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Broad spectrum antibacterial activity of granaticinic acid, isolated from *Streptomyces thermoviolaceus* NT1; an endophyte in *Catharanthus roseus* (L.) G. Don

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ABSTRACT

Searching for endophytic actinomycetes, strain NT1 was isolated from surface sterilized stem of *Catharanthus roseus* (L.) G. Don collected from Paschim Medinipur, India. The strain was identified as *Streptomyces thermoviolaceus* NT1 on the basis of morphological, biochemical and 16s rDNA based phylogenetic analysis. It showed potential antagonism against several Gram positive and Gram negative bacterial pathogens along with drug resistant *Staphylococcus aureus*. Maximum antibacterial production was obtained in ISP2 media at pH 7.2, 35 °C for 10 days. The active antibacterial substance was purified by Silica gel column chromatography and activity guided TLC. IR and NMR analysis identified the active compound as granaticinic acid of m/z 463.26 [M^+H]. These results suggest that the antimicrobial produced by the isolated endophytic strain will be useful in near future.

INTRODUCTION

Screening of antimicrobial compound producing microorganisms is a continual field of research and actinomycetes are playing a promising role as the source of such compounds. Over two third of present day antibiotics were obtained from Streptomyces (Baltz, 1998), a special group within actinomycetes but presently isolation of such compounds are unable to meet the demand for a new class of bioactive compounds. Moreover emergence of drug resistant pathogens, outbreak of opportunistic bacteria and throttle success of combinatorial chemistry creates the situation more severe (Strobel, 2003). Endophytic microorganisms have taken this opportunity as a good source of future antimicrobial compounds. Endophytes reside within the plant's internal tissues which are still less explored ecosystem harboring diverse microbes. Some of them act synergistically by producing secondary metabolites antagonistic to plant pathogens (Strobel and Daisy, 2003) or by producing plant growth promoting substances (Bhattacharya and Jha, 2012).

Actinomycetes are also found in endophytic association like fungi (Kharwar *et al.*, 2008) and other bacteria and can serve as a prime source of novel class antimicrobial compounds, as chances of getting new compound is relatively higher from new strain and isolation of new strain is advantageous from unexplored or less explored habitat (Stierle *et al.*, 1993). Several studies describe the isolation and identification of endophytic actinomycetes producing metabolites against fungal plant pathogens but few are reported against bacterial pathogens especially drug resistant or opportunistic bacterial pathogens (Castillo *et al.*, 2002; Castillo *et al.*, 2003; Taechowisan *et al.*, 2005, Taechowisan *et al.*, 2014).

Present study illustrates the isolation and identification of endophytic *Streptomyces thermoviolaceus* NT1 from *Catharanthus roseus*. Purification of metabolites produced by this strain afforded granaticinic acid, which showed broad spectrum antibacterial property and was also active against drug resistant pathogens.

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MATERIALS AND METHODS

Endophytic actinomycetes isolation

Healthy and young plants of *Catharanthus roseus* (L.) G. Don. were collected from various places of Psachim Medinipur, India (22.57° N – 87.11° E). Stems (about 1 cm length) of *C. roseus* were surface sterilized by sequential ethanol and NaOCl treatment and finally washed three times with autoclaved distilled water (Coombs and Franco, 2003).

Authenticity of surface sterilization was verified by plating the final sample washed water on nutrient agar media. The bark was removed of samples and tissues were soaked in 10 % $NaHCO_3$ for 5 min to suppress fungal contamination. Inner tissues were then aseptically transferred to ISP2, ISP5 (International Streptomyces Project) agar and actinomycetes isolation agar media (Himedia, Mumbai) supplemented with cycloheximide and streptomycin (50 µg/ ml). Plates were incubated at 28 ºC for 14 days. Isolated strains were pure cultured and preserved in glycerol based liquid media at -20 ºC.

In vitro antimicrobial assay

Isolated strain, NT1 was checked for Antimicrobial potency by agar diffusion methods. This strain was grown in ISP2 broth and 100 µl cell free cultures was applied in wells of Muller Hinton agar media that was previously seeded with bacterial pathogens. Inhibition zones were recorded after 24 h incubation at 35 ºC for each pathogen.

