Journal of Innovations in Pharmaceuticals and Biological Sciences
www.jipbs.com
JIPBS
ISSN: 2349-2759

Research article

Antidermatophytes from bioactive secondary metabolites of local *Streptomyces* spp.

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Abstract

Dermatophytes were considered as pathogenic fungi that invade human skin, nails and hairs and cause dermatomycosis. One hundred and eleven were isolated from both marine and terrestrial Egyptian habitats were screened for their ability to produce bioactive secondary metabolites with antidermatophytic this has been done by cultivating them on ISP2 liquid media for 10 days. The antdermatophytic activity was tested for the produced extracts by well-diffusion methods. Extracts from isolates A1, A3, A10, G5 and P4 exhibited potent antidermatophytic activity against trichophyton mentagrophytes (RCMB 09285), Microsporumcanis (RCMB 07321) and Microsporumgypseum (RCMB 07336). These potent isolates were identified by studying their morphological, physiological and biochemical characteristics and microscopical studies.

Key words: Antidermatophytes, Bioactive secondary metabolites, *Streptomyces*.

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1. Introduction

Natural products (secondary metabolites) considered the most important source of potential drug leads to be used in drug discovery [1], furthermore there is an urgent need to discover new antibiotic to counter and reverse the spread of antibiotic resistant pathogens [2] and to combat life-threatening diseases [3]. Even though there are a considerable progress within the fields of chemical synthesis and engineered biosynthesis of antimicrobial compounds, nature still remains the most important source for new antibiotics [4].

Actinomycetes, the cultivable group of Gram-positive, filamentous bacteria from diverse ecological niches, they are saprophytes and are responsible for the degradation of complex biopolymers [5]. *Actinomycetes* considered as one of the most important suppliers of antibiotics. Further, they can produce an array of secondary metabolites, many of which have antibacterial or antifungal properties. In fact, most antibiotics developed from human pharmaceutical use are produced by *actionmycetes,* and

many being derived from *Streptomyces* sp. [6]. *Streptomyces* is the most predominant genera representing about 57% of the total soil actinobacteria population], and is widely distributed in terrestrial and aquatic habitats [7]. This member of the order Actinomycetales with complex life cycle involving three stages of differentiation. It is thought that the morphological and physiological differentiation and onset of secondary metabolites production result from common elements of regulation [8]. D*ermatomycosis* is a skin mycotic disease of skin caused by *Dermatophytes*. Pathogenic fungi, which invade the keratinized and cutaneous areas of the body (nail, hair and skin). Most of them belong to the *Hyphomycetes* but several are now known to have perfect states (teleomorphs) in the family *Gynmoascaceae* of the order *Eurotiales* [9,10]. The dermatophytes are represented by three genera: *Microsporum, Trichophyton*, and *Epidermophyton* on the basis of morphology of their macro and microconidia. The teleomorphs of the *Microsporum* and *Trichophyton* spp. are called nannizia and arthroderma respectively, however an asexual form of *Epidermophyton floccosunt* is unknown[11]. As the dermatophytes have developed resistance to antimycotic drugs [12], there is an urgent need to discover safe and cost-effective antidermatophytes drugs [13]. This study is undertaken with the aim of isolating, cultivating and screening of *Streptomyces* spp. Extracts for their ability to work as antidermatophytic agents. The potent Streptomyces isolates were further identified by studying the morphological, cultural physiological as well as the biochemical characteristic.

2. Materials and Methods

Collection of marine and soil samples

Marine Samples were collected from different locations of the Red sea (Ghardga), Marine. Ain Sokhna Sediment and Ras Sedr sediment. Terrestrial samples are collected from Mansoura governorate, National research, Dokki, Giza NRC and Pyramids zone, Giza soil. All the samples were kept in sterile tubes in refrigerator at 4^{o}C for further analysis.

