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ISOLATION OF A NEW MODERATELY HALOPHILIC BROAD-SPECTRUM ANTIBIOTIC PRODUCING *ACTINOBACTER*

Hamedi J*, Mohammadipanah F, Amoozegar MA, Dehghan S

Microbial Biotechnology laboratory, Department of Microbiology, School of Biology, College of Science, University of Tehran, Tehran, Iran

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Abstract

In a screening program for antibiotic producer rare-actinobacteria from soil of Iran, a novel moderately halophilic *actinobacter* (Q70) was isolated. The strain had the ability of antibiotic production against methicillin resistant *Staphylococcus aureus*. Maximum antibiotic production was obtained in the medium containing 10% (w/v) NaCl. The strain Q70 had also activity against *Enterococcus faecalis*, *Bacillus subtilis* and *Pseudomonas aeruginosa.* The antibiotic extracted from fermentation broth had low toxicity (681/3 µg/ml LC_{50}) against *Artemia salina* in brine shrimp lethality test. The antibiotic produced by strain Q70 had polar structure. The strain Q70 is proposed as a new species with the name of *Nocardiopsis iraniensis* with considering the significant morphological, physiological, molecular and chemotaxonomical differences between Q70 and other species of *Nocardiopsis.*

Keywords:

Actinobacteria, Antibiotic, Halophile, MRSA, *Nocardiopsis*, *Pseudomonas aeruginosa*.

Introduction

Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research. *Actinobacteria* are of great importance in biotechnological industries because of their ability to produce a large number of antibiotics and other bioactive secondary metabolites. Today more than 90% of pharmaceutical antibiotics are originated from action-bacteria (1). Isolation and characterization of *actinobacteria* from unexplored habitats is of value for establishing novel strains with pharmaceutical and industrial appli-cations (2). One strategy for enhancing the likelihood of obtaining novel antibiotic compounds and other secondary meta-bolites is to analyze uncommon eco-systems which exist under extreme conditions and to consider *actinobacteria* that have been poorly studied in the past. Many of these strains may represent new species and thus can provide a valuable resource for access to new lead antibiotic compounds (3). Halophilic bacteria have optimal growth in the presence of 3-15% total salt. These bacteria like other extremophiles groups such as thermophiles and alkalophiles have considerable advantages in biotechnological process (4).

In the present study, we describe the attempt for the isolation of uncommon halophilic *actinobacteria* from saline habitats with the aim of finding antibiotic compound against methicillin resistant *Staphylococcus aureus* (MRSA) and advanced work carried out on identifi-

*E-mail: jhamedi@khayam.ut.ac.ir

cation of the isolate with polyphasic approach. Also antibacterial spectrum, production conditions and toxicity of the antibiotic were determined.

Materials

Microbial strains

The MRSA strains used was obtained from clinically isolated *Staphylococcus aureus* (Daneshvari hospital, Tehran, Iran). The bacterial characteristics of this strain were confirmed by doing morphological and physiological tests. According to National Committee for Clinical Laboratory Standards documents *Staphylococcus aureus* strains with less than 9mm inhibition zone against 5µg content methicillin disks are resistant to methicillin (5). The MRSA used throughout this study showed no inhibition zone against methicilin disk. Other test organisms used were *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* NCTC8213, *Pseudomonas aeruginosa* ATCC9027, *Salmonella entrica* subsp. *Typhi* NCTC5761 and *Escherichia coli* ATCC8739.

Soil sample collection and preparation

Soil Samples were taken up to 20cm depth from several diverse saline soils of Iran after removing approximately 3cm of the soil surface. Each sample was grounded and passed through a 2mm-mesh sieve (6).

Isolation medium

Isolation of halophilic actinobacteria was done by starch-casein agar containing 10% (w/v) NaCl [10g/L soluble starch, 0.3g/L casein vitamin free, 2g/L $K(NO_3)_2$, 2g/L NaCl, 2g/L K_2HPO_4, 0.05g/L $MgSO_4.7H_2O$, 0.02g/L $CaCO_3$, 0.01g/L $FeSO_4.7H_2O$, 18g/L agar, pH 7]. Isolation media were supplemented by cycloheximide (100µg/ml) to inhibit growth of fungi (7). Isolation and identification of the actinomycete strains were done in the laboratory of microbial biotechnology, University of Tehran.

