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ISOLATION, CHARACTERIZATION, AND ANTIMICROBIAL POTENTIAL OF MELANIN FROM FUNGI ASSOCIATED WITH MARINE MACROALGAE

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Background: *The marine ecosystem provides an abundant array of distinctive enzymes and pigments. Macroalgae and their accompanying microorganisms are essential to the ecological and biochemical dynamics of coastal water.* ***Method****: The study entailed the collection of diverse macroalgae from three coastal sites in Egypt, from autumn 2022 to summer 2023. This study investigated the diversity of epiphytic fungi on marine macroalgae and their potential for melanin pigment production. Then melanin investigation was conducted using UV-visible and Fourier Transform Infrared (FTIR) spectroscopy. We subsequently explored the antimicrobial efficacy of melanin against various pathogenic microorganisms.".* ***Results:*** *- Seventeen fungal species from five genera were discovered. Aspergillus niger was the predominant species throughout all seasons, comprising 32.5% in winter, 39.4% in spring, and 37% in summer. In contrast, Cladosporium oxysporum , Trichoderma viride, and Curvularia chlamydospora were the least common. Ten identified epiphytic fungal species were observed to synthesize melanin pigment, including Aspergillus niger, A. flavus, A. terreus, A. fumigatus, Trichoderma viride, Cladosporium oxysporum , Curvularia chlamydospora, Alternaria alternata, Penicillium chrysogenum , and Rhizopus stolonifer. Subsequent investigations employing UV-visible spectroscopy and Fourier Transform Infrared (FTIR) spectroscopy validated the identity of the pigments produced as melanin. The isolated melanin exhibited differing degrees of antimicrobial efficacy against various pathogenic bacteria and fungi, such as Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhi, and Candida albicans. Nevertheless, it demonstrated no efficacy against Aspergillus fumigatus.* ***Conclusion:*** *-this findings highlighted the potential of marine epiphytic fungi as a source of significant bioactive chemicals, especially melanin.*

Keywords: *Macroalgae, Epiphytic fungi, Melanin production*

INTRODUCTION

The marine microbial population, comprising bacteria, plankton, and fungi, is regarded as a crucial biological component of the marine environment. Fungi associated with seaweed have been extensively investigated for biotechnological applications. Certain investigations investigated intriguing metabolites and extracellular enzymes that are probable to decompose complicated polymers. Macroalgae are essential in nutrient cycling by ingesting dissolved elements from the water column, specifically nitrogen and phosphorus, which frequently serve as limiting variables in marine ecosystems. The absorption of nutrients can enhance water clarity and quality. Marine macrophytes as macroalgae, seagrasses, and mangroves significantly influence coastal ecosystems and their biochemical processes[1]. The study of marine macrophytes has established close associations with microorganisms from major life domains[2,3]. Bacteria and fungi living on these marine macrophytes provide essential substances including hormones, vitamins, minerals, carbon

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dioxide, and unique bioactive metabolites. These substances play crucial roles in the macrophytes' development, growth, and immune defense. Many scientists have studied the microbial communities on the surfaces of land plants and their interactions with the plants themselves [4].

Biological pigments are natural substances occurring in the environment and can be classified into two main categories: simple pigments and complex pigments. Simple or monomeric pigments are made up of single molecules such as luciferin, carotenoids, flavonoids,and heme/porphyrin-based pigments including chlorophylls, hemoglobin, and hemocyanin) and while complex or polymeric pigments are composed of multiple molecules as melanins, tannins, and humic compounds. These pigments play a vital role in various biological functions, such as camouflage, cosmetics, absorbing solar energy for metabolic processes and shielding against radiation damage.[5.]

Melanin is an intricate organic polymer formed by a chemical process involving phenols and quinones. Based on its structure, melanin can be categorized into three main types: allomelanin, pheomelanin, and eumelanin, Eumelanin and allomelanin are responsible for dark brown pigmentation in cells, while pheomelanin, commonly found in animals, produces a yellow or red color [6-8]**.** Melanin, a pigment found in all living organisms from microorganisms to plants and animals, plays a crucial role in protection or defense mechanisms [9,10]**.** Fungi, in particular, produce all types of melanin found in nature. These pigments are formed by combining phenolic compounds or polymerizing phenolic compounds **[11].** One of their key functions is to shield organisms from the harmful effects of ultraviolet radiation. Additionally, melanin acts as an antioxidant, removing harmful free radicals, can bind to metal ions, and helps organisms tolerate heat and dry conditions[10,12].

Melanin plays a dual role in biological systems. It helps maintain the structural integrity of the cell wall and can also bind to heavy metals[7,13]. Melanin is widely utilized in the derma-cosmetic sector for its ability to block harmful UV rays, making it a key ingredient in sunscreens and other skin protection products.[6,10]. In addition, melanin has demonstrated various health benefits, including anti-cancer, antibacterial, anti-inflammatory, and hepatoprotective capabilities[9, 14, 15]. Coastal regions in Egypt extend along the Eastern Mediterranean and the Red Sea for over 3,500 kilometers. The Suez Canal, a man-made waterway connecting the East and West, was inaugurated in 1869. While the Suez Canal serves as a primary navigation channel, it has also become a corridor for migrating invasive marine macroalgae species between the Red Sea and the Mediterranean Sea. Since the canal's opening[16] hundreds of alien Indo-Pacific species have migrated eastward, a phenomenon known as "Lessepsian migration". Over three decades, researchers at Suez Canal University conducted in-depth studies on the Red Sea, Eastern Mediterranean, and Suez Canal macroalgae[17-18]. Various marine macroalgae were found, leading to an abundance of certain species. Therefore, this research aims to extract and characterize melanin from fungi associated with marine macroalgae using various physicochemical techniques, such as UV-Vis spectroscopy and FTIR analysis, while assessing its antimicrobial efficacy against pathogenic microorganisms.”

MATERIALS AND METHODS

Study area and seaweed collection

Eleven abundant marine macroalgal samples were collected manually at the intertidal zone during the low tide from three sites of the Suez Canal, Egypt during a year-long study from November 2022 to August 2023 **(Table. 1 and Fig.1).** The macroalgal species were conveyed to the laboratory in a sterile polyethylene bag. for subsequent analysis. The selected macroalgae were identified according to[19-22]. The collected samples included four green macroalgae and six red macroalgae in addition to one brown macroalga **(Table. 2 and Fig. 2).**

Table 1: The studied sites, areas and their coordinates.

Site	Area	Coordinates
The Hunting Club I	Port Said, Mediterranean Sea	31.26941" N, 32.31513" E
Aldunfah Beach Club II	Ismailia, Suez Canal	30.58973"N, 32.30508"E
Fayed III	Ismailia, Suez Canal	30.3261° N, 32.2986° E

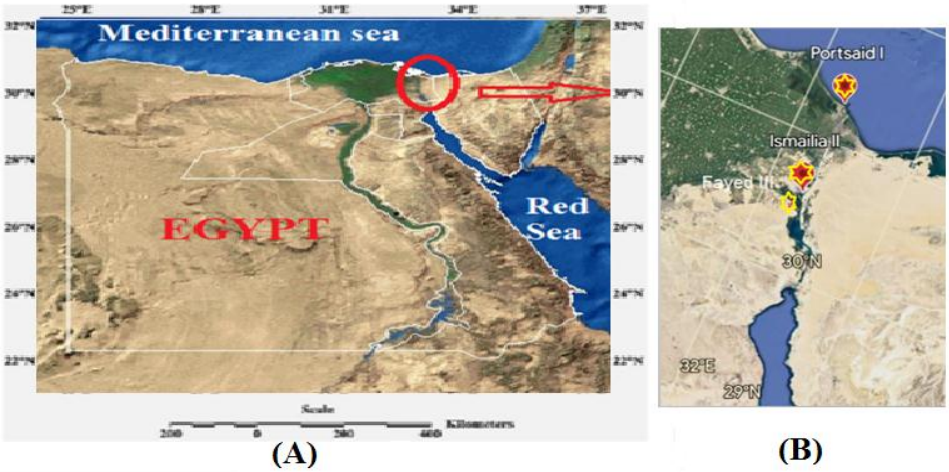

Fig. 1 : (A) Map of Egypt showing the study area, and (b) The study area showing the selected sites.

