Iranian Journal of Fisheries Sciences
http: doi.org/ 10.22092/ijfs.2026.135842 2026, 25(4) 617-635

*Research Article*

# Anti-Alzheimer and antioxidant activities of bacteria isolated from the Persian Gulf sponges

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**Keywords**

Alzheimer disease,
Acetylcholinesterase,
Enzyme inhibitors,
Persian Gulf,
Sponge-associated bacteria

**Article info**

Received: August 2025
Accepted: May 2026
Published: July 2026



**Abstract**

Acetylcholinesterase (AChE) inhibitors are vital in the management of Alzheimer's disease. This study aimed to identify bacteria isolated from sponge samples collected near Hengam Island and evaluate their acetylcholinesterase inhibitory activity. Sponge samples of *Halicona* sp. and *Axinella* sp. were collected by scuba diving. A total of 131 bacterial strains were isolated using Marine Zobell agar medium. *Bacillus* and *Vibrio* species were the most frequently identified bacteria from the sponge samples. Analysis of the extracted metabolites revealed that four isolates could inhibit acetylcholinesterase activity, with $IC_{50}$ values between 203.60 and 385.62 µg/ml. According to the MTT cell proliferation assay, HH 151 extract exhibited cytotoxic effects on the HUVEC cell line. The AChE inhibitory extracts also showed DPPH radical scavenging activity between 78.12 µg/mL and 237.54 µg/mL. The 16S rRNA sequence analysis showed about 99 to 100% homology between HH 127, HH 151, HA 315, and HA 338 strains with *Vibrio azureus, Streptomyces nigrescens, Bacillus pumilus*, and *Bacillus subtilis* species. These findings provided new candidates of the acetylcholinesterase inhibitors from sponge-associated bacteria for *in vivo* studies.

## Introduction

Alzheimer's disease (AD) is the most common form of dementia, affecting mainly older adults. The disease is characterized by progressive memory loss and cognitive decline. Significant neurodegenerative changes are observed in the brains of affected individuals (Singh and Kumar, 2024). The enzyme acetylcholinesterase (AChE) plays a critical role in the pathophysiology of Alzheimer's disease, particularly in the cholinergic neurotransmitter and beta-amyloid (Aβ) deposition. This enzyme is responsible for the hydrolysis of acetylcholine, a key neurotransmitter in patients with AD. AChE dysfunction leads to cognitive decline and memory impairment. Progressive loss of cortical AChE activity is correlated with cognitive decline (Gajendra *et al.*, 2024). Acetylcholinesterase inhibitors play a critical role in the management of Alzheimer's disease by reversing the cholinergic deficit that is characteristic of this disorder. The major action of AChE inhibitors involves preventing the breakdown of acetylcholine. This inhibition leads to an increase in synaptic acetylcholine concentration, enhancing cholinergic transmission. AChE inhibitors are used as a clinical neuroprotective treatment for neurological disorders such as senile dementia, ataxia, myasthenia gravis, and Alzheimer's disease. By preventing the hydrolysis of acetylcholine, altering AChE activity may help restore cholinergic balance, slow the progression of Alzheimer's disease, and improve cognition (Eldufani and Blaise, 2019). Donepezil remains a cornerstone medication, FDA-approved for mild to severe AD due to its reversible, brain-selective AChE inhibition that sustains cholinergic transmission (Nakamura *et al.*, 2025). Donepezil and similar AChE inhibitors provide established symptomatic value in AD pharmacotherapy, their transition toward disease-modifying roles via multifunctional next-generation agents underscores a vital evolution in clinical practice (Li *et al.* 2023; Grabowska *et al.*, 2025).

Marine sponges (Porifera) are a significant source of bioactive compounds with pharmaceutical potential. Recent investigations have highlighted the complex interactions between sponges and their associated microorganisms, which often contribute to the production of bioactive compounds (Nasiri *et al.*, 2024). Bioactive compounds derived from the marine sponge microbiome have received considerable attention for their therapeutic applications, especially in drug discovery (Raymond and Rakotondraibe, 2025). Symbiotic bacteria in sponges actively contribute to the biosynthesis of various natural products that could be of medicinal importance. These products include enzyme inhibitors, anticancer, antimicrobial compounds are investigated for potential therapeutic applications (Li *et al.*, 2023; Alborz *et al.*, 2025).

Numerous researchers are focusing on discovering enzyme inhibitors to treat Alzheimer's from under-exploited sources, such as marine bacteria (Ozturk *et al.*, 2025). Recent studies have identified several types of marine bacteria that produce acetylcholinesterase inhibitors; this is a crucial finding given the

