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Original article

Isolation and characterization of 2-hydroxy-9,10-anthraquinone from *Streptomyces olivochromogenes* (ERINLG-261) with antimicrobial and antiproliferative properties

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ABSTRACT

Currently *Streptomyces* is one of the most important antibiotic producing microorganisms against several diseases. In the present study *Streptomyces olivochromogenes* ERINLG-261 was isolated from the soil samples of the Mudumalai hills, Western Ghats, India. Morphological, physiological, biochemical and 16S rRNA studies strongly suggested that this isolate belonged to the genus *Streptomyces*. ERINLG-261 showed good antimicrobial activity against different bacteria and fungi in *Micromonospora* fermentation medium. The active ethyl acetate extract was packed in column chromatography over silica gel which led to the isolation of 2-hydroxy-9,10-anthraquinone as the active principle. The isolated compound showed good antimicrobial activity against tested bacteria and fungi in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) studies. The compound showed moderate *in vitro* antiproliferative activity against A549 and COLO320 cells. The compound was subjected to molecular docking studies for the inhibition of Topoisomerase, TtgR and Beta-lactamase enzymes which are targets for antimicrobials. Docking results of the compound showed low docking energy with these enzymes indicating its usefulness as antimicrobial agent. This is the first report of antimicrobial and antiproliferative activity of 2-hydroxy-9,10-anthraquinone isolated from *Streptomyces olivochromogenes* along with molecular docking studies.

Introduction

Actinomycetes have been especially useful to the pharmaceutical industry for their seemingly unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Actinomycetes are Gram-positive filamentous bacteria, characterized by a complex morphologic differentiation cycle accompanied by the production of numerous extracellular enzymes as well as many kinds of bioactive secondary metabolites having great structural and functional diversity (Williams et al., 1983). Actinomycetes are known producers of structurally diverse metabolites namely, β-lactam antibiotics, thienamycin, macrolides, streptomycin, erythromycin, anthracyclines, daunorubicin, doxorubicin, polyketides, rapamycin, FK-506, peptide antibiotics, virginiamycin, pristinamycin, aminoglycosides, gentamicin and kanamycin (Demain, 1999). Secondary metabolites are potent antibiotics which have made *Streptomyces* the primary antibiotic producing organisms exploited by the pharmaceutical industry (Raja and Prabakaran, 2011). *Streptomyces* is one of the most major sources of antibiotic producing microorganisms and back-bone for curing important diseases. *Streptomyces* are known to be producers of many secondary metabolites which have different biological activities such as antibacterial, antifungal, antiparasitic, antitumor, inflammatory responses and

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immunosuppressive actions (Demain, 1999; Sanghvi et al., 2014; Rambabu et al., 2014; Balachandran et al., 2015). In recent times, *Streptomyces* has been called as antibiotic store room or library. More than 23,000 bioactive secondary metabolites produced by microorganisms have been reported and over 10,000 of these compounds are produced by actinomycetes (Raja and Prabakaran, 2011). Among actinomycetes, around 7600 compounds are produced by *Streptomyces* species (Raja and Prabakaran, 2011). Aouiche et al. (2014) had reported saquayamycins isolated from *Streptomyces* spp. PAL114 which showed good activity against *Candida albicans* M3 and *Bacillus subtilis* ATCC 6633. Huang et al. (2015) reported the isolation of new compounds and four known compounds were isolated from the marine *Streptomyces griseus* RSH0407 such as butyl homononactate, butyl nonactate, 8-actyl homononactic acid, homononactic acids, nonactic acid, homononactyl nonactate, homononactyl homononactate. Among these seven compounds butyl homononactate showed good cytotoxic properties against HCT-8, A2780, BGC-823, BEL-7402, and A549 cells.

Naturally occurring anthraquinones form the largest group of plant and microbial secondary metabolites. Anthraquinone derivatives are well recognized as important biologically active components from microbes and plants (Anke et al., 1980). The anthraquinones type of compounds showed activity against certain diseases including antifungal, antibacterial, anticancer, antioxidant, antiviral, anti-inflammatory and antihuman cytomegalovirus (Barnard et al., 1995; Agarwal et al., 2000; Iizuka et al., 2004; Chen et al., 2007; Ifesan et al., 2009). In the present study antibacterial, cytotoxic and molecular docking properties of 2-hydroxy-9,10-anthraquinone (**1**) isolated from *Streptomyces olivochromogenes* (ERINLG-261) were assessed.

Materials and methods

Isolation of Streptomyces olivochromogenes

The soil samples were collected from the depth of 5–15 cm at Mudumalai hills, Nilgiris, Western Ghats of Tamil Nadu, India. Isolation of *Streptomyces olivochromogenes* was performed by serial dilution using dilution plate technique (Balachandran et al., 2014a).

Morphological and biochemical observations

Cultural and morphological features of ERINLG-261 were characterized following the directions given by the ISP (Shirling and Gottlieb, 1966) and the Bergey's Manual of Systematic Bacteriology. Cultural characteristics of pure isolates in various media (ISP 1–7) were recorded after incubation at 30 °C for 7–14 days. The shape of cell, Gram-stain, color, the presence of spores and colony morphology were assessed on solid ISP agar medium. Biochemical reactions, different temperatures, NaCl concentration, pH level, pigment production, enzyme reaction and acid or gas production were done following the methods of Balachandran et al. (2012, 2014a,b).

16S rRNA gene amplification

Genomic DNA of ERINLG-261 was isolated using the methods of Hipura *Streptomyces* DNA spin kit-MB 527-20pr from Hi-media. The 16S ribosomal RNA gene was amplified by PCR method using primers 27f (5^1AGTTTGATCCTGGCTCAG3^1) and 1492r (5^1ACGGCTACCTTGTTACGACTT3^1). Each PCR mixture in a final volume of 20 μl contained 10 mM Tris–HCl (pH.8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer, 50 ng of genomic DNA and 1 U of Taq DNA Polymerase (New England Biolabs Inc). PCR amplification was detected by 1% agarose gel electrophoresis and was visualized by ultraviolet (UV) fluorescence after ethidium bromide staining. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystem, and USA). The same primers as above were used for this purpose. The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks using the NCBI BLAST available at http://www.ncbinlm-nih.gov/. The partial 16S rRNA gene sequence of isolate ERINLG-261 has been deposited in the GenBank database under accession number KF061091. A phylogenetic tree was constructed using the neighbor-joining DNA distance algorithm using software MEGA (version 4.1) (Tamura et al., 2007).

Primary antimicrobial screening

Primary antimicrobial activity was evaluated on Modified Nutrient Glucose Agar medium (MNGA) by the cross streak method against various pathogenic microorganisms (Balachandran et al., 2014b).

Media optimization

Four fermentation media were used for media optimization such as *Streptomyces* media-1 (tryptone: 17 g, peptone: 3 g, NaCl: 5 g, K_2HPO_4: 1.25 g, pH 7 and H_2O: 1000 ml), Nutrient Glucose media-2 (glucose: 10 g, peptone: 5 g, yeast extract: 3 g, NaCl: 3 g, beef extract: 3 g, pH 7 and H_2O: 1000 ml), Bennett media-3 (glucose: 10 g, peptone: 2 g, yeast extract: 1 g, malt extract: 1 g, pH 7 and H_2O: 1000 ml), *Micromonospora* media-4 (glucose: 10 g, starch: 24 g, peptone: 3 g, meat extract: 3 g, yeast extract: 5 g, $CaCO_3$: 4 g, pH 7 and H_2O: 1000 ml). Active culture ERINLG-261 was inoculated in these four fermentation media and incubated for 0 day, 2nd day, 4th day, 6th day, 8th day, 10th day and 12th day. After incubation secondary metabolites were extracted using $CHCl_3$, EtOAc and butanol (1:1 v/v). All the extracts were checked for antimicrobial activity against bacteria and fungi.