Table 2: The selected macroalgal species in the studied area.

Macroalgal species	Area & Site
Chlorophyta	
1. *Ulva lactuca* Linnaeus	Ismailia II , Fayed III
2. *Ulva fasciata* Delile	Port said I, Ismailia II
3. *Cladophora crinalis* Harvey	Port said I, Fayed III
4. *Enteromorpha compressa* (Linnaeus) Nees	Port said I, Fayed III
Rhodophyta	
5. *Sarconema filiforme* (Sonder) Kylin	Ismailia II
6. *Hypnea cornuta* (Kützing) J.Agardh	Ismailia II
7. *Acanthophora spicifera* (M.Vahl) Børgesen	Fayed III
8. *Ceramium gracillimum* Griffiths & Harvey ex Harvey	Ismailia II
9. *Gracilaria verrucosa* (Hudson) Papenfuss, nom. rejic.	Fayed III
10. *Laurencia obtusa* (Hudson) J.V. Lamouroux	Fayed III
Phaeophta	
11. *Dictyota dichotoma* (Hudson) J.V. Lamouroux	Ismailia II

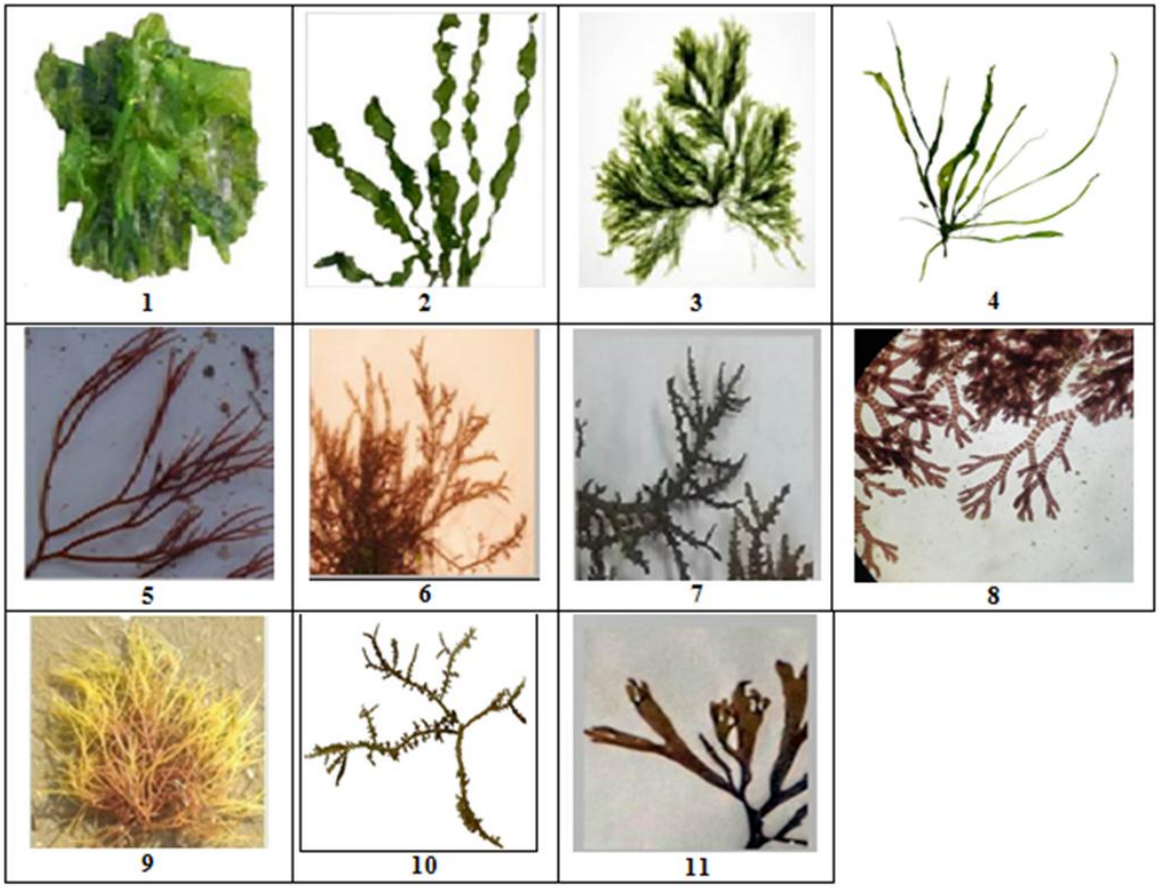

Fig. 2 : Photos of the selected abundant macroalgal species belonging to the three divisions
1**.** *Ulva lactuca* 2. *Ulva fasciata* 3. *Cladophora crinalis* 4. *Enteromorpha compressa* 5. *Sarconema filiforme* 6. *Hypnea cornuta* 7..*Acanthophora spicifera* 8..*Ceramium gracillimum* 9 .*Gracilaria verrucose* 10..*Laurencia obtuse* 11.. *Dictyota dichotoma*
Chlorophytes species from (1-4) Rhodopytes species from (5-10) Phaeophytes species (11)

Fungal Isolation

Epiphytic fungi were isolated through plating Tanique. Five fragments (1 cm each) of the selected macroalgae were sampled on potato dextrose agar medium (PDA), which was treated with 0.01% w/v chloramphenicol. The plates were incubated at 28 ± 1°C for 7 days and examined every 48 hours for fungal growth. The proliferating fungus was transferred to a new PDA medium for further cultivation[23-24]. Macro- and micro-morphological analyses were employed to identify the isolated fungi to the genus level, encompassing colony coloration, texture, and pigment synthesis, in addition to microscopic characteristics such as hyphae, conidia, stipes, and phialides inspection. Fungi were classified utilizing standard mycological and taxonomy keys.[25-26].

Propagation of fungal mycelium for melanin synthesis

Fungi were grown in a sterile potato dextrose broth solution (PDB). Small pieces of fungal material (5mm) were added to the broth in Erlenmeyer flasks and then incubated at a controlled temperature of 28±1°C for three weeks. During this time, the broth turned dark brown as the fungi produced a pigment. After the incubation period, the fungi were removed from the broth through a filtering process.

Extraction and purification of Melanin pigment

An acid-alkali method to extract melanin pigment from the fungal biomass was employed. The dried fungal material was immersed in 1N potassium hydroxide solution and autoclaved at 121°C for 20 minutes. The resulting liquid was filtered and then acidified

with 3N hydrochloric acid until the pH reached 2.5. The precipitated melanin was collected through centrifugation, washed, and extracted with a solvent mixture. After drying, the melanin powder was dissolved in a potassium hydroxide solution for further analysis. This multi-step process effectively isolated and purified the melanin pigment from the fungal source [27-28].

Characterization of fungal melanin
Physiochemical characterization

The solubility of the extracted melanin pigment was evaluated using distilled hot and cold water, along with other organic solvents such as ethanol, acetone, methanol, chloroform, hexane, ethyl acetate, DMSO (dimethyl sulfoxide), acetic acid, and petroleum ether. Additionally, 1 mol/L HCl and 1% (w/v) FeCl3 precipitate were evaluated and subsequently exposed to a strong oxidizing agent, specifically 30% H2O2[29]. L-DOPA melanin was used as standard.

