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Research Article

Biodiversity and screening of antimicrobial, antioxidant, and cytotoxic activity of bacteria isolated from the Persian Gulf sponge (*Halicona oculata*)

Nasiri N.[1*], Taherizadeh M.R.[1], Gozari M.[2], Tamadoni Jahromi S.[2]

1 Department of Marine Biology, Faculty of Marine Science and Technology, University of Hormozgan, Bandar Abbas, Iran
2 Persian Gulf and Oman Sea Ecology Research Center, Iranian Fisheries Science Research Institute, Agricultural Research, Education and Extension Organization, Bandar Abbas, Iran
*Correspondence: n.nasiri1389@gmail.com

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Abstract

Screening of marine bacteria for developing new drugs is an emerging field in marine biotechnology. The purpose of this study was to investigate the diversity of sponge-associated bacteria from Kish and Larak islands (Persian Gulf) and to determine their antimicrobial, antioxidant, and cytotoxic activities. After sampling, bacteria were grown on the marine sponge agar medium. The isolated bacteria were characterized by polyphasic methods. The antimicrobial activity of the isolated bacteria was determined by the microdilution broth method. Cytotoxic activity was evaluated by MTT (3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide) assay on human cell lines. Antioxidant activity was performed by inhibiting DPPH free radicals. Among 121 bacterial isolates, *Vibrio* and *Bacillus* genera were the dominant frequency. The minimum inhibitory concentration of the extracted metabolites was recorded in the range of 64 to 512 µg/mL. The IC_{50} of antioxidant activity varied from 73.42 to 670.90 µg/mL. The cytotoxic activity of the extracted metabolites ranged from 40.57 to 181.80 µg/mL against SW 480 cell line and 141.30 to 359.70 µg/mL against HepG2 cell line. The HL 15, HL 85, and HK 5 extracts showed less toxicity against human umbilical vein endothelial cells. The results of genetic identification based on the comparison of 16S rRNA gene sequence showed that the potent strains HL 15, HL 24, HL 85, HK 5, and HK 36 belonged to *B. safensis*, *V. alginolyticus*, *V. rotiferianus*, *B. aureus* and *Pseudomonas paralactis*, respectively. The present study provided a new understanding of the diversity pattern and biological activity of the bacteria associated with *Haliclona oculata*.

Introduction

Drug resistance has become a major global crisis in recent years, reducing the efficacy of treatments for infectious diseases and cancers. The discovery of new drugs is crucial to combat the crisis posed by the growing number of drug-resistant infections and tumors (Kurt Yilmaz and Schiffer, 2021). Marine sponges (phylum Porifera) have attracted the attention of researchers as a source of natural products to find novel drugs for the treatment of infectious agents, cancer, and other diseases (Esposito *et al.*, 2022). The diversity, novelty, and complexity of sponge compounds have made them a valuable source for drug discovery (Nazemi *et al.*, 2017; Liu *et al.*, 2022). The sponges and their symbionts produce various metabolites with diverse biological activities, such as cytotoxic, antibiotic, and antioxidant activities through the chemical defense mechanism (Gavriilidou *et al.*, 2021).

Many studies have focused on the isolation and screening of bacteria associated with marine sponges. Bacteria are an important part of the sponge microbiome and have a great biosynthetic potential to produce bioactive secondary metabolites (Liang *et al.*, 2023). The high level of bacterial diversity and chemical adaptation to environmental conditions have resulted in genetic and metabolic diversity, including the creation of new biosynthetic pathways and the production of secondary metabolites with unique structures (Loureiro *et al.*, 2022).

Screening studies have evaluated the antimicrobial and cytotoxic activity of sponge-associated bacteria to identify potential antibiotics and anticancer drugs. Liang *et al.* (2023) reviewed 270 antimicrobial secondary metabolites against a range of pathogenic strains reported from 2012 to 2022. Among them, 31 % were originated from bacteria. In a related study, Skariyachan *et al.* (2016) isolated bacterial strains associated with Indian sponges that synthesized several types of secondary metabolites. These compounds were effective against a wide spectrum of pathogenic bacteria, such as *Pseudomonas aeruginosa,* methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi,* and *Klebsiella pneumoniae*. Furthermore, cytotoxic activity of sponge-associated bacteria was investigated in different studies. For example, Gozari *et al.* (2019b) isolated different species of actinobacteria from the Persian Gulf sponge *Dysedia avara.* They isolated *Streptomyces* sp. strain 85 that produced a new analog of anticancer agent olivomycin A. Several anticancer agents isolated from sponge-associated bacteria are in final clinical trial studies. For instance, Salinosporamide A is an anticancer agent, commercially known as marizomib, produced by *Salinispora tropica.* This bacteria was isolated from the sponge *Pseudoceratina clavata* (Fenical *et al.*, 2009; Jensen, 2022).

