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Antimicrobial, antibiofilm and antioxidant activities of bioactive secondary metabolites of marine *Scarus ghobban* gut-associated *Aspergillus niger*: In-vitro and in-silico studies

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Fungal extracts have garnered significant interest in recent years for their diverse applications in pharmaceutical field. This research focused on isolating fungi from the gut of *Scarus ghobban* for the first time and evaluate their biological activities *Aspergillus niger* was successfully isolated and identified using morphological and molecular techniques. Gas chromatography–mass spectrometry (GC-MS) analysis of the ethyl acetate extract (EA) of *A. niger* revealed eight compounds, with diisooctyl phthalate (54.32%) and 1,2-benzenedicarboxylic acid, bis (2-methoxyethyl) ester (26.32%) as the most abundant. High-performance liquid chromatography (HPLC) analysis identified catechol (15.41 µg/mL) and syringic acid (13.25 µg/mL) as prominent phenolic compounds in the extract. The EA extract exhibited significant antibacterial activity toward pathogenic bacterial strains, with the highest inhibition zone (32 ± 0.1 mm) and minimum inhibitory concentration (MIC) of 7.8 µg/mL against *Bacillus subtilis*. Additionally, it showed antifungal activity against *Candida tropicalis* (MIC 7.8 µg/mL) and *Candida albicans* (MIC 31.25 µg/mL). The extract also demonstrated potential antibiofilm activity against *Salmonella typhimurium*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Escherichia coli*, with inhibition percentages exceeding 87%. Moreover, it exhibited potent antioxidant activity IC₅₀ 8.17 µg/mL. Transmission electron microscopy revealed severe structural damage in *B. subtilis*, emphasizing the extract's antibacterial effectiveness and potential for therapeutic applications. Eventually, docking studies and computational calculations have been utilized to demonstrate the reactivity of the molecules. In conclusion, the ethyl acetate extract of *A. niger* from gut of *S. ghobban* demonstrates significant antibacterial, antibiofilm, and antioxidant activities, highlighting its potential as a valuable resource for developing effective antimicrobial agents and therapeutic applications.

Keywords Fish gut associated -fungi, Antibacterial, Antibiofilm, Gas chromatography–mass spectrometry, Transmission electron microscopy, Docking study

Pathogenic bacterial contamination has emerged as a major public health concern in the food, medical, agricultural, and environmental sectors, resulting in severe human diseases and financial losses on a worldwide scale^{1–3}. It's believed that 80% of pathogenic infections are attributed to difficult-to-eliminate biofilms, which significantly enhance the resistance of pathogenic and spoilage bacteria to conventional antibiotics and disinfectants, rendering them 10 to 1000 times more resilient against these treatments. This underscores the critical need for novel therapeutic approaches, such as those derived from natural sources like fungal extracts, to effectively combat biofilm-associated infections⁴. It's important and challenging to treat persistent medical infections, rapid food spoilage and associated diseases attributable to repeated contamination of bacterial biofilm⁵. For removal or

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destruction of biofilm, chemical techniques have been widely studied, but there are many important limitations including high toxicity, low efficacy and drug resistant, especially antibiotic resistance⁶. This emphasizes how vital it is to create antibiofilm agents that are safe for the environment. In addition to their ecological and economic significance, coral reef habitats are well-known for their high biodiversity⁷. Coral reef fish, one of the most significant communities in coral reef ecosystems, are vital to the reef resilience and functioning of the ecosystem⁸. The intestinal tract of fish contains an abundance of microorganisms where a complex symbiotic relationship between host and microorganisms was formed in a long-term natural evolution process^{9,10}. A reproductive environment for intestinal microorganisms is provided by the fish gut. Furthermore, immune metabolism, nutrition, growth and physiological health of the host depend on these intestinal microbes^{11,12}. Therefore, Our knowledge about the complex interaction between the host and the microorganisms that inhabit the fish gut greatly enriches due to study the microflora inhabiting the fish gut in many species of coral reef fish¹³.

In marine environments, *Aspergillus* is a genus of fungi that is widely found^{14,15}. *Aspergillus fumigatus*, *A. niger*, *A. versicolor*, *A. flavus*, *A. ochraceus*, *A. ticus*, and *A. terreus*, etc. are examples of prevalent species. Azolones, flavonoids, steroids and other bioactive natural compounds can be produced by marine *Aspergillus* which is a valuable source^{16–18}. These metabolites exhibit a variety of biological activities including lipid-lowering, antibacterial anticancer, antiviral, anti-inflammatory, and anti-diabetic effects. They also have a variety of different structures^{19–22}. According to recent studies, *A. niger* has been confirmed to be an essential source of bioactive natural compounds with a variety of biological activities²³. Rasouli et al. reported that *A. niger* has antibiofilm activity on clinical *staphylococcus epidermidis* and *Pseudomonas aeruginosa*²⁴. The aim of this study was to isolate fungi from the gut of *Scarus ghobban* fish and evaluate their antibacterial, antibiofilm, and antioxidant activities, with the goal of exploring their potential applications in pharmaceutical applications.

Materials and methods

Collection of fish sample

Scarus ghobban fish was collected from the Suez Gulf region, Egypt, situated along the Red Sea, (28° 45' 0" N latitude and 33° 0' 0" E) in March 2023. The Gulf of Suez extends about 314 km with an average depth of 40 km. The collected fish was directly placed in sterile Ziploc plastic bags, an ice box was used for transportation to the laboratory, and maintained at 4 °C for fungal isolation.

Isolation of fungi from *Scarus ghobban* gut

After the fish was collected, it was transferred in a dissecting tray and its entire body was cleaned with 75% alcohol. After using dissecting scissors to cut the fish in an upward arc along the anus, The gut was removed aseptically and put into a 15 mL centrifuge tube (Eppendorf, Germany) and diluted with 3 ml of sterile water. An analog vortex mixer (OHAUS Corporation, United States of America) (OHAUS, USA) was used to shake the tubes vigorously. On sterile petri dishes with Glucose Yeast Peptone Agar (GYPA) medium (0.5% glucose, 0.1% yeast extract, 0.5% peptone, and 2% agar) containing chloramphenicol (0.2 g/l) to inhibit bacterial growth, a 100 µL dilution of the sample was applied. A sterilized plates culture media was used as a negative control and plated under the same conditions to ensure that no contamination occurred during the isolation process. At 25 °C, plates were incubated and the growth of fungal hyphae was observed every day. In order to get pure cultures, fungal colonies found on the medium were recultured into fresh Potato Dextrose Agar (PDA) (20% potato, 2% glucose, 2% agar). The isolated fungal strain was kept for further investigation at 4 °C²⁵.

Morphological and molecular identification of the fungal isolate

The Morphological identification of the fungal isolate was performed by detecting the macroscopic features including color, texture, and appearance as well as microscopic features by utilizing a light microscope. Additionally, the examined fungus was molecularly identified using 28 S rRNA gene sequencing in accordance with the manufacturer's methodology used by Sigma Scientific Services Company (Giza, Egypt). National Center for Biotechnology Information's nucleotide Basic Local Alignment Search Tool (NCBI n-BLAST) search software was utilized to compare the sequence with similar sequences that were obtained from DNA databases <https://www.ncbi.nlm.nih.gov/genbank/>. In MEGA version 11, phylogenetic and evolutionary studies were carried out^{26,27}.

Extraction of fungal active compounds

In 2000-mL Erlenmeyer flasks, the fungal isolate was placed in 1000 mL of PDB and maintained at 28 °C for 21 days under static conditions. To get the fungal mycelia, the broth medium was filtered. In order to extract the filtrate with ethyl acetate, it was combined with an equivalent volume of the solvent, shaken on a vortex shaker for 10 min, and then allowed to settle for 5 min to create two separate layers. Then, the ethyl acetate layer was separated using a separating funnel and evaporated in an oven set at 60 °C. After dissolving the resultant crude fungal extract in 1% Dimethyl sulfoxide (DMSO) to reach the last concentration of 1 mg/mL, it was kept for use in subsequent studies at -20 °C²⁸.

Gas chromatography-mass spectrometry (GC-MS)

The chemical composition of the EA extract of *A. niger* was analyzed using a trace Gas Chromatography-Triple Quadrupole (GC-TSQ) mass spectrometer (Thermo Scientific, Texas, USA). The setup includes a direct capillary column TG-5MS. The ion source temperature was maintained at 200 °C, and the mass spectra of the extract were compared with those in the WILEY 09 and National Institute of Standards and Technology (NIST) 14 mass spectrometry databases^{29,30}.