Culture method and Maintenance

Soil samples (2g) were dissolved and homogenized in 20 mL normal saline. Appropriate dilutions of the samples were plated on isolation media and were incubated at 28°C for 21 days in aerobic condition (6). Actinomycete colonies (colonies having compact and leathery or powdery appearance) were selected and purified on ISP2[1] agar plates. Spore suspensions of selected antibiotic producing strains were kept at -20°C and freeze dried for further investigations.

Screening of isolates for antibacterial activity

Antibiotic production activity of the isolated *actinobacteria* was assessed against test bacteria and strain with the most antibacterial activity was selected for further investigations. The isolates were cultured on antibiotic production medium Hickey-Tresner agar (8) [10g/L dextrin, 1g/L meat extract, 1g/L yeast extract, 2g/L trypton, 0.02g/L $CoCl_2$, 15g/L agar, pH 7.2] that was selected in previous studies (9). The plates were incubated at 28°C for 10 days in aerobic condition. Then, the plates were covered by 3mL of top agar layer (Nutrient agar 0.6%) containing approximately 1.5×10^8 cell/ml methicillin resistant strain of *Staphylococcus aureus* or other bacterial test strains. After incubation at 34°C for 24 h, the plates were examined for inhibition zones around actinomycetes colonies and Diameter of inhibition zones appeared around them was measured (10).

1- ISP2, International Streptomyces Project Medium No. 2.

Determination of NaCl requirement for growth

Necessity of NaCl for growth was determined for the strains with the most activity against MRSA. The growth of the strain in the minimal broth media containing different concentrations of NaCl (0%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20%) [10g/L glucose, 2g/L peptone, 0.2g/L K_2HPO_4, pH 7] was compared. Occurrence or absence of turbidity in the broth was used for the assessment of growth (4).

Selection of the seeding and fermentation media for antibiotic production

Antibiotic production of the strain Q70 was assessed in 3 seeding (Table 1) and 5 metabolites production media (Table 2) containing 2.5% (w/v) NaCl to providing required NaCl. Also, antibiotic production was assessed in selected seeding and fermentation media with different concentrations of NaCl (0%, 2.5%, 5%, 7.5%, 10%, 15% and 20%). Two mL of spore suspension was inoculated into 1000mL Erlenmeyer flasks containing 80mL seeding medium and incubated at 28°C for 42 h with 200rpm in aerobic condition. The amounts of 1mL of appropriate seeding cultures were added into 100mL Erlenmeyer flasks containing 20mL fermentation medium. The flasks were incubated at 28°C for 7 days with 220rpm in aerobic condition.

Table 1: Seeding media used for growth of the strain Q70 (g/L)

I	II	III
Malt extract 10.0	peptone 10.0	Soy meal 30.0
Yeast extract 4.0	Malt extract 10.0	Glucose 10.0
Glucose 4.0	Glycerol 10.0	Glycerol 10.0
		$(NH_4)_2 SO_4$ 3.5
		$(NH_4)_2HPO_4$ 1.0
		$CaCO_3$ 5.0
pH 7	pH 7	pH 7
Ref (11)	Ref (12)	Ref (13)

Table 2: Fermentation media used for antibiotic production by the strain Q70 (g/L)

Modified Starch medium	Starch medium	Hickey-Tresner medium	Glycerol medium	Glycerol-Soy meal medium
Starch 20	Starch 30	Dextrin 10	Glycerol 30	Glycerol 15
Rape seed oil 10	Soya meal 15	Meat extract 1	Casein peptone 2	Soy meal 10
Soya meal 15	Corn steep liquor 5	Yeast extract 1	NaCl 1	NaCl 5
Corn steep liquor 5	Yeast extract 2	Trypton 2	K_2HPO_4 1	$CaCO_3$ 1
K_2HPO_4 0.5	Trace element solution 1ml	$CoCl_2$ 0.02	$MgSO_4.7H_2O$ 0.5	$CoCl_2.7H_2O$ 0.001
$MgSO_4.7H_2O$ 0.25	pH 7	pH 7.2	Trace element solution 5ml	pH 7
Trace element solution 1ml			pH 6.8	
pH 7				
Ref(12)	Ref (12)	Ref (7)	Ref (12)	Ref (12)

Antimicrobial assay of fermentation broth

The amount of 100µL of ~1.5×10^8 cell/mL concentration of 24h-old cultures of the MRSA was spread on the surface of plates containing 25mL Mueller-Hinton agar. The fermentation broth was filtered (0.22 µm). 100µL of the filtrate was poured in stainless steel cylinders with standard dimensions (10 mm height, 6 mm inner diameter and 8 mm outer diameters) and incubated at 34°C for 24h. Inhibition zone diameters were measured by digital vernier (±0.1mm) and the average of the data was recorded.

