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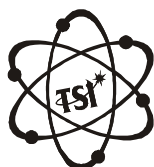

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Antibacterial activity of some isolates of actinomycetes from Yemeni soils

Amina El-Shaibany*, Gamal El-Didamony

Department of Pharmacognosy, Faculty of Pharmacy, University of Sana'a, Sana'a, (YEMEN)

E-mail : aminaalshaibani@yahoo.com

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ABSTRACT

Actinomycetes are one of the most attractive sources of antibiotics and other biologically active substances of high commercial value. It accounts roughly for two-thirds of the total known antibiotics. This study was undertaken to characterize antibacterial activity of some actinomycete isolates occurring in Yemeni soils. Fourteen soil samples were collected from cultivated soil in six Governorates of Yemen. Sixty three actinomycete isolates were obtained by using the dilution plate technique method. Using diffusion methods twenty isolates gave antibacterial activities. Two isolates namely YI(l)d and YI(8)g gave the highest antibacterial activities against the tested bacterial strains. The bioutography revealed that the antibacterial activity against tested organisms was due to the presence of antibiotic. Ethyl acetate and acetone were found to be the best solvents to extract the antibiotic substance(s) from actinomycete isolates number YI(l)d and YI(8)g respectively. The extracts were concentrated by evaporation under vacuum and the residues were tested for activity. Further purification was done by using preparative thin layer chromatography that gave more than one spot i.e. there was more than one antibiotic produced by isolate number Yl(l)d.
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KEYWORDS

Actinomycetes;
Antibacterial activity;
Yemeni soil.

INTRODUCTION

Actinomycetes particularly members of the genus Streptomyces produce 60% of all known antibiotics including most useful ones of this group of microorganisms still remain an important source of antibiotic[6,13]. Actinomycetes are gram-positive bacteria which form branching hyphae at same stage of their development and may produce a spore-bearing mycelium[9]. There are a world wide search for antibiotic from various terrestrial substances and geographical regions[11,29]. Actinomycetes are numerous and widely distributed microorganisms in nature. They are the best common source of novel antibiotics[25]. Actinomycetes synthesize a wide range of antimicrobial compound with diverse chemical structure, and different mechanism of antimicrobial action. These components include amphotericin B, rifampycin, streptomycin, neomycin, novobiocin, tetracycline and vancomycin[7]. Four novel cyclic homodecapeptide antibiotics, Streptocidins A ~ D from mycelium extract of *Streptomyces sp.*Tu 6071 were isolated[8]. Recently Niladevi et al. (2007)[23] and Bussari et al. (2008)[4] isolated Taccse and

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Cephamycin C from *Streptomyces clavulrgerus* Nt4 and *Streptomyces psammoticus*, respectively using solid-state fermentation.

Earlier studies revealed that saltpans are rich in antibiotic producing actinomycetes[14,15,17,28]. Moreover, Shiburgi (2003)[31] analyzed 120 soil samples for antibiotic producing actinomycetes from Neyyar wild life sanctuary of Kerala state. These samples showed an average of 18.265 x 10^6 numbers of actinomycetes per gram of soil and further isolated four actinomycetes isolates with broad spectrum anti-microbial activity. Screening for antimicrobial activity among 50 isolates of actinomycetes isolated from farming soils of Turkey was studied by Oskay et al. (2004)[26]. They showed that 34% of all isolates were active against at least one of 7 tested organisms. Also 86 isolates of bacteria isolated from a mason basin in Brazilin were screened for antimicrobial activity by Motta et al. (2004)[21]. They reported that antimicrobial against at least one indicator strain was detected for 59 isolates (68.6%). It is, therefore, an ongoing endeavour to search new ecological niches for novel actinomycetes. Target directed screening is being used for screening of antibiotic producing actinomycetes.

