PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

IDENTIFICAÇÃO MOLECULAR DE ASPERGILLUS NIGER E DETECÇÃO DA CAPACIDADE DE BIOSSÍNTESE DE MELANINA E SEU PAPEL CONTRA MICRORGANISMOS PATOGÊNICOS

MOLECULAR IDENTIFICATION OF ASPERGILLUS NIGER AND DETECTION OF THE ABILITY OF MELANIN BIOSYNTHESIS AND ITS ROLE AGAINST PATHOGENIC MICROORGANISMS

التشخيص الجزيئي لفطر Aspergillus Niger وتقييم التخليق الحيوي للميلانين وفعاليته المضادة للميكروبات

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RESUMO

Introdução: Pigmentos hidrofóbicos de alto peso molecular conhecidos como melaninas são reconhecidos pelo seu potencial antimicrobiano de amplo espectro contra vários patógenos. **Objetivo**: Este estudo investigou a capacidade de biossíntese de melanina de isolados de Aspergillus niger do solo e avaliou a eficácia da melanina extraída contra bactérias e fungos isolados de casos clínicos. **Métodos**: A melanina foi extraída do micélio fúngico utilizando um método de hidrólise alcalina (1 M KOH, 120°C) seguido de precipitação ácida. Os isolados foram identificados molecularmente via PCR. Os efeitos da temperatura, pH e concentração de biomassa no rendimento da melanina foram otimizados. A atividade antimicrobiana foi avaliada pelo método de difusão em poços nas concentrações de 10%, 25% e 50%. **Resultados**: Foram observadas variações significativas na produção de melanina entre os isolados, sendo que o Isolado Nº 13 apresentou o maior rendimento (0.30 mg/L). A biossíntese ótima ocorreu a 0.80 mg/L e 20°C e pH 6, e a produção aumentou com maiores concentrações de biomassa. A melanina extraída demonstrou potente atividade antimicrobiana. Na concentração de 50%, zonas de inibição de 29 mm, 25 mm e 20 mm foram registradas contra Klebsiella pneumoniae, Staphylococcus aureus e Escherichia coli, respectivamente. Contra os isolados fúngicos, a mesma concentração produziu zonas de inibição de 16 mm, 15 mm e 13 mm para Microsporum canis, Mucor sp. e Candida albicans, respectivamente. **Conclusão**: O Aspergillus niger é uma fonte viável de melanina biocompatível. O pigmento exibe propriedades antimicrobianas significativas, sugerindo o seu potencial como agente terapêutico natural.

Palavras-chave: *Aspergillus niger, Staphylococcus aureus, Melanina, PCR*

ABSTRACT

Background: High-molecular-weight hydrophobic pigments known as melanins are recognized for their broad-spectrum antimicrobial potential against various pathogens. **Aim**: This study investigated the melanin biosynthesis capacity of Aspergillus niger soil isolates and evaluated the efficacy of the extracted melanin against bacteria and fungi isolated from clinical cases. **Methods**: Melanin was extracted from fungal mycelia using an alkaline hydrolysis method (1 M KOH, 120°C) followed by acid precipitation. The isolates were molecularly identified via PCR. The effects of temperature, pH, and biomass concentration on melanin yield were optimized. Antimicrobial activity was assessed using the well diffusion method at concentrations of 10%, 25%, and 50%. **Results**: Significant variations in melanin production were observed among isolates, with Isolate No. 13 exhibiting the highest yield (0.30 mg/L). Optimal biosynthesis occurred at biomass of 0.80 mg/L, 20°C, and pH 6, and production increased with higher biomass concentrations. The extracted melanin demonstrated potent antimicrobial activity. At a 50% concentration, inhibition zones of 29 mm, 25 mm, and 20 mm were recorded against Klebsiella pneumoniae, Staphylococcus aureus, and Escherichia coli, respectively. Against fungal isolates, the same concentration yielded inhibition zones of 16 mm, 15 mm, and 13 mm for Microsporum

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canis, Mucor sp., and Candida albicans, respectively. **Conclusion**: Aspergillus niger is a viable source for biocompatible melanin. The pigment exhibits significant antimicrobial properties, suggesting its potential as a natural therapeutic agent.

Keywords: *Aspergillus niger*, *Staphylococcus aureus*, Melanin, PCR.

