Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 27(5): 249 – 263 (2023)
www.ejabf.journals.ekb.eg

Isolation, Antimicrobial and Cytotoxic Activity of Bioactive Secondary Metabolites from Marine Sponge-Associated Bacteria

Haiam M. Aboul-Ela[1*], Aida M. Farag[2], Ibrahim M. El-sherbiny[3], Mamdouh Abdel-Mogib[4], Ahmed. S. Shehata[2,5]

[1]College of Fisheries and Aquacuture Technology, Arab Academy for Science, Technology and Maritime Transport, AbuQir, Egypt
[2]Marine Biotechnology and Natural Product (MBNP) Lab., Marine Environment, National Institute of Oceanography and Fisheries (NIOF), Alexandria, Egypt
[3]Department of Nanomaterial Science, Zewil City, Egypt
[4]Department of Chemistry, Faculty of Science, Mansoura University, Egypt
[5]Green Material Technology, Environment and Natural Materials Research Institute (ENMRI), City of Scientific Research and Technological Applications (SRTA-City), New Borg El- Arab City, 21934, Alexandria, Egypt

[*]**Corresponding Author: haiam.morsy@aast.edu**

ARTICLE INFO

Article History:
Received: Sept. 3, 2023
Accepted: Sept.11, 2023
Online: Sept. 20, 2023

Keywords:
Marine Bacteria,
Cytotoxic activity,
Symbiotic bacteria,
Antimicrobial Activity

ABSTRACT

Sponge-associated bacteria represent a potential promising source for new bioactive compounds. These compounds can be useful for the development of new antimicrobial and anticancer agents. In the present study, the endosymbiotic bacteria associated with marine sponge species; *Amphimedon ochracea*, *Hyrtios erecta*, *Amphimedon* sp., *Ircinia echinata*, *Ircinia muscarum* and *Ircinia fasciculate*, collected from Hurghada, the Red Sea and Alexandria, the Mediterranean Sea, Egypt were screened for antimicrobial and cytotoxic activities. Seventeen bacterial crude extracts were tested for antimicrobial activities; five were the most potent. Two of these most promising bacterial extracts (R4 and R14) showed the highest activities. On the other hand, the crude extracts of the tested five bacterial extracts showed growth inhibition effects against the cancer cell lines [Hepatocellular carcinoma (HepG2), breast cancer (MCF7), and colon cancer (HCT)]. The bacterial isolate (R4) crude extract showed the most promising cytotoxic activity against MCF7 and HCT, while R14 showed the highest activity against HepG2. The bacterial species were identified based on the phylogenetic analysis of the nucleotide sequences of their 16S rDNA genes. The most potent bacterial isolate (R4) was identified as *Bacillus gottheilii* MSB1 by using conventional techniques and DNA sequencing of the 16S rRNA gene. The partial fractionation depending on the polarity n-hexane (H), dichloromethane (M), ethyl acetate (E), and n-butanol (B) yielded four cytotoxic-active fractions from *Bacillus gottheilii* MSB1 crude extract, the fraction M was the most promising one against HepG2, MCF7, and HCT, while fraction B showed a weak cytotoxic activity against different cell lines.

INTRODUCTION

The marine environment has proven to be a very rich source of extremely potent compounds that have demonstrated significant activities as antitumor, anti-inflammatory, analgesic, immunomodulation, anti-allergy and anti-viral **(Vinatoru, 2001; Putz & Proksch, 2018)**. Marine natural products display not only novel characteristics but also complexity in terms of chemical structures **(Schäfer & Wink, 2009)**. The needs for novel therapeutically active compounds are urgent, as well as the new chemical entities decline in drug **(Cragg & Newman, 2013)**. Most marine natural products involved in clinical or preclinical trials are produced by invertebrates, viz., sponges, tunicates, bryozoans or mollusks, which is in contrast to the compounds derived from the terrestrial environment where plants by far exceed animals with respect to the production of bioactive metabolites **(Proksch *et al*., 2002)**.

Numerous marine sponges are associated with dense and phylogenetically diverse microbial consortia including bacteria, archaea and single-celled eukaryotes (fungi and microalgae) that can account for nearly half of the animal's biomass **(Ganesan, 2008)**. The symbiotic microbial consortia also prove to be a source of bioactive compounds with pharmaceutical potential, and there is a growing recognition that marine invertebrates and marine plants surface are usually populated with enormous quantities of associated or symbiotic microorganisms **(Kabiru & Garba, 2014)**; they have produced a wide range of secondary metabolites such as polyketides, alkaloids, fatty acids, peptides and terpenes of various medical importance, such as antimicrobial, antiprotozoal, antiviral, anticholesterol, antitumor, anthelminthic, anticancer and immune-suppressant **(Gerwick & Fenner, 2013)**.

Among the population of marine sponges, *Bacillus* species are reported to be the most abundant forms. *Bacillus* strains are of major interest in bacteriocin research since this genus produces a diverse array of antimicrobial peptides, with different basis chemical structure **(Molinski, 2010)**. They are producers of most of the known bioactive metabolites. In addition, they include potentially useful and most promising array of pharmacologically and agriculturally active compounds **(Sumi *et al*., 2015)**. Recently, symbiotic bacteria associated with marine invertebrates have gained increasing attention from a medical perspective since they are suspected as the true producers of many rare bioactive secondary metabolites.

MATERIALS AND METHODS

1. Chemicals

All chemicals used in the study were of the highest purity and used without further purification. The chemicals were purchased from Sigma- Aldrich & Alfa Aesar, Germany and microbiological media from Becton Dickinson, Sparks, MD, USA and LobaChemie PVT. LTD, Mumbai, India.