The aim of this study was to investigate the presence and antibacterial activity of actinomycetes in soil samples from various locations in Yemen as well as extracting and partially purifying the antibiotic substance(s) from the highest producing actinomycetes isolate.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from different locations, belonging to six Governorates in Yemen such as Sana'a, Ibb, Taiz, Damar, Amran, Hodidah (TABLE 1). The surface of soil and all plant materials were first removed, then soil samples was taken at 2 to 6 inches below the surface. Samples were usually taken when soil was reasonably dry. A representative quantity (0.5 Kg) of the mixed samples, after sifting was kept in a plastic bag. These bags were tied and labelled with letter codes and serial numbers including the basic information of each samples, i.e. locality and cultivated crop.

TABLE 1 : Total actinomycetes content of collected soil sample

Number of sample	Locality	Crops: Scientific name	Total content of actinomycetes ($x10^2$ CFU/g soil)
1	Ibb-Wadi Bana	*Sorghum bicofor* L moench	1.00
2	Ibb-wadi Bana	*Zea mays*	1.51
3	Sana'a - Hiziaz-Alsawad	*Punica granatum*	6.50
4	Sana'a - Hiziaz-Alsawad	*Ficus carica*	6.50
5	Sana'a - Hiziaz-Alsawad	*Pruns persica (*Firsikh*)*	7.20
6	Sana'a	*Phoenix dactylifera*	6.10
7	Ibb	*Ficus carica*	12.00
8	Ibb	*Punica granatum*	21.00
9	Ibb - Ketab	*Zea mays*	18.00
10	Ibb - Yareem	*Solanum tuberosum*	9.00
11	Damar	*Sorghum bicolour L.* Moench	33.00
12	Taiz	*Triticum vulgaris*	27.00
13	Amran - Khamer	*Zea mays*	17.00
14	Hodida	*Cynodon dactylon*	22.00

Isolation

a) Medium used for isolation

Starch-nitrate medium prepared according to Tadashi (1975)[33] with following composition (g/ L): Soluble starch, 20; NaN03, 2; K2HP04, 1; KCL, 0.5; MgS04.7H20, 0.5; FeS04.7H20, 0.01; CaC03, 2; agar-agar, 15 and 1000 ml distilled water, pH value of the medium was adjusted to 7.2 before sterilization.

b) Isolation methods

A suspension of soil samples were prepared by shaking 10 g of soil in 100 ml of sterile distilled water in flask (250 ml), allowed to stand for 10 min. Serial dilution were carried out from 10^{-1} to 10^{-5} using sterile distilled water. Only 0.1 ml of each dilution is transferred to starch-nitrate agar medium in Petri dishes and spread by a glass spreader under aseptic conditions and then incubated for 7 days at 37°C. The developed colonies of actinomycetes were counted and characterized according to their colour of aerial and substrate mycelia. Selected colony was further purified by streaking on the starch-nitrate agar plate. Single colony was picked up into starch-nitrate agar slant(s). After incubation at 37°C for 7 days some of these slants were used for

preparation of spore- suspension.

Preparation of seeded plates by tested bacteria

Tested bacteria: (*Bacillus subtilies*, *Sarcina sp*, *Pseudomonas aeruginosa*, *Escheria coli* and *Serratia*) were obtained from Dr. A. AL-Baze, Department of microbial genetics, Faculty of science, Monofia University, Egypt.

Media and cultures: Nutrient agar medium prepared according to Shiriling and Gottlieb (1966)[32] with the following composition (g/l) was used: Sodium chloride 5; yeast extract 3; beef extract 3; agar-agar 20 and distilled water 1000 ml, pH was adjusted to 7.2. Nutrient agar was prepared in conical flasks (100 ml/ each flask), while broth was prepared in test tubes (10 ml tube). These media were sterilized by autoclave at 121°C for 15 min. Broth culture from each bacterium was prepared by transferred a lapful from bacterial culture (slant) to tube containing nutrient broth and incubated at an incubator shaker (150 rpm) for 19 hours. After liquefaction of solid nutrient-agar medium and left to reach 30°C, 5 ml of this broth culture were transferred to flask and poured into Petri dishes (10 ml/ plate).