Following pathogenic bacteria were selected in this study: methicillin resistant *Staphylococcus aureus*, penicillin resistant *Staphylococcus aureus* (clinical isolate), *Bacillus subtilis* (ATCC 11774), *Bacillus cereus* (ATCC 14579), *Vibrio parahemolyticus* ATCC 1782, *Pseudomonas aeruginosa* (ATCC 9027), *Shigella flexnerii* (ATCC 12022), *and Escherichia coli* (clinical isolate).

Morphological, physiological and biochemical characterizations

Characterization of the strain was done based on cultural characteristics, morphological, physiological and biochemical properties. Cultural morphology was established after growing the organism at various media (Shirling and Gottlieb, 1966; Williams *et al.*, 1983) and cellular morphology was determined by the compound and scanning electron microscopy (Vega© TESCAN, Czech Republic). Extracellular enzyme production and sugar utilization test (Gordon *et al.*, 1974) were made by standard methods. The strain was grown in ISP 2 broth at various incubation temperature and pH conditions for determining its optimum growth temperature and pH requirement.

Genomic DNA isolation and amplification of 16s rRNA gene

Cells were lysed of 5 days old culture by lysozyme and SDS treatment (5 mg/ ml and 10 % respectively). Genomic DNA was extracted with phenol-chloroform and precipitated with isopropanol. Finally genomic DNA was dissolved in 100 µl TE (pH 8) buffer. The 16s rRNA gene was amplified using the primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Santhi and Solomon, 2011) in a thermocycler (Eppendorf). PCR products were purified with Hi-PurA™ PCR product purification spin kit (Himedia Laboratories, India). Forward and reverse DNA sequencing of amplicon was carried out with the same primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Phylogeny construction and sequence submission

Consensus sequences of 1412 bp rRNA gene were edited and assembled from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST with the 'nr' database of NCBI Genbank to determine operational taxonomic units. Based on maximum identity score sequences were selected and aligned using the multiple alignment software program, Clustal W. Distance matrix was generated using RDP (Ribosomal Database Project) database and the phylogenetic tree were constructed using MEGA 6 (Tamura *et al.*, 2007) (Molecular Evolutionary Genetics Analysis). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987).

The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980). DNA sequences were deposited in GenBank under accession number KJ486841.1

Antimicrobial production at optimum conditions

Optimization of antibacterial production from NT1 was done by culturing it in various physical and chemical conditions. The strain was grown in a wide range of media pH (5- 10) and incubation temperature (20 – 45 ºC). Optimum values were determined by measuring zone of inhibition produced by culture filtrates of each variant against *B. cereus*. Optimum additional carbon source was determined by adding glucose, galactose, fructose, maltose, lactose and sucrose (1 %, w/v) in ISP2 broth. Tryptone, peptone, soya meal (0.5 %, w/v), KNO_3 NH_4NO_3 and NH_4Cl were additionally (0.2 %, w/v) added to the ISP broth to find most suitable nitrogen source for antibacterial production by the strain NT1.

A 1 cm diameter agar plug, full of NT1 spores was inoculated in 30 ml ISP2 broth and cultured at 28 ºC for 3 days. 5 % culture was transferred to 100 ml ISP2 broth in 500 ml conical flask and cultured for 3 days more and used as seed for next step larger production. Finally, 5 % seed was inoculated in 1.5 L ISP2 broth and cultured for 10 days at 28 ºC with 150 rpm. Cell mycelia were separated by filtration with Whatman paper No. 1 and then centrifuged at 12000 rpm for 15 min. to obtain a cell free culture medium. Culture filtrate was extracted with equal volume ethyl acetate and organic fraction was made water free by adding sufficient anhydrous Na_2SO_4. It was then dried under vacuum in a rotary evaporator at 40 °C (HS – 2005S, HAHNSHIN, Korea) and antibacterial activity was determined by disk diffusion assay.

