Screening of membrane active antimicrobial metabolites produced by soil actinomycetes using membrane models

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ABSTRACT

The focus of this study was antimicrobial membrane-activity of actinomycetes isolated from some soils of Iran. In this work, soil samples were collected from desert and farming zones of Northern and Central Iran. A total number of 45 actinomycetes were isolated from the soil samples. In the primary screening performed to evaluate antimicrobial activity, isolated microorganisms were analyzed in terms of their general inhibition effects to indicator strains *E. coli*, *C. albicans*, and *S. cervisae*. It has been found that 12 actinomycetes, were effective against test microorganisms. In the secondary screening to determine membrane-active metabolites producing microorganisms, isolates having an inhibitory effect against test microorganisms, were analyzed for membrane activity using a Rapid Chromatic Detection method. Based on color changes that are easily identified by the naked eye and recorded by UV-vis spectrophotometery, two actinomycetes had membrane-activity effect and were stored for the sake of further study and identification.

Keywords: Actinomycetes; Soil; Antimicrobial Activity; Membrane

INTRODUCTION

The prevalence of antimicrobial resistance among key microbial pathogens is presently an urgent focus of research [1-3]. Beside the resistance problem, over the past 30 years a number of new infectious diseases have been discovered [4&5]. With the continuation of this process, it is important to continue to find new anti-infective drugs in particular, with new and less resistant targets to antimicrobial activity [6 &7]. Membrane as a new and potential target is noteworthy for antibiotics action because of lower tendency to develop resistance [8&9]. Most antibiotics in clinical use are direct natural products or semi-synthetic derivatives from natural microorganisms [10&11]. Soil, in particular, is an intensively exploited ecological niche the inhabitants of which, to produce many useful biologically active natural products, including clinically important antibiotics [6&12]. This study was undertaken with an aim of highlighting the detection of antimicrobial membrane-activity of actinomycetes isolated from farming and desert zones of Northern and Central parts of Iran. The isolated Microorganisms of these areas were not studied yet for representing membrane-activity.

MATERIALS AND METHODS

Samples selection and used strains

From 2008 to 2009, soil samples were collected from several parts of northern and central Iran. These areas included the mineral soils around Meshkinshahr (38˚29.545˝ N and 047˚44.886˝ E), Ardabil and desert soils around Damghan (35˚54.509˝ N and 053˚57.832˝ E), Semnan. The samples were taken up from a depth of 15 cm from soil surface. The samples were placed in sterile bags, closed tightly and stored in a refrigerator.

The indicator strains used for screening antimicrobial activity were provided from American Type Culture Collection and were: *Escherichia coli* ATCC 25922, *Candida albicans* ATCC 10231, *Saccharomyces cervisiae* BY4743.

Isolation and storage of the isolates

For each collected sample, one gram of the soil was suspended in 10 ml of physiological water (Na Cl 9 gl^{-1}) then incubated in an orbital shaker incubator at 28°C with shaking at 200 rpm for 30 min. An aliquot of 0.1 ml of each dilution was taken and spread evenly over the surface of inorganic salt starch agar

(Difco, USA) for actinomycetes isolation that was complemented with Rifampicin (5 µg/ml) and Amphotericin B (100 µg/ml) to inhibit bacterial and fungal contamination, respectively. After incubation at appropriate time and temperature, actinomycetes colonies and aerial and substrate mycelia production were identified according to morphological characteristics by light microscopy (100 x). Repeated streaking on the same agar plates led to purify actinomycete colonies. Plates containing pure cultures were stored at 4°C during two months until further examinations, and in a freezer at -70°C in the presence of glycerol (50%, v/v) for a longer period.

Primary screening of isolates for antimicrobial activity

Primary screening for antibiotic production was done by conventional spot inoculation method on agar medium and overlay agar technique [6]. Pure isolates were spot inoculated on the same agar medium. The plates were incubated at 28 °C for 4-6 days for growing actinomycetes. Colonies were then covered with a 7.5-8% agar layer of Sabouraud dextrose medium (Difco, USA) for growing yeasts and brain-heart infusion medium (Merck, Germany) for bacteria which previously seeded with one of the test organisms. The antimicrobial activity was observed after 24 h incubation at 37°C for bacteria and 48 h incubation at 28 °C for yeast [13]. The antimicrobial activities were measured by the determination of the size of the inhibition zone. Active isolates were selected for secondary screening.

