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Isolation and identification of secondary metabolites producer *Nocardia* spp. from Iraqi soil

Enas A. Bady[1], Amin A. Al-Sulami[2]* and Kawther H. Mehdi[3]

[1]College of Pharmacy, University of Basra, Basra, Iraq.
[2]College of Education, University of Basra, Basra, Iraq.
[3]Collage of Science, University of Basra, Basra, Iraq.

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One hundred and sixty nine *Nocardia* isolates were recovered from 111 Iraqi soil samples on six cultivation media, using paraffin baiting and dilution techniques. The North of Iraq soil was the richest in *Nocardia.* Paraffin baiting technique was more efficient in the isolation of *Nocardia* than dilution technique. Glucose asparagine agar was more suitable for cultivating *Nocardia* isolates followed by Sabouraud dextrose agar. All the 169 presumptive Nocardia isolates recovered were subjected to primary screening of antibacterial activity, with 10-55 mm inhibition zones against standard *Staphylococcus aureus* (NCTC 6571) and 10-38 mm inhibition zones against standard *Escherichia coli* (ATCC 25922). Eleven isolates with the highest antibacterial activities were selected for chemo-taxonomic studies. These isolates exhibited high similar features of 87-89% with *Nocardia brasiliensis.* Therefore, it is suggested to give proposed names for these new taxa: *Nocardia* sp.1, *N.* sp. 2, *N.* sp. 3, *N.* sp. 4, *N.* sp. 5, *N.* sp. 6, *N.* sp. 7, *N.* sp. 8, *N.* sp. 9, *N.* sp. 10 and *N.* sp. 11.

Key words: Secondary metabolite, *Nocardia* sp., antimicrobial activity.

INTRODUCTION

Actinobacteria, especially when isolated from soil produce many important industrial and commercial antitumor agents, bioactive metabolites and enzymes (Demain and Lancini, 2006). Filamentous nocardiform bacteria represent continuous reservoir of pharmacological, medicinal and agricultural important compounds (Berdy, 2005). Among them, Transvalencin Z as a strong antibiotic against Gram positive bacteria, fungi and tumor cells was recovered from *Nocardia transvalensis* (Akira et al., 2006) and cepha-mycin C was produced by *Nocardia lactemdura* (Kagliwal et al., 2009). Also, Speitling et al. (1998) and Mukai et al. (2009) reported the moderate activities of the metabolites of *Nocardia pseudobrasiliensis* (new anthrax-cycline, dimethyl mutactimycins) against Gram positive bacteria and their cytotoxic activities against P388, L1210 and Hela tumor cells. They also reported novel thiopeptide antibiotic and nocardithiocin with strong activity against rifampicin resistance and sensitive *Mycobacterium tuberculosis* and *Gordonia* sp.

Sakagami et al. (2005) demonstrated that nocobactin

*Corresponding author. E-mail: aminabdulah@yahoo.com.

NAa and NAb from *Nocardia farcinica had* antitumor potential against human tumor cells; whereas gastric adenocarcinoma, breast carcinoma and hepatocellular carcinoma were inhibited by nocardichelins from *Nocardia* strain Acta 3026, which was isolated from mangrove soil (Schneider et al., 2007). The aim of this study is to isolate and identify *Nocardia* spp. from Iraqi soil with high secondary metabolites production.

MATERIALS AND METHODS

Sample collection

One hundred and eleven soil samples used in this study were collected from April-November 2009 from different regions in Iraq. Temperature was measured at the same time of collection; calcium carbonate, salinity and pH of each sample were measured in Marine Science Centre, Basra University.

Isolation technique

One gram of dried sifted soil sample was suspended in 9 ml of saline solution by shaking vigorously for 5 min. Serial decimal dilutions of the supernatant were prepared (El-Nakeeb and LeChevalier, 1962), in which 0.1 ml from each dilution was spread over the surface of six types of media: Glucose asparagine agar (GAA), Sabouraud dextrose agar (SDA), Glycerol agar (GA), Nutrient agar (NA), Yeast extract agar (YEA), modified Czapeks agar (MCA). Antifungal agent, cyclohexamide (actidione) of 50 µg /ml and anti-bacterial agent, chloramphenicol of 50 µg/ml were added to the sterilized media at 46°C (Nazar et al*.,* 1986). Plates were then incubated at 28-30°C for 7-14 days.

