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Fermentation, Extraction and Characterization of Antimicrobial Agent from Marine *Saccharomonospora viridis* AHK 190

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STUDY is aimed to ferment, isolate and characterize a significant antimicrobial metabolite from the marine thermophilic, *Saccharomonospora viridis* AHK 190. Maximum yield of a wide spectrum antimicrobial agent was optimized and fermented by *Saccharomonospora viridis* AHK 190. Extraction, purification and molecular formula elucidation of the fermented agent were also assayed. A diversity of human microbial pathogens was applied as indicators in the antibiosis assay. Thin layer chromatography technique was employed for partial purification of the antimicrobial agent. Spectral and elemental analysis studies were as well screened for molecular formula elucidation of the purified compound. Actinomycete optimum growth and maximum antibiotic fermentation were confirmed after 3 days of incubation at 55^{o}C under the effect of pH at 8.0 and shaking at 180 rpm. In addition, maximum antibiotic production was achieved with galactose, aspartic acid, 3.0% NaCl and 0.11% K_2HPO_4; while pyridoxine, starch, 5.0% NaCl and 0.11% K_2HPO_4 encouraged the actinomycete optimum growth. Elucidated molecular formula of the under-test antimicrobial compound was found as $C_{31}H_{34}O_4S_3$ of molecular weight = 567. It is of a new chemical formula different from other current main classes of antibiotics.

Keywords: Marine actinomycetes, Antitumor activity, *Saccharomonospora viridis*, Antibiosis.

Introduction

Actinomycetes are continuous bio-sources for active metabolites; they are able to produce cosmetics, vitamins, nutritional materials, herbicides, antibiotics, pesticides, antitumor agents, immunosuppressive agents, anti-parasitic and enzymes. Variable studies were screened for bioactive applications of these valuable marine and terrestrial prokaryotic organisms (Reddy et al., 2011; Valli, 2012; Sharma, 2014; Kafilzadeh & Dehdari, 2015 and Sheik et al., 2017). It is presumed that marine microorganisms exhibit unique structural and bioactive properties that encourage their survival in extremes of pressure, salinity and temperature. Marine mesophilic actinomycetes were investigated for different types of novel bioactive secondary metabolites not observed in terrestrial inhabitant microorganisms (Abdelfattah et al., 2012; Sengupta et al., 2015; Subramanian et al., 2017 and Tuncer & Bizsel, 2017). In fact, antibiotic substances from thermophilic marine actinomycetes were not focused in review of literature (Ibrahim, 2017); so it was selected as our study target. Polysaccharides, proteins, nucleic and fatty acids are primary metabolites common in all biological systems. However, secondary metabolites are of low molecular weight and highly diversified chemically and taxonomically with mysterious function, characteristic mainly to some specific, distinct types of organisms (Berdy, 2005). They include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immuno-modulating agents, receptor antagonists' and agonists, pesticides, antitumor agents and growth promoters of animals and plants (Demain, 2000). Secondary metabolites amazing structural diversity makes them a promising field for structure elucidation and synthesis. Synthesis of secondary metabolites is detected under suboptimal growth conditions in the stationary phase or near the exponential growth phase end. Antibiotic compounds may fermented by living organisms primary or rather a secondary metabolism. Fermentation is a term has been extended by industrial microbiologists to characterize any process for product production by the mass culture of microorganism (Stanbury et al., 1995). Antibiotics are compounds that inhibit the microbial growth or kill other microbiota; moreover, they proved to have a cytotoxic activity (Soria et al., 2005 and Gamal, 2016).

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The present study was undertaken to ferment a wide spectrum antimicrobial agent by marine *Saccharomonospora viridis* AHK190 under the optimum conditions of variable cultural and environmental parameters; as well extraction, purification, characterization and structure elucidation of the purified compound were investigated.

Materials and Methods

Antibiosis Test microorganisms

Fusarium moniliform (ATCC/60858), *Trichoderma lignorum* (ATCC/1313), *Penicillium chrysogenum* (ATCC/11709), *Aspergillus terreus* (ATCC/28301) and *Candida albicans* (ATCC/10231), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC/27853), *Pseudomonas aeruginosa* (ATCC/15442), *Bacillus cereus* (ATCC /10876) and *Bacillus subtlis* (ATCC 14028) were the indicator strains of antibiosis activity evaluation.

Bacterial strains were cultivated overnight at 37°C on nutrient broth (NB) (diffco) for 24 h; while fungal strains at 28°C on Sabaroud's broth (diffco) (SB) for 4 d for fungi. Cultures were then standardized to O.D. of 0.1 at 620 and 530 nm for bacteria and fungi, respectively and were used as inocula for further studies.

Optimization of antibiotic fermentation

Antimicrobial metabolite fermentation from *Saccharomonospora viridis* AHK190 was optimized under the effect of variable environmental and cultural parameters (Table 1). The marine actinomycete strain was previously isolated and studied by Ibrahim (2017); it registered a wide spectrum antimicrobial activity. It was obtained and cultured on a starch casein agar (SCA) with rifampin: 5.0 mg L^{-1}, nystatin: 50 mg L^{-1} and artificial sea water (Ibrahim, 2017) for 3.0 d at 55°C. After the incubation period, spores were harvested and homogenized in 0.5% tween 20 (0.2 OD) then used as inocula. Starch casein broth SCB was applied as the antibiotic fermentation medium; inocula of 1.0 ml spore suspension /100 ml SCB were applied for each under-test parameter. Sets of 250 ml conical flasks (in triplicate) containing 100 ml SCB/flask were sterilized and examined successively under the effect of the estimated optimum level of the former tested parameter (s) jointly with the current one. After the incubation period, actinomycete broth cultures were filtered using Whatman No.1 filter paper. Then, *Saccharomonospora viridis* AHK190 growth was estimated as dry mycelial weight in a fixed volume of culture broth by drying the cell mass in a hot air oven at 80°C for 18-24 h (Kandula & Terli, 2013); and expressed as growth dry weight (mg/100 ml culture). Antibiosis activities of the filtrates were assessed based on agar-well diffusion test (ADT) in accordance with the Clinical and Laboratory Standards Institute (CLSI) and growth inhibition zone diameter (mm). The parameters corresponding to the highest growth dry weight of actinomycete strain and/or widest inhibition zone diameter were indicated as the optimum conditions.

TABLE 1. Tested environmental and nutritional parameters for optimization of antibiotic production by *Saccharomonospora viridis* AHK190..