Secondary screening of isolates for detection of membrane activity

Isolates that showed antimicrobial activity against test organisms in agar medium were grown in submerged culture in 500 ml flasks containing 100 ml of malt extract-yeast extract broth. A two-day-old broth culture grown on malt extract-yeast extract broth was used to inoculate the flasks. These cultures were grown in a rotary shaker (Adolf Kuhner AG, Switzerland) at 200 rpm, 28 °C, for 14days. The resulting culture broths obtained following growth of each isolate in the culture media were separated from the biomass by centrifugation at 4000 rpm (Sigma, Germany) for 15 min. The supernatant was filtered through 0.45 µm membrane filter. Filtrate was used for extracellular antimicrobial activity by agar well diffusion method against test microorganisms[6]. Sterile cork borer was used for punching the wells in appropriate agar medium plates previously seeded with one of the test microorganisms. A volume of 100 µl of supernatant of each isolate was dispensed in each well. Plates were kept at 4 °C for at least 2 h to allow the diffusion of produced antimicrobial metabolites. The diameter of inhibition zone was determined after 24 h of incubation at 37 °C for bacteria, and after 48 h at 28 °C for yeasts. The antimicrobial activities were analyzed by the determination of the size of the inhibition zone. Antimicrobial compound was recovered from the culture of each active isolate by solvent extraction with ethyl acetate. Ethyl acetate was added to the centrifuged broth at the ratio 1:1 (v/v) along three times and shaken vigorously for 5 min in each time. The organic layers were collected and the organic solvent was evaporated to dryness in a rotary vacuum evaporator at 40 °C. The remainder was dissolved in 1ml dimethylsulfoxid (DMSO). Resulting solution was studied for membrane activity.

Vesicle preparation

The constituents include Dimyristoylphosphatidylcholine (DMPC) and the diacetylenic monomer 10, 12-tricosadiynoic acid (both purchased from Sigma) were separately dissolved in dichloromethane (1mg/ml). Vesicles containing DMPC and 10, 12-tricosadiynoic acid (2:3 molar ratio) were prepared at a concentration of 1 mM. The lipids were dried together *in vacuo*. Following evaporation, distilled water was added and the suspension was sonicated at 70°C for 8-9 min. The resultant vesicle solution was cooled at 4°C overnight and then polymerized by irradiation at 254 nm for 40-60 s, resulting in intense blue color appearance due to polymerization of the diacetylene units [14].

Interaction of phospholipid/PDA vesicles solution with antimicrobial extracts for detection of membrane-active metabolites

In order to determine the effect of the extract of each strain showing antimicrobial activity that was dissolved in DMSO, phospholipid/PDA vesicles were used as the membrane model to detect membrane-active metabolites.

Test tubes containing 300-400 µl vesicle solutions and 2mM Tris solution pH 8.5 got the volume of 1000 µl by adding bioactive extracts of strains. The pH in the solutions was 8.5 in all experiments [15&16]. Tubes incubated at 28°C for 1 h. Amphotericin B was used as positive control. Color changes identified qualitatively by the naked eye. Also color transition was determined quantitatively by UV-vis measurements and calculating CR% (colorimetric response) [17].

UV-vis measurements: A quantitative value for the extent of blue-to-red color transition is given by the colorimetric response (%CR), which is calculated from the visible absorbance spectra acquired for the vesicle solutions. The colorimetric response is defined:

$$CR = \frac{PB_0 - PB_1}{PB_0} . 100$$

where $PB = \frac{A_{blue}}{A_{blue} + A_{red}}$

A is the absorbance at either the "blue" component in the UV-vis spectrum (640nm) or "red" component (500nm), PB0 is the red/blue ratio of the control sample (before induction of color change), and PBI is the value obtained for the vesicle solution after addition of the tested compound [17].

Characterization of membrane-active isolates

Actinomycete colonies were characterized morphologically and physiologically following the methods given in the international Streptomyces project (ISP) [18]. These isolates were identified as species belonging to the genus Streptomyces by analyzing their morphological characteristics [19] and by biochemical tests [20]. The isolates were identified morphologically to the genus level by comparing the morphology of spore bearing hyphae with entire spore chain as described in Bergey's Manual [19]. This was done by using cover slip method in which sterile square cover slips were inserted at an angle of 45° in sterile ISSA medium in petridishes. Individual cultures were transferred to the intersection of the medium and cover-slip. The cover slips were removed after seven days of incubation, air-dried and observed under high power magnification. Morphological characters were noted.