High-performance liquid chromatography (HPLC) analysis

By Using Agilent series 1100 HPLC technique (Agilent, USA), the analysis of phenolic compounds of EA fungal extract was carried out. HPLC system included solvent degasser, auto-sampling injector, two 1100 series Liquid Chromatography) LC (pumps, ChemStation software, as well as UV/Vis detector adjusted at 250 nm. The gradient program began with 100% Solvent B (1:25 solution of acetic acid in water) for 3 min, then 50% Solvent A (methanol) for 5 min, then elevated to 80% Solvent A for two minutes, and finally decreased to 50% for the final 5 min. With injection volumes of 25 μ L, the separation was performed at 25 °C and the flow rate of the solvent was 1 mL/min^{31,32}.

Antibacterial assay

In accordance with Clinical and Laboratory Standards Institute (CLSI) guideline M51-A2³³, the agar well diffusion method was used to assess the antibacterial activity of the EA extract of *A. niger* against *Escherichia coli* ATCC 8793, *Salmonella typhi* ATCC 6538, *Klebsiella pneumoniae* ATCC13883, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Enterococcus faecalis* ATCC 29212. Tested bacterial strains were cultured individually on Muller Hinton agar plates. 100 μ L of EA fungal extract (1 mg/mL), gentamicin (1 mg/mL) and DMSO was put in each well (7 mm). The plates were refrigerated for 2 h and then incubated for 24 h at 37 °C. After that, the diameter of the inhibition zone was measured^{34,35}.

Antifungal assay

The antifungal activity of EA extract of *A. niger* against unicellular fungi including (*Candida tropicalis* and *Candida albicans*) using agar well diffusion method. fungal suspensions were regularly dispersed across Sabouraud Dextrose Agar (SDA) plates individually. After that, 100 μ L of EA fungal extract (1 mg/mL), positive control fluconazole (1 mg/ mL) and DMSO as negative control were added to agar wells (7 mm) separately. For 3 days at 30 °C, All plates were incubated then we measured the diameter of inhibition zones³⁴.

Determination of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and minimum fungicidal concentration (MFC)

The broth microdilution method (CLSI, 2020) was used to determine the MIC and MBC of EA extract of *A. niger*. The MIC index (MIC_i) was calculated using Eq. 1, which provided clarification on the interpretation of the effect of tested fungal extract, whether bacteriostatic/ fungistatic or bactericidal/ fungicidal. The extract exhibited bacteriostatic/ fungistatic effect when its MIC_i value was ≥ 4 , and bactericidal/ fungicidal activity when it was ≤ 2 ^{36,37}.

$$\text{MIC}_i = \frac{\text{MBC/MFC}}{\text{MIC}} \quad (1)$$

Antibiofilm assay

Antibiofilm activity of EA extract of *A. niger* was evaluated using 96-well polystyrene flat-bottom plates. In summary, each microplate well received 300 μ L of newly inoculated trypticase soy yeast (TSY) broth at 10^6 CFU/mL. Sublethal extract concentrations of 75, 50, and 25% of the MIC were then added to the microplates. Control wells contained just medium and no extract^{38,39}. The inhibition of bacterial film (IBF) formation was calculated using the following equation:

$$\% \text{IBF} = 1 - \frac{\text{Treated Ab} - \text{Blank Ab}}{\text{Control Ab} - \text{Blank Ab}} \times 100$$

Blank Ab represented the absorbance of the media only. While the control Ab showed bacteria absorption without any treatment with fungal extract. Also, treated Ab represented the absorbance of the test organism after treatment.

Transmission electron microscopy

To examine the impact of EA extract from *A. niger* on the ultrastructure of the most susceptible bacteria, bacterial cells were centrifuged at 4000 rpm for 10 min. Following a wash with distilled water, the samples underwent post-fixation in a potassium permanganate solution for 5 min after being fixed in 3% glutaraldehyde and rinsed in phosphate buffer at room temperature. Following 15 min of dehydration in each ethanol dilution, ranging from 10 to 90%, the samples underwent an additional 30 min of dehydration in absolute ethanol. Samples were infiltrated with acetone and epoxy resin in a graded sequence, followed by infiltration with pure resin, and ultrathin sections were obtained using copper grids. Subsequently, uranyl acetate was employed following lead citrate to achieve twofold staining of the sections. Ultimately, stained sections were analyzed at RCMB, Al-Azhar University, utilizing a JEOL-JEEM 1010^{40,41}.

Antioxidant activity using DPPH

The evaluation of antioxidant activity of EA extract from *A. niger* was conducted using the DPPH assay, following the methodology outlined in references^{42,43}. One milliliter of a 0.1 mM DPPH solution in ethanol was mixed with three milliliters of fungal extract at concentrations varying from 1,000 to 1.95 μ g/mL. The solution was permitted to equilibrate at ambient temperature for 30 min following thorough agitation. Following that, we measured the absorbance at 517 nm with a Milton Roy UV-VIS spectrophotometer. Ascorbic acid was utilized as the reference standard. The percentage of inhibition or DPPH scavenging activity (%) is determined using the formula below:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

where A0 and A1 stand for the control (DPPH solution) and sample absorbances, respectively.

Ferric reducing antioxidant power (FRAP) assay

To evaluate the impact of solvent polarity on the total reducing capacity of the extracts, modified potassium ferricyanide and trichloroacetic acid approach⁴⁴ was used and adaptable for microplate application⁴⁵. Ferric reducing antioxidant power (FRAP) will henceforth be called total reducing power (TRP). 40 mL of the sample was put into each labeled Eppendorf tube, which was then diluted with 50 mL of 0.2 mol/L sodium phosphate dihydrate (Na₂HPO₄·2H₂O) buffer and 50 mL of 1% potassium ferricyanide (K₃Fe (CN)₆), as well as 50 mL of 10% trichloroacetic acid. For 10 min, the mixture was centrifuged at 3,000 rpm. Subsequent to centrifugation, 33.3 mL of 1% ferric chloride (FeCl₃) was mixed with 166.66 mL of each sample's supernatant that had been transferred to a 96-well plate. The absorbance was measured at 630 nm by using microplate reader (Biotek ELX800; Biotek, Winooski, VT, USA). The positive control was ascorbic acid (1 mg/mL), while the negative control was DMSO. The results were presented as microgram of ascorbic acid equivalent (AAE) per milligram of extract.

Computational procedures

The Gaussian 09 W program was employed to conduct calculations of Density Functional Theory (DFT) using the hybrid functional B3LYP (Becke's three-parameter hybrid functional combined with the BLYP correlation functional) and the 6-31G(d) basis set, employing the Berny method⁴⁶.

Molecular modeling and docking

The molecular modeling of compounds 1, 2-benzen dicarboxylic acid, bis (2-methoxyethyl) ester (comp. 2) and 5-hydroxy-2,2-dimethyl-5,6-bis-(2-oxopropyl)-cyclohexanone (comp. 5) against *B. subtilis* (ATCC 6633), *E. faecalis* (ATCC 29212) and *S. aureus* (ATCC 6538). As well, anticancer activity breast cancer MCF-7 and hepatic HepG2 has studied and fabricated using standard bond lengths and energy, with the Auto Dock Vina and detected by Discovery Studio Client (version 4.2)⁴⁷.

Results and discussion

Isolation and identification of fish gut fungus

In this study, fungal isolate S1 was isolated from the gut of *S. ghobban*. Identification of this fungal isolate was performed using morphological and molecular techniques. The morphological examination indicated that the colonies proliferate swiftly, attaining a size of 40 mm within 4 days at 28 °C on PDA, characterized by powdery black colonies with a faint yellow reverse, as illustrated in (Fig. 1). Microscopic examination revealed that mycelium is hyaline and septate, whereas, conidiophores are non-septate, erect, and smooth-walled, globose vesicles containing hyaline conidia. Conidia are smooth and spherical. Then, molecular identification was carried out using 28 S rRNA gene. Molecular identification revealed that the fungal strain was identified genetically as *Aspergillus niger* which is related to fungal strain *A. niger* strain AL-27 KC341933.1 that was deposited in the NCBI database with similarity percentages of 99%. The gene bank recorded the fungal strain *A. niger*, which was identified in the current study, under the accession number PV017292.

In a previous study, intestinal fungi particularly *Aspergillus* spp isolated from Three Species of Coral Reef Fish, where these fungi included *Aspergillus niger*, *A. medius*, *A. aculeatinus*, *A. ochraceopetaliformis*, *A. pseudoglaucus*, *A. restrictus* and *A. sydowii*⁴⁸. Likewise, Long, Wu⁴⁹ isolated *Aspergillus* from intestine of Black Carp and Grass Carp. Also, Ekanem, Itah⁵⁰ reported that, isolated *Aspergillus* and *Penicillium* from fish gut of fish species (*Pseudotolithus typus* and *Chrysichthys nigrodigitatus*) from Qua Iboe River Estuary.

