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Antioxidant and cytotoxic activities of metabolites produced by a new marine *Streptomyces* sp. isolated from the sea cucumber *Holothuria leucospilota*

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Abstract

Marine microorganisms are important sources for novel natural products. Hence, the aim of this study was to introduce marine microorganisms with the capability of producing antioxidant and cytotoxic metabolites. For this purpose, ten live sea cucumbers (*Holothuria leucospilota*) were collected from Larak Island, Persian Gulf. Then, their intestine contents were serially diluted and treated by heating (55°C). 100 µL of treated samples were inoculated on starch casein nitrate agar medium, which is supplemented with nalidixic acid and cycloheximide. The plates were incubated at 28 °C for 4 weeks and the colonies were purified. The antioxidant activity of extracted metabolites from the isolated actinobacteria was evaluated using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity assay and the cytotoxic activity was screened by Brine-Shrimp micro well cytotoxicity method. In addition, the cytotoxic effect was evaluated against HCT 116 and SW 480 cell lines by MTT cell proliferation assay. A new strain of marine actinobacterium represented maximum antioxidant and cytotoxic activity among 17 actinobacterial isolates. The ethyl acetate culture extract of the isolate scavenged DPPH radicals with IC_{50} value of 211.2 µg mL^{-1}. In addition, the extract exhibited high toxicity against HCT 116 and SW 480 tumor cell lines with IC_{50} values of 26.48 and 18.53 µg mL^{-1} respectively. The isolate was identified as *Streptomyces* sp. strain SC 156 and showed 98% similarity with type strains in NCBI database. These results suggested that *Streptomyces* sp. strain SC 156 could be considered as promising candidate for antioxidant and cytotoxic compound discovery.

Keywords: Antioxidant activity, Cytotoxic activity, *Holothuria leucospilota,* Marine *Streptomyces*

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Introduction

Marine organisms are important sources for novel natural products (Blunt *et al.*, 2013). Production of secondary metabolites as a defense strategy is well developed in marine invertebrates like sea cucumbers (Bogatyrenko and Buzoleva, 2016). Among the metabolites they could produce antioxidant compounds to protect themselves against elevated levels of reactive oxygen species (ROS) that are exposed to in their extreme environments (Velho-Pereira *et al.*, 2015). They also produce cytotoxic substances to combat against predators and pathogens in their habitat. It is suggested that some of these secondary metabolites are produced by their symbiotic bacteria however for the confirmation of the above mentioned hypothesis comprehensive studies need to be done (Nyholm and Graf, 2012). Sea cucumbers integrate close interaction with bacterial communities in marine microenvironments through ingestion and filtration of marine sediments and seawater respectively (Slater *et al.*, 2011). Consequently, this complicated interaction facilitate nutrient digestion for sea cucumbers and modulate their immune and defensive responses (Amaro *et al.*, 2012; Amaro *et al.*, 2009; Hess *et al.*, 2011; Ray *et al.*, 2012; Warnecke *et al.*, 2007). Many researchers have reported the presence of diverse groups of bacteria including: *Proteobacteria*, *Bacteroidetes*, *Firmicute* and *Actinobacteria* from sea cucumbers (Gao *et al.*, 2014). Among them, *Actinobacteria* have outstanding position in the microbial biotechnology. These group of bacteria exhibited high physiological diversity and produce almost half of the discovered bioactive metabolites which are reported as antitumors, antibiotics, antioxidants, enzymes and other pharmaceutical natural products (Manivasagan *et al.*, 2014). Many anticancer drugs including anthracyclines, peptides, enediynes, aureolic acids, antimetabolites, carzinophilin, mitomycins were originated from Actinobacteria (Newman and Cragg, 2007; Olano *et al.*, 2009). In relation to antioxidant compounds, Motohashi *et al.* (2010) have isolated two newly modified Indole-containing peptides, JBIR-34 and JBIR-35, from a sponge-associated *Streptomyces* species. The *Streptomyces*, *Amycolatopsis*, *Micromonospora*, *Saccharopolyspora* and *Actinoplanes* genera are the major bioactive producing Actinobacteria (Subramani and Aalbersberg, 2012). Among them the members of *Streptomyces* members produce about 80% of the discovered natural products from actinobacteria (Watve *et al.*, 2001). Although, those organisms are interesting for scientists because of their ecological roles and their taxonomy, but very limited research have focused on the bacterial flora of the sea cucumbers and their product's bioactivities. Among the very limited reports, Ye *et al.* (Ye *et al.*, 2016) isolated a new cytotoxic curvularin glycoside which is synthesized by a marine actinobacterium: *Pseudonocardia* sp. HS7. In addition to the above report, a novel lineage of