Purification of active component

Residual crude was dissolved in 5 ml chloroform, adsorbed by Silica gel and loaded onto a silica gel column (230-400 Å mesh). It was then fractionated with graded hexane – chloroform (60:0, 50:10, 30:30,10:50, 0:60) and then with chloroform-methanol (60:0, 55:5, 50:10, 40:20, 30:30, 20:40, 0:60). About 60 ml of each fraction was collected. Fractions were concentrated and checked for antibacterial property by disk diffusion technique on MHA plates. Active fraction was re-fractioned with more finely graded solvent system (chloroform and methanol; 60:0, 59:1, 58:2, 57:3, 56:4, 55:5, 50:10, 0:60) and active fraction was determined by similar assay.

TLC and bioautogram analysis

Purified fraction was concentrated and analyzed by thin layer chromatography with chloroform and methanol (5:1). Separated components were located on UV exposure. Autobiogram was done with *B. cereus* to find active component on TLC plates. After TLC analysis the plate was overlaid with molten MHA media (45 ºC) containing 50 µl aliquots of *B. cereus* (10^6 cfu/ ml). TLC plate was incubated at 37 ºC overnight and inhibition zone was located by methylthiazoletetrazolium (MTT-5 mg/ ml).

Spectroscopic analysis of active compound

Structural information of active component was gathered by UV, IR, NMR, and mass spectral data. λ_{max} was determined of active compound after dissolving in chloroform by UV-Vis spectrophotometer (UV-1800, SHIMADZU). FTIR spectra were taken in FT-IR spectrophotometer (Spectrum T, Perkin Elmer). The sample was dissolved in $CDCl_3$ and analyzed for ^1H and ^{13}C NMR spectra at 600 MHz (Bruker, Avance 600). ESI Mass was analyzed with positive ion mode.

RESULTS AND DISCUSSIONS

The isolated endophytic strain was gram positive, filamentous actinobacteria that grow well aerobically. Substrate mycelia nicely emerge within 3 day after inoculation and aerial formations were grey that turns to white. The strain produces soluble violet pigment in ISP2 media. It produced slow-growing, dry and hard colonies on ISP2 agar. Scanning electron microscopy of the organism revealed the presence of numerous aerial filaments and few smooth, cylindrical spores (0.8 µm in length and 0.2 µm in diameter) that were produced in short curved (Fig. 1).

Other morphological, and biochemical properties of strain NT1 were summarized in table 1. Comparative 16S rRNA gene sequence analysis (Fig. 2) showed that the strain was taxonomically belong to the species of *Streptomyces* and highest similarities being found with the sequences of *Streptomyces thermoviolaceus* (AB184685.1), *Streptomyces thermocyaneomaculatus (AB184583.1), Streptomyces thermocyaneoviolaceus (AB184582.1), Streptomyces thermophilus* (AB184358.1) about 99 % and with *Streptomyces thermodiastaticus* (AJ001434.1), *Streptomyces* sp. E1125 (DQ303453.1), *Streptomyces* sp. SDCB6 (JN617215.1) 98%. It is evident from the phylogenetic tree based on the neighbor-joining method that the strain NT1 clusters with the nearest neighbors *S. thermoviolaceus* NBRC 13387 in a separate branch with high bootstrap support.

Table 1: Cultural and physiological characteristics of endophytic *Streptomyces* themoviolaceus NT1.

Colony morphology	**Elevated, rough surface, dry, brownish to white colony**
Growth on ISP2	Good
Substrate mycelia	Brownish
Aerial mycelia	White
Soluble pigment	Redish violet
Growth on ISP4	Moderate
Substrate mycelia	Yellowish
Aerial mycelia	White
Soluble pigment	Violet
Growth of ISP5	Very less
Substrate mycelia	Gray
Aerial mycelia	White
Soluble pigment	No
Growth on ISP7	Very poor
Substrate mycelia	Yellowish
Aerial mycelia	White
Soluble pigment	No
Spore morphology	3- 5 spores at tip of the filaments Cylindrical, smooth surface About 0.8 to0.2 µm diameter
Cell morphology	Highly branched filamentous, Gram positive
Extra cellular enzymes	Cellulase, amylase, protease (+)
Carbon source utilization	Dextrose, fructose, galactose, lactose, maltose, sucrose, starch, mannitol (+), xylose, inositol, rhamnose (-)
Growth temperatue	
20 ºC	-
28 ºC	++
35 ºC	++ (optimum*)
40 ºC	-
Growth pH	
5	-
6	+
7	++ (optimum*)
8	+
9	+
10	-

(*Optimum has been determined as mean of triplicate study).