الخلاصة

التمهيد الأصباغ الكارهة للماء ذات الوزن الجزيئي العالي، والتي تُعرف باسم الميلانين، بقابليتها المضادة للميكروبات الواسعة النطاق ضد الكائنات الممرضة . **الهدف** :حققت هذه الدراسة في قدرة عزلات الفطر *Aspergillus niger* المعزولة من التربة على التخليق الحيوي للميلانين، وقيمت فعالية الميلانين المستخلص منه ضد البكتيريا والفطريات المعزولة من الحالات السريرية .**الطرائق** :تم استخلاص الميلانين من الغزل الفطري باستخدام طريقة التحلل القلوي (1 M KOH, 120°C)تلاه الترسيب الحامضي .تم تحديد العزلات بواسطة تشخيصها جزيئياً باستخدام تقنية تفاعل البوليميريز المتسلسل (PCR) . حُسنت المؤثرات لتصل الى الحد المثالي وهي كل من درجة الحرارة، الأس الهيدروجيني (pH) وتركيز الكتلة الحيوية من ناحية مردودها على انتاج الميلانين .قُيم النشاط المضاد للميكروبات باستخدام طريقة الأنتشار بالحفر وبتراكيز10% ،25% و50%. **النتائج** :لوحظ وجود فروق معنوية في إنتاج الميلانين بين العزلات، حيث أظهرت العزلة رقم 13 أعلى قيمة (0.30 ملغم/لتر). كان التخليق الأمثل عند الكتلة الحيوية 0.80 ملغم/ لتر , درجة الحرارة المئوية 20 ودرجة الحموضة 6 ، إذ زاد الإنتاج مع ارتفاع تراكيز الكتلة الحيوية .أظهر الميلانين المستخلص نشاطاً قوياً مضاداً للميكروبات .عند تركيز50% ، سُجلت مناطق تثبيط 29 ملم، 25 ملم و 20 ملم ضد *Klebsiella pneumoniae* ، و*Staphylococcus aureus*، و*Escherichia coli*، على التوالي . أما بالنسبة لعزلات الفطريات، إذ أدى نفس التركيز إلى مناطق تثبيط بلغت 16 ملم، و 15ملم، و 13ملم لـ*Microsporum canis* ، *Mucor* sp. و *Candida albicans* على التوالي .**الاستنتاجات** :يعتبر فطر **Aspergillus niger** مصدراً مهماً للميلانين المتوافق حيويا وأظهرت الصبغة خصائص هامة مضادة للميكروبات ، مما يوحي بإمكانية استخدامها كعامل علاجي طبيعي.

الكلمات المفتاحية: العفن الاسود, المكورات العنقودية الذهبية, الميلانين, تفاعل البوليميريز المتسلسل

1. INTRODUCTION:

Melanins are high-molecular-weight pigments that are black or brown in color and are produced when indolic or phenolic compounds undergo oxidative polymerization (Pralea *et al.,* 2019). Melanin is a secondary metabolite composed of complex, heterogeneous polymers. Melanin found in fungi is regarded as a biodegradable, natural pigment with a variety of functional properties and biological activities. Its unique qualities make it an anti-radiation, antioxidant, photoreceptor, and adsorption agent. Furthermore, melanin in hair, skin, and eyes primarily accounts for their apparent pigmentation (Hearing, 2011). Indeed, melanin and the processes involved in its formation could be useful targets for the development of antimicrobial drugs. Thus, a comprehensive understanding of melanin structure will help discover novel strategies to target this mysterious polymer (Joshua *et al.,* 2015).

Several natural antimicrobial compounds have shown promising activity against pathogenic bacteria. Essential oils such as *Citrus latifolia* (Everton *et al.,* 2018) and plant extracts, including clove and hibiscus (Sahi *et al*., 2024), have shown significant antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Klebsiella pneumoniae,* suggesting the potential use of natural products as alternatives to conventional antibiotics.

Due to their ability to function as microbial cellular factories, produce large quantities of metabolites with diverse chemical structures, and are easily cultivated on a large scale, fungi are among the microbial species that provide an important economic source of these chemicals (Chambergo and Valencia, 2016). Many pathogenic microorganisms have been linked to virulence through melanin production. Melanin is believed to contribute to microbial virulence by reducing the pathogen's sensitivity to the host's antimicrobial immune mechanisms and altering the host's immune response to infection. Therefore, melanin and the processes involved in its production are promising targets for antimicrobial drug development. Notably, both host-produced and microbial melanin can bind drugs, potentially influencing the efficacy of antimicrobial therapy. Fungal melanins are widely known for their potent antibacterial properties. For example, the growth of bacteria such as *E. coli, Proteus sp., Klebsiella pneumoniae,* and *Pseudomonas fluorescens* was significantly inhibited by melanin from *Schizophyllum commune* (Arun *et al.,* 2015). Similarly, the growth of *Lactobacillus vulgaris*, *E. coli*, and *Vibrio cholerae* was significantly inhibited via melanin extracted from *Hortaea werneckii* (Helan *et al.,* 2013). Furthermore, melanins extracted from *Armillaria mellea* were shown to inhibit the growth of *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus cereus,* and *B. subtilis*, while melanins extracted from *Exidia nigricans* and *Scleroderma citrinum* inhibited the growth of the latter two species. (łopusiewicz, 2017; łopusiewicz, 2018).

The highly productive filamentous fungus *Aspergillus niger* is of particular interest in this endeavor for several reasons, including its presence in the gut microbiota of healthy humans (Peters *et al.,* 2017). This fungus can efficiently convert renewable plant biomass into a wide range of products (such as organic acids, proteins, enzymes, and secondary metabolites), ultimately leading to the production of food, biofuels, textiles, and pharmaceuticals. Therefore, it is considered one of the most important biotechnological microorganisms on Earth (Cairns *et al.,* 2018). It thrives in environments that mimic microgravity (Cortesão *et al.,* 2022).