Bioactive secondary metabolites from sponge-associated bacteria have been reported from various ecosystems (Amelia *et al.*, 2022). However, few studies investigated the biological activity of sponge-associated bacteria in the Persian Gulf (Gozari *et al.*, 2019b; Ansarizadeh *et al.*, 2023). The Persian Gulf has unique physicochemical characteristics and its

temperature and salinity are higher than open seas (Riegl and Purkis, 2012; Akbarzadeh-Chomachaei *et al.*, 2023). These factors considered the Persian Gulf as a climate change laboratory to evaluate the diversity and chemical diversity of the sponge-associated bacteria and their bioactive compounds. Approximately, 55 genera of the sponges have been recorded in the Persian Gulf (Najafi *et al.*, 2018). Therefore, this distinct population is a potential target for screening studies. The present study aimed to evaluate the diversity of bacteria associated with the sponge *Haliclona* around the Persian Gulf islands, Larak and Kish, and to measure their antimicrobial, antioxidant, and cytotoxic activities.

Material and methods

Collection of sponge samples

In this study, 32 samples of marine sponge, *Haliclona oculata,* were collected around Kish and Larak Islands in the Persian Gulf by scuba diving at depths between 5-10 meters in October 2022. The sponge samples were placed in sterile glass bottles containing sterile seawater and were transferred to the Persian Gulf and Oman Sea Ecological Research Center laboratory. The voucher specimens (HOL 1024, HOK 2045) were deposited in the sponge collection at the Persian Gulf and Oman Sea Ecological Research Center.

Isolation and characterization of bacteria

The sponge samples were rinsed with sterile seawater to clean exogenous organisms and loosely attached microorganisms. Then, samples were cut, homogenized, and serially diluted in sterile seawater (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) (Gozari *et al.*, 2019a; Ansarizadeh *et al.*, 2023). After vortex mixing, 100 µl of each dilution was spread on the surface of the modified marine sponge agar medium. The isolation medium consisted of 10 g raffinose, 1 g L-histidine, 1 g dipotassium hydrogen phosphate 0.5 g calcium carbonate, 0.01 g ferrous sulfate, 15 g agar, 100 mL aqueous *H. oculata* extract in 900 mL of seawater (Gozari *et al.*, 2019b). The culture media were kept at 2 at 28°C for up to 4 weeks. The bacterial colonies were passaged on the same sterile medium and the purity of the bacteria was checked by microscopic observation (Pourmozaffar *et al.*, 2023). The morphological characteristics of the colonies including, texture, color, spores, and growth properties on the media were used to categorize the isolated bacteria. Furthermore, microscopic features of the colonies such as shape and gram reaction were employed for preliminary characterization (Brenner *et al.*, 2005; Goodfellow *et al.*, 2012). Biochemical and Physiological identification of the isolates was performed according to Bergey's manual of systematic bacteriology (Brenner *et al.*, 2005; Vos *et al.*, 2011).

Extraction of bacterial metabolites

The isolated bacteria were cultured in trypticase soy broth medium prepared with seawater and were incubated at 28˚C in a shaking incubator at 200 rpm. After incubation for 72h, fermentation broths were centrifuged at 3000 g for 10 min and the harvested supernatants were extracted by equal volumes of ethyl acetated twice. Consequently, the ethyl

acetate phases were evaporated in a rotary evaporator at 37 ˚C and the crude extracts were kept for bioassay analysis (Bucar *et al.*, 2013; Abdelmohsen *et al.*, 2022).