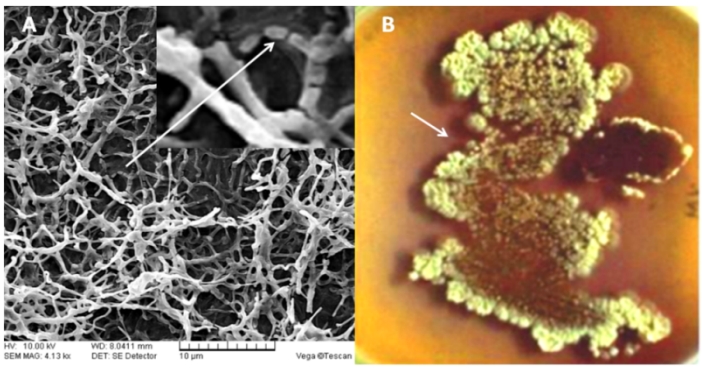

Fig. 1: Endophytic *Streptomyces thermoviolaceous* NT1; A: SEM view (arrows indicate spores, magnified view), B – colony morphology on ISP2 (arrow indicate redish violet pigmentation).

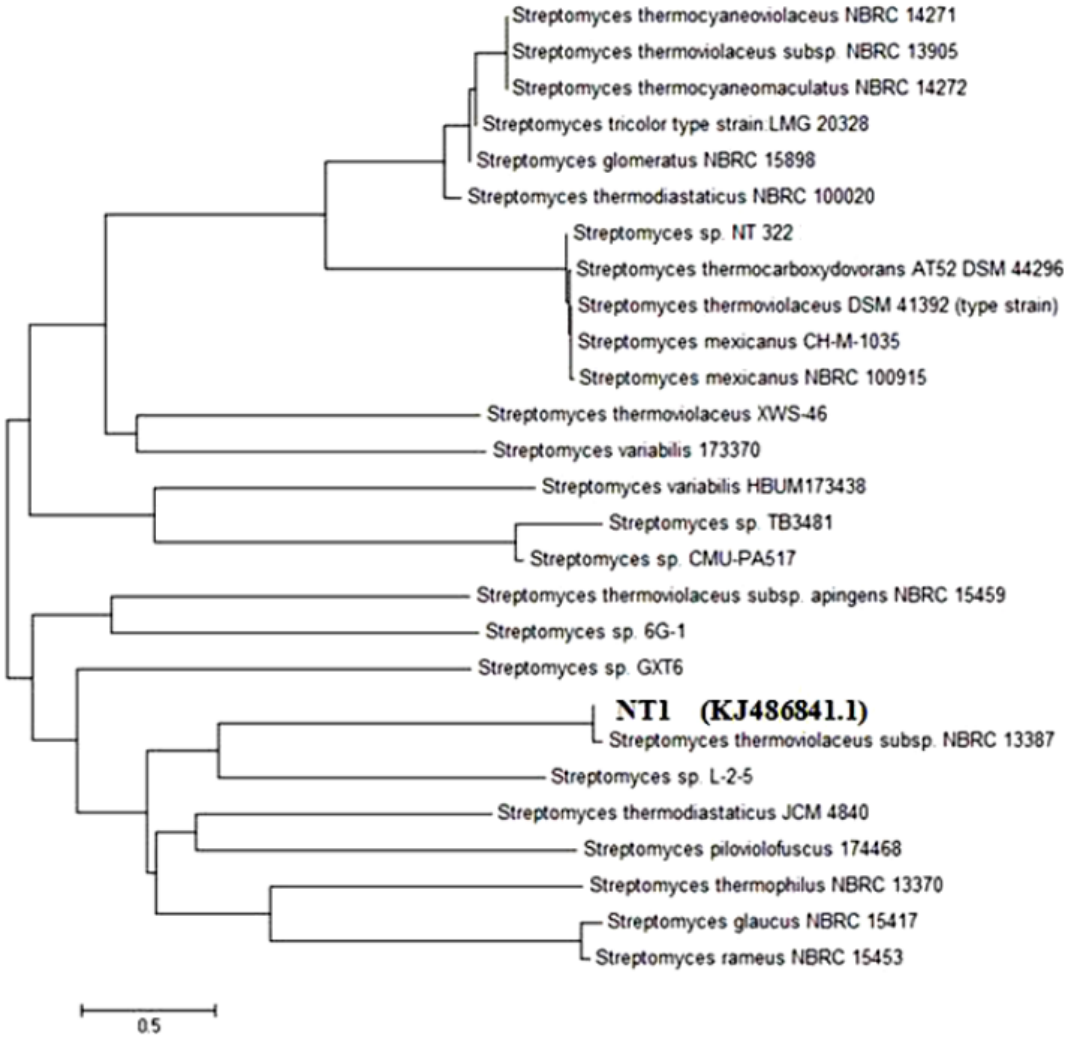

Fig. 2: Neighbor-joining tree based on 16s ribosomal RNA gene sequences showing relationship between NT1 and members of the genus *Streptomyces*.

Table. 2: Pathogen growth inhibition by NT1 culture filtrate and column purified active fraction.

Sample	Zone inhibition (mm) (Results are represented as means ± standard deviation of three replicates)							
	PRSA	**MRSA**	***B. cereus***	***B. subtilis***	***V. parahemolyticus***	***P. aeruginosa***	***S. flexnerii***	***E. coli***
Culture filtrate†	13.6±0.5	13±0.8	17.3±1	18±0.4	9.6±0.9	8.83±0.8	10.5±1.4	12.5±1
Crude extract#	29.3±1.2	27.6±1.2	30.8±2	33±0.9	13±0.5	16.3±0.8	19.8±0.4	15±0.6

†, At the amount of 100 µl of 14 days old culture supernatant in agar well.
#, At the amount of 25 µl on paper discs soaked with tested substance.

The strain fitted, in all respects, the taxonomic position within genus *Streptomyces*. Cellular and colony morphology, soluble pigments on media and biochemical characterization like enzyme profile and molecular characterization strongly suggest our isolate to be a new strain, *Streptomyces thermovilaceous* NT1. Few or modest spore production was found from this isolate in different media may be due to some biochemical