GC-MS analysis

GC-MS is a potent analytical technique that is extensively employed to separate and identify volatile and semi-volatile compounds in a variety of samples^{41,51}. In the current study, GC-MS was applied for fungal extract of *A. niger* to detect its compounds as shown in (Fig. 2; Table 1). Table 1 shows presence 8 compounds in the ethyl acetate extract of *A. niger* using GC-MS analysis. The most abundant compounds identified were Diisooctyl phthalate and 1,2-benzen dicarboxylic acid, bis (2-methoxyethyl) ester with percentages 54.32 and 26.32% respectively. Moreover, other low-percentage compounds found were hexadecanoic acid (5.43%), oleic acid (4.84%), 9-octadecanoic acid (Z)- (2.20%), Tetradecanoic acid (2.18%) and 2,2-dideutero octadecanal (0.98%). El-Enain et al.⁵² reported that, diisooctyl phthalate was the dominant and exhibited promising antimicrobial activity. Likewise, Al-Askar et al.⁵³ confirmed that, diisooctyl phthalate is powerful bioactive compound where it showed potential antifungal activity. Furthermore, the bioactive compounds identified 1,2-benzenedicarboxylic acid and hexadecanoic acid are consistent with the research conducted by Siddiquee et al.⁵⁴. Moreover, Lukitaningsih and Rumiyati⁵⁵ reported that hexadecanoic acid has antioxidant, anti-inflammatory, and antimicrobial activities. Furthermore, tetradecanoic acid has Antimicrobial, antioxidant, nematocidal, anticancer activities^{55,56}. Moreover, 9-octadecanoic acid (Z)- showed antibacterial and antioxidant activity^{57,58}.

Phenolic compounds in EA extract of *A. niger*

Phenolic compounds have different biological activities, making them beneficial for reducing oxidative stress and preventing chronic diseases⁶⁵. Thus, quantities of phenolic compounds in EA extract of *A. niger* were determined using HPLC as shown in (Fig. 3). Results revealed that, the most abundant phenolic compounds in EA extract of *A. niger* were catechol (15.41 µg/mL), syringenic (13.25 µg/mL) as shown in (Table 2). Also,

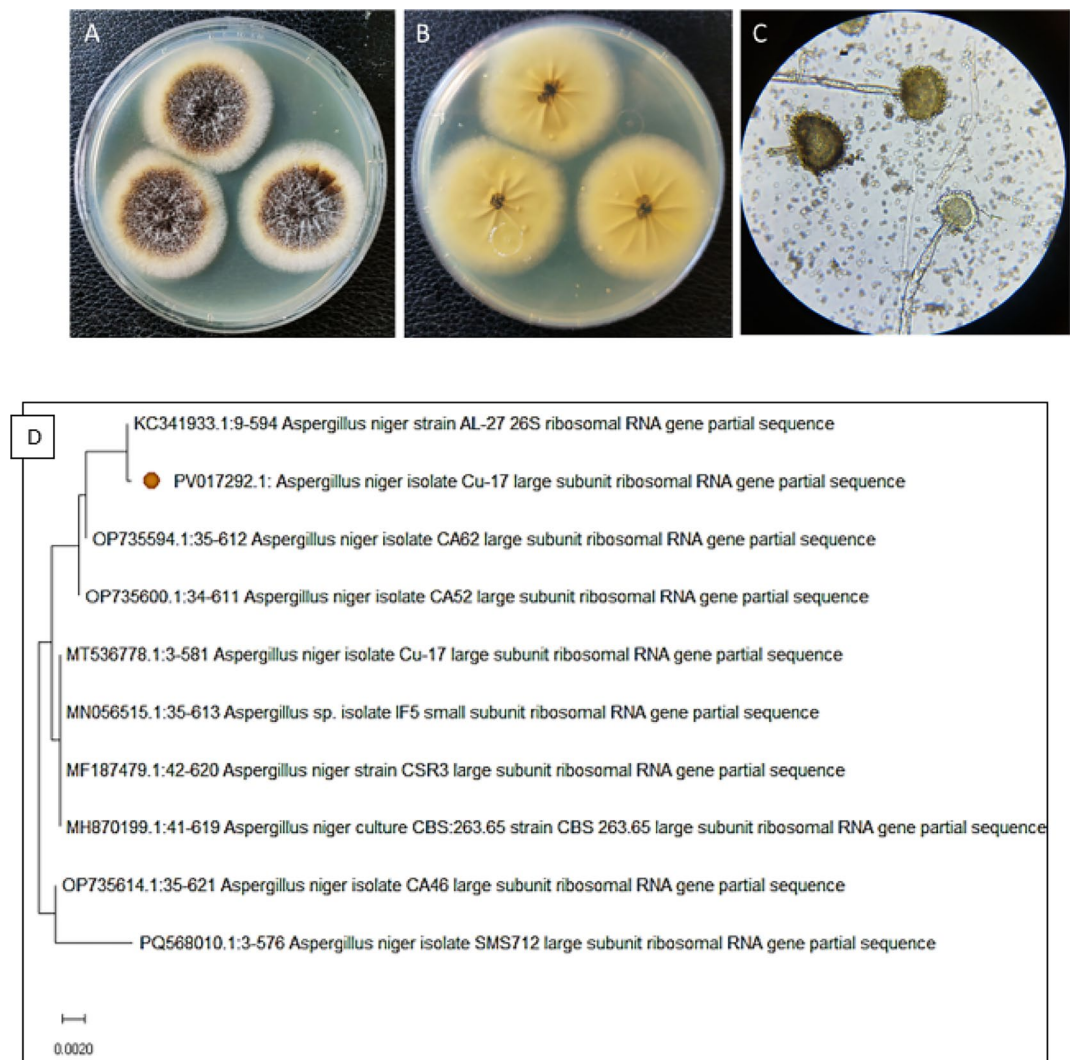


Fig. 1. (A) surface colony of *A. niger* on PDA grown; (B) reverse colony; (C) light microscope showing conidiophore, and conidia of *A. niger* (400X); (D) Phylogenetic tree of *A. niger*.

some of phenolic compounds were detected, cinnamic, caffeic, salicylic, pyrogallol, Chlorogenic and ferulic acid with percentages 7.41, 5.38, 4.65, 3.19, 2.55 and 2.49 $\mu\text{g/mL}$ respectively. Surana et al.⁶⁶ reported that catechol framework plays a crucial role in medicinal chemistry as it is found in numerous naturally occurring compounds with diverse biological activities. Furthermore, Caffeic acid has antimicrobial and antioxidant activities⁶⁷. Feriotto et al.⁶⁸ found that caffeic acid exhibits both anti-inflammatory and anticancer properties, specifically targeting certain leukemia cell lines. This suggests potential therapeutic applications for caffeic acid in treating leukemia by reducing inflammation and inhibiting cancer cell proliferation. Other researchers demonstrated that salicylic acid is a natural and safe antimicrobial agent⁶⁹. Bio-functions of chlorogenic acid were reported, including antioxidant, anti-bacterial, anti-tumor, and anti-inflammatory, besides other therapeutic properties⁷⁰. Ferulic acid was detected in *Aspergillus* sp. and exhibited antioxidant and antifungal activity⁷¹.

Antimicrobial activity

Fungi represent a varied collection of life forms that are crucial to ecosystems, especially in the processes of nutrient cycling and decomposition⁷². Many fungi exhibit remarkable antimicrobial properties, generating a diverse range of bioactive substances that can inhibit the proliferation of bacteria, viruses, and other harmful microorganisms^{73,74}. The potential of these antimicrobial properties is especially significant given the rising issue of antibiotic resistance, with marine fungi presenting an encouraging avenue for discovering new antimicrobial agents. In the current work, the antibacterial efficacy of EA extract of *A. niger* was carried out as shown in (Fig. 4). Results revealed that, EA extract of *A. niger* showed promising antibacterial activity against all bacterial tested strains compared to gentamicin. The most significant growth inhibition observed was 32 ± 0.1 mm in *B. subtilis*, with *E. faecalis* following closely at 30 ± 0.2 mm. The least growth inhibition observed was 22 ± 0.1 mm against *S. aureus*. Additionally, the extract from *A. niger* exhibited antifungal properties against *C. tropicalis* and *C. albicans*, showing inhibition zones of 28 ± 0.2 mm and 27 ± 0.2 mm, respectively (Table 3).