Extraction of secondary metabolites

Culture inoculate of the isolate ERINLG-261 was taken in 500 ml Erlenmeyer flasks containing 150 ml of media-4 and incubated at 30 °C in a shaker (200 rpm) for 10 days. After 10th day the culture broth was centrifuged at 8000 × *g* for 20 min to remove the biomass. Equal volume of $CHCl_3$, EtOAc and butanol (1:1 v/v) were added and shaken in a separating funnel. The process was repeated thrice and the extracts were combined. The extract was dried over anhydrous sodium sulphate and distilled in a rotary evaporator and the red residue obtained was finally dried in vacuum. The secondary metabolite production was continued up to 20 l.

Column chromatography

The active EtOAc extract (9 g) was subjected to silica gel column chromatography (Acme's 100–200 mesh) (column size-60 cm length/2.5 cm). The column was successively eluted with hexane, hexane:EtOAc mixtures with increasing polarity and finally with EtOAc:MeOH and MeOH (each fraction being 100 ml). Based on thin layer chromatography (TLC) profiles, the fractions were combined to give seventeen fractions. All these fractions were subjected to antimicrobial screening against bacteria and fungi. Among the seventeen fractions, fraction number 6 showed good antimicrobial activities against tested bacteria and fungi. In further active fraction 6 (yield 560 mg) was subjected to preparative high-performance liquid chromatography (HPLC) analysis. HPLC with isocratic elution capability H_2O alliance system was used. Column length was

Table 1
Culture characteristics of *Streptomyces olivochromogenes* (ERINLG-261) in different media.

Medium	Growth	Substrate mycelium	Aerial mycelium	Spores	Pigment color
ISP 1	Good	Golden yellow	Dark gray	Present	Red
ISP 2	Good	Golden yellow	Dark gray	Present	Red
ISP 3	Good	Yellow	Gray	Moderate	Red
ISP 4	Good	Yellow	Gray	Present	Light red
ISP 5	Good	Yellow	Gray	Present	Red
ISP 6	Good	Yellow	Gray	Present	Light red
ISP 7	Poor	Poor	Gray	Poor	Moderate
MNGA	Good	Yellow	Dark gray	Present	Light red

ISP 1–7: International *Streptomyces* Project; MNGA: modified nutrient glucose agar; +: present. –: absent.

250 mm with internal diameter of 6.0 mm and was filled with silica particles of 15 μm diameter bonded with octadecylsilane (YMC pack ODS A (250 × 6.0 mm), 15 μm). The mobile phase was composed of ACN and aqueous HOAc (15:85, v/v); it was isocratically eluted at a flow-rate of 3 ml/min and injection volume was 100 μl. Elution was monitored at 254 nm and peak fraction was collected according to the elution profile. The pure compound was obtained as yellow crystal from MeOH (97.66%) (retention time 14.008 and elution time 20 min).

Microbial organisms

The following Gram negative and Gram positive bacteria, clinical isolates and fungi were used for the experiment. Seven Gram negative bacteria: *Enterobacter aerogenes* MTCC 111, *Shigella flexneri* MTCC 1457, *Salmonella paratyphi-B, Klebsiella pneumonia* MTCC 109, *Pseudomonas aeruginosa* MTCC 741, *Proteus vulgaris* MTCC 1771 and *Salmonella typhimurium* MTCC 1251; four Gram positive bacteria: *Bacillus subtilis* MTCC 441, *Micrococcus luteus* MTCC 106, *Staphylococcus aureus* MTCC 96 and *Staphylococcus epidermidis* MTCC 3615; seven clinical isolates (isolated from patient's urine samples): *Escherichia coli* (ESBL-3984, Extended Spectrum Beta Lactamase), *Escherichia coli* (ESBL-3904), *Klebsiella pneumoniae* (ESBL-3971), *Klebsiella pneumoniae* (ESBL-75799), *Klebsiella pneumoniae* (ESBL-3894), *Klebsiella pneumoniae* (ESBL-3967) and *Staphylococcus aureus* (MRSA– methicillin resistant, clinical pathogen). The reference cultures were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India-160 036; *Candida albicans* MTCC 227, *Malassesia pachydermatis* and *Aspergillus flavus* were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Bacterial inoculums were prepared by growing cells in Mueller Hinton broth (MHB) (Himedia) for 24 h at 37 °C. The filamentous fungi were grown on Sabouraud dextrose agar (SDA) slants at 28 °C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on Sabouraud dextrose broth (SDB) at 28 °C for 48 h.

Antimicrobial assay

Antibacterial and antifungal activities were carried out using disk diffusion method (Balachandran et al., 2013). Zones of inhibition were recorded in millimeters and the experiment was repeated thrice.

Minimum inhibitory concentration (MIC)

MIC studies of the isolated compound were performed according to the standard reference methods for bacteria (Balachandran et al., 2014a,b), filamentous fungi (CLSI, 2008), and yeasts (NCCLS, 1999, 2002). The required concentrations (100, 50, 25, 12.5, 6.25 and 3.125 μg/ml) of the compound were dissolved in DMSO.

Minimum bactericidal concentration (MBC)

Freshly prepared tubes containing serial twofold dilutions of the compound in 5 ml of MHB (range, 100, 50, 25, 12.5, 6.25 and 3.13 μg/ml) were inoculated beneath the surface with 5×10^5 to 1×10^6 cells in 0.1 ml of MHB, mixed by flushings and incubated without shaking or agitation. After 20 h of incubation, all broths were examined for visual turbidity or growth of small colonies at the bottom of tubes and again vortexed. The tubes were re-incubated for a further 4 h and vortexed again until all tubes were found to be without visual turbidity. The MBC was considered as the lowest concentration of isolated compound which prevented growth and reduced the inoculum by ≥99.9% within 24 h, irrespective of counts of survivors at higher antibiotic concentrations and

Table 2
Physiological and biochemical characteristics of *Streptomyces olivochromogenes* (ERINLG-261).

Characteristics	Results
Gram staining	Positive
Shape and growth	Filamentous aerial growth
Production of diffusible pigment	+
Range of temperature for growth	25–37 °C
Optimum temperature	30 °C
Range of pH for growth	6–10
Normal pH	7
H_2S production	–
Amylase	+
Chitinase	+
Protease	+
Gelatinase	–
Indole production	+
Growth in the presence of NaCl	1–7%
Sugar analysis	
Glucose	++
Galactose	+
Lactose	–
Mannitol	–
Sucrose	++
Xylose	+
Rhamnose	++
Ribose	+
Arabinose	–
Mannose	++
Maltose	++
Starch	++
Standard antibiotics	
Ciprofloxacin	S
Gentamicin	S
Ampicillin	S
Cephaloridine	S
Streptomycin	S
Erythromycin	S
Vencomycin	S
Amikacin	S
Penicillin	S
Rifamycin	S
Norfloxacin	S

+: presence; –: absence; S: sensitive; R: resistance.