Antimicrobial activity assays

Minimum inhibitory concentrations (MICs) of the secondary metabolites extracted from the most potent isolates were determined against the microbial test strains using the standard microdilution method (CLSI, 2015). The crude extracts were dissolved in 5% dimethyl sulfoxide. The stock concentration was 1 mg/mL. Further concentrations were prepared by twofold serial dilution. Each well was inoculated with 5 µl of 10^8 CFU/mL and 10^4 spore/mL of the bacterial or the fungal suspensions, respectively. Ketoconazole (Sigma-Aldrich) and Streptomycin (Sigma-Aldrich) were used as the standard positive control for the fungal and bacterial strains, respectively. The inoculated trays were incubated for 24 h at 37˚C for the bacteria and 27˚C up to 7 days for the fungi. After the incubation period, the absorbance at 550 nm was measured by a microplate reader (BioTek® Instruments). The MICs of the samples were determined based on the minimum concentration of the crude extracts that effectively inhibited the growth of the test strains (CLSI, 2015).

Antioxidant activity assay

The DPPH radical scavenging activity of the culture extracts of the distinct isolates was determined by the microdilution method at the final concentration of 1250 µg/mL. IC_{50} of the most potent isolates with >90% scavenging activity was determined at seven final concentrations (1250, 625, 312, 156, 78, 39, 19.5 µg/mL). Five microliters of each primary concentration were added to 195 µl of DPPH solution at 100µM concentration in methanol. The 96 well microplates were incubated at room temperature in the dark for 30 minutes. The absorbance of samples was measured by a Microplate Reader (BioTech instrument) at 517 nm. Ascorbic acid was used as the standard positive control. The scavenging activity percentage of the samples was calculated by the following equation (Leong and Shui, 2002; Faust, 2019):

Scavenging activity% $=(I_0)-(I_s)/I_0\times 100$

Where, I_0 is the absorbance of the untreated DPPH solution, I_s is the absorbance of the samples or the standard control in the DPPH solution.

Cytotoxicity assay against human cell lines

The cytotoxic activity of the culture extracts were determined against colon cancer cell line (SW480) (NCBI Code: C506) and hepatoma G2 cells (HepG2) (NCBI Code: C158) as well as normal cell human umbilical vein endothelial cells (HUVECs) (NCBI Code: C554) by MTT (3-4,5-dimethylthiazol-2yl -2,5-diphenyltetrazolium bromide) assay (Das *et al.*, 2014). 100 µl of the cell suspensions were transferred to 96-well microplates at a density of 10^4 cells per well in DMEM or RPMI media. The microplates were incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Then the cultured cell lines were treated with 100 µl of each culture extract at final concentrations of 800, 400, 200, 100, 50, 25, and 12.5 µg/mL and incubated for an additional 36 h. Then, 50 µL of the MTT solution (5 mg/mL) was added to each well and it was incubated for

a further 4 h. After removal of the MTT-treated media, 100 µl of DMSO: ethanol (4:1) solution was added to each well and shaken in a microplate shaker (200 rpm) for 1 hour to dissolve the insoluble formazan dye. The optical density (OD) of each well was measured at λ 550 nm using the microplate reader. The cell viability percentage was measured by the equation 1:

$$\text{Cell viability (\%)} = [(\text{OD test}) - (\text{OD Blank})] / [(\text{OD control}) - (\text{OD Blank})] \times 100 \quad \text{(Eq. 1.)}$$

Molecular identification and Phylogenetic analysis

The Genomic DNA of the putative isolates was extracted according to the CTAB procedure described by Kieser (Kieser, 2000). Consequently, their 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R as described by Heuer (Heuer *et al.*, 1997; Tamadoni Jahromi *et al.*, 2021). After purification, the amplified 16S rRNA gene was sequenced by Macrogen (Seoul, Korea). The 16S rRNA sequences were compared to the same genes deposited in NCBI (National Centre for Biotechnology Information) by the Blastn program (Zhang *et al.*, 2000). The sequences were aligned with most similar 16S rRNA gene sequences in Genbank and the phylogenetic tree was constructed using The MEGA X program according to the neighbor-joining model (Kumar *et al.*, 2018).

Statistical analyses

All of the experiments were performed in triplicates (three independent experiments). The statistical significance of the data was analyzed with one-way ANOVA followed by the least significant difference (LSD) test using SPSS program (Version 24) and the significance level was set at $p<0.05$. The cytotoxic activity was expressed as mean IC_{50}±standard error (SE). The IC_{50} values of the samples were calculated using the linear regression between the final concentration of the samples and respective cell viability obtained from Eq. 1 by the software GraphPad PRISM version 6 (GraphPad Software, San Diago, CA). The statistical significance of the resultant phylogenetic tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates with MEGA X.