	Factor	Factor variations
Environmental factors	Incubation period (h.)	24-48-72-96-120-144
	Temperature (ºC)	35-40-45-50-55-57-60
	Shaking (rpm)	60-100-140-180-220
	pH	6-6.4-6.8-7.2-7.6-8-8.4-8.8-9.2
Nutritional factors	Carbon source (1.0g /100 ml)	arabinose-fructose-galactose-glycerol-lactose-maltose-mannitol-mannose-meso-inisitol-starch-sucrose-xylose-glucose-raffinose-inulin-control**
	Nitrogen source (at equimolecular amount)*	ammonium nitrate-ammonium sulphate -potassium nitrate -sodium nitrate-urea- aspartic acid-glutamic acid-phenyl alanine- glycine-pyridoxine-beef extract-peptone- no nitrogen source (basic medium starch casein without potassium nitrate)
	NaCl concentration (%)	0-2-3-5-10-15-20
	K_2HPO_4 Concentration (%)	0.001-0.003-0.006-0.009-0.011-0.013-0.015

*: at equimolecular amount with 2.0 g KNO_3
**: basic medium with no carbon source (starch casein agar without starch)

Statistical analysis

The means, standard deviations and least significant difference (LSD at 5%) of the inhibition zones diameters and growth dry weights were calculated. After testing the data for normality, it was analyzed by analysis of variance (ANOVA). The significance of variations was assessed according to SPSS software (SPSS, 2006).

Fermentation and extraction

Under achieved optimum fermentation conditions, a total of 8 liters SCB was inoculated by previously prepared spore suspension of actinomycete strain (5.0 ml inoculum/100ml SCB) then incubated. After the incubation period, crude metabolites were extracted and the dried solvent extract was bio-assayed for antibiosis activity and conducted for purification analysis via thin layer chromatography technique.

Ten variable organic solvents/solvent systems: Ethyl acetate, diethyl ether, benzene, petroleum ether, chloroform, toluene, benzene:chloroform (3:1) (v/v), benzene:methanol (19:1) (v/v) and n-hexane:ethyl acetate (1:1) (v/v) were screened for the proper extractant; 20 ml out of previously performed culture supernatant was extracted through manual shaking thrice with equal volume of the tested solvent. Each organic solvent layer was collected, dried over anhydrous $NaSO_4$, desorbed in methanol, concentrated and tested for antibiotic activity. The organic solvent which produced a crude extract of the widest inhibition zone diameter was selected for extraction assay.

Purification by thin layer chromatography

Partial purification assay of crude solvent-extract was undertaken by analytical ascending thin layer chromatography (TLC) technique on 1.0 mm thick silica gel 60 (Merck Ltd.). Elution was optimized with 8 different running organic solvent/solvent system(s) (v/v); toluene: petroleum ether (3:1), toluene:chloroform (3:1), toluene:chloroform (3:2), toluene:chloroform (1:1), toluene:ethyl acetate (1:1), toluene:acetone (1:1) and toluene:glacial acetic acid (1:1). TLC plates were prepared, spotted with the crude extract sample, developed by running the mobile phase then observed under UV light at 254 and 365 nm (Model, MOPEL ENF-260C, New York, U.S.A). After ultraviolet light visualization, spots were separately scraped off carefully, pooled, dissolved in methanol, centrifuged at 10,000 g for 10 min to remove silica and concentrated; antimicrobial activity was then evaluated. Active TLC spot-extract was conducted to further studies.

Physical properties

Physical properties such as colour, consistency, melting point and solubility of the TLC spot-extract were surveyed.

Spectroscopic analysis

The TLC spot-extract was inspected under spectroscopic studies. Infrared spectra were measured on Nicolet IS10 FT-IR spectrophotometer and twelve libraries were searched for matches: Georgia state crime Lab sample libraries, HR Nicolet sampler libraries1-6, Aldrich condensed phase sample libraries 1&2, Hummel polymer sample libraries and Siga biological sample libraries 1&2 (Egyptian Petroleum Research Institute, Cairo, Egypt). UV-visible absorption spectra were recorded on UV-1600 spectrophotometer (Micro analytical Center, Faculty of Science, Cairo University, Giza, Egypt). Proton magnetic resonance (^{1}HNMR) was measured on Varian Genini-300 MHz spectrophotometer (Micro analytical center, Faculty of Science, Cairo University, Giza, Egypt). Mass spectrum (EI-MS) was measured on HP MODEL GC MS-QPL000EX mass spectrometer (Shimadeu) at 70 eV (The national research center, Giza, Egypt). Elemental analysis of C, H, N, and S was carried out in the Micro analytical centre, Faculty of Science, Cairo University, Giza, Egypt.

Minimum inhibitory and bactericidal concentrations

Determination of both minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations of the under-test substance was designed in accordance with (CLSI). A 100 mg of the substance was dissolved in 10 ml of 20% Dimethyl sulfoxide (DMSO) to get a stock solution of concentration 10 mg ml^{-1}. Bacterial broth cultures were prepared and subsequently each one was diluted 1:100 in nutrient broth (NB) and again cultivated until it reached the exponential growth phase then standardized to 0.1 O.D.at 620 nm. Then, the substance stock was diluted using the standardized bacteria as a diluent to obtain different concentrations (from 1000 to 62.5 µg ml^{-1}) following two-fold serial dilution method in accordance with (CLSI). Diluted stocks were incubated at 37°C for 24 h; MIC values of the pure antibiotic compound produced was measured by visual comparison method expressed in turbidity differences between

stock cultures as a result of bacterial growth. MIC is the lowest concentration of a substance showing clarity in which growth is not observed and was expressed as µg ml^{-1}. MBC of the pure compound was also evaluated by inoculating sterile NA plates from clear tubes that showed no growth via streak manner. The lowest antibiotic concentration causing no growth was considered as the MBC and expressed as µg ml^{-1}.

MIC was also screened against variable fungal strains. The antifungal assay was assessed based on ADT and inhibition zone evaluation. A two-fold serial dilutions of the stock substance were prepared using sterile saline solution as a diluent. A 100 µl of each dilution was transferred into a hole made in Sabaroud's agar (SA) plate already seeded with 100 µl of a fungal inoculum. Inoculated plates were incubated at 30°C for 4.0 d; obtained fungal inhibition zones and MIC were recorded.

Antitumor activity assay

Antitumor activity of the partial purified substance was measured using a colourimetric cytotoxicity assay (Skehan et al., 1990 and Soria et al., 2005). HCT-116 colon carcinoma cell line was collected from a culture at exponential phase; it was counted and plated for 24 h as a cell monolayer in 96 multi-well plates (104 cells/well). Then different concentrations of the purified compound (0, 5, 12.5, 25 and 50 µg L^{-1}) were added to the wells in triplicate. After 48 h of incubation at 37°C in an atmosphere of 5.0% CO_2, cells were fixed, washed then stained with Sulfo-Rhodamine-B (SRB) stain. Excess stain was washed with acetic acid and the attached stain was soaked with Tris–EDTA buffer. Colour intensity was evaluated by using ELISA reader. The relation between survival fraction and antibiotic concentration was plotted to get the tumour cell line survival curve after the specified antibiotic dilution. The LC_{50} (the minimum concentration which reduces the initial cell number to half) was calculated.