2. Sponge collection, processing and identification

A diverse variety of marine sponges were collected from the Red & Mediterranean Seas, Egypt. Sponge samples beonged to six species, with three samples

collected from El-Gouna region at a depth of 1.5m. (N: 27 22 39.98, E: 33 40 58.95) in May 2012. One sample was collected from the National Institute of Oceanography and Fisheries station, Hurghada at a depth of 2m (N: 27 17 07.45, E: 33 46 26.50) in May 2016. Whereas, the other three sample were collected from Abou-Quir, Alexandria (N: 31 17 60.00, E: 30 09 60.00) and Marsa-Matrouh (N: 31 21 10.44, E: 27 14 14.10) at a depth of 5m during May 2016. The specimens were kept in sterilized- aged seawater (ASW) to remove loosely associated microorganisms from inner and outer sponge surfaces (Sundaram *et al*., 2010). Samples were surface cleaned with ASW and surface sterilized with 70% alcohol to eliminate epiphytic microorganisms. Fresh specimen of each sponge was used for isolating the sponge-associated bacteria.

The different kinds of sponges from the Red sea were taxonomically identified as *Amphimedon ochracea,* (Class Demospongiae, Order Haplosclerida, Family Niphatidae) and *Hyrtios erecta,* (Order Dictyoceratida, Family Thorectidea) by Prof. Rob. W. M. van Soest at the Department of Marine Zoology, Netherlands Center for Biodiversity, the Netherlands; undescribed species of *Amphimedon* (Order Haplosclerida: Family Niphatidae) and *Ircinia echinata* (order Dictyoceratida: Family Irciniidae) were taxonomically identified by Prof. Michele Kelly at National Institute of Water and Atmospheric Research (NIWA) Ltd., Auckland, New Zealand **(Keller, 1889; van Soest *et al*., 1991)**, and the species from the Mediterranean sea were taxonomically identified as *Ircinia muscarum* (Order Dictyoceratida: Family Irciniidae) and *Ircinia fasciculate* (Order Dictyoceratida: Family Irciniidae) by Dr. Hamdy Omar Ahmed (Invertebrate Lab., Aquaculture Division, National Institute of Oceanography and Fisheries (NIOF), Egypt).

3. Preparation of the culture media

The medium used to culture sponge symbiotic bacteria was the marine nutrient agar (MNA), which was used as general rich medium to grow many heterotrophic marine bacteria and the ISP medium was prepared for 2 Agar **(Mondol *et al.*, 2013)**. All above media were prepared according to manufacturer's instructions. Each medium was modified to contain 2% (w/v) NaCl. The pH was adjusted to 7.0 with 0.1 N NaOH or 0.1 HCl. Agar (2%) was added as a solidifying agent to the constituted media when solid media were needed. The media were sterilized by autoclaving for 20min at 121°C. After sterilization, the media were allowed to cool to 50°C before adding antibiotics. 25µg/ml nystatin was added to suppress fungal growth **(Errakhi *et al*., 2007)**. Plates were then poured, and the plates were inverted and stored at 4°C until needed.

4. Isolation and purification of bacterial strains

A fresh sponge sample (1 cm^3) was placed in a sterile mortar with 10 ml of sterile ASW and thoroughly ground for 2- 3min. The sample was diluted and 100µl of each dilution was placed on each isolation medium. Plates were incubated at 30°C for about 1-2 weeks in aerobic condition. Each distinct colony morphotype was picked and plated on successive plates until pure cultures were achieved, as estimated by colony homogeneity.

5. Screening of antimicrobial agents of the isolated strains

One ml (OD_{600}=0.1) of each bacterial isolate was inoculated in 250ml Erlenmeyer flasks, with each containing 100ml of sterilized liquid ISP medium 2. The cultures were grown for 48hrs at 37°C under shaking condition (160 x*g*). After incubation, the culture

media were centrifuged at 5000 x *g* for 15min on cooling centrifuge. 100µl of the cell-free culture supernatants (filter, 0.2µm sterilized supernatant) was used.

6. The antimicrobial activity (agar well diffusion assay)

Antimicrobial activity of bacterial isolates was estimated by using agar well diffusion method **(Singh *et al*., 2018)**. 100µl of each target pathogenic bacteria culture was mixed into separate sterile, molten, cool Müller-Hinton agar medium (MHA), mixed well and poured into sterile petri plates. Each plate was punched to make wells of a 6mm diameter, with the help of sterile cork borer at different sites of the plates. 100µl of each supernatant was pipette into the well in assay plates under aseptic conditions. Plates were incubated at 37°C for 24hrs. After incubation, plates were observed for the inhibition zones, diameters of which were measured in mm.

7. *In vitro* cytotoxicity assay of potential bacterial strains against cancer cell lines

The cell lines (Human Breast Cancer (MCF-7), hepatocellular carcinoma (HepG2), and colon carcinoma (HCT-116) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium, supplemented with 10% inactivated fetal calf serum and 50 µg/ml gentamycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two times a week.

The antitumor activity was evaluated on carcinoma cell lines at the Regional center for Mycology & Biotechnology, Al-Azhar University, Cairo, Egypt. Briefly, the cell lines grew as monolayers in growth medium supplemented with 10% inactivated fetal calf serum and 50µg/ ml gentamycin. The monolayer of 10,000 cells adhered at the bottom of the wells in a ninety six-well microtiter plate (Falcon, NJ, U.S.A) was incubated for 24h at 37°C in a in a humidified incubator with 5% CO_2. The monolayers were then washed with sterile phosphate buffer saline (0.01 M pH 7.2), and simultaneously the cells were treated with a hundred µl from totally distinctive dilutions of tested compound in new maintenance medium and incubated at 37°C. A control of untreated cells was created within the absence of tested compound. A positive control containing doxorubicin drug was also tested as reference drug for comparison. Six wells were used for every concentration of the test sample. Every 24h, the observation under the inverted microscope was made. The number of the living cells was determined by staining the cells with crystal violet, followed by cell lysing using 33% glacial acetic acid and examining the absorbance at 590nm wih the usage of ELISA reader (SunRise, TECAN, Inc., U.S.A) once well mix. The absorbance values from untreated cells were considered as 100% proliferation, and the percentage of viability was calculated as [1-(ODt/ODc)] x100% where ODt is the mean optical density of wells treated with the tested compounds, and ODc is the mean optical density of untreated cells. The 50% inhibitory concentration (IC_{50}), the concentration required to cause toxic effects in 50% of intact cells, was estimated from graphic plots **(Zhishen *et al*., 1999; Wilson, 2000; Gangadevi & Muthumary, 2007)**.