Results

Isolation and diversity of the sponge-associated bacteria

In total, 121 bacterial colonies were isolated from the collected sponge samples. Among these, 51 isolates were from around Kish Island, and 70 isolates were isolated from Larak Island. The biodiversity pattern of the isolated bacteria showed that the isolates belonging to the *Vibrio* and *Bacillus* genera were the dominant species in the sponge collected around the Kish and Larak Islands. Other genera isolated with less abundance included *Nocardiopsis*, *Micromonospora*, *Streptomyces*, *Photobacterium*, *Psychrobacter*, *Virgibacillus,* and *Micrococcus* (Fig. 1). The frequency of *Vibrio* and *Bacillus* isolates in the sponge collected from Kish were 33.33 and 25.49%. However, *Vibrio* and bacillus isolate with a frequency of 38.57 and 25.49% formed a significant part of the isolated bacteria from Larak's sponges. After that, 5 genera *Streptomyces*, *Photobacterium*, *Pseudomonas*,

Virgibacillus, and *Micrococcus* formed the existing isolates with less frequency (Fig. 2).

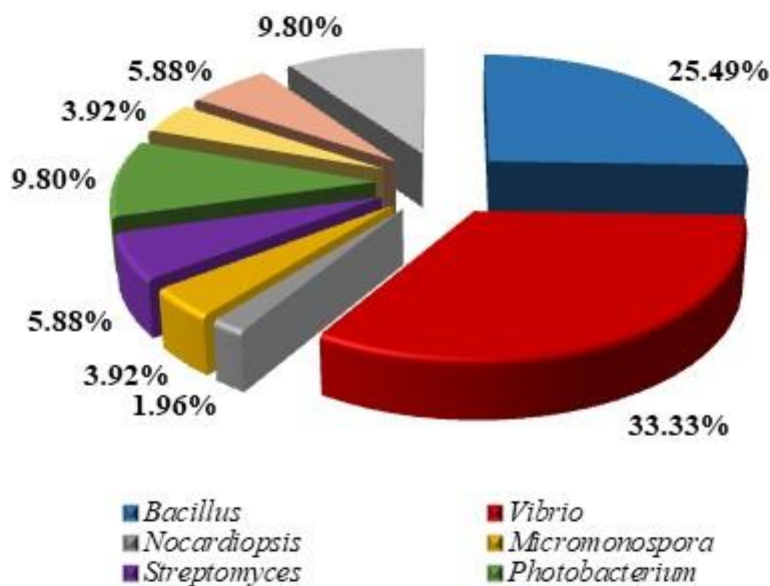

Figure 1: Diversity of bacteria isolated from *Haliclona* collected from the Kish Island.

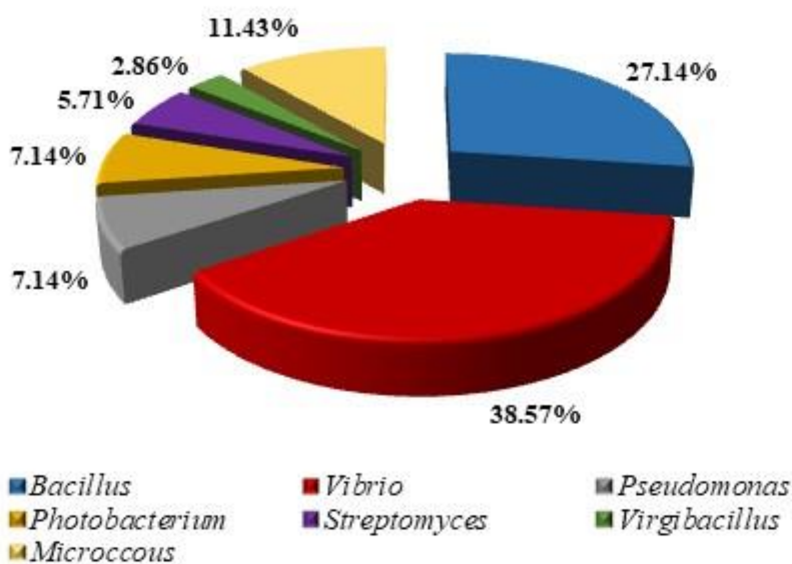

Figure 2: Diversity of bacteria isolated from sponge samples collected from the Larak Island.

