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Kosinostatin, a Major Secondary Metabolite Isolated from the Culture Filtrate of *Streptomyces violaceusniger* Strain HAL64

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During a screening program, an actinomycete strain isolated from the Egyptian soil was investigated for its potential to show antimicrobial activity. The identification of this isolate was performed according to spore morphology and cell wall chemo-type, which suggested that this strain is a streptomycete. Further cultural, physiological characteristics and the analysis of the nucleotide sequence of the 16S rRNA gene (1480 bp) of this isolate indicated that this strain is identical to *Streptomyces violaceusniger* (accession number EF063682) and then designated *S. violaceusniger* strain HAL64. In its culture supernatant, this organism could produce one major compound strongly inhibits the growth of Gram-positive but the inhibition of Gram-negative indicator bacteria was lower. The antibiotic was separated by silica gel column chromatography and then purified on a sephadex LH-20 column and finally the purity was checked by HPLC. The chemical structure of the purified compound was determined using spectroscopic analyses (molecular formula of $C_{33}H_{32}N_2O_{10}$ and molecular weight of 617.21) and found to be identical to the kosinostatin, a quinocycline antibiotic which is known to be produced by *Micromonspora* sp. TP-A0468 (Igarashi *et al.*, 2002) and to quinocycline B isolated from *Streptomyces aureofaciens* (Celmer *et al.*, 1958). Although the antibiotic is known, the newly isolated strain was able to produce the antibiotic as a major product providing an important biotechnological downstream advantage.

Keywords: *S. violaceusniger* strain HAL64, kosinostatin, 16S rDNA, PCR

Natural products have a major impact on human health during the second half of this century. The discovery of naturally occurring antibiotics had a major impact on the control of infectious diseases and the development of pharmaceutical industry. In the last decades, the pharmaceutical industry has not only continued to screen microbial extracts for antibacterial activity but has successfully extended to include other disease areas (Kremer *et al.*, 2000) such as the 'statins' (cholesterol-lowering) and cyclosporin (immunosuppressant), in addition to the antibacterials augmentin, clarithromycin and ceftriaxone (Cardenas *et al.*, 1998). In drug discovery, for example, novel natural-product chemotypes with interesting structures and biological activities continue to be reported. Without such discoveries "there would be a significant therapeutic deficit in several important clinical areas, such as neurodegenerative disease, cardiovascular disease, most solid tumors, and immune-inflammatory diseases" (Price *et al.*, 2001).

Filamentous soil bacteria belonging to the genus *Streptomyces* is identified as a major source of bioactive natural products representing some 70-80% of the all isolated compounds (Bull *et al.*, 1992; El-Naggar *et al.*, 2003; Berdy, 2005; El-Naggar *et al.*, 2006). The streptomycetes form a distinct clade within the radiation encompassed by the high-GC Gram-positive bacteria in the 16S rDNA tree. There is an evidence that specific metabolites, such as clavulanic acid, may be synthesized by strains in a specific clade and that the ability to synthesize, for example, streptomycin and related metabolites appear to be randomly distributed across the whole genus (Chater, 1998). The specific/infra-specific relationships in the streptomycetes and the way they are reflected in the biosynthetic potential to produce bioactive compounds could significantly influence strategies for search and discovery, screening, and bioprocess development. To extend whole-genome studies to more streptomycetes would reveal these relationships in a comprehensive way, which would enable validation of current methodologies (from 16S rDNA phylogenies to DNA-DNA pairing) and lead to new understanding of speciation, phylogenetic relationships, and genome function in secondary metabolism (Woese, 1998; Butler *et al.*, 2003).

The present work signifies downstream biotechnological importance of isolation and identification of an actinomycete strain, producing one major bioactive compound. The latter was isolated, purified and its structure was elucidated.

Materials and Methods

Antibiotic producer and culture conditions

S. violaceusniger strain HAL64 was isolated from the Egyptian soil. Soil samples were taken down to a 10 cm depth into the soil surface after the removing of approximately 3 cm of the soil surface. This strain was isolated using starch-casein agar (Okazaki and Okami, 1972) containing cycloheximide and nystatin (50 μg/ml each) in order to minimize fungal contamination. The characterization of the isolated strain followed the guidelines adopted by the International *Streptomyces* Project (Shirling and Gottlieb, 1966). Colours were assessed on the scale adopted by Kornerup and Wanscher (1978). Diaminopimelic acid (A_2pm) isomers in the cell-wall were analyzed by using the methods of Becker *et al.* (1965) and Lechevalier and Lechevalier (1980). The electron micro-

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scope study was carried out using scanning electron microscope (JEOL JSM 5300, JEOL Technics Ltd., Japan).