Solvent selection for extraction of antibiotic

After selection of optimum seeding and fermentation media for antibiotic production, 600 mL of fermentation broth of the strain Q70 was prepared. The fermentation broth was centrifuged at 4000rpm for 20min in 4°C. The supernatant was filtered, divided to 5 equal volumes and each part mixed with the equal volume dichloromethane, chloroform, ethyl acetate, n-hexane and diethyl ether. Extraction process repeated 4 times. The organic fractions were collected, dried with $MgSO_4$ and concentrated at 34 °C in low pressure.

Antimicrobial assay of extracts

Antimicrobial assay was performed by standard disk-diffusion method using methicillin resistant *S. aureus*. 100µL volume of ~1.5×10^8 cell/mL concentration of 24h-old cultures of the MRSA was spread on the surface of the plate containing 25mL Mueller-Hinton agar. Measuring of the antibiotic concentration in the fermentation broth was very difficult, because the strain was isolated from the soil and there was no information about its antibiotic purity, structure, potency and etc. So, the antibiotic activity was measured in the same volume of the organic fraction obtained from the same volume of fermentation broth. One mL of the each organic fraction was added to each standard paper disk (Padtan Teb Co.). Each paper disk was placed in the well with same diameter of the disk. Then, 100µL of the organic fraction was added, the well was stand in room temperature and the solvent was evaporated. This work was repeated ten times. The dried disks were placed on the surface of MRSA-inoculated agar plate. Also, One mL of each organic solvent was added to the paper disks. After evaporation of the solvents, the disks were used as solvent control disks. This experiment was done in triplicates. After 24h incubation at 34°C, diameter of inhibition zone for each sample was measured by digital vernier (±0.1mm) and the average of the triplicates was recorded (14).

Evaluation of extracts cytotoxicity

Brine shrimp lethality test method by *Artemia salina* was used. Brine shrimp eggs were hatched in a polyethylene bottle filled with artificial sea water at 25°C under illumination and aeration. Appropriate volumes of the extracts were added into microplate wells containing artificial sea water. After 48h, five phototrophic nauplii were collected and transferred into the well containing artificial sea water. The plates were incubated at 25°C under illumination for 24h and survival of the napulii was studied under microscope. Also, aqueous solution of 0.5M potassium dichromate and artificial sea water was used as positive and negative controls, respectively (15).

Morphological and physiological characteristics of the isolates

The intact arrangement of hyphae, notably aerial hyphae and spore chains, was observed on ISP2 agar after 14 days at 28°C using the cover-slip technique (16). Morphological and cultural characteristics and pigment production of actinobacterial isolates were examined (17). Assimilation

of carbon sources, utilization of nitrogen sources, and enzymes activities, degradation activity, growth temperatures and pH was determined as described by Williams et al. (18).

Chemotaxonomic characterization
The strain was cultured at 28°C for 10 days in BHI broth. Diaminopimelic acid (DAP) isomers and whole-cell sugars were analyzed using thin layer chromatography according to the method of Staneck and Roberts (19).

Analysis of 16S rDNA sequence
The isolates were grown at 28°C for 5 days in nutrient broth. The mycelia was obtained by centrifugation and washed twice with double-distilled water. Chromosomal DNA was extracted using genomic extraction kit (Sigma Co.). The DNA purity and its quantity were checked by gel electrophoresis and spectrophotometer at 260 and 280 nm. The fragments with 1.5-kb 16S rDNA were amplified using modified universal primers: 9f (5'-AAGAGTTTGATCATGGCT-CAG-3') (20) and 1541r (5'- AGGAG-GTGATCCAACCGCA-3'). The PCR products were purified (Bioneer purification Kit) and used as the sequencing template. The sequencing reactions were performed with automated fluorescent Taq cycle sequencing using the ABI 3130XL automatic DNA Sequencer, according to the manufacturer's protocol (21).