UV Spectrophotometric analysis

A UV–visible spectrophotometer (T90+UV/VIS Spectrophotometer) examined the fungal melanin's 200–420nm absorption spectra. A standard L-DOPA melanin was used to measure it.[27]. L-DOPA melanin serves as a valuable standard for comparison with natural melanin due to its chemical similarity, reproducibility, purity, and utility as a model system in scientific research. provided the method for dissolving fungal melanin (1mg) in 10% 1M KOH (20 mL). The reference blank was 10% 1M KOH. The Spectrophotometric analysis study was conducted at the Central Laboratory and Toxicology Research Unit of Suez Canal University.

Fourier transform infrared spectrum FTIR analysis

The chemical compositions of fungal and L-DOPA melanin were analyzed by mixing the purified samples with potassium bromide and pressing them into discs according to [7]**.** Fourier Transform Infrared Spectroscopy (FTIR) was then performed on these discs using a BRUKER ALPHA 11 spectrometer. The spectra were obtained at a resolution of $4cm^{-1}$ in the 400-4,000cm^{-1} range. FTIR analysis was conducted at the Central Laboratory and Toxicology Research Unit of Suez Canal University.

Antimicrobial activity of tested fungal melanin extracts

The antimicrobial activity of melanin extracts from five fungal isolates (*Cladosporium oxysporum.*, *Curvularia chlamydospore*, *Aspergillus niger*, *Aspergillus flavus*, and *Alternaria alternata)* was assessed using the agar well diffusion method against four of pathogenic bacteria (*Bacillus subtilis*, ATCC 6633; *Staphylococcus aureus*, ATCC 6538; *Escherichia coli*, ATCC 8739 & *Salmonella typhi*, ATCC 6539) and two of fungi (*Candida albicans*, ATCC 10221 & *Aspergillus fumigatus*). Melanin extracts were prepared from fungal cultures and tested at a concentration of 50 µg/well. Gentamicin and Fluconazole served as positive controls, while saline solution acted as a negative control. The diameter of the inhibition zones around each well was measured to evaluate the antimicrobial efficacy of the melanin extracts. Each experiment was repeated three times, and the mean diameter of the inhibition zones was calculated.[30]

Statistical analysis

Boxplots for data analysis were conducted using PAST: Paleontological Statistics (Version 2.17). Multivariate and cluster analyses were performed to categorize the 10 examined fungal species using PAST based on all acquired quantitative traits [31].

RESULTS AND DISCUSSION

Composition of Epiphytic Fungal Communities

In the present study, it was revealed that 17 fungal species from 5 genera were associated with 11 abundant macroalgal species across the three study sites over a year-long period from (autumn 2022 to summer 2023). It

revealed that only 10 species (including *Aspergillus niger*, *A. flavus*, *A. terreus*, *A. fumigatus*, *Trichoderma viride*, *Cladosporium oxysporum* ., *Curvularia chlamydospora*, *Alternaria alternata*, *Penicillium chrysogenum sp.*, and *Rhizopus stolonifera*) were capable of producing melanin **(Fig. 3)**. Most of these melanin-producing fungi belong to the phylum Ascomycota, while *Rhizopus stolonifera* is a member of the phylum Zygomycota. The total frequency % of the fungal species occurrence isolated from the selected abundant marine macroalgae during the study period at three sites is summarized in **(Table. 3)**. The seasonal variation of the fungal community indicated that spring represented the highest frequency (98.4%), followed by summer (96.2%), autumn (95.6%), and winter (92.9%). The seasonal variation may attribute to changes in environmental conditions (temperature, light, nutrients). Host-microbe interactions and community dynamics. External factors like climate and human activities.

This study demonstrated that the macroalga *Laurencia obtusa* is the predominant algal species linked to a significant proportion of epiphytic fungus species in spring and winter, accounting for 14.6% and 14.7%, respectively. During summer and autumn, *Ulva fasciata* and *Hypnea cornuta* were regarded as the predominant algal species associated with a significant proportion of epiphytic fungal species at 23.0% and 13.9%, respectively. This study, with Site 1 exhibiting various species throughout the year. Site 2 displayed a restricted fungal community, with *A. flavus* and *Cladosporium oxyporum* regularly prevailing. Site 3 had a comparatively constant fungal composition, characterized by the prevalence of *A. niger* and *A. terrus*. This Site-Specific variation may be due to Seasonal changes or fluctuations in water quality and nutrient levels which can lead to different fungal assemblages at different times of year. Also, local human activities, such as pollution or coastal development, may influence the fungal communities present at specific sites, leading to variations in epiphytic fungal populations.

Aspergillus niger exhibited the highest cumulative frequency percentage throughout three seasons. Consequently, winter (S3) constituted 32.5% isolated from *Cladophora crinalis.* During spring and summer, constituted 39.4% and 37%, respectively from *Ulva fasciata* Additionally, *Cladosporium oxysporum* represented the lowest total frequency (1.1%) during autumn (S3) from *Ulva lactuca*, *A. terrus* constituted 1.2% in winter (S2) from *Ceramium gracillimum*, *Trichoderma viride* represented 3.3% in spring (S3), and *Curvularia chlamydospore* comprised 2.2% in summer (S2) from *Ulva lactuca* **(Table. 3)**. The current work corroborates the findings of[32-33]. who demonstrated that fungi constitute a significant and diverse group of microorganisms within marine microbiological communities and are essential for nutrient cycling. Furthermore, these data agree with **[34].** who indicated that fungi derived from algae can be linked to various algal types, including brown (e.g., *Agarum clathratum, Fucus sp., Laminaria sp., Sargassum sp.),* green *(e.g., Ulva sp., Enteromorpha sp., Flabellia sp.),* and red *(e.g., Chondrus sp., Dilsea sp., Ceramium sp.)* algae. This current study corroborates previous findings by [35]. which reported that the most frequently documented fungi associated with algae belong to the Ascomycota phylum and encompass a diverse array of genera, including *Acremonium, Alternaria, Aspergillus, Cladosporium, Phoma, Penicillium chrysogenum , Trichoderma, Emericellopsis, Retrosium, Spathulospora, Pontogenia, and Sigmoidea*. Numerous studies have confirmed the existence of various microbiological bacterial and fungal symbionts on the surfaces of macroalgae and seagrasses, which contribute to morphological development and defense mechanisms[36]**.** This study revealed a clear seasonal fluctuation in the fungus flora over the four seasons. This conclusion is corroborated by **[3].** who indicated that seasonal fluctuations, geographical disparities, and environmental factors affect the epiphytic microbial composition of macroalgae and seagrasses. Moreover**, [37.]** revealed that the composition of epiphytic microorganisms associated with macroalgae and seagrasses differs according to the host species.

The boxplot in **(Fig. 4)** illustrates the total frequency % in the different fungal species' prevalence from autumn 2022 to summer 2023. There was a wide range of frequencies among the different fungal species. Some genera, such as *A. niger* and *Afumigatus*, exhibited significantly higher frequencies in all seasons.

In contrast, other species, like *Rhizopus stolonifera* and *Curvularia chlamydospore*, demonstrated more pronounced seasonal fluctuations. The diversity of the isolated fungi proposes a dynamic fungal community with a range of species adapting to varying environmental conditions.

The provided dendrogram **(Fig. 5)** represents a hierarchical clustering analysis of fungal species across different seasons. The dendrogram reveals distinct clusters among the seasons. The closest clustering is observed between winter 2023 and spring 2023, suggesting a similarity in their fungal composition. The next closest cluster was formed by the summer 2023 and autumn 2022. This indicates a moderate level of similarity between these seasons. The overall structure of the dendrogram suggests a clear seasonal pattern in fungal occurrence. The clustering suggests that the fungal community in winter 2023 is more closely related to spring 2023 than to summer 2023 and autumn 2022 communities.

Table 3: Total frequency % of fungal species isolated from the selected abundant marine macroalgae during the study period.

