



Anti-*Helicobacter pylori* Activity of Red Sea *Hyrtios erectus* sponge and micro-organisms Extracts

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ABSTRACT

Helicobacter pylori were considered one of the most bacterial infections in the world. Recently, the resistance of antibiotic against *H. pylori* is increasing and hence, it is necessary to find new and natural effective agents. Marine organisms and microorganism produce complex secondary metabolites and some of these compounds are antimicrobials agents. This study aimed to provide preliminary data to determine if marine *Hyrtios erectus* sponge, actinomycetes, and fungi extracts can inhibit the growth of *H. pylori*. In this study, the methanol extracts of the three samples of *Hyrtios erectus* sponge and the ethyl acetate extracts of seven actinomycetes strains and four fungi strains were used to determine the antibacterial effect against a standard strain of *H. pylori*. The samples of actinomycetes and fungi were identified by using 16S rRNA amplification for actinomycetes and Internal Transcribed Spacer-PCR for fungi using Genetic Analyzer 3500, and then the DNA sequences were compared through BLAST and alignment with other actinomycetes and fungi which already present in the GenBank from various study areas. Results indicated that the anti-*H. pylori* screening showed that only 13 from 14 extracts exhibited anti-*H. pylori* effect. Among them, the methanol extract of *Hyrtios erectus* (1), the ethyl acetate extract of actinomycete strain S7 and the ethyl acetate extracts of fungi strains M1, M2, M3, M4 exhibited strong effect against *H. pylori* more than other extract with minimal inhibitory concentrations 3.9, 15.63, 3.9, 15.63, 3.9 and 7.81 $\mu\text{g/ml}$, respectively. The 16S rRNA and ITS rRNA gene sequences and phylogenetic analysis provided strong evidence for the isolates of actinomycetes and fungi, respectively. The fungi isolates were identified as three species that belong to *Aspergillus* genus and one species that belong to *Penicillium* genus.



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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative bacterium (Oleastro and Ménard, 2013). *H. pylori* infect the stomach and duodenum mucus lining. It can penetrate the stomach mucous layer and then can be found in several locations such as overlying mucus gel, inside vacuoles or lies in the interface between the mucus gel and surface of the gastric epithelial cells (Karlık et al., 2009; Bakri, 2013). It inhibits the gastric epithelium mucosal layer (Abadi, 2014). This bacterium is only known as a micro-

organism that can survive in the highly acidic environment of the stomach (Karlık et al., 2009).

Warren and Marshall (1983) isolated *H. pylori* and since that time it has been considered as one of the most common infections caused by bacteria in the world and its infection is one of the most common chronic infections in the world (Al-Shraim et al., 2013; Hasosah et al., 2015). *H. pylori* are not deadly like some bacteria, but it is the most successful pathogen in the history of humanity because it infects 50% of the world. The prevalence of *Helicobacter pylori* in developing countries is the highest (Oleastro and Ménard, 2013).

Although the most infection by *H. pylori* is asymptomatic (~85%) but it's reported to play a significant role to induce some related disease like ulcer disease, gastritis, gastric carcinoma and mucosa-associated lymphoid tissue lymphoma (Oleastro and Ménard, 2013; Hasosah et al., 2015). In 1994, *Helicobacter pylori* infection by the International Agency for Research on Cancer (IARC) (World Health Organization (WHO)) was classified as a type I carcinogen (Oleastro and Ménard, 2013).

The host's immune system can't clear the infection, and it persists unless treated. So, the cure of *Helicobacter pylori* infection done by many treatment regimens such as standard triple therapy (most common therapeutic regimen), sequential therapy, bismuth quadruple therapy, levofloxacin-based triple therapy, concomitant therapy, hybrid therapy, rifabutin-based triple therapy, and levofloxacin-based subsequent therapy. The triple treatment consists of two antibiotics and a proton pump inhibitor (PPI) (Chua et al., 2015; Smith, 2015). Unfortunately, *Helicobacter pylori* are resistant to one or more of the antimicrobial drugs. So, there is a need to look for a new and natural treatment effective against *H. pylori* (Amin et al., 2013).

As we know, oceans are highly complex environments and harbour. It contains many different types of organisms (Selvam et al., 2013). Marine organisms and micro-organisms live and survive within complex communities. They produce secondary metabolites as a reaction to ecological compressions like tide difference, competition for space and predation. Also, it can prevent the development or limit the outgrowth of other micro-organisms like bacteria, viruses and fungi that contention with it because these compounds have antimicrobials property (Abad et al., 2011; Selvam et al., 2013; Pérez et al., 2016). Marine organisms, such as sponges are among the richest sources of pharmacologically-active chemicals from other organisms in the marine ecosystem.

Sponges and their associated micro-organisms have more than 5300 various products and about 200 novel metabolites from sponges are reported every year (Abad et al., 2011). Marine sponges that belong to the genus *Hyrtios* (Kingdom: Animalia, phylum: Porifera, class: Demospongiae, order: Dictyoceratida, family: Thorectidae) are reported to be rich sources of biologically active secondary metabolites (Youssef et al., 2013; Shady et al., 2017). Among the *Hyrtios* genus, the *Hyrtios erectus* sponge (*H. erectus*) is the most frequently investigated source of biologically active natural products. The *H. erectus* sponge has been collected from different marine environments like Okinawa in Japan and the Red Sea in Egypt (Shady et al., 2017). *Hyrtios* species produce important groups of bioactive secondary metabolites like sesquiterpenes, macrolides, sesterterpenes, acyclic triterpenes, β -carboline alkaloids, and indole (Youssef et al., 2013; Shady et al., 2017; Kaweetripob et al., 2018). Many of these compounds possess anti-inflammation agent, antimicrobial activities, anti-cancer activity, antioxidant activities, and antifeedant (Youssef et al., 2013; Kaweetripob et al., 2018).

Marine micro-organisms like actinobacteria have provided many important bioactive compounds with high commercial valuable antibiotics like salinosporamide and abyssomycin. There are about 10.000 of bioactive compounds isolated from actinobacteria (Selvam et al., 2013). *Streptomyces* is the largest genus of actinobacteria and considered as a significant source of new biologically active compounds. They produce about two-thirds of natural antibiotics (Fan et al., 2011).

Marine-associated fungi is also a type of marine micro-organisms that produce different types of secondary metabolites which is beneficial in a medical field (Bajpai, 2016). *Penicillium* and *Aspergillus* are the most abundant types of filamentous fungi that reported from a marine. *Penicillium* and *Aspergillus* species are renowned producers of biologically active compounds (Nicoletti and Vinale, 2018).

Therefore, exploration of some highly effective and safer anti-*H. Pylori* derived from marine organisms and micro-organisms are becoming important as an alternative therapy.

