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Gas Chromatography-mass Spectrometry (GC-MS) Analysis of Bioactive Compounds Extracted from *Streptomyces antibioticus* by Ethyl Acetate

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Sequence of the isolate was submitted in gene bank and provided with accession number KF996507 and named as *Streptomyces antibioticus* strain ess_amA8. The GC-MS analysis revealed that presence of 2-methyl propanamide, n-hydroxymethylacetamide, hexanamide, 1,2 enzenedicarboxylic acid, hexadecyl-oxirane, 4-hydroxytetradec-2-ynal, 8-pentadecanone, phenylpropanedioic acid, phenylethyl alcohol and 3-methyl-but anamide. Ethyl acetate Extract of *Streptomyces antibioticus* was most effective against *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 70060, *Salmonella typhi* ATCC 6539 and *Pseudomonas aeruginosa* ATTC 27853 respectively.The findings obtained from this study may contribute to development of potentially effective and environmentally safer alternative antibacterials.

Key words: *Streptomyces antibioticus,*GC-MS, Ethyl acetate, hexadecyl-oxirane.

The cultivation, isolation, and identification of natural product-producing bacteria are necessary tasks to pursue the identification of novel compounds for drug discovery. One way to search for novel natural products begins with finding unique strains of microorganisms. Multitudes of actinobacterial strains are noteworthy both as human pathogens and as essential producers of antibiotics. Natural products produced by actinomycetes most importantly include those with antibacterial activity against resistant strains of pathogens, which have been on the rise since the introduction of modern antibiotic chemotherapy in the 1940s. Actinomycetes are widely distributed in the natural environment, and synthesize numerous natural products. These natural products, or derivatives thereof are widely used in medicine to fight bacterial, viral and fungal infections, as well as cancer and immune system disorders (Bredholdt *et al*. 2007). The discovery of the antituberculosis agent streptomycin from the culture broth of *Streptomyces griseus* by Waksman in 1953 provided the foundation for targeting the genus *Streptomyces* and related Actinomycetales (Waksman, 1953). Actinomycetes have gained prominene in recent years because of their potential for producing antibiotics (Kumar *et al*., 2005). Streptomycin, gentamicin, rifamycin are some of the antibiotics which are in use presently and erythromycin are the product of actinomycetes. The actinomycetes are important in the field of pharmaceutical industries and also the agriculture. Previous study showed that actinomycetes isolated from Malaysia soil have the potential to inhibit the growth of several plant pathogens (Jeffrey *et al*., 2007). The aim of this study was to isolate and identify *Streptomyces antibioticus* from some insects; evaluated their antagonistic

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activities against selective human pathogenic bacteria and identified contents of the extract by GC-MS.

MATERIALS AND METHODS

Isolation and purification of *Streptomyces*

Actinobacterial isolate obtained by employing serial dilution followed by pour plate techniques using starch casein agar. Inoculated plates incubated at 28°C for 5 days. The bacterial colonies grown on the solid medium then observed, and cultures with colonies of the same morphology on starch casein agar considered as pure culture. A single colony of different morphologies were aseptically selected and streaked onto a separated starch casein agar plate and allowed to grow at 30°C for 2-3 days. Repeated single colony isolation was carried out until the culture purity was ensured.

Preparation of preliminary test organisms

The test pathogens used for preliminary screening were Gram positive (*Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923) and Gram negative (*Salmonella typhi* ATCC 6539, *Pseudomonas aeruginosa* ATTC 27853 and *Klebsiella pneumonia* ATCC 700603). Test bacteria were grown in 5.0 ml Nutrient broth for 24hrs, and then standardized to McFarland standard of 0.1 at OD 600nm.

Primary screening of actinobacterial isolate for bioactive compounds production

Actinomycete isolate were inoculated on SCA plates and incubated at 28 ± 2°C for 7 days. For this purpose 20 ml of sterilized Mueller Hinton agar (MHA) in McCartney bottles was seeded with 100 µl of standardized test bacteria swirl gently and aseptically poured into petri plate and allowed to solidify. Sterile cork borer (6 mm diameter) was used to bore holes in the plate. Agar discs of well grown actinobacterial cultures were placed on plates seeded with the following test strains; Gram positive (*Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923) and Gram negative (*Salmonella typhi* ATCC 6539, *Pseudomonas aeruginosa* ATTC 27853 and *Klebsiella pneumonia* ATCC 700603). The plates were incubated at 37°C for 24 h for bacteria in Fig. (3). Antimicrobial activity was determined by the inhibition zone. The isolate had been have antimicrobial activity against the test pathogens, were selected for the present study.