Season & Total frequency %	Site	Fungal general& Frequency% Abundant / Macroalgae	*A. niger*	*A. fumigatus*	*A. terrus*	*A. flavus*	*Trichoderma viride*	*Rhizopus stolonifera*	*Curvularia chlamydospora*	*Cladosporium oxysporum*	*Alternaria alternata*	*Penicillium chrysogenum* sp.
Autumn 2022 95.6 %	S1	*Ulva Fasciata*	0.5	1.8	3.8	-	5	-	-	-	-	5
		Cladophora cronalis	3.3	-	4	3.3	-	2.7	2.7	-	-	-
	S2	*Ulva lactuca*	1.2	2.2	-	-	2.4	4	-	-	-	0.7
		Sarconema filiformae	1.1	1.7	-	-	-	-	-	-	2.7	-
		Hypnea cornuta	0.6	3.3	-	4.5	-	4	1.5	-	-	-
		Dictyota dichotoma	2.2	1.1	2.7	3.3	-	-	-	-	2.4	-
	S3	*Acanthophora spicifera*	2.7	1.1	2.7	3.1	-	-	-	-	2.4	-
		Ulva lactuca	2.2	1.1	3.1	-	-	-	-	1.1	2.4	-
Total frequency %			**13.8**	**12.3**	**16.3**	**14.2**	**7.4**	**10.7**	**4.2**	**1.1**	**9.9**	**5.7**
Winter 2023 92.9 %	**S1**	*Ulva fasciata*	3	-	-	-	0.6	0.6	2.5	-	-	3.7
		Enteromorpha compressa	3.7	-	-	-	1.2	-	-	-	-	5
	S2	*Ulva fasciata*	1.8	-	-	-	1.2	0.6	-	1.2	-	3.7
		Ceramium gracillimum	3	-	1.2	7.5	0.6	-	-	-	-	-
	S3	*Ulva lactuca*	3.0	-	-	-	-	-	-	-	2.5	-
		Enteromorpha compressa	3.7	--	--	0.5	1.2	-	-	-	-	3.7
		Gracilaria verrucose	3.7	-	-	3	-	0.6	-	-	3.5	-
		Laurencia obtusa	5.0	-	-	-	1.8	-	1.8	1.8	-	4.3
		Cladophora cronalis	5.6	-	-	3	0.6	-	-	-	2.5	-
Total frequency %			**32.5**	**0**	**1.2**	**14**	**7.2**	**1.8**	**4.3**	**3**	**8.5**	**20.4**
Spring 98.4 %	**S1**	*Ulva Fasciata*	7.7	3.5	-	4..1	1.1	-	-	1.7	-	-
		Enteromorpha compressa	5.9	-	4.7	-	1.1	-	-	-	-	--
		Ulva lactuca	5.3	2.9	-	-	1.1	-	-	4.7	-	-
	S2	*Ceramium gracillimum*	2.9	-	4.1	1.7	-	-	-	1.7	-	-
		Hypnea cornuta	5.9	-	3.5	-	-	-	2.3	-	-	-
	S3	*Ulva lactuca*	3.5	-	2.9	2.3	-	-	-	-	2.3	-
		Enteromorpha compressa	3.5	-	-	1.7	-	-	-	2.9	2.9	-
		Laurencia obtusa	4.7	-	5.3	-	-	-	2.9	1.7	-	-
Total frequency %			**39.4**	**6.4**	**20.5**	**5.7**	**3.3**	**0**	**5.2**	**12.7**	**5.2**	**0**
Summer 96.2%	**S1**	*Ulva fasciata*	7.3	5.6	-	6.2	-	-	-	2.8	1.1	-
		Enteromorpha compressa	4.5	-	2.2	-	-	-	-	-	1.1	4.5
	S2	*Ulva fasciata*	5	2.8	-	-	-	-	-	2.2	1.6	-
		Ceramium gracillimum	5.6	-	2.2	1.6	-	-	-	1.6	-	-
		Ulva lactuca	4.5	-	3.3	-	-	-	2.2	-	-	-
	S3	*Ulva lactuca*	5.6	-	2.8	2.2	--	-	-	-	3.3	-
		Enteromorpha compressa	4.5	-	-	3.3	-	-	-	3.3	3.3	-
Total frequency %			**37**	**8.4**	**10.5**	**13.3**	**0**	**0**	**2.2**	**9.9**	**10.4**	**4.5**

Where, S1: Port Said, S2: Ismailia, S3: Fayed

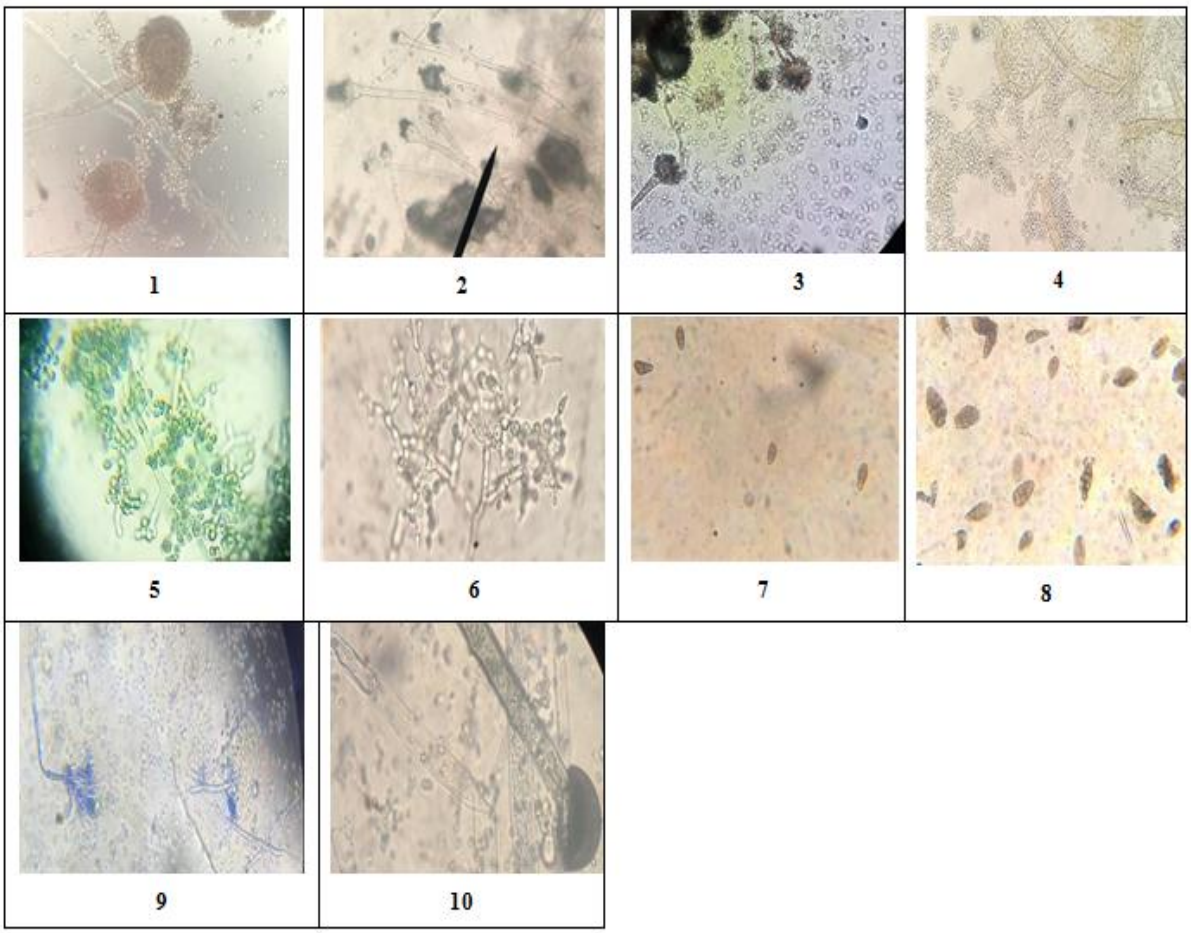

Fig. 3 : Some types of fungi associated with the abundant marine macroalgae on 40X 1. *Aspergillus niger* 2. *A. fumigitus* 3. *A.terrus* 4. *A.flavus* 5. *Trichoderma viride* 6. *Cladosporium oxysporum* 7. *Curvularia chlamydospore* 8. *Alternaria alternata* 9. *Penicillium chrysogenum* 10. *Rhizopus stolonifera*