Antimicrobial activity of the sponge-derived bacteria

The extracted metabolites from the HL 15 strain showed broad-spectrum activity against microbial strains. MIC value of this

extract varied from 64 µg/mL against *M. luteus* and *S. aureus* to 512 µg/mL against *P. aeroginosa and A. niger*. The MBC or MFC values of this strain ranged from 218 µg/mL to more than 1250 µg/mL. The HL 85 extract exhibited selective activity against the gram positive bacteria and fungi. The highest MIC value of this extract was 128 µg/mL against *M. luteus* and *C. albicans.* The highest MBC or MFC values of this extract was 256 µg/mL against these gram positive bacteria and *C. albicans.* The extract of the HL 24 exhibited broad spectrum activity against test strains. This extract showed highest antimicrobial activity against *M. luteus* and *C. albicans* with a MIC value of 64 µg/mL. MBC and MFC of this extract varied from 256-512 µg/mL. The HK 5 extract also showed broad spectrum activity and exhibited the highest MIC and MBC values against *M. luteus* at 64 and 128 µg/mL, respectively. The extract the HK 36 exhibited specific antimicrobial activity against the gram-positive bacteria *M. luteus* and *S. aureus* with MIC values of 64 and 256 µg/mL, respectively. The MBC value of this extract against *M. luteus* was 256 µg/mL (Table 1).

Table 1: Antimicrobial activity of extracted metabolites from the most potent isolates.

Isolates	*S. aureus*		*M. luteus*		*E. coli*		*P. aeroginosa*		*C. albicans*		*A. niger*	
	MIC[1]	*MBC*[2]	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*	*MIC*	*MBC*
HL 15	64	128	64	128	512	512	512	NA	128	128	512	NA
HL 85	256	256	128	256	NA	NA	NA	NA	128	256	512	512
HL 24	128	256	64	256	256	512	128	256	64	256	256	512
HK 5	128	256	64	128	256	512	256	512	128	256	NA	NA
HK 36	256	512	64	64	NA	NA	NA	NA	256	512	NA	NA
Streptomycin	8	8	8	8	8	8	16	16	-	-	-	-
Ketoconazole	-	-	-	-	-	-	-	-	16	16	16	16

[1]Minimum inhibitory concentration (µg/mL)
[2]Minimum bactericidal concentration (µg/mL)

Antioxidant activity of the sponge-derived bacteria

The results of the antioxidant activity assay showed that 12 out of 120 isolates scavenged DPPH free radicals at concentrations less than 1000 µg/mL. The IC_{50} values of the antioxidant metabolites ranged from 73.42 µg/mL produced by strain HL 24 to 670.90 µg/mL produced by HL 56 strain (Table 2).

Table 2: DPPH Radical Scavenging Activity of *Haliclona*-isolated bacteria.

Isolates	IC_{50} ±SE (µg/mL)	Isolates	IC_{50} ±SE (µg/mL)
HL 15	163.20±20.69	HL 81	538.80±25.01
HL 23	497.40±29.36	HL 85	239.40±37.03
HL 24	73.42±3.69	HK 5	89.34±0.59
HL 31	546.70±27.24	HK 14	414.80±15.56
HL 56	670.90±26.11	HK 36	207.30±20.17
HL 77	447.80±19.32	HK 41	610.0±49.31

Cytotoxic activity of extracted metabolites against human cell lines

The extracted metabolites from 5 bacterial isolates showed high cytotoxic activity against 3 human cell lines. As seen in Table 3, isolate HK 36 showed the highest cytotoxicity against SW490 cell line with a IC_{50} value of 40.57 µg/mL. Cytotoxic bioassay showed that isolate the HL 85 exhibited maximum cytotoxic activity toward HepG2 cell line with IC_{50} value 141.30 µg/mL. Interestingly, the HK 36 and the HL 85 showed minimum cytotoxicity against HUVEC cell line with IC_{50} values of 301.80 and 234.10, respectively.

Table 3: Cytotoxic activity of extracted metabolites from potent isolates.