Paraffin baiting technique

Twenty five gm of each soil sample was transferred into a sterile conical flask. Into each of these conical flasks, a paraffin-coated sterile glass rod was introduced and covered with cotton and aluminum foil. The conical flask was incubated at 37°C for 4 weeks with some modifications (Mishira and Randhawa, 1969). Paraffin rod was scraped by cotton swab and streaked on different isolation media (GAA, SDA, GA, NA, YEA, and MCA); plates were incubated at 28-30°C for 7-10 days. After incubation period, colonies were streaked on yeast-malt extract agar (YMA) for sub culturing. After that, colonies were stained with Gram staining and acid-fast staining using the method of Ziehl-Neelsen (Benson, 2002).

Morphological and colonial characteristics

For identification of *Nocardia*, the microscopical features of bacterial cells growing on YMA such as cells shape, arrangement and stain were recorded. Also, colonial characteristics of culture isolates in different media were noted.

Primary screening of bacterial isolates

Presumptive *Nocardia* isolates have been screened for production of bioactive metabolites against standard isolates of *Staphylococcus aureus* and *Escherichia coli* using paper disc agar plate diffusion method (Bauer et al., 1966).

Biochemical tests

To know the Nocardia isolates with high antimicrobial activities, motility test, gelatin hydrolysis test, catalase test (Alexander and Strete, 2001), growth tests, tyrosine hydrolysis test, testosterone hydrolysis test, antibiotic resistance test, utilization of organic compounds as carbon source and utilization of some compounds as nitrogen source (Williams et al., 1983), determination of meso-diamino pimelic acid (DAP) and whole sugars in cell wall and melanin production test (Cross, 1981),citrate utilization test, urea's test, nitrate reduction test, starch hydrolysis test, casein hydrolysis test and esculin hydrolysis test (Benson, 2002) and lysozyme resistance test (Forbes et al., 2007) were done. Molecular method used for identification, which is crucial for confirming strain identity was not used due to the unavailability of its tools. However, selected isolates will be sent abroad for sequencing.

RESULTS

Physical and chemical parameters of soil samples

Table 1 showed that soil samples collected for this study have temperatures of 21-49.6°C and pH of 6.8-8.1, respectively; calcium carbonate and salinity ranges were 0.65-12.22 mg/l and 1.03-53.27 ppt.

Isolation techniques

Paraffin baiting technique was more efficacious than dilution technique for isolation of *Nocardia* from soil sample. Paraffin baiting technique gave the highest isolation rate of 400%, while dilution technique gave the highest isolation rate of 100%. Therefore, all other samples were treated with paraffin baiting technique.

Cultivation and distribution of isolates

An isolation rate of the following deceasing order was noticed: GAA 41.42%> SDA 21.30%> NA 14.79%> GA 11.83%> MCA 7.6%9> YEA 2.95%. Results showed that S-N soil samples recorded the highest isolation rate of 244.4% for recovery of isolates and A-S soil samples recorded the lowest isolation rate of 20% for recovery of isolates.

Morphological identification of isolates

One hundred and sixty nine isolates collected in this study were identified as Gram positive filamentous *Nocardia* fragmented into rod to coccid cells with diameter 1.2 µm (Figure 1). The results of acid fast staining divided *Nocardia* into three groups depending on the reaction mode with acid fast stain: acid fast, partially acid fast and non- acid fast.

The results of culturing isolates on different cultivation media showed that *Nocardia* colonies had chalky, dried cerebroid, wrinkled or heaped, domed and smooth with regular or filamentous margin; consistency may be

Table 1. Physical and chemical parameters of soil samples.

Soil type	Collection site	Symbol	Number of samples	Temperature (°C)	pH	$CaCO_3$ (mg/l)	Salinity (ppt)
Agriculture	Garden	A-G	41	41	21.3	12.22	4.28
	Missan sugar cane field	A-S	5	5	37.2	3.76	4.11
River margin	Garmat-Ali	R-G	7	7	21.1	7.54	1.03
	Al- Ashar	R-AS	3	3	21	9.64	1.39
	Al-Shafi	R-SH	3	3	41.6	8.16	5.77
Sandy	Rumaila	S-R	10	10	24.5	8.94	5.24
Oil-contaminated	5-mile	O-M	15	15	49.6	0.65	6.50
Salty	Garmat-Ali	S-G	8	8	23.2	4.38	53.27
Manure	Garmat-Ali	M-G	10	10	28	2.24	13.18
Sulphur saturated	North of Iraq	S-N	9	33.3	7.9	4.3	3.31

Figure 1. Gram staining of *Nocardia.*

mucoid, pasty and cartilaginous or leathery with different diameters range from 2-8 mm. Colonies appeared with different colours as white, creamy, red, yellow, pink, orange, pale tan and purple. Soluble brown pigment was produced by some isolates on different media. Colonies had the ability to grow as convoluted fold on some media as yeast-malt extract agar (Figure 2).