8. Identification and characterization of selected bacterial strains

All the bacterial strains (isolated from the marine sponges) were identified to genus Bacillus according to morphological, microscopic examination, and biochemical analysis; in addition, the most active antimicrobial and anticancer producing isolates (R4 and R14) were further identified by 16S rRNA gene sequence analysis. Polymerase chain

reaction (PCR) amplification of 16S rRNA gene was carried out using specific primer 27f (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492r (5'-GGTTACCTTGTTACG ACTT-3') (Li *et al*., 2012). Total genomic DNA was amplified through Creacon (Holland, Inc) Polymerase Chain Reaction (PCR) system cycler. PCR for amplified genomic DNA was carried out. The reaction consists of 40 cycles, each cycle consisted of denaturation at 94°C for 30sec, followed by annealing at 30°C for 30sec and extension at 72°C for 30sec. There was an initial delay for 15min at 95°C at the beginning of the first cycle and 10min delay at 72°C at the end of the last cycle, as a post extension step. The product was stored at –20 or 4°C.The reaction mixture contained the following reagents in a 17.8µl sterile nuclease free water, 2.5µl 10x Taq buffer, 2.5µl 4mM PCR nucleotide mix, 1.0µl (5pmol/µl) primer, 0.2µl (5u/µl) Taq DNA polymerase and 1.0µl (50ng/µl) genomic DNA extracted sample.

9. Extraction and identification of secondary metabolites

According to antimicrobial and cytotoxic screening, the most potent bacterial isolate *Bacillus gottheilii* MSB1 (KU199821) that showed strong antimicrobial and anticancer activity was chosen for further studies including the characterization of its metabolites, thus this strain was cultivated in liquid ISP2 medium under optimized conditions. After incubation, the culture was centrifuged at 5000xg rpm for 15 minutes and 1liter of the supernatant was used for successive extractions with different solvent having different polarities, n-hexane (250 ml), dichloro- methane (250 ml), ethyl acetate (250 ml) and n-butanol (250 ml). The respective organic phases were collected and dried with anhydrous sodium sulphate and concentrated by the evaporation of solvent, and then 15, 20, 16 and 14 mg crude n-hexane, dichloromethane, ethyl acetate and n-butanol extracts were obtained.

RESULTS

Six sponge species were collected from the Red and Mediterranean Seas. Samples were coded as A, B, C, D, E and F. They were identified as *Amphimedon ochracea*, *Hyrtios erecta*, undescribed species of Amphimedon sp., *Ircinia echinata*, *Ircinia muscarum* and *Ircinia fasciculate*, respectively. Samples, A, B, C, and D were collected from the Red Sea; whereas, samples E and F were collected from the Mediterranean Sea (Fig. 1).

A total of 17 bacterial strains were isolated from sex species (seven samples) of sponges. Total count ranged from a highest of 2×10^5 to a lowest of 3×10^3 CFU per gram of sponge tissue (Table 1).

Table 1. Total count of bacteria associated with the different species of sponges

Code	Sponge species	Total bacterial count (CFU/g)
RS1 (A)	*Amphimedon ochracea*	2×10^5
RS2 (B)	*Hyrtios erecta*	3×10^4
RS3 (C)	*Amphimedon* sp.	5×10^3
RS4 (D)	*Ircinia echinata*	4×10^4
MS1 (E)	*Ircinia muscarum*	4×10^3
MS2 (F)	*Ircinia fasciculate*	3×10^3

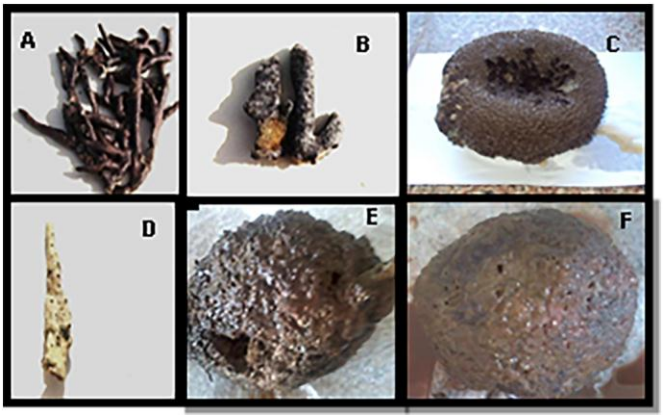

Fig. 1. Sponge samples, Sample 1 (A); Sample 2 (B); Sample 3(C); Sample 4 (D); Sample 5 (E) and Sample 6 (F).

The selected phenotypically different bacterial isolates, representing the community structure in different types of sponges, were examined to determine their ability to produce antimicrobial activities against tested pathogenic bacteria (Gram-positive and negative bacteria) and fungi. Their activities were tested using the well–cut diffusion technique to test the ability of the bacterial isolates to inhibit the growth of tested pathogenic bacteria. After incubation period (24hr), the diameter of inhibition zone around each well was determined for the 17 isolates; they were found to be antimicrobial agent producers. The antimicrobial agent activity differed from strain to strain, and both broad-spectrum and species-specific activities were noted (Table 2).