Isolation of *Streptomyces spp.*

Streptomyces spp. were isolated by serial dilution technique [14] in which Sample were added to a sterile saline solution (0.85% NaCl) in a percent of 1:10(w/v or v/v) starch casein (SCA) of the following constituents (g/l): Soluble Starch (10); KNO_3 (2.0); Casein (0.3) $MgSO_4.7H_2O$ (0.05); $FeSO_4.7H_2O$ (0.01); $CaCO_3$ (0.02); agar, 20.0 and distilled water (1000 ml) in case of soil isolation and filtered aged sea water in case of marine isolation the pH was adjusted to 7.2. Proper antibacterial (Penicillin G) and antifungal (Nystatin) agents were added. After sterilization at 121^{o}C for 20 min. the media were poured into Petri dishes and left over night to prevent moisture film [15]. 100 µl from each dilution were used to inoculate a Petri dish of 10 cm diameter containing about 20 ml medium and the inoculums was spread on the dish top using a sterilized glass rod. The inoculated plates were incubated at 28^{o}C for 7-14 days and noticing any growth. The isolation of *streptomycetes* based on their special morphological characteristics (deep sitting colonies, sporulation, characteristic color, etc.) The plates that showed countable single colonies were selected and purified by a streak plate technique.

Fermentation and extraction

Streptomyces spp. were cultivated on ISP2 medium (g/l): Glucose (4.0), Yeast extract (4.0), Malt extract (10.0). The pH was

adjusted to 6.8. One liter volume of Erlenmeyer flasks each containing 200 ml of ISP2 medium were inoculated with 5 ml spore suspension. The inoculated flasks were incubated on a rotary shaker with 150 rpm rotation at 30ºC for 10 days. The *Streptomyces* cells were removed by centrifugation at 5000 rpm for 20 min, and the culture supernatants were extracted using ethyl acetate Table (1), the ethyl acetate phase was evaporated till dryness and used for further studies.

Antidermatophytic activity

The antidermatophytic activity of ethyl acetate extracts were tested using the agar well diffusion method [16], 100 µl of each extract culture was placed in a well made with a sterile cork borer on Sabouraud dextrose agar plates (pH 5.6) seeded with the test fungal cultures *Trichophyton mentagrophytes (RCMB 09285), Microsporum canis (RCMB 07321) and Microsporum gypseum (RCMB 07336)*. The plates were incubated at 28ºC and observed for antibiosis after 3-4 days [17] Results are shown in Table (2). Amphotericin B was used as a control. Twenty-one isolates were primarily selected as they exhibited interesting antidermatophytic activities. Ten isolates (A1, A3, A8, A10, A12, G5, P2, P4, P7, P9) exhibited antidermatophytic activity by a clear zone of inhibition against all the test dermatophytes. While nine isolates (B13, B17, F5, F7, F12, F20, S1, S5, S14) exhibited antidermatophytic activity by a clear zone of inhibition against *Microsporumcanis (RCMB 07321) and Microsporumgypseum (RCMB 07336*) and two isolates(H3, H10) exhibited antidermatophytic activity by a clear zone of inhibition against (*Trichophyton mentagrophytes (RCMB 09285)* and *Microsporumcanis (RCMB 07321).* Taxonomic identification: The diagnostic properties of the strain were compared with those reported for identification of *Streptomyces* species according to Shirling and Gottleib [18-20]. Alternatively, the strains were rather identified using the Bergey's Manual of Determinative Bacteriology [21] and Bergey's Manual of Systematic Bacteriology [22].

3. Results and discussion

Results

Isolation of actinomycete One hundred and eleven of actinomycetes was isolated from different marine and terrestrial habitats including Ghrgada marine sea, Ras Sedr sediment, Ain Sokhna. Results in Table (1) indicate Highest number of strains were isolated from Hurgada sea water (20 %) followed by Ras sedr sediments (16 %) and soil isolates from Mansoura (17 %).

Table 1. Distribution and percent of *streptomyces* spp. isolated from different marine and terrestrial localities.

Location	Isolates account	Percentage incidence (%)
Marine sea water Hurgada	22	20 %
Marine algae	13	12 %
Ras Sedr sediments	18	16 %
Ain Sokhna sediment	13	12 %
Marine algae 2	5	4 %
Soil from Mansoura	19	17 %
Soil from NRC garden	10	9 %
Pyramids soil	11	10 %
Total isolates	111	100 %

Antidermatophytic activity test: Result in Table 2 revealed that twenty-one isolates exhibited antidermatophytic activities. Ten extracts from. (A1, A3, A8, A10, A12, G5, P2, P4, P7, P9) showed antidermatophytic activity against all test dermatophytes: *Trichophyton mentagrophytes (RCMB*

09285), Microsporum canis (RCMB 07321) and Microsporum gypseum (RCMB 07336).While the most potent *streptomyces* spp. extracts were (A1, A3, A10, G5 and P4)vsubjected to identification using physiological, biochemical and cultural characterization.