Primary screening of isolates

Primary screening of the 169 isolates from the different soil samples showed antibacterial activities with inhibition zones against standard isolates of *S. aureus* NCTC 6571 (10-55 mm) and *E. coli* ATCC 25922 (10-38 mm). The primary screening enables us to select *Nocardia* isolates with secondary metabolites of high antimicrobial activity (Table 2) and finally subjected them to conventional biochemical tests to know (Table 3) their growth characteristics (Table 4).

All isolates were able to utilize glucose, sucrose, lactose, fructose, galactose, cellobiose, arabinose, xylose, rhamnose, raffinose, mannose, maltose, mannitol, glycerol, sodium acetate, sodium citrate, butanol, sorbitol, trehalose, inositol and adonitol as a sole source of carbon and potassium nitrate, lysine, threonine, serine, methionine, asparagines and phenylalanine as a sole source of nitrogen. The structure of *Nocardia* cell wall contains amino acid (meso-diaminopimelic acid with green spot and high R_f(0.9), sugars as arabinose with red brown spot and R_f (0.53) and galactose with brown spot and R_f (0.38).

According to principal taxonomic characters in Sykes and Skinner (1973), Cowan et al. (1974), Holt et al. (1994) , Kageyama et al. (2004) , Kageyama et al. (2005), Watanabe et al. (2006) and Forbes et al. (2007), these isolates showed a range of chemical properties consistent with their classification in the genus *Nocardia* and not corresponding with any published *Nocardia* sp. But these

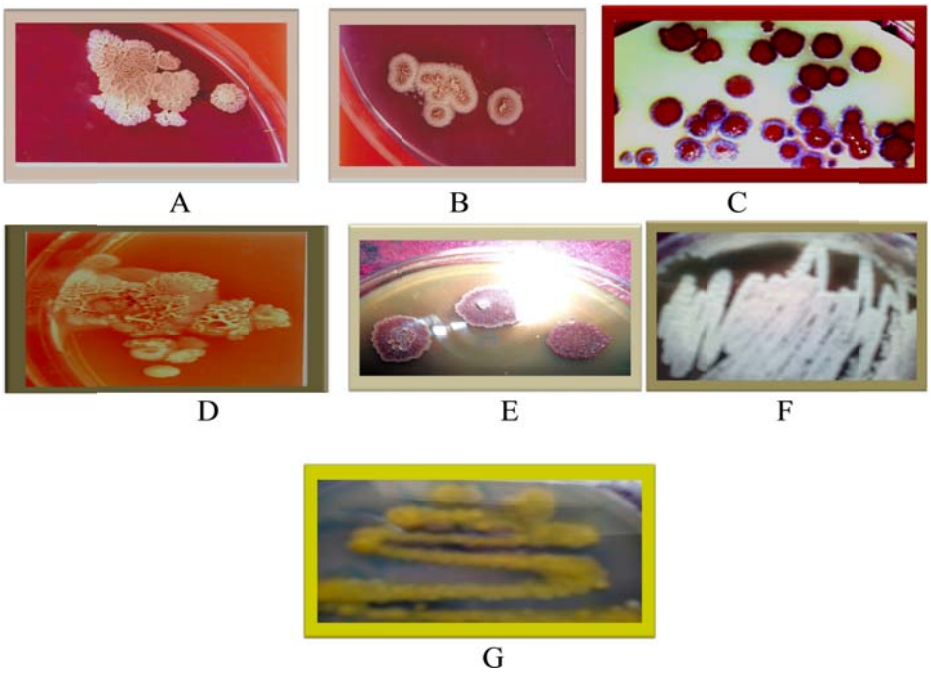

Figure 2. Colonies of *Nocardia.* (A) White yeast-malt extract agar with crystal violet, (B) pale tan, (C) red yeast-malt extract agar, (D) Creamy, (E) pink, (F) chalky growth on yeast-malt extract agar, (G) yellow.