The seventeen bacterial isolates were microscopically identified to Gram- positive bacteria (57%) and Gram-negative bacteria (43%). The results in Table (2) reveal that most bacterial isolates with an antagonistic effect against both Gram-positive, Negative bacteria and tested pathogenic fungi (35.29% of the total bacterial isolates), compared to Gram-negative bacteria and pathogenic fungi (11.76% of the total bacterial isolates),Gram-negative bacteria and pathogenic fungi (11.76% of the total bacterial isolates), Gram-negative bacteria (17.65% of the total bacterial isolates), Gram-positive bacteria (11.76% of the total bacterial isolates) and pathogenic fungi (11.76% % of the total bacterial isolates), exhibiting inhibition zones ranging from 4 to 22 mm. A higher antimicrobial agent production was shown by two isolates (R4 and R14), which produced inhibition zones on all the tested pathogenic bacteria and fungi.

According to the previous results, two bacterial (R4, MSB1) and (R14, MSB2), isolated from *Hyrtios erecta* (B) and *Amphimedon ochracea* (A), respectively, were selected potent organisms producing antimicrobial activity to complete the next further study and screened for anticancer activity since they showed the highest diameter of inhibition zone (antimicrobial agent production) on both tested pathogenic bacteria and fungi.

Table 2. The antagonistic effect of different bacterial isolates against some pathogenic bacteria and fungi.

Sponge	Isolates	Diameter of inhibition zone (mm)								
		P. aeruginosa	***Aeromonas***	***E. coli***	***Vibrio sp.***	***S. aureus***	***B. subtilis***	***Enterococcus fecalis***	***C.albicans***	***A. flavius***
RS4 (D)	R1	13	7	9	6	7	7	8	10	8
	R2	8	5	5	6	0	0	0	5	8
	R3	7	6	5	5	0	0	0	0	0
RS2 (B)	R4	22	5	10	5	10	10	8	17	10
	R5	10	5	5	6	6	7	6	10	6
	R6	12	4	5	9	0	0	0	7	5
RS3 (C)	R7	10	4	4	4	0	0	0	6	7
	R 8	0	0	0	0	0	0	0	5	7
	R9	0	0	0	0	7	6	5	0	0
RS1 (A)	R10	12	4	5	6	0	0	0	0	0
	R11	0	0	0	0	6	6	7	8	8
	R12	9	4	4	7	7	5	5	10	9
	R13	13	6	7	8	9	8	7	15	8
	R 14	20	5	9	10	10	7	10	20	12
MS1 (E)	M 15	0	0	0	0	0	0	0	5	7
MS2 (F)	M16	0	0	0	0	5	5	4	0	0
	M 17	5	5	0	0	0	0	0	0	0
	Ciprofloxacin	24	18	20	26	13	31	17		

1. Cytotoxic activity of the five bacterial crude extracts against different cell lines

The crude extracts of all the five bacterial isolates, each at six different concentrations (50- 1.56µg/ ml), were used to screen the cytotoxicity against hepatocellular carcinoma (HepG2), breast cancer (MCF7) and colon cancer (HCT). Cytotoxic effect of microbial crude extracts on cell viability of cancer cell lines was observed. All the tested crude extracts showed growth inhibition effects against the tested cell lines as shown in Table 3, bacterial extract from isolate R4 showed the most promising strong cytotoxic activity against (MCF7) and (HCT), but bacterial extract from isolate R14 showed a strong cytotoxic activity against (HepG2).

Table 3. IC_{50} values of the five tested bacterial isolates crude extracts against different cancer cell lines.

Cancer cell line	IC_{50} of the bacterial crude extracts (µg/ml)				
	R1	**R4**	**R5**	**R13**	**R14**
HepG2	> 50	24.1	37	32.8	19.6
MCF-7	> 50	16.5	46.8	42.1	23.2
HCT	70	20.3	161	22.8	34.8

2. Phylogenetic analysis of the selected isolate R4

The identification of the selected isolate was done on bases of 16s rRNA gene sequences. The nucleotide sequence analysis of the sequence was performed at BlastN site at NCBI server. The isolate R4 has 1369 bp (Fig. 2) and was identified as *Bacillus*

gottheilii MSB1 (Fig. 3**)**. The sequence of the identified bacterial strain was deposited in GenBank with an accession number of KU199821.

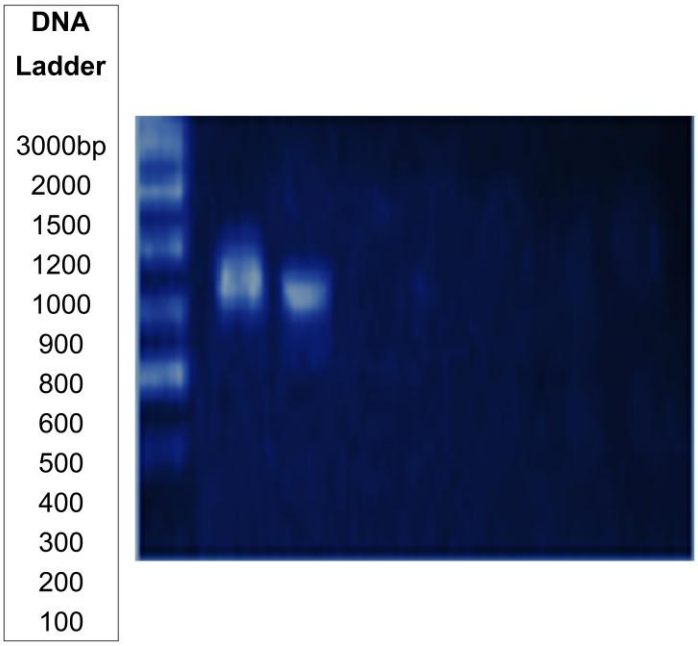

Fig. 2. Agarose gel electrophoresis of the amplified region of the isolate for two *Bacillus* sp. specific genes with approximately 1369 and 1190 bp (R4, R14), respectively.