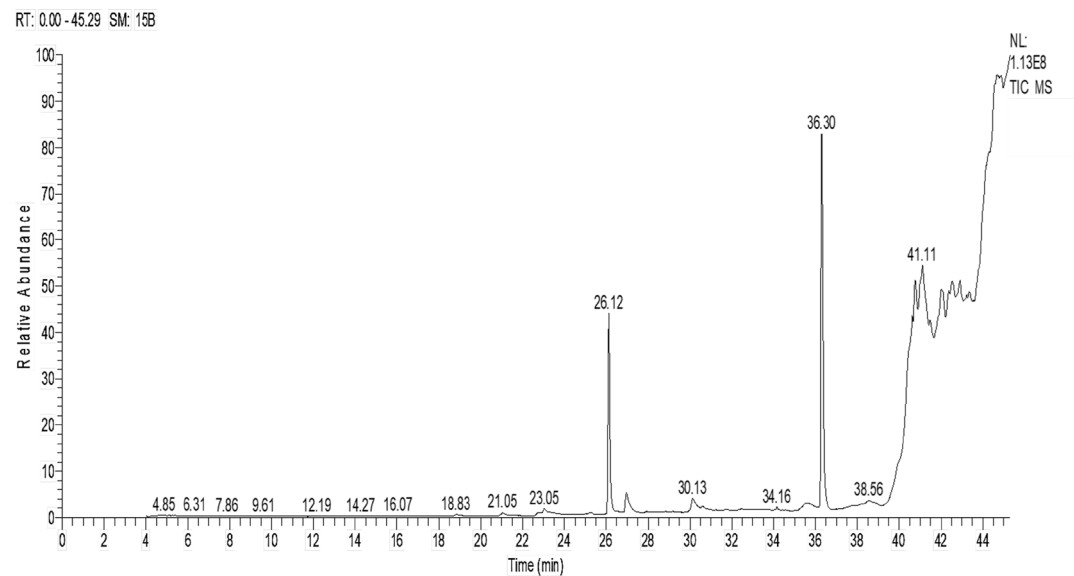


Fig. 2. GC-MS chromatogram of EA of *A. niger*.

Compound	RT	Peak area%	Mwt	MF	Activity	Reference
Tetradecanoic acid	23.04	2.18	288	C14H28O2	Antimicrobial, antioxidant, nematocidal, anticancer activities	55,56
1,2-benzen dicarboxylic acid, bis (2-methoxyethyl) ester	26.12	26.32	278	C16H22O4	Antibacterial activity	59
Hexadecanoic acid	26.96	5.43	256	C16H32O2	Antioxidant, antimicrobial, anti-inflammatory activity	55
Oleic acid	30.12	4.84	282	C18H34O2	Anti-inflammatory, antifungal, antibacterial, antioxidant activity	60–62
5-hydroxy-2,2-dimethyl-5,6-bis-(2-oxopropyl)-cyclohexanone	35.54	1.61	254	C14H22O4		
Diisooctyl phthalate	36.30	54.32	390	C24H38O4	Antimicrobial, scavenging capacity	52,53
9-octadecanoic acid (Z)-	38.57	2.20	258	C16H34S	Antibacterial and antioxidant activity	57,58
2,2-dideutero octadecanal	39.91	0.98	270	C18H34D2O	Antimicrobial activity	63,64

Table 1. GC-MS of the EA extract of *A. niger*.

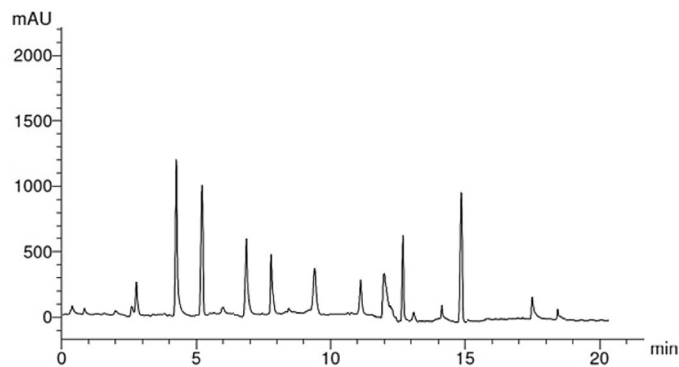


Fig. 3. HPLC chromatogram of phenolic compounds identified in ethyl acetate extract of *A. niger*.

Liao et al.⁴⁸ reported that *A. niger* isolated from the intestine of *Trachinotus blochii* fish exhibited high antibacterial activity. Additionally, they found other species of *Aspergillus* including *A. ochraceopetaliformis* and *A. pseudoglaucus* which are isolated from the intestine of *Lutjanus argentimaculatus*, and *Lates calcarifer* fish, respectively showed antibacterial activity against *vibrio alginolyticus* bacterium. Marine-derived *Aspergillus* sp. was isolated from the viscera of the barracuda and exhibited antibacterial effects against *B.subtilis*, from

RT	Compound	Concentration (µg/mL)
2.9	Chlorogenic	2.55
4.0	Catechol	15.41
5.0	Syringenic	13.25
7.0	Cinnamic	7.41
8.0	Caffeic	5.38
9.0	Pyrogallol	3.19
11.0	Ferulic	2.49
12.0	salicylic	4.65

Table 2. Phenolic compounds of EA extract of *A. niger* by HPLC.

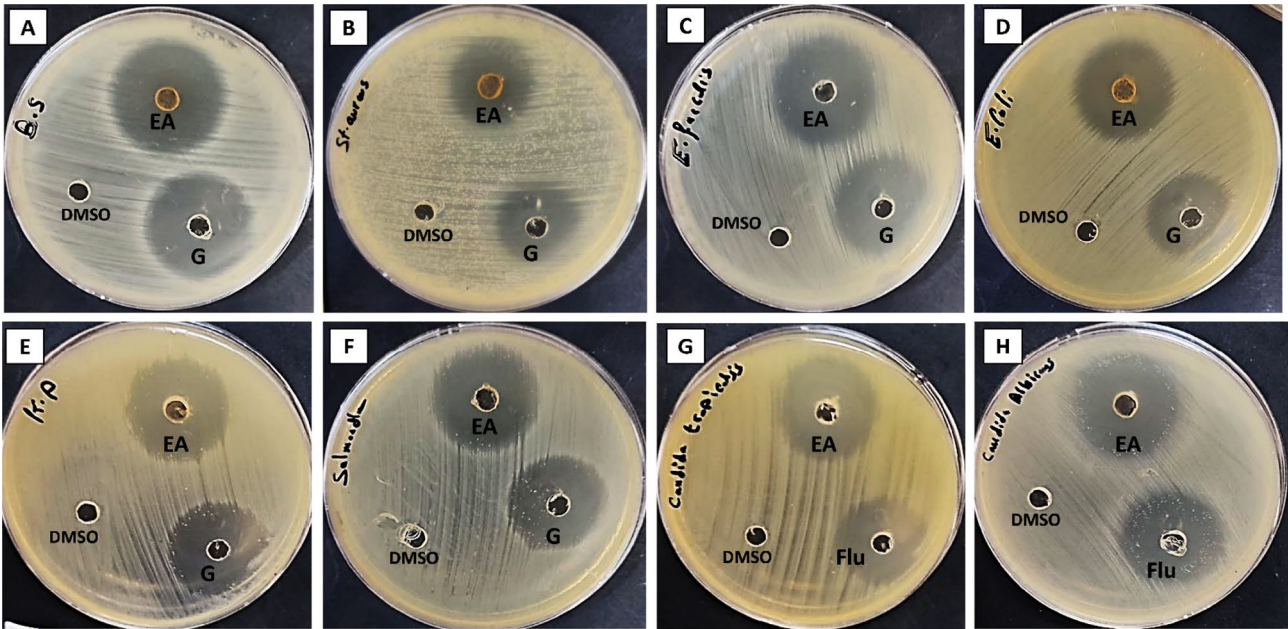


Fig. 4. Antimicrobial activity of EA extract of (A) *niger* (EA), positive control (Gentamycin (G)/Fluconazole (Flu)), and DMSO against (B) *subtilis* (A), *S. aureus* (B), *E. faecalis* (C), *E. coli* (D), *K. pneumoniae* (E), *S. typhimurium* (F), *C. tropicalis* (G) and *C. albicans* (H).

Test microbial strains	Diameter inhibition zone in mm	
	<i>A. niger</i> EA extract	Gentamycin/fluconazole (1 mg/1 mL)
<i>B. subtilis</i>	32 ± 0.1	30 ± 0.2
<i>E. faecalis</i>	30 ± 0.2	26 ± 0.2
<i>S. aureus</i>	22 ± 0.1	24 ± 0.2
<i>E. coli</i>	27 ± 0.1	25 ± 0.1
<i>S. typhi</i>	28 ± 0.2	25 ± 0.2
<i>K. pneumoniae</i>	27 ± 0.2	24 ± 0.1
<i>C. tropicalis</i>	28 ± 0.2	22 ± 0.2
<i>C. albicans</i>	27 ± 0.2	27 ± 0.2

Table 3. Antimicrobial activity of EA crude extract of *A. niger* on tested bacterial and fungal strains.

which four butyrolactones and methyl 2,4-dihydroxy-3,5,6-trimethylbenzoate compounds were identified. These compounds showed antibacterial effects against *S. aureus*, *Enterobacter aerogenes*, *B. subtilis*, and *E. coli*⁷⁵. Previous studies suggested that the extraction via ethyl acetate exhibited the highest activity compared to others⁷⁶. Similarly, research on fungal extracts demonstrated that EA extracts displayed the highest antimicrobial activity compared to those from methanol, water, and n-hexane^{77,78}.

