Isolation and Characterization of Antibacterial Agent Produced by *Streptomyces avermilitis*, SK60-8 Active against pathogenic bacteria isolates from follicular fluid collected from Inferitility Egyptian Women

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Abstract: Natural products have a major impact on human health during the second half of this century. During the screening program for new antimicrobial agent active against follicular fluidbacterial pathogens complaining of Egyptian Woman Infertility an antibacterial agent produced by *Streptomyces avermilitis*, SK60-8 isolated from a soil sample collected from Kilo 60, Seuz governorate, Egypt. In its culture supernatant, this organism could produce one major bioactive compound exhibited strong antibacterial activity against the isolated Bacterial follicular fluid pathogens. *Escherichia coli* ES-1, *Staphylococcusaureus* ES-2, *Propionibacterium* ES-3, *lactobacillus Acidophilus* ES-4, *lactobacillus plantarum* ES-5, *lactobacillus ruminis* ES-6, *lactobacillusparacasei* ES-7, *Streptococcus agalactiae* ES-8, Enterococcus faecalis ES-9, *Enterococcus hirae* ES-10, & *Proteus mirabilis* ES-11; with mean diameter of inhibition zone 28.0, 30.0, 26.0, 20, 25.0,22.0,25.0,21.0,25.0,22.0 and 19.0 mm respectively, these activities were found to be similar to that of ofloxacin, antibiotic and it had a better therapeutic effect among the antibiotics tested. The active compound was separated by silicagel column chromatography and then purified on sephadex LH-20 column. The physico-chemical characteristics of the active agent viz. color, melting point, solubility and spectroscopic characteristics (UV, FTIR, Mass and 1HNMR spectra and elemental analysis) have been investigated. These analyses indicate a suggested empirical formula of C20H17CIF21N3O3S. The purified compound was found to be belonging to macrolide antibiotics and identified as a derivative of Monbactams. Although this antibiotic is known, the newly isolated strain was able to produce the antibiotic as a major product providing an important biotechnological downstream advantage.

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Keywords: Streptomyces avermilitis, SK60-8, follicular fluid, antibacterial antibiotic, ofloxacin.

1. Introduction

The broad involvement of bacterial species in infertility, as a key factor contributing to the failure of Intracytoplasmic sperm injection (ICSI) and embryo transfer procedures in women with asymptomatic anaerobic infections **(Campiscian *et al*.,2017).** The presence of microorganisms within ovarian follicles may be a consequence of prior infertility treatment protocols, with bacteria being introduced into the ovary at the time of oocyte retrieval. Alternatively, bacteria from other sites, such as the oral cavity or the respiratory tract, could access the ovary after haematogenous dissemination and subsequently colonize the ovary **(Cohen *et al*., 2003).**

In the ovary, the Follicle Stimulating Hormone (FSH) receptor is essential for follicular development and oocyte maturation. Such inhibition would prevent the normal hormonal functioning of FSH. It is therefore plausible that the presence of microorganisms in human follicular fluid may result in inhibition of the functioning of FSH, damage to the cumulus oocyte complex, the subsequent immune response within the follicular fluid during folliculogenesis or in the uterus at the time of implantation either by the microorganisms themselves, or the microbial products of metabolism. Identification of bacteria colonizing the follicular fluid in couples experiencing a prolonged failure to conceive may present the clinician with an opportunity to initiate antimicrobial treatment prior to the next attempt at conception **(Toth Aand Toth AB.,2011).** the emergence of multi-resistant microorganisms tomost all available antibiotics leaded many researchers are focused now on discovering novel antimicrobials from many natural resources such as those produced by actinomycetes especially those isolated from many undiscoverable or poorly explored environments **(Undabarrena *et al*., 2016).** Although the soil is a

natural reservoir of actinomycetes which produce antibiotics for pharmaceutical, industryunexplored habitats and niches have attracted considerable attention in recent years. Actinomycetes are Gram-positive, saprophytic filamentous bacteria that are responsible for the production of over 20,000 natural products extensively used in pharmaceutical and agrochemical industry **(Charousova *et al*.,2017).** Egyptian soil reservoir is considered as a poorly investigated source for actinobacteria, and very few reports were published **(Rifaat*et al*., 2013).** The present work was conducted in order to develop a new antibiotic active against pathogenic bacteria isolates from follicular fluid in 56 Inferitility Egyptian Women.