therapeutic potential of these compounds (Yadav *et al.*, 2022). Notable genera discovered include *Psychrobacter, Microbacterium, Stenotrophomonas, Planococcus, Nocardia, Streptomyces, Leucobacter, Bacillus, Virgibacillus, and Brevibacterium*. These microorganisms have been isolated from diverse marine environments including sponges, soft corals, and sediments, representing a rich source of bioactive metabolites in marine ecosystems (Nguyen *et al.*, 2023). Despite the importance of these studies, the AChE inhibitory activity of sponge-associated bacteria in the Persian Gulf has not been reported so far. Key limitations include the logistical difficulties of accessing and sampling marine habitats, coupled with the structural complexity of many marine metabolites (Abuhijjleh *et al.*, 2021). These factors render isolation, characterization, and synthesis technically demanding. Moreover, lengthy discovery pipelines characterized by high risk, combined with limited short-term funding, further impede systematic exploration of marine natural products (Mehbub *et al.*, 2024). The Hengam Island has a diverse sponge population and provides great potential for screening programs as a less- explored habitat. Environmental stressors such as heavy metals and oil pollution might induce some adaptations in the sponges and their microbiome in these habitats (Akbarzadeh-Chomachaei *et al.*, 2023; De Castro-Fernandez *et al.*, 2023). These adaptations to the environment can lead to genetic diversity and the creation of new metabolites. The aims of this study were to identify the bacteria isolated from the sponge samples collected around Hengam Island and assess their acetylcholinesterase inhibitory activity.

## Material and methods

*Sampling*

Marine sponges were sampled in February 2021 from 5 to 10 meter depth in Hengam Island which is located at 27° 97′ N latitude and 56° 49′ E longitude in the Persian Gulf. About 30 samples were collected by scuba diving and were kept in sterile plastic bags on ice and were transferred to the laboratory. The collected sponges were identified based on morphologic and morphometric properties by the Persian Gulf and Oman Sea Ecology Research Institute. Fresh specimens undergo examination of external features (shape, color, surface texture, oscules) and internal skeletal architecture (Spicule types, dimensions, arrangement) are measured and compared against sponge keys and the World Porifera Database (Hooper, 2003; De Voogd *et al.*, 2022).

*Isolation and characterization of bacteria*

The collected samples were rinsed with sterile seawater to remove attached organisms and sand. Then, the samples were cut into 1 $cm^3$ pieces under aseptic conditions and homogenized in a sterile mortar. Subsequently, the homogenized samples were diluted serially with 0.2 µm filtered sterile seawater up to $10^{-5}$ (Pourmozaffar *et al.*, 2023). 100 µL of each dilution were inoculated on Marine Zobell agar medium and spread with a sterile glass rod. Finally, the cultures were incubated at 30°C for a week. The bacterial colonies were purified by subculturing on the

isolation medium and checked by microscopic observation. The purified isolates were characterized based on phenotypic properties, including morphological, biochemical and physiological experiments (Vos *et al.*, 2011).

*Extraction of secondary metabolites from the isolated bacteria*

The isolated bacteria were cultured in a modified Tryptic soy broth as a fermentation medium at $10^5$ CFU/mL density. The fermentation medium was incubated at 28°C for 48h in a shaking incubator (220 rpm). After incubation, the fermented culture was filtered using a vacuum pump and extracted with an equal volume of ethyl acetate twice. Then, the ethyl acetate phase was dried by a rotary evaporator under vacuum at 37°C and stored at -20°C for further experiments (Seidel, 2012).

*Acetylcholinesterase inhibitor experiment*

The inhibitory activity of the extracted metabolites on AChE enzyme activity was determined based on the improved Ellman method using the Acetylcholinesterase Inhibitor Screening Kit (MAK324; Sigma-Aldrich) (de Almeida *et al.*, 2022). The extracts from each bacterial isolate were prepared at successive concentrations (1250, 625, 312.5, 156.25, 78.12, 39 µg/ml). The extracts were prepared in sodium phosphate buffer. 45 µL of pure AChE enzyme with a concentration of 400 Units/L was added to each well. Also, as a negative control, 45 µL of the kit assay buffer was added to the control wells instead of the enzyme. These 3 wells were considered as 100% inhibition. The other 3 wells were also free of inhibitor. To these wells containing purified AChE enzyme and also to the negative control wells, 5 µL of phosphate buffer was added. Physostigmine hemisulfate at the same concentrations as the test extracts was used as a positive control to validate the assay. The microplates were incubated for 15 min at 25°C. Then, 150 µL of the reaction mix was added to each well containing the sample, control (without enzyme) and control without inhibitor. The reaction mix consisted of 154 µL of assay buffer, 1 µL of substrate and 0.5 µL of DNTB (5,5 dithiobis-2-nitrobenzoic acid). The microplates were gently shaken. The optical absorbance of each well was measured using a microplate reader at 412 nm at times 0 and 10 min. The inhibition percentage was calculated according to the following equation. In this equation, by calculating and subtracting the difference in absorbance of the samples at the time of incubation and 10 minutes, any increase in sample absorbance because of the color of the extracts or spontaneous hydrolysis of the substrate will be compensated:

$$\% \text{ Inhibition} = (1 - \Delta A_{\text{Test Cpd}} / \Delta A_{\text{No Inhibitor}}) \times 100\%$$

$\Delta A_{\text{Test Cpd}}$ = the $A_{412}$ value of a test compound well at 0 minutes subtracted from the $A_{412}$ value of the same well at 10 minutes

$\Delta A_{\text{No Inhibitor}}$ = the $A_{412}$ value of the No Inhibitor Control well at 0 minutes subtracted from the $A_{412}$ value of the No Inhibitor Control well at 10 minutes.

