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Research Article

Evaluation of the antibacterial and anticancer activities of marine *Bacillus subtilis* ESRAA3010 against different multidrug resistant *Enterococci* (MDRE) and cancer cell lines

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Abstract

Fifty nine isolates belonging to six species of *Enterococci* namely, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterococcus raffinosus*, *Enterococcus durans*, *Enterococcus mundtii* and *Enterococcus avium* (*n* = 35, 15, 4, 3, 1 and 1 isolates, respectively) were obtained from different clinical specimens including urine, pus, blood, wound, sputum and synovial fluid. The highest numbers of *enterococci* were recorded from the pus (20 isolates, 33.90%) followed by urine (12 isolates, 20.34%) while the lowest frequency was observed with synovial fluid samples (2 isolates, 3.39%). These isolates showed different multidrug resistant patterns with the lowest resistant for linezolid (*n* = 5, 8.48%), followed by teicoplanin (*n* = 14, 23.73%) and vancomycin (*n* = 20, 33.90%) while they exhibited the highest resistant against penicillin (*n* = 53, 89.83%), oxacillin (*n* = 50, 84.75%), erythromycin (*n* = 49, 83.05%) and streptomycin (*n* = 47, 79.66 %). On the other hand, a free living marine bacterium under isolation code ESRAA3010 was isolated from seawater samples obtained from the fishing area Masturah, Red Sea, Jeddah, Saudi Arabia. The phenotypic, chemotaxonomic, 16S rRNA gene analyses and phylogenetic data proved that isolate ESRAA3010 is very close to *Bacillus subtilis* and then it was designated as *Bacillus subtilis* ESRAA3010. It gave the highest antagonistic activity against all clinical *Enterococcus faecalis, Enterococcus faecium, Enterococcus raffinosus, Enterococcus durans, Enterococcus mundtii* and *Enterococcus avium* isolates under study with minimum inhibitory concentration (MIC) ranged from 4 to 56 µg/mL, 4 to 12 µg/mL, 4 to 8 µg/mL, 4 to 8 µg/mL, 8 µg/mL and 4 µg/mL, respectively as well as minimum bactericidal concentration (MBC) (8 to 64 µg/mL, 4 to 16 µg/mL, 4 to 12 µg/mL, 4 to 16 µg/mL, 12 µg/mL and 8 µg/mL, respectively). Moreover it showed anti-proliferative activity against colon (HCT-116), liver (HepG-2), breast (MCF-7) and lung (A-549) carcinomas with IC_{50} equal to 39, 50, 75 and 19 µg/mL, respectively which indicates its prospective usage in the upcoming decades.

More Information

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Introduction

Enterococcus species are Gram-positive cocci usually present in the human and sometimes become severely infectious agents; particularly they are frequently find novel mechanisms to evade the antibiotics treatments [1,2]. They developed multidrug resistance to different antibiotics in common use (MDR) with markedly increasing prevalence by contacting with contaminating surfaces and apparatus or infected persons [3]. They gain increasing concern due to their facility for withstanding the influence of various antibacterial agents, accordingly limit the drug of choices and leads to higher mortality and morbidity [4]. Therefore, finding of alternate powerful, inexpensive and harmless natural agents against multidrug resistant bacteria can be potent way for solving this serious global problem [5-7]. Marine bacteria are promising reservoirs of diverse effective bioactive natural products and many of them are being used in chemotherapy to treatment human diseases especially with the continuing need for new potent compounds against drug-resistant pathogens and

managing of distressing cancers with high selective activity and less toxicity [8,9]. Red sea host diverse and abundant free living microorganisms have the ability to produce promising bioactive marine natural products [10]. Marine *Bacillus* species produce multipurpose biologically active compounds including lipopeptide, polypeptide, macrolactone, fatty acid, polyketide and isocoumarin metabolites [11,12] with wide variety of antifungal, antibacterial, antioxidant and antiproliferative activities [13,14]. The capability of *Bacillus* species to biosynthesis different antibiotics with varied structures has been demonstrated by numerous genetic studies, and genetic analysis of *Bacillus* strains has shown that about 8% of the genome is dedicated to antibiotic synthesis [15,16]. This work was aimed to assess the incidences and distributions of *Enterococci* among patient admitted to El-Demerdash teaching hospital (Cairo, Egypt) along with determining their antibiotic sensitivity profiles against a panel of antibiotics to select the multidrug resistant *Enterococci* (MDRE). Moreover, this study aimed to explore potent marine *Bacillus* species that able to produce biologically active substances against these clinical isolates and diverse malignant cell lines including colon, liver, breast and lung carcinomas.

Materials and methods

Clinical specimens

Different samples including urine, pus, blood, wound, sputum and synovial fluid (5 samples for each) collected from patients ranging from 1 to over 60 years old (15 females and 15 males) admitted to I.C.U (intensive care unit), surgery, diabetic, skin and venereal disease, hematology, emergency, E.N.T (ear, nose and throat) and neurology units of El-Demerdash teaching hospital, Cairo, Egypt. Samples were brought to the laboratory under iced conditions and promptly processed. Fifty nine *Enterococci* isolates obtained were involved in this work. Moreover, *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Enterococcus raffinosus* ATCC 49427, *Enterococcus durans* ATCC 19432, *Enterococcus mundtii* ATCC 43186 and *Enterococcus avium* ATCC 14025 used as reference strains.