The target of the present research was to explore new and natural compounds that can be used as a therapeutic system to treat infected people with *H. pylori* with high capacity to eradicate the growth of *Helicobacter pylori*. Also, to study the inhibitory effect of the extracts of *Hyrtios erectus* sponge and marine micro-organism against *H. pylori*.

MATERIALS AND METHODS

Three samples of *H. erectus* sponge were collected from the Red Sea of SharmElshikh (27°91' N, 34°33' E), Yanbu (24°09' N, 38°06' E) and Obhur in Jeddah (21°54' N, 39°20' E), using scuba diving at a depth of about 30 m. The sponge specimens were later identified to be *Hyrtilos erectus* by Dr Sameh Elhady (Faculty of Pharmacy, King Abdulaziz University). A voucher specimen was preserved at the Faculty of Pharmacy, King Abdulaziz University, under Registration Number SEE-1 Figure 1.

The sediment samples and *Stylissa carteri* sponge were obtained from Obhur Red Sea in Jeddah (21°54' N, 39°20' E), using scuba diving at a depth of about 30 m. The sponge specimen was later identified to be *Stylissa carteri* by Dr Sameh Elhady (Faculty of Pharmacy, King Abdulaziz University). A voucher specimen was preserved at the Faculty of Pharmacy, King Abdulaziz University, under Registration Number SEE-2 Figure 2.



Figure 1: *H. erectus* sponge SEE-1



Figure 2: *S. carteri* sponge SEE-2

Preparation of *H. erectus* sponge extract

The fresh *H. erectus* sponge materials were frozen immediately after collection. The sponge materials were cut into small pieces. 200 g of sponge materials were extracted with methanol (MeOH) until exhaustion at room temperature. The combined crude extract was concentrated under reduced pressure to give a viscous crude extract. The crude extracts

were dissolved with dimethylsulfoxide (DMSO), and water then stored at – 20°C until required.

Isolation of actinomycetes from sediment

The marine sediment was subsequently plated out on appropriate agar plates (ISP medium 2). The plates were incubated at 30°C for 4-8 weeks. After the growth of microbe, a small swap was taken by a loop from plate to obtain a pure isolate by using (ISP medium 2). The plates were incubated at 30°C for 2-8 weeks (Abdelmohsen *et al.*, 2010).

Isolation of fungi from *S. carteri* sponge

A specimen of *S. carteri* sponge was washed in sterile normal saline and then cut into pieces. The pieces thoroughly were vortexed with normal saline. The pieces and supernatant subsequently plated out on appropriate agar plates (ME medium) supplemented with anti-bacterial (10% chloramphenicol). The plates were incubated at 30°C for 1-3 weeks. Distinct colony morphotypes were picked and re-cultured until visually free of contaminants to obtain pure isolates (Zhang *et al.*, 2012; Kalyani *et al.*, 2017).

Isolation of fungi from sediment

The marine sediment was subsequently plated on appropriate agar plates (ME medium) supplemented with anti-bacterial (10% chloramphenicol). Then the inoculated plates were incubated at 30 °C for 1–3 weeks. Distinct colony morphotypes were picked and re-cultured until visually free of contaminants to obtain pure isolates (Zhang *et al.*, 2012; Kalyani *et al.*, 2017).

Extraction of selected actinomycetes metabolites from growth media

The selected pure isolates of actinomycetes Figure 3 were cultured in 250 ml Erlenmeyer flasks contained 100 ml of appropriate broth medium (ISP2). The inoculated liquid media were incubated for 14 days at 30 °C while shaking at 150 rpm by shaking incubator. After that, the media, containing microorganisms, were centrifuged at 5500 × g for 12 min. at 4°C to separate the growth media from cells. The cells were stored at – 20°C until required and 80 ml of ethyl acetate (EtOAc) was added to each flask, containing growth medium, and left overnight to stop cells growth. Each growth medium was extracted three times (3x80ml) with distilled EtOAc. The combined extracts were evaporated under vacuum by using Buchi Rotary evaporator to obtain EtOAc crude extract. Finally, the crude extracts were dissolved with dimethylsulfoxide (DMSO), and water then stored at – 20°C until required (Abdelmohsen *et al.*, 2010).

Extraction of selected fungi metabolites from growth media

The selected four pure isolates of fungi Figure 4 were cultured in 250 ml Erlenmeyer flasks contained 100 ml of appropriate broth medium (ME), supplemented with chloramphenicol. The liquid media were incubated for 30 days at 30 °C while shaking at 150 rpm by shaking incubator. After that, the media, containing micro-organisms, were filtered to separate growth media from cells. The cells were stored at - 20°C until required and 80 ml of ethyl acetate (EtOAc) was added to each flask, containing growth medium, and left overnight to stop cells growth. Each growth medium was extracted three times (3x80 ml) with distilled EtOAc. The combined extracts were evaporated under vacuum by using Buchi Rotary evaporator to obtain EtOAc crude extract. Finally, the crude extracts were dissolved with dimethylsulfoxide (DMSO), and water then stored at - 20°C until required (Zhang *et al.*, 2012; Kalyani *et al.*, 2017).

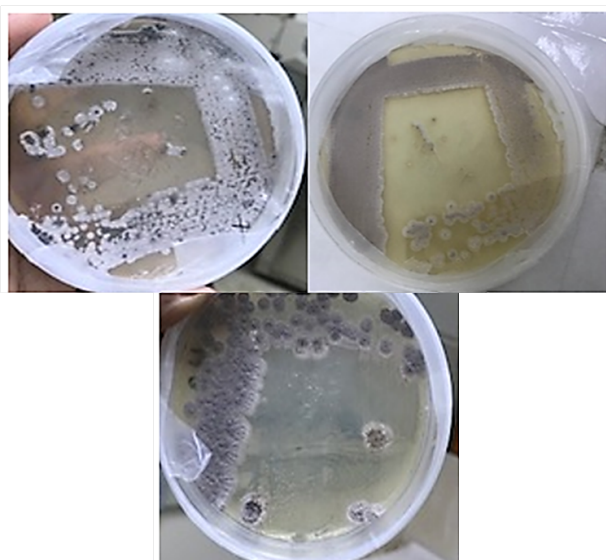


Figure 3: Some of actinomycetes pure isolates

DNA Genomic Extraction from pure Actinomycetes and Fungi isolates

The DNA extraction of pure actinomycetes and fungi isolates was performed using the Gene JET Genomic DNA Purification Kit Bacteria obtained from the Technolab solutions company. Extracted samples of DNA were stored at -20 °C until used.