relationship with its host plant. Previous isolates of this bacterium were of thermophilic range that could be cultivated at 50 ºC with antimicrobial production (James and Edwards, 1989) but NT1 does not grow over 40 ºC. Internal plant tissue is a specialized ecosystem may be responsible of such alteration in gene sequence during co-evolution (Germaine *et al.,* 2004).

Here is the opportunity of getting variations in metabolites from similar micro-organism isolated from other common habitats. Surprisingly an endophytic fungus, *Fusarium oxysporum* of *C. roseus* was interestingly found to produce vinblastine and vincristine the same natural products as host plants which opens a new probability in drug discovery (Kumar *et al.,* 2013).

The isolated endophytic strain may be a good source for the antimicrobial compound due to its broad spectrum antibacterial property and its potency against drug resistant pathogens. Culture filtrate of endophytic *S. thermoviolaceus* NT1 showed broad spectrum antibacterial activity to all selected pathogens. Table: 2 represent inhibition zones produced by culture filtrate and crude ethyl acetate extract against test pathogenic bacteria. It was found

that the antimicrobial compound was more effective for Gram positive bacteria than Gram negative bacteria. *S. thermoviolaceus* WR-141, a previous isolate of non-endophytic origin (St. Pyrek *et al.*, 1977) was found to produce antimicrobial that solely inhibited Gram positive.