Testes microbial strains	A. niger EA extract (µg/mL)		
	MIC	MBC/ MFC	(MBC or MFC)/MIC
B. subtilis	7.8	15.62	2
E. faecalis	31.25	62.5	2
S. aureus	125	500	4
E. coli	62.5	125	2
S. typhi	62.5	125	2
K. pneumoniae	15.62	15.62	1
C. tropicalis	7.8	15.62	2
C. albicans	31.25	62.5	2

Table 4. MIC and MBC of EA extract of *A. niger* against tested pathogenic microbes.

Pathogenic strains	Antibiofilm activity (%)		
	75% of MIC	50% of MIC	25% of MIC
S. typhimurium	94.49 ± 0.37	87.72 ± 0.51	85.97 ± 0.36
S. aureus	90.36 ± 0.35	82.87 ± 0.55	32.85 ± 0.27
E. faecalis	87.81 ± 0.39	81.91 ± 0.59	66.98 ± 0.15
E. coli	88.80 ± 0.47	76.82 ± 0.58	48.08 ± 0.34

Table 5. Antibiofilm activity of EA extract of *A. niger* against tested pathogenic bacteria.

Minimum inhibitory, bactericidal, and fungicidal activity of EA extract of *A. niger*

MIC and MBC assessments were conducted on all evaluated bacterial and fungal strains, as depicted in the (Table 4). The results indicated that the EA extract of (*A*) *niger* exhibited the lowest MIC of 7.8 µg/mL against *B. subtilis* and the lowest MBC of 15.62 µg/mL against *B. subtilis*, *K. pneumoniae*, and *C. tropicalis*. *E. coli* and *S. typhimurium* had the greatest MIC of 62.5 µg/mL, whereas *S. aureus* demonstrated the highest MBC of 500 µg/mL. The determined MBC/MIC index values demonstrated the bacteriostatic effect of *A. niger* extract on *S. aureus* and its bactericidal/fungicidal effect on other species. In a previous study, *Aspergillus* sp. From marine source exhibited antimicrobial activity toward MRSA, *E. faecalis*, *S. aureus*, and *K. pneumonia* where MIC values were 0.45–7.8 µg/mL⁷⁹. Furthermore, the marine fungus *Aspergillus* sp.LS57 was shown to have antibacterial properties, with MIC values of 64 µg/mL against *S. aureus* and 128 µg/mL against (*B*) *subtilis* and *E. coli*^{80,81}.

Anti-biofilm activity

Microbial biofilms are compact aggregates of bacteria that attach to surfaces and are encased in a self-generated extracellular matrix, rendering them resistant to environmental stresses and antimicrobial treatments⁸². One effective approach to destructing biofilms is the application of antimicrobial agents, which can penetrate the biofilm matrix and target the embedded microorganisms. Antimicrobial agents, such as antibiotics, disinfectants, and essential oils, are used to disrupt the biofilm’s integrity and inhibit microbial growth⁸³. In this study, EA extract of *A. niger* was assessed for antibiofilm activity toward some of tested bacterial strains as shown in (Table 5). Results illustrated that, EA extract of *A. niger* showed promising antibiofilm activity toward *S. typhimurium* with percentages 94.49, 87.72, and 85.97% at 75, 50 and 25% of MIC respectively. Moreover, it exhibited antibiofilm activity toward *S. aureus*, *E. faecalis* and *E.coli* but less than *S. typhimurium* with percentages (90.36, 82.87, 32.85%), (87.81, 81.91, 66.98%), and (88.80, 76.82, 48.08%) at 75, 50 and 25% of MIC respectively (Table 5). In a previous study, *A. niger* exhibits notable antibiofilm effectiveness, achieving inhibition rates of 95.3%, 74.9%, 77.1%, and 93.6% against *E.coli*, *P. aeruginosa*, *Proteus mirabilis*, and MRSA, respectively⁸⁴. The findings align with the data presented by Hamed et al.⁸⁵, which showed that an ethyl acetate crude extract from *Aspergillus* sp. SO12, sourced from marine environments, displayed significant biofilm inhibitory effects against *S. aureus*, *E. coli*, *B. subtilis*, and *P. aeruginosa*, achieving percentages of 70.23%, 45.25%, 35.23%, and 20.02%, respectively. Also, several MDR bacteria were shown to have their biofilm production inhibited by Aspulvinones B, H, R, and S, which are produced from the marine fungus *Aspergillus* sp^{81,86}.

The effect of EA extract of (*A*) *niger* on (*B*) *subtilis* under TEM

To confirm the antibacterial activity of EA extract of (*A*) *niger*, TEM was employed to examine the ultrastructure of *Bacillus subtilis* treated with this extract as illustrated in (Fig. 5). The transmission electron micrograph of typical (*B*) *subtilis* reveals rod-shaped cells characterized by a smooth, continuous cell wall and an intact cell membrane. The cytoplasm appears homogeneous and electron-dense, indicating robust cellular integrity. Additionally, a normal electron-lucent zone is observed between the cell wall and the cell membrane, which is indicative of healthy cellular structure. These features highlight the typical morphology of *B. subtilis*, providing a baseline for comparison with treated cells, especially in studies assessing the effects of antibacterial agents (Fig. 5A). In contrast, *B. subtilis* treated with EA extract of *A. niger* exhibited significant alterations in cellular structure, as evidenced by transmission electron microscopy. The treated cells showed an enlarged periplasmic

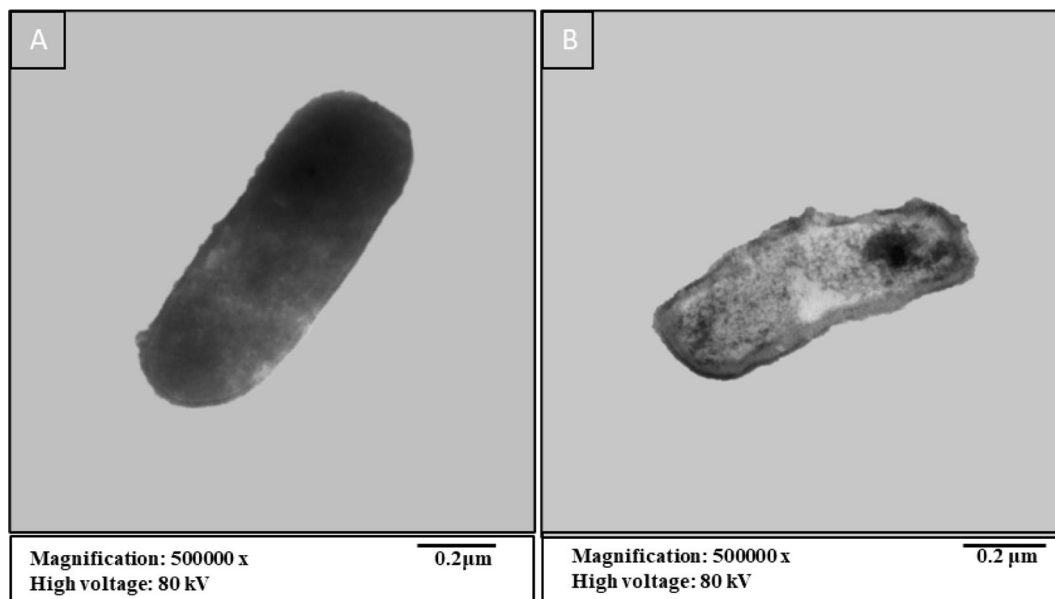


Fig. 5. Transmission electronic micrographs of *B. subtilis*. (A) control (untreated) and (B) treated cell with EA extract of *A. niger*.

region between the outer membrane and the cytoplasmic membrane, indicating disruption of the normal cellular architecture. Severe damage was evident in lysed cells, which displayed a disintegrated cell wall and ruptured cytoplasmic membrane, leading to the leakage of cytoplasmic contents. These destructive changes underscore the antibacterial effectiveness of the EA extract, highlighting its potential to compromise bacterial cell integrity, as illustrated in (Fig. 5B). According to Yassein, Hassan⁸⁴ the crude extract of *A. niger* induced distinct morphological changes in various tested bacteria. In *E. coli*, the cells became shorter and smaller, indicating a reduction in size and possibly viability. For *Proteus mirabilis*, the cells exhibited a curved shape and showed signs of division, suggesting alterations in their growth dynamics. In the case of *P. aeruginosa*, the cells were distorted, transitioning from a bacillus shape to a more spherical form, which reflects significant morphological disruption. Finally, MRSA cells began to swell and adopt an irregular spherical shape, indicating severe cellular stress and potential loss of structural integrity.

