

Inhibitory bioactive metabolites produced by actinomycetes isolated from mangrove ecosystem of Khor Kalba' shores, UAE

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Abstract. In this study, marine sediment habitat of mangrove ecosystem of Khor Kalba' Shores, Emirate of Sharjah-UAE was investigated for the presence of inhibitory and antiangiogenic compounds-producing actinomycetes. Marine sediments were enriched to recover actinomycetes on starch casein nitrate (SCN) agar medium. The potential of the recovered colonies to inhibit the growth of different Gram positive and negative multi-resistant bacterial pathogens was tested by the agar disc diffusion method. Data showed that all recovered isolates were able to produce inhibitory bioactive compound(s) against one or more of the tested bacterial pathogens with zones of inhibition ranging between 4 and 55 mm in diameter. However, three isolates KM1, KM2 and KM3 showed maximal inhibition against *Pseudomonas aeruginosa* with 55, 48 and 45 mm diameter zones of inhibition, respectively. It is noted that the isolate KM18 exhibited an inhibitory action against all tested bacterial pathogens except *Klebsiella* sp., with zones of inhibition ranging between 18 and 42 mm diameter. After extraction of KM1 metabolites with the solvent butanol:ethylacetate, the concentrated extract showed antiangiogenesis activity as reflected by the reduction of the neovascular index from 3.1/cm² to 0.5/cm². Partial molecular characterization of the bioactive metabolites-producing actinomycetes by PCR analysis using universal and Actino specific primers for the 16S rRNA gene fragment showed a single 500 and 350 bp band, respectively. Based on the results of this study, it can be concluded that the inhibitory effect of the recovered actinomycetes isolates from unique habitats in Sharjah (UAE) may suggest the novelty of the inhibitory/antiangiogenic compound(s) produced by KM1 strain and/or the other promising strains.

Keywords: Actinomycetes, bioactivity, antiangiogenesis, antibacterial, metabolites, PCR.

INTRODUCTION

Multidrug resistant bacteria is becoming a worldwide problem posing health risks for all spectrum of patients and therefore, limiting therapy options are expected (Sathish Kumar and Bhaskara Rao 2012; Dhayanithi et al, 2012). There are several mechanisms by which bacteria are resisting antibiotics (Livermore, 2003; Sathish Kumar and Bhaskara Rao, 2012), and due to the problems, the search for new antibiotics is highly needed. In addition, cancer treatment is still far from optimum and

the quest for new anti-cancer drugs is in high demand.

Marine environment is one of the habitats that has been relatively unexplored, and which may provide novel actinomycetes with the ability to produce new bioactive compounds (Gottlieb, 1973; Goodfellow and O'Donnell, 1989; Takizawa et al., 1993; Tresner et al., 1968). The isolation of actinomycetes from marine environment represent an alternative to the traditional approach of isolation from terrestrial soils, with initiation of screening

programs from this environment may lead to isolation of novel inhibitory bioactive compound(s)-producing actinomycetes.

The coastal wet land mangroves forests are mainly found in the intertidal zone of estuaries, back waters, deltas, creeks, lagoons, marshes and also mud flats of the tropical and subtropical latitudes (Sahoo and Dhal, 2009). The mangrove marine ecosystem in UAE coastal area and particularly Kalba' mangrove forest are located along the Oman coast which can be seen about 12 km southeast of the mangrove forest called Khor Kalba'. The environment of the mangrove ecosystem is saline, and highly rich in an organic matter because of its various microbial enzymatic and metabolic activities (Kizhekkedathu and Parukuttyamma, 2005).

Marine microorganisms have become important in the study of novel bioactive compounds with antimicrobial, antifungal, antiviral, antitumor as well as anticoagulant and cardioactive properties (Austin, 1989; Kokare et al., 2004; Marderosian, 1969; Mangamuri et al., 2012; Molinski, 1993, Usha et al., 2010). Several studies have reported the isolation of bioactive actinomycetes from mangrove sediments with antiangiogenic, anti cancer, and antibacterial activity (Baskaran et al., 2011; Janardhan et al., 2012; Hong et al., 2009; Usha et al., 2010). Janardhan et al. (2012) in their screening for bioactive compounds from actinomycetes isolated from mangrove soil found that some strains have wound healing property in embryonated egg. Therefore, this study was carried out to search for actinomycetes isolated from the mangrove ecosystem of Khor Kalba', Sharjah-UAE and to test their anti-microbial and antiangiogenic activity. Morphological and molecular characterizations of the most potent isolates were also investigated.