Target organisms

The following target organisms were used for the determination of minimum inhibitory concentration (MIC) of the antibiotic: *Aspergillus terreus* (Local isolate), *Bacillus subtilis* (ATCC 6633), *Candida albicans* (Local isolate), *Enterobacter cloacae* (ATCC 13047), *Escherichia coli* (NCIB 1186), *Klebsiella pneumonia* (Local isolate), *Mucor miehei* (Tü 284), *Pseudomonas fluorescens* (ATCC 13525), *Saccharomyces cerevisiae* (Local isolate), *Sarcina leutea* (NCIB 495), *Staphylococcus aureus* (209 P FDA), *Staphylococcus aureus* (Smith), and *Staphylococcus aureus* MRSA.

Antibiotic bioassay

To test for the antibacterial activity, *S. aureus* FDA 209P was used as a test organism. Mueller-Hinton agar medium was used as an assay medium. The agar medium at 45°C was mixed with 0.1 ml bacterial suspension containing approximately 10^5 cfu/ml. The mixture was poured into 9 cm Petri dish and allowed to solidify. Sterile paper discs (6 mm) were placed on the dried surface of the medium. Each disc received 30 µl of the culture filtrate. The Petridishes were kept in a refrigerator for 2 h at 4°C to allow the diffusion of the bioactive compound. Petri dishes were incubated at 37°C for 18 h. The inhibition zone, if any, was measured in mm diameter. The minimum inhibitory concentration (MIC) of the purified antibiotic was determined by 2-fold dilution method using Mueller-Hinton agar (Difco) as an assay medium at pH 7.0 and the MICs were read in µg/ml after overnight incubation at 37°C. Yeast extract-malt extract medium was used for yeast, which was incubated at 28°C and fungi at 30°C.

Total genomic DNA isolation

S. violaceusniger strain HAL64 was grown on a slant of inorganic salts-starch agar for 14 days at 30°C. Two milliliter of a spore suspension were inoculated into the starch-nitrate broth and incubated on a shaker incubator at 200 rpm and 30°C for 3 days to form pellet of vegetative cells (pre-sporulation). The total genomic DNA preparation was carried out according to Sambrook *et al.* (1989).

PCR amplification, sequencing and analysis of the 16S rRNA gene

PCR amplification of the 16S rRNA gene of the local actinomycete strain was performed using two primers StrepF; 5′-ACGTGTGCAGCCCAAGACA-3′ and StrepR; 5′-ACAA GCCCTGGAAACGGGGT-3′ according to Edwards *et al.* (1989). The PCR mixture consisted of 30 picomoles of each primer, 100 ng of chromosomal DNA, 200 µM dNTPs and 2.5 units of Taq polymerase in 50 µl of polymerase buffer. The amplification was carried out for 30 cycles of 94°C for 1 min, annealing at 53°C for 1 min and extension at 72°C for 2 min. The PCR reaction mixture was analyzed by agarose gel electrophoresis and the remnant mixture was purified using QIA quick PCR purification reagents (Qiagen).

16S rRNA gene was sequenced on both strands by dideoxy chain termination method according to Sanger *et al.* (1977). The 16S rRNA gene (1.5 kb) sequence for the PCR product was obtained using Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The sequence data have been deposited in the GenBank database under the accession number EF063682.

Blast program (www.ncbi.nlm.nih.gov/blst) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program (Page, 1996).

Fermentation and isolation of the bioactive compound

S. violaceusniger strain HAL64 grown on agar slants (14-day-old) was used to inoculate twenty five Erlenmeyer flasks (1000 ml) each containing 400 ml sterile starch-nitrate medium. It contained (g/L): Starch; 10.0, $NaNO_3$; 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 [$CuSO_4 \cdot 5H_2O$ (0.64 g/L), $FeSO_4 \cdot 7H_2O$ (0.11 g/L), $MnCl_2 \cdot 4H_2O$ (0.79 g/L), and $ZnSO_4 \cdot 7H_2O$ (0.15 g/L)], and distilled water, 1.0 litre. The medium was adjusted to the initial pH 7.0 prior to sterilization using 0.1 N NaOH or 0.1 N HCl solution. The inoculated flasks were incubated on a rotary shaker at 30°C and 300 rpm for 7 days (where the maximum antibacterial activity and growth were achieved; data not shown).