1.1. Aims

The objectives of this study are to determine the ability of *Aspergillus niger to produce melanin, list several factors that affect melanin production, and evaluate the antimicrobial activity* of melanin synthesized against some pathogenic microorganisms.

2. MATERIALS AND METHODS:

2.1. Materials

2.1.1. Reagents and Solutions:

1. Sabouraud Dextrose Agar (SDA)
2. Potato Dextrose Broth (PDB)
3. Potassium Hydroxide (KOH)
4. Hydrochloric Acid (HCl)
5. Distilled Water (D.W.) or reagent water type IV.
6. EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit (Bioneer)
7. Maxime PCR PreMix kit (iNtRON)
8. Ethidium Bromide dye
9. TBE buffer (1X)

2.1.2. Materials and Equipment:

1. Erlenmeyer flasks (500 mL)
2. Centrifuge tubes
3. Petri dishes
4. Microcentrifuge tubes
5. PCR tubes
6. Pipette tips
7. Centrifuge
8. Autoclave
9. Incubator
10. PCR Thermocycler
11. Gel Electrophoresis apparatus
12. UV Transilluminator
13. Vortex mixer
14. pH meter
15. Analytical balance
16. Micropipettes

2.1.3. Samples:

1. Soil samples from Karbala University for isolation of *Aspergillus niger*
2. Bacterial isolates: *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli*
3. Fungal isolates: *Microsporum canis, Mucor sp., Candida albicans*

2.1.4.Others:

1. DNA Ladder (100 bp)
2. Forward and Reverse primers for 18S RNA (ITS1) gene of *Aspergillus niger*
3. Agarose powder for gel electrophoresis

2.2. Methods

2.2.1 Isolation

A. niger was isolated from soil samples collected at Karbala University. The soil depth for collection was 1 cm, and 50 samples were collected from different college soils. Fungal mycelial plugs collected from developing colonies on SDA at 25 ˚C for three weeks were placed into 250 mL of PDB, pH 6.0, in each 500 mL Erlenmeyer flask. For three weeks, the cultivation was carried out in the dark at 25 ˚C and 125 rpm on a reciprocal shaker. To extract the fungal mycelia, the cultures were centrifuged for 15 minutes at 11000 rpm after incubation. After being dried at 60˚C for 48 hours, the fungal mycelia were weighed and stored at 4˚C in the dark. After that, they were cooled for 20 minutes (Samson *et al.,* 2014; Youngchim *et al.,* 2004).

2.2.2. Melanin extraction

Mycelial plug was cut from colonies grown on culture medium, boiled in 5ml of D.W for five minutes, and centrifuged for five minutes (5000 cycles). After the pellet was washed, 3 ml of 1 M KOH was autoclaved with it for 20 minutes at 120°C to extract the melanin. The addition of 1 M KOH was required before any more pigment could be removed. The extracted melanin was refined via acid hydrolysis (5 ml of 7 M HCl) in a sealed glass vial for 2 hours at 100°C, after which it was rinsed three times with D.W and dried overnight at 20°C in a dehumidified atmosphere.

Once the pigment had cooled, it was dried and cleaned three times with distilled water (Sun *et al.,* 2016; De Souza *et al.,* 2018).

2.2.3. Molecular identification

2.2.3.1. DNA extraction

DNA extraction from fungal colonies was performed using the EZ-10 Spin Column Fungal Genomic DNA Mini-Preps Kit supplied by Bioneer company and primers used for amplification as shown in Table 1 (White *et al.,* 1990).

2.2.3.2. Preparation of PCR master mix

The PCR reaction mixture was prepared using the Maxime PCR PreMix kit from the Korean company iNtRON, according to the company's instructions as shown in Table 2.

2.2.3.3. PCR Thermocycler conditions

A test of the PCR Thermocycler was performed for each gene, as shown in Table 3.

2.2.4. Determining factors affecting melanin production

Several factors affect the production of melanin by the fungus *Aspergillus niger*, including temperature (15, 20, 25, and 30 °C), pH (4, 6, 8, and 9), and Biomass (0.50, 0.60, 0.70, and 0.80 mg/L) (Liu *et al.,* 2022).

2.2.5.Antimicrobial effect of melanin

This test was performed by preparing a series of gradient-concentration melanin solutions. The stock solution was prepared according to the method described in Liu *et al*. (2022), and a series of dilutions was made and settled at the following concentrations: 0% (control), 10%, 25%, and 50%. The test was performed using the well diffusion method, a widely validated approach for evaluating natural antimicrobial compounds (Sahi *et al.,* 2024). Three replicates were incubated at 28 °C for a week, after which the inhibition zones were measured (CLSI, 2018).

2.2.6. statistical analysis

LSD was used to detect significant differences ($P<0.05$) in the effects of different melanin concentrations on both bacterial and fungal isolates.

3. RESULTS AND DISCUSSION:

3.1. Results

3.1.1. Detection of melanin production

This study showed a large variation of melanin production among fifty fungal isolates in which isolate No. 13 was very efficient in production by 0.30 mg/L, then isolate No. 10 by 0.274 mg/L, No. 9 and No.44, which gave a production amount of 0.253 mg/L, while the least productive isolates were isolate No. 2 and No. 3 by (0.11 mg/L), and finally No.1 and No.16 (0.002 mg/L) Table 4.