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Actinobacteria, *Iamia majanohamensis* was isolated from abdominal epidermis of a sea cucumber: *Holothuria edulis* (Kurahashi *et al.*, 2009). Therefore, considering the importance of the actinobacterial flora of the sea cucumbers prompted us to isolate and identify the marine bacteria from *H. leucospilota* and evaluate their exudate in their culture media for antioxidant and cytotoxic activities.

Materials and methods

Sample location

The samples were collected from Larak Island, which is located at 26° 82′ 33"N latitude and 56° 33′ 46" E longitude in the Persian Gulf (Fig. 1).

Figure 1: Sample location in Larak Island, Persian Gulf (Google Maps, 2014).

Sample collection and isolation

Ten live samples of sea cucumber of the species *H. leucospilota* were collected from Larak Island, Persian Gulf, by scuba diving at a depth of 10-15 meters in February 2016. The animals were dissected and the samples were collected from their intestine contents after rinsing with sterile seawater, they were dissected and the samples were collected from their intestine contents. One gram of intestine content was serially diluted with sterile-filtered seawater and then treated by heating at 55 °C for 6 min (Jensen *et al.*, 2005). Then 100 µL of the treated samples were inoculated on starch casein nitrate agar medium which was prepared with seawater and supplemented with nalidixic acid (20 mg L^{-1}) and cycloheximide (100 mg L^{-1}) (Maldonado *et al.*, 2005). The plates were incubated at 28 °C for 4 weeks and the colonies were picked up and purified by sub-culturing onto the same media. Then, the suspected colonies were preliminarily characterized using macroscopic and microscopic observations and they were purified for further experiments (Goodfellow *et al.*, 2012).

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Production and extraction of bioactive metabolites

The isolates were inoculated in marine broth medium (Himedia) and incubated at 28 ˚C under shaking incubator (220 rpm) conditions for 5 days. The filtrated fermentation broths were extracted with ethyl acetate twice (1:1 v/v) and after evaporation of the solvent, the culture extracts were kept for the subsequent experiments (Bucar *et al.*, 2013).

Antioxidant bioassay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity was assayed according to microdilution method (Leong and Shui, 2002). The ethyl acetate culture extracts were diluted in methanol at seven final concentrations (1250, 625, 312, 156, 78, 39, 19.5 µg mL^{-1}). Five microliters of each concentration were added to 195 µL of DPPH solution at 100µM concentration in methanol. The microplate was incubated at room temperature in the dark place for 30 minutes. The absorbance of each well was measured by microplate reader (BioTech instrument) at 517 nm. Ascorbic acid was used as standard control and the scavenging activity of the samples was calculated using the following equation:

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

I_0 is the absorbance of 195 µL DPPH plus 5 µL methanol

I_s is the absorbance of sample or standard control

Finally, IC_{50} of potent isolate crude extract was calculated by the software GraphPad PRISM version 6 (GraphPad Software, San Diago, CA).

Brine-shrimp cytotoxicity assay

The cytotoxic activity of all Actinobacterial extracts was screened by Brine-shrimp micro well cytotoxicity method (Atta-ur-Rahman, 2001). The ethyl acetate culture extracts were diluted to four final concentrations in seawater (1000, 500, 250, 125 µg mL^{-1}). Water insoluble extracts were dissolved in 1mL DMSO prior to serial dilution. 100 µL of *Artemia franciscana* nauplii suspension (10-15 nauplii 100 μL^{-1}*)* was added to 100 µL of each dilution in each 96-well microplate. After incubation at 25°C for 24 hours, the number of live and dead nauplii was recorded and LC_{50} of each crude extract was calculated.