The fermentation broth (ca. 20 L) was centrifuged at 5°C for 20 min at 6,000 rpm to separate the mycelium cake, and the supernatant fluid was extracted twice with an equal vol-

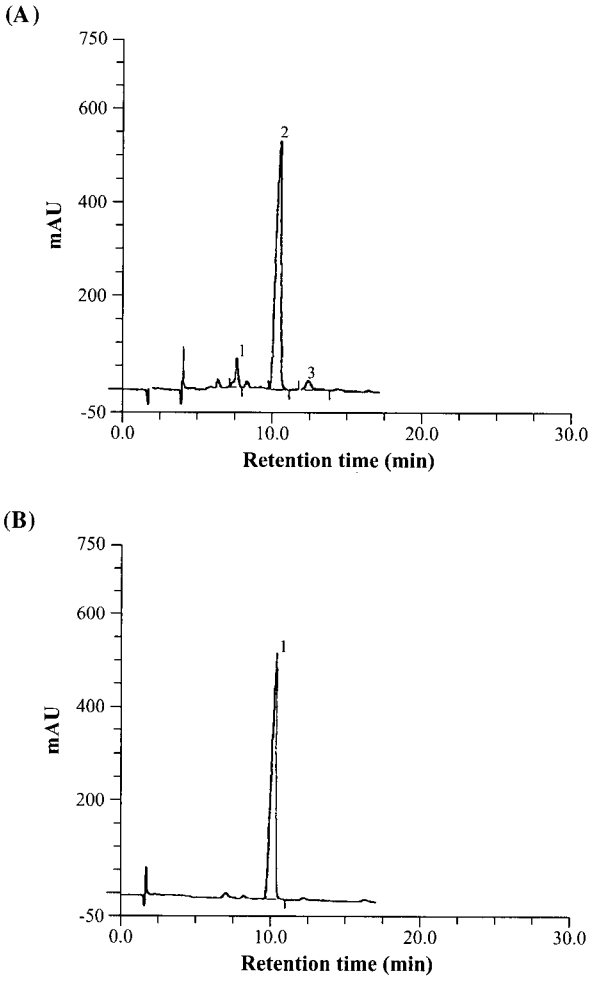

Fig. 1. Analytical HPLC chromatograms showing (A) the fraction A3 profile after the column chromatography. The total relative areas recorded for the three peaks 1 (A3-1), 2 (A3-2; major compound) and 3 (A3-3) were 7.60, 87.73 (major compound) and 4.67% respectively. (B) Shows the retention time of the pure major product with a 100% total relative area.

Table 1. Cultural characteristics of *S. violaceusniger* strain HAL64

Medium	Growth	Aerial mycelium	Reverse side
Yeast extract-malt extract agar	Good	Abundant, gray	Brown
Oatmeal agar	Good	Abundant, gray	Yellowish brown
Inorganic salts-starch agar	Good	Abundant, gray	Light brown
Glycerol-asparagine agar	Moderate	Moderate, white	Light brown
Peptone-yeast extract iron agar	Moderate	None	Yellowish brown
Tyrosine agar	Poor	Moderate, white	Brown
Sucrose nitrate agar	Good	None	Light brown
Sucrose asparagine agar	Good	moderate, white and gray	Light brown