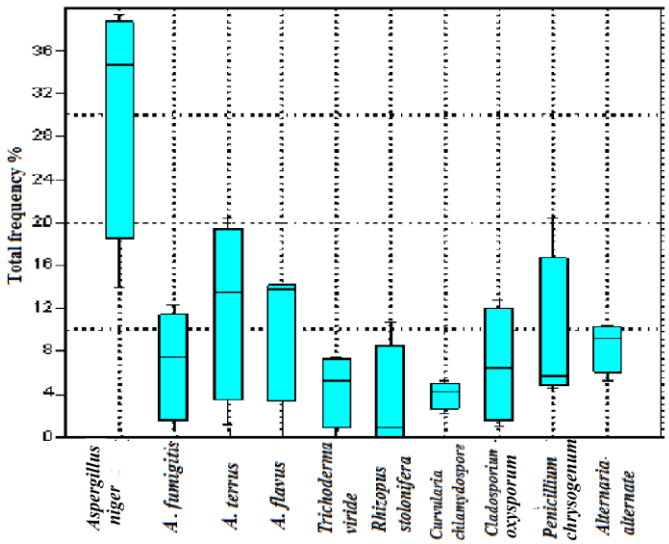

Fig. 4 : Boxplot of the total frequency % of the isolated fungal species during the study period**.**

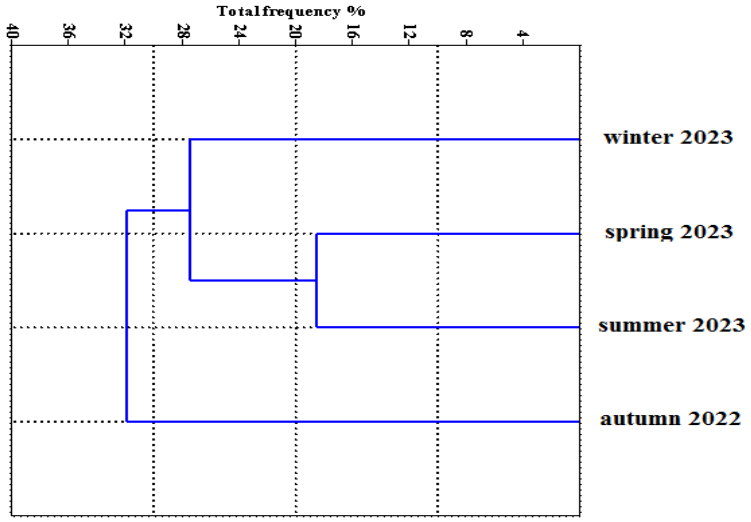

Fig. 5 : Cluster analysis of the fungal species across different seasons during the study period**.**

Screening of melanin-producing fungi

The relative abundance of the isolated fungal species concerning their crude melanin pigment production was depicted in **(Fig. 6).** Among the examined fungi, *A.alternata* , *Curvularia chlamydospore*, *Cladosporium oxysporum* ., and *Rhizopus stolonifera* which were identified as the principal producers of crude melanin pigment, yielding 0.9, 0.8, 0.7, and 0.6 mg/250 ml, respectively. *A. niger* and *A. terrus* provide modest yields of 0.38 mg and 0.5 mg/250 ml, respectively. The remaining species, comprising *A. f.umigatus*, *Penicillium chrysogenum sp.*, *Trichoderma viride*, and *A. flavus*, exhibited less melanin pigment production. Certain species, like *A. fumigatus* (0.06 mg/250 ml), contributed relatively little yields to the total output. After 21 days of incubation at 28±1°C, the 10 fungal species generated a brownish-black mycelial mat accompanied by a droplet of diffusible black pigment in the PDB broth **(Fig. 7)**.

Melanin is a versatile bioactive molecule that holds promise for a wide range of applications in biotechnology, environmental science, and medicine. Its potential uses span cosmetics, antioxidant activities, drug delivery, antimicrobial treatments, and cancer therapy [6-7]**.** Traditional methods of melanin synthesis have struggled to keep pace with growing global demand. As a result, [10] are actively exploring innovative approaches for large-scale production. Microorganisms, known for their sustainability and cost-effectiveness, have emerged as a promising bioresource for melanin synthesis. Studies have investigated various microbial sources, from diverse biological environments, for their ability to produce melanin[7,13].

Physicochemical characteristics of the extracted melanin

(Table. 4) summarized the physiochemical properties of extracted fungal melanin pigments from the different fungal species and compared them to a standard DOPA melanin. The properties evaluated include color, solubility in various solvents, and reaction with ferric chloride ($FeCl_3$). Most of fungal melanin pigments exhibited a blackish-brown color, similar to the standard DOPA melanin. However, *Penicillium chrysogenum* . and *Trichoderma viride* produced pigments with a darker green color. The fungal melanin pigments generally exhibited limited solubility in common solvents. They were soluble in 1M KOH, but insoluble in chloroform, acetone, methanol, ethyl acetate, and distilled water. However, they are partially soluble (P.S.) in dimethyl sulfoxide (DMSO).

All the fungal melanin pigments precipitate (PPT) upon adding 1% $FeCl_3$, as DOPA melanin standard that confirmed the extracted pigment was melanin. The studied physicochemical parameters of the extracted melanin were confirmed by previous documents [8,15,38,39]. Our findings agree with [8,40,41]. who indicated that melanin synthesized by fungi insoluble in water, ethyl acetate, and chloroform, exhibits partial solubility in DMSO and is fully soluble in KOH solution.

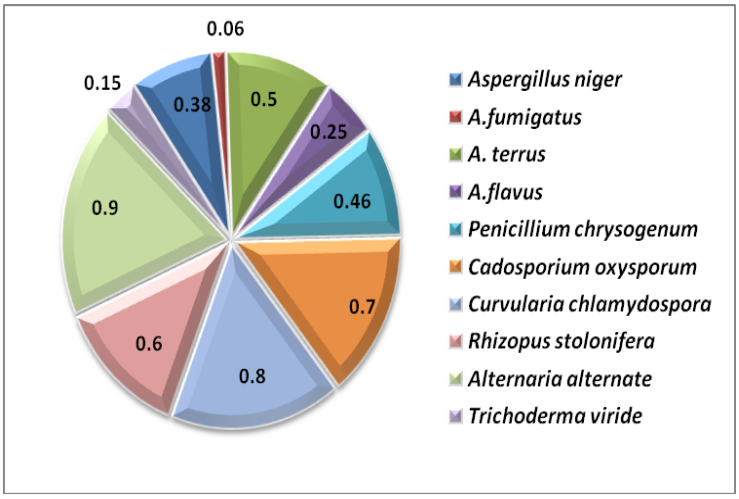

Fig. 6 : Average yields of crude melanin pigment mg/250 ml**.**

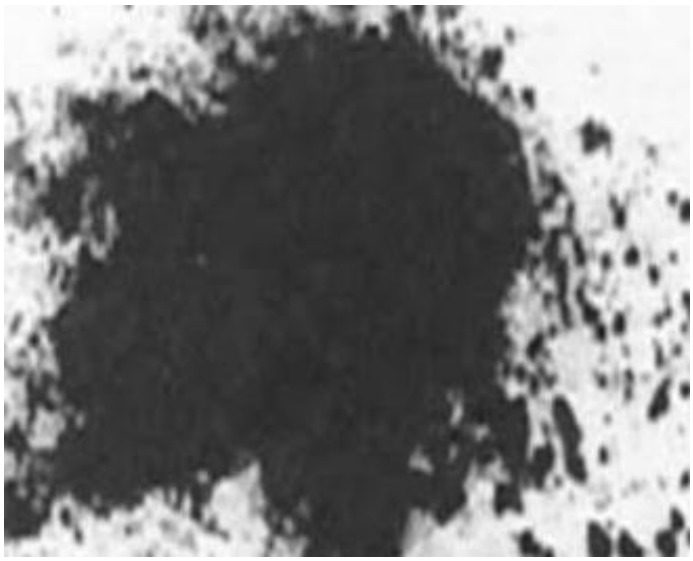

Fig. 7: Pellets of fungal melanin after extraction and purification.

