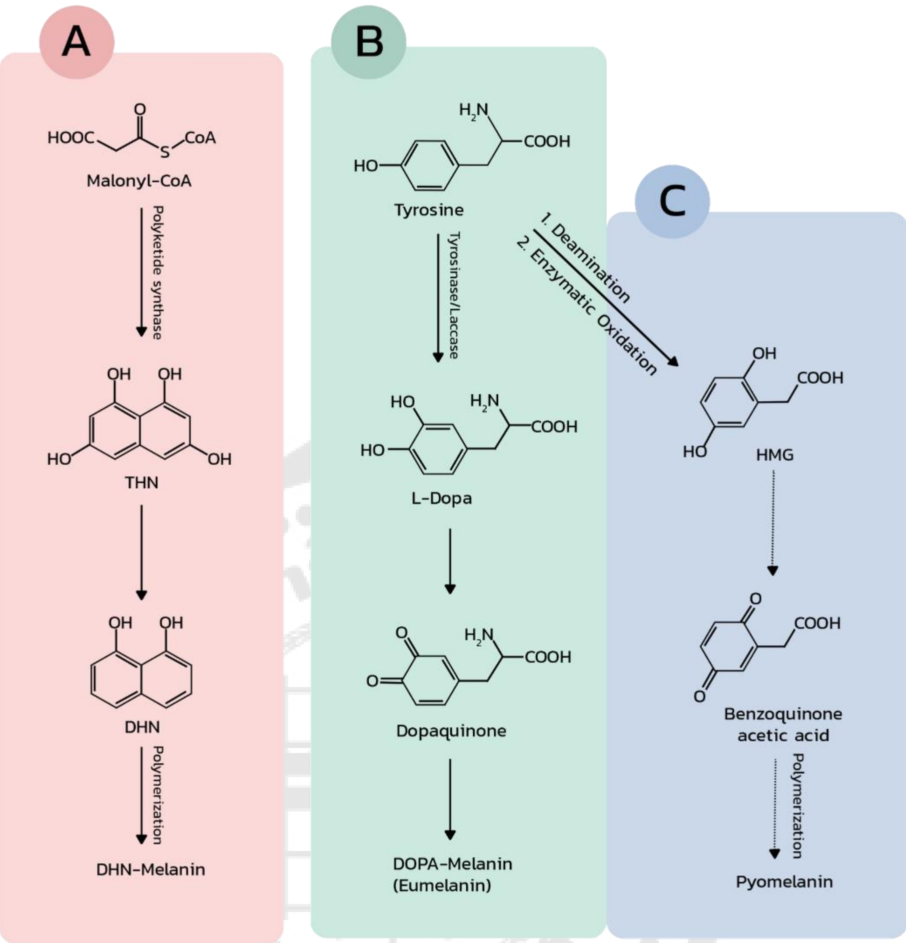


Figure 4: Diagram of melanin biosynthesis pathways in bacteria and fungi highlighting core chemical reactions.

A) DHN-pathway; B) DOPA-pathway; C) Enzymatic imbalances may result in alterations of metabolic pathways, consequently producing different types of melanin.

In fungi, melanin production via the DHN pathway is generally not favored for high yields, as the pigment is synthesized internally and remains firmly bound to the inner layer of the cell wall.[78] The strong binding of melanin to the cell wall renders its extraction highly challenging and may introduce artifacts due to the use of harsh chemicals. Conversely, melanogenesis through the DOPA pathway enables microorganisms to detoxify environmental phenolic compounds, including those produced during host defense responses. As a result, many microbes depend on

exogenous tyrosine or its derivatives as substrates for melanin synthesis. This extracellular mode of melanin production has garnered significant scientific interest, as it circumvents the need for aggressive extraction techniques.[79, 80] The optimization of microbial melanin production will be displayed in table 4.[77]

Table 4 Studies focused on optimization of microbial melanin production.

Microorganisms	Melanin type	Max. melanin production/g L^1,(Incubation time / days)	Tyrosine added	Metal ions added	Substrates	References
Actinoalloteichus sp. MA-32	DOPA	0.1(7)	Yes	Fe,Mg	Glycerol	Manivasagan et.al.[60]
Bacillus safensis	DHN	0.9(24 h)	None	None	Fruit waste extract	Tarangini and Mishra 2014[53]
Brevundimonas sp. SGJ	DOPA	0.8(54 h)	Yes	Cu	Tryptone	Surwase et.al.[81]
Nocardiopsis alba MSA10	DHN	3.4(7)	Yes	Nd	Sucrose	Kiran et.al. [82]
Pseudomonas sp. WH001 55	DHN	7.6(6)	Yes	None	Strach, yeast	Kiran et.al. [83]
P. stutzeri HMGM-7	DOPA	7.2(3)	Yes	None	Nutrient broth in sea water	Ganesh Kumar et.al. [71]
S. glaucescens NEAE-H	DOPA	0.4(6)	Yes	Fe	Protease peptone	El-Naggar and El-Ewasy[38]

Moreover, these characteristics provide significant control over the yield and type of resulting melanin. While tyrosine is recognized as the primary melanin substrate, other catecholamines like dopamine and norepinephrine can also serve as alternative substrates. It's worth noting that melanin produced from different substrates may exhibit structural differences due to various catabolic processes involving different enzymes. This variability offers opportunities for tuning the physicochemical properties and optimizing the production of microbial melanin.

Melanin formation depends on the regulation of enzymes involved in its synthesis, which is affected by diverse nutritional factors and physicochemical conditions. Common carbon and nitrogen sources, such as peptone, glucose, and yeast extract, are utilized in melanin production.[84-86] Agricultural residues like fruit waste extract, corn steep liquor, and wheat bran extract have also been explored to enhance yield and reduce production costs. Copper plays a crucial role in melanin production as a cofactor for laccases and tyrosinases.[87-89] Varying copper concentrations can lead to irregular pigmentation in fungi and bacteria.[90] Additionally, other metals, such as iron and nickel, have been found to enhance tyrosinase activity and melanin production.[91] Metal presence may induce stress responses in microbes, leading to melanin formation.[92] Stressors like high temperature, nutrient-poor growth media, and hyperosmotic pressure can also promote melanin synthesis.[93-95] Given the numerous factors affecting melanin biosynthesis, there is no single standardized culture medium or condition suitable for all melanogenic microorganisms. Instead, the formulation and proportion of medium components must be customized for each microbial strain. Environmental parameters such as temperature, pH, oxygen availability, aeration, light exposure, and irradiation during cultivation play critical roles in influencing cell growth and pigment production, necessitating careful optimization. Experimental designs employing statistical approaches like the Taguchi method, Plackett-Burman design, and Response Surface Methodology are frequently utilized to systematically assess the effects of these variables. Furthermore, genetic engineering strategies aimed at enhancing the innate melanogenic abilities or developing novel melanin-producing strains primarily through modification of genes

encoding melanin biosynthetic enzymes are commonly applied to improve microbial melanin yields. Among these, efforts often concentrate on upregulating tyrosinase activity.[38, 81, 96, 97]

9. Properties of melanin

In fungi and bacteria, melanin has traditionally been associated with the virulence of pathogenic species, serving as a protective agent against environmental stressors and host immune responses. However, recent scientific and technological advances have shifted attention toward their broader utility. Melanin pigments are now being explored as multifunctional materials in diverse sectors, including green technology, materials science, biomedicine, cosmetics, and environmental remediation. Within these fields, melanin contributes to sustainable innovations, advanced material development, targeted drug delivery, skincare formulations, and pollution mitigation, highlighting its growing relevance and versatility.[93, 98]

9.1 Antimicrobial activity

Crude melanin pigment has exhibited notable potential in combating infectious diseases. For example, melanin obtained from *Streptomyces* species has shown antibacterial activity against *L. vulgaris* and *E. coli*.[99] Furthermore, melanin complexes have been reported to possess inhibitory effects on pure cultures of *Candida albicans* and *Helicobacter pylori*, underscoring the promise of melanin-based therapeutics in addressing a broad spectrum of pathogenic infections.[100]

9.2 Anti-free radicals

Melanin demonstrates potent free radical scavenging activity and significant electron transfer capacity.[6] Electron spin resonance (ESR) spectroscopy studies have shown that melanin from *Cryptococcus neoformans* can accept electrons from free radical species in solution,[91] with similar ESR profiles observed in melanins from other fungal sources. Additionally, melanin from *C. neoformans* contributes to the reduction of Fe^{3+} to Fe^{2+},[91] promoting redox cycling by exporting electrons extracellularly to generate Fe^{2+}.

This mechanism helps sustain the reducing power of extracellular redox buffers.[101] Figures 5 has been adapted from the original published diagrams.[4]

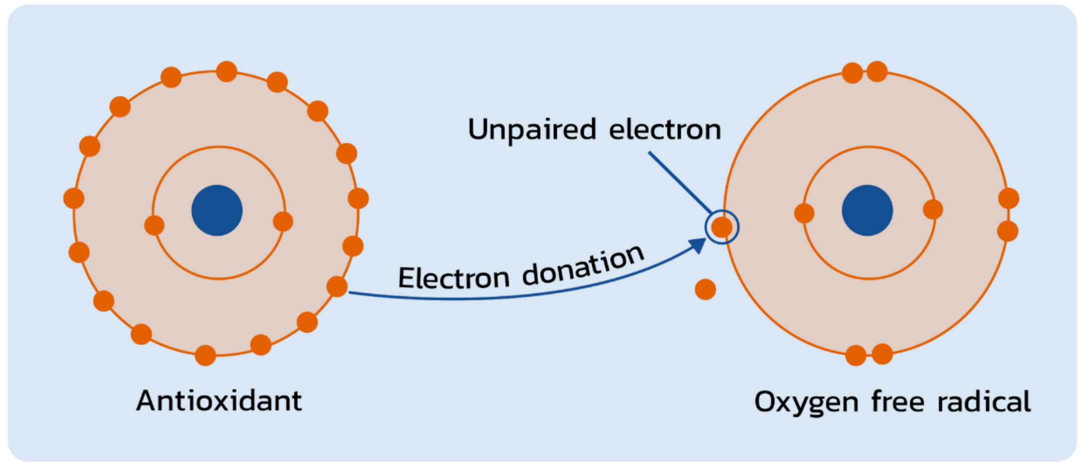


Figure 5: Mechanisms of melanin antioxidant activity

9.3 Anti-ultraviolet radiation

Melanin provides significant protection against UV radiation by absorbing a wide range of electromagnetic spectrum, thereby minimizing photo-induced cellular damage.[102] This photoprotective property supports its use in commercial applications such as sunscreens and optical lenses, including eyeglasses. In addition to human protection, melanin plays a crucial role in shielding certain fungi and bacteria from harmful solar, UV, and gamma radiation.[103] UV radiation reaching the Earth's surface ranges from 200 to 400 nm and is classified into three regions: UV-C (200–280 nm), UV-B (280–320 nm), and UV-A (320–400 nm). The potential for biological damage increases as the wavelength decreases, with UV exposure linked to sunburn, skin aging, immune suppression, and higher risk of skin cancer.[77] Although melanin in human skin cannot completely block UV rays, it significantly reduces their penetration to cell nuclei, thereby protecting against cellular damage.

9.4 Ability to chelate metal ions

Melanin exhibits a strong capacity to bind heavy metals commonly found in the environment. This ability arises from various functional groups within the melanin polymer such as carboxyl, phenolic, hydroxyl, and amine groups that act as binding and

biosorption sites for metal ions.[95] For example, in *C. neoformans*, melanized cells demonstrate greater resistance to the toxic effects of silver nitrate compared to non-melanized cells, likely due to melanin's chelation of silver ions which neutralizes its toxicity.[95]

9.5 Anti-apoptotic and anti-inflammatory

Inflammation is the body's inherent response to tissue damage or infection and is closely linked to antioxidative mechanisms. Melanin possesses antioxidant properties that mitigate the generation of reactive oxygen species (ROS), thereby attenuating the inflammatory response. Experimental studies involving the topical application of melanin on formalin-induced paw edema in rats have revealed significant anti-inflammatory effects, highlighting its potential as a therapeutic anti-inflammatory agent.[104] Melanin inhibits the activities of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, both critical in regulating inflammation. COX converts arachidonic acid into prostaglandins linked to inflammatory diseases, while LOX produces leukotrienes involved in inflammation pathology. The antioxidant effects of melanin correlate with decreased inflammation, reflecting their interconnected nature.[105]

Moreover, melanin shows anti-apoptotic effects and promotes cell viability. For example, water-insoluble melanin from *I. hispidus* can clear intracellular ROS, improving the survival of embryonic liver cells exposed to hydrogen peroxide and exhibiting strong antioxidant activity.[8] Similarly, melanin from *Ophiocordyceps sinensis* neutralizes ROS in various cells and inhibits hydrogen peroxide-induced apoptosis in HEK293 cells. Although the antioxidant activity of *O. sinensis* is mainly attributed to peptides and polysaccharides, cellular studies highlight melanin as a key component in combating intracellular ROS and their damaging effects, underscoring its biological significance.[10]

9.6 Radioprotective therapy

Melanin has shown significant effectiveness in reducing radiation-induced damage, especially in preclinical models. In mouse studies, administering eumelanin at 15 mg/kg body weight one hour before exposure to radiation markedly decreased hematopoietic injury by inhibiting splenic apoptosis, reducing hepatic oxidative stress, and restoring

immune balance.[106, 107] Additionally, melanin can selectively protect healthy tissues during radiotherapy without affecting the destruction of cancer cells, allowing for higher radiation doses to improve tumor control. Recent advances include melanin-coated silica nanoparticles designed to protect bone marrow from ionizing radiation during cancer treatment, highlighting promising biomedical applications.[108] Outside clinical use, melanin-based nanostructures like melanin nanoshells have been incorporated into protective fabrics and materials to provide passive defense against environmental or occupational radiation exposure.[106]

9.7 Nanoparticles

Melanin biopolymers including pigments derived from L-DOPA act as effective reducing and stabilizing agents in the synthesis of metallic nanostructures. For instance, L-DOPA melanin can convert silver nitrate and chloroauric acid into silver and gold nanoparticles respectively. The silver nanoparticles produced display strong antifungal activity and have potential as functional additives in paints.[109] Leveraging these properties, polydopamine nanoparticles, synthetic melanin analogs, have been investigated for biomedical applications due to their powerful antioxidant effects. In vitro studies using an oxygen-glucose deprivation model showed that mesenchymal stem cells (MSCs) pretreated with polydopamine nanoparticles significantly improved neuronal survival following hypoxic ischemic injury compared to treatment with MSCs or nanoparticles alone. This neuroprotective effect is linked to scavenging reactive oxygen species, inhibiting neuronal apoptosis by deactivating caspase 3, downregulating proapoptotic Bcl-2–associated X protein (Bax), and upregulating antiapoptotic B-cell lymphoma 2 (BCL-2 proteins). These results highlight the potential of polydopamine nanoparticles to enhance MSC mediated neuroprotection and offer promising therapeutic avenues for ischemic stroke.[110]