Antioxidant activity of fungal extract

Compounds that act as antioxidants are essential for protecting cells from oxidative stress by countering harmful free radicals, which can damage critical cellular components such as DNA, proteins, and lipids. Antioxidants reduce the risk of chronic diseases, such as cancer, cardiovascular issues, and neurodegenerative disorders, by scavenging and neutralizing these free radicals, which in turn mitigates oxidative damage. This protective function emphasizes the significance of antioxidants in the preservation of cellular health and the enhancement of overall well-being^{87,88}. In this study, the antioxidant activity of the ethyl acetate (EA) extract of *A. niger* was assessed at different concentrations using the DPPH method. The findings demonstrated that the EA extract showed notable antioxidant activity, presenting an IC₅₀ value of 8.17 μg/mL, in contrast to ascorbic acid (AA), which recorded an IC₅₀ of 2.97 μg/mL (Fig. 6). FRAP method was also used to evaluate antioxidant activity of the *A. niger* extract. Results revealed that, *A. niger* extract exhibited antioxidant activity with ascorbic acid equivalent (AAE) 732.5 ± 3.4 μg/mg. Yang et al.⁸⁹ observed that marine *A. versicolor* SH0105 exhibited powerful reduction of Fe³⁺ with the FRAP value of 9.0 mM under the concentration of 3.1 μg/mL, which was more potent than ascorbic acid. In prior research, they found that *A. niger* extract is high in phenolic compounds and has strong antioxidant activity in vitro⁹⁰. *Aspergillus* sp. isolated from Barracuda fish exhibited good scavenging activity with an EC₅₀ value of 2.8 mg/mL against DPPH free radicals⁹¹. Also, rubrolide R a natural product derived from *Aspergillus* sp. which isolated from the viscera of *chelon haematocheilus* fish exhibited antioxidant activity with IC₅₀ 1.3 Mm⁷⁵. The findings robustly endorse the utilization of ethyl acetate crude extract of *A. niger* as an effective natural antioxidant for health maintenance against various oxidative stress linked to degenerative diseases.

Computational procedures

DFT calculations are a valuable method employed to investigate the reactivity of chemicals. The optimized geometries of 1,2-benzenedicarboxylic acid, bis(2-methoxyethyl) ester (compound 2), and 5-hydroxy-2,2-dimethyl-5,6-bis(2-oxopropyl)cyclohexanone (compound 5) were computed using DFT B3LYP/6-31G(d) methodology. Table 6 presents metrics that indicate the reactivity and stability of molecules, including total energy (ET), energy of the highest occupied molecular orbital (EHOMO), energy of the lowest unoccupied molecular orbital (ELUMO), and the energy gap (Eg). Figure 7 illustrates the molecular structure of compounds

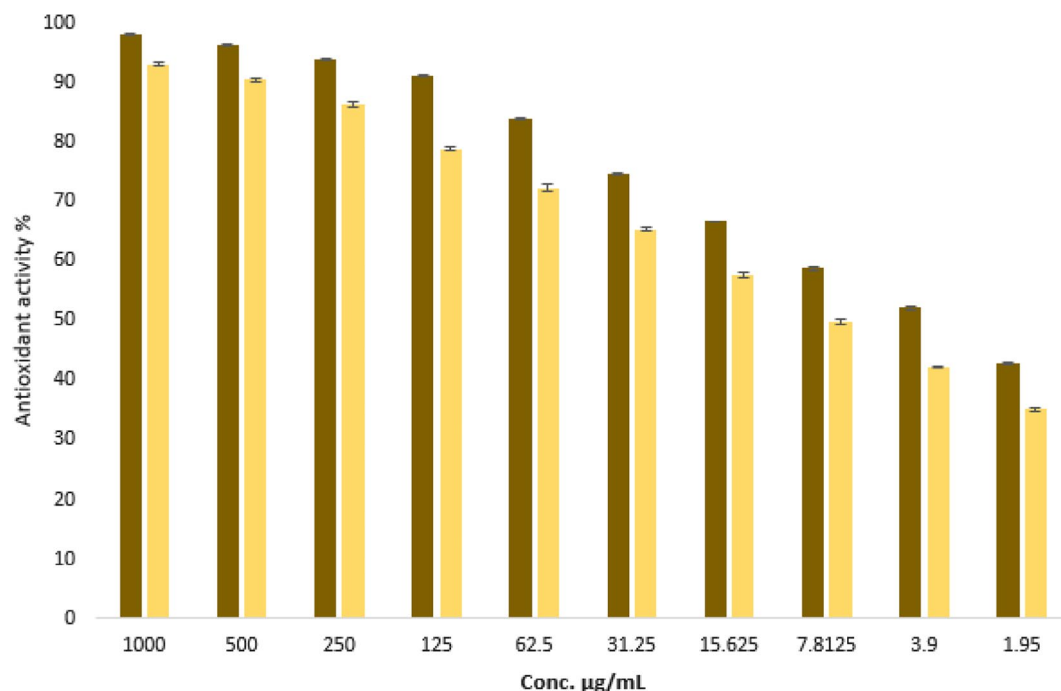


Fig. 6. Antioxidant activity of EA extract of *A. niger* using DPPH method.

Parameter	Comp. 2	Comp. 5
	DFT 3LYP/6-311G (d)	DFT 3LYP/6-311G (d)
ET (au)	−847.390	−995.3505
EHOMO(eV)	−0.23556	−0.2497
ELUMO (ev)	−0.03979	−0.06816
ΔE (ev)	0.19577	0.18154
μ (Debye)	6.3034	3.2325

Table 6. Optimized geometries of (comp. 2 & 5).

2 and 5, together with the reactivity of the isolated compounds. As the energy gap (Eg) diminishes, it facilitates the passage of electrons from lower orbitals to higher ones, hence enhancing the likelihood of reactions in the compounds^{92,93}.

$$\Delta E = ELUMO - EHOMO$$

Molecular modeling and docking

The growth inhibition of pathogenic microorganisms by EA crude extracts of (*A. niger*) has been examined. The molecular modeling of (*B. subtilis* (ATCC 6633) (PDB: 2hq7) crystal structure of the LuxS-Quorum sensor molecular complex from *Salmonella typhi*, *S. aureus* (ATCC 6538) (PDB: 3e6e), and the crystal structure of the Protein related to general stress protein 26 (GS26) of *B. subtilis* (pyridoxinephosphate oxidase family), *E. faecalis* (ATCC 29212) (PDB: 5e68) (Fig. 8). The crystal structure of Alanine racemase from *E. faecalis* in complex with cycloserine, with prediction of anticancer activity of breast cancer MCF-7 (PDB: 4XO7) and hepatic HepG2 (PDB: 4FM9) as a protein receptor against comp. 2 & 5 as a ligand has studied via molecular docking investigation with good bond lengths and energy as in (Table 7). As the bond length decrease the ligand became closer to the receptor and more reactive. A perfect bond length reaches 0.8 Å and 1.0 Å in case breast cancer MCF-7 (PDB: 4XO7) and hepatic HepG2, so we expect the compounds have anticancer activity⁹⁴.

Conclusion

In the current study, *A. niger* was isolated from the gut of *Scarus ghobban* for the first time. The ethyl acetate extract of *A. niger* has significant antibacterial, antibiofilm, and antioxidant characteristics, making it a suitable option for the development of new antimicrobial agents. The identification of essential bioactive components using GC-MS and HPLC investigations emphasizes its medicinal potential. The extract's efficacy against both Gram-positive and Gram-negative bacteria, together with notable antifungal properties, underscores its adaptability in addressing various diseases. Additionally, the observed potent antioxidant activity suggests that *A. niger* could