Morphological and physiological characterization of actinobacterial isolate

Traditional methods for identification of actinomycetes were occurred by use of morphological, physiological, and biochemical characters. The classical method described in the identification key by Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (1994 and 2012) is very much useful in the identification of actinomycetes.

Amplification of 16S rDNA and its sequencing

The 16S ribosomal DNA gene was amplified by PCR using the universal primer pair Star-F $^{5-}$AGAGTTTGATCGTGGCTCAG^{-3} and 1387-R $^{5-}$CGGGCGGTGTGTACAAGG^{-3} (Sivakumar 2001). The amplified products were analyzed by GATC Biotech, European Custom Sequencing Centre, D-51105 Cologne, Germany. DNA sequence analysis was then performed by BLAST network services at the NCBI. Sequences of 16S ribosomal DNA had provided with a phylogenetic tree that allows the investigation of evolution of actinomycetes and also provided the basis for identification; the 16S rRNA gene sequences of the isolate were aligned with reference sequences obtained from gene Bank using CrustalW (Thompson *et al*., 1997). Phylogenetic tree was generated using the maximum likelihood method with MEGA 5 package (Saitou &Nei 1987 and Tamura *et al*., 2011). The evolutionary distance matrix was derived with Jukes and Cantor model (Jukes & Cantor 1969). Topology of phylogenetic tree was evaluated by bootstrap analysis based on 500 replicates (Felsenstein 1985).

Physiological characterization of actinobacterial isolate

The isolate will be characterized for their physiological (temperature; 4, 28, 37 and 45°C; salinity; 1, 4,7, 10 and 14% and pH; 5, 7 and 10). The assay for enzymatic activity was performed according to Hopwood (1957); for examples starch hydrolysis, casein hydrolysis, cellulase, protease and lipase activity. In the physiological tests, selected isolates were grown in a 250 ml of Erlenmeyer flask containing 100 ml of ISP-2 broth (SCB) for 3 days with shaking of 200 rpm. Each of tests was performed in triplicate way; otherwise, all listed the tests were accompanied under pH 7.2.

Secondary screening of ethyl acetate extract for antibiotic production

Based on the results of preliminary screening, *Streptomyces* isolate were selected for the fermentation and assessment of antibiotic production. Fermentation of actinomycete isolate was carried out in a 2 liter conical flask (5 liter) in a complex medium that consisted of (per liter deionized water) glucose 10 g, starch soluble 20 g, yeast extract 5 g, casein peptone 5 g and $CaCO_3$ 1 g; the pH was adjusted to 7.0 (HCl) prior sterilization. Each flask was inoculated with 5 % by volume of culture grown in ISP-1 medium at 28 ± 2 °C in 100 ml-Erlenmeyer flasks for 3 days on a rotary shaker at 120 rpm. The fermentation was carried out for 7 days with agitation at 250 rpm and at 28 ± 2 °C. At the end of the incubation period, the culture was harvested by centrifugation at 13000 rpm for 15 min. The culture supernatant was extracted three times with equal volumes of ethyl acetate (1:1 v/v) and vaporized to dryness in a rotary evaporator at 40 °C (Sambannuihy & Ellaiah 1974). The crude extracts were dissolved in ethanol at known concentrations of extracts as stock for further studies.

Determination of minimum inhibitory concentration (MIC) of the extract

To determine the antimicrobial activity of the crude extract by agar disk diffusion method (Kirby *et al*., 1957);1.5 ml individual bacterial and fungal cultures were poured with 300 mla nutrient agar medium and then were poured in petri plates (90 mm), sterilized filter paper discs (Whatman No. 3; 6 mm in diameter) soaked in different beakers containing the dissolved extracts at different concentrations in case of bacterial strains 5, 10, 40, 80 µg per disc. Soaked discs were taken out with sterilized forceps and air-dried and placed on petri plates with the different microbes. The plates were incubated at 37°C for 24 h for bacterial strains. Control was carried out by soaking paper disks of ethyl acetate in against each test organism to ensure that it does not have activity against test bacteria The plates were triplicates in all the experiments.

Gas chromatography-Mass spectrophotometer (GC-MS) analysis

The actinobacterial extract were dissolved in the organic solvent till it dissolved completely and analyzed by GC-MS on GC Clarus 500 perkin Elmer using the following experimental conditions: column type-Elite-5 ms (5% diphenyl-95% dimethylpolysiloxane); Column dimension 30 m X 250 µm; carrier gas-helium moves at 1ml/min through the column **(**Usha, 2011).