*Assessment of the cytotoxic activity of inhibitory extracts*

The cytotoxic activity of the inhibitory extracts were investigated at different concentrations against normal human umbilical cord endothelial cell lines HUVECs (NCBI Code: C554) by MTT cell proliferation assay (Peng and Zhao, 2009). 100 µL of the cell suspensions were transferred in 96-well Microplates at a density of $10^4$ cells /well in DMEM medium. The microplates were kept at 37°C for 24 h in a humidified 5% $CO_2$ atmosphere. Then the cultured cells were treated with 100 µL of each final concentration (800, 400, 200, 100, 50, 25, 12.5 µg/mL) and incubated for an additional 36 hours. After the incubation period, 50 µL of the MTT solution (5 mg/mL) was added to each well and incubated for another 4 hours. After removal of the MTT solution, 100 µL of DMSO/ethanol (4:1) solution was added to each well and kept 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 550 nm by a 96-well microplate reader. The cytotoxic activity percentage was measured by the following equation:

$$\text{Cell viability (\%)} = [(OD_{test}) - (OD_{Blank})/ (OD_{control}) - (OD_{Blank})] \times 100$$

*Assessment of antioxidant activity of the inhibitory extracts*

Antioxidant activity of the inhibitory extracts was determined based on the DPPH radical scavenging activity according to the microdilution Method (Leong and Shui, 2002; Gozari *et al.*, 2019). The inhibitory extracts were serially diluted in methanol at different concentrations (1250, 625, 312, 156, 78, 39, 19.5 µg/mL). Five microliters of each concentration was added to 195 µL of DPPH solution at 100µM concentration in methanol. The Microplate was kept at 21°C in a dark place for 30 minutes. The absorbance of each well was measured by a Microplate Reader (BioTech instrument) at 517 nm. The ascorbic acid was used as a standard control, and the scavenging activity of samples was calculated using the following equation:

$$\text{Scavenging activity} = (I_0) - (I_s)/I_0 \times 100$$

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

*Molecular identification of inhibitory isolates*

Genomic DNA extraction of inhibitory isolates was performed using the CTAB extraction method (Doyle and Doyle, 1987). The quantity of extracted DNA were determined by spectrophotometry method at 230, 260, and 280 nm. The quality of the extracted DNA was evaluated using 1% agarose gel electrophoresis (Tamadoni Jahromi *et al.*, 2021). Genetic identification of inhibitory isolates were investigated based on 16S rRNA sequencing. The 16S rRNA gene was amplified by PCR using universal primers 27 F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1492R (5′ ACGGCTACCTTGTTACGA

3′) (Heuer *et al.*, 1997; Tamadoni Jahromi *et al.*, 2021). 16S rRNA gene sequencing was performed using the Sanger method and a Dye Deoxy Terminator Cycle Sequencing kit technique by Amitis gene company. Phylogenetic analysis of the inhibitory isolates were calculated by comparing them with the most similar strains in the NCBI nucleotide database by Blastn analysis (Zhang *et al.*, 2000). The phylogenetic tree was constructed by MEGA X software based on the neighbor-joining model (Kumar *et al.*, 2018). The 16S rRNA gene sequences were depositedin the NCBI GenBank database with following accession numbers PQ013041, PQ013043, PQ013044, PQ013045, PQ013048, PQ013049, PQ013050 and PQ013051.

*Statistical analysis*

All the experiments were performed in triplicate. The statistical significance of the data was analyzed with one-way ANOVA followed by LSD using SPSS program (Version 24) and the significance level was set at $p<0.05$. The cytotoxic activity is 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 cytotoxic activity obtained from the previously mentioned equation 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 based on 1000 replicates with MEGA X (Kumar *et al,* 2018).

## Results

*Biodiversity of bacteria isolated from sponge samples*

We obtained 83 bacterial isolates from the sponge *Haliclona* sp. and 48 isolates from sponge *Axinella* sp. collected from Hengam Island. The results of bacterial identification showed that the diversity patterns were different in the two sponge species (Fig. 1).