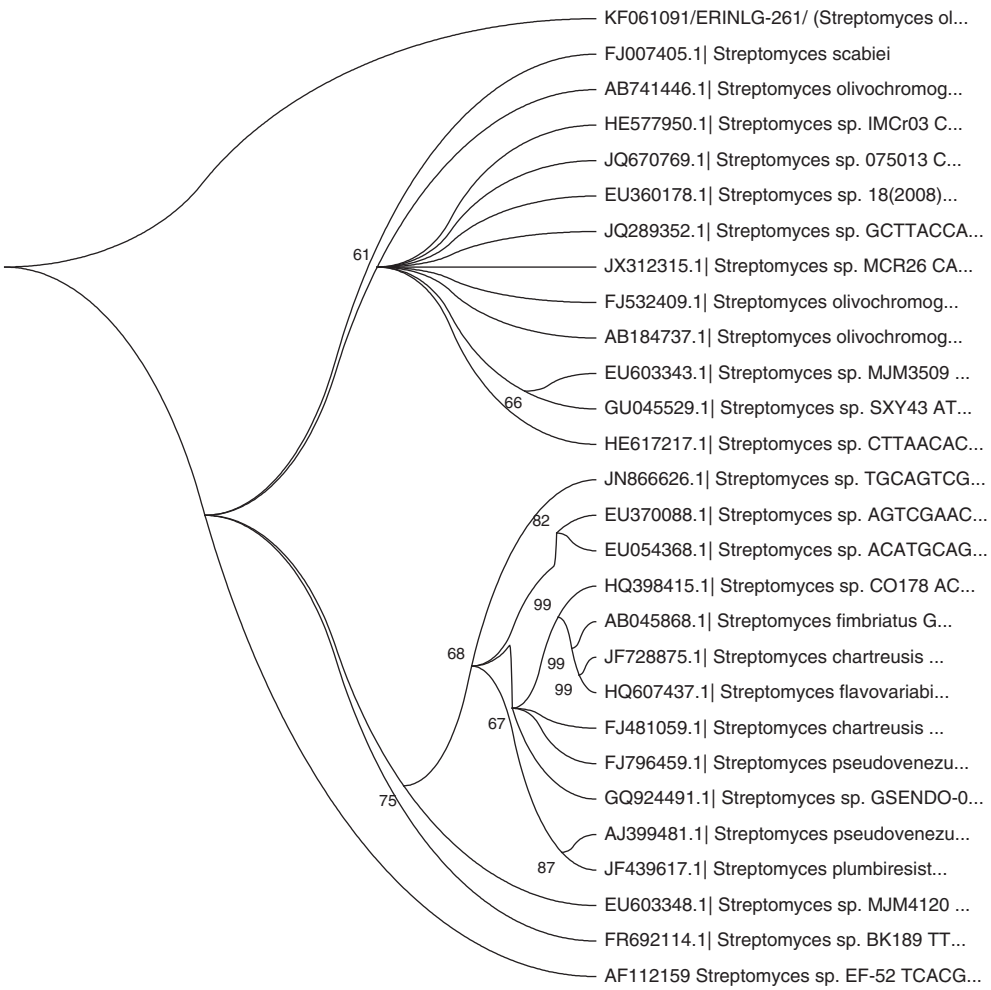


Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship between *Streptomyces olivochromogenes* (ERINLG-261) and the other species belonging to the genus *Streptomyces* constructed using the neighbor-joining method. Bootstrap values were expressed as percentages of 1000 replications.

the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate. For fungi, the plates were incubated for 48–72 h at 28 °C and for bacteria the plates were incubated for 24 h at 37 °C (Chennakesava Rao et al., 2014).

Cytotoxic properties

A549 lung adenocarcinoma cancer cell line and COLO320 cancer cell line were obtained from National Institute of Cell Sciences, Pune. A549 cell line was maintained in complete tissue culture medium Dulbecco's Modified Eagle's Medium (DMEM) and COLO320 cancer cell line Roswell Park Memorial Institute medium (RPMI) with 10% Fetal Bovine Serum and 2 mM L-Glutamine, along with antibiotics (about 100 International Unit/ml of penicillin, 100 μg/ml of streptomycin) with the pH adjusted to 7.2. The cytotoxicity was determined according to the method (Saravana Kumar et al., 2014) with some changes. Cells (5000 cells/well) were seeded in 96 well plates containing medium with different concentrations such as 500, 400, 300, 200, and 100 μg/ml. The cells were cultivated at 37 °C with 5% CO_2 and 95% air in 100% relative humidity. After various durations of cultivation, the solution in the medium was removed. An aliquot of 100 μl of medium containing 1 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was loaded in the plate. The cells were cultured for 4 h and then the solution in the medium was removed. An aliquot of 100 μl of DMSO was added to the plate, which was shaken until the crystals were dissolved. The cytotoxicity against cancer cells was determined by measuring the absorbance of the converted dye at 540 nm in an enzyme linked immune sorbant assay reader. Cytotoxicity of each sample was expressed as the half maximal inhibitory concentration (IC_{50}) value. The IC_{50} value is the concentration of test sample that causes 50% inhibition of cell growth, averaged from three replicate experiments. The percentage of growth inhibition was calculated using the following formula; inhibition (%) = A − B/A × 100 (A – Control group and B – Treated group).

Molecular docking studies

Molecular docking studies were done using the AutoDock Tools (ADT) version 1.5.6 and AutoDock version 4.2.5.1 docking program. Docking studies were performed by Intel® core i5 CPU (2.53 GHz) with Windows 7 operating system.

Protein structure preparation

Docked receptor structures of DNA Topoisomerase IV (PDB ID: 4EMV), TtgR (PDB ID: 2UXO) and beta lactamase (PDB ID: 4NK3) were obtained from the Protein Data Bank. The co-crystallized ligand in the receptor crystal structure was removed. Then the polar hydrogen atoms were added, lower occupancy residue structures were deleted, and any incomplete side chains were replaced using the ADT. Further ADT was used to remove crystal water; Gasteiger charges were added to each atom, and merged the non-polar hydrogen atoms to the protein structure. The

distance between donor and acceptor atoms that formed a hydrogen bond was defined as 1.9 Å with a tolerance of 0.5 Å, and the acceptor–hydrogen–donor angle was not less than 120°. The structures were then saved in PDBQT file format, for further studies in ADT.

Ligand structure preparation

Ligand 2D structure was drawn using ChemDraw Ultra 12.0 (ChemOffice, 2010). Chem3D Ultra 12.0 was used to convert 2D structure into 3D and the energy was minimized using