2. Materials and Methods

1. Testing the susceptibility of the obtained isolates to the different antibiotics.

The isolated bacterial cultures; *Escherichia coli* ES-1, *Staphylococcus aureus*, ES-2, *Propionibacterium* ES-3, *lactobacillusacidophilus* ES-4, *lactobacillus plantarum* ES-5, *lactobacillus ruminis*, ES-6, *lactobacillus paracasei* ES-7, *Streptococcus agalactiae* ES-8, *Enterococcus faecalis* ES-9, *Enterococcushirae* ES-10 & *Proteus mirabilis* ES-11. were tested with antibiotics to check for their sensitivity pattern using the antibiotic discs methods. The antibiotics used and their concentration per disc are as follows: Gentamycin (CN 10µg), Tetracycline (TE 10µg), Cloxacillin (OB 5µg), Augmentin (Aug 30µg), Amoxycillin (Amx 25µg), Chloramphenicol (C 30µg), Cotrimoxazole (SXT 25µg), Erythromycin (E 5µg), Bacitracin (B 25µg), Sulfadiazine (SD 10µg), Streptomycin (S 15µg), Nalidixic Acid (NA 30µg), Erythromycin (E 15µg) and Methicillin (ME5 10µg) by diffusion plate methods **(Cooper, 1963 and 1972).**

2. Testing antibacterial activity of actinomycete isolate, SK60-8

Antibacterial activity ofactinomycete isolate, SK60-8 was carried out by diffusion plate methods **(Cooper, 1963 and 1972)**, which based on the observation of inhibition clearing zone of bacterial growth on agarized media through the following techniques:

2.1. Direct bioassay method (Agar-culture disc).

In this method, plugs are cut out from the culture plates or medium under aseptic conditions using sterile cork borer and subsequently put on plates of agarized media seeded with the bacterial test organism.

2.2. Agar well method.

The seeded plates with target test organisms were cut by sterile cork borer to make holes (9 mm in diameter). Only 0.1 ml of the filtrate of actinomycete growth was transferred into each hole under aseptic conditions.

2.3. Paper disk method.

Paper disk (Whatman No.3, 6.0 mm in diameter) was saturated with proper volume of the filtrate of actinomycete growth until dryness, then the disc was placed on the surface of the agar medium seeded with target organisms.

The plates were kept in a refrigerator for 1-2hrs to permit diffusion of the metabolites before the growth of the test organism takes place. Subsequently, the plates were incubated and then examined for antibacterial activity.

3. Fermentation

A loopful of *Streptomyces avermilitis*, SK60-8, 6-day old culture was used to inoculate sterile seeding medium (liquid starch-nitrate) which consisted of (g/L); starch:20.0; $NaNO_3$:2.5; K_2HPO_4:1.0; KH_2PO_4:1.0; $MgSO_4 \cdot 7H_2O$:0.5; KCl:0.5; trace salt solution: 1.0 ml [(g/L): $CuSO_4 \cdot 5H_2O$:0.64; $FeSO_4 \cdot 7H_2O$:0.11; $MnCl_2 4H_2O$:0.79 and $ZnSO_4 \cdot 7H_2O$:0.15]; natural sea water: 500ml and distilled water: 500 ml, incubated at 30°C for 3 days on a rotary shaker (200 rpm). One percent of the vegetative inoculum was then used to inoculate the fermentation flasks, each containing 50 ml of the fermentation medium have the same composition. pH value of the medium was adjusted at 7–7.4 before sterilization. After inoculation, the flasks were fixed to a rotary shaker of 250 rpm and the fermentation was carried out at 30°C for 7 days.

4. Extraction

The culture broth obtained after fermentation was filtrated and extracted by a mixture of chloroform: methanol (2:1 v/v), the mixture was added to the filtrate at the ratio (1:1 v/v) respectively, in a separating funnel, shaken well and left for 10 min until complete separation. The organic phase was collected, evaporated under reduced pressure using a rotary evaporator until the crude extract was obtained.

5. Purification of active compound

The obtained crude extract was then subjected to silica gel column (22 X 5 cm, Silica gel 60; Merck) using a gradient polarity solvent system of ethyl acetate: methanol (10:1 to 1:10). 30ml of each fraction were collected and the fractionation process was monitored through TLC analysis. The fractions that exhibited similar TLC profiles were combined to give 7 fractions (F_1-F_7). Bioactivity of the obtained fractions was evaluated to identify antibacterial activity against *Escherichia coli, Es-1 & Staphylococcus aureus Es2*as a most two sensitive test isolates by plate diffusion assay **(Cappuccino and Sherman, 2004).** Fractions that exhibited antibacterial activity were applied on sephadex LH-20 column (2X15cm) using 100% methanol as the eluent solvent (300 ml) and lastly 3 ml of the fractions were collected.