MATERIALS AND METHODS

Samples collection

Two marine sediment samples were collected in Feb. 2012 from Khor Kalba' mangrove ecosystem situated along the north coast of Oman. They were collected by scraping off an approximately 3 cm of surface material with a spatula and taking an approximately 1000 g sample at 10 cm below the surface. Samples were placed in plastic bags, transferred to the laboratory and stored at 4°C until use for analysis. Sea water sample of 5 L, was also collected from Khor Kalba' shore to be used in further experiments.

Samples treatment and enrichment

Marine sediment samples were air-dried at room temperature for one week. One gram of sediment

samples was placed in a crucible dish then heated in an oven (Philip Harris Ltd, England) at 45°C for 12 h. After drying, soil samples were mixed with 0.1 g of CaCO₃ and incubated at 26°C for 7 days in a water bath (Saadoun and Muhana 2008).

Isolation technique

One gm of each of the enriched sediment was suspended in 99 ml filtered 50% sea water, incubated in orbital shaker incubator (MaXQ 5000, Thermo Scientific-Canada) at 28 ± 1°C with shaking at 140 rpm for 30 min. Mixtures were allowed to settle then serial ten-fold dilutions were prepared. 0.1 ml was taken from each dilution and spread evenly over the surface of starch casein nitrate agar (SCNA) (EI-Nakeeb and Lechevalier, 1963; Küster and Williams, 1964) plates (in triplicate) with sterile L-shaped glass rod, and incubated at 28 ± 1°C for 10 days. Nalidixic acid (20 µg ml⁻¹) was added to the media after autoclaving in order to retard general bacterial growth (Usha et al., 2010). Plated dilutions that gave 20 to 200 colonies at 28°C were chosen for estimation of actinomycetes count and for further isolations. Typical actinomycetes colonies recovered on SCNA plates were selected on morphological basis (Shirling and Gottlieb, 1966). They were picked out, and then repeatedly streaked on SCNA plates to purify bacterial colonies that showed actinomycetal-like appearance.

Antibacterial bioactivity assay by agar disc diffusion method

Antimicrobial activity was tested by the Bauer–Kirby method (Bauer et al., 1966) against eight pathogenic bacteria (*Pseudomonas* sp., *Escherichia coli*, *Proteus* sp., *Salmonella* sp., *Staphylococcus aureus*, *Klebsiella* sp., *Streptococcus pneumoniae*, and *Streptococcus pyogenes*) that are reported to be sensitive or resistant to different antibiotics and are commonly used in hospitals. They were identified and kindly provided by the Clinical Laboratory at the Sharjah University Hospital, Sharjah, UAE. Each pathogen was grown in 250 ml Erlenmeyer flask containing 50 ml of nutrient broth (Hi-media/India) (pH 7.2) with shaking for overnight at 100 rpm and 37°C. Turbidity of organisms in the broth was adjusted to be equal to or greater than 0.5 McFarland turbidity standards (1.5 × 10⁸ cfu/ml). The test organisms were homogeneously inoculated by a sterile cotton swab on the surface of two freshly prepared Mueller-Hinton agar (Hi-media, India) then 3 agar discs (5 mm in diameter) were cut out from each actinomycete culture that has been grown on oatmeal agar (ISP-3) (Shirling and Gottlieb, 1966); then transferred by a flame-sterilized needle and placed onto the surface of Mueller-Hinton

agar plates. Plates that were seeded only by the tested pathogens were considered as negative controls. Plates were then incubated at $28 \pm 1^\circ\text{C}$. The actinomycetes isolates that showed inhibition zones with a diameter more than or equal to 5 mm were considered as active producers. Inhibition zones were visually detected after 24 h.

Antiangiogenesis activity test

A seed culture was prepared by growing the most potent KM1 isolate on oatmeal agar at $28 \pm 1^\circ\text{C}$ for 14 days. After growth, the whole aerial mycelium was scrapped by a loop and then suspended in 10 ml of sterile distilled water. Aliquot of 0.5 ml of the spore suspension was inoculated in 250 ml Erlenmeyer flasks containing 50 ml of SCN broth (SCNB). KM1 seed culture was grown at $28 \pm 1^\circ\text{C}$ with shaking at 100 rpm for 10 days in incubator shaker (MaXQ 5000, Thermo Scientific-Canada).