Table 4: Physiochemical properties of the extracted fungal melanin pigment from different fungal species and standard DOPA melanin standard.

Fungal species / Test	*A. niger*	*A. fumigitius*	*A. flavus*	*A. terrus*	*Cladosporium oxysporum*	*Curvularia chlamydospora*	*Penicillium chrysogenum*	*Rhizopus stolonifera*	*Alternaria alternata*	*Trichoderma viride*	DOPA
Color	Blackish brown	Blackish brown	Blackish brown	Brown	Brown	Blackish brown	Dark green	Blackish brown	Blackish brown	Dark green	
Solubility test	+	+	+	+	+	+	+	+	+	+	+
1M KOH	-	-	-	-	-	-	-	-	-	-	-
Chloroform	-	-	-	-	-	-	-	-	-	-	-
Acetone	-	-	-	-	-	-	-	-	-	-	-
Methanol	-	-	-	-	-	-	-	-	-	-	-
Ethyl acetate	-	-	-	-	-	-	-	-	-	-	-
Dist. water	-	-	-	-	-	-	-	-	-	-	-
DMSO	P.S	P.S	P.S	P.S	P.S	P.S	P.S	P.S	P.S	P.S	P.S
1% $FeCl_3$	PPT	PPT	PPT	PPT	PPT	PPT	PPT	PPT	PPT	PPT	PPT

Where, +: Sign denotes soluble, -: Sign denotes Insoluble, P.S: Partially soluble and PPT: Precipitated.

Spectrophotometric characterization

The UV-visible spectrum (200-600 nm) of the purified melanin from various fungal isolates exhibited a prominent absorption peak in the ultraviolet region, specifically between 226 and 232 nm **(Fig. 8)**. As the wavelength increased, the absorbance significantly decreased, aligning with the characteristic absorption pattern of melanin. The plot of the logarithmic optical density (OD) of the melanin solution against wavelength resulted in a linear curve with a negative slope, further confirming the presence of melanin.

The extracted melanin displayed a characteristic UV absorption peak within the 226-232 nm range, which may be attributed to complex conjugated aromatic chemical compounds within their molecular composition [42]. The observed linear decrease in absorption intensity with increasing wavelength is a well-established property of melanin [43] This linear relationship between wavelength and logarithmic optical density, represented by a negative slope in a plot, serves as a diagnostic tool for identifying melanin [39]. In agreement with these findings [44] reported comparable negative slopes (−0.0015 to −0.0030) for melanin extracted from various terrestrial and endophytic fungi.

Fourier transform infrared spectrum FTIR analysis

Fourier-transform infrared spectroscopy (FTIR) was employed to analyze the functional group composition of melanin pigments produced by various fungal species as shown in **(Fig. 9)**. The FTIR spectra of the studied melanin fungal samples revealed characteristic absorption peaks associated with specific functional groups. A prominent absorbance band was observed around 3300 cm^{-1}, indicating the O-H stretching vibration of alcohols and carboxylic acids. Additionally, a strong absorption peak near 2920 cm^{-1} corresponded to the N-H stretching vibration of amine salts. The presence of carbonyl groups (C=O) in acids, esters, and ketones was confirmed by a distinct peak at 1720 cm^{-1} in the spectra of *Cladosporium oxysporum* ., *Curvularia chlamydospora*, and *Rhizopus stolonifera*. This carbonyl band is particularly significant for melanin detection and is associated with a conjugated quinone structure. These FTIR results provide valuable insights into the chemical composition of fungal melanin pigments and highlight the presence of key functional groups that contribute to their unique properties.

The functional groups, particularly conjugated alkenes (C=C stretching) observed at multiple wavelengths (e.g., 1631, 1636, 1621, 1629, 1638, 1619, and 1627 cm^{-1}), support the designation of the pigment as melanin. Melanin is known to absorb light broadly across the UV-visible spectrum due to its complex, heterogeneous structure. This broad absorbance is another hallmark of melanin and supports its identification. This data is consistent with earlier studies by[8 , 15,] who observed similar FTIR absorption peaks at 3,348, 2,890, and 1,634 cm^{-1} in the melanin extracted from *Thermothelomyces hinnuleus* SP1. These peaks correspond to hydroxyl (-OH and -NH) and aromatic (C=C and CO) groups, confirming the melanic nature of the pigment.

This study investigated epiphytic fungi associated with macroalgae as a potential novel source of melanin. Other researchers have reported that the fungal genera *Aspergillus*, *Penicillium chrysogenum* , and *Curvularia* produce melanin[15]. Our findings corroborate prior studies demonstrating the melanin-producing capabilities of *Aspergillus*, *Alternaria*, *A. terreus*, and *Penicillium chrysogenum species* when cultured on various media[15,45]. The variation in melanin production observed can be chiefly ascribed to interspecies differences and strain-specific variations in pigment synthesis [46] Although extraction methods, growth media, and cultivation conditions may affect pigment yield, it is broadly acknowledged that certain fungal species inherently exhibit a greater capacity for melanin production than others.[15,39] .

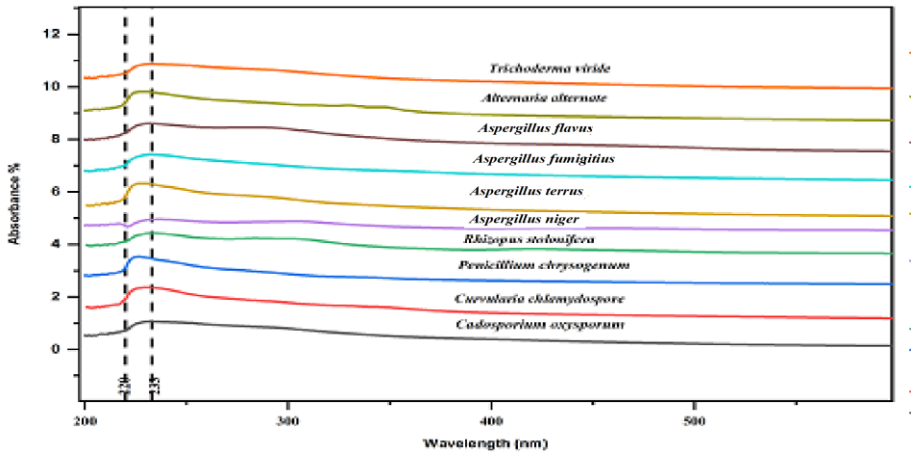

Fig. 8 : Ultraviolet-visible (UV–visible) absorbance spectrum of melanin pigment from epiphytic fungal species.