3.1.2. (PCR) product

The results of PCR analysis showed that all the isolates under study that were characterized phenotypically contained a single band of extracted DNA utilizing (DNA Ladder) of (100) bp, which was utilized as an indicator of the size of DNA fragments that may appear after replication via (PCR), as in Figure 1.

3.1.3. Melanin production affecting factors

The study showed that environmental factors affect melanin production by the fungus, with the highest production at 20 °C, where the amount reached 0.30 mg/L (Figure 2).

Regarding the effect of pH on melanin production, the optimum pH was 6, yielding 0.30 mg/L, followed by 4 (0.25 mg/L), 8 (0.10 mg/L), and 9 (0.02 mg/L). Figure 3.

As for biomass production, the study showed that production increases with biomass size, with the least productive biomass (0.50 mg/L) yielding 0.12 mg/L, while biomass (0.80 mg/L) yielded 0.30 mg/L (Fig. 4; El-Naggar *et al.,* 2020).

3.1.4. Antimicrobial effect of melanin

The study showed significant differences in the effects of different melanin concentrations on both the bacterial and fungal isolates. The bacterial and fungal isolates that were tested were subjected to different effects of melanin. The 50% concentration was the most effective, yielding zones of inhibition of 29, 25, and 20 mm for *K. pneumoniae*, *E. coli*, and *Staphylococcus aureus*, respectively. (Table 5)

Regarding fungal isolates, the 50% concentration was the most effective, with inhibition zones of 16, 15, and 13 mm for *M. canis*, *Mucor*, and *C. albicans*, respectively. (Table 6)

These inhibition zones for bacterial isolates (29, 25, and 20 mm for K. pneumoniae, S. aureus, and E. coli, respectively) are comparable to or exceed those reported for other natural antimicrobial agents. Sahi *et al*. (2024) reported maximum inhibition zones of 18-21 mm with plant extracts, while Everton *et al*. (2018) reported zones of 10-21 mm with essential oils against similar bacterial species, suggesting that melanin exhibits superior antimicrobial potential.

3.2. Discussion

Melanin is a biocompatible, biodegradable, and light-absorbing substance that can be synthesized in large quantities in vitro (Vasileiou and Summerer, 2020). There is exciting potential for *A. niger*, a biotechnologically modified cell factory, which will be utilized in the next space missions. It can be a major player in space biotechnology as a multifunctional cell factory that generates a wide range of chemicals and compounds (Cairns *et al.,* 2018). A. niger can spontaneously create and secrete pyomelanin on a g/L scale as a secondary metabolite, in addition to its ability to synthesize conservatives, proteins, enzymes, and antibiotics. This type of melanin may be utilized to shield materials, people, and habitable areas on the Moon or Mars from cosmic and solar radiation. Additionally, because melanins are known to interact with and bind to metals (Cordero and Casadevall, 2017), A. niger's capacity to create melanin may be used to remediate and preserve the environment on Earth and in space.

In a number of fields, including materials science, healthcare, green technology, cosmetics, and environmental remediation, melanin pigments can now be converted into useful materials.

From a physicochemical perspective, melanin functions as a natural "sunscreen," absorbing the UV–visible light spectrum in its broadband form. This pigment not only blocks UV light but also has strong antioxidant properties. Additionally, melanin displays hydration-dependent, semiconductor-like properties. Therefore, it is assessed as a part of organic electronic devices (Kim *et al.,* 2013; Bothma *et al.,* 2008). The bioavailability, biocompatibility, and biodegradability of microbial melanin are further benefits that make it a viable option for biomedical applications, such as implantable devices (Vahidzadeh *et al.,* 2018).

The antimicrobial properties demonstrated in this study further expand the potential applications of melanin. The inhibition zones observed against K. pneumoniae (29 mm), S. aureus (25 mm), and E. coli (20 mm) compare favorably with those of other natural antimicrobial agents such as clove extract (18-20 mm) and hibiscus extract (16-21 mm) reported by Sahi *et al*. (2024), and essential oils (10-21 mm) described by Everton *et al*. (2018).

Melanin has been used in an alternative application to create ecologically safe silver nanostructures. These melanin-mediated silver nanostructures have potential applications in the health and food sectors and exhibit broad-spectrum antibacterial activity against food pathogens (Kiran *et al.,* 2014; Patil *et al.,* 2018). Melanin is used in cosmetic and dermal applications, such as hair coloring and sunscreen. In environmental applications, melanin can act as a metal chelator. Up to 94% of Pb(II) in water systems can be removed by using fungal melanin in combination with other polymers, such as polycaprolactone and polyurethane, to create melanin-based composites (Tran-Ly *et al*. 2020).