After fermentation, the supernatant from the flask was separated from the mycelium by centrifugation at 4000 rpm for 30 min at 4°C . Whole broth was extracted with 1:1 butanol: ethylacetate (v:v), overnight at 4°C . After extraction, the pellet was discarded and the supernatant was evaporated in a rotor evaporator (Heidolph, Germany), after dehydration with anhydrous Na_2SO_4 (Usha et al., 2010). The dry weight of the organic evaporate extract was determined and the extract was then suspended in least volume of sterile water.

In this study, the antiangiogenesis activity of the most active KM1 isolate's extracted metabolite was conducted on fertile chicken eggs. These eggs were cleaned with surgical alcohol and a hole was punctured aseptically. Two different concentrations (100 and 200 μl) of the crude KM1 isolate metabolite extract were injected into the hole, then was covered with a tape. Starch casein nitrate broth extract as well as untreated eggs were used as controls. The eggs were incubated for 3 days at $37 \pm 1^\circ\text{C}$ (Aisha et al., 2010). The neovascular indices (average number of blood vessels/ cm^2) were measured at different places using a dissecting microscope at 40X (Srinivasan et al., 2008).

Molecular characterization of the isolates

Extraction of genomic DNA from pure actinomycetes isolates

The genomic DNA used for the PCR was prepared from single colonies grown on SCNA medium for 3 days. Genomic DNA extraction was conducted using PureLink® Genomic DNA Miniprep Kit (Invitrogen, USA) as per manufacturer's instructions. The isolated DNA was checked for purity and quantified according to standard procedures (Sambrook et al., 1989).

Actinomycetes-specific PCR primers

For molecular identification of the recovered most active metabolite-producing actinomycetes, two sets of primers were used in this study. The first set (Actino specific-Forward1/Actino specific-Revers1) was adopted from Nilsson and Storm (2002) while the second set (Actino specific-Forward2/Actino specific-Revers2) was adopted from Mangamuri et al. (2012). Both sets amplify 16S rRNA gene fragment found only in actinomycetes. Primers were synthesized by Invitrogen Life Technologies (USA).

Amplification of 16S rRNA gene fragment of the active metabolite-producing actinomycetes

The reaction mixture for PCR amplification was prepared in 50 μl volume containing the following: Actino-F; 0.2 μM , Primer Actino-R; 0.2 μM , 1X Taq OCR master mix (Qiagen, USA), 0.25 μg template DNA and Nuclease-Free water (Qiagen, USA) was used to bring the reaction volume to 50 μl .

PCR amplification was carried out in 0.2 ml thin walled, nucleases free PCR tubes (Treff Lab, Switzerland) using iCycler thermocycler (Bio-Rad, USA) programmed as follows: a hot start of 94°C for 3 min, followed by 30 cycles of amplification at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and extra extension at 72°C for 5 min. Finally, the tubes were held at 4°C for direct use, or stored at -20°C until needed.

Electrophoresis and photography

PCR products were checked for DNA by standard electrophoresis procedure (Sambrook et al., 1989) with 1% (w/v) agarose gels (PROMEGA, USA) and detected by staining with ethidium bromide (EB) (Acros Organic, USA) at 0.5 $\mu\text{g}/\text{ml}$ final concentration. Electrophoresis was carried out at 110 V for 45 min. The size of the PCR products was estimated using 1 Kb DNA ladder (Promega, USA).

Gels were viewed and photographed using Gel Doc-It-310 (Imaging System, UVP-USA).

RESULTS

Isolation of actinomycetes

By employing the enrichment medium method, a total of 14 different actinomycetes-like colonies were recovered from two mangrove marine sediment samples that were collected from Khor Kalba', Sharjah-UAE. All of these isolates were selected based on their colony morphology on SCNA after 10 days of incubation at 27°C . The colony

Table 1. Geographical designation and total number of Actinomycetes isolates collected from sediments of Kalba' mangrove.

Sample no.	Location	Average no. of actinomycetes (CFU × 10 ⁴)	No. of actinomycetes isolates	Texture	Color
I	Kalba' mangrove sediment sample 1	8.98	6	Wet	Gray
II	Kalba' mangrove sediment sample 2	7.99	8	Wet	Black- gray

Table 2. Antibacterial activity of the isolates against Gram positive and Gram negative bacterial strains by using agar diffusion assay.