RESULTS

Isolation of actinomycetes

Using the selective medium and cultivation conditions described previously a total of 45 actinomycetes, were recovered from soil samples collected from mineral and desert zones of northern and central parts of Iran from 2008 to 2009. All of the strains were isolated on culture medium supplemented with appropriate antibiotics.

All isolates grew on ISSA medium showing morphology characteristics of actinomycetes [19]. Colony appearance, size and color were visible by the naked eye. Colonies were slow growing, aerobic and had different appearance such as chalky, glabrous and folded. Aerial and substrate mycelia production were studied by optical microscope. All of the selected colonies indicated to possess substrate mycelia, and some showed to have aerial mycelia. Colonies had variety of colors, sizes and appearance.

Antimicrobial activities

In this study, the total number of 45 isolated strains was screened on agar medium and a spectrum of antimicrobial activity was observed in 26% of the strains (Table 1). Antibacterial activity against *E. coli* was observed in 83% of active isolates and antifungal activity against *C. albicans* and *S. cerevisiae* was observed in 50% and 33% of active isolates, respectively (Table 1).

Detection of antimicrobial membrane activity

Active isolates of primary screening were studied for antimicrobial activity in liquid medium. Determination of the size of the inhibition zone is shown in Table 2.

As shown in Figure 1, zones of inhibition produced by two potent strain 1-6 and 05-3-17 (according to Table 2) against indicator microorganisms.

In order to detect antimicrobial membrane-activity of microorganisms, using ependorf tubes containing prepared vesicle solution, extracts added to the vesicle solution. After incubating the tubes for 1 h, color changes were easily identified by the naked eye and recorded by UV-vis spectrophotometery [14].

Color changes that were apparent in our assay are illustrated in Figure 2 and the absorbance of blue and red components with calculation of colorimetric response are depicted in Figure 3 and 4, respectively.

Table 1. Primary screening of soil isolates for antimicrobial activity by agar overlay technique.

Zone	Total strains isolated	Number of Active isolates (% total)	Total number of active isolates against test Microorganisms (% total)		
			E. coli ATCC 25922	*C. albicans* ATCC 10231	*S. cerevisiae* ATCC BY4743
mineral soil	26	8	6	4	2
Desert soil	19	4	4	2	2
total	45	12 (26%)	10 (83%)	6 (50%)	4 (33%)

Table 2. Antimicrobial activities of soil actinomycetes grown in liquid medium against three test microorganisms

Isolate no.	The test microorganism and inhibition zone diameters (mm)		
	Escherichia coli	*Candida albicans*	*Saccharomyces cerevisiae*
mineral soil			
08-24-1	-	16	-
08-24-2	12	-	14
08-24-3	8	-	-
08-24-4	16	20	-
08-24-5	20	14	-
08-24-6	-	32	34
08-24-7	6	-	-
08-24-8	12	-	-
Desert soil			
05-3-17	26	-	-
05-3-10	6	20	20
05-3-11	14	-	-
05-3-12	18	14	18

