Biolife
AN INTERNATIONAL QUARTERLY JOURNAL OF BIOLOGY & LIFE SCIENCES
3(2):476-482
ISSN (online): 2320-4257
www.biolifejournals.com
BIOLIFE RESEARCH ARTICLE

Production and characterization of antimicrobial compound produced by *Streptomyces atrovirens* H33

Alaa-ElDin M S Hosny[1], Donia H Sheir[2]*, Ahmed I El-Diwany[3], Nayera A M Abdelwahed[4], Adyary Fallarero[5] and Pia M Vuorela[6]

[1] *Department of Microbiology and Immunology, Faculty of Pharmacy, Kasr El-Aini St, POB11562, Cairo University, Cairo, Egypt*
[2-4]*Division of Pharmaceutical Industries, Department of Chemistry of Natural and Microbial Products,33 Al Behouth., POB 12311, National Research Centre, Dokki, Giza, Egypt*
[5-6]*Division of Pharmaceutical Biosciences, Faculty of Pharmacy, POB 56 (Viksbågen 5E), FI-00014,University of Helsinki, Finland*

E-mail: donia_sheir@yahoo.com

ABSTRACT

Streptomyces atrovirens was isolated from soil samples in Egypt and showed broad spectrum antimicrobial activity. It was identified as *Streptomyces atrovirens* (strain H33) based upon16S rRNA gene sequencing. It was deposited in the GenBank database under accession number of KJ435269. *Streptomyces atrovirens* (strain H33)was cultivated by submerged fermentation bioreactor to produce the antimicrobialmetabolites. Purification and identification of antimicrobial compound was carried out. The antimicrobial compound exhibited low cytotoxic effect on human epithelial HL cells.

Key words: 16S rRNA, bioreactor; cytotoxicity, HPLC MS/MS, *Streptomyces atrovirens* H33.

INTRODUCTION

Actinomycetes are the most economically and biotechnologically valuable prokaryotes. They are responsible for the production of about half of the discovered bioactive secondary metabolites (Berdy, 2005), notably antibiotics (Berdy, 2005), antitumor agents (Kim et al., 2008), immunosuppressive agents (Mann, 2001) and enzymes (Oldfield et al., 1998).

How to cite this article:
Alaa-ElDin M S Hosny, Donia H Sheir, Ahmed I El-Diwany, Nayera A M Abdelwahed, Adyary Fallarero and Pia M Vuorela (2015). Production and characterization of antimicrobial compound produced by *Streptomyces atrovirens* H33. Biolife, 3(2), pp 476-482.

Streptomyces is the largest genus of actinomycetes which produce two-thirds of the clinically useful antibiotics and antifungals e.g. nystatin, amphotericin B, natamycin produced by *S. noursei*, *S. nodosus* and *S. natalensis* respectively (Raja and Prabakarana, 2011). Pharmaceutically important antimicrobial agent generated by Streptomyces can be produced by submerged fermentation in bioreactors (Vandamme,1983)which act as biological factories for the synthesis of bioactive compounds at high concentrations (Su, 2006).

MATERIALS AND METHODS

Microorganisms:

Streptomyces atrovirens was kindly obtained from National Research Center culture collection.

Standard strains:

Gram-positive bacteria (*Bacillus subtilis* NRRL-B-4219, *Staphylococcus aureus* (ATCC 29213); Gram-negative bacteria (*Escherichia coli* (ATCC 25922): *Pseudomonas aeruginosa* (ATCC 27953));

filamentous fungi (*Aspergillus niger* NRRL 363); Unicellular fungi (*C. albicans* ATCC 10231).

Screening of antimicrobial activity of the extract:

After extraction using different solvents, all the crude extracts were tested against the standard test strains using disk diffusion method (Garrod and Waterworth, 1971). The agar plate and incubated at 37 ºC for 24 h for bacteria and also at 28ºC for 24 h and 48 h in case of fungal species.