Isolates	IC_{50} ±SE (µg/mL)		
	SW490	HepG2	HUVECs
HL 15	181.80±5.34	359.70±22.95	248.60±9.97
HL 24	138.30±8.18	308.60±18.68	181.50±9.90
HL 85	70.60±4.30	141.30±6.97	234.10±19.11
HK 5	97.35±4.53	255.00±23.46	71.43±4.51
HK 36	40.57±2.02	200.30±6.97	301.80±15.86
Doxorubicin	2.83±0.64	10.10±1.20	0.73±0.11

Identification of the most potent isolates

The results of identification revealed that the most potent isolates belonged to *Bacillus*, *Vibrio,* and *Pseudomonas* genera. The HL 15 and the HK 5 isolates showed typical characteristics of *Bacillus* genus. They were gram-positive and spore-forming bacilli that showed the same carbon and nitrogen utilization profile except that the HL 15 utilized fructose, raffinose and valine while, HK 5 could not utilize these sources. In addition, the HL 15 tolerated NaCl concentration up to 10% while HK 5 could grow up to 9%. Identification results showed that the HL 85 and the HL 24 represented phenotypic properties of *Vibrio* genus. Both strains showed the same characteristics except that the HL 85 could utilize asparagine, grew at 25-40°C and tolerated to 3-6% of NaCl concentration. However, HL 24 could not metabolize asparagine, grew at broaden temperature (10-40°C), and salinity ranges (3-10%). Our results demonstrated that HK 36 strain belonged to *Pseudomonas* genus (Table 4).

Molecular identification based on 16S rRNA gene sequence revealed that the strains HL 15 and HK 9 showed the highest homology with *Bacillus safensis* strain NBRC 100820 and *Bacillus aerius* strain 24K with 99.11% and 98.83 %, respectively. These results demonstrated that HL 24 had maximum similarity (98.61%) with *Vibrio alginolyticus* strain NBRC 15630. While HL 85 showed complete homology (100%) with *Vibrio rotiferianus* strain CAIM 577. These analyses showed that the 16S rRNA of strain HK 36 had 99.33% similarity with *Pseudomonas lactis* strain DSM 29167.

The results of the phylogenetic analysis showed that there were close evolutionary relationships between the potent strains studied and the strains isolated from marine sponges in previous studies. The

phylogenetic tree drawn based on the 16s rRNA gene sequence and using the neighbor-joining pattern confirmed that the strains were placed in two separate clades. HL 15 and HK 5 strains were placed in a clade with other *Bacillus* strains, which were isolated from sponges in previous studies. However, the branching pattern of the phylogenetic tree showed that these strains are placed in different clusters but they are derived from a common ancestor. In the second clade, *Vibrio* and *Pseudomonas* strains were placed in two different clusters. *Pseudomonas* bacteria were placed in the first cluster of the second clade, and *Vibrio* strains were placed in the second cluster of this clade with other strains recorded in the NCBI gene bank. The branching pattern in this cluster showed that *Vibrio* strains have undergone a different evolutionary pattern despite having a common ancestor (Fig. 3).

Table 4: Morphological, biochemical, and physiological characterization of potent isolates.

Characters		Isolates				
		HL 15	**HL 24**	**HL 85**	**HK 5**	**HK 36**
Gram reaction		+	-	-	+	-
Spore		+	-	-	+	-
Carbon sources utilization	Glucose	+	+	+	+	+
	Fructose	+	+	+	-	+
	Xylose	+	-	-	+	-
	Arabinose	+	-	-	+	-
	Rhamnose	-	-	-	-	-
	Sucrose	+	+	+	+	-
	Raffinose	+	-	-	-	-
	Galactose	+	+	+	+	-
	Manitol	+	+	+	+	+
	Inositol	-	-	-	-	-
Nitrogen sources utilization	Valine	+	ND	ND	-	+
	Arginine	-	-	-	-	-
	Ornithine	-	+	+	-	-
	Asparagine	-	-	+	-	-
Growth temperature range		5-45 °C	10-40 °C	25-40 °C	5-45 °C	15-45 °C
Growth pH range		5-11	6-9	6-9	5-11	7-9
NaCl Tolerance		0-10	3-10	3-6	0-9	0-8
H_2S production		-	-	-	-	-
Voges-proskauer		+	-	-	+	-
Gelatinase		-	+	+	-	+
Arginine dihydrolase		-	-	-	-	-
Ornithine decarboxylase		-	+	+	-	ND
Oxidation/Fermentation		+/-	+/+	+/+	+/-	+/+
Lysine decarboxylase		-	+	+	-	ND
Catalase production		+	+	+	+	+
Oxidase production		+	+	+	+	+
Citrate utilization		-	+	+	-	+
Indole production		-	+	+	-	-
Nitrate reduction		-	+	+	-	+