In general, fungal production varies due to internal factors related to the fungus and external factors, such as the environment. Among the internal factors, the fungus's genotype has genes responsible for production, and more of these genes are well. Production is expressed in abundance, and vice versa when it is not expressed. Here, gene expression is weak and significantly affects cellular metabolism that produces melanin (El-Batal and Al Tamie, 2016). It was stated that many factors affect melanin production in A. oryzae, including tyrosine, dry weight, and pH. At the same time, Gonçalves *et al*. (2012) indicated that isolates differ in melanin production. It was found that some isolates produce abundantly compared to others, such as *A. nidulans*.

Temperature directly affects the structural and metabolic reactions and pathways that lead to melanin production. Pombeiro-Sponchiado *et al*. (2017) stated that the optimum temperature for growth is the same as that suitable for the production of melanin, and that 25 °C is the appropriate temperature for the melanin production by *A.nidulans*, while 30°C is the

appropriate temperature for the production of the fungus Monoascus sp.

The pH is an important environmental factor because it regulates enzyme activity. If the pH is appropriate, the fungus produces a high level of melanin (Liu *et al.,* 2022).

The relationship between biomass and melanin production is positive, and the logical explanation is that the larger the inoculum size, the faster the fungal growth, the greater the number of cells, and the greater the melanin production (Fomina *et al.,* 1999).

Infections with bacteria or fungi can damage medical equipment and food storage facilities, thereby adversely affecting human health. In addition, the emergence of antibiotic resistance in bacteria poses a serious challenge to the management of illnesses. Liu and colleagues have identified melanin as a possible antibacterial agent that can combat gram-positive and gram-negative microorganisms (Liu *et al.,* 2014).

According to Laxmi *et al*. (2016), melanin produced by the marine isolate Providencia rettgeri significantly reduced the biofilm formed by foodborne pathogens, including Bacillus pumilus, Staphylococcus warneri, and *Pseudomonas aeruginosa*.

The antibacterial efficacy of melanin against *K. pneumoniae, S. aureus*, and *E. coli* demonstrated in the present study is consistent with the growing body of evidence supporting the use of natural antimicrobial compounds. When compared with plant extracts tested by Sahi *et al*. (2024) using the same agar-well diffusion methodology, melanin showed equal or superior performance, particularly against K. pneumoniae, achieving 29 mm of inhibition compared to 18 mm for the most effective plant extract.

Extracellular melanin from the Schizophyllum commune mushroom exhibited strong antifungal and antibacterial action against *Trichophyton rubrum* and *Trichophyton simii*, as well as considerable antibacterial activity against common multiple drug-resistant infections (Arun *et al.,* 2015). Other studies found that intracellular melanin from Lachnum YM30 (Xu *et al.,* 2017) and melanin purified from the saprophytic fungus *Exidia nigricans* (Lopusiewicz, 2018) showed antibacterial activity against Salmonella typhi, Bacillus megaterium, Listeria monocytogenes, Vibrio parahaemolyticus, and *E. coli*. These investigations demonstrated the effectiveness of melanin as a strong bacteriostatic agent, with broad applications in infectious disease and biomedical settings for the functionalization of biomaterials.

Melanin affects bacterial cells by disrupting the cell membrane, allowing the release of cell contents, leading to bacterial cell death, and may also interfere with protein production and cell division. Melanin had little effect on fungi because they are eukaryotic organisms with extensive genetic material and considerable genetic variation, which allows them to withstand harsh conditions. Previous research has shown that melanin affects the cell wall and cell membrane of fungal cells (Kukuminato *et al.,* 2021).

Table 7 presents a comparative analysis of melanin's antimicrobial activity with that of other natural antimicrobial agents, tested using similar methodologies against the same pathogenic bacteria.

The superior performance of melanin against *K. pneumoniae* (29 mm vs 18 mm) and its comparable activity against *S. aureus* and *E. coli* demonstrate its potential as a strong natural antimicrobial alternative, particularly against Gram-negative bacteria.

4. CONCLUSIONS:

Aspergillus niger, a highly productive and easy-to-grow fungus, can synthesize melanin. pH 6 was the most effective for melanin production, with a temperature of 20°C also the most effective, and a biomass of 0.80 yielding the highest melanin quantity. Melanin pigments exhibit strong antimicrobial properties, with inhibition zones ranging from 20 to 29 mm against the tested pathogenic bacteria (*K. pneumoniae, S. aureus*, and *E. coli*) at 50% concentration. The highest antimicrobial activity was observed against *K. pneumoniae* (29 mm), followed by *S. aureus* (25 mm) and *E. coli* (20 mm). These results demonstrate melanin's potential as a natural antimicrobial agent, particularly effective against Gram-negative bacteria, and support its further investigation for biomedical applications.

5. DECLARATIONS

5.1. Study Limitations

This study has some limitations. Financial constraints limited the scope of the investigation, and difficulties in obtaining extraction kits and

primers affected the experimental timeline. Future research should address these limitations by exploring the mechanism of action of melanin against microorganisms, testing its activity against a broader range of pathogens, and investigating its potential synergistic effects with other antimicrobial agents.

5.2. Acknowledgments

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5.4. Conflicts of Interest

The authors declare no conflicts of interest.

5.5. Data Availability

All data presented in this study are available in the manuscript tables and figures. Raw data are available upon request from the corresponding author.