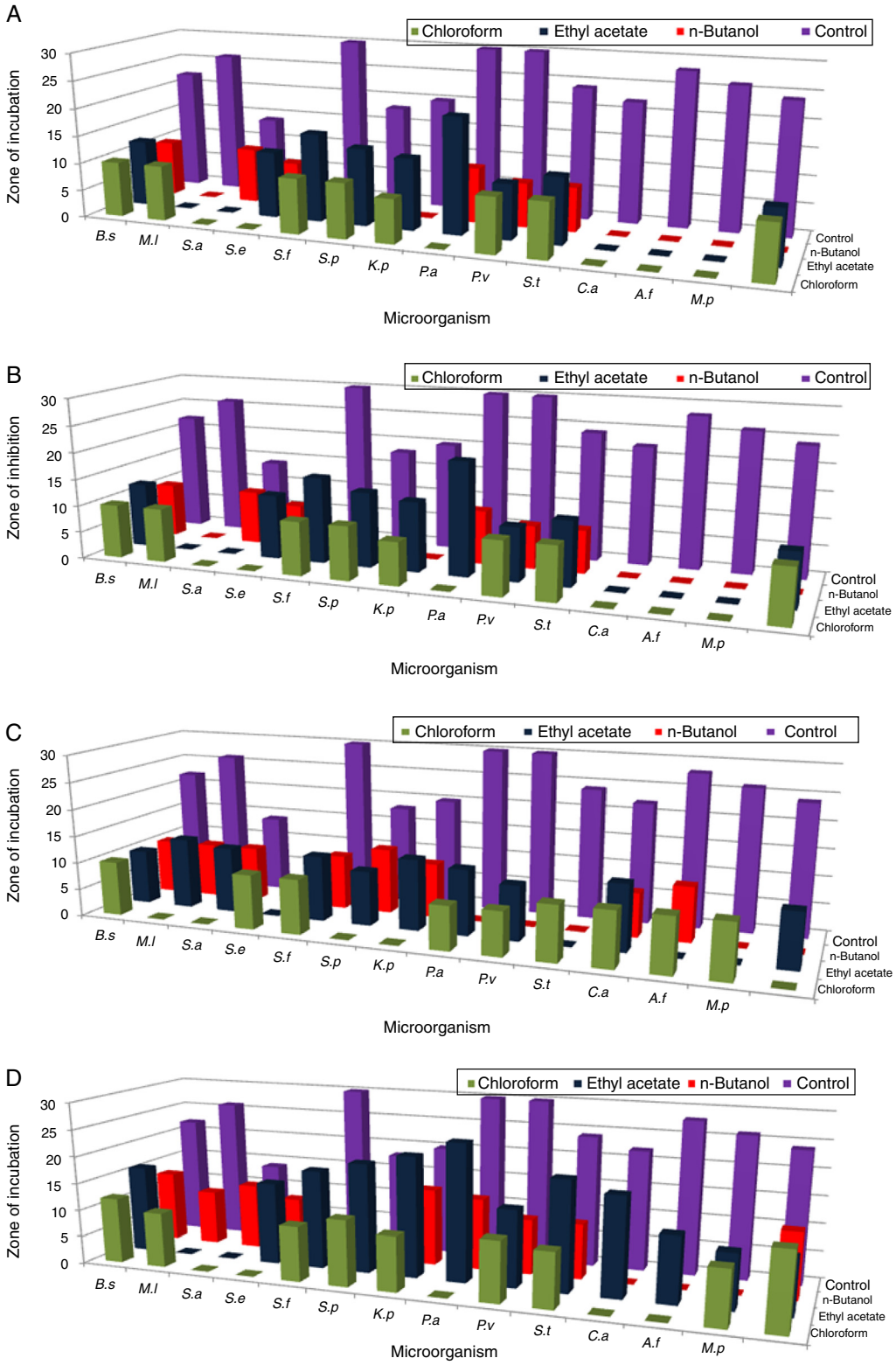


Fig. 2. Antimicrobial activities of chloroform, ethyl acetate and butanol extract of *Streptomyces olivochromogenes* (ERINLG-261) extracted from different fermentation media-1 to -4 using disk diffusion method (zone of inhibition in mm) (5 mg/disk). (A) Fermentation media-1, (B) Fermentation media-2, (C) Fermentation media-3 and (D) Fermentation media-4. Values in each column followed by the same alphabets are not significantly different by Duncan test at $p \leq 0.05$. Control-Streptomycin – standard antibacterial agent; ketoconazole – standard antifungal agent.

semi-empirical AM1 method. Minimized energy to minimum RMS gradient of 0.100 was set in each iteration. All structures were saved as PDB file format for input to ADT. All the ligand structures were then saved in PDBQT file format, to carry out docking in ADT.

Grid formation

A grid box with dimension of $40 \times 40 \times 40$ Å^3 with 0.375 Å spacing and centered on (*x*,*y*,*z*) 14.789, 29.446, 7.080; 0.856, 34.778, 13.333 and 5.475, 13.239, 18.265 was created around the binding site of ligand on DNA Topoisomerase IV (PDB ID: 4EMV), TtgR (PDB ID: 2UXO) and beta lactamase (PDB ID: 4NK3), respectively, using ADT. The center of the box was set at ligand center and grid energy calculations were carried out.

Docking protocol

For the AutoDock docking calculation, default parameters were used and 50 docked conformations were generated for each compound. The energy calculations were done using genetic algorithms. The outputs were exported to PyMol for visual inspection of the binding modes and interactions of the compounds with amino acid residues in the active sites (PyMOL, 2010).

Statistical analysis

Antimicrobial and cytotoxic activities of 2-hydroxy-9,10-anthraquinone (**1**) were statistically analyzed by Duncan multiple range test at $p = 0.05$ with the help of SPSS 11.5 version software package.

Table 3
Minimum inhibitory concentration and minimum bactericidal concentration of 2-hydroxy-9,10-anthraquinone from *Streptomyces olivochromogenes* (ERINLG-261) against bacteria and fungi.

Organism	MIC (μg/ml)	MBC (μg/ml)	Streptomycin
Gram negative			
E. aerogenes	50	50	25
K. pneumoniae	50	50	25
P. vulgaris	12.5	25	6.25
P. aeruginosa	12.5	12.5	25
S. paratyphi-B	25	25	6.25
S. typhimurium	25	25	25
S. flexneri	12.5	12.5	6.25
Gram positive			
B. subtilis	>100	>100	12.5
M. luteus	>100	>100	6.25
S. aureus	>100	>100	6.25
S. epidermidis	50	100	–
Clinical isolates			
E. coli (ESBL-3984)	25	25	25
E. coli (ESBL-3904)	50	100	25
K. pneumoniae (ESBL-3971)	12.5	12.5	6.25
K. pneumoniae (ESBL-75799)	50	50	25
K. pneumoniae (ESBL-3894)	>100	>100	6.25
K. pneumoniae (ESBL-3967)	>100	>100	25
S. aureus (MRSA)	50	100	6.25
Fungi			Ketoconazole
C. albicans	50	100	25
A. flavus	>100	>100	12.5
M. pachydermatis	25	50	15

Streptomycin – standard antibacterial agent; ketoconazole – standard antifungal agent.

Results and discussion

Morphology and biochemical studies

We were isolated 25 strains from different soil samples of the Mudumalai hills, Nilgiris, Western Ghats of Tamil Nadu, India using humic acid vitamin agar medium. Among the 25 isolates ERINLG-261 strain showed good antimicrobial activity in preliminary screening. This strain was Gram-positive filamentous bacterium. The color of the substrate mycelia was golden yellow (Table 1). ERINLG-261 showed good growth on medium amended with sodium chloride up to 7%; no growth was seen at 9%. The temperature for growth ranged from 25 to 37 °C with optimum of 30 °C and the pH range was 6–10 with optimal pH of 7. Utilization of various carbon sources by ERINLG-261 indicated a wide pattern of carbon source assimilation. Starch, maltose, mannose, rhamnose, sucrose and glucose supported the growth of the strain. ERINLG-261 showed sensitivity in all tested antibiotics (Table 2). The culture, morphological characteristics and antimicrobial activities of different *Streptomyces* isolates have been reported by several investigators (Oskay et al., 2004).