Taxonomical study of the most potent isolates

The chromatographic analysis of the cell wall hydrolysate revealed that the five isolates contain LL-DAP and no characteristic sugars (Table 3). The first isolate (A1) identified as *Streptomyces violaceus* [23]. The strain characterized by rectiflexibiles spore chains (Figure 1a) and smooth spore surface (Figure 1b). Melanoid pigments are generally produced at tyrosine agar. The color of spore mass is yellow to brown and diffusible pigments are produced hydramycini from *Streptomyces violaceus*[24]. Also, isolate (A3) was detected as *Streptomyces somaliensis* [25]. The strains are characterized with filamentous, Straight aerial hyphae (Figure 2a) and smooth spore surface (Figure 2b). Melanoid pigments are generally not produced. Diffusible pigments may be produced on some media. In addition, isolate (A10) was detected as *Streptomyces antibioticus* [26]. The strains are characterized with spiral spore chains (Figure 3a) and hairy spore surface (Figure 3b). Melanoid pigments are generally produced, Diffusible pigments not produced. The spore mass is grey. *Streptomyces antibioticus* Tü 6040 was used in the production of simocyclinones. Finally, isolate (G5) identified as *Streptomyces exfoliatus* [27,28]. This isolate is characterized by rectiflexibiles spore chains (Figure 4a) and the spore surface is smooth (Figure 4b). Melanoid pigments are not produced and the spore mass is usually yellow. Exfoliazone was extracted from *Streptomyces exfoliatus* BT-38, showed antifungal activity against *V. ceratosperma* [29]. Taxonomic identification of the most potent actinomycetes strain (P4) has been restricted to *Streptomyces* sp. The strains are characterized with spiral spore chains (Figure 5a) and warty spore surface (Figure 5b). Melanoid pigments are generally produced. The strain was deposited in the Department of Microbial Chemistry Collection, NRC, Egypt.

Figure 1a. Photomicrograph showing rectiflexibiles sporophores hyphae (isolate A1, × 400).

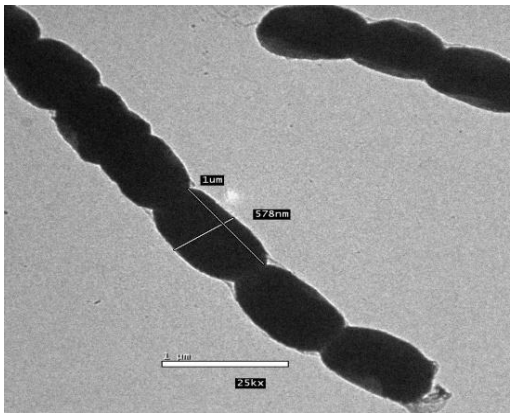

Figure 1b. TEM photomicrograph showing smooth spore surface (isolateA1, × 25000).

Discussion

Natural products are organic compounds that are not directly involved in the normal growth, development, or reproduction of an organism [30]. They play a very important role in everything of our life such as food, cloths, buildings etc. Beside this, they

provide a significant protection against various diseases. For many years, natural sources are important in the development of new active molecules. The discovery of penicillin by Fleming opened the door to a variety of new natural products "miracle drugs" that have saved the lives of millions. But before the discovery of penicillin, the only treatments available for microbial infections were quinine, arsenic and sulfa drugs. All of these were highly toxic (poisonous). As a result of the Fleming discovery and with the continuous research of the past, in our days ten thousands of antibiotics derived from microbial sources are known [31]. In the "heroic" or "golden era" in the forties and early fifties, when almost all groups of important antibacterial antibiotics (tetracyclines, cephalosporins, aminoglycosides, macrolides) were discovered, the success story had continued. It seemed that the main problems of chemotherapy had been solved. Antibiotics discovered in this period were mainly isolated from *Streptomyces* species representing some 70 to 80% of the all isolated compounds they were primarily active against bacteria and fungi. In this period the discovery of antitumor, antiviral and non-antibiotic – enzyme inhibitory – metabolites, had just started [31].