Isolation and identification of clinical bacteria

Enterococcus isolates were isolated and characterized according to the traditional methods and biochemical key were previously reported [17-23].

Antibiotics susceptibility profile of clinical *Enterococcal* isolates

Antibacterial susceptibility test of *Enterococci* species was done following disk diffusion technique using Muller Hinton agar (MH) based on World Health Organization [24] and adopted as consensual standard by the Clinical Laboratory Standards Institute [25,26] and European Committee on antimicrobial susceptibility testing [27] against a panel of thirteen antibiotics including penicillin 10 IU, ciprofloxacin 5 µg, streptomycin 10 µg, vancomycin 30 µg, gentamicin 10 µg, tetracycline 30 µg, kanamycin 30 µg, linezolid 10 µg, chloramphenicol 30 µg, teicoplanin 30 µg, nitrofurantoin 100 µg, erythromycin 15 µg and oxacillin 5 µg (Oxoid, Basingstoke, Hampshire, England in µg/disk).

Marine samples and isolation of marine *Bacillus* species

Ten samples of seawater from the fishing area Masturah, Red Sea, Jeddah, Saudi Arabia (latitude: 23°5031.4600N/ longitude: 38°49017.5200E) were collected in August 2018 at different depths in sterile screw cap bottles under iced conditions. Collected samples were taken to the laboratory, gathered and processed instantly. The isolation medium and process were prepared and done following the method of Ivanova, et al. [28] by plating serial dilutions of water sample individually to Petri dishes of nutrient agar (NA) supplemented with 100 µg/mL nystatin and cycloheximide, incubated at 30 °C for 3 days and recognized bacterial single colonies were transferred periodically to NA at 30 °C for 48 h and included in this study. Bacterial isolates were preserved on NA at 4 °C till using.

Antibacterial activity of marine bacterial isolates against different MDR-*Enterococci*

Muller Hinton agar (MH) plates were inoculated with the clinical MDR-*Enterococci* isolates, individually and paper assay discs loaded with 30 µL of marine bacterial isolates supernatants separately were plated on the top of inoculated medium, incubated at 37 °C for 24 h and then antimicrobial activity of bacterial isolates was determined against the MDRE isolates by using the routine diffusion plate technique via evaluating the inhibition zone diameters in mm [data determined as no antagonistic activity (-),weak antagonistic activity (<10 mm, +), moderate antagonistic activity (10 - 15 mm, ++) and excellent antagonistic activity (16 - >20 mm, +++)] [11,29].

Phenotypic and chemotypic properties of marine bacterial isolate ESRAA3010

ESRAA3010 strain was specified by conventional taxonomic procedures by means of API 20E and API 50CH methods along with other phenotypic and chemotypic characters [29-32]. *Bacillus subtilis* ATCC 6051T used as standard strain.

Molecular identification of isolate ESRAA3010 by 16S rDNA sequence analysis

DNA extraction, PCR amplification of 16S ribosomal RNA (rRNA) gene, purification of the PCR products, gel electrophoreses, and the 16S rDNA sequence analysis were performed based on previous reports [8,31,33-35] followed by aligning the 16S rRNA gene sequence of isolate ESRAA3010 with published sequences in NCBI GenBank database (http://www.ncbi.nih.gov). The tree topology was assessed through neighbor-joining method and bootstrap analyses based on 500 replications with MEGA-X [36-38].

Extraction of bioactive metabolites from marine *B. subtilis* ESRAA3010

Bacillus subtilis ESRAA3010 was inoculated into Erlenmeyer flasks containing tryptic soy broth medium then incubated for 24 h at 30 °C and 100 rpm, after incubation period the fermented broth of ESRAA3010 strain (5 L) was collected and the supernatant was separated under reduced pressure and then extracted twice with ethyl acetate (1:1, pH 4.5 under overnight shaking). The EtOAc extract obtained evaporated to dryness giving light brownish oil (6.41 g).

Determination of minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *B. Subtilis* ESRAA3010 extract against *Enterococci* strains.

The MIC and MBC of the extracted secondary metabolites were estimated in µg/mL against MDRE isolates as described by Cappuccino and Sherman [39] and Lavermicocca, et al. [40].

Determination of anti-proliferative activity (MTT assay) of the *Bacillus subtilis* ESRAA3010 on the colon (HCT- 116), liver (HepG-2), Breast (MCF-7) and lung (A-549) carcinomas.

Cell viability test was measured by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) to purple formazan [41,42]. HCT-116, MCF-7, HepG-2 and A-549 were achieved from Cancer Center, Karolinska Institute, Stokholm, Sweden; DMEM medium, RPMI 1640 medium and 1% antibiotic-antimycotic (10,000 U/mL potassium penicillin; 10,000 µg/mL streptomycin sulfate and 25 µg/mL amphotericin B) were achieved from Life Technologies/Gibco (Grand Island, NY, USA). The effect of diverse amounts of *B. subtilis* ESRA-3010 extract ranging between 25 and 300 µg/mL on the cytotoxicity and cell viability of these tumor cell lines was measured as stated by Mosmann [41] and Wilson [42] in 96-well microplate at 37 °C under 5% CO_2 using a water jacketed CO_2 incubator (Sheldon, TC2323, Cornelius, OR, USA) for 48 h followed by aspirating medium, adding 40 µL MTT salt (2.5 µg/mL) to wells, incubating for 4 h at 37 °C as mentioned above, ending the reaction along with dissolve the resulted crystals by adding 200 µL of 10% sodium dodecyl sulfate to wells and thus incubating overnight at 37 °C. The absorbance was estimated at 595 nm by the microplate multi-well reader (Bio-Rad Laboratories Inc. model 3350, Hercules, California, USA). The IC_{50} (the amount of the extract that decreased cell viability by 50%) compared to the control (wells contain only solvent without any extract) were determined using SPSS 11 program. Percentages (%) of cell viabilities were estimated based on the formula: [(Absorbance of treated cell lines with extract / Absorbance of negative control) -1] X 100.