ume of ethyl acetate:methanol (1:4) as the best solvent mixture. The combined organic layers were concentrated *in vacuo* under reduced pressure using rotary evaporator (Büchi, R-114, Switzerland at a temperature does not exceed 50°C) to give 2 g of crude extract. The latter was dissolved in 3 ml methanol and applied to a column chromatography using silica gel (3 i.d.×25 cm) as stationary phase. The column was developed with a solvent mixture of ethyl acetate:methanol (10:1 - to - 1:10). Eight fractions (A-H) were collected (500, 200, 100, 30, 10, 60, 17, and 15 mg, in order). Only, the first (A) and fourth (D) fractions proved active against *S. aureus*. The first fraction (A) was re-purified on Sephadex LH-20 column (3 i.d.×25 cm) using ethyl acetate:methanol (1:5) as eluent. Five fractions (A1-A5) were collected and only fraction A3 (400 mg) was found to be active against *S. aureus* with R_f values of 0.25 when ethyl acetate:methanol (15:85) mixture was used. The homogeneity of this fraction was checked with the analytical high performance liquid chromatography (HPLC) by injecting 20 µl into the analytical (C_{18}) column (25.0×0.46 cm) packed with 5 µ hypersile octadecyl silan (ODS), at a flow rate of 1.0 ml/min and chart speed of 1 cm/min with both ultraviolet (UV) at a wavelength of 254 nm and refractive index (RI) detection. An aqueous solution of acetonitrile (75%) was used as a mobile phase eluent. The 3 peaks (Fig. 1A) with the total relative areas of 7.60 (A3-1), 87.73 (A3-2; major compound) and (A3-3) 4.67%, respectively were recorded. Re-purification to this major compound was carried out using column chromatography as described above with Sephadex LH-20 column (3 i.d.×25 cm) using ethyl acetate: methanol (3:5) as the solvent system. The three fractions (A3-1 to A3-3) were individually separated. The major fraction (A3-2; major compound) was amounted to 350 mg. The purity of the pure major bioactive compound was reconfirmed (Fig. 1B) with the analytical HPLC as described above. The pure major bioactive compound eluted at a retention time of 10 min with a 100% total relative area.

The pure fraction (350 mg) was subjected to the spectroscopic analyses: 300 MHz ^{1}H NMR, 75 MHz ^{13}C NMR (Varian mercury VX-300 NMR spectrophotometer). Elemental analysis, IR (Shimadzu FT-IR 8101 PC infrared spectrophotometer), UV absorption (Shimadzu spectrophotometer), FABMS spectra were measured on JEOL DX303 and JMS-HX110A spectrometers.

Results

The producer strain and its molecular phylogeny

The cultural characteristics of the local actinomycete strain are given in Table 1. This strain grew well with a hygroscopic appearance on most of the organic and synthetic media tested. Diffused melanoid pigments and other soluble pig-

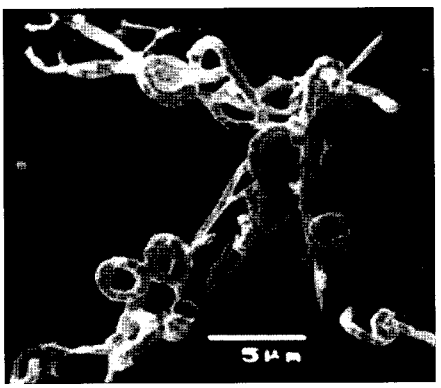

Fig. 2. Scanning electron micrograph showing the spore chains of *S. violaceusniger* HAL64 grown on inorganic salts-starch agar for 14 days at 30°C.

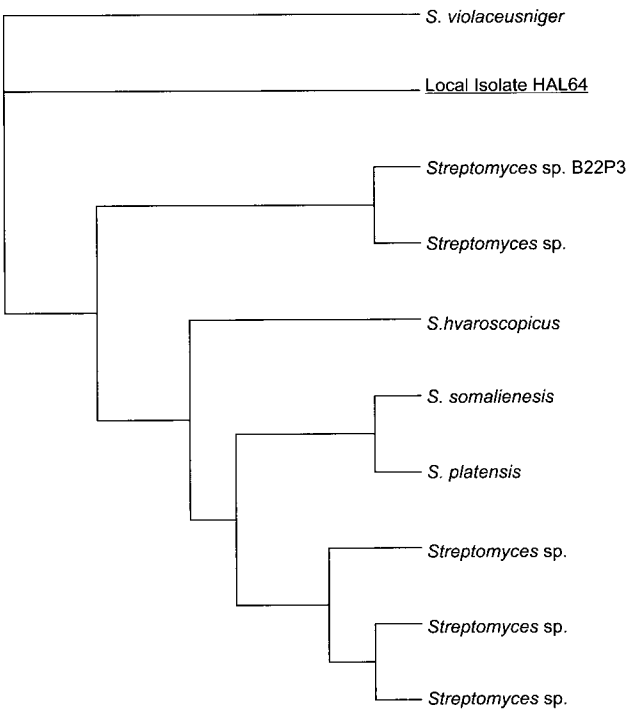

Fig. 3. The phylogenetic position of *S. violaceusniger* strain HAL64 among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences.