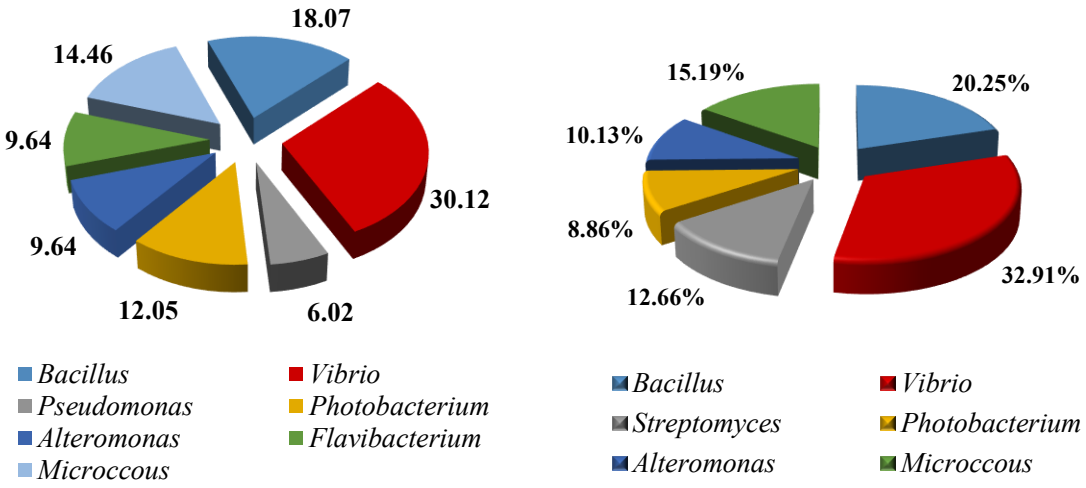


**Figure 1: Bacterial diversity in *Haliclona* sp. (Left) and *Axinella* sp. (Right) samples collected from Hengam Island.**

*Inhibition of acetylcholinesterase activity*

Evaluation of the inhibitory activity of metabolites extracted from 131 isolates obtained from sponge samples showed that 4 isolates producing acetylcholinesterase inhibitory compounds with $IC_{50}$ values were less than 1250 g/mL. The $IC_{50}$ inhibitory properties of the metabolites extracted from the potent isolates are listed in Table 1. Among the extracted metabolites, the highest inhibitory activity belonged to HH 127 isolate with an $IC_{50}$ value of 203.6 µg/mL. The metabolites extracted from the HA 338 isolate had the lowest $IC_{50}$, with 385.62 µg/mL (Table 1).

**Table 1: Assessment of AChE inhibitory activity of the extracted metabolites.**

| Isolate | $IC_{50}$ ±SE (µg/mL) |
|---|---|
| HH* 127 | 203.60±4.71 |
| HH 151 | 253.80± 14.49 |
| HA† 315 | 227.21± 9.08 |
| HA 338 | 385.62± 10.91 |
| Physostigmine | 3.68± 0.14 |

*HH (Hengam Island, *Haliclona* sp.), †HA (Hengam Island, *Axinella* sp.)

*Antioxidant activity of AChE inhibitory isolates*

Evaluation of the antioxidant activity of metabolites extracted from the inhibitory isolates showed that all of four potent isolates produced antioxidant agents. These potent isolates showed scavenging activity ranging from 78.12 µg/mL by HH 127 isolate to 237.54 µg/mL by HA 338 isolate (Table 2).

**Table 2: Assessment of antioxidant activity of AchE inhibit.**

| Isolate | $IC_{50}$ ±SE (µg/mL) |
|---|---|
| HH 127 | 119.60±3.33 |
| HH 151 | 78.12±4.81 |
| HA 315 | 133.31±3.51 |
| HA 338 | 237.54±13.38 |
| Ascorbic acid | 61.37±3.19 |

*Cytotoxic activity of the AChE inhibitory metabolites*

The results of the cytotoxic activity assay of the extracted AChE-inhibiting metabolites showed that HH 127, HA 315 and HA 338 isolates were not toxic against the HUVEC cell line. These results also revealed that the extracted metabolites from the HH 151 isolate was toxic against the cervical HUVEC cell line. The $LC_{50}$ value of the extracted metabolites of HH 151 isolate was equal to 590.8 µg/mL (Table 3).

**Table 3: Determination of $LC_{50}$ cytotoxic activity of extracted metabolites against the HUVEC cell line.**

| Isolate | $LC_{50}$± SE (µg/mL) |
|---|---|
| HH 127 | NT* |
| HH 151 | 590.80± 31.53 |
| HA 315 | NT |
| HA 338 | NT |

*NT: Not toxic

Examination of microscopic images of treated cells and treatment without treatment confirmed a significant reduction in the number of living cells and their morphological changes (Fig. 2).

*Genetic identification of inhibitory isolates*

The results of genomic DNA extraction of the inhibitory isolates confirmed the extraction process. The agarose gel electrophoresis revealed that the length of the extracted DNA were more than 10 kbp. These results showed that the integrity of genomic DNA structure of the inhibitory isolates were protected during extraction process (Fig. 3).

The results of PCR gene electrophoresis of 16S rRNA of selected isolates indicated the production of sequences with a length of about 1500 bp. These results showed that the 16S rRNA gene amplification process was performed specifically by the polymerase chain reaction (Fig. 4).

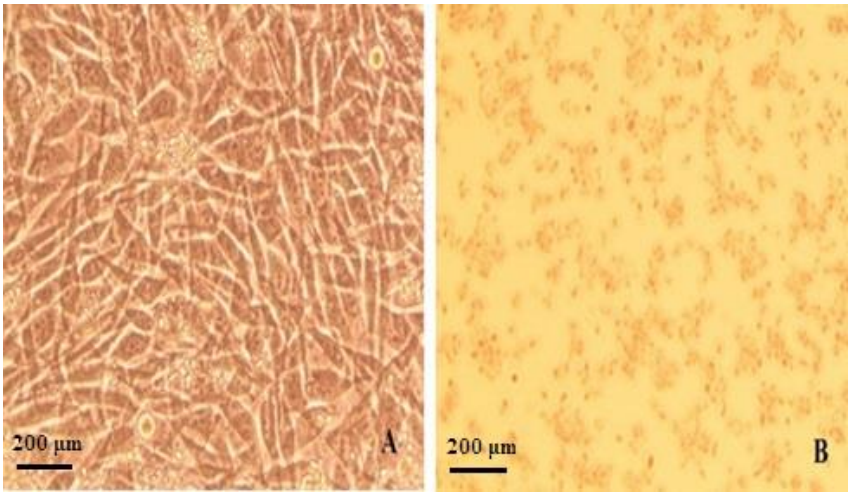


**Figure 2: Effect of extracted metabolites from HH 151 isolate on the Human Umbilical Vein Endothelial cell line. A. Untreated HUVEC. B. Treated HUVEC. (Magnification: 200x).**