Results and discussion

Isolation, characterization and occurrence of clinical *Enterococci* isolates

A total of fifty nine isolates of Gram-positive *Enterococci* isolates including 16, 11, 8, 5, 10 and 9 isolates were isolated from the age groups 1-6, >6-12, >12-20, >20-40, >40-60 and over 60 years old (Table 1). The largest number of MDRE (27.12%) was collected from ages ranging from 1 to 6 years. Furthermore, 33 isolates (55.93%) were obtained from male samples while 26 isolates (44.07%) were obtained from female participants (Table 1) from El-Demerdash teaching hospital. The presence of resistant *Enterococci* isolates in human societies acts as a source for hospital infections [1]. In line with our results, Karna, et al. [4] revealed that the predominance of *Enterococci* isolates were achieved from age group 0-10 (20.9%), after that age group 20-30 (19.8%) along with detection of the high incidence of infection in male participants. *Enterococci* are recognized as unique reasons of hospital infections in patients with weakened immune systems. On the other hand, Table 2 showed the different department from which specimens were collected, 17 (28.81%); 16 (27.12%); 8 (13.56%); 7 (11.86%); 5 (8.48%); 4 (6.78%); 1 (1.70%) and 1 (1.70%) isolates were obtained from I.C.U (intensive care unit), surgery, diabetic, skin and venereal disease, hematology, emergency, E.N.T (ear, nose and throat) and neurology departments, respectively which indicating that hospitalization in the intensive care unit is an important risk factor for MDRE colonization and occurrence. Similar distribution observation were reported previously in resistant *Enterococci* collated from different humanoid

Table 1: Specimens recovered from different gender and age groups.

Demographic and clinical conditions	No. of clinical isolates	% of clinical isolates
Gender		
Males	33	55.93
Females	26	44.07
Age		
1-6	16	27.12
>6-12	11	18.64
>12-20	8	13.56
>20-40	5	8.48
>40-60	10	16.95
>60	9	15.25

Table 2: Incidence of *Enterococci* isolates in various departments at El-Demerdash teaching hospital.

Department	No. of *Enterococci* isolates	% of *Enterococci* isolates
Surgery	16	27.12
I.C.U.	17	28.81
Diabetic	8	13.56
Emergency	4	6.78
E.N.T.	1	1.7
Neurology	1	1.7
Haematology	5	8.48
Skin and venereal disease	7	11.86
Total	59	100

infections [19], clinical samples in Kashmir; North India [43], health care setting [3], tertiary care center of Eastern Nepal [4] and Turkey [1].

Characteristics of clinical *Enterococcus* strains

Following the standard guideline of species specification [2,17-20], six different species of *Enterococci* isolates were identified, namely *E. faecalis, E. faecium, E. raffinosus, E. durans, E. mundtii* and *E. avium* (n = 35, 15, 4, 3, 1 and 1, respectively, Table 3. All isolates were Gram-positive, non-motile, positive for Voges-Proskauer reaction, negative for catalase activity and β-glucoronidase, produce acid from sorbitol and lactose as well as grown at 45 °C and pH 9.6. Only *E. mundtii* was able to produce yellow pigment which is a key feature of this species in addition to produce acid from trehalose; *E. durans* was the only negative strain for acid production from rhamnose, melezitose, arabinose and mannitol and *E. avium* was the only strain failed to produce acid from amidon but gelatin hydrolysis ability was only recorded in *E. faecalis* strains (Table 3). The other details of phenotypic characterizations and chemotypic features of these species obtained in the current study are presented in table 3. Interestingly *E. faecalis* and *E. faecium* (Figures 1 and 2) together constitute more than 84.74% of total isolates and their increased proportion in current work might be attributed to their capability to attain and developed different resistance patterns against multiple antibiotics. In the majority of previous reports, *E. faecalis* has been documented as the main *Enterococci* species, followed by *E. faecium*. For example Karna, et al. [4] reported that among seven different identified species of *Enterococci* the highest frequency of strains among total isolates was reported for *E. faecalis* followed by *E. faecium* and together, they made up over 90% of the total isolates. Nevertheless, this result is higher than other studies [44,45]. These differences in bacterial occurrence can be attributed to the differences in geographic site, sample, period of hospitalization, and drugs used [46].