Isolate #	Antibacterial activity (mm diam. inhibition zone)							
	<i>Pseudomonas</i>	<i>E. coli</i>	<i>Proteus</i>	<i>Salmonella</i>	<i>S. aureus</i>	<i>Klebsiella</i>	<i>S. pneumonia</i>	<i>S. pyogenes</i>
Mangrove sediment sample 1 isolates								
KM1	55	16	14			15		
KM2	48	16	14			16		
KM3	45	19	13			13		
KM4	4	21	15			14		14
KM14	4	20	18	20			21	
KM16	44	17	14					
Mangrove sediment sample 2 isolates								
KM18	42	20	20	20	14		20	18
KM23	37	20	17	20	20			23
KM28	40	18	14					
KM30	33	17	15	13				16
KM32								
KM34	34	16	14		13	12		
KM35	30	19	16	20				
KM37	31	14				13		20

morphology indicated that they were small (1 to 10 mm diameter), discrete and leathery, initially relatively with smooth surface but later developed a weft of aerial mycelium that appeared granular, powdery and velvety.

Data indicated that the actinomycetal count was 8.98×10^4 cfu/g in the mangrove sediment of sample 1 and 7.99×10^4 cfu/g in the mangrove sediment sample 2 (Table 1).

Antibacterial activity

Actinomycetes isolates were screened for production of inhibitory bioactive compound(s). Data showed that all recovered actinomycetes isolates were able to produce inhibitory bioactive compound(s) against one or more of the tested bacterial pathogens with an inhibition zone diameter ranged between 4 and 55 mm (Table 2). However, three isolates KM1, KM2 and KM3 showed maximal inhibition against *Pseudomonas* with inhibition zone diameter of 55, 48 and 45 mm, respectively. Data revealed that the isolate KM18 was able to inhibit all tested pathogens except *Klebsiella*, with inhibition zone diameter ranged between 18 and 42 mm. The isolates

had a wide spectrum of inhibition against both Gram negative and Gram positive bacteria (Table 2).

Antiangiogenesis activity

The starch casein nitrate broth extract and the untreated eggs showed an average neovascular index of around 2.1 and 3.1/cm², respectively. By comparison of the KM1 extracted metabolites at concentrations of 100 and 200 µl/egg, data showed the indices of 1.5 and 0.5/cm², respectively (Figure 1).

Analysis of the 16S rRNA gene fragment of actinomycetes isolates

PCR assay was used as a selective method for identification of the active metabolites-producing actinomycetes KM1, KM2, KM 14 and KM 18 isolates. Performing the PCR reactions using Actino-F1/Actino-R1 primers gave the expected single 500 bp band (Figure 2), and Actino-F2/Actino-R2 primers gave the expected single 350 bp band (Figure 3).