Cytotoxicity assay

Cytotoxic effect of the potent crude extract against human colon cancer cell lines (HCT 116) and (SW 480) was determined by MTT cell proliferation assay (Peng and Zhao, 2009). Four different final concentrations (100, 50, 25, 12.5 µg mL^{-1}) were prepared by two-fold serial dilution in the respected culture media. 100 µl of HCT 116 or SW 480 was cultured in 96-well microplates at a density of 10^4 cells per well in 100 µL complete DMEM or RPMI 1640 media, respectively. The cells were incubated at 37°C for 24 h in a humidified 5% CO_2 atmosphere. Then the cultured cell lines were treated with 100 µL of each concentration of crude extract and incubated for an additional 48 h. After the incubation period 20 µL of the MTT solution (5 mg mL^{-1}) was added to each well and incubated for another 4 h. The formazan crystal were

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dissolved in 150 µL DMSO per well. The absorbance of each well was measured at 570 nm by a microplate reader (Micura, England) and cell viability was calculated according to the following formula: Dose-response curve and IC_{50} related to cytotoxic effect of extracts were calculated using GraphPad PRISM 6 software.

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

A_{test} is the absorbance of treated well

A_{Blank} is the absorbance of culture medium well

$A_{control}$ is the absorbance of untreated well

Identification of the isolate

Morphological, biochemical and physiological identification

Structure and arrangement of spores and mycelia were determined by cover slip method. Growth properties such as color of mycelia, spores and pigment production were visually monitored (Goodfellow *et al.*, 2012; Shirling and Gottlieb, 1966). Biochemical and physiological identification of the specific isolates was performed by International Streptomyces project media (ISP) (Shirling and Gottlieb, 1966). Assimilation of carbohydrates was tested by growth on ISP 9 medium supplemented with 1% various carbon sources. Utilization of nitrogen sources, H_2S and enzyme production were evaluated according to the method described by Williams (Williams, 1989) and the growth temperature, pH and salinity (0-15%) ranges were determined on ISP II medium.

Chemotaxonomical analysis

Determination of diaminopimelic acid (DAP) isomers in the whole cell hydrolysates were analyzed by thin layer chromatography (TLC) procedure (Goodfellow *et al.*, 2012; Staneck and Roberts, 1974).

Molecular identification and phylogenetic analysis

Genomic DNA of putative isolate was extracted according to CTAB procedure described by Kieser (2000). Consequently, the 16S rRNA gene was amplified by PCR using universal primers 27F and 1492R as described by Desai *et al.* (2016). Thermal cycle reactions were as follows: (94°C for 4 min, 94°C for 1 min, 60°C for 1 min and 72°C for 2 min) ×35 cycles followed by final extension at 72°C for 10 min. After purification, amplified 16S rRNA gene was sequenced by Macrogen (Seoul, Korea). The 16S rRNA sequences were compared to same genes deposited in NCBI (National Centre for Biotechnology Information) by blastn program (Zhang *et al.*, 2000). The sequences were aligned with most similar 16S rRNA gene sequences in Genbank and a phylogenetic tree was constructed using MEGA 7 program (Kumar *et al.*, 2016) according to the neighbor joining model. The 16S rRNA gene sequence was registered to NCBI GenBank database with the following accession number KY249897.

Statistical analyses

All of the experiments were implemented in triplicates. The results

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of isolation were reported as percentage. The results of assays were expressed as mean±standard deviation (SD). IC_{50} and LC_{50} values and their 95% confidence intervals were analyzed by non-linear regression using GraphPad Prism 6. Statistical analyses were conducted using Microsoft ™ Excel 2013 statistical software (Microsoft, Seattle, WA). The statistical significance of the resultant phylogenetic tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1000 replicates with MEGA7.