9.8 Food industry

Microbial melanin pigments have attracted considerable attention due to their beneficial biological activities in food applications. Melanin is increasingly used as a natural food colorant and nutritional supplement, providing an ecofriendly alternative to chemically

synthesized pigments that often cause environmental harm.[111] For example, melanin produced by the sponge-associated actinobacterium *Nocardiopsis alba* has been applied in the green synthesis of silver nanostructures, which show promise for sustainable food packaging materials.[82] These silver nanostructures exhibit broad antimicrobial activity against common foodborne pathogens, highlighting their potential for use in food packaging and as preservatives for stored fruits and food products.[77]

9.9 Other Uses

Melanin pigments perform diverse biological, environmental, and technological roles. Melanogenesis is vital for many free-living organisms, offering protection against ecological stresses and enhancing survival.[2] In plants, melanin reinforces cell walls, contributing to structural strength.[62] Eumelanin and pheomelanin are notable for their strong metal ion binding abilities, effectively chelating metals such as Ca(II), Zn(II), Cu(II), and Fe(III).[112, 113] This capacity enables melanin to sequester toxins, heavy metals, and other harmful substances, regulating their cellular uptake.[98, 114] Microbial melanin also aids detoxification by inhibiting mycotoxin secretion and has potential as a bioinsecticide in agriculture.[67] Industrially, melanin finds use in bioplastics, paints, and varnishes and shows promise in bioelectronics as a natural semiconductor material.[115, 116]

10. Future applications of melanin

Melanin, natural biopolymers contributing to various biological processes and environmental protection, have gained prominence in recent years for their applications in organic semiconductors, bioelectronics, drug delivery, photoprotection, and environmental bioremediation. Despite substantial progress in these fields, real-world applications of melanin remain limited, primarily due to the constrained and costly supply of natural melanin. However, recent biotechnological strides have enabled large-scale production of microbial melanin, offering a potential replacement for existing commercial melanin sources.[117] From a physicochemical perspective, melanin acts as a natural sunscreen, absorbing a broad spectrum of UV-visible light and serving as a potent

antioxidant. Its hydration-dependent semiconductor-like behavior positions it as a viable component for organic electronic devices.[118, 119] Microbial melanin, with added benefits of bioavailability, biocompatibility, and biodegradability, It has emerged as a promising candidate for various biomedical applications, including implantable devices.[120] In a separate application, melanin has been employed for the eco-friendly synthesis of silver nanostructures, exhibiting broad-spectrum antimicrobial activity against foodborne pathogens and showing promise for applications in the food and health industries.[82, 121] Dermal and cosmetic applications capitalize on melanin's properties, utilizing it for sunscreen formulations and hair dyeing. Additionally, melanin has metal-chelating capabilities to find applications in environmental contexts. Incorporating natural melanin with polymers like polycaprolactone and polyurethane has shown promise in removing up to 94% of Pb (II) in water systems.[77]

Recent research in melanin biosynthesis has emphasized the significance of statistical methods like Plackett-Burman designs (PBD) and response surface methodology (RSM) for optimizing production conditions. These approaches involve the screening of influential factors and subsequent detailed optimization, often employing methods such as Central composite design (CCD).[122, 123] Despite limited research in the past decade, these statistical methods have proven effective in significantly increasing melanin production. The integration of artificial intelligence (AI), particularly artificial neural networks (ANN), holds promise for further optimization by mimicking human brain functions, yet literature on its application in melanin production optimization is notably lacking.[124, 125] Another strategy, the economy substrate approach, advocates for the use of agro-industrial byproducts as a sustainable and cost-effective medium for melanin production, emphasizing the need for studies directed towards semi-industrial production with simplified cultivation processes.[3] The recombinant microbes' approach involves genetic engineering to enhance melanin yield, utilizing microorganisms like *E. coli* and *S. glaucescens.*[38] Finally, the green nano-melanin approach explores the eco-friendly synthesis of melanin nanoparticles (NPs) with advanced capabilities, including drug delivery and photothermal therapy.[49, 126] Despite these promising avenues, the

commercialization of melanin applications remains in the developmental stage, highlighting the ongoing transition towards sustainable materials in microbial melanin research that is yet to reach its full potential.

Consequently, to investigate the physicochemical and biological activity of *S. spectabilis* strains AQ2 and CQ2, with the objective of identifying a potential new source for melanin production. If the melanin derived from *S. spectabilis* strains AQ2 and CQ2 exhibits biological activity aligned with the research objectives, either comprehensively or in specific aspects, it could pave the way for the development of materials that are destined to be sustainable in the future. Figure 6 has been adapted from the original published diagrams.[77]

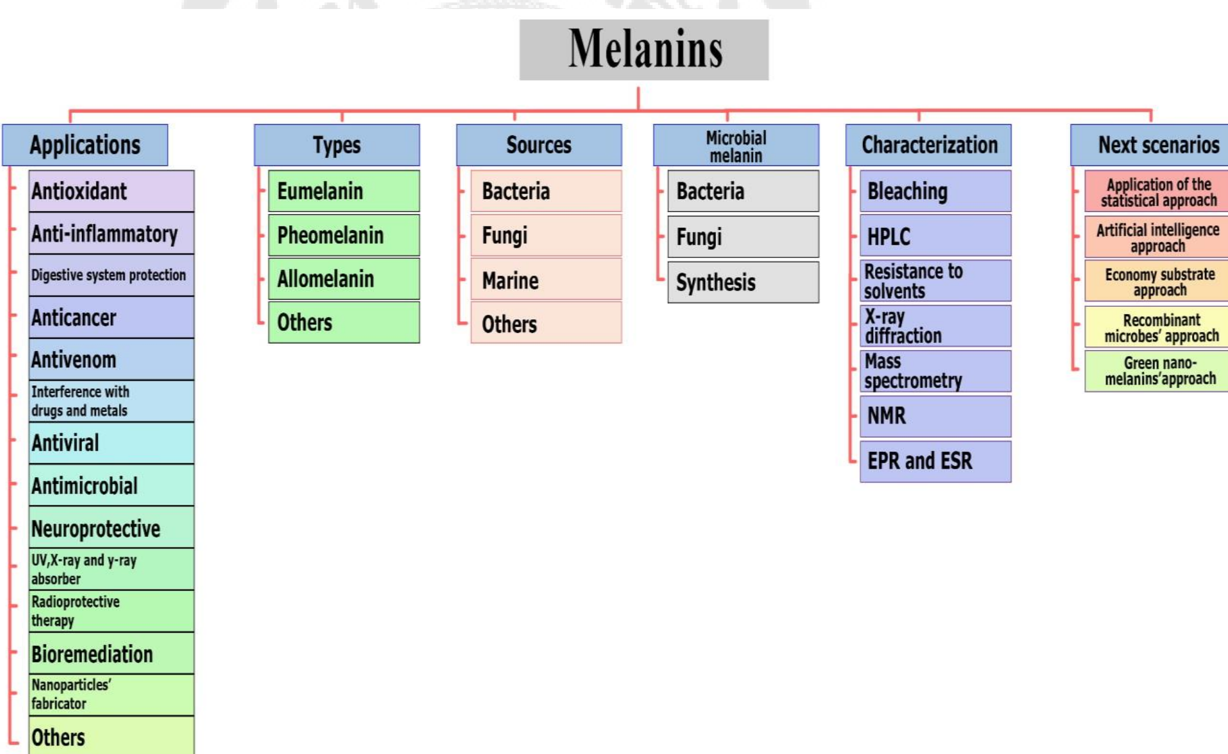


Figure 6: Diagrammatic representation of the various applications, microbial origins, and potential future directions for melanin production.

CHAPTER 3

MATERIALS AND METHODS

Materials

1. Analytical balances (Adam, England)

2. Centrifuge (Sorvall^{TM}, Germany)

3. Fourier transform infrared spectrometer (PerkinElmer, USA)

4. Filter paper disc

5. Incubator (POL-EKO APARATURA, Poland)

6. Microplate reader (Bio-Tek, USA)

7. pH Meter (OHAUS, USA)

8. Spectrophotometer (NEXBIO, Thailand)

9. Shaker incubator (BIOBASE, China)

10. UV-Visible spectrophotometer (BIOBASE, China)

11. Vilber Lourmat UV-Crosslinker (BIO-LINK, Germany)

Chemicals

1. L-ascorbic acid, Ascorbic acid (Sigma-Aldrich, Switzerland)

2. Dimethyl sulfoxide (Sigma-Aldrich, USA)

3. Dulbecco's modified eagle medium (GIBCO, USA)

4. 2',7'-Dichlorodihydrofluorescein diacetate, DCFH-DA (Sigma-Aldrich, USA)

5. 2,2-diphenyl-1-picrylhydrazyl, DPPH (Sigma-Aldrich, Germany)

6. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid,Trolox (Sigma-Aldrich, Switzerland)

7. 70%,95% Ethanol, EtOH

8. Fetal bovine serum, FBS (HyClone^{TM}, USA)

9. Hydrochloric acid, HCl

10. Nonessential amino acid (Millipore Sigma, USA)

11. 95% Methanol, MeOH

12. Potato dextrose agar, PDA (HIMEDIA, India)

13. Potato dextrose broth, PDB (HIMEDIA, India)

14. Penicillin-streptomycin (HyClone^{TM}, Austria)

15. Dulbecco’s phosphate-buffered saline, DPBS (Caisson, USA)

16. Resazurin solution (Sigma-Aldrich, USA)

17. Synthetic melanin (Sigma Aldrich, United Kingdom)

18. Sodium hydroxide, NaOH

19. Ethylenediaminetetraacetic acid, EDTA

20. Trypsin (HiMedia, India)

21. 0.01% Tween 80 (AMRESCO, Canada)

22. Manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$)

23. Iron (II) sulfate heptahydrate ($FeSO_4 \cdot 7H2O$)

24. Copper (II) sulfate ($CuSO_4$)

25. Yeast malt agar, YMA (HIMEDIA, India)

Bacteria, fungi, and cell line

1. *S. spectabilis* AQ2 (soil isolated strain)

2. *S. spectabilis* CQ2 (soil isolated strain)

3. *C. albicans*

4. *E. coli* (ATCC 25922)

5. *S. aureus* (ATCC 25423)

6. Human keratinocyte cell line (HaCaT, Germany)

7. Mouse melanocyte cell line (B16F10, ATCC)

Antibiotic

1. Ampicillin disc µL (Le Pont de Claix, France)

2. Chloramphenicol disc 30 µL (Spark, USA)

3. Tetracycline disc 30 µL (Le Pont de Claix, France)

Methods

1. Cultivation of *S. spectabilis* strains

The cultivation of *S. spectabilis* strains was performed as previously describe.[15] *S. spectabilis* strains AQ2 and CQ2, which were isolated from soil samples and preserved in glycerol stock at -20°C, will first be reactivated by cultivation on yeast malt agar (YMA) medium at 28°C for 6 days. This activation step allows the bacterial strains to recover and proliferate, ensuring optimal physiological conditions for subsequent cultivation. Following activation, the strains will be harvested with 0.01% tween 80 and transferred into potato dextrose broth (PDB) and incubated at 28°C with continuous agitation at 200 rpm for 7 to 14 days. This setup facilitates bacterial growth and metabolic activity under conditions favorable for melanin biosynthesis. In addition to culturing, an important aspect of the experimental protocol involves quantifying the bacterial inoculum to evaluate the relationship between cell density and melanin production. Bacterial biomass will be assessed by drying the cultures and measuring the dry weight, which will be compared with the corresponding melanin yield at defined inoculum levels. Accurate determination of inoculum size contributes to a better understanding of melanin biosynthesis under defined conditions and facilitates subsequent analytical procedures related to pigment characterization.

2. Melanin extraction

After the cultivation process, the supernatant solution will be collected by centrifugation at 4500 × g for 15 minutes to separate the liquid from the microbial biomass. Following this, a 6 M Hydrochloric acid (HCl) solution will be added to the supernatant to adjust the pH to 2. The supernatant will then be incubated at 28°C for 24 hours to allow for the precipitation of crude melanin. After incubation, the crude melanin will be collected by centrifugation at 4500 × g for 15 minutes. The precipitate will then be washed thoroughly with deionized water (dH_2O) to remove any remaining impurities. This washing process will be repeated four times to ensure the removal of unwanted proteins, carbohydrates, lipids, and other contaminants that may have co-precipitated with the melanin. Once purification is complete, the resulting pure melanin will be dried at 70°C for 2 days in an

oven to ensure complete moisture evaporation. Finally, the amount of purified melanin obtained will be quantified using high-precision analytical balances. This thorough process ensures the isolation of high-quality melanin, free from impurities, and ready for further analysis and characterization. To quantify the bacterial biomass, after the melanin extraction process, the bacterial culture will be subjected to centrifugation to separate the cells from the culture medium. The resulting pellet will be transferred and dried at 70°C for 24 hours in an oven to ensure complete dehydration. Once the biomass is dried, it will be accurately weighed using an analytical balance. The weight of the dried biomass will provide an estimate of the bacterial biomass, which will then be compared with the amount of melanin produced.

