Antimicrobial activities of some alkaliphilic and alkaline-resistant microorganisms isolated from Wadi Araba, the eastern desert of Egypt

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Abstract: Thirty soil samples from different six localities representing Wadi Araba, Egypt were collected for isolation of alkaliphilic and alkaline-resistant microorganisms as a possible source of new antimicrobial compounds. The climatic factors and soil analysis of the study area are given. The soil samples were sandy and varied from slightly to moderate alkaline, and also from non-saline to slightly saline. The number of microbial colonies from the different soil samples varied from 10^2 to 10^4 CFU/g of soil. It was obvious that the viable microbial counts were affected by the organic matter content and the pH of the soil. A total of 117 alkaliphilic and alkaline-resistant microorganisms were isolated. Among them, 73 isolates were bacteria, 40 isolates were actinomycetes and the remaining 4 isolates were fungi. The purified alkaliphilic and alkaline-resistant microorganisms were investigated for their antimicrobial activities and the results revealed that 23 isolates of bacteria, 22 isolates of actinomycetes and two fungal isolates have antimicrobial activities. Therefore, the results recommended the screening of extremophiles as possible source of new secondary metabolites.

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1. Introduction

Since more than 12000 antibiotics have already been found to date, there may be increasing difficulty in discovering new antibiotics under normal cultural conditions. Screening for antibiotics by using an extreme cultural condition is one possible way to overcome this difficulty (Sato *et al*., 1983, Hozzein *et al*. 2011). Both eukaryotic and prokaryotic microorganisms capable of growth at several values of pH above neutrality have long been known. Having been often considered as oddities they have received little attention until the last two decades.

The term "alkaliphiles" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and 12, but cannot grow or grow only slowly at the near-neutral pH value of 6.5 (Horikoshi, 1999). Alkaliphiles can be separated into two broad categories: alkaline-tolerant organisms, which show optimal growth in the pH range 7- 9 but cannot grow above pH 9.5, and alkaliphilic organisms, which show optimal growth between pH 10 and 12 (Krulwish & Guffanti, 1989).

The Eastern Desert of Egypt occupies the area extending from the Nile Valley eastward to the Gulf of Suez and the Red Sea, which is about 223000 km^2, i.e. 21% of the total area of Egypt. It is located on an Eocene calcareous substratum more than 1000 m thick (Said, 1990). It is a highland terrain dissected by numerous wadis (valleys) running eastward to the Red Sea or westward to the Nile valley.

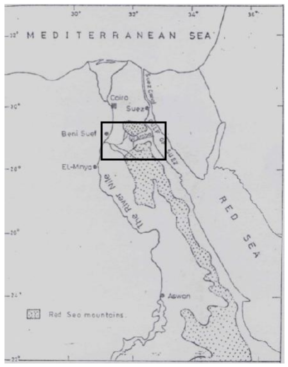
A wadi system is the assemblage of arid and hyperarid zone biotypes and often associated with a dry drainage system sometimes used by rare and unpredictable floods (Fossati *et al.*, 1998). Wadi Araba is one of the largest wadis (valleys) in the Eastern Desert of Egypt. This valley is about 30 km wide (N – S) (Zahran and Willis, 1992). It lies between 28º 28′- 29º 19′N. Lat. and 31º 50′- 32º 38′ E. Long.

In the course of our screening of soil microorganisms for the production of new antimicrobial compounds, we choose to collect soil samples from Wadi Araba, Egypt. Also, the high alkaline media as an extreme condition for isolation of alkaliphilic and alkaline-resistant microorganisms as a possible source of new antibiotics was tested.

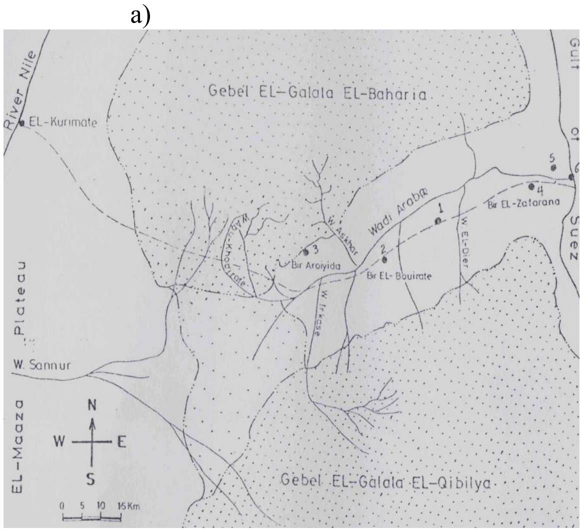
2. Material and Methods

Collection of soil samples:

Thirty soil samples from six different localities represented Wadi Araba, Egypt were collected (Fig. 1). Each soil sample was taken at a depth of 5- 20cm with a collecting spatula. Most of the collected samples were obtained from the rhizosphere of the dominant plants in clean sterilized plastic bags and then stored in a refrigerator at 4°C. Samples were analyzed as quickly as possible.



a)



b)

Figure 1. Two maps showing (a) the location of the study area (Wadi Araba), and (b) the location of the different six localities from which the soil samples were collected.