Results

A total of 17 distinct Actinobacteria colonies were recovered from the intestine of ten *H. leucospilota* samples. Among the isolated Actinobacteria, 12 isolates (71%) were determined as *Streptomyces* genus and five non-*Streptomyces* genus isolates (29%) were characterized (Figure 2). Actinobacteria isolates exuduted their secondary metabolites in marine broth medium during their incubation period. Antioxidant bioassay of all ethyl acetate extracts revealed that SC 156, SC 160 and SC 166 isolates could scavenge DPPH radicals with IC_{50} values of 211.2, 686.3 and 822.5 (µg mL^{-1}), respectively (Table 1, Fig. 3). Among all Actinobacteria isolates only SC 156 showed cytotoxic activity against *Artemia franciscana* nauplii with LC_{50} value of 446.7 µg mL^{-1}. Therefore, SC 156 isolate was selected for cytotoxicity assay against tumor cell lines. The crude extract of SC 156 showed a concentration dependent effect against human colon cancer HCT 116 and SW480 cell lines with the IC_{50} values of 26.48 and 18.53 µg mL^{-1}, respectively (Fig. 4). Microscopic observation showed the dramatic decrease in viability and morphological changes of the cancerous cells (Fig. 5). After preliminary characterization of the isolated Actinobacteria, the SC 156 isolate was identified according to biochemical, physiological and molecular methods. The cultural characteristics of SC 156 are illustrated in Table 2. Chemotaxonomical investigation revealed that SC 156 contained LL- diaminopimelic acid in its cell wall. Biochemical and physiological experiments showed that SC 156 isolate utilized all tested carbon sources except raffinose and sucrose. SC 156 isolate assimilated arginine and asparagine as sole nitrogen source, but it could not utilize valine and ornithine. This potent isolate produced diffusible pigment in the absence of melanoid ones. SC 156 showed mesophilic growth condition and was able to grow at pH 5-9. Moreover, this isolate tolerated NaCl concentration up to 10% with capability to produce catalase and nitrate reductase (Table 2). Furthermore, SC 156 isolate showed 98 % similarity with *Streptomyces chartreusis*, *S. variabilis* and *S. labedae* (Table 3). Interpretation of the constructed phylogenetic tree based on 16S rRNA gene demonstrated that SC 156 strain positioned into the same clade by their most closely type strains although SC 156 strain developed a different lineage along with the most similar strains (Fig. 6).

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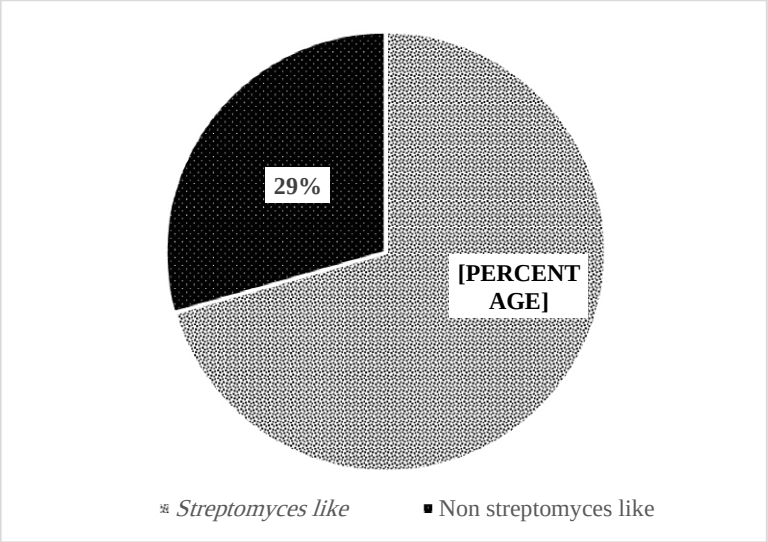

Figure 2: Percentage of isolated Actinobacteria from *Holothuria leucospilota.*

Table 1: Antioxidant activity of isolated Actinobacteria from *Holothuria leucospilota* .

Isolate No	DPPH radical scavenging activity IC_{50} (µg mL^{-1})	Isolate No	DPPH radical scavenging activity IC_{50} (µg mL^{-1})
SC 150	>1250	SC 159	>1250
SC 151	>1250	SC 160	686.3 (543.0-882.4)
SC 152	>1250	SC 161	>1250
SC 153	>1250	SC 162	>1250
SC 154	>1250	SC 163	>1250
SC 155	>1250	SC 164	>1250
SC 156	211.2 (167.7-254.7) *	SC 165	>1250
SC 157	>1250	SC 166	822.5 (667.9-1040.7)
SC 158	>1250		