Screening of antibacterial activity of isolated actinomycetes

a) Cut-cup diffusion method

One ml of spore suspension (9.2 x 10^9 CFU/ ml) of actinomycete was transferred to plate containing starch-nitrate agar medium. After spreading of spore suspension, this plate was incubated for 7 days at 28-30°C. Under a septic conditions and using sterile cork borer discs of diameter 5 cm from each actinomycetes culture were cut and impregnated upside down on the surface of seeded target organism plates. Plates were kept in refrigerator for 2 hours before incubation. These plates were incubated for 18-24 hours at 37°C. After incubation period, the antibacterial activity of the actinomycete isolates was detected as result of clear inhibition zone around the discs.

b) Disc diffusion method

Five mm filter paper discs were impregnated with fermentation broth before extraction or soaked in the obtained concentrated extracts after extraction, dried and then placed over agar surface plates freshly seeded with tested bacteria (a control test for each solvent was also performed). The plates were kept in a refrigerator for 2 hours and then incubated at 37°C for 16-24 hours.

Isolation and purification of antibacterial metabolites

a) Fermentation

Actinomycete isolates with highest antibacterial activity were selected and cultured in 100 ml of starch-nitrate broth (pH 7.2) in Erlenmyer flasks (250 ml). After inoculation by 3 ml of spore suspension (8.2 x 10^6 spore/ ml) of old of culture (7 days), flasks were incubated under shaken (150 rpm) for 7 days at 28°C. Ten flasks were prepared for each isolate.

b) Extraction

The culture broth was collected and filtrated through Whatman No.1. The clear filtrated was mixed thoroughly with equal volumes of petroleum ether by shaking in 250 ml capacity separating funnel and allowed to stand for 30 min. Two layers were separated, the aqueous layer and the organic layer containing the antibacterial agent. The aqueous layer was mixed with chloroform and separated the organic layer; the aqueous layer was further mixed with ethyl acetate and then repeated with acetone. Organic layer from each solvent was concentrated by evaporation under vacuum to the least volume. Antibacterial activity was determined by the disc diffusion method using *Bacillus subtilis* and *E. coli* as tested bacteria.

Purification using thin layer chromatography

According to the antibacterial activity each selected extract was screened by thin layer chromatography (TLC) using different solvent systems to demonstrate the chromatography pattern of different fractions. All fractions were purified by preparative thin layer chromatography (PTLC) on silica gel plates and the bands were visualized under UV light (254 nm), then the zones were scrapped off and eluted with a mixture of suitable solvents according to the polarity of each fraction, after concentrated of each eluted band, antibacterial activity was determined using paper-disc method.

RESULTS

Isolation of actinomycetes

The results presented in TABLE 1 show that, the

collected cultivated soil samples significantly differed in their total contents of actinomycetes when starch-nitrate medium used for isolation. The fourteen soil samples represented six Governorates as following: 4 samples from Sana'a, 6 from Ibb and one sample from Taiz, Amran, Hodidah and Damar. All soil samples were taken from cultivated soil with the exception of the sample from Hodidah. Soil cultivated by *Sorghum bicolor* in Damar gave the highest count of actinomycetes (33 x 10^2 CFU/ g soil), while the lowest count of actinomycetes was obtained from soil cultivated by the same crop in lbb-Wadi Bana (1 x 10^2 CFU/ g soil). Collected soil samples, which grown by monocotyledons plants were 8 samples, while other samples were dicotyledons plants. Generally soil samples collected from soil cultivated by monocot plants (such as sorghum in Damar and triticum in Taiz) contain highest count of actinomycetes (33 x 10^2 and 27 x 10^2 CFU/ g soil respectively) compared with other tested soil samples.

Preliminary characterization of actinomycete isolates according to colour of aerial mycelium and presence of soluble pigments

TABLE 2 show the range of colours of aerial and substrate mycelia and their frequency of occurrence in the actinomycetes isolated from experimental soils. Nine main groups of colour were observed, often with colour intergrades seen within a group. The main colours were gray, pink, red, white, violet, creamy, olive, yellow and brown, with the gray colour group being dominated. Results also revealed that, about 66% of isolates produced soluble pigments of various colours, while 34% of the isolates did not produce any soluble pigments. Brown coloured pigments were dominated in these isolates.