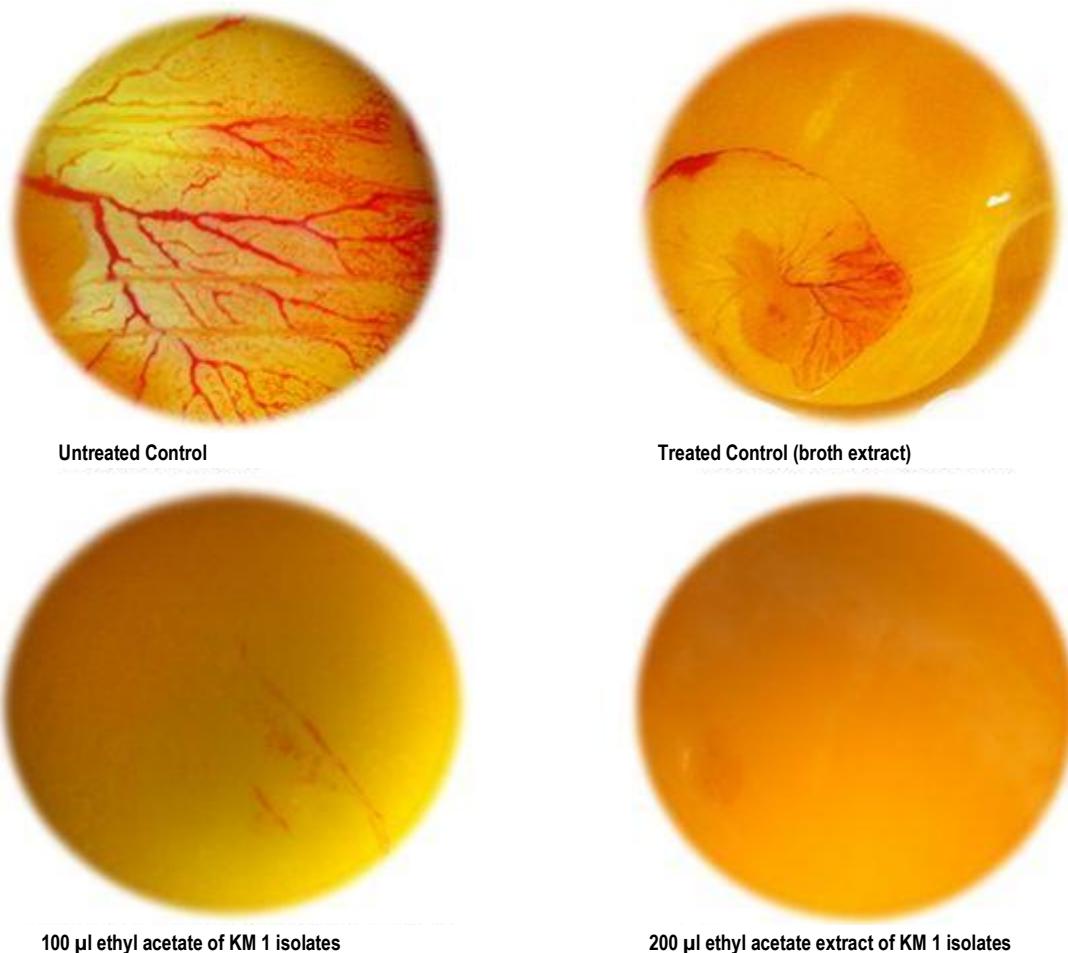


Figure 1. Antiangiogenesis activity of the extracted metabolites of KM1 isolate. A. Untreated egg (control), B. Treated egg with broth medium, C-Treated egg with 100 μ l ethyle-acetate, D-Treated egg with 200 μ l ethyle acetate, disappearance of neovascular.

DISCUSSION

With the development of antibiotic resistant strains of bacteria, there is a trend to expand programs for novel inhibitory bioactive compounds produced by actinomycetes and some other microorganisms from unique environments with the possibility of finding novel drugs produced by new species. Moreover, antiangiogenic therapeutic drugs for treatment of human cancer are extremely important and still under investigation (Wu et al., 2008). Traditionally, soil actinomycetes are searched for inhibitory bioactive compounds (Sweetline and Usha, 2013).

However, occurrence and distribution of actinomycetes in the mangrove environment is poorly understood (Mangamuri et al., 2012; Vikineswary et al., 1997; Usha et al., 2010). Nevertheless, this study demonstrated the occurrence of actinomycetes from the unique marine mangrove sediment habitat with their potentiality to produce inhibitory bioactive compounds which may serve as model systems in the discovery of new drugs (Bernan

et al., 1997; Fenical, 1997). Similar to the soil actinomycetes, the SCNA media appeared suitable for the isolation of the actinomycetes from the mangrove area (Sweetline and Usha, 2013). In contrast, Satish Kumar and Bhaskara Rao (2012) reported that the SCNA media was not the optimum for the isolation of marine actinobacteria thus highlighting high diversity of growth needs of the different marine flora. When the isolates were subjected to the primary screen for antibacterial activity, three of them (KM1, KM2 and KM3) appeared promising with high inhibition zone. The ability of some of the isolates (KM18) to inhibit multiple pathogens is striking where seven of eight pathogens were suppressed.

Similarly, Satish Kumar and Bhaskara Rao (2012), reported the isolation of many *Streptomyces* strains from marine salt pan producing inhibitory bioactive compounds. These results appeared similar to reports of actinomycetes isolated from soil (Sweetline and Usha, 2013). These results are indeed promising as they expand and diversify the locations for actinomycetes