Enterococci species distribution in various clinical specimens

Among a total number of 59 *Enterococci* isolates obtained, 12 (20.34%), 20 (33.9%), 9 (15.25%), 10 (16.95%), 6 (10.17%) and 2 (3.39%) were recovered from urine, pus, blood, wound, sputum and synovial fluid samples, respectively (Table 4). After analyzing the distribution of the six *Enterococci* species in the various clinical specimens obtained, we found that 8 (13.56%), 11 (18.64%), 6 (10.17%), 5 (8.48%), 3 (5.09%) and 2 (3.39%) of *E. faecalis* (n = 35, 59.32%) as well as 3 (5.09%), 5 (8.48%), 3 (5.097%), 3 (5.097%), 1 (1.70%) and 0 (0.00%) isolates of *E. faecium* (n = 15, 25.42%) along with 1 (1.70%), 2 (3.39%), 0 (0.00%), 1 (1.70%), 0 (0.00%) and 0 (0.00%) of *E. raffinosus* (n = 4, 6.78%) were obtained from urine, pus, blood, wound, sputum and synovial fluid samples, respectively. Moreover only 3 isolates of *E. durans* (1 isolate from pus and 2 isolates from sputum), *E. mundtii* (1 isolate from pus) and *E. avium* (1 isolate from wound) were isolated (Table 4).

Table 3: Characteristics of clinical *Enterococcus* strains.

Characteristic	No. of positive *Enterococci* strains					
	E. faecalis n = 35	*E. faecium* n = 15	*E. raffinosus* n = 4	*E. durans* n = 3	*E. mundtii* n = 1	*E. avium* n = 1
Gram stain	35	15	4	3	1	1
Motility	0	0	0	0	0	0
Pigment (yellow)	0	0	0	0	1	0
Voges-Proskauer reaction	35	15	4	3	1	1
Hippurate hydrolysis	29	12	3	0	0	0
α-Galactosidase	0	15	2	0	1	0
β-Galactosidase	11	14	0	0	1	0
Arginine dihydrolase	35	15	0	3	1	0
β-Glucuronidase	0	0	0	0	0	0
Acid from						
Amidon	35	15	3	3	1	0
Glycogen	7	0	0	0	1	0
Sucrose	31	14	2	0	0	1
Sorbose	13	0	0	0	0	1
Rhamnose	32	3	1	0	1	1
Melibiose	9	14	4	0	1	0
Melezitose	0	13	4	0	1	1
L-Arabinose	33	12	4	0	1	1
Mannitol	34	0	4	0	1	1
Sorbitol	35	15	3	3	1	1
Lactose	35	15	4	3	1	1
Trehalose	0	0	0	0	1	0
Inulin	0	15	0	0	1	0
Raffinose	30	1	1	0	0	1
D-xylose	25	5	1	0	1	0
Adonitol	0	0	2	0	0	1
Gelatinase production	24	0	0	0	0	0
H_2S production	0	0	0	0	1	1
β-hemolysis	10	3	0	2	0	0
Catalase	0	0	0	0	0	0
Growth at						
4 °C	0	15	2	1	1	0
45 °C	35	15	4	3	1	1
50 °C	0	11	4	0	1	0
pH 9.6	35	15	4	3	1	1
0.01% Tetrazolium	35	0	2	0	0	0

In agreement with our results, Karna, et al. [4] analyzed ninety one isolates of *Enterococcus* obtained from numerous clinical samples, among them the highest *Enterococci* isolates incidence reported from urine then pus and blood (61.5%, 19.8% and 5.5%, respectively).

Antibiotic susceptibility pattern in the *Enterococci* isolates

In the current work, the susceptibility patterns in the

Enterococci isolates against antibiotics in common use by the disc diffusion method in table 5 displayed that all *Enterococci* isolates were multidrug resistant (MDRE) due to they showed co-resistant to many classes of antibiotics at the same time. The multidrug resistant (MDR) considered particular phenotypic characteristics of the clinical *Enterococci* strains [1,47]. The highest resistant was reported for penicillin (n = 53, 89.83%), oxacillin (n = 50, 84.75%), erythromycin (n = 49, 83.05%), streptomycin (n = 47, 79.66%), ciprofloxacin (n = 42, 71.19%), kanamycin (n = 41, 69.49%), tetracycline (n = 40, 67.80%), gentamicin (n = 33, 55.93%), chloramphenicol (n = 31, 52.54%) and nitrofurantoin (n = 29, 49.15%) but the lowest resistant was detected with linezolid (n = 5, 8.48%), followed by teicoplanin (n = 14, 23.73%) and vancomycin (n = 20, 33.90%) (Table 5). Karna, et al. [4] stated that highest frequency of susceptibility among the isolates of *Enterococci* was noted for linezolid after that teicoplanin and then gentamicin (97.8%, 95.6% and 81.3%, respectively). Interestingly 94.29%, 88.57%, 42.86%, 85.71%, 80.0%, 31.43%, 77.14%, 100.00%, 11.43%, 25.71%, 57.14%, 85.71% and 94.29% of total *E. faecalis* isolates compared to 93.33%, 80.00%, 20.00%, 20.00%, 33.33%, 60.00%, 66.70%, 86.67%, 6.67%, 26.66%, 46.67%, 53.33% and 86.67% of total *E. faecium* isolates showed resistant to penicillin, streptomycin, vancomycin, gentamicin, tetracycline, nivtrofurantoin, ciprofloxacin, erythromycin, linezolid, teicoplanin, chloramphenicol, kanamycin and oxacillin, respectively but *E. raffinosus*, *E. durans*, *E. mundtii* and *E. avium* species showed less degree of resistant to these antibiotics (Table 5). The higher positive resistance rate to all antibiotic under study demonstrated in our work agreed well with other studies [2,3,20] that could be attributed to the low affinity between these antibiotics and protein binding sites of *E. faecium*, *E. raffinosus*, *E. durans, E. mundtii* and *E. avium* or/and the presence of plasmid-encoded β-lactamase and other antibiotics degrading enzymes in some strains than others [6,48]. Our findings are in agreement with many studies reported *E. faecalis* as the most frequently species obtained from hospitalized patient with multidrug resistance against different antibiotics including vancomycin [44,49]. Conversely Karna, et al. [4] suggested that *E. faecium* strains were higher resistant to all antibiotics under study than *E. faecalis* but *E. durans* showed no resistant to any of the tested antibiotics.