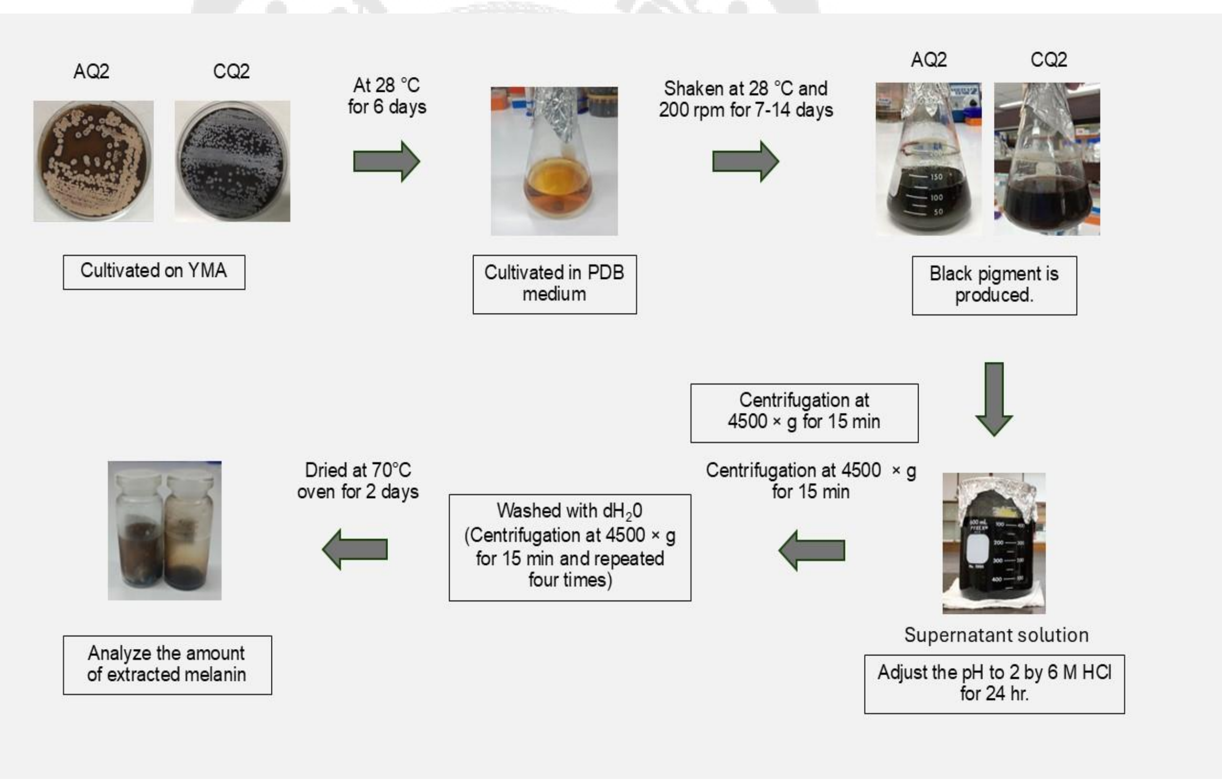


Figure 7: The melanin extraction process *from S. spectabilis* strains AQ2 and CQ2

3. Spectroscopy analysis

3.1 Ultraviolet-visible spectroscopy

The extracted melanin from *S. spectabilis* strains AQ2 and CQ2 will be dissolved in DMSO, and its concentration will be adjusted to 1 mg/mL by adding 95% ethanol (EtOH). The UV-visible absorption spectrum will be recorded in the wavelength range of 200–900 nm using a UV-visible spectrophotometer. Synthetic melanin (0.5 mg/mL) will be used as a standard for comparison. This analysis will provide valuable data on the absorbance characteristics of the melanin, enabling the identification and quantification of specific absorption peaks within this range.

3.2 Fourier transform infrared spectroscopy

The extracted melanin from *S. spectabilis* strains AQ2 and CQ2 will be analyzed using Fourier transform infrared spectroscopy in the scanning range of 4500–400 cm^{-1}. Synthetic melanin will be used as a standard for comparison. This technique will provide information on the infrared absorption bands corresponding to functional groups and molecular composition, thereby offering insights into the chemical structure of the melanin sample.

4. Biological activity

4.1 Antibacterial activity of melanin

The antibacterial activity of *S. spectabilis* strains AQ2 and CQ2 melanin against bacteria, such as *E. coli* and *S. aureus*, will be investigated. The antibacterial assays will be performed using the disc diffusion method. The stock solutions of melanin AQ2 and CQ2 will be prepared by weighing the melanin using an analytical balance and dissolving it in dimethyl sulfoxide (DMSO). A sonicator may be used to facilitate the dissolution of melanin. Once the melanin is completely dissolved, its concentration will be adjusted to 12.5, 25, and 50 mg/mL for testing. A volume of 1 mL of bacterial suspension (0.5 McFarland, 1.5×10^8 CFU/mL) will be prepared by culturing *E. coli* and *S. aureus* for 2 days on nutrient agar (NA) at 28°C. After incubation, the bacterial suspension will be adjusted to a concentration of 0.5 McF in 0.01% Tween 80 using a

spectrophotometer at OD = 600. Next, 4 mL of nutrient agar will be poured into each well of a six-well plate, followed by the addition of 1 mL of the bacterial suspension containing 0.01% Tween 80. After the agar solidifies, an antibiotic disc will be placed at the center of each well. The melanin AQ2 and CQ2 at concentrations of 12.5, 25, and 50 mg/mL will be applied to the disc with 20 µL per disk. Ampicillin 10µL, Chloramphenicol 30µL, and Tetracycline 30µL will be used as positive controls. The plates will then be incubated at 28°C for 24 hours. After incubation, antibacterial activity will be evaluated by observing the clear zones around the wells.

4.2 Free radical scavenging activity of melanin

The antioxidant activity was determined by DPPH free radical scavenging as previously described.[127] The ability of *S. spectabilis* strains AQ2 and CQ2 melanin to scavenge free radicals will be assessed using the DPPH assay. Different concentrations of AQ2 and CQ2 melanin will be prepared in DMSO at 1, 2, 3, 4, and 5 mg/mL. Trolox and ascorbic acid, serving as positive controls, will be prepared at concentrations of 0.025, 0.05, 0.075, 0.1, and 0.125 mg/mL.

To prepare the DPPH stock solution, DPPH powder will be carefully weighed and transferred into a 250 mL bottle using a small spoon. Approximately 100 mL of methanol will be added, and the mixture will be thoroughly mixed to produce a deep purple solution. The optical density (OD) at 515 nm will be measured using a fluorescent microplate reader to ensure it falls between 1.0 and 1.2. If the OD exceeds 1.2, the solution will be diluted with methanol; if the OD is below 1.0, additional DPPH will be added to adjust the concentration.

Subsequently, 180 µL of the DPPH solution will be transferred to each well of a 96-well plate, followed by 20 µL of the test sample (melanin). The plate will be incubated in the dark for 15 minutes. After incubation, the absorbance at 515 nm will be measured to determine the free radical scavenging activity. Methanol will serve as a negative control, while ascorbic acid will be used as a positive control for comparison in this experiment.

The ability to scavenge the DPPH radical will be calculated by using the following equation:

Scavenging rate (%) = [(A0 – A1)/A0] × 100

where A0: DPPH + Methanol.; A1: DPPH + melanin.

4.3 Anti-ultraviolet radiation

4.3.1 UV absorption capacity of melanin variant concentration

The melanin extracted from *S. spectabilis* strains AQ2 and CQ2 will be dissolved in DMSO, and its concentration will be adjusted using 95% EtOH to achieve final concentrations of 12.5, 25, 50, 100, and 200 µg/mL. UV absorption will be measured at selected UV-A wavelengths (315, 330, 360, and 400 nm) and UV-B wavelengths (290, 300, and 315 nm) using a UV-Vis spectrophotometer. This analysis will help determine the optimal melanin concentration for subsequent cell culture experiments.

4.3.2 Cytotoxicity evaluation of melanin on HaCaT and B16F10 cell lines

The Cytotoxicity activity was determined by resazurin assay as previously describe.[128] The cell lines utillzed in this study include the human keratinocyte cell line (HaCaT, Germany) and the mouse melanocyte cell line (B16F10, ATCC). These cell lines will be cultured in a growth medium containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 IU/mL streptomycin, and non-essential amino acids. The cultures will be maintained in a humidified incubator at 37°C with 5% CO_2 to support optimal cell growth. Once the cells reach approximately 80% confluence or form a confluent monolayer on the culture vessel surface, they will be detached using 0.125% trypsin and resuspended. The cells will then be collected, counted, and sub-cultured into new culture flasks containing 20 mL of complete medium for further expansion and maintenance. This procedure ensures consistent cell viability and readiness for experimental assays.

To evaluate the cytotoxicity of melanin, cells at approximately 80% confluence will be gently washed with sterile Dulbecco’s phosphate-buffered saline (DPBS), detached with 0.125% trypsin, and seeded onto 96-well plates at a density of 2×10^4 cells per well for B16F10 and 5×10^4 cells per well for HaCaT. After cell attachment, different concentrations of melanin will be applied to the wells and incubated for 24

hours. DMSO-treated wells will serve as control groups. After a 2-hour incubation with resazurin solution, cell viability will be measured using a fluorescence microplate reader with an excitation wavelength of 530 nm and emission at 590 nm.

4.3.3 Cell viability assay after UV exposure in HaCaT and B16F10 cells

The cell viability activity was determined by resazurin assay as previously describe.[128] For the determination of UV radiation effects on cells, when the cells reach approximately 80% confluence, they will be gently washed with sterile DPBS, detached using 0.125% trypsin, and seeded onto 6-well plates at a density of 6×10^5 cells per well for B16F10 and 1×10^6 cells per well for HaCaT. Two different concentrations of melanin will be applied to the cells and incubated for 24 hours. DMSO-treated cells will serve as a control. After melanin treatment, the cells will be directly irradiated with UV-A at 800 mJ/cm² and UV-B at 80 mJ/cm² using a Vilber Lourmat UV-Crosslinker. Following UV exposure, the cells will be incubated for an additional 6 hours. Cell viability will then be assessed using the resazurin assay, and fluorescence will be measured at 530 nm (excitation) and 590 nm (emission) using a microplate reader.

4.3.4 Intracellular ROS level after UV exposure in HaCaT and B16F10 cells

The intracellular ROS level was determined by DCFH-DA as previously describe.[129] To determine the levels of ROS in cells following UV radiation treatment, cells will first be cultured to approximately 80% confluence. They will then be gently washed with sterile DPBS, detached using 0.125% trypsin, and seeded onto 6-well plates at a density of 6×10^5 cells per well for B16F10 and 1×10^6 cells per well for HaCaT. Two different concentrations of melanin will be applied to the cells and incubated for 24 hours. DMSO-treated cells will serve as a control. Prior to UV radiation exposure, cells will be incubated with 10 µM of DCFH-DA in growth medium for 20 minutes in the dark to allow for intracellular probe loading. After incubation, cells will be washed again with DPBS to remove excess probe. The cells will then be exposed to UV-A at 800 mJ/cm² and UV-B at 80 mJ/cm² using a Vilber Lourmat UV-Crosslinker. Following irradiation, intracellular ROS levels will be quantified by measuring fluorescence at 485 nm (excitation) and 535 nm (emission) using a microplate reader.[10]

4.4 Ability to chelate metal ions

The ability to chelate metal ions was determined by viability assay as previously describe.[10] Metal salt solutions (200 mM $MnCl_2 \cdot 4H_2O$, 300 mM $FeSO_4 \cdot 7H_2O$, and 60 mM $CuSO_4$) will be prepared using distilled water. These metal salt solutions will be individually combined with melanin powder (1 mg and 5 mg) in separate reaction tubes. Each mixture will be thoroughly vortexed and incubated at 25 °C for 24 hours to allow interaction between the metal ions and melanin. After incubation, the mixtures will be centrifuged at 10,000 × g for 10 minutes. The resulting supernatants, containing the soluble metal–melanin complexes, will be carefully collected.

Supernatants from both the pre-incubation (T1) and post-incubation (T2) stages will be mixed with Potato dextrose agar (PDA) at a volume ratio of 1:15. The mixture will then be poured and spread evenly over PDA plates. *C. albicans* (OD600 = 1, 1×10^8 CFU/mL) will be inoculated onto the surface of the plates to assess the effects of metal ions on fungal growth. The plates will be incubated under suitable conditions, and a 10-fold dilution of the supernatant solutions will also be tested to evaluate the influence of concentration on fungal stress response. This experimental setup enables the investigation of metal ion-induced stress on *C. albicans* and the potential role of melanin in mitigating this stress.

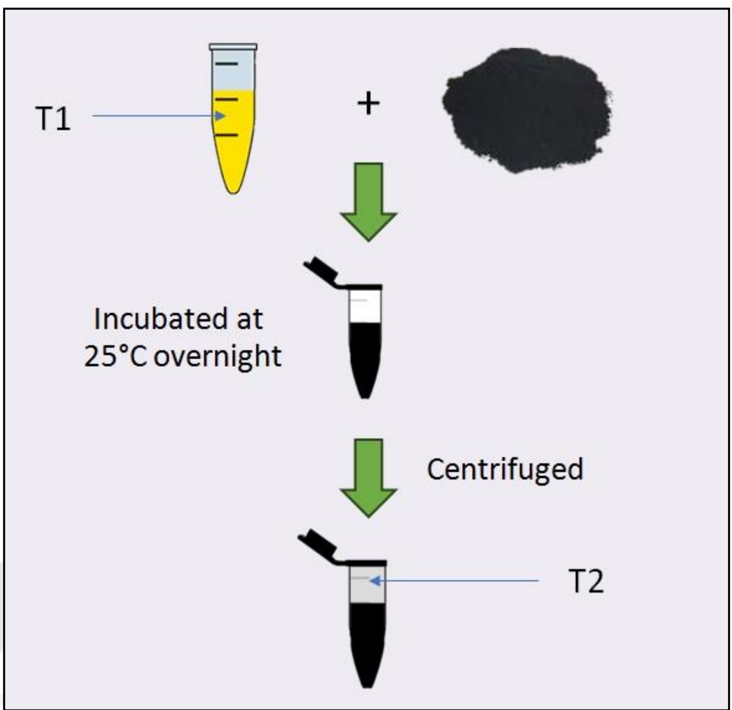


Figure 8: Schematic representation of the metal ion chelation assay

Statistical analysis

The experimental data will be analyzed utilizing GraphPad 9 Software (GraphPad Software, LLC). Each experiment underwent four repetitions to compute the average results. The measured data will be presented in the form of mean ± standard error of the mean ($\bar{x}$ ± SD) for DPPH assay. In addition, the measured data will be presented in the form of mean ± standard error of the mean ($\bar{x}$ ± SEM), providing information on both the central tendency and the variability of the results. To assess the significance of the data, a one-way analysis of variance (ANOVA) followed by Tukey's test will be employed, and a significance level of $P < 0.05$ will be considered indicative of a statistically significant difference.

