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 munditii 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 munditii 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 12 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 IC₅₀ equal to 39, 50, 75 and 19 μg/mL, respectively which indicates its prospective usage in the upcoming decades.

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 ICU (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 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, Stockholm, 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* ESRAA3010 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% CO2 using a water jacketed CO2 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 multiwell reader (Bio-Rad Laboratories Inc. model 3350, Hercules, California, USA). The IC50 (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 Enterococcus 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-motive, 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 (5.09%), 3 (5.09%), 1 (1.70%) and 0 (0.00%) isolates of *E. faecium* (*n* = 15, 12.86%) 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 over 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).

| Characteristic | No. of positive Enterococci strains |
|---------------|------------------------------------|
| Gram stain    | *E. faecalis* = 35, *E. faecium* = 15, *E. raffinosus* = 4, *E. durans* = 3, *E. mundtii* = 1, *E. avium* = 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-Glucoronidase | 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-xylene | 25 5 1 0 0 1 |
| Adonitol | 0 0 2 0 0 1 |
| Gelatinase production | 24 0 0 0 0 0 |
| H2S production | 0 0 0 0 1 1 |
| β-hemolysis | 10 3 0 2 0 1 |
| 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, nitrofurantoin, 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 [23,20] 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.

Isolation and evaluation of antagonistic activity of different marine Bacillus strains 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, polyketyde and isocoumarin metabolites that have demonstrated a wide array of
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-frucrose; citrate utilization; hydrolysis of starch, gelatin, casein and tween 80 as well as assimilation of L-arabinose, D-xyllose, 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 aminopeptidase 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 | Enterococci Total (n = 59) | Enterococcus faecalis (n = 35) | Enterococcus 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 |
| 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 Enterococcus faecalis, C = % of total Enterococcus 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 Enterococcus 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 (+)          | 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 (+)          | 1 (+)          |
| Bacillus sp. ESRAA3008              | (-)                                                          | (-)               | (-)               | (-)               | (-)            | (-)            | (-)            |
| Bacillus sp. ESRAA3009              | (-)                                                          | (-)               | (-)               | (-)               | (-)            | (-)            | (-)            |
| Bacillus sp. ESRAA3010              | 35 (++ to ++++)                                              | 15 (+++)          | 4 (+++)           | 3 (+++)           | 1 (+)          | 1 (+++)        | 1 (+++)        |
| Bacillus sp. ESRAA3011              | (-)                                                          | (-)               | (-)               | (-)               | (-)            | (-)            | (-)            |
| Bacillus sp. ESRAA3012              | 35 (+ to ++++)                                               | 15 (+ to +++)     | 3 (+++)           | 1 (+)             | 1 (+++)        | 1 (+++)        | (-)            |

* (-) no inhibitory activity, (+) weak inhibitory activity, moderate inhibitory activity (+++) and (++++) excellent inhibitory activity
Evaluation of the antibacterial and anticancer activities of marine *Bacillus subtilis* ESRAA3010 against different multidrug resistant *Enterococci* (MDRE) and cancer cell lines

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-Arabinose aminopeptidase | + |
| N-benzoyl-L-leucine aminopeptidase | - |
| L-Tryptophan aminopeptidase | - |
| Spores | + |
| VP reaction | + |
| **Growth at** | | |
| 45 °C | + |
| 50 °C | - |
| 60 °C | + |
| pH 5.5 | + |
| NaCl 5% | + |
| NaCl 10% | + |

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/mL, 4 to 8 μg/mL, and 4 to 8 μg/mL, respectively.

Figure 3: Gram staining of isolate ESRAA3010 under microscope.
they demonstrated the antagonistic activity of secondary metabolites derived from marine Bacillus species as potential antimicrobial agents against multidrug-resistant bacteria.

**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 IC50 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     | 12          | 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 |
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 activity of Bacillus species against colon, lung, liver and breast adenocarcinomas with IC50 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 antibiotic and/or anticancer agents against deadly infectious agents and cancers.

**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 IC50 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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