*95% Confidence Intervals

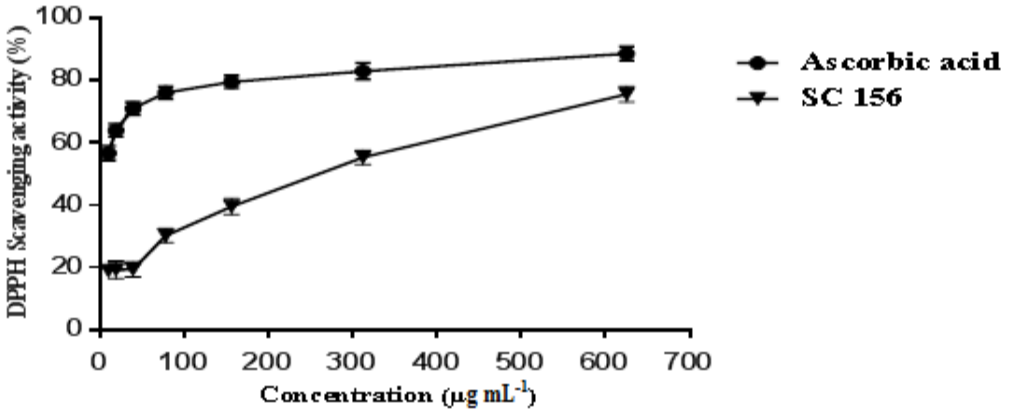

Figure 3: Dose -response curve for DPPH radical scavenging activity of SC 156 crude extract compared with ascorbic acid.

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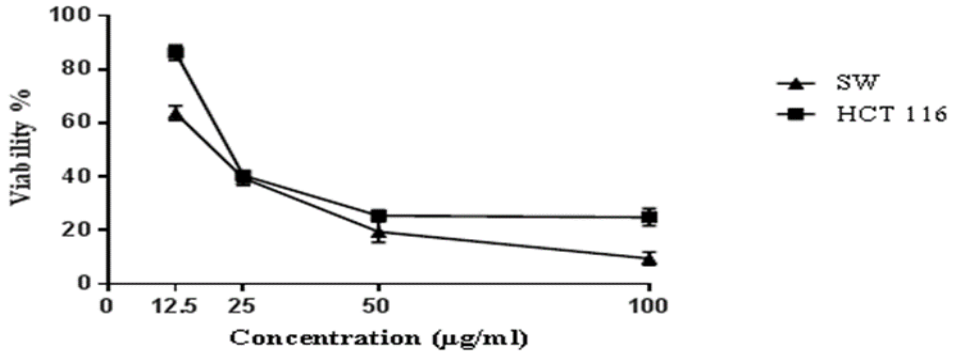

Figure 4: Dose -response curve for cytotoxic activity of SC 156 crude extract against SW 480 and HCT 116 tumor cell lines.

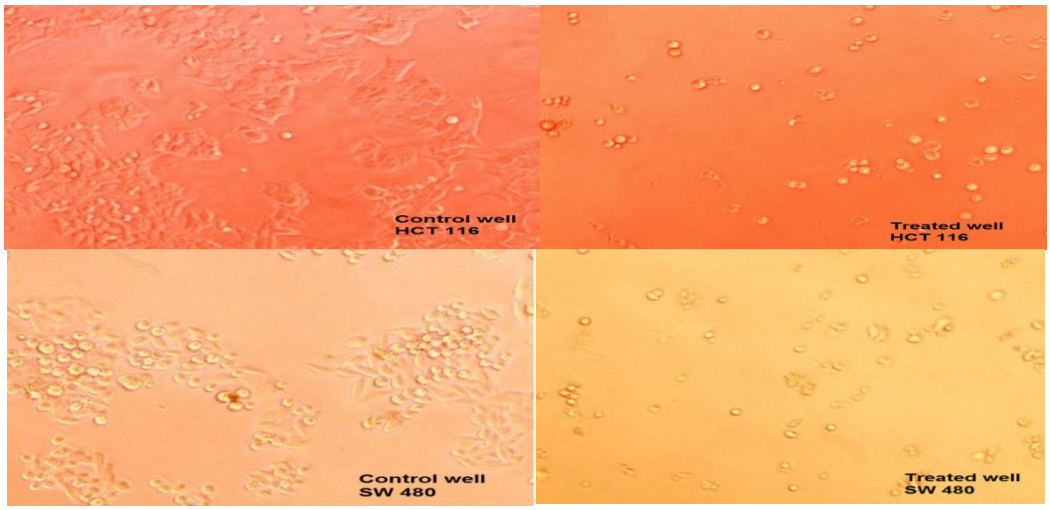

Figure 5: Cytotoxic activity of SC 156 crude extract against SW 480 and HCT 116 tumor cell lines (Control wells were shown on the left).