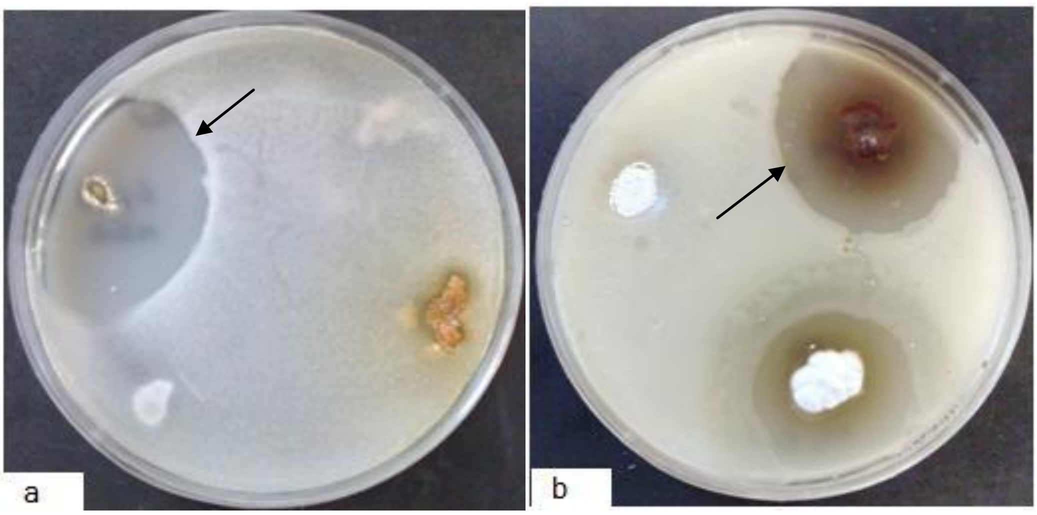

Figure 1. Antimicrobial activity by agar diffusion method. Zones of inhibition produced by a) strain 08-24-6 and b) strain 05-3-17 against test microorganisms

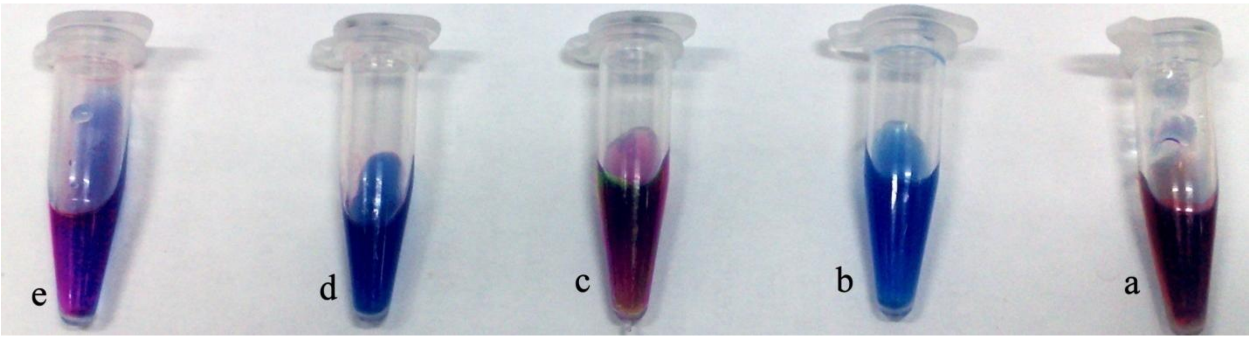

Figure 2. Color changes produced by interacting of vesicle solution with membrane active metabolites secreted by active microorganisms. Color changes after adding:
a) antimicrobial extract of a non-membrane active microorganism 05-3-2 (according to Table 1).
b) antimicrobial extract of a membrane active microorganism 10-34-6 (according to Table 1).
c) antibiotic amphotericin B as the positive control.
d) deionized water (control solution).