16S rRNA amplification of actinomycetes

The genetic level identification of potential actinobacteria was carried out. The 16S rRNA gene of the strains was amplified using the 16S rRNA primers, forward primer (5'-GAGTTTGATCCTGGCTCAG-3') and reverse primer

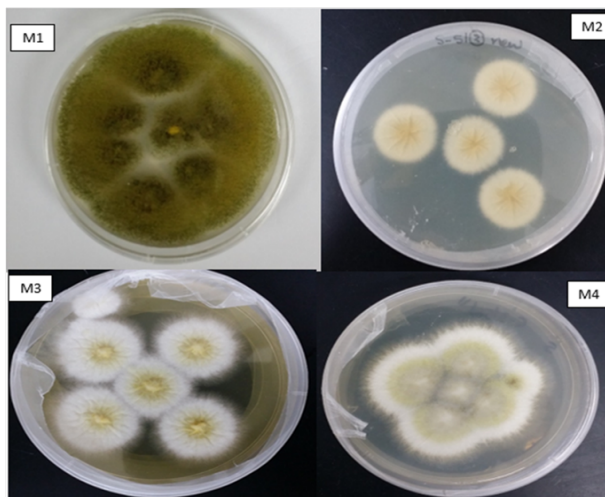


Figure 4: The four pure isolates of fungi

(5'-ACGGCTACCTTGTTACGACTT-3') (Selvam *et al.*, 2013).

Transfer 5 μ l of nuclease-free water into PCR tube then 13 μ l of the master mix, 2 μ l of forwarding primer, 2 μ l of reverse primer and 3 μ l (50 ng/ μ l) of extracted DNA were added. Samples were then placed in a thermal cycler. PCR was running for 2 hours. The standard PCR protocol included an initial denaturation step for 5 min. at 94°C then 35 cycles of 1min. at 94°C, 1min. at 53°C and 1min. at 72°C. The final step was performed for 10 min, at 72°C. PCR reaction without DNA template was used as a negative control in each run. The resulting products were separated on an agarose gel Figure 5.

Internal Transcribed Spacer (ITS)-Polymerase Chain Reaction (PCR) of fungi

The genetic level identification of potential fungi was carried out.

The primers used for the identification of the fungal species were universal primers for fungal amplification: ITS1 and ITS4 primers, ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Zhang *et al.*, 2012).

Transfer 12 μ l of the master mix into PCR tube than 2 μ l of forwarding primer (ITS1), 2 μ l of reverse primer (ITS4) and 9 μ l (25 ng/ μ l) of extracted DNA was added. Samples were then placed in a thermal cycler. PCR was running for 2 hours.

The standard PCR protocol included an initial denaturation step for 5 min. at 94°C then 35 cycles of 1 min. at 94°C, 1 min. at 53°C and 1 min. at 72°C. The final step was performed for 10 min, at 72°C.

PCR reaction without DNA template was used as a negative control in each run. The resulting products were separated on an agarose gel Figure 6.

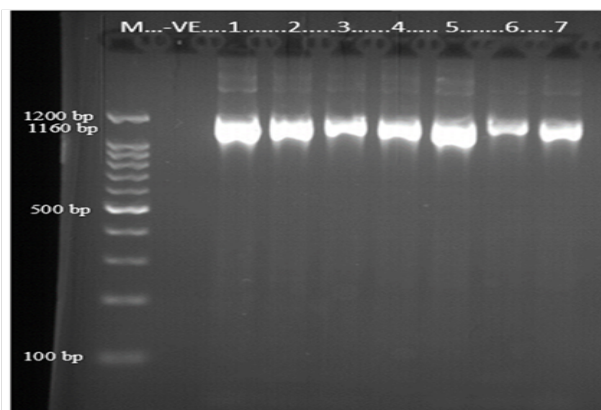


Figure 5: PCR amplification of actinomycetes DNA. Lanes 1-7 represent specific DNA fragments of actinomycetes 16S rRNA gene at approximately 1160 bp

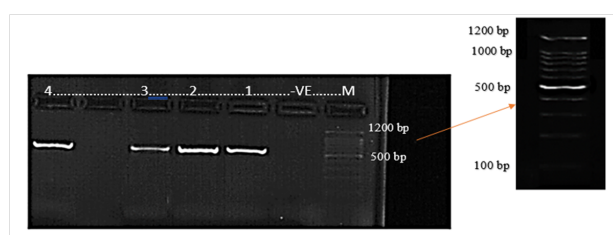


Figure 6: PCR amplification of fungi DNA. Lanes 1-4 represent specific DNA fragments of fungi ITS rRNA gene at 500 bp

DNA Sequencing Protocol

The sequencing analysis involved two main steps: 1. purification of the PCR products and cycle sequencing; and 2. purification of cycle sequencing products and drying and denaturing. The purified sequencing samples were then loaded into a 96-Well microtiter sequencer plate and run on a genetic analyzer for data interpretation (3500). The nucleotide sequence data were examined for comparison with a reference sequence retrieved from the National Center for Biotechnology Information and the Basic Local Alignment Search Tool database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

H. pylori strain

H. pylori ATCC 700392 was obtained from the American Type Culture Collection (U.S.A.). The bacterium was cultured in *H. pylori* selective supplement (DENT) at 37°C for three days, under microaerophilic conditions in 5% O₂, 10% CO₂, 85% N₂ atmosphere by using CampyGen CN025 (Atmosphere Generation Systems).

Determination of the Minimal Inhibitory Concentration (MIC)

The minimal inhibitory concentration (MIC) values, which represent the lowest marine extracts and

standard clarithromycin concentrations that completely inhibit the growth of *H. pylori* ATCC700392, were determined by a micro-well dilution method.

RESULTS

Actinomycetes and fungi isolates

Seven actinomycetes isolates (S1, S2, S3, S4, S5, S6 and S7) and three of fungi isolates (M1, M3 and M4) were isolated from the sediment samples that were collected from the Red Sea of Jeddah.

One fungus isolate (M2) was isolated from *S. carteri* sponge (Red Sea, Jeddah).

Preparation the extracts

This study included 14 extracts obtained from *H. erectus* sponge samples, actinomycetes and fungi. The three *H. erectus* sponge samples were extracted by methanol.

The extracts of actinomycetes strains (S1, S2, S3, S4, S5, S6 and S7) and the extracts of fungi strains (M1, M2, M3 and M4) were extracted by ethyl acetate.

DNA amplification, sequencing, and phylogenetic analysis for actinomycetes and fungi

All actinomycetes and fungi isolates were identified by using 16S rRNA gene and ITS rRNA gene sequencing analysis, respectively after that, the sequences of DNA were compared through BLAST and alignment with other actinomycetes and fungi that already present in the GenBank from various study areas. The phylogenetic analysis provided strong evidence for the actinomycetes and fungi isolates, and results were presented in phylogenetic trees, as shown in (Figure 7, Figure 8, Figure 9, Figure 10, Figure 11, Figure 12, Figure 13, Figure 14, Figure 15, Figure 16 and Figure 17). The percentages of sequence identity of actinomycetes strains and fungi strains were also determined Table 1 and Table 2.