1. GC-MS identification of the organic fractions from *Bacillus gottheilii* MSB1 crude extract

The chemical compositions of the n-hexane, dichloromethane, ethyl acetate and n-butanol fractions from *Bacillus gottheilii* MSB1 crude extract were studied by using GC/MS analysis. The most abundant constituents of n-hexane fraction were 2,6,10,15-tetramethylhepta decane (R_t 13.54 min), 2,4-ditert-butylphenol (R_t 13.87 min), dodecanoic acid, ethenyl ester (R_t 15.19 min), 2,2-dimethyl-N-phenethyl propion amide (R_t 17.6 min), 2,6,10-trimethyltetradecane (R_t 18.59 min), 9-octadecenamide (R_t 23.87 min), 2-methyl-1-hexadecanol (R_t 27.64 min), heptacosane (R_t 29.18 min) and 17-pentatriacontene (R_t 30.64 min). The most abundant constituents of dichloromethane fraction were 2-benzyl-1-[(4-methylphenyl) sulfonyl]-3-azetidinone (R_t 6.99 min), 4-methyl-1H-indene-1,2(3H)-dione (R_t 8.04 min), N-hydroxymethyl-2-phenyl acetamide (R_t 12.36 min),2,2-dimethyl-N-(2-phenylethyl) propan amide (R_t 17.65 min), ethyl 14-oxotetradecanoate (R_t 19.64 min), 3-Isobutyl hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (R_t 21.45 min), ethyl hexadecanoate (R_t 22.12 min), ethyl Z-9-octadecenoate (R_t 25.38 min) and 1-[(2Z)-4-(4-Chlorophenyl)-3-phenyl-2 butenyl] pyrrolidine (R_t = 28.96 min). While, the most abundant constituents of ethyl acetate fraction were 2,4-di-tert-butylphenol (R_t 13.89 min), ethyl 14-methyl-hexadecanoate (R_t 19.68 min), ethyl 12-oxododecanoate (R_t 22.14 min), 1,2-Benzenedicarboxylic acid, bis(6-methylheptyl) ester (R_t 31.49 min) and 1-Butyl 2-octyl phthalate (R_t 20.08 min). On the other hand, the most abundant constituents of n-butanol fraction were 3-methylbutaneamide (R_t 5.64 min), 2-

propyl-1-heptanol (R_t 6.1 min), 4-methylpentanamide (R_t 7.33 min), 1-butoxy-1-isobutoxy-butane (R_t 9.39 min), 2-phenylacetamide (R_t 12.42 min), n-hexadecanol (R_t 15.14 min), i-propyl 10-methyl-dodecanoate (R_t 19.54 min), 4-amino-6-hydroxy-2-methyl-5-nitroso-pyrimidine (R_t 21.5 min), butyl 13-methyltetradecanoate (R_t 23.15 min) and bis(2-ethylhexyl) 1,2-benzenedicarboxylate (R_t 31.50 min)**.**

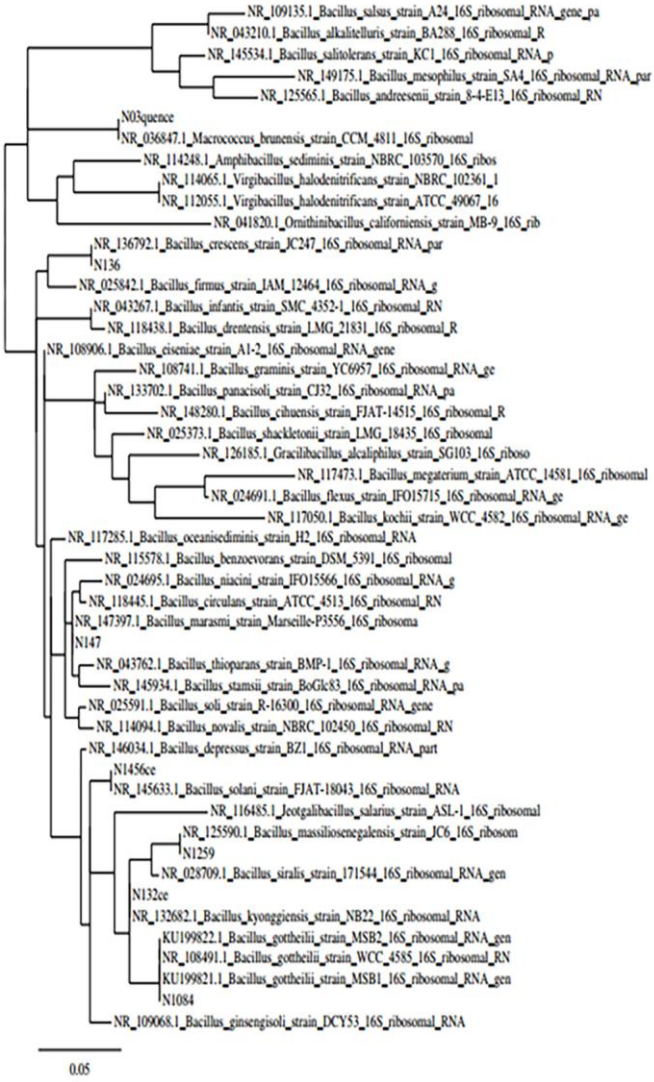

Fig. 3. Phylogenetic tree for two bacterial isolates (R4 and R14) according to specific gene sequencing data

2. Cytotoxic activity of organic fractions from *Bacillus gottheilii* MSB1 crude extract against cancer cell lines

The four fractions, n-hexane (H), methylene chloride (M), ethyl acetate (E), and n-butanol (B) of bacterial crude extract, each at six different concentrations (500 - 15.6 μg/ml) were used to screen the cytotoxicity against hepato-cellular carcinoma (HepG2),

breast cancer (MCF7) and colon cancer (HCT). Cytotoxic effect of microbial extracts on cell viability of cancer cell lines was recorded. All the tested fractions showed growth inhibition effects against the tested cell lines, as shown in Table (4). Bacterial extract fraction M showed the most promising cytotoxic activity against HepG2, MCF7 and HCT, while fraction B showed weak cytotoxic activity against different cell lines.