16S rRNA gene amplification

The result of the sequencing of ERINLG-261 was obtained in the form of rough electrophoregrams. The phylogenetic tree obtained by applying the neighbor joining method is illustrated in Fig. 1. Culture characteristics and 16S rRNA studies strongly suggested that our isolate ERINLG-261 belonged to the genus *Streptomyces*. Studies on the microbial diversity by 16S rRNA gene analysis showed that a group of high-GC Gram-positive bacteria (actinomycetes) were dominant in the soil (Urakawa et al., 1999). The identification of isolate ERINLG-261 was confirmed as *Streptomyces olivochromogenes* with homology of 100%.

Antimicrobial activity of extracts

Streptomyces olivochromogenes (ERINLG-261) was grown in different fermentation media-1 to -4 and extracted with $CHCl_3$, EtOAc and butanol. Each extracts (fermentation media 1–4) of $CHCl_3$, EtOAc and butanol were tested against bacteria and fungi. The EtOAc extract (fermentation media-4) showed good antibacterial and antifungal activities against tested bacteria and fungi compared to $CHCl_3$ and butanol extracts (5 mg/ml) (Fig. 2). Secondary metabolite production was checked in different days of incubations with fermentation media-4. Maximum secondary metabolite production was observed on 10th day and it showed good antimicrobial activity (Fig. 3).

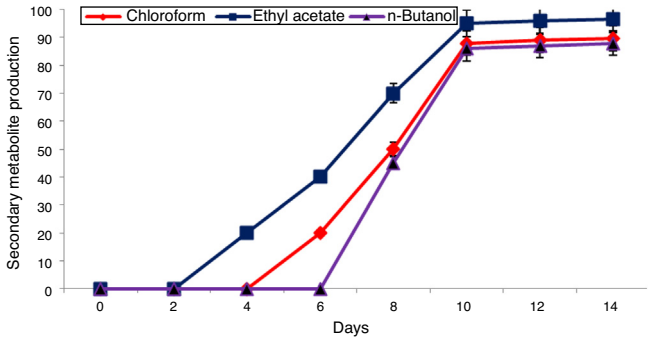
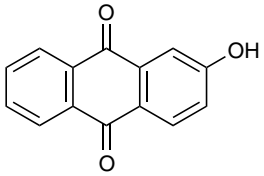


Fig. 3. Selection of secondary metabolite production using fermentation media-4 (*Micromonospora*) at different days of incubation. 10th day showed good secondary metabolite production and antimicrobial activity.

Isolation of active principle

The active principle obtained by preparative HPLC from fraction 6 as the major compound gave yellow crystals from methanol (50 mg); it gave a blue color with alcoholic $FeCl_3$ for phenol and pink color with alcoholic NaOH. On TLC over silica gel with EtOAc:MeOH (9:1) as the developing system it gave a single spot (R_f 0.39), yellow in color which on exposure to ammonia vapour turned pink. The compound was $C_{14}H_8O_3$ $[M+H]^+$, *m/z* 225, on the basis of ^1H NMR and ^{13}C NMR (dept) and Mass. mp 301–302 °C [lit. 298–299 °C]. UV: λ_{max} MeOH nm: 225, 249, 278 and 322. IR: υ_{max} KBr cm^{-1}: 3413 (hydroxyl), 2952, 2821, 1671 (quinone carbonyl), 1618, 1570, 1536, 1373, 1331, 1256, 1223, 1175, 1148, 1023, 871, 815, 785 (aromatic). ^1H NMR (δ, $CDCl_3$, 400 MHz): 8.35 (2H, *m*, H-5 and H-8), 7.84 (2H, *m*, H-6 and H-7), 7.42 (1H, *d*, H-1), 6.82 (1H, *d*, H-3), 7.08 (1H, *s*, H-4), 5.14 (1H, *s*, OH). ^{13}C NMR (δ, $CDCl_3$, 100 MHz): 181.2 (C-9, C-10), 152.5 (C-3), 135.1 (C-6, C-7), 133.5 (C-8a, C-10a), 126.6 (C-9a, C-4a), 131.2 (C-5, C-8), 130.2 (C-1), 118.5 (C-2), 117.5 (C-4). The ^1H and ^{13}C NMR showed the compound to be 2-hydroxy-9,10-anthraquinone (**1**). On the basis of the physical and spectroscopic data the compound was identified as 2-hydroxy-9,10-anthraquinone. Physical and spectroscopic data (UV, FT-IR, ^1H NMR, ^{13}C NMR and MASS) were compared with literature (Saha et al., 2013).



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MIC and MBC values of isolated compound

The compound showed potent antibacterial and antifungal activities. The MIC and MBC values of isolated compound were seen against *E. aerogenes*, *S. flexneri*, *S. paratyphi-B, K. pneumoniae*, *P. aeruginosa*, *P. vulgaris* and *S. typhimurium*; some clinical isolates were *E. coli* (ESBL-3984), *E. coli* (ESBL-3904), *K. pneumoniae* (ESBL-3971), *K. pneumoniae* (ESBL-75799), *S. aureus* (MRSA) and fungi *M. pachydermatis* and *C. albicans* (Table 3). Maximum growth and pigment production were observed in glucose as the sole source of carbon. The optimum temperature of 30 °C was found to be effective for growth and pigment production. Maximum antimicrobial compound was obtained at pH 7.0. Earlier report showed that twelve actinomycetes strains were isolated from the soil samples of the Himalayas and ERIH-44 showed both antibacterial and antifungal activity (Duraipandiyan et al., 2010). Normally antibiotic production was higher in medium having glucose (1%) as carbon source. *Streptomyces olivochromogenes* (ERINLG-261) showed

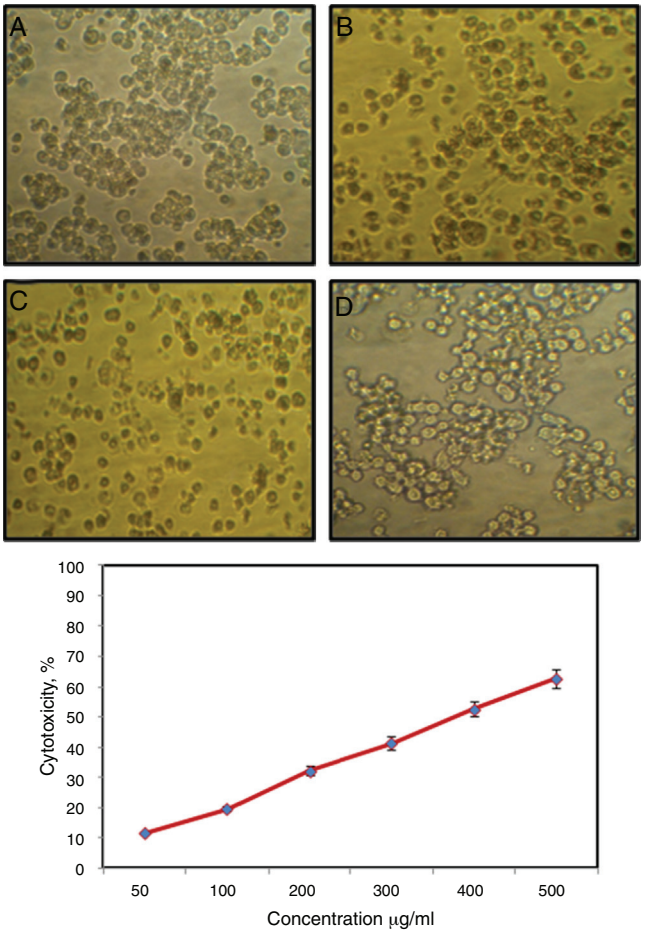

Fig. 4. Cytotoxicity properties of 2-hydroxy-9,10-anthraquinone (**1**) against COLO320 cells. Data are mean ± SD of three independent experiments with each experiment conducted in triplicate. Positive control-Cyclophosphamide at a concentration of 90 ± 0.00156 µg/ml (IC_{50}).