Table 2: Morphological, biochemical and physiological characterization of SC 156 isolate.

Characters		Results	Characters		Results
Color	Vegetative mycelia	Gray	Nitrogen sources utilization	Valine	-
	Aerial mycelia	Gray		Arginine	+
Growth and sporulation on	ISP II	Good		Ornithine	-
	ISP III	Good		Asparagine	+
	ISP IV	Good	Carbon sources utilization		
	ISP V	Good		Glucose	+
	ISP VI	Good		Fructose	+
	ISP VII	Good		Xylose	+
	CDA	Good		Arabinose	+
	BA	Good		Rhamnose	+
	NA	Good		Sucrose	-
Spore morphology		Spirals		Raffinose	-
Melanoid pigment		-		Galactose	+
Diffusible pigment		+		Manitol	+
H2S production		-		Inositol	+
Catalase production		+	Growth temperature range		10-40 °C
Oxidase production		-	Growth pH range		5-9
Nitrate reduction		+	NaCl Tolerance		<10%
DAP-isomer		LL			

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Table 3: Comparison of 16S rRNA gene sequence homology between *Streptomyces* sp SC 156 and closest type strains in NCBI database.

Description	Max score	Total score	Query coverage	E value	Ident	Accession
Streptomyces chartreusis strain NBRC 12753	1847	1847	95%	0.0	98%	NR_118341.1
Streptomyces variabilis strain NRRL B-3984	1847	1847	95%	0.0	98%	NR_043840.1
Streptomyces labedae strain NBRC 15864	1847	1847	95%	0.0	98%	NR_041192.1
Streptomyces erythrogriseus strain NBRC 14601	1847	1847	95%	0.0	98%	NR_112438.1
Streptomyces matensis strain NBRC 12889	1847	1847	95%	0.0	98%	NR_041088.1
Streptomyces griseoincarnatus strain NBRC 12871	1847	1847	95%	0.0	98%	NR_112312.1

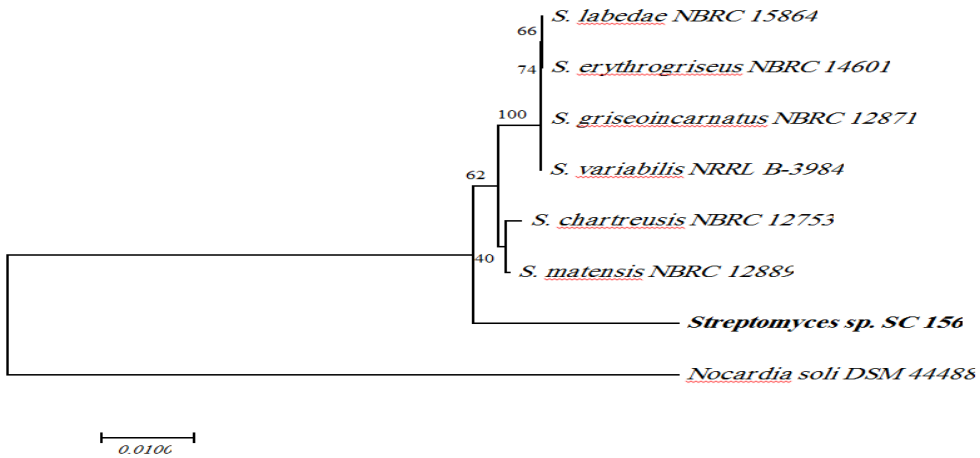

Figure 6: Phylogenetic tree based on 16S rRNA gene sequence analysis, reconstructed from evolutionary distances by using the neighbor-joining method, showing the phylogenetic position of potent strains and the most closely related strains. N. soli was used as an out-group. Bootstrap values are indicated at the relevant branching points. Numbers of branch node are bootstrap value based on 1000 resampling. Bar: 0.01 substitutions per nucleotide position.