Table 4. IC_{50} values of four organic fractions from *Bacillus gottheilii* MSB1 crude extract tested against cancer cell lines

Cancer cell line	IC_{50} of the fractions of bacterial extract (µg/ml)			
	Bacterial extract			
	H	M	E	B
HepG2	53.5	30.8	47	186
MCF-7	96.3	59.7	81.9	231
HCT	100	43.2	60.7	233

DISCUSSION

Bio-prospecting is the effort to discover natural compounds with therapeutic and biological applications **(Ashforth *et al.*, 2010)**. The significance of microbial communities from marine sponges has been reported **(Brinkmann *et al.*, 2017)**. Therefore, sponges can be an untapped source of microbes that can be used as the source of antimicrobial substances and generally bioactive compounds. The objective of this study was to isolate bacteria associated to sponges, identify and screen them for their potential against different bacterial, fungal pathogens and cytotoxic activity. Six different types of sponge were collected from different marine environment (the Red and Mediterranean Seas) and used as sources for isolation of marine antimicrobial and anticancer agent procedures bacteria **(Shaala *et al.*, 2017; Shreadah *et al.*, 2018)**. A total of 17 bacterial strains were isolated from the samples of sponges. Total bacterial count ranged from 2×105 (*Amphimedon ochracea*) to 3×103 (*Ircinia fasciculate*) CFU /gram of sponge tissue.

The results revealed that most bacterial isolates have antagonistic effect against both Gram-positive, Negative bacteria and tested pathogenic fungi (35.29% of the total bacterial isolates), compared to Gram-negative bacteria and pathogenic fungi (11.76% of the total bacterial isolates), Gram-negative bacteria and pathogenic fungi (11.76% of the total bacterial isolates), Gram-negative bacteria (17.65% of the total bacterial isolates), Gram-positive bacteria (11.76% of the total bacterial isolates) and pathogenic fungi (11.76% % of the total bacterial isolates), exhibiting inhibition zones ranging from 4 to 22 mm. The higher antimicrobial agent production was obtained by five isolates (R1, R4, R5, R13, and R14) in addition to two isolates (R4) and (R14) showing the highest inhibition zones on all the tested pathogenic bacteria and fungi. Based on these findings, five bacterial isolates were selected to complete the next experiments as potent organisms since they showed the highest diameter of inhibition zone (antimicrobial agent production) on the tested microbes (bact. & fungi). Cytotoxic effect of microbial crude extracts of the most potent five bacterial isolates on cell viability of different cancer cell lines was observed. All the tested crude extracts showed growth inhibition against the tested cell lines, as shown in Table (3); bacterial extract from isolate R4 showed the most

promising strong cytotoxic activity against (MCF7) and (HCT). On the other hand, R14 bacterial extract showed a strong cytotoxic activity against (HepG2). Sponge associated bacteria can produce bioactive compounds that are expected to have some of the medicinal property, especially anticancer activity. The bacterial crude extracts were tested for the cytotoxic effect on liver cancer (HepG2), breast cancer (MCF-7) and cervical cancer (HeLa) **(Safari *et al*., 2016; Priyanto *et al*., 2017)**.

Based on physiological, biochemical characteristics and 16S rRNA sequence analysis, bacterial isolate R4 (isolated from Hyrtios erecta) was identified as **Bacillus gottheilii** MSB1. In accordance with the present results, *Bacillus gottheilii* MSB1 showed the most promising strong cytotoxic activity against MCF7 and HCT. It is well known that several species of Bacillus are capable of producing biologically active metabolites, having anticancer, antimicrobial, antiviral, antiprotozoal, anti-inflammatory and antioxidant activities **(Devi *et al.*, 2010; Flemer *et al.*, 2012; Ye *et al*., 2012; Phelan *et al.*, 2013)**.

This is the first isolation of *B. gottheilii* MSB1 from marine sponge. However, it was previously isolated for the 1st time from the mangrove sediment in peninsular Malaysia **(Auta *et al*., 2017)**. It is a Gram-positive, rod-shaped, motile, strictly aerobic, endospore forming bacterium, which can be used in pharmaceutical production line **(Seiler *et al*., 2013)**.

Isolation, identification and cytotoxic activity of the antimicrobial agent produced from *B. gottheilii* MSB1 culture were also examined. All the tested fraction extracts showed growth inhibition effects against the tested cell lines, with methylene chloride (M) fraction showing the most promising cytotoxic activity against HepG2, MCF7 and (HCT) due to the anticancer potential of pyrrolopyrazine derivatives **(Sureshan & Sahadevan, 2014; Lalitha *et al*., 2016)**. On the other hand, n-butanol (B) fraction showed weak cytotoxic activity against three different cell lines. It was documented that *Bacillus gottheilii* species are considered as a source of interesting bioactive secondary metabolites and most promising candidates for some applications in food industry **(Subbalaxmi & Murty, 2016)**.