The NT1 is also active against *P. aeruginosa*, an opportunistic pathogen that has become a major cause of nosocomial infections worldwide and a serious issue of public health. Moreover, only a few drugs are available to treat very serious infection of drug resistant Staphylococci. In this regard anti MRSA and anti PRSA activity of this strain indicates its promising role in future drugs. It was found that this strain produced the antibacterial substance best level at media pH-7, and 35 °C (Fig. 3) with 1 % additional glucose and tryptone (0.5 %). In a separate experiment, *S thermoviolaceous* NCIB 10076 was found to produce antibacterial substances optimally at pH-7 and at 45 °C incubation temperature (James and Edwards, 1989).

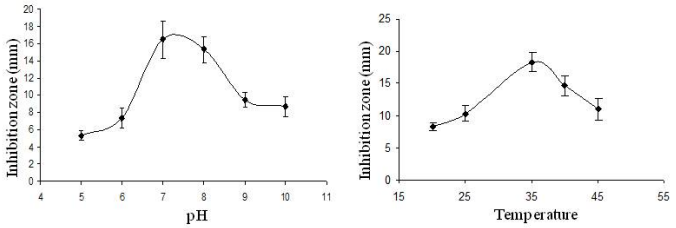

Fig. 3: Most suitable pH and temperature for optimum antibacterial production by NT1.

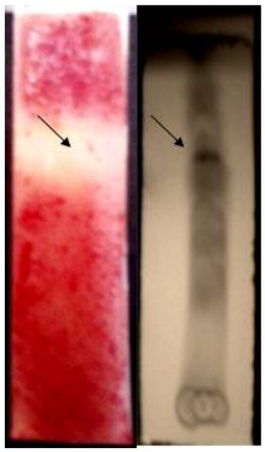

Fig. 4: Thin layer chromatography and autobiogram analysis of purified substance. Arrows indicate presence of active component.

After repeated column chromatography and activity guided TLC purification (Fig. 4), the active compound was found as amorphous red substance and changes its color from red to blue by pH change. It absorbs UV strongly with UV λ_{max} ($CHCl_3$) nm (log ε) 223 (1.3), 236 (0.32), 523 (1.24), 558 (1.32), 612 (1.43); IR ν_{max} were found with (KBr) cm^{-1}: 3600 ~ 3400, 3000 ~ 2850, 1712, 1665, 1610, 1458 (Fig. 3); MS m/z (relative intensities): 463.26 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ7.812 (doublet, j = 7.8 Hz), 7.714 (doublet, j = 5.4), 7.541 (triplet, $J_1 = J_2$ = 7.2 Hz), 7.455 (triplet, $J_1 = J_2$ = 7.8 Hz), 7.269, 6.427 (doublet, j = 5.4), 3.648, 3.597, 2.374, 1.471 - 0.843; 13C NMR ($CDCl_3$) δ170.11 (-COOH), 154.26, 132.13, 129.07, 128.63, 127.34 (C aromatic), 113.04 (C), 42.44, 41.23 (CH), 35.01, 34.05, 33.70, 31.90, 29.67, 29.14, 27.18, 24.82, 22.67 (CH_2), 19.47, 18.81, 14.10, 11.25 (CH_3) (Fig. 5).