TABLE 2 : Preliminary characterization of actinomycete isolates according to colour of aerial mycelium and presence of soluble pigments

Group No.	Growth	Colour mycelia		Number of isolates	%of isolates
		Aerial	Substrate		
1	Good	Red/pink	Dark brown	4	6.9
2	Very good	White/pink	Brown	2	3.4
3	Very good	Pale violet	Yellow	12	20.7
4	Good	White/gray	Olive	4	6.9
5	Good	Gray	Violet	6	10.3
6	Very good	White/gray	Pale yellow	15	25.9
7	Very good	Creamy	Dark brown	3	5.2
8	Good	White/gray	Red/pink	4	6.9
9	Good	Gray	Not clear	8	13.8
Total	6 groups produce soluble pigments			58	100

Antibacterial activity of the actinomycete isolates

All isolates of actinomycetes were screened for antibacterial activity, by agar-disc diffusion method based

TABLE 3 : Screening inhibition spectrum (mm) of the isolated actinomycetes against the test bacteria

Bacterial strain	Antibacterial activity of isolated actinomycetes expressed as inhibition zone (mm)																											
	YI(1)								YI(2)						YI(7)		YI(8)							YI(9)			YI(10)	
	a	b	c	D	e	f	h	H	a	b	c	D	e	F	a	b	a	b	c	d	e	F	g	a	b	c	a	b
Bacillus subtilis	-	-	-	2+	3+	2+	-	2+	-	-	-	-	3+	-	-	-	-	-	-	-	-	2+	4+	-	-	-	-	-
Sarcina	-	-	-	3+	3+	3+	-	2+	-	3+	2+	-	2+	-	-	-	-	-	1+	-	-	-	3+	-	-	-	-	-
Serratiae marcescens	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-	-
E.coli	-	-	-	3+	3+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4+	-	-	-	-	-
Pseudomonas aeruginosa	-	3+	-	2+	2+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Bacterial strain	Antibacterial activity of isolated actinomycetes expressed as inhibition zone (mm)																																		
	YS(3)					YS(4)			YS(5)			YS(6)			YD(11)							YT(12)		YA(13)						YH(14)					
	a	b	c	D	e	a	b	c	a	b	c	a	b	c	a	b	c	d	e	f	g	a	b	a	b	c	d	e	f	a	b	C	d	e	F
Bacillus subtilis	-	-	2+	-	-	-	-	2+	-	-	4+	-	3+	-	-	-	-	-	-	4+	-	-	-	-	-	-	-	-	-	-	3+	-	-	-	-
Sarcina	-	-	2+	-	4+	-	-	-	-	-	5+	-	2+	-	-	-	-	-	-	4+	-	-	-	-	-	-	-	-	-	-	2+	1+	-	-	2+
Serratiae marcescens	-	-	2+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E.coli	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-

1+ = 13 - 15 mm **YI = Yemen – Ibb** **2+ = 16 - 20 mm** **YS = Yemen - Sana'a**
3+ = 21 – 25 mm **YD = Yemen – Damar** **4+ = 26 – 30 mm** **YT = Yemen - Taiz**
5+ = 31 – 35 mm **YA = Yemen – Amran** **- = No inhibition zone** **YH = Yemen – Hodida**

on the observation of inhibition zones around the disc loaded with antibacterial agents on seeded agar containing the target bacterial (*Bacillus subtilis*, *Sarcina*, *Serratia marcescens*, *E. coli* and *P. aeruginosa* were used as target organisms). These bacteria covered the G +ve and G -ve bacteria (TABLE 3 and Figure 1).