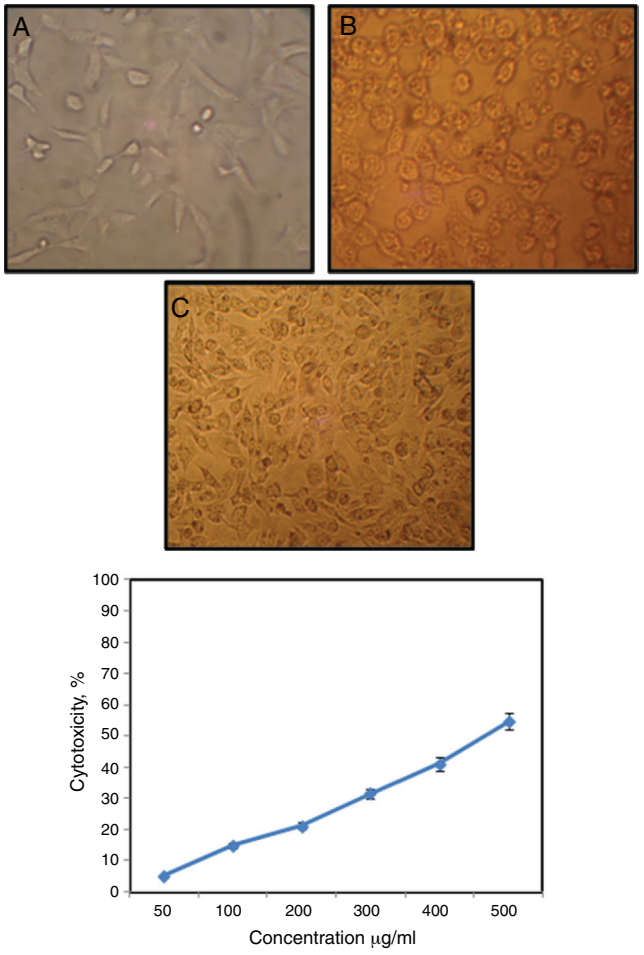

Fig. 5. Cytotoxicity properties of 2-hydroxy-9,10-anthraquinone (**1**) against A549 cells. Data are mean ± SD of three independent experiments with each experiment conducted in triplicate. Positive control 9.80 ± 0.43 µm (IC_{50}) (cisplatin).

good antimicrobial activity in *Micromonospora* medium and indicated that the antimicrobial compounds were extracellular. Most of the secondary metabolites and antibiotics were extracellular in nature and extra cellular products of actinomycetes showed potent antimicrobial activities (Bernan et al., 1994; Hacene et al., 2000). The study of the influence of different nutritional media and culture conditions on antimicrobial compound production indicated that the highest biological activities were obtained when *Micromonospora* medium was used as a base. In fact, it has been shown that the nature of carbon and nitrogen sources strongly affected antibiotic production in different organisms and the antibiotic production was increased by glucose rich medium (Cruz et al., 1999). *P. aeruginosa* has emerged as one of the most problematic Gram-negative pathogen, with an alarmingly high antibiotic resistance rate (Bacq-Calberg et al., 1999). Even with the most effective antibiotics against this pathogen, namely carbapenems (imipenem and meropenem), the resistance rate was found to be 15–20.4% amongst 152 *P. aeruginosa* strains (Savafi et al., 2005). The present study showed that the isolated compound was active against *P. aeruginosa.* This activity might be due to their ability to complex with bacterial cell wall (Cowan, 1999) thus, inhibiting the microbial growth and the membrane disruption could be suggested as the mechanism of action (Arvind et al., 2004). The antimicrobial compound from *Streptomyces olivochromogenes* (ERINLG-261) was recovered using ethyl acetate solvent. Most of the antimicrobial compounds are extracted using ethyl acetate (Sosio et al., 2000). Moreover, three bioactive compounds of 3-phenylpropionic acid, anthracene-9,10-quinone and 8-hydroxyquinoline showed strong antibacterial and antifungal activities (Narayana et al., 2008). Balachandran et al. (2014) had reported 2,3-dihydroxy-9,10-anthraquinone isolated from *Streptomyces galbus* (ERINLG-127) which showed good antimicrobial activity against tested bacteria and fungi. Duraipandiyan et al. (2014) had reported novel 1,5,7-trihydroxy-3-hydroxy methyl anthraquinone isolated from terrestrial *Streptomyces* sp. (ERI-26) which showed significant antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Bacillus subtilis*, *Epidermophyton floccosum*, *Aspergillus niger*, *Aspergiller flavus*, *Trichophyton rubrum* and *Botrytis cinerea*.

Cytotoxic properties of isolated compound

The isolated compound 2-hydroxy-9,10-anthraquinone (**1**) showed moderate cytotoxic activity *in vitro* against A549 lung and COLO320 cells. It showed 62.7% activity at the dose of 500 μg/ml with IC_{50} value of 400 μg/ml against COLO320 cells (Fig. 4). Isolated compound showed 54.7% cytotoxicity against A549 cells at the dose of 500 μg/ml (Fig. 5). All concentrations used in the experiment decreased the cell viability significantly ($P < 0.05$) in a concentration dependent manner. A number of anthraquinones have been reported to possess tumor cell inhibitory effects and are currently utilized as clinical anticancer agents. Anthraquinones have been shown to inhibit cancer cells through a variety of mechanisms including induction of apoptosis, intercalation and binding with cellular DNA, redox-cycling radical formation, and inhibition of topoisomerase (Patterson et al., 1983; Fisher et al., 1990; Barasch et al., 1990; Mueller and Stopper, 1999; Lee et al., 2001; Lee, 2001). A new, highly oxygenated angucyclin one gephyromycin was isolated from an extract of a *Streptomyces griseus* strain. Gephyromycin exhibited glutaminergic activity toward neuronal cells (Bringmann et al., 2005).