Results

Optimization of antibiotic fermentation

Environmental parameters

Collected data showed that the most significant growth and antibiotic fermentation by *Saccharomonospora viridis* AHK190 were noticed after 3.0 d of incubation at 55ºC under shaking at 180 rpm and pH 8.0 (Table 2 and Fig.1). *Saccharomonospora viridis* AHK190 exhibited a narrow range of incubation temperature for a relatively good growth and antibiotic production.

Cultural parameters

The effect of cultural parameters on *Saccharomonospora viridis* AHK190 growth and antibiosis bioactivity (Table 2 and Fig.1) revealed that optimum antagonistic action against all tested bacteria and fungi was evaluated at 3.0% NaCl; while it's optimum growth rate was observed at 5.0% NaCl. However, a further increase in the salt concentration reduced the antagonism behaviour. *Saccharomonospora viridis* AHK190 was able to utilize all tested carbon sources; whereas, starch stimulated highest growth intensity. Optimum antibiosis activity was reported with galactose followed by starch then raffinose; whereas, actinomycete did not show any antimicrobial activity with fructose, glycerol, lactose, maltose, mannose, glucose and inulin. On the other hand, cultures containing aspartic acid as a nitrogen source enhanced highest antibiotic activity followed by beef extract, urea and potassium nitrate. It is interesting to notice that pyridoxine and K_2HPO_4 at 0.11% were the high potent treatments either for growth or antagonism.

Statistical analysis

Antibiosis efficacy evaluations and calculated averages and SD values of estimated inhibition zone diameters and growth dry weights were illustrated in Fig. 1 and Table 2 for all screened parameters. It was found that SD values of recorded inhibition zone diameters were significantly different among all the tested parameters.

Antibiotic extraction

Solvent extraction assay indicated that toluene was the most appropriate solvent for antibiotic(s) primary extraction followed by ethyl acetate, petroleum ether then benzene extracts. Toluene extract gave the highest antagonism activities against both the tested bacteria and fungi. The obtained crude antibiotic extract was brown solid crystals.

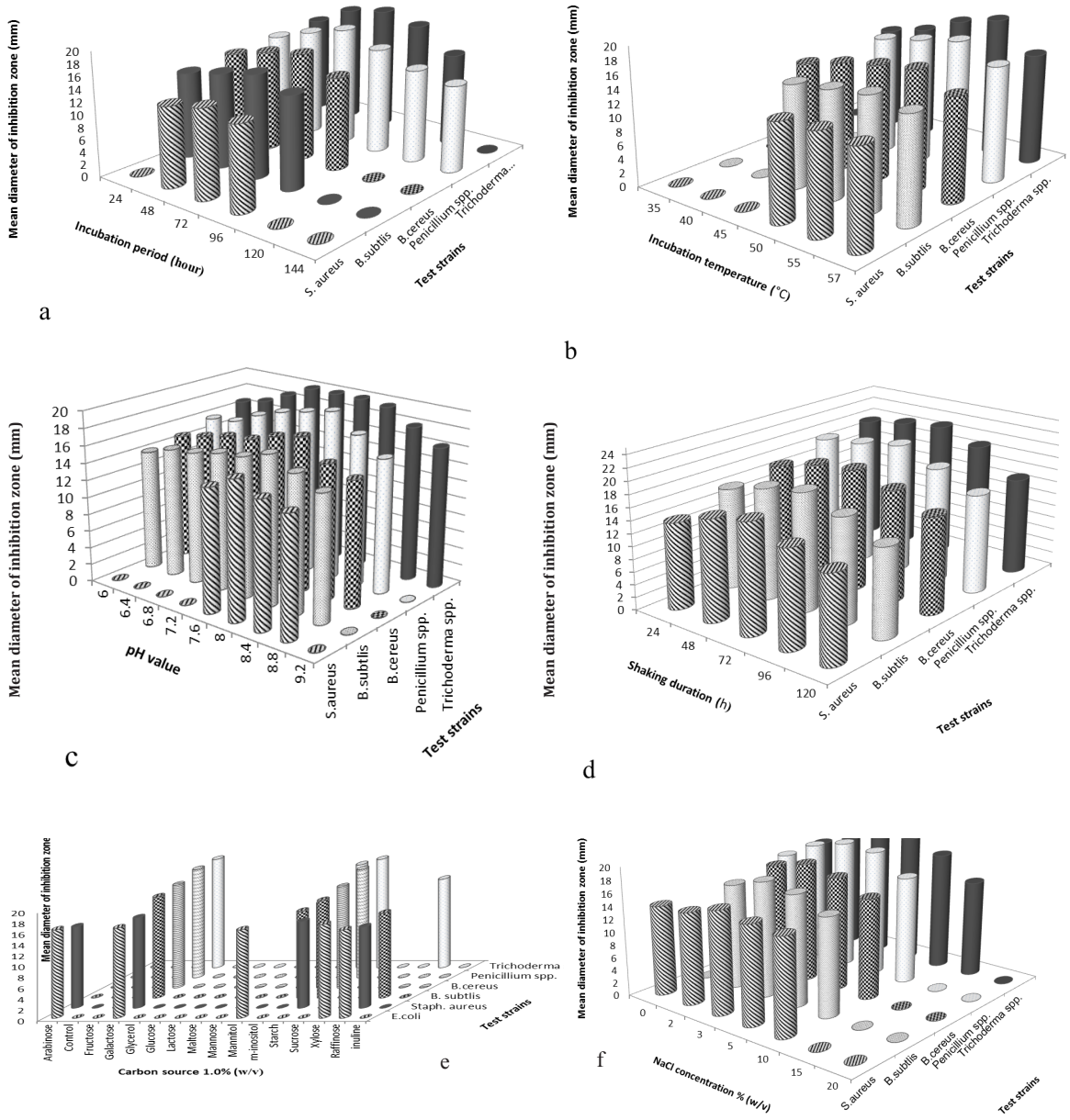
Purification by thin layer chromatography

Among the eight solvent systems applied in TLC assay, Toluene:Chloroform (3:2 v/v) was found to be the best solvent system for antimicrobial metabolites separation at Rf value = 0.95. Only one spot was developed on TLC assay under the effect of UV light at 254 and 365 nm. This active spot appeared as violet color at 254 nm and red colour at 365 nm.

TABLE 2. Effect of variable environmental and nutritional parameters on growth of *Saccharomonospora viridis* AHK 190 expressed as dry weight (mg/100ml).