CONCLUSION

This study confirmed that Bacillus species are still considered as a proliferative agent for antimicrobial and anticancer compounds. It can be used as a producer of antimicrobial, anticancer and biocontrol agents in pharmaceutical and agriculture fields.

ACKNOWLEDGEMENTS

Many thanks to Prof. Rob. W. M. van Soest at Department of Marine Zoology, Netherlands Centre for Biodiversity, the Netherlands, Prof. Michele Kelly at National Institute of Water and Atmospheric Research (NIWA) Ltd., Auckland, New Zealand for the helping in the taxonomy of sponge samples and deep thanks to Prof. Faida Hassan Ali Bamane Professor of Organic Chemistry, Department of Clinical Biochemistry, Faculty of Medicine, Jeddah, King Abdullaziz University, K. S. A. for her kind help and vital support to achieve the goals of this work. This work was experimentally supported by Marine Biotechnology and Natural Product Lab, Marine Environment, National Institute

of Oceanography and Fisheries. Thanks to the Regional Center for Mycology and Biotechnology, Al-Azhar University, for the help in the evaluation of antitumor activity.

FUNDING INFORMATION

No specific grant was given to this research by funding organizations in the public, private, or not for profit sectors.

CONFLICTS OF INTEREST

Authors declare that there is no conflict of interest.

REFERENCES

Ashforth, E.J.; Fu, C.; Liu, X.; Dai, H.; Song, F.; Guo, H. and Zhang, L. (2010). Bioprospecting for antituberculosis leads from microbial metabolites. *Natural Product Reports* 27(11): 1709–1719, https://doi.org/10.1039/C0NP00008F.

Auta, H.S.; Emenike, C.U. and Fauziah, S.H. (2017). Screening of Bacillus strains isolated from mangrove ecosystems in Peninsular Malaysia for microplastic degradation. *Environmental Pollution* 231: 1552–1559, https://doi.org/10.1016/j.envpol.2017.09.043.

Brinkmann, C.M.; Marker, A. and Kurtböke, D.İ. (2017). An Overview on Marine Sponge-Symbiotic Bacteria as Unexhausted Sources for Natural Product Discovery. *Diversity* 9(4): 40, https://doi.org/10.3390/d9040040.

Cragg, G.M. and Newman, D.J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica Et Biophysica Acta* 1830(6): 3670–3695, https://doi.org/10.1016/j.bbagen.2013.02.008.

Devi, P.; Wahidullah, S.; Rodrigues, C. and Souza, L.D. (2010). The Sponge-associated Bacterium Bacillus licheniformis SAB1: A Source of Antimicrobial Compounds. *Marine Drugs* 8(4): 1203–1212, https://doi.org/10.3390/md8041203.

Errakhi, R.; Bouteau, F.; Lebrihi, A. and Barakate, M. (2007). Evidences of biological control capacities of Streptomyces spp. against Sclerotium rolfsii responsible for damping-off disease in sugar beet (Beta vulgaris L.). *World Journal of Microbiology and Biotechnology* 23(11): 1503–1509, https://doi.org/10.1007/s11274-007-9394-7.

Flemer, B.; Kennedy, J.; Margassery, L.M.; Morrissey, J.P.; O'Gara, F. and Dobson, A.D.W. (2012). Diversity and antimicrobial activities of microbes from two Irish marine sponges, Suberites carnosus and Leucosolenia sp. *Journal of Applied Microbiology* 112(2): 289–301, https://doi.org/10.1111/j.1365-2672.2011.05211.x.

Ganesan, A. (2008). The impact of natural products upon modern drug discovery. *Current Opinion in Chemical Biology* 12(3): 306–317, https://doi.org/10.1016/j.cbpa.2008.03.016.

Gangadevi, V. and Muthumary, J. (2007). Preliminary studies on cytotoxic effect of fungal taxol on cancer cell lines. *African Journal of Biotechnology* 6(12), https://doi.org/10.4314/ajb.v6i12.57553

Gerwick, W.H. and Fenner, A.M. (2013). Drug Discovery from Marine Microbes. *Microbial ecology* 65(4): 800–806, https://doi.org/10.1007/s00248-012-0169-9.

Kabiru, A. and Garba, S. (2014). Fundulopanchax gardneri Test: A Convenient Method of Bioassay for Active Constituents of Natural Products. *Natural Products Chemistry & Research* 2, https://doi.org/10.4172/2329-6836.1000133.

Keller, C. (1889). Die spongienfaunades. Rothen Meeres (I. Halfte). *Z. Wiss. Zool.* 48: 311–405.

Lalitha, P.; Veena, V.; Vidhyapriya, P.; Lakshmi, P.; Krishna, R. and Sakthivel, N. (2016). Anticancer potential of pyrrole (1, 2, a) pyrazine 1, 4, dione, hexahydro 3-(2-methyl propyl) (PPDHMP) extracted from a new marine bacterium, Staphylococcus sp. strain MB30. *Apoptosis: An International Journal on Programmed Cell Death* 21(5): 566–577, https://doi.org/10.1007/s10495-016-1221-x.

Li, W.; Zhang, Y.; Liu, Y.; Yue, F.; Lu, Y.; Qiu, H.; Gao, D.; Gao, Y.; Wu, Y.; Wang, Z.; Huang, R. and Zhang, C. (2012). In vitro kinetic evaluation of the free radical scavenging ability of propofol. *Anesthesiology* 116(6): 1258–1266, https://doi.org/10.1097/ALN.0b013e3182567dcc.

Molinski, T.F. (2010). NMR of natural products at the "nanomole-scale." *Natural Product Reports* 27(3): 321–329, https://doi.org/10.1039/b920545b.