Oven temperature program

2 µl of sample of the extract was injected and the column temperature was controlled at 50 °C min^{-1} up to 150 °C at the rate of 10 °C min^{-1} min hold, up to 250 °C at the rate of 8 °C min-1 min hold, up to 300 °C at the rate of 15 °C min-5 min hold. Injector temperature: 280 °C, total GC running time:33 min; and Split: 10:1, Detector: Mass detector: turbo mass gold-perkin Elmer software: tubomass 5.4.2.

MS program

The identification of the chemical constituents was based on matching their recorded mass spectra with those obtained from the NIST version-year 2005; Inlet line temperature: 200 °C.; source temperature: 200 °C; electron energy: 70 eV; mass scan: 45-450 m/z.;total MS running time was 36 min.

RESULTS AND DISCUSSION

Morphologically the selected isolate appeared as gray to off white according to different media, circular, smooth large colonies in Agar media Fig 2 . Under the microscope, the gram staining results revealed that strain ess_amA8 appeared to be Gram positive as shown in table 1, 2 and Fig. 2. Its cellular arrangement is spore chain as shown in Fig. 1. It is possibly related to *Streptomyces species*. Contig sequence obtained from 16S rRNA sequencing followed by basic local alignment search tool (BLAST) analysis showed that the isolates had close 16S rRNA database similarity with known *streptomyces* spp. multiple sequences were selected and aligned using MultAlin software (CLUSTAL 2.1). A phylogenetic tree/dendogram demonstrated that the isolated strain was a member of Streptomyces. Sequence similarity calculation indicated that the closest relative of isolate were *Streptomyces antibioticus* from the phylogenetic analysis, it was clear that, based on good 16S rRNA sequences, the potential bacterial strain ess_amA8 belonged to the genus *streptomyces* (Stackebrandt & Goebel, 1994) and shows significant similarity with *"Streptomyces*

antibioticus". Sequence of the isolate was submitted in gene bank and provided with accession number KF996507 and named as *Streptomyces antibioticus* strain ess_amA8. The obtained results showed that the extract affected the linear growth of the treated with significant variation. All bacterial pathogens were highly inhibited in their growth once treated with extract at its applied concentrations (Fig. 3 and Fig. 4). Generally, extract was most effective against *Streptococcus pyogenes* ATCC 19615, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumonia* ATCC 70060, *Salmonella typhi* ATCC 6539, *Pseudomonas aeruginosa* ATTC 27853 respectively. In addition, the results indicated that there were significant differences in the inhibition rate at 40 and 80 µg per disc, while at 5, 20 µg per disc, there was no significance as shown in Fig. 4. These results were agreement with earlier reports (Sahin, and Ugur, 2003). This results discrepancy with the results of Ahmet *et al*. who reported that the hexane extract showed a stronger and broader spectrum of antibacterial activity, followed by the methanol and ethanol extracts (Ahmet *et al*. 2005). The main constituents of the effective actinobacterial extract from *Streptomyces antibioticus* identified by GC-MS analysis are summarized in Table 3 and Fig. 5 according to their retention indices (RI) and percentage composition. 2-methyl propanamide (30.62%), n-hydroxymethylacetamide (18.94%), hexanamide (9.13%), 1,2 enzenedicarboxylic acid (12.02%), hexadecyl-oxirane (13.96%), 4-hydroxytetradec-2-ynal, 8-pentadecanone (10.13%), phenylpropanedioic acid (1.5 %), phenylethyl

Table 1. Physiological and biochemical properties of *Streptomyces* antibioticus strain ess_amA8

Name of the test	*Streptomyces*
Carbon sources	
Rhaffinose	-
Fructose	±
Sucrose	-
Arabinose	+
lactose	±
D-galactose	+
D-xylose	+
Citrate	-
Nitrogen sources	
L-Cysteine	+
L-Valine	+
L-Phenylalanine	±
L-Histidine	++
Enzyme activity	
amylase	+
Protease	+
Chitinase	-
catalase	+
DNase	+
Hydrolysis of esculin	+
Lecithin hydrolysis	-
H_2S production	-
Nitrate reduction	+
Urea hydrolysis	+
Lipid hydrolysis	-
Growth at different conc. NaCl	
1 %	++
4 %	+
7 %	-
10 %	-
14 %	-
Growth at different pH	
5	-
7	+
10	-
Growth at different temperatures	
4	-
26	++
30	+++
37	+
45	-
Antibiotic resistance	
E Erythromycin (15 µg)	+
GM Gentamamicin (10 µg)	+
PG Pencillin G (10 µg)	+
RP Rifampicin (30 µg)	-
K Kanamycin (1000 µg)	+
VA Vancomycin (5 µg)	-
CO Colisttinsulphate (10 µg)	+
AK Amikacin (30 µg)	-
ATM Aztreonam (30 µg)	-
C Chloramphenicol (30 µg)	-
CAZ Ceftazidime (30 µg)	+
IMI Imipenem (10 µg)	-
CIP Ciprofloxacin (1 µg)	+
PRL Piperacillin (100 µg)	+
T Tetracycline (30 µg)	-