Characteristics of the study area:

Climatic parameters of the study area including temperature, rainfall and relative humidity are given here as obtained from Bir Araiyida station (cover an area with 50 km radius at elevation of 290 m ASL).

Particle size is determined using a series of sieves ranging from 2mm till 0.05mm. Thin silt and clay are separated using pipette method (Piper, 1944). Organic matter content was determined by using the wet combustion method of Walkley and Black as described by Tan (1996). Total carbonates were determined by the rapid titration method according to Allen *et al*. (1974), bicarbonates by titration with standard potassium bisulfate and chlorides by titration with silver nitrate. The electric conductivity corresponding to the total soluble salts was determined in 1: 5 soil–water extracts according to Jackson (1967). The soil reaction (pH) was determined in 1: 5 soil–water extracts by using Bekman pH meter.

Isolation of alkaliphilic and alkaline-resistant microorganisms:

Soil dilution plate technique (Johnson *et al*., 1959) was used for such a purpose. The isolation medium was Sato medium "A", which was recommended by Sato *et al*. (1983) for isolation of microorganisms on high alkaline pH. The pH of the medium was 10 - 10.5.

Tenfold serial dilutions were made to cover the range of 10^{-1} to 10^{-6}. The plates were then incubated for 14 days at 30ºC. The most suitable dilutions for counting were selected and colonies of bacteria, actinomycetes and fungi formed on a plate were counted. These colonies were isolated, purified and then subcultured on slants of the same medium. The bacterial and fungal isolates were selected according to differences in cultural characteristics. The actinomycete isolates were selected at random except that apparent duplicate isolates, according to the color grouping method of Williams *et al*. (1969).

Antimicrobial activities of the alkaliphilic and alkaline-resistant microorganisms:

The isolates were inoculated to Sato medium "A" (Sato *et al*., 1983) and then incubated for 3 days on a rotary shaker of 200rpm at 30ºC. One ml suspension of each culture was transferred aseptically to the modified starch-nitrate medium (Tadashi, 1975) used for antibiotic production which contains (g/l): Soluble starch, 20; $NaNO_3$, 2; K_2HPO_4, 1; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; and $FeSO_4.7H_2O$, 0.01. The pH was adjusted to 10 after sterilization by addition of sterilized 10% Na_2CO_3 solution.

After incubation for 2 days for bacterial isolates and 7 days for actinomycete and fungal isolates on a rotary shaker of 200rpm at 30ºC, testing of the antimicrobial activities was carried out by using the classical diffusion method (Cooper, 1963 & 1972) by loading analytical paper disks (5mm in diameter for bacteria and yeast and 12mm for fungi) with the fermentation broth after neutralization with dilute HCl and aseptically placed on the surface of the seeded plates with the different test organisms. Controlled disks were made by loading the basal fermentation medium after neutralization to the disks. The following microbial cultures were used as test organisms for such a purpose: *Bacillus subtilis* NCTC 10400, *Bacillus pumillus* NCTC 8214, *Staphylococcus aureus* NCTC 7447, *Micrococcus luteus* ATCC 9341, *Escherichia coli* NCTC 10416, *Pseudomonas aeroginosa* ATCC 10145, *Klibsella penuminae* NCIB 9111, *Saccharomyces cerevisiae* CBS 1171, *Candida albicans* IMRU 3669, *Penicillium chrysogenum* ATCC 12960 and *Aspergillus flavus*. The plates were then incubated at 37ºC for 24 hours for bacteria, and at 30ºC for 24- 48 hours for yeast, and 7days at 27ºC for fungi.

3. Results

Characteristics of the study area:

All climatic parameters are summarized in table (1) as obtained from Bir Araiyida station. Table (1) shows that, The annual mean temperature is 21.4ºC. The absolute minimum temperature was recorded in February and reaches 1ºC and the absolute maximum temperature was recorded in June and reaches 45ºC. Rainfall is so variable and table (1) shows that during four years (1995- 1998), the mean annual rainfall is 1mm/year and occurred during the months December-March (i.e. winter rainfall). The summer months are completely rainless. The most humid months are from December to February.

Table 1. Average of monthly mean temperature, rainfall and relative humidity of Wadi Araba, Egypt (records of Bir Araiyida station during the period 1995-1998).