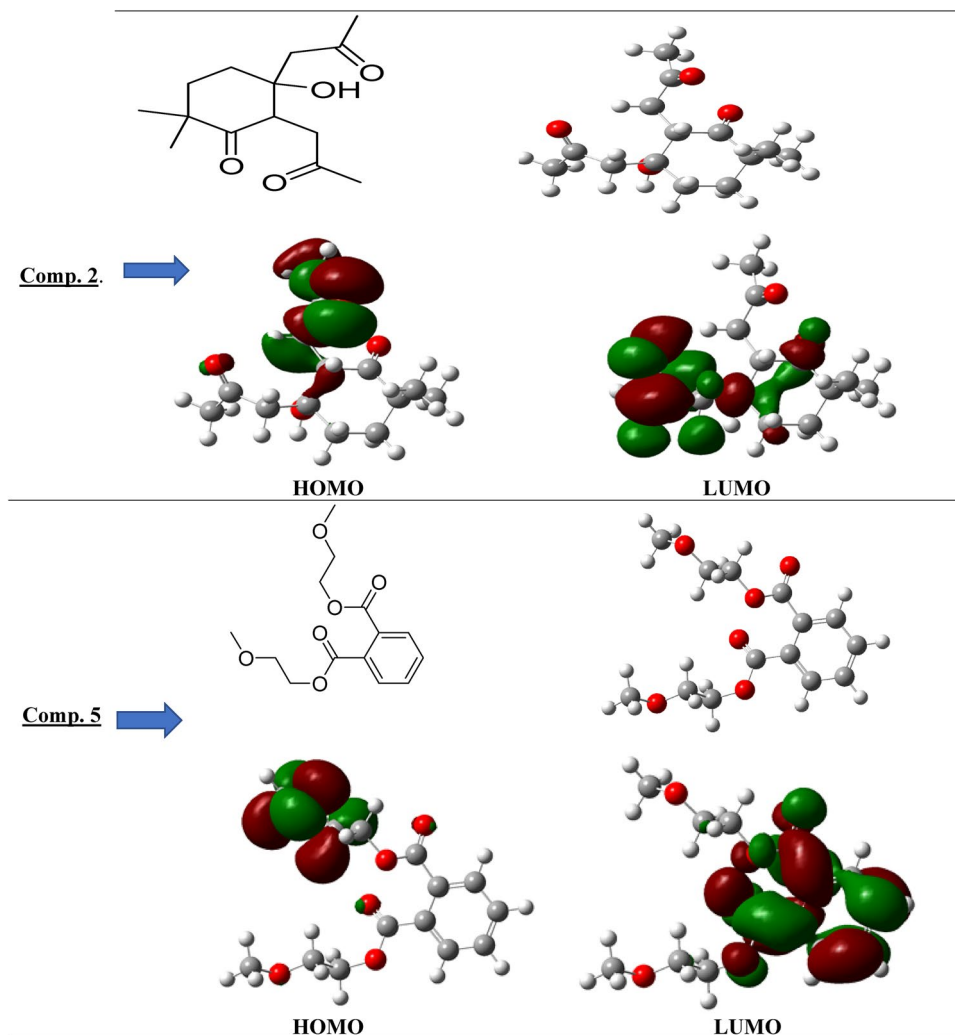


Fig. 7. The optimized geometry and numbering system of comp. 2 & 5 and Gap energy (HOMO–LUMO).

play a crucial role in addressing oxidative stress-related conditions. These findings not only contribute to the growing body of research on fungal extracts but also pave the way for future studies aimed at harnessing *A. niger* for innovative applications in pharmaceuticals and biotechnology. DFT calculation and molecular docking showed good compounds reactivity and good prediction for cancer cell with breast cancer MCF-7 (PDB: 4XO7) and hepatic HepG2 with short bond length.

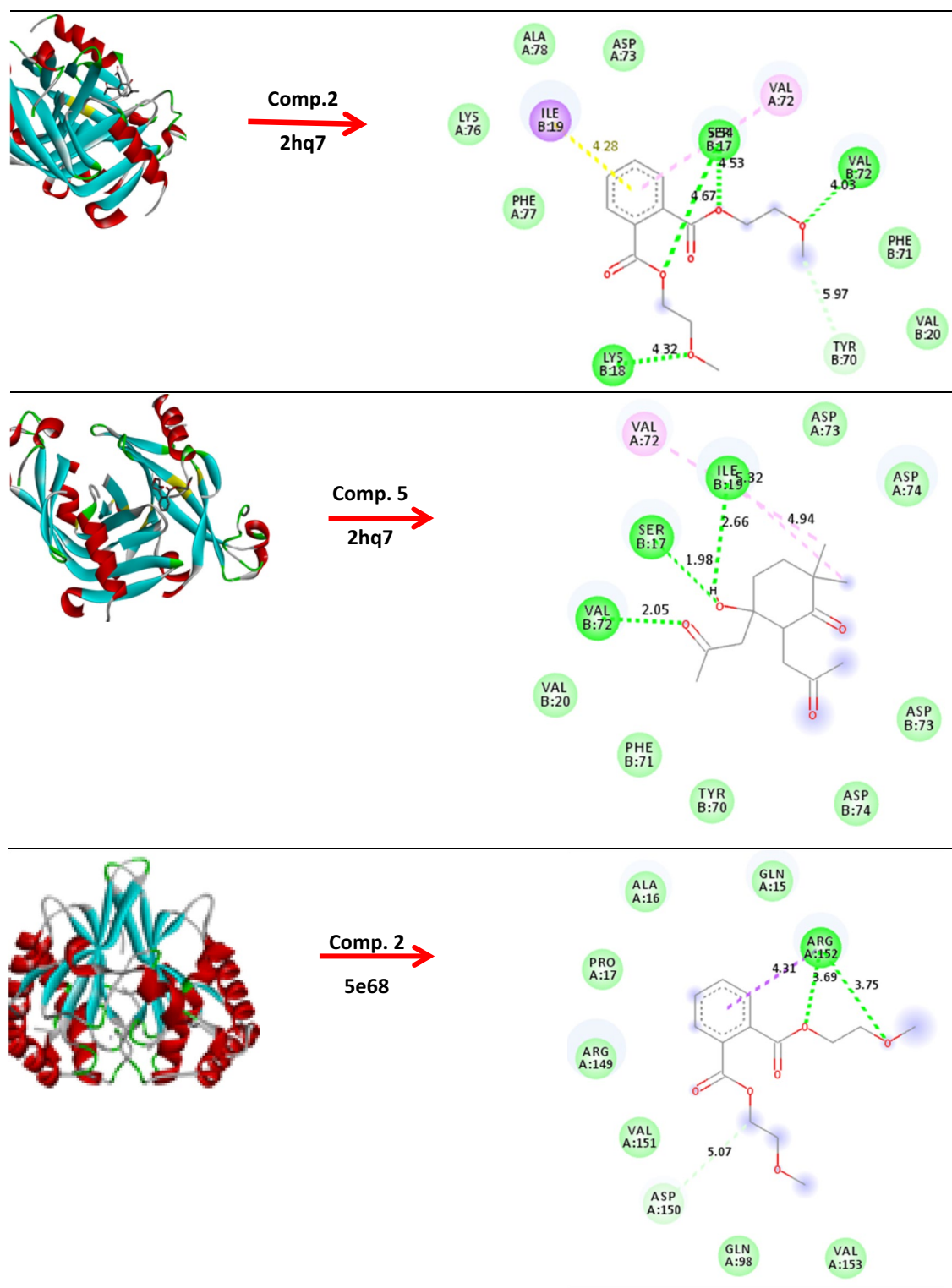


Fig. 8. Molecular docking of comp. 2 & 5 with different protein.