Table 2. Antidermatophytic activity of the best *streptomyces* spp. ethyl acetate extracts

Isolate no.	Antidermatophytic activity (clear zone, mm)		
	***Trichophyton mentagrophytes* (RCMB 09285)**	***Microsporum canis* (RCMB 07321)**	***Microsporum gypseum* (RCMB 07336)**
A1	17.6± 0.52	19.8± 0.35	13.2± 0.13
A3	13.1± 0.13	16.4± 0.35	11.4± 0.28
A8	13.6± 0.37	14.7± 0.13	9.3± 0.23
A10	18.1± 0.12	20.3± 0.34	12.4± 0.21
A12	15.6± 0.32	11.1± 0.16	10.1± 0.26
B13	NA	12.8± 0.22	11.6± 0.35
B17	NA	15.6± 0.13	13.4± 0.32
F5	NA	16.8± 0.24	15.3± 0.27
F7	NA	17.6± 0.46	13.9± 0.13
F12	NA	18.4± 0.29	14.8± 0.19
F20	NA	19.3± 0.12	19.1± 0.39
G5	17.2± 0.26	18.1± 0.12	16.3± 0.35
H3	10.3± 0.13	13.2± 0.52	NA
H10	9.9± 0.13	10.6± 0.24	NA
S1	NA	13.1± 0.19	9.3± 0.34
S5	NA	15.7± 0.13	11.4± 0.26
S14	NA	13.4± 0.25	12.5± 0.12
P2	13.6± 0.16	15.2± 0.19	11.2± 0.27
P4	20.8± 0.25	23.7± 0.18	21.3± 0.26
P7	13.8± 0.21	13.9± 0.19	14.2± 0.33
P9	15.7± 0.23	11.3± 0.43	15.4± 0.26
Amp.	22.9± 0.12	25.3± 0.19	23.6± 0.31

Table 3. Physiological and chemo-taxanomical properties of the selected streptomycetes isolates.

Isolate no.		**A1**	**A3**	**A10**	**G5**	**P4**
Melanin pigment production	Pepton iron agar	-	-	-	-	-
	Tyrosine agar	+	-	+	-	+
Enzyme activities	proteolysis	+	+	+	+	+
	lipolysis	-	+	-	-	+
	lecithinase	+	-	-	+	-
Utilization of different carbon source	No suger (-)	+	+	+	+	+
	D-Glucose (+)	+	+	+	+	+
	D-Fructose	+	+	+	+	+
	Sucrose	-	+	+	+	+
	Rhamnose	-	-	+	+	+
	D-Mannitol	-	+	+	-	+
	D-Xylose	-	-	+	+	+
	Raffinose	-	-	+	+	+
	I-inositol	-	-	+	-	+
	Galactose	-	+	+	+	+
	L-Arabinose	+	+	+	-	+
Whole cell	Type of sugar	-	-	-	-	-
	DAP*	+	+	+	+	+
Nitrate reduction		+	+	+	+	+
H2S production		-	-	+	+	+
Starch hydrolysis		+	+	+	+	+
Cellulose decomposition		-	-	+	+	-
Gelatin liquification		+	+	+	+	+

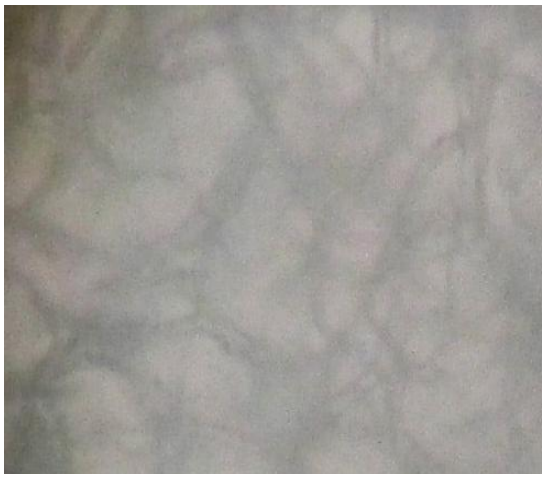

Figure 2a. Photomicrograph showing Straight aerial sporophores hyphae (isolate A3, × 400).

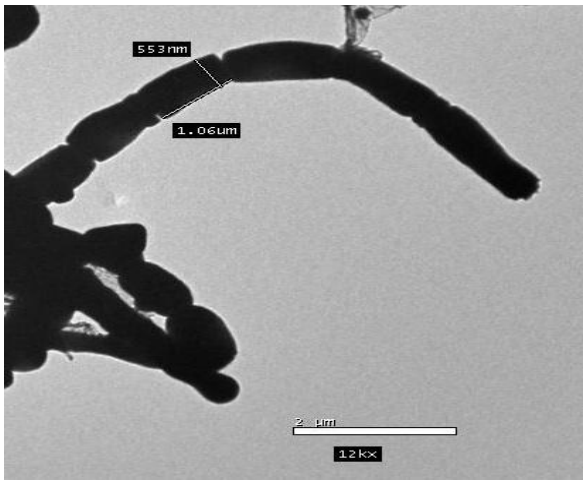

Figure 2b. TEM photomicrograph showing spiny spore surface (isolateA3, × 12000).