6. **Physico-chemical Properties of the purified compound**

The physicochemical characteristics of the purified compound such as: solubility, color, melting point and behavior toward acids and behavior toward bases were studied. Structural characterization of the obtained pure compound was achieved via spectroscopic analyses: UV spectra in methanol using a 20 UV/VIS spectrophotometer (Perkin Elmer, Ohio, U.S.), Fourier Transform Infrared (FTIR) using KBr method of (JASCO FT/IR–6100) spectrophotometer. Nuclear magnetic resonance (NMR) spectra using a Varian Mercury VX-300 NMR spectrometer operated at 300 MHz for ^{1}H. Electron Ionization Mass Spectroscopy (EIMS) spectra were obtained with a Direct Inlet part DI-50 connected to the mass analyzer m Shimadzu GC/MS-QP5050. The elemental analysis C, H, O, N, Sand I were carried out at Micro-Analytical Center, Faculty of Science, Cairo University, Cairo, Egypt.

7. **Characterization of the antibacterial agent**

The antibacterial agent produced by *Streptomyces avermilitis*, SK60-8 was identified by consulting antibiotics recommended identification keys **Umezawa, (1977**), **Berdy, (1980a, b & c).**

3. Results and Discussion

3.1. Testing the susceptibility of the obtained isolates against the different antibiotics

In this study eleven different bacterial species; *Escherichia coli* ES-1, *Staphylococcus aureus* ES-2, *Propionibacterium* ES-3, *lactobacillusacidophilus* ES-4, *lactobacillus plantarum* ES-5, *lactobacillus ruminis* ES-6, *lactobacillus paracasei* ES-7, *Streptococcus agalactiae* ES-8, *Enterococcus faecalis* ES-9, *Enterococcus hirae* ES-10 & *Proteus mirabilis* ES-11. were tested for their susceptibility to 20 different antibiotics by diffusion plate methods using paper disc technique. It was found that; Ofloxacin (Ofx 20µg), Kanamycin (K 30µg), Ampicillin (AM 10µg) and Cloxacillin (OB 5µg) had a better therapeutic effect among the antibiotics tested while the other tested antibiotics exhibited activities against some of the isolated strains. **Momoh *et al.*, (2011)** reported that, ofloxacin had a better therapeutic effect among the antibiotics tested. Less than 50% of all the isolates showed resistance to ofloxacin, where as, more than 50% of all isolated strains showed resistance to tetracycline. Results of susceptibility of the obtained isolates to the different antibiotics are recorded in table (1).

3.2. Testing antibacterial activity of actinomycete isolate, SK60-8.

The active metabolites produced by actinomycete Streptomyces avermilitis, SK60-8 exhibited various degrees of activities against the isolated follicular fluid pathogens;. *Escherichia coli* ES-1, *Staphylococcus aureus*, ES-2, *Propionibacterium* ES-3 *lactobacillus acidophilus* ES-4, *lactobacillus plantarum*, ES-5 *lactobacillus ruminis*, ES-6, *lactobacillus paracasei* ES-7, *Streptococcus agalactiae* ES-8, *Enterococcus faecalis* ES-9, *Enterococcus hirae* ES-10, & *Proteus mirabilis* ES-11. with a mean diameter of inhibition zone 28.0, 30.0, 26, 20, 25.0,22.0,25.0,21.0,23.0,22.0 and 19.0mm respectively.

Arifuzzaman *et al*., (2010) reported that, twenty actinomycetes isolates produced antibiotic against one or more Gram-negative pathogenic bacteria such as *Shigellaboydii, Shigella flexneri-AN-31153, Shigelladysenterriae* type-1, *Vibrio cholerae-0139, Salmonella typhi-Ao-12014, Vibrio cholerae-OGET* and *Escherichia coli-186LT*..

Fermentation, extraction and purification of bioactive compound.