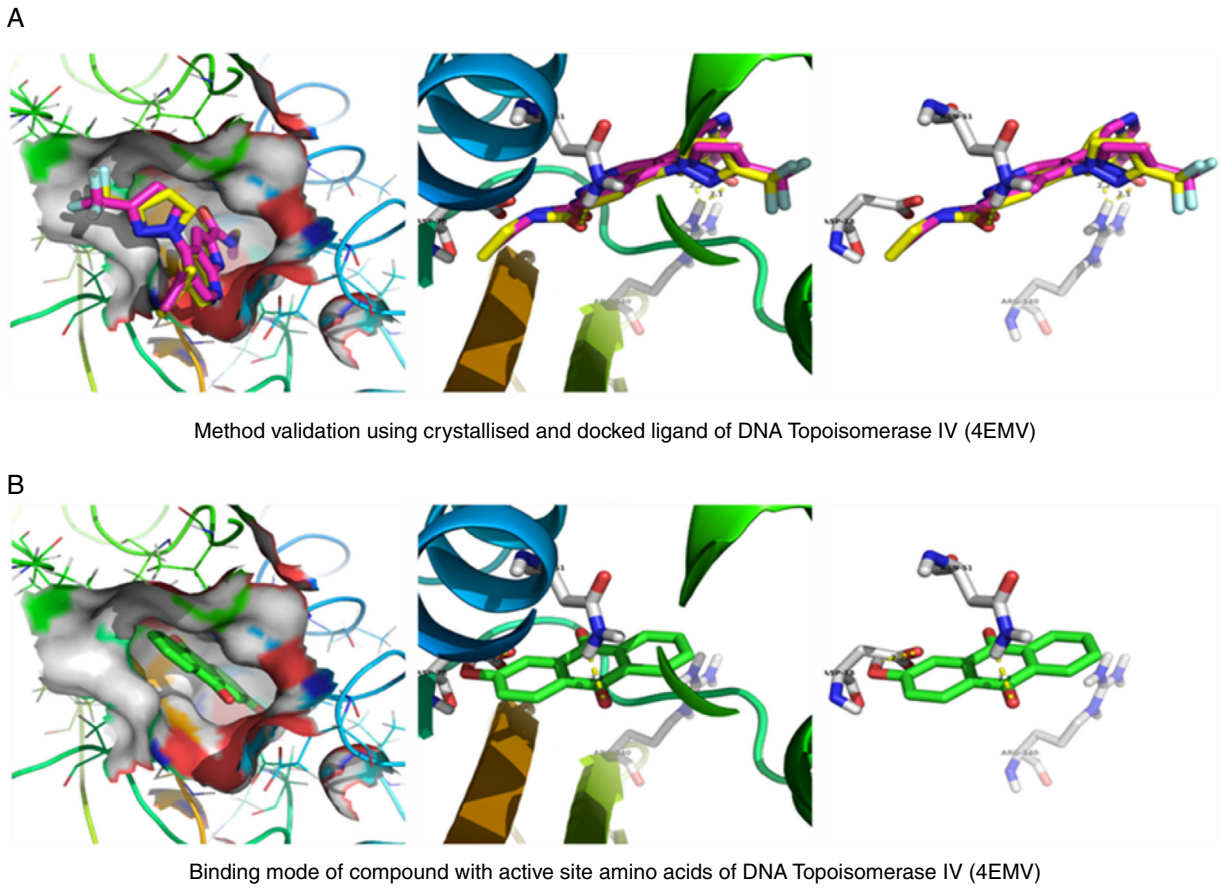

Fig. 6. Putative binding pose of compound 2-hydroxy-9,10-anthraquinone (**1**) with topoisomerase. Docking of compound 2-hydroxy-9,10-anthraquinone with Topoisomerase (A – method validation and B – docking with amino acids).

Molecular docking analysis

The compound was subjected to molecular docking studies using the AutoDock Tools (ADT) version 1.5.6 and AutoDock version 4.2.5.1 docking program (Sanner, 1999) to investigate the potential binding mode of inhibitor. Molecular docking was performed with the DNA Topoisomerase IV (PDB ID: 4EMV), TtgR (PDB ID: 2UXO) and beta lactamase (PDB ID: 4NK3) receptors. DNA topoisomerase IV receptor is required for maintenance of proper DNA topology during transcription and replication in bacteria. TtgR is important target for antibiotic drugs because antibiotic resistance is a major problem in antimicrobial drug synthesis. One major mechanism that underlies antibiotic resistance in bacteria is the active extrusion of toxic compounds through the membrane bound efflux pumps that are often regulated at the transcriptional level. TtgR represses the transcription of TtgABC, a key efflux pump, which is highly resistant to antibiotics (Manchester et al., 2012). Beta-lactams kill bacteria by inhibiting the cell wall construction enzymes known as PBPs. However, some bacteria have developed enzymes that can destroy beta-lactams before they can inactivate the PBPs. These enzymes, known as beta-lactamases, thus enable the bacteria to survive even in the presence of high concentrations of beta-lactams. Hence targeting this beta-lactase enzyme is vital in the antibacterial drug design (Alguel et al., 2007).

In order to verify the reproducibility of the docking calculations, the bound ligand was extracted from the complex and submitted for one-ligand run calculation. This reproduced top scoring conformation falling within root-mean-square deviation (rmsd) value of 0.65 Å, 0.74 Å and 1.14 Å from bound X-ray conformation of 4EMV, 2UXO and 4NK3 respectively, suggesting that this method is valid enough to be used for docking studies of other compounds.

Docking of the compound to DNA Topoisomerase IV, TtgR and beta lactamase was performed using AutoDock, following the same protocol used as in that of validation study. Docking was taken into 2.5 million energy evaluations were performed for the test molecule. Docked ligand conformation was analyzed in terms of energy, hydrogen bonding, and hydrophobic interaction between ligand and receptor. Detailed analyses of the ligand receptor interactions were carried out, and final coordinates of the ligand and receptor were saved. PyMol software was used for display of the receptor with the ligand binding site. From the docking scores, the free energy of binding (FEB) of the compound was calculated.

Molecular docking of compound with DNA Topoisomerase IV (4EMV) receptor showed the binding energy value of −7.04 kcal/mol with two hydrogen bonds. In the compound, hydrogen of O–H interacts with the C=O oxygen of amino acid (ASP-78) and forms a hydrogen bond with the bond length of 2.2 Å. Furthermore, oxygen of the one of C=O interacts with NH_2 hydrogen of amino acid (ASN-51) and forms a hydrogen bond with the bond length of 2.2 Å (Fig. 6). The compound showed the binding energy value of −6.85 kcal/mol with two hydrogen bonds with the docked TtgR (2UXO) receptor. In the compound, oxygen of O–H interacts with the N–H hydrogen of amino acid (ASP-172) and forms a hydrogen bond with the bond length of 2.7 Å. Also, hydrogen of the O–H interacts with the C=O oxygen of the amino acid (PHE-168) and forms a hydrogen bond with the bond length of 2.1 Å (Fig. 7).