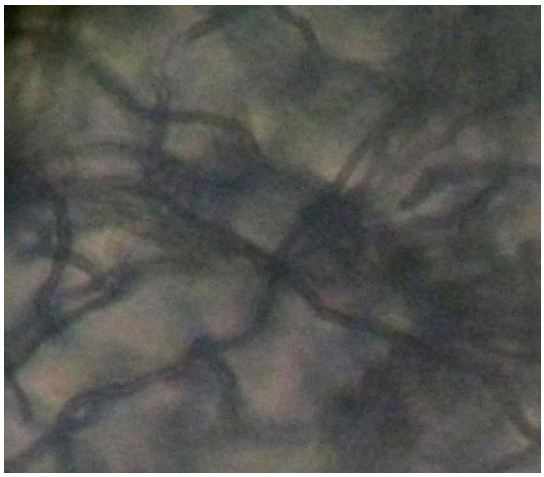

Figure 3a. Photomicrograph showing spiral sporophores hyphae (isolate A10, × 400).

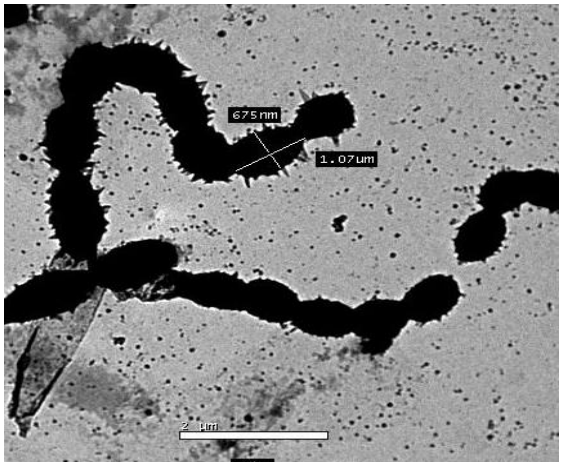

Figure 3b. TEM photomicrograph showing hairy spore surface (isolate A10, × 25000).

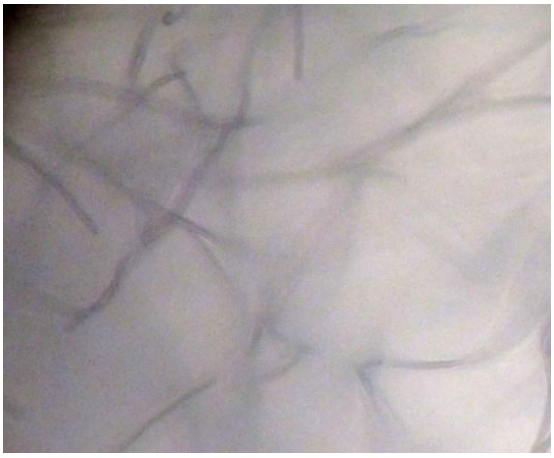

Figure 4a. Photomicrograph showing rectiflexibiles sporephores hyphae (isolate G5, × 400).

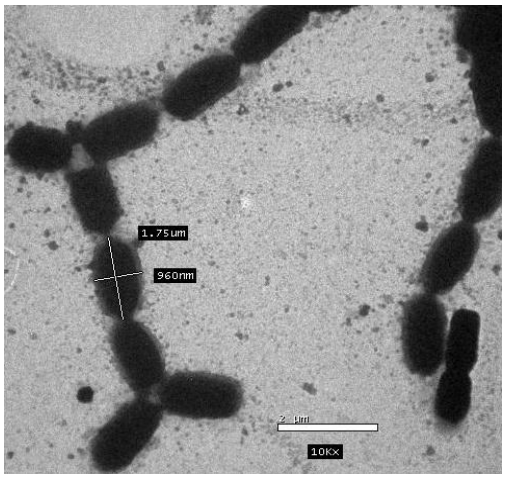

Figure 4b. TEM photomicrograph showing smooth spore surface (isolateG5, × 10000).