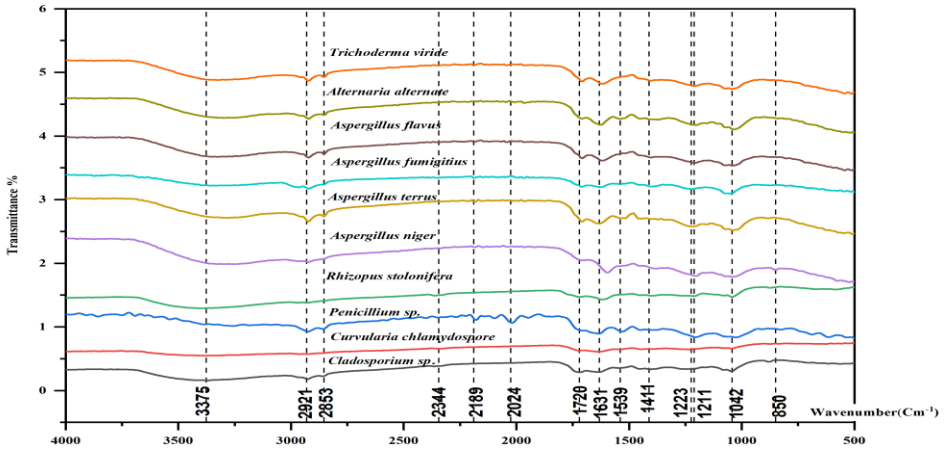

Fig. 9 : FTIR spectrum of melanin pigment synthesized by different epiphytic fungal species.

Antimicrobial activity of melanin

The extracted melanin from several fungi exhibited varying degrees of antimicrobial activity against distinct clinical bacterial and fungal isolates **(Table. 5)**. Our findings indicated that melanin extract effectively inhibits the proliferation of diverse clinical bacteria with varying potency. The mean inhibition zones were as follows: 25.00 to 28.00 mm against *B. subtilis*; 20.00 to 25.00 mm against *S. aureus*; 22.00 to 27.00 mm against *E. coli*; 20.00 to 23.00 mm against *S. typhi;* 27.00 to 39.00 mm against *C. albicans*; and no effect against *A. fumigates*. The importance of melanin extract as an effective agent against Gram-positive and Gram-negative bacteria was attributed to its offers as a potential solution to antibiotic resistance bacteria, it was considered as a natural, biocompatible, and sustainable material., and it provides insights into novel antimicrobial mechanisms that could inspire new therapies. A significant antimicrobial efficacy was demonstrated compared to the positive controls Gentamycin and Fluconazole **(Fig. 10)**. The results indicated that fungal-extracted melanin was an intriguing antimicrobial agent, demonstrating notable antibacterial and antifungal properties. However, *Candida albicans* recorded a high inhibition zone diameter (mm).in case of *Alternaria alternata* melanin exteact (39±0.2), *Curvularia chlamydospore* (32±0.2), and *Aspergillus flavus* (31±0.2) melanin extracts respectively**. (Fig. 11)**.

Table 5: Antimicrobial activity of fungal melanin pigment extract against different pathogenic isolates using well diffusion (inhibition zone, mm) **.**

Melanin test from selected fungal species (mm)& control	-Ve Control (vehicle)	+Ve Control	*Cladosporium oxysporum*	*Curvularia chlamydospore*	*Aspergillus niger*	*Aspergillus flavus*	*Alternaria alternata*
pathogenic species	**100 µl (50 µg/well)**						
Bacillus subtilis	**Nil**	**26±0.2**	**27±0.1**	**28±0.1**	**25±0.1**	**28±0.2**	**27±0.1**
Staphylococcus aureus	**Nil**	**20±0.2**	**24±0.2**	**25±0.2**	**22±0.1**	**22±0.2**	**20±0.2**
Escherichia coli	**Nil**	**21±0.1**	**24±0.2**	**27±0.2**	**24±0.2**	**24±0.1**	**22±0.1**
Salmonella typhi	**Nil**	**20±0.2**	**20±0.1**	**23±0.2**	**22±0.1**	**21±0.1**	**21±0.1**
Candida albicans	**Nil**	**27±0.2**	**27±0.2**	**32±0.2**	**30±0.1**	**31±0.2**	**39±0.2**
Aspergillus fumigatous	**Nil**	**24±0.1**	**Nil**	**Nil**	**Nil**	**Nil**	**Nil**

Where, Nil: No activity, Control for Bacteria was Gentamycin, and for fungi was fluconazole

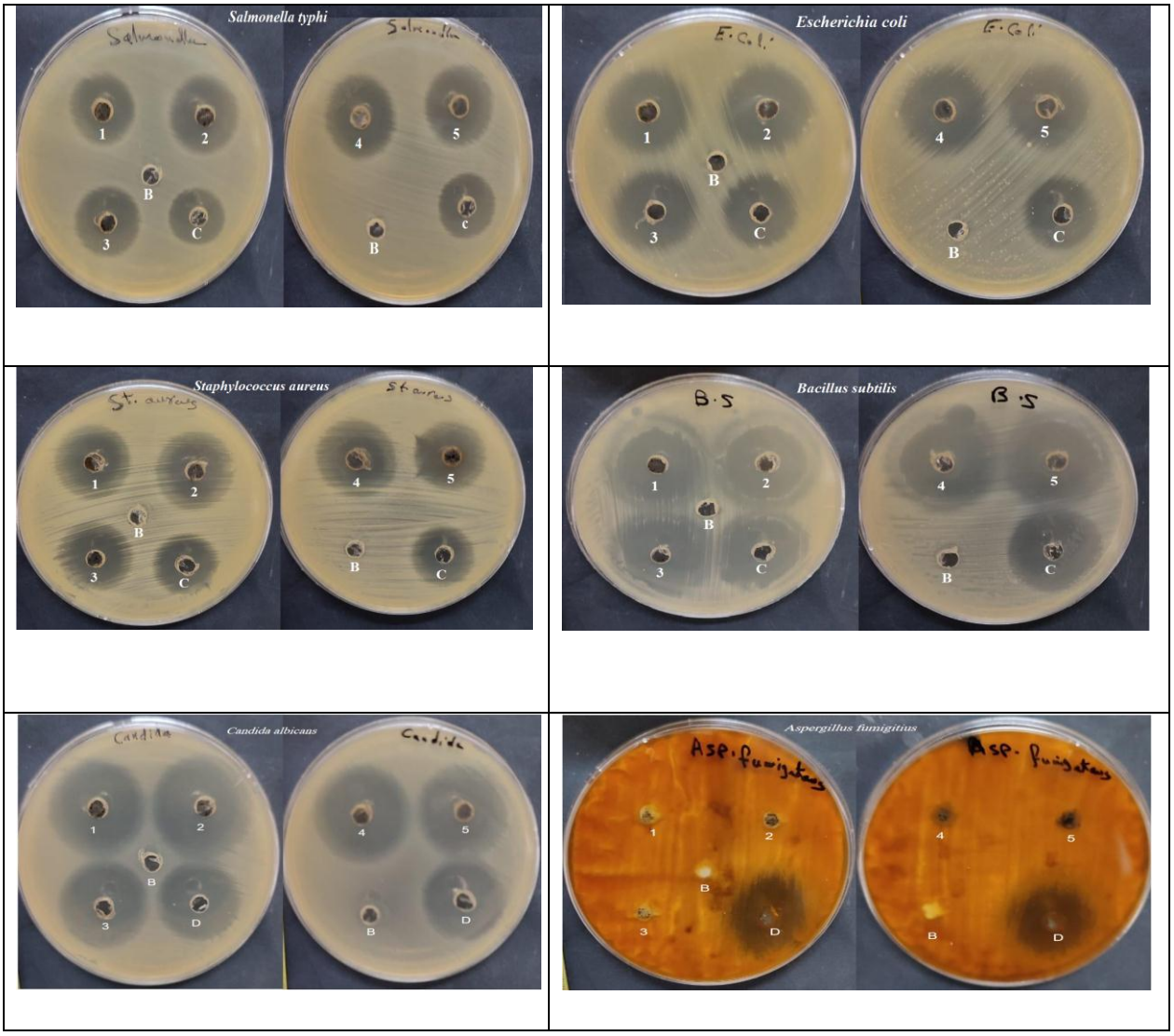

Fig. 10 : Photos of inhibition zones (mm) of fungal extracted melanin antimicrobial activity: 1- *Cladosporium oxysporum* , 2- *C. chlamydospore, 3- A. niger , 4- A. flavus, 5- Alternaria alternata , B- Blank, C- Gentamycin and D- Fluconazole.*