CHAPTER 4
RESULTS

1. Melanin production of *S. spectabilis* strains AQ2 and CQ2

The results of the cultivation of *S. spectabilis strains AQ2 and CQ2* over a 7-day period for AQ2 and a 14-day period for CQ2 demonstrate that both strains produced melanin in quantities that were quite similar. In the case of the AQ2 strain, when cultured in 1000 mL of PDB medium, the biomass amounted to 4.234 grams, which resulted in the production of 0.03756 g of extract, corresponding to a yield of 0.89%. On the other hand, the CQ2 strain, cultured under the same conditions for 14 days, produced a biomass of 6.14 grams and yielded 0.05188 g of extract, which is equivalent to a 0.84% yield. When comparing the two strains

S. spectabilis AQ2

S. spectabilis CQ2

Figure 9: Colony morphology of *S. spectabilis* grown on YMA agar

Table 5: Comparison of melanin production and yield from different strains

Microorganisms	Culture Media	Max. melanin production/g L^1,(Incubation time / days)	Percentage Yield	Biomass	(mg/g Biomass)	(mg/mL Culture Broth)
S. spectabilis AQ2	PDB	0.03756 g (7-8)	0.89%	4.23 g	8.87 mg/g	0.0376 mg/mL
S. spectabilis CQ2	PDB	0.05188 g (12-14)	0.84%	6.14 g	8.46 mg/g	0.0518 mg/mL

From the table, *S. spectabilis* AQ2 produces melanin at a higher rate per gram of biomass (8.87 mg/g), despite having a lower total biomass (4.234 g). In contrast, *S. spectabilis* CQ2 exhibits a higher biomass (6.14 g) but produces melanin at a slightly lower rate (8.46 mg/g). Although CQ2 yields more biomass, AQ2 demonstrates greater efficiency in melanin production per unit biomass. When comparing production across different culture volumes, CQ2 consistently produces higher total melanin amounts, such as 5.18 mg from 100 mL of medium, compared to 3.76 mg from AQ2. However, CQ2 requires a longer cultivation period (12–14 days) compared to AQ2 (7–8 days). These results suggest that AQ2 is more efficient and faster in melanin production, whereas CQ2 may be more suitable for large-scale production requiring higher biomass, despite the extended cultivation time. Ultimately, the choice between the two strains depends on specific production goals, balancing efficiency, yield, and time.

2. Physicochemical properties

2.1 Fourier transform infrared spectroscopy

The experimental results revealed that the melanin extracted from both *S. spectabilis* strains exhibited chemical characteristics closely resembling those of synthetic melanin used as a standard. Fourier transform infrared spectroscopy analysis showed absorption bands around 3200–3300 cm^{-1}, indicating the presence of –NH groups hydrogen-bonded with –OH groups within the indole ring, a fundamental component of melanin. This absorption pattern suggests hydrogen bonding, which contributes to the stability of the melanin structure. Additionally, absorption peaks at 1620–1650 cm^{-1} correspond to asymmetric deformation vibrations associated with C=O and N–H groups in the amide moiety, indicating the presence of peptide bonds and supporting the complex polymeric nature of melanin. Moreover, absorption bands in the 600–800 cm^{-1} region were observed, characteristic of aromatic rings substituted with a conjugated system involving low-frequency aromatic hydrogen. This pattern is indicative of melanin's aromatic backbone and its highly conjugated system, which contribute to its light absorption properties. Collectively, these spectroscopic features confirm that the chemical structure of melanin extracted from *S. spectabilis* is remarkably similar to that of synthetic melanin.

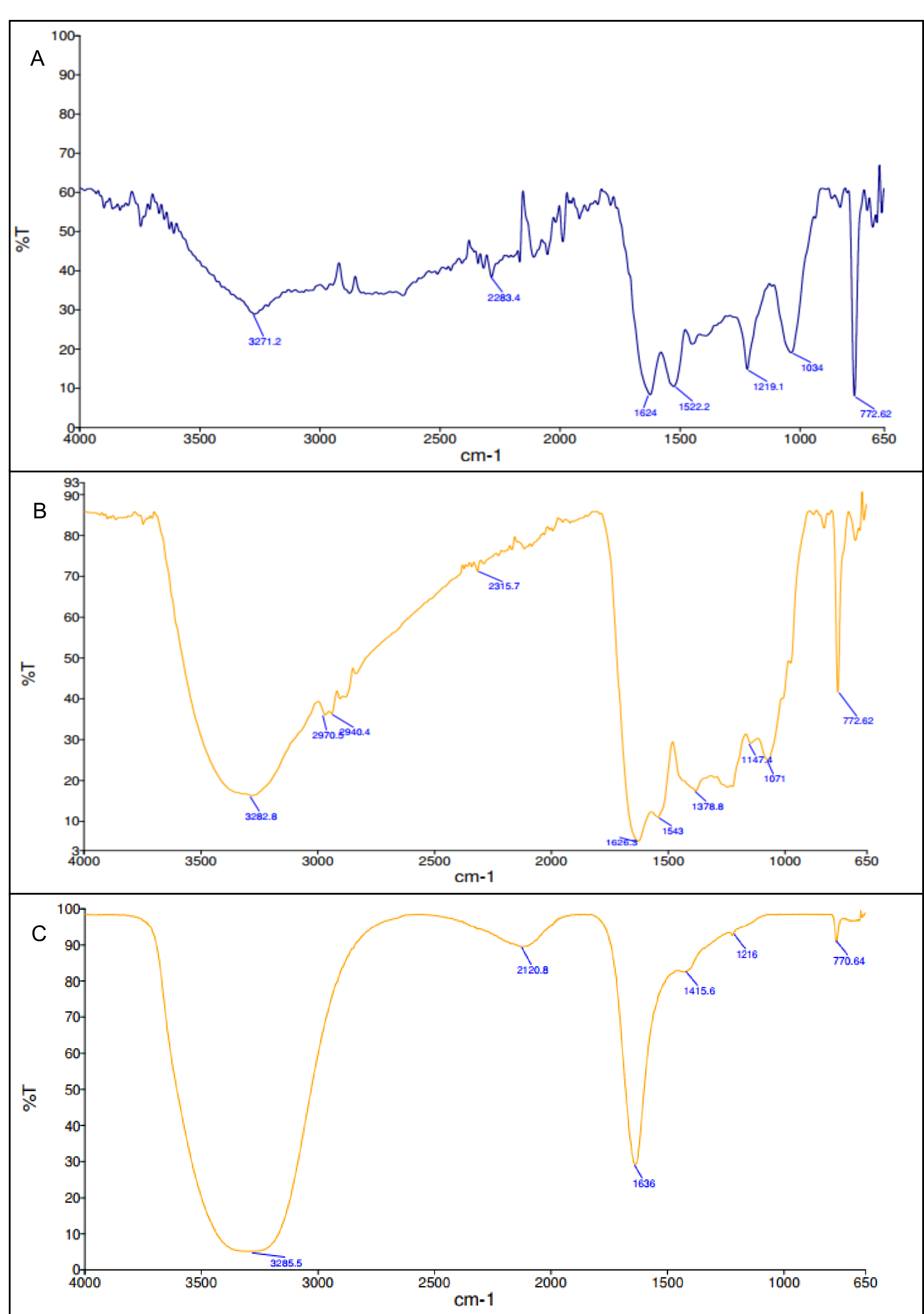


Figure 10: Fourier transform infrared spectrometer analysis of melanin

A: Melanin from *S. spectabilis* AQ2 B: Melanin from *S. spectabilis* CQ2

C: Synthetic melanin (Control)

2.2 Ultraviolet-visible spectroscopy

The experimental results on the UV absorption properties of melanin extracted from *S. spectabilis* strains AQ2 and CQ2 provided valuable insights into the characteristics of naturally derived melanin. The findings revealed that melanin from *S. spectabilis* exhibited UV absorption behavior strikingly similar to that of synthetic melanin, which was used as a standard for comparison. Specifically, the UV absorption spectra showed distinct peaks within the wavelength range of approximately 230–250 nm, closely matching the absorption pattern observed in synthetic melanin. This similarity suggests that the natural melanin extracted from *S. spectabilis* shares key structural features with its synthetic counterpart. These results underscore the potential of *S. spectabilis* derived melanin as a viable natural alternative to synthetic melanin, particularly for applications requiring UV protection.

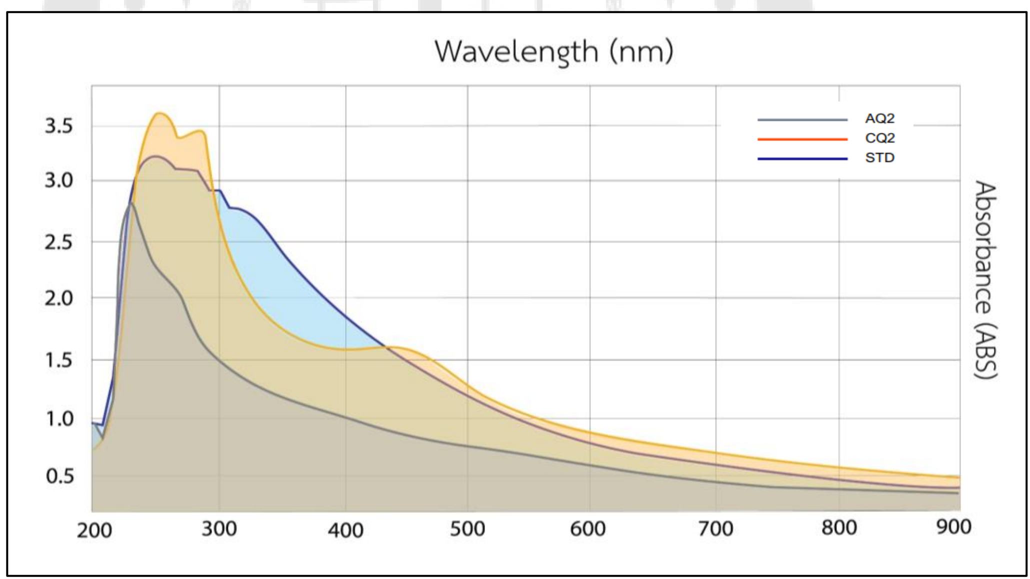


Figure 11: Ultraviolet-visible spectroscopy of melanin extracted

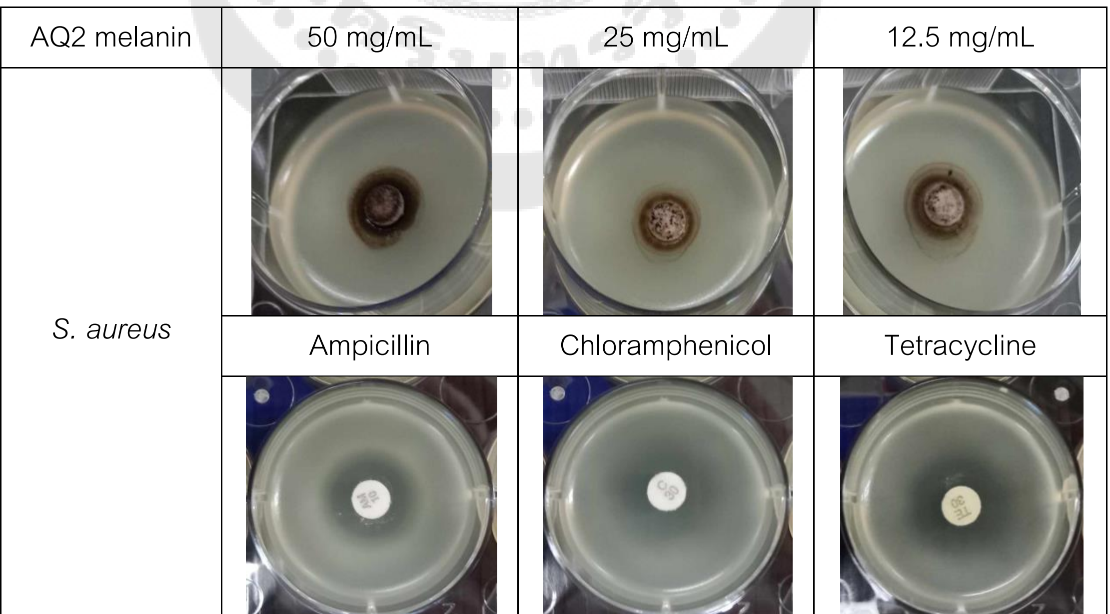
3. Biological activity

3.1 Antibacterial activity

The experimental results evaluating the antibacterial activity of melanin extracted from *S. spectabilis* strains AQ2 and CQ2 at concentrations of 12.5, 25, and 50 mg/mL against *S. aureus* and *E. coli* showed that melanin from both strains and exhibited no antibacterial effects. No inhibition of bacterial growth was observed at any tested concentration. In contrast, the positive controls Ampicillin, Chloramphenicol, and Tetracycline produced clear zones of inhibition measuring 7–8 mm in diameter, effectively suppressing the growth of both bacterial strains.

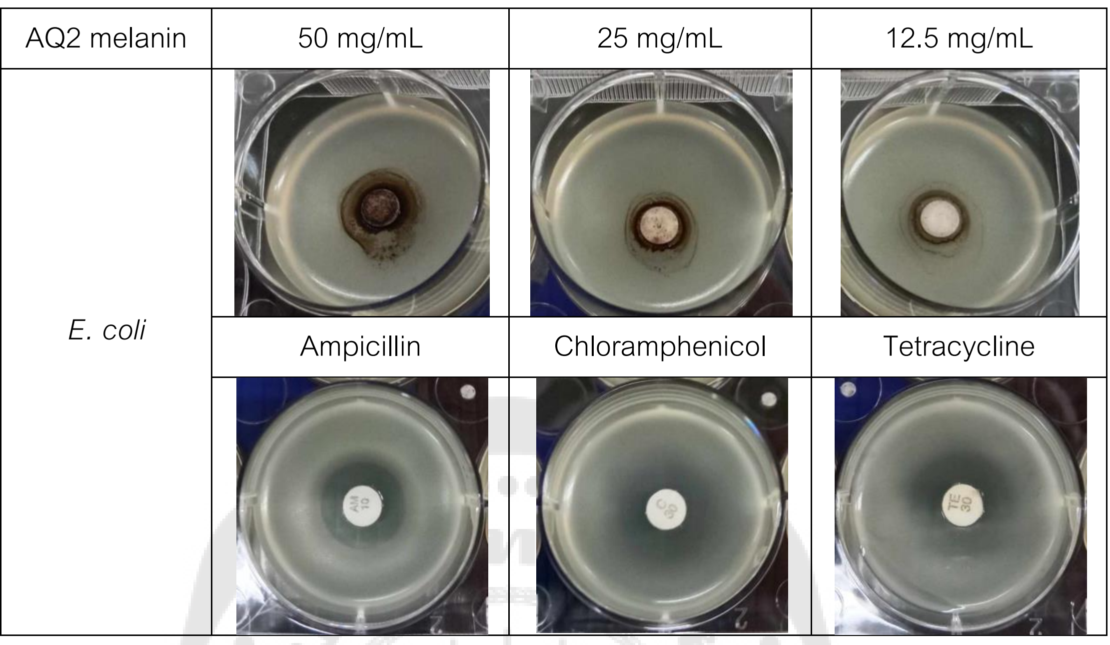
These findings indicate that melanin extracted from *S. spectabilis* strains AQ2 and CQ2 lacks antibacterial activity against *S. aureus* and *E. coli* at the tested concentrations. This suggests that, under the conditions of this study, melanin may not serve as an effective antimicrobial agent. However, further investigations using different concentrations, extraction methods, or target organisms may be warranted to fully assess its antimicrobial potential.