Phylogenetic analysis
The 16S rDNA sequence of Q70 was aligned with sequences published (EMBL/GenBank/DDBJ databases) of related species using the program clustal X (22) and phylogenetic tree viewed by Mega version 3.0 (23). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings (24).

Results

About 100 soil samples were examined from several diverse saline soils of Iran. From which, the strain Q70 was isolated from desert soil, Qom, Iran.

Antibacterial spectrum of Q70
The strain Q70 had antibacterial activity against all tested Gram positive bacteria and *Pseudomonas aeruginosa* (Fig. 1 and Table 3).

Table 3: Antibacterial activity of the strain Q70 (mm)

Test strain	Inhibition zone (mm)
Escherichia coli ATCC8739	-
Salmonella entica subsp. Typhi NCTC5761	-
Pseudomonas aeruginosa ATCC9027	30
Enterococcus faecalis NCTC8213	21
Bacillus subtilis ATCC 6633	52
methicillin resistant *Staphylococcus aureus*	68

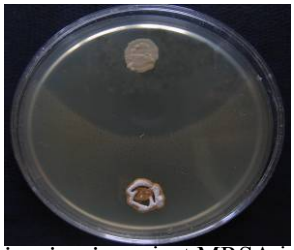

Fig. 1: Activity of *Nocardiopsis iraniensis* against MRSA in Hickey-Tresner medium containing 10 %(w/v) NaCl.

Requirement of Q70 to NaCl

Strain Q70 was moderately halophilic and no growth was seen in minimal medium without NaCl and in 20% (w/v) NaCl. Good growth was obtained in the medium containing 5-12.5% (w/v) NaCl.

Relation between antibiotic production and NaCl concentration

Maximum antibiotic production was obtained in Hickey-Tresner medium supplemented with 10% (w/v) NaCl. However, antibiotic production occurred in various concentrations of NaCl (Fig. 2).

Selection of the seeding and fermentation medium for the antibiotic production

Among examined seeding and fermentation media, seeding medium (I) and Hickey-Tresner fermentation medium had the most antibiotic production (Fig. 3). Therefore, these media were selected

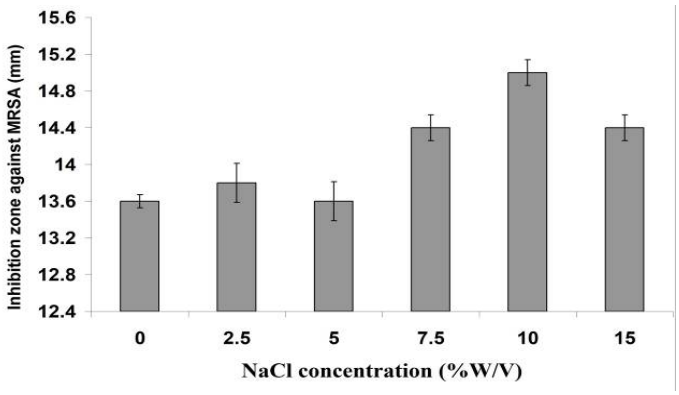

Fig. 2: Antibiotic production against MRSA in Hickey-Tresner medium with different NaCl concentration (mm).

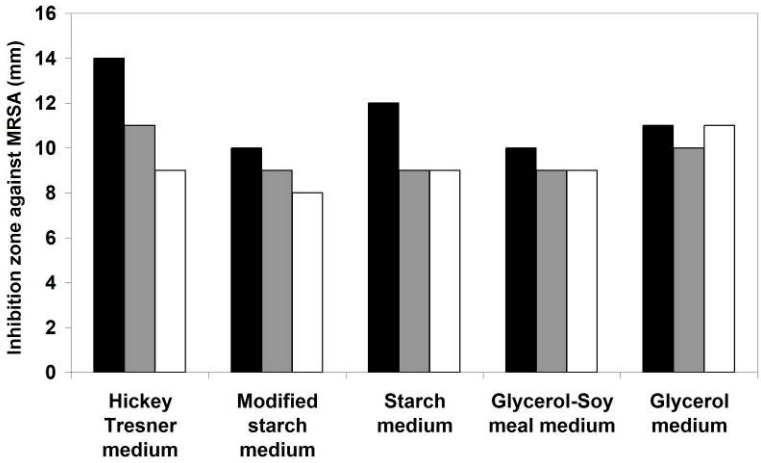

Fig. 3: Antibiotic production by strain Q70 against MRSA in different seeding (medium 1 (■), medium 2 (■), medium 3 (□) and fermentation media (mm).