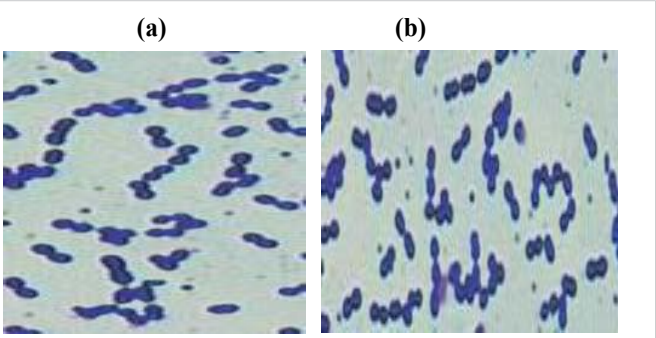

Figure 1: *E. faecalis* (a) and *E. faecium* (b) under microscope.

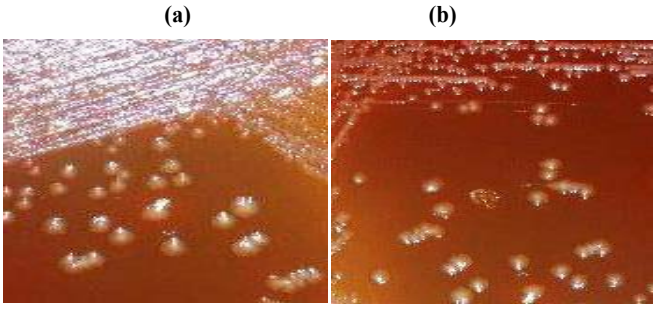

Figure 2: Appearance of *E. faecalis* (a) and *E. faecium* (b) on MacConkey agar.

Isolation and evaluation of antagonistic activity of different marine *Bacillus* isolates against different *Enterococci* strains

Twelve isolates of free living marine *Bacillus* species were isolated, cultivated, and then their antagonistic activity toward *Enterococci* strains under study was evaluated and tabulated in table 6. Data clearly indicated that the isolate under the isolation code ESRAA3010 was the hyperactive strain that showed inhibitory activity against all *Enterococci* strains under study with inhibition power ranged from good (++) to excellent (+++) followed by ESRAA3012 isolate showed inhibitory activity toward 93.22% of all isolates (Table 6). Then ESRAA3010 strain was selected for the further studies. Our results supported the previous finding of Lv, et al. Mondol, et al., and Freitas-Silva, et al. [13,14,50] they documented that marine *Bacillus* strains can biosynthesis multipurpose compounds comprising lipopeptide, carotenoid, polypeptide, macrolactone, fatty acid, polyketide and isocoumarin metabolites that have demonstrated a wide array of

Table 4: Distribution of *Enterococcus* species in the clinical specimens.

Specimen	Total no. of Enterococcus *n*, (%)	Enterococci species *n*, (%)					
		E. faecalis	*E. faecium*	*E. raffinosus*	*E. durans*	*E. mundtii*	*E. avium*
Urine	12 (20.34)	8 (13.56)	3 (5.09)	1 (1.70)	0 (0.00)	0 (0.00)	0 (0.00)
Pus	20 (33.90)	11 (18.64)	5 (8.48)	2 (3.39)	1 (1.70)	1 (1.70)	0 (0.00)
Blood	9 (15.25)	6 (10.17)	3 (5.09)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Wound	10 (16.95)	5 (8.48)	3 (5.09)	1 (1.70)	0 (0.00)	0 (0.00)	1 (1.70)
Sputum	6 (10.17)	3 (5.09)	1 (1.70)	0 (0.00)	2 (3.39)	0 (0.00)	0 (0.00)
Synovial fluid	2 (3.39)	2 (3.39)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total	59 (100.00)	35 (59.32)	15 (25.42)	4 (6.78)	3 (5.09)	1 (1.70)	1 (1.70)

bioactivities including antibacterial, antifungal, antitumor and antioxidant properties. Consequently, there is a potential for using these marine *Bacillus* species metabolites as promising medicines and in other biological treatments.