*ND: non-diagnostic

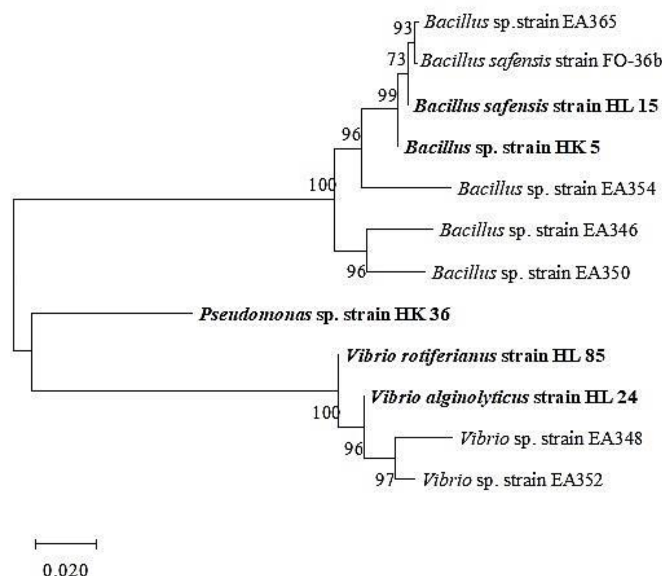

Figure 3: Phylogenetic dendrogram based on 16S rRNA gene sequence analysis, reconstructed from evolutionary distances by using the neighbour-joining method, showing the phylogenetic position of potent strains and the closest related strains. *N. soli* was used as an out-group. Bootstrap values are indicated at the relevant branching points. The numbers of branch nodes are bootstrap values based on 1000 resampling. Bar: 0.02 substitutions per nucleotide position.

Discussion

In recent years, extensive exploration programs have been implemented to isolate natural product-producing bacteria from the microbiome of marine animals (Osman and Weinnig, 2022; Wilson *et al.*, 2023). In the current study, we selected sponge populations around Lark and Kish islands as the isolated source for bacteria. The results obtained from the identification tests showed that *Vibrio* and *Bacillus* genera were predominantly present in the sponge samples. However, the abundance of other species was less than 10%. Isolation of diverse bacteria with high frequency allows the exploitation of more biosynthetic genes and metabolic pathways, which results in an increase in the hit rate of screening programs for potent isolates (Hegemann *et al.*, 2023). The diversity pattern of sponges associated bacteria depends on complex variables such as porosity and chemical composition of the sponge tissue, metabolism, physiology and filtration rate of the sponge, geographical location, physicochemical conditions, seasonal changes and climate. The predominant presence of *Vibrio* and *Bacillus* species in marine sponges has been reported in previous studies. For example, Bibi *et al.* (2020) showed that *Bacillus* strains constituted 55% of the isolated bacteria from sponges collected from the Red Sea. In contrast, other studies have reported a different biodiversity pattern. For example, Gozari (2020) reported that 25.43% of the isolated bacteria from the *Haliclona* sponge belonged to actinobacteria, of which 70% were *Streptomyces* species. One of the important reasons for this difference in the diversity pattern was the implementation of the selective isolation process including physical and chemical treatments and supplementation of antibiotics in the

aforementioned study. However, the purpose of the present study was to screen all cultivable bacteria associated with the sponge.

The results of the antimicrobial activity screening showed that 9.92% of the isolated bacteria showed antimicrobial activity against at least one of the pathogenic test strains. These results confirmed the presence of antibiotic-producing bacteria in the host sponge. The results of recent studies showed that bacteria, as an important part of the sponge's microbiome play an important role in their adaptation and survival in the marine ecosystem (Freitas-Silva *et al.*, 2023). In this regard, the theory of symbiosis of bacteria and sponges has been presented based on scientific evidence of these mutual interactions (Longford *et al.*, 2019; Hadfield, 2021). In this regard, our results provided another evidence for the presence of antibiotic-producing bacteria in marine sponges. This evidence can strengthen the possibility of their participation in the chemical defense mechanism of the host sponge against microbial pathogens. The results of MIC and MBC assays showed that all of the extracted metabolites exhibited maximum antimicrobial activity against *M. luteus* in ranges of 64 to 128 µg/mL and 64 to 256 µg/mL, respectively. These results demonstrated the high antimicrobial activity of the extracted metabolites against pathogenic microbial agents. Although the MIC and MBC values of the extracted metabolites were significantly less than the control antibiotics. This lower antimicrobial activity can be attributed to the lack of purity of the extracted metabolites compared to the high purity of commercial antibiotics.