H. pylori strain

H. pylori ATCC 700392 colonies appeared after three days on *H. pylori* selective supplement (DENT) medium.

The Minimal Inhibitory Concentration (MIC)

The MIC determination showed that only 13 of 14 extracts exhibited inhibition activity against the standard strain of *H. pylori* (ATCC 700392). These 13 marine extracts showed a variety of anti-*H. pylori* activity, as shown in (Table 3, Table 4, Table 5, Table 6, Table 7, Table 8 and Table 9) (Graph 1, Graph 2, Graph 3 and Graph 4)

Table 1: Phylogenetic identification of actinomycetes samples

| No. | Actinomycetes Samples | Closest Matches Identification | Sequence Identity |
|-----|-------------------------------|--|-------------------|
| S1 | <i>S. rochei</i> (S1) | <i>S. rochei</i> strain SCSIOZ-SH09 | 97.97% |
| S2 | <i>S. enissocaesilis</i> (S2) | <i>S. enissocaesilis</i> strain 10D | 95.39% |
| S3 | <i>S. rochei</i> (S3) | <i>S. rochei</i> strain S145 | 97.62% |
| S4 | <i>S. rochei</i> (S4) | <i>S. rochei</i> strain SCSIOZ-SH05 | 98.62% |
| S5 | <i>S. enissocaesilis</i> (S5) | <i>S. enissocaesilis</i> strain APBSMLB182 | 86.31% |
| S6 | <i>S. avidinii</i> (S6), | <i>S. avidinii</i> strain KSR1 | 99.08% |
| S7 | <i>S. enissocaesilis</i> (S7) | <i>S. enissocaesilis</i> strain 2C | 98.38% |

Table 2: Phylogenetic identification of fungi samples

| No. | Fungi Samples | Closest Matches Identification | Sequence Identity |
|-----|----------------------------|---------------------------------------|-------------------|
| M1 | <i>A. flavus</i> (M1) | <i>A. flavus</i> strain RG01 | 96.55% |
| M2 | <i>P. rubens</i> (M2) | <i>P. rubens</i> strain XQ3 | 96.58% |
| M3 | <i>A. tubingensis</i> (M3) | <i>A. tubingensis</i> strain Hoba5-41 | 93.86% |
| M4 | <i>A. niger</i> (M4) | <i>A. niger</i> isolate 78 | 94.61% |

Table 3: Anti- *H. pylori* ATCC700392 activity as MICS ($\mu\text{g/ml}$) of methanol extracts of *H. erectus* sponge samples

| Samples | Obtained from | MICS ($\mu\text{g/ml}$) |
|-----------------------|-----------------------|---------------------------|
| <i>H. erectus</i> (1) | Sharm Elshikh Red Sea | 3.9 |
| <i>H. erectus</i> (2) | Jeddah Red Sea | 31.25 |
| <i>H. erectus</i> (3) | Yanbu Red Sea | 125 |
| St. Clarithromycin | | 0.98 |

All determinations were carried out in triplicate manner. St. Clarithromycin: Positive control. NA: No anti- *H. pylori* activity

Table 4: Anti- *H. pylori* ATCC700392 activity as MICS ($\mu\text{g/ml}$) of ethyl acetate extracts of actinomycetes that obtained from sediment of Obhur Red Sea in Jeddah

| Isolate No. | Actinomycetes | MICS ($\mu\text{g/ml}$) |
|-------------|-------------------------------|---------------------------|
| S1 | <i>S. rochei</i> (S1) | 31.25 |
| S2 | <i>S. enissocaesilis</i> (S2) | 125 |
| S3 | <i>S. rochei</i> (S3) | 31.25 |
| S4 | <i>S. rochei</i> (S4) | NA |
| S5 | <i>S. enissocaesilis</i> (S5) | 125 |
| S6 | <i>S. avidinii</i> (S6) | 125 |
| S7 | <i>S. enissocaesilis</i> (S7) | 15.63 |
| | St. Clarithromycin | 0.98 |

All determinations were carried out in triplicate manner. St. Clarithromycin: Positive control. NA: No anti- *H. pylori* activity

Table 5: Anti- *H. pylori* ATCC700392 activity as MICS ($\mu\text{g/ml}$) of fungi ethyl acetate extracts

| Isolate No. | Fungi | Obtained from | MICS ($\mu\text{g/ml}$) |
|-------------|----------------------------|--|---------------------------|
| M1 | <i>A. flavus</i> (M1) | Sediment of the Red Sea of Obhur in Jeddah | 3.9 |
| M2 | <i>P. rubens</i> (M2) | <i>Stylissa carteri</i> sponge (Obhur Red Sea in Jeddah) | 15.63 |
| M3 | <i>A. tubingensis</i> (M3) | Sediment of the Red Sea of Obhur in Jeddah | 3.9 |
| M4 | <i>A. niger</i> (M4) | Sediment of the Red Sea of Obhur in Jeddah | 7.81 |
| | St. Clarithromycin | | 0.98 |

All determinations were carried out in triplicate manner. St. Clarithromycin: Positive control. NA: No anti- *H. pylori* activity

Table 6: Micro dilution mean inhibitory % \pm SD of methanol extracts of different *H. erectus* samples against *H. pylori*

| Sample conc. ($\mu\text{g/ml}$) | <i>H. erectus</i> (1) | <i>H. erectus</i> (2) | <i>H. erectus</i> (3) |
|-----------------------------------|-----------------------|-----------------------|-----------------------|
| 125 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 62.5 | 100 \pm 0.00 | 100 \pm 0.00 | 90.16 \pm 2.4 |
| 31.25 | 100 \pm 0.00 | 100 \pm 0.00 | 69.15 \pm 1.3 |
| 15.63 | 100 \pm 0.00 | 90.34 \pm 0.76 | 42.16 \pm 0.92 |
| 7.81 | 100 \pm 0.00 | 76.16 \pm 2.2 | 21.47 \pm 0.58 |
| 3.9 | 100 \pm 0.00 | 61.32 \pm 1.6 | 19.32 \pm 1.8 |
| 1.95 | 81.34 \pm 1.3 | 28.35 \pm 0.58 | 9.32 \pm 0.96 |
| 0.98 | 59.14 \pm 1.2 | 8.34 \pm 1.6 | 0.00 \pm 0.00 |
| 0 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| MIC | 3.9 | 31.25 | 125 |

All determinations were carried out intriplicate manner. The MIC value is defined as the lowest concentration to inhibit 100% of microbial growth under the assayed conditions. NA: No activity