Test parameter	Mean dry weight (mg/30ml)	Test parameter	Mean dry weight (mg/30ml)	Test parameter	Mean dry weight (mg/30ml)	Test parameter	Mean dry weight (mg/30ml)
Incubation period (h)		**Incubation Temperature (°C)**		**N-source (at equimolecular amount)***		**Carbon source (1%)**	
24	230.00 (±3.00)	35	220.00 (±9.53)	Ammonium sulphate	355.0 (±7.0)	Arabinose	340.00 (±7.00)
48	250.00 (±8.60)	40	260.00 (±5.56)	Potassium nitrate	350.0 (±10.00)	Control	230.00 (±14.17)
72	300.00 (±2.00)	45	320.00 (±6.55)	Ammonium nitrate	220.0 (±10.5)	Fructose	240.00 (±12.49)
96	285.00 (±5.00)	50	330.00 (±8.18)	Sodium Nitrate	330.0 (±4.0)	Galactose	340.00 (±10.00)
120	275.00 (±6.00)	55	350.00 (±6.08)	Urea	230.0 (±7.2)	Glycerol	230.00 (±5.00)
144	250.00 (±1.00)	57	250.00 (±10.00)	Beef extract	270.0 (±16.0)	Glucose	210.00 (±10.00)
LSD at 5%	65.20	LSD at 5%	74.00	Peptone	220.0 (±5.0)	Lactose	260.00 (±5.29)
Shaking (rpm)		**pH**		Pyridoxine	480.0 (±2.0)	Maltose	250.00 (±10.14)
60	114.00 (±7.50)	6	390.00 (±8.18)	Phenyl alanine	330.0 (±8.6)	Mannose	280.00 (±9.16)
100	155.00 (±7.20)	6.4	410.00 (±5.56)	Glycine	240.0 (±10.0)	Mannitol	270.00 (±5.00)
140	207.33 (±6.40)	6.8	410.00 (±2.00)	Glutamic acid	240.0 (±10.0)	Meso-inositol	450.00 (±10.00)
180	303.33 (±6.70)	7.2	420.00 (±5.00)	Aspartic acid	360.0 (±8.7)	Starch	550.00 (±4.58)
220	243.33 (±5.80)	7.6	440.00 (±8.71)	LSD at 5%	48.00	Sucrose	410.00 (±10.00)
LSD at 5%	47.65	8	460.00 (±5.29)	**K_2HPO_4 Concentration (%)**		Xylose	290.00 (±6.08)
NaCl concentration (%)		8.4	470.00 (±6.08)	0.001	220.00 (±10.0)	Raffinose	250.00 (±4.58)
0	250.00 (±8.71)	8.8	480.00 (±5.29)	0.003	240.00 (±17.3)	inulin	330.00 (±5.29)
2	320.00 (±4.35)	9.2	480.00 (±5.56)	0.006	270.00 (±9.0)	LSD at 5%	44.00
3	330.00 (±4.58)	LSD at 5%	27.00	0.009	290.00 (±10.0)		
5	330.00 (±1.00)			0.011	300.00 (±8.7)		
10	280.00 (± 5.00)			0.013	280.00 (±10.0)		
15	260.00 (± 8.00)			0.015	265.00 (±10.0)		
20	220.00 (± 5.00)			LSD at 5%	29.00		
LSD at 5%	14.00						

*: at equimolecular amount with 2.0 g KNO_3



LSD at 5% NS: non-significant value (-): not assessed

Figure symbol	Test parameter	Test strain						
		E. coli	*Staphy. aureus*	*B. subtlis*	*B. cereus*	*P. chrysogenum*	*Tricho. lignorum.*	*F. moniliform*
		LSD at 5%						
a	Incubation period	-	NS	1.20	1.95	2.01	1.62	-
b	Incubation temp.	-	NS	NS	1.54	1.17	1.86	-
c	pH	-	1.54	1.66	2.10	1.58	1.84	-
d	Shaking (Aeriation)	-	1.35	1.82	2.14	2.18	2.11	-
e	Carbon sources	NS	NS	2.19	2.88	NS	1.68	-
f	NaCl conc.	-	1.96	1.66	2.14	2.32	2.25	

Fig. 1. Effect of variable environmental and cultural parameters on antibiotic efficacy of *Saccharomonospora viridis* AHK190 against some fungal and bacterial strains

Antibiotic characterization and structure elucidation

Physical properties

The purified substance was of brown colour, crystals of melting point >360ºC, soluble in DMSO, sparingly soluble in water and insoluble in ethanol, methanol, toluene, butanol, diethyl ether and dimethyl formamide.

Structure elucidation

Results of spectral studies comprised IR, UV, ^{1}HNMR, ^{2}HNMR and Mass spectrum (MS) were represented in Fig. 2, 3, 4, 5 and 6, respectively. The infrared spectrum (KBr) of the pure substance plotted a diagnostic peak at 3396.08 cm^{-1}; it was OH group indicative. However, the peak at 1629.81 cm^{-1} was assigned to (C=O) group. The clarified peak at 1145.21 cm^{-1} was assigned to (C–S) group. The peak formed at 617.06 was assigned to aliphatic C–H bend (CH_3 attached to an aliphatic chain) (Fig. 2). Twelve libraries were screened for IR matches (Georgia state crime Lab sample libraries, HR Nicolet sampler libraries1-6, Aldrich condensed phase sample libraries 1 & 2, Hummel polymer sample libraries and Siga biological sample libraries 1 & 2) but didn't give any noticeable matches (>50%). The UV visible spectrum of the purified active compound (dissolved in DMSO) indicated the presence of conjugated structure with absorptions at λ max=268 and 230 nm (Fig. 3). The ^{1}HNMR and ^{2}HNMR spectra of the pure compound (Fig.4 and 5, respectively) confirmed the presence of OH which was observed as multiplets at 6.076 - 6.449 ppm. Methyl group protons were observed at 1.271 & 1.231 ppm. The aliphatic protons appearing at 3.651 & 4.90, 5.125 and 3.512 ppm were assigned to CH, CH_3 and CH_2, respectively. The molecular formula of the antibiotic was deduced as $C_{31}H_{34}O_4S_3$ based on the results of elemental analysis (Anal. Cal. for $C_{31}H_{34}O_4S_3$:C, 65.69%; H, 6.05%; S, 16.97%; O, 11.29; found C, 69.3%; H, 5.61%; S, 15.18%). The electron impact (EI) mass spectrum confirmed the molecular weight of the antibiotic was 567. According to the described chemical assignments that obtained from the spectral and elemental analysis, this compound is not identical with similar antibiotics described before. Its probable structure and IUPAC name is given in Fig. 7.