The fermentation process was carried out for seven days at 30 °C using liquid starch nitrate as fermentation medium. Thirty-liters total volume filtered was conducted followed by centrifugation at 5000 rpm for 20 min. The clear filtrates containing the active metabolite was adjusted to pH 7.0 then extraction process was carried out using by a mixture of chloroform: methanol (2:1 v/v) **(Khalifa, 2008)**. The organic phase was collected and evaporated under reduced pressure using rotary evaporator until deep red residues (3.0g) was obtained as a crude extract.

Bioactivity-guided fractionation of the active crude extract (3.5g) was dissolved in the least amount of the eluting solvent then applied to silica gel column (2.5X50 cm), a total of F7 fractions (F1-F7) were tested for antibacterial activity against Escherichia coli, Es-1 & Staphylococcus aureus, ES-2. The bioactive fractions (F-4, F-5 & F-6) were applied on TLC precoated plates using different solvent systems. It was found that, among these fractions only one major brown band at Rf =0.50 was obtained by applying solvent system; Toluene: Ethyl acetate: Formic acid (5:4:1 v/v) this band was scratched, collected in a clean glass beaker and eluted by ethyl acetate as a pure active compound.

3.3. Characterization of the active pure compound

3.3.1. Physicochemical Characteristics

The purified compound was isolated as greenish brown crystal, good acceptable odour and melting point is 170-175°C, orange in acidic greenish yellow in alkaline solution. The compound is freely soluble in chloroform, ethyl acetate, ethyl alcohol and methanol and insoluble in petroleum ether, hexane, benzene and water.

3.3.2. Spectroscopic studies on the major bioactive compound

UV spectrum, IR spectrum, 1HNMR spectrum and mass spectrum.

Table (1): Susceptibility of the obtained isolates to the different antibiotics using diffusion method (paper disctechnique).

No.	Antibiotics	Mean diameter of inhibition zone (mm) of the tested bacterial strains										
		E. Coli ES-1	***S. aureus ES-2***	***Propioni bacterium ES-3***	***L.acidophilus ES-4***	***L.plantarum ES-5***	***L.ruminis ES-6***	***L.paracasei ES-7***	***S.agalactiae ES-8***	***E.faecalis ES-9***	***E.hirae ES-10***	***P.mirabilis ES-11***
1	**Gentamycin (CN 10µg)**	0	20	16	18	15	0	0	13	12	20	0
2	**Tetracycline (TE 10µg)**	22.5	23	18	24.5	16.9	0	20	19	17	10	3
3	**Cloxacillin (OB 5µg)**	25	23	26	27	24	21	22	23	26	15	14
4	**Augmentin (Aug 30µg)**	18	17	23	15	0	0	0	15	17	14	12
5	**Amoxycillin (Amx 25µg)**	3	0	22	18	26	27	10	12	15	17	14
6	**Chloramphenicol (C 30µg)**	20	23.5	20	23	0	0	15	12	10	14	8
7	**Cotrimoxazole (SXT 25µg)**	0	0	0	0	0	0	0	0	0	0	0
8	**Erythromycin (E 5µg)**	16	15	20	0	24	22	13	12	10	14	8
9	**Bacitracin (B 25µg)**	17	16	18	15	22	19	20	13	12	17	3
10	**Sulfadiazine (SD 10µg)**	0	0	0	0	0	0	0	0	0	0	0
11	Streptomycin (S 15µg)	6	19	16	18	15	0	12	0	14	13	5
12	Nalidixic Acid (NA 30µg	20.5	23	20	18.5	16.9	0	16	15	13	10	9
13	Erythromycin (E15µg	24	21	26	27	24	21	20	22	12	15	13
14	Methicillin (ME5 10µg)	12	15	23.5	21	16	20	12	14	18	16	6
15	Ampicillin (AM 10µg)	0	0	14	21	16	20	12	14	18	16	6
16	Ciprofloxacin (CIP 10µg)	23	20.5	14	23	0	0	10	0	0	14	7
17	Colistin (CL 20µg)	0	0	0	0	0	0	0	0	0	0	0
18	Kanamycin (K 30µg)	22	24	26	23	20	22	10	17	15	20	10
19	Polymyxin B (PB 10µg)	22	19	18	15	22	19	10	12	13	10	5
20	Ofloxacin (Ofx 20 µg)	26	28	27	30	25	23	21	25	28	22	16