Identification of the hyperactive bacteria ESRAA3010 by phenotypic, chemotypic and 16S rDNA analysis

Strain ESRAA3010 showed a grayish-white, roundish, opaque, flatted and medium size colonies ranged from drying on LB agar and nutrient agar to smooth and moist on tryptic soy agar in addition to complete hemolytic activity on blood agar (Table 7). It was Gram positive rods, spore forming, cell diameter estimated to be 0.8–0.9 and 2.7–3.2 µm in width and length, respectively (Figure 3). It was positive for oxidase, catalase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, β-fucosidase, α-arabinosidase, L-arginine aminopeptidase, VP reaction and nitrate reduction; acid production form D-glucose, L-arabinose, D-xylose, D-mannitol and D-fructose; citrate utilization; hydrolysis of starch, gelatin, casein and tween 80 as well as assimilation of L-arabinose, D-xylose, meso-Inositol, sorbitol, methyl-D-glucoside, D-melibiose, D-raffinose and glycogen (Table 7). On the other hand, it was negative for KOH test, indol reaction, aminopeptidase, phenylalanine deaminase, arginine dihydrolase, N-benzoyl-L-leucine amino peptidase and L-tryptophan aminopeptidase; utilization of propionate and assimilation of galactose, rhamnose, methyl-D-mannoside, N-acetyl-glucosamine, dextrin and gluconate (Table 7).

Table 5: Antibiotic resistant profiles of the *Enterococcus* strains.

Antibiotics	*Enterococcus* strains									
	Total *Enterococci*		*E. faecalis* (*n* = 35)			*E. faecium* (*n* = 15)			Other *Enterococci* (*n* = 9)	
	No. of total resist *Enterococci*	Percentage of resistance	No. of resist isolates	% of resistance*		No. of resist isolates	% of resistance*		No. of resist isolates	% of resistance*
				A	B		A	C		D
Penicillin	53	89.83	33	55.93	94.29	14	23.73	93.33	6	66.67
Streptomycin	47	79.66	31	52.54	88.57	12	16.95	80	4	44.44
Vancomycin	20	33.9	15	25.42	42.86	3	5.09	20	2	22.22
Gentamicin	33	55.93	30	50.85	85.71	3	5.09	20	0	0
Tetracycline	40	67.8	28	47.46	80	5	8.48	33.33	7	77.8
Nitrofurantoin	29	49.15	11	18.64	31.43	9	15.25	60	9	100
Ciprofloxacin	42	71.19	27	45.76	77.14	10	16.95	66.7	5	55.6
Erythromycin	49	83.05	35	59.32	100	13	22.03	86.67	1	11.11
Linezolid	5	8.48	4	6.78	11.43	1	1.7	6.67	0	0
Teicoplanin	14	23.73	9	15.25	25.71	4	4.21	26.66	1	11.11
Chloramphenicol	31	52.54	20	33.9	57.14	7	11.87	46.67	4	44.44
Kanamycin	41	69.49	30	50.85	85.71	8	13.56	53.33	3	33.33
Oxacillin	50	84.75	33	55.93	94.29	13	22.03	86.67	4	44.44

*A = % of total *Enterococci*, B = % of total *E .faecalis*, C = % of total *E. faecium*, D = % of total other *Enterococci* (*E. raffinosus*, *E. durans*, *E. mundtii* and *E. avium*).

Table 6: Antagonistic activity of marine *Bacillus* isolates against clinical *Enterococcus* strains.

Antagonistic marine *Bacillus* isolates	No. of sensitive *Enterococci* strains / inhibition activity range*					
	E. faecalis *n* = 35	*E. faecium* *n* = 15	*E. raffinosus* *n* = 4	*E. durans* *n* = 3	*E. mundtii* *n* = 1	*E. avium* *n* = 1
Bacillus sp. ESRAA3001	20 (+ to +++)	9 (+ to ++)	3 (+ to ++)	(-)	(-)	(-)
Bacillus sp. ESRAA3002	(-)	(-)	(-)	1 (++)	1 (+)	1 (+)
Bacillus sp. ESRAA3003	4 (+ to ++)	6 (+ to ++)	3 (+ to +++)	(-)	(-)	1 (++)
Bacillus sp. ESRAA3004	11 (+ to ++)	6 (+ to ++)	3 (+)	3 (+ to ++)	(-)	(-)
Bacillus sp. ESRAA3005	9 (+ to +++)	5 (++)	(-)	2 (++)	1 (+)	1 (++)
Bacillus sp. ESRAA3006	25 (+ to ++)	13 (++)	1 (+++)	(-)	(-)	(-)
Bacillus sp. ESRAA3007	18 (+ to +++)	14 (++ to +++)	(-)	1 (++)	1 (++)	1 (++)
Bacillus sp. ESRAA3008	(-)	(-)	(-)	(-)	(-)	(-)
Bacillus sp. ESRAA3009	(-)	(-)	(-)	(-)	(-)	(-)
Bacillus sp. ESRAA3010	35 (++ to +++)	15 (+++)	4 (+++)	3 (+++)	1 (+++)	1 (+++)
Bacillus sp. ESRAA3011	(-)	(-)	(-)	(-)	(-)	(-)
Bacillus sp. ESRAA3012	35 (+ to +++)	15 (+ to ++)	3 (+++)	1 (+++)	1 (+++)	(-)

* (-) no inhibitory activity, (+) weak inhibitory activity, moderate inhibitory activity (++) and (+++) excellent inhibitory activity

In addition, the sequencing of the 16S rRNA gene exhibited 99.93% similarity to various *B. subtilis* strains (DSM 10, JCM 1465 and NBRC 13719). The phenotypic, chemotaxonomic, 16S rRNA gene analyses and phylogenetic data in table 7 and figures 3,4 showed that isolate ESRAA3010 is very close to *B. subtilis* as previously reported [8,29-35]. Then it was designated as *B. subtilis* ESRAA3010.

Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *B. subtilis* ESRAA3010 extract against different *Enterococci* strains

Data in table 8 indicated that EtOAc extract of *B. subtilis*

Table 7: Identification and characterization of marine isolate ESRAA3010.

Physiological and biochemical test	Characteristics
Growth performance on	
TSA agar + 5% fetal calf serum	Grayish white, roundish, opaque, thick ridges, smooth, moist and medium size colonies
LB agar	Grayish white, roundish, opaque, flat, dry and medium size colonies
Rabbit blood agar	Grayish white, roundish, complete hemolysis, opaque, flat, dry and medium size colonies
Nutrient agar	Grayish white, roundish, opaque, flat, dry and medium size colonies
Shape	Rod
Gram stain	Positive
Cell diameter	0.8–0.9 X 2.7–3.2 µm
Indol reaction	-
Aminopeptidase	-
KOH test	-
Oxidase	+
Catalase	+
Phenylalanine deaminase	-
Arginine dihydrolase	-
α-Galactosidase	+
β- Galactosidase	+
α- Glucosidase	+
β-Glucosidase	+
β-Fucosidase	+
α-Arabinosidase	+
L-Arginine aminopeptidase	+
N-benzoyl-L-leucine amino peptidase	-
L-Tryptophan aminopeptidase	-
Spores	+
VP reaction	+
Growth at	
45 °C	+
50 °C	-
60 °C	+
pH 5.5	+
NaCl 5%	+
NaCl 10%	+
NaCl 15%	+
Acid form	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Mannitol	+
D-Frucrose	+
Utilization of	
Citrate	+
Propionate	-
Nitrate	+
Hydrolysis of	
Starch	+
Gelatin	+
Casein	+
Tween 80	+
Assimilation of	
L-Arabinose	+
D-Xylose	+
Galactose	-
Rhamnose	-
meso-Inositol	+
Sorbitol	+
Methyl-D-mannoside	-
Methyl-D-glucoside	+
N-acetyl-glucosamine	-
D-Melibiose	+
D-Melibios	+
D -Raffinose	+
Dextrin	-
Starch	+
Glycogen	+
Gluconate	-

ESRAA3010 showed potent antagonistic activity against all *Enterococci* species under study. It showed MIC against *E. faecalis*, *E. faecium*, *E. raffinosus*, *E. durans*, *E. mundtii* and *E. avium* (35, 15, 4, 3, 1 and 1isolates, respectively) ranged from 4 to 56 µg/mL, 4 to 12 µg/mL, 4 to 8 µg/mL, 4 to 8 µg/

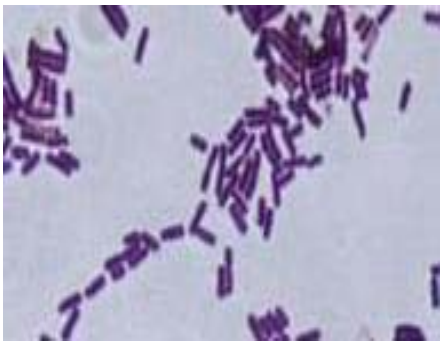

Figure 3: Gram staining of isolate ESRAA3010 under microscope.

mL, 8 µg/mL and 4 µg/mL, respectively and MBC reached 8 to 64 µg/mL, 4 to 16 µg/mL, 4 to 12 µg/mL, 4 to 16 µg/mL, 12 µg/mL and 8 µg/mL, respectively. Our data are consistent with Kizhakkekalam, et al. and Freitas-Silva, et al. [11,50] they demonstrated the antagonistic activity of secondary metabolites derived from marine *Bacillus* species as potential antimicrobial agents against multidrug-resistant bacteria.

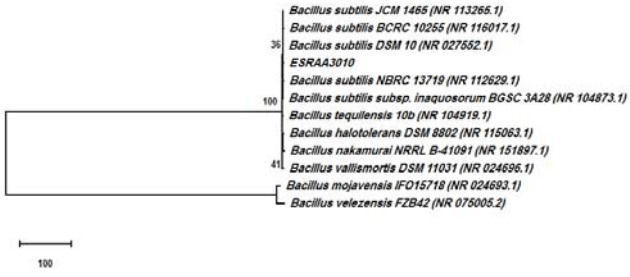

Figure 4: Phylogenetic dendrogram of isolate ESRAA3010 based on 16S rDNA sequence analysis, constructed using the neighbor-joining method.

Anti-proliferative activity of *B. subtilis* ESRAA3010 extract

In vitro anti-proliferative activity of EtOAc extract of *B. subtilis* ESRAA3010 strain on different tumor cell lines was evaluated by MTT cell viability assay and illustrated in figure 5. Data clearly indicated that the cells viability of A-549 carcinoma was completely inhibited after treatment with ESRAA3010 extract at a concentration of 50 µg/mL (Figure 5). Furthermore, HCT-116 and HepG-2 carcinomas totally inhibited at 100 µg/mL but the growth of MCF-7 cell line was completely inhibited at a concentration of 200 µg/mL with IC_{50} equal to 39, 50, 75 and 19 µg/mL against colorectal (HCT-116),

Table 8: Minimum inhibitory (MIC) and minimum bactericidal concentrations (MBC) of *B. subtilis* ESRAA3010 extract against different clinical *Enterococci* strains.