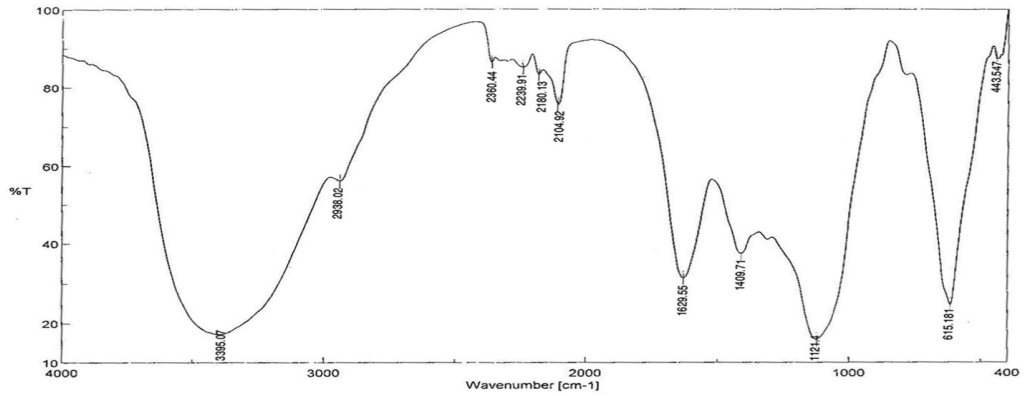


Fig. 2. Infrared (IR) spectrum (KBr) of the purified compound.

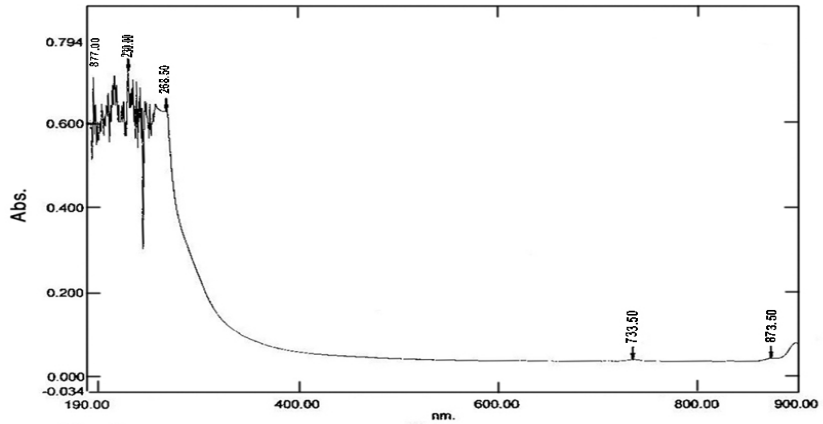


Fig. 3. Ultraviolet (UV) spectrum (DMSO) of the purified compound.

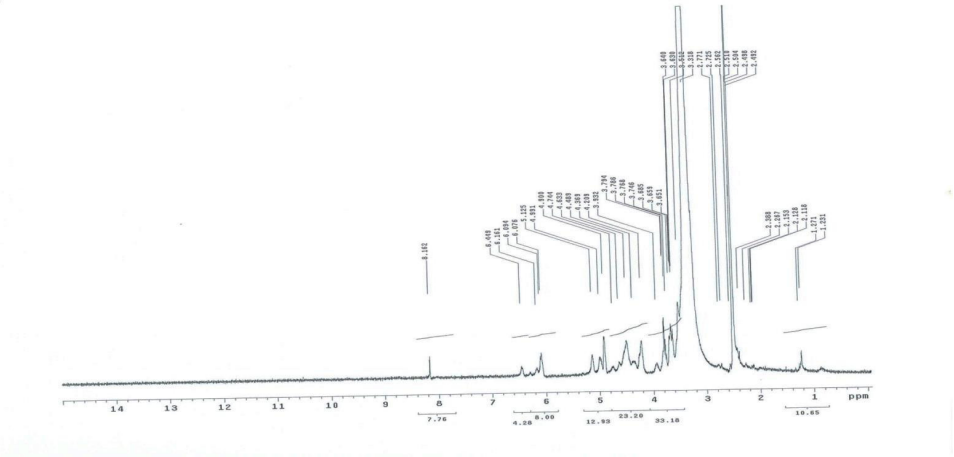


Fig. 4 . [1]HNMR spectrum (DMSO) of the purified compound.

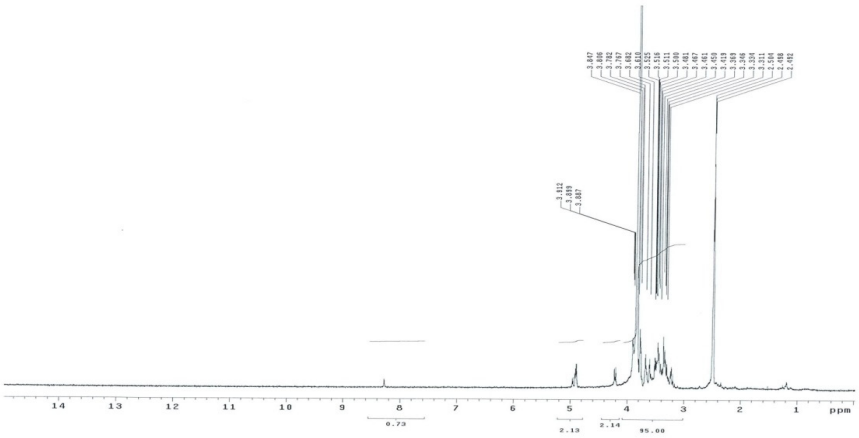


Fig. 5. [2]HNMR spectrum (DMSO-D2O) of the purified compound.

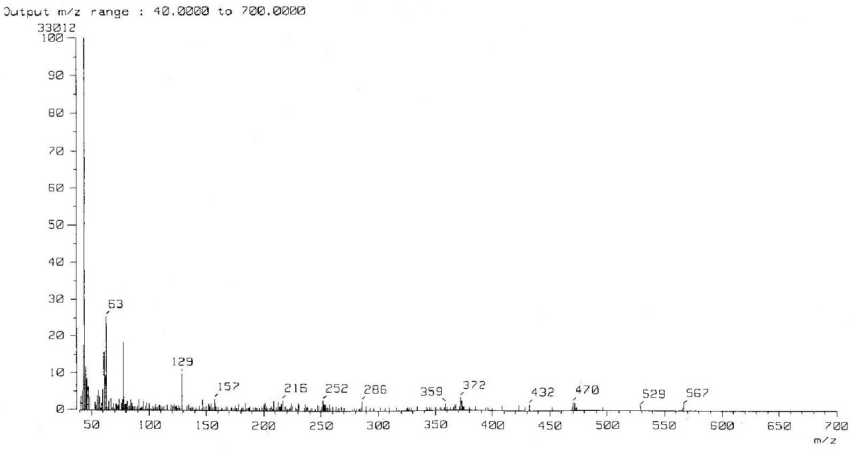


Fig. 6. Mass spectrum (EI-MS) of the purified compound.