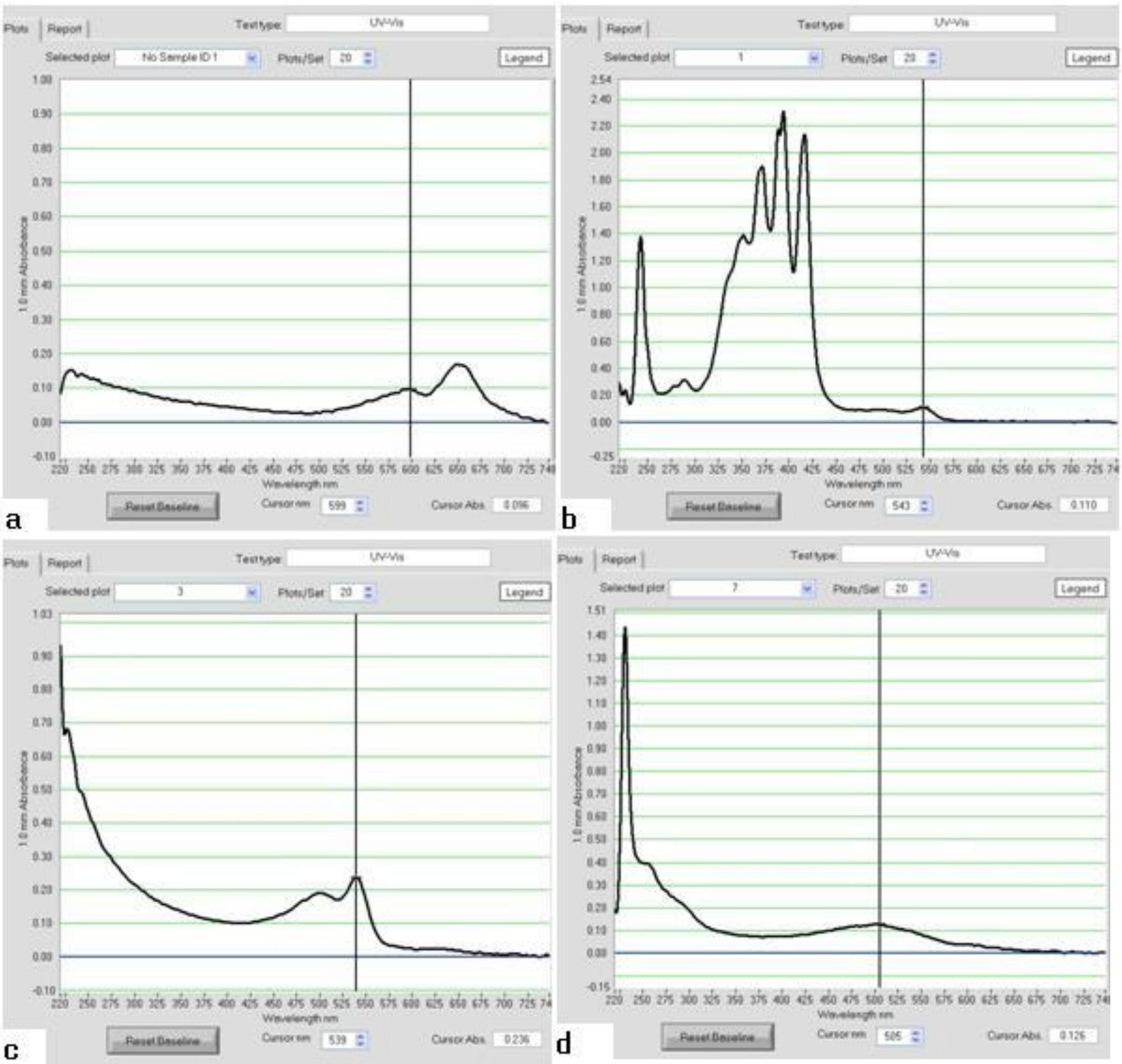

Figure 3. UV-vis spectral responses of color changes vesicle solutions under effect of membrane active compounds produced by active isolates. a) control: de-ionized water; b)Amphotericin B; c) Strain 08-24-2 (according to Table 2); d) Strain 05-3-17 (according to Table 2).

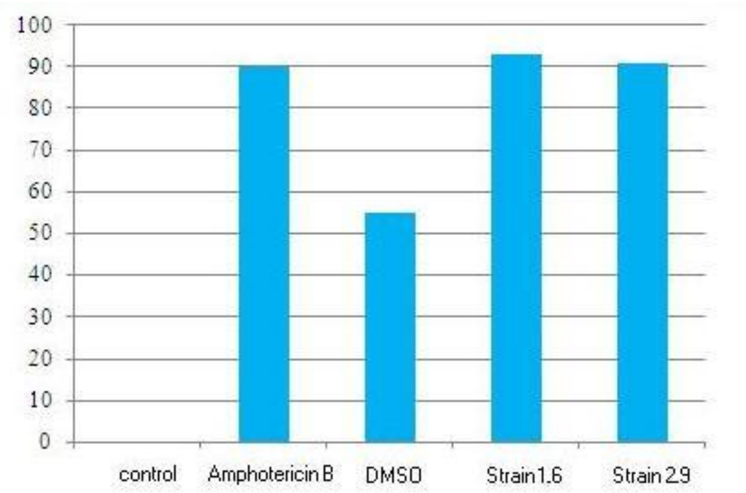

Figure 4. Bar chart of CR% (Y-axes) of color transition in vesicle solutions under effect of membrane active compounds produced by active isolates

As shown in Figure 3, strongest color transition is related to microorganism 08-24-6 and 05-3-17 (according to Table 2) respectively.
Considering the above mentioned results of color changes, 2 actinomycetes 08-24-6 and 05-3-17 were selected as isolates producing membrane active metabolites.