Molecular characterization and phylogenetic analyses of *Streptomyces atrovirens*:

The extraction of genomic DNA was performed according to the protocol recommended for the DNA purification of Gene JET™ genomic DNA purification kit (Thermo Fisher Scientific).PCR amplification was performed using Maxima Hot Start PCR Master Mix (ThermoFisher Scientific) in 50 µl of reaction system containing (25µl) Maxima Hot Start PCR master mix (2X), (1 µl) 20 µM 16S rRNA forward primer (5'-AGA GTT TGA TCC TGG CTC AG -3'), (1 µl) 20 µM 16S rRNA reverse primer (5' -GGT TAC CTT GTT ACG ACT T-3') (Lane et al., 1985), (5µl) DNA template, (18µl) water, nuclease - free. The initial denaturing was at 95°C for 10 min; and then 35 cycle 95°C for30 s; 65 °C for 60 s; 72 °C for90 s; and final extension at 72°C for 10 min. Purification of PCR product was using Gene JET™ PCR Purification kit.

Finally, the sequencing of the PCR product was done by ABI 3730xl DNA sequencer (GATC, Germany).For phylogenetic analysis, the determined sequences were compared with the sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank data base (www.ncbi.nlm.nih.gov) by BLAST. The alignment was manually verified and adjusted prior to the construction of phylogenetic tree. The phylogenetic tree was constructed by using neighbour-joining (Saito and Nei, 1987) in the MEGA program version 6 (Tamura et al., 2013).The confidence values of branches of the phylogenetic tree were determined using the bootstrap analyses based on 1000 resamplings (Felsenstien, 1985).

Antifungal production from *Streptomyces* strain in a bioreactor:

The inoculum was prepared as follows: 2 L shake flask containing 250 mLof starch casein broth medium was inoculated with spore suspension from 7 days old SC agar plates and incubated for 2 days on 200 rpm orbital shaker (New Brunswick) at 30 °C (Hobbs et al., 1989).The submerged fermentation was carried out by the vegetative inoculum 5% (v/v) of the 2 L shake flask in a bench-top stirred tank reactor (STR) (New Brunswick Scientific Bioflo & celligen 310) under the following conditions: 5 L working volume of SC broth medium, temperature of 28-30ºC, aeration rate 5.0 v/v.min and agitation speed 400 rpm. The fermentation medium pH was adjusted to 7.0 before sterilization. Samples were collected every 6 hours, and inhibition zone (I Z) for antifungal activity was determined against *C. albicans* ATCC (American Type Culture Collection) 10231. Dissolved oxygen concentration (DO %), pH were determined using pH and DO polarographic electrodes (MettlerIngold, Germany), respectively. CDW, cell dry weight (g/L). Samples were taken and filtered through the (Whatman No.1) filter paper and then dried at 70 ºC for a constant weight. The weight of the dried cells was measured by calculation of the difference between the weight of filter paper before and after filtration.

Extraction and isolation of antimicrobial compounds:

After optimal antimicrobial production using starch casein medium (Hosny et al., 2015), the culture filtrate was extracted with different organic solvents such as n-hexane, ethyl actetate, chloroform and n-butnol by twice volume. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum to dryness. The crude extracts obtained were subjected to Thin-layer chromatography combined with bioautography (TLC-Bio) for detection of active compound against test strains. Samples were spotted on 20 cm × 20 cm silica gel plates (Merck, Silica gel $60F_{254}$, Germany), which were developed with butanol: methanol: water (4: 5: 1, v/v) and then air-dried overnight at 37ºC to remove solvents. Two plates were prepared for each fractionation experiment. The first plate was used to localize the bioactive compounds based on their retention factor (R_f). These compounds were visualized by UV 365. The second plate was placed in a plastic bioassay dish (23 cm × 23 cm × 2.2 cm, Fisher Scientific, Labosi), overloaded with 35 mL of nutrient agar previously seeded with test strains spores and incubated for 24 h at 37°C. The clear areas revealing the inhibition of test strains growth indicate the location of antimicrobial compounds, which were correlated to their R_f on the reference TLC plates. Also these bands were compared with streptomycin, erythromycin, mitomycin, kanamycin, vancomycin, neomycin).

Spectral analysis and structural elucidation of the pure isolated compound:

The pure isolated compound was subjected to chemical characterization by using modern analytical techniques such as UV-VIS, IR spectrophotometry, ^{1}HNMR and LC MS/MS spectrometry.