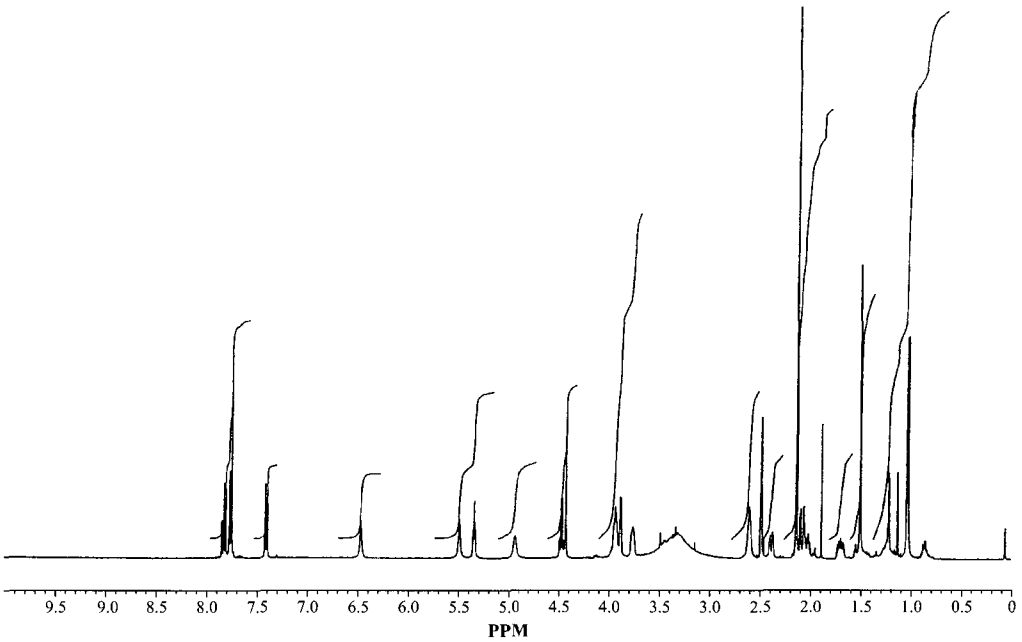

Fig. 4. ^{1}H NMR spectrum of Kosinostatin (DMSO) of the purified substance extracted from the culture filtrate of *S. violaceusniger* strain HAL64.

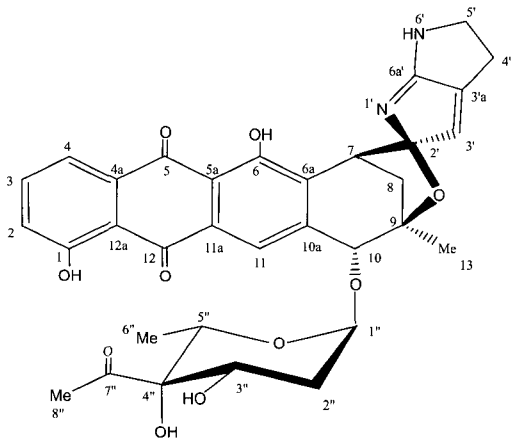

Fig. 5. The molecular structure of the kosinostatin produced by the local *S. violaceusniger* strain HAL64.

ments were not produced in any agar medium tested. The local actinomycete strain formed well developed and branching substrate and aerial mycelia, but the fragmentation of the substrate mycelium was not observed. The spore chains of the strain were spirals (Fig. 2) and the spore mass was gray, moist with maturity. Each spore was characterized by its rugose surface. Neither special structures nor zoospores were observed. Whole cell hydrolysate of this strain contained LL-A_2pm, and this indicates the strain has a chemotype I cell wall. This strain could utilize D-glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, raffinose, sucrose and galactose, but the utilization of inositol was doubtful (data not shown). The results of the utilization of carbon compounds were compared to the data published for *S. violaceusniger* (Lechevalier *et al.*, 1989).

To confirm the identification of the isolated strain, 16S rDNA sequence of the local isolate was compared to sequences of 9 *Streptomyces* spp. The phylogenetic tree (displayed by the Tree View program) showed that the locally isolated strain is closely related to *S. violaceusniger* (Fig. 3). Multiple sequence alignment was done between the sequences of the 16S rDNA genes of *S. violaceusniger*, *S.* sp. NRRL 27, *S. hygroscopicus* and *S.* sp. B22P3 and the local isolate. Computer assisted DNA similarly searches against bacterial database revealed that 16S rDNA sequence was 99% identical to *S. violaceusniger*.