Table 7: Micro dilution mean inhibitory % \pm SD of ethyl acetate extracts of different actinomycetes samples against *H. pylori*

| Sample conc. ($\mu\text{g/ml}$) | <i>S. rochei</i> (S1) | <i>S. enissocaesilis</i> (S2) | <i>S. rochei</i> (S3) | <i>S. rochei</i> (S4) |
|-----------------------------------|-----------------------|-------------------------------|-----------------------|-----------------------|
| 125 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 | 0.00 \pm 0.00 |
| 62.5 | 100 \pm 0.00 | 93.25 \pm 1.6 | 100 \pm 0.00 | 0.00 \pm 0.00 |
| 31.25 | 100 \pm 0.00 | 65.24 \pm 2.2 | 100 \pm 0.00 | 0.00 \pm 0.00 |
| 15.63 | 81.35 \pm 0.66 | 24.15 \pm 2.3 | 83.21 \pm 1.9 | 0.00 \pm 0.00 |
| 7.81 | 59.31 \pm 2.3 | 8.32 \pm 1.7 | 67.32 \pm 1.6 | 0.00 \pm 0.00 |
| 3.9 | 50.16 \pm 1.8 | 0.00 \pm 0.00 | 44.16 \pm 0.75 | 0.00 \pm 0.00 |
| 1.95 | 24.35 \pm 0.72 | 0.00 \pm 0.00 | 21.34 \pm 0.91 | 0.00 \pm 0.00 |
| 0.98 | 5.61 \pm 1.2 | 0.00 \pm 0.00 | 13.54 \pm 1.6 | 0.00 \pm 0.00 |
| 0 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| MIC | 31.25 | 125 | 31.25 | NA |

All determinations were carried out in triplicate manner. The MIC value is defined as the lowest concentration to inhibit 100% of microbial growth under the assayed conditions. NA: No activity

Table 8: Micro dilution mean inhibitory % \pm SD of ethyl acetate extracts of different actinomycetes samples against *H. pylori*

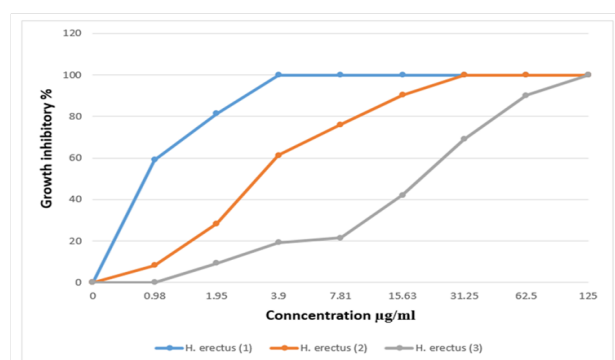
| Sample ($\mu\text{g/ml}$) | conc. | <i>S. enissocaesilis</i> (S5) | <i>S. avidinii</i> (S6) | <i>S. enissocaesilis</i> (S7) |
|-----------------------------|-------|-------------------------------|-------------------------|-------------------------------|
| 125 | | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 62.5 | | 88.74 \pm 1.3 | 91.35 \pm 1.3 | 100 \pm 0.00 |
| 31.25 | | 62.14 \pm 0.58 | 77.35 \pm 0.95 | 100 \pm 0.00 |
| 15.63 | | 36.25 \pm 0.93 | 61.32 \pm 2.3 | 100 \pm 0.00 |
| 7.81 | | 20.14 \pm 1.5 | 49.37 \pm 1.8 | 84.31 \pm 2.1 |
| 3.9 | | 9.32 \pm 1.6 | 31.28 \pm 0.95 | 56.38 \pm 1.6 |
| 1.95 | | 0.00 \pm 0.00 | 20.14 \pm 1.4 | 33.73 \pm 1.9 |
| 0.98 | | 0.00 \pm 0.00 | 10.32 \pm 2.2 | 17.98 \pm 2.4 |
| 0 | | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| MIC | | 125 | 125 | 15.63 |

All determinations were carried out in triplicate manner. The MIC value is defined as the lowest concentration to inhibit 100% of microbial growth under the assayed conditions. NA: No activity

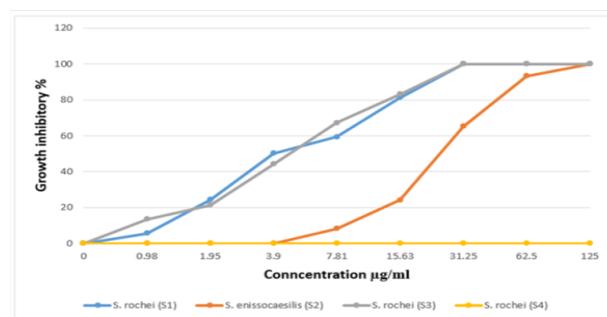
Table 9: Micro dilution mean inhibitory % \pm SD of ethyl acetate extracts of different fungi samples against *H. pylori*

| Sample ($\mu\text{g/ml}$) | conc. | <i>A. flavus</i> (M1) | <i>P. rubens</i> (M2) | <i>A. tubingensis</i> (M3) | <i>A. niger</i> (M4) |
|-----------------------------|-------|-----------------------|-----------------------|----------------------------|----------------------|
| 125 | | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 62.5 | | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 31.25 | | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 15.63 | | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 | 100 \pm 0.00 |
| 7.81 | | 100 \pm 0.00 | 72.34 \pm 1.6 | 100 \pm 0.00 | 100 \pm 0.00 |
| 3.9 | | 100 \pm 0.00 | 46.31 \pm 2.1 | 100 \pm 0.00 | 92.16 \pm 1.5 |
| 1.95 | | 88.75 \pm 1.8 | 21.35 \pm 1.7 | 83.25 \pm 1.2 | 69.25 \pm 2.1 |
| 0.98 | | 59.32 \pm 0.84 | 12.32 \pm 0.92 | 61.34 \pm 0.92 | 24.69 \pm 1.3 |
| 0 | | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 | 0.00 \pm 0.00 |
| MIC | | 3.9 | 15.63 | 3.9 | 7.81 |

All determinations were carried out in triplicate manner. The MIC value is defined as the lowest concentration to inhibit 100% of microbial growth under the assayed conditions. NA: No activity



Graph 1: Micro dilution mean inhibitory % \pm SD of methanol extracts of different *H. erectus* samples against *H. pylori*



Graph 2: Micro dilution mean inhibitory % \pm SD of ethyl acetate extracts of different actinomycetes samples against *H. pylori*

DISCUSSION

H. pylori is a common bacterium that infected millions of people in the worldwide, and its prevalence



Figure 7: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces rochei* (S1) using the neighbor-joining algorithm