Table 2. Designated *Nocardia* with high antibacterial activity against standard *S. aureus* and *E. coli.*

Nocardia isolate designate	Soil sample	Cultivation media	IZ (mm) of antibacterial activity against *S. aureus*	IZ (mm) of antibacterial activity against *E. coli*
1	A-G	NA	21	30
2	A-G	YEA	40	30
3	A-G	YEA	24	38
4	A-G	GAA	55	10
5	R-G	GA	16	25
6	O-M	NA	10	20
7	A-G	SDA	13	15
8	S-N	GA	23	10
9	S-N	NA	13	14
10	O-M	SDA	30	10
11	S-N	GA	14	30

isolates exhibited similar character ranging from 87-89 % with *N. brasiliensis* (SPSS 2007). Therefore, it is suggested to give proposed names for these taxa including *Nocardia* sp.1, *N.* sp. 2, *N.* sp. 3, *N.* sp. 4, *N.* sp. 5, *N.* sp. 6, *N.* sp. 7, *N.* sp. 8, *N.* sp. 9, *N.* sp. 10 and *N.* sp. 11.

DISCUSSION

Isolation of *Nocardia* from soil

Paraffin baiting technique led to an unequivocal isolation and identification of *Nocardia* from soil due to the ability of *Nocardia* enzymes to degrade paraffin and utilize it as a sole source of carbon Ayyar et al., 1992; Narang et al., 2004; Kaur and Oberoi, 2005). This conforms with Kurup et al. (1968) who confirmed that this technique was successful for the isolation of *Nocardia* from soil. Many *Nocardia* species were isolated from soil by paraffin baiting technique such as *N. brasiliensis, Nocardia asteroids and Nocardia caviae* from Argentina soil (Komaid et al., 1987) and Delhi soil (Kumar and Mohaptra, 1968).

Jayabarath et al. (2010) established that salty soil is a

Table 3. Biochemical characteristics for the identification of the 11 isolates of *Nocardia*.

Test \ Isolate	1	2	3	4	5	6	7	8	9	10	11
Catalase	+	+	+	+	+	+	+	+	+	+	+
Urea's	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+
Melanin	+	+	W	+	+	-	+	+	+	-	-
Hydrolysis of Casein	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+
Tyrosine	-	-	-	-	-	-	-	-	-	-	-
Testosterone	-	-	-	-	-	-	-	-	-	-	-
Esculin	+	+	+	+	+	+	+	+	+	+	+
Liquification of gelatine	+	+	-	+	+	-	+	+	+	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+

+ = good growth, - = no growth, w = weak.

Table 4. Growth characteristics of the 11 isolates of *Nocardia.*

Test \ Isolate	1	2	3	4	5	6	7	8	9	10	11
Soluble pigments	Cr-G	Cr+R	P+R	Cr-G	Cr-G	R	Cr-G	Cr+R	Cr	Cr	R
Acid-fast	-	Par	+	-	+	+	Par	Par	-	Par	-
Motility	-	-	-	-	-	-	-	-	-	-	-
Growth at											
10°C	+	+	+	+	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+	+	+	+	
45°C	+	+	+	+	+	+	+	+	+	+	+
Growth at PH											
5	w	w	w	w	w	w	w	w	w	w	w
7	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+
Growth at NaCl (%)											
4% NaCl	++	++	++	++	++	++	++	++	++	++	++
7% NaCl	++	++	++	++	++	++	++	++	++	++	++
10% NaCl	++	++	++	++	++	++	++	++	++	++	++
13% NaCl	++	++	++	++	++	++	++	++	++	++	++
Growth at the presence of											
Sodium azide 0.01%	++	++	*	++	++	++	++	++	++	++	+
Sodium azide 0.02%	w	w	W	w	w	+	w	w	+	+	+
Crystal violet 0.001%	++	+	++	++	++	+	++	+	++	+	++
Phenol 0.1%	w	-	+	w	w	W	-	w	W	-	-

++ = very good growth, + = good growth, - = no growth, w = weak, Cr = creamy, P = pink, G = green, R = red, par = partial.

good reservoir for *Nocardia amarae, Nocardia farcinica* and *Nocardia vaccini*. *Nocardia* was considered as one of the most common aerobic bacteria responsible for degradation of hydrocarbons in petroleum-contaminated soil (Pucci et al., 2000; Barathi and Vasudevan, 2001; Baek et al., 2004; Alquati et al., 2005; Wyszkowska and Kucharski,