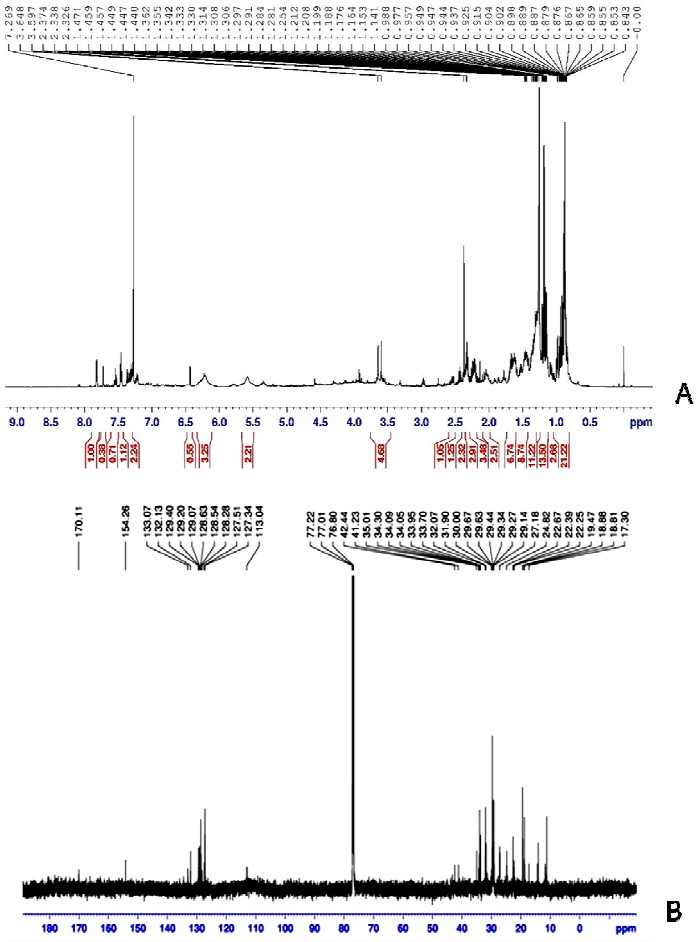

Fig. 5: NMR spectra of active antibacterial substance; A- ^{1}H spectra, B- ^{13}C spectra.

Granaticins are a series of polyketide-derived pyranonapthoquinone antibiotics (Fleck *et al.*, 1980). UV absorbance at 223 nm is the most important measurement of granaticin like compound, beside that granaticin absorbs 285, 410, 490, 525 and 562 nm dissolved in an acidified ethanol (0.1 M HCl in ethanol) (James and Edwards 1989). Similar UV absorbances were recorded at 223, 236, 523, 558, and 612 nm for compound dissolved in chloroform. Granaticin B contain L-rhodinose sugar moiety with granticin A which is absent in Granaticinic acid and concomitant γ-lactone cleavage of Granaticn A lead to the formation of granaticinic acid (St. Pyrek *et al.*, 1977). Granaticinic acid is a red pigmented amorphous substance and changes its color with change of pH. This compound with the molecular formula, $C_{22}H_{22}O_{11}$ and molecular weight, 462.41 were isolated from a thermophilic *Streptomyces* sp. XT-11989 (Maehr *et al.*, 1979) along with granaticin (Mr 444.39). Mass analysis of purified substance in this study represent 463.26 $[M^+H]$ for granaticinic acid solely and no other masses related to granaticin or its other

derivative were noticed in the spectrum. The IR spectrum shows typical OH stretching vibrations of the phenolic and carboxylic acid at 3600 - 3400 and 3000 - 2850 cm^{-1}. Peak at 1715 cm^{-1} is indicative of carbonyl vibrations of a saturated aliphatic carboxylic acid, which is also similar to granaticinic acid reported (US patent). IR absorbance at 1610 cm^{-1} is indicative of quinine carbonyl with strong intermolecular hydrogen bonds. In ^{1}H NMR, δ 7.269 strongly suggest the presence of p- disubstituted aromatic, δ 2.374 indicates H of adjacent carbon to carboxyl group and δ 1.471 - 0.843 suggest the presence of H in primary or secondary aliphatic carbon. δ 170.11 in ^{13}C NMR clearly represent for a C=O in acid group where as δ 154.26 stands for =C< in aromatic ring structure. So all physical and spectral evidences confirmed that the active antibacterial component is granaticinic acid produced by *S. thermoviolaceus* NT1.

CONCLUSION

This study demonstrates isolation of a new strain *Streptomyces thermoviolaceus* NT1 that colonized the inner tissues of *C. roseus*. It also proposes that the strain might be a significant source of antibacterial compound. Granaticinic acid was a major active component in cell free culture filtrate of NT1 which showed effective antagonism to drug resistant pathogens. This is the first report of isolation and identification of an endophytic (stems of *C. roseus*) *Streptomyces thermoviolaceus* producing granaticinic acid.

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