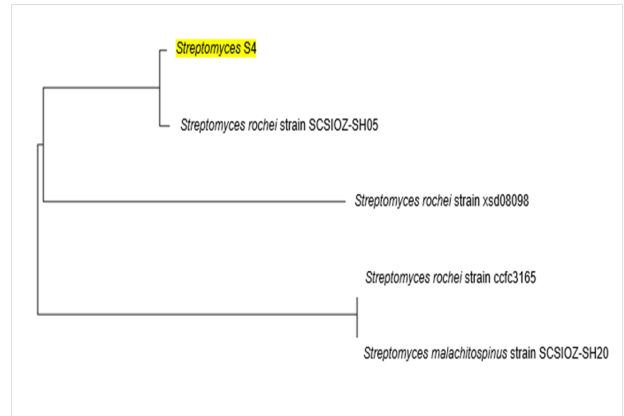


Figure 10: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces rochei* (S4) using the neighbor-joining algorithm

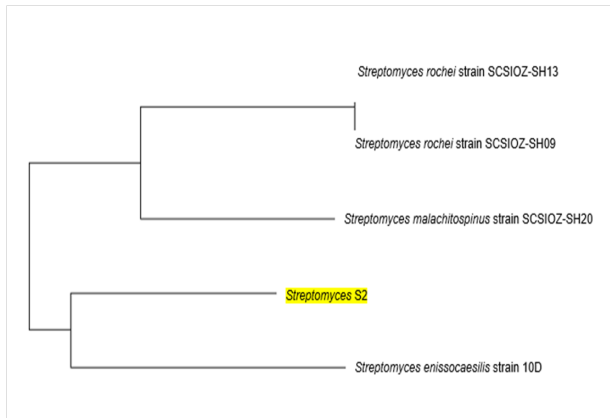


Figure 8: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces enissocaesilis* (S2) using the neighbor-joining algorithm

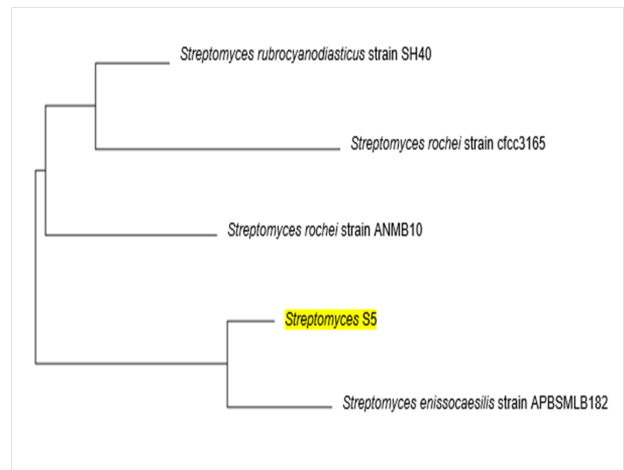


Figure 11: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces enissocaesilis* (S5) using the neighbor-joining algorithm

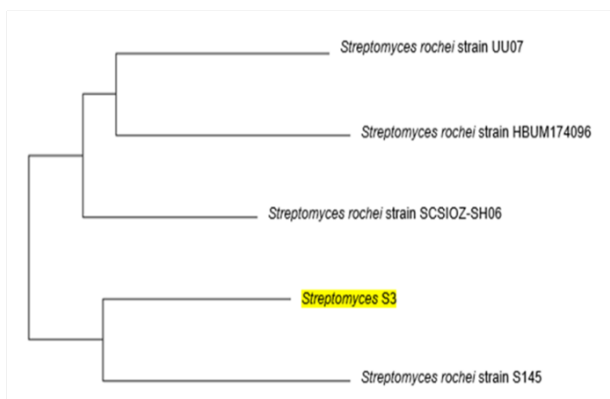


Figure 9: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces rochei* (S3) using the neighbor-joining algorithm

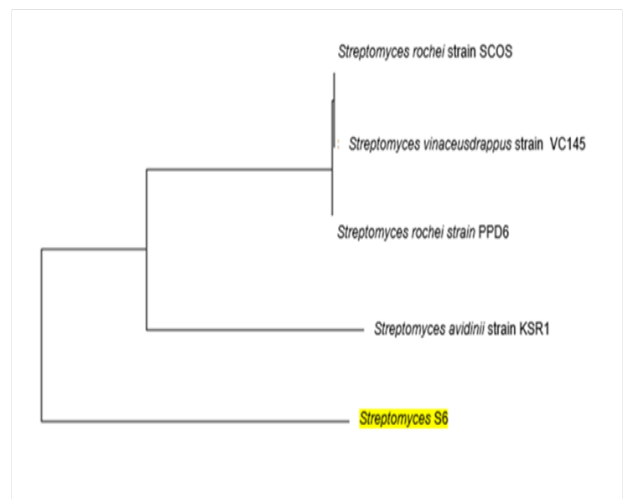


Figure 12: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces avidinii* (S6) using the neighbor-joining algorithm

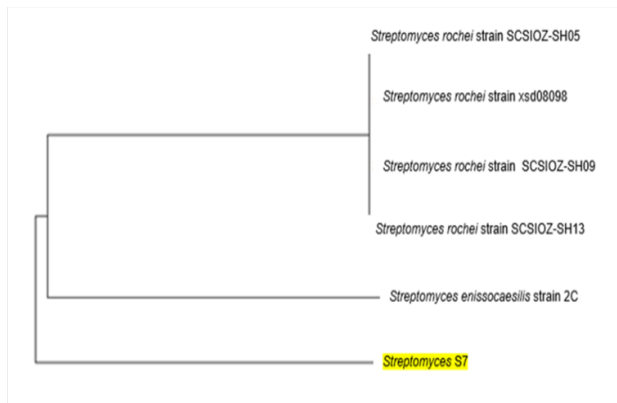


Figure 13: The phylogenetic tree for backbone sequences of actinomycete, *Streptomyces enissocaesilis* (S7) using the neighbor-joining algorithm

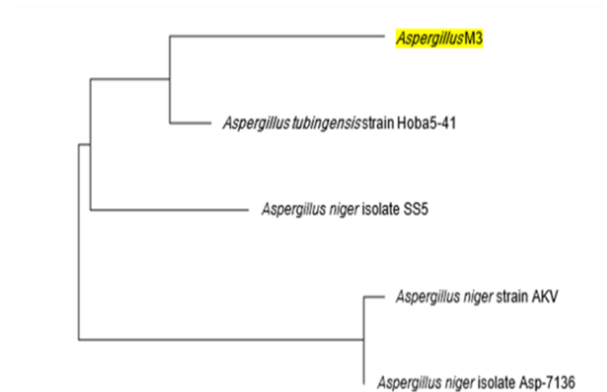


Figure 16: The phylogenetic tree for backbone sequences of fungus, *Aspergillus tubigenensis* (M3) using the neighbor-joining algorithm