The results of antioxidant activity assay revealed that 12 isolates showed significant activity in scavenging DPPH free radicals. The production of antioxidant secondary metabolites by marine invertebrates, including sponges, has been identified as a defense strategy (Alves *et al.*, 2021). The presence of high levels of reactive oxygen species (ROS) in marine environments is a potential threat for invertebrates such as sponges. Therefore, sponges develop defense mechanisms against high levels of ROS, such as the production of antioxidant compounds (Hu *et al.*, 2021). The antioxidant activity of metabolites extracted from sponges has been proven in different studies (Campos *et al.*, 2020; Martignago *et al.*, 2023). In the present study, the IC_{50} values of the antioxidant activity of the active metabolites ranged from 73.42 µg/mL produced by the HL 24 strain to 670.90 µg/mL produced by the HL 56 strain. Recent studies in this field have also reported the antioxidant activity of marine bacteria. For example, Poongodi *et al.* (2014) reported the IC_{50} of the antioxidant activity of the metabolite extracted from *Nocardiopsis* sp. isolated from the sediments of the Gulf of Mannar at 58.20 µg/mL (Poongodi *et al.*, 2014). In another study, *Nocardiopsis* sp. S-1 isolated from the coast of Jeju Island in South Korea showed a high activity of up to 53% in terms of free radical scavenging (DPPH) (Kim *et al.*, 2014).

The results of cytotoxic activity assay revealed that the extracted metabolites exhibited high cytotoxicity against 2 human cancer cell lines SW 480 with IC_{50} values

ranging from 40.57 to 181.80 µg/mL. These results showed that the IC_{50} value of the extracted metabolites against the HepG2 cell line was in the range of 141.30 to 359.70 µg/mL. However, the extracted metabolites exhibited cytotoxic activity against the HUVEC cell line as human normal cells. Examining the microscopic images of cells treated with extracted metabolites and untreated cells confirmed a significant decrease in the number of living cells and their morphological changes (Fig. 3). Interestingly, the cytotoxic effect of metabolites extracted from HL 24, HL 85, and HK 36 strains was less than their toxicity against cancer cell lines with higher therapeutic index. Other studies also reported the cytotoxic activity of bacteria associated with sponges. Gozari *et al.* (2019b) showed that the secondary metabolites extracted from *Streptomyces* sp. strain SP 85 isolated from the sponge *Dysedia avara* showed cytotoxic activity against SW 480 and HUVEC cell lines with IC_{50} value of 19.21 and 410.60 µg/mL, respectively.

Isolation of bacteria with cytotoxic activity from the sponge *Haliclona* in the present study strengthened the possible role of the sponge microbiome in applying chemical defense mechanisms against predators and fouling organisms in the light of holobiont theory. According to this theory, the origin of some of the secondary metabolites in sponges is their symbiotic microorganisms (Stévenne *et al.*, 2021). Therefore, certain types of symbiotic bacteria must be permanently and specifically associated with the host organism and perform their function (Biggs *et al.*, 2023). Based on the results of the bioassays, we selected 5 potent strains for polyphasic identification. The results of molecular identification showed that the potent isolates HL 15, HL 24, HL 85, HK 5, and HK 36 had 98 to 100% similarity with *Bacillus safensis*, *Vibrio alginolyticus*, *Vibrio rotiferianus*, *Bacillus aerius* and *Pseudomonas paralactis*, respectively. Comparison of morphological, biochemical and physiological characteristic with molecular results confirmed the identification results. Phylogenetic analysis showed that the potent strains were clustered in two separate clades. *Bacillus safensis* strain HL 15 and *Bacillus* sp. strain HK 5 were located in the same clade as their closest strains, which evolutionally derived after them from a common ancestor. The constructed dendrogram showed the isolated Vibrios derived faster from the common ancestor than the strains isolated in other studies. These results can show the evolutionary dynamics of potent strains associated with the population of sponges in the Persian Gulf.

Conclusions

The present study provided a new understanding of the biodiversity and biological activity of the bacteria associated with the sponge *Haliclona*. These results confirmed the presence of bacteria as a major part of the culturable microbial community in the sponge *Haliclona*. The results of biological activity provided another evidence to verify the active role of bacterial populations associated with sponges. The metabolites extracted in this study had strong cytotoxic activity with an appropriate therapeutic index against human cancer cell lines. Further studies in

the field of in vivo effects of extracted metabolites are being conducted.

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Conflicts of interest
The authors declare that there is no conflict of interest.

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