Table 5: Antibacterial activity of melanin from AQ2 against *S. aureus*



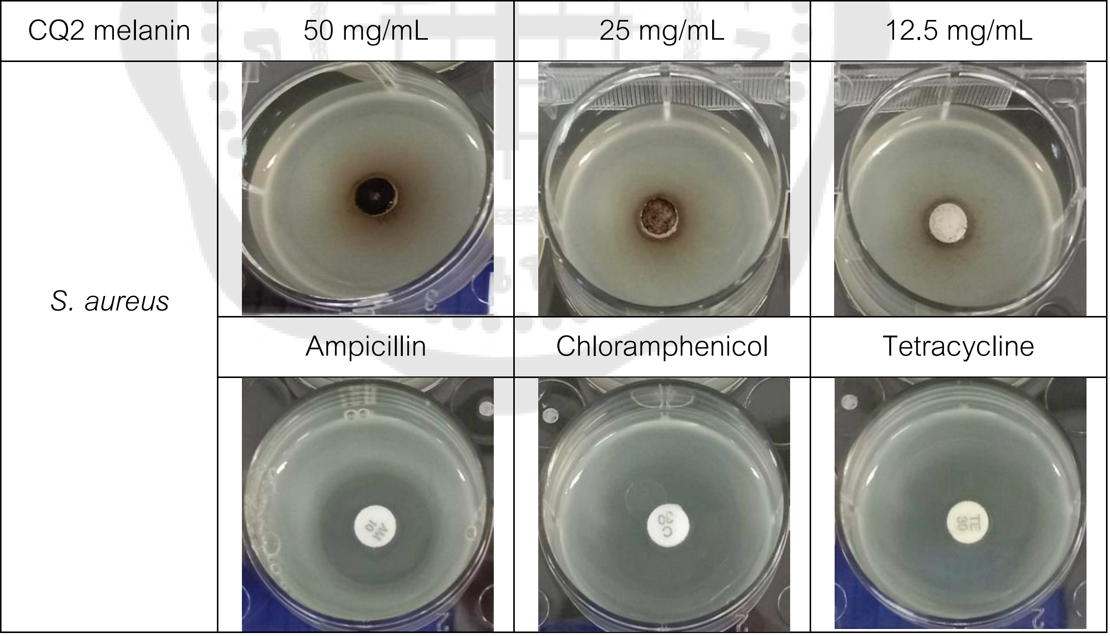
AQ2 melanin	50 mg/mL	25 mg/mL	12.5 mg/mL
S. aureus			
	Ampicillin	Chloramphenicol	Tetracycline

Table 6: Antibacterial activity of melanin from AQ2 against *E. coli*



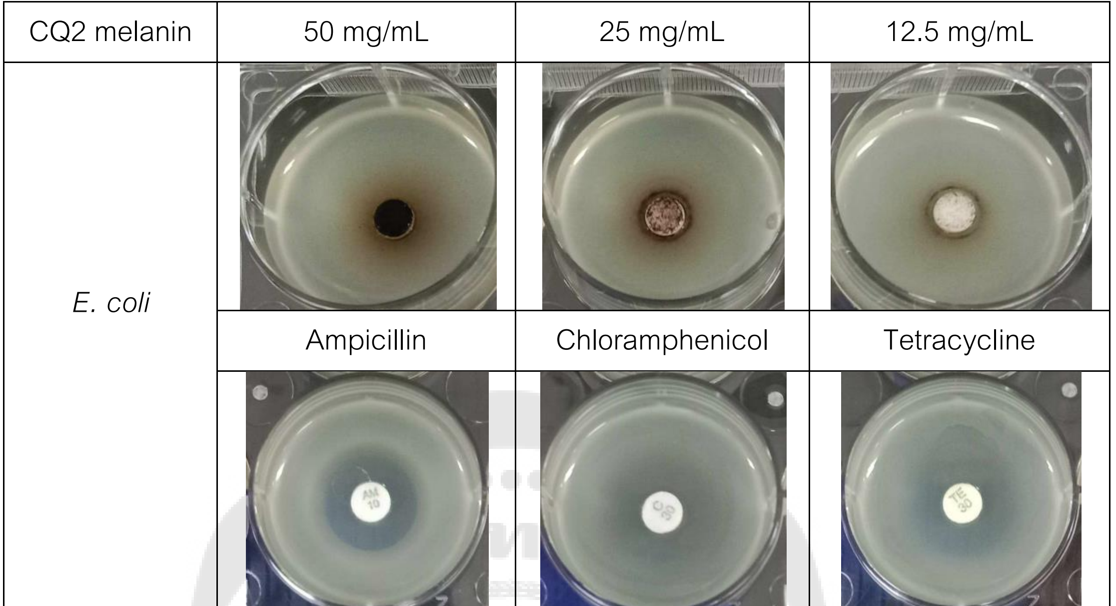
AQ2 melanin	50 mg/mL	25 mg/mL	12.5 mg/mL
E. coli			
	Ampicillin	Chloramphenicol	Tetracycline

Table 7: Antibacterial activity of melanin from CQ2 *against S. aureus*



CQ2 melanin	50 mg/mL	25 mg/mL	12.5 mg/mL
S. aureus			
	Ampicillin	Chloramphenicol	Tetracycline

Table 8: Antibacterial activity of melanin from CQ2 against *E. coli*



CQ2 melanin	50 mg/mL	25 mg/mL	12.5 mg/mL
E. coli			
	Ampicillin	Chloramphenicol	Tetracycline

3.2 Antioxidant activity

The evaluation of the antioxidant activity of melanin extracted from *S. spectabilis* strains AQ2 and CQ2 demonstrated that both strains possess free radical scavenging capabilities, indicating their potential as natural antioxidants. Among the two, melanin derived from strain CQ2 consistently exhibited higher antioxidant activity across all tested concentrations (1, 2, 3, 4, and 5 mg/mL), suggesting a strain-dependent difference in radical-scavenging efficiency. However, when compared to standard antioxidants such as Trolox and Ascorbic acid, the antioxidant performance of both melanin samples was significantly lower. The positive controls showed markedly stronger free radical neutralization, underscoring their superior efficacy. These findings indicate that, while melanin extracted from *S. spectabilis* strains AQ2 and CQ2 demonstrates measurable antioxidant activity, its potency remains moderate relative to established antioxidants. Further research may investigate strategies to enhance the antioxidant potential of microbial melanin or explore its synergistic effects with other antioxidant agents for possible applications in pharmaceuticals, cosmetics, or food preservation. The antioxidant activity of all tested substances is summarized as;

Ascorbic acid > Trolox > CQ2 melanin > AQ2 melanin

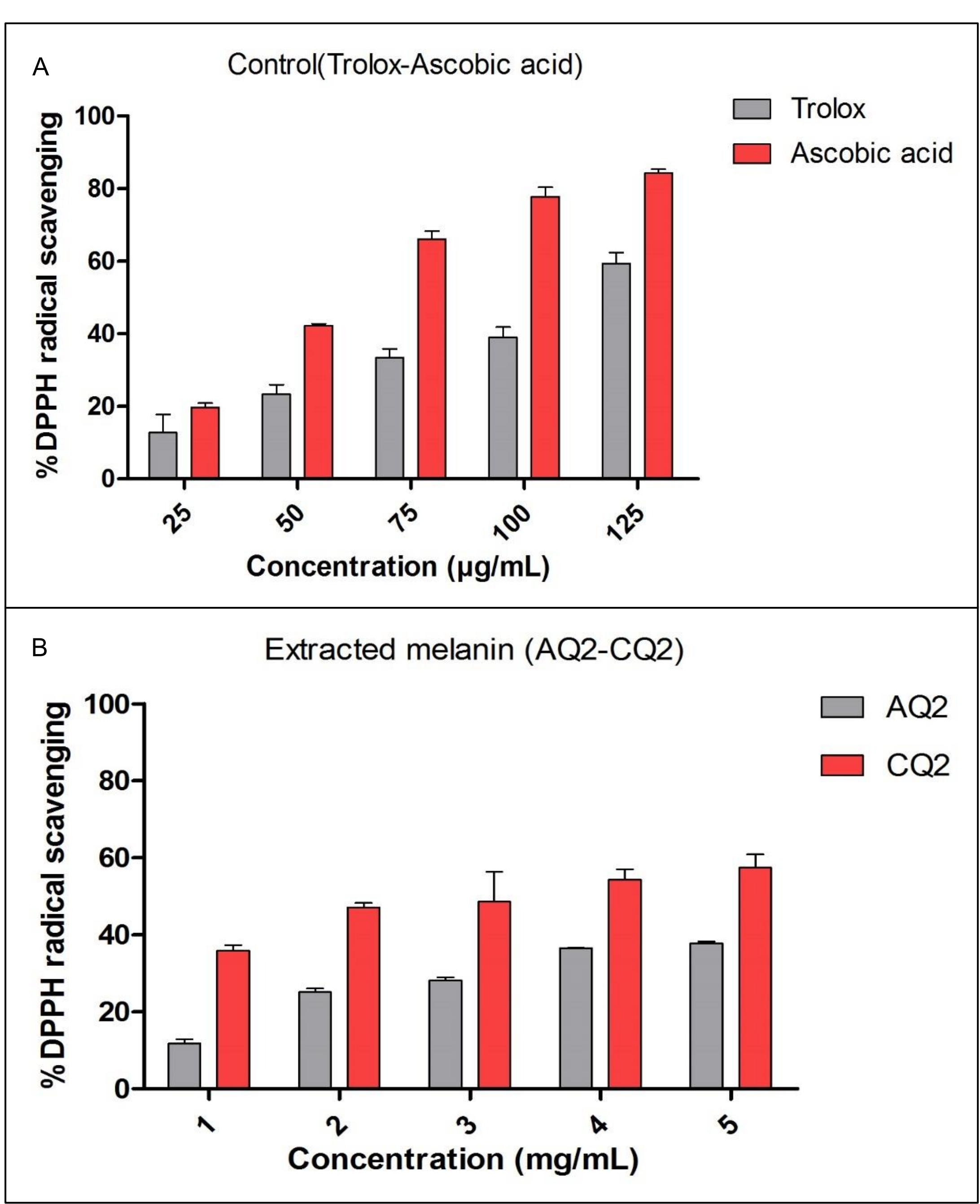


Figure 12: The antioxidant activity (DPPH) of melanin

A: Trolox and Ascorbic acid (Control) B: Melanin from *S. spectabilis*

3.3 Anti-Ultraviolet radiation

3.3.1 UV absorption capacity

The results of the UV absorption test on melanin extracted from AQ2 and CQ2 strains at varying concentrations revealed that melanin from both strains absorbed UV-B light significantly more effectively than UV-A light. Among the two, melanin from the CQ2 strain exhibited superior UV absorption efficiency compared to the AQ2 strain. Moreover, both strains demonstrated notable UV absorption efficiency at a concentration of 25 mg/mL. These findings highlight the promising potential of melanin from both strains as effective UV protectants and emphasize the direct relationship between melanin concentration and UV absorption efficiency. The slightly better performance of the CQ2 strain suggests its greater suitability for applications requiring UV protection, such as in the cosmetics and pharmaceutical industries.

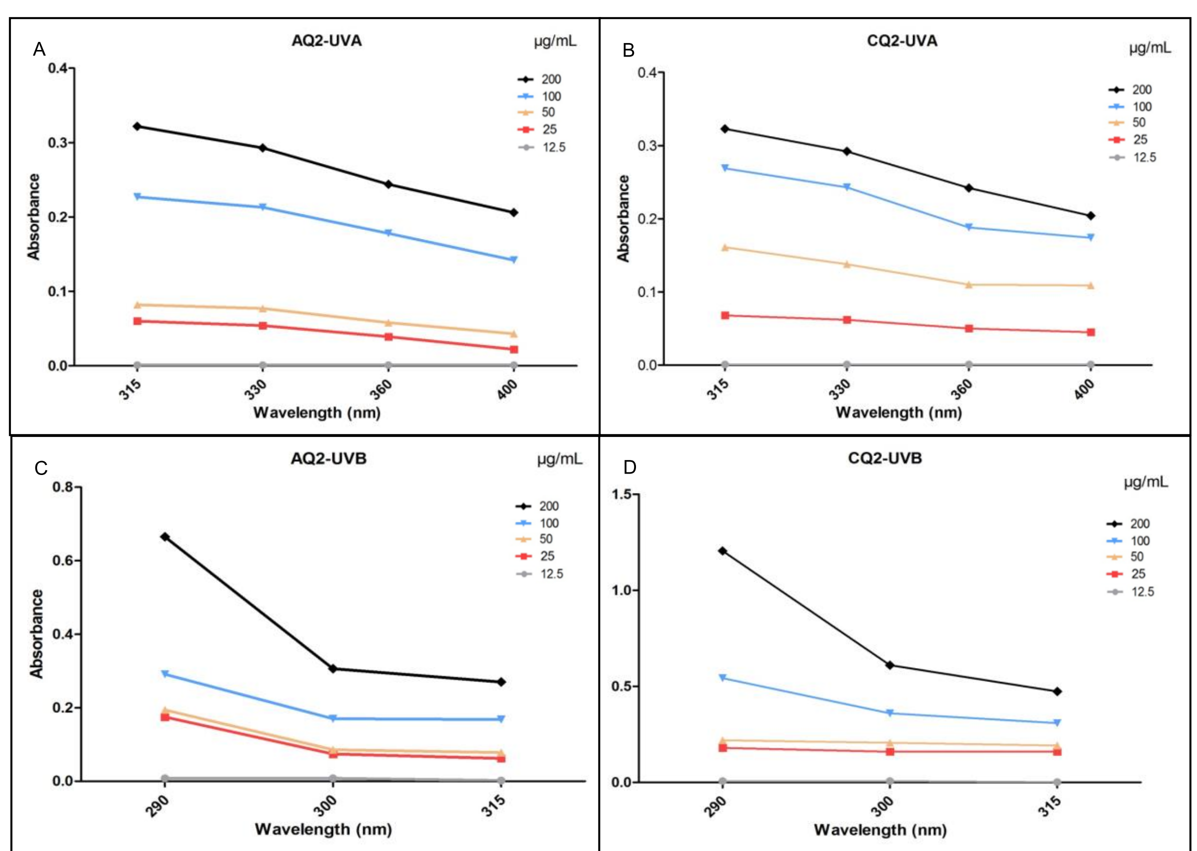


Figure 13: UV absorption of melanin from AQ2 and CQ2 strains

A: UV-A absorption of AQ2-melanin B: UV-A absorption of AQ2-melanin

C: UV-B absorption of CQ2-melanin D: UV-B absorption of CQ2-melanin

3.3.2 Cytotoxic activity

The cytotoxicity tests performed to assess the appropriate dosage of melanin for evaluating UV protection involved testing a range of concentrations, including 0.125, 0.25, 0.5, 1, 3.125, 6.25, 12.5, 25, and 50 µg/mL. The results indicated that melanin derived from *S. spectabilis* AQ2 began to exhibit cytotoxic effects on melanocyte cells (B16F10) at a concentration of 25 µg/mL.(Fig.14A) But in HaCaT cells melanin extracted from *S. spectabilis* AQ2 displayed cytotoxic effects only at a higher concentration of 50 µg/mL.(Fig.14C) This finding suggests that, at this threshold, the melanin starts to induce toxic effects that could compromise cell integrity and function. As such, concentrations below 25 µg/mL are considered safer for subsequent in vitro testing and are more suitable for assessing melanin's UV-protective efficacy while maintaining cell viability.