LC MS/MS:

Determination of the bioactive compound by MS/MS analyses were performed on an Agilent 6460 Triple Quadrupole LC/MS system equipped with an electrospray ionization source (ESI) (Agilent Technologies). The LC Pump system were

performed at 27 °C using a Zorbas XDB column (150 x 4.6 mm; C18, 5mm) purchased from Agilent Technologies, USA. Samples were injected using High Performance autosampler with a 30 uL injection volume. Separations were carried out by using water ultrapure as solvent A and acetonitrile as solvent B at a flow rate of 1 ml min^{-1}. A linear gradient from 10% to 100% acetonitrile in 30 min, at a flow rate of 1 ml min^{-1}, was used (Olano et al., 2004). The gradient program is given in Table 1. The HPLC system was coupled directly to an Agilent 6460 Triple Quadrupole LC/MS system with Agilent Jet Stream ESI ionization source. Instrument control and data acquisition were performed using Agilent MassHunter Workstation Software. ESI was operated in the positive and negative ion mode. The conditions were as follows: temperature: 27 °C, nebulizer pressure: 40 psi, flow rate: 1 mL/min, cell accelerator voltage: 7 V, fragmentor voltage: 135 V. Full mass scan spectra were recorded over a range of 50–1000 m/z.

FT-IR:

IR spectra were recorded on Portable FT-IR Spectrometer as powder analyzer (Burker mobile IR, Germany), scan range 500-4000 cm^{-1} range. The infrared spectrum within 4000 - cm^{-1}400 cm^{-1}.

NMR:

The NMR spectra was recorded in DMSO-d6using a Varian Gemini300 MHz spectrometer. All spectra were locked to deuterium resonance of DMSO. The error in ppm values was 0.01. All ^{1}H NMR measurements were carried out at the operating frequency300.0117 MHz; flip angle, pw 45°; spectral width, sw 6000 Hz; acquisition time, at 3.1 s; relaxation delay, d1 1.0 s; T = 298.1 K.

Cell viability assays:

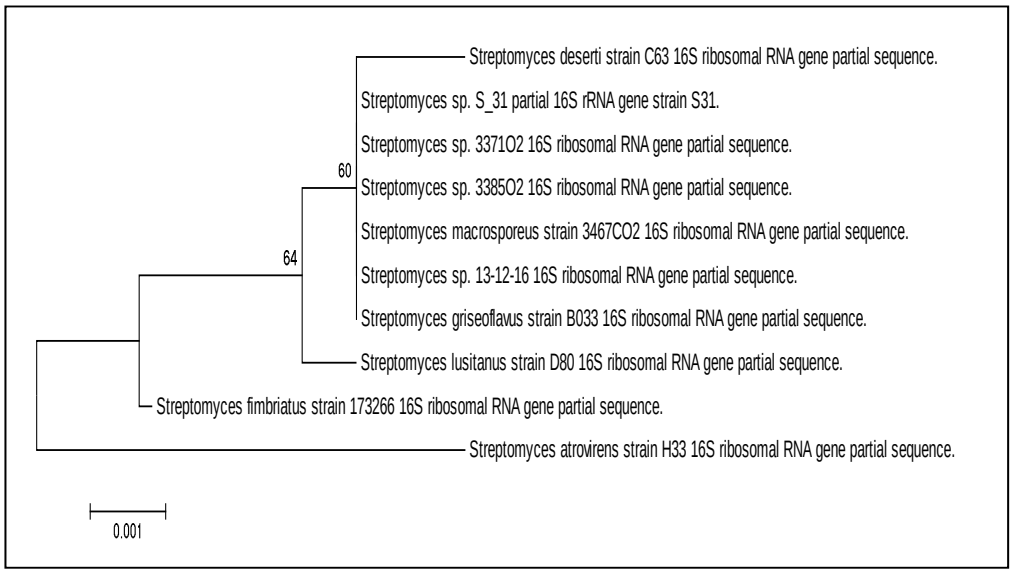
Human epithelial HL cells were used to assess cell viability using the resazurin assay as in (Karlsson et al., 2012). Cellular density of 60.000 cells /well was used and the plates were incubated for 24 h.

RESULTS AND DISCUSSION

The morphological, biochemical, physiological characterization studies strongly suggested that this strain belongs to the genus *Streptomyces* (Hosny et al., 2015). Nowadays, detection and classification of *Streptomyces* is also commonly performed by molecular approaches (Locatelli et al., 2002). *Streptomyces* sp. 4 exhibited similarity level of 98% with *Streptomyces atrovirens* strain NRRL B-16357, designated as H33 and it was deposited in the GenBank nucleotide sequence database under accession number KJ435269. For data analysis, the phylogenetic tree of the strain is presented in Figure 1.