2005; Chikere et al., 2009; Gomes et al., 2009; Nih-Cong et al., 2010) and in gasoline-contaminated aquifers (Rosenberg and Gutnick,1981). Cain (1958) isolated *Nocardia erythropolis* from manure heaps. His results supported that of manure samples. Sandy and agriculture soils (garden and sugar cane) represented a rich source of *Nocardia* (Orchard and Goodfellow, 1974; Padoley et al., 2009). Bredholdt et al. (2007) isolated *Nocardia* from shallow water sediments of the Trondheim fjord (Norway); all these explain the results of Figure 2.

Glucose asparagine agar exhibited a good growth and more numbers of *Nocardia* from marine sediment and Thailand soil, respectively, but less numbers of action-mycetes were recorded with Nutrient agar (Kokare et al., 2004; Srivibool and Sukchotiratana, 2006). Sabouraud dextrose agar containing cyclohexamide was used for isolating *Nocardia* species from Iran soil (Aghamirian and Ghiasian, 2009). Glycerol agar containing soil extract represented a perennial source of organic matter, carbon, nitrogen, minerals and vitamins and a natural medium for growth of many organisms and glycerol, which is important in the growth of actinomycetes (El-Nakeeb and Lechevalier, 1962). Therefore, Xu et al. (1996) isolated *Nocardia* from 4,200 China soil by glycerol asparagine agar medium.

Morphological identification of *Nocardia*

The results of microscopical and cultural tests of *Nocardia* have revealed that they are Gram positive filamentous rod or cocci cells (Figure 1). This is correlated with other studies conducted by Whitmore et al. (1961) and Hattori et al. (2003). *Nocardia* also showed different response to acid fast staining; isolates were divided into non- acid fast, partial acid fast and acid fast due to the average carbon numbers of the mycolic acids. Each species of *Nocardia* possesses a characteristic profile of mycolic acid composition (Yano et al., 1978). Zhang et al. (2004) and Xu et al. (2005) demonstrated that *Nocardia xishanensis* and *Nocardia polyresistens* respectively isolated from soil were partially through acid-fast staining. The colonies' texture varies among isolates (Figure 2) depending on the degree of mycelia development; and the colonies exhibited multiple shapes and colors due to carotenoid pigments (Cowan et al., 1974). Many studies agreed with this result such as that of Whitmore et al. (1961) who showed that *Nocardia* exhibited gray, red, yellow, white, smooth, domed colonies with rough powdery surface. Zhang et al. (2004) established *Nocardia* colonies as yellow to orange, convex to irregular with filamentous margin. Kurup and Schmitt (1970) noticed colonies of *Nocardia* on the paraffin bait as yellow, white, brown, pink. Hattori et al. (2003) isolated *N. africana* with orange wrinkled colonies.

Primary screening of *Nocardia* isolates

Primary screening was necessary for evaluating the antimicrobial activities of *Nocardia* isolates against microorganisms tested including *S. aureus* and *Candida albicans* (Anansiriwattana et al., 2006; Vengadesh et al., 2011; Vinhot et al., 2011) due to their ability to produce bioactive metabolites against them (Chandrashekhara, 2010). *Nocardia iowensis* isolated from garden soil of Osceola, Iowa, USA represented a source of antibiotic production (Lamm et al., 2009). The appropriate conditions characterized this soil as neutral pH and suitable temperature in the presence of calcium carbonate played a good role in the exuberance of *Nocardia* from this soil (Table 3).

Biochemical identification of *Nocardia*

As a result of primary screening, eleven of 169 isolates with high antibacterial activity were selected and identified (Tables 2 to 4). The biochemical features of the 11 isolates did not correspond with any *Nocardia* species but exhibited high similar features (87-89 %) with species of *N.brasiliensis*. Therefore, it was suggested to name them as *Nocardia* sp.1*, N.* sp. 2*, N.* sp. 3*, N.* sp. 4*, N.* sp. 5*, N.* sp. 6*, N.* sp. 7*, N.* sp. 8*, N.* sp. 9*, N.* sp. 10 and *N.* sp. 11. This was in accordance with other studies that recorded new species of *Nocardia* (Kageyama et al., 2004, 2005). The results were obtained by comparing the biochemical features of new isolates with published ones.

Conflict of Interests

The authors have not declared any conflict of interests.

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