In contrast, melanin extracted from *S. spectabilis* CQ2 displayed cytotoxic effects only at a higher concentration of 50 µg/mL in both B16F10 melanocytes and HaCaT call (Fig.14B and Fig.14C) This indicates that CQ2-derived melanin possesses a lower cytotoxic profile at similar or lower doses in melanocyte cell line, making it potentially more favorable for applications that may require higher or more frequent administration. The differing toxicity profiles between AQ2 and CQ2 strains underscore the necessity of precise dosage optimization when employing melanin as a UV-protective agent.

These observations emphasize the importance of selecting appropriate melanin concentrations for UV protection testing. Since the toxicity threshold varies between strains, it is essential to carefully adjust the dosage, particularly for AQ2-derived melanin, in order to preserve cell viability and ensure reliable and reproducible results while still evaluating protective efficacy against UV radiation

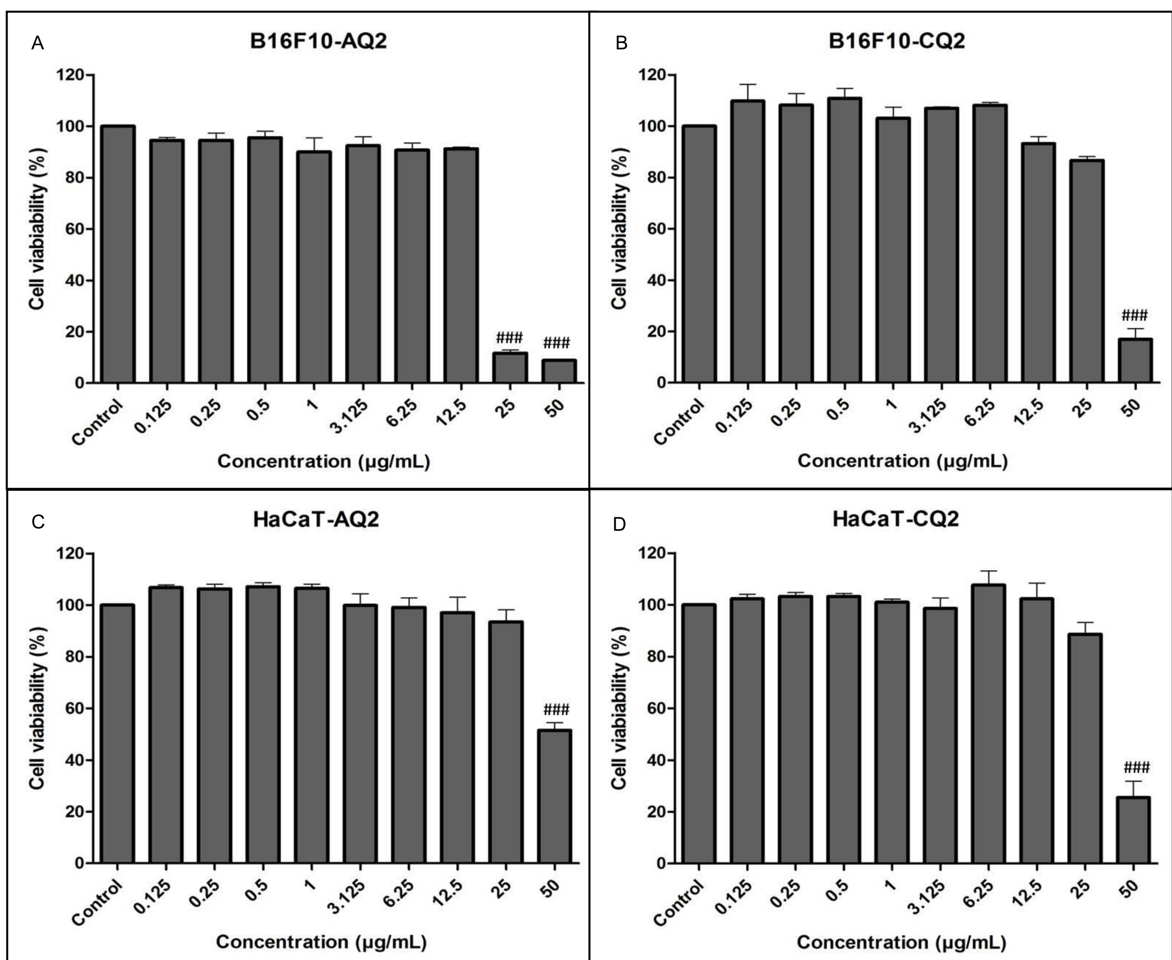


Figure 14: Cytotoxic activity of melanin from AQ2 and CQ2 strains

Cytotoxicity effects of various concentrations of AQ2 and CQ2 melanin on B16F10 cell lines after 24 h (A, B) and HaCaT cell lines after 24 h (C, D). The results are represented as mean ± SEM (n = 4) and analyzed with Tukey's test. ### $P < 0.001$ versus the control group.

3.3.3 Cell viability after UV exposure

The cell viability was measured in both melanocyte (B16F10) and keratinocyte (HaCaT) cell lines following UV-A and UV-B exposure to evaluate the cell protections of melanin derived from *S. spectabilis* strains AQ2 and CQ2. The results of the cell viability assay after UV exposure on melanocyte cell lines showed that melanin derived from *S. spectabilis* AQ2 exhibited slight protective effects. Specifically, when cells were treated with AQ2 melanin at concentrations of 7.25 and 12.5 µg/mL, the survival rates of cells exposed to both UV-A and UV-B radiation were slightly higher compared to the untreated control groups. which exhibited significant increase viability in B16F10 cells both of UV-A and UV-B (Fig.15A and 16A). This suggests that AQ2 melanin has some effect in enhancing the resistance of melanocyte cells against UV-induced damage at these concentrations, although it does not exhibit strong protective efficacy. In contrast, for melanin derived from *S. spectabilis* CQ2, the results showed a different pattern. Cells treated with CQ2 melanin at concentrations of 12.5 and 25 µg/mL exhibited lower survival rates when exposed to both UV-A radiation and UV-B radiation (Fig.15B and 16B) compared to untreated cells. This indicates that CQ2 melanin not only failed to protect the cells but also may have contributed to increased cytotoxicity under UV exposure. These findings suggest that while AQ2 melanin demonstrates some protective effects against UV-induced cell death, CQ2 melanin lacks this protective capability at the tested concentrations. This could be due to differences in the structural properties or composition of the melanin from the two sources. Therefore, AQ2 melanin shows greater potential for use as a UV-protective agent, whereas further investigation is needed to understand the mechanisms behind CQ2 melanin's lack of effectiveness. Additionally, when considering the effects of AQ2 and CQ2 melanin at concentrations of 12.5 and 25 µg/mL on HaCaT cells, a similar pattern was observed. Melanin from AQ2 exhibited a slight increase in survival rates compared to untreated cells, indicating some degree of protection in keratinocytes as well. However, in HaCaT cells, although viability increased, the effect was not statistically significant. (Fig.15C and 16C) In contrast, CQ2 melanin at the same concentrations led to lower survival rates under both UV-A and UV-B exposure,

mirroring the findings in HaCaT (Fig.15D and 16D). All these results further confirm the differential protective effects of AQ2 and CQ2 melanin, with AQ2 showing a slight protective effect, while CQ2 may have a detrimental impact on cell viability under UV stress.

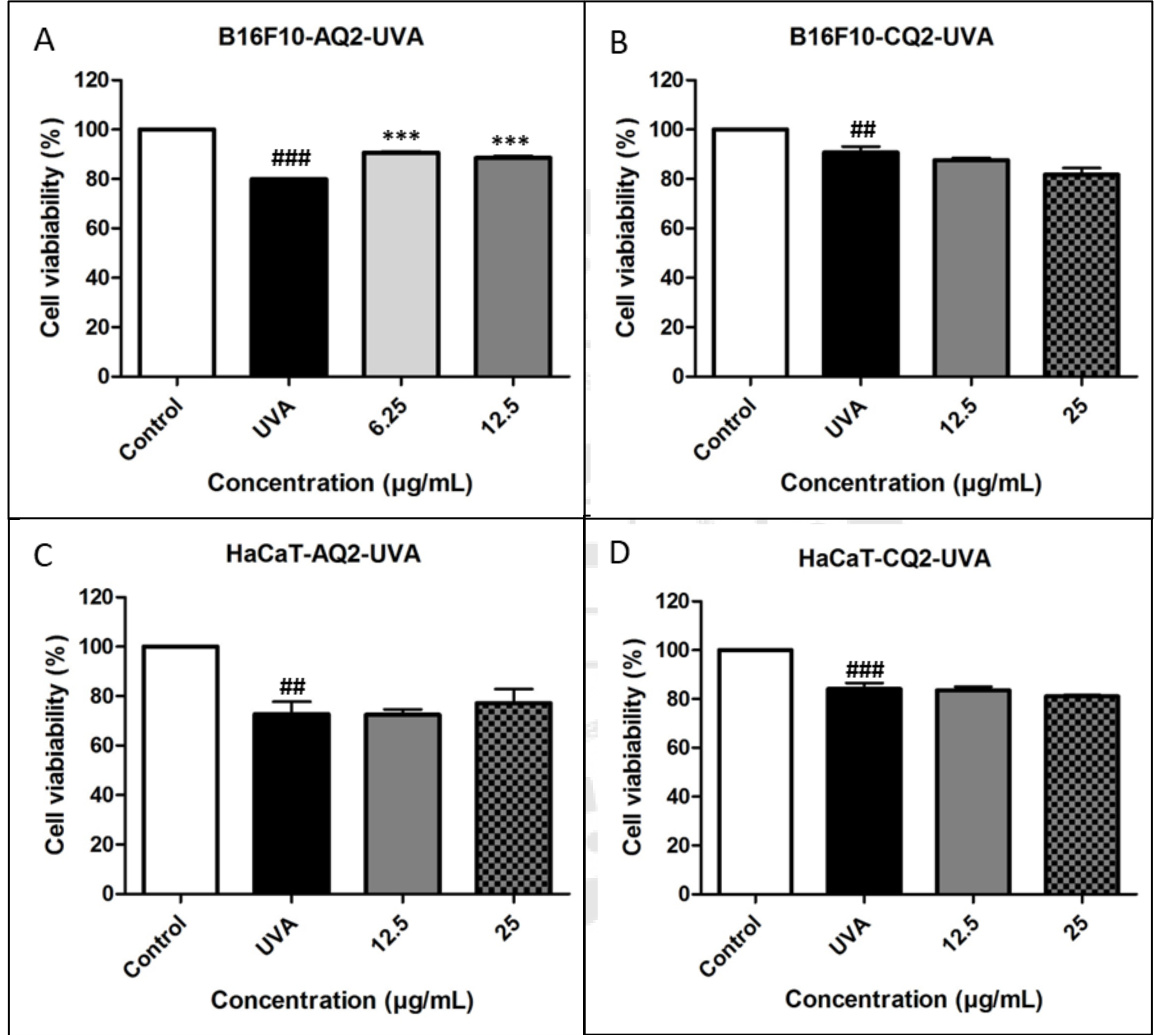


Figure 15: The relative percent cell viability after UV-A Exposure

(A, B) shows the cell viability after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on B16F10. (C, D) shows the cell viability after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on HaCaT. The relative percent cell viability, which are presented as mean ± SEM (n = 4) and analyzed with

Tukey's test. ## $P < 0.01$. and ### $P < 0.001$ versus the control group. *** $P < 0.001$ versus UV irradiated cells alone.