It showed antimicrobial activity against Gram-positive (*B. subtilis*), Gram-negative (*E. coli, P. aeruginosa* and *A. faecalis*) and antifungal (*C. albicans* ATCC 10231*, A. niger*) using agar well diffusion assay (Hosny et al., 2015). Searches of the literature have revealed that *S. atrovirens* is one of the potential species that produce some important metabolites e.g. cytotoxic benzamides that have antimicrobial and anticancer activities isolated from

Figure-1. Phylogenetic tree is based on the nucleotide sequence of 16S rRNA genes. The Neighbour-Joining algorithm tree was constructed by MEGA 6 for *S. atrovirens*. Tree derived from 16S rRNA gene sequence data. Numbers at branching points indicate bootstrap values that are expressed as a percentage of 1,000 replications.



soil sample (Shaaban et al., 2012) and benzaldehyde compounds that have antibacterial activities isolated from marine seaweed rhizosphere (Pranitha et al, 2014 and Cho and Kim, 2012).

Antimicrobial production from *S. atrovirens* in a bioreactor. Submerged fermentation in bioreactors is the common method used for production of pharmaceutically important antibiotics from Streptomycetes in larger amounts (Su, 2006 and Vandamme, 1983), which are needed subsequently for purification and identification process. For antifungal production in bioreactor, SC broth medium was found to be good for inducing antifungal activity for all streptomycetes isolates (Shirling and Gottlieb, 1966). In addition, vegetative inoculum was used to obtain higher productivity of antibiotics compared with spore inoculums (Elibol et al., 1995) and minimized the lag phase. However, the maximum antifungal metabolites biosynthesis by *S. atrovirens* began after the consumption of the dissolved oxygen in the production medium where the IZ of the antifungal metabolites rose to the maximum of 32 mm against *C. albicans* ATCC 10231 at 66 h, and maintained constant until 72 h (Figure 2).

Extraction and isolation of antimicrobial agent:

Submerged fermentation in bioreactors is the common method used for production of pharmaceutically important antibiotics from Streptomycetes in larger amounts (Su, 2006 andVandamme, 1983), which are needed subsequently for purification and identification process. For antifungal production in bioreactor, SC broth medium was found to be good for inducing antifungal activity for all streptomycetes isolates (Shirling and Gottlieb, 1966). Chloroform was the best solvent able to extract the antifungal compound. The crude extract was separated on Thin Layer chromatography (TLC) eluted by solvent system butanol: methanol: water (4: 4: 5), where the fraction was separated into 4 bands. These bands were compared with reference compounds (tetracycline, amphotericin B, streptomycin, erythromycin, mitomycin, kanamycin, vancomycin, neomycin). The R_f of bioactive compound was at 0.15 (Table-1).

Table 1. Thin Layer Chromatography (TLC) of chloroform extract of *S. atrovirens* H33 compared with reference compounds

Compounds	R_f
Antimicrobial agent *S. atrovirens* H33	0.15
Tetracycline	0
Neomycin	0
Mitomycin	0.68
Anisomycin	0.33
Vancomycin	0
Erythromycin	0
Streptomycin	0
Amphotericin B	0.23, 0.56
Kanamycin	0

Chemical characterization of antimicrobial agent:

In order to characterize the effective antimicrobial several spectroscopic techniques were carried out as follows:

Figure-2. Batch fermentation for antifungal production by (a) *S. atrovirens.* closed diamond: DO (dissolved oxygen) (%), closed triangle: biomass concentration (g/L), closed square: IZ (mm).