Mondol, M.A.M.; Shin, H.J. and Islam, M.T. (2013). Diversity of Secondary Metabolites from Marine Bacillus Species: Chemistry and Biological Activity. *Marine Drugs* 11(8): 2846–2872, https://doi.org/10.3390/md11082846.

Phelan, R.W.; Barret, M.; Cotter, P.D.; O'Connor, P.M.; Chen, R.; Morrissey, J.P.; Dobson, A.D.W.; O'Gara, F. and Barbosa, T.M. (2013). Subtilomycin: A New Lantibiotic from Bacillus subtilis Strain MMA7 Isolated from the Marine Sponge Haliclona simulans. *Marine Drugs* 11(6): 1878–1898, https://doi.org/10.3390/md11061878.

Priyanto, J.A.; Astuti, R.I.; Nomura, J. and Wahyudi, A.T. (2017). Bioactive Compounds from Sponge Associated Bacteria: Anticancer Activity and *NRPS-PKS* Gene Expression in Different Carbon Sources. *American Journal of Biochemistry and Biotechnology* 13(4): 148–156, https://doi.org/10.3844/ajbbsp.2017.148.156.

Proksch, P.; Edrada, R.A. and Ebel, R. (2002). Drugs from the seas - current status and microbiological implications. *Applied Microbiology and Biotechnology* 59(2–3): 125–134, https://doi.org/10.1007/s00253-002-1006-8.

Putz, A. and Proksch, P. (2018). Chemical Defence in Marine Ecosystems (From APR Volume 39). In *Annual Plant Reviews online*, pp. 162–213. John Wiley & Sons, Ltd, https://doi.org/10.1002/9781119312994.apr0419

Safari, W.F.; Chasanah, E. and Wahyudi, A.T. (2016). Antibacterial And Anticancer Activities Of Marine Bacterial Extracts And Detection Of Genes For Bioactive Compounds Synthesis. *International Journal of Pharmacy and Pharmaceutical Sciences* 8(2): 55–59.

Schäfer, H. and Wink, M. (2009). Medicinally important secondary metabolites in recombinant microorganisms or plants: progress in alkaloid biosynthesis. *Biotechnology Journal* 4(12): 1684–1703, https://doi.org/10.1002/biot.200900229.

Seiler, H.; Wenning, M.; Schmidt, V. and Scherer, S. (2013). Bacillus gottheilii sp. nov., isolated from a pharmaceutical manufacturing site. *International Journal of Systematic and Evolutionary Microbiology* 63(Pt 3): 867–872, https://doi.org/10.1099/ijs.0.036277-0.

Shaala, L.A.; El Hady, S.S.; Schmidt, E.W. and Youssef, D.T.A. (2017). Red Sea Sponges of the Genus Hyrtios as a Source of Symbiotic Fungi with Antimicrobial Activities. *Records of Pharmaceutical and Biomedical Sciences* 1(1): 73–80, https://doi.org/10.21608/rpbs.2018.9854.

Shreadah, M.A.; Monem, N.M.A.; Yakout, G.A. and Ela, H.M.A. (2018). Bacteria from Marine Sponges: A Source of Biologically Active Compounds. *Biomedical Journal of Scientific & Technical Research* 10(5): 001–020, https://doi.org/10.26717/BJSTR.2018.10.002025.

Singh, P.; Sharma, R.; Shukla, A.K. and Singh, R. (2018). Isolation of Bacillus spp. from Soil for Antimicrobial Production and Antibiotic Resistance. *Advances in Biotechnology* 8(4): 555741, https://doi.org/10.19080/AIBM.2018.08.555741.

van Soest, R.; Hooper, J. and Hiemstra, F. (1991). Taxonomy, phylogeny and biogeography of the marine sponge genus Acarnus (Porifera: Poecilosclerida). *Beaufortia* 42: 49–88.

Subbalaxmi, S. and Murty, V.R. (2016). Process optimization for tannase production by Bacillus gottheilii M2S2 on inert polyurethane foam support. *Biocatalysis and Agricultural Biotechnology* 7: 48–55, https://doi.org/10.1016/j.bcab.2016.05.004.

Sumi, C.D.; Yang, B.W.; Yeo, I.-C. and Hahm, Y.T. (2015). Antimicrobial peptides of the genus Bacillus: a new era for antibiotics. *Canadian Journal of Microbiology* 61(2): 93–103, https://doi.org/10.1139/cjm-2014-0613.

Sundaram, R.; Krishnakumar, S.; Inbaneson, S. and Gnanadesigan, M. (2010). Antagonistic activity of marine actinomycetes from Arabian Sea coast. *Archives of Applied Science Research* 2: 273–280.

Sureshan, S. devi and Sahadevan, R. (2014). Anticancer activity of Pyrrolo (1, 2-alpha) pyrazine 1, 4 dione hexahydro 3-(2-methylpropyl) metabolite from marine Streptomyces cirratus SRP11. *Research Journal of Chemistry and Environment* 18: 22–27.

Vinatoru, M. (2001). An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrasonics Sonochemistry* 8(3): 303–313, https://doi.org/10.1016/S1350-4177(01)00071-2.

Wilson, A.P. (2000). *Cytotoxicity and viability assays in animal cell culture: A Practical Approach*. 3rd ed. (ed. Masters, J. R. W.). Oxford University Press.

Ye, Y.; Li, Q.; Fu, G.; Yuan, G.; Miao, J. and Lin, W. (2012). Identification of Antifungal Substance (Iturin A2) Produced by Bacillus subtilis B47 and Its Effect on Southern Corn Leaf Blight. *Journal of Integrative Agriculture* 11(1): 90–99, https://doi.org/10.1016/S1671-2927(12)60786-X.

Zhishen, J.; Mengcheng, T. and Jianming, W. (1999). Research on antioxidant activity of lavonoids from natural materials. *Food Chem.* 64: 555–559.