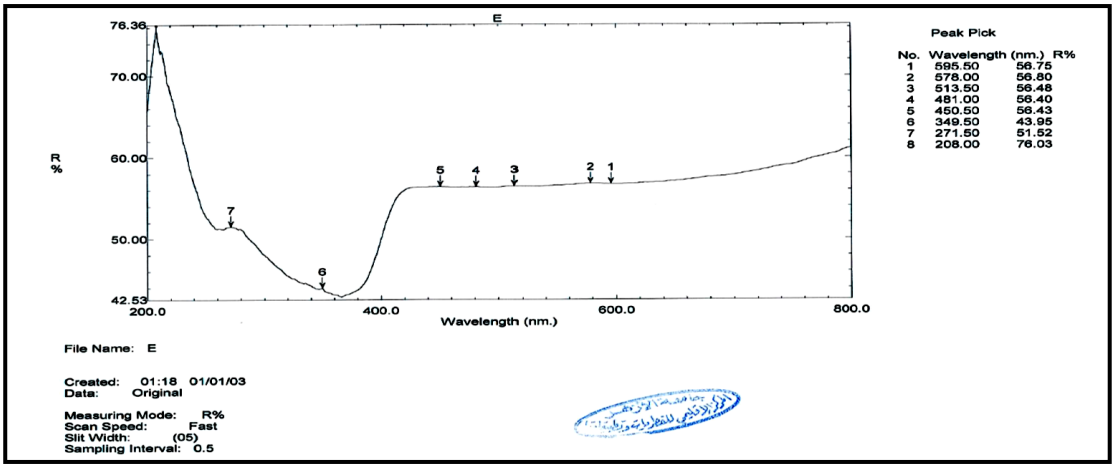

Figure (1): UV-spectrum peaks of purified bioactive compound, SK60-8 produced by Streptomyces avermilitis, SK60-8.

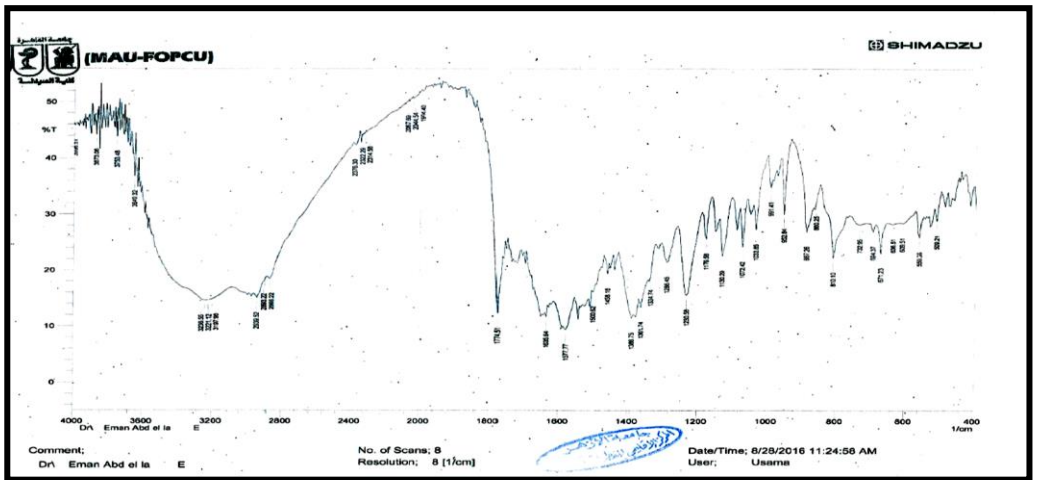

Figure (2): IR-spectrum peaks of purified bioactive compound, SK60-8 that produced by *Streptomyces avermilitis*, SK60-8.