Month	Temperature (ºC)			Average rainfall	Average Relative humidity (%)
	Mean max.	Mean min.	Mean		
Jan	19.3	5.2	12.3	0.2	49.97
Feb	20.1	5.7	12.9	0	48.03
Mar	25.7	7.4	16.6	0.4	41.76
Apr	28.9	12.2	20.6	0	33.5
May	35	18.1	26.6	Trace	29.9
Jun	36.5	19.5	28	0	24.6
Jul	37.2	21	29.1	0	32.1
Aug	36.6	20.4	28.5	0	35
Sep	35.5	18.8	27.2	0	34.3
Oct	30.8	15.2	23	0	39.4
Nov	25.6	10.3	18	0	43.37
Dec	20.8	6.2	13.5	0.4	48.5
Average total	29.3	13.3	21.4	1	37.43

All the soil samples collected from the six localities representing Wadi Araba are sandy soils. The values of organic matter content are widely varied from 0.06% in the soil around Bir Araiyida to 0.77% in the soil from the main valley bed. We must take into consideration that the amount of organic matter content in the soil may depend on the density and type of plant cover or whether soil was collected from the rhizosphere of plants or from the nearby zone. Total carbonates (CO_3^{--}) varied greatly from 12.4 % in the main valley bed to 33.4% in the coastal region. Bicarbonates (HCO_3^-) are ranging from 0.063 % to 0.088 %. Chlorides (Cl^-) varied from 0.11% in the main valley bed to 0.60 % in the coastal region. The electric conductivity was varied greatly from 0.26 mmohs cm^{-1} in the main valley bed to 1.80 mmohs cm^{-1} in the coastal region. The pH values of the tested soil samples from the different six localities showed slight variations, all of them fall in the alkaline range. The lowest value (7.64) was for the main valley bed and the highest value (8.45) for the coastal region.

Isolation of alkaliphilic and alkaline-resistant microorganisms:

The number of microorganisms grown on high alkaline medium was counted (Table 2), and it was estimated to be $10^2 \sim 10^4$ CFU per one gram of soil. In general, among colonies on Sato medium A, 67 % of the colonies were bacteria, 30 % were actinomycetes and the remaining 3 % were fungi. By using Sato medium A, 117 alkaliphilic and alkaline-resistant microorganisms were isolated from 30 soil samples from different six localities represented Wadi Araba, Egypt. Among the 117 microorganisms isolated, 73 isolates were bacteria, 40 isolates were actinomycetes and the remaining 4 isolates were fungi.

Table 2. Average microbial counts (CFU/gram of soil) of six different localities representing Wadi Araba, Egypt.

Locality	Bacteria	Actinomycetes	Fungi
Valley bed	24.3×10^{-4}	86.6×10^{-2}	3.3×10^{-2}
Bir Bouirate	93.5×10^{-3}	12.5×10^{-2}	2.5×10^{-2}
Bir Araiyida	48.7×10^{-3}	2.5×10^{-2}	0.0
Bir Zafarana	4.5×10^{-3}	15.0×10^{-2}	2.5×10^{-2}
Littoral region	19.5×10^{-3}	17.5×10^{-2}	0.0
Coastal region	11.0×10^{-3}	9.0×10^{-2}	0.0

Antimicrobial activities of the alkaliphilic and alkaline-resistant microorganisms:

The results of the antimicrobial activities of the isolated alkaliphilic and alkaline-resistant microorganisms by the paper disk method revealed that 23 isolates of bacteria, 22 isolates of actinomycetes and two fungal isolates were active. Among the 23 isolates of bacteria, 23 isolates showed antibacterial activities and 5 isolates showed antifungal activities. Among the 22 isolates of actinomycetes, 22 isolates showed antibacterial activities and 4 isolates showed antifungal activities. The two fungal isolates showed antibacterial activities only (table 3).

Table 3. Grouping of the microorganisms isolated on high alkaline medium from Wadi Araba, Egypt and their antimicrobial activities.

Microbial group	Isolated strains	Active strains	Types of antimicrobial activities				
			I	II	III	IV	V
Bacteria	73	23	16	2	0	4	1
Actinomycetes	40	22	15	3	0	3	1
Fungi	4	2	2	0	0	0	0

I: Strains with activity against Gram-positive bacteria; II: Strains with activity against Gram-positive & Gram-negative bacteria; III: Strains with activity against Gram-negative bacteria; IV: Strains with activity against Gram-positive bacteria and also fungi; and V: Strains with activity against Gram-positive & Gram-negative bacteria as well as fungi.