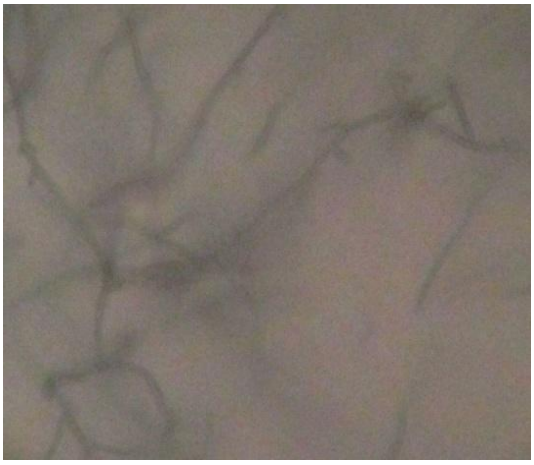

Figure 5a. Photomicrograph showing straight sporophores hyphae (isolate P4, × 400).

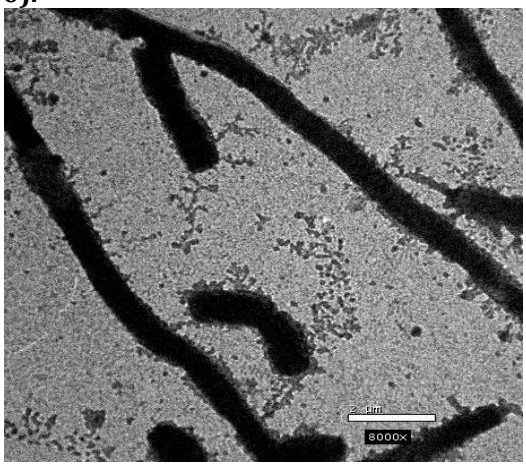

Figure 5b. TEM photomicrograph showing warty spore surface (isolateP4, × 8000).

The ability of *Streptomyces* species to produce secondary metabolites has attracted great attention among researchers [32]. This ability is generally due to the existence of the clusters of the *Streptomyces* strains, which encode enzymes for a lot of secondary metabolic pathways [33]. However, secondary metabolite production in microbes is also strongly controlled by some nutritional factors and growth conditions [34]. Modification of the environmental conditions for a strain growth, such as variation of medium composition, could also influence the patterns and increase the productivity of secondary metabolites. Many species of *actinomycetes*, particularly those belonging to the genus *Streptomyces*, are well known as antifungal biocontrol agents that inhibit several pathogenic fungi [35]. The antagonistic activity of *Streptomyces* to fungal pathogens is usually related to the production of antifungal compounds [36].

The present work was focused on the isolation of *streptomycetes* from marine and terrestrial sources collected from different location in Egypt. Soil actinomycetes were isolated using starch-nitrate agar medium provided with nystatin (anti-fungal) as described [37], and for the marine samples, starch-casein agar medium (SCA) were used [38,39]. The importance of marine sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last decade and was high lightened in many articles by using well-agar diffusion method [17,40-42]. Amphotericin B was used as controls. Five isolates coded (A1, A3, A10, G5 and P4) were selected as potent antidermatophytic producers as they exhibit interesting antidermatophytic activity. Colonies which are usually round, convex, shaped colonies, with deeply rooting growth into the medium and covered with spore masses, they are dry and powdery. The isolated strains have been maintained on starch-nitrate agar slants and kept at 4^{O}C until use. Numerous attempts have recently been done to overcome the diversity of criteria and techniques. The chemical composition of the cell wall allows a clear separation of *Streptomyces* and other *actinomycetes* from fungi [43,44, 45], indicated that the diamine acids of the cell wall peptidoglycan could prove useful in the diamination of the genera. Streptomyces strains contained LL-diaminopimelic acid (LL-DAP) and glycine. Whole cell hydrolyzates was used to determine isomers of DAP and sugar patterns[48]. They separated *actinomycetes* genera into Chemotypes and *Streptomyces* was placed in type I, having LL-DAP, glycine and no characteristic sugar pattern. In the present investigation, the identification of the experimental isolates was carried out according to the latter approach [46]. Taxonomic identification has been restricted to several *Streptomyces* isolates. Based on The morphological, biochemical characteristics and chemo-taxonomical methods according to International Streptomycete Project (ISP) and the keys of Nonomura [47] or Bergey's Manual[18, 19]. The selected isolates were identified as (A1) *Streptomyces violaceus*, (A3) *Streptomyces somaliensis,* (A10) *Streptomyces* antimycoticus, (G5) *Streptomyces exofoliatus*, (P4) *Streptomyces sp*.

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