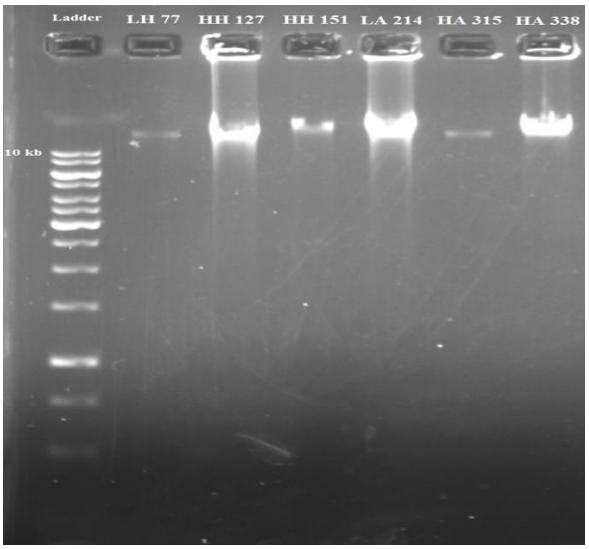


**Figure 3: Agarose gel electrophoresis of Genomic DNA extracted from the inhibitory strains.**

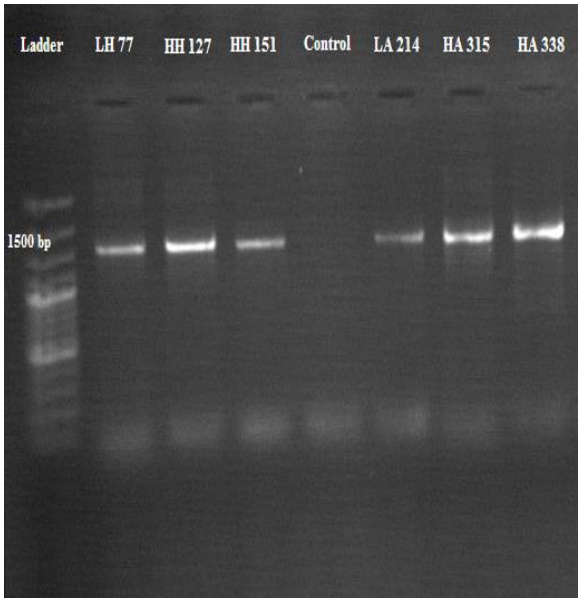


**Figure 4: Agarose gel electrophoresis of amplified 16S rRNA genes of the inhibitory strains.**

*16S rRNA sequence analysis of the inhibitory strains*

The Blastn analysis of inhibitory strains showed about 99 to 100% similarity in 16S rRNA sequence with the type strains submitted in the NCBI gene bank. These results revealed that the HH 127 strain showed complete homology with the *Vibrio azureus* strain LC2-005. HH 151 strain showed the highest homology at 99.52% with *Streptomyces nigrescens* strain NRRL ISP-5276. The 16S rRNA sequence of the HA 315 strain showed 100% similarity with *Bacillus pumilus* strain NCTC10337. These results also showed that the HA 338 strain had the highest similarity with the *Bacillus subtilis* strain M18SP4Q with 99.78%.

*Phylogenetic analysis of the inhibitory strains*

The results of phylogenetic analysis based on 16s rRNA gene sequences showed that the inhibitory strains and their most similar strains were placed in 3 different clusters. The HA 338 and HA 315 strains were in a common clade, although the HA 315 strain derived in a distinct evolutionary pathway. *Vibrio azureus* strain HH 127 was located on a separate evolutionary path with its most similar strains (Fig. 5).