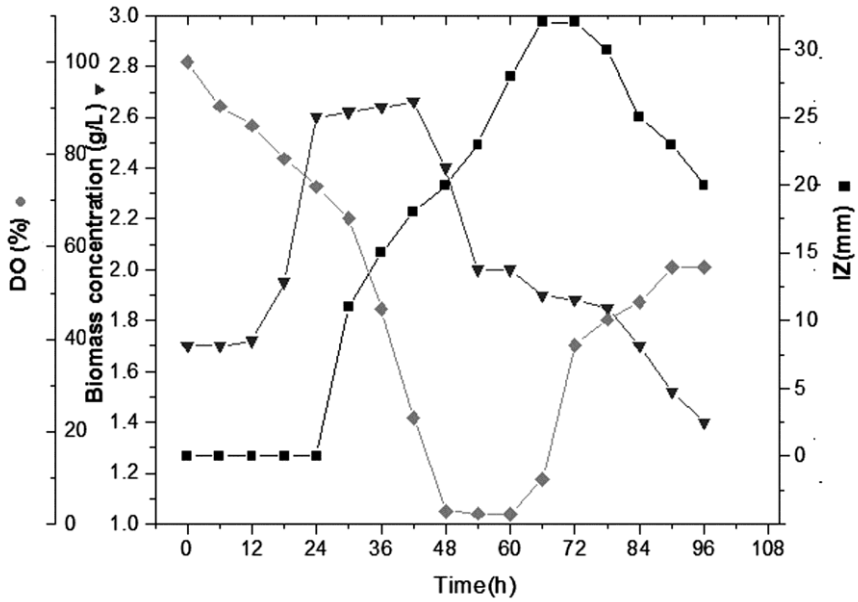
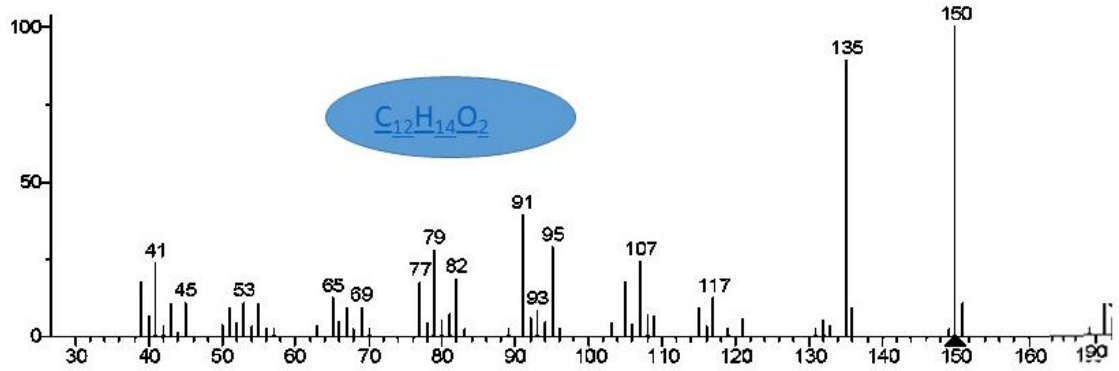
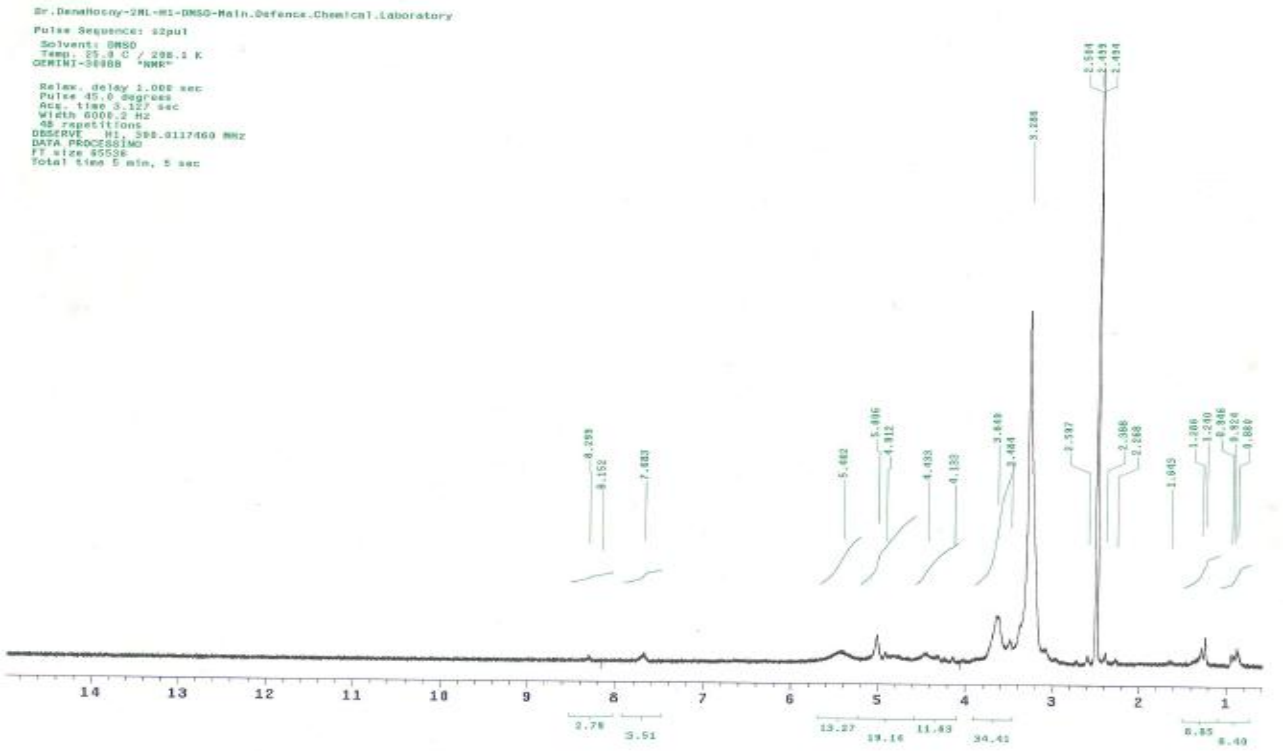