5.6. Author Contributions

Ihsan Ali (IA): Led conception and design (CD), wrote manuscript (MW), approved final version (FA). Majid Kadhim (MK): Collected data (DC), performed analysis (DAI), approved final version (FA).

5.7. AI and Computational Tools Declaration

The authors declare that no generative artificial intelligence tools or computational language models were used in the conception, design, execution, data collection, data analysis, interpretation, manuscript writing, or any other aspect of this research or manuscript preparation.

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6. STUDIES INVOLVING HUMAN AND ANIMAL SUBJECTS

6.1. Ethics Committee Approval

This study did not involve human subjects or vertebrate animals. The research utilized fungal isolates from soil samples and bacterial/fungal cultures from existing collections. Therefore, ethics committee approval was not required.

6.2. Informed Consent

Not applicable.

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Table 1. Primers used for gene amplification

18S rRNA (ITS1) gene	F	TTGTACCCTGTTGCTTCGGC	450 bp
Aspergillus niger	R	TTCAGCGGGTATCCCTACCT	

Table 2. PCR master mix

PCR master mix	**Volume**
DNA template	5µL
Forward primer (10pmol)	1µL
Reverse primer (10pmol)	1µL
Free nuclease water	13µL
Total	**20µL**

Table 3. PCR Thermocycler conditions

PCR Step	**Repeat cycle**	**Temperature**	**Time**
Initial denaturation	1	95 ºC	5min
Denaturation		95 ºC	5sec.
Annealing	30	59.3 ºC 61.3 ºC	30sec
Extension		72 ºC	1 min
Final extension	1	72 ºC	5min
Hold	**-**	**4** ºC	

***Table 4**. A.niger isolates and their production of melanin (concentrations mg/L)*

No. of isolate	melanin concentration mg/L	Standard error
1	0.002	0.00058
2	0.011	0.00058
3	0.011	0.00058
4	0.1577	0.00393
5	0.1003	0.00088
6	0.1507	0.00233
7	0.0547	0.00318
8	0.0707	0.00348
9	0.2533	0.00333
10	0.274	0.00208
11	0.016	0.00058
12	0.0267	0.00167
13	0.30	0.00153
14	0.0133	0.0012
15	0.1467	0.00441
16	0.002	0.00058
17	0.248	0.002
18	0.0053	0.00088
19	0.0767	0.00088
20	0.0113	0.00033
21	0.1557	0.00233
22	0.2517	0.00441
23	0.1453	0.00033
24	0.0167	0.00033
25	0.047	0.00173
26	0.025	0.00289
27	0.2157	0.00067
28	0.1127	0.00088
29	0.0077	0.00067
30	0.0437	0.00033
31	0.0213	0.00176
32	0.1317	0.00441
33	0.0097	0.00033
34	0.176	0.00058
35	0.131	0.001
36	0.2333	0.00167
37	0.002	0
38	0.014	0.00115
39	0.024	0.00058
40	0.0123	0.00067
41	0.019	0.00058
42	0.1317	0.00088
43	0.0777	0.00033
44	0.2533	0.00167
45	0.0073	0.00033
46	0.018	0.00058
47	0.1127	0.00145
48	0.0013	0.00033
49	0.0163	0.0012
50	0.0213	0.00067
Calculated P value	**<0.0001**	
LSD (P<0.05)	**0.0051**	

Table 5. Effect of different concentrations of melanin against Bacterial isolates

Bacterial isolates	**Control**	**Concentrations**		
		10%	**25%**	**50%**
			inhibition zone (mm)	
K. pneumonia	0±0	10.33±0.57	22.66±0.57	28±1
S.aureus	0±0	15.33±0.66	17.66±0.57	24±0.57
E. coli	0±0	11±1	15±1	19.66±0.57
LSD(P<0.05)			**1.25**	

Table 6. Effect of different concentrations of melanin against Fungal isolates

Fungal isolates	**Control**	**Concentrations**		
		10%	**25%**	**50%**
			inhibition zone (mm)	
M. canis	0±0	3.33±0.57	8.66±0.57	15±1
Mucor	0±0	5±1	7.66±0.57	14.66±0.57
C. albicans	0±0	3.66±0.57	6±1	13.33±0.57
LSD*(P<0.05)*			**1.08**	

Table 7. Comparison of inhibition zones (mm) of melanin with other natural antimicrobial agents against pathogenic bacteria.