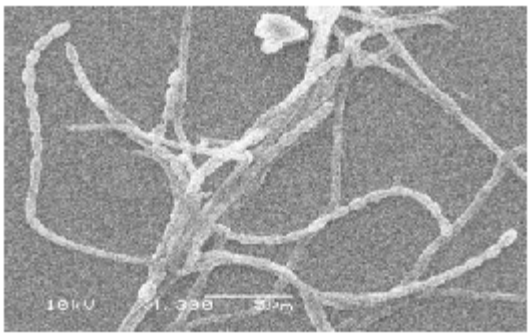

Fig. 1. Image of the *Streptomyces antibiotics* strain ess_amA8 under scanning electron microscope

Fig. 2. Growth of *Streptomyces antibioticus* strain ess_amA8 on Starch casein agar plate

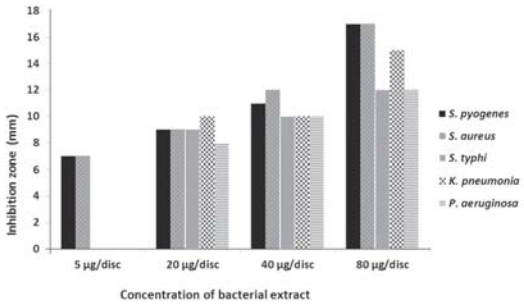

Fig. 3. Effect of different concentrations of extract on tested pathogenic bacteria

Fig. 4. Inhibition of bacterial growth by *Streptomyces antibioticus*

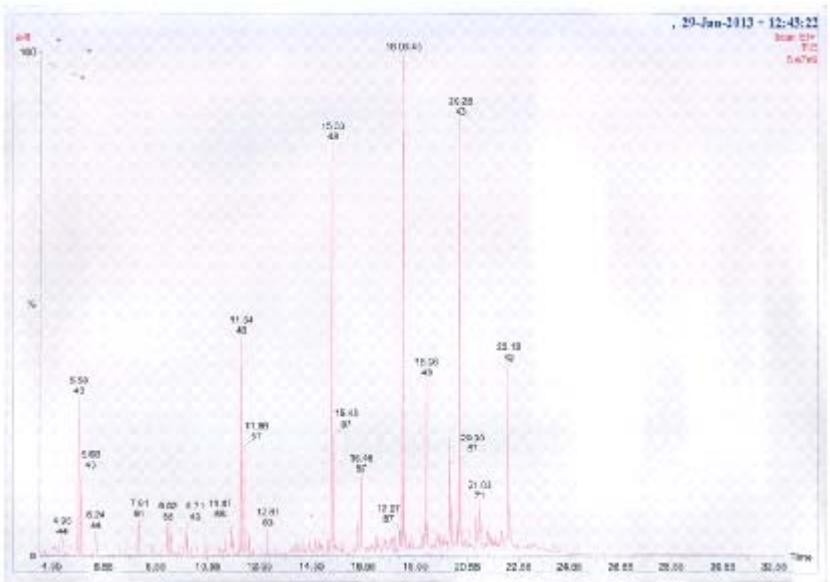

Fig. 5. The gas chromatogram of *Streptomyces antibioticus* ethyl acetate crude extract shows the appearance of the ten major compounds at different retention times

Table 2. Cultural characterization of *Streptomyces* antibioticus strain ess_amA8 on different culture media

Parameter Medium	Color of aerial mycelium	Color of substrate mycelium	AM/SM	pigmentation	Melanin production	growth	Form of spore chain
ISP-2	Gray	brown	A M	Brown	-	good	Retinaculum apertum
ISP-4	White black	yellow	A M	-	-	good	Retinaculum apertum
ISP-5	white	Light grey	A M	-	-	moderate	Retinaculum apertum
ISP-6	Creamy gray	Light white	Am	Brownish black	+ ve	good	Retinaculum apertum
ISP-7	Dark white	Light green	Am	-	-	good	Retinaculum apertum
Czapex Dox	Light white	Light white	Am	-	-	poor	Retinaculum apertum
Starch casein agar	Gray	Light white	Am	-	-	good	Retinaculum apertum