Figure-3. Mass spectrum LC MS/MS spectroscopic analysis of antimicrobial compound biosynthesized by *S. atrovirens* H33



Figurfe-4. I.R spectrum of antimicrobial compound produced by *S. atrovirens* H33



LC MS/MS:

The mass spectrum also revealed that the compound properly had the Formula: $C_{12}H_{14}O_2$ and molecular weight: 190.

Infrared- red (IR) spectroscopic analysis:

The IR spectrum (Figure-4) had a peak at 3234 cm^{-1} indicates the presence of hydroxyl stretch, and hydrogen bond which is function group for phenolic compounds. The peak at 2924 cm^{-1} indicates presence of C-H stretch which is Alkan function group. The peak at 2328 cm^{-1} indicates H-C=O: C-H stretch which is a function group for aldehyde. The peaks appearing at 1725 and 1647 cm^{-1} indicates C=O stretch and -C=C-. The peaks at 1563 cm^{-1} indicates C-C stretch aromatic ring. The peak at 1371 cm^{-1} indicates C-H rock alkanes. The peaks at 853, 842, 757 and 707 indicates C-H bond (alkenes). The peaks at 932 cm^{-1}indicates O-H carboxylic acid. Finally, the peaks at 668 and 653 cm^{-1} can be assigned to aromatics C-H ¨Oop¨

Figure-5. H1 NMR spectrum of the antimicrobial compound produced by S*. atrovirens* H33

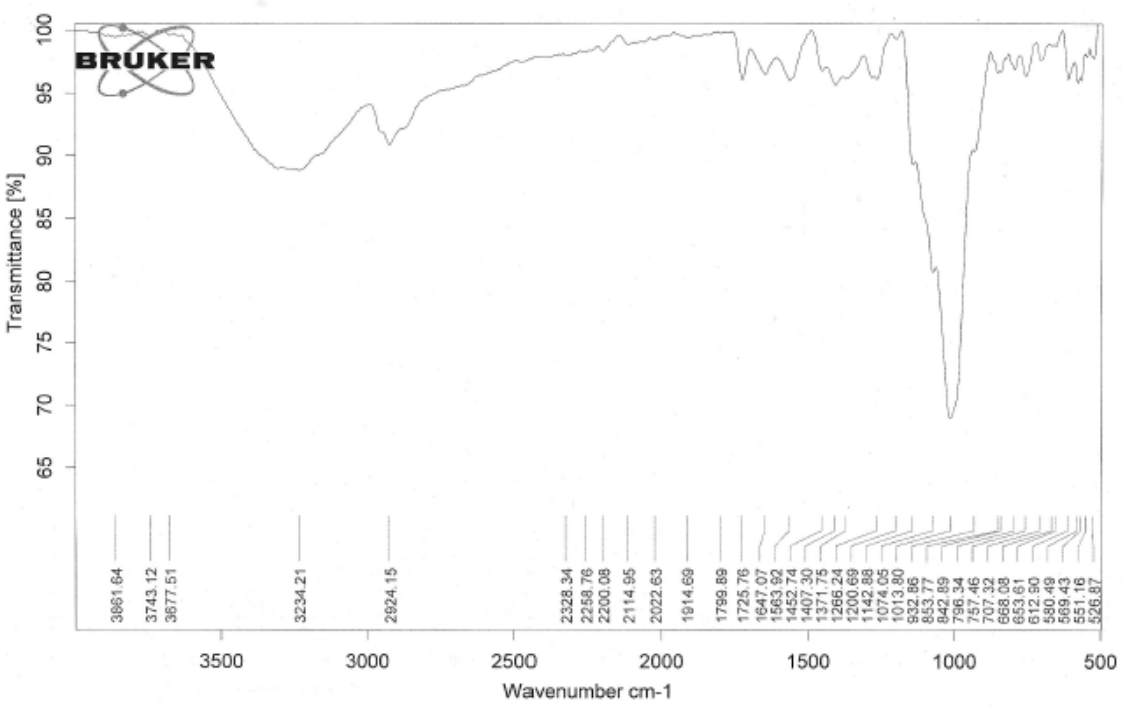
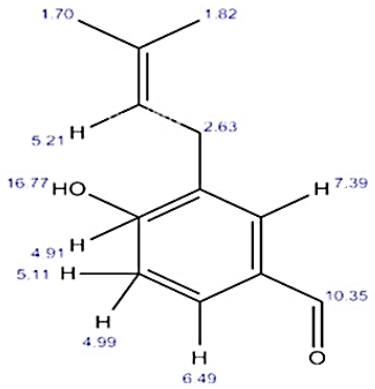