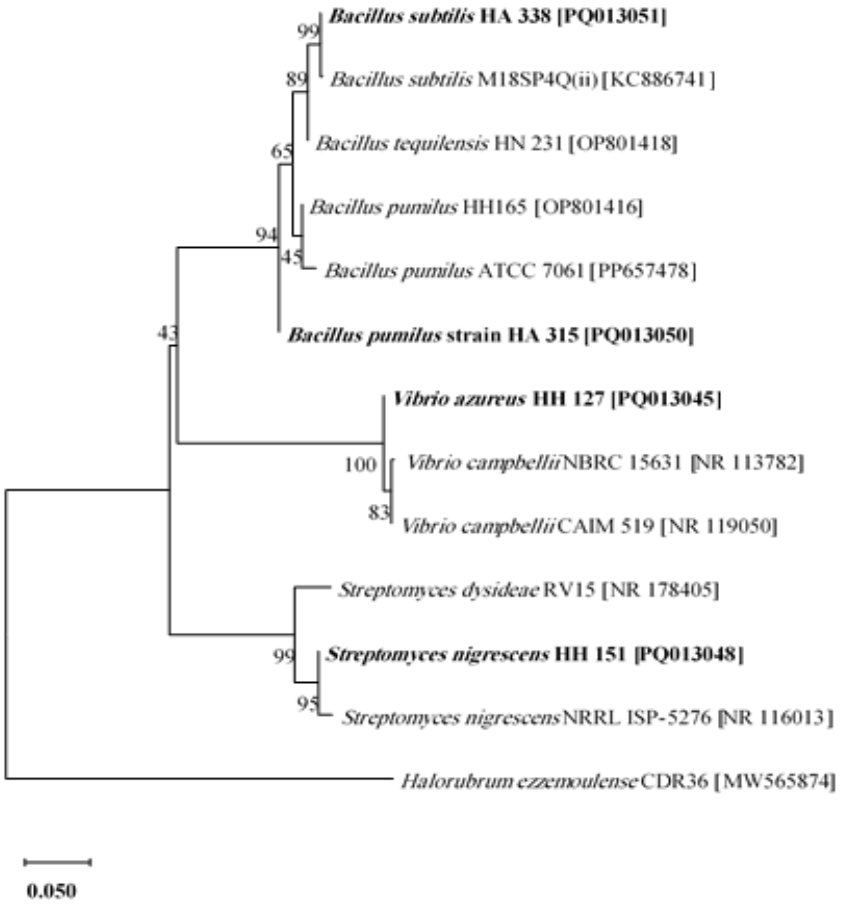


**Figure 5: The neighbor-joining phylogenetic tree of the selected strains (Bold) and most similar strains. The numbers shown next to the nodes indicate the Bootstrap value. The scale bar indicates the replacement of 5 nucleotides per 100 nucleotides.**

**Discussion**

Recent research has highlighted the significance of marine microorganisms as a novel source of medicinal compounds with promising activities, including anti-Alzheimer's disease. Marine natural compounds can be explored as unique and novel scaffolds for medicinal chemistry research to discover potent and effective treatments for this disease (Gozari *et al.*, 2019). A review study showed that among 233 marine-derived compounds for the treatment of Alzheimer's disease, 102 compounds had inhibitory activity against the enzyme acetylcholinesterase (Hafez Ghoran and Kijjoa, 2021). Therefore, the aim of the present study is important in order to obtain bacteria that produce compounds that inhibit the acetylcholinesterase enzyme. Selection of a habitat is one of the important strategies to increase the chance of success in screening programs of marine natural products. We isolated bacteria from the sponge population around Hengam Island in the Persian Gulf.

We identified seven distinct bacterial genera in the sponge *Haliclona* sp. from Hengam Island.

These results for *Axinella* sp. sponge samples from Hengam Island indicated the isolation of 6 different bacterial genera. In the same context, the results of the study by Ansarizadeh *et al.* (2023b) showed that 10 bacterial genera were isolated and identified from *Haliclona* sp. sponges around Qeshm and Hormoz Islands. Also, in another study, 6 and 7 bacterial genera were isolated from *Niphates* sp. sponge samples from Qeshm and Hormoz Islands, respectively (Ansarizadeh *et al.*, 2023a). However, it should be noted that the results obtained from the biodiversity pattern of the isolated bacteria in the present study, like other similar studies, only include culturable bacteria. Therefore, it does not represent the pattern of the entire microbiome of the studied sponges, including non-culturable bacteria.

The results of the present study also showed that the *Bacillus* and *Vibrio* isolates were predominantly present in the sponge samples. *Bacillus* species are prevalent among cultivable bacteria associated with marine sponges, frequently comprising 8–16% of isolates across diverse taxa and geographic regions, owing to their spore-forming resilience in nutrient-poor marine conditions (Riyanti *et al.*, 2020). This dominance is evident in studies of Indo-Pacific sponges such as *Fasciospongia cavernosa*, where *Bacillus* ranked alongside *Vibrio* as a primary genus producing bioactive metabolites like AchE inhibitors, and in South African *Siphonodictyon coralliphagum* microbiomes (Pandey *et al.*, 2014). In other study Ansarizadeh *et al*. (2023b) also showed that the most bacteria isolated from the sponges *Haliclona* sp. and *Niphates* sp. of Hormuz and Qeshm islands were *Bacillus* and *Vibrio* bacteria. In several studies designed and implemented with the aim to screen bacteria that produce bioactive compounds, the dominance of these two genera has also been reported. For example, another study found that the genus *Bacillus* comprised 55% of the culturable bacteria of the sponge *Suberea mollis* collected from the Red Sea (Bibi *et al.,* 2020). This study also reported that *Pseudovibrio* bacteria with 13% and