Table 3. List of compounds detected by GC/MS

A-8	Name of compound	RT	Area %
1	2-methyl propanamide	4.93	30.62
2	n-hydroxymethylacetamide	7.19	18.94
3	hexanamide	8.43	9.13
4	1,2 benzenedicarboxylic acid	19.8	12.02
5	hexadecyl-oxirane	20.6	13.96
6	4-hydroxytetradec-2-ynal	22.5	10.13
7	8-pentadecanone	16.4	2.5
8	phenyl propanedioic acid	9.9	1.5
9	phenylethyl alcohol	7.91	19.31
10	3-methyl-butanamide	6.23	38.73

alcohol (19.31%) and 3-methyl-butanamide (38.73%) respectively. Various aliphatic acids and aromatic compounds were also identified in the studied fractions. Compounds identified by GC-MS analysis possess various pharmaceutical applications. Alkylate phenol and its derivatives are known for their antimicrobial properties. Palmitic acid and Hexadecanoic acid possess potent antioxidant, anticancer and antimicrobial properties (Sudha and Masilamani, 2012).

CONCLUSION

In conclusion, *Streptomyces antibioticus* is a rich source of actinomycetes species producing the antibacterial metabolites. *Streptomyces antibioticus* strain ess_amA8 showed antioxidant activity, helpful in preventing or impedance the progress of various oxidative stresses related disorders. This spectral study confirms that ethyl acetate extract of *Streptomyces antibioticus* strain ess_amA8 have an antibacterial metabolites. It could be considered as a potential source for antibacterial drug development. The purification and structural elucidation of the antibacterial compound produced are under investigation

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REFERENCES

1. Ahmet AZ, Medine G, Meryem S., Hatice O, Fikrettin S, Isa K, Antimicrobial Effects of *Ocimum basilicum* (Labiatae) Extract, *Turk J Biol*, 2005; **29**: 155-160.
2. Bredholdt, Harald, *et. al. Environ. Microbiol.* 2007; **9**: 2756-2764.
3. Jeffrey, L.S.H, Sahilah , A.M, Son R, Tosiah S., Isolation and screening of actinomycetes from Malaysian soil for their enzymatic and antimicrobialactivities, *J Trop Agricult Food Sci*. 2007; **35**: 159-164.
4. Kumar,S.V, Sahu, M.K and Kathiresan .K., Isolation and characterization of streptomycetes producing antibiotics from a mangrove environment, *Asian J Microb Biotech Env. Sc*. 2005; **7** (3): 457-464.
5. Kumar.K.S, Haritha.R, Mohan.Y.S.Y.V and Ramana.T, Screening of marine Actinobacteria for Antimicrobial Compounds, *Res J Microbiol* 2011; **6**(4): 385-393.
6. Sahin N, Ugur A, Investigation of the antimicrobial activity of some Streptomyces isolates. *Turkish J Biol* 2003; **27**: 79-84.
7. Sahin, N. and A. Ugur, Investigation of the Antimicrobial activity of some Streptomyces isolates *Turk. J. Biol*., 2003; **27**: 79-84.
8. Smoker, J.A., Barnum, S.R., Rapid small-scale DNA isolation from filamentous cyanobacteria. *FEMS. microbiol. Let*., 1988; **56**(1): 119-122.
9. Srikesavan S, Masilamaniselvam M. Actinomycetes from marine sediment Screening for cytotoxicity identification and analysis of bioactive constituents by GCMS ICBBHS 2012: 32-45.
10. Sudha S, Masilamani SM Characterization of cytotoxic compound from marine sediment derived actinomycete Streptomyces avidinii strain SU4 *Asian Pacific J Trop Biomed* **2**(10): 770-773.
11. Tamura, K., Peterson, D., Peterson, N., Stecher, G., *et al.*, MEGA5, Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods.*Biol. Evol.*, 2011; **28**: 2731–2739.
12. Waksman, S. A. *Science*. 1953; **118**: 259–266.
13. Waksman, S.A., The Actinomycetes classification, identification and determination of genera and species. The Williams and Wilkins Co., Baltimore, 1961; **2**: 1-363.
14. Williams, Wilkins, Bergey's manual of determinative bacteriology. Holt (ed.). Co., Baltimore, 1994.