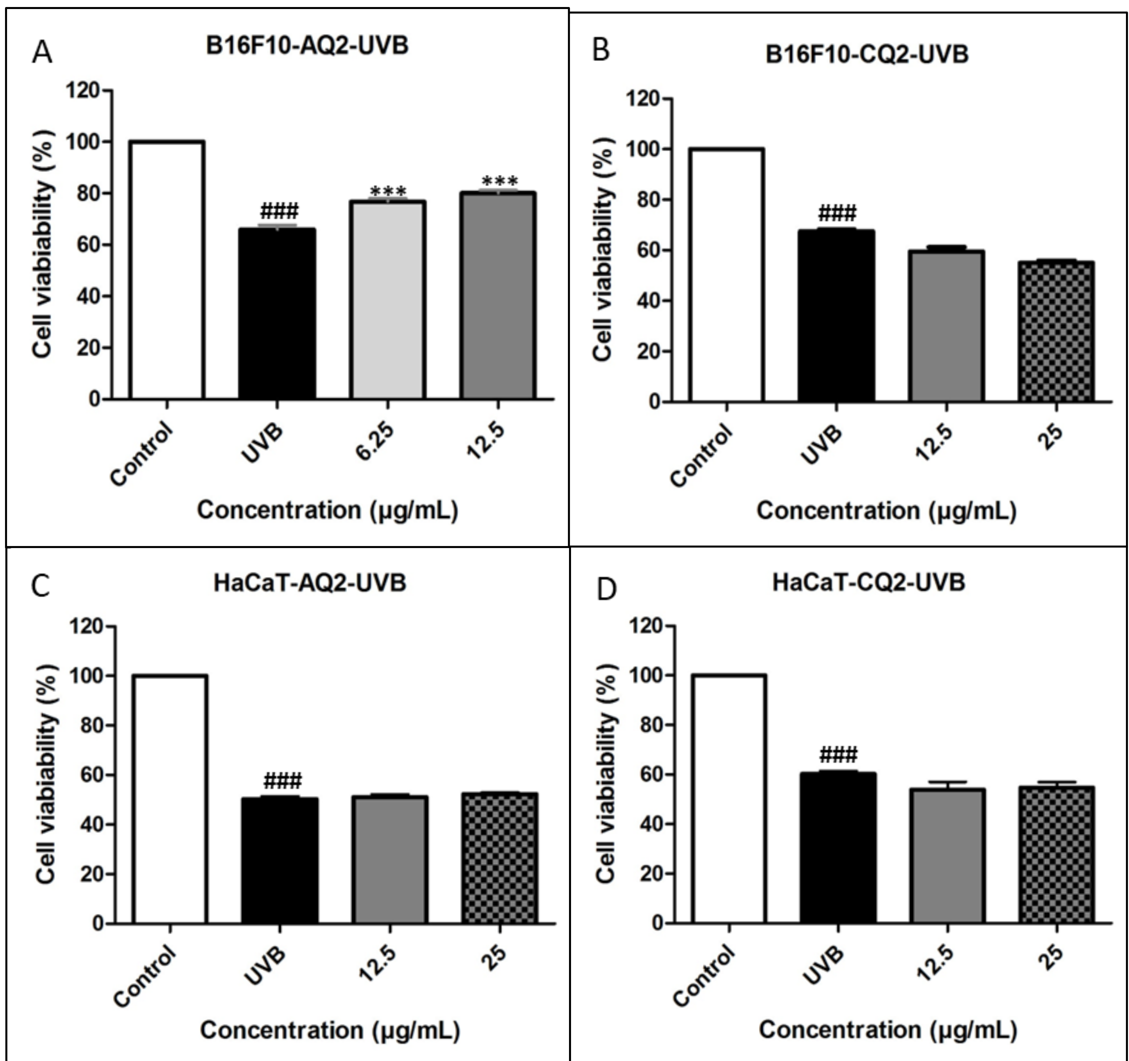


Figure 16: The relative percent cell viability after UV-B Exposure

(A, B) shows the cell viability after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on B16F10. (C, D) shows the cell viability after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on HaCaT. The relative percent cell viability, which are presented as mean ± SEM (n = 4) and analyzed with Tukey's test. ## $P < 0.01$. and ### $P < 0.001$ versus the control group.

3.3.4 Intracellular ROS after UV exposure

The intracellular ROS levels were measured in both melanocyte (B16F10) and keratinocyte (HaCaT) cell lines following UV-A and UV-B exposure to evaluate the antioxidant potential of melanin derived from *S. spectabilis* strains AQ2 and CQ2. Results indicated that UV-A radiation induced higher levels of intracellular ROS than UV-B, highlighting UV-A's greater role in oxidative stress.

In melanocyte cells, melanin extracted from strain AQ2 at concentrations of 6.25 and 12.5 µg/mL led to reduction in ROS levels after exposure to both UV-A and UV-B. (Fig.17A and 18A) The decrease was more pronounced under UV-A exposure. A significant reduction was observed when cells were exposed to UV-A; however, under UV-B exposure, although a reduction was also noted, it was not statistically significant. This may be attributed to the lower melanin concentration used in melanocyte cells compared to other groups. Additionally, in B16F10 cells, treatment with melanin derived from strain CQ2 at concentrations of 12.5 and 25 µg/mL significantly reduced intracellular ROS levels under both UV-A and UV-B exposure (Fig.17B and 18B). These findings indicate that melanin from both AQ2 and CQ2 strains can reduce intracellular ROS levels in melanocyte cells following UV-A and UV-B irradiation.

In keratinocyte cells, both AQ2 (Fig.17C and 18C) and (Fig.17D and 18D) melanin showed a comparable ability to reduce intracellular ROS levels following UV-A exposure and UV-B exposure, with more notable reductions under UV-A. The antioxidant effects observed in HaCaT cells were statistically significant when compared to untreated controls. These findings indicate that melanin derived from both AQ2 and CQ2 can reduce intracellular ROS levels in HaCaT cells under both UV-A and UV-B exposure.

Collectively, these findings suggest that melanin derived from both *S. spectabilis* strains AQ2 and CQ2 possesses antioxidant properties that help alleviate UV-induced oxidative stress by reducing intracellular ROS levels in both melanocytes and keratinocytes. Both types of melanin exhibited comparable efficacy in their protective effects.

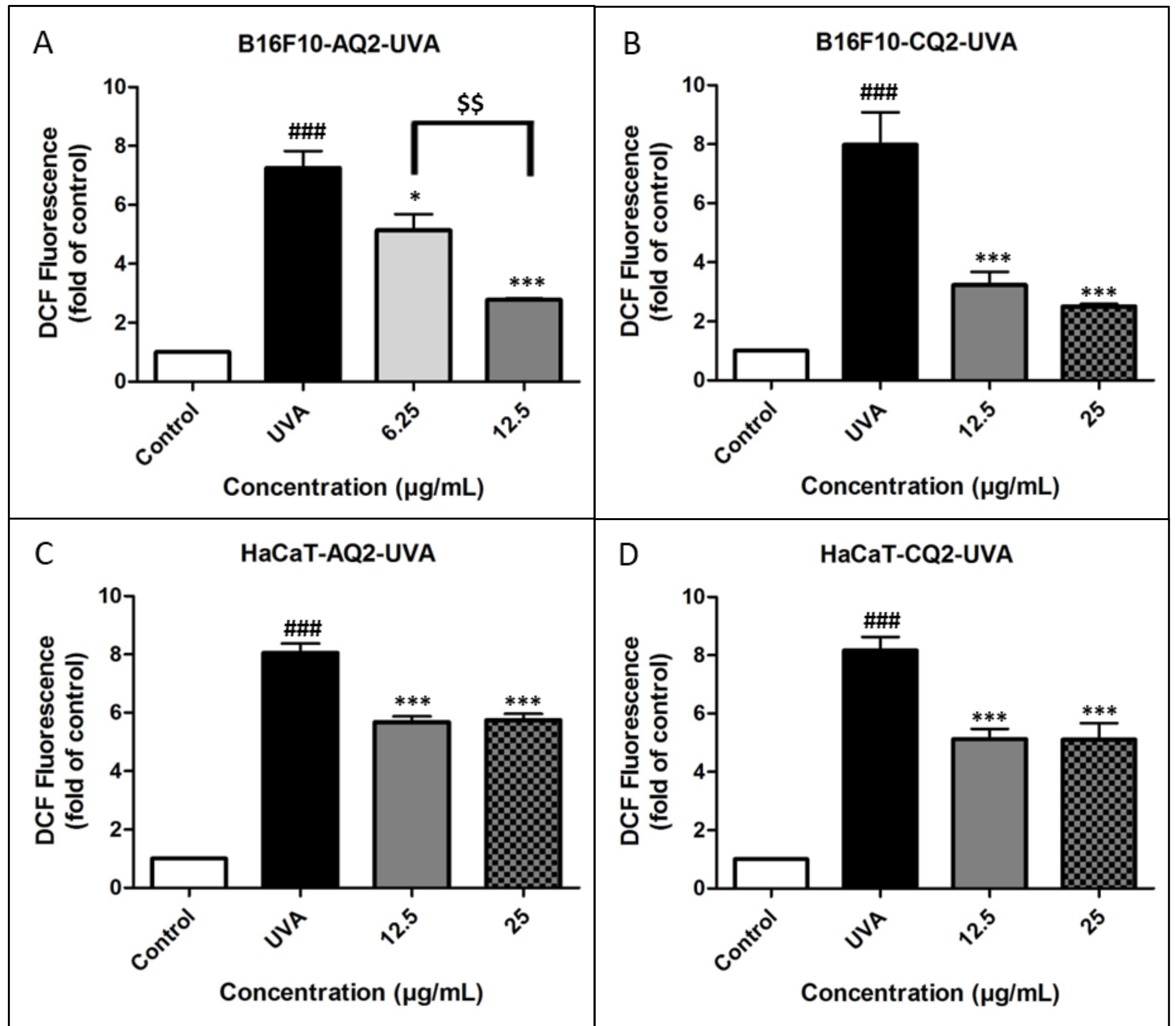


Figure 17: The relative level of intracellular ROS after UV-A exposure

(A, B) shows the DCF fluorescent after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on B16F10. (C, D) shows the DCF fluorescent after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-A irradiation, on HaCaT. The relative fluorescence intensities, which are presented as mean ± SEM (n = 4) and analyzed with Tukey's test. ### $P < 0.001$ versus the control group, * $P < 0.05$, and *** $P < 0.001$ versus UV irradiated cells alone. The statistical difference between the melanin concentration was at $ $ $P < 0.01$.

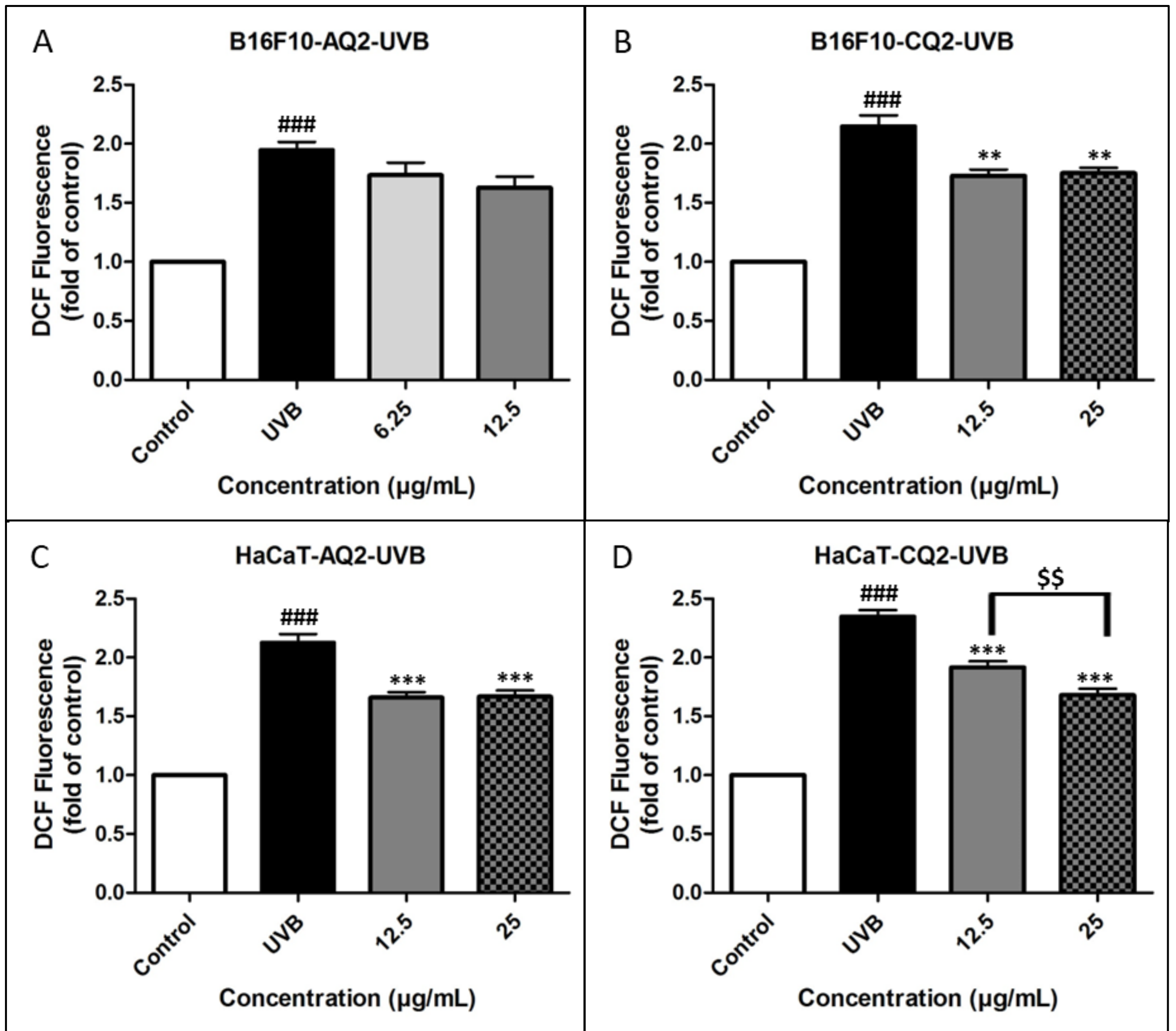


Figure 18: The relative level of intracellular ROS after UV-B exposure

(A, B) shows the DCF fluorescent after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-B irradiation, on B16F10. (C, D) shows the DCF fluorescent after pre-incubating with the AQ2 CQ2 melanin, exposed to single UV-B irradiation, on HaCaT. The relative fluorescence intensities, which are presented as mean ± SEM (n = 4) and analyzed with Tukey's test. ### $P < 0.001$ versus the control group ** $P < 0.01$, and *** $P < 0.001$ versus UV irradiated cells alone. The statistical difference between the melanin concentration was at $ $ $P < 0.01$.

3.4 Chelate metals ions activity

The results of the heavy metal chelation assay demonstrated that melanin derived from both *S. spectabilis* strains AQ2 and CQ2 exhibited comparable efficacy in adsorbing heavy metals at a concentration of 5 mg. However, at the lower concentration of 1 mg, neither type of melanin showed sufficient chelating activity, as their binding capacity was inadequate for all tested metals at this level. Upon increasing the concentration to 5 mg, melanin from both AQ2 and CQ2 strains effectively chelated all the heavy metals under investigation, as assessed by the survival rate of *C. albicans* growth on agar plates. The experiment was divided into four groups. The first group served as control, where *C. albicans* demonstrated normal growth. The most prominent and statistically significant results were observed in the $CuSO_4$ group, where melanin from both AQ2 and CQ2 clearly exhibited strong chelation of copper ions (Cu^{2+}), as evidenced by a marked reduction in *C. albicans* survival compared to groups without melanin treatment. However, in the groups treated with $FeSO_4 \cdot 7H_2O$ and $MnCl_2 \cdot 4H_2O$, *C. albicans* showed relatively higher survival rates despite melanin exposure, although a slight decrease was still noted. Notably, the chelating effect was considerably more pronounced in the copper ion group.