Morphological, cultural and biochemical characteristics of active isolates

Both colonies were slow growing, aerobic, with aerial and substrate mycelia of different colors and possessed an earthy odor. Strain 08-24-6 was chalky and strain 05-3-17 had a glabrous colony. A confirmatory identification to genus was based on acid-fastness, gram-stain, and hydrolysis of starch and degradation of casein, tyrosine and xantine. Both Streptomycetes strains were acid-fast negative and gram-stain positive. 2, 6-diaminopimelic (DAP) in the cell wall of two strains was analyzed by using thin layer chromatography (TLC) according to the procedure described by stanek [21]. It was found that the cell wall of both strains contained L, and L-DAP isomer. The cultural characteristics (pigment production), melanoid production, morphological characteristics of two isolates are presented in Table 3. Melanoid production was examined on tyrosine agar (ISP 7) and to examine the reverse side color and soluble pigment media used were those recommended by Shirling and Gottlieb [18]. According to the shape of the spore chains observed under light microscopy, isolates 08-24-6 and 05-3-17 were grouped as Rectus-Flexibilis (RF) and Spiral (S), respectively (Table 3) [19]. Two strains were identified as genus streptomyces. However, Strain 05-3-17 had been identified by chemical methods in our previous work [22].

Table 3 Morphological, Cultural and biochemical characteristics of active isolates

properties	Strain 08-24-6	Strain 05-3-17
Morphology of spore chain	Spiral (s)	Rectus-Flexibilis (RF)
The colour of the substrate mycelium	creamy	orange
The colour of the aerial mycelium	white	white
Gram stain	+	+
Acid-fastness	-	-
Degradation of casein	+	-
Degradation of tyrosine	+	+
Degradation of xantine	+	-
Hydrolysis of starch	+	+
Melanoid production	-	+
Production of Soluble pigment	-	+ (orange-violet pigment)
Cell wall DAP	L,L	L,L

DISCUSSION

From mineral and desert soil samples collected from northern and central parts of Iran, 45 isolates of actinomycetes were obtained. Approximately 26% (12) of the isolates produced antibiotics (Table 1), included among these were broad and narrow spectra ones. 83% (10) of active isolates produced antibacterial substances against *E. coli*, 50% (6) and 33% (4) of active isolates produced antifungal substances against *C. albicans* and *S. cerevisiae*, respectively (Table 1). The studied areas are the zones protected from human activity, and could have the conditions that are excellent for certain microbial strains that produce metabolites with targeted property. Production of such metabolites can be due to competition among the microbial flora and be influenced from environmental conditions that enhance the competition for survival and the production of vital substances.

This study was undertaken with an aim of highlighting the detection of antimicrobial membrane-activity of microorganisms isolated from soils of Iran. As mentioned previously, it was demonstrated by Jelinek *et al*. that PDA polymer presented in vesicle model, undergoes dramatic visible blue-to-red transformations that can be recorded by UV-vis spectrophotometery [14&17]. According to the results shown in Figures 3 and 4, two actinomycetes (namely numbers 08-24-6 and 05-3-17, according to Table 2) isolated from mineral and desert soil respectively, had membrane-activity effect. The results in Figure 3 indicate that membrane active antimicrobial metabolites of strain 08-24-6 (according to Table 2) have intensively interacted with and disrupted the surface of vesicles tructure [17]. Also, according to Figure 4, the highest amount (93%) of colorimetric response was represented by strain 08-24-6. Strain 05-3-17 and amphotericin B produced CR amounts of 91% and 90%, respectively. DMSO as a solvent control, showed a value of 55% in CR. DMSO, by itself, has a chemical structure that causes, to certain extent, a forcing effect on model vesicles, hence resulting in a low color change and colorimetric response of vesicle solution.

Two active isolates were selected and identified. Morphological, cultural, and biochemical characteristics clearly indicate that these belong to the *Streptomyces* genera and *Streptomycetaceae* family.

It was concluded that increasing number of duplications in screening for antimicrobial metabolites from natural products and the urgent demand for new leading structures in pharmacology have enforced the search for metabolites in so far untouched habitats and with potent targets in the mechanism of action [1]. Our interest focused on microorganisms producing membrane active antimicrobial metabolites as a new and potent target. In our laboratory among isolated actinomycetes, 2 strains 08-24-6 and 05-3-17 that were isolated from the mineral and desert soils of Iran, exhibited membrane activity against *Candida albicans/ saccharomyces cervisae* and *Escherichia coli*, respectively. Mentioned isolates were stored for further study and identification.

ACKNOWLEDGMENTS

The financial support from Drug Design and Bioinformatics Unit in Medical Biotechnology Department of Pasteur Institute of Iran is gratefully acknowledged.

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