for antibiotic production and providing fermentation broth for antibiotic extraction

Determination of antibiotic extraction solvent

The fermentation broth of Q70 was extracted by dichloromethane, chloroform, ethyl acetate, n-hexane and diethyl ether and the results were shown in Table 4. As seen, chloroform was the best solvent for antibiotic extraction between the solvents used. No antibiotic activity was seen by n-hexane, aqueous fractions and solvent control disks.

Evaluation of cytotoxicity of extracts

The concentration that in which 50% of the nauplii was died after 24h, were used as LC_{50} (15). Artificial sea water and organic solvents had not any toxic effect after 24h. But, all of the nauplii were died after 40min in the wells containing potassium dichromate (positive control). Ethyl acetate extracts with LC50 68.1 µg/mL had high toxicity for eukaryotic cells, but chloroform and dichloromethane extracts with the same LC_{50} (681.3 µg/mL) had minimum toxicity (Table 5).

Identification of the strain

Microscopic morphology

Strain Q70 is a Gram positive bacterium and had long-branched substrate mycelia. The aerial mycelium completely fragmented to long-chains of spores with rectangular shape (Fig. 4).

Table 4: Diameter of inhibition zones of the fermentation broth fractions extracts (mm)

Aqueous phase	-
Chloroform	29
Dichloromethane	28
Ethyl acetate	21.5
n-hexane	-
Diethyl ether	11

Table 5: Death percent of the fermentation broth fractions extracted by organic solvent against *Artemia salina* after 24h

Fractions	10 µg/ml	100 µg/ml	1000 µg/ml
Ethyl acetate	20	80	100
Chloroform	0	0	0
Dichloromethane	0	0	0

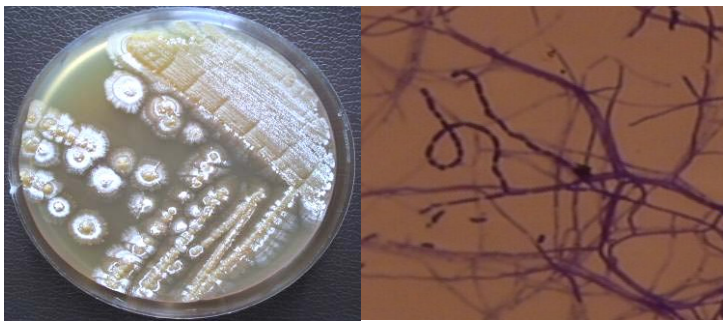

Fig. 4: Macroscopic morphology (right) and arrangement of spore chain (left) of *Nocardiopsis iraniensis* on ISP2 medium with 10 %(w/v) NaCl.

Cultural properties
Strain Q70 was able to growth on all ISP media and was sporulated on most of them. This strain was not able to produce any pigment on the media tested.

Physiological properties
Strain Q70 was not able to growth at 10°C and 40°C and had optimal growth at 28°C. This strain is able to growth in pH 6-12 and 2.5-15% (w/v) NaCl. Versatile compounds can be used by strain Q70 as sole carbon and nitrogen sources (Table 6).

Determination of cell wall components
meso-Diaminopimelic acid was detected as the only DAP of the peptidoglycan. Whole-cell hydrolysate contained no diagnostic sugar that represents C type of sugar pattern.

Comparison of 16S rDNA nucleotides of Q70 with related strains
The similarity of 16S rDNA nucleotides of Q70 with related species in *Nocardiopsis* genus is shown in the Fig. 5. Strain Q70 has the most phylogenetic relationship with *Nocardiopsis halotolerans* DSM44410 (95.17%).