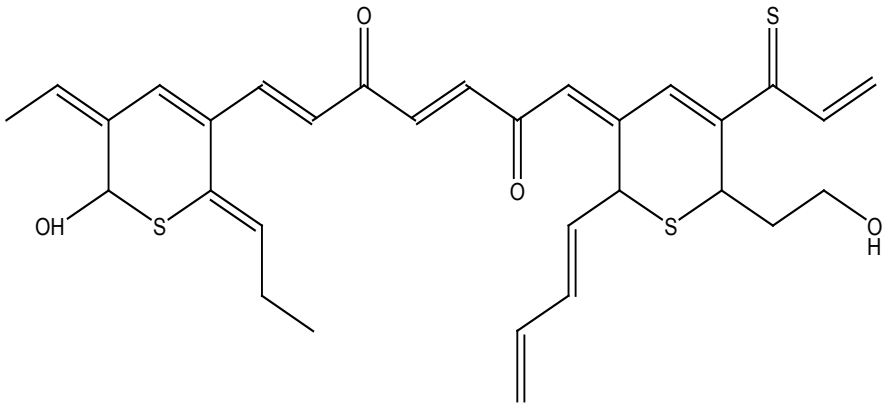


Fig. 7. Proposed structure of the active compound from *Saccharomonospora viridis* AHK190 and its IUPAC name: (1Z, 3E, 6E)-1-(2- ((E)-buta-1, 3-dienyl)-6-(2-hydroxyethyl)-5-(prop-2 enethioyl)-2H-thiopyran-3 (6H)-Ylidene)-7-((5Z, 19Z)-5-ethylidene-5, 6-dihydro-6-hydroxy-2-propylidene-2H-thiopyran-3-yl) hepta-3, 6-diene-2, 5 dione.

Electronic database search

ChemID plus (Advanced) database (containing 370,000 compound) was surveyed with classification codes such as "antibiotic", "anti-cancer", "poison" and "hazardous" in the molecular weight range 550-567. However, the survey didn't matched the properties of the under-test compound. Another database, Novel Antibiotic Database, was also screened; no bioactive compound was found matching described properties of the under-test substance.

Antimicrobial assay

The pure substance showed a broad spectrum of antibiosis activity against both Gram positive and Gram negative bacteria. Evaluated MIC values were the same against all strains (125 µg ml^{-1}) except for *Bacillus subtlis*, it was 250 µg ml^{-1}; MBC estimations were found either 250 or 500 µg ml^{-1} depending on the tested bacteria (Table 3). As well, the pure compound showed antifungal activity against all tested strains; detected MIC values were 250 or 500 µg ml^{-1} depending on fungal strain (Table 4).

TABLE 3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the purified antibiotic against the tested bacteria.

Test bacteria	Gram reaction	MIC (µg ml^{1-})	MBC (µg ml^{1-})	MBC/MIC
Escherichia coli (ATCC 10536)	-	125	250	2
Staphylococcus aureus (ATCC 6538)	+	125	250	2
Bacillus subtlis (ATCC 14028)	+	250	500	2
Bacillus cereus (ATCC /10876)	+	125	500	4
Pseudomonas aeruginosa (ATCC/15442)	-	125	250	2
Salmonella typhimurium (ATCC 14028)	-	125	250	2

TABLE 4. Minimum inhibitory concentration (MIC) of the purified antibiotic against the tested fungi.

Test fungi	MIC (µg ml^{-1})
Aspergillus terreus (ATCC/28301)	250
Fusarium moniliform (ATCC/60858)	500
Trichoderma lignorum (ATCC/1313)	500
Penicillium chrysogenum (ATCC/11709)	250
Candida albicans (ATCC/10231)	250

Antitumor activity assay

The survival fraction of colon (HCT116) cell line was plotted against the different concentrations (5–50 µg ml^{-1}) of the pure compound(s) (Fig.8). The evaluated concentration which reduced survival of carcinoma cell line of the colon to 50% was 24.2µ ml^{-1}.

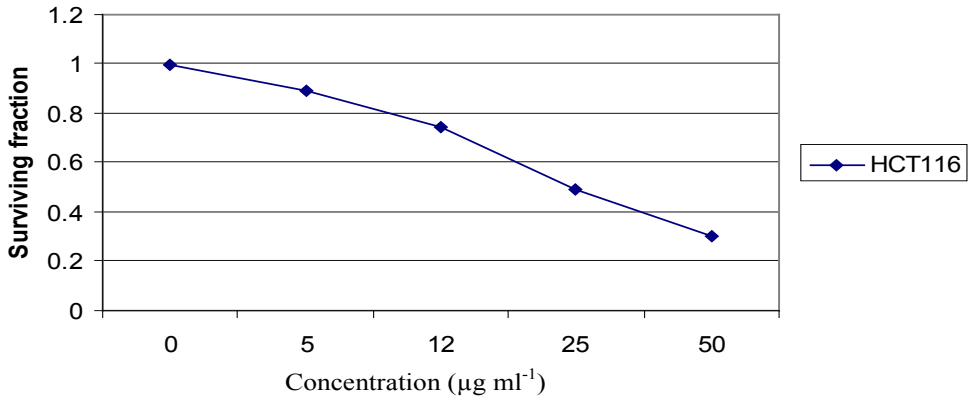


Fig. 8. Potential antitumor activity of the purified antibiotic on human cell line HCT116 (colon carcinoma) with IC_{50} value.

Discussion

Rapid growth rate of thermophilic actinomycetes and the reduction in contamination due to the elevated growth temperature make these organisms particularly useful for industrial fermentation (Tendler & Burkholder, 1960). Performance of a supportive production medium is an essential parameter for successful and effective fermentation of a specific bioactive compound and achievement of high product yield. Abdelaziz et al. (2012) and Messaoudi et al. (2015) estimated that antibiotic production is highly depended on the medium carbon/nitrogen ratio; high ratio encourages optimum growth and metabolites fermentation of nearly all actinomycetes strains. Kandula & Terli (2013) reported that proteins, peptides and amino acids in the medium supply the balanced use of C and N sources forming the basis for pH control as buffering capacity. James et al. (1991) documented that amino acids enhanced growth and antibiotic production, also in present foundation, pyridoxine and aspartic acid influenced growth and antibiosis activity. Presence of starch and casein in SCA stimulated the growth as well as antibacterial activity actinomycetes (Meklat et al., 2013 and Ranjan & Jadeja, 2017), which was in parallel with present study in which SCB showed high growth and antibiosis activity. Elements promoted antimicrobial fermentation by actinomycetes was classified by Trabelsi et al. (2016) into three main groups necessary for antibiotics fermentation: Organic nutrients such as starch, asparagine, sucrose; inorganics salts (Ca, Mg, K, Na, NH_4) and Trace elements (Mn, Zn, Fe). This was in agree with current assay in which the SCB medium contained starch, casein, KNO_3, $MgSO_4.7H_2O$, $FeSO_4.7H_2O$, $CaCO_3$ and NaCl; moreover, addition of K_2HPO_4 in SCB medium was found strongly yield and metabolite production enhanced which was coincide with Kandula & Terli (2013). Optimum antibiotic fermentation by *Saccharomonospora viridis* AHK190 was noticed after 3 d of incubation which supported that the fermentation was started at the late exponential phase and the stationary phase which revealed that product must be a secondary metabolite; also Kandula & Terli (2013) recorded the highest antibiotic yield in the late exponential phase and the stationary phase. Trabelsi et al. (2016) and Ranjan & Jadeja (2017) recorded that the optimum antagonism started during stationary phase.