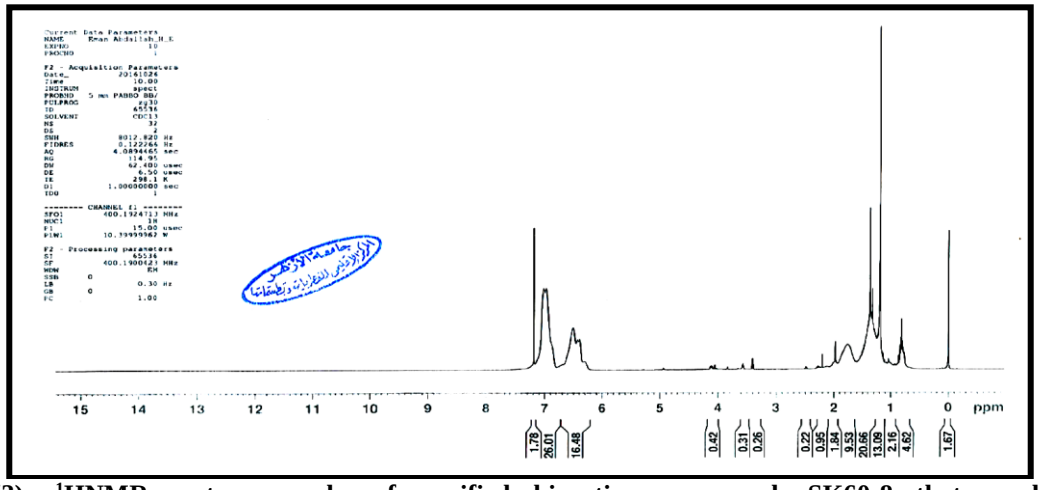

Figure (3): ^{1}HNMR-spectrum peaks of purified bioactive compound, SK60-8 that produced by *Streptomycesavermilitis*, SK60-8

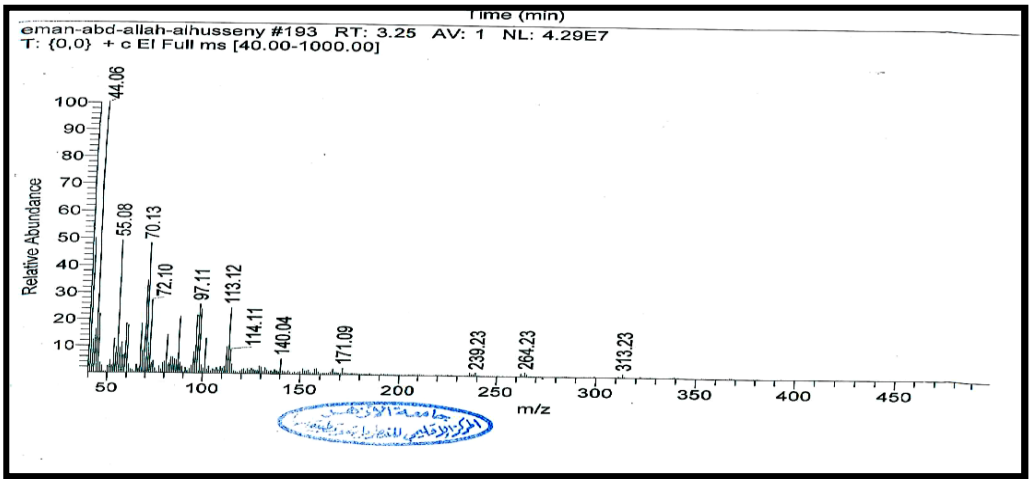

Figure (4): Mass-spectrum peaks of purified bioactive compound,SK60-8that produced by *Streptomyces avermilitis,* SK60-8.

Analysis of the elements content of the obtained bioactive compoud, SK60-8that produced by Streptomyces avermilitis, SK60-8. revealed that it contained (% w/w): C= 54.46 %; H = 4.2 %; O = 10.55 %; F= 8.2 %; N=9.50%; S=10.7%; I=2.39% from the above listed contents, the empirical formula of the isolated compound was C20H17CIF21N3O3S.

3.3.3. Identification of the bioactive compound

On the basis of the recommended keys for the identification of antibiotics such as: Umezawa **(1977)**, Berdy **(1980a, b, c)** and in view of the comparative study of the recorded properties of the antibacterial agent, it could be stated that the produced active compound is being belonging to B-lactamantibiotics (Berdy, 1989), as a derivative of monobactams which was isolated from *Streptomyces avermilitis* **(Macdougall,2011and Dalhoff*et al*.,2006)**

Conclusion

In conclusion, the data obtained in the present work reported that, the isolation of antibiotic as a major bioactive compound that produced in the culture filtrate of *Streptomyces avermilitis*, SK60-8 provides a biotechnological advantage in the industrial downstream processing and lead to improvements in bioprocess control for existing products in large-scale fermentations.

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Ethics statement

Ethical approval was obtained from the the International Islamic Center for Population Studies and Research (IICPSRC), Azhar University, Cairo, Egypt. All patients provided informed written consent for their follicular fluids to be used in this study and gave permission for researchers to access medical records to obtain their reproductive history.

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