Table 6: Differentiating Characteristics of the strain Q70 and *Nocardiopsis halotolerans* DSM44410

Characteristics	Strain Q70	*Nocardiopsis halotolerans* DSM44410
Aerial mycelium	Dirty white	Yellow-gray
Substrate mycelium	Dirty yellow	Beige-brown
Pigment production	-	-
Indicative sugars	-	Glucose , Ribose
Growth in 0% (w/v) NaCl	-	+
Growth in 10% (w/v) NaCl	+	+
Growth in 15% (w/v) NaCl	+	+
Growth in 20% (w/v) NaCl	-	-
Growth at 10°C	+	+
Growth at 45°C	-	-
Glucose	+	+
Adonitol	-	-
Mannose	+	+
Arabinose	-	-
Galactose	-	+
Sucrose	+	+
Mellibiose	-	+
Xylose	+	-

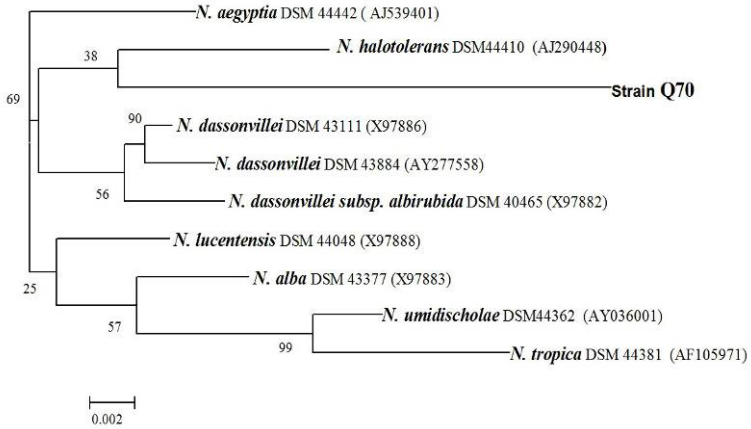

Fig. 5: Dendrogram of phylogenetic position of *Nocardiopsis iraniensis* (strain Q70) and related *Nocardiopsis* species

Discussion

The resistance of numerous pathogenic bacteria to commonly used bioactive secondary metabolites is presently an urgent focus of research, and new antibacterial molecules are necessary to combat these pathogens (10,25). One strategy to increase the possibility of discovering novel chemical entities is to screen microorganisms that previously were under-represented in natural product screening. So, isolation of such strains may lead to the obtaining of new potent antibiotics (26). There is few article published on antibiotic production by halophilic actinomycetes (27) and this is the first report in the world on the biotechnological ability of halophilic *Nocardiopsis* species on antibiotic production. Also strain Q70 was a new species in the rare genus *Nocardiopsis.* This strain is related to *Nocardiopsis halotolerans* DSM44410 in phylogenetic tree according the result of 16S rDNA nucleotide sequence analysis (28). By consideration of significant differences in 16S rDNA nucleotide sequence (4.83%), physiological properties (xylose utilization and inability in utilization of galactose and mellibiose), whole cell sugar pattern (absence of determinative sugar), and aerial mycelium morphology (difference in pigment production) (28), strain Q70 proposed as novel species with the name *Nocardiopsis iraniensis* and was stored in Persian Type Culture Collection as *Nocardiopsis iraniensis* PTCC 1708. Due to that the similarities of the 16S rRNA gene sequences of the type strains to the other *Nocardiopsis* species are far below the new value proposed by Stackebrandt and Ebers. They could show in their studies that a sequence similarity range above 98.7-99 % should be mandatory for testing the genome uniqueness of a novel isolate (29).

Q70 has some useful biotechnological properties. Industrial antibiotic production was normally done by high quality low salt content water. Therefore, using of halophilic strains, such as *Nocardiopsis iraniensis* can reduce the operation costs. Also, the antibiotic produced by *Nocardiopsis iraniensis* has low toxicity (LC_{50} 681.3 µg/ml) and has wide spectrum antibacterial activity, especially against methicillin resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*, two serious opportunistic pathogens.

The n-hexane extract (non-polar solvent) had not antibiotic activity, but, fractions extracted by chloroform and dichloromethane (polar solvents) had maximum antibacterial activity. It can be concluded

that the antibiotic produced by *Nocardiopsis iraniensis* has polar structure. Further investigations are under way to define the specification of antibiotic produced by *Nocardiopsis iraniensis*.

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