*Rugeria* bacteria with 10% were the most abundant in the sponges. The results of another study showed that the genera *Vibrio, Bacillus, Pseudomonas and Marinobacter* were isolated from four species of sponges collected from the Gulf of Mannar (India) (Anand *et al.*, 2006). In the same context, the study by Mohan *et al*. (2016) reported the dominance of *Bacillus* and *Vibrio* bacteria in 21 species of *Demospongea* sponges collected from around Agatti Island, India. Although there are other studies that have reported a different pattern. In this context, the study of Pandey *et al*. (2013) showed that the biodiversity of bacteria isolated from the sponge *Aka coralliphaga* included the phyla Firmicutes (12.70%), Actinobacteria (4.97%), Proteobacteria (3.86%), and Bacteroides (0.5%). In this context, the study conducted by Gozari *et al.* (2020) indicated a different bacterial pattern in the sponge *Haliclona* sp. with a predominance of the genus *Streptomyces*. Another study that differed from the present results was the study of Zhang *et al*. (2006), which showed that species belonging to the genus *Streptomyces* made up 73.6% of the bacteria isolated from the sponge *Hymeniacidon perleve* (Zhang *et al.,* 2006). In another study, *Streptomyces* bacteria were reported as the largest population of bacteria isolated from the sponge *Iotrochota* sp.(Jiang *et al.*, 2008). Also, the prevalence of *Streptomyces* bacteria was reported to be 23.91% in the sponge *Dendrilla nigra* (Selvin *et al.,* 2009). In another study, Chen *et al*. (2022) reported that out of 52 strains isolated from the sponge *Ophlitaspongia* sp. collected from the Yellow Sea, most of the strains belonged to Actinobacteria (Chen *et al*., 2022). Several reasons can be given for this difference in the bacterial pattern. The first reason for this difference can be explained in the objectives of the studies and implementing various isolation strategies, including different culture media and physical and chemical treatments. In the first group of studies, such as the present study, general culture media were used, while in the second group, selective treatments and specific culture media were used to isolate species capable of producing specific medicinal compounds. The second reason for this difference in the pattern of bacterial biodiversity is a set of morphological characteristics of the host sponge, such as its porosity and tissue type. The physiological function of a sponge is influenced by its porosity. The large diameter and number of pores in the sponge tissue increase its porosity and facilitate the entry and exit of bacteria. As a result, colonization of the sponge microbiome is performed more efficiently (Granito *et al.*, 2017). The type of tissue and its constituent compounds can play a role in the sponge's diversity microbiome. Among the compounds that affect the permeability of sponge tissue are fats and sterols. Various studies in this field have shown the effect of fatty acids on the diversity of the sponge microbiome (de Oliveira *et al.*, 2020). Another reason for this difference in biodiversity pattern could be the difference in the metabolism of sponges and their need for interactions with their microbiome (Botté *et al.*, 2019). Therefore, sponges exposed to stress factors have more complex and extensive requirements for their microbiome and are likely to have a

different biodiversity pattern than sponges living in low-stress environments. The innate immune system of the host sponge also has a profound effect on the uptake and acceptance of bacteria and their microbiome pattern (Posadas *et al.*, 2022). Other factors affecting the biodiversity of the sponge microbiome is the water filtration capacity, which can explain the difference in the biodiversity pattern between *Haliclona* and *Axinella* sponges in the present study on Hengam Island. Therefore, the microbial flora of water and sediments can affect the biodiversity pattern in these areas. Environmental variables can also affect the difference in biodiversity.

Several studies have used the acetylcholinesterase enzyme as a screening target for finding drug-inhibitory compounds (Dimitrova *et al.*, 2025). Therefore, this enzyme was also the target of bioassay tests in the present study. The results of the present study showed that 2 isolates derived from *Haliclona* sp. produced inhibitory compounds against the acetylcholinesterase enzyme. While 2 isolates obtained from *Axinella* sp. produced acetylcholinesterase inhibitory metabolites. The $IC_{50}$ values of these metabolites ranged from 203.6 to 385.62 µg/mL. This range of inhibition is comparable to the $IC_{50}$ value of inhibitory activity of metabolites extracted from *Streptomyces lateritus* strain UTMC1334 of 360 µg/mL (Almasi *et al.*, 2018). Also, in another study, the EPSR4 compound extracted from *Bacillus subtilis* showed a dose-dependent inhibitory effect and inhibitory activity with an $IC_{50}$ of 786.38 µg/mL(Abdel-Wahab *et al.*, 2022). In another study, the EPSR5 compound was extracted from the marine bacterium *Kocuria* sp. It had an inhibitory activity of 797.02 µg/mL (Alshawwa *et al.*, 2022). The screening process for enzyme inhibitor compounds of marine origin has complexities and challenges. Screening marine-derived enzyme inhibitors faces multiple challenges: (1) low success in isolating productive bacteria due to the unculturable nature of sponge microbiomes; (2) instability of bacterial composition from environmental fluctuations (Nowak *et al.*, 2024). and (3) poor therapeutic potential of new AChE inhibitors stemming from digestive absorption and bioavailability issues (Alharbi *et al.*, 2023).