Antimicrobial Agent	*K. pneumoniae*	*S. aureus*	*E. coli*	Reference
Melanin (50%)	29	25	20	This study
Clove extract	18	18	20	Sahi *et al.,* 2024
Hibiscus extract	16	21	18	Sahi *et al.,* 2024
Lemon juice	14	14	13	Sahi *et al.,* 2024
C. latifolia oil	14	14	13	Everton *et al.,* 2018

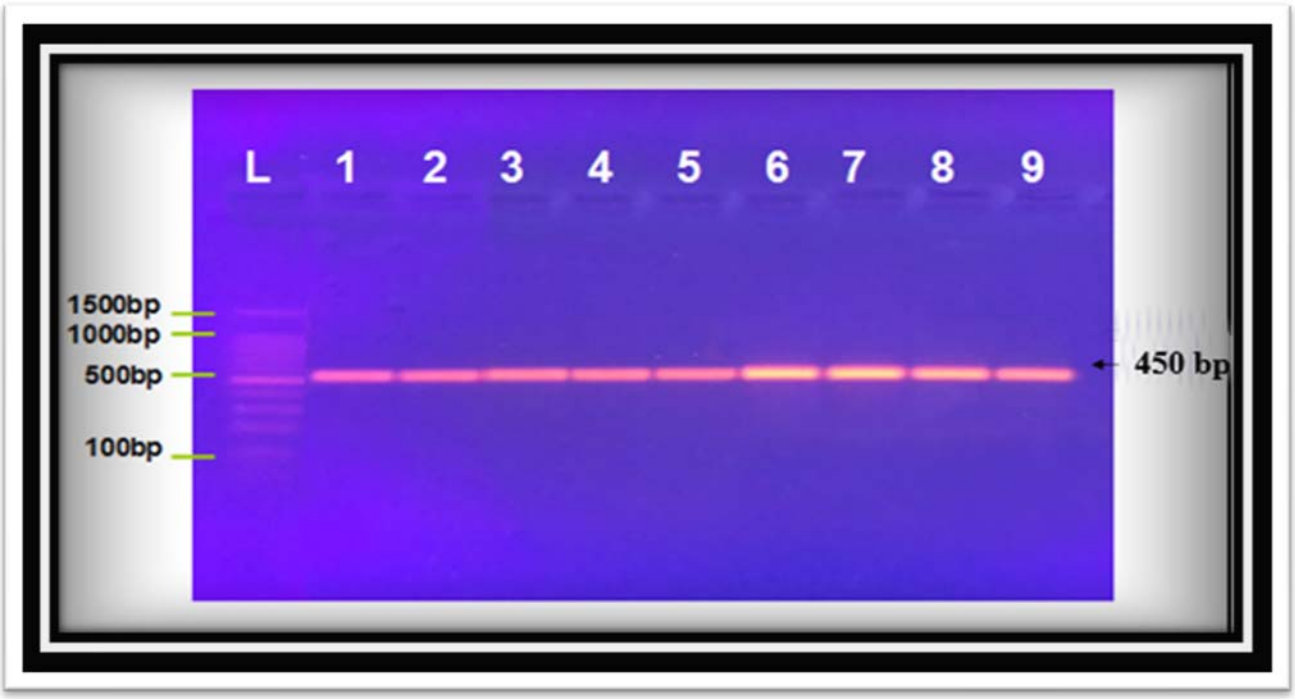

Figure 1. *Electrophoresis of the PCR product. Amplification of 18S rRNA (ITS1) of different fungal isolates. The letter L indicates the ladder marker 100 bp. Electrophoresis conditions: 100 V / for 30 minutes, Gel concentration: 1% (w/v) (w/v), Buffer used: TBE buffer (1X), ethidium bromide dye*