Spectroscopic studies on the major bioactive compound

In the present work, the antibiotic produced by the local isolate, is a major bioactive constituent isolated from the culture filtrate, and its structure was determined via spectroscopic techniques. The Infrared spectrum (KBr) of the pure brown bioactive compound showed a diagnostic peak at 3340, 2950, 1720, 1630, and 1950 per cm, whereas the UV visible spectrum of the purified active compound (dissolved in methanol) indicates the presence of aromatic nucleus with the absorption maxima at λ_{max} 228, 258, 291, and 423 nm. The ^{1}H NMR (Fig. 4) and ^{13}C NMR spectra assignments are summarized in the supplementary Tables I and II. The molecular formula of the antibiotic (Fig. 5) was determined to be $C_{33}H_{32}N_2O_{10}$ based on the high resolution FAB-MS and its molecular weight was found to be 617.21.

Antimicrobial spectrum of the antibiotic

The minimum inhibitory concentrations (MICs) of the purified compound are shown in Table 2. The antibiotic proved active against a number of Gram-positive and Gram-negative bacteria but, Gram-positive bacteria were more sensitive to the antibiotic than Gram-negative representatives. On the other hand, the antibiotic has a weaker effect against *S. cerevisiae* and *C. albicans* whereas, *M. miehei*, and *A. terreus* were ever less susceptible to the antibiotic.

Table 2. Antimicrobial activities of the antibiotic kosinostatin produced by *S. violaceusniger* strain HAL64

Test organism	MIC (μg/ml)
1-Bacteria	
Bacillus subtilis ATCC 6633	1.00
Enterobacter cloacae	3.2
Escherichia coli	1.9
Klebsiella pneumonia	3.0
Pseudomonas fluorescens	1.8
Sarcina leutea	1.5
Staphylococcus aureus FDA 209P	0.5
Staphylococcus aureus MRSA	1.5
Staphylococcus aureus Smith	0.75
2-Yeasts	
Candida albicans	>50
Saccharomyces cerivisiae (local isolate)	>50
3-Fungi	
Aspergillus terreus	>200
Mucor miehei	>200

Discussion

At the turn of the century, a shift driven by new technologies is occurring in the way to search for exploitable biology. This shift is exemplified by the extent of biodiversity now revealed and recognized by molecular biology, information and data-rich methods for characterizing organisms and for defining taxon-property relationships, high-throughput screening, the PCR and DNA sequencing. The locally isolated *Streptomyces* strain was identified as *S. violaceusniger* according to 16S rDNA technology, in addition to the cell wall chemotype, cultural, and physiological characteristics. The cultural and physiological properties of the isolated strain were compared to those reported for actinomycetes as described in Bergey's Manual of Determinative Bacteriology and found that this strain belongs to the genus *Streptomyces* (Lechevalier *et al.,* 1989). The comparison of the physiological characteristics of the local isolate to those published in Bergey's Manual of Determinative Bacteriology for *S. violacusniger* clearly revealed that these two strains are identical. It has long been known that some actinomycete strains of the same species could produce different antibiotics, whereas some other strains belonging to different species produced the same antibiotics (Lechevalier, 1975). Antibiotic production by actinomycetes, therefore, may not be species-specific but strain-specific. A number of the *S. violaceusniger* strains have been isolated and reported as antibiotic producers, and the antibiotics generated by this species are WA8242A_1, A_2, and B (Youshimura *et al.,* 1998); Macrolide antibiotics RS-22A, B, and C (Ubukata *et al.,* 1995); WA9326A (Hayashi *et al.,* 1992); and BE-24566B (Kojiri *et al.,* 1995).

The identification of previously discovered compounds can often be accomplished quickly by taking advantage of the available databases of natural product structures. So, the spectral data for the bioactive molecule combined with the taxonomy of the producing organism were used to conduct a search using the AntiBase database (Laatsch, 2003) as well as a survey of the available literature, the proposed molecular structure of the antibiotic with its molecular weight generated by this local strain in the present investigation proved to be similar to chemical structure of the antitumour antibiotic kosinostatin generated by *Micromonospora* sp. TP-A0468. Kosinostatin is a stereoisomer of the isoquinocycline B produced by *S. aureofaciens* (Celmer *et al.,* 1958). But this antibiotic has not been reported to be produced by any of the *S. violaceusniger* known strains.

In conclusion, the data obtained in the present work report the isolation of the antibiotic kosinostatin as a major bioactive compound produced in the culture filtrate of the newly isolated from a different species in the genus *Streptomyces* (*S. violaceusniger* strain HAL64). Finding an organism with a potential to produce only one major bioactive compound 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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