4. Discussion

The list of novel microorganisms and products found in microbiologically poorly explored areas of the world stresses the advantage of investigated new habitats (Nolan and Cross, 1988). Therefore, Wadi Araba in the Eastern Desert of Egypt was selected for collection of the samples as a microbiologically poorly explored area.

In qualitative and quantitative terms, the microbial flora is governed by the surrounding habitat (Alexander, 1983). The stage of the life cycle predominates, the size of the community, its biochemical transformations, and the genera and species found are determined by the forces acting within the ecosystem. The primary ecological influences include the organic matter content, pH, moisture and the temperature. Therefore, the ecological characteristics of the study area were reported here to show their effects on the microbial flora.

The climatic parameters recorded revealed that this area locates in the hyperarid zone with a mean annual temperature of 21.4ºC and a mean annual rainfall of 1mm/year. The physicochemical characteristics of the soil samples showed that all of them are sandy soils. Based on soil pH values, the soil varied from slightly alkaline (pH = 7 - 8) to moderately alkaline (pH = 8 - 9) according to Tan (1982) and Brady (1990). Based on percentage of chlorides and electric conductivity, the soil samples varied from non-saline to slightly saline according to Jackson (1958). Our present results are in accordance with those of Sharaf El-Din and Shaltout (1985) who studied an area of $10km^2$ of the main bed of Wadi Araba.

The mechanisms that allow the organism to adapt to extreme environments are one of the most interesting subjects for microbiologists. Micro-organisms that prefer unusual extreme conditions to normal conditions offer an important research tool for investigating the relationships and interactions between environmental factors and microbial life (Gould and Corry, 1980). In general microorganisms that proliferate and grow under the most extreme conditions are obligately adapted to their particular environment (Horikoshi and Grant, 1991), and fail to grow at lower intensities of the same environmental factor.

The number of microbial colonies grown on high alkaline medium from the different soil samples varied from 10^2 CFU g^{-1} to 10^4 CFU g^{-1}. On a neutral pH agar plate with sodium carbonate omitted from Sato medium A, $10^5 \sim 10^7$ colonies appeared in one gram of the same soil. Therefore, the numbers of colonies capable of growing on a high alkaline medium seemed to decrease by about 10^{-3} of that on a neutral pH medium. We think that, this count is far from the actual count and in general it is lower than the real count. This is because of the efficiency of the dilution plate technique used is markedly influenced by the composition of the nutrient medium (Williams and Cross, 1971). This decrease in count is in accordance with Sato *et al*. (1983) who reported that, the number of colonies capable of growing on a high alkaline medium seemed to decrease by about 10^{-2} of that on a neutral pH medium.

It is obvious from the recorded results that the viable microbial counts are greatly affected by the organic matter content and the pH of the soil. The highest microbial counts were from soil samples of the main valley bed (O.C. = 0.77% and pH = 7.64) and the lowest microbial counts were from soil samples around Bir Araiyida (O.C. = 0.06% and pH = 8.21). Therefore, the viable microbial counts increased with increasing the organic matter content and decreasing the pH. These results are in accordance with many authors (Ragab, 1993; Mishra, 1996, Hozzein *et al*., 2008) who stated that the organic matter content greatly affected the microbial content. Also, Alexander (1983) reported that the size of the community depends on the soil type, particularly on certain of the physical characteristics, organic matter content and pH of the environment.

The results of the antimicrobial activities of the isolated alkaliphilic microorganisms showed that, 31.5% of bacteria, 55% of actinomycetes and 50% of fungi were biologically active. Hence, the actinomycetes are the most active group among the isolated alkaliphilic microorganisms. Recent reports showed that this group of bacteria continues to be a major source of novel and useful compounds (Basilio *et al*., 2003; Singh *et al*., 2012).

Some surveys have attempted to show that there is a relation between the percentage of antibiotic-producing strains and the sites of collection of soil samples. Two extreme views have crystallized, concerning the distribution of antibiotic-producing microorganisms in nature. Some authors think that ecological factors have only a little or no influence on synthesizing different types of antibiotics. On the other hand, many investigators stress the importance of ecological factors. There may be such a relation but many of the data are unconvincing (Porter, 1971). Accordingly, we do not study here the relation between the distribution of antibiotic-producing isolates and the soils from which they are collected.

The present study aimed to isolate new microorganisms with potentially useful biological activities from different soil samples collected from Wadi Araba, Egypt. From this study we can conclude that: (1) The microbiologically poorly explored areas can harbor new microorganisms and they should be investigated for their microflora, (2) Microorganisms which live under extreme alkaline conditions, could be candidates for production of new antibiotics; and (3) Much interest should be oriented to the microorganisms isolated under extreme cultural conditions as a possible source of new natural products.

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