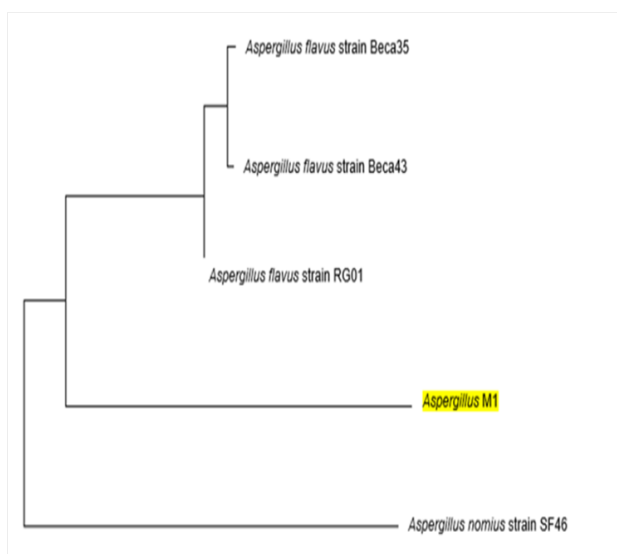


Figure 14: The phylogenetic tree for backbone sequences of fungus, *Aspergillus flavus* (M1) using the neighbor-joining algorithm

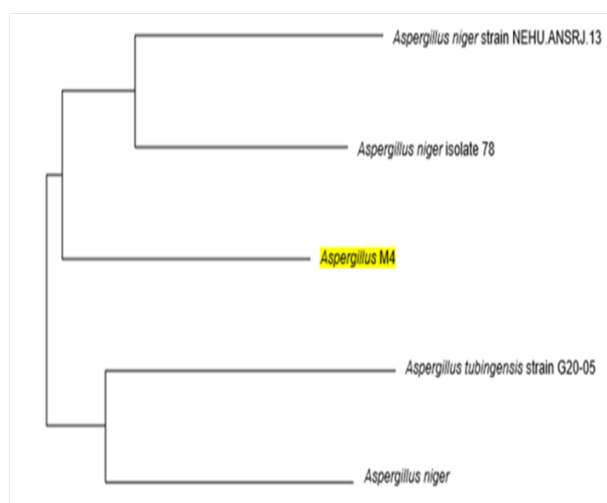


Figure 17: The phylogenetic tree for backbone sequences of fungus, *Aspergillus niger* (M4) using the neighbor-joining algorithm

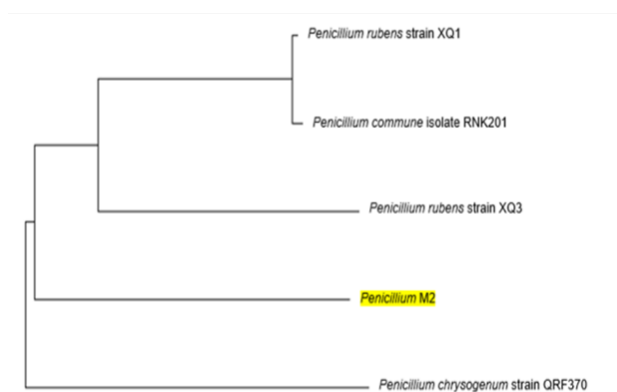
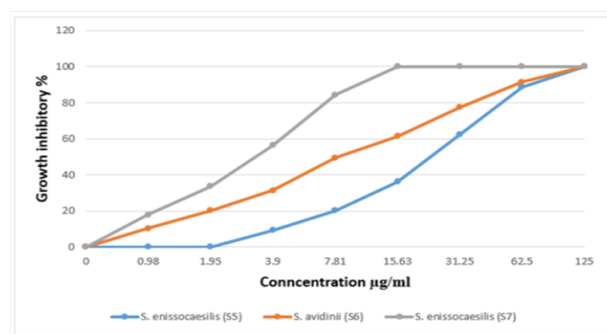
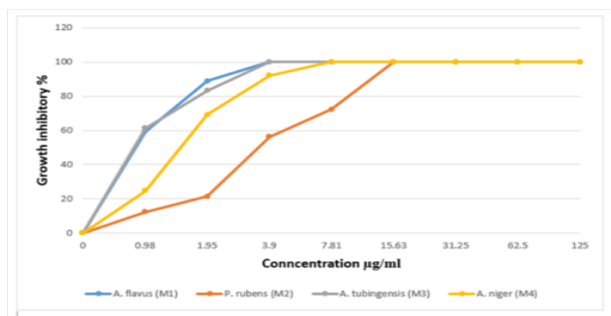


Figure 15: The phylogenetic tree for backbone sequences of fungus, *Penicillium Rubens* (M2) using the neighbor-joining algorithm



Graph 3: Micro dilution mean inhibitory % ± SD of ethyl acetate extracts of different actinomycetes samples against *H. pylori*



Graph 4: Micro dilution mean inhibitory % \pm SD of ethyl acetate extracts of different fungi samples against *H. pylori*

is very different among various countries, ethnic groups, age groups and societies even within the same state (Kaur and Naing, 2003; Hildreth et al., 2008; Hanafi and Mohamed, 2013). Because *H. pylori* are considered as class 1 carcinogen by the WHO, the efforts are focused on eradicating *H. pylori* by many therapies (Amin et al., 2013).

H. pylori treatment is associated with numerous problems such as bacterial resistance, adverse side effects, and high pre treatment cost. In the developing countries, *H. pylori* resistance to MNZ and TET is alarmingly increasing (Amin et al., 2013). So, due to the increase in resistance of antibiotic, it is essential to explore new and natural compounds from natural resources to be used as therapeutic agents against *H. pylori*.

The secondary metabolites produced by marine organisms and micro-organisms are different from the secondary metabolites produced by the terrestrial organisms because the environment is significantly different (Nazim et al., 2014). Sponges are a rich source of a vast diversity of secondary compounds that discovered over 50 years, and they have a benefit to humans (Nazim et al., 2014; Anjum et al., 2016). Also, some sponges-associated micro-organisms are the sources of bioactive compounds which are useful in the production of drugs and antibiotics for the treatment of different diseases (Nazim et al., 2014; Vimala and Innocent, 2015).

In the marine ecosystem, there is a unique group of actinomycetes that can produce a variety of secondary metabolites. The genus *Streptomyces* have a vast range of secondary metabolites (Selvam et al., 2013). The bioactive metabolites of actinomycetes are responsible for the production of antitumor agents, antibiotics, enzymes and immunosuppressive agents (Lam, 2006). Marine-derived fungi also produce a varied range of bioactive secondary metabolites, and it is considered as

one of the essential sources for novel biologically active compounds like anti-bacterial, anti-fungal, antiviral, anti-cancer, anti-inflammatory, immunosuppressants and enzyme inhibitors (Bajpai, 2016; Fouillaud et al., 2017).