The antimicrobial profile of an actinomycete depends on both culture medium and extraction performance (Trabelsi et al., 2016); and the best extraction solvent is depending on the actinomycete strain and on the produced substance (Abdelaziz, et al., 2012). Aouiche et al. (2012) achieved that some antimicrobial compounds fermented by certain strains are very hydrophilic and cannot be extracted by solvents moderately polar as ethyl acetate. The recent study showed that toluene followed by ethyl acetate was the best solvents for antibiotic extraction; while Messaoudi et al. (2015) results indicated that ethyl acetate and butanol were the most appropriates

for antibiotics extractions. Abdelaziz et al. (2012) mentioned that they obtained two different crude extracts in *n*-butanol and dichloromethane from culture filtrates of two isolates; in addition, crude extracts with hexane of the two isolates showed a very significant antifungal activity.

TLC is an appropriate technique to get enough antibacterial agents and efficient method for separation of antibiotic. In the current assay, toluene: chloroform (3:2 v/v) was proved as the best TLC purification solvent system that identified a single active spot under UV light at 254 and 365 nm; which indicated that it is only one compound. As well, Messaoudi et al. (2015) detected only one spot with solvent system (ethanol-ammonia-water) at 256 and 355 nm. Whereas, chloroform: methanol (24:1, v/v) was found to be the best solvent system for the separation of antibacterial metabolites in Ranjan & Jadeja (2017) work. Characterization of recent purified substance via spectroscopic and elemental analysis confirmed the presence of variable chemical groups and a conjugated structure. The most useful classification of antibiotics is based on chemical structure. The main classes of antibiotics are: Beta-Lactams (Penicillins and Cephalosporins), Macrolides Fluoroquinolones, Tetracyclines and Aminoglycosides. While each class is composed of multiple drugs, each drug is unique in some way. According to evaluated tasks, present compound is not identical chemically or physically with any of antibiotics related to these main antibiotic classes; it seems to be a novel agent. Microorganisms often transformed into resistant strains to current antibiotics, so new one may be useful in these cases.

Collected resent study data of MICs and MBCs revealed the broad spectrum of the purified antimicrobial agent activity against Gram+ve and -ve bacteria and fungi. Variable tested MICs from different marine *Streptomyces* strains were also examined by Henrietta (2010) and El-Gendy et al. (2008) . Cytotoxic assay of the present compound was found to have a LC_{50} value of 24.2 µg/ml; dissimilar, Feling et al. (2003) deduced IC_{50} of 80 µg ml^{-1} and Soria et al. (2005) evaluated variable IC_{50} values of 2.40, 0.97, 1.84 for three different antibiotics.

Conclusion

According to the chemical and physical characterization, it was considered that this isolated antimicrobial agent is a novel broad spectrum compound. It is of new chemical formula different from other current main classes of antibiotics. This agent must be tested medically for its effect on human health; if it is safe, it can be used as a human drug against many microbial pathogens. Thermophilic marine actinomycetes are a new unique antibiotic source of microbiota and hopefully a promise amazing bio-source for many active secondary metabolites.

Compliance with Ethics Requirement: This article does not contain any studies with human or animal subjects.

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References

Abdelaziz, M., Azzeddine, B., Mouloud, K. and Said, B. (2012) Taxonomy and study of antimicrobial activity of two halophilic actinomycetes AH1 and AH2 isolated from a saline soil of Bejaia (Algeria). *Res. Rev. BioSciences*, **6**(11), 328-334.

Abdelfattah, M.S., Elmallah, M.I.Y., Hawas, U.W., Abou El-Kassema, L.T., Aouiche, A., Sabaou, N., Meklat, A., Zitouni, A., Mathieu, F. and Lebrihi, A. (2012) Antimicrobial activity of a Saharan *Streptomyces* spp. PAL111 strain against various clinical and toxinogenic microorganisms resistant to antibiotics. *J. of Med. Mycology,* **22**(1), 42-51.

Berdy, J. (2005) Bioactive microbial metabolites. *Rev. J. Antibiot*. **28**(1),1-26.

Demain, A.L. (2000) Small bugs, big business: The economic power of the microbe. *Biotechnol.Adv.***18**, 499-514.

El-Gendy, M.M., Shaaban, M., EL-Bondkly, A.M. and Shaaban, K.A. (2008) Bioactive benzopyrone derivatives from new recombinant fusant of marine Streptomyces. *Appl. Biochem. and Biotech*. **150**(1), 85-96.

Feling, R.H., Buchanan, G.O., Mincer, T.J., Kauffman, C.A., Jensen, P.R. and Fenical, W. (2003) Salinosporamide A: A highly cytotoxic proteasome inhibitor from a novel microbial source, a marine bacterium of the new genus Salinospora. *Angew Chem. Int. Ed. Engl.* **42,** 355-357.

Gamal, M.A. (2016) Isolation and characterization of marine-derived actinomycetes with cytotoxic activity from the Red Sea coast. *Asian Pac. J. Trop. Biomed.* **6**(8), 651–657.

Henrietta, O.I.N. (2010) Assessment of antibiotic production by some marine *Streptomyces* isolated from the Nahoon beach. *Master of Science Thesis*, Fort Hare Alice University. South Africa 108p.

Ibrahim, S.Y. (2017) Isolation, identification and antibiosis efficacy of marine thermophilic actinomycetes. *Egypt. J. Microbiol.* **52,** 113-128

James, P.D.A., Edwards, C. and Dawson, M. (1991) The effects of temperature, pH and growth rate on secondary metabolism in *Streptomyces thermoviolaceus* grown in a chemostat. *J. Gen. Microbiol*. **137,** 1715-1720.