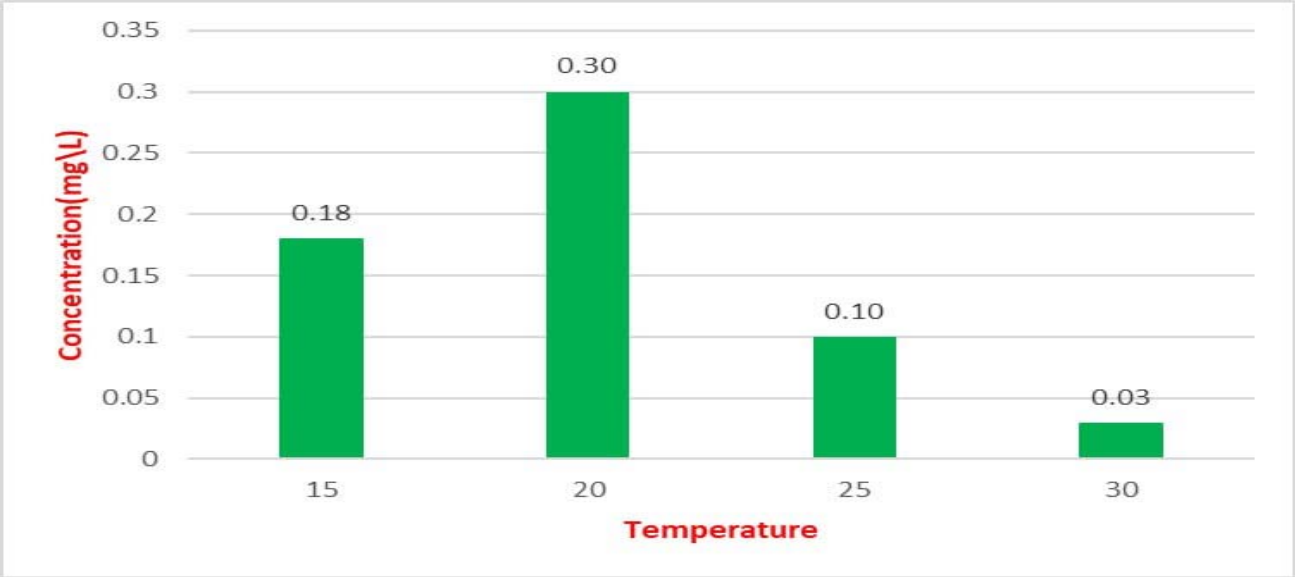

Figure 2. *Effect of temperature on melanin production*

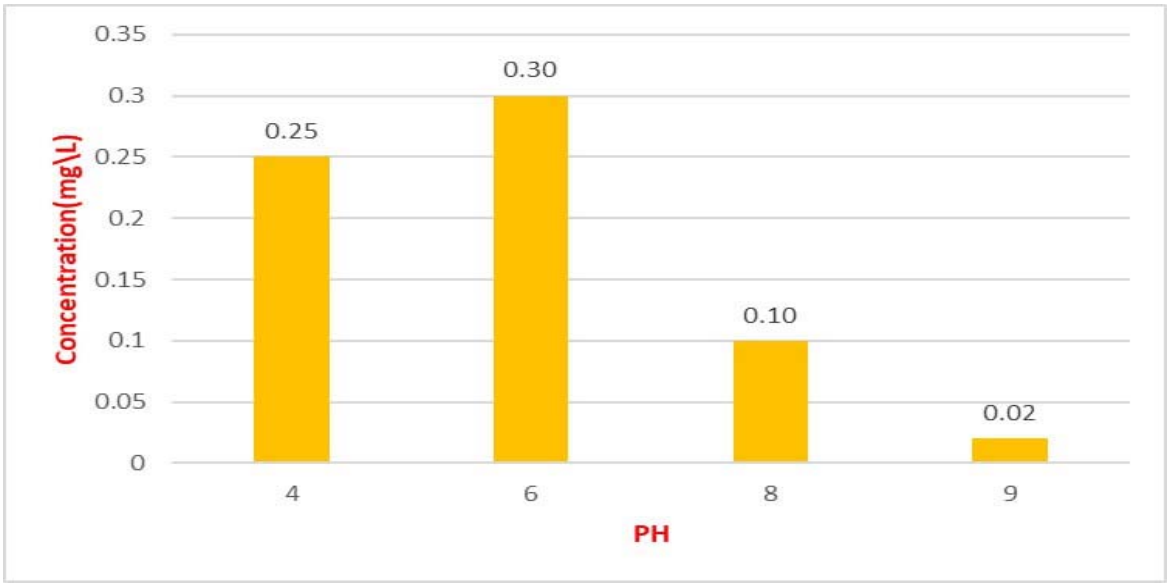

Figure 3. *Effect of pH values on melanin production*

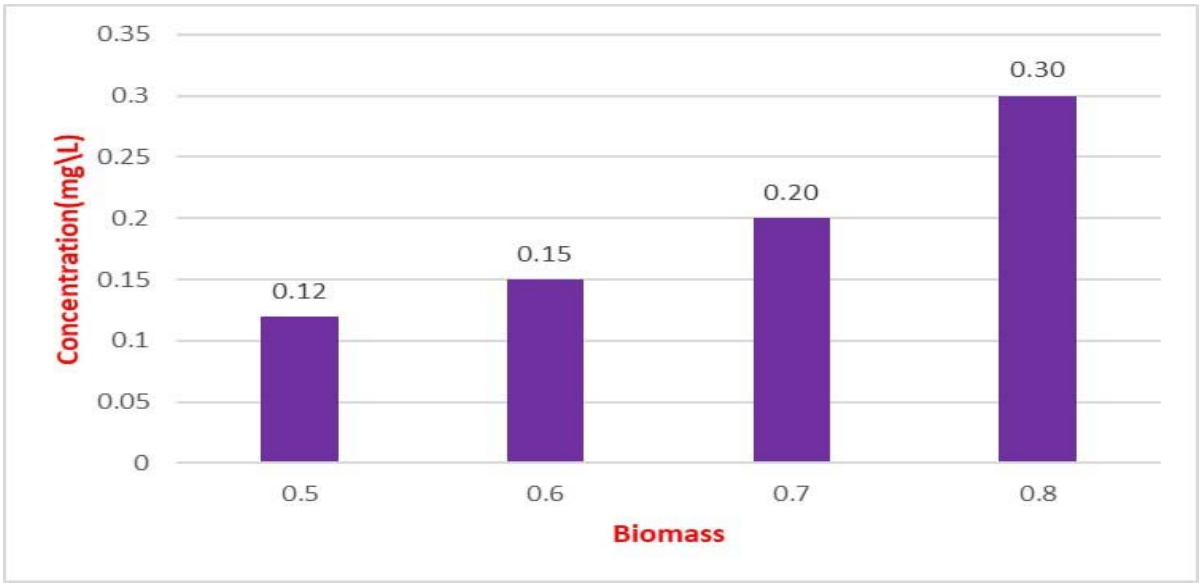

Figure 4. *Effect of biomass of fungal isolates on melanin production*