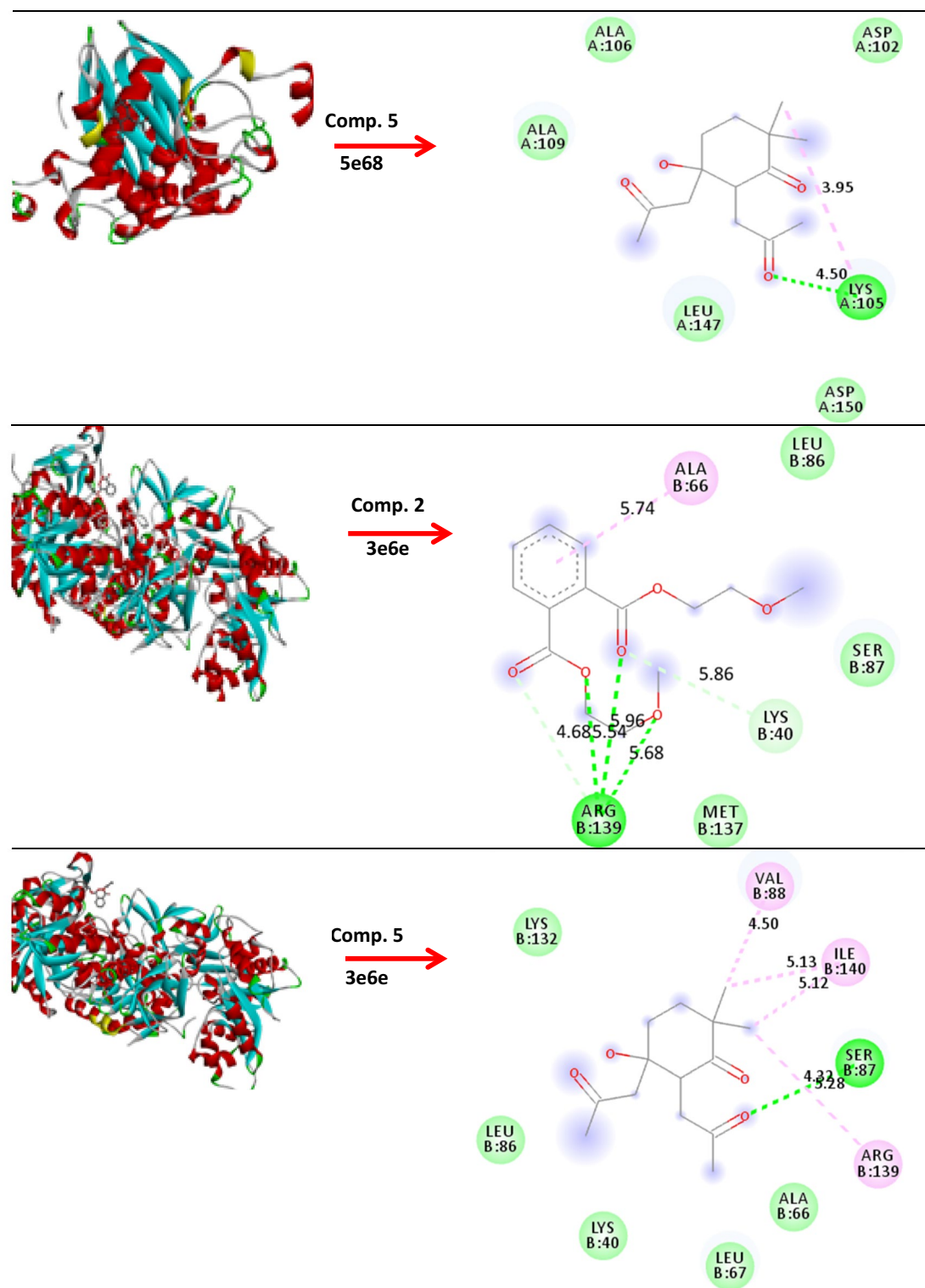


Fig. 8. (continued)

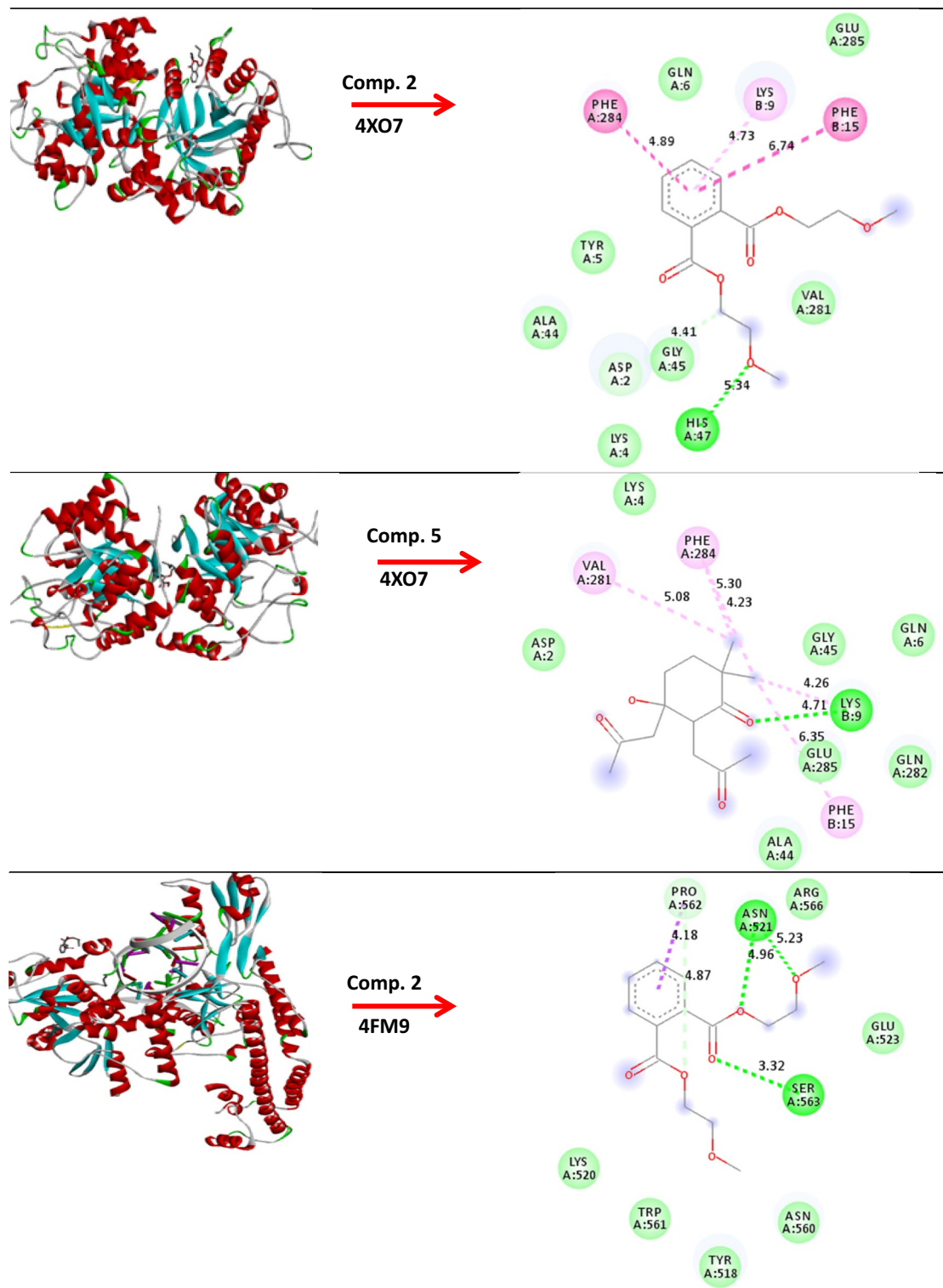


Fig. 8. (continued)

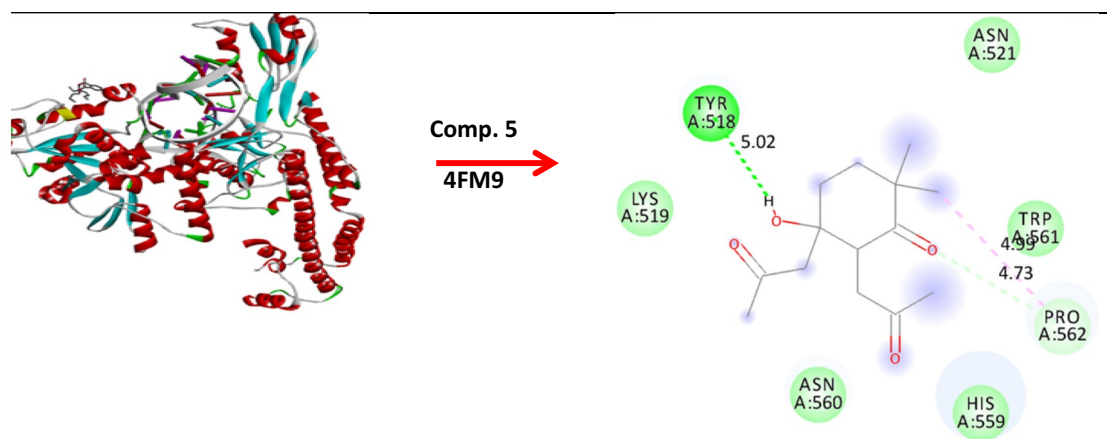


Fig. 8. (continued)

Protein(PDB: ID)	Comp. 2	Comp. 5
2hq7	(−5.3 1.518 Å)	(−5.4 1.695 Å)
5e68	(−3.5 1.755 Å)	(−3.6 1.572 Å)
3e6e	(−4.8 1.837 Å)	(−4.0 2.143 Å)
4XO7	(−4.5 1.618 Å)	(−5.4 0.841 Å)
4FM9	(−4.1 1.316 Å)	(−4.0 1.001 Å)

Table 7. Molecular docking of comp. 2 & 5 with bond lengths and energy.

Data availability

The datasets analyzed during the current study are available in the NCBI GenBank database repository with the accession number of PV017292, <https://www.ncbi.nlm.nih.gov/nucleotide/PV017292>.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

The fish study was approved by Ethics Committee of Faculty of Science, Banha University (Code: BUFS-REC-2025-332 Bot).

Accordance and arrive guidelines statement

Accordance: We confirmed that all experiments in this study were performed in accordance with the relevant guidelines and regulations. *Arrive:* All the procedure of the study is followed by the ARRIVE guidelines.

Additional information

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