Kafilzadeh, F. and Dehdari, F. (2015) Amylase activity of aquatic actinomycetes isolated from the sediments of mangrove forests in south of Iran. *Egypt. J. Aquatic Res*. **41,** 197-201.

Kandula, S.K. and Terli, R. (2013) Production, purification and characterization of an antimicrobial compound from marine *Streptomyces coeruleorubidus* BTSS-301 *J. Pharm. Res*. **7**, 397-403

Meklat, A., Bouras, N., Zitouni, A., Mathieu, F., Lebrihi, A., Schumann, P., Sproer, C., Klenk, H.P. and Sabaou, N. (2013) Actinopolyspora mzabensis sp. nov., a halophilic actinomycete isolated from an Algerian Saharan soil. *Int. J. Syst. Evol. Microbiol.* **63**(10), 3787-92.

Messaoudi, O., Bendahou, M., Benamar, I. and Abdelwouhid, D. (2015) Identification and preliminary characterization of non-polyene antibiotics secreted by new strain of actinomycete isolated from sebkha of Kenadsa, *Algeria. Asian Pac. J. Trop. Biomed.* **5**(6), 438-445.

Ranjan, R. and Jadeja, V. (2017) Isolation, characterization and chromatography based purification of antibacterial compound isolated from rare endophytic actinomycetes *Micrococcus yunnanensis*. *J. Pharm. Anal.* **7,** 343-347.

Reddy, N.G., Ramakrishna, D.P.N. and Gapol, S.V.R. (2011) A morphological, physiological and biochemical studies of marine *Streptomyces rochei* (MTCC10109) showing antagonistic activity against selective human pathogenic microorganisms. *Asian J. of Biol. Sci*. **4**(1), 1-14.

Sharma, M., Dangi, P. and Choudhary, M. (2014) Actinomycetes: Source, identification, and their applications. *Int. J. Curr. Microbiol. App. Sci.* **3**(2), 801-832.

Sengupta, S., Pramanik, A., Ghosh, A. and Bhattacharyya, M. (2015) Antimicrobial activities of actinomycetes isolated from unexplored regions of Sundarbans mangrove ecosystem. *MC Microbiol*. **15,** 170.

Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J.T., Bokesch, H., Kenney, S. and Boyd, M.R. (1990) New colorimetric cytotoxicity assay for anti-cancer drug screening. *J. Natl. Cancer Inst.* **82**, 1107-1112.

Sheik, G.B., Maqbul, M.S., Shankar, G.S. and Ranjith M.S. (2017) Isolation and characterization of actinomycetes from soil of ad-dawadmi, saudi arabia and screening their antibacterial activities. *Int. J. of Pharm. and Pharmaceutical Sci.* Original Article ISSN- 0975-1491 **Vol 9,** Issue 10

Soria, I.E., Prieto, A., Jensen, P.R. and Fenical, W. (2005)Antibiotic terpenoid chloro-dihydroquinones from a new marine actinomycete. *J. Nat. Prod.* **68**, 904-910.

SPSS base 15.0 User's guide (2006) SPSS Inc., Chicago, USA.

Stanbury, P.F., Whitaker, A. and Hall, S.J. (1995) "*Principles of Fermentation Technology*". 2nd ed, Pergamon, 93-116, 43-65.

Subramanian, D., Kim, M., Kim, D. and Heo, M. (2017) Isolation, characterization, antioxidant, antimicrobial and cytotoxic effect of marine actinomycete, *Streptomyces carpaticus* MK-01, against fish pathogens. *Braz. Arch. Biol. Technol.* **60**, e170539

Tendler, M.D. and Burkholder, P.R. (1960) Studies on the thermophilic actinomycetes: Taxonomy. *The Soc. Am. Bacteriol*. **9**(8), 174–178.

Trabelsi, I., Oves, D., Magan, B.G., Manteca, A., Genilloud, O. and Nour, M. (2016) Isolation, characterization and antimicrobial activities of actinomycetes isolated from a Tunisian saline wetland. *J. Microbiol. Biochem. Technol.* **8**(6), 465-473.

Tuncer, I. and Bizsel, N. (2017) Phylogeny and physiology of actinomycetes and biogeochemical parameters in sediments of eastern Mediterranean Sea. *Inter. J. Environ. Sci. Dev.* **8**(8), 581-585.

Valli, S., Suvathi, S.S., Aysha, O.S., Nirmala, P., Vinoth, K.P., Reena, A. (2012) Antimicrobial potential of *Actinomycetes* species isolated from marine environment. *Asian Pac. J. Trop. Biomed.* **2**(6), 469-473.

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تخمر واستخلاص ودراسة خواص مضاد ميكروبي مُنتج بواسطة الأكتينوميسيتس سكارومونوسبورا قيريدس 190 المعزول من بيئة بحرية

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تهدف الدراسة إلى تخمير وعزل وتوصيف مضاد ميكروبي مميز من الكائن الأكتينوميسيتي 190 AHK *Saccharomonospora viridis* المحب للحرارة العالية والمعزول من بيئة بحرية. تم تحت أفضل الظروف تخمير وإنتاج أكبر عائد من هذا المضاد المميز واسع الطيف ضد الميكروبي من 190 AHK *Saccharomonospora viridis*. كما تم استخلاص المضاد وتنقيته وفحص التركيب الجزيئي له.

وقد تم استخدام مجموعة متنوعة من مسببات أمراض الإنسان الميكروية كمؤشرات فحص كفاءة التضاد للمركب المُنتج. ثم استُخدمت تقنية الكروماتوجرافي للتنقية الجزئية للمضاد الميكروبي. وقد تم دراسة التحليل الطيفي والعنصري لمعرفة الصيغة الجزيئية للمركب المُنقى، وتم الحصول على النمو الأمثل للأكتينوميسيتس وأكبر إنتاج للمضاد الحيوي بعد ٣ أيام من التحضين عند 55 درجة مئوية تحت تأثير الرقم الهيدروجيني 8.0 والرج عند 180 دورة في الدقيقة . بالإضافة إلى ذلك، تم تحقيق أكبر إنتاج المضاد الحيوي مع الجلاكتوز وحمض الأسبارتيك و 3% من كلوريد الصوديوم و 0.1% من K_2HPO_4 . في حين أن البيريدوكسين والنشا و 5.0% من كلوريد الصوديوم و 0.11% من K_2HPO_4 شجع على النمو الأمثل للكائن. تم استنباط الصيغة الجزيئية للمركب تحت الاختبار المضاد للميكروبات كالأتي: $C_{31}H_{34}O_4S_3$ وكذلك وُجِد الوزن الجزيئي له = 567. ولوحظ من الصيغة الكيميائية للمضاد الحيوي أنه جديد ويختلف عن تلك الأقسام الرئيسية للمضادات الحيوية الحالية.