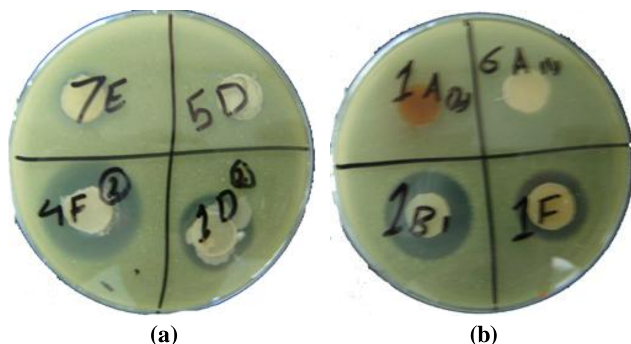

Figure 1 : Antibacterial activity of some isolated actinomycetes by agar-disc diffusion against (a) *B. sbtilitis* and (b) *E. coli*

Of the 63 actinomycete isolates, 18 isolates (28.57%) showed antibacterial activity and 4 of them were active against G +ve and G –ve (broad spectrum). Also soils No. 1 collected from sorghum field in lbb (Wadi-Bana) gave the lowest actinomycetes contents (1.00 X 10^2 CFU/ g soil). It gave 8 colonies, 5 of them, showed antibacterial activity of (62%). Four of them were with strong antibacterial activity producing inhibition of G +ve and G -ve bacteria.

Isolate YI(8)g, which was isolated from soil with wield plant (*Olea europaea*) in Ibb Governorate produced the strongest antibacterial activity against *B. Subtilis*, *Sarcina*, *Sarratia marcescens* and *E. coli* compared to others, producing an average inhibition zone of 16–30 mm diameter, while isolate YI(1)d & e inhibited the growth of G +ve (*B. subtilis* and *Sarcina*) and G –ve (*E. coli* and *P. aeruginosa*) producing an average inhibition zones reaching 16–25 mm in diameter. On the other hand, isolates YS(5)c and YD(11)f gave the maximum range of inhibition zone diameter (26–35 mm) with G +ve bacteria *(B. subtilis* and *Sarcina*). Among the tested bacteria (*B. subtilis* and *Sarcina*) were the most sensitive bacteria than others with an inhibition of 27% and 12 % respectively. Gram –ve bacteria were less sensitive than Gram +ve bacteria whereby they were inhibited by only 5% of the actinomycetes isolates. In addition, *P. aerugenosa* was inhibited by 4 isolates of actionomycetes producing an inhibition zone reaching 16–25 mm.

Extraction and purification of antibiotic substance(s)

Isolates YI(1)d and YI(8)g which gave the highest antibacterial activity against the target bacteria were selected for the extraction and purification of antibacterial product using chromatographic separation. The results obtained in TABLE 4 revealed the ethyl acetate and acetone to be able to extract the antibiotic from actinomycete isolates YI(1)d and YI(8)g respectively. These two solvent extract gave the highest zone against inhibition target bacteria (*B. subtilis* and *E. coli*) with an inhibition zones averaged 14–21 mm and 10–13 mm respectively.

TABLE 4 : Antibacterial activity of selected isolates YI $_{1B(1)}$ and YD9 broth extracted by different organic solvent

Bacterial strains	Inhibition zone (mm) of different organic solvents extracted from actinomycetes broth							
	YI$_{1B(1)}$				YD9(5)			
	Petroleum ehter	Chloroform	Ethy1 acetate	Acetone	Petroleum ether	Chloroform	Ethy1 acetate	Acetone
E. coli	-	-	2+	-	-	-	-	1+
Bacillus subtilis	-	-	3+	-	-	-	-	2+

The antibacterial substances extracted from the broth of both isolates were appeared at different R_f values. Further purification of the antibiotics has been carried out by preparative thin layer chromatograpgy (PTLC). Concerning the ethyl acetate extract of YI(1)d the de-