Figure-6. 4-hydroxy-3-(3-methylbut-2-enyl) benzaldehyde



Nuclear magnetic resonance spectroscopy (H^1NMR analysis):

According to the described chemical assignments obtained from the IR, MS and NMR, the suggested structure for the purified compound was 4-hydroxy-3-(3-methylbut-2-enyl) benzaldehyde with molecular formula of $C_{12}H_{14}O_2$ and molecular weight of 190.

Cell viability assays:

To further characterize the biological activity of the antimicrobial compound, it was evaluated for its effect on human epithelial cells. It was nontoxic at 10 and 20 $mgmL^{-1}$ (>80% viability).

CONCLUSION

Streptomyces spp. are considered as a robust source for production of antimicrobial. *S. atrovirens* was able to produce a broad spectrum antimicrobial against Gram-positive, Gram-negative bacteria and fungi. It was successfully cultivated by submerged fermentation in a bioreactor. The Purification and identification of antimicrobial compound was carried out using several modern spectroscopic techniques such as, IR spectrophotometery,^{1}HNMR and LC MS/MS spectrometery. The pure compound was suggested to be 4-hydroxy-3-(3-methylbut-2-enyl) benzaldehyde with molecular formula of $C_{12}H_{14}O_2$ and molecular weight of 190 with low cytotoxic effect on human epithelial HL cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

1. **Bauer, A.W., Kirby, W.M. M., Sherris, J.C., and Turck, M. (1966).** Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.***45**, 493-496.