Discussion

Different researchers are interested in sea cucumbers because of their great potential in producing bioactive natural compounds, particularly anticancer agents (Liu *et al.*, 2016; Wijesinghe *et al.*, 2013). Their symbiotic bacteria, especially Actinobacteria have integral role in the sea cucumber digestive tract and this make them interesting for research of their bioactivities. We have purified 17 Actinobacteria isolates from the intestine of *H. leucospilota* collected from the Persian Gulf. while Zhang *et al.* (2012) characterized 55 bacterial species in *H. leucospilota*, most of which belonged to *Vibrio* and *Bacillus.* Whereas, in the present study application of the selective isolation growth media and the heat treatment reduced non-actinobacterial isolates like Proteobacteria and Firmicutes drastically. In addition, in this project the vast majority (71%) of the isolated Actinobacteria belonged to *Streptomyces* species, which is comparable to previous reports obtained from marine sediments of the Persian

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Gulf (Gozari *et al.*, 2016a; Gozari *et al.*, 2016b). It is reported that there is high similarity between bacterial composition of the sea cucumbers intestine and their surrounding sediments except some minor changes (Gao *et al.*, 2014). Radical scavenging activity of the culture extracts of the isolates showed that three *Streptomyces* isolates could scavenge DPPH radicals with IC_{50} values under 1 mg mL^{-1}. Among these isolates, SC 156 exhibited the lowest IC_{50} value of 211.2 µg mL^{-1}. Furthermore, antioxidant activity of SC 156 isolate was dose dependent, and gradually increased with elevation of concentration. In this context, Poongodi *et al.*(2014) showed that a marine Actinobacterium, *Nocardiopsis* sp., which is isolated from sediment samples of Mannar Gulf had the ability to produce a strong antioxidant compound with IC_{50} value of 58.2 µg mL^{-1}. On the other hand, the cytotoxicity assay played an important role in biodiscovery studies for new marine anticancer drugs during the past 30 years (Hu *et al.*, 2015). Therefore, in this project, the cytotoxic activity of SC 156 crude extract by MTT cell proliferation was evaluated and the results indicated that the SC 156 crude extract expressed high cytotoxic activity with the IC_{50} values of 26.48 and 18.53 µg mL^{-1} against HCT 116 and SW 480 cell lines respectively (Fig. 4). However, a new Actinobacterium species, *Salinospora arenicola* CNR-005 which is isolated from marine sediments produced arenicolides. Arenicolide exhibited cytotoxicity toward HCT 116 cell line with an IC_{50} of 30 µg mL^{-1} (Williams *et al.*, 2007). In this regard, other scientists showed that *Pseudonocardia* sp. HS7 *moebii* also produced a new curvularin glycoside with IC_{50} values ranging from 0.59 to 3.39mM against six cancer cell lines (Ye *et al.*, 2016). Polyphasic identification of SC 156 isolate confirmed the preliminary characterization results. Comparison of phenotypic characteristics of SC 156 with the closest 16S rRNA sequence type strain *S. chartreusis*, revealed that SC 156 could not utilize raffinose and sucrose whereas the *S. chartreusis* metabolizes these carbon sources (Goodfellow *et al.*, 2012). Moreover, SC 156 did not produce melanoid pigment and grew in salinities of up to 10%. These physiological differences clearly differentiate the SC 156 from *S. chartreusis* hence it might be considered as a candidate for a new species. Phylogenetic analysis showed that SC 156 developed a disparate lineage along with the most closely type strains, therefore, any mutation could happen during the adaptation process of the isolate with marine microenvironment conditions (Orr, 2005). Accordingly, it is completely possible that these genetic changes took place in biosynthetic gene clusters and developed or modified its biosynthetic pathways. Hence, it is essential to elucidate the structure of cytotoxic produced compound by SC 156 strain to determine its novelty. Finally, it could be concluded that *H. leucospilota* can be consider as a source of bioactive-producing Actinobacteria. Likewise, SC 156 is a promising strain

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for the discovery of novel cytotoxic agents. Purification and structural elucidation of the antioxidant and cytotoxic compounds are under investigation. This study also revealed valuable information about the diversity of culturable actinobacteria in sea cucumber *H. leucospilota.*

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