Determination of the cytotoxicity of the extracted metabolites on a human cell line is essential to ensure the safety. In this study, human umbilical cord endothelial cells (HUVEC) were used as an in vitro model for toxicity detection because of their ease of culture and necessary sensitivity. Also, this cell line, due to its widespread use in other studies, allows for comparison of the results of different studies (Cao *et al.*, 2017). In the present study, the MTT cell proliferation assay was used to evaluate the cytotoxicity of compounds extracted from bacteria. This method has proven its effectiveness in exploratory research and screening of drugs of natural origin (Hu *et al.*, 2015). However, cytotoxicity assays have inherent limitations, whereby observed effects may arise from assay-specific conditions rather than intrinsic compound toxicity. To address these limitations, this study followed a standard protocol by

incorporating blanks, triplicate replicates and dose-response curves to differentiate methodological artifacts from biologically relevant cytotoxicity. The results of the cytotoxicity showed that only HH 151 extracts were toxic to the HUVEC cell line with $LC_{50}$ equal to 590.80 µg/mL. For this reason, this strain was excluded from further studies. The toxicity of this extract is debatable from various perspectives. It can be confirmed that compounds that disrupt cellular metabolism are present in the extracts of these metabolites. However, the mechanism of action of this toxicity cannot be determined precisely because there are various mechanisms, including induction of apoptosis, destruction of the cell wall, inactivation and inhibition of the activity of key enzymes involved in cell proliferation and metabolism processes, and interference in cellular exchanges for cell death. One of the modes of action in this field could be the inhibitory effect of AchE-inhibiting compounds on other vital cell enzymes. The cytotoxic activity of enzyme inhibitors against cancer cells has been shown in various studies. For this reason, future studies can examine the effects of these compounds on cancer cells and calculate the therapeutic index of these compounds by comparing their effects on normal HUVEC cells. The production of these toxic compounds by bacteria isolated from sponges can be another evidence of their symbiotic hypothesis and the active participation of these isolates in the sponges' defense strategy against foreign agents such as predators and pathogens (Busch *et al.*, 2024).

Based on the comparison of 16S rRNA gene sequences with strains registered in the NCBI database, genetic identification revealed a similarity of 99–100%. These results showed that the majority of the selected isolates were distinct strains from the strains registered in the NCBI. The 16s rRNA gene is considered as a marker of phylogenetic analysis as a molecular clock or an orthologous gene and is studied in phylogenetic analyses. This is because of the conservation of the sequence of this gene formed during the evolution of bacteria and its sequence order provides valid data for classification, identification of bacteria, and introduction of new species (Abellan-Schneyder *et al.*, 2021). The phylogenetic analysis was performed based on distance and character parameters. The results of the phylogenetic analysis based on the Neighbor-joining distance method showed that the *Vibrio* isolates producing AChE inhibitory compounds had a close evolutionary relationship with other strains isolated from sponges. The phylogenetic tree drawn based on the Neighbor joining model indicated the evolutionary commonality of the isolated *Vibrio* strains and their derivation from a common ancestor. However, evolutionary divergence was observed among the created clusters. For example, the *Vibrio azureus* strain HH 127 had a separate evolutionary path from the other two tested strains and was placed in a common cluster with the *Vibrio campbellii* NBRC 15631 strain. This result indicates the possibility of mutations and indels occurring in the evolution of these two strains. These two strains may have undergone mutations in evolutionary genes during adaptation to their host and habitat conditions. Considering that these mutations could

occur in biosynthetic genes besides evolutionary genes, alterations in the biosynthesis pathways of secondary metabolites are possible. Evaluation of the phylogenetic tree drawn based on the Neighbor joining model showed that *Bacillus subtilis* strain HA 338 and *Bacillus pumilus* strain HA 315 were placed in the same clade but in different clusters. These results confirm the rate of divergence between HA 315 and HA 338. Based on the morphology of the phylogenetic tree, strain HA 315 showed a faster divergence rate. This result could be due to the difference in mutation rates and genetic bottlenecks because of selective pressures on the 16S rRNA gene. The bootstrap statistical test also confirmed the correctness of the topology of the drawn tree and its phylogenetic validity. Phylogenetic analyses of *Streptomyces* strains and other closely related strains reported showed close evolutionary relationships between these strains. These results and interpretation of the branching patterns indicated a faster derivation of strain HH 151 compared to the closest strain to itself, namely strain ISP-5276. This study provided the first insight into the biodiversity pattern of bacteria isolated from *Haliclona* and *Axinella* sponges in Hengam Island. These results can be used in biotechnology studies. Another important aspect of this study explains the inhibitory potential of sponge-associated bacteria against the AChE enzyme. The achievement of these inhibitory metabolites, besides their ability to be measured in other studies, demonstrates the high potential of this group of marine bacteria.

## Conclusion

This study provided further evidence of the active role of the sponge microbiome in the framework of the holobiont theory. As a result, this study introduced 4 bacterial strains capable of producing AChE inhibitory compounds. These bacteria represent valuable resources for structural elucidation of inhibitory compounds and in vivo assays in future studies. The antioxidant activity of these metabolites showed their role in improving the immune system and reducing oxidative stress. Overall, the results of this exploratory study provide potential options for anti-Alzheimer's drug development research.

## Conflicts of interest

The authors declare that they have no conflict of interest**.**

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