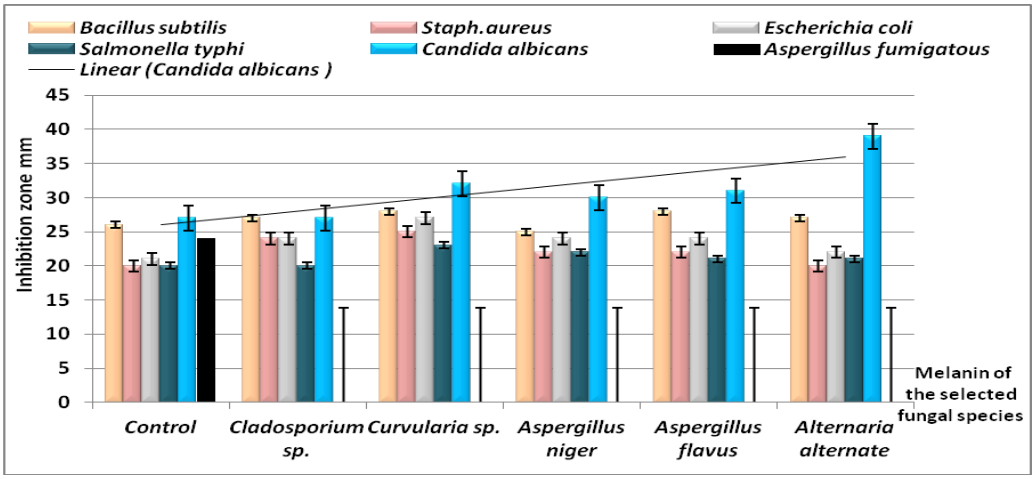

Fig. 11 : Effect of Fungal extracted melanin on different microbes. Values are expressed as mean±S.E.

The data was analyzed in collaboration with another researcher. [47]. who discovered that the mushroom fungus *Schizophyllum commune* produces melanin, which exhibits significant antibacterial action against *E. coli, Proteus sp., Klebsiella pneumoniae,* and *Pseudomonas fluorescens*, as well as antifungal activity against *Trichophyton simii* and *T. rubrum*. The synthesis of melanin by the endophytic fungus *Phoma sp.* RDSE17 was investigated for its antibacterial efficacy against *Bacillus subtilis, Staphylococcus aureus, E. coli,* and *Salmonella typhi*, as well as its antifungal efficacy against *A. flavus, A. niger, Rosellinia sp.*, and *Ustilaginoidea virens* [15].

The data indicated that melanin appeared more effective against Gram-negative and Gram-positive bacteria.[48]. Melanin pigment can successfully treat microbial infections. Melanin synthesized by *Streptomyces sp.* shown antibacterial activity against *Lactobacillus vulgaris* and *E. coli* [49]. Furthermore, melanin has been identified to be antibacterial against *Candida albicans* and *Helicobacter pylori* [50]. Comparable research has elucidated the antifungal efficacy of black melanin against A. *niger, A. oryzae,* and *Penicillium chrysogenum sp* [8]. Furthermore, melanin has shown significant antifungal effectiveness against the dermatophyte fungus, *Trichophyton rubrum,* and *T. simii*[47]. Finally, this study demonstrated the significant antimicrobial potential of melanin pigments extracted from fungi associated with marine macroalgae. The isolated and characterized melanin exhibited inhibitory activity against a broad spectrum of pathogenic microorganisms, including bacteria and fungi. These findings emphasized the promising future of marine fungal melanin as a natural antimicrobial agent.

Conclusion

The study examined the diversity of epiphytic fungi linked to macroalgae from three coastal sites in Egypt over the course of a year. Fungal communities reached their highest abundance in spring, followed by summer, autumn, and winter. The predominant phyla were Ascomycota and Zygomycota. The isolated fungal melanin was analyzed by UV-Vis spectroscopy and FTIR, which validated its identification. Fungal melanin showed differing levels of antimicrobial efficacy against several pathogenic pathogens, encompassing both gram-positive and gram-negative bacteria, and also revealed antifungal properties against *Candida albicans*, but not against Aspergillus fumigatus. Despite more than a century of investigation, the biological functions, taxonomy, and ecological relationships of marine fungi remain predominantly unexamined. Additional research is required to investigate the potential of marine fungi as a biological control agent.

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عزل و توصيف و استقصاء النشاط المضاد للميكروبات للميلانين المستخلص من الفطريات المصاحبه للطحالب البحريه الكبيره

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توفر البيئة البحرية مصدرا غنيا للإنزيمات والصبغات الفريدة. وتلعب الطحالب البحرية والكائنات الدقيقة المرتبطة بها دورًا حاسمًا في العمليات البيئية والبيوكيميائية للمياه الساحلية. وتهدف هذه الدراسة إلى استكشاف تنوع الفطريات الطفيلية التي تنمو على الطحالب البحرية الكبيرة وإمكاناتها في إنتاج أصباغ ميكروبية. وقد تضمن البحث جمع أنواع مختلفة من الطحالب البحرية الوفيرة من ثلاث مواقع ساحلية في مصر على مدار عام (خريف ٢٠٢٢ إلى صيف ٢٠٢٣). تم التعرف على سبعة عشر نوعًا فطريًا تنتمي إلى خمسة أجناس، واعتبرت مصادر محتملة لصبغة الميلانين البيولوجية النشطة. وكان *Aspergillus niger* هو النوع الأكثر شيوعًا في جميع الفصول، حيث بلغت نسبته ٣٢.٥٪ في الشتاء و ٣٩.٤٪ في الربيع و ٣٧٪ في الصيف. بينما كان *Aspergillus terreus* وفيرًا في الخريف، حيث شكل ١٦.٣٪. على النقيض من ذلك، كانت . *Cladosporium oxysporum* و *Aspergillus terreus* و *Trichoderma viride* و *Curvularia chlamydospora* هي الأقل شيوعًا، بنسب إجمالية ١.١٪ و ١.٢٪ و ٣.٣٪ و ٢.٢٪ على التوالي في جميع الفصول. تم التعرف على عشرة من أصل سبعة عشر نوعًا فطريًا نباتيا تنتج صبغة الميلانين. شملت هذه الفطريات المنتجة للميلانين *Aspergillus niger*، *A. flavus*، *A. terreus*، *A. fumigatus*، *Trichoderma viride*، *Cladosporium oxysporum* . *Curvularia chlamydospora*، *Alternaria alternata*، *Penicillium chrysogenum* و *Rhizopus stolonifera* sp.. وقد أظهرت دراسة التوصيف الفيزيائي الكيميائي للصبغات الفطرية ومعيار الميلانين الاصطناعيDOPA أن قابلية ذوبانه محدودة في DMSO ولكن له قابلية ذوبان كاملة في 1M KOH. وقد أكد المزيد من التحليل باستخدام مطيافية الأشعة فوق البنفسجية المرئية (UV) ومطيافية الأشعة تحت الحمراء (FTIR) هوية الصبغات المُصنّعة على أنها ميلانين. كما أظهر الميلانين المستخلص مستويات متفاوتة من النشاط المضاد للميكروبات ضد العديد من البكتيريا والفطريات الممرضة، بما في ذلك *Bacillus subtilis* ، *Staphylococcus aureus*، *Escherichia coli*، *Salmonella typhi* و *Candida albicans*. ومع ذلك لم يظهر أي تأثير ضد *Aspergillus fumigatus* . وتؤكد هذه النتائج على إمكانات الفطريات البحرية كمصدر قيم للمركبات البيولوجية النشطة، وخاصة الميلانين. وهذا قد يمهد المزيد من البحث في هذه الكائنات لإنشاء عوامل مبتكرة مضادة للميكروبات واستخدامات بيوتكنولوجية أخرى.