2. **Berdy, J. (2005).** Bioactive microbial metabolites. *J. Antibiot.* (Tokyo), **58**, 1-26.
3. Cho, J. Y. and Kim, M. S. (2012) Antibacterial benzaldehydesproduced by seaweed-derived *Streptomyces atrovirens* PK288-21. *Fish. Sci.*, **78**, 1065-1073.
4. **Elibol, M., Ulgen, K., Kamarulzaman, K., andMavituna, F. (1995)**. Effect of inoculum type on actinorhodin production by *Streptomyces coelicolor*. *Biotechnol*. *Lett*.,**17**, 579-582.
5. **Felsenstien, J. (1985).** Confidence limits on phylogenies: an approach usingthe bootstrap.*Evolution*, **39,** 783-791.
6. **Garrod, L. P. and Waterworth, P. M. (1971)**. A study of antibiotic sensitivity testing with proposals for simple uniform methods. *J. Clin. Pathol.*,**24**, 779-789.
7. **Hobbs, G., Frazer, C.M., Gardner, D.J.C., Cullum, J.A., and Oliver, S.G. (1989).** Dispersed growth of *Streptomyces* in liquid culture. *Appl. Microbiol. Biotechnol*.,**31**, 272-277.
8. **Hosny, A.M. S., Sheir, D. H., Abdelallah,N. A., Amin, D. H. M., El-Diwany,A. I., Abdelwahed, N.A. M., El-Beih,A. A., Fallarero,A., and Vuorela,P. M. (2015).** Isolation of*Streptomyces* sp. producing antifungal agent. Vol. 4 (2) April-June, *Curr.Sci. Int.* (In press).
9. **Karlsson, D., Fallarero. A., Brunhofer, G., Mayer, C., Prakash, O.et al. (2012).** The exploration of thienothiazines as selective butyrylcholinesterase inhibitors. European Journal of Pharmaceutical Sciences, 47,190-205.
10. **Kim,K-J., Kim,M-A., and Jung,J-H. (2008).** Antitumor and antioxidant activity of protocatechualdehyde produced from *Streptomyces lincolnensis* M-20. *Arch. Pharm. Res.*, **31**, (12), 1572-1577.
11. **Lane, D., Pace, B., Olsen, G., Stahl, D., Sogin , M. et al. (1985).** Rapid determination of 16S ribosomal RNA sequence for phylogenetic analyses. *Proc. Natl. Acad. Sci.U. S. A.*, **82**, 6955 –6959.
12. **Locatelli, L., Tarnawski, S., Hamelin, J., Rossi,P., Aragno, M. et al. (2002).** Specific PCR amplification for the genus Pseudomonas targeting the 3 half of 16S rDNA and the whole 16S- 23S rDNA spacer. *Syst*. *Appl. Microbiol*.,**25**, 220-227.
13. **Mann, J. (2001).** Natural products as immunosuppressive agents. *Natural Product Reports*, **18**, 417-430.
14. **Olano,C., Moss, S. J., Braña, A. F., SheridanR. M., MathV. et al. (2004).** Biosynthesis of the angiogenesis inhibitor borrelidin by *Streptomyces parvulus* Tü4055: insights into nitrile formation. *Mol. Microbiol.*,**52** (6), 1745–1756.
15. **Oldfield, C., Wood, N. T., Gilbert, S. C., Murray, F. D., and Faure, F. R. (1998).** Desulphurisation of benzothiophene and dibenzothiophene by actinomycete organisms belonging to the genus *Rhodococcus*, and related taxa. *Antonie Van Leeuwenhoek*, **74**,119-132.
16. **Pranitha, G. Krishna and M.A Singara Charya** (2014). Evaluation of antibacterial and anifungal activity of fruiting Body extracts of *trametes versicolar. Biolife.* 2(4):1181-1184.
17. **Raintala, H., Nvalainen, A., Ronka, E., and Suutari, M. (2001).** PCR primers targeting the 16S rRNA gene for the specific detection of *Streptomyces*. *Mol. Cell. Probes*,**15**, 337-347.
18. **Raja A. and Prabakarana P. (2011).** Actinomycetes and drug-An overview. *Am. J. of Dru. Dis. and Dev.*, **1**, 75-84.
19. **Saito, N. and Nei, M. (1987).** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.*, **4**, 406-425.
20. **Shaaban, K.A., Shepherd, M.D., Ahmed, T.A., Nybo, S.E., Leggas, M. et al. (2012).** Pyramidamycins A-D and 3-Hydroxyquinoline-2-carboxamide; Cytotoxic Benzamides from *Streptomyces* sp. DGC1.*J.Antibiot.* (Tokyo),**65**(12): 615-622.
21. **Shirling, E.B. and Gottlieb, D. (1966).** Methods for characterization of *Streptomyces* sp. *Int. J. Syst. Bacteriol.,***16**, 313-340.
22. **Su, W. W. (2006).** Bioreactor engineering for recombinant protein production using plant cell suspension culture. In: Plant Tissue Culture Engineering, S. Dutta Gupta and Y. Ibaraki (Eds), Springer, Printed in the Netherlands, pp. 135-159.
23. **Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S.(2013).** MEGA6: Molecularevolutionary genetics analysis version 6.0. *Mol. Biol. Evol*., **28**(10), 2731-2739.
24. **Vandamme, E.J. (1983).** Peptide antibiotic production through immobilized biocatalyst technology. *Enzyme Microb. Technol*.,***5***, 403–416

DOI: https://dx.doi.org/10.5281/zenodo.7269694
Received: 6 April 2015;
Accepted; 12 May 2015;
Available online : 5 June 2015