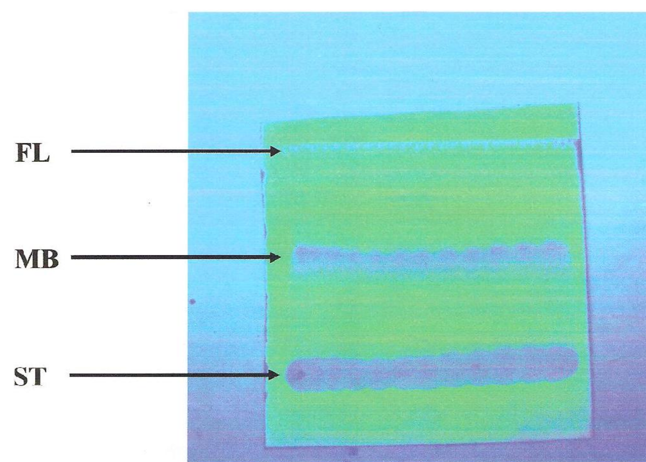

Figure 2 : PTLC of acetone extract of the isolate YI(8)g broth using solvent system (6:4 ethyl acetate:methanol). Double run visualized by UV lamb (254nm).

velopment system was (9:1 chloroform : ethyl acetate) showing four clear bands at R_f 0.9, 0.78, 0.52 and 0.25 while the acetone extract isolate YI(8)g broth gave one major band at R_f 0.62 (Figure 2) using solvent system (6:4 ethyl acetate : methanol) double run. According to the polarity of the isolated bands the third band of the ethyl acetate extract of isolate YI(1)d with R_f 0.52 gave antimicrobial activity against both bacteria (*E. coli* and *B. subtilis*) with an inhibition zone of 14 mm and 12 mm respectively. On the other hand, the acetone gave major band at R_f 0.62 producing an inhibition zone of 18 mm with previously tested bacteria.

DISCUSSION

The development of drug resistance in human pathogenic bacteria such as *Staphylococcus*, *Mycobacterium*, *Streptococcus*, *Enterococcus* and others has promoted a search for more and better antibiotics, especially as diseases caused by these bacteria represent a clear and growing threat to world health[22,27,30]. There is also an increasing need for more and better antimycotics, especially as human population is developing more fungal infections, particularly in HIV/AIDS patients and patients with organ transplant who must take immunosuppressive drugs[34-36]. Actinomycetes produce numerous substances essential for health as antibiotics[20]. Soil is very rich source of actinomycetes, where selection pressure leads to the enrichment of a particular group of actinomycetes and in competition these groups are expected to express certain phenotype, which confer them an advantage over other[19].

Our actinomycete isolates were grouped into 9 groups according to the colour of aerial and substrate mycelia. These were gray, pink, red white, violet, creamy, olive, yellow and brown. Occurrence of these colours has been documented previously[18,32]. Along the same line, Ndonde and Semu (2000)[24] and Ahmed (2003)[1] grouped their isolate of actinomycetes (isolated from Tanzanian and Yemeni soil, respectively) according to the colour mycelia. Our results also indicated that, total count of actinomycetes in soil was varied according to crops but this variation not affected on the antibacterial activity. Whereas results of screening of antibacterial activity shows that the lowest soil (1 X 10^2 CFU/g soil) of actinomycetes (Ibb-Wadi bana) gave the highest number (8 from 9) of actinomycetes with antibacterial activity against G +ve and G -ve. This might be due to increase in organic carbon and total nitrogen contents of rhizosphere which results from the root exudates[3,16]. Castillo et al. (2002)[5] obtained Munumbicins A, B, C and D from Streptomyces NRRL 3052, which is endophytic in the medicinal plant snakevine (*Kennedia nigricans*), native to the Northern territory of Australia. These antibiotics were considered new with a wide spectrum of activity against many human as well as plant pathogenic fungi and bacteria, and *Plasmodium sp*. In parallel with this finding our results indicated that the isolates with the strongest antibacterial activity against both G +ve and G -ve or G +ve only were isolated from soil cultivated by *Ficus carica*, *Sorghum bicolour* and *Pruns prscia*, which are crops of potential medicinal importance.

Our results further demonstrate the presence of the active antibacterial agent in both the ethyl acetate and acetone extracts of the broth from the two isolates [YI(1)d and YI(8)g] with the highest antibacterial activity against G +ve and G -ve bacteria. These findings are in agreement with previous studies[1,10,12,20].

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