Enterococci strain	MIC (µg/mL)	MBC (µg/mL)	*Enterococci* strain	MIC (µg/mL)	MBC (µg/mL)
Clinical strains			**Clinical strains**		
E. faecalis 1	24	32	*E. faecalis* 34	16	20
E. faecalis 2	16	24	*E. faecalis* 35	32	32
E. faecalis 3	32	40	*E. faecium* 1	4	4
E. faecalis 4	8	16	*E. faecium* 2	4	8
E. faecalis 6	32	40	*E. faecium* 3	8	8
E. faecalis 7	12	56	*E. faecium* 4	12	16
E. faecalis 8	56	64	*E. faecium* 6	12	12
E. faecalis 9	16	16	*E. faecium* 7	12	16
E. faecalis 10	32	40	*E. faecium* 8	4	4
E. faecalis 11	12	24	*E. faecium* 9	8	12
E. faecalis 12	16	24	*E. faecium* 10	8	8
E. faecalis 13	8	24	*E. faecium* 11	4	4
E. faecalis 14	56	64	*E. faecium* 12	4	8
E. faecalis 15	12	20	*E. faecium* 13	8	16
E. faecalis 16	12	20	*E. faecium* 14	12	12
E. faecalis 17	12	28	*E. faecium* 15	12	16
E. faecalis 18	16	32	*E. raffinosus* 1	4	8
E. faecalis 19	24	30	*E. raffinosus* 2	8	8
E. faecalis 20	8	12	*E. raffinosus* 3	4	4
E. faecalis 21	28	50	*E. raffinosus* 4	8	12
E. faecalis 22	50	64	*E. durans* 1	4	4
E. faecalis 23	16	16	*E. durans* 2	4	8
E. faecalis 24	20	24	*E. durans* 3	8	16
E. faecalis 25	8	12	*E. mundtii* 1	8	12
E. faecalis 26	12	20	*E. avium* 1	4	8
E. faecalis 27	4	8	**Reference strains**		
E. faecalis 28	12	24	*E. faecalis* ATCC 19433	12	20
E. faecalis 29	36	40	E. faecium ATCC 19434	4	8
E. faecalis 30	16	32	*E. raffinosus* ATCC 49427	4	4
E. faecalis 31	20	40	*E. durans.* ATCC 19432	8	12
E. faecalis 32	24	64	*E. mundtii* ATCC 43186	8	12
E. faecalis 33	12	16	*E. avium* ATCC 14025	4	4

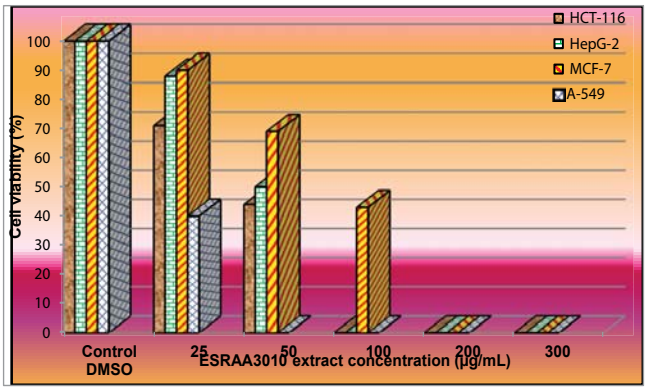

Figure 5: Effect of *B. subtilis* ESRAA3010 extract against HCT-116, HepG-2, MCF-7 and A-549 carcinomas.

hepatocellular (HepG-2), breast (MCF-7) and lung (A-549) carcinomas. Similar to our results Vo, et al., Koim-Puchowska, et al. and Mondol, et al. [7,12,14] reported the anticancer activities of *Bacillus* species extracts on a large number of carcinomas. Also, Zhao, et al. [16] reported that treating carcinomas with products from cultured *Bacillus* strains had significant inhibitory effects on ovarian and colorectal carcinomas proliferation in a dose dependent manner.

Conclusion

Marine ecosystems in Egypt have proven to be prolific resource for various types of marine bacteria, especially *Bacillus* species that produce different stimulating biological metabolites against infectious agents which widely distributed as multidrug resistant *Enterococci* strains and cancer. The ethyl acetate extract of *B. subtilis* ESRAA3010 showed potent anti-MDRE activity against all clinical *Enterococci* isolates under study including *E. faecalis*, E. *faecium*, *E. raffinosus*, *E. durans*, *E. mundtii* and *E. avium* with MIC 4 to 56, 4 to 12, 4 to 8, 4 to 8, 8 and 4 µg/mL, respectively and MBC 8 to 64, 4 to 16, 4 to 12, 4 to 16, 20 and 8 µg/mL, respectively. *Bacillus subtilis* ESRAA3010 extract exhibited anti-proliferative activity against colon, lung, liver and breast adenocarcinomas with IC_{50} equal to 39, 50, 75 and 19 µg/mL, respectively. Our data supported the potential application of *B. subtilis* ESRAA3010 extract in drug delivery and industry as novel and promising antibacterial and/or anticancer agents against deadly infectious agents and cancers.

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