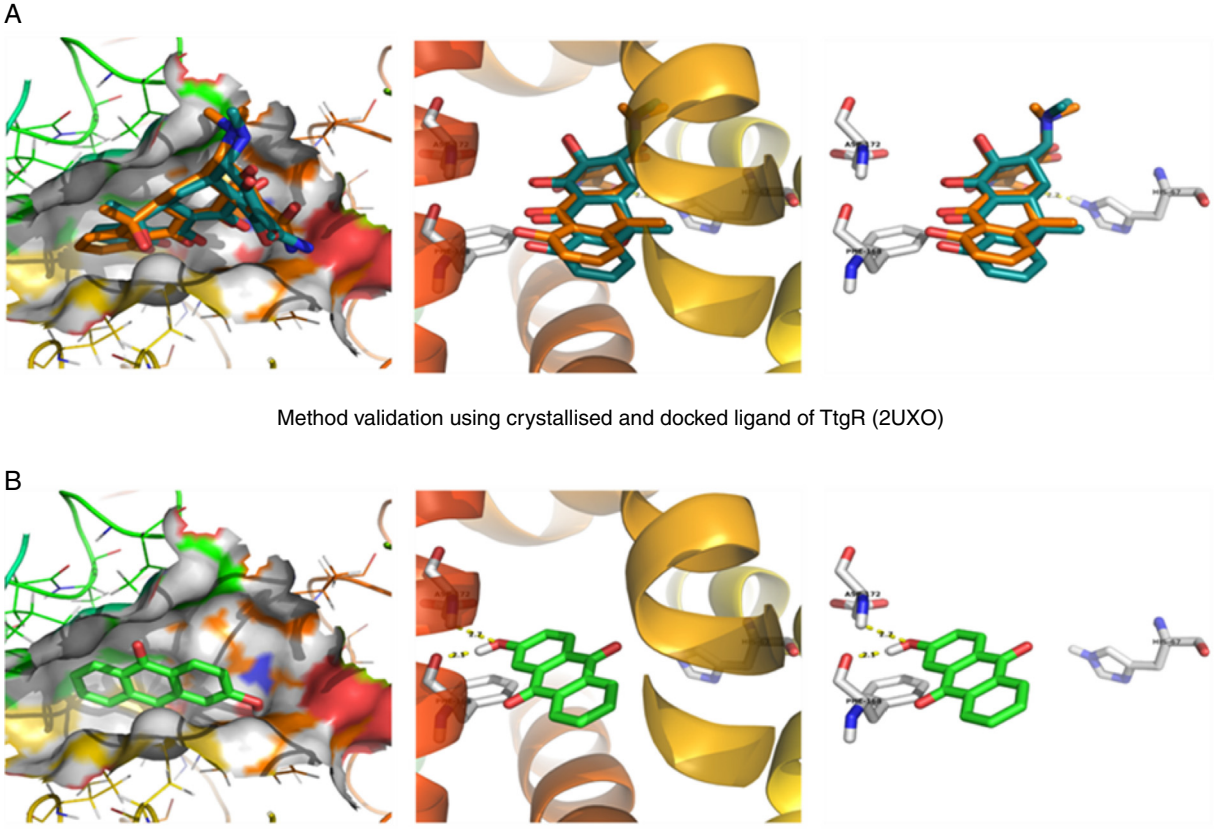

Fig. 7. Putative binding pose of compound 2-hydroxy-9,10-anthraquinone (**1**) with TtgR. Docking of compound 2-hydroxy-9,10-anthraquinone with TtgR (A – method validation and B – docking with amino acids).

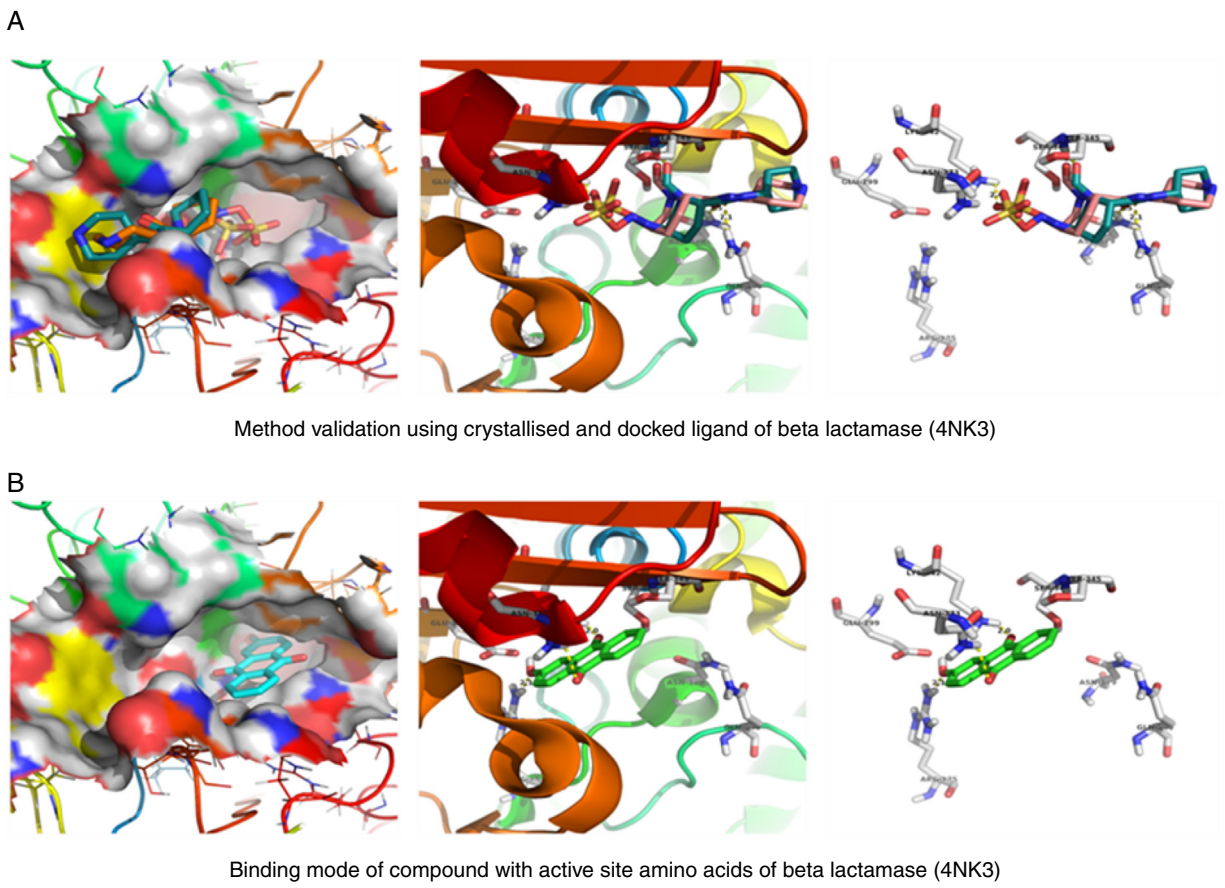

Fig. 8. Putative binding pose of compound 2-hydroxy-9,10-anthraquinone with Beta-lactamase (4NK3). Docking of compound 2-hydroxy-9,10-anthraquinone with Beta-lactamase (A – method validation and B – docking with amino acids).

The compound showed the binding energy value of −6.26 kcal/mol with two hydrogen bonds with the docked beta lactamase (4NK3) receptor. In the compound, oxygen of O–H interacts with the N–H hydrogen of amino acid (ARG-175) and forms a hydrogen bond with the bond length of 2.1 Å. Also, oxygen of the C=O interacts with the NH_2 hydrogen of amino acid (ASN-373) and forms a hydrogen bond with the bond length of 2.4 Å (Fig. 8).

Conclusion

Streptomyces olivochromogenes (ERINLG-261) was isolated from the soil samples of the Mudumalai hills, Nilgiris, Western Ghats of Tamil Nadu, India. Ethyl acetate extract of ERINLG-261 showed significant antimicrobial activities against tested Gram positive and Gram negative bacterial pathogens and filamentous fungal pathogens. The bioactivity guided fractionation of the ethyl acetate led to the isolation of 2-hydroxy-9,10-anthraquinone (**1**) as the active principle. The 2-hydroxy-9,10-anthraquinone was subjected to antimicrobial activity: it showed good antimicrobial activity against tested bacteria and fungi. The 2-hydroxy-9,10-anthraquinone was also tested against COLO320 and A549 lung adenocarcinoma cancer cells. The 2-hydroxy-9,10-anthraquinone showed moderate cytotoxic properties against tested cells. Molecular docking studies of isolated compound 2-hydroxy-9,10-anthraquinone with enzyme Topoisomerase, TtgR and Beta-lactamase showed low binding energy. This is the first report for the antimicrobial and cytotoxic properties of 2-hydroxy-9,10-anthraquinone isolated from *Streptomyces olivochromogenes* (ERINLG-261).

Authors' contributions

Conceived and designed the experiments: CB, VD, YA, BS, NE, NAAD, SI, YI, AO and PTP. Performed the experiments: CB, VD, YA, and BS. Analyzed the data: CB, VD, YA, BS, NE, NAAD, SI, YI, AO and PTP. Prepared the manuscript, CB, VD, YA, BS and SI. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.12.003.

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