These findings indicate that melanin from both strains possesses a notable affinity for copper ions and may be effectively utilized as a chelating agent, particularly against $CuSO_4$. This underscores the potential application of these melanins in detoxification processes and environmental remediation in the future.

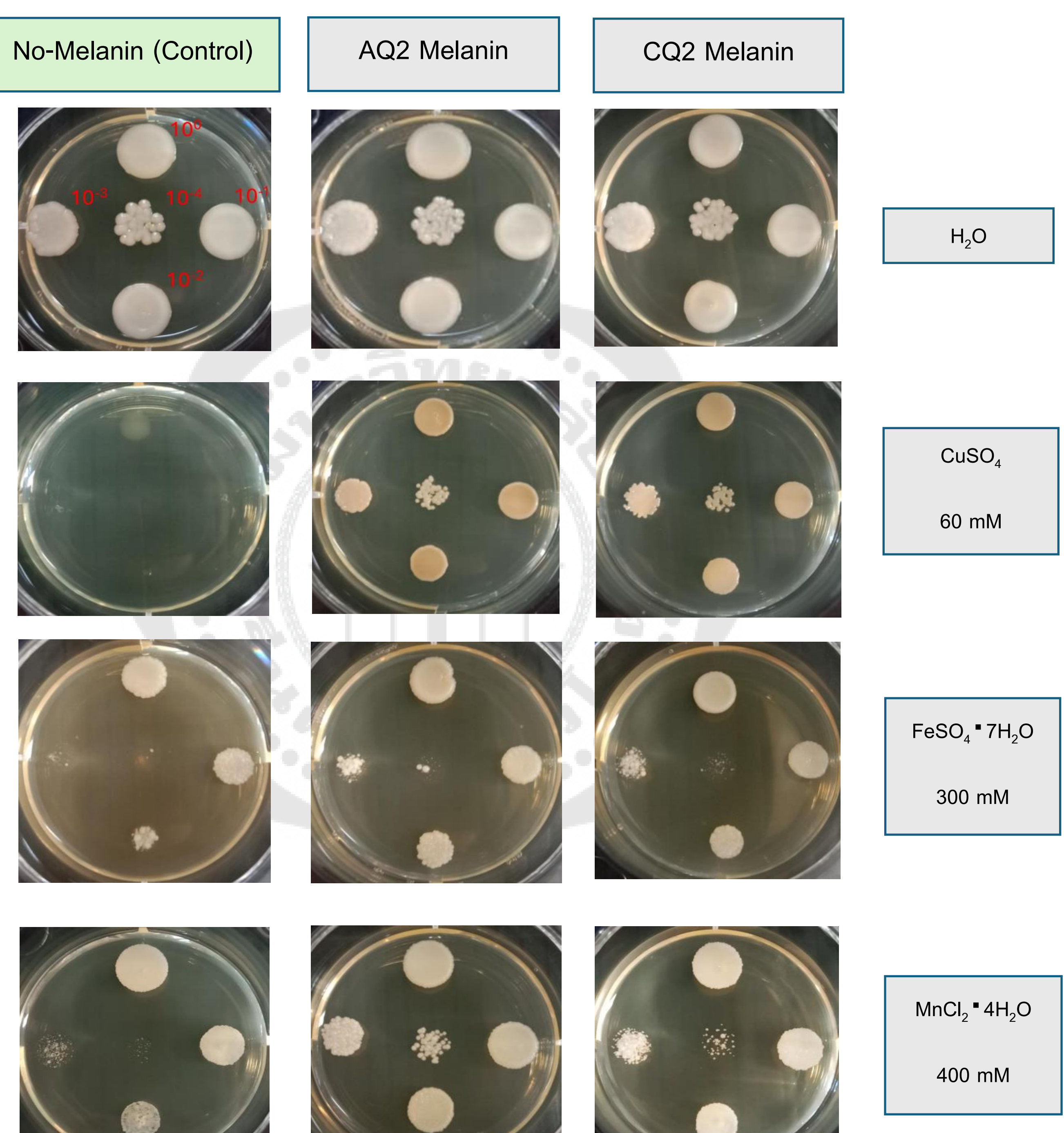


Figure 19: Melanin attenuates metal ion inhibition of *C.albicans* growth

(No-Melanin: The control group without incubation of heavy metals with melanin)

CHAPTER 5
DISCUSSION AND CONCLUSION

Discussion

This study aimed to evaluate the physicochemical properties and bioactivities of melanin extracted from *S. spectabilis* strains AQ2 and CQ2 to explore their potential applications in various fields in the future. The results indicated that although both strains were capable of producing melanin, they showed similar yields and bioactivities. This highlights that the extracted substances from both strains are indeed melanin and exhibit bioactivities comparable to melanin reported in diverse current studies, which is a crucial factor determining their suitability for various applications.

From a production perspective, the AQ2 strain exhibited greater melanin production efficiency relative to its biomass, suggesting it may be more cost-effective for large-scale applications that require rapid synthesis. In contrast, the CQ2 strain produced higher overall melanin yields over a longer cultivation period, making it potentially better suited for processes that allow extended incubation and prioritize maximizing final output. This variation in melanin biosynthetic capacity likely arises from strain-specific enzymatic activities, differences in metabolic flux, or regulation within the melanogenesis pathway. Further genetic and transcriptomic analyses could help clarify the underlying mechanisms and facilitate metabolic engineering to optimize melanin production in each strain. However, our study also found that melanin yields from both AQ2 and CQ2 strains were relatively lower compared to those reported from other microbial sources.[71, 73] Despite this, previous studies have demonstrated that microbial melanin production can be significantly enhanced through various optimization strategies, including tyrosine supplementation, metal ion addition, and increased substrate concentrations. Therefore, accurately identifying the specific type of melanin produced is crucial for guiding future research and improving extraction and synthesis methods.[77]

The melanin extracted from both *S. spectabilis* strains exhibited chemical properties that closely paralleled those of synthetic melanin, suggesting its structural authenticity and functional relevance. Fourier transform infrared spectroscopy analysis provided evidence of this similarity through the identification of characteristic absorption bands around 3200–3300 cm^{-1}. which correspond to –NH and –OH groups associated with indole rings. These signals suggest the presence of hydrogen bonding, a key factor in stabilizing the melanin structure. Further analysis revealed peaks at 1620–1650 cm^{-1}. indicative of C=O and N–H stretching vibrations from amide groups, supporting the presence of peptide bonds and confirming the polymeric nature of the pigment. Additionally, distinct absorption signals in the range of 600–800 cm^{-1} were observed, corresponding to low-frequency aromatic hydrogen vibrations, which further validate the aromatic and highly conjugated backbone typical of melanin.(10, 50, 65) UV–visible spectroscopy results aligned with the FTIR findings, showing that melanin from *S. spectabilis* displayed UV absorption, with notable peaks between 230–250 nm closely resembling the UV absorbance profile of synthetic melanin standards.(38, 50, 65).These spectroscopic characteristics collectively affirm that *S. spectabilis*-derived melanin shares both the structural and photoprotective properties of synthetic melanin. Therefore, this natural pigment demonstrates promising potential as a viable, bio-based alternative for use in applications requiring UV protection.

The evaluation of antioxidant activity revealed that melanin derived from *S. spectabilis* strains AQ2 and CQ2 possesses measurable free radical scavenging capacity, reinforcing its potential as a natural antioxidant. This finding aligns with previous reports in which melanin isolated from *Spissiomyces* and other *Streptomyces* species also demonstrated similar radical-scavenging properties,(38, 50, 65) suggesting that this functionality may be a conserved feature among microbial melanins. Notably, melanin extracted from strain CQ2 consistently exhibited greater antioxidant activity than that from AQ2 at all tested concentrations (1–5 mg/mL), indicating that the efficiency of radical scavenging may be strain-dependent. This variation could be attributed to differences in melanin structure, degree of polymerization, or the presence of functional

groups that influence electron-donating capacity. Such inter-strain variability highlights the importance of strain selection when considering microbial melanin for antioxidant applications. However, when benchmarked against known antioxidant standards such as Trolox and ascorbic acid, the antioxidant activity of both CQ2 and AQ2 melanin was significantly lower. These positive controls exhibited markedly stronger free radical neutralization, underscoring the comparatively moderate antioxidant capacity of microbial samples. While this does not negate the potential utility of *S. spectabilis*-derived melanin, it does suggest that its use as a standalone antioxidant may be limited in high-demand applications.

The evaluation of intracellular ROS levels following UV-A and UV-B exposure in melanocyte (B16F10) and keratinocyte (HaCaT) cell lines highlighted the antioxidant potential of melanin extracted from *S. spectabilis* strains AQ2 and CQ2. Overall, UV-A irradiation induced higher ROS levels than UV-B, indicating UV-A as a more significant contributor to oxidative stress. Treatment with AQ2-derived melanin at concentrations of 6.25 and 12.5 µg/mL significantly reduced intracellular ROS levels in melanocytes, particularly under UV-A exposure. Similarly, CQ2-derived melanin at 12.5 and 25 µg/mL also led to a marked decrease in ROS levels, with CQ2 showing slightly greater ROS reduction than AQ2 in melanocytes. Likewise, under UV-B exposure, CQ2-derived melanin showed -a slightly stronger ROS-reducing effect than AQ2 in melanocytes. The lower efficacy in reducing ROS observed with AQ2 melanin may be attributed to the lower concentration of melanin used in melanocytes compared to the other groups, which could account for the observed differences. In keratinocytes, both AQ2 and CQ2 melanin treatments effectively decreased ROS generation following UV-A and UV-B irradiation, with comparable antioxidant efficacy between the two melanin types. These reductions in ROS were statistically significant in melanin treatment relative to untreated controls and were more pronounced under UV-A-induced oxidative stress.

Although cell viability assays were performed following UV exposure to assess the protective potential of the melanin samples, the results showed that AQ2 melanin provided only modest protection, while CQ2 melanin offered no effective protection

against UV-induced cytotoxicity, the ROS assays clearly demonstrated the antioxidative capabilities of both melanin samples. These findings suggest that despite limited impact on overall cell survival, melanin derived from *S. spectabilis* strains AQ2 and CQ2 can substantially mitigate UV-induced oxidative stress by reducing intracellular ROS levels. This is consistent with previous studies which reported that melanin derived from *O. sinensis* and *I. hispidus* exhibits the ability to reduce intracellular ROS levels.[10, 130] This antioxidant property underscores their potential utility as bioactive ingredients in UV-protective and skin-care formulations aimed at minimizing oxidative damage.

The results of the metal ion chelation assay revealed that melanin extracted from *S. spectabilis* strains AQ2 and CQ2 exhibited comparable efficacy in binding heavy metals, particularly at a concentration of 5 mg. The chelation efficiency was evaluated based on the growth inhibition of *C. albicans* on agar plates supplemented with metal solutions pretreated with melanin. Among the tested metal ions, $CuSO^4$ demonstrated the most pronounced effect, with both AQ2- and CQ2-derived melanin showing strong copper-binding capacity, as evidenced by a significant increase in *C. albicans* growth. This result implies effective removal of toxic copper ions from the medium. These findings are consistent with previous studies reporting that melanin derived from *O. sinensis* also possesses high copper-chelating activity.[10, 113] Therefore, melanin extracted from both AQ2 and CQ2 strains of *S. spectabilis* shows promising potential as a natural chelating agent, particularly for copper ions. This suggests possible applications in detoxification and environmental remediation.[9] These findings support the concept of microbial melanin as a viable biotechnological tool for mitigating heavy metal pollution in ecological systems.

Importantly, the diverse functional properties of melanin make it a highly versatile biopolymer. Its combined abilities in UV absorption, antioxidant activity, and heavy metal chelation render it a promising candidate for applications across a wide range of industries. In the cosmetic industry, melanin can be incorporated into sunscreens, anti-aging creams, or protective lotions, especially those marketed as natural or organic. In the pharmaceutical sector, melanin-based formulations may offer protection against

radiation-induced skin damage or serve as adjuncts in antioxidant therapies. In industrial applications, melanin can be used to coat nanomaterials for use in biosensors, smart textiles, or packaging materials designed to prevent UV degradation and microbial contamination.[64, 77] Several key challenges remain before melanin can reach commercial viability. Addressing the stability, scalability, and reproducibility of the extraction and formulation processes is essential for reliable production. The observed variability in melanin properties across different microbial strains, as shown in this study, emphasizes the importance of developing standardization protocols to maintain product consistency. In addition, future research should prioritize assessments of biocompatibility, metabolic stability, and in vivo performance to ensure safe and effective use across intended applications.

Conclusion

Melanin produced by *S. spectabilis* strains AQ2 and CQ2 exhibited distinct physicochemical characteristics that indicate promising potential for diverse applications, particularly in ultraviolet protection and heavy metal chelation. Both strains yielded comparable amounts of melanin; however, strain AQ2 demonstrated greater efficiency in melanin production per unit of biomass, whereas strain CQ2 produced higher overall quantities during extended cultivation periods. The extracted melanins possessed chemical and structural properties analogous to those of synthetic melanin, including strong UV absorption and notable antioxidant activity, thereby confirming their potential as natural alternatives for UV protection.

Despite the antioxidant capacity demonstrated by both AQ2 and CQ2 melanins, their efficacy in mitigating oxidative stress varies. The DPPH assay revealed that CQ2 melanin exhibited significantly stronger free radical scavenging activity compared to AQ2, indicating a more potent antioxidant effect at elevated concentrations. Complementary results from the DCFH-DA assay further confirmed that melanins derived from both strains effectively reduced intracellular ROS levels following UV irradiation. Notably, CQ2 melanin induced a marked decrease in ROS levels in both melanocytes

and keratinocytes, especially under UV-A exposure. Although AQ2 melanin exhibited slightly lower efficacy in melanocytes, it nonetheless significantly reduced ROS accumulation in both cell types after UV exposure. These results underscore the potential of melanins from both AQ2 and CQ2 strains as natural antioxidants capable of alleviating UV-induced oxidative stress, thereby positioning them as promising candidates for UV-protective applications.

Moreover, both AQ2 and CQ2 melanins demonstrated substantial metal ion chelation activity, with a pronounced affinity for copper ions. This property highlights their prospective utility in environmental detoxification and bioremediation strategies. Collectively, the bioactive properties exhibited by melanin derived from *S. spectabilis* strains AQ2 and CQ2 suggest broad applicability across multiple fields, including cosmetics, pharmaceuticals, and environmental remediation. Nevertheless, further research is required to optimize their protective and chelation functions and to evaluate their suitability for commercial applications.

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