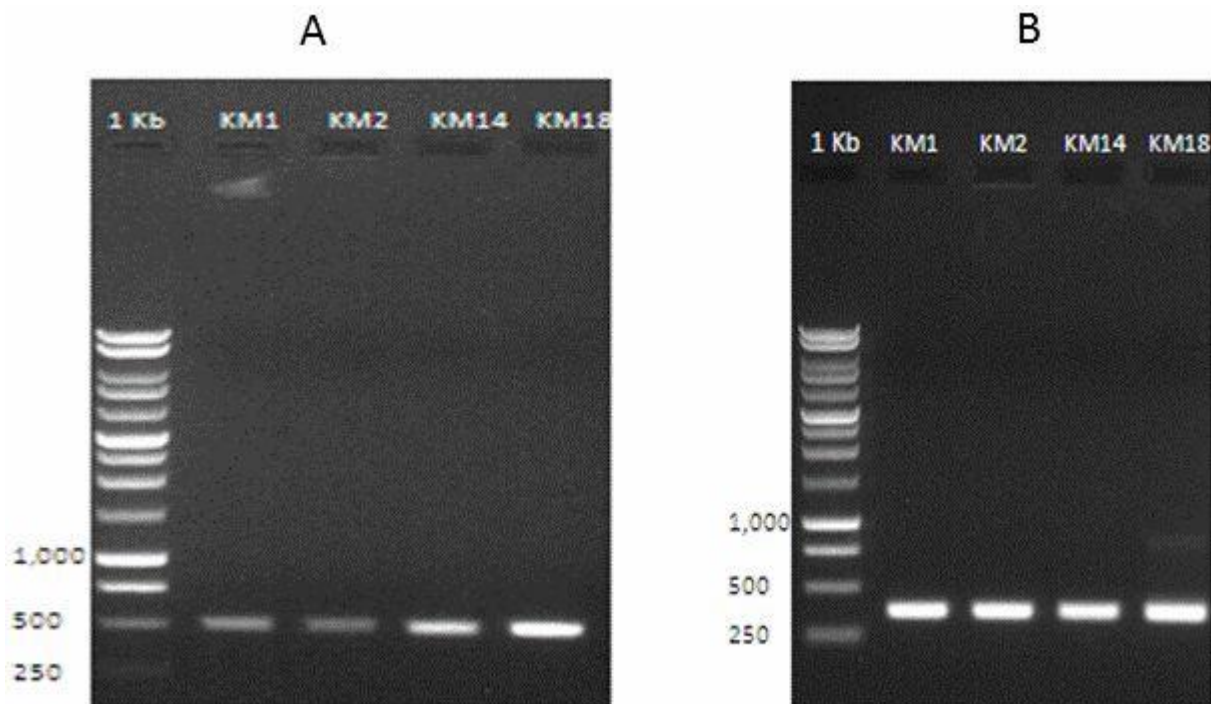


Figure 2A and B. 1% agarose gel electrophoresis of PCR amplification of 16S rRNA of the four bioactive metabolites-producing actinomycetes using their DNA as a template and the two sets for primers; Actino-F1/Actino-R1 and Actino-F2/Actino-R2 primers. Lane 1: 1 Kb DNA ladder; lanes 2 to 5: active metabolites-producing actinomycetes isolates KM1, KM2, KM14 and KM18.

isolation to mangrove areas rather than being restricted to soil areas.

When the secondary metabolites from the same isolates were tested for identifying anti-cancer agents, they exhibited promising results. The observed reduction in the formation of new blood vessels in the chorioallantoic membranes was promising and was 1.5 to 3 times more than what Usha et al. (2010) reported. Indeed these results support the possible antiangiogenic activity of KM1 extracted metabolites. In the same token, Saisivam et al. (2008) reported antiangiogenic activity by measuring the neovascular indices of ethyl acetate extract of a soil *Streptomyces* culture filtrate with 5.5 and 4.5/cm² at two different concentrations. These results are promising and as such they may possess remarkable therapeutic action for combating multi-resistant pathogens as well as certain malignancies.

The primer sets Actino-F1/Actino-R1 and Actino-F2/Actino-R2 showed specificity in detecting actinomycetes, demonstrating the reliable use of these primer pairs. The primer pairs was able to identify the most potent mangrove marine sediment actinomycetes isolates more simply, accurately and rapidly, confirming the results of the conventional description and identification of the recovered actinomycetes colonies on the agar medium. Further, they can be applied for differentiation in screening projects, taxonomic characterization, and phylogenetic analysis.

In conclusion, our study indicated the inhibitory effect of the recovered actinomycetes isolates from unique

habitats in Sharjah-UAE, and may suggest the novelty of the inhibitory/antiangiogenic metabolites produced by KM1 strain and the other promising strains.

It is expected that this attempt to isolate actinomycetes from mangrove area will be useful for identification of new bioactive compound against cancer and the increasingly challenging pathogens. Further studies on optimization, purification and elucidation of chemical structure of active compound(s) in addition to the molecular identification of the distinct actinomycetes isolates will be conducted at a later stage.

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