In this study, 14 marine extracts, obtained from deferent *H. erectus* sponge samples, actinomycetes and fungi were used to show their inhibition effect against the standard strain of *H. pylori* ATCC700392. Three methanol extracts were obtained from three different samples of *H. erectus* sponge; that were obtained from Sharm Elshikh Red Sea, Jeddah Red Sea and Yanbu Red Sea.

Also, seven ethyl acetate extracts were isolated from different actinomycetes strains (*S. rochei* (S1), *S. enissocaesilis* (S2), *S. rochei* (S3), *S. rochei* (S4), *S. enissocaesilis* (S5), *S. avidinii* (S6) and *S. enissocaesilis* (S7)) which were isolated from the sediment of Red Sea of Jeddah. Four ethyl acetate extracts were obtained from four strains of fungi (*A. flavus* (M1), *P. Rubens* (M2), *A. tubingensis* (M3) and *A. niger* (M4)) that were obtained from Jeddah Red Sea (sediment and *S. carteri* sponge).

The three methanol extracts, obtained from various *H. erectus* sponge samples, had exhibited different inhibition effect against *H. pylori*. The methanol extract of *H. erectus* sponge (1) (Red Sea of Sharm Elshikh) had shown a high anti-*H. pylori* activity at a concentration of 3.9 µg/ml since it inhibited *H. pylori* at concentrations less than 20 µg/ml. The methanol extract of *H. erectus* sponge (2) (Red Sea of Jeddah) exhibited anti-*H. pylori* at a concentration of 31.25 µg/ml while the methanol extract of *H. erectus* sponge (3) (Red Sea of Yanbu) displayed anti-*H. pylori* activity at a concentration of 125 µg/ml. Our results revealed that the *H. erectus* sponge samples extracts inhibited *H. pylori* growth. Similar to our finding, Alahdal et al. (2018) reported that the five compounds, obtained from Red Sea marine sponge *Hyrtios erectus* sesterstatin 7 (1) scalarolide(3), 12-epi-24-deoxyscalarin (4), 19 acetylsesterstatin 3 (6) and 12b,20a-dihydroxy-16b-acetoxy-17-scalaren-19,20-olide (10), had possessed potent anti-*H. pylori* activity with MIC 4.39, 10.10, 9.11, 8.78, 8.47 µM, respectively.

In this present study, the ethyl acetate extracts of different actinomycetes strain exhibited different levels of anti-*H. pylori* activity. The ethyl acetate extracts of actinomycete *S. enissocaesilis* (S7) showed a high inhibition activity against *H. pylori* because it inhibited *H. pylori* at a concentration of 15.63 µg/ml (less than 20 µg/ml). While, the ethyl acetate extracts of *S. rochei* (S1) and *S. rochei* (S3) had both inhibited *H. pylori* at a concentration of

31.25 µg/ml. The ethyl acetate extracts of *S. enissocaesilis* (S2), *S. enissocaesilis* (S5) and *S. avidinii* (S6) displayed anti-*H. pylori* activity at a concentration of 125 µg / ml. The ethyl acetate extract of *S. rochei* (S4) did not exhibit any inhibition activity against *H. pylori*.

Our study revealed that all the extracts of actinomycetes (*S. rochei* (S1), *S. enissocaesilis* (S2), *S. rochei* (S3), *S. enissocaesilis* (S5), *S. avidinii* (S6) and *S. enissocaesilis* (S7)) exhibited anti-*H. pylori* activity, except the extract of actinomycete *S. rochei* (S4), did not show any inhibition activity against *H. pylori*. In agreement with an earlier study in 2013, the ethyl acetate extracts of actinobacteria *S. acrimycini* NGP, *S. albogriseolus* NGP, *S. variabilis* NGP and isolate 4, obtained from marine sediment of South Indian coastal region, showed anti-bacterial activity against *H. pylori* with inhibition zones 16±0.32, 15±0.33, 10±0.67 and 7±0.56 mm, respectively. The ethyl acetate extract of actinobacteria isolates 3, obtained from the same place of the other isolates, didn't show any anti-*H. pylori* activity (Selvam et al., 2013).

In this study, also four fungi extracts were used to show their activity against *H. pylori*. The extracts of fungi were extracted by using ethyl acetate. The ethyl acetate extracts of *A. flavus* (M1), *P. Rubens* (M2), *A. tubingensis* (M3) and *A. niger* (M4) have shown a high anti-*H. pylori* activity because they inhibited *H. pylori* at a concentration of 3.9 µg/ml, 15.63 µg/ml, 3.9 µg/ml and 7.81 µg/ml, respectively.

Until now, there is no study to prove the anti-*H. pylori* effect of marine fungi extracts, but our results are related to other studies that reported the anti-bacterial activity of marine-derived fungi against different bacterial species (Gram-positive and negative).

In 2017, the ethyl acetate extract of *Aspergillus fumigatus* (MF-1), isolated from marine soil, Bay of Bengal, India, exhibited anti-bacterial activity against *Staphylococcus aureus* (30 mm), *Sphingomonas paucimobilis* (32 mm), *Bacillus subtilis* (29 mm), *Klebsiella pneumonia* (24 mm), *Corynebacterium glutamicum* (35 mm), *Bacillus coagulans* (30mm), *Streptococcus pyrogens* (34 mm) and *E.coli* (36 mm) (Kalyani et al., 2017).

According to this study the methanol extract of *H. erectus* sponge (1), the ethyl acetate extract of actinomycete *S. enissocaesilis* (S7) and all the ethyl acetate extracts of all fungi strain possessing potent activities in the anti-*H. pylori* bioassay because they inhibited *H. pylori* at concentrations less than 20 µg/ml (Fouillaud et al., 2017; Alahdal et al., 2018).

The cell toxicity of marine extracts and also the characterization of crude extracts was not discussed in this study.

The crude extracts of *H. erectus* sponge samples, actinomycetes and fungi strains that completely inhibited *H. pylori* may be attributed to the inclusion of one or more of bioactive compounds such as terpenoids, steroids, phenolic compounds, alkaloids, polysaccharides, peptides, polyketides or fatty acids (Abad et al., 2011).

CONCLUSION

Generally, most of the marine extracts in this present study have anti-bacterial activity against *H. pylori*. One of the most worldwide concern is the increasing development of antibiotic resistance in *H. pylori* against current drugs. Therefore, using marine organisms and marine micro-organisms extracts may have potential benefits against *H. pylori* infection. Therefore, aquatic organisms and micro-organisms can be explored as rich sources to the isolation of natural anti-*H. pylori*. Also, this study has shown that marine organisms and micro-organisms are potent sources of anti-*H. pylori* activity. Accordingly, their extracts and compounds can be useful as an antimicrobial activity for other bacterial species.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

S.A.O. methodology, analysis and writing; F.A.A. review and editing; S.S.E. and M.A.E. collected the